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First step towards a quantitative model describing Czc-mediated heavy metal resistance in *Ralstonia metallidurans*

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Abstract

Quantitative models were derived to explain heavy metal resistance in *Ralstonia metallidurans*. A Δ*czcA* deletion of the gene for the central component of the $Co^{2+}/Zn^{2+}/Cd^{2+}$ efflux system CzcCBA combined with the expression level of *czcCBA* as studied with a Φ(*czcC-lacZ-czcBA*) operon fusion demonstrated that CzcCBA was the only prerequisite for resistance to $Co^{2+}/Zn^{2+}/Cd^{2+}$ at concentrations of 200 μM and above. The cellular content of the CzcA protein (resistance nodulation cell division protein family RND) determined by Western blot was used to model the CzcCBA expression level as the response to various metal concentrations. These data and experimentally determined uptake velocities were used to derive a flow equilibrium model that describes the cytoplasmic content c_i of the cells as an interaction between cation uptake and CzcCBA-mediated efflux. Alternatively, binding of heavy metals to inactivated *R. metallidurans* cells was described with a modified Freundlich's equation. The metal content of growing *R. metallidurans* cells was determined and compared to the predictions of both models. High amounts of zinc precipitates, exclusively formed by living cells, prevented a model validation for zinc. An additional net efflux activity let to lower amounts of cell-bound Co^{2+} than predicted. The flow equilibrium model described cadmium resistance sufficiently for *R. metallidurans* growing in the presence of 0.2–1 mM Cd^{2+}. Description of cadmium resistance in early stationary cells requires the binding model in addition to the flow equilibrium model. Thus, it was possible to simulate physiological events in growing cells by quantitative models that are derived from the biochemical data of the interacting transport proteins.

Abbreviations: TrisGlo – Tris-buffered mineral salts medium containing 2 g/l sodium gluconate as the carbon source; EDX – energy-dispersible X-ray analysis; RND – resistance-nodulation-cell division protein family

Introduction

Future biology could by a combination of bioinformatics, classical biochemistry/molecular biology and "omics" (e.g. genomics, proteomics) with the ultimate goal to obtain a holistic view of life processes on the basis of systems theory (Kitano 2002). We use the expression "bionomy" ($\beta\iota o\sigma$, life; $\nu o\mu o\sigma$, number) for such an approach to understand life processes and try to describe physiological functions of bacterial cells as the result of the interaction of cellular subsystems in "bionomical models".

Once bionomical models of bacteria have reached a certain degree of complexity, wet lab testing can hardly examine all levels and numbers of cellular subsystems. Instead, differences between two cellular conditions have to be exploited. To obtain differences, cells have to be disturbed. Heavy metal ions are useful disturbing agents, since they act on cells over a wide range of concentrations from pM to mM, yielding

cellular responses from starvation to toxicity. Heavy metals are not produced or degraded, and with a few exceptions (e.g. copper, iron, mercury, chromium) not reduced or oxidized. Every heavy metal ion is unique in its biochemical effect, but these effects may also overlap with other heavy metal ions or major bioelements (Nies 1999, 2000).

Ralstonia metallidurans strain CH34 [previously *Alcaligenes eutrophus*, (Goris et al. 2001)] is adapted to survive several forms of heavy metal stress (Nies 1999, 2000). Therefore, it is our subject to develop bionomy by looking at heavy metal disturbance of cellular processes. This bacterium shows a unique combination of advantages not present in this form in other bacteria. (i) Its genome has been fully sequenced (Preliminary, unnotated sequence data were obtained from the DOE JointGenome Institute (JGI) at http://www.jgi.doe.gov/tempweb/JGI_microbial/ html /index.html). (ii) It is non-pathogenic, therefore, models of the cell can also be tested in artificial environments similar to the natural habitats of this bacterium. (iii) It is related to the plant pathogen *R. solanacearum* (Salanoubat et al. 2002). (iv) It is of ecological importance since related bacteria are predominant in mesophilic heavy metal-contaminated environments (Diels et al. 1995a; Goris et al. 2001). (v) It is of industrial importance and used for heavy metal remediation and sensing (Nies 2000). (vi) It is an aerobic chemolithoautotroph, facultatively able to grow in a mineral salts medium in the presence of H_2, O_2 and CO_2 without an organic carbon source (Mergeay et al. 1985). The energy providing subsystem of the cell under these conditions is composed only of the hydrogenases, the respiratory chain and the F_1F_0-ATPase. This keeps this subsystem simple and clearly separated from the anabolic subsystems that starts with the Calvin cycle for CO_2-fixation. (vii) It is able to degrade xenobiotics even in the presence of high heavy metal concentrations (Springael et al. 1993).

(viii) Finally, strain CH34 is adapted to the outlined harsh conditions by a multitude of heavy metal resistance systems that are encoded by the two indigenous megaplasmids pMOL28 and pMOL30 or the bacterial chromosome(s) (Nies 1999, 2000). This paper describes the border conditions for a first simple bionomical model, develops the model, and delivers the first wet lab tests. It is therefore the very first step towards the realization of bionomy.

Material and methods

Bacterial strains, plasmids and growth conditions

Ralstonia metallidurans strains used were AE128(pMOL30) and the metal-sensitive, plasmid-free strain AE104. Strain AE128(pMOL30-9) contains a Φ(*czcC-lacZ-czcBA*) fusion on a plasmid pMOL30 derivative (Große et al. 1999). All *R. metallidurans* strains were cultivated in Tris-buffered mineral salts medium (Mergeay et al. 1985) containing 2 g/l sodium gluconate (TrisGlo). Analytical grade salts of the heavy metal chlorides were used to prepare 1 M stock solutions, which were sterilized by filtration. Solid Tris-buffered media contained 2 g/l agar. *Escherichia coli* strains were routinely cultivated in Luria broth (Sambrook et al. 1989). The minimal inhibitory concentrations were determined as published (Mergeay et al. 1985).

Uptake experiments

As published (Nies & Silver 1989b), *R. metallidurans* cells were cultivated in TrisGlo. The cells were harvested by centrifugation, washed and suspended into 10 mM TrisHCl buffer, pH 7.0, or into fresh TrisGlo. $^{65}Zn^{2+}$, $^{57}Co^{2+}$ or $^{109}Cd^{2+}$ (Amersham, Braunschweig, FRG or NEN, Köln, Germany) was added to various final concentrations (1 μM to 10 mM, c_o) and incubation was continued with shaking at 30 °C. The cellular dry weight (d.w.) was determined by the turbidity and a calibration curve (Nies & Silver 1989b). The total concentration of metals was determined in 100 μl of the cell suspension with a scintillation counter (LS6500, Beckman, München, Germany). At various time points, 200 μl of cells were filtered through a membrane filter (pore diameter 0.45 μm, Sartorius, Göttingen, Germany), washed twice with a tenfold volume of 10 mM TrisHCl, pH 7.0, containing 10 mM $MgCl_2$, and the amount of metals bound to the cells was measured with the scintillation counter.

Binding experiments

To determine metal binding to inactivated cells, *R. metallidurans* strain AE104 cells were cultivated in TrisGlo. The cells were harvested by centrifugation, washed and suspended into 10 mM TrisHCl buffer, pH 7.0, or into fresh TrisGlo. The cells were pasteurized for 15 min at 80 °C, cooled down on ice, and diluted three- or ninefold. No living cells remained

after this treatment as shown by cultivation on nutrient broth agar plates, and no cell aggregations were visible under the microscope. The cell density was determined by the turbidity and a calibration curve. $65Zn^{2+}$, $57Co^{2+}$ or $109Cd^{2+}$ were added to various final concentrations (1 μM to 10 mM, c_o). The total concentration of metals in the sample was determined in 100 μl of the cell suspension with a scintillation counter (Beckman, München, Germany). For the determination of the amount of metals loosely attached to the cells (m_a), 500 μl of the cell suspension was removed after 1 h at 23 °C, micro-centrifuged, and the metal concentration in 100 μl of cell-free supernatant was determined. m_a was the difference of total metals minus metals remaining in the cell-free supernatant, all divided by the dry weight of the cells in 100 μl.

Additionally, 200 μl of cells were filtered through a membrane filter (pore diameter 0.45 μm, Sartorius, Göttingen, Germany), washed twice with a tenfold volume of 10 mM TrisHCl, pH 7.0, containing 10 mM $MgCl_2$, and the amount of metals bound to the cells was measured. m_b was this amount of metals bound divided by the dry weight of the cells used. m_a was plotted against m_b to obtain q and w by $m_a = \theta \cdot m_b^w$. Second, c_o was plotted against the reciprocal dry weight of the cell suspension x used following $c_b = c_o^u \cdot K_f + c_o^u \cdot K_c/x$. Third, the resulting 1/x-dependent and -independent terms were plotted against the outside concentrations used yielding u, K_f and K_c.

Analytical electron microscopy

Cells grown overnight in TrisGlo with added Zn^{2+} (1 mM) were chilled in liquid propane (JFD 030, Balzers, Liechtenstein), freeze substituted in acetone (CSauto, Leica, Bensheim, Germany) and embedded in ERL (Plano, Wetzlar, Germany). Sections (~100 nm) were collected on Ti-grids without supporting film and analysed with an EM912 OMEGA transmission electron microscope (LEO, Oberkochen, Germany) equipped with an EDX-system (energy-dispersible X-ray analysis, Link eXIII, Oxford Instruments, High Wycombe, Bucks, UK) in the spot mode (100 nm spot size at 80 KeV, 20 μA emission current). For analysis a computer program was used considering the net counts, excitation probability and section thickness, determined with the in-column filter.

Induction experiments

R. metallidurans strain AE128[pMOL30-9, Φ(*czcC-lacZ-czcBA*)] was cultivated overnight at 30 °C in TrisGlo. The culture was diluted to a turbidity of 30 Klett units, cultivated with shaking at 30 °C until a turbidity of 75 Klett units was reached and induced after 10 min with various concentrations of heavy metal cations. Incubation was continued with shaking. After induction, samples were taken every 10 min up to 3 h. β-galactosidase activity was determined in permeabilized cells as published previously with 1 U defined as the activity forming 1 nmol of *o*-nitrophenol per min at 30 °C (Pardee et al. 1959; Ullmann 1984).

Genetic techniques

Standard molecular genetic techniques were used (Nies et al. 1987; Sambrook et al. 1989). Transformation of *E. coli* strains was conducted as previously described (Nies et al. 1987). The gene *czcA* was deleted from megaplasmid pMOL30 by recombination as described (Grass et al. 2000) leading to the derivative plasmid pMOL30-38. Insertional mutagenesis was done to mutate the genes for the polyphosphate kinase (gene 2109 on contig 548 of the *R. metallidurans* sequence project), the exopolyphosphatase (gene 2112 on contig 548), and the *pit* gene that encodes the phosphate inorganic transport system [gene 4794 on contig 653, unnotated, preliminary sequence data was obtained from the DOE JointGenome Institute (JGI) at http://www.jgi.doe.gov/tempweb/JGI_microbial/ html /index.html] as published (Juhnke et al. 2002). A piece of 300 bp in the middle of the target gene was amplified by PCR from total DNA of *R. metallidurans* AE104 and cloned into pLO_2 (Lenz et al. 1994). The resulting plasmid was conjugated into *R. metallidurans* strains. Kanamycin resistance encoded by pLO_2 was used to screen for insertion of pLO_2 into the target gene by single cross recombination. Correct knockouts were verified by PCR.

Construction of strain AE128(pMOL30-20)

In this bacterium, expression of the *czcCBA* resistance operon was removed from its native control and brought under control of the *tac* promoter that is a constitutive low-level promoter in *R. metallidurans*. Megaplasmid pMOL30-20 was produced by recombinational insertion of plasmid pECD557 into megaplasmid pMOL30-9 after electroporation of plasmid pECD557 into *R. metallidurans* strain AE128(pMOL30-9). Electroporation was done using 2 mm cuvettes (peqlab Biotechnologie GmbH, Erlangen, Germany) in a gene pulser (BIO-RAD, München,

Germany) at 200 Ω, 25 μF, 2.48 kV as described (Taghavi et al. 1994).

Plasmid pECD557 containes *lacI*^{q} and the 295 base pair (bp) 5′ end of *czcC* under control of a *tac* promoter. The single components were amplified by PCR and cloned into plasmid pECD553 which is a derivative of pUC19 with kanamycin resistance instead of ampicillin resistance. The *lacI*^{q} gene was amplified from chromosomal DNA of *E. coli* strain K12 (ATCC 23716) using the upstream primer 5′-AAACTGCAGGAATGGTGCAAAACCTTTCGCG-3′ (*Pst* I site underlined, makes *lacI* expression constitutive) and the downstream primer 5′-ATTATACGAGCCGATGATGATTAATTGTCAAC-AG/GAAGCATAAAGCCTGGGGT-3′ (the "slash" separates the 5′ sequence with a part of a *tac* operator/promoter region and the 3′ region required for *lacI* amplification). The 295 bp 5′ part of *czcC* was amplified from plasmid pMOL30 DNA using the upstream primer 5′-GATAACAATTTCACACAGGAGGCAGCT/ATGA-TGCGAAGACTATTTCTGCCGCTCGG-3′ (the "slash" separates the 5′ part for *czcC*′ amplification and the 3′ part with a part of a *tac* operator/promoter region) and the downstream primer 5′-AAAGGATCCTGAGGATGATGATGTTCAGCGA-ATTAATTAACTTCCAA AA-3′ (the *Bam*HI site is underline and is followed by a stop codon which was underlined). Two additional, overlapping primer were used to construct the *tac* operator/promoter region by a PCR filling-in reaction: 5′-CTGTTGACAATTAATCATCGGCTCGTATAATGT-CTGTGGAATTGTGAGCGGATA-3′ and 5′-CATAGCTGCCTCCTGTGTGAAATTGTTATCCG-CTCACAATTCCACACATT-3′. All three fragments were used as templates in an threefold overlap extension PCR with the *PstI*-site and the *Bam*HI-site containing primer pair, digested with the respective restriction endonucleases, cloned into plasmid pECD553, and verified by DNA sequencing.

Quantitative western blot experiments

R. metallidurans cells were cultivated under various conditions and the cell density was determined from the turbidity with an equilibration curve (Nies & Silver 1989b). A sample representing a dry weight of 25 μg was mixed with threefold cracking buffer (final concentrations 50 mM Tris-HCl, pH 6.8, 10 g/l SDS; 10 ml/l β-mercaptoethanol, 2 mM EDTA, 0.1 g/l bromphenole blue, 100 g/l sucrose), incubated for 20 min at 40 °C and loaded onto a SDS gel (Laemmli 1970). Purified CzcA-strep-tag (Goldberg et al. 1999) protein was used as standard. The gel was blotted with a tank blot (Biometra, Göttingen, Germany) in 0.1 M cyclohexylaminopropane sulfonic acid (pH 11.0; Roth, Karlsruhe, Germany), 100 ml methanol/l for 1.5 h, 100 V, 250 mA onto a PVDF-membrane (Roche, Mannheim, Germany). The membrane was dried by air and then blocked in PBS (4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl), 5 ml Tween20 and 50 g skimmed milk per l with shaking at 4 °C for 16 h. The PVDF-membrane was washed 3 times 5 min with PBS-Tween (PBS, 1 ml Tween20/l) and further 5 min with PBS at 23 °C. The membrane was incubated for 1 h at 23 °C with shaking with the first antibody [polyclonal CzcA-antibody 1:10000, diluted in PBS-Tween, antibody raised in rabbits after CzcA purification as described (Goldberg et al. 1999)]. The membrane was washed three times for 15 min with PBS-Tween and 5 min with PBS, and incubated with the second antibody [a monoclonal anti-rabbit-IgG conjugated with horseradish peroxidase (Sigma-Aldrich, Deisenhofen, Germany) and diluted 1:50000 in PBS-Tween] for 1 h at 23 °C with shaking. Unbound antibody was washed off the membrane 3 times for 15 min with PBS-Tween and once 5 min with PBS. For detection, the PVDF-membrane was incubated 1:1 with solution 1 [0.1 M Tris-HCl (pH 8.5), 0.4 mM p-Coumaric acid, 2.5 mM luminol) and solution 2 [0.1 M Tris-HCl (pH 8.5), 5.4 M H_2O_2] for 1 min. The unnecessary liquid was wiped off and the membrane was exposed for 5 sec to 5 min to ECL-hyperfilm (Amersham Pharmacia Biotech, Uppsala, Sweden).

