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# Identification of a gene cluster, *czr*, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa* ☆

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**Abstract**

*Pseudomonas aeruginosa* CMG103 was isolated from a metal-polluted river in Pakistan and displayed a high level of Zn and Cd resistance. An Ω-Km transposon mutant of strain CMG103, which showed a substantial decrease in resistance to Zn and Cd, was obtained. A 12.8 kb region determining Zn and Cd resistance in strain CM103 was cloned by complementing the mutant strain, and its nt sequence was determined. Five genes, *czrSRCBA*, involved in Zn and Cd resistance, were identified. The predicted gene products of *czrCBA*show a significant similarity with the proteins encoded by the plasmid borne metal resistant determinants *czc*, *cnr* and *ncc* of *Ralstonia* strains, which determine a chemiosmotic cation-antiporter efflux system. The predicted CzrS and CzrR proteins show a significant similarity to the sensor and regulatory protein, respectively, of two component regulatory systems, such as CopS/CopR and PcoS/PcoR involved in the regulation of plasmid-borne Cu-resistant determinants, and CzcS/CzcR involved in the regulation of *czc*. The cloned *czr*region contained downstream of *czrCBA* additional ORFs whose predicted gene products are similar to proteins involved in catabolism of aromatic compounds. DNA–DNA hybridization indicated strong conservation of *czr* in other enviromental *P. aeruginosa* isolates and in the *P. aeruginosa*type strain PAO1, a clinical isolate. This was confirmed by a comparison of the sequence of the CMG103 *czr* region with the currently available genome sequence of strain PAO1. A high sequence identity (till 99% at the nt level) and organizatory conservation of the *czr* region of CMG103 was found in PAO1 as well regarding coding sequences as intervening sequences between ORFs. The *czr* locus was localized between coordinates 2400 and 2550 kb on the physical map of the chromosome of PAO1. 

*Keywords:* Chromosome; DNA sequence; Polluted water sample; *Ralstonia eutropha*-like

## 1. Introduction

The genetic determinants for Cd and Zn resistance have been well studied in the Gram-positive bacterium, *Staphylococcus aureus*, where Cd resistance is encoded by the *cadCA* operon (Endo and Silver, 1995) and in the facultative chemolithotrophic Gram-negative bacterium, *Alcaligenes eutrophus* CH34, recently renamed *Ralstonia eutropha*-like CH34 (Brim et al., 1999), where Cd, Zn and Co resistance is encoded by the *czc* gene cluster (Mergeay et al., 1985; Nies et al., 1989).

The *czcNICBADRS* gene cluster contains eight ORFs encoding the polypeptides CzcN, CzcI, CzcC, CzcB, CzcA, CzcD, CzcR and CzcS (Nies et al., 1989; van der

Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); *czc*, cadmium, zinc and cobalt resistance determinant of *R. eutropha*; *czr*, cadmium and zinc resistance determinant of *P. aeruginosa*; kb, kilobase(s); Km, kanamycin; mM, millimolar; MTC, maximum tolerable concentration; nt, nucleotide(s); PFGE, pulsed field gel electrophoresis; OD, optical density; Ω-Km, Omegon-Km; ORF, open reading frame; SDS, sodium dodecyl sulfate; Sm, streptomycin; SSC, 0.15 M NaCl/0.015 M $Na_3$.citrate, pH 7.6; Tc, tetracycline.

☆ The DNA sequence of the *Pseudomonas aeruginosa* region containing the *czr* determinant has been submitted to EMBL under the Accession No. Y14018.

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Lelie et al., 1997). The CzcC, CzcB and CzcA proteins constitute a three-component chemiosmotic antiporter efflux system that actively transports the cations out of the bacterial cell. In this efflux system, CzcA is a central protein and functions as a pump (chemiosmotic cation/$H^+$ antiporter) driven by a $H^+$ gradient. CzcB and CzcC are associated with CzcA and apparently influence the degree and specificity of the metal efflux (Nies and Silver, 1995). The regulatory genes of the *czc* determinant are arranged in an upstream regulatory region consisting of *czcN* and *czcI* and a downstream regulatory region consisting of *czcD*, *czcR* and *czcS* (van der Lelie et al., 1997).

However, little is known about Zn and Cd resistance in fluorescent *Pseudomonas* (Horitsu et al., 1986) although this genus, and especially *Pseudomonas aeruginosa*, shows a fairly high resistance to these metals (Wang et al., 1997). In this paper, we report on new environmental isolates of *P. aeruginosa*, displaying a high resistance to Zn and Cd, and the identification and analysis of the genetic determinant involved in resistance to these metal ions. We show that the genetic determinant for Zn and Cd resistance is strongly conserved in both environmental and clinical isolates of *P. aeruginosa*.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids and culture media

The bacterial strains and plasmids used in this study are listed in Table 1. Zn-resistant strains CMG101–CMG107 were isolated from different metal-polluted water samples of the Malir river of Karachi. The Malir river is a small coastal river that passes through an important industrial area of Karachi and ends in the Arabian sea. The water samples were taken at the site of entry of industrial and sewage waste. All isolates were identified as *P. aeruginosa* using Gram staining and API20NE (Biomerieux, France). *P. aeruginosa* and *R. eutropha*-like strains were grown at 30°C, and *Escherichia coli* was grown at 37°C. Luria–Bertani medium (Sambrook et al., 1989) and Nutrient Broth 3 medium (Difco Laboratories) were used as rich media. Resistance to heavy metal salts was tested on Tris mineral medium supplemented with 0.2% gluconate (Mergeay et al., 1985). When necessary, media were supplemented with the appropriate concentration of antibiotics from stock solutions sterilized by filtration.

