A Copper-Activated Two-Component System Interacts with Zinc and Imipenem Resistance in *Pseudomonas aeruginosa*[▽]

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The effects of copper (Cu) on trace metal and antibiotic resistance of *Pseudomonas aeruginosa* have been investigated. Cu treatments induced resistance not only to this metal but also, surprisingly, to zinc (Zn). Quantitative reverse transcription-PCR (qRT-PCR) revealed that after Cu treatment the transcription of the *czcRS* two-component system (TCS) operon was enhanced as well as that of the *czcCBA* operon encoding an efflux pump specific for zinc, cadmium, and cobalt. Cu treatments at the same time caused a decrease in the production of OprD porin, resulting in resistance to the carbapenem antibiotic imipenem. The CzcR regulator was known to repress *oprD*. However, Cu was still able to decrease the production of OprD and induce imipenem resistance in a *czcRS* knockout mutant. This strongly suggested that another Cu-dependent regulatory system was acting negatively on *oprD* expression. TCS regulator genes *copR-copS* have been shown to be involved in Cu tolerance in *P. aeruginosa*. qRT-PCR showed that overproduction of the CopR or of the CzcR regulator resulted in increased transcription of the *czcC* gene as well as in a decrease in *oprD* gene transcription, either in the wild-type strain or in the *czcRS* knockout mutant. Overproduction experiments suggest that a metal-dependent mechanism operates at the posttranscriptional level to control the production of the CzcCBA efflux pump. This study shows that CopR is a new negative regulator of OprD porin and that it links Zn, Cu, and imipenem resistances by interacting with the CzcRS TCS.

Zinc (Zn) and copper (Cu) are required in trace amounts for bacterial growth. Their homeostasis has to be tightly regulated, because they are toxic when present in excess. Zn binds to free thiol groups, affecting protein function (4). Cu exerts its toxicity by generating reactive oxygen species via Cu-mediated redox cycling. It also binds to sulfhydryl groups, especially in its Cu(I) form (35). Both metals are also major trace-metal pollutants in the environment. Bacteria have several mechanisms allowing them to thrive in environments contaminated with toxic levels of metals. Active efflux of metal cations is a key aspect of resistance. Previous studies indicated that a network of different families of transporters coordinates efflux, including CBA systems that mediate proton-driven efflux, P-type ATPases, and cation diffusion facilitators (25).

Pseudomonas aeruginosa, a gram-negative bacterium, is an extremely versatile organism that grows in many diverse terrestrial, marine, and freshwater habitats. It is also an opportunistic pathogen frequently encountered in the hospital, causing severe infections in immunocompromised hosts and cystic fibrosis patients (32). *P. aeruginosa* is characterized by an intrinsically high level of resistance to antimicrobial agents and trace metals, which can be accounted for by a combination of low outer membrane permeability and the presence of multiple efflux pumps (26). Resistance to Cu of the PAO1 wild-type strain of *P. aeruginosa* is due to the action of a Cu(I)/Ag(I) P-type ATPase and possibly of two resistance nodulation cell division (RND) efflux systems. The CopRS two-component

system (TCS) is also involved. CopR appears to be a key regulator involved in Cu resistance (38). It is also essential for the activation of *ptrA* (for “*Pseudomonas* type III repressor A”) in response to Cu signal (10). In *Escherichia coli* (9), the multicopper oxidase CueO is implicated in intrinsic copper resistance. In *P. aeruginosa*, the *pcoA* gene encodes a multicopper oxidase that is involved in the oxidation of Fe(II) to Fe(III). However, its role in copper tolerance is unclear (16). Intrinsic Zn resistance in this bacterium is mainly due to the activity of an RND type of efflux pump, called CzcCBA, that is under the control of the TCS CzcRS (11).

In bacteria, TCSs are widely used as signal transduction systems in response to environmental changes or stresses. Classically, TCSs are constituted by a membrane-located sensor kinase and a cytoplasmic transcriptional regulator. The sensor protein is autophosphorylated on a histidine residue once it has bound its ligand. This phosphate is then transferred to an aspartate residue on the regulator protein that becomes active for the transcriptional activation of target genes (14). When *P. aeruginosa* cells are treated with Zn, the expression of the *czcRS* operon is strongly enhanced, leading to the transcriptional activation of *czcCBA* (30). At the same time, the Zn-induced increased production of the CzcR regulator was found to negatively regulate *oprD*. This gene codes for a specific porin, OprD, that belongs to a large family of porins (37). OprD is a basic amino acid-peptide channel and a primary route of entry for carbapenems, such as imipenem, in *P. aeruginosa* (40). This coregulation was responsible for the observed cross-resistance between trace metals and the antibiotic imipenem.

