Pseudomonas aeruginosa is an environmental bacterium involved in mineralization of organic matter. It is also an opportunistic pathogen able to cause serious infections in immunocompromised hosts. As such, it is exposed to xenobiotics including solvents, heavy metals, and antimicrobials. We studied the response of P. aeruginosa upon exposure to heavy metals or antibiotics to investigate whether common regulatory mechanisms govern resistance to both types of compounds. We showed that sublethal zinc concentrations induced resistance to zinc, cadmium, and cobalt, while lethal zinc concentrations selected mutants constitutively resistant to these heavy metals. Both zinc-induced and stable zinc-resistant strains were also resistant to the carbapenem antibiotic imipenem. On the other hand, only 20% of clones selected on imipenem were also resistant to zinc. Heavy metal resistance in the mutants could be correlated by quantitative real time PCR with increased expression of the heavy metal efflux pump CzcCBA and its cognate two-component regulator genes czcR-czcS. Western blot analysis revealed reduced expression of the basic amino acid and carboxapenem-specific OprD porin in all imipenem-resistant mutants. Sequencing of the czcR-czcS DNA region in eight independent zinc- and imipenem-resistant mutants revealed the presence of the same V194L mutation in the CzcS sensor protein. Overexpression in a susceptible wild type strain of the mutated CzcS protein, but not of the wild type form, resulted in decreased oprD and increased czc expression. We further show that zinc is released from latex urinary catheters into urine in amounts sufficient to induce carbapenem resistance in P. aeruginosa, possibly compromising treatment of urinary tract infections by this class of antibiotics.

Pseudomonas aeruginosa is a Gram-negative bacterium thriving in environments polluted with organic matter. It is also an opportunistic pathogen frequently encountered in the hospital, causing morbidity and mortality in immunocompromised and cystic fibrosis patients (1). P. aeruginosa is characterized by an intrinsically high level of resistance to xenobiotics including antimicrobial agents, solvents, and heavy metals (2), which can be accounted for by a combination of its low outer membrane permeability and the presence of multiple efflux pumps (3). These pumps belong to the resistance, nodulation, cell division (RND)³ transporter family, present in many Gram-negative bacteria (4). To extrude substrates from the cytoplasm across the two membranes, these systems are composed of a proton antipporter located in the cytoplasmic membrane, a membrane fusion protein spanning the periplasmic space, and an outer membrane protein (5). Members of the RND family, namely the Mex pumps, have recently gained increasing interest. In particular, the constitutively expressed MexAB-OprM (6, 7) and the inducible MexXY (8) efflux pumps endow the PA01 reference strain and other clinical isolates (9) with a natural resistance to a wide range of antimicrobial agents. Proton-driven RND type efflux pumps conferring heavy metal resistance have been described in Ralstonia metallidurans (for a recent review, see Ref. 10) and include the Cnr system (nickel/cobalt) (11), the Ncc system (nickel/cobalt/cadmium) (12), and the Ccz system (cobalt/zinc/cadmium) (13, 14). In P. aeruginosa, an RND type efflux pump called CzrCBA was recently described in an environmental isolate where it contributes to the intrinsic resistance to zinc and cadmium (15). Cross-resistance between heavy metal and antibiotic pumps has not been reported so far. In the few cases where associations have been observed they were either plasmid-mediated (16) or resulted from uncharacterized multiple resistance mechanisms (17, 18). In P. aeruginosa, the question of heavy metal and antibiotic resistance is of particular concern since this organism is a possible candidate for bioremediation processes where selection of antibiotic resistance upon heavy metal exposure is undesirable. On the other hand, zinc was found to be released from urinary catheters resulting in antibiotic resistance (19, 20). Therefore the possibility of cross-resistance selection by either heavy metals or antibiotics is of concern for both environmental and clinical issues. In the present study, we addressed this question by exposing the P. aeruginosa reference strain PA01 to either zinc or to the antibiotic imipenem. Surprisingly exposure to zinc selected strains that were resistant to both heavy metals (zinc, cadmium, and cobalt) and to the carbapenem antibiotic imipenem. Analysis of the underlying mechanism revealed a co-regulation between carbapenem influx and heavy metal efflux. A single amino acid change located in the two-component sensor protein CzcS, regulating heavy metal efflux pump expression, was found to be responsible for the observed cross-resistance in P. aeruginosa.
Heavy Metal and Carbapenem Resistance in P. aeruginosa

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Bacterial strains used in this study are listed in Table I. Strain BD4 was isolated from soil of a heavily metal-contaminated site (zinc and lead) near Geneva (Bedou Bay) by successive subculturing in tryptone-yeast extract-glucose (TYG) medium (21) provided with increasing ZnCl₂ concentrations (10–35 mM; dose increase, 5 mM). Strain BD4 was confirmed as _P. aeruginosa_ using an API20E gallery (Biomerieux, Marcy l’Etoile, France). Luria-Bertani (LB) (22) and TYG medium were used as rich media. Some experiments were performed in liquid mineral medium (13) supplemented with 0.4% glucose and 50 mM sodium HEPES, pH 7.0, instead of Tris buffer. The phosphate content (0.64 mM) of this HEPES-buffered minimal medium minimized the interference of phosphate with heavy metals. Cultures were grown at 37 °C on a rotary shaker in 200-ml Erlenmeyer flasks containing 25 ml of minimal medium.