The hyperfilm was scanned (Apple Computer, Ismaning, Germany) and the intensities of the signals were determined with NIH Image (National Institutes of Health, USA). The standards were used to construct an equilibration curve for each gel and this curve was used to calculate the amount of CzcA per 25 μg d.w. of *R. metallidurans* cells. This number was divided by the molecular masses of the CzcA-strep-tag protein, multiplied by the Lohschmidt number $6.023 \cdot 10^{23}$ mol^{-1} and finally multiplied with the mass of a single *R. metallidurans* cell, 902 fg/cell as determined by a plating experiment (data not shown).

Table 1. Metal-resistance of a Δ*czcA* mutant strain[a]

Bacterial strain	Relevant genotype	Complementing genes *in trans*	Zn^{2+} [mM]	Co^{2+} [mM]	Cd^{2+} [mM] [mM]
AE128(pMOL30)[b]	Wild type	none	10	10	2.0
AE128(pMOL30, pVDZ′2[c])	Vector control	none	10	10	2.0
AE104[b]	Plasmid-free	none	0.2	0.3	0.3
AE104(pVDZ′2)	Vector control	none	0.2	0.3	0.3
AE104(pDNA278[d])	Complementation control	czcBAD′	5.0	1.0	0.4
AE104(pDNA280[d])	Complementation control	czcAD′	2.0	0.5	0.4
AE128(pMOL30-38)	Δ*czcA*	none	0.1	0.2	0.1
AE128(pMOL30-38, pDNA278)	Δ*czcA*	czcBAD′	10	10	2.0
AE128(pMOL30-38, pDNA280)	Δ*czcA*	czcAD′	10	7.5	2.0

[a]The cells were incubated for 3 days at 30 °C on TrisGlo. The experiment was reproducible with identical results. Indipendently isolated Δ*czcA* strains gave also identical results.
[b]Mergeay et al. (1985).
[c]Deretic et al. (1987).
[d]Rensing et al. (1997).

Results and discussion

Border conditions for simple models

Heavy metal homeostasis is the product of three cellular processes: (i) binding of a heavy metal ion to the cell; (ii) transport into the cytoplasm; (iii) export by heavy metal efflux systems; and (iv) cytoplasmic precipitation and sequestration. The cytoplasmic concentration c_i of a heavy metal is in a flow equilibrium that is the product of uptake and efflux processes. Therefore, it can be described in a flow equilibrium model. Additionally, the pool m_b of bound heavy metal ions has to be considered. This part of the bacterial heavy metal interaction can be described with a binding model.

The first problem to solve on the way to a flow equilibrium model is the multitude of possible heavy metal efflux systems present in *R. metallidurans*. With respect to $Co^{2+}/Zn^{2+}/Cd^{2+}$, there are three efflux proteins of the CDF [cation diffusion facilitator, T.C.2.A.4.1.1–2, (Paulsen & Saier 1997; Saier, 2000)] family, three of the CPx-type heavy metal ATPases of the zinc/cadmium/lead-subgroup [(T.C. 3.A.3, (Rensing et al. 1999)], and 12 genes encoding putative heavy metal-transporting proteins of the RND [T.C.2.A.6.1.1, (Paulsen et al. 1996, 1997)] family. Together with possible metal-binding components and unknown factors, these systems allow *R. metallidurans* to grow in the presence of high concentrations of heavy metal cations (Mergeay et al. 1985).

Czc alone is responsible for high level resistance to cobalt, zinc and cadmium

If a specific set of conditions (e.g. a concentration window) exists that solely requires the function of only one efflux system, the flow equilibrium model can be easily derived. Deletion of plasmid pMOL30 decreased resistance of the resulting plasmid-free strain AE104 down to MIC values of about 0.2 mM $Co^{2+}/Zn^{2+}/Cd^{2+}$, respectively (Mergeay et al. 1985). Constitutive expression of the *czcCBA* operon (Nies et al. 1989), cloned on the broad host range vector plasmid pVDZ′2 (Deretic et al. 1987), gave back a wild type resistance level, however, the effect of the different copy numbers of pMOL30 and pVDZ′2, and the constitutive expression of *czcCBA* under control of the *lac* promoter may have produced artifacts. Moreover, plasmid pMOL30 contains in addition to *czc a ncc*-determinant similar to the nickel-cobalt-cadmium resistance determinant from *Achromobacter xylosoxidans* (Schmidt & Schlegel 1994) and a CPx-type ATPase as part of the lead resistance determinant (Borremans et al. 2001). To understand the effect of these two and possible other genes on pMOL30 on the cobalt-zinc-cadmium resistance mediated by this plasmid, the *czcA* gene was deleted from pMOL30 in strain AE128(pMOL30) leading to the mutant plasmid pMOL30-38.

The MIC values for AE128(pMOL30-38) were even smaller than the MICs for the plasmid-free strain AE104 (Table 1). The DczcA mutation could be complemented in trans nearly to full wild type resistance

Table 2. Expression data for AE128(pMOL30-9)[a]

Metal cation	C_{max}, [U] mg d.w.	Kr [μM]	Q_{ind}-fold	c_s [μM]
Zn^{2+}	435 ± 26	133 ± 8	10.3	14.3
Co^{2+}	107 ± 19	277 ± 50	2.5	180
Cd^{2+}	270 ± 37	94 ± 13	6.4	17.4

[a]Induction experiments were done and the specific β-galactosidase activities was plotted against time. The data points were used to calculate the maximum induction level reached and the concentration required for half maximum induction (C_{max}, K_r). The values Q_{ind} and c_s were calculated from the other data to evaluate the flexibility and competence of the induction system. Q_{ind} is the fold maximum induction and is C_{max}/42.2 U/mg d.w., c_s gives as the sensitivity of the system the lower induction limit, is the concentration formally needed for Cmax = 42.2 U/mg d.w., and is defined as $c_s = K_r\,[1/(Q_{ind} - 1)]$.

by *czcAD′* or *czcBAD′*. Thus, czc alone is necessary and essential for the top layer of cobalt, zinc and cadmium resistance in strain AE128(pMOL30) at concentrations of 200 μM and above, although other factors may support the Czc system in its action.

Czc expression levels

For additional verification of the fact that Czc is solely responsible for the outer shell of cobalt-zinc-cadmium resistance, regulation of Czc expression was studied with a *lacZ* operon fusion. Strain AE128[pMOL30-9 Φ(*czcC-lacZ-czcBA*)] (Große et al. 1999) was induced with various concentrations of Co^{2+}, Zn^{2+} or Cd^{2+} and the increase in β-galactosidase activity was determined (Table 2). In all the experiments, β-galactosidase activity reached a constant level within 2 h of incubation time (data not shown). The actual level reached was a function of the concentration of the respective metal used. Double reciprocal plots of the maximum level against the concentration gave linear functions, and for each heavy metal, a maximum expression level C_{max} and a concentration of half-maximum expression K_r could be calculated (Table 2). The β-galactosidase level before induction was 42.2 ± 12.7 U/mg d.w. as a mean value from all experiments.

Three conclusions could be drawn from this experiment. First, Co^{2+} was a very bad inducer of Czc. Best induction by zinc was tenfold, by cadmium sixfold and by cobalt only twofold (Table 2). Second, the Czc regulatory system (Große et al. 1999) had theoretical threshold levels (c_s, Table 2) of about 15 μM of Zn^{2+} or Cd^{2+}, but of 200 μM of Co^{2+}. Below these (outside) concentrations, Czc seemed not to be needed at all. Finally, Czc was at least 50% expressed at Zn^{2+}/Cd^{2+} concentrations above 100 μM of each metal, but 300 μM Co^{2+} were needed to reach half maximum expression. These confirmed that the CzcCBA efflux system is responsible for resistance at 200 μM Co^{2+}/Zn^{2+}/Cd^{2+} and above.

The subunits of the CzcCBA protein complex are the cation-proton-antiporter CzcA located in the cytoplasmic membrane, the periplasmic efflux or membrane fusion protein CzcB, and the outer membrane associated protein CzcC (Rensing et al. 1997). All three proteins are necessary for full resistance, and the activity of the efflux complex was studied in inside-out vesicles (Nies 1995). Finally, the central pump protein CzcA, which belongs to the RND protein family, was purified and characterized in proteoliposomes (Goldberg et al. 1999). Thus, sufficient biochemical data for the Czc system are available, and a flow equilibrium model could describe Co^{2+}, Zn^{2+}, and Cd^{2+} resistance in *R. metallidurans* as the interaction between uptake and CzcCBA-mediated efflux.

