



Zinc-Induced Transposition of Insertion Sequence Elements Contributes to Increased Adaptability of *Cupriavidus metallidurans*

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Bacteria can respond to adverse environments by increasing their genomic variability and subsequently facilitating adaptive evolution. To demonstrate this, the contribution of Insertion Sequence (IS) elements to the genetic adaptation of *Cupriavidus metallidurans* AE126 to toxic zinc concentrations was determined. This derivative of type strain CH34, devoid of its main zinc resistance determinant, is still able to increase its zinc resistance level. Specifically, upon plating on medium supplemented with a toxic zinc concentration, resistant variants arose in which a compromised *cnrYX* regulatory locus caused derepression of CnrH sigma factor activity and concomitant induction of the corresponding RND-driven *cnrCBA* efflux system. Late-occurring zinc resistant variants likely arose in response to the selective conditions, as they were enriched in *cnrYX* disruptions caused by specific IS elements whose transposase expression was found to be zinc-responsive. Interestingly, deletion of *cnrH*, and consequently the CnrH-dependent adaptation potential, still enabled adaptation by transposition of IS elements (*ISRme5* and *IS1086*) that provided outward-directed promoters driving *cnrCBAT* transcription. Finally, adaptation to zinc by IS reshuffling can also enhance the adaptation to subsequent environmental challenges. Thus, transposition of IS elements can be induced by stress conditions and play a multifaceted, pivotal role in the adaptation to these and subsequent stress conditions.

Keywords: metal ions, resistance, *Cupriavidus*, insertion sequence element, adaptation

INTRODUCTION

Bacteria continuously evolve to survive different environmental challenges and the field of evolutionary biology has long been interested in the interplay between generation of genetic diversity and natural selection favoring the best adapted organism. The central dogma that evolution proceeds through natural selection of heritable mutations was first proposed by Darwin (Darwin, 1910). In addition, however, Darwin also suggested that environmental stress might generate a diversity of genetic variants, upon which subsequent natural selection works (Darwin, 1910). The claim that mutation rate might be influenced by stress is, however, still under debate (Rosenberg and Hastings, 2004; Roth, 2011). The classic experiments of Luria and Delbrück (1943) and Lederberg (1989) demonstrated for the first time that mutations can arise without the influence

of selective stress. However, the lethal selections they used could likely not have detected mutations induced by selective conditions, and others have described genetic systems in which selective conditions indeed seemed to increase the mutation rate (Shapiro, 1984; Cairns et al., 1988; Hall, 1990; Foster, 2007; MacLean et al., 2013; Ram and Hadany, 2014). In addition, the coupling of potential mutation-generating systems to local events such as stress-induced transcription of a specific subset of genes (Wright, 2004) might form an example of stress-directed mutation where the mutation is specifically induced by the stress condition that the mutation relieves (Cairns et al., 1988). However, non-selected mutations readily occur during selective conditions disputing the existence of non-random mutations (Foster, 2007).

One frequently encountered type of mutation results from the hopping of transposable elements from the donor to the target site, potentially leading to important phenotypic changes (Mahillon and Chandler, 1998; Mahillon et al., 1999). Insertion sequence (IS) elements, which are widely distributed in the genomes of bacteria as well as higher organisms (Siguiet et al., 2006, 2014), are of interest as they constitute an important driving force for genome plasticity (Kazazian, 2004). On the one hand, intra-genomic transposition of these small elements (<2.5 kb), which generally encode no other functions than those involved in their mobility, can both inactivate or activate adjacent genes by physically interrupting coding sequences or encoding regulatory sequences, respectively. The activation can be caused by a promoter encoded by the element and directed outward to the adjacent gene (Ciampi et al., 1982; Zhang and Saier, 2009; Wang and Wood, 2011; Saier and Zhang, 2014) or by the formation of a hybrid promoter in which the -35 region at the end of the IS element is correctly situated close to the -10 region of the adjacent gene (Jaurin and Normark, 1983; Prentki et al., 1986). On the other hand, homologous IS elements within the chromosome can become substrates of the cell's recombination machinery, which can result in complex chromosomal rearrangements (e.g., inversions, duplications, or deletions) depending on the orientation of the involved IS elements (Louarn et al., 1985; Schneider et al., 2000a; Parkhill et al., 2003).

Selective conditions can promote the activation and redistribution of IS elements that exist in the chromosome. Indeed, stress factors were found to induce the transposition of mobile genetic elements (MGEs) in plants (McClintock, 1984; Studer et al., 2011), flies (González et al., 2010), yeast (Stanley et al., 2010), and bacteria (Ohtsubo et al., 2005; Drevinek et al., 2010). The acquisition of phenotypic traits caused by transposition of IS elements has been well-documented, particularly in bacteria. Different conditions such as UV light, low frequency magnetic fields or sub-inhibitory concentrations of antibiotics were shown to promote transposition of IS elements in *Escherichia coli* (Eichenbaum and Livneh, 1998; Del Re et al., 2003; Lartigue et al., 2006). Transposition of IS elements in response to a selective condition has also been described in other gram-negative genera, e.g., *Pseudomonas* and *Burkholderia* in response to high temperature, conjugation or oxidative stress (Ohtsubo et al., 2005; Christie-Oleza et al., 2009;

Drevinek et al., 2010). Active redistribution of IS elements was also observed in the gram-positive *Deinococcus radiodurans* after exposure to radiation (Pasternak et al., 2010) and in the gram-positive *Bacillus subtilis* under competence-inducing conditions (Takahashi et al., 2007).

Cupriavidus metallidurans strains are, next to metal-contaminated soils (Diels and Mergeay, 1990; Brim et al., 1999; Goris et al., 2001), being increasingly recovered from other anthropogenic environments not typified by metal contamination (Van Houdt et al., 2012; Mijnenonckx et al., 2013), including medically-relevant sources (Coenye et al., 2005; Langevin et al., 2011; D'Inzeo et al., 2015). They are studied for their resistance and adaptation to toxic levels of metal ions. Type strain *C. metallidurans* CH34 counteracts metal ion toxicity via a battery of resistance mechanisms, including transporters belonging to the Resistance Nodulation cell Division (RND), the cation diffusion facilitator (CDF) and the P-type ATPase families, with an important role for its two mega-plasmids as most of the heavy metal resistance mechanisms are encoded on either pMOL28 or pMOL30 (Mergeay et al., 1985, 2003; Nies and Silver, 1995; Nies, 1999, 2003; Monchy et al., 2007; von Rozycki and Nies, 2009; Janssen et al., 2010). In addition, *C. metallidurans* CH34 harbors 21 distinct IS elements belonging to 10 different IS families (Mijnenonckx et al., 2011). Out of the 21 distinct IS elements, active transposition has been observed for *ISRme1*, *ISRme3*, *IS1086*, *IS1087B*, *IS1088*, and *IS1090* (Dong et al., 1992; Collard et al., 1993; Grass et al., 2000; Ma-e-Talat, 2000; Schneider et al., 2000b; Tibazarwa et al., 2000). In fact, active involvement of *IS1087B* in metal resistance has been shown for *C. metallidurans* AE126, which is a derivative of CH34 sensitive to zinc as it only carries plasmid pMOL28 and not plasmid pMOL30 (Collard et al., 1993; Tibazarwa et al., 2000). Spontaneous zinc-resistant AE126 mutants constitutively expressing *cnrCBAT* coding for the RND-driven efflux system *CnrCBAT* appear when AE126 is grown in the presence of 0.8 mM Zn²⁺ (Collard et al., 1993; Grass et al., 2000; Tibazarwa et al., 2000). The *cnrCBAT* operon is regulated by the upstream *cnrYXH* locus coding for the membrane-bound anti-sigma factor *CnrY*, the sensor protein *CnrX*, and the ECF (Extracytoplasmic Function) family sigma factor *CnrH*. The latter is released from the *CnrYX* transmembrane anti-sigma factor complex in the presence of inducers (Ni²⁺ or Co²⁺) and subsequently promotes transcription of both *cnrYXH* and *cnrCBAT* (Grosse et al., 2007; Trepreau et al., 2011, 2014; Maillard et al., 2015). Analysis of two of such derivatives indicated that the inactivation of the gene coding for the anti-sigma factor *CnrY* (by *IS1087B* and a frameshift mutation), and concomitant release of *CnrH* increased transcription of the structural *cnrCBAT* cluster and led to increased (non-specific) Zn²⁺ efflux (Collard et al., 1993; Grass et al., 2000; Tibazarwa et al., 2000).

These observations indicate that IS transposition may play a dynamic role in the genetic adaptation of *C. metallidurans* AE126 to toxic zinc concentrations. Therefore, in this study, we thoroughly scrutinized the mutations leading to, and the genetic determinants essential for increased zinc resistance in AE126. The involvement of IS elements was dissected via the identification of the concerned IS elements, their target sites, and

the effect elicited by their transposition as well as via analysis of transposase expression upon metal ion exposure.

MATERIALS AND METHODS

Strains, Media, and Culture Conditions

Bacterial strains and plasmids used in this study are listed in **Table 1**. *C. metallidurans* strains were routinely cultured at 30°C in Tris-buffered mineral medium supplemented with 0.2% (w/v) sodium gluconate (MM284) as described previously (Mergeay et al., 1985). *E. coli* strains were routinely cultured at 37°C in Lysogeny Broth (LB). Liquid cultures were grown in the dark on a rotary shaker at 150 rpm, for culturing on agar plates 2% agar (Thermo Scientific, Oxoid) was added. When appropriate, the following chemicals (Sigma-Aldrich or Thermo Scientific) were added to the growth medium at the indicated final concentrations: kanamycin [50 µg/ml for *E. coli* (Km⁵⁰) or 1500 µg/ml for *C. metallidurans* (Km¹⁵⁰⁰)], tetracycline (20 µg/ml), chloramphenicol (30 µg/ml), Zn²⁺ (0.1 or 0.8 mM; as zinc sulfate heptahydrate), Ni²⁺ (0.1 mM; as nickel chloride hexahydrate), Cd²⁺ (0.01 mM; as cadmium chloride hemipentahydrate), Co²⁺ (0.05 mM; as cobalt chloride hexahydrate), 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal; 40 µg/ml), and isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.1 mM).

Determination of Growth and Minimal Inhibitory Concentration

The susceptibility of *C. metallidurans* strains to different heavy metals was determined via minimal inhibitory concentrations (MICs). The strains were cultivated in triplicate by inoculating 2 ml MM284 supplemented with different concentrations of heavy metals with 20 µL of a stationary phase culture at 30°C. The MIC is defined as the lowest concentration of heavy metal that will inhibit visible growth of the culture after 2 days of incubation. Growth experiments were performed by 1:100 dilution of stationary phase cultures in fresh MM284 and subsequently for each sample (biological replicates), 200 µL was added to a 96-well white cell culture plate (Nunc Flat-bottom, Thermo Scientific) which was placed into a CLARIOstar® (BMG LABTECH). Plates were incubated at 30°C with shaking for 48 h and at 30-min time intervals, the optical density at 600 nm was measured. The growth data were fitted by the model of Baranyi and Roberts (1994) using the Microsoft Excel add-in package DMFit (Institute of Food Research, Norwich, United Kingdom). Statistical comparison of the growth curve parameters was performed using a one-way ANOVA analysis, followed by a *post-hoc* Tukey test.

Isolation of Zinc-Resistant Mutants

C. metallidurans AE126 was cultivated in MM284 at 30°C up to stationary phase, 10⁹ cells were pelleted and cell suspensions (100 µL) of a serial ten-fold dilution in saline were spread on MM284 agar plates containing a final concentration of 0.8 mM Zn²⁺ and incubated at 30°C. Colony forming units (CFU) were counted after day 2 up to day 5 and survival frequency was calculated as viable cell count on MM284 0.8 mM Zn²⁺ agar

plates divided by viable cell count on MM284 agar plates. The variance-to-mean ratio and mutation rate in a fluctuation assay with 20 independent cultures was calculated according to Luria and Delbrück (1943) and using Fluctuation Analysis CalculatOR (FALCOR) (Hall et al., 2009), respectively.

Analysis of IS Transposition

Transposition of IS elements in the regulatory locus of the *cnr* operon (**Supplementary Figure 1**) was identified by colony PCR (DreamTaq DNA polymerase, Thermo Scientific) with primers CnrFw and CnrRv (**Supplementary Table 1**) and sequencing. A *sacBR* IS trap (Gay et al., 1985; Dong et al., 1992) was used to compare the adaptation potential of AE126^{R4}, which carries an *ISRme5* copy inserted in *cnrX* in the same transcriptional orientation as *cnrYX*, with AE126. Sucrose-resistant derivatives of *C. metallidurans* AE126 and AE126^{R4} carrying pJV240 (Gay et al., 1985; Dong et al., 1992) were selected on LB plates without NaCl and supplemented with 10% (w/v) sucrose and 1500 µg/ml kanamycin. The insertion of *ISRme5* in pJV240 was identified by colony PCR with primer pairs *ISRme5_Fw* and *SacR_Rv* or *pISRme5_Rv* and *SacR_Rv*, respectively (**Supplementary Table 1**).

Whole Genome Gene Expression Microarrays

The whole-genome expression profile of AE126^{R2} and AE126^{R3} were compared to its parental strain AE126 by whole genome oligonucleotide microarrays. The strains were cultivated by inoculating 30 ml of MM284 in biological triplicates with 300 µL of a stationary phase culture at 30°C. These subcultures were allowed to grow until an OD₆₀₀-value of around 0.6 was reached. Next, cells were harvested by centrifugation for 2 min at 10,000 rpm and the bacterial pellets were flash frozen by immersion into liquid nitrogen. The bacterial pellets were kept frozen at -80 °C until total RNA extraction was performed using the SV Total RNA Isolation system (Promega Corporation). The quantity of extracted RNA was measured using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific). The RNA quality was determined with a Bioanalyzer (Agilent 2100 Electrophoresis Bioanalyzer Agilent Technologies). Only extracted RNA with a RNA integrity number (RIN number) of higher than eight was accepted for further analysis (Schroeder et al., 2006). Twenty micrograms of RNA were reverse transcribed following the instructions provided with the Pronto kit (Promega). Microarray slide spotting, RNA labeling, hybridization, and data analysis were performed as previously described (Monsieurs et al., 2011). The full description of the microarray data have been deposited at the Gene Expression Omnibus website (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE74091.

cnrH Mutant Deletion Construction

The *cnrH* gene of *C. metallidurans* AE126 was amplified by PCR (Phusion High-Fidelity DNA polymerase) with primer pairs *cnrH_Fw-Rv* (**Supplementary Table 1**), providing *HindIII/EcoRI* restriction sites. Afterwards, these PCR products were cloned as a *HindIII/EcoRI* fragment into the mobilizable

TABLE 1 | Strains and plasmids used in this study.

Strain or plasmid	Genotype/Relevant characteristics	Reference
STRAIN		
C. Metallidurans		
AE126	pMOL28 <i>cnrYXHCBAT</i> , Zn ^S	(Mergeay et al., 1985)
AE126 ^{R1}	Nonsense mutation in <i>cnrX</i> , 2d	This study
AE126 ^{R2}	Frameshift, 1-bp deletion in <i>cnrX</i> , 5d	This study
AE126 ^{R3}	Frameshift, 5-bp duplication in <i>cnrY</i> , 5d	This study
AE126 ^{R4}	IS <i>Rme5</i> insertion in <i>cnrX</i> , 5d	This study
AE126 Δ <i>cnrH::tet</i>	Deletion of <i>cnrH</i> , Tc ^R	This study
AE126 Δ <i>cnrH::tet</i> ^{R1}	IS <i>Rme5</i> insertion between Δ <i>cnrH::tet</i> and <i>cnrC</i>	This study
AE126 <i>cnrH::tet</i> ^{R2}	IS1086 insertion between Δ <i>cnrH::tet</i> and <i>cnrC</i>	This study
AE104	Ni ^S Co ^S Zn ^S	(Mergeay et al., 1985)
E. coli		
DG1	<i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i> , modification-, restriction-) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i>	Eurogentec (Belgium)
HB101	F ⁻ <i>mcrB mrr hsdS20(rB- mB-) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 glnV44 λ-</i>	Laboratory collection
PLASMID		
pRK600	Helper plasmid; Cm ^R <i>tra</i>	Laboratory collection
pK18mob	<i>lacZα</i> Km ^R <i>oriT oriV</i>	(Katzen et al., 1999)
pK18mob- <i>cnrH</i>	<i>cnrH</i> Km ^R	This study
pK18mob- <i>cnrH::tet</i>	<i>cnrH::tet</i> Tc ^R Km ^R	This study
pJV240	IncQ <i>sacBR</i> , Km ^R	(Dong et al., 1992)
pBBR1MCS2	<i>ori</i> pBBR1 <i>oriT lacZα</i> , Km ^R	(Kovach et al., 1995)
pBBR1MCS2- <i>cnrYXH</i>	<i>cnrYXH</i> of AE126, Ni ^S Co ^S Zn ^S	This study
pBBR1MCS2- <i>cnrYXH</i> ^R	<i>cnrYXH</i> of AE126 ^{R1} , Ni ^S Co ^S Zn ^S	This study
pBBR1MCS2- <i>cnrYXHCBAT</i>	<i>cnrYXHCBAT</i> of AE126, Ni ^R Co ^R Zn ^S	This study
pBBR1MCS2- <i>cnrYXHCBAT</i> ^R	<i>cnrYXHCBAT</i> of AE126 ^{R1} , Ni ^R Co ^R Zn ^R	This study
pBBR1MCS2- <i>cnrYXΔH::tetCBAT</i>	Deletion of <i>cnrH</i> , Ni ^S Co ^S Zn ^S Tc ^R	This study
pBBR1MCS2- <i>cnrYXΔH::tetCBAT</i> ^R	<i>cnrYXΔH::tetCBAT</i> ^R of AE126 Δ <i>cnrH::tet</i> ^{R1} , Ni ^S Co ^S Zn ^R Tc ^R	This study
pGLR1	Km ^R <i>ori</i> pBBR1 <i>oriT</i> ; GFP- <i>luxCDABE</i> reporter	(Benedetti et al., 2012)
pGLR1-P _{IS<i>Rme5</i>} - <i>gfpluxCDABE</i>	Promoter IS <i>Rme5</i> (91 bp)	This study
pGLR1-P _{IS<i>Rme5a</i>} - <i>gfpluxCDABE</i>	Promoter IS <i>Rme5a</i> (Rmet_1251; 209 bp)	This study
pGLR1-P _{IS<i>Rme5b</i>} - <i>gfpluxCDABE</i>	Promoter IS <i>Rme5b</i> (Rmet_1280; 257 bp)	This study
pGLR1-P _{IS<i>Rme5c</i>} - <i>gfpluxCDABE</i>	Promoter IS <i>Rme5c</i> (Rmet_1301; 297 bp)	This study
pGLR1-P _{IS<i>Rme5d</i>} - <i>gfpluxCDABE</i>	Promoter IS <i>Rme5d</i> (Rmet_4152; 239 bp)	This study
pGLR1-P _{IS1088} - <i>gfpluxCDABE</i>	Promoter IS1088 (85 bp)	This study
pGLR1-P _{IS1087B} - <i>gfpluxCDABE</i>	Promoter IS1087B (89 bp)	This study
pGLR1-P _{<i>cnrC</i>} - <i>gfpluxCDABE</i>	Native <i>cnrC</i> promoter AE126	This study
pGLR1-P _{IS<i>Rme5cnrC</i>} - <i>gfpluxCDABE</i>	Fragment <i>cnrC</i> promoter AE126 Δ <i>cnrH::tet</i> ^{R1} (including 3' IS <i>Rme5</i> fragment without its endogenous transposase promoter)	This study
pGLR1-P _{IS1086<i>cnrC</i>} - <i>gfpluxCDABE</i>	Fragment <i>cnrC</i> promoter AE126 Δ <i>cnrH::tet</i> ^{R2} (including 3' IS1086 fragment without its endogenous transposase promoter)	This study

^Ssensitive, ^Rresistant, 2d, and 5d: isolated from the first and second population after 2 and 5 days of exposure, respectively.

suicide vector pK18mob. The resulting pK18mob *cnrH* plasmid from an *E. coli* DG1 transformant selected on LB Km⁵⁰ was further confirmed by sequencing prior to amplifying of the flanking sequences of *cnrH* by inverse PCR (Phusion High-Fidelity DNA polymerase) with primer pair *cnrH_tet_Fw-Rv* (Supplementary Table 1), providing *BcuI/XbaI* restriction sites. At the same time the *tet* gene from pACYC184 (Table 1; Chang and Cohen, 1978) was amplified by PCR (Phusion High-Fidelity DNA polymerase) with primer pair Tet_Fw-Rv

(Supplementary Table 1), providing *BcuI/XbaI* restriction sites. Afterwards, this PCR product was cloned as a *BcuI/XbaI* fragment into the former inverse *cnrH* PCR product. The resulting pK18mob-*cnrH::tet* plasmid from an *E. coli* DG1 transformant selected on LB Tc²⁰ Km⁵⁰ was further confirmed by sequencing prior to conjugation (triparental with *E. coli* HB101 pRK600 as helper) to *C. metallidurans* AE126. The resulting transformants selected on MM284 Km¹⁵⁰⁰ were replica plated on MM284 Tc²⁰ and MM284 Km¹⁵⁰⁰. AE126Δ*cnrH::tet* cells

resistant to Tc²⁰ but sensitive to Km¹⁵⁰⁰ were further confirmed by sequencing.

Identification of *cnrCBAT* Transcription Start Site

The transcription start site of *cnrCBAT* in wild type AE126 and in the two zinc-resistant derivatives AE126Δ*cnrH::tet*^{R1} and AE126Δ*cnrH::tet*^{R2} were identified using the cRACE method (Maruyama et al., 1995; Dallmeier and Neyts, 2013). The strains were cultivated by inoculating 30 ml of MM284 with 300 μL of a stationary phase *C. metallidurans* AE126, AE126Δ*cnrH::tet*^{R1}, or AE126Δ*cnrH::tet*^{R2} culture at 30°C. The cultures were allowed to grow until an OD₆₀₀-value of around 0.6 was reached. Next, cells were harvested by centrifugation for 2 min at 10,000 rpm and the bacterial pellets were flash frozen by immersion into liquid nitrogen. The bacterial pellets were kept frozen at -80°C until total RNA extraction was performed using the SV Total RNA Isolation system (Promega Corporation). The quantity of extracted RNA was measured using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific). The RNA quality was determined with a Bioanalyzer (Agilent 2100 Electrophoresis Bioanalyzer Agilent Technologies). Only extracted RNA with a RNA integrity number (RIN number) of higher than eight was accepted for further analysis (Schroeder et al., 2006). One microgram of RNA was reverse transcribed following the instructions provided with the GoScript™ Reverse Transcription System (Promega) with a gene-specific 5'-phosphorylated oligonucleotide *cnrC_R1* (Supplementary Table 1). Subsequently, three volumes of TE buffer (10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid, pH 8.0) containing 4 μg/ml RNase A (Promega) was added to the reaction. Successively, 28 μL of the resulting cDNAs was circularized by 20 U of T4 RNA ligase (Thermo Scientific) in the presence of 15% (w/v) polyethylene glycol (PEG4000; Thermo Scientific) in a total volume of 50 μL at 37°C for 60 min. Next 1.5 U of Pfu DNA polymerase (Thermo Scientific) was added to the reaction at 37°C for 30 min employing its 3'-5' exonuclease activity to remove unreacted residual first-strand primers and cDNAs. Finally, a 2 μL aliquot was directly used as template for the amplification by PCR (DreamTaq DNA polymerase) with primer pair *cnrC_F1-R2* (Supplementary Table 1), the PCR product was cloned by TOPO cloning (Thermo Scientific) and the transcription start site was identified by sequencing.

Construction Of Plasmids

The *cnr* locus (*cnrYXHCBAT*) and its regulatory part (*cnrYXH*) from *C. metallidurans* AE126 and the zinc-resistant derivative AE126^{R1} were amplified by PCR (Phusion High-Fidelity DNA polymerase) with primer pairs *CnrIns_Fw-CnrIns_Rv* and *CnrIns_Fw-CnrT_Rv* (Supplementary Table 1), respectively, providing *SacI/BcuI* restriction sites. Afterwards, these PCR products were cloned as a *SacI/BcuI* fragment into pBBR1MCS2. The resulting pBBR1MCS2-*cnrYXH*, pBBR1MCS2-*cnrYXH*^R, pBBR1MCS2-*cnrYXHCBAT*, and pBBR1MCS2-*cnrYXHCBAT*^R plasmids from *E. coli* DG1 transformants selected on LB Km⁵⁰ were further confirmed by sequencing prior to transformation to *C. metallidurans* AE104. In addition, the *cnr* locus

(*cnrYXΔH::tetCBAT*) from *C. metallidurans* AE126 Δ*cnrH::tet* (see above) and the zinc-resistant derivative AE126 Δ*cnrH::tet*^{R1} (Table 1) were amplified by PCR (Phusion High-Fidelity DNA polymerase) with primer pair *Cnr_Fw-RvpBBR1MCS2* (Supplementary Table 1). At the same time, the pBBR1MCS2 (Table 1) plasmid was linearized by PCR (Phusion High-Fidelity DNA polymerase) with primer pair pBBR1MCS2_Fw-Rv (Supplementary Table 1), providing homologous ends with the former amplified *cnr* locus. Afterwards, these PCR products were cloned using the GeneArt® Seamless Cloning and Assembly Enzyme Mix (Thermo Scientific) and the resulting pBBR1MCS2-*cnrYXΔH::tetCBAT* and pBBR1MCS2-*cnrYXΔH::tetCBAT*^R plasmids from a *E. coli* DG1 transformant selected on LB Km⁵⁰ were further confirmed by sequencing prior to conjugation (triparental with *E. coli* HB101 pRK600 as helper) to *C. metallidurans* AE104.

Monitoring of Transcription

For quantitatively assaying endogenous promoter activity, *C. metallidurans* AE126 carrying luminescence reporter constructs (Table 1) were constructed to analyze expression from the *ISRme5*, *IS1088*, and *IS1087B* transposase promoters. DNA fragments comprising the IS endogenous promoter (from the left inverted repeat until the transposase start codon) were amplified by PCR (Phusion High-Fidelity DNA polymerase) for *ISRme5* (91 bp), *IS1088* (85 bp), and *IS1087B* (89 bp) with primer pairs, p*ISRme5_Fw-Rv*, p*IS1088_Fw-Rv*, and p*IS1087B_Fw-Rv*, respectively (Supplementary Table 1), providing *EcoRI/XbaI* restriction sites. Similarly, fragments comprised of the sequence upstream of the transposase start codon up to the adjacent gene sequence were amplified for all four *ISRme5* copies [209 bp for *ISRme5a* (Rmet_1251), 257 bp for *ISRme5b* (Rmet_1280), 297 bp for *ISRme5c* (Rmet_1301), and 239 bp for *ISRme5d* (Rmet_4152)] with primer pairs p*ISRme5a/b/c/d_Fw* and p*ISRme5_Rv*, respectively (Supplementary Table 1). Afterwards, these PCR products were cloned as *EcoRI/XbaI* fragments into pGLR1. The resulting pGLR1-P*ISRme5/a/b/c/d-gfpluxCDABE*, pGLR1-P*IS1088-gfpluxCDABE*, and pGLR1-P*IS1087B-gfpluxCDABE* plasmids from transformants selected on LB Km⁵⁰ were further confirmed by sequencing prior to conjugation (triparental with *E. coli* HB101 pRK600 as helper) to *C. metallidurans* AE126 (Table 1). The luminescence reporter constructs were inoculated (in triplicate) into 4 ml of MM284 Km¹⁵⁰⁰ and grown for 48 h. These stationary phase cells were diluted 1:100 (v/v) into fresh MM284 Km¹⁵⁰⁰ and grown for 4 h. Next, exponential phase cell suspensions were divided and supplemented with different concentrations of Zn²⁺, Cd²⁺, Ni²⁺ or Co²⁺. For each sample, 200 μL was added to a 96-well black cell culture plate (Nunc Flat-bottom, Thermo Scientific) which was placed into a CLARIOstar® (BMG LABTECH). Plates were incubated at 30°C with shaking for several hours. At 30-min time intervals, the optical density at 600 nm and luminescence were measured. Data are shown as the ratio of the relative light unit to optical density (RLU/OD₆₀₀) normalized to the MM284 control.

The expression of *cnrCBAT* from two zinc-resistant derivatives, AE126 Δ*cnrH::tet*^{R1} and AE126 Δ*cnrH::tet*^{R2}

(**Table 1**), was quantitatively assayed via a transcriptional reporter fusion. A 953 and 853-bp DNA fragment comprising the *cnrC* promoter of AE126 $\Delta cnrH::tet^{R1}$ (harboring also a 3' fragment of *ISRme5* without its transposase promoter) and AE126 $\Delta cnrH::tet^{R2}$ harboring also a 3' fragment of *IS1086* without its transposase promoter) were amplified by PCR (Phusion High-Fidelity DNA polymerase) with primer pairs *ISRme5_Internal-pCnrC_Rv* and *IS1086_Internal-pCnrC_Rv*, respectively (**Supplementary Table 1**). In addition, as control, the native *cnrC* promoter of AE126 was amplified by PCR (Phusion High-Fidelity DNA polymerase) with primer pair *pCnrC_Fw-Rv* (**Supplementary Table 1**). At the same time, the pGLR1 (**Table 1**) plasmid was linearized by PCR (Phusion High-Fidelity DNA polymerase) with primer pair *pGLR1_Fw-Rv* (**Supplementary Table 1**), providing homologous ends with the former amplified fragments. Afterwards, these PCR products were cloned using the GeneArt[®] Seamless Cloning and Assembly Enzyme Mix (Thermo Scientific) and the resulting pGLR1-*P_{ISRme5cnrC}-gfpluxCDABE*, pGLR1-*P_{IS1086cnrC}-gfpluxCDABE*, and pGLR1-*P_{cnrC}-gfpluxCDABE* plasmids from an *E. coli* DG1 transformant selected on LB Km⁵⁰ was further confirmed by sequencing prior to conjugation (triparental with *E. coli* HB101 pRK600 as helper) to *C. metallidurans* AE104 (**Table 1**). The luminescence reporter constructs were inoculated (in triplicate) into 4 ml of MM284 Km¹⁵⁰⁰ and grown for 48 h. These stationary phase cells were diluted 1:100 (v/v) into fresh MM284 Km¹⁵⁰⁰ and grown for 4 h. Next, 200 μ L exponential phase cell suspensions were added to a 96-well black cell culture plate (Nunc Flat-bottom, Thermo Scientific) which was placed into a CLARIOstar[®] (BMG LABTECH). Plates were incubated at 30°C and the optical density at 600 nm and luminescence were measured.

RESULTS

Isolation and Phenotypic Characterization of Zinc-Resistant AE126 Derivatives

The minimal inhibitory concentration (MIC) of Zn²⁺ for *C. metallidurans* AE126, which is type strain CH34 cured from its pMOL30 mega plasmid encoding the *czc* operon (cadmium/zinc/cobalt resistance), is much less than its parent, being 0.2 and 12 mM, respectively. However, AE126 has the capability to spontaneously acquire additional zinc resistance, i.e., the ability to grow in the presence of 0.8 mM Zn²⁺ at a frequency of $\sim 10^{-6}$ (**Table 2**; Collard et al., 1993). Interestingly, using a zinc plate assay two populations of zinc-resistant AE126 derivatives were observed that appeared at different incubation times. The first population, which arose after 2 days of incubation, showed a high variance-to-mean ratio (26 CFU) in a fluctuation assay with 20 independent cultures and a calculated mutation rate to zinc resistance of $0.105 \pm 0.022 \times 10^{-7}$ per generation. However, new colonies emerged on the selective plate in the ensuing days and we defined derivatives specifically emerging at day 5 as the second population. A higher mutation rate to zinc resistance of $0.307 \pm 0.056 \times 10^{-7}$ per generation and lower variance-to-mean ratio (3 CFU) was

calculated for the second population. The lower mutation rate and high variance-to-mean ratio of the first population, with most plates yielding a variable number of mutant colonies while occasional plates showed a large number of mutants (so-called “jackpots”), suggested that the first population was pre-existing while the second population emerged on the plate itself.

Cultured separately in liquid MM284 without zinc demonstrated similar growth rates for AE126 and zinc-resistant derivatives from the first (AE126^{R1}) and second (AE126^{R3}) population (**Table 3**). Moreover, the zinc-resistant derivatives exhibited a similar lag phase that was shorter than that of AE126 (**Table 3**). In addition, both derivatives grew equally fast in the presence of 0.8 mM Zn²⁺ but displayed a prolonged lag phase compared to MM284 without zinc (**Table 3**). Next to increased Zn²⁺ resistance (a 10-fold increase of the MIC), the characterized zinc-resistant AE126 derivatives also displayed an increased resistance to Ni²⁺, Co²⁺ and Cd²⁺ (3, 2.5 and two-fold increase of the MIC, respectively). However, the level of zinc and cadmium resistance of these mutants is still considerably lower than those from the type strain *C. metallidurans* CH34 (**Table 2**).

Genetic Characterization of Zinc-Resistant AE126 Derivatives

In previous work, only two zinc-resistant AE126 derivatives were analyzed (Collard et al., 1993; Grass et al., 2000). Therefore, we scrutinized *cnrY* and *cnrX* of 498 randomly selected zinc-resistant AE126 isolates, of which 296 belong to the first population and 202 belong to the second population, by PCR and sequencing to determine if *cnrY* and *cnrX* were indeed the sole targets leading to increased zinc resistance of AE126 derivatives (**Supplementary Figure 1**). This analysis confirmed that all 498 randomly selected isolates either had an IS element inserted or another mutation (point mutation, small insertions, or deletion) in *cnrY* or *cnrX* (**Figure 1**). Interestingly, however, the first population (46.3%) had a significantly lower contribution of IS elements than the second population (68.8%) as determined by a Fisher Exact test with a $p < 0.05$ (**Figure 1**), indicating that transposition of these IS elements might be promoted by the selective zinc condition.

Characterization of IS-Independent Zinc-Resistant AE126 Mutants

Regarding the IS-independent mutations in *cnrYX*, it is clear that only mutations resulting in inactivation of either *CnrY* or *CnrX* are selected in the zinc plate assays. The IS-independent mutations are dispersed throughout the *cnrYX* locus and include frameshift mutations, non-sense mutations and larger deletions. For instance, a 360-nucleotide deletion was observed including the complete *cnrY* gene.

Two zinc-resistant IS-independent AE126 derivatives, AE126^{R2} (harboring a frameshift mutation caused by a single nucleotide deletion in *cnrX*) and AE126^{R3} (harboring a five nucleotide 5'-CGCGA-3' duplication in *cnrY*), both belonging to the second population of zinc-resistant AE126 derivatives, were selected for whole-genome expression profiling to further

TABLE 2 | Phenotypic characterization of *C. metallidurans* strains carrying different parts of the *cnr* operon and their relation with resistance to different heavy metals.

Strains	Presence of			Cell survival frequency		MIC (mM)		
	CnrYX	CnrH	CnrCBAT	2d	5d	MIC Zn ²⁺	MIC Ni ²⁺	MIC Co ²⁺
AE104	–	–	–	0	0	0.2	0.3	0.3
AE126	+	+	+	10 ^{–6}	10 ^{–6}	0.2	2.5	5
AE126 Δ <i>cnrH::tet</i>	+	–	+	0	10 ^{–8}	0.2	0.3	0.3
CH34 (<i>czc</i> operon)	+	+	+	1	1	12	2.5	5
AE126 ^{R1} , AE126 ^{R2} , AE126 ^{R3} , AE126 ^{R4}	–	+	+	1	1	2	9	12
AE126 Δ <i>cnrH::tet</i> ^{R1} , AE126 Δ <i>cnrH::tet</i> ^{R2}	+	–	+	1	1	2	9	12
AE104 pBBR1MCS2	–	–	–	0	0	0.2	0.3	0.3
AE104 pBBR1MCS2- <i>cnrYXH</i>	+	+	–	0	0	0.2	0.3	0.3
AE104 pBBR1MCS2- <i>cnrYXH</i> ^R	–	+	–	0	0	0.2	0.3	0.3
AE104 pBBR1MCS2- <i>cnrYXHCBAT</i>	+	+	+	10 ^{–6}	10 ^{–6}	0.2	2.5	5
AE104 pBBR1MCS2- <i>cnrYXHCBAT</i> ^R	–	+	+	1	1	2	9	12
AE104 pBBR1MCS2- <i>cnrYX</i> Δ <i>H::tetCBAT</i>	+	–	+	0	10 ^{–8}	0.2	0.3	0.3
AE104 pBBR1MCS2- <i>cnrYX</i> Δ <i>H::tetCBAT</i> ^R	+	–	+	1	1	2	9	12

Cell survival frequency after exposure to 0.8 mM Zn²⁺ was determined after 2 and 5 days of incubation, respectively. Minimum inhibitory concentrations (MICs) of different heavy metals were determined after 2 days of incubation.

TABLE 3 | Growth parameters of different *C. metallidurans* strains in MM284 medium with and without 0.8 mM Zn²⁺.