**Isolation of Spontaneous Mutants Resistant to Heavy Metals or Imipenem**—For selection of zinc-resistant mutants, PT5 was inoculated on a final concentration of about 2 × 10⁷ cells/ml in 5 ml of liquid TYG medium in test tubes that were incubated for 5 days at 30 °C. The medium was supplemented with 15 (maximal tolerable concentration (MTC)), 20, 25, or 30 mM ZnCl₂. From each condition, clones were isolated on TYG medium by serial dilutions. 50 clones were tested for zinc resistance after growth for 48 h at 30 °C on TYG plates containing 20 × 10⁻⁶ M ZnCl₂ independent clones; then, treated antibiotic susceptibility for zinc resistance and tolerance to other heavy metals. PT5 was also inoculated on Mueller-Hinton agar plates and exposed to imipenem-impregnated disks (10 μg, Biomerieux). After overnight incubation at 37 °C, 42 imipenem-resistant clones appearing inside the inhibition zone were picked, streaked on TYG medium, and tested for zinc resistance. Resistant mutants were then tested for tolerance to other heavy metals (cadmium, cobalt, copper, nickel) and antibiotics (penicillin, ticarcillin, carbencillin, nalidixic acid, ciprofloxacin, norfloxacin, tetracycline, amikacin, chloramphenicol, and polymyxin B).

**Determination of Heavy Metal and Antibiotic Resistance**—The MTCs of heavy metals were determined on solidified TYG medium containing different concentrations of heavy metal salts. The MTCs were scored in TYG medium in test tubes that were incubated for 5 days at 30 °C in TYG medium was supplemented with 15 (maximal tolerable concentration) 20, 25, or 30 mM ZnCl₂. From each condition, clones were isolated on TYG medium by serial dilutions. 50 clones were tested for zinc resistance after growth for 48 h at 30 °C on TYG plates containing 20 × 10⁻⁶ M ZnCl₂ independent clones; then, treated antibiotic susceptibility for zinc resistance and tolerance to other heavy metals.

**Killing Curves in Presence of Zinc**—PT5 was grown for 48 h at 37 °C in phosphate-deficient (0.1 mM) minimal medium to increase bioavailability of heavy metals. Cultures were performed in the absence (uninduced control cells) or in the presence of 1 mM ZnCl₂ (metal-induced cells). Treated cultures were found to exhibit a lag phase of about 24 h with shaking (150 rpm) for 5 h. Kinetics of killing of uninduced versus metal-induced cells was then followed. At defined intervals, samples were removed, diluted in TYG medium, and plated on the same medium for viable counts.

**DNA Manipulations**—Standard techniques were used for DNA manipulation (22). The gene encoding the CzcC protein was amplified by PCR from PT5 genomic DNA (Pfu DNA polymerase, Promega) with primers czcR-F and czcR-R (see below). The 900-bp product was cloned into the SmaI site of vector pMMB66EH (23) under the tac promoter, yielding plasmid pRWT. The _czcC_ gene from PT5 and PT1105 was amplified by PCR using primers S58 (5'-cggatcctgcatgatcagcttcg) and S59 (5'-cggatcctgcgagacgggttcg), containing an EcoRI and a HindIII site, respectively. The 1,600-bp product was EcoRI/HindIII-digested into the EcoRI site of pMMB66EH under the tac promoter, yielding plasmids pSWT and pSV194L, respectively. Correct orientation of the genes was verified by sequencing. Plasmids were electroporated into _P. aeruginosa_ strain PT5 (24). To delete the _czaA_ gene from strain PT5, a 600-bp PCR fragment corresponding to the 5′-end of _czaA_ was generated with primers 48 (5'-ccggatccgctgcgtggctggtg) and 49 (5'-gtctgctcggcggctggtggtg) and ligated to an 800-bp PCR fragment containing the 3′-end of _czaA_ generated with primers 50 (5'-gggatcctgcatgatcagcttcg) and 51 (5'-gtctgctcggcggctggtggtg). The gentamicin resistance cassette from plasmid pPS858 (25) was inserted between the two parts in the BamHI site. This construct was cloned as a PCR fragment into the HindIII-claved plasmid pEX18Ap (25). After transfer and homologous recombination into strain PT5, excision of the gentamicin cassette was performed as described (25). The resulting strain PT1173 carries a 1,700-bp deletion within the _czaA_ gene as verified by PCR.

