A Zur-mediated transcriptional regulation of the zinc export system

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Abstract:

The control of cellular zinc (Zn) concentrations by dedicated import and export systems is essential for the survival and virulence of *Pseudomonas aeruginosa.* The transcription of its many Zn transporters is therefore tightly regulated by a known set of transcription factors involved in either the import or the export of Zn. In this work, we show that the Zur protein, a well-known repressor of Zn import, plays a dual role and functions in both import and export processes. In a situation of Zn excess, Zur represses Zn entry, but also activates the transcription of *czcR*, a positive regulator of the Zn export system. To achieve this, Zur binds at two sites, located by DNA footprinting in the region downstream the *czcR* transcription start site. In agreement with this regulation, a delay in induction of the efflux system is observed in the absence of Zur and Zn resistance is affected. Surprisingly, deletion of the entire *czcR* 5' untranslated region or impairing Zur binding in the region is sufficient to restore the growth defect of a *zur* mutant in excess of zinc, disclosing an important link between Zur and zinc export. The discovery of this regulation highlights a new role of Zur as global regulator of Zn homeostasis in *P. aeruginosa*.

Introduction:

Metal ions are essential to the functioning and survival of all known forms of cellular life. Among all trace elements, zinc (Zn) is of particular importance because it functions as a cofactor of many essential enzymes and is, more generally, needed to sustain protein structure. Zn is found at the top of the Irving-Williams series (Mg < Ca < Mn < Fe < Co < Ni < Cu > Zn) that ranks metal ions according to their ability to form complexes with proteins (1). This is why, when present in excess inside the cell, Zn tends to outcompete other metals and cause protein mismetallization, the major cause of its cellular toxicity (2). The intracellular concentration of Zn has therefore to be tightly and finely controlled by complex homeostasis mechanisms. In bacteria, the cytoplasmic Zn concentration is maintained within the range of 10^{-3} to 10^{-4} M, making it the second most abundant metal after iron (3).

In the Gram-negative bacterium *Pseudomonas aeruginosa*, several systems are involved in Zn homeostasis (4,5). Zn import into the periplasm is mediated by TonB-dependent importers such as ZnuD. A P-type ATPase and three ABC transporters help with the uptake of Zn from the periplasm into the cytoplasm. In case of metal excess, Zn is exported by a specific P-type ATPase CadA, a Resistance-Nodulation-Cell Division (RND) efflux pump (CzcCBA), and two cation diffusion facilitators (CDF) that play a minor role in Zn resistance (3,6). Control of Zn import and export is extremely important for some bacterial pathogens, especially for their interaction with their host, whether an animal (7) or a plant (8). *P. aeruginosa*, for example, is able to grow and thrive in Zn-poor environments such as the bloodstream, where trace metals are chelated to prevent microbial growth (3). Conversely, in a phagolysosome, high concentrations of Cu and Zn have antimicrobial properties and can eliminate the pathogen (9).

Several regulators of Zn homeostasis have been identified in *P. aeruginosa*. The major regulator of Zn import is the Zinc Uptake Regulator, hereafter Zur, protein (formerly Np20, (10)), a Fur (Ferric Uptake Regulator)-like protein (11). In the presence of an excess of cytoplasmic Zn, Zur binds to the metal ion, dimerizes, and, in its dimeric form, binds to specific Zur boxes on the DNA to repress genes involved in Zn import (4,10). Zn export is controlled by two other regulators: CadR, a MerR-type regulator that activates the transcription of the P-type ATPase CadA (12), and CzcR, a response regulator (RR) of the two-component system CzcRS, which, when phosphorylated by CzcS, activates the transcription of the efflux pump CzcCBA that expels Zn from the cell (13). Until now, the regulatory networks controlling Zn import and Zn export have been considered essentially independent from one another and no crosstalk between them has been substantiated.

In this study, we discovered that the Zur protein is needed for the full expression of the Zn efflux system when *P. aeruginosa* switches from a low-Zn environment to an excess-Zn situation. We show that Zur accelerates *czcR* transcription, and thus activates Zn efflux, by binding to two sites downstream of the *czcR* promoter, in a region corresponding to its 5' untranslated region (5'-UTR). Interestingly, the increase in *czcR* transcription following the removal of this region in the chromosome restores the growth defect of a *zur* mutant in presence of Zn-excess. This new function makes Zur the keystone of Zn homeostasis in *P. aeruginosa*, acting not only as a repressor of Zn import but also as activator of Zn export.

Results:

Zur binds to the czcRS promoter

The Zur protein, when concentrations of Zn are sufficient or excessive, binds to a 17 bp DNA Zur box to repress genes involved in Zn import (14). In a global bioinformatics search on the *P. aeruginosa* genome, we found, as expected, Zur boxes in the promoter regions of genes involved in the import of Zn, but also, intriguingly, in *czcRS* promoter region (5). This suggested a possible direct regulatory link, mediated by Zur, between the import and export of Zn. In order to validate the *in silico* analysis, we purified the Zur protein and performed an electrophoretic mobility shift assay (EMSA) on the *czcR* promoter. A clear DNA shift was observed in the presence of Zur (Fig. 1A). The interaction is Zn-dependent as no shift was detected in presence of TPEN, a Zn chelator. DNA footprint analysis revealed two boxes located approximately 40 bps upstream of the translation start codon of *czcR* (Fig 1B, 1C and S1A). The transcription start site of *czcR* was determined by 5'RACE and it was found 300 bps upstream of the *czcR* translation start, revealing a 292-nucleotide 5'-UTR (Fig 2A, S1B). Promoter fusions (-*gfp*) confirmed that a region longer than 292 bps upstream of the ATG is required to sustain *czcRS* transcription in the presence of Zn (construct #1 and #2 in Fig 2B).