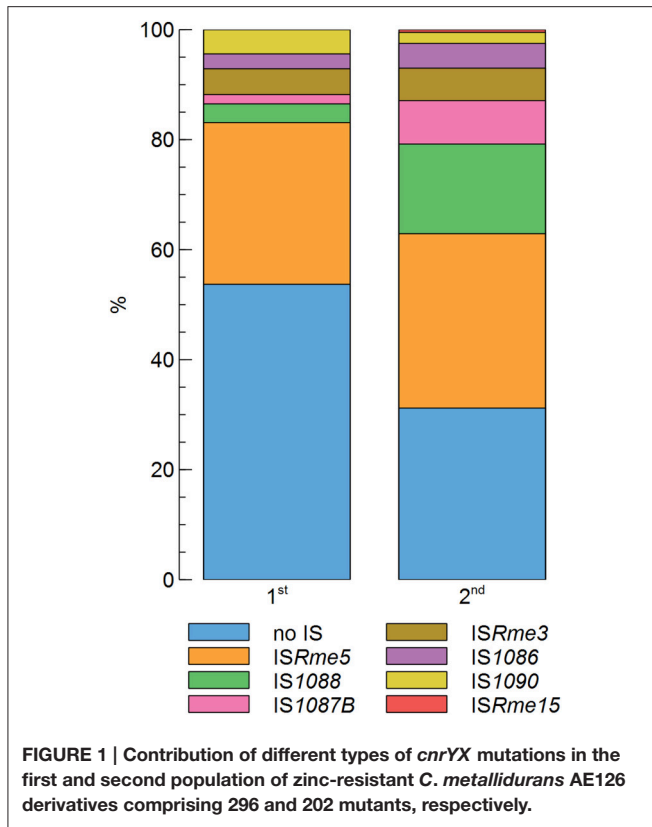
<i>C. metallidurans</i>	MM284		MM284 0.8 mM Zn ²⁺	
	Lag phase (h)	Growth rate (1/h)	Lag phase (h)	Growth rate (1/h)
AE126	22.02 ± 1.19	0.04 ± 0.01	No growth	
AE126 ^{R1}	17.62 ± 0.35* [§]	0.05 ± 0.00	29.54 ± 2.66* [§]	0.11 ± 0.07* [§]
AE126 ^{R3}	18.32 ± 0.32* [§]	0.05 ± 0.00	26.00 ± 3.18* [§]	0.09 ± 0.06* [§]
AE126 Δ <i>cnrH::tet</i>	23.27 ± 2.79	0.06 ± 0.02	No growth	
AE126 Δ <i>cnrH::tet</i> ^{R1}	18.65 ± 0.27* [§]	0.05 ± 0.00	28.91 ± 3.11* [§]	0.11 ± 0.09* [§]

Parameter values are means ± standard deviations. Strains for which the estimates are significantly different (based on ANOVA and post-hoc Tukey) from AE126 and AE126 Δ *cnrH::tet* are marked with * and [§], respectively.

confirm the *cnr*-dependency underlying toxic zinc stress and subsequent acquired zinc resistance. Although, IS-independent mutations are discussed here, the five nucleotide duplication in *cnrY* of AE126^{R3} might suggest an insertion and subsequent excision event of an IS element resulting in a direct target repeat in *cnrY*. Both AE126^{R2} and AE126^{R3} bear mutations leading to inactivation of the anti-sigma CnrYX complex, and whole-genome expression profiling showed 219 and 86 open reading frames (ORFs) that were significantly differentially expressed (>2-fold with an adjusted $p < 0.05$) in comparison with the parental AE126 strain, respectively (**Supplementary Table 2**). Twenty ORFs were commonly differentially expressed in both zinc-resistant derivatives. The mutated regulatory genes *cnrY* and *cnrX* were the most upregulated (11- and 15-fold in AE126^{R2}, and 33- and 57-fold in AE126^{R3}, respectively) followed by the structural locus *cnrCBAT* (nine-fold average for AE126^{R2} and 18-fold average for AE126^{R3}). Transcription of *cnrH* was three- or six-fold upregulated in AE126^{R2} and AE126^{R3}, respectively (**Supplementary Table 2**). As such, it seems that constitutive overexpression of the *cnr* pump is responsible for the acquired zinc resistance. The high amount of other differentially expressed

genes and the fact that most of these genes (93%) were unique for one of both zinc-resistant derivatives indicate that probably different mutations, not involved in the acquired zinc resistance, occurred during the selective exposure to Zn²⁺, which thus may comprise a more general response to stress with mutations randomly happening, supporting zinc-induced mutagenesis for the second population of zinc-resistant derivatives.

To further and unambiguously confirm the essential role of the *cnrYXHCBAT* locus in the capability to spontaneously acquire zinc resistance, complementation assays were performed in the zinc-sensitive and plasmidless *C. metallidurans* CH34 derivative, i.e., strain AE104 (**Table 2**). The findings of Collard et al. (1993) that strain AE104 can never be evolved to withstand the toxic zinc concentration used in zinc plate assays (0.8 mM Zn²⁺) was confirmed. Complementation of *C. metallidurans* AE104 with the complete *cnr* locus, thus both the regulatory and structural genes, resulted in the ability to acquire zinc resistance (frequency of $0.96 \pm 0.38 \times 10^{-6}$ after 48 h exposure to 0.8 mM Zn²⁺), whereas complementation of AE104 with the complete but modified *cnr* locus from AE126^{R1} resulted in full resistance (frequency of 100%; **Table 1**). To test if the CnrH



sigma factor could drive other potential resistance genes as well, the parental regulatory *cnrYXH* locus, and the mutated *cnrYXH* locus from AE126^{R1} were introduced in AE104 but no zinc resistance could be acquired. Thus, the presence of the *cnrCBAT* genes encoding the RND-driven efflux system was essential to acquire zinc resistance (Table 2). These complementation assays showed that the *cnrYXHCBAT* cluster is necessary for acquiring zinc resistance at high frequency in *C. metallidurans* AE104 or AE126 and that the inactivation of *cnrYX* and concomitant CnrH-mediated derepression of *cnrCBAT* is responsible for this zinc resistance.

Characterization of IS-Dependent Zinc-Resistant AE126 Mutants

Focusing further on the IS-dependent zinc-resistant mutants, *ISRme5* appeared to be the main contributor to the obtained insertions both in the first (29.4%) and second (31.7%) population (Figure 1). Other identified IS elements included *ISRme3*, *ISRme15*, *IS1090*, *IS1088*, *IS1086* and *IS1087B*. Comparing the contribution of specific IS elements in the zinc-resistant AE126 population to their abundance in the genome showed that some IS elements were clearly more represented than would be expected from their copy number (Figure 2). *ISRme5* (four identical copies in the genome) had a significant higher contribution to both populations, and *IS1087B* (two identical copies in the genome) to the second population as determined by a Fisher Exact test with a $p < 0.05$. In addition, *IS1088* (nine identical copies in the genome) was significantly

more represented in the second than in the first population (Fisher Exact test; $p < 0.05$), but comparing its contribution to the second population to its abundance in the genome revealed no significant higher contribution than would be expected from its copy number (Figure 2).