**Sequence of _czcRS_**—The DNA region from position 2,843,235 to 2,845,974 on the PAO1 chromosome (26) including the promoter region of _czcR-czcC_ and the _czcS-czcC_ operon was amplified using four different sets of primers: procZcR-F (5'-gggatcctgcgagacgggttcg) and procZcR-R (5'-gtctgctcggcggctggtggtg); _czcR-F_ (5'-gggatcctgcgagacgggttcg) and _czcR-R_ (5'-gtctgctcggcggctggtggtg); _czcS-F_ (5'-gtctgctcggcggctggtggtg) and _czcS-R_ (5'-gtctgctcggcggctggtggtg). DNA sequencing was performed on double-stranded DNA templates obtained from genomic DNA by PCR amplification. Sequencing reactions were performed by the core facility of the Medical School of the University of Geneva using an Applied Biosystems (Foster City, CA) capillary sequencing machine (model 3100).

**Western Blot Analysis**—An overnight preculture of _P. aeruginosa_ grown in minimal medium was diluted 100-fold in the same medium and grown at 37 °C to an A₅₇₀ of 1. Total protein was solubilized by vigorously shaking a bacterial pellet in 1 × SDS gel loading buffer; samples were boiled 5 min and centrifuged 10 min in a microcentrifuge to remove bacterial debris. A duplicate sample was used for protein quantification with the Lowry method (27) on NaOH-solubilized extracts (28). 10 μg of total protein were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Blots were incubated with anti-OpD1, anti-OpD2, or anti-Hp70 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.

**Real Time PCR Analysis**—For RNA isolation, cultures were stored in 5 ml of minimal glucose medium described above or in LB medium and grown at 37 °C to midexponential growth phase. 0.25 ml of this culture, corresponding to 5 × 10⁷ cells, was added to 0.5 ml of RNasey Protect bacteria solution (Qiagen, Hildesheim, Germany), and total RNA was isolated with RNeasy columns according to the instructions of the supplier. Residual RNA was eliminated by DNase treatment using 20 units of RQ1 RNase-free DNase (Promega). After removal of DNase by phenol/chloroform extraction, RNA was precipitated, and the pellet resuspended in 50 μl of RNase-free H₂O. For cDNA synthesis, 1 μg of RNA was reverse-transcribed using random hexamer primers (Promega) and ImProm-II reverse transcriptase (Promega) according to the supplier’s instructions. Reverse transcriptase was inactivated by incubation at 70 °C for 15 min, and the obtained cDNAs were stored at −20 °C until use.

The following primer sequences for the PCR amplification of _czaA_ were designed using the Primer3 program²: czcR-F (5'-gtctgctcggcggctggtggtg) and czcR-R (5'-gtctgctcggcggctggtggtg), _czcS-F_ (5'-gtctgctcggcggctggtggtg) and _czcS-R_ (5'-gtctgctcggcggctggtggtg). A RotorGene real time PCR machine (model RG9000, software version 4.6.6.7) was used for the quantification of _czaA_. PCR experiments were performed using a Sybr Green Quantitect kit (Qiagen, Hilden, Germany) according to the instructions of the supplier. To check for residual contaminating DNA, control reactions without the template were amplified in the real time PCR apparatus using the rpsL–primers. The PCR product was analyzed in the real time PCR apparatus using the rpsL–primers. No amplification signal above the non-template control was detected indicating that the RNA samples were free of contaminating DNA. The cDNA samples were diluted 10-fold, and 3 μl of this diluted solution served as the template in the PCRs that were performed in duplicate for each sample. To correct for differences in the starting material, the ribosomal _rpsL_ gene was chosen as a reference gene. Results are presented as ratios of gene expression between the target gene and the reference gene (rpsL), which were obtained according to the following equation: ratio = [E_target gene]_target_ [E_reference gene]_target_ [E_target gene]_uninduced_ [E_reference gene]_uninduced_. The threshold for Real Time PCR efficiency for the _rpsL_ gene and the reaction was set at 20% (29).

² See www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi.
**Characterization of the CzcCBA Heavy Metal Efflux Pump from PAO1**—An efflux pump of the RND transporter family, called CzcCBA, was recently shown to produce tolerance to zinc and cadmium as well as to cobalt (Table II). A killing rate experiment showed that uninduced PT5 cells were killed after 3 h in the presence of 5 mM ZnCl₂, while about 50% of cells, preincubated in the presence of 1 mM ZnCl₂, stayed viable after 5 h of incubation (Fig. 1). Therefore, the induced cells are strongly protected by the CzcCBA efflux pump against a challenge with lethal heavy metal concentrations.