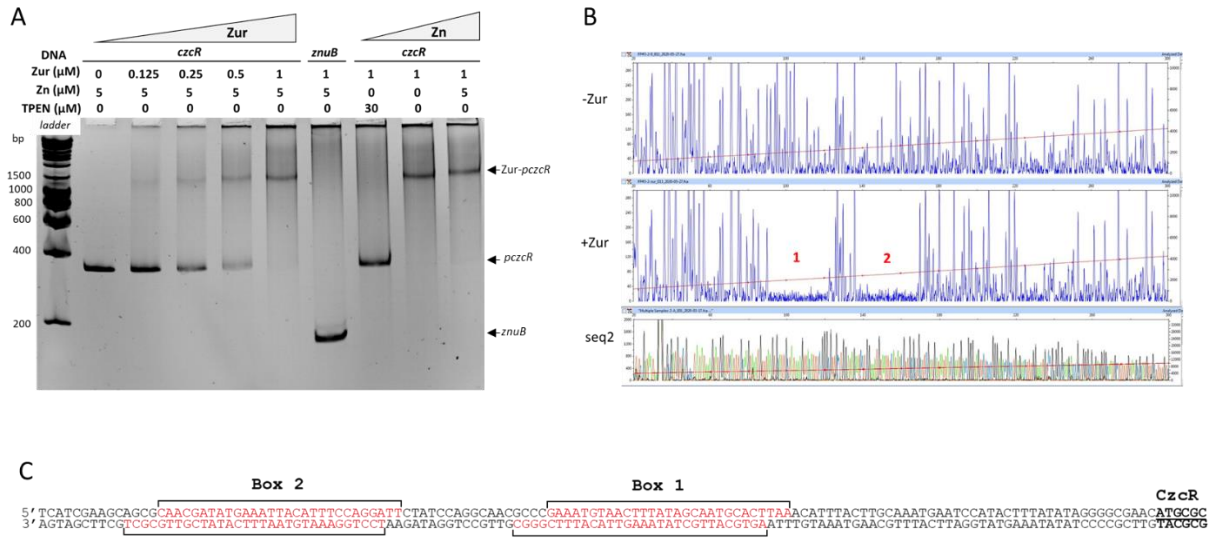

Figure 1: Zur binds to the *czcR* 5'-UTR. **A)** Electrophoretic Mobility Shift Assay (EMSA) using purified Zur protein and *czcR* (5'-UTR) or *znuB* DNA region as negative control. 25 ng of DNA per reaction was used. The Zur protein, Zn and TPEN (Zn chelator) concentrations are indicated at the top of the figure. **B)** DNAse I footprinting of *czcR* promoter in the absence or presence or of 1 µM Zur protein. DNA fragments were analyzed by capillary electrophoresis. The two Zur boxes are indicated by numbers 1 and 2 in red. The sequencing reaction is visible below the figure (seq2). C) Sequence of the two boxes as determined by the footprinting experiments (Fig 1B coding strand; Fig S1A template strand).

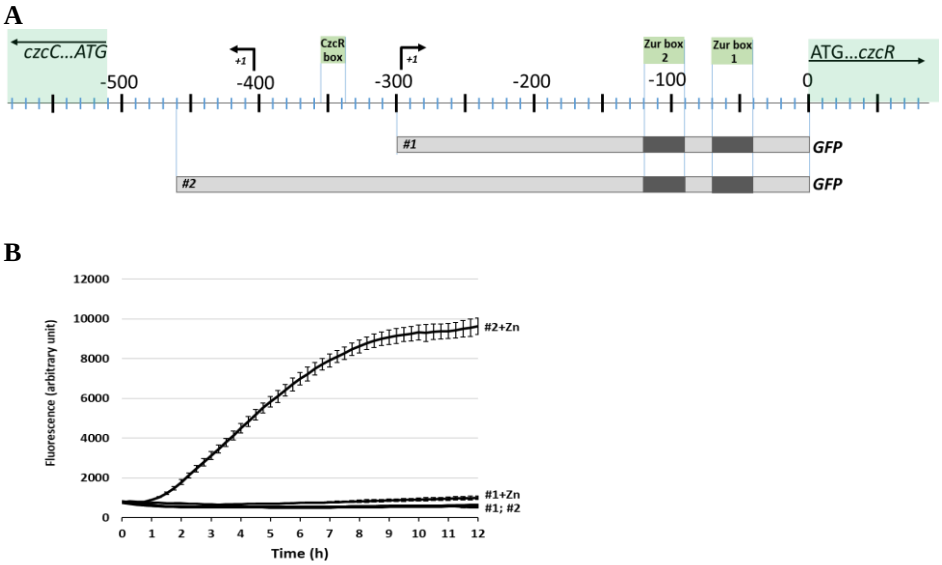

Figure 2: GFP reporter of the *czcR* promoters. **A)** Map of the *czcR* promoter and 5'-UTR region with the two Zur boxes, the CzcR DNA binding site (according to (15)) and the *czcR* and *czcC* transcription start sites (+1). The number represents the nucleotide position relative to the translation start of *czcR* gene. **B)** GFP promoter fusions #1 and #2 transformed into the wt PAO1 strain. Time 0 corresponds to the addition of 2 mM $ZnCl_2$. Standard deviations of the triplicates are indicated.

To determine whether the binding at the two Zur boxes was independent, we repeated the band shift assay using different versions of the *czcR* promoter, where box1 (mut1), box 2 (mut2), or both (mut1+2) Zur boxes were mutated at key positions (Fig S2A). A higher shift was obtained when the two Zur boxes were present compared to the presence of only one, and no shift was detected when the two boxes were mutated (Fig S2B), indicating that the two boxes support the binding of the regulator independently.

Zur is involved in zinc resistance

In order to investigate whether Zur had a role in the regulation of Zn export, we characterized the phenotype of a Δ*zur* mutant. We found that, in the absence of Zur, *P. aeruginosa* is more sensitive to Zn excess than the parental strain (Fig. 3A). The phenotype is milder than in a Δ*czcRS* mutant, but more severe than in a Δ*cadR* mutant (12).

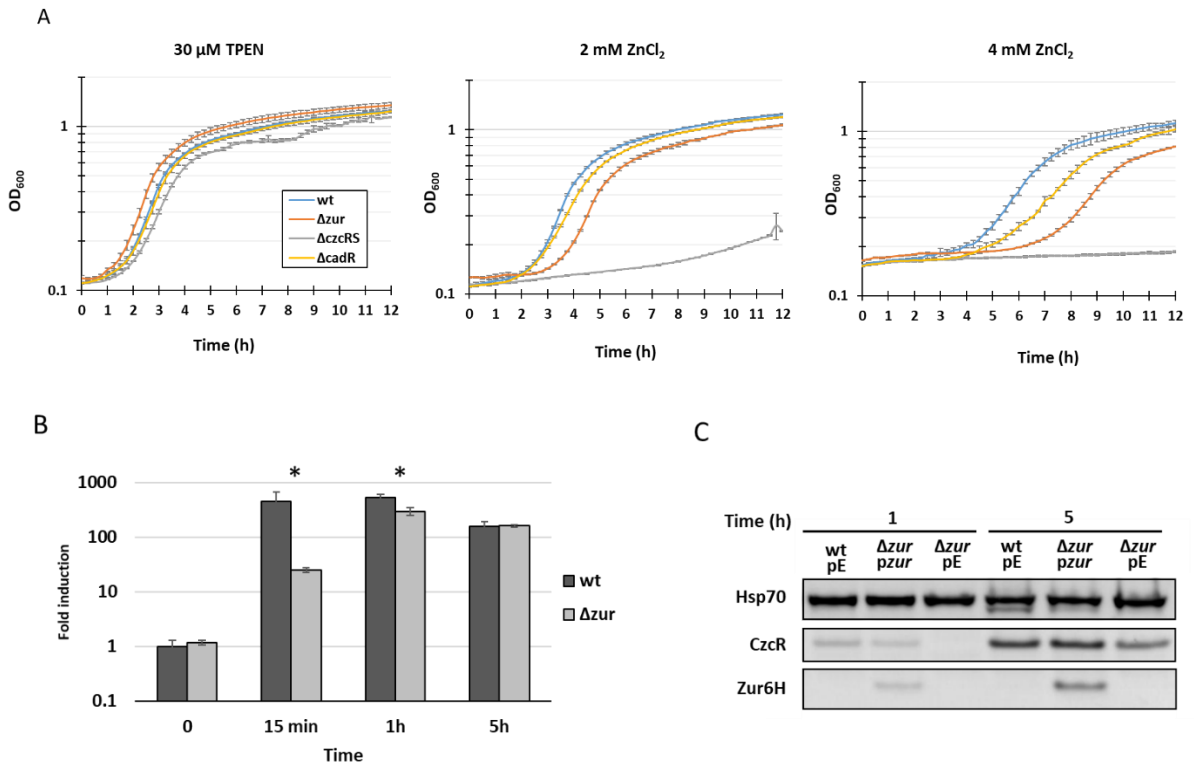

Figure 3: Zur is involved in CzcR expression. **A)** Growth curves of *P. aeruginosa* wt, Δ*zur*, Δ*czcRS* and Δ*cadR* mutants in Zn-depleted medium (30 µM TPEN), in 2 mM and 4 mM $ZnCl_2$, as indicated. **B)** Expression of *czcR* measured using qRT-PCR. RNAs were extracted at 0, 15 min, 1h and 5h after addition of 2 mM $ZnCl_2$ as indicated. Fold expressions are compared to the wt strain at t0 and normalized by *oprF* expression. Standard deviations of the triplicates are indicated. Statistical analyses were performed according to the Student's *t*-test and *p*-values are given as follows: ≤0.05 (*). **C)** Western blot analysis of total proteins sampled 1h and 5h after addition of 2 mM Zn. wt and Δ*zur* mutant containing the empty plasmid (pE) or the IPTG inducible *zur:6His* gene (p*zur*). Blots were decorated with anti-CzcR, anti-6His for Zur detection and anti-Hsp70 as loading control.

The effect of the *zur* deletion on *czcR* transcription was assessed using qRT-PCR. In the absence of Zur, *czcR* expression decreased significantly compared to the control (Fig 3B). The difference was obvious 15 minutes (400-fold decrease) after Zn addition, still visible after 1h (230-fold decrease), but no longer present after 5h of induction. An immunoblot analysis confirmed this expression pattern at the protein level (Fig 3C). As expected, the delay in *czcR* expression was abolished when *zur:6His* was overexpressed *in trans* by the addition of 0.2 mM IPTG (Fig 3C). In agreement with our model, the transcription of *czcCBA* and *czcD,* which are dependent on CzcR (4), was also affected in the Δ*zur* mutant (Fig S3). Conversely, the P-type ATPase CadA, which is known to be overexpressed in the absence of CzcR (12), showed increased expression in the Δ*zur* mutant (Fig S3). Altogether, these results strongly suggest that Zur is required to bring about the full expression of *czcR* and mount the proper response to a Zn excess.

Modulation of *czcR* transcription is fundamental for the Zur-mediated Zn resistance

In order to confirm the direct effect of Zur on *czcR* expression, we modified the two Zur boxes at key positions on the PAO1 wild type and Δ*zur* mutant chromosomes and analyzed the growth of the mutants in excess of Zn. Unexpectedly, mutations in both Zur boxes restored the growth of the *zur* mutant in the presence of 2 and even 4 mM Zn since the same growth rate was obtained in both the wt and the Δ*zur* mutant in the absence of Zur boxes (Fig 4A). This result suggests that a relevant effect of the Zur-mediated regulation consists in the stimulation of Zn export upon Zn excess, since the absence of Zur boxes in the *czcR* promoter is sufficient to abrogates the *zur* mutant phenotype. In agreement, deleting the Zur regulated Zn-import permease *znuB* has no effect on the *zur* mutant growth phenotype in the presence of Zn (Fig. 4B).

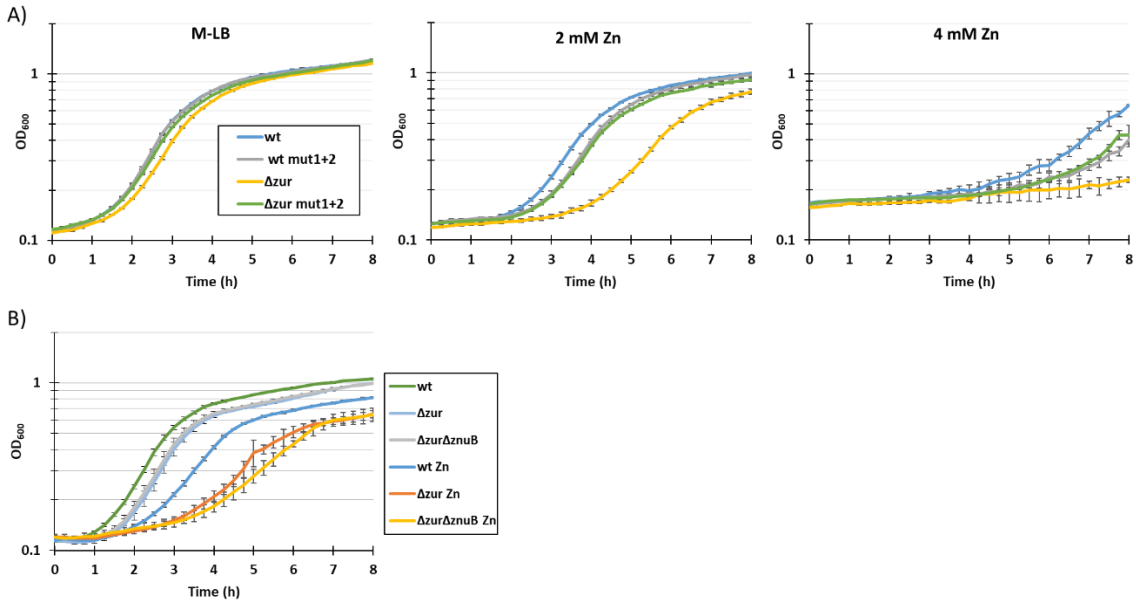

Figure 4 : Effect of *zur* box mutations on the Zn response. **A)** Growth curve of the wt PAO1 strain and the Δ*zur* mutant without or with the two mutated zur boxes (mut1+2). Strains were grown in the absence (M-LB) or in the presence of 2 or 4 mM $ZnCl_2$, as indicated. **B)** Growth curves of the wt PAO1 strain, the Δ*zur* mutant and the Δ*zur*Δ*znuB* double mutant without or in presence of 2 mM $ZnCl_2$ (Zn). Standard deviations of the triplicates are indicated.

The locus containing the two Zur boxes is involved in the repression of *czcR* expression

To further demonstrate the involvement of the 5'-UTR region of *czcR* in Zn-resistance, we deleted the entire region, leaving only 10 nucleotides after the transcription start and 40 nucleotides before the *czcR* ATG (see Materials and Methods). Similarly to the Zur boxes mutants, this deletion was constructed in the wild type and the Δ*zur* genetic background, creating the Δ5'-UTR and the Δ*zur* Δ5'-UTR strains, respectively. Surprisingly, deletion of the entire 5'UTR allowed for better growth of both the strains in the presence of 4 mM $ZnCl_2$ as compared to the wild type and the Δ*zur* mutant strains (Fig 5A). No effect on growth was observed in Zn-depleted medium containing 100 µM TPEN, nor in M-LB in the different strains tested.

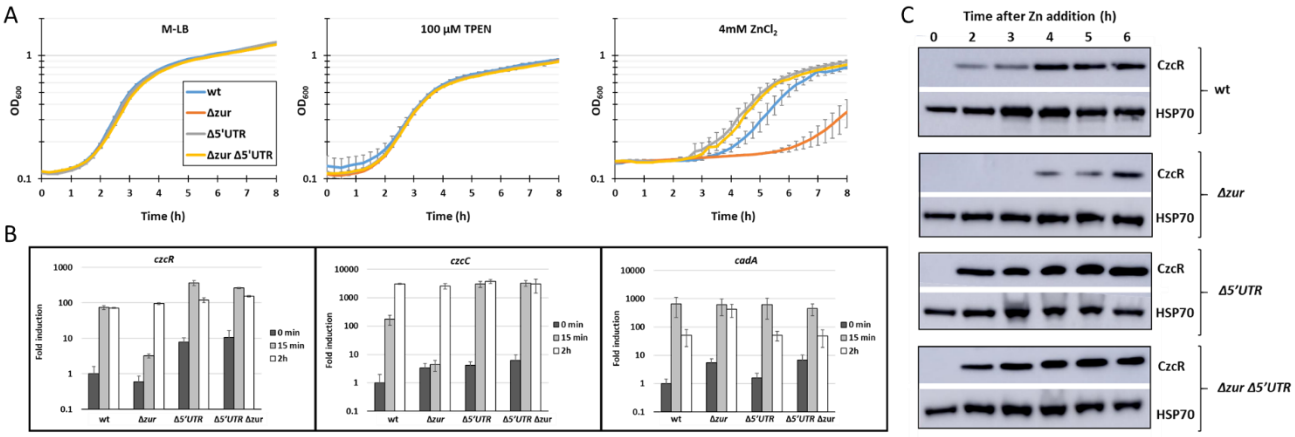

Figure 5: The 5'-UTR of *czcR* represses its own transcription. A) Growth curves of *P. aeruginosa* wt, Δ5'UTR, Δ*zur* and Δ*zur*Δ*5'UTR* strains in M-LB, in M-LB supplemented with 100 µM TPEN and in M-LB containing 4 mM $ZnCl_2$, as indicated. B) qRT-PCR of *czcR*, *czcC* and *cadA* on RNA extracted before Zn addition (t0) and after 15 minutes (15min) and 2h of 2 mM $ZnCl_2$ as indicated, in the wt, Δ5'UTR, Δ*zur* and Δ*zur*Δ*5'UTR* strains. Fold expressions are compared to the t0 of the wt strain and normalized by *oprF* expression. Standard deviations of the triplicates are indicated. C) Immunoblot analysis of CzcR regulator on total protein extract of the wt, the Δ5'UTR, Δ*zur* and Δ*zur*Δ*5'UTR* strains before (t0) and after different times (as indicated) of 2mM $ZnCl_2$ addition. Blots were exposed to anti-CzcR and anti-Hsp70 (loading control) antibodies.

The expression of *czcR* in the absence of a 5'-UTR was also measured by qRT-PCR (Fig 5B). The induction delay observed in the absence of Zur, as measured after 15 min, became non-significant when the 5'-UTR of *czcR* was deleted. Likewise, when the *czcR* 5'-UTR was removed, *czcC*, which is directly activated by CzcR, reached similar high levels in the wild type and the Δ*zur* strain, whereas the expression of *cadA*, which is indirectly repressed by CzcR, reached similar low levels in the wild type and the Δ*zur* strain after 2 hours (Fig 5B) (12). The amount of CzcR protein followed the mRNA levels of the different strains, meaning that the amount of protein was higher and appeared more rapidly in the absence of the 5'-UTR, even in the absence of Zur (Fig 5C). All together, these results suggest that the 5'-UTR region of the *czcR* transcript exerts negative control on its own transcription. This negative effect is partly bypassed by the presence of the Zur protein or by deleting or modifying the region in the vicinity of Zur boxes.

Discussion

Zinc is an essential element whose cellular concentration is tightly regulated (16). It is vital for cells and must always be present in sufficient amounts, but can become toxic as soon as it exceeds a threshold (17). In the bacterium *P. aeruginosa*, eight zinc import and four zinc export systems have been described (4). In case of zinc excess, the Zur protein represses the expression of many import systems, thus preventing too much Zn from entering the cell. In this work we described a novel key function of the Zur protein in *P. aeruginosa*, being involved not only in the repression of the import of Zn, but also in the early and fast induction of the CzcCBA Zn export system. Zur is therefore a keystone of Zn homeostasis in *P. aeruginosa*, integrating both the import and export of the ion.