Environmental and clinical strains of *P. aeruginosa* were shown to be functionally equivalent with respect to several

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
<i>P. aeruginosa</i>		
PT5	PAO1 wild type	Laboratory collection
$\Delta czcA$	PT5 $\Delta czcA$	PT1173 (30)
$\Delta czcRS$	PT5 $\Delta czcRS$	This study
Plasmids		
pMMB66EH	Expressing vector, Cb ^r	8
pCzcR	<i>czcR</i> gene on pMMB66EH, Cb ^r	pRWT (30)
pCopR	<i>copR</i> gene on pMMB66EH, Cb ^r	This study

^a Cb^r, carbenicillin resistant.

clinically relevant properties (1), in agreement with the highly conserved genome encountered among such isolates (43). These findings suggest that Zn-contaminated environments could constitute reservoirs of harmful, antibiotic-resistant strains of *Pseudomonas aeruginosa*. Such reservoirs could be, for example, soils to which Zn-contaminated sewage sludge from wastewater treatment plants has been applied. Like Zn, Cu is spread in the environment as a consequence of agricultural practices. It is used as a fungicide and is often present in animal manure. Moreover, it is released from the washout of copper pipes and roofs. Field experiments showed that Cu amendment of agricultural soils selects for Cu resistance and further coselects for antibiotic resistance (3). This prompted us to examine whether Cu treatments of *P. aeruginosa* also resulted in cross-resistance to antibiotics. In the present article, we showed that such exposures also caused resistance to the antibiotic imipenem due to a decrease in the level of OprD and that CopR is a new regulator of the expression of the *oprD* gene. We further revealed an interaction between the CopR-CopS TCS involved in Cu resistance of *P. aeruginosa* (10, 38) and the CzcR-CzcS TCS controlling Zn resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Luria-Bertani (LB) (34) and Mueller-Hinton (MH) media were used as solid media. LB liquid medium was used in the absence of trace metal. Liquid cultures containing Cu or Zn were performed in HEPES-buffered mineral medium (21) supplemented with 0.4% glucose and 50 mM sodium HEPES, pH 7.0, instead of Tris buffer. Liquid cultures were grown at 37°C on a rotary shaker (160 rpm) in 200-ml Erlenmeyer flasks containing 30 ml of minimal medium.

Determination of trace metal and antibiotic MIC. The maximal tolerable concentrations (MTCs) of trace metals were determined as previously described (30), with slight modifications. Briefly, 10 μ l of culture at an optical density at 600 nm (OD₆₀₀) of 1 were spotted on LB agar medium containing different concentrations of trace-metal salts. The MTCs are defined as the highest metal concentrations at which growth was observed after 48 h of incubation at 30°C. Resistance to antibiotics was determined by the Kirby-Bauer method as follows. An overnight liquid culture grown at 37°C in MH medium was diluted 1:500 in 0.9% NaCl, and 2 ml of the suspension was spread on MH agar plates containing different concentrations of trace-metal salts. The plate was air dried, and antibiotic-impregnated disks (Biomérieux) were applied. Plates were incubated overnight at 37°C. Inhibition zones were measured and compared with those of the reference strain PT5.

DNA manipulations. To delete the *czcRS* genes from strain PT5, we generated a 549-bp PCR fragment corresponding to the promoter and the first 90 bp of *czcR* gene with primers OC1 (5'-CGGAATTCGCAACCGTTCATCGGCG), containing an EcoRI restriction site (in boldface), and OC2 (5'-GGCGCGGTCGACGATGTAGCCG), including a SalI restriction site. This fragment was cloned into the pKS-Bluescript plasmid (Stratagene) in the EcoRI/SalI sites. A second PCR fragment of 650 bp corresponding to the last 88 bp of *czcS* gene was produced with primers SOC3 (5'-ACGCGTCGACCTACCACGGCGGCCGCG

CCG), containing a SalI restriction site, and SOC4 (5'-GCCGGTACCGGTCA CCAACCAGGCCACCG), containing a KpnI restriction site, and cloned into the same pKS-Bluescript in the SalI/KpnI restriction sites. A gentamicin-green fluorescent protein resistance cassette from plasmid pPS858 was then recovered with SalI restriction enzyme and added to pKS-Bluescript containing both fragments in the SalI restriction site. A 3-kbp PCR fragment from pKS-Bluescript containing the three fragments was produced with primers HindIII-Lup (5'-CC CAAGCTTGTAACGACGCGCCAGTGAAT) and HindIII-Lrp (5'-CCCAAG CTTAACAGCTATGACCATGATTAC), both containing a HindIII restriction site, and cloned into the HindIII-cleaved plasmid pEX18Ap. After transfer and homologous recombination into strain PT5, excision of the gentamicin cassette was performed as described previously (13). The resulting $\Delta czcRS$ strain (Fig. 1) carries a 1,900-bp deletion inside the *czcRS* genes as verified by PCR.

The gene encoding the CopR protein was amplified by PCR with primers 122 (5'-CGGAATTCGGTCGGCATGGCCGGC) and 123 (5'-CGGGATCCGGAC ATCCGCGAGCCG), containing an EcoRI and a BamHI site, respectively. The 750-bp product was cloned into the EcoRI/BamHI sites of pMMB66EH under the *tac* promoter, yielding the pCopR plasmid. Plasmids pCopR and pCzcR (30) were transferred into *P. aeruginosa* by electroporation (7). Bacteria were then cultivated in the presence of 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to induce the transcription of the *copR* or *czcR* gene.

Western blot analysis. Cultures were grown at 37°C to an OD₆₀₀ of 1 in minimal medium or LB. Cells were then washed with 0.9% NaCl and resuspended in 2 $\times$ sodium dodecyl sulfate (SDS) gel loading buffer at 2 mg/ml of protein and an OD₆₀₀ of 1, corresponding to 0.175 mg/ml of protein. Samples were boiled for 5 min and centrifuged for 10 min. Ten micrograms of total protein was separated on an SDS-10% polyacrylamide gel and transferred to nitrocellulose membranes. Blots were incubated with anti-OprD or anti-Hsp70 antibodies and revealed by chemiluminescence. All antibody incubations and washes were performed in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) supplemented with 5% powdered milk.

RT-PCR analysis. For quantitative reverse transcription-PCR (qRT-PCR), total RNA was extracted using the RNA bacteria protect solution and the RNeasy kit (QIAGEN, Hildesheim, Germany) according to the manufacturer's instructions. Residual DNA was eliminated by DNase treatment using 20 U of RQ1 RNase-free DNase (Promega) followed by phenol-chloroform extraction. The RNA was precipitated with ethanol and resuspended in RNase-free water. For cDNA synthesis, 1 μ g of RNA was reverse transcribed using random hexamer primers and Imprim-II reverse transcriptase (Promega) according to the supplier's instructions. Reverse transcriptase was inactivated by incubation at 70°C for 15 min, and obtained cDNAs were stored at -20°C until use. cDNAs were quantitatively measured with a Bio-Rad iCycler machine using a Sybr Green Quantitect kit (QIAGEN, Hildesheim, Germany). The primer pairs that were used for qRT-PCR were designed using the primer3 program (33) and are shown in Table 2.

RESULTS

Copper induces both copper and zinc resistance in *P. aeruginosa*. The PT5 wild-type strain and two mutant strains affected in the CzcCBA efflux system (Table 1) have been used to investigate *P. aeruginosa* tolerance to copper (Cu) and zinc (Zn). Table 3 shows that the PT5 wild-type strain becomes Cu tolerant after a prolonged treatment with 30 μ M Cu, as shown

FIG. 1. Map of the *czcRS* region in the wild-type (wt) strain and in the $\Delta czcRS$ mutant. The latter carries a 1,900-bp deletion in the *czcRS* locus joining the first 80 nucleotides of *czcR* to the last 90 nucleotides of *czcS*. It still contains the intergenic region between *czcR* and *czcC*, including the *czcRS* promoter and the *cop* box region.

TABLE 2. Primers used for qRT-PCR

Amplicon	Primer	Sequence (5'-3')	Position in gene	Product length (bp)
<i>rpsL</i>	rpsL F	GCAAGCGCATGGTCGACAAGA	35	201
	rpsL R	CGCTGTGCTCTTGCAGGTTGTGA	235	
<i>oprD</i>	oprD1	ATCTACCGCACAAACGATGAAGG	772	156
	oprD2	GCCGAAGCCGATATAATCAAACG	927	
<i>czcR</i>	czcR1	GTCATCACCCGGACGCAGATCAT	502	153
	czcR2	GTAGCCGACGCCGGAATGGTAT	654	
<i>czcS</i>	czcS1	TACGCCAGCTCTCGCAGTTCTCC	740	201
	czcS2	TGTCCACCTGCACCAGGAACAGC	940	
<i>czcC</i>	czcC1	GGTCAGCATCGGCAGCAAGTACG	834	206
	czcC2	GGTCGTAGGCCTGTACCGCTTCG	1039	
<i>copR</i>	copR L	ACCTCGAACTCGATTGCTG	395	166
	copR R	TCGCTGTCTGAAGTTCATGTC	560	
<i>copS</i>	copS L	GTTCGACCGGTTCTATCGTG	1158	160
	copS R	AGCGAAATCGATGACGAAAC	1317	

by an increase in the MTC. Quite unexpectedly, the same Cu treatment also rendered the cells resistant to Zn. In *P. aeruginosa*, tolerance to Zn is mainly due to the induction of the *czcCBA* operon regulated by the CzcR-CzcS TCS and is specific for the efflux of zinc, cadmium, and cobalt (11, 30). Cu induced Zn tolerance by expressing *czcCBA*, as revealed by qRT-PCR. Addition of 30 μ M Cu to the culture minimal medium resulted in the up-regulation of the *czcR* and *czcS* genes as well as in that of the *czcC* gene coding for the outer membrane protein of the efflux pump (Fig. 2A) (90-, 9-, and 130-fold induction for *czcR*, *czcS*, and *czcC* genes, respectively).

Zn (300 μ M) was not found to induce Cu tolerance (Table 3). Moreover, a mutant strain deleted in the *czcA* gene, encoding the motor of the efflux pump, displayed Zn sensitivity as expected (MTC of 5 mM) (Table 3). However, its intrinsic resistance to Cu was not affected, and tolerance to this cation was still inducible in this mutant. These observations clearly indicate that the CzcCBA efflux pump does not expel Cu.

Copper, like zinc, induces resistance to the carbapenem antibiotic imipenem. OprD porin is involved in the entry of basic amino acids and of carbapenem antibiotics like imipenem in *P. aeruginosa* cells (40, 41). The induction of *czcCBA* caused by exposure to Zn resulted in cross-resistance to imipenem due

to decreased OprD production (30). However, trace-metal resistance is not caused by the decrease in OprD, since an *oprD* knockout mutant displayed the same susceptibility to Zn or Cu as its parent strain, PT5 (30). Moreover, overproduction of OprD in the wild-type PT5 strain did not affect tolerance to these metals (data not shown). We observed in both PT5 and Δ *czcA* strains that Cu, like Zn, was very efficient in inducing imipenem resistance, while it had no effect on ciprofloxacin sensitivity (Table 3). The weak imipenem resistance displayed by the Δ *czcA* mutant cultivated in the presence of Zn (Table 3) was probably due to the growth inhibition caused by the metal treatment. The imipenem resistance was associated with a decrease of *oprD* expression as revealed by qRT-PCR. The levels of *oprD* mRNA dropped to 50% in the presence of 30 μ M Cu in the medium and to 10% after a 300 μ M Zn treatment (Fig. 2A).

As negative posttranscriptional processes have been observed in *oprD* expression (18, 19), the amount of OprD was determined by Western blot analysis (Fig. 2B). The OprD protein was undetectable at 10 μ M Cu or Zn, which accounted for the imipenem-resistant phenotype observed (Table 3).