**Selection of Mutants Resistant to Either Zinc or Imipenem**—We attempted to select stable, heavy metal-resistant mutants from our wild type strain. We therefore exposed PT5 to elevated zinc concentrations (20–25 mM). Sixteen independent clones displayed resistance to zinc, cadmium, and cobalt with MTCs comparable to those of the induced PT5 strain (data not shown). The resistance phenotype was stable even after several passages in the absence of zinc. Since the CzcCBA pump belongs to the RND transporter family that also includes all the Mex multidrug efflux pumps, we tested the susceptibilities of the 16 zinc-selected mutants to various antibiotics (see “Experimental Procedures”). Surprisingly all the tested mutants displayed increased imipenem resistance, while susceptibility to the other antibiotics was unaffected (data not shown). One zinc- and imipenem-resistant mutant, called PT1108, was selected for further experiments (Table II).

To investigate a possible link between heavy metal and carbapenem resistance, the reciprocal experiment was performed by exposing strain PT5 to imipenem. Spontaneous mutants appearing after 24 h in the inhibition zone around the imipenem disk were picked, and their resistance profiles were analyzed. Two groups of mutants were identified. Members of the first group were resistant only to imipenem (24 of 30 mutants), while those of the second group were resistant to both heavy metals (zinc, cadmium, and cobalt) and imipenem (6 of 30 mutants). One mutant from the first group (PT1102) and one from the second (PT1105) were selected for subsequent experiments. All imipenem-resistant mutants showed resistance levels between those of the wild type and the oprD knock-out strain PT364 (Table II). Zinc- and imipenem-resistant mutants, like PT1105 and PT1108, were termed zir. The fact that mutants selected independently on either zinc or imipenem may display the same resistance profile suggested the existence of a common regulatory mechanism in P. aeruginosa.

**Expression of oprD in Zinc-induced Strains and in Heavy Metal-resistant Mutants**—In P. aeruginosa carbapenem resistance is known to result mainly from mutations affecting expression of the oprD porin (30) involved in the facilitated diffusion of basic amino acids (31) and carbapenem antibiotics (32). We therefore investigated OprD expression in the wild type strain PT5 in the presence of zinc and subsequently in the mutants obtained under zinc or imipenem selection. Western blot analysis revealed that in the wild type strain OprD expression strongly decreased at 3 μM ZnCl₂ and was undetectable at

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**Results**

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**Table I**

| Strains | Relevant characteristics | Reference or source |
|---------|-------------------------|---------------------|
| PT5     | PAO1 wild type          | Laboratory collection |
| PT364   | PT5 oprD::T1C           | 57                  |
| PT1173  | PT5 czcA               | This study          |
| PT1102  | PT5, IPTζ, selected on imipenem | This study |
| PT1105  | PT5, IPTζ, Znζ, Cdζ, Coζ, selected on zinc | This study |
| PT1108  | PT5, IPTζ, Znζ, Cdζ, Coζ, selected in zinc | This study |
| PT1152  | PT5 (pMMB66EH)         | This study          |
| PT1151  | PT5 (pSWT), overexpression of CzcR-WT | This study |
| PT1153  | PT5 (pSWT), overexpression of CzcS-WT | This study |
| PT1154  | PT5 (pSV194L), overexpression of CzcS(V194L) | This study |
| BdB4    | P. aeruginosa environmental isolate | This study |
| BdB4-3  | BdB4, IPTζ, Znζ, Cdζ, Coζ, selected on zinc | This study |

* See www.pseudomonas.com.
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TABLE II

| Strains      | Selection | MTCs | Zones of inhibition |
|--------------|-----------|------|---------------------|
|              |           |      | zinc | Cadmium | Cobalt | Copper | Nickel | IPM | CIP |
| PT5          | NA        | -2-3 | 6    | ND     | ND     | 3      | 25     | 32  |
| PT1173 (ΔczcA) | NA        | -2-3 | 6    | ND     | ND     | 3      | 27     | 30  |
| PT5 + zincab | NA        | 2-3  | 6    | 3      | 3      | 14ab   | 31b    |
| PT5 + cadmiumab | NA      | 2-3  | 6    | 3      | 3      | 13     | 34    |
| PT1102       | IPM       | 3    | 6    | 3      | 3      | 14     | 34    |
| PT1105       | IPM       | 3    | 6    | 3      | 3      | 14     | 34    |
| PT1108       | Zinc      | 2-3  | 6    | 3      | 3      | 14     | 34    |
| PT364        | NA        | 3    | 6    | 3      | 3      | 14     | 34    |
| BdB4         | NA        | 2-3  | 6    | 3      | 3      | 14     | 34    |
| BdB4-3       | Zinc      | 4    | 6    | 3      | 3      | 14     | 34    |

ab Induced overnight at 37 °C (2 mM zinc or 0.4 mM cadmium).