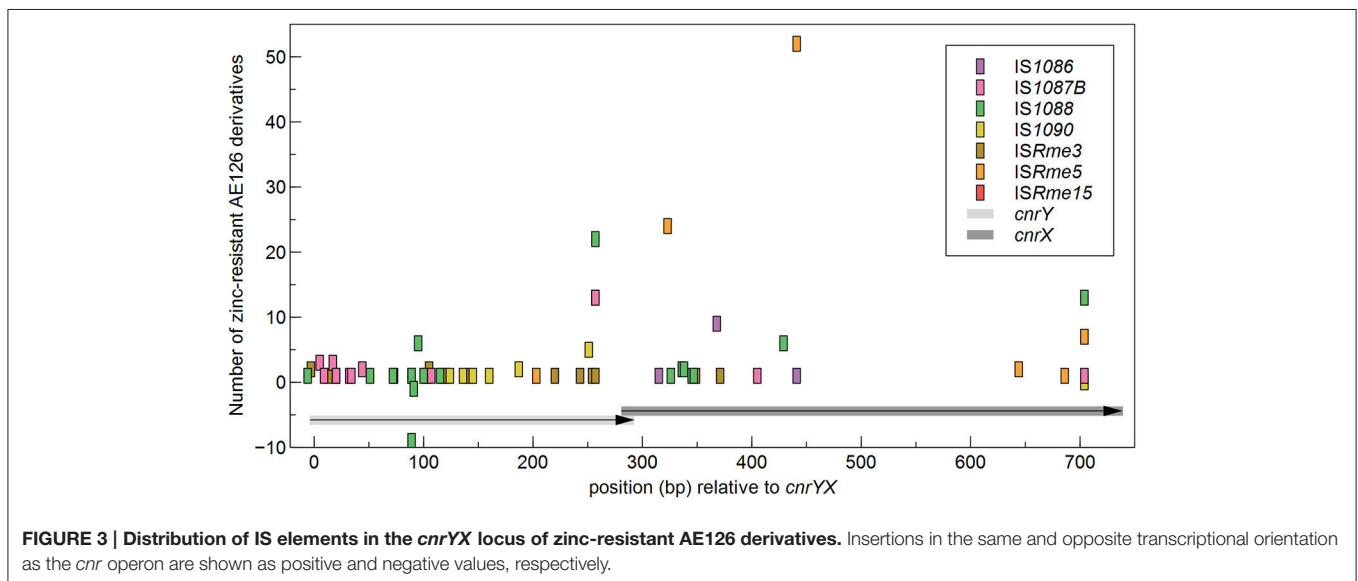
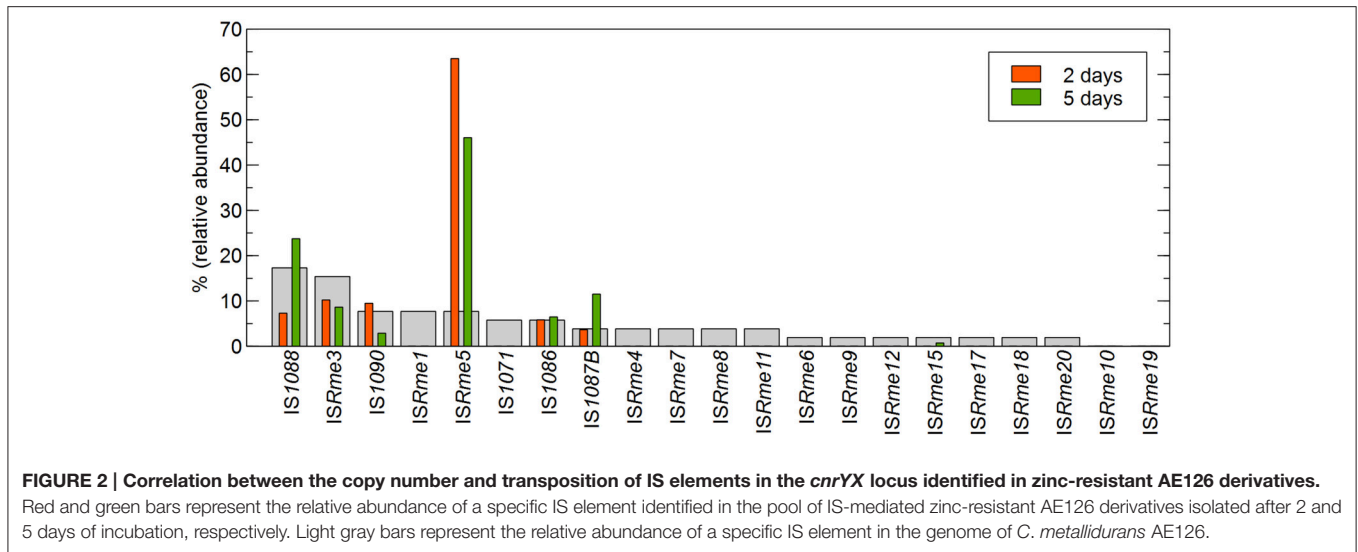
Next, the identified IS insertion sites were scrutinized. A different use of target genes (i.e., *cnrY* or *cnrX*) was detected for the identified IS elements, although this had no influence on zinc resistance as determined by cell survival at 0.8 mM Zn^{2+} and by MIC testing (data not shown). Most IS elements inactivated *cnrY* with the exception of *ISRme5* and *IS1086*. In general, all IS elements, except *IS1088*, inserted exclusively in the same transcriptional orientation as the disrupted gene. No difference in target site use was detected between mutants from the first or second population. *ISRme5* used six different insertion sites throughout the target locus and two were used preferentially (Figure 3).

A position-specific scoring matrix was derived from the multiple sequence alignment of all *ISRme5* target sites (Figure 4). This position-specific scoring matrix was used to scan the genome sequence of *C. metallidurans* CH34 via the RSAT motif scan (Turatsinze et al., 2008) and resulted in numerous hits (Supplementary Table 3). Besides *cnrX*, other genes involved in regulation are found in the list composed of the top 100 hits, including genes encoding for transcriptional regulators, receptor proteins, a sensor histidine kinase, and a signal transduction protein. In addition, the family of transporters is with 21 genes also overrepresented in the motif scan. The other identified IS elements in *cnrYX* showed a more random insertion pattern although *IS1087B* preferentially inserted into the 5'-region of the regulatory locus.

Characterization of *cnrH*-Independent Zinc-Resistant AE126 Mutants

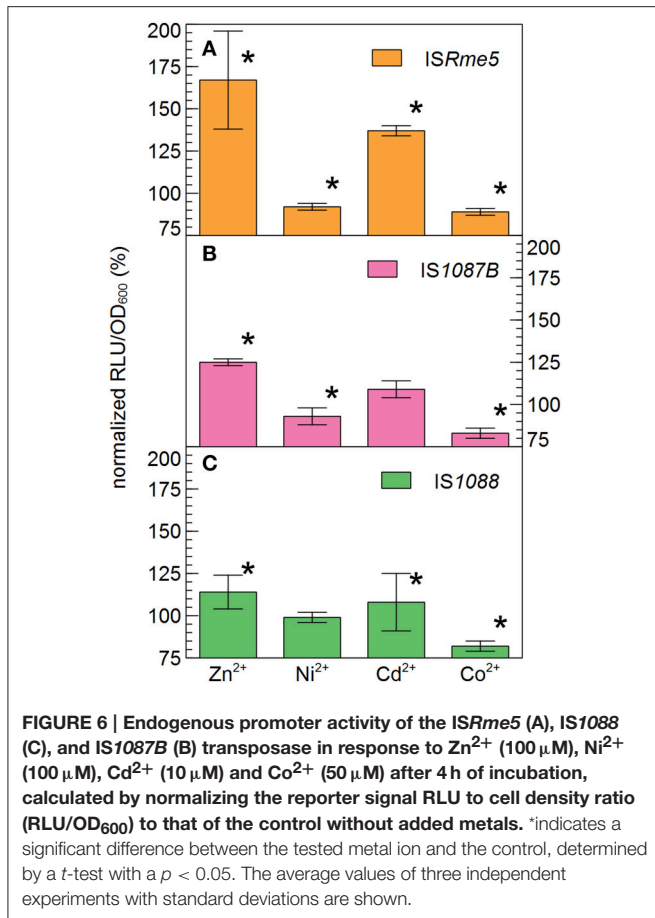
To further analyze the adaptation capacities of *C. metallidurans* AE126 to toxic zinc concentrations, a *cnrH* deletion strain was created to assess if other resistance mechanisms than CnrH-mediated expression of *cnrCBAT* could result in increased zinc resistance. No difference in zinc resistance was observed between the parental and $\Delta cnrH$ deletion strain, however, as expected, the *cnrH* deletion resulted in a drastically decreased cobalt and nickel resistance (Table 2). Interestingly, zinc-resistant AE126 $\Delta cnrH::tet$ derivatives could be isolated after exposure to 0.8 mM Zn^{2+} , but only after a minimum exposure time of 96 h and at a frequency 100-fold less compared with AE126 (Table 2). Cultured separately in liquid MM284 without zinc demonstrated similar growth rates for AE126, AE126 $\Delta cnrH::tet$, and the zinc-resistant derivative AE126 $\Delta cnrH::tet^{R1}$ (Table 3). In addition, again, the zinc-resistant derivative exhibited a lag phase that was shorter than that of AE126 $\Delta cnrH::tet$ and AE126 (Table 3). Moreover, AE126 $\Delta cnrH::tet^{R1}$ displayed the same growth rate in the presence of 0.8 mM Zn^{2+} than the CnrH-dependent zinc-resistant AE126^{R1} and AE126^{R3} (Table 3).

Phenotypic characterization of zinc-resistant AE126 $\Delta cnrH::tet$ derivatives further indicated an increased resistance



to Ni^{2+} (22-fold) and Co^{2+} (32-fold; **Table 2**), indicative for a role of the *cnr* locus. Genetic characterization of six independent zinc-resistant AE126 $\Delta\text{cnrH}::\text{tet}$ derivatives revealed for five derivatives an insertion of *ISRme5* at the same target site 25 bp upstream the *cnrC* start codon (**Figure 5**). One representative was further used and designated AE126 $\Delta\text{cnrH}::\text{tet}^{\text{R1}}$. The other derivative (AE126 $\Delta\text{cnrH}::\text{tet}^{\text{R2}}$) carried an *IS1086* insertion 29 bp upstream of the *cnrC* start codon (**Figure 5**). To exclude any other possible involvement of additional resistance determinants, complementation assays were performed where the complete but modified *cnr* operon of AE126 $\Delta\text{cnrH}::\text{tet}$ and AE126 $\Delta\text{cnrH}::\text{tet}^{\text{R1}}$ were transferred to *C. metallidurans* AE104, respectively. These complementation assays demonstrated that *cnrYX* $\Delta\text{cnrH}::\text{tetCBAT}^{\text{R}}$ mediated the increased zinc resistance (**Table 2**). Furthermore, transcriptional reporter fusions with