### 2.2. Determination of the MTC of heavy metals

The MTCs of heavy metals were determined by streaking the strains on solidified Tris-gluconate medium containing different concentrations of heavy metal salts. The plates were incubated at 30°C and checked for growth for up to 4 days. The MTC is the highest metal concentration at which growth was still observed.

### 2.3. Molecular biology

Standard recombinant DNA techniques were used for DNA extraction, cloning and subcloning (Sambrook et al., 1989). Plasmid extraction from *P. aeruginosa* was performed as described by Kado and Liu (1981). PFGE was performed as described previously (Römling and Tümmler, 1991). DNA fragments to be used as probes were extracted from the agarose gel using the Gene clean II purification kit of BIO101, La Jolla, CA. DNA labelling was carried out using a multiprime DNA labelling reagent kit of Amersham with [$^{32}$P]dCTP. Southern blotting, using Gene Screen Plus membranes (Dupont, NEN Research Products, Boston, MA), was carried out as described by the manufacturer. Hybridization was performed at 65°C, and the membranes were washed twice at 65°C in 0.1 × SSC containing 0.1% SDS. The genomic library of CMG103 was constructed in the IncP broad host range cosmid vector pLAFR3 as described by Staskawicz et al. (1987). The recombinant DNA molecules were packaged into phage particles using the Gigapack II DNA Packaging extract kit (Stratagene, La Jolla, CA). Recombinant plasmids were electroporated into *E. coli* strain DH10B using a Bio Rad Gene pulser (BioRad Laboratories, Richmond, CA) according to the manufacturer's instructions.

### 2.4. Conjugative matings and transposon mutagenesis

Matings were performed on solid medium. Matings between *E. coli* and *P. aeruginosa* were performed at 42°C. The recipient *P. aeruginosa* strain was pregrown overnight in LB at 42°C without aeration, to minimize its restriction capacity. *E. coli* HB101 (pRK2013) was used as helper strain to supply transfer functions (Figurski and Helinski, 1979). Zn-sensitive mutants of *P. aeruginosa* CMG103 were obtained by delivery of suicidal vector, pJFF350 carrying the Ω-Km transposon (Fellay et al., 1989). $Km^R$ transconjugants strains were selected on Tris medium containing 1000 μg/ml of Km. Five hundred $Km^R$ colonies were replica-plated on Tris gluconate agar plates containing 1000 μg/ml of Km and/or different concentrations of $ZnCl_2$or $CdCl_2$ to check the loss of Zn and/or Cd resistance.

### 2.5. Growth studies

Growth studies were performed at 30°C on a rotary shaker in 125 ml Wiame flasks (side-armed) containing 20 ml of Tris medium. Heavy metal salts were supplemented at final concentrations of 1 mM of Zn and 0.8 mM of Cd. Cells were inoculated at an initial $OD_{660}$ of 0.05 from overnight precultures ($OD_{660}$ =

Table 1
Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics[a] | Reference or source |
|---|---|---|
| *P. aeruginosa* strains | | |
| CMG101 | $Zn^R$ , $Cd^R$ | This study |
| CMG102 | $Zn^R$, $Cd^R$, pMOL1033 | This study |
| CMG103 | $Zn^R$, $Cd^R$ | This study |
| CMG104 | $Zn^R$, $Cd^R$, pMOL1034 | This study |
| CMG105 | $Zn^R$, $Cd^R$, pMOL1035 | This study |
| CMG106 | $Zn^R$, $Cd^R$, pMOL1036 | This study |
| CMG107 | $Zn^R$, $Cd^R$, pMOL1037 | This study |
| CMG103#13 | $Zn^S$, $Cd^S$, $Km^R$, *czr*-13::ΩKm | This study |
| DS178 | $Zn^R$, $Cd^R$ | L. Diels |
| PAO1 | $Zn^R$, $Cd^R$ | Holloway et al. (1994) |
| *R. eutropha*-like strains | | |
| CH34 | pMOL28, pMOL30, $Cd^R$, $Zn^R$, $Co^R$, $Ni^R$, $Cu^R$, $Hg^R$, $Cr^R$ | Mergeay et al. (1985) |
| AE104 | Plasmid-free derivative of strain CH34 | Mergeay et al. (1985) |
| *E. coli* strains | | |
| DH10B | $F^-$, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), ϕ80d*lacZ*Δ*M15*, *ΔlacX74*, *deoR*, *recA1*, *endA1*, *araD139*, Δ(*ara*, *leu*)*7697*, *galU*, *galK*, $\lambda^-$, *rpsL*, *nupG* | Gibco BRL |
| HB101 | $F^-$, *hsdR20* ($R^-M^-$), *supE44*, *ara-14*, *galK12*, *lacY1*, *proA2*, *rpsL20* ($Sm^R$), *xyl-5*, *mtl-1*, *recA13*, *mcrB*, *mrr*, *thi*, *leuB6* | Gibco BRL |
| *Plasmids* | | |
| pJFF350 | 5.3 kb, carries Ω-Km, $Mob^+$, ColE1 | Fellay et al. (1989) |
| pUC19 | 2.7 kb, contains the *lac* promotor and *lacZ*, ColE1, $Ap^R$ | Gibco BRL |
| pLAFR3 | 22 kb, cosmid, contains the *lac* promotor and *lacZ*, $Mob^+$, IncP1, $Tc^R$ | Staskawicz et al. (1987) |
| pRK2013 | $Km^R$, $Tra^+$, repE1 | Figurski and Helinski (1979) |