Fig. 1. Kinetics of killing by high zinc concentration. Colony forming units (cfu) were determined at different time points after incubation with (Z1 and Z2) or without (C1 and C2) a lethal zinc concentration of 5 mM in phosphate-limiting minimal medium. C1 and Z1 were preincubated in the same medium devoid of zinc, and C2 and Z2 were incubated in the presence of 1 mM ZnCl2.

10 μM ZnCl2 (Fig. 2A). Expression of the major outer membrane protein OprF was not affected. The amount of oprD mRNA was determined by quantitative real time PCR (qRT-PCR). In the presence of 1 μM ZnCl2, oprD mRNA levels dropped to 60% and decreased to 20% when 10 μM ZnCl2 was added. These results demonstrate that ZnCl2 has a negative effect on oprD transcription in a dose-dependent manner, resulting in carbapenem resistance in P. aeruginosa. However, heavy metal resistance is not caused by OprD down-regulation per se since PT364, an oprD knock-out mutant, displayed the same heavy metal susceptibility as its parent strain PT5 (Table II).

We further investigated by Western blot analysis the amount of OprD in the three strains obtained by either zinc or imipenem selection. A strongly reduced but still detectable amount of OprD was observed in all three strains (Fig. 2B). These results were confirmed by oprD mRNA transcript analysis using qRT-PCR. The level of oprD mRNA in strains PT1102, PT1105, and PT1108 dropped, respectively, to 30, 2, and 24% of those in the wild type. From these data, we conclude that imipenem resistance in the three mutant strains can be directly attributed to decreased oprD expression.

Involvement of CzcCBA Efflux Pump in Heavy Metal Resistance—We investigated the possible involvement of the CzcCBA efflux system in the heavy metal resistance by qRT-PCR. When wild type strain PT5 was grown in the presence of 5 mM zinc, the amount of czcC mRNA was increased 215-fold (Table III). Thus, zinc is able to strongly induce the czcCBA efflux system. Cadmium was less effective than zinc as an inducer, while, in contrast to R. metallidurans (33), cobalt and nickel had no measurable effect on transcription of czcC (data not shown).

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brane prediction program TMHMM-2.0 suggested the presence of two membrane-spanning segments (Fig. 3). According to this program, residues Val-194 and Gly-197 are located just outside the second transmembrane segment on the cytosolic side.

To verify that the V194L mutation in the CzcS sensor was responsible for the observed zir phenotype, we cloned the mutated czcS gene as well as the wild type czcS and czcR genes under the control of the inducible tac promoter in plasmid pMMB66EH. When the mutated CzcS protein was overexpressed in the wild type strain PT5, the level of OprD decreased significantly, while overexpression of the wild type CzcS protein had no effect (Fig. 4). In parallel, an increase in heavy metal resistance occurred upon expression of the CzcS protein carrying the V194L mutation, while expression of the normal CzcS protein had no influence (Table IV). MTCs of nickel were not affected. In agreement with decreased OprD expression, resistance to imipenem increased when CzcS(V194L) was expressed in the wild type strain, leading to a decrease of the inhibition zone from 26 to 19 mm (Table IV). Heavy metal and imipenem resistance levels obtained upon plasmid-directed expression of the mutated CzcS protein were lower than those in the mutant strains (PT1105 and PT1108), a fact that can be explained by competition with the wild type CzcS protein encoded on the chromosome. These results clearly demonstrate that the resistance to both heavy metals and imipenem in the selected mutants is due to the V194L mutation in CzcS (zirS mutants).

Effect of CzcR Overexpression—Since czcR transcription was strongly increased in the zirS mutants and upon zinc induction in the wild type strain, we also cloned the wild type czcR gene in vector pMMB66EH. When overexpressed in strain PT5, CzcR reduced OprD expression (Fig. 4) and rendered cells resistant to imipenem (Table IV). Surprisingly, CzcR overexpression did not result in increased resistance to zinc, cadmium, or cobalt but instead rendered bacteria more susceptible to these heavy metals (Table III). Thus, overexpression of wild type CzcR per se allows repression of OprD but is not sufficient to cause increased expression of the CzcCBA efflux pump.

