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The RcnRA (YohLM) system of *Escherichia coli*: A connection between nickel, cobalt and iron homeostasis

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Abstract

The transporter RcnA has previously been implicated in Ni(II) and Co(II) detoxification in *E. coli* probably through efflux. Here we demonstrate that the divergently described *rcnA* and *rcnR* gene products constitute a link between nickel, cobalt and iron homeostasis. Deletion of the *rcnA* gene resulted in increased cellular nickel, cobalt and iron concentrations. Expression of *rcnA* was induced by Ni(II) or Co(II). Overproduction of *rcnR* inhibited induction of *rcnA* by metal cations but RcnR did not bind to the *rcnA* promoter *in vitro*. When *rcnR* or *fur*, the gene of the global repressor of iron homeostasis, was deleted, expression of *rcnA* was also induced by iron. The promoter region of *rcnA* was positive in a Fur titration (FURTA) *in vivo* assay indicative of Fur binding. Thus, *rcnA* is part of the Fur regulon of *E. coli*. The implications of a connection between the homoeostasis of closely related transition metals are discussed.

Introduction

Iron, cobalt and nickel are located proximately in the periodic system of the elements. All three metals are transition elements, which are highly suited to form complex compounds because their incompletely filled d-orbitals enable additional bonding possibilities with ligands. The number of d-electrons increases from Fe(II) to Ni(II) from 6 to 8, while the ionic radii of the divalent or trivalent ions in comparable complexes is quite similar (Housecroft & Constable 2006).

Despite this similarity, electrochemical transition between the divalent and trivalent cations in water is easy in case of iron ($E^\circ = +0.771$ V, $E^{\circ\prime} = 0.351$ V at pH = 7), difficult for cobalt ($E^\circ = +1.83$ V in 2 M H_2SO_4) and not possible for the free cation in case of nickel (Weast 1984). This assigns all three cations different roles in the cellular biochemistry, iron as one electron donor/acceptor in non-heme and heme-containing enzymes, cobalt as central cation in B_{12} catalyzing rearrangements of C–C and C–H bonds, and nickel as bond former and splitter in hydrogenase, urease, superoxide dismutase, and many enzymes in anaerobic bacteria (Nies 2004). This leaves the cell with the tremendous task to sort out three sterically very similar cations, each a possible competitive inhibitor of the other two. This metal homeostasis network must reliably function in environments, where the resources of each metal might vary considerably between toxic mM and limiting nM concentrations.

Ni(II) and Co(II) are taken up into *Escherichia coli* by transport systems with broad substrate specificity, such as the magnesium transporter CorA (Hmiel *et al.* 1989; Moncrief & Maguire 1999) or the ZupT protein that belongs to the ZIP protein family (Grass *et al.* 2005a). Additionally, the specific ABC uptake system NikACBED supplies Ni(II) at low environmental nickel concentrations especially for hydrogenase biosynthesis

(De Pina *et al.* 1999). As a counterpart of the unspecific Ni(II) and Co(II) uptake systems, the YohM metal cation transporter of *E. coli* has been designated RcnA and described as the first cobalt and nickel efflux system of this bacterium (Rodrigue *et al.* 2005). Expression of *rcnA* was specifically induced by Co(II) or Ni(II) (Rodrigue *et al.* 2005) and RcnA seems to be required for nickel and cobalt homeostasis. *E. coli* is able to maintain a cellular "quota" of about 180 μM iron, 5 μM nickel and 0.5 μM cobalt (Outten & O'Halloran 2001). This means that *E. coli* maintains a 36-fold higher iron than nickel concentration and a 360-fold higher iron than cobalt concentration. Therefore, we wondered how *E. coli* is able to induce *rcnA* expression specifically by nickel and cobalt despite the high background concentration of the chemically very similar iron cation.

The *rcnA* gene is flanked by two genes of unknown function, *yohL* upstream and on the other DNA strand, *yohN* downstream and in the same direction of transcription (Figure 1). The gene downstream of *yohN*, *yehA*, is part of a putative fimbrial protein cluster on the other DNA strand. The gene *yohL* is followed by the *thiMD* thiamin biosynthesis operon (http://www.ecocyg.org). Therefore, the two genes *yohL* and *yohN* that are adjacent to *yohM* might be additional genes involved in metal resistance in *E. coli*, but probably not *thiM* or *yehA*.

BLAST (Altschul *et al.* 1997) analysis of the predicted YohL protein revealed that YohL was conserved in many bacteria. A paralogue of YohL in *E. coli* is YaiN = FrmR (Herring & Blattner 2004), a probable repressor of the formaldehyde degradation operon *fmrRAB*. In the present study we elucidated that expression of *rcnA* was influenced by YohL, which we therefore denominated RcnR. In addition the global regulator of iron homeostasis, Fur, was also required to prevent induction of *rcnA* by iron, exemplifying how the control circuits of metal homeostasis systems overlap to gain specificity on regulation.

Materials and methods

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in Luria–Bertani (LB) medium or in Tris-buffered mineral salts medium (Mergeay *et al.* 1985) containing 2 ml glycerol and 3 g casamino acids per liter. Solid media contained 20 g agar/l. Antibiotics (20 μg/ml chloramphenicol, 25 μg/ml kanamycin, 125 μg/ml ampicillin) and metal chlorides were added where appropriate.

Dose–response growth experiments

Overnight cultures of *E. coli* strains were diluted 1:400 into fresh LB medium. After 2 h they were diluted 1:400 into fresh LB medium with increasing Co(II) or Ni(II) concentrations and cultivated for 16 h with shaking at 37 °C. Turbidity was measured at 600 nm using a SmartSpec3000 photometer (Bio-Rad, Munich, Germany).

