Molecular characterization of an operon, *cueAR*, encoding a putative P1-type ATPase and a MerR-type regulatory protein involved in copper homeostasis in *Pseudomonas putida*

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The authors have characterized a chromosomally localized two-gene operon, *cueAR*, which encodes a putative P1-type ATPase, CueA, and a MerR-type metalloregulatory protein, CueR, in *Pseudomonas putida* PNL-MK25. Disruption of *cueAR* by the insertion of mini-Tn5::*gfp* into the wild-type strain led to a mutant strain with a sixfold reduction in its tolerance to copper; however, the tolerance of this mutant strain to the other seven related transition metals tested was not affected. The sensitivity of the mutant strain was attributed to a higher level of accