Environ Sci Pollut Res
DOI 10.1007/s11356-015-5580-6

IN-LINE MULTIPLEXED BIOSENSING

Pushing the limits of nickel detection to nanomolar range using a set of engineered bioluminescent *Escherichia coli*

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Received: 3 August 2015 / Accepted: 7 October 2015

Abstract The detection of nickel in water is of great importance due to its harmfulness for living organism. A way to detect Ni is the use of whole-cell biosensors. The aim of the present work was to build a light-emitting bacterial biosensor for the detection of Ni with high specificity and low detection limit properties. For that purpose, the regulatory circuit implemented relied on the RcnR Ni/Co metallo-regulator and its *rcnA* natural target promoter fused to the *lux* reporter genes. To convert RcnR to specifically detect Ni, several mutations were tested and the C35A retained. Deleting the Ni efflux pump *rcnA* and introducing genes encoding several Ni-uptake systems lowered the detection thresholds. When these constructs were assayed in several *Escherichia coli* strains, it appeared that the detection thresholds were highly variable. The TD2158 wild-type *E. coli* gave rise to a biosensor ten times more active and sensitive than its W3110 *E. coli* K12 equivalent. This biosensor was able to confidently detect Ni concentrations as little as 80 nM (4.7 μg l^{-1}), which makes its use compatible with the norms governing the drinking water quality.

Keywords Biosensor · Whole bacteria · Bio-luminescence · Metal · Water control

Responsible editor: Philippe Garrigues

Julien Cayron and Elsa Prudent contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11356-015-5580-6) contains supplementary material, which is available to authorized users.

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Introduction

Among the pollutants to be monitored in water, metals are of great importance because of their toxicity, abundance and diversity. While several chemical or analytical physical methods exist and are normalized, the detection of metals in water using whole-cell biosensors has proven to be an alternative solution (van der Meer and Belkin 2010). The requirement to build such sensors is a genetic circuit that can convert the input signal (metal) into an easily detectable output signal (fluorescence, luminescence…). The challenge for the detection of metals is to set up a genetic circuit whose response is strictly restricted to a given metal. Indeed natural genetic systems involved in the detection of metals often respond to several species (Bereza-Malcolm et al. 2014). The aim of the present work was to construct a whole-cell bacterial biosensor for the specific detection of nickel. For the reason of their chemical similarity, Co and Ni are often handled by the same homeostatic systems in bacteria. These systems involve efflux pumps that are able to extrude both metals. The synthesis of these pumps is regulated at the transcriptional level by metallo-regulators also able to sense both metals (Ma et al. 2009). However, a few regulators were shown to be specific for Ni. For instance, the *nrsRS* two-component regulatory

Published online: 24 October 2015

system of *Synechocystis* sp. PCC 6803 controls the expression of the *nrsBACD* operon involved in Ni tolerance by a yet unknown mechanism (Lopez-Maury et al. 2002). A sensor was built by cloning the promoter of the *nrsBACD* operon in fusion with the *lux* reporter genes, along with *nrsR* on a plasmid (Peca et al. 2008). This sensor detected Ni specifically in the μM range and elicited no response to Co or other divalent cations. This bioreporter strain detected Ni only when the cyanobacteria were incubated in light. The strain showed no detectable luminescence when incubated in darkness, most likely for the reason of the silencing of expression from the P*nrsB* promoter. This could clearly constitute a limit for the use of such a strain. Another attempt to build a Ni sensor was the use of the *cnrYXH* genes from *Cupriavidus metallidurans* CH34, formerly *Ralstonia eutropha* CH3 (Tibazarwa et al. 2001). These genes code for a sigma/anti-sigma system that controls the expression of the CnrCBA efflux pump involved in the efflux of Ni and Co (Grass et al. 2005). The sensor strain consisted of a plasmid, containing the promoter of *cnrXYH* fused to the *luxCDABE* reporter genes, hosted by *C. metallidurans*. This sensor responded to Ni, but also to Co, in the micromolar range.

Apart from the *nrsRS* regulatory system of *Synechocystis* cited above, the only described Ni-specific regulator is NikR. In *Escherichia coli*, NikR represses the expression of the *nikABCDE* Ni-uptake system in response to Ni (De Pina et al. 1999). In *Helicobacter pylori*, it acts as either a repressor or an activator of a variety of genes (Contreras et al. 2003). However, either in *E. coli* or in *H. pylori*, the NikR-regulated genes are often co-regulated by other signals such as iron, pH and oxygen. These multilayered regulations are expected to generate more fluctuations in a sensor strain based on the detection of Ni by NikR. In order to design a robust Ni sensor, the *rcn* system of *E. coli* was investigated in this work. The *rcnAB* operon encodes a Ni and Co efflux system (Bleriot et al. 2011). Ni or Co represses the expression of *rcnA via* the RcnR repressor (Iwig et al. 2006). *rcnR* and *rcnA* are expressed from a shared divergent promoter and *rcnR* also controls its own expression (Blaha et al. 2011). At high Ni or Co concentrations, RcnR binds Ni or Co and dissociate from the DNA, allowing transcription of *rcnAB* and *rcnR* (Iwig et al. 2006). Interestingly, several point mutations in RcnR were shown to change its specificity towards metal sensing. Some of these mutations were described to render RcnR blind to Co while still sensing Ni (Iwig et al. 2008).

The first challenge of this work was to construct a sensor strain able to specifically detect Ni by introducing these mutations in RcnR and then by testing the response of the system in the presence of Ni or Co. A second challenge was to build a stable sensor strain with improved properties towards Ni sensing. On the one hand, stability was obtained by constructing reporter strains deprived of plasmid. On the other hand, mutating efflux systems and introducing uptake transporters in order to increase the intracellular accumulation of Ni achieved improvement of Ni detection. Finally, several *E. coli* backgrounds were tested to select the most sensitive sensor.

Materials and methods

Bacterial strains, plasmids and culture conditions

Strains used in this work are summarized in Table 1 and Fig. S1. Bacterial cells were grown in LB medium or M63 minimal medium supplemented with 0.4 % glucose. Cultures were performed at 37 °C unless otherwise stated. Where required, antibiotics purchased from Sigma Aldrich were used at the following concentrations: kanamycin at 50 μg/ml and ampicillin at 100 μg/ml.

Construction of mutants and gene reporter strains

Standard genetic methods were used (Sambrook et al. 1989). The W3110 C35A-*rcnR* (WC35A) strain was constructed as follows: the recipient strain, EGE65 (W3110 Δ*rcnR*), resulted first in the transduction of the Δ*rcnR-cat* cassette from strain DB150 (Blaha et al. 2011) to strain W3110, resulting in strain WRCR. The WRCR strain was then transformed with the thermo-sensitive plasmid pCP20 expressing the λ red recombinase to remove the cat cassette (Datsenko and Wanner 2000). Correct removal of the *cat* cassette between the FRT sites was checked by PCR and sequencing. The C35A-*rcnR* allele was obtained by two overlapping PCR amplifications, the first one with primers thiM_1 and rcnR_3 and the second with primers rcnR_4 and rcnA_2. The products of the two PCR were mixed and submitted to PCR amplification with primers thiM_1 and rcnA_2. The PCR product was cloned into suicide plasmid pKO3 and transformed in strain EGE65 (Link et al. 1997). The *E. coli* W3110 C35A-*rcnR* mutant was then constructed following the method described previously (Link et al. 1997). Correct replacement of the *rcnR* gene was checked by PCR and by sequencing. The strain was named WC35A. The amplification of the *PrcnA-luxCDABE-kanR-'rcnA* cassette from plasmid p1b was performed using primers RTMup and 2KanR-rcnA (Table 2). Insertional inactivation of *rcnA* was accomplished using the one-step mutagenesis method (Datsenko and Wanner 2000) by recombining the *PrcnA-luxCDABE-kanR-'rcnA* cassette into strains W3110 and WC35A to give rise to strains WRCAlux and WC35Alux, respectively. The [C35A-*rcnR* allele-*PrcnA-lux*-Kan-'*rcnA*] locus was moved into the strains

Table 1 Strains and plasmids

Strain or plasmids	Genotype or description	Source, reference
Strains		
W3110	*E. coli* K12 wild type	Laboratory stock
DB150	NM522, Δ*rcnR*-cat	(Blaha et al. 2011)
WRCR	W3110, Δ*rcnR*-cat	This study
EGE65	W3110 Δ*rcnR*	This study
WRCA1	W3110, Δ*rcnA-cat*	(Bleriot et al. 2011)
WRCAlux	W3110, Δ*rcnA PrcnA::lux kan*	This study
WC35A	W3110, C35A-*rcnR*	This study
WC35Alux	W3110 C35A-*rcnR* Δ*rcnA PrcnA::lux kan*	This study
TD2158	Wild type	(Dhillon et al. 1998)
TDRAlux	TD2158, C35A-*rcnR* Δ*rcnA PrcnA::lux kan*	This study
Plasmids		
pmerlux	p15A ori, *luxCDABE P. luminescens*, Kan^R, for lux transcriptional fusions	(Prévéral et al. 2015)
p1b	Promoter of *rcnA* in plux	This study
p6a	*rcnR* -P*rcnA* in plux	This study
pAlux	C35A-*rcnR* -P*rcnA* in plux	This study
pDlux	H60A-*rcnR* -P*rcnA* in plux	This study
pKO3	repA(ts), CmR, M13ori, sacB, suicide vector	(Link et al. 1997)
pKO3-C35A-*rcnR*	C35A-*rcnR* in pKO3	This study
pNiCoT (or pIG49)	P*tac*-nicoTB in pSB1C3 (pUC19 derived), NiCoT from *Novosphingobium aromaticivorans*	(Duprey et al. 2014)
pNik-BS	*nikABCDE* from *B. suis* in pUC18	(Jubier-Maurin et al. 2001)
pFS45	*ureH* (NiCoT family) from *Y. pseudotuberculosis* 32777 in pUC18Ω	(Wu et al. 1991)
pLW21	*nikABCDE* from *E. coli* in pUC19	(Sebbane et al. 2002)

TD2158 via generalized phage transduction using P1 *vir* to give rise to strain TDRAlux as described by Miller (1992).

Plasmid construction

Reporter constructions were all introduced in the plux plasmid containing the *luxCDABE* genes from *Photorhabdus luminescens* and a p15A low copy number origin of replication. The plux plasmid derives from a prototype bioluminescent mercury biosensor encoded in the pCC306 plasmid (Condee and Summers 1992). The *luxAB* genes from the *Vibrio harvei* luciferase in pCC306 were replaced (between BamHI and EcoRI restriction sites) by the *Photorhabdus luminescens* luciferase *luxCDABE* operon obtained from pUTmini-Tn5-luxCDABE-Tc (Winson et al. 1998), giving rise to pmerlux (Prévéral et al. 2015). For cloning purposes, the *mer* region is replaced by the insert of interest. The primers used for cloning are listed in Table 2. Plasmid p6a contains the *rcnR* gene, the *rcnR-rcnA* intergenic region and the first 200 bp of *rcnA* upstream of the *lux* genes. The *rcn* region was amplified using Pr_Rcn_NcoI and Pr_Rcn_ApaI primers and pAR123 as a template (Rodrigue et al. 2005). Plasmid p1b contains the first 118 bp of *rcnR* , the *rcnR-rcnA* intergenic region and the first 200 bp of *rcnA* upstream of the *lux* genes amplified from pAR123 using Pr_Rcn2_NcoI and Pr_Rcn2_ApaI primers. Site-directed mutagenesis of *rcnR* was performed using the QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene) and the primers listed in Table 2. Plasmids pAlux and pDlux are variants of plasmid p6a encoding for C35A-RcnR and H60A-RcnR, respectively. The plasmids are outlined in Fig. S1.

Nickel and cobalt susceptibility testing

Metal sensitivity assays were conducted as follows: first, bacteria were grown until mid-log phase in LB medium. The cultures were inoculated in 0.4 % glucose M63 minimal media tubes containing increasing concentrations of metal. The tubes were incubated at 37 °C for 24 h, with vigorous shaking, and the number of bacteria was

Table 2 Primers used in this study

Primers	Sequence 5′–3′	Purpose
Pr_Rcn_Nco1	CAATCCATGGCGGCAGTTTTACAATCGCG	*rcnR PrcnA* cloning (plasmid p6a)
Pr_Rcn_Apa1	ATATGGGCCCATTCTTAGTATTAATTCGGCAATCTG	*rcnR PrcnA* cloning (plasmid p6a)
Pr_Rcn2_F_Nco1	CAATCCATGGGTAAAACTGCAGCGCATTCG	*PrcnA* cloning (plasmid p1b)
Pr_Rcn2_R_Apa1	ATATGGGCCCCCACGGTTCTGCTGATTGAG	*PrcnA* cloning (plasmid p1b)
pRcN-Cys35Ala-F	GTTGTAAAACTGCAGCGGCTTCGTGCGGCTCGTC	C35A-*rcnR PrcnA* cloning (plasmid pAlux)
pRcN-Cys35Ala-R	GACGAGCCGCACGAAGCCGCTGCAGTTTTACAAC	C35A-*rcnR PrcnA* cloning (plasmid pAlux)
pRcN-His60Ala-F	CGATGTGTTCCGTCAGAGCACCTTTAATCACTTCCCG	H60A-*rcnR PrcnA* cloning (plasmid pDlux)
pRcN-His60Ala-R	CGGGAAGTGATTAAAGGTGCTCTGACGGAACACATCG	H60A-*rcnR PrcnA* cloning (plasmid pDlux)
RTMup	CCG AAT TTA CAA CTC TTC TTC AGC	P*rcnA::luxCDABE*-kan^R-*rcnA* 3′ amplification
2KanR-rcnA	TAAGCCAATCAACAGACTGGAAAAATAGGGGGCG CGTTTAGCGAGAGTGTTAAATCCGCTCCAGCGTT TTGCGACCTGCT CAACAAAGCCACGTTGTGT	P*rcnA::luxCDABE*-kan^R-*rcnA* 3′ amplification
thiM_1	CTG ACT GGC CTC TTC GGA TCC GAT AAC CAT CGC TGG	Construction of C35A-*rcnR* mutant strain
rcnR_3	CGA CGA GCC GCA CGA AGC CGC TGC AGT TTT ACA AC	Construction of C35A-*rcnR* mutant strain
rcnR_4	GTT GTA AAA CTG CAG CGG CTT CGT GCG GCT CGT CG	Construction of C35A-*rcnR* mutant strain
rcnA_2	CTG CGG AAA TCA GCT GGA TCC ACG GTT CTG CTG ATT G	Construction of C35A-*rcnR* mutant strain

estimated using OD_{600nm}. In each set of experiments, the OD_{600nm} value measured from the culture without nickel or cobalt was taken as the 100 % value to normalize the data. Growth in the presence of nickel or cobalt is expressed as a percentage of the growth without these metals.

Luminescence assays

The assays were conducted in 96-well plates. The wells were filled with 200 μL of 0.4 % glucose M63 minimal media containing increasing concentrations of metals, inoculated with 10^6 bacteria harvested at OD_{600} of 0.6. The plate was sealed using gas-permeable Breathe Easy membrane (Sigma Aldrich) and placed into a "TECAN Infinite Pro" plate reader equilibrated at 37 °C and programmed to measure OD_{600nm} and luminescence every 20 min, after a 1-min period of shaking, during 12 to 20 h. Background OD and luminescence (values at time =0) were subtracted to each data point. To calculate the maximal activity, for each well, the ratio (luminescence/OD) was plotted as a function of time, and the maximal value was conserved. Then, this maximal activity was plotted as a function of metal concentration. By doing so, we took into account the eventual growth lag between two conditions. To calculate the specific maximum activity, the activity (luminescence/OD) was plotted as a function of time. Then, for each metal concentration, the maximal slope was calculated from the curves. This maximal specific activity was finally plotted as a function of metal concentration.

Results

The *rcnRA* system as a candidate sensor for the detection of Ni and Co

We previously showed, by quantifying mRNA or by using *gfp* as a reporter gene, that the expression of *rcnA* is dependent on extracellular concentrations of Ni or Co (Blaha et al. 2011) (Bleriot et al. 2011). In this study, two plasmid-borne constructs were assayed in a first attempt to build a sensor strain. Both constructs were cloned in the plux plasmid containing a low copy number origin of replication and the *luxCDABE* genes from *Photorhabdus luminescens*. The use of the entire *luxCDABE* operon dispenses with the need to add exogenously a long-chain fatty aldehyde substrate for the luciferase. The p1b plasmid contains the P*rcnA*::*luxCDABE* fusion, whereas the p6a plasmid contains the *rcnR* gene in addition (Fig. S1). These plasmids were introduced in the wild-type strain W3110. Luminescence of the cultures was measured during 12 h in minimal media containing increasing amounts of Ni or Co, and the maximal activity (luminescence A. U./OD_{600nm}), recorded during the kinetics, was plotted (Fig. 1a, b). Both constructs responded gradually to Ni and Co. The dynamic response interval was 1–40 μM for Ni and 0.1–1 μM for Co in the tested conditions. The graphs also show that p1b generates more activity and sensitivity than p6a. Because *rcnR* is in multicopy in the W3110/p6a strain, more RcnR is produced; thus, the RcnR/(Ni or Co) ratio is higher. The consequence is that more metal-free (active) RcnR is present at higher Ni or Co concentration; this apo-RcnR then can

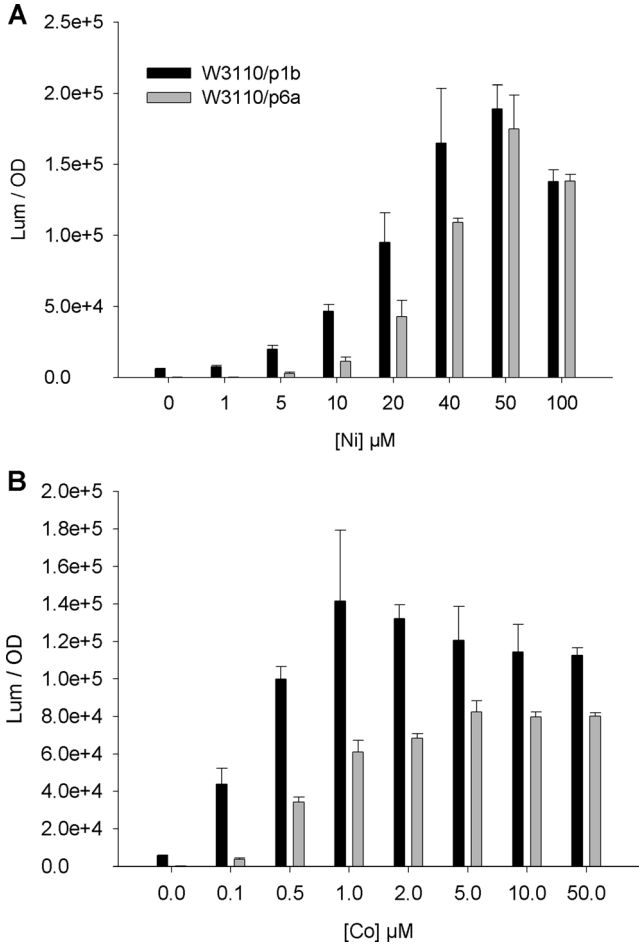

Fig. 1 Response of the P*rcnA*::*lux* fusion. W3110 (WT) strain containing plasmids p1b (P_{rcna}::*lux*) (*black*) or p6a (*rcnR*- P_{rcna}::*lux)* (*grey*) was grown in the presence of **a** $NiCl_2$ or **b** $CoCl_2$. The luminescence was recorded during 8 h; the value of the maximum activity is plotted on the graph (luminescence (A.U.)/$OD_{600\ nm}$). The experiments were repeated at least three times; SD are presented on the *graph*

still repress the P*rcnA*. It can be seen also that p1b generates more luminescence when no metal was added. These constructs were highly specific to Ni or Co and did not display activity when assayed in the presence of $CuCl_2$, $ZnSO_4$, $CdCl_2$, $HgCl_2$, $PbCl_2$ (Fig. S2).

Amino acid substitutions in RcnR that affect cell physiology and sensor specificity

Several point mutations were previously described that affected either Co or Ni responsiveness of RcnR (Iwig et al. 2008). Among them, mutation of residues C35, H60 and H64 conferred to RcnR the same responsiveness to Ni than did the wild type RcnR, while they abolished its response to Co. Derivatives of p6a were constructed, with the difference that pAlux encodes for C35A-RcnR and pDlux encodes for H60A-RcnR. Both plasmids were transformed in W3110 wild-type strain. As expected, both strains responded gradually to Ni (Fig. 2a). Interestingly, the strain containing pAlux totally lost its ability to detect Co (Fig. 2b). The strain harboring pDlux responded gradually to Co, in contrast to previously published results (Iwig et al. 2008). These results clearly indicate that the couple C35A-RcnR/P*rcnA* is a good candidate for the design of a Ni-specific sensor, even if the C35A mutation in RcnR entails a loss in the detection limit for Ni for concentrations below 10 µM (compare W3110/p1b (Fig. 1a) versus W3110/pAlux (Fig. 2a). It is interesting to note that in the presence of the C35A-*rcnR* allele expressed from a plasmid, the regulation by the wild-type chromosomal copy of *rcnR* seems to be abolished (compare W3110/p1b vs W3110/pAlux on Figs. 1a, b and 2a, b respectively). It suggests that overexpressed C35A-RcnR fully titrate P*rcnA*.

As shown in Fig. 1a, strains expressing RcnR from a plasmid displayed less activity than strains expressing RcnR only from the chromosome. Moreover, the presence of plasmids has several drawbacks: less stable expression from plasmid-borne genes, the obligation to maintain a selective pressure and the eventual dissemination of antibiotic resistance genes. To circumvent this, the gene encoding C35A-RcnR was recombined into the chromosome to give rise to WC35A strain. The Ni response kinetics of the WT and WC35A strains containing plasmid p1b are shown in Fig. 3a, b. The kinetics were similar, with a luminescent activity showing a maximum slope after 30 min of incubation and a maximum of luminescence after 5 h. The major difference between the two strains was the detection limit. The limit was 1 µM for W3110/p1b and 10 µM for WC35A/p1b. Concerning Co, the response was very fast for the wild-type strain within 30 min (Fig. 3c). As expected, Co elicited no response from the WC35A strain (Fig. 3d).

The mutations in *rcnR* are predicted to affect the metal resistance profile of the strains as RcnR controls the Ni and Co RcnA efflux pump. The mutated strains should lose their adaptation ability to external Co, while they should retain their adaptation to Ni. To measure this phenomenon, minimal inhibitory concentrations (MICs) to Ni and Co were assayed. For that purpose, cultures were conducted in test tubes with vigorous shaking. As expected the WC35A strain was as resistant as the W3110 strain when exposed to Ni (Fig. 4a). The MIC values (10 µM) in these conditions were far less important than the one we observed when the bacteria were grown in 96-well plates with discrete shaking. The resistance to Co of the wild-type strain was higher, with MICs of circa 20 and 30 µM for WC35A and W3110, respectively (Fig. 4b).

Ni sensing improvement

The efflux pump RcnA is a major actor for the maintenance of Ni or Co intracellular concentrations in *E. coli*. It has been proven that the deletion of *rcnA* enhances Ni and Co intracellular accumulation (Bleriot et al. 2011) (Duprey et al. 2014).

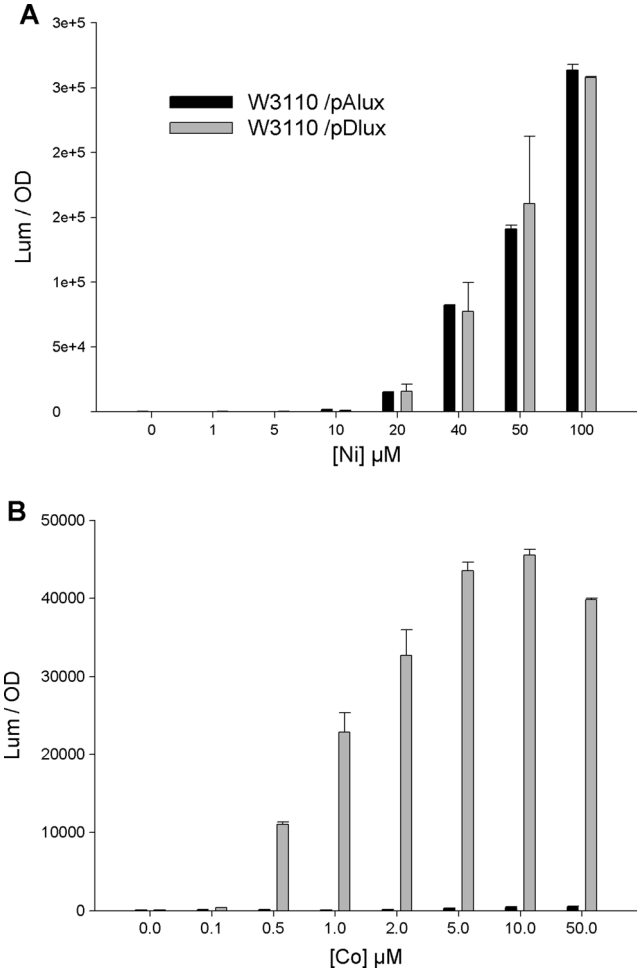

Fig. 2 Co detection by mutated RcnR. W3110 (WT) strain containing plasmids pAlux (C35A-*rcnR*) (*black*) or pDlux (H60A-*rcnR*) (*grey*) was grown in the presence of **a** $NiCl_2$ or **b** $CoCl_2$. The luminescence was recorded during 8 h; the value of the maximum activity is plotted on the graph (luminescence (A.U.)/$OD_{600\ nm}$). The experiments were repeated at least three times; SD are presented on the *graph*

The deletion of *rcnA* is thus expected to increase the response of the Ni sensor. To test that, the wild-type strain and its Δ*rcnA* derivative were transformed with pAlux (C35A-*rcnR* P*rcnA*) and cultured in the presence of Ni. Indeed the induction of the sensor was stronger in the strain lacking *rcnA*, indicating that more Ni was present in the cell (Fig. 5a). However, the detection limit remained the same for the two strains (20 μM). It seems like there is a threshold and that other systems allowing Ni homeostasis exist in addition to *rcnA* or that concentrations below 10 μM in the external medium are not sufficient for Ni to accumulate in the cytoplasm.

Given that, in order to both have a *rcnA* mutant and a stable chromosomal fusion, the P*rcnA*::lux fusion was recombined in the chromosome in replacement of *rcnA*, resulting in strain WRCAlux. The fusion was then transduced to strain WC35A, resulting in strain WC35Alux (W3110 C35A-*rcnR* P*rcnA*::lux Δ*rcnA*). Then, in the same C35A-*rcnR* genetic context, the plasmid-borne and chromosomal P*rcnA*::lux fusions were tested. To gain a more dynamic view of the response, the specific activity (lum/OD_{600}/time) was plotted as a function of the Ni concentration (Fig. 5b). The activity of the chromosomal sensor gave rise to a curve with two phases, a first line for concentrations comprised between 0 and 20 μM and a second between 20 and 40 μM. Above 40 μM, the specific activity increased more slightly. For the vector-borne sensor, the curve also displayed two phases, between 5 and and 30 μM and above 30 μM. It appeared that, for concentrations below 20 μM, the induction fold (as compared with the condition without added metal) is higher when the fusion is present on the plasmid, whereas the induction fold of the fusion hosted by the chromosome is higher for concentrations above 20 μM.

The importance of the chassis

In order to test the robustness of the sensor, these different constructions were then assayed in several "domesticated" *E. coli* K12 strains, such as DH10ß, TOP10 and W3110. Differences in luminescence intensity as well as detection thresholds were noticed along with the genetic background (data not shown, see Brutesco et al. (2015) in this special issue for complementary results). Ni import, Ni efflux and luciferase activity may vary from one strain to another and influence the sensor properties. Finally, the sensor was introduced into the *E. coli* TD2158 strain. This strain was isolated from sewage (Dhillon et al. 1998). This strain was proven to be more resistant to lyophilisation (see Brutesco et al. (2015) in this special issue). This latter property constitutes a major quality for the packaging and storage of the sensors.

C35A-rcnR, P*rcnA*::lux transcriptional fusion and Δ*rcnA* mutations were introduced in the TD2158 chromosome (strain TDRAlux) and assayed in the presence of increasing concentrations of Ni. The kinetics of Ni induction was very quick for concentrations above 10 μM (Fig. 6a). For concentrations below 10 μM, the response was delayed, as if the cells needed to accumulate trace ions before eliciting a response. The calculated specific activity was plotted as a function of Ni concentration and compared to the equivalent strain in the W3110 genetic background (WC35Alux). Figure 6b plot clearly shows boosted capacities for the TDRAlux strain as compared to the WC35Alux. The maximal activity was tenfold higher. Most interestingly, the detection limit was lowered to 2 μM instead of 20 μM.

In order to expand the boundaries of this strain, one parameter was still to be tested: the uptake of Ni. Two classes of transporters ensure Ni-specific uptake in bacteria: ABC transporters or secondary transporters of the NiCoT family (Eitinger and Mandrand-Berthelot 2000) (Eitinger et al. 2010). In *E. coli*, the NikABCDE, ABC transporter, mediates Ni uptake. In order to enhance Ni accumulation, the strain TDRAlux was transformed with plasmid-borne Ni uptake

