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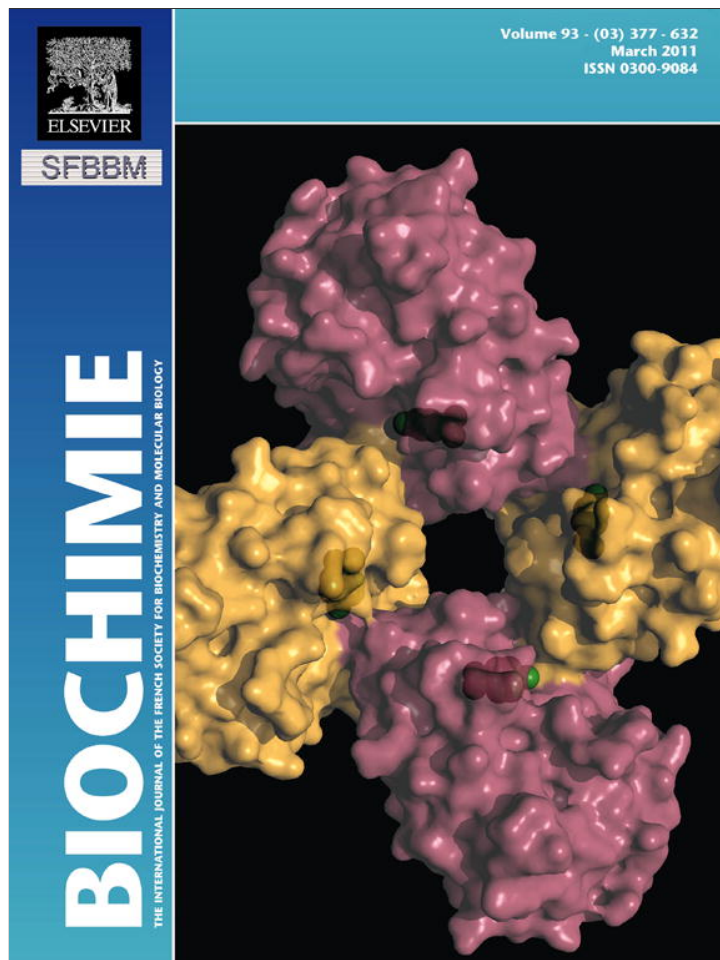
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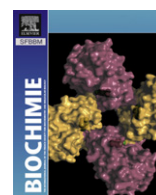
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## Research paper

# The *Escherichia coli* metallo-regulator RcnR represses *rcnA* and *rcnR* transcription through binding on a shared operator site: Insights into regulatory specificity towards nickel and cobalt

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## ABSTRACT

RcnA is an efflux pump responsible for Ni and Co detoxification in *Escherichia coli*. The expression of *rcnA* is induced by Ni and Co via the metallo-regulator RcnR. In the present work, the functioning of the promoter–operator region of *rcnR* and *rcnA* was investigated using primer extension and DNase I footprinting experiments. We show that the promoters of *rcnR* and *rcnA* are convergent and that apo-RcnR binds on symmetrically located sequences in this intergenic region. Moreover, RcnR DNA binding is specifically modulated by one Ni or Co equivalent and not by other metals. In addition to *rcnA*, RcnR controls expression of its own gene in response to Ni and Co, but the two genes are differentially expressed.

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## 1. Introduction

Nickel and, to a lesser extent, cobalt are obligate nutrients in bacteria because they fulfill important biological functions [1,2]. The intrinsic properties of these metals make them unique catalysts for a subset of specialized, vital functions. Biological systems have overcome the reactivity of these metals by evolving a large set of metal-coordinating proteins dedicated to metal transport and insertion [3]. Ni and Co are ubiquitous and are often co-localized in natural environments [4]. When present in excess concentrations, both metals are expelled out of the bacterial cell to ensure detoxification. In *Escherichia coli*, one Ni and Co export system, the RcnA efflux pump, has been reported. RcnA confers resistance to Ni and Co, and its expression is induced by these two metals and not by other divalent cations [5]. The *rcnR* gene, which is located upstream of the *rcnA* gene and is divergently transcribed, encodes a repressor that controls *rcnA* expression in response to Ni and Co [6].

RcnR binds the intergenic *rcnR*–*rcnA* region on the palindromic sequence TACT-G6-N-AGTA, which is repeated twice and interacts

with flanking DNA regions, possibly resulting in DNA wrapping [7]. RcnR is thought to act as a tetramer, composed of a dimer of dimers [7] and to bind one Ni or Co ion per monomer, these metals being coordinated by a set of common residues as well as specific ligands [6]. RcnR is structurally similar to CsoR, a Cu-sensing repressor whose crystallographic structure reveals a homodimeric state in which Cu(I) is coordinated at the interface of the two monomers [8]. CsoR from *Bacillus subtilis* binds Ni(II), Zn(II) and Co(II) with high affinity *in vitro*. However, this binding does not affect the DNA binding affinity of CsoR, as these metals are coordinated at different positions relative to that of Cu(I) [9]. The metal specificity of the RcnR regulatory pathway was not clearly defined and the promoter elements of the *rcnR*–*rcnA* intergenic region were not determined. The aim of the present work was therefore to gain insight into the regulatory features of the *rcnR* and *rcnA* genes via RcnR by using a combination of genetics and biochemistry.

## 2. Materials and methods

## 2.1. Bacterial strains, plasmids and oligonucleotides

The *E. coli* strains, plasmids and oligonucleotides used in this study are listed in Table 1. Gene deletions of *rcnR* and *rcnA* were constructed in the *E. coli* BW25113 strain according to [10] using the primers listed in Table 1. For phenotypical analysis, the *rcnR* deletion was transferred in *E. coli* NM522 by P1 phage transduction.

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**Table 1**  
Bacterial strains, plasmids and primers used in this study.

Strain or plasmids	Genotype or description	Source, reference
<b>Strains</b>		
BW25113	<i>lacI<sup>q</sup> rrnB<sub>T14</sub> ΔlacZ<sub>WJ16</sub> hsdR514 ΔaraBAD<sub>AH33</sub>ΔrhaBAD<sub>LD78</sub></i>	[10]
NM522	<i>Δ(lac-proAB), thi, hsdS, supE, [P<sub>proAB</sub>+, lacI, lacZ ΔM15]</i>	Stratagene
B834	<i>F<sup>-</sup> ompT hsdS<sub>B</sub> (r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal [dcm] met (DE3)</i>	[20]
DB150	<i>As NM522, ΔrcnR-cat</i>	This study
ARY023	<i>As MC4100, rcnA::uidA-Kan<sup>R</sup></i>	[5]
ARY024	<i>As NM522, rcnA::uidA-Kan<sup>R</sup></i>	P1 transduction from ARY023
<b>Plasmids</b>		
pGEM-T Easy	Cloning vector, Amp <sup>R</sup>	Promega
pET30	Overexpression vector, T7 promoter, Kan <sup>R</sup>	Novagen
pDB1	<i>rcnR</i> cloned into pET30	This study
pDB2	<i>rcnRA</i> regulatory region cloned into pGEM-T Easy	This study
pAR123	<i>yohL-yohN</i> region in pUC18	[5]
p127	<i>rcnA::gfp</i> in pPROBE	[21]
<b>Primers</b>		
Youp	Sequence 5'–3' CGAGTCGTTTGCATCCGATGATGATAAATCG	Purpose Cloning of <i>rcnR</i>
Yolup	AGGTGTCATATGTCTCATAC	Cloning of <i>rcnR</i>
Yompromdwn	GCTGAAGAAGAGTTGTAATTCGG	PrcnRA probe
yompromup	TCACTCCCCGATCAGACC	PrcnRA probe
yolFRTup1	ATTTGATATATGAATCCAGCACCTTCAGAACGACATCCAGTGTGAGGCTGGAGCTGCTTC	<i>rcnR</i> mutant
yolFRTdwn1	AAGAAATGCTCGACGAGCCGACCAATGCGCTGCGATTTTCATATGAATATCTCTCTTAG	<i>rcnR</i> mutant
YoMATG	AATTCGGTCATGATAAATTC	Primer ext. <i>rcnA</i>
Yol4	CACGTTTTAGTCTATCCC	Primer ext. <i>rcnR</i>
Rpob1FQ	GAAGGGCAGTATGGTCTGGA	qRT-PCR <i>rpoB</i>
Rpob1RQ	TCACCAGACGAGTTAACG	qRT-PCR <i>rpoB</i>
Rpoa 1FQ	TGATGTCGAAATCGTCAAGC	qRT-PCR <i>rpoA</i>
Rpoa 1RQ	TGTAGGCAATACGCTCCAC	qRT-PCR <i>rpoA</i>
Yohm2FQ	GAAACGCCTGGTTTCTCATCC	qRT-PCR <i>rcnA</i>
Yohm2RQ	AGGCAATTAACCAGACCACTG	qRT-PCR <i>rcnA</i>
yohl1FQ	TCGGCAATCTGATTCTACTCC	qRT-PCR <i>rcnR</i>
yohl1RQ	CTGGCCCTGAATCTTACTGG	qRT-PCR <i>rcnR</i>