Formation of the flow equilibrium model

Number of CzcCBA efflux complexes per cell as a function of the heavy metal concentration

To connect the β-galactosidase activity of the Φ(*czcC-lacZ-czcBA*) fusion with the actual number of CzcCBA efflux complexes present in the cell, antibodies against the CzcA protein were generated in rabbits. These antibodies were used to determine the number of CzcA proteins per cell under various conditions in a quantitative western blot experiments (Figure 1). When the β-galactosidase activity was blotted against the number of CzcA molecules per cell (Figure 1B), the result was a linear function with an increase of 53.7 ± 17.5 CzcA per 1 U/mg d.w. β-galactosidase activity. In an additional control using anti-β-galactosidase antibodies, the number of LacZ molecules was also determined in four cultures. All four data points gave a ratio of CzcA:LacZ close to one (Figure 1C). Both proteins had a half-life much larger than the duplication time of the *R. metallidurans* cells and could thus be considered as stable proteins (data not shown). Therefore, CzcA and LacZ polypeptides were expressed from the Φ(*czcC-lacZ-czcBA*) fusion in equimolar ratios and the number of CzcA proteins per cell could be calculated from the β-galactosidase activity.

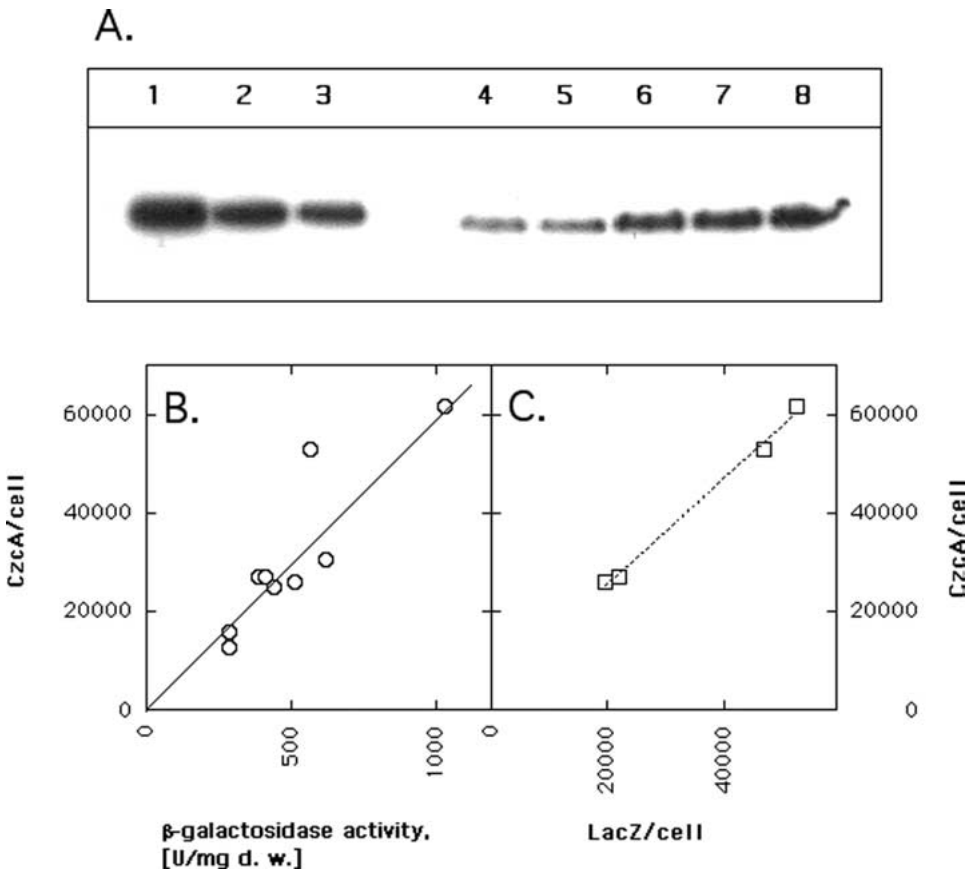

Figure 1. The number of CzcA proteins per cell corresponds to the specific β-galactosidase activity in AE128(pMOL30-9, Φ(*czcC-lacZ-czcBA*)) The number of CzcA and of LacZ proteins per *R. metallidurans* strain AE128(pMOL30-9) cell was determined by Western blot analysis. Panel A gives the data for one Western blot experiment. Strain AE128(pMOL30-9) was cultivated in the presence of 0.5 mM Zn^{2+} (lane 4), 1 mM Zn^{2+} (lanes 5 and 6) and 2 mM Zn^{2+} (lanes 7 and 8) to the early (lanes 5 and 7) or late exponential (lanes 4, 6, 8) phase of growth. The cells were harvested, and the proteins of the total crude extract corresponding to 25 μg of dry weight of these cultures was separated by polyacrylamide gel electrophoresis and blotted onto a PVDF membrane. Purified CzcA-strep-tag protein was used as standard in lanes 1 (602 μg), 2 (301 μg) and 3 (150 μg) The blot was treated with rabbit anti-CzcA antibodies and visualised with the ECL system. After densitometric analysis of the bands, an equilibration curve was constructed with the standards and used to calculate the amount of CzcA per lane, which was than used to calculate the number of CzcA per cell. In panel B, this number (circles) is plotted against the specific β-galactosidase activity measured in the same cultures at the same time. The slope obtained by linear regression was 57.3 ± 17.5 CzcA/cell per U/mg d.w. Panel C shows the plot of the number of CzcA proteins per cell against the number of LacZ proteins per cell in four experiments. The slope in this curve (dashed line) was 1.08 ± 0.01 CzcA/LacZ.

By multiplication of the 53.7 CzcA/cell per U/mg with the Czc baseline expression level of 42.2 U/mg and with the C_{max} values for cobalt, zinc and cadmium, the number of CzcA proteins per cell under these conditions could be obtained. These were 2300 CzcA/cell in non-induced cells and a maximum number of 5700 (Co^{2+}), 23,400 (Zn^{2+}) or 14,500 (Cd^{2+}) CzcA/cell after induction. The turn-over number of CzcA-driven transport for the respective metals as determined in proteoliposomes (Goldberg et al. 1999) was 100 sec^{-1} (Co^{2+}), 385 sec^{-1} (Zn^{2+}) and 28 sec^{-1} (Cd^{2+}), respectively, leading to a maximum turn over of 5.75×10^5 (Co^{2+}), 8.99×10^6 (Zn^{2+}) and 4.06×10^5 (Cd^{2+}) metal cations per induced cell and second. These turn-over numbers per cell were multiplied with 60 sec/min, divided by the Lohschmidt number 6.023×10^{23} mol^{-1} and finally divided by the dry mass of a *R. metallidurans* cell (902 fg d.w./cell) to obtain a maximum efflux velocity v_p that was 63.5 μmol min^{-1} g^{-1} d.w. (Co^{2+}), 993 μmol min^{-1} g^{-1} d.w. (Zn^{2+}) or 44.8 μmol min^{-1} g^{-1} d.w. (Cd^{2+}). The value of the maximum efflux velocity is under the assumption that the transport rates determined with reconstituted CzcA protein in vitro are identical with

the transport rates of the CzcCBA protein complex in vivo. If this is not true, an *in vivo* to *in vitro* quotient "q" has to be introduced and the actual maximum efflux velocity of the cells is $q \times v_p$.