In some other bacteria, including *Xanthomonas campestris*, *Streptomyces coelicolor* and *Caulobacter crescentus*, Zur also acts as an activator of zinc efflux genes (18-20). The localization of the Zur boxes on the promoter region of its target genes, along with the ability of the protein to multimerize at high zinc concentrations, appear to determine whether the regulator functions as a repressor or an activator (6). Other processes, not directly related to metal homeostasis, have also been found to be positively regulated by Zur such as the synthesis of secondary metabolites in *Streptomyces avermitilis* or the type VI secretion system in *Yersinia pseudotuberculosis* (21,22). In the case of *P. aeruginosa*, we found that the Zur protein binds in a Zn-dependent manner to two DNA regions (Zur box1 and 2) located downstream of the *czcR* promoter. These boxes are essential for the positive effect of Zur on *czcR* transcription and, in general, for the Zur-mediated Zn resistance. This suggests that the growth defect of the Δ*zur* mutant upon Zn excess condition is not only due to the absence of import inhibition, but mainly to a direct action of Zur on *czcR* transcription.

Removing the entire *czcR* 5'-UTR region, including the Zur boxes, results in higher *czcR* expression, even in the absence of Zur. What could be the feature in this region that negatively affects *czcR* transcription? And what is the molecular action of Zur, a DNA-binding protein? Deciphering the regulation occurring at the *czcR* 5’-UTR will also help shedding the light on the Zur mechanism of action. Some 5'-UTRs in bacteria are known to encode for small peptides (23). Looking at the recently performed proteomic analysis (4), no small peptides encoded were detected nor putative ORFs were bioinformatically predicted in this region, but at the moment we cannot fully exclude this possibility. Some 5’-UTR are also known to transcribe regulatory RNAs such as small RNAs (sRNAs) or antisense RNAs (asRNAs) (24). These regulatory RNAs could affect transcription by mechanism called transcription interference, attenuation (25) or

by the interaction between the asRNA and the mRNA (26). Zur could also compete with binding of a transcriptional repressor in this region, revealing an alternative start of transcription. For example, numerous TCS RRs have recently been detected in the intergenic region between *czcR* and *czcC* (27). Alternatively, Zur could modify a secondary structure at the DNA limits the full transcription of *czcR*. In *P. chlororaphis* 30-84, the 5'-UTR of *phzX*, involved in phenazine biosynthesis, contains secondary structures that modulate negatively the abundance of the transcript (28). A deletion in this 5'-UTR facilitates *phzX* gene expression. In *P. aeruginosa*, a hairpin encompassing the two Zur boxes was also predicted *in silico* (Fig. 6A). The removal, or mutations in this structure, de-represses the full transcription of *czcR*. A conceivable model, shown in Fig. 6B, is that the Zur protein, by binding to the *czcR* promoter region in the presence of excess Zn, abolishes the inhibitory activity that the 5'UTR has on *czcR* transcription. By mutating the Zur boxes (mut 1+2 strain) or by deleting the 5'-UTR region, the secondary structure is lost and the repressive activity of the hairpin disappears.

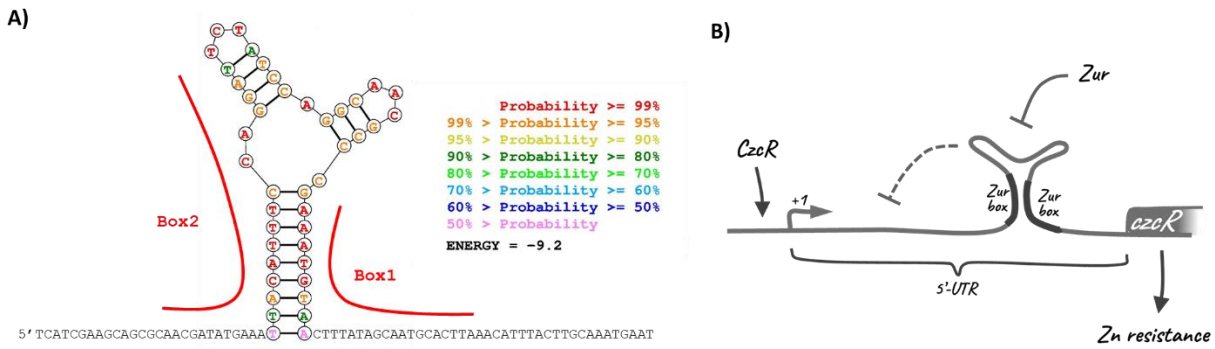

Figure 6: Model of transcriptional regulation of *czcR*. **A)** Representation of the putative secondary structure formed with the two Zur boxes. The structure was determined using the server for DNA or RNA secondary structure prediction (https://rna.urmc.rochester.edu/RNAstructureWeb/). The colors represent the probability of pairing. The two boxes are indicated with the red lines. **B)** Sketch representing a putative model of regulation based on the secondary structure acting negatively on *czcR* transcription. Zur, by binding to this hairpin, would prevent the structure from having a negative effect on transcription.