Imipenem Resistance Induced by Zinc Release from Urinary Catheters—Recently Conejo et al. (20) showed that zinc eluted from siliconized latex urinary catheters decreases OprD expression in P. aeruginosa causing carbapenem resistance. The authors carried out their experiments using segments of siliconized latex urinary catheters incubated in Mueller-Hinton broth (34). To determine whether zinc was also released from latex urinary catheters (LUCs) into urine, we grew strain PT5 for 48 h at 37 °C in LUCs containing filter-sterilized urine. As determined by atomic absorption spectroscopy, zinc reached a concentration of about 1 mM in urine (Table V). This concentration was sufficient to induce wild type cells to resist the subsequent challenge by 20 mM zinc (Table V). This tolerance was accompanied by a 33-fold increased expression of the CzcCBA efflux system as determined by qRT-PCR (Table V). As expected, zinc released from LUCs decreased OprD expression.

| Gene | PT5 | PT5 + Zn | PT1102 | PT1105 | PT1108 |
|------|-----|---------|--------|--------|--------|
| czcC | 1.0 | 214.8 ± 29.4 | 0.3 ± 0.03 | 190.9 ± 12.2 | 217.5 ± 3.2 |
| czcR | 1.0 | 20.7 ± 1.9 | 0.4 ± 0.04 | 55.6 ± 3.0 | 61.5 ± 9.3 |
| czcS | 1.0 | 4.5 ± 0.5 | 0.5 ± 0.07 | 9.5 ± 0.1 | 7.8 ± 1.2 |

* Levels of mRNA are expressed as fold change relative to those in the wild type strain PT5.

** PT5 incubated in LB in the presence of 5 mM zinc.

** See www.cbs.dtu.dk/services/TMHMM-2.0.
of the heavy metal pumps could be obtained upon exposure to zinc and cadmium (36) or by constitutive expression resulting, as we show here, from mutations in the two-component sensor CzcS. Zinc in the growth medium not only induced the czcCBA efflux pump operon but at the same time transcription of the two-component regulator genes czcR and czcS. This positive autoregulation loop could allow the cells to respond rapidly to the presence of small amounts of heavy metals in the environment.

Analysis of mutants constitutively expressing the CzcCBA pump surprisingly revealed that all of them were also resistant to carbapenem antibiotics. This resulted from decreased expression of the porin OprD mediating facilitated diffusion of this class of antibiotics. However, OprD is not directly involved in heavy metal resistance since an oprD knock-out mutant was as susceptible as the parent to heavy metals. Hence OprD decrease is a secondary effect linked to CzcCBA overexpression. While only 20% of the analyzed strains selected on imipenem were co-resistant to heavy metals, it nevertheless strongly suggests the existence of a common regulatory mechanism that connects czcCBA to oprD expression. This hypothesis is supported by the identification of two mutational events found in the same region of the CzcS sensor kinase in zinc-selected derivatives from PT5 and from an environmental isolate. Moreover the V194L mutation was selected in PT5 either upon zinc or imipenem exposure (zirS mutants). Therefore, the sensor kinase CzcS via its cognate regulator CzcR is involved in the regulation of both heavy metal and antibiotic resistance. How could the mutations in CzcS lead to constitutive expression of the CzcCBA efflux pump and reduced expression of OprD? According to sequence analysis, CzcS, like EnvZ in Escherichia coli, belongs to class I histidine kinases. In this class the catalytic and ATP-binding domain is separated in two regions (37). The A domain is involved in the dimerization, phosphotransfer, and phosphatase activity, while the B domain binds ATP (38, 39). These histidine kinases transfer a phosphoryl group on an aspartate residue in the cognate regulator protein (OmpR in the case of EnvZ). The activated regulator protein then triggers expression of specific target genes. Many histidine kinases also possess phosphatase activities that allow dephosphorylation and hence inactivation of the regulator protein (37). In the case of EnvZ/OmpR, the level of phosphorylation of OmpR is important for the tight control of osmoregulation (40). Mutations in EnvZ that specifically affect either kinase or phosphatase activities were localized in separate structural domains (41). This bifunctional enzymatic activity of EnvZ is further regulated by a linker region containing two helices (42). Upon signal input, the interaction between these two helices is modified, thereby altering the relative position of the domain A and B and hence the ratio of kinase/phosphatase

![Efflux system](image)

![Resistance](image)

![Phenotype](image)

TABLE IV

| STRAINS          | MTCs | Zones of inhibition |
|------------------|------|--------------------|
|                  | Zinc | Cadmium | Cobalt | Nickel | IPM | CIP |
| PT5 (pMMB66EH)  | 15   | 6       | 2–3    | 3      | 26  | 34  |
| PT5 (pRWT)      | 10   | 4       | 1      | 3      | 13  | 32  |
| PT5 (pSWT)      | 15   | 6       | 2–3    | 3      | 26  | 33  |
| PT5 (pSV194L)   | 20   | 8       | 3      | 3      | 19  | 34  |

TABLE V

| Growth conditions | Zinc concentration | MTC of zinc | czcC | oprD | zirS |
|------------------|------------------|-------------|------|------|------|
|                  | mM               | mM          | -Fold expression over PT5
| Urine            | 0.026            | 15          | 1    | 1    |
| Urine inside LUCs| 1.1              | 20          | 33.2 | 0.15 |

* Values are means of triplicate measurements; the standard deviation was less than 12% of the mean.
activity of the catalytic and ATP-binding domain (42). The mutated Val-194 and Gly-197 residues in CzcS are located in this linker region that connects the putative catalytic and ATP-binding domain to the second transmembrane segment (Fig. 4). The V194L and the G197D mutations could therefore cause a conformational change in CzcS that is normally induced by the signal (heavy metals), causing CzcS to be permanently autophosphorylated. Alternatively the change in conformation could decrease the phosphatase activity of CzcS, thereby preventing or reducing dephosphorylation of CzcR.