Uptake of heavy metal cations under physiological conditions

To investigate the uptake part of the uptake/efflux-governed flow equilibrium, uptake of $Co^{2+}/Zn^{2+}/Cd^{2+}$ by *R. metallidurans* was re-investigated. For an efficient detoxification by efflux, the maximum efflux power of the cell has to be larger than the uptake velocity. The kinetical constants for metal uptake in *R. metallidurans* were determined (Nies & Silver 1989a), however, only in Tris-buffer and at low metal cation concentrations. Therefore, net metal ion uptake was determined again for strain AE104 in 10 mM TrisHCl, pH 7.0, buffer and in TrisGlo, the medium routinely used to cultivate the bacterium. In TrisGlo, uptake could only be measured at concentrations above 100 μM (data not shown). The velocity of zinc uptake did not change from 500 μM to 5 mM and was 53 ± 22 μmol/min g d.w. (Table 3). So, no kinetical analysis was possible for TrisGlo. Careful analysis of the uptake data for Zn^{2+} in Tris buffer revealed the presence of at least two uptake systems functioning mainly at low ($\leq$100 μM) concentrations ($v_{max-up} = 3.9 \pm 0.1 \mu$mol/min g d.w., $K_{m-up} = 74 \pm 1 \mu$M) and at high concentrations ($v_{max-up} = 67 \pm 1 \mu$mol/min g d.w., $K_{m-up} = 2.74 \pm 0.01$ mM). Similarly, at least two systems seemed to be responsible for the uptake of Co^{2+} and Cd^{2+} (Table 3). The slow uptake system displayed kinetical parameters similar to the published data. The fast uptake system, however, seemed to be the active counterpart of Czc at high environmental cation concentrations.

Quantitative flow equilibrium

The apparent cytoplasmic metal cation concentration c_i is assumed to be in an flow equilibrium between uptake (velocity v_{up}) and efflux (velocity v_{ef}). Uptake follows

$$v_{up} = v_{max-up} \cdot c_o/(K_{m-up} + c_o) \quad (1)$$

with the outside concentration c_o and the kinetical constants v_{max-up} and K_{m-up} of the uptake system. Efflux by Czc follows a Hill-type kinetic with Hill coefficients n = 2 for Zn^{2+} and Co^{2+} and probably n = 1 for Cd^{2+} (Goldberg et al. 1999; Nies 1995):

$$v_{ef} = vmax - ef \cdot c_i^n/(K_{50}^n + c_i^n) \quad (2)$$

In a flow equilibrium, $v_{up} = v_{ef}$ leading to

$$v_{max-up} \cdot c_o/(K_{m-up} + c_o) = v_{max-ef} \cdot c_i^n/(K_{50}^n + c_i^n) \quad (3)$$

which gives

$$c_i = K_{50} \cdot [v_{max-ef}/v_{max-up} \cdot (K_{m-up}/c_o + 1) - 1]^{-1/n} \quad (4)$$

As decribed above, the CzcCBA-dependent efflux velocity of the cells depends on the maximum efflux power v_p and regulation of expression of Czc (given as the concentration K_r of half-maximum induction) and is

$$v_{max-ef} = q \cdot v_p \cdot c_o/(K_r + c_o) \quad (5)$$

Combination of equation 4 and equation 5 gives

$$c_i = K_{50} \cdot [q \cdot v_p/v_{max-up} \cdot (K_{m-up} + c_o)/(K_r + c_o) - 1]^{-1/n} \quad (6)$$

that describes the cytoplasmic concentration c_i as the result of the flow equilibrium model. To obtain the amount of metals m_i bound to the cytoplasmic space, c_i has to be multiplied with the cellular volume V_c, which is 3 ml/g d.w. for *R. metallidurans* – like bacteria (Li et al. 1994).

Mathematical consequences resulting from the flow equilibrium model

Under the assumption that the flow equilibrium model alone describes the reality, some conclusions could be drawn after the measured constants were introduced into Equation (6). For Co^{2+}, $K_r \approx K_{m-up}$ (Tables 2 and 3, K_{m-up} values for the fast uptake system in Tris buffer). This leaves the term "$(K_{m-up} + c_o)/(K_r + c_o)$" always close to 1, thus, for Co^{2+}, Equation (6) can be simplified to $c_i = K_{50} \cdot [q \cdot v_p / v_{max-up} - 1]^{-1/n} = 18.5$ mM $\cdot [4.88]^{-0.5} = 8.4$ mM or 25 μmol/g d.w. if q = 1. The Czc system should keep the amount of cobalt in the cell at about 25 μmol/g d.w. because the expression level of the efflux pump is up-regulated with a half-maximal expression level similar to the half-maximum uptake concentration. This would explain why Czc is able to detoxify cobalt efficiently although cobalt is a relatively bad inducer for Czc.

For Zn^{2+}, on the other hand, the K_{50} value of Equation (6) is divided by the square root of a large

Table 3. Uptake of heavy metals by *R. metallidurans* strain AE104

Metal cation	Zn^{2+}	Co^{2+}	Cd^{2+}
Published:[a]			
v_{max-up} [μmol/min g]	3.7±2.1	1.9±0.3	3.4±1.2
K_{m-up} [μM]	137±87	40±29	136±22
Tris buffer, slow:			
v_{max-up} [μmol/min g]	3.9±0.1	3.9±0.8	8.1±0.1
K_{m-up} [μM]	74±1	23±5	61±1
Tris buffer, fast:			
v_{max-up} [μmol/min g]	67±1	10.8±0.2	143±2
K_{m-up} [μM]	2740±10	305±5	3520±50
TrisGlo:			
v_{max-up} [μmol/min g]	53±22	9.7±2.4	at 5 mM: 137

[a]Published in Nives & Silver (1989a).

number which results from the fact that $K_{m-up} > K_r$ and that $v_p > v_{max-up}$. The apparent cytoplasmic concentration (at q = 1) should therefore be always below the K_{50} value of 6.6 mM or 20 μmol/g d.w.

For Cd^{2+}, the term "v_p/v_{max-up}" is smaller than 1, in fact 0.313 if q = 1. At an outside concentration of 1.5 mM Cd^{2+}, which is close to the MIC value of this cation, the term "$[v_p/v_{max-up} \cdot (K_{m-up} + c_o)/(K_r + c_o) - 1]$" theoretically reaches zero and the c_i-values approaches indefinite: this models closely the fact that at above 1 mM Cd^{2+}, *R. metallidurans* cells are no longer able to efflux cadmium sufficiently (Nies & Silver 1989b). This might also serve as evidence that q is actually about 1, because otherwise cadmium detoxification by Czc should reach a critical level earlier ($q \ll 1$) or much later ($q \gg 1$) than at $c_o = 1.5$ mM Cd^{2+}.

Binding model

Binding of heavy metals to R. metallidurans cells

Theoretically, the whole cell content of heavy metal ions m_t should be the sum of metals located in the cytoplasm as described by the flow equilibrium model ($m_i = V_c \cdot c_i$) and metals bound to the cell (m_b). However, the pool of cations bound could be influenced by cation efflux, especially since metals attached to the surface of the cell are usually removed during the washing steps of the uptake experiment. Since CzcCBA may be a transenvelope efflux system (Rensing et al. 1997), metals bound within the periplasmic space, e.g. to the cell wall, could be reached and detoxified by CzcCBA. Therefore, it is not certain, if a specific heavy metal pool can be detoxified by CzcCBA or not. Of the totally available binding sites for heavy metals, only a portion "p" may continue to bind heavy metals in the presence of an active efflux system. Metals bound to the remaining binding sites could be reached by the efflux system and efficiently detoxified. Thus, the total amount of metals bound to the cell can be described as

$$m_t = V_c \cdot c_i + p \cdot m_b \qquad (7)$$

The total amount of metals bound to *R. metallidurans* (that was not removed by washing with magnesium chloride) was studied in pasteurized cells of the plasmid-free strain *R. metallidurans* AE104. The resulting m_b value could be described using Freundlich's equation (Weber 1972), however, with a variation taking competition of cells for metal ions into account:

$$m_b\ [\mu\text{mol/g d.w.}] = c_o^u\ (K_f + K_c/x) \qquad (8)$$

with K_f being the Freundlich's constant, K_c an additional competition constant, x the dry weight in mg/l and c_o the concentration in μM. Table 4 gives the results for the m_b determination in Tris-buffer and TrisGlo. In addition to m_b, metal might be loosely attached to the cell. The amount of this m_a was also determined (Table 4), it is m_a [μmol/g d.w.] = $\theta \cdot m_b^w$. There was no difference in metal ion binding between cells of AE104 and AE128(pMOL30) cells which were pre-cultivated in medium containing 2.5 mM Zn^{2+} (data not shown).