Two regulators, CzcR and CopR, act negatively on OprD production. We previously showed that exposure of *P. aeruginosa* to Zn caused an up-regulation of the *czcR* regulator gene

TABLE 3. Metal and antibiotic resistance of wild-type *P. aeruginosa* PT5 and of mutant strains affected in the CzcCBA efflux system^a

Strain	Treatment	MTCs (mM) of:		Metal resistance profile	Inhibition zones (mm) for:		IPM resistance profile
		Zn	Cu		IPM	CIP	
PT5	None	15	6	Reference	27.5	32.5	S
PT5	Zn	25	6	Zn R	15.7	33	R
PT5	Cu	20	8	Zn R; Cu R	9	34.5	R
Δ <i>czcA</i>	None	5	6	Zn S	27	31.5	S
Δ <i>czcA</i>	Zn	5	6	Zn S	21	35.5	R
Δ <i>czcA</i>	Cu	5	8	Zn S; Cu R	8.5	31.3	R
Δ <i>czcRS</i>	None	2.5	6	Zn S	28.2	34.2	S
Δ <i>czcRS</i>	Zn	NA	NA	Zn S	26	32.7	S
Δ <i>czcRS</i>	Cu	2.5	8	Zn S; Cu R	8.5	35.2	R

^a MTCs for Cu and Zn were recorded after 48 h of growth at 30°C on LB medium containing different concentrations of metals. Zn and Cu pretreatments were 300 μ M and 30 μ M, respectively, in liquid minimal medium before MTC determinations. Determination of antibiotic resistance was completed according to the Kirby-Bauer method after overnight growth at 37°C in liquid MH medium. Inhibition zones were measured in MH agar medium containing 200 μ M Zn or 2.5 mM Cu. MTC and inhibition zone values are the averages of two determinations. IPM, imipenem; CIP, ciprofloxacin. R and S correspond to resistant and weakly resistant phenotypes, respectively, relative to the sensitive reference strain PT5. NA, not applicable.

FIG. 2. Both Cu and Zn induce the *czcCBA* operon and repress *oprD* expression. (A) Transcription of *czcRS*, *czcC*, and *oprD* genes analyzed by qRT-PCR in the wild-type PT5 strain grown in the presence of 300 μM ZnCl₂ or 30 μM CuCl₂. Localizations of the primer pairs used for the amplifications are shown in Table 2. The amount of mRNA is represented relative to the PT5 strain cultivated in the absence of metal. Experiments were performed in duplicate on two independent occasions. Error bars represent the standard deviations of two determinations. (B) Immunoblot analysis of OprD porin on total protein extract of the PT5 strain cultivated in HEPES-buffered mineral medium in the presence of increasing concentrations (0, 3, 10, and 30 μM) of ZnCl₂ or CuCl₂. Blots were exposed to anti-OprD or anti-Hsp70 (loading control) antibody.

which led to repression of *oprD* expression, thus causing imipenem resistance (30). In order to determine whether Cu repressed this porin via the same regulatory mechanism, we constructed a *czcR czcS* double knockout mutant as shown in Fig. 1. As expected, Zn tolerance and the Zn-induced suppression of OprD were abolished in this mutant strain (Table 3; Fig. 3). Surprisingly, however, the decrease of OprD was maintained after a Cu exposure, resulting in imipenem resistance as revealed by inhibition zone measurements and by Western blot analysis (Table 3; Fig. 3). Clearly, in the presence of this trace metal, another system independent from the *czcR-czcS* regulatory sequences acts negatively on *oprD* expression.

A candidate was the *copR-copS* TCS that has been shown to be involved in Cu tolerance of *P. aeruginosa* (10, 38). CopR was found to display 73% similarity and 55% identity with the CzcR regulator, as determined by pairwise sequence alignment. We therefore cloned the *copR* regulatory gene under an inducible promoter. Overproduction of either CzcR or CopR

resulted in a strong decrease in the levels of *oprD* mRNAs, both in the wild type PT5 (Fig. 4A) and in the $\Delta czcRS$ mutant (Fig. 4B). The production of the OprD protein was also abolished (Fig. 4C). Overproduction of both regulators up-regulated the *czcC* and the *czcR-czcS* genes (Fig. 4A). However, the overproduction of CopR did not affect *copS* transcription. Altogether these results show that the CopR regulator, like CzcR, negatively regulates *oprD* and positively regulates the *czcCBA* operon (Fig. 5).