Gene deletions and lacZ-reporter constructions

Genes were deleted by the insertion of Cam^r or Kan^r cassettes using the λ Red-recombinase system (Datsenko & Wanner 2000). For *lacZ*-fusions the resistance cassette vector pKD3 (Datsenko & Wanner 2000) was modified as follows: immediately

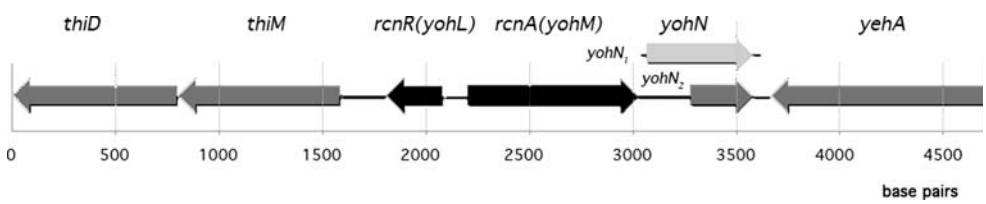

Figure 1. Structure of the *thiDM-rcnRA-yohN-yehA* gene region. The arrows in various shades of gray represent size and direction of transcription of the genes *thiDM*, *rcnR, rcnA*, *yohN* and *yehA*, which are located in the genome of *E. coli* K12 between nucleotide positions 2,181,738 and 2,186,432 (http://www.ecocyc.org). Proteins related to the putative YohN protein can be found in many other bacteria (data not shown), however, most amino acid identities start with methionine 61 of YohN. Therefore, *yohN* might start as annotated (*yohN*$_1$) or 243 bp downstream (*yohN*$_2$). The latter $YohN_2$ contains a typical leader peptide for translocation into the periplasm at its amino terminus, which on the other hand led to annotation of $YohN_1$ as a "membrane protein".

Table 1. E. coli strains and plasmids used in this study.

Strains	Plasmids	Relevant genotype	Source/Reference
W3110		Wild-type	Bachmann (1972)
BW25113	pKD46	*lacI*^{q}, *rrnB*$_{T14}$, ΔlacZ*WJ*16, *hsdR*514, Δ*araBA-D*$_{AH33}$, Δ*rhaBAD*$_{LD78}$	Datsenko & Wanner (2000)
ECA333		Δ*rcnR*::*cat*	This study
ECA334		Δ*rcnA*::*cat*	This study
ECA335		Δ*yohN*::*kan*	This study
ECA336		Δ(*rcnRA-yohN*)::*cat*	This study
ECA348		Δ*corA*, Δ*zupT*::*cat*	This study
ECA349		Δ*corA*, Δ*rcnA*, Δ*zupT*::*cat*	This study
ECA350		Δ*lacZYA*, Δ*rcnR*::(*lacZ-cat*)	This study
ECA351		Δ*lacZYA*, Δ*rcnA*::(*lacZ-cat*)	This study
ECA352		Δ*lacZYA*, Δ*yohN*(1)::(*lacZ-cat*)	This study
ECA354		Δ*lacZYA*, Δ*rcnA*::*lacZ* Δ*rcnR*::*cat*	This study
ECA355		Δ*fieF* Δ*fur*, Δ*lacZYA*::*kan*, Δ*rcnR*::(*lacZ-cat*)	This study
ECA356		Δ*fieF* Δ*fur*, Δ*lacZYA*::*kan*, Δ*rcnA*::(*lacZ-cat*)	This study
ECA357		Δ*fieF* Δ*fur*, Δ*lacZYA*::*kan*, Δ*yohN*(2)::(*lacZ-cat*)	This study
ECA373		Δ*lacZYA*, Δ*rcnR*::(*lacZ-cat*), Δ*nikR*::*kan*	This study
ECA374		Δ*lacZYA*, Δ*rcnA*::(*lacZ-cat*), Δ*nikR*::*kan*	This study
H1717		Δ*lacZYA*, Δ*aroB*, *fhuF-lacZ*	Hantke (1987)
	pCP20	Heat inducible FRT-dependent flipase gene	Datsenko & Wanner (2000)
	pGEM®-T Easy	Cloning vector	Promega, Mannheim, Germany
	pECD955	pGEM::*rcnA*	This study
	pECD956	pGEM::(*rcnRA-yohN*)	This study
	pASK-IBA3	Expression vector (C-terminal Strep-tag)	This study
	pASK-IBA7	Expression vector (N-terminal Strep-tag)	This study
	pECD964	pASK-IBA3::*rcnR*	This study
	pECD966	pASK7::*rcnR*	This study
	pGP1-2	Heat inducible T7-RNA-polymerase-biosynthesis	Tabor & Richardson (1985)

downstream of the distal *FRT*-site a promoter-less *lacZ* gene was introduced by PCR. Thus, primers used for deletion of genes can be used in parallel for construction of Δ"target gene"-*lacZ* fusions. In this pKD3-derivative PCR amplifies not only the *FRT*-sites-flanked chloramphenicol resistance gene (*cat*) as in the parental pKD3 plasmid but also *lacZ*. Subsequent λ Red-mediated recombination results in replacement of the target gene by a "*lacZ-FRT-cat-FRT*"-cassette. Initial deletions in *E. coli* strain BW25113 comprising a chloramphenicol (*cat*), or kanamycin (*kan*) resistance cassette or *lacZ-cat* reporters were transferred by general transduction with phage P1 into *E. coli* strain W3110. Multiple deletions were constructed by FRT-dependent elimination of the respective resistance cassette assisted by Flipase from plasmid pCP20 (Datsenko & Wanner 2000) and subsequent general phage P1 transduction. In "*lacZ-FRT-cat-FRT*"-constructs, removal of the resistance cassette in final constructs is also possible, while retaining the *lacZ*-reporter.

Plasmid construction

Individual *rcnA*, *rcnR* or *yohN* genes or the *rcnRA-yohN* region were amplified by PCR and cloned into the pGEM®-T Easy (Promega, Mannheim, Germany) or pASK-IBA (IBA GmbH, Göttingen, Germany) vectors as were promoter-regions of *rcnA* or *fhuF*, respectively.

Metal uptake

Uptake experiments were performed by the filtration-method (Munkelt *et al.* 2004) with minor changes. Cells were grown overnight in LB medium, diluted 1:400 into Tris mineral salts medium, grown overnight and diluted to 30 Klett units into

fresh medium. After 1 h 20 μM $CoCl_2$ or 500 μM $NiCl_2$ were added to induce *rcnA*. After growth for an additional hour, cells were washed twice with 10 ml wash-buffer I (10 mM Tris–HCl, pH 7.0), and metal uptake was started by addition of a mixture of $CoCl_2$ labeled with $^{57}CoCl_2$ (final cobalt concentration, 5 μM) or $NiCl_2$ labeled with $^{63}NiCl_2$ (final nickel concentration, 5 μM). Cells were incubated at 30 °C with shaking, and 0.4 ml aliquots were filtered through nitrocellulose membranes (0.45 μm) at various times and immediately washed with 5 ml wash-buffer II (10 mM Tris–HCl, pH 7.0, and 10 mM $MgCl_2$). The membranes were dried, and radioactivity was measured using a liquid scintillation counter (LS6500; Beckman, München, Germany). $^{63}NiCl_2$ (370 GBq/g) and $^{57}CoCl_2$ (148 TBq/g) were from Perkin–Elmer (Boston, MA).

β-galactosidase assay

E. coli cells containing a gene *lacZ*-fusion on the bacterial chromosome were grown overnight in LB medium, diluted 1:400 into Tris mineral salts medium, grown overnight and diluted 1:100 into fresh medium. After 2 h of growth, increasing concentrations of metals were added. Incubation was continued with shaking for 2 h at 37 °C, and the β-galactosidase activity was determined (Miller 1972).

Fur titration assay (FURTA)

FURTA was performed as described (Stojiljkovic *et al.* 1994). High-copy-number pGEM®-T Easy vector derivatives containing the promoter-regions of *rcnA* or *fhuF* were transformed into *E. coli* strain H1717 (Hantke 1987), which has a chromosomal *fhuFp-lacZ* fusion. *E. coli* H1717 transformants were grown in LB medium and streaked on MacConkey lactose agar or MacConkey lactose agar supplemented with 200 μM 2,2′-dipyridyl (DIP) or 30 μM $Fe(NH_4)_2(SO_4)_2$. After incubation for 18 h at 37 °C, the Lac phenotype was recorded. When β-galactosidase was synthesized from the *fhuFp-lacZ* fusion, cells could utilize lactose provided by the MacConkey agar and the resulting acid stained the agar red. When the *fhuFp-lacZ* fusion was not induced the sugar could not be used and the cells remained colorless. Strain H1717 containing plasmid pGEM®-T Easy without promoter-region was used as a negative control and the cloned *fhuF* promotor region was used as a positive control.

Atomic absorption spectroscopy (AAS)

Cells were grown overnight with shaking in LB medium, diluted 1:400 into Tris mineral salts medium without or with 200 μM $NiCl_2$, $FeSO_4$ or 25 μM $CoCl_2$. After 16 h of growth at 37 °C the turbidity was measured at 600 nm and 0.2 ml aliquots were washed 3-fold with 0.5 ml ice-cold wash-buffer (10 mM Tris–HCl, pH 7.0, 100 μM EDTA). The pelleted cells were broken in 0.1 ml 10 M HNO_3 and mineralized by the addition of 0.05 ml 10 M H_2O_2. The volume was filled up with H_2O to 0.2 ml, and the metal-content measured by AAS (ZEEnit® 600/650, Analytik Jena AG). Values from control experiments determined from blanks without cells were subtracted.

Genetic techniques

Standard molecular genetic techniques were used (Sambrook *et al.* 1989). PCR was performed with *Taq* or Taq/*Pwo* DNA polymerase (Roche, Mannheim, Germany). All primer sequences can be obtained upon request.

Results

RcnR and YohN did not influence RcnA-mediated nickel- or cobalt resistance

The arrangement of the *rcnRA-yohN* genes on the *E. coli* chromosome (Figure 1) suggested that these genes were organized as a divergon. To investigate a function in metal resistance *rcnA, rcnR, yohN*, or *rcnRA-yohN* were deleted from the *E. coli* genome and metal resistance of the mutant strains was analyzed on solid medium and in liquid culture (Table 2, Figure 2). Deletion of *rcnA* or *rcnRA-yohN* decreased nickel and cobalt resistance of *E. coli* strain W3110 similarly as published (Rodrigue *et al.* 2005). The phenotype could be complemented *in trans* (Table 2). As a control the impact of other divalent metal cations on growth of the Δ*rcnA* deletion mutants were also investigated. Zn(II) or Cd(II) resistance was not affected in this strain. Also, deletion of *rcnR* or of *yohN*

Table 2. Metal cation resistance of *E. coli* strains carrying deletions in the *rcnRA-yohN* gene region.

Strains, relevant genotype	Minimal inhibitory concentration (mM)[a]			
	Co(II)	Ni(II)	Zn(II)	Cd(II)
Wild-type strain W3110	2.2	2.2	1.4	1.2
Δ*rcnA*	1.6	1.9	1.4	1.2
Δ(*rcnRA-yohN*)	1.6	1.9	n.d.	n.d.
Δ*rcnR*	2.2	2.2	n.d.	n.d.
Δ*yohN*	2.2	2.1	n.d.	n.d.
W3110(pGP1-2, pGEM)	2.2	2.2	n.d.	n.d.
Δ*rcnA*(pGP1-2, pGEM)	1.6	1.9	n.d.	n.d.
Δ*rcnA*(pGP1-2, pGEM::*rcnA*)	2.3	2.6	n.d.	n.d.
Δ*rcnA* (pGP1-2, pGEM::(*rcnRA-yohN*)	3.2	3.9	n.d.	n.d.

n.d., not done.[a]Strains were grown for 16 h in LB, diluted 1:100 and streaked on LB agar plates with increasing metal concentrations. Growth was monitored for 2 days at 37 °C. The results were confirmed by at least three independent replicates.

had no consequence on cobalt or nickel resistance, neither on solid medium (Table 2) nor in liquid culture (Figure 2). This indicated that RcnR or YohN were not required for RcnA-mediated metal resistance.

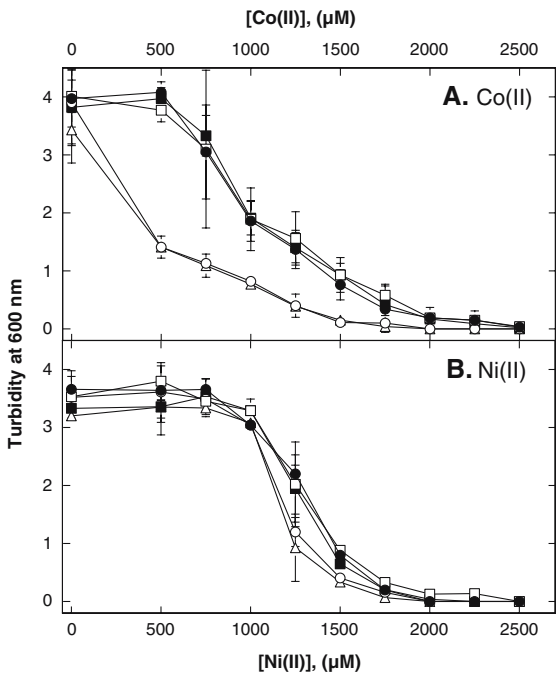

Figure 2. Metal resistance of *E. coli* strains containing deletions in genes in the *rcnRA-yohN* region. Dose–response curves show the turbidity at 600 nm of parallel *E. coli* cultures with increasing cobalt (Panel A) or nickel (Panel B) concentrations after growth for 16 h at 37 °C in LB medium. *E. coli* strain W3110 (closed circles, ●), derivatives Δ*rcnR* (open squares, □), Δ*rcnA* (open circles, ○), Δ*yohN* (closed squares, ■), or Δ(*rcnRA-yohN*) (open triangles, Δ). Triple determinations with standard deviations are shown.