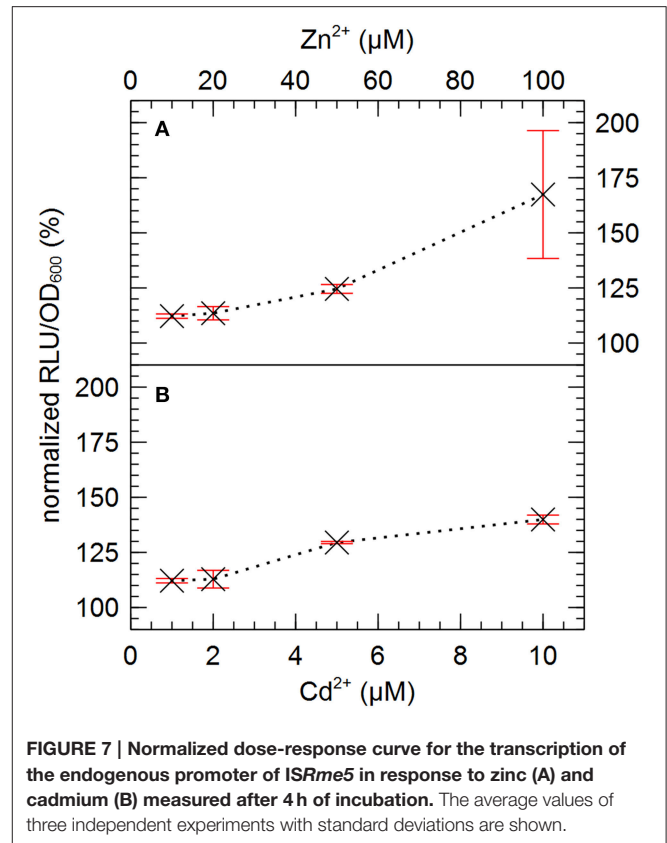
cnrC promoter fragments of AE126 $\Delta\text{cnrH}::\text{tet}^{\text{R1}}$ (953 bp fragment harboring also a 3' fragment of *ISRme5* without its transposase promoter) and AE126 $\Delta\text{cnrH}::\text{tet}^{\text{R2}}$ (853 bp fragment harboring also a 3' fragment of *IS1086* without its transposase promoter) confirmed the constitutive expression of *cnrCBAT* (**Supplementary Figure 2**). Therefore, the insertion of these IS elements in the intergenic region between $\Delta\text{cnrH}::\text{tet}$ and *cnrC* resulted in a constitutively increased transcription of *cnrCBAT*, which was also confirmed by microarray analysis of AE126 $\Delta\text{cnrH}::\text{tet}^{\text{R1}}$ (**Supplementary Table 4**). Since read-through expression from the transposase promoter did not account for this increased expression, the transcription start site of *cnrCBAT* in AE126 $\Delta\text{cnrH}::\text{tet}^{\text{R1}}$ (*ISRme5* insertion) was identified and showed that expression is driven by an outward-directed promoter positioned at the 3' extremity of *ISRme5* (**Figure 5**). This could also be deduced for *IS1086* based



copies exerts a negative effect on *ISRme5* endogenous promoter activity. This is especially true for *ISRme5b* where almost no expression is detected, i.e., <0.5% (Figure 8). Furthermore, although the reporter construct composed of only the promoter region of *ISRme5*, thus without additional genomic sequences, is Zn^{2+} -inducible, no Zn^{2+} induction was detected for the reporter constructs composed of upstream genomic content of *ISRme5a*, *ISRme5b* and *ISRme5d*. The reporter construct of *ISRme5c*, however, was more than two-fold induced for Zn^{2+} (Figure 8).

Enhanced Adaptation Potential to Other Stress Challenges

The effect of IS transposition in the *cnrYX* genes on the general fitness was analyzed. In this test, the parental AE126 and the zinc-resistant *C. metallidurans* AE126^{R4} strain were used. AE126^{R4} carries an additional *ISRme5* element inserted in *cnrX* in the same transcriptional orientation as the *cnr* operon, yielding a continuous read-through *ISRme5* transposase expression as whole-genome expression verified that expression of *cnrYX* is induced in CnrH-dependent zinc-resistant AE126 derivatives (Supplementary Table 2). The pJV240 plasmid was introduced into AE126 and AE126^{R4}, and biological triplicates were subsequently exposed to 10% sucrose. Sucrose-resistant derivatives were isolated and the pJV240-encoded *sacBR* locus

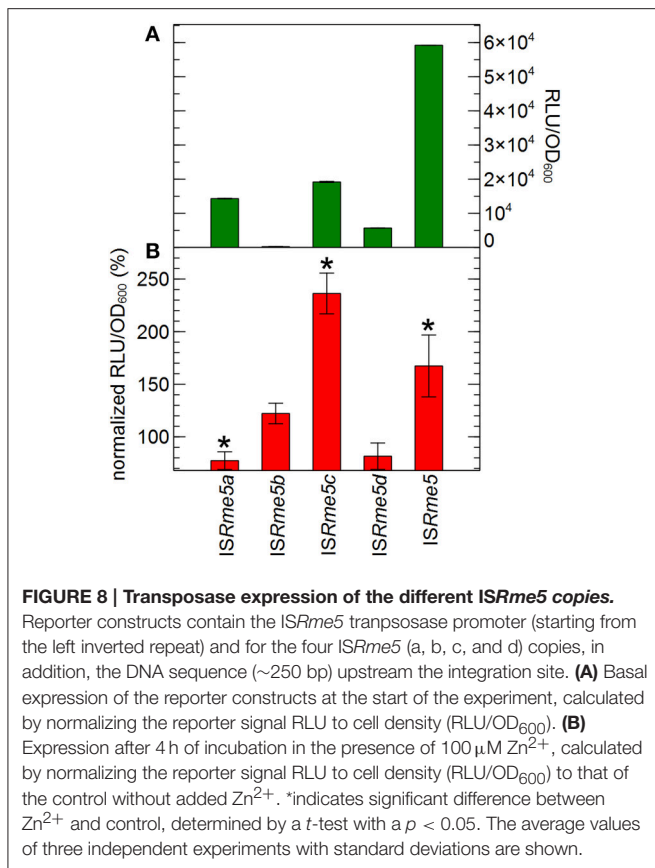


was screened by PCR to determine the involvement of *ISRme5*. AE126^{R4} pJV240 displayed a higher survival frequency ($4.78 \pm 1.58 \times 10^{-6}$) than AE126 pJV240 ($1.42 \pm 0.30 \times 10^{-7}$) and *ISRme5* was found to disrupt *sacBR* in sucrose-resistant AE126^{R4} pJV240 derivatives (3/20) opposed to sucrose-resistant AE126 pJV240 derivatives (0/20).

DISCUSSION

MGEs have been shown to provide their hosts with a wide diversity of adaptive traits. Although, transposition of IS elements mostly results in detrimental mutations, a fraction of insertions can be beneficial and lead to increased survival. The simplest effect of IS transposition is the inactivation of a gene at a particular target site. In addition, IS transposition can also lead to altered expression of adjacently located genes by co-transcription or by providing a (hybrid) promoter or other protein binding elements located inside the IS element.

It was previously demonstrated that *C. metallidurans* AE126 could readily acquire increased zinc resistance (Collard et al., 1993). We observed that zinc-resistant AE126 derivatives arose at different time points after exposure to 0.8 mM Zn^{2+} and all 498 tested derivatives had mutations targeting the regulatory locus of the RND-driven CnrCBA efflux system. The first population arose after an exposure time of 48 h and new derivatives kept appearing in the ensuing days with a second population defined as derivatives that arose after an exposure time of 120 h. The



first population displayed a high variance-to-mean ratio, with most plates yielding a variable number of mutant colonies while occasional plates showed a large number of mutants (so-called “jackpots”), indicating the involvement of pre-existing mutations (Luria and Delbrück, 1943). Interestingly, the second population displayed a higher mutation rate and lower variance-to-mean ratio. Although, the latter was still three times the theoretical value of one when only stress-induced mutagenesis is considered, the much lower value and higher mutation rate are indicative for the involvement of zinc-induced mutagenesis in the second population. Furthermore, mutants from both populations have the same growth rate on zinc medium suggesting once more that the second population may originate from selection on the selective zinc plate. Adaptive mutation and accumulation of colonies throughout incubation time has been described for the activation of a promoterless phenol degradation operon in starving *Pseudomonas putida*. Although, phenol-utilizing mutants already appeared on phenol-minimal plates on day 2, the majority of the colonies began to appear on day 3 and continued to accumulate during the next 7 days (Kasak et al., 1997). In addition, mutations to ciprofloxacin resistance also continually occurred in non-dividing *E. coli* cells during a 7-day exposure to ciprofloxacin in agar and the late-appearing resistant mutants had no growth rate defect compared to wild type or early-appearing mutants (Riesenfeld et al., 1997). Moreover, *E. coli* cells could utilize glycerol in the absence of Crp by activation of the silent *glpFK* glycerol utilization operon by transposition

of IS5 upstream of *glpFK*. The transposed IS5 element harbors a permanent bend and IHF binding site at its 3'-end that promoted the expression of the adjacently located *glpFK* operon (Zhang and Saier, 2011). When *crp*⁻ cells of *E. coli* are incubated on solid glycerol minimal medium, Glp⁺ colonies first appeared after 3 days of incubation and new colonies continued to appear in the ensuing days. However, wild type and *crp* Glp⁺ cells plated on the same solid medium formed already colonies in less than 2 days, indicating that the Glp⁺ mutants that arose from the *crp* cells on glycerol minimal medium plates were not present in the cell culture before plating (Zhang and Saier, 2011).

For acquiring zinc resistance at high frequency (10⁻⁶), inactivation of anti-sigma factor CnrYX complex is essential to liberate CnrH and increase *cnrCBAT* expression. Although the efflux pump CnrCBAT is necessary to mediate zinc efflux, the role of the sigma factor CnrH in promoting *cnrCBAT* expression is not. In the absence of *cnrH*, expression of *cnrCBAT* and acquisition of zinc resistance could be mediated by the correct insertion of *ISRme5* or *IS1086* carrying an outward-directed promoter positioned at their 3' extremity, albeit to a lower frequency (10⁻⁸), and only after a minimum exposure time of 96 h. These mutants displayed the same growth rate on zinc medium than CnrH-dependent zinc-resistant AE126 derivatives from the first population, which were isolated after 48 h, suggesting that these CnrH-independent zinc-resistant derivatives may originate from selection on the selective zinc plate. In addition, using the cRACE method, the wild type promoter transcription start site of the *cnrCBAT* structural resistance genes was found to be located downstream of *cnrH* as previously reported by Grass et al. (2000) and not upstream as previously reported by Tibazarwa et al. (2000).