Interestingly, by removing this 5'-UTR region, which acts as a brake in *czcR* induction, cells are more resistant to high doses of Zn and no difference in growth compared to the wild type strain is observed in a Zn-deficient medium. Fine tuning of the efflux system could be essential in environmental conditions not tested in the laboratory. For instance, rapid changes in Zn concentration are known to occur during infection, because of nutritional immunity and metal excess in the phagolysosome (29,30). The tight efflux pump control might also be important in the environment, where rapid changes in metal concentrations might also occur. Interestingly,

among the *Pseudomonas* species analyzed for the presence and location of Zur boxes (i.e. *P. chlororaphis, P. fluorescens, P. putida, P. stuzeri* and *P. syringae*), only *P. aeruginosa* possesses boxes upstream of the *czcR* gene (5). This characteristic could be related to its ability to proliferate in presence of high metal concentrations. Thus, in *P. aeruginosa*, Zur appears to be the central cog in Zn homeostasis since it directly controls both the import and export of this metal. CzcR is also known to be capable of controlling the expression of the gene coding for the OprD porin, involved in imipenem resistance, or the *lasI* gene involved in quorum sensing (13,31). The importance of the 5'-UTR in the control of *czcR* expression could therefore be linked to other non-Zn-related phenotypes. Altogether, this positive action on CzcR expression highlights a novel and important role of Zur in the interaction between Zn homeostasis, virulence factor expression and antibiotic resistance in *P. aeruginosa*.

Materials and Methods

Bacterial Strains and Culture Media. The bacterial strains are listed in supplementary table S1. The modified Luria-Bertani medium (M-LB) used for this work was prepared as mentioned previously (12). When required, antibiotics were added to the medium at the following concentrations: 50 µg/mL tetracycline (Tc, Axxora), 200 µg/mL carbenicillin (Cb, phytotechlab), 50 µg/mL Gentamycin (Gm, AppliChem) for *P. aeruginosa* or 100 µg/mL ampicillin (Ap, AppliChem) and 15 µg/mL Tc or Gm for *E. coli*.

Genetic Manipulations. The primers used for cloning are detailed in supplementary table S2. Restriction and ligation enzymes (Promega), or fragment insertion using the Gibson assembly Cloning kit (New England Biolabs) were employed according to the supplier's instructions. Plasmids were transformed into *E. coli* DH5α by heat shock, verified by sequencing before being electroporated into the *P. aeruginosa* wild type or *Δzur* strains (32). For transcriptional *gfp*-fusion assays, the two *czcR* promoter regions (#1 and #2 in Fig 2A) were obtained by PCR amplification. Fragments were then digested with KpnI and BglII enzymes and ligated into the corresponding sites of the pBRR1-*gfp* vector. For Zur6HIS overexpression assays, the full *zur* gene was amplified by PCR with a reverse primer containing the 6xHIS tag. The fragment was digested with the EcoRI and BamHI enzymes and ligated into the pMMB66EH plasmid. The pME6001-*czcRS* vector was used as a template for the sequencing reactions of the 5'RACE experiments. For this construction, the full *czcRS* operon and its promoter region were amplified by PCR and cloned into the pME6001 vector with the XhoI and HindIII restriction sites. Genomic modifications were performed by homologous recombination. Two 500bp-fragments flanking the

gene of interest were amplified by PCR either from the *P. aeruginosa* wt genomic DNA (for the *zur* or 5'-UTR of *czcR* deletions) or from *in vitro* synthetized DNA containing the *zur* box modifications (#mut1+2, Fig S2A, GeneArt, Thermo Fisher Scientific). For the *Δ5'UTR* and #mut 1+2 mutants, fragments were inserted into the suicide vector pME3087 using the Gibson assembly Cloning kit. For the *Δzur* mutant, an insert product consisting of the two amplicons was obtained by megaprimer amplification, digested with EcoRI and BamHI restriction enzymes and ligated into the plasmid according to standard procedure (33). After verification, the resulting plasmids were transformed into the *P. aeruginosa* wt strain or in the *Δzur* mutant (for the *Δzur Δ5'UTR* double mutant). Merodiploids were selected as described previously (34) and deletions were confirmed by sequencing.

Growth experiments. To monitor the growth of the wt and the mutant strains, overnight cultures were diluted in M-LB medium to an OD600 of 0.05, supplemented with N,N,N′,N′-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN; Brunschwig) or $ZnCl_2$ (Fluka) as indicated in figures 3A and 4. Cultures were incubated at 37°C with shaking in a microplate reader (BioTek Instruments) and absorbance at 600 nm was measured every 15 min, for 12h. For strains carrying the pBRR1 plasmid with *gfp* fusions #1 or #2, overnight cultures were diluted to an OD600 of 0.1 and cultured for 2h30 in the presence of 30 µM TPEN before adding $ZnCl_2$. Absorbance at 600 nm and fluorescence at 528 nm were then monitored every 15 min. Arbitrary units indicted in the figure 2B correspond to the fluorescence values normalized with the cell density.

Immunoblot analyses. Immunoblot analyses were performed as previously described (31). Briefly, overnight cultures were diluted to an OD 0.1 and incubated 2h30 in M-LB supplemented with 30 µM TPEN. Isopropyl-1-thio-D-galactopyranoside (IPTG, Sigma Aldrich) was added at a final concentration of 0.2 mM to cultures complemented with Zur6HIS on the pMMB66EH or the empty plasmid. 1 ml was collected and centrifuged immediately prior to $ZnCl_2$ addition (t0) and after several time points, as specified in figures 3C or 5C. Pellets were solubilized in the appropriate volume of 2× β-mercaptoethanol gel-loading buffer (an OD_{600} of 1 gives 0.175 mg/ml of protein) to obtain a final concentration of 2 mg/ml and loaded onto SDS PAGE, using 4-12% precast gels (Thermofisher Scientific). Transfers were performed with iBlot2 transfer stacks (Invitrogen) and nitrocellulose membranes were incubated with the anti-CzcR (35), anti-penta HIS (Invitrogen) and anti-HSP70 (31) antibodies. Blots were revealed by chemiluminescence using the Amersham Imager 680 System.