A *cop* box most likely accounts for the copper-induced regulation of the CzcCBA efflux system. In *Pseudomonas syringae*, the CopR regulator binds to a *cop* box located upstream of the *copABCD* operon (22). A similar *cop* box has been identified in the promoter region of the *P. aeruginosa czcR* gene (6). It is therefore very likely that the *P. aeruginosa* CopR regulator binds to this *cop* box, thus activating the transcription of the *czcR-czcS* genes which led to that of the *czcCBA* operon (Fig. 2A and 5). CopR overproduction resulted in the up-regulation of the *czcC* gene in the wild type as well as in the $\Delta czcRS$ mutant strain (Fig. 4A and B). Overproduction in the absence of sensor ligand probably gave rise to the unphosphorylated form of the CopR regulator and resulted in Cu tolerance (Table 4). This suggests that high levels of unphosphorylated CopR can bind to the *cop* box and directly activate the transcription of the *czcCBA* operon (Fig. 5). However, after exposure of $\Delta czcRS$ mutant to Cu, no increase in Zn tolerance was noticed (Table 3). This indicated that in the presence of the metal, the active, phosphorylated CopR regulator induced only the transcription of the *czcRS* regulatory sequences and acted on the *czcCBA* operon via the CzcR regulator.

FIG. 3. Cu is able to inhibit OprD production independently of the TCS CzcR regulator controlling the transcription of the *czcCBA* operon. Immunoblot analysis of OprD porin on total protein extract of the $\Delta czcRS$ strain cultivated in HEPES-buffered mineral medium in the presence of increasing concentrations (0, 3, 10, and 30 μM) of ZnCl₂ or CuCl₂. Blots were exposed to anti-OprD or anti-Hsp70 (loading control) antibody.

FIG. 4. Overproduction of the CzcR and CopR regulators and its effects on the transcription the *copRS*, *czcRS*, *czcC*, and *oprD* genes (A and B) and on OprD porin production (C). Transcription was determined by qRT-PCR in the wild-type (wt) PT5 strain (A) or in the $\Delta czcRS$ strain (B). (C) Immunoblot analysis of OprD porin on total protein extract of the same two strains. Bacteria were cultivated in LB in the presence of 0.1 mM IPTG to induce the transcription of the *copR* or *czcR* gene. Localizations of the primer pairs used for the amplifications are shown in Table 2. Overproduction of the CzcR and CopR regulators was performed in both strains transformed with plasmids pCzcR and pCopR, respectively. The amount of mRNA is represented relative to bacteria containing the empty pMMB66EH-expressing vector (control). Experiments were performed in duplicate on two independent occasions. Error bars represent the standard deviations of two determinations.

Evidence for a posttranscriptional regulation of the CzcCBA efflux system. The repression of the expression of *oprD* was most likely caused by phosphorylated CzcR in the presence of Cu or Zn (Fig. 2) and possibly by unphosphorylated forms of each regulator, as overexpression experiments were performed in the absence of metal (Fig. 4A and B). Strains overproducing CzcR or CopR were analyzed for Zn and Cu resistance (Table 4). High levels (1,000-fold induction) of both regulators were

found to strongly up-regulate the *czcC* gene, both in the wild type and in the $\Delta czcRS$ strain (Fig. 4A and B). While overproduction of CopR was able to induce Cu resistance in the PT5 strain (Table 4), it did not increase Zn tolerance. Overproduction of CzcR in the wild type was even slightly inhibitory (Table 4). Overproduction of either CzcR or CopR in the $\Delta czcRS$ strain increased Zn resistance (Table 4). However, the tolerance to the metal was much lower than that expected from the

FIG. 5. Complex regulatory circuits link Zn and Cu tolerance to imipenem resistance in *P. aeruginosa*. Zn and Cu induce the expression of the *czcRS* and *copRS* operons, respectively. The active, phosphorylated CzcR regulator induces the expression of *czcCBA*, leading to zinc, cadmium, and cobalt resistance (30). Cu resistance is thought to be due to up-regulation of P-type ATPase and cation diffusion facilitator transporter genes (38). The CopR regulator positively activates the expression of the *czcRS* and *czcCBA* genes, most likely by binding to a *cop* box located between these gene sequences (6). It also up-regulates the *ptrA* gene, a repressor of the type III secretion system genes (10). OprD porin that facilitates the entry of basic amino acids (a.a.) as well as of the antibiotic imipenem is tightly regulated. Basic amino acids, such as arginine, induce *oprD* expression by way of the transcriptional activator ArgR (27), while salicylate or benzoate exerts a negative effect (28). MexT, the positive regulator of the *mexEF-oprN* operon coding for an efflux pump that leads to antibiotic resistance, negatively regulates *oprD* (18). Both CopR and CzcR regulators also down-regulate *oprD* expression either directly or via an undetermined mechanism, leading to imipenem resistance.

very high amounts of *czcC* mRNAs (Fig. 4B). These results suggest that the presence of Cu or Zn is essential for a functional CzcCBA system and that a posttranscriptional mechanism regulates this efflux pump.