Since overexpression of wild type CzcR only reduced OprD expression without increasing heavy metal resistance, it can be concluded that at least the unphosphorylated form of CzcR negatively regulates OprD, while the amount of active phosphorylated CzcR is critical for triggering czcCBA transcription.

Simultaneous overexpression of an efflux pump and down-regulation of a porin pathway seems to be an efficient mechanism to prevent intracellular accumulation of toxic molecules. This type of co-regulation has been described in nfxC type mutants of P. aeruginosa (43). These mutants overexpress the multidrug efflux pump MexEF-OprN (44), conferring resistance to quinolones, chloramphenicol, and trimethoprim, and are resistant to carbapenems due to decreased OprD expression (44, 45). Interestingly this resistance phenotype can be obtained in the susceptible wild type PT5 upon plasmid-mediated overexpression of MexT, the transcriptional activator of the MexEF-OprN efflux pump (46, 47). MexT overexpression was shown to decrease both transcriptional (46, 47) and post-transcriptional expression of oprD (46). Hence both CzcR and MexT are able to repress OprD expression, which could occur either directly or via another regulator (Fig. 5). Since in PT5 as well as in other PAO1 strains the oprD gene (52) is controlled by environmental signals and subsequent infection. It should take into account an eventual colonization by carbapenem-resistant P. aeruginosa and subsequent infection.