Deletion of *rcnA* led to increased accumulation of Ni(II) by *E. coli* (Rodrigue *et al.* 2005). This could be validated (Figure 3). Involvement of RcnA in cobalt detoxification has previously been shown only indirectly (Rodrigue *et al.* 2005). Measurements of ^{57}Co(II) uptake by *E. coli* W3110 and its *rcnA* deletion mutant strain ECA334 were not successful (data not shown), probably caused by interference of other Co(II)-transporters. Therefore, a double deletion strain was generated that lacked the two possible Co(II) uptake systems CorA (Hmiel *et al.* 1989) and ZupT (Grass *et al.* 2005a). In this Δ*corA* Δ*zupT* mutant strain, additional deletion of *rcnA* led to increased accumulation of ^{57}Co(II), but only after 10 min of incubation (Figure 3).

An ΔrcnA mutant exhibited increased cellular steady-state nickel, cobalt and also iron concentrations

Wild-type and Δ*rcnA* mutant cells cultivated in Tris-buffered mineral salts medium contained similar amounts of Ni(II), Co(II), Fe(II) and Cd(II) (Table 3). This medium contained 80 nM Ni(II), 84 nM Co(II), 4.3 μM Fe(II) and no Cd(II) (Mergeay *et al.* 1985). When additional 200 μM Ni(II) were added to this medium, wild-type cells doubled their nickel content while the Ni(II) content in Δ*rcnA* cells increased 4-fold (Table 3). Thus, RcnA did not change the steady state Ni(II) concentration in cells cultivated at

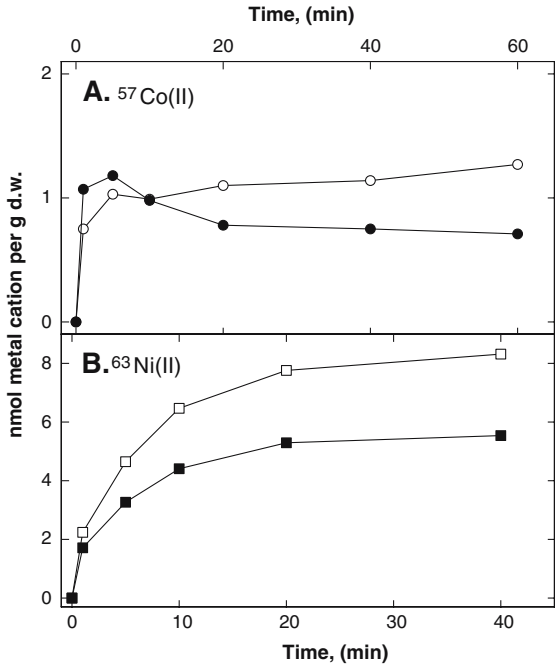

Figure 3. Metal cation uptake by *E. coli* cells containing deletions in the *rcnA* gene. Uptake of 5 μM or ^{57}Co(II) (Panel A) or of ^{63}Ni(II) (Panel B) by *E. coli* cells was determined by the filtration method. Strains used were (A) ECA348 (Δ*corA* Δ*zupT*, closed circles, ●) and its Δ*rcnA* mutant derivative ECA349 (open circles, ○) for cobalt uptake or (B) strain W3110 wild-type (closed squares, ■) and ECA334 (Δ*rcnA*) (open squares, □) for nickel uptake. Mean values of triple determinations shown.

low nickel concentrations, but lack of RcnA clearly increased the cellular nickel content at high but non-toxic nickel concentrations.

Table 3. Metal content of an *E. coli* Δ*rcnA* mutant[a].

Metal	Relevant genotype	Metal content (μg/g dry weight)	
		No metal added	Metal added
Ni(II)	Wild-type	95 ± 23	213 ± 46
	Δ*rcnA*	102 ± 25	420 ± 44
Co(II)	Wild-type	6.6 ± 0.5	44.6 ± 5.8
	Δ*rcnA*	7.1 ± 0.6	57.1 ± 5.3
Fe(II)	Wild-type	752 ± 369	3350 ± 425
	Δ*rcnA*	956 ± 545	4560 ± 846
Cd(II)	Wild-type	0.7 ± 0.1	6890 ± 1,446
	Δ*rcnA*	2.0 ± 0.2	4850 ± 757

[a]Cells were incubated in Tris-buffered mineral salts medium at 37 °C with shaking for 16 h in the presence of 200 μM of $NiCl_2$, $FeSO_4$ or $CdCl_2$. $CoCl_2$ was supplied only at 25 μM to prevent growth defects of the mutant strain. Shown are averages with standard deviations of three independent AAS measurements.

Similarly, the cobalt content of the Δ*rcnA* mutant strain was increased when 25 μM Co(II) was added to the growth medium (Table 3). Higher Co(II) concentrations could not be applied because growth of the Δ*rcnA* mutant strain was negatively affected under such conditions. Surprisingly, also the iron content of the Δ*rcnA* mutant strain increased slightly in response to iron-replete growth conditions than in the wild-type strain. In contrast the Δ*rcnA* mutant cells did not accumulate more cadmium than the wild-type cells (Table 3).

The rcnA and rcnR genes were induced by Ni(II) or Co(II)

Induction of individual genes in the *rcnRA-yohN* gene region was studied by *lacZ* reporter gene fusions. Therefore, each gene was replaced by a promoterless *lacZ* gene in the Δ*lacZYA E. coli* W3110 derivative strain ECA150. Expression of *rcnA* was induced by Ni(II) and an increase in β-galactosidase activity of the Δ*rcnA*::*lacZ* fusion was observed in response to increasing Ni(II) concentrations (Figure 4A). When Co(II) was used for induction a different expression pattern was observed. From 0 to 10 μM Co(II), expression increased sharply, but leveled off thereafter (Figure 4B).