## 2.2. Overexpression and purification of the RcnR Protein

The coding region of *rcnR* was cloned into pET30 using primers Youp and Yolup to generate pDB1 (Table 1). Overproduction of the native RcnR was carried out in *E. coli* B834 strain. Cells were grown at 29 °C in LB medium containing ampicillin until OD 600 nm reached 0.6, 1 mM IPTG was added and cells were grown for an additional 6 h at 29 °C. Cells were collected by centrifugation, washed and resuspended in 10 mM sodium phosphate pH 7 (binding buffer). Crude protein extracts were obtained by disrupting the bacterial cells with a French pressure cell (Aminco) and the lysed cells were centrifuged at 20000 × *g* for 30 min to obtain clear lysates. The protein solution was first applied to a 5 ml HiTrap heparin column HP (GE Healthcare), the loaded column was washed with the binding buffer and bound proteins were eluted using a NaCl linear gradient (0.1–2.5 M). RcnR eluted in the 0.3 M NaCl fraction. The RcnR fraction dialysed against 50 mM sodium phosphate pH 6.5 was loaded onto 1 ml HiTrap cation exchange column (GE Healthcare). The bound proteins were eluted with NaCl linear gradient (0.1–1 M) in 50 mM sodium phosphate pH 6.5. The RcnR protein was efficiently eluted with 0.2 M of NaCl, this fraction was buffer-exchanged on NAP-10 column (Amersham Biosciences) into complexation buffer (24 mM Hepes, 8 mM Tris–HCl, 140 mM KCl, 10% glycerol, pH 7.4). The protein concentration was determined by the method of Bradford (BioRad kit) and by measurement of OD at 280 nm ( $\epsilon = 1490 \text{ M}^{-1} \text{ cm}^{-1}$ ).

## 2.3. In vitro DNA–protein interaction

The *E. coli rcnR–rcnA* regulatory region (RRrcnA) was amplified by PCR using the yompromdwn and yompromup primers. The

resulting 325-bp amplification product was cloned into the pGEM-T Easy Vector (Promega). The RRrcnA DNA fragment obtained by a restriction digest with *Sall* and *SacII* was end labeled by filling the *SacII* end in the presence of ( $\alpha$ -<sup>32</sup>P) dCTP and DNA polymerase. DNase I footprinting was performed using a modified version of a procedure previously described [11]. About 50 000 cpm of DNA probe was incubated for 15 min at 30 °C with 10–200 nM of RcnR into complexation buffer. The reaction mixtures were adjusted to 10 mM MgCl<sub>2</sub>, 5 mM CaCl<sub>2</sub> and 0.1% (v/v) Nonidet P-40 (Roche Applied Science), supplemented with metals at the concentrations indicated in the figures and incubated for 15 min at 30 °C. DNase I was added ( $5 \times 10^{-3}$  units; Roche) and incubated at 30 °C for 15 s. The digestion was blocked by the addition of 25  $\mu$ l of stop solution (100 mM EDTA, pH 8), and 50  $\mu$ l of ice-cold TE (50 mM Tris–HCl, 1 mM EDTA, pH 7.5) was then added to increase the volume of the mixture. After phenol–chloroform extraction, DNA fragments were ethanol precipitated, resuspended in 10  $\mu$ l of formamide–dye mixture and separated by electrophoresis on a 6% polyacrylamide sequencing gel. Bands were detected by autoradiography.

## 2.4. Primer extension

Total RNA was extracted from *E. coli* NM522 cells by the TRIzol reagent method (Invitrogen). Total RNA was treated by DNase (Ambion), and 5  $\mu$ g RNA was annealed with approximately  $6 \times 10^4$  cpm of a <sup>32</sup>P-end-labeled 19–20-mer oligonucleotide (YoMATG for *rcnA* and Yol4 for *rcnR*) in annealing buffer (Invitrogen). Extension reactions were performed with 1  $\mu$ l of SuperscriptII RT (Invitrogen), and products were fractionated on sequencing gels. Dideoxy sequencing reactions performed with the

same oligonucleotide and the plasmid pAR123 were used as size markers.