[a] $Km^R$, $Ap^R$, $Tc^R$, $Sm^R$, $Ni^R$, $Co^R$, $Cu^R$, $Hg^R$, $Zn^R$, $Cd^R$: resistance to kanamycin, ampicillin, tetracycline, streptomycin, Ni, Co, Cu, Hg, Zn and Cd, respectively; $Zn^S$, $Cd^S$: sensitivity to Zn and Cd, respectively.

0.5–0.8). Uninduced precultures were grown in Tris gluconate medium. Metal-induced precultures were grown in Tris gluconate medium containing 0.2 mM of Zn or 0.1 mM of Cd. $OD_{660}$ was determined in a Spectrometer (Milton Ray Company/Analis, Gent, Belgium).

### 2.6. Nucleotide sequence determination and analysis

Double-stranded DNA sequence determination was performed by the Sanger dideoxy chain termination method, using the Auto Read Sequencing Kit of Pharmacia (Pharmacia, Uppsala, Sweden) on a Pharmacia Automated Laser Fluorescent A.L.F. DNA sequencer apparatus. The nt sequences were analysed with the Genetics Computer Group (University of Wisconsin) (version 8.0) program using FASTA and the non-redundant GenBank–EMBL database.

## 3. Results

### 3.1. Determination of the metal resistance phenotype of the Malir river P. aeruginosa isolates

The MTCs of different heavy metal ions were determined for the *P. aeruginosa* isolates CMG101 to CMG107 and compared with the MTCs of reference strains *P. aeruginosa* DS178, *P. aeruginosa* PAO1, *R. eutropha*-like CH34 and its metal-sensitive and plasmid-free derivative AE104 (Table 2). The CMG strains demonstrated a significant metal resistance to Zn and Cd and a lower level of resistance against Co and Ni. *P. aeruginosa* CMG103 showed the highest resistance to Zn and Cd, i.e. 8.0 and 2.0 mM, respectively, among all examined *P. aeruginosa* strains and displayed a slightly higher resistance than strain CH34. Therefore, strain CMG103 was chosen for further analysis of its Zn and Cd resistance in growth studies with metal-induced and uninduced inocula. Cells of CMG103 precultured in the absence of metals exhibited a lag phase when grown in the presence of Zn or Cd, while Zn or Cd precultured cells did not show any lag phase when grown in the presence of metals (data not shown). These observations suggest the existence of a genetically controlled active resistance mechanism in strain CMG103. Moreover, Zn was able to induce Cd resistance, and Cd was able to induce Zn resistance, indicating that Cd and Zn resistances are linked. *P. aeruginosa* strains CMG101 and CMG103 were found to be plasmid-free. The other new *P. aeruginosa* isolates displayed a plasmid band of an approximate molecular weight of 300 kb (data not shown).

### 3.2. Cloning of a genetic determinant involved in Zn/Cd resistance in P. aeruginosa CMG103

Ω-Km transposon mutagenesis was applied to generate Zn-sensitive mutants of strain CMG103. One trans-

Table 2
MTCs (in mM) of different heavy metals at 30°C for the *P. aeruginosa* CMG strains, *P. aeruginosa* DS178, *P. aeruginosa* PAO1, *R. eutropha*-like CH34, *R. eutropha*-like AE104, and CMG103 mutant strain CMG 103#13 and diploids

| Strain | MTC (mM) | | | | Relevant genotype/phenotype |
|---|---|---|---|---|---|
| | Zn | Cd | Co | Ni | |
| CMG101 | 7.0 | 1.5 | 0.4 | 0.2 | *czr*$^+$ |
| CMG102 | 5.0 | 1.5 | 0.4 | 0.2 | *czr*$^+$ |
| CMG103 | 8.0 | 2.0 | 0.4 | 0.2 | *czr*$^+$ |
| CMG104 | 6.0 | 1.0 | 0.4 | 0.2 | *czr*$^+$ |
| CMG105 | 5.0 | 2.0 | 0.4 | 0.2 | *czr*$^+$ |
| CMG106 | 7.0 | 2.0 | 0.4 | 0.2 | *czr*$^+$ |
| CMG107 | 7.0 | 1.5 | 0.4 | 0.2 | *czr*$^+$ |
| DS178 | 4.0 | 1.0 | 0.4 | <0.2 | *czr*$^+$ |
| PAO1 | 4.0 | 1.0 | 0.4 | <0.2 | *czr*$^+$ |
| CH34 | 7.0 | 1.0 | 5.0 | 2.5 | *czc*$^+$ |
| AE104 | 0.6 | 0.6 | 0.4 | 0.6 | Czc$^-$ |
| CMG 103#13 | 0.6 | 0.4 | <0.2 | <0.2 | *czr-13* |
| CMG 103#13 (pMOL864) | 8.0 | 2.0 | 0.2 | 0.2 | *czr-13*/*czr*$^+$ |
| CMG 103#13 (pMOL865) | 8.0 | 2.0 | 0.2 | 0.2 | *czr-13*/*czr*$^+$ |
| CMG 103#13 (pMOL866) | 8.0 | 2.0 | 0.2 | 0.2 | *czr-13*/*czr*$^+$ |
| CMG 103#13 (pMOL867) | 8.0 | 2.0 | 0.2 | 0.2 | *czr-13*/*czr*$^+$ |
| CMG 103#13 (pMOL888) | 8.0 | 1.5 | 0.2 | 0.2 | *czr-13*/*czr*$^+$ |