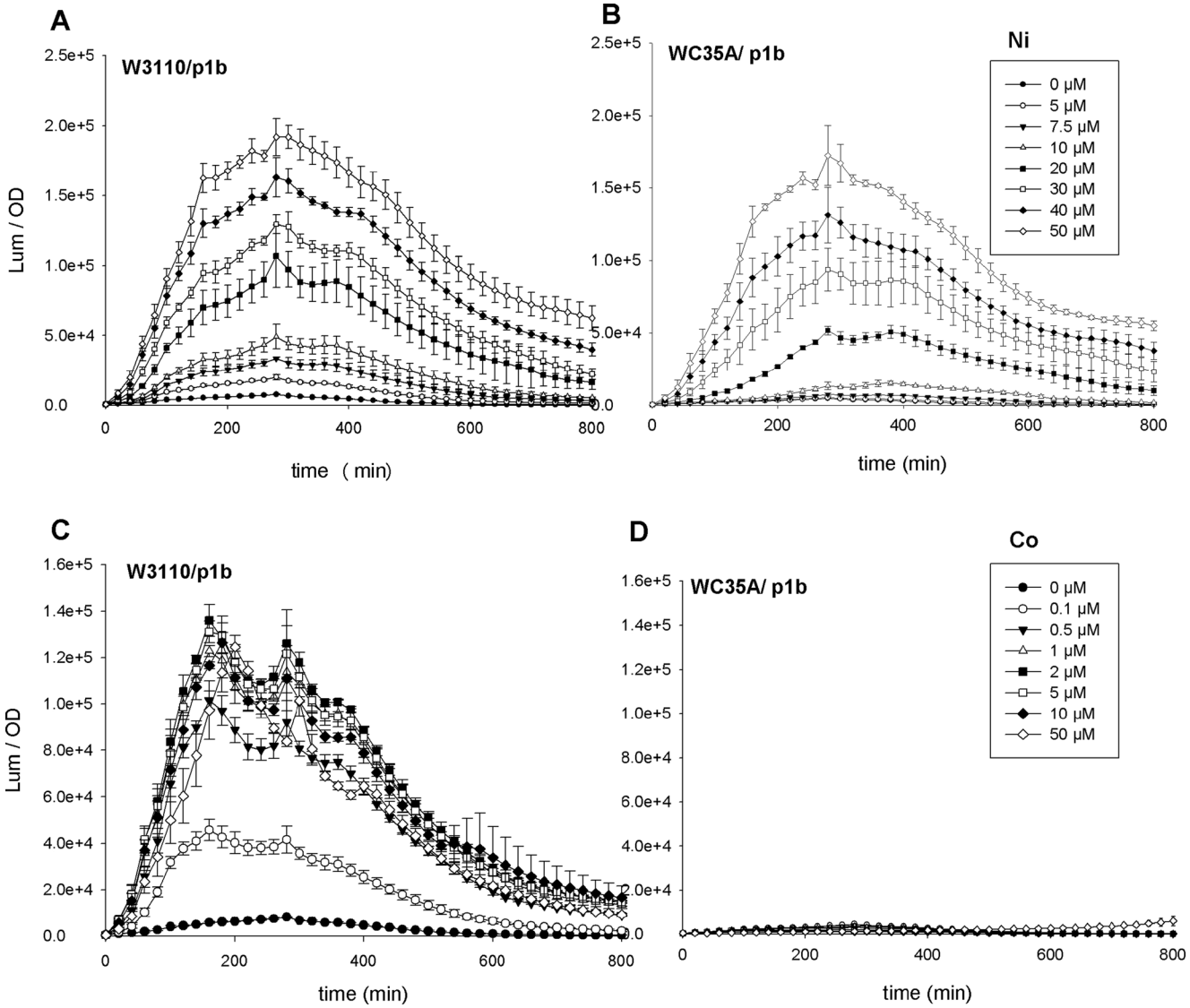

Fig. 3 Compared kinetics of the WT strain and its C35A-*rcnR* derivative. W3110 (WT) and WC35A strains containing plasmid p1b were grown in the presence of **a**, **b** $NiCl_2$ or **c**, **d** $CoCl_2$. The luminescence was recorded during 16 h; the activity is presented on the *graph* (luminescence (A.U.)/ $OD_{600\ nm}$). The experiments were repeated at least three times; SD are presented on the *graph*

systems: the *nikABCDE* genes from *E. coli* (pLW21) or *Brucella suis* (pNik) or NiCoT transporters gene from *Novosphingobium aromaticivorans* (pNiCoT) and *Yersinia pseudotuberculosis* (pFS45). Figure 7 displays the maximum luminescence of the different sensors induced by Ni. Above 5 μM, no significant differences were observed between the strains (Fig. 7a), in contrast to lower concentrations. Between 0.625 and 5 μM, pNiCoT, pFS45 and pNik conferred detection levels significantly different from the WT strain and from the background luminescence (Student *T*-test). Very interestingly, pNik conferred specific detection thresholds for concentrations as low as 78 nM (Fig. 7b). Thus, the use of additional Ni transporter is proven to be useful to lower the detection limit. The pLW21 plasmid that also encodes the NikABCDE uptake system, but from *E. coli*, gave no advantage to the strain which displayed a highly fluctuating activity for the low concentrations (Fig. 7b). This might be due to interferences with the endogenous *nik* system (membrane insertion, competition for the substrate…).

Discussion

The goal of the present work was to engineer a sensor strain for the detection of Ni. Many microbial sensors have been designated for the detection of metals until now (Bereza-Malcolm et al. 2014), but only two were described for the detection of Ni (Tibazarwa et al. 2001). In the present work we chose to work on single-input biosensors. For that purpose the minimal requirement is a sensor protein that also acts as a

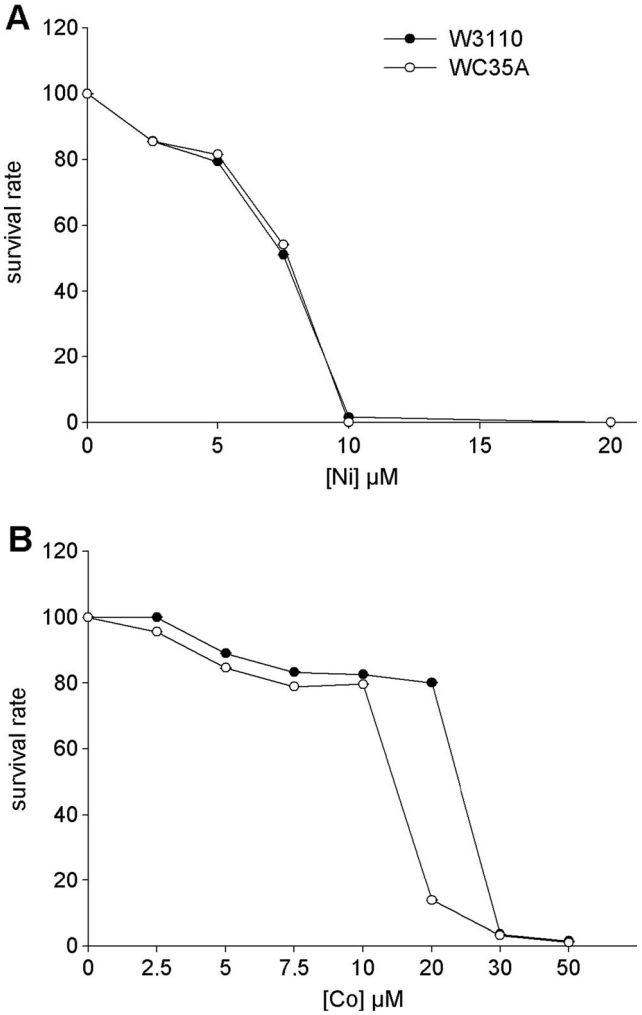

Fig. 4 Impact of the C35A-*rcnR* mutation on the growth. The wild-type strain (W3110) or C35A-*rcnR* isogenic mutant was grown in M63 minimal medium supplemented with 0.4 % glucose and increasing amounts of $NiCl_2$ (**a**) or $CoCl_2$ (**b**). OD_{600nm} was recorded after 16 h of aerobic incubation at 37 °C. The experiments were repeated at least three times. The data shown are from a single representative experiment