2.5. cDNA synthesis and real-time PCR

Total RNA was extracted using the RNeasy kit (Qiagen). RNA was treated with DNase (Ambion). cDNA synthesis was performed with the SuperscriptII kit (Invitrogen). The real-time PCR experiments were performed using the Master<sup>PLUS</sup> SYBRGreen I kit (Roche Applied Science). The *rpoB* and *rpoA* genes were chosen as reference genes for data normalization. Amplification and detection of the specific products were carried out with the LightCycler System, and data analysis was performed with the Lightcycler Relative Quantification software (Roche Applied Science). Relative expression was calculated as the ratio of the normalized value of each

sample relative to that of the corresponding untreated wild-type cells according to [12].

3. Results

3.1. Transcription of *rcnA* and *rcnR*: convergent promoters for a shared operator

The *E. coli rcnA* and *rcnR* genes are divergently orientated. Their translation start codons are separated by 120 bp. The transcription start site for each gene was mapped by primer extension experiments. A single primer extension product was detected for *rcnA* or *rcnR* (Fig. 1A, B). The *rcnA* start site is located 90 bp upstream of the ATG, and the *rcnR* start site is located 107 bp upstream from the ATG. The two transcript starts are thus separated by 75 bp. A striking feature of these genes is that the two promoters are