Table 4. Constants which determine metal-binding to the surface of *R. metallidurans*

Metal ion	Solution	u	Kf/L/g d.w.	Kc · 10^{-3}	θ	w
Zn^{2+}	Buffer	0.760 ± 0.047	0.137 ± 0.006	38.62 ± 0.42	12.1 ± 1.6	0.77 ± 0.10
Zn^{2+}	Medium	0.736 ± 0.017	0.668 ± 0.010	58.7 ± 2.0	2.50 ± 0.18	1.07 ± 0.08
Co^{2+}	Buffer	0.813 ± 0.097	0.061 ± 0.001	65.38 ± 0.13	6.4 ± 2.0	1.06 ± 0.33
Co^{2+}	Medium	0.911 ± 0.042	0.032 ± 0.001	20.43 ± 0.06	1.75 ± 0.12	1.08 ± 0.07
Cd^{2+}	Buffer	1.000 ± 0.032	0.021 ± 0.001	17.92 ± 0.38	1.71 ± 0.15	1.12 ± 0.10
Cd^{2+}	Medium	0.711 ± 0.022	0.561 ± 0.016	82.11 ± 0.16	1.84 ± 0.15	1.08 ± 0.09

Binding of the metal cations was determined with $^{65}Zn^{2+}$, $^{57}Co^{2+}$ or $^{109}Cd^{2+}$ in 10 mM TrisHCl buffer (Buffer), pH 7.0 or in Tris-buffered mineral salts medium (Medium) containing 2 g sodium gluconate/l and pasteurized (15 min 80 °C) cells of *R. metallidurans* strain AE104. After 1 h at 23 °C, the cells were washed twice with a tenfold volume of 10 mM TrisHCl, pH 7.0, containing 10 mM MgCl2 to determine m_b in μmol/g d.w. The values were adapted to the function $m_b = c_o^u$ (K_f + K_c/x) with u, K_f and K_c given above, c_o the concentration in μM and x the dry weight in mg/l used for the experiments. In addition to m_b, the loosely cell-associated metal ion amount m_a was determined by comparing the concentration of metals remaining in the supernantant after the cells were spinned down. m_a could be calculated from c_b using the equation $m_a = \theta \cdot m_b^w$.

Verification and comparison of the models

Equation (6) describes the cytoplasmic metal cation concentration in a flow equilibrium of uptake and efflux. The velocity of uptake was determined directly, the velocity of efflux at a given concentration could be predicted by the flow equilibrium model from the data of the number of CzcCBA complexes per cell at a given c_o and the turn over number of the CzcA protein determined in vitro. The only unknown parameter is the in vivo/in vitro coefficient q that was assumed as q = 1. The alternative binding model described in Equation (8) adds another aspect. Equation (7) describes how both processes may interact to give the cellular metal content m_t. The parameter p defined in Equation (7) is a test parameter for the simulation quality of both models. Arithmetical isolation of p gives

$$p = (m_t - V_c \cdot c_i)/m_b \quad (9)$$

The values c_i and m_b are the results of the flow equilibrium and the binding model, respectively, and m_t can be determined experimentally. If only the flow equilibrium model is correct, p = 0. If, on the other hand, only the binding model describes reality, p = 1. If 0 > p > 1, both processes contribute. If p < 0, net efflux of metal cations is faster than assumed by the flow equilibrium model. Finally, if p > 1, living washed cells have to bind more metal than inactivated, washed cells.

Metal-content of R. metallidurans cells at various concentrations

Cells of strain AE128(pMOL30-9) were cultivated in the presence of various concentrations of heavy metals and the amount of cell-bound cations m_t was determined with the filtration method and radioactive metal isotopes. The experiment was done at concentrations starting at 0.2 mM of each metal, and up to 1 mM (Cd^{2+}) or 5 mM (Co^{2+}, Zn^{2+}). At least three independent growth curves were done for each concentration. Higher cadmium concentrations could not be tested since the cells did not grow at concentrations of 2 mM or higher. From these m_t data and the simulated c_i and m_b results at the respective medium concentrations c_o, the p-values were calculated and plotted against the cell density x (Figure 2).

Zinc

In the presence of Zn^{2+}, washed exponentially growing cells contained much more zinc than predicted to be even loosely associated to inactivated cells (Figure 2A). Two p-maxima could be observed. The first peak at p close to 60 was associated with early exponential cells (150 mg d.w./L = Klett 60), the second (Figure 2B) when the cells entered the stationary phase of growth (p-values around 8, about 650 mg d.w./L = Klett 230). This amount of bound zinc decreased during the growth phase (Figure 2A). Most p-values for cells growing in the presence of 0.2 mM or 0.5 mM Zn^{2+}, however, arrived in a region between 0 and 1 when the cells reached the stationary phase of growth (Figure 2B).

The huge amount of zinc exclusively associated to washed, living, early exponential cells of *R. metallidurans* was much higher than even loosely associated to inactivated cells. The m_a/m_b quotient θ was smaller than 2.5 in TrisGlo and smaller than 12 in Tris buffer