Exposure of AE126 to 0.8 mM Zn²⁺ revealed transposition of seven different IS elements in the *cnrY* or *cnrX* gene (occurrence of transposition *ISRme5* > *IS1088* > *ISRme3* > *IS1087B* > *IS1090* > *IS1086* > *ISRme15*). Active IS transposition in *C. metallidurans* was already observed for *ISRme1*, *ISRme3*, *IS1086*, *IS1087B*, *IS1088* and *IS1090* (Dong et al., 1992; Collard et al., 1993; Grass et al., 2000; Ma-e-Talat, 2000; Schneider et al., 2000b; Tibazarwa et al., 2000; Mijnenonckx et al., 2011), but not for *ISRme5* and *ISRme15*. Overall, IS elements contributed more to the second population that arose after five days of incubation on plate. *ISRme5* was the main IS contributing to both populations, whereas *IS1088* and *IS1087B* showed a higher contribution to the second compared with the first population. Most of the transposed IS elements (>95%) inserted exclusively in the same transcriptional orientation as *cnrCBAT*, although inactivation of CnrYX is sufficient to acquire zinc resistance. It is unclear why a given site should be used in only one orientation (Lampe et al., 1998). Most transposases did not show insertion site specificity, only for *ISRme5* two hotspot loci were identified that were used preferentially and specifically by *ISRme5*. Scanning the genome of *C. metallidurans* CH34 with the generated target sequence matrix resulted in numerous hits, with various hits in genes involved in regulation and transport processes, indicating the evolutionary power that *ISRme5* might exert as inactivation of those genes mostly results in significant phenotypic alterations. In some cases transposases prefer a particular DNA structure, most notably

bent DNA, as target site (Craig, 1997). It seems that this was the case for the *IS1087B* transposase as it mostly inserted in the 5'-region of *cnrY*, indicating that the *IS1087B* transposase does not recognize target DNA in any sequence-specific manner but that certain sequences surrounding the insertion site may allow local structures to form that are conducive for *IS1087B* insertion. There is precedence for this phenomenon in the non-random use of target sequences of P transposable elements, where the local target preference depends more on DNA structure than on primary sequence (Liao et al., 2000).

Our study showed for the first time that the endogenous promoters of the *ISRme5*, *IS1088* and *IS1087B* transposases can be induced by metal ions. This increased promoter induction was specific for zinc and cadmium but not nickel and cobalt. The dual zinc and cadmium induction could be explained by the fact that both metal ions have many similar (bio)chemical characteristics. Other examples of transposase transcriptional expression induced by environmental stimuli include *Tn4652* and *IS1246* in *P. putida* KT2440 by zinc, cadmium and nickel but not cobalt (Haritha et al., 2009), and *ISFtu1* and *ISFtu2* by spermine in *Francisella tularensis* (Carlson et al., 2009). Elevated transpositional activity of several IS elements was also observed at high temperature in *Burkholderia multivorans* (Ohtsubo et al., 2005). However, the induced transpositional activity in the examples above did not mediate enhanced tolerance to the inducer. Although, the *ISRme5* transposase promoter internal to the element is inducible by zinc and cadmium, the genomic region upstream IS integration has a strong regulatory effect on the expression of the IS element. The upstream genomic region resulted in reduced promoter activity of all *ISRme5* copies, especially for *ISRme5b*, and only the *ISRme5c* copy was found to be metal-inducible. In addition, the transposase of *ISRme5* (*IS481* family member) has no stop codon within the element. Consequently, the *ISRme5b* transposase has a prolonged carboxy-terminal end compared with the other copies, generated by its insertion site, which might influence its enzymatic stability and hence, could contribute to the possible diminished transposition activity of this copy compared to the other *ISRme5* elements. Therefore, the evolutionary success of *ISRme5*, as probably for all IS elements, depends not only on the element itself, but also on its genomic integration site. Taking into account that many transposases preferentially act in *cis* (Mahillon and Chandler, 1998), which reduces the probability that transposase expression from a particular element would activate transposition of related copies elsewhere in the genome, the higher basal expression level and zinc-inducibility of the *ISRme5c* transposase indicate that most *ISRme5*-mediated *CnrYX* inactivation events are probably the result of *ISRme5c* transposition. Repression or enhancement of promoter activity by the upstream flanking sequence is also observed for other MGEs (Lavie et al., 2004).

Bacteria respond to environmental changes by adapting the expression of key genes, however, such reprogramming requires time and energy. Interestingly, all tested zinc-resistant derivatives showed a considerably shorter lag phase in liquid MM284 without zinc compared to wild type, rendering the

evolved population better suited to quickly respond to other environmental challenges. For instance, in conditions where the lag phase is long, adaptations reducing the lag phase are highly beneficial as the evolved population can start their exponential growth earlier and consume most of the environmental nutrients before the wild type exits the lag phase (Oxman et al., 2008). Furthermore, adaptation to zinc by IS reshuffling can have an additional impact on the adaptation to subsequent stress challenges. Insertion of an IS element in *cnrYX* in the same transcriptional orientation as the *cnr* operon will probably result in read-through transposase expression as whole-genome expression indeed verified that expression of *cnrYX* is induced in *CnrH*-dependent zinc-resistant AE126 derivatives. Thus, redistribution of IS elements yielding IS-dependent zinc-resistant derivatives, hence harboring an additional copy of the IS element in a constitutively transcribed region, might result in an increased pool of the transposase in the cell and subsequent enhance the adaptation potential to new environmental challenges, such as was shown here for *ISRme5* and *SacB*-mediated sucrose hypersensitivity. The higher survival frequency of the IS-dependent zinc-resistant derivative compared to wild type can be explained by (i) an increased pool of *ISRme5* transposase resulting in *ISRme5*-mediated *sacBR* disruption, (ii) the possible insertion and subsequent excision of *ISRme5*, resulting in various possible frameshift deletions (Ooka et al., 2009) or duplications (Mahillon and Chandler, 1998) inactivating *sacBR*, however, the presence of these mutations was not investigated, and (iii) the uncharacterized genetic background of the IS-dependent zinc-resistant derivative, which likely, consistent with other derivatives from the second population, harbors more mutations.

To summarize, it was shown that zinc induced the expression of IS elements and that these elements mediate the adaptation of *C. metallidurans* to zinc stress by modulating its transcriptional network or by providing outward-directed mobile promoters.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: MM, AA, and RVH. Performed the experiments: JV. Analyzed the data: all authors. Wrote the paper: JV, AA, and RVH.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00359>

Supplementary Figure 1 | Schematic representation of the *cnr* operon of megaplasmid pMOL28 from *C. metallidurans* extracted from the MaGe platform (<https://www.genoscope.cns.fr/agc/microscope/mage/>) with *cnrYX* encoding two membrane-bound anti-sigma factors, *cnrH* a sigma factor, *cnrCBA* a RND efflux pump, and *cnrT* a cation diffusion facilitator.

Black line represent the amplicon generated with the primer pair Cnr_Fw and Cnr_Rv used for screening the *cnrYXH* structural locus.

Supplementary Figure 2 | Promoter activity of the *cnrC* promoter of AE126 Δ *cnrH::tet*^{R1} and AE126 Δ *cnrH::tet*^{R2}. Data are represented as fold change of the report signal normalized to cell density (RLU/OD₆₀₀) obtained for AE104 pGLR1-P_{ISRme5cnrC-gfpLuxCDABE} (953 bp fragment harboring also a 3' fragment of *ISRme5* without its transposase promoter) and AE104 pGLR1-P_{IS1086cnrC-gfpLuxCDABE} (853 bp fragment harboring also a 3' fragment of *IS1086* without its transposase promoter) compared with the control AE104 pGLR1-P_{cnrC-gfpLuxCDABE}.

Supplementary Table 1 | Primers used in this study.

Supplementary Table 2 | Whole-genome microarray analysis of gene expression in two zinc-resistant AE126 derivatives (AE126^{R2} and AE126^{R3}). Data are represented as fold change relative to the parental AE126. Only open reading frames (ORFs) that were significantly expressed (>2-fold with an adjusted *p* < 0.05) are shown. **(A)** ORFs differentially expressed in both derivatives, **(B)** ORFs differentially expressed in AE126^{R2} and **(C)** ORFs differentially expressed in AE126^{R3} with ND not determined. The full description of

the microarray data have been deposited at the Gene Expression Omnibus website (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE74091.

Supplementary Table 3 | Scan of the *C. metallidurans* CH34 genome sequence (+ for forward and – for reverse strand) with the position-specific scoring matrix obtained from the multiple sequence alignment of the *ISRme5* target sites in 87 zinc-resistant AE126 derivatives using RSAT. The weight score $W_s = \log(P(S|M)/P(S|B))$ is the probability for the sequence segment *S* to occur according to the motif model *M*, whereas *P(S|B)* is the probability for the same sequence *S* to occur under the background model *B*. The weight score is thus the log-ratio of the likelihood of *S* in these two respective models. Top 100 targets are shown.

Supplementary Table 4 | Transcriptional expression of the *cnrCBAT* cluster, determined by whole-microarray analysis, in two zinc-resistant AE126 derivatives (AE126^{R2} and AE126^{R3}) and in a zinc-resistant AE126 Δ *cnrH::tet* derivative (AE126 Δ *cnrH::tet*^{R1}). Data are represented as fold change relative to the parental AE126 and AE126 Δ *cnrH::tet*, respectively. The full description of the microarray data have been deposited at the Gene Expression Omnibus website (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE74091.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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