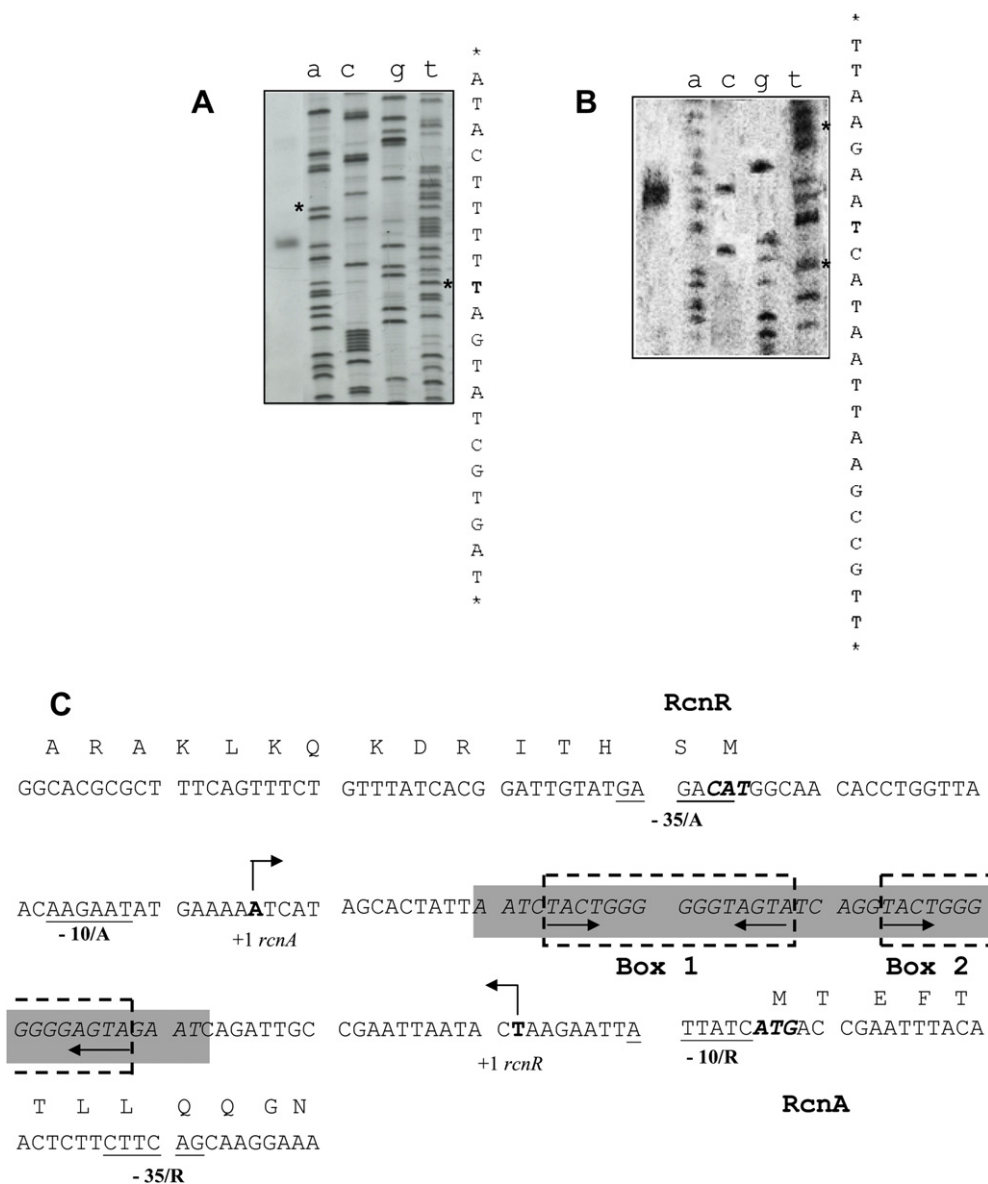


Fig. 1. Intergenic region organization of *rcnA* and *rcnR*. Determination of transcription initiation start sites of (A) *rcnA* and (B) *rcnR* using primer extension experiments. The DNA sequence surrounding the initiation site is written on the right, the +1 is in bold letter. (C) Sequence and organization of the region. The transcription start sites are indicated by bent arrows. The deduced -10 and -35 sequences are underlined (/A refers to *rcnA* and/R refers to *rcnR*). The protected region in the footprinting experiments is shaded gray (see Fig. 2A). Inverted repeated sequences are underlined with arrows. The two repeated RcnR binding boxes are framed with dashed lines.



convergent, an unusual topology among divergently transcribed genes.

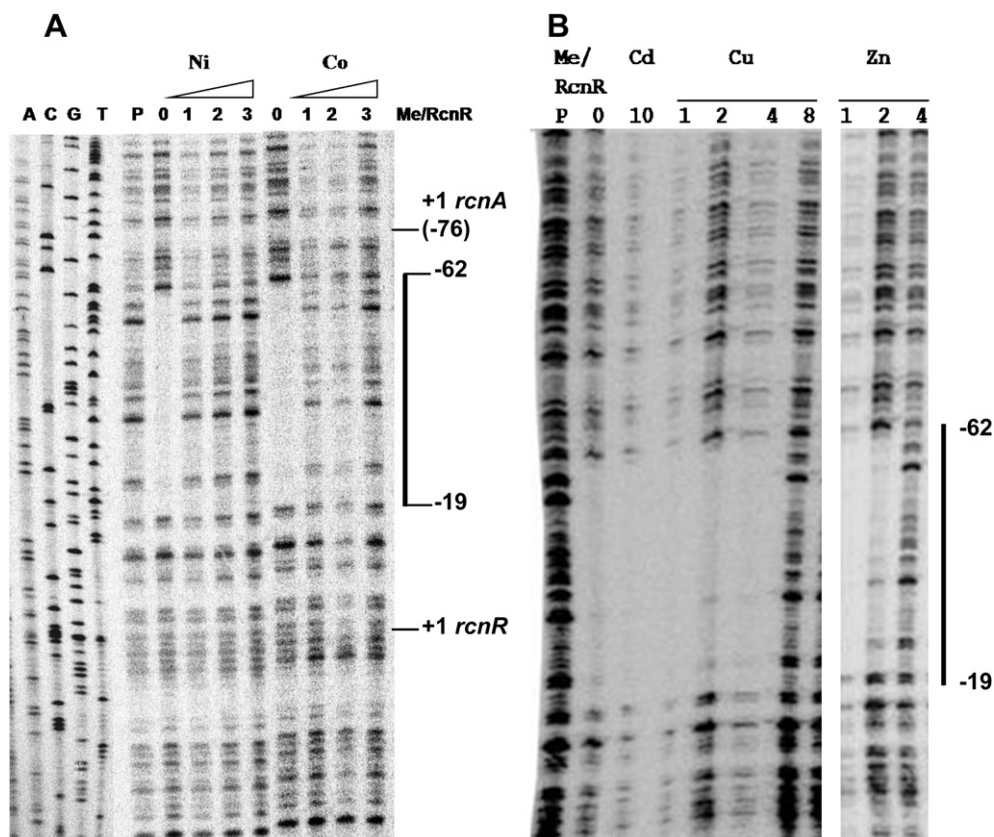
The sequence surrounding the two start sites contains a palindromic sequence, TACT-N7-AGTA recognized by RcnR [7], which is repeated twice, with the two directly repeated dyad symmetry sequences separated by 5 bp. These two repeated sequences are located in between the *rcnA* and *rcnR* + 1, downstream of each site (Fig. 1C). DNase I footprints in the presence of purified RcnR confirmed the occupancy of a 43-bp region covering the two boxes (Fig. 2A) using here a 325-bp probe located from –166 to +159 bp relative to the ATG of *rcnR* [7]. Our determination of the transcription start sites highlights the symmetrical organization of RcnR binding sites towards the *rcnA* and *rcnR* promoters. The fact that RcnR binds downstream of both transcription start sites suggests that repression of transcription hampers RNA polymerase progression in both directions.