conjugant, i.e. CMG103#13, showed a substantial decrease in resistance from 8 to 0.6 mM Zn and from 2 to 0.4 mM Cd and also a decreased tolerance to Ni and Co (Table 2). A genomic library of strain CMG103, constructed in cosmid pLAFR3, was used to complement strain CMG103#13 for Zn resistance. Tris gluconate agar plates containing 1 mM Zn and 200 μg/ml of Tc were used to select for the complementation of CMG103#13, and Tris gluconate agar plates with 200 μg/ml of Tc were used to check the transfer of the vector. Zn-resistant transconjugants were recovered in 2% of the tested Tc$^R$transconjugants. Restriction endonuclease site analysis of recombinant cosmid DNA isolated from randomly chosen Zn-resistant transconjugants showed different restriction patterns with common insert fragments. The physical maps of four representative recombinant cosmids, i.e. pMOL864, pMOL865, pMOL866 and pMOL867, were compiled. The inserts displayed an overlapping fragment of 15.8 kb (Fig. 1A). The presence of the metal-resistance determinant on the recombinant cosmids was confirmed by reintroduction of the recombinant cosmids into CMG103#13, with 100% restoration of the Zn and Cd resistance phenotype and of wild-type tolerance to Ni and Co (Table 2). *Bam*HI-, *Hin*dIII- and *Eco*RI-digested fragments of pMOL864 were further subcloned into pLAFR3 and screened for the complementation of strain CMG103#13. The strain containing pMOL888, which carries a 12.8 kb *Hin*dIII fragment of pMOL864, displayed wild-type resistance to Zn and Cd (Fig. 1A) and to other metals and showed a Zn and Cd resistance induction pattern similar to the wild-type strain (data not shown).

### 3.3. Nucleotide sequence analysis of the Cd- and Zn-resistance locus of P. aeruginosa CMG103

Subcloning and DNA sequence analysis of the 12.8 kb fragment of pMOL888 revealed the presence of eight ORFs with significant similarities to sequences present in the EMBL and non-redundant GenBank databases.

Three ORFs (Fig. 1B) were identified encoding proteins with significant similarity to the CzcC, CzcB and CzcA proteins encoded by the plasmid borne metal resistance determinant, *czc*, of the metallotolerant strain *R. eutropha*-like CH34 (Nies et al., 1989) (Table 3). Due to the similarities with *czcCBA*, the three ORFs are proposed to be the structural genes of the metal-resistance determinant in *P. aeruginosa* CMG103 and were named *czrC*, *czrB*, and *czrA*.

Like the *R. eutropha*-like CH34 CzcC, CnrC and NccC proteins, CzrC showed a significant homology with other outer membrane factors involved as auxiliary proteins in the secretion or efflux of different metabolites or drugs from the bacterial cell (Saier et al., 1994) (Table 3). The putative aa sequence of CzrC displays at its N terminus a putative lipoprotein signal peptidase cleavage site between residue 4 and 17 (Wu and Tokunaga, 1986) which seems to be a significant characteristic of outer membrane proteins.

The second protein specified by the *czrCBA* gene cluster, CzrB, shows outside CzcB notable identity with other members of a family of bacterial membrane fusion proteins that facilitate the tranporter proteins to efflux or export cations/drugs/metabolites from the cell (Saier et al., 1994) (Table 3). In comparison with CzcB, CzrB is shorter at its N terminal site and thus misses the two