DISCUSSION

In *P. aeruginosa*, the CzcR-CzcS two-component system (TCS) controls zinc (Zn) tolerance (11, 30) while a copper (Cu)-responsive TCS, CopR-CopS, is responsible for Cu resis-

tance (10, 38). Our results demonstrate that the regulator CopR links Cu resistance to Zn tolerance by activating the *czcRS* operon. A connection between two distinct TCS has already been described in *E. coli*, where the CpxA-CpxR TCS acts in concert with EnvZ-OmpR, another TCS that regulates OmpF and OmpC porins (2). In *Pseudomonas syringae*, CopR is a transcriptional activator protein that binds to a conserved domain (*cop* box) in the promoter of the chromosomally carried operon responsible for Cu resistance (22). The fact that a putative *cop* box has been identified in *P. aeruginosa* between the *czcR* regulatory gene and the *czcC* gene of the *czcCBA* operon (Fig. 5) (6) suggests that CopR can bind to this region and regulate the transcription of the two operons.

When overproduced in the absence of trace metal, CopR is able to activate directly the transcription of the *czcCBA* operon, as shown by the finding that the *czcR-czcS* regulatory sequences were not necessary for induction (Fig. 4B). However, in bacteria exposed to Cu, CopR did not activate directly the *czcCBA* operon, as shown by the Zn-sensitive phenotype displayed by the Δ *czcRS* mutant (Table 3). In the presence of Cu, a major part of the CopR protein was presumably phosphorylated (CopR-P) by the ligand-activated CopS sensor kinase. CopR-P then acted on *czcCBA* indirectly by activation of the *czcRS* operon, thus increasing the amount of the CzcR regulator (Fig. 4A and 5). Possibly the latter was phosphory-

TABLE 4. Tolerance to Zn and Cu in PT5 and Δ *czcRS* strains after overproduction of CzcR and CopR regulator proteins^a

Strain	MTCs (mM)		Metal resistance profile
	Zn	Cu	
PT5 + pMMB66EH	15	6	Reference
PT5 + pCzcR	10	6	Zn S
PT5 + pCopR	15	8	Cu R
Δ <i>czcRS</i> + pMMB66EH	2.5	6	Zn S
Δ <i>czcRS</i> + pCzcR	5	6	Zn S
Δ <i>czcRS</i> + pCopR	5	8	Zn S; Cu R

^a MTC determinations were performed as described for Table 3. Bacteria were cultivated in the presence of 0.1 mM IPTG to induce the transcription of the *czcR* and *copR* genes on pMMB66EH expressing vector. R and S correspond to resistant and weakly resistant phenotypes, respectively, relative to the sensitive reference strain PT5.

lated by phospho-donors like acetyl phosphate (20) or by cross-talk between the CopS sensor kinase and the CzcR regulator.

The overproduction of the unphosphorylated forms (in the absence of sensor ligands) of the two regulators, CopR and CzcR, was found to activate the transcription of the *czcRS* and *czcCBA* operons (Fig. 4A and B). A reasonable explanation for this observation is that very high levels of unphosphorylated regulators could activate the transcription of these operons. However, when grown in the absence of metal, the PT5 wild-type strain overproducing the regulators CopR and CzcR was not resistant to Zn (Table 4), which indicated that it did not possess a functional CzcCBA efflux system. A 33% decline in Zn resistance was observed even when CzcR was overproduced. These results strongly suggest that CzcCBA production is also regulated at the posttranscriptional level by a metal-dependent mechanism. Antisense regulation or repression of translation has already been observed in the case of TCS target genes. For example, in *E. coli*, the two major porins, OmpC and OmpF, are regulated transcriptionally by the TCS EnvZ-OmpR and at the posttranscriptional level by two small RNAs, MicC and MicF (5, 23). In *P. aeruginosa*, the GacS-GacA TCS positively activates quorum-sensing genes in combination with the posttranscriptional regulator small RNAs RsmY and RsmZ as well as RsmA, a small RNA binding protein repressing translation (12, 17, 24). It cannot, however, be ruled out that high amounts of unphosphorylated regulators might be inhibitory to *czcCBA* mRNA translation or to other posttranscriptional mechanisms.