### 3.2. Transcriptional control of *rcnA* and *rcnR* genes by Ni and Co via *rcnR*

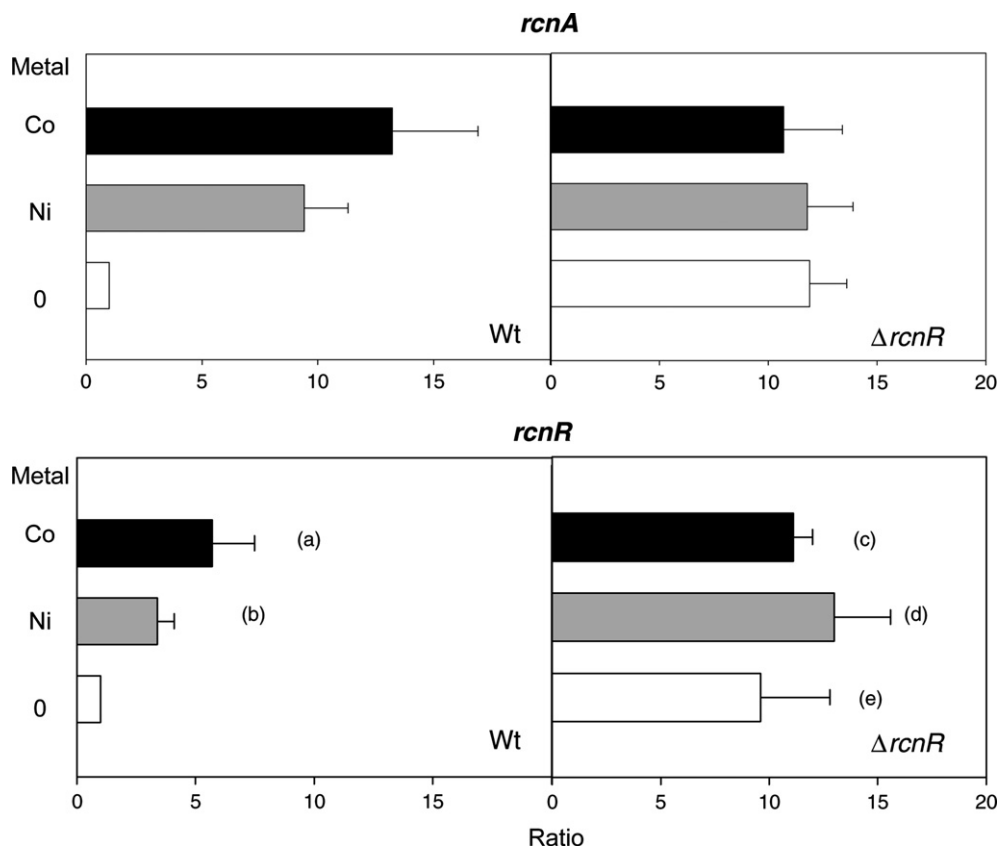
DNase I experiments were further carried out in the presence of Ni or Co (Fig. 2A). As expected, RcnR dissociates from its cognate binding site upon the addition of one or the other metal. This dissociation has been previously shown but using EMSA experiments and in the presence of a large excess of Ni or Co ([metal]/[protein] > 10<sup>3</sup>) [13]. In the present work, after the addition of one metal equivalent of either metal, a release of 80–100% of RcnR from P<sub>*rcnR-A*</sub> was assessed by profile density image analysis. Ni or Co binding by RcnR was monitored using UV–visible spectroscopy.

The primary band for Co binding was at 308 nm, and the primary band for Ni binding was at 278 nm. We observed a stoichiometry of one Ni(II) or Co(II) binding to each RcnR monomer (data not shown), in agreement with data published elsewhere [6]. Overall, these results show that RcnR responds similarly to Ni and Co *in vitro*.