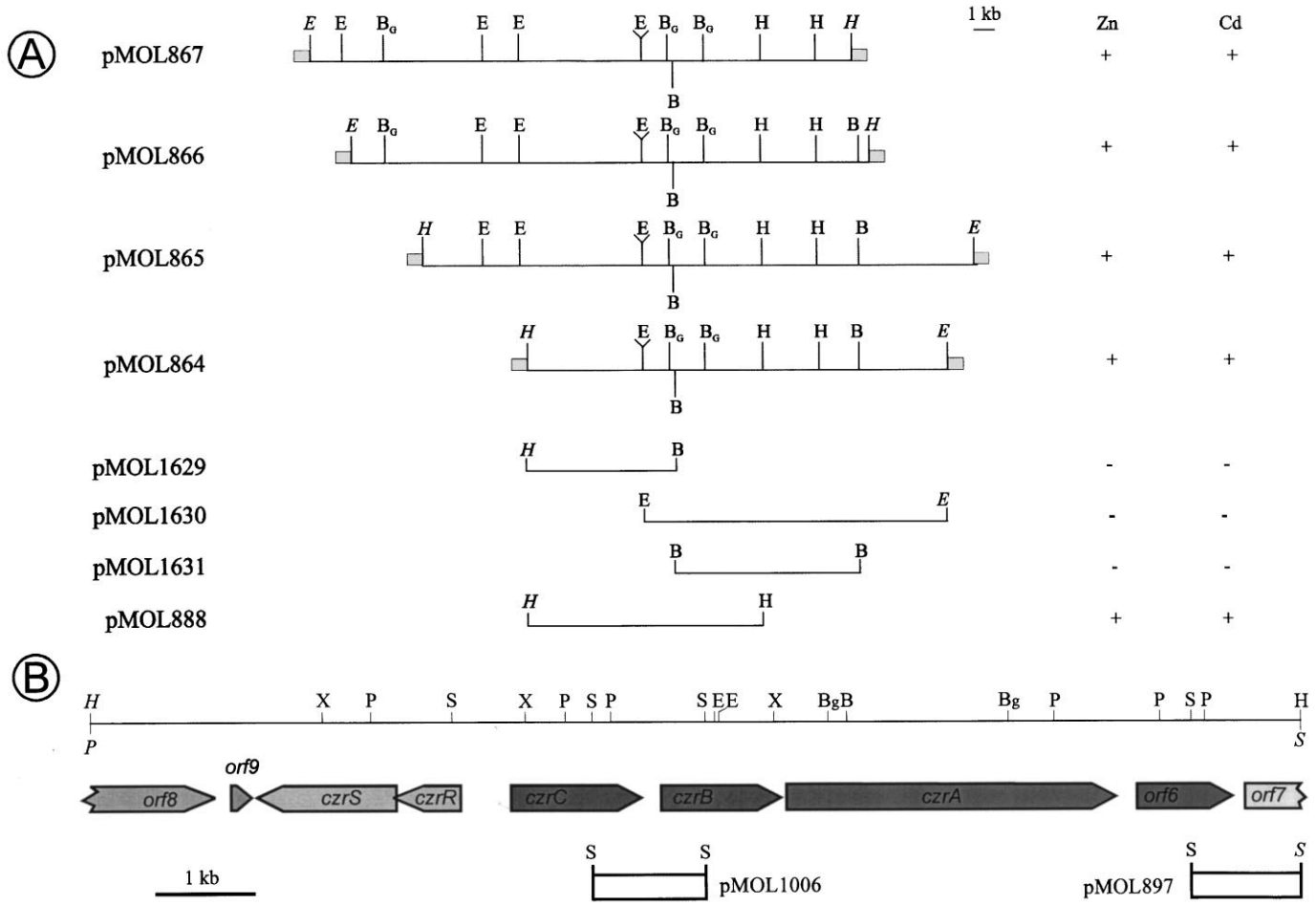


Fig. 1. Cloning and gene organization of the Zn and Cd resistance locus of *P. aeruginosa* CMG103. (**A**) Restriction maps of *czr* containing pLAFR3 recombinant cosmids, pMOL864, pMOL865, pMOL866 and pMOL867, subclones of pMOL864, and complementation of CMG103#13 for Zn and Cd resistance (+, growth in the presence of metals 2 mM Zn and 1 mM Cd; −, no growth in the presence of metals). (**B**) Detailed restriction map of pMOL888 and position of the identified ORFs. The direction of transcription of the ORFs is indicated. Abbreviations for restriction enzyme sites are as follows: X, *Xho*I; P, *Pst*I; S, *Sal*I; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII and E, *Eco*RI. Restriction sites indicated in italics belong to the vector.

Table 3
Similarity (identity) comparisons of the putative aa sequences of the gene products of *czrS*, *czrR*, *czrC*, *czrB* and *czrA* of *P. aeruginosa* CMG103 with corresponding deduced aa sequences of gene products from other relevant gene clusters[a]

| | CzrS | CzrR | CzrC | CzrB | CzrA |
|---|---|---|---|---|---|
| *czcNICBADRS* | 42.6 (33.7) | 69.5 (55.6) | 47.5 (39.5) | 61.7 (53.5) | 82.1 (74.6) |
| *cnrYXHCBA* | NR[b] | NR | 36.5 (30.2) | 36.6 (29.4) | 59.7 (48.8) |
| *nccYXHCBA* | NR | NR | 35.7 (29.5) | 35.5 (28.5) | 60.7 (49.8) |
| *helCBA* | NR | NR | 35.0 (25.2) | 42.5 (33.9) | 70.9 (62.1) |
| *mexABoprM* | NR | NR | 36.6 (29.0) | 35.8 (28.3) | 37.1 (26.1) |
| *mexCDoprJ* | NR | NR | 31.3 (22.8) | 37.2 (29.5) | 36.7 (26.6) |
| *mexEFoprN* | NR | NR | 30.5 (23.9) | 33.3 (26.1) | 36.0 (25.7) |
| *copABCDRS* | 51.0 (43.8) | 66.8 (55.6) | NR | NR | NR |
| *pcoABCDRS* | 44.5 (36.1) | 64.7 (53.4) | NR | NR | NR |

[a] *czcNICBADRS* and *cnrYXHCBA* are from *R. eutropha*-like CH34 (Liesegang et al., 1993; van der Lelie et al., 1997); *nccYXHCBA* is from *A. xylosoxidans* 31A (Schmidt and Schlegel, 1994); *helCBA* is from *Legionella pneumophila* (Arroyo et al., 1994); *mexABoprM*, *mexCDoprJ* and *mexEFoprN* are from *P. aeruginosa* PAO1 (Poole et al., 1996a,b; Köhler et al., 1997); *copABCDRS* is from *Pseudomonas syringae* pv. *tomato* (Brown et al., 1992); *pcoABCDRS* is from *E. coli* (Mills et al., 1994). The relevant genes in the gene clusters for comparison are underlined; in the *mex* operons, the equivalents of CzrC, CzrB and CzrA are Opr(M, J and N), Mex(A, C and E) and Mex(B, D and F), respectively.