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REFERENCES

1. Rosenfeld, M., Ramsey, B. W., and Gibson, R. L. (2003) Curr. Opin. Pulm. Med. 9, 492–497
2. Wang, C. L., Michels, P. C., Dawson, S. C., Kitasakkul, S., Baross, J. A., Keasling, J. D., and Clark, D. S. (1997) Appl. Environ. Microbiol. 63, 4075–4078
3. Nikaido, H. (1994) Science 264, 382–388
4. Saier, M. H., Tam, R., Reizer, A., and Reizer, J. (1994) Mol. Microbiol. 11, 841–847
5. Nikaido, H. (1996) J. Bacteriol. 178, 5853–5859
6. Poole, K., Krebes, K., McNally, C., and Neeshat, S. (1993) J. Bacteriol. 175, 7363–7372
7. Li, X.-Z., Nikaido, H., and Poole, K. (1995) Antimicrob. Agents Chemother. 39, 1543–1553
8. Ramos-Aires, J., Kohler, T., Nikaido, H., and Plesiat, P. (1999) Antimicrob. Agents Chemother. 43, 2624–2628
9. Ziba-Zarifi, I., Llanes, C., Kohler, T., Pechere, J. C., and Plesiat, P. (1998) Antimicrob. Agents Chemother. 43, 287–291
10. Merguey, M., Monchy, S., Vallaeys, T., Auquier, V., Benotmane, A., Bertin, P., Toghiavi, S., Dunn, J., Carette, J., Delerie, L., and Wattiez, R. (2003) FEMS Microbiol. Rev. 27, 385–410
11. Liesegang, H., Lemke, K., Siddiqui, R. A., and Schlegel, H. G. (1993) J. Bacteriol. 175, 767–778
12. Schmidt, T., and Schlegel, H. G. (1994) J. Bacteriol. 176, 7045–7054
13. Merguey, M., Nies, D., Schlegel, H. G., Gerits, J., Charles, P., and Van Gijseghem, P. (1985) J. Bacteriol. 162, 328–334
14. Nies, D., Merguey, M., Friedrich, B., and Schlegel, H. G. (1987) J. Bacteriol. 169, 4865–4868
15. Hassan, M. T., van der Lelie, D., Springael, D., Remling, U., Ahmed, N., and Merguey, M. (1989) Gene 79, 417–421
16. Marques, A. M., Congregado, F., and Simon-Pujol, D. M. (1979) J. Appl. Bacteriol. 47, 347–350
17. Filali, B. K., Tanukij, J., Zouali, Y., Dazai, F. Z., Tabli, M., and Blaesign, H. (2000) Curr. Microbiol. 41, 151–156
18. de Vicente, A., Aviles, M., Codina, J. C., Corrojo, J. R., and Romero, P. (1990) J. Bacteriol. 168, 625–629
19. de Haan, K. E., Woroniecka, U. D., Boxma, H., de Groot, C. J., and van den Hamer, C. J. (1990) Burns 16, 393–395
20. Conejo, M. C., Garcia, I., Martinez-Martinez, L., Picaben, L., and Pascual, A. (2003) Antimicrob. Agents Chemother. 47, 2313–2315
21. Merguey, M., Gerits, J., and Houba, C. (1978) C. R. Seances Soc. Biol. Fیلد 172, 575–579
22. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. Purste, J. P., Pansegrea, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M., and Lanks, E. (1986) (Amst.) 48, 119–131
24. Smith, A. W., and Iglesweski, B. H. (1989) Nucleic Acids Res. 17, 10509
25. Hoang, T. T., Karkhoff-Schweizer, R. R., Kutcheva, A. J., and Schweizer, H. P. (1998) Gene (Amst.) 212, 77–86
26. Stover, C. K., Pham, X. Q., Erwin, A. L., Minzoguchi, S. D., Warrenner, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Couliot, S. N., Polger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, K. G., Wu, Z., and Paulsen, I. T. (2000) Nature 406, 959–964
27. Lowery, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
28. Hanson, R. S., and Merguey, M. (1992) Curr. Opinion in Pulmonary Medicine, 8, 349–353
29. Trias, J., and Nikaido, H. (1999) J. Bacteriol. 181, 4079–4082
30. Trias, J., and Nikaido, H. (1999) Antimicrob. Agents Chemother. 44, 99–103
31. Nies, D. H. (1992) J. Bacteriol. 174, 8110–8112
32. Martinez-Martinez, L., Pascual, A., Conejo, M. C., Picaben, L., and Perea, E. J. (1989) Antimicrob. Agents Chemother. 33, 397–399
33. Rensing, C., Pribyl, T., and Nies, D. H. (1997) J. Bacteriol. 179, 6871–6879
34. Nies, D. H., and Silver, S. (1989) J. Bacteriol. 171, 896–900
35. Taghavi, S., Dunn, J. J., and Merguey, M. (1999) Antimicrob. Agents Chemother. 43, 395–407
36. Park, H., Saha, S. K., and Inouye, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6728–6732
37. Zhu, Y., Qin, L., Yoshida, T., and Inouye, M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7803–7813
38. Yoshida, T., Cai, S., and Inouye, M. Microbiol. 46, 1283–1294
39. Hsing, W., Russo, F. D., Bernd, K. K., and Silhavy, T. J. (1998) J. Bacteriol.
42. Zhu, Y., and Inouye, M. (2003) J. Biol. Chem. 278, 22812–22819
43. Fukuda, H., Hosaka, M., Hirai, K., and Iyobe, S. (1990) Antimicrob. Agents Chemother. 34, 1757–1761
44. Köhler, T., Michea-Hamzehpour, M., Henze, U., Gotoh, N., Kocjancic-Curty, L., and Pechère, J. C. (1997) Mol. Microbiol. 23, 345–354
45. Masuda, N., and Ohya, S. (1992) Antimicrob. Agents Chemother. 36, 1847–1851
46. Köhler, T., Epp, S. F., Kocjancic-Curty, L., and Pechère, J. C. (1999) J. Bacteriol. 181, 6300–6305
47. Ochs, M. M., McCusker, M. P., Bains, M., and Hancock, R. E. (1999) Antimicrob. Agents Chemother. 43, 1085–1090
48. Maseda, H., Saito, K., Nakajima, A., and Nakae, T. (2000) FEMS Microbiol. Lett. 192, 107–112
49. Andersen, J., and Delihas, N. (1990) Biochemistry 29, 9249–9256
50. Tanaka, T., Hori, T., Shibayama, K., Sato, K., Ohsuka, S., Arakawa, Y., Yamaki, K., Takagi, K., and Ohta, M. (1997) Microbiol. Immunol. 41, 697–702
51. Delihas, N., and Forst, S. (2001) J. Mol. Biol. 313, 1–12
52. Esterling, L., and Delihas, N. (1994) Mol. Microbiol. 12, 639–646
53. Cooper, G. L., Louie, A., Baltch, A. L., Chu, R. C., Smith, R. P., Ritz, W. J., and Michelsen, P. (1998) J. Clin. Microbiol. 36, 2368–2370
54. Daly, J. S., Dodge, R. A., Glew, R. H., Soja, D. T., DeLuca, B. A., and Hebert, S. (1997) J. Clin. Microbiol. 35, 1027–1029
55. Baxter, I. A., and Lambert, P. A. (1997) J. Antimicrob. Chemother. 39, 838–839
56. Talja, M., Saarela, K., Ruutu, M., Andersson, L. C., and Alfthan, O. (1993) Ann. Chir. Gynaecol. Suppl. 206, 74–79
57. Epp, S. F., Köhler, T., Pléziat, P., Michea-Hamzehpour, M., Frey, J., and Pechère, J. C. (2001) Antimicrob. Agents Chemother. 45, 1780–1787