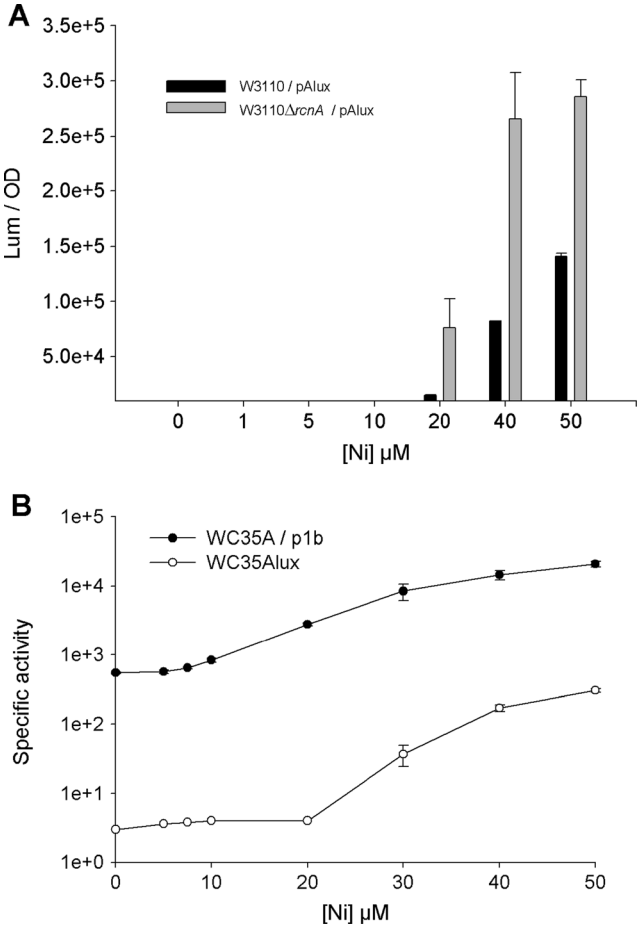

Fig. 5 Ni accumulating sensors. **a** The W3110 (WT) strain (*black*) or its Δ*rcnA* derivative (WRCA1) (*grey*) were transformed with plasmid pAlux (C35A-*rcnR* P*rcnA*::*lux*) and grown in the presence of increasing concentrations of Ni. The luminescence was recorded during 8 h; the value of the maximum activity is plotted on the graph (luminescence (A.U.)/$OD_{600\ nm}$). The experiments were repeated at least three times; SD are presented on the graph. **b** The WC35A strain (C35A-*rcnR*) transformed by plasmid p1b (*PrcnA*::*lux*) (*black circles*) or WC35Alux strain (C35A-*rcnR PrcnA::lux* Δ*rcnA*) (*white circles*) were grown in the presence of increasing concentrations of Ni. The luminescence was recorded during 8 h. The value of the maximum specific activity is plotted on the graph (luminescence (A.U.)/$OD_{600\ nm}$/time (min)). The experiments were repeated at least three times; SD are presented on the *graph*

transcriptional regulator and a target gene regulated by the sensor and fused to reporter genes.

The Ni regulators described so far belong to seven structural families (Pal et al. 2014). These are the ArsR, anti-sigma/sigma 70, CsoR/RcnR, NikR, two-component, PadR and Fur families that all act as repressors (Ma et al. 2009; Pal et al. 2014). The *rcnR* regulator is part of the RcnR/CsoR family (Iwig et al. 2008) (Liu et al. 2007). In *E. coli*, RcnR controls the expression of *rcnA* as well as its own expression by a derepression mechanism (Blaha et al. 2011). RcnR is predicted to act as a dimer losing its affinity for DNA upon binding two Ni or two Co per dimer, involving the conserved motif H3, C35, H60 and H64 (Iwig et al. 2008; Liu et al. 2007).

Here, first, the *rcnA* gene was fused to the *luxCDABE* genes on a plasmid under the control of *rcnR* either expressed from the chromosome or from the same plasmid. The measures of luminescence confirmed that the *rcn* system is specific to Ni and Co and respond gradually to these metals when added at 0.1–50 µM. The response was more sensitive when *rcnR* was expressed from the chromosome. In this case, it is expected that less RcnR is present and is more readily in interaction with Ni or Co and thus alleviates the repression of P*rcnA* for smaller concentrations of metals. The choice of the *rcn* system has been made because several mutations were previously described that changed the specificity of RcnR and rendered it specific to Ni (Iwig et al. 2008). Indeed we show here that C35A-RcnR loses Co detection while retaining Ni sensing (Fig. 2a, b). However, this mutation also impacted Ni detection by rendering the sensor strain slightly less

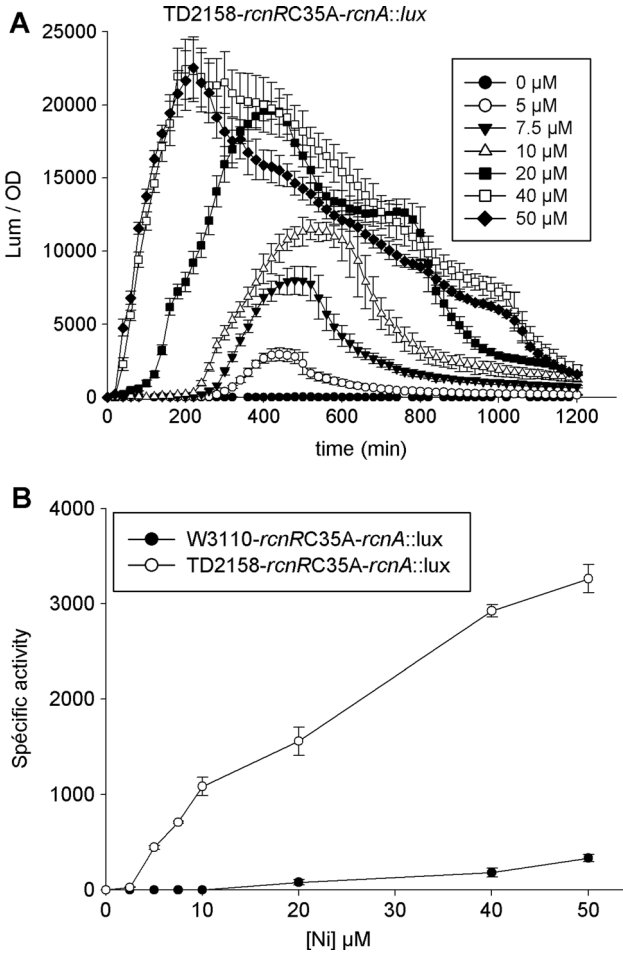

Fig. 6 The detection of Ni is influenced by the genetic background. **a** Kinetics response of the TDRAlux strain grown in the presence of increasing $NiCl_2$ concentration. The luminescence was recorded during 20 h; the activity is presented on the graph (luminescence (A.U.)/$OD_{600\ nm}$). The experiments were repeated at least three times; SD are presented on the graph. **b** Comparison of two *E. coli* sensor strains. TDRAlux strain and WC35Alux strain were grown in the presence of increasing concentrations of Ni. The luminescence was recorded during 16 h. The value of the maximum specific activity is plotted on the graph (luminescence (A.U.)/$OD_{600\ nm}$/time (min)). The experiments were repeated at least three times; SD are presented on the *graph*