[b] NR: not relevant.

histidine-rich motifs that were in CzcB proposed to form the Zn-binding site (Nies and Silver, 1995). Yet, these histidine-rich motifs in the CzcB protein were found to be not essential for cation detoxification by CzcCBA (Rensing et al., 1997).

Interestingly, the Czr proteins are apparently more related to the corresponding *R. eutropha*-like Czc proteins than to the corresponding *R. eutropha*-like Cnr/Ncc proteins. This is also the case regarding the general organization of the structural genes. The *czcCBA*, *nccCBA*, *cnrCBA* and *czrCBA* determinants display the same gene organization, but in the case of *ncc* and *cnr*, an overlap exists between the termination codons and start codons of the *C* and *B* genes and of the *B* and *A* genes (Liesegang et al., 1993; Schmidt and Schlegel, 1994). These overlaps do not exist in the *czc* and *czr* gene clusters. In *czrCBA*, a 194 bp gap exists between *czr*C and *czrB*, while this gap is 18 bp between *czcC* and *czcB* (Nies et al., 1989). A gap of 16 and 23 bp exists between *czcB* and *czcA* and between *czrB* and *czrA*, respectively. Analysis of the nt sequence upstream of the *czrC* gene revealed the presence of a consensus sequence (YSTTGR17-18YRTAAT) (Ronald et al., 1992) for the binding of the transcription initiation factor σ70, but only a 16 bp space exists between the putative TTG and TAAT boxes.

Five hundred base pairs upstream of *czrC*, two ORFs were identified, in an orientation divergent of the structural genes (Fig. 1B). The ORFs encoded predicted proteins of 224 and 472 aa with significant similarities with the two proteins of bacterial two-component autophosphorylating (sensor kinase protein)-transphosphorylating (transducers/DNA binding protein) regulatory systems (Parkinson and Kofoid, 1992) (Table 3). The highest similarities were obtained with the plasmid-encoded two-component regulatory system proteins CopS and CopR of the Cu-resistance operon of *Pseudomonas syringae*, with PcoS and PcoR of the Cu resistance operon of *E. coli*, and with CzcS and CzcR of the *czc*determinant of *R. eutropha*-like CH34 (Brown et al., 1992; Mills et al., 1994; van der Lelie et al., 1997). The two ORFs are therefore believed to encode the regulatory system of the *czrCBA* genes and were named *czrS*and *czrR*. The predicted CzrS aa sequence displayed the three functional important regions previously identified in sensor proteins of other two-component systems including the histidine kinase autophosphyrylation site at residue 259. The CzrR aa sequence displayed at residue 51 the conserved aspartic acid phosphorylation site necessary for regulatory activation (Mills et al., 1994). A major difference between the *czr* system and its *czc*, *cop* and *pco* counterparts is the location of regulatory genes and their direction of transcription. *copRS*, *pcoRS*and *czcRS* are located downstream of the metal resistance genes and are transcribed from a constitutive promoter as an operon of two genes in the same direction as their resistance operons (Brown et al., 1992; Mills et al., 1994; van der Lelie et al., 1997). In CMG103, *czrSR* lies upstream of the *czrCBA* genes and is transcribed in the opposite direction. *czrSR* of CMG103 is also the only regulatory gene cluster detected up to now near *czrCBA*, while the regulation of the *czcCBA* determinant in *R. eutropha*-like CH34 is operated by two regulatory regions (van der Lelie et al., 1997). These observations indicate the combination of separate regulatory and structural gene blocks to form different cation resistance systems.

Downstream of *czrA*, a sixth ORF (*orf6*) starts, corresponding with a predicted protein of 318 aa which shows 62–74% similarity with the XylS proteins, the transcriptional activators of the benzoate/*m*-toluate *meta*-cleavage pathways encoded by different TOL plasmids (Assinder et al., 1993). A seventh but incomplete ORF (*orf7*) starts 20 bp downstream of *orf6* and displays significant similarity with the *xylX* and *benA* genes determining the α-subunit of the *m*-toluate/benzoate multicomponent 1,2-dioxygenase of *Acinetobacter calcoaceticus* and *P. putida*, respectively (Harayama et al., 1991). Downstream of *czrSR* and bordering the sequenced fragment, a part of an ORF (*orf8*) was found, whose predicted aa sequence showed significant similarity with OprM (45% similarity) of the *mexAB-oprM*-multidrug efflux system of *P. aeruginosa* PAO1. Between *orf7* and *czrS*, a short ORF, *orf9*, could be recognized with no similarity to ORFs present in the non-redundant GeneBank–EMBL database. Both *orf8* and *orf9* are transcribed in the opposite direction of the *czrSR* genes.

The G+C contents of *orf8*, *orf9*, *czrS*, *czrR*, *czrC*, *czrB*, *czrA*, *orf6* and *orf7* of CMG103 were 69, 73, 69, 65, 71, 70, 67, 68 and 63%, respectively, which is in agreement with other genes from *P. aeruginosa* (60–70%) (Poole et al., 1996a,b; Köhler et al., 1997). The codon usage patterns of all genes were similar and reflected those observed with other *P. aeruginosa* genes.