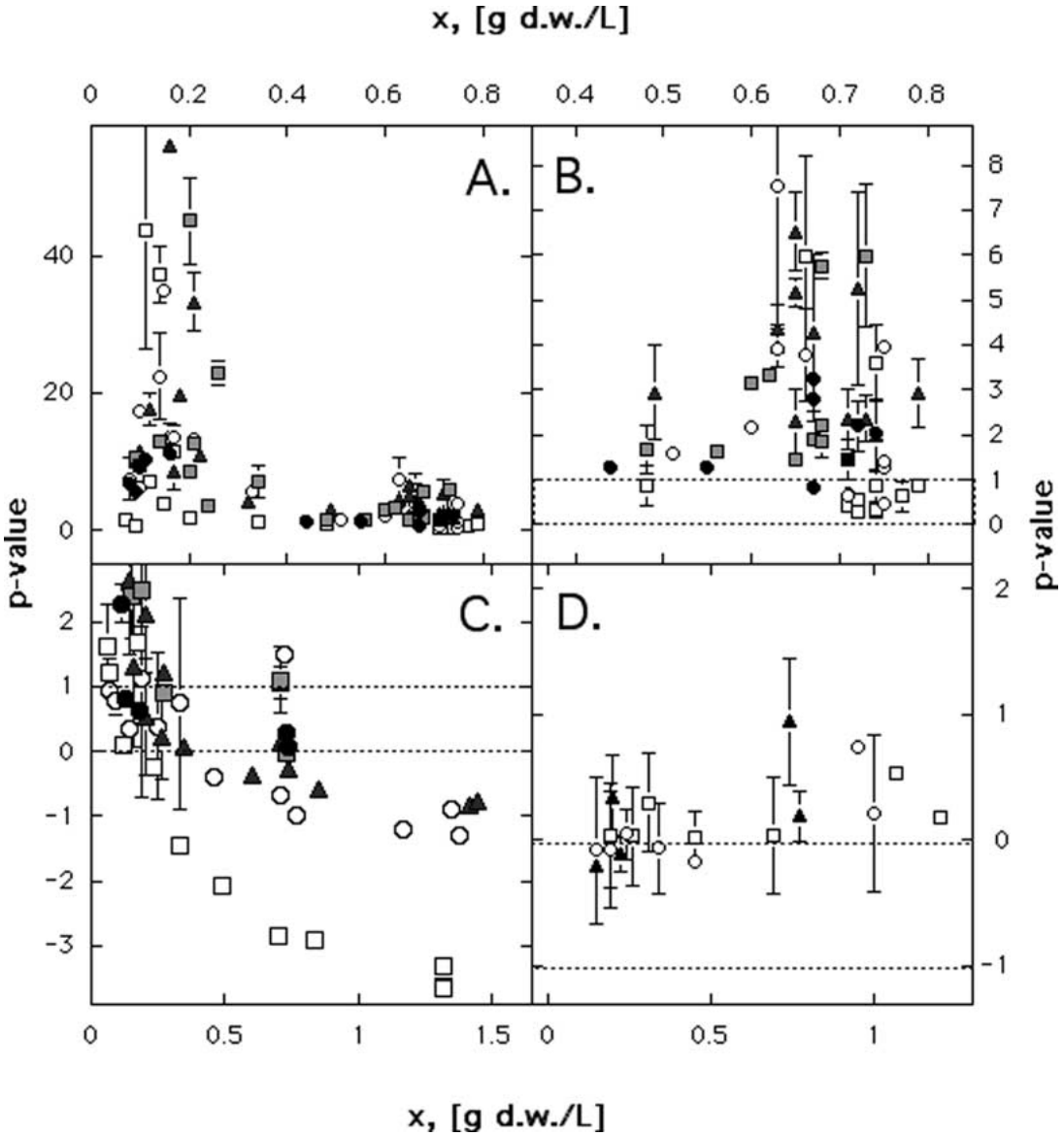

Figure 2. Comparison of the models and the reality. Strain AE128(pMOL30-9) was cultivated in TrisGlo in the presence of 5 mM (closed circles), 2 mM (closed squares), 1 mM (closed triangles), 0.5 mM (open circles) or 0.2 mM (open squares) of Zn^{2+} (panel A, an explosion of panel A is shown in panel B), Co^{2+} (panel C) or Cd^{2+} (panel D) In at least three independent growth curves, double determinations of the cellular metal content m_t was performed by the filtration assay. The cytoplasmic metal content $m_i = V_c \cdot c_i$ as calculated by the flow equilibrium model was substracted from m_t, and the resulting value was divided by the amount of metals m_b as calculated from the binding model to give the p-values, which were plotted against the cell density of the respective culture. The dashed box indicates the region between p = 0 and p = 1 in panels B, C and D. The standard deviation bars are the percentage of the deviation of the experimental m_t determination multiplied with the respective p-value.

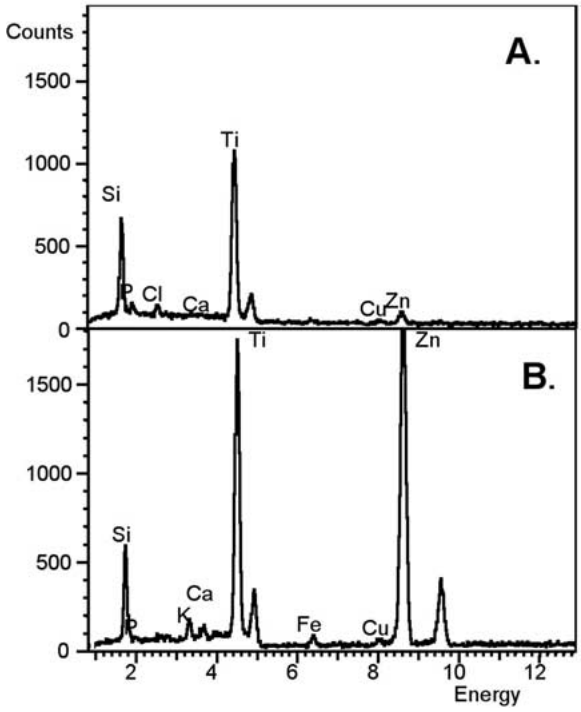

Figure 3. EDX analysis of the amount of zinc in *R. metallidurans* cells and in precipitates outside of the cells. *R. metallidurans* was cultivated in the presence of 1 mM Zn^{2+} in TrisGlo and exponentially growing cells were analyzed with the electron microscope. Using EDX analysis, the amount of zinc inside the cells (A) and in precipitates outside of the cells (B) was analyzed.

only (Table 4). This zinc-binding could be the result of an unexpectedly low activity of the CzcCBA efflux system, of binding to cytoplasmic components like polyphosphate as has been described for mercury and other (Pan-Hou et al. 2002) cations or of precipitations outside of the cells.

To investigate the influence of the phosphate transport systems (Aiking et al. 1984; Beard et al. 2000) and of polyphosphate (Kornberg 1995) on an intracellular precipitation of zinc, the genes for the polyphosphate kinase, the exopolyphosphatase and the phosphate inorganic transport system Pit were interrupted on the *R. metallidurans* chromosome by insertional mutagenesis. None of these mutations, however, had any effect on the zinc content (data not shown). Moreover, *R. metallidurans* cells were cultivated in the presence of 1 mM Zn^{2+} and the zinc content of these early exponential cultures was analyzed by EDX analysis (Figure 3). The zinc content within the cells was low (Figure 3A), but the zinc formed precipitates outside the cells (Figure 3B).

To find out why living cells associated more Zn^{2+} than inactivated cells under the same conditions, the influence of the phosphate concentration on this effect was studied. Parallel cultures were cultivated in the presence of different phosphate concentrations and of 1 mM Zn^{2+}, labeled as $^{65}Zn^{2+}$ or $^{32}PO_4^{3-}$, respectively (Figure 4). An increase or a decrease of the phosphate concentration (642 μM in TrisGlo) by a factor of three (1.93 mM, 214 μM) did not influence growth (Figure 4A) or the amount of cell-associated zinc (Figure 4C) or phosphate (Figure 4D) in the cells, although the available amount of phosphate in the 214 μM phosphate culture reached zero at the end of the experiment (Figure 4B). The molar ratios of zinc:phosphate in the growth medium was between 1:2 and 1:0.2, a tenfold difference that made no effect. The molar amounts of zinc and phosphate associated to living cells was always about 6:1 (Figure 4 C and D), which ruled out simple $ZnHPO_4$ or $Zn_3(PO_4)2$ precipitates.

Thus, living *R. metallidurans* cells formed zinc precipitates in the early exponential phase of growth. These complexes could be mixed phosphate, hydroxide or carbonate complexes (from sodium gluconate assimilation, degradation and respiration), and they dissolved during growth. Active complexation of heavy metals by *R. metallidurans* has been used for biotechnological purposes (Diels et al. 1995a, b; Nies 2000; Taghavi et al. 1997; van der Lelie 1998), so the occurrence of the precipitates fits into the picture. Nevertheless, these precipitates make it impossible to measure the cytoplasmic zinc content with $^{65}Zn^{2+}$, and other methods have to be applied to validate the model for zinc at these concentrations. Currently, however, suitable methods are not available (Maret 2002).

Cobalt

In the presence of Co^{2+}, early exponential cells contained also more cobalt than predicted by the binding model (Figure 2C), but the p-values were smaller than 3, twenty times smaller than the respective p-values for zinc. During growth, p reached negative values. Especially low values (close to –4) were reached at 0.2 mM Co^{2+}, values close to –1 at 0.5 mM and 1 mM Co^{2+}. In these cases, additional efflux activity seems to occur in the cells. Interestingly, cells growing in the presence of elevated cobalt concentrations between 0.2 mM and 1 mM reached much higher cell densities than all other cells. Probably, even the low activity level of CzcCBA for Co^{2+} led to Co^{2+} starvation that