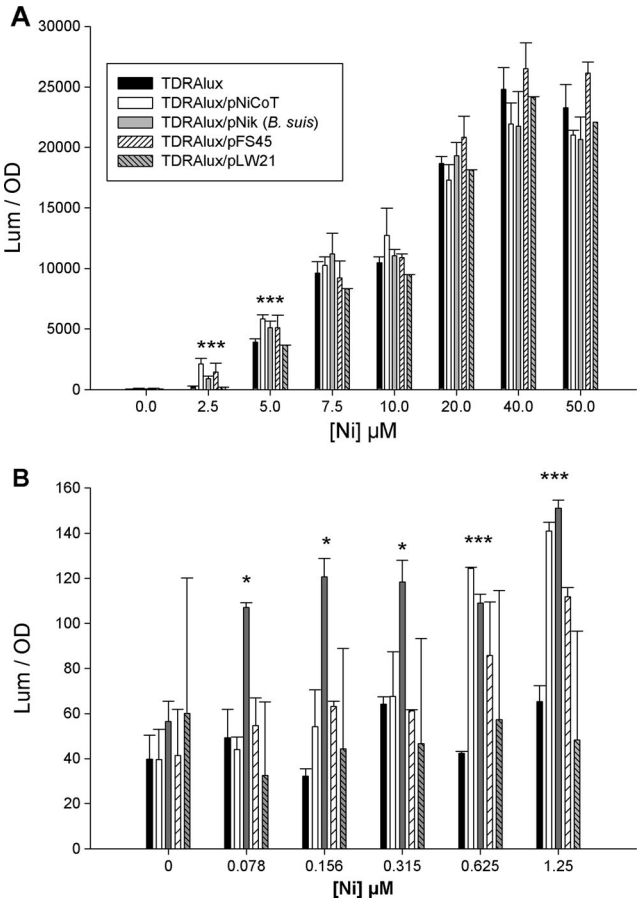

Fig. 7 Contribution of additional Ni importers. The TDRAlux strain (*black*) was transformed with pNiCoT (*white*), pNik-B. suis (*grey*), pFS45 (*hatched white*) or pLW21 (*hatched grey*). **a** Growth was performed with Ni concentrations in the micromolar range. **b** Growth was performed with Ni concentrations in the nanomolar range. The luminescence was recorded during 8 h; the value of the maximum activity is plotted on the graph (luminescence (A.U.)/$OD_{600\ nm}$). The experiments were repeated at least three times; SD are presented on the graph. Significant differences between the different strains for a given concentration (Student *T* test, *P* value<0.05) are marked by *asterisks*

sensitive (compare p6a on Fig. 1a with pAlux on Fig. 2a). When the C35A-*rcnR* allele was recombined in the chromosome in place of the wt-*rcnR* allele, again a slight loss in sensitivity was observed (Fig. 3a, b). As RcnR regulates the synthesis of the efflux pump RcnA, this mutation could impact not only the sensitivity of the sensor but also the survival of the strain in the presence of Ni or Co. It was expected that this strain could be slightly less resistant to Ni and less resistant to Co. Figure 4 shows that the MIC for Ni was not significantly modified, whereas that for Co was diminished. The higher sensitivity to Co toxicity could constitute a limit for the use of the sensor strain as it could promote cell death. However, it is unlikely because for their use as a sensor, bacteria will not be used as fresh cultures. They will first be grown in the absence of metal, then lyophilized and stored. Finally, at the moment of their use, they will be rehydrated and incubated with the liquid solution to be assayed for a short period of time (see Prévéral et al. (2015) and Brutesco et al. (2015) in this special issue). In these conditions, there are little chances for Co to be toxic.

As said previously, the introduction of the C35A mutation in *rcnR* and its recombination into the chromosome rendered the sensor less sensitive to Ni. To overcome this problem, we also engineered a Δ*rcnA* strain able to accumulate more Ni and thus to lower detection limits. Indeed this strain emitted more luminescence, suggesting that more Ni was present, but the detection threshold remained unchanged, in agreement with RcnA being an efflux pump functioning when excess metal is present. Then, a *rcnA::lux* fusion was constructed and inserted into the chromosome in order to (a) create an *rcnA* mutant and (b) construct a stable *rcnA::lux* fusion. This chromosomal fusion displayed a behavior similar to the fusion expressed from

the plasmid (Fig. 5b), with less emitted luminescence but a superior signal/background ratio (considering the condition with no added metal as the background).

Once the genetic circuit was validated, the last parameter to be adjustable was the strain in which it would give the best detection limits. Several *E. coli* K12 strains were tested. Minor to large variations in luminescence were detected, but with roughly the same detection thresholds. Finally, the TD2158 strain was tested. This strain isolated from sewage is a true wild-type *E. coli* and is non-pathogenic. Moreover, the strain was tested for its revival capacities after lyophilization and displayed higher rate of survival as well as better luminescence expression once rehydrated than *E. coli* K12 (Brutesco et al. (2015) in this special issue). The C35A-*rcnR* and *rcnA::lux* gene modification were recombined into the chromosome of TD2158. The kinetics response to Ni of strain TDRAlux displayed two phases (Fig. 6a). For concentrations above 20 μM, the response was very quick (≤20 min). For concentrations below 10 μM, the time of response was 200 min. However, the detection limit was much lower than in the corresponding WC35lux strain (derived from W3110). The behavior of the kinetics suggests that there might exist a different Ni homeostasis system in this strain than in other *E. coli*. The genome of strain TD2158 is being sequenced and the annotation not yet finished (M. Ansaldi, personal communication). However, we searched the genome for the Ni homeostasis genes and retrieved the *nikR/nikABCDE* ABC transporter and the *rcnR/rcnA* efflux system. No NiCoT homolog was found. It suggests that either there exist Ni homeostatic genes yet unknown or that the Ni-handling genes are similar to other *E. coli*. In this case, some other parameters like external membrane transport, cross-talk with other metal influx or efflux systems would change the Ni intracellular concentration. Still the improvement of the strain towards Ni sensing was investigated by adding heterologous Ni uptake systems. Transporters of the ABC type, *nikABCDE*, or of the NiCoT type were tested. No significant differences appeared when the *nik* system of *E. coli* K12 was expressed from a plasmid (Fig. 7a). This suggests that this system may compete with the endogenous *nik* transporter of TD2158. By contrast, when the NiCoT from *Y. pseudotuberculosis* or from *N. aromaticivorans* were expressed, the boundaries of the detection limit were significantly lowered to reach the value of 0.6 μM (35 μg l^{-1}). This detection limit is of particular interest in the frame of the European Drinking Water Directive, Council Directive 98/83/EC, that is 20 μg/L (0.34 μM) for Ni (EDW Directive 1998). It is even more suitable considering the WHO norm for drinking water of 70 μg/L (1.2 μM) (WHO 2011). Most interestingly, the detection limit reached 80 nM (4.7 μg L^{-1}) when the cells expressed the *nik* system from *B. suis*, providing a comfortable margin towards the drinking water directives. The last improvement of the strain would be to insert the *nik* genes from *B. suis* in the *E. coli* chromosome or to constitutively express the *nik* genes of *E. coli* by replacing the native promoter by a strong promoter using the promoter swapping technique.

As a conclusion, the biosensor constructed in this study is a promising tool for the rapid and easy detection of alert levels of Ni in drinkable water. It can also be used as an alert system in natural aquatic environments.

Acknowledgments This work was supported by the French National Agency ANR Ecotech, COMBITOX.

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