### 3.4. Conservation of czr in the clinical isolate P. aeruginosa PAO1 and other environmental P. aeruginosa strains

To determine whether *czr* and the surrounding region are conserved in other *P. aeruginosa* strains, the left end 8.4 kb *Hin*dIII/*Bam*HI fragment of pMOL888, common to all *czr*recombinant cosmids (Fig. 1A), was hybridized with *Eco*RI-digested total genomic DNA from *P. aeruginosa* CMG103, CMG103#13, CMG106, DS178 and PAO1. The restriction enzyme *Eco*RI was used to verify the internal double *Eco*RI site present in *czrB* of CMG103 (Fig. 1A and B). A *Not*I fragment containing *czc* of *R. eutropha*-like CH34 was included in the hybridization experiment. The autoradiogram shows the presence of DNA similar to *czr* in the genome of all *P. aeruginosa* strains tested including the clinical isolate

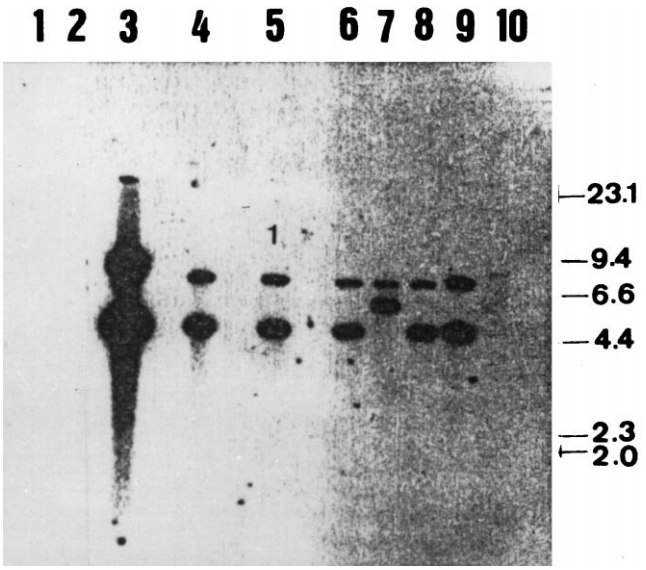


Fig. 2. Presence of *czr* in other *P. aeruginosa* isolates. *Eco*RI-digested genomic DNAs of different *P. aeruginosa* isolates were Southern blot-hybridized with the 8.4 kb *Hin*dIII/*Bam*HI fragment containing *czr* of pMOL864 as a probe (see Fig. 1). Lanes 1 and 10, λ *Hin*dIII size marker; lane 2, *Not*I fragment of pMOL30 containing *czc* of *R. eutropha*-like CH34; lane 3, *Eco*RI-digested pMOL867; lane 4, *P. aeruginosa* PAO1; lane 5, *P. aeruginosa* DS178; lane 6, *P. aeruginosa* CMG106; lane 7, *P. aeruginosa* CMG103#13; lane 8, *P. aeruginosa* CMG107; lane 9, *P. aeruginosa*CMG103.

Table 4
Comparison of nt sequence identity and aa sequence similarity (identity) between the *czr* region of CMG103 and the *czr* region of PAO1

| Sequence | DNA sequence identity | aa sequence similarity (identity) |
|---|---|---|
| *orf8* | 99.3 | 98.7 (98.7) |
| Intervening sequence *orf8–orf9* | 99.2 | |
| *orf9* | 97.4 | 97.4 (96.1) |
| Intervening sequence *orf8–czrS* | 100 | |
| *czrS* | 99.1 | 99.6 (99.4) |
| *czrR* | 99.9 | 100 (100) |
| Intervening sequence *czrR–czrC* | 99.2 | |
| *czrC* | 98.7 | 99.3 (99.1) |
| Intervening sequence *czrC–czrB* | 99.0 | |
| *czrB* | 99.6 | 99.8 (99.8) |
| Intervening sequence *czrB–czrA* | 100 | |
| *czrA* | 99.3 | 99.5 (99.2) |
| Intervening sequence *czrA–orf6* | 98.5 | |
| *orf6* | 99.3 | 100 (100) |
| Intervening sequence *orf6–orf7* | 99.1 | |
| *orf7* | 98.7 | 99.5 (99.0) |

and *P. aeruginosa* type strain PAO1 (Fig. 2). The two bands of hybridization are in agreement with the presence of the double internal *Eco*RI site in the 8.4 kb fragment used as a probe and with the restriction map of the *czr*region compiled from cosmid clones pMOL864, pMOL865, pMOL866 and pMOL867. These two bands are conserved in all strains. The difference in DNA fragment sizes hybridizing with the *czr* probe in the genomic DNA of mutant strain CMG103#13 and of parental strain CMG103 can be explained by the insertion of the Ω-Km transposon in *czr* of the mutant strain and shows that the *czr*-13:: Ω-Km locus is located to the left of the two *Eco*RI sites. No clear cross-hybridization was found with the *czc* gene cluster of *R. eutropha*-like strain CH34. The hybridization patterns indicate that both *czrSR* and *czrCBA* as well as their relative locations to each other are conserved in all tested *P. aeruginosa* strains.