In contrast, expression of Δ*rcnR*::*lacZ* was only slightly induced by Co(II) but not by Ni(II). The maximum induction factor was about 1.6 (Figure 4). Expression of neither Δ*rcnA::lacZ* nor of Δ*rcnR*::*lacZ* changed when chelator EDTA (up to 100 μM) was added to sequester divalent cations from the growth medium (data not shown). Thus, these *rcn* genes were induced by nickel and cobalt but were unresponsive to metal-deprivation. In contrast, expression of a Δ*yohN*::*lacZ* fusion was neither altered by Ni(II) or Co(II) (Figure 4) nor by EDTA (data not shown). Together with the results of the metal-resistance studies (Table 2, Figure 2) this suggested that RcnA and maybe RcnR, but not YohN, are involved in Ni(II) or Co(II) homeostasis of *E. coli*.

RcnR influenced induction of rcnA by Ni(II) and Co(II)

The divergent transcription of *rcnR* and *rcnA* suggested that RcnR could be regulator of *rcnA*

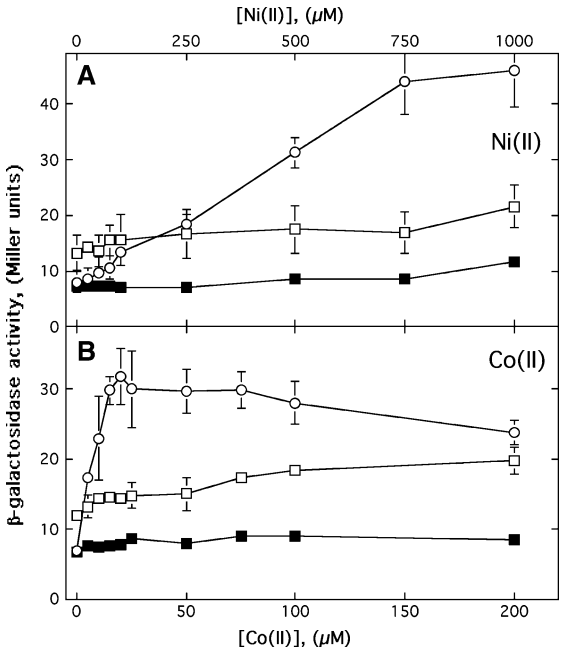

Figure 4. Induction of the *rcnR, rcnA or yohN* genes by Ni(II) or Co(II). Exponentially growing cells of three *E. coli* reporter strains carrying Δ*rcnR*::*lacZ* (open squares, □), Δ*rcnA*::*lacZ* (open circles, ○), or Δ*yohN*::*lacZ* (closed squares, ■) fusions were incubated in the presence of increasing $NiCl_2$ (Panel A) or $CoCl_2$ (Panel B) concentrations in Tris-buffered mineral salts medium for 2 h with shaking at 37 °C and the *β*-galactosidase activity was determined. Averages of triplicate determinations with standard deviations are shown (except for Δ*yohN*::*lacZ* fusions, here $n = 1$).

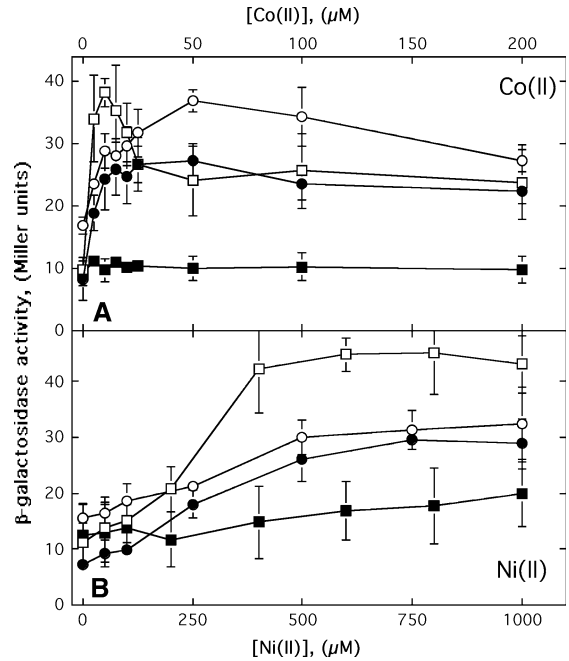

Figure 5. Induction of a Δ*rcnA*::*lacZ* reporter gene fusion by Ni(II) or Co(II) in the presence or absence of RcnR. *E. coli* reporter strains carried a Δ*rcnA*::*lacZ* reporter gene fusion alone (closed circles, ●) or were additionally deleted in Δ*rcnR* (open circles, ○). Alternatively, *rcnR* was expressed from a high copy number vector *in trans* (closed squares, ■) and an empty vector served as control (open squares, □). Exponentially growing cells were incubated in the presence of increasing $CoCl_2$ (Panel A) or $NiCl_2$ (Panel B) concentrations in Tris-buffered mineral salts medium for 2 h with shaking at 37 °C and the *β*-galactosidase activity was determined. Averages of triplicate determinations with standard deviations are shown.

expression. To explore this possibility *rcnR* was deleted without altering the intergenic region between *rcnR* and *rcnA*, and the induction of *rcnA* by metal cations was reinvestigated. Basal expression of Δ*rcnA*::*lacZ* in a Δ*rcnR* mutant strain was higher than in the wild-type strain but the reporter fusion was still induced by Co(II) (Figure 5A) and by Ni(II) (Figure 5B). Conversely, expression of *rcnR* from high copy number vector pASK-IBA7 (N-terminal Strep-tag fusion, Figure 5) but not from pASK-IBA3 (C-terminal fusion, data not shown) led to down-regulation of metal-dependent induction of *rcnA*::*lacZ* by either metal. This suggested that RcnR was involved in expression control of *rcnA* and that RcnR probably serves as a negative regulator for *rcnA* expression. However, RcnR alone was not sufficient for this regulatory process because *rcnA* was still induced by metal cations in the absence of RcnR.

To investigate if RcnR exerts repression of *rcnA*-expression through direct binding to the promoter-region upstream of *rcnA*, the RcnR protein was purified as N-terminal as well as C-terminal strep-tag fusion protein. However, in electrophoretic mobility shift assays this protein did not bind to the putative 200 bp promoter sequence upstream of *rcnA*, neither in the presence nor in the absence of 100 μM Co(II) or Ni(II) (data not shown). Because this is negative evidence, it cannot be ruled out that RcnR might bind to the *rcn*-promoter region as a repressor of *rcnA* transcription under different experimental conditions. Thus, the mechanism by which RcnR represses RcnA expression remains elusive.

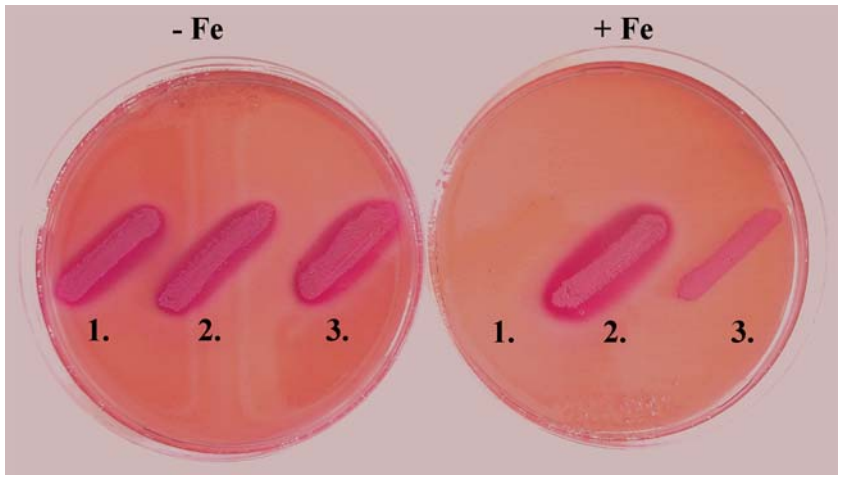

Figure 6. Fur titration assay (FURTA) of the *rcnA* promoter. Overnight cultures of *E. coli* strain H1717 carrying plasmids pGEM® T-Easy (1), pGEM::*fhuFp* (2) or pGEM::*rcnAp* (3) grown in Luria–Bertani broth were streaked on MacConkey agar plates without (panel A) or 30 μM $Fe(NH_4)_2(SO_4)_2$ (B). When provided on a high-copy plasmid, a DNA-region comprising a Fur-binding box titrated free Fur from the cytoplasm. This caused induction of a chromosomal *fhuFp-lacZ* fusion in *E. coli* strain H1717. The β-galactosidase-activity turned colonies and surrounding medium red. Without a high-copy Fur-binding box *in trans* cells remained colorless due to lack of β-galactosidase-activity. Plates were incubated at 37 °C for 18 h.

Fur interacted with the rcnAp promoter in vivo

The intergenic region between *rcnR* and *rcnA* contained a putative binding-box for the global iron uptake regulator Fur. This 19 nt sequence "aAT TAAT AC TAA GA ATTAT T a" was conserved in 13 positions (shown in boxes) to the *E. coli* Fur consensus sequence "tGA TAAT GA TAA TC ATTAT C a" (Baichoo & Helmann 2002). To investigate if this sequence was recognized by Fur *in vivo*, a Fur titration assay (FURTA) was employed to screen for interaction of the iron uptake regulatory protein with the promoter *rcnAp*. *E. coli* strain H1717 (Stojiljkovic *et al.* 1994) allowed detection of such an interaction by measuring induction of a chromosomal *fhuFp-lacZ* operon fusion. In this bacterium, the Fur-repressed *fhuF*-promoter was fused to a promoterless *lacZ* gene. In the presence of another promoter, which was cloned in a high copy number vector, Fur repressor protein was titrated away from the chromosomal *fhuFp-lacZ* fusion, leading to increased β-galactosidase activity.

On solidified MacConkey medium, expression of high levels of β-galactosidase resulted in red colonies, indicative of a plasmid carrying a Fur-binding promoter (Figure 6). In contrast, without a plasmid carrying a Fur-binding promoter, low β-galactosidase expression led to in white colonies. This indicated that Fur bound to the *rcnR-rcnA* intergenic region (Figure 1) *in vivo*. When the chromosomal *rcnR* gene was deleted from strain H1717 and the FURTA assay was repeated, this result did not change (data not shown), the plasmid-encoded *rcnA* promoter region still titrated Fur from the chromosomal *fhuF-lacZ*-reporter. This indicated that Fur exerted its interaction with the *rcnA* promoter region without involvement of RcnR.

The rcnR gene was induced by iron, but rcnA and yohN were not

Titration of Fur by the *rcnA* promoter region suggested a regulatory role of Fur in *rcn*-expression. Therefore, induction of expression by iron-depletion (EDTA) or iron-repletion of the three genes in the *rcnRA-yohN* region was examined (Figure 7A). While neither Δ*yohN*::*lacZ* nor Δ*rcnA*::*lacZ* showed any change in expression in response to changing iron concentrations, Δ*rcnR*::*lacZ* expression was induced by iron (Figure 7A, and data not shown for EDTA). Expression of the *rcnR* gene was up-regulated about 2.3-fold by 50 μM Fe(II). Iron is thus the best inducer of *rcnR* because induction was only about 1.6-fold either by Ni(II) or by Co(II) (Figure 4). This positive iron-regulation was somewhat surprising, because Fur exerts its mode of regulation by repression.

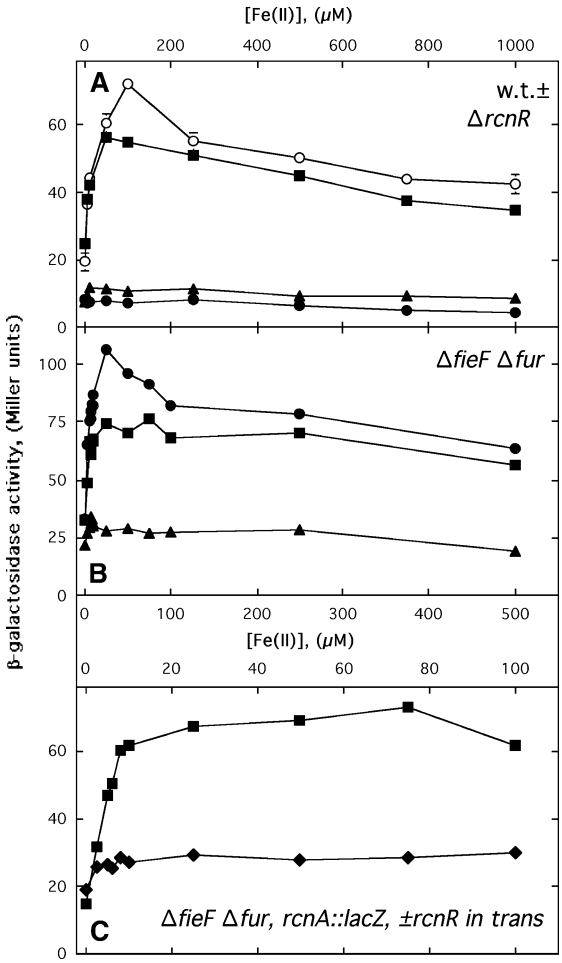

Figure 7. Induction of the *rcnRA-yohN* genes by iron. Exponentially growing cells of *E. coli* reporter strains were incubated in the presence of increasing Fe(II) concentrations in Tris-buffered mineral salts medium for 2 h with shaking at 37 °C and the *β*-galactosidase activity was determined. Host background was either (Panel A) *E. coli* strain W3110 {with (closed symbols) or without the *rcnR* gene (open circles, ○)} or (Panel B, C) a Δ*fieF* Δ*fur* deletion derivative of strain W3110. Reporter gene fusions were in Panels A and B Δ*rcnR*::*lacZ* (closed squares, ■), Δ*yohN*::*lacZ* (closed triangles, ▲), or Δ*rcnA*::*lacZ* {this fusion either in the wild-type (open circles, ●) or Δ*rcnR* mutant background (open circles, ○)}. In Panel C, *rcnR* was expressed from a plasmid in a Δ*rcnA::lacZ* Δ*fieF* Δ*fur* background (closed diamonds, ♦) or vector pASK-IBA7 was used as a control (closed square, ■). Triple determinations are shown.

The rcnA gene was induced by iron in the absence of RcnR

Gene expression of *rcnR* responded to iron and RcnR may function as a negative regulator. Nonetheless, expression of a Δ*rcnA*::*lacZ* fusion was not induced by Fe(II) (Figure 7A). Likely, high iron conditions repressed *rcnA* expression through an increase of RcnR protein levels. To investigate this, Δ*rcnA*::*lacZ* expression was examined in a Δ*rcnR* mutant strain. In this strain expression of Δ*rcnA*::*lacZ* was up-regulated by iron, up to 3.7-fold at 100 μM Fe(II) (Figure 7A). This indicated that RcnR was involved in negative regulation of *rcnA* by iron.

In the absence of Fur rcnA and rcnR were both induced by iron

To investigate the complex regulation of the genes of the *rcnRA-yohN* region by Fe(II) and Fur, induction was re-examined under conditions when iron-uptake was derepressed and iron-efflux was diminished. These conditions were provided by using an *E. coli* strain deleted in the genes for the iron efflux system FieF (Grass *et al*. 2005b) and for Fur. Expression of Δ*yohN*::*lacZ* was still not up-regulated by Fe(II) giving further evidence that *yohN* was not part of the *rcnRA* divergon. In contrast, expression of Δ*rcnR*::*lacZ* still was inducible by iron (Figure 7B). Thus, induction of *rcnR* expression by iron did not depend on Fur.

Under these conditions, however, expression of Δ*rcnA*::*lacZ* also became up-regulated by iron (Figure 7B). This suggested that not only RcnR but also Fur was involved in iron-dependent negative regulation of *rcnA* and that the presence of both RcnR and Fur led to repression of Δ*rcnA*::*lacZ* in the presence of iron. Consequently, expression of *rcnR in trans* strongly decreased iron-dependent up-regulation of the Δ*rcnA::lacZ* expression (Figure 7C).

Deletion of *fur* and *fieF* did not influence induction of Δ*rcnR*::*lacZ* or Δ*rcnA*::*lacZ* by Ni(II) (data not shown). Also, the induction pattern of either gene by Co(II) was changed little and only at high cobalt concentrations (data not shown).

Discussion

In this study a novel connection between nickel/cobalt and iron homeostasis of *E. coli* was elucidated. Such a link can be deduced and probably results from the chemical similarities of these three transition metals.

Iron is essential for *E. coli* and is present in the cell with a quota (total cellular concentrations;

division of the moles per cell by a maximum volume for a cell) of 180 μM (Outten & O'Halloran 2001). The cellular nickel quota is 5 μM and thus 36-fold smaller. Ni(II) is required for glyoxalase I but not essential for the methylglyoxal shunt to D-lactate because an alternative route via glyoxalase III does not require a nickel-containing enzyme (http://www.ecocyc.org). However, nickel is an essential part of the active center of the large subunits of all four hydrogenases of *E. coli*.

Interestingly, Co(II) seems not to be essential for *E. coli*. There are only few catabolic reaction chains that require B_{12}/cobalamin and do not function without this cobalt-containing cofactor. In addition there are 20-odd enzymes listed in the EcoCyc data base, which were activated by or contain Co(II) as a cofactor, but probably none uses Co(II) as the sole choice of a divalent transition metal cation. Thus, *E. coli* is probably not dependent on Co(II). This fits to the low cobalt "quota" of 0.5 μM (Outten & O'Halloran 2001). Such a low cellular cobalt content could solve a problem that originates from a special feature of Co(II): it forms octahedral complexes similar to Fe(II). This makes Co(II) a dangerous putative inhibitor of Fe(II) and Ni(II)-dependent reactions, explaining why *E. coli* avoids elevated concentrations of this potentially detrimental metal.

In many other bacteria specific Ni(II)/Co(II) detoxification systems are known (Nies 2003). Conversely, so far no *bona fide* Ni(II)/Co(II) efflux system is known for *E. coli* but data presented here and elsewhere (Rodrigue *et al.* 2005) strongly suggest that the function of RcnA is to rid the cells of surplus nickel and cobalt ions. In contrast to the study by Rodrigue *et al.* (2005), however, in our hands overproduction of RcnA in a Δ*rcnA* strain only conferred a mild phenotype, not a 100-fold nickel resistance increase. While these differences might be attributed to different experimental settings, the induction pattern of *rcnA* by Ni(II) or Co(II) (Figure 5) is intriguing: Co(II) led to increased expression of *rcnA* at low (< 50 μM) concentration but no further increase at higher concentration was observed, whereas increasing Ni(II) concentrations resulted in increasing gene expression. This might suggest that different regulatory mechanisms are at play for these metals.

Furthermore, only a rather small decrease in Co(II) resistance of the Δ*rcnA* mutant in comparison to the wild-type was observed [Table 2 and Rodrigue *et al.* (2005)]. If RcnA was an intrinsic Co(II) efflux transporter for detoxification, high gene expression at elevated metal concentrations would be expected. This way the cells can ward off toxic high cytoplasmic Co(II) concentrations as is probably the case for Ni(II). Possibly, this Δ*rcnA::lacZ* induction may be caused by displacement of Ni(II) by Co(II) from ligands within the cytoplasm, resulting in elevated cytoplasmic "free" Ni(II) that in turn would induce the reporter. In this scenario Co(II) induction is indeed indirect Ni(II) induction. However, cells deleted in *rcnA* were clearly more sensitive to Co(II) than to Ni(II) (Table 2, Figure 2). A reason for this discrepancy might be the higher toxicity of Co(II). Perhaps the complicated metal-dependent regulation of *rcnR* and *rcnA* can shed some further light on this puzzling situation.

Data presented in this study suggested that the proteins of the *rcnRA* divergon are a link between iron, cobalt and nickel homeostasis of *E. coli* in which RcnR is a negative regulator and RcnA is most likely an efflux system for Co(II) and Ni(II) (Rodrigue *et al.* 2005) (Figure 3) but only to a small extend, if at all, an efflux system for Fe(II) (Table 3). At least two factors modulated *rcnA* expression, the iron uptake regulator Fur and RcnR, the latter encoded upstream of *rcnA* on the other DNA strand (Figure 1). Overproduction of RcnR decreased induction of *rcnA* by Co(II) and Ni(II) (Figure 5) and also by Fe(II) in a Δ*fur* background (Figure 7C). This expression of *rcnR* from a high-copy number vector may have led to a constitutively repressed *rcnA* gene, making it probable that apo-RcnR (rather than a hypothetical metallo-RcnR) is the active regulator configuration.

This fits to results obtained with a paralogue of RcnR in *E. coli*, YaiN (now termed FmrR). The latter protein controls induction of an operon involved in glutathione-dependent degradation of formaldehyde and expression of wild-type *frmR* results in repression of this operon (Herring & Blattner 2004). RcnR and FrmR share 40% sequence identity and both may be repressors that uncover their specific promoters in the presence of different inducers, metal cations or formaldehyde, respectively. However, so far for neither protein substrate binding or promoter-binding was experimentally proven. Interestingly, both RcnR and FrmR appear to be sensitive to C-terminal

extensions because proteins modified in this way were rendered inactive *in vivo* (Herring & Blattner 2004), (data not shown for RcnR).

Unexpectedly, C-terminally extended RcnR was only inactive for nickel or cobalt dependent induction of *rcnA* but overproduction of RcnR (either with N- or C-terminal strep-tag extensions) prevented iron-dependent regulation of *rcnA* in a Δ*fur* background (Figure 7C and data not shown). Conversely, the addition of Fe(II) did not alter *rcnA* expression in *fur* wild-type when RcnR was over-produced (data not shown). This precluded a physical interaction with Fur as prerequisite for RcnR-mediated regulation.

Putative RcnR-like regulators are also broadly distributed amongst bacterial metal-resistant determinants. For instance, in the β-proteobacterium *Cupriavidus metallidurans* 31A an orthologue, NreA, is a putative regulator of the gene for the Ni(II) efflux pump, NreB (Grass *et al.* 2001). Orthologs of RcnR are also part of other nickel/cobalt resistance determinants in enterobacteria such as *Klebsiella oxytoca* (Stoppel *et al.* 1995), *Hafnia alvei* (Park *et al.* 2003, 2004), or *Serratia marcescens* (Marrero *et al.* 2006). All of these *rcnR* orthologs were located upstream of *rcnA* orthologs, but in contrast to *E. coli* in the same direction of transcription. In the δ-proteobacterium *Bdellovibrio bacteriovorus* strain HD101 a gene for a putative iron-transporter of the ferroportin (solute carrier 39) family (NP_968874) is accompanied by another *rcnR*-orthologue (NP_968873). However, while *rcnR*-like genes are frequently encoded in close vicinity to a metal efflux pump gene, there is no experimental evidence for a function in direct gene regulation such as DNA-binding.

In *E. coli* RcnR alone does not function as a metal-dependent repressor of *rcnA* expression because *rcnA* is still induced by metals in a Δ*rcnR* deletion strain (Figure 5). This might be the underlying reason why purified RcnR failed to bind to the *rcnAp* promoter *in vitro* (data not shown). Involvement of Fur in expression control of *rcnA* suggested that RcnR–Fur interaction is required for promoter binding or for functional alteration of Fur except for overproduction of RcnR in a Δ*fur* strain still inhibited *rcnA* expression. Thus, Fur and RcnR regulation of *rcnA* expression is not interdependent. Nevertheless, in a wild-type strain both RcnR and Fur had to be present to inhibit induction of *rcnA* by iron (Figures 5, 7).

The Fur protein of *E. coli* binds two different divalent metal cations; a Zn(II) is responsible for the structural integrity of the protein (Jacquamet *et al.* 1998), while the second site complexes the co-repressor iron in a distorted octahedral coordination (Adrait *et al.* 1999; Pohl *et al.* 2003). Fur proteins from various bacteria not only bind Fe(II) to this regulatory site, but also Mn(II), Co(II), Cd(II), to a lesser extend Ni(II) and *in vitro* Zn(II) (Adrait *et al.* 1999; Zheleznova *et al.* 2000; Pohl *et al.* 2003). Therefore, Fur may not only be triggered by iron but also by other divalent transition metals (Van Vliet *et al.* 2001; Contreras *et al.* 2003; Delany *et al.* 2005; Ikeda *et al.* 2005). However, there is no evidence that Ni-Fur is the regulator of *rcnA* and this would not explain the observed positive regulation by metals except for interplay of Ni-Fur with RcnR that seems not to occur (Figures 5, 7).

NikR is the only known Ni(II)-dependent regulator of *E. coli*. NikR represses expression of the NikACBED ABC-uptake system in the presence of elevated intracellular Ni(II)-concentrations (De Pina *et al.* 1999) and co-expression of this uptake-system with RcnA would lead to an expensive futile cycle of nickel import and export. In contrast to *Helicobacter pylori*, NikR from *E. coli* is not involved in positive gene-regulation (Ernst *et al.* 2005). As a consequence deletion of *nikR* in our *rcnR::lacZ* reporter strains did not abrogate Ni(II)-dependent expression (data not shown). Moreover, expression was not constitutive, which would have been expected when NikR was involved in repression of *rcnA*. However, the expression pattern changed and higher induction levels were reached in a Δ*nikR* background (data not shown). This is most probably caused by disturbed Ni(II) homeostasis when NikR was missing and the NikABC-transporter is constitutively switched on.

Taken together, data presented here and by others (Rodrigue *et al.* 2005) demonstrate that RcnA is probably an efflux system for Ni(II) and Co(II). Regulation of *rcnA* gene expression stems from interplay of nickel, cobalt and iron homeostasis. The regulator Fur and the putative regulator RcnR are involved in this process and each is necessary for metal-dependent regulation.

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