The physiological significance of the Cu-induced activation of the *czcCBA* operon is unclear. The CzcA RND protein is specifically involved in the export of zinc, cadmium, and cobalt (25). The finding that the *czcA* knockout mutation had no influence on Cu tolerance (Table 3) is in agreement with the idea that the CzcCBA efflux system does not expel Cu and with the results of Teitzel et al. (38) attributing *P. aeruginosa* Cu resistance to a Cu(I)/Ag(I) P-type ATPase and, possibly, to two resistance nodulation cell division efflux systems. So why is this pump activated by Cu treatments? One possible explanation for the induction of the CzcCBA efflux system by Cu is that in the presence of this trace metal there is a need for a decrease in the intracellular Zn concentration. Interactive toxic effects between Cu and Zn were found to be synergistic in the gram-negative bacterium *Vibrio fischeri* (42). Zn excretion would then minimize toxic synergistic effects of the two metals. Another hypothesis is that the Cu-induced activation of the CzcCBA system may be part of a protection mechanism involving multiple responses to metallic stress and allowing cells to cope with polluted environments contaminated with several metals.

OprD porin is involved in the uptake of basic amino acids and small peptides containing these amino acids across the outer membrane of *P. aeruginosa* (41). Besides this specific function, OprD also has a nonspecific role in the uptake of small compounds like gluconate (15). The presence of this porin when *P. aeruginosa* is grown in minimal medium containing glucose (Fig. 2 to 4) suggests that OprD may have another still unknown function(s). There are several negative regulatory mechanisms acting on *oprD* (Fig. 5). It is repressed by MexT, the inducer of the MexEF-OprN efflux system (18), salicylate (28), succinate due to catabolite repression (27), and trace metals via response regulators CzcR (30) and CopR (this

study). On the other hand, *oprD* expression is strongly induced when arginine and other amino acids serve as the sole source of carbon. The arginine-mediated induction depends on the regulatory protein ArgR (27). The multiple systems involved in the regulation of *oprD* expression suggest that it plays a crucial role in *P. aeruginosa* growth and resistance to various stresses.

What is the physiological purpose of coregulation of *oprD* with a broad-specificity antibiotic efflux operon through MexT and with genes involved in trace-metal efflux via regulators CzcR and CopR? The repression of *oprD* associated with the activation of RND efflux pumps may be part of a general mechanism involving downregulation of primary metabolism and upregulation of protective mechanisms to cope with the stress caused by antibiotics or high amounts of trace metals. Such regulations have been observed in *P. aeruginosa* submitted to oxidative stress (29). On the other hand, the suppression of OprD porin may represent a more specific mechanism decreasing the intracellular concentration of basic amino acids or peptides that could be inhibitory to resistance mechanisms. A common feature between the MexT, CzcR, and CopR regulators is that they all regulate the transcription of operons involved in the formation of RND efflux systems (18, 30, 38). Interference of basic amino acids or peptides with these protective mechanisms is not unlikely. Indeed, a transcriptome analysis of the response of *P. aeruginosa* to oxidative stress revealed an 18-fold decrease in the mRNA levels of an arginine/ornithine transport protein in cells treated with H₂O₂ (29). In this regard, oxidative stress is the primary mechanism of Cu toxicity in *E. coli* (39). Moreover, RND efflux systems of *P. aeruginosa* are affected by efflux pump inhibitors containing basic amino acids like arginine or ornithine (31). It is therefore reasonable to assume that arginine or other basic amino acids act as or could be the precursors of an intracellular, diamino-containing efflux pump inhibitor.

There is increasing concern about trace-metal contamination of the environment. In freshwater habitats, exposure to anthropogenic metals rather than antibiotics is thought to be more important in selecting for antibiotic-resistant bacteria, including opportunistic human pathogens (36). In vitro, Zn was found to give rise to spontaneous mutants of *P. aeruginosa* affected in the *czcS* sensor gene constitutively resistant to the antibiotic imipenem (30). Similarly, Cu may produce such resistant strains by mutations in the *copS* sensor gene. Future research will determine whether constitutive coregulatory resistance is induced in metal-contaminated environments and represents a novel antibiotic resistance mechanism. These studies will contribute to assessments of the role of metal contaminants as a selective force in maintaining and propagating a pool of antibiotic-resistant determinants in reservoirs of clinically important microorganisms.

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