### 3.5. Comparison of the CMG103 czr region sequence with the available P. aeruginosa PAO1 genome sequence

Recently, approximately 83% of the genomic DNA sequence of *P. aeruginosa* PAO1 was released on the Internet. To confirm the existence of a strongly conserved *czr* region in *P. aeruginosa* PAO1, the sequence of the CMG103 *czr* region was compared with the currently available PAO1 genome sequence. *orf8*, *orf9*, *czrS*, *czrR*, *czrC*, *czrB*, *czrA*, *orf6*, *orf7* and their intervening sequences of CMG103 were compared separately with the PAO1 sequence and showed identities of more than 99% at the nt level with nine ORFs present on one contiguous stretch of DNA (Table 4). The corresponding ORFs were organized on the PAO1 chromosome in the same order as found for *orf8orf9czrSRCBAorf6orf7* in *P. aeruginosa* CMG103, confirming the strong conservation of this DNA region in *P. aeruginosa* PAO1.

### 3.6. Mapping of the czc locus on the P. aeruginosa PAO1 chromosome

The 1.15 kb *Sal*I fragment of pMOL1006 containing sequences unique to *czrBC*and the 1.08 kb *Sal*I fragment of pMOL897 containing sequences unique to ORF5 and ORF6 (Fig. 1B) were used separately as gene probes to hybridize with the chromosomal *Spe*I and *Dpn*I fragments of *P. aeruginosa* PAO1, separated by PFGE. Both gene probes hybridized with *Spe*I fragment Q and *Dpn*I fragment B of the *P. aeruginosa* PAO1 chromosome (data not shown). These results correspond to a location of the *czc* locus between position 2400 and 2550 kb on the physical map of PAO1 (Holloway et al., 1994).

The mapping of *xylX*/*benA* on *Spe*I fragment Q is in agreement with the fact that this region is known to contain the *ben* and *cat* markers involved in the *ortho*-cleavage catabolic pathway for benzoate degradation (Holloway et al., 1994). In that pathway, BenA makes

part of a multi-enzyme dioxygenase complex oxidizing benzoate into catechol. Interestingly, an *xylS*-like gene, normally involved in regulation of a catechol *meta*-cleavage pathway (Assinder et al., 1993) seems to be combined with genes involved in *ortho*-cleavage in the *P. aeruginosa* chromosome.

## 4. Discussion

The *czrSRCBA*gene cluster is the first reported Zn- and Cd-resistant determinant in *P. aeruginosa* and in γ-proteobacteria. The predicted CzrC, CzrB, CzrA proteins encoded by the CMG103 *czrCBA* genes show remarkable similarities with the proteins encoded by the *R. eutropha*-like CH34 plasmid borne metal resistance operon, *czcCBA*, which determines a cation-antiporter efflux system for metal resistance. Therefore, it can be suggested that a similar kind of efflux system is functional for Zn and Cd in *P. aeruginosa*CMG103. However, the organization of the *czc* and *czr* gene clusters regarding regulatory modules is different. Moreover, in *R. eutropha*-like CH34 and related strains, *czc* is plasmid-bound (Mergeay et al., 1985), whereas in *P. aeruginosa*, *czr*is chromosomally bound.

Hybridization studies revealed the presence of CMG103 *czr* homologous sequences in the genome of all *P. aeruginosa*strains tested including the clinical isolate PAO1 and likely at a similar position of the genome flanked by the same gene sequences. The latter observation was confirmed for strain PAO1 by the comparison of the CMG103 *czr* region sequence with the available *P. aeruginosa* PAO1 genome sequence. A strong identity in the nt sequence was found between strain CMG103 and PAO1 as well of the *czr*region as for the *czr* flanking regions. Although only one *P. aeruginosa* strain of clinical origin, i.e. type strain PAO1, was considered in this study, these observations might indicate conservation of this region in *P. aeruginosa* and the involvement of *czr* in the intrinsic fairly high Cd- and Zn-resistance properties of that species. The phenotypic differences in Cd/Zn resistance between the different examined strains might be explained by discrete mutations arising as a response to changes in the selection pressure.

The identification of *czrCBA* in PAO1 determines a fourth ABC transporter system in *P. aeruginosa*. Other efflux systems in *P. aeruginosa* are MexAB-OprM, MexCD-OprJ, and MexEF-OprN, which are responsible for the efflux of drugs in *P. aeruginosa* (Poole et al., 1996a,b; Köhler et al., 1997). The *mexAB-oprM* and *mexCD-oprJ* operons were physically mapped on the *P. aeruginosa* PAO1 chromosome to regions that are different from the region that contains *czr*(Poole et al., 1996a; Poole et al., 1996b). Amino acid similarities of the *P. aeruginosa czrCBA* and *mexAB-oprM, mexCD-oprJ* and *mexEF-oprN* operons suggest that they have a common ancestor. However, the gene organization of *mexAB-oprM*, *mexCD-oprJ* and *mexEF-oprN* operons are different from *czrCBA*, and the three operons are combined with a regulatory system different from the supposed regulatory system of *czrCBA* (Poole et al., 1996b; Köhler et al., 1997). Furthermore, on the basis of aa similarities, *czr* of *P. aeruginosa* is more related to *czc* of *Ralstonia* than to *mexAB-oprM*, *mexCD-oprJ* and *mexEF-oprN*. These observations indicate that *czr* and *mex* probably do not originate from an endogenous duplication and that one of them was probably acquired by horizontal gene transfer.

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