The respective expression of *rcnA* and *rcnR* in the presence of either metal was assessed at the mRNA level by quantitative real-time PCR (Fig. 3). Cells were grown in LB medium in the presence of either Ni or Co from the start of the culture until mid-log phase. The chosen concentrations of Ni and Co (1 mM NiSO<sub>4</sub> and 0.1 mM CoCl<sub>2</sub>) gave the highest responses with the *rcnA::uidA* gene reporter fusion ([5] and data not shown). This difference between effective concentrations of Ni and Co has been reported elsewhere [14] and may be explained in terms of actual free metal concentrations in the cytoplasm. As expected, the expression of *rcnA* in a wild-type background was induced by either Ni or Co (Fig. 3). The induction by these two metals was nearly the same, approximately tenfold. In the  $\Delta$ *rcnR* derivative, similar expression ratios were obtained for *rcnA* in the three tested conditions, LB, LB +Ni, LB +Co, reaching the level of expression obtained in the wt strain grown in LB supplemented with either Ni or Co. This confirms that *rcnA* regulation by Ni or Co is mediated by RcnR at the transcriptional level. In the wt strain, the addition of Ni or Co led to a similar level of induction (approximately fivefold) of *rcnR*. Interestingly, in an *rcnR* mutant, *rcnR* expression was the same for the three conditions (LB, LB +Ni, LB +Co), but the levels of expression were twofold higher than that of the wt strain grown in the presence of either Ni or Co. Thus, full expression of *rcnR* is only attained in the absence of



**Fig. 2.** RcnR dissociation from cognate DNA upon metal binding. A) Footprinting experiments in the presence of Ni or Co. Lane P: probe, lanes 0–3: probe + 200 nM RcnR + 0, 1, 2 or 3 equivalents of Ni or Co. The region protected by RcnR (–62 to –19) is indicated relative to *rcnR* transcription start site. B) RcnR metal specificity testing. Footprinting experiments in the presence of Cd, Cu or Zn. Lane P: probe, lanes 0, 1, 2, 4, 8, 10: probe + 200 nM RcnR + 0, 1, 2, 4, 8 or 10 metal equivalents, with the corresponding metal indicated above.



**Fig. 3.** Expression of *rcnR* and *rcnA* in response to Ni and Co. Quantification of the expression of the *rcnR* and *rcnA* genes was performed by qRT-PCR after growth of the wild-type strain NM522 and its  $\Delta rcnR$  derivative until mid-log phase in LB (white bars), LB + 1 mM NiSO<sub>4</sub> (gray bars) or LB + 0.1 mM CoCl<sub>2</sub> (black bars). Expression ratios are relative to the data obtained with the wild-type cells grown in LB. Data are the mean of at least three independent datasets, SD are presented in the figure. There were significantly different values between groups (a, b) and (c, d, e), with *P*-values: (a, c) =  $1.4 \times 10^{-4}$ ; (b, d) =  $4 \times 10^{-7}$ ; (b, e) = 0.01.

RcnR and is not attained by the sole addition of Ni or Co, as observed for *rcnA*. This shows that RcnR controls its own expression and suggests that supplementary regulatory events may control the expression of *rcnR* and therefore metal efflux.

### 3.3. Regulation of the divergent operon is highly specific to Ni and Co

We have previously reported that *in vivo* regulation of the expression of the transcriptional *rcnA::uidA* fusion is specifically induced by Ni or Co and that addition of Cd, Cu or Zn did not have any effect on the transcription of *rcnA* [5]. To test the *in vitro* specificity of the system, footprinting experiments were performed in the presence of Cd, Cu or Zn (Fig. 2B). As expected the addition of up to 10 metal equivalents of Cd triggered no release of RcnR from the *rcnR-rcnA* intergenic region. For Cu and Zn, RcnR dissociated from the probe with the addition of 8 and 4 metal equivalents, respectively.

## 4. Discussion

We demonstrate in this work that *rcnA* and *rcnR* are coordinately expressed in *E. coli* via RcnR and we provide insight into the molecular mechanism of this process. We have previously shown that the *in vivo* induction of *rcnA* is dependent on Ni or Co ions [5]. Here, using *in vitro* footprint experiments, we present evidence that addition of one Ni or Co equivalent leads to the release of RcnR interaction from the two recognition sites lying in the *rcnR-rcnA*

promoter–operator region. Furthermore, the other transition metals Cd, Cu and Zn were not able to regulate *rcnR-rcnA* operator DNA binding or were active at far higher concentrations which may not be physiologically compatible. These results are in full agreement with the phenotypes associated with *rcnA* mutants, namely resistance to Ni and Co and not to other divalent cations [5]. At both functional and regulatory levels, this system is dedicated to the detoxification of Ni and Co. Until recently, *E. coli* RcnA was the only described member of this family of efflux pumps. However a new member, MdrH, has recently been described in *Pseudomonas putida* which confers resistance to Cd, Zn and Ni; its expression is induced by Cd, Ni, Zn and Co [15]. The *mdrH* gene is thought to be regulated by the upstream gene *mreA*, which has the same expression pattern as the target gene. The RcnA family might therefore have broad substrate specificity and may have co-evolved as a metal transporter and a dedicated metallo-regulator.