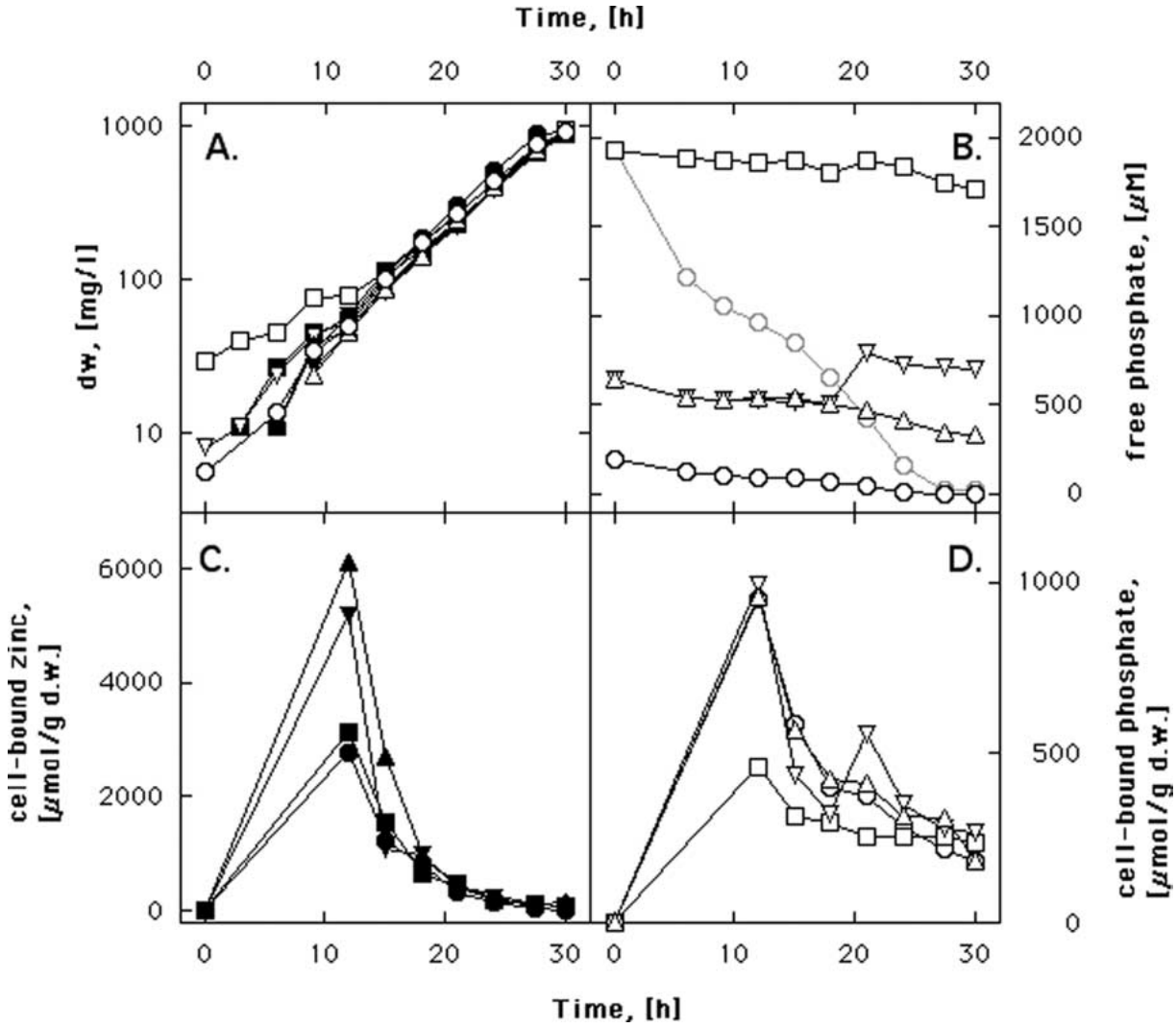

Figure 4. Enhanced zinc-binding by growing cells of *R. metallidurans* does not depend on the phosphate concentrations. *R. metallidurans* strain AE128(pMOL30-9) was cultivated in the presence of 1 mM Zn^{2+} and various concentrations of phosphate in Tris-buffered mineral salts medium containing 2 g/l of sodium gluconate. Phosphate concentrations were 624 μM as routinely used with this medium (triangles), the threefold amount (squares), one-third of the amount (circles) and 624 μM plus another 624 μM added after 20 h (inverse triangles) For each phosphate concentration, two parallel cultures were labeled either with $^{65}Zn^{2+}$ (closed symbols) of $^{32}PO_4^{3-}$ (open symbols) Panel A shows the growth curves of the eight cultures, panel B. the concentration of free phosphate in the medium. The gray open circles indicate the amount of free phosphate in the 1/3 phosphate culture, but tenfold exploded. Panels C and D give the amount of total cell-bound zinc and phosphate as determined with the filtration method.

was overcome by the increased cobalt concentrations in the growth medium. This would explain why Co^{2+} is such a bad inducer for Czc and Cnr.

Cobalt concentrations of 2 and 5 mM led to no increased growth yield and to p values around 0 in stationary phase cells (Figure 3C). Thus, with the exception of some binding to early exponential cells, the combination of both models explains cobalt homeostasis at 2 mM and 5 mM sufficiently. At 0.2 mM to 1 mM Co^{2+}, the activity of another efflux system, a higher in vivo activity of CzcCBA, unexpected elevated expression of CzcCBA or down-regulation of metal uptake (Chamnongpol & Groisman 2002) has to be included to explain the observed data.

To rule out at least one of these possibilities, expression of *czcCBA* was brought under control of the *tac* promoter. Plasmid pECD557 was based on the *colE1* replicon that makes it a suicide vec-

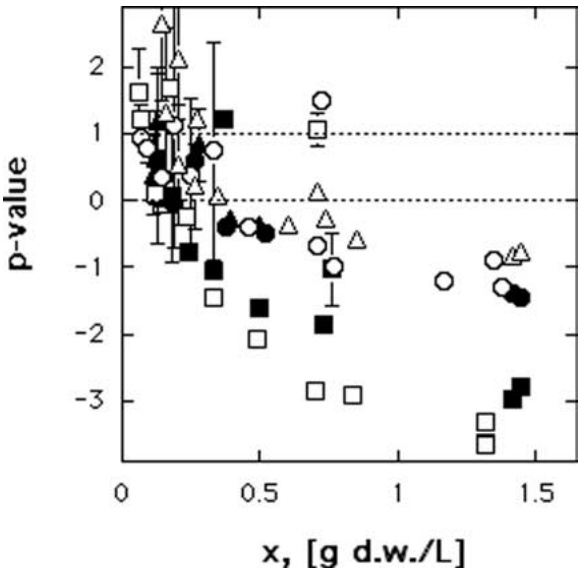

Figure 5. Comparison of the p-values of strains with different control of Czc expression in the presence of cobalt. As described in Figure 2, strain AE128(pMOL30-9) (open symbols) and strain AE128(pMOL30-20) (closed symbols) were cultivated in the presence of 0.2 mM (squares), 0.5 mM (circles) or 1 mM (triangles) of Co^{2+}, and the p-values were determined. Strain AE128(pMOL30-9) expressed Czc under native control, strain AE128(pMOL30-20) constitutively under control of the weak *tac* promoter.

tor in *R. metallidurans* (Nies et al. 1987). The plasmid contains the 5′ part of *czcC* under control of the *tac* promoter and with the $lacI^q$ gene upstream. After electroporation, transconjungants were selected by their kanamycine resistance and verified by PCR. Strain AE128(pMOL30-20) expressed the Φ(*czcC-lacZ-czcBA*) operon constitutively with a β-galactosidase activity of 50 U/mg. Although the *lacI* gene on plasmid pMOL30-20 was constitutively expressed, the expression level did not depend on the $Co^{2+}/Zn^{2+}/Cd^{2+}$ concentration nor on the IPTG level even at increased IPTG concentrations (data not shown).

To model the flow equilibrium for strain AE128 (pMOL30-20), equation 4 and a constant value of $v_{max-ef} = 29.65\ \mu mol\ min^{-1}$ g d.w. was used to calculate c_i. Although expression of czcCBA could not be up-regulated in strain AE128(pMOL30-20), the p data for this strain were not much different from the p data for strain AE128(pMOL30-9) (Figure 5). Thus, the negative p-values in the validation experiment for cobalt were not the result of an elevated expression of the *czcCBA* operon.

Cadmium

The cadmium content matched the predictions of the flow equilibrium model, but only in growing cells (Figure 2D). Early stationary cells contained more cadmium with p-values between 0 and 1. Thus, the flow equilibrium model describes successfully cadmium resistance in growing *R. metallidurans* cells at cadmium concentrations between 0.2 and 1 mM Cd^{2+}.

Conclusions

As the very first step to understand the heavy metal resistance of a bacterial cell on a quantitative level, a theoretical model was derived that describes the cytoplasmic metal ion content as a flow equilibrium between uptake and efflux processes, plus binding of metals to the cell. The model was derived for Czc-mediated cobalt/zinc/cadmium resistance at cation concentrations of 0.2 mM and above. It described successfully cadmium resistance of *R. metallidurans* cells growing exponentially in the presence of 0.2, 0.5 or 1 mM Cd^{2+}. Moreover, the model predicted mathematically a failure of cadmium detoxification at concentrations higher than 1.5 mM Cd^{2+}. This prediction fits to the MIC of Cd^{2+} at around 2 mM. The model could not be validated for zinc due to heavy zinc precipitates that formed exclusively but transiently in the vicinity of early exponential *R. metallidurans* cells. These complexes contained less than 20 mol percent of phosphate and could be mainly zinc carbonate/hydroxide complexes since sodium gluconate metabolism of growing cells is likely to form the two ligands OH^- and CO_3^{2-}. The model did also not describe cobalt resistance correctly, but indicated the presence of an unexpectedly high net cobalt efflux in *R. metallidurans* cells. In any case, this complex quantitative model was useful to uncover previously unknown processes that interact with high-level heavy metal resistance in *R. metallidurans*.

Acknowledgements

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http://www.jgi.doe.gov/tempweb/JGI_microbial/ html /index.html

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