EMSA. The ability of Zur to bind the 5'-UTR of *czcR*, was determined by electrophoretic mobility shift assays (EMSA). The *znuB* gene, the 5'-UTR of *czcR*, wt or containing the mutated *zur* box-es (#mut1, #mut2 or #mut 1+2), were amplified by PCR from either the *P. aeruginosa*

wt genomic DNA or from the *in vitro* synthetized mutated DNA (GeneArt, Thermo Fisher Scientific, Fig S4). Primers used for PCR amplification are indicated in supplementary table S2. Amplicons were then purified on agarose gel. Binding assays were performed as previously described (4). Results were analyzed by staining the gel with 0.1% ethidium bromide and revealing with UV light using a NuGenius instrument.

DNAse I Footprinting assays. The *czcR* promoter was amplified by PCR from the wild type *P. aeruginosa* genomic DNA with 5' Fluorescein (6FAM) primer as indicated in supplementary table S2. DNA, with the plus or minus strand labeled independently, were gel purified. 50 ng of DNA fragments were mixed with or without 1 µM Zur in 40 µL of EMSA binding buffer (4) supplemented with 5 µM zinc. The reaction was incubated for 30 min at room temperature, followed by partial DNAse I digestion as described previously (12). The sequence of the pME6001-*czcRS* plasmid, determined with the same FAM-labeled primers and the Thermo Sequenase Dye kit (Thermofisher Scientific) was used to align the peaks. All fragments were analyzed by capillary sequencing (Microsynth AG, Switzerland) using ILS600 as a size standard, then peaks were assessed using PeakScanner2 software (Thermofisher Scientific).

RNA extraction. Overnight cultures of the *P. aeruginosa* wt or mutant strains were diluted to an OD600 of 0.1 in M-LB complemented with 30 µM TPEN and cultured for 2h30 before being induced with 2mM $ZnCl_2$. For RT-qPCR, 0.5 ml of culture was mixed with 1ml of RNA protect (Qiagen) immediately prior to metal addition (t0) and after several time points as indicated in the figures. The *P. aeruginosa* wt culture intended for the 5'RACE experiment was collected after 5h of 2 mM $ZnCl_2$ induction. Total RNA extractions were performed as mentioned in previous publications (35). Briefly, pellets were resuspended in 100 µL Tris-EDTA buffer supplemented with 5 mg/mL Lysozyme (Fluka) and incubated at room temperature for 10 min. The following steps were carried out with the RNeasy mini kit (Qiagen), according to the manufacturer's directives. Residual genomic DNA was removed by treating with 10 units/sample of RNAse-free RQ1 DNAse (Promega) for 1h at 37°C followed by phenol-chloroform extraction and ethanol precipitation. Total RNA was then resuspended in RNAse-free water.

RT-qPCR. Quantitative RT-PCR were performed as previously described (36). Briefly 500 ng of total RNA were reverse transcribed using random primers (Promega) with the ImProm-II reverse transcriptase (Promega) according to the manufacturer's instructions. Quantitative PCR was performed on a tenfold dilution of resulting cDNA and using the SYBR Select Master Mix (Applied Biosystem), with the primers listed in table S2. Results were analyzed as formerly mentioned (37) and standardized with *oprF*.

5'RACE. The transcription starts of the *czcR* and *czcC* genes, were determined using the Rapid amplification of cDNA-5'ends (5'RACE) kit (Roche). Briefly, 5 µg of total RNA from the *P. aeruginosa* wt were reverse transcribed using the specific primers sp1R for *czcR* mRNA or sp1C for *czcC* mRNA. Resulting cDNAs were purified with the Wizard SV Gel and PCR Clean-Up System (Promega) and tailing with dATP and TdT. Tailed-cDNA strands were then amplified with the dT-Anchor primer (Roche) and the specific primer sp2R or sp2C (for *czcR* cDNA and *czcC* cDNA respectively). A twenty-fold dilution of the first PCR was used as template for a second PCR with primers Anchor (Roche) and sp3R for *czcR* cDNA or sp2C for *czcC* cDNA. PCR products were then purified by agarose gel electrophoresis and subcloned into a pCR2.1 vector with the TA Cloning kit (Life Technologies) according to the supplier's instructions. Plasmids isolated from *E. coli* clones were analyzed by sequencing. The transcription starts of *czcR* or *czcC* indicated in figure 2A correspond to the first nucleotide following the polyA tail.

Experimental relevance and Statistical Data. Regarding the graph representations, the mean values of at least three independent experiments are indicated in the figures, along with the corresponding standard deviations. When specified, statistical analyses were performed according to the Student's *t*-test and significance *p*-values were set at $p \leq 0.05$(*) or $p \leq 0.001$ (***). For other figures, data points represent the mean of triplicate values.

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