We show here that the induction of *rcnR* transcription occurs by direct binding of Ni or Co on RcnR. Each monomer of RcnR can accommodate either one Ni or one Co ion, as reported here and elsewhere [6]. However, *in vivo* Co concentrations tenfold lower than those of Ni induced comparable effects on transcription. It can be hypothesized that the cellular requirement for Co differs from that of Ni. Co is indeed much less used by biological systems than Ni, especially in *E. coli* where Co utilization seems restricted to vitamin B12 import and use [2,16]. This was further supported by measuring the increase of the *rcnA::uidA* gene fusion after growth in LB medium supplemented by Ni or Co. An increase in the  $\beta$ -glucuronidase activity was observed for Ni concentrations up

to 1 mM, whereas the maximum was reached for 0.03 mM Co. The toxic effect of these ions on the bacterial growth was recorded for Co and Ni concentrations greater than 0.1 mM and 0.5 mM respectively (data not shown). These data indicate that a similar amount of Ni or Co added to the growth medium results in different intracellular concentrations. Another clue is the respective harmfulness of the two metals. Although the effects of these two metals at the molecular level are not yet fully understood, one striking difference is the capacity for Co to compete with Fe in Fe-metallocenters, leading for instance to inactivation of iron–sulfur clusters [17]. The bacterial cell might therefore be inclined to control Co levels closely and export excess of the metal cation.

Transcription of *rcnA* was induced tenfold by Ni or Co, as observed from qRT-PCR experiments. The same induction level was observed in the *rcnR* mutant, indicating that saturating concentrations of Ni or Co fully alleviate repression via unbinding of Ni- or Co-loaded RcnR. The regulation of *rcnR* seems more complex than regulation of *rcnA*. Indeed, induction levels with Ni or Co reached roughly fivefold. The difference in induction level of *rcnA* and *rcnR* might be due to different properties of each promoter, such as promoter strength. However, when measured in an *rcnR* background, the induction level of *rcnR* increased tenfold, indicating that this regulation is mediated by RcnR. Thus, transcription of *rcnR* includes an additional layer of regulation relative to the transcription of *rcnA*. Previous work has suggested that iron might induce *rcnR*, but in a *fur*-independent pathway [14]; this point needs further investigation.

The peculiar topology of the *rcnR* and *rcnA* promoters could also shed light on our understanding of these differential patterns of expression. We show here that the two promoters are convergent and that the two RcnR DNA binding boxes are symmetrically located from each transcription start site. This topology is not frequent and provides functional constraints on the transcription of the divergent genes. Convergent promoters in themselves were shown to trigger transcriptional interference of one gene over the divergent one, leading to increased expression of one of the genes and resulting in so-called “weak” and “strong” promoters. The competition between the “weak” and the “strong” promoters might be the result of two phenomena: (i) RNAP bound to one promoter decreases the interaction between RNAP and the second promoter and (ii) when both promoters are occupied by RNAP, transcription initiation occurs faster on one of the promoters [18,19]. We identified this kind of conserved promoter organization in *rcnA/rcnR* systems of beta, gamma and delta Proteobacteria (data not shown). However the topology of the RcnR binding box is diverse as genes under its control may also be transcribed in the same orientation. Similarly tandem repeats of RcnR binding site is not an absolute rule, a single site being present in many cases (not shown). It has been shown experimentally that RcnR from *E. coli* can bind one site *in vitro* when the other one is missing [7]. The diversity in operator site organization may thus provide diversity in gene regulation among RcnR homologs which are involved in metal sensing and in oxidative stress response.

This work provides new insights on molecular mechanisms of transcriptional control of the efflux pump RcnA, but our genetics data suggest that additional yet unknown effectors modulate the regulation of *rcnR*. Further work is needed to identify these factors.

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## Appendix. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biochi.2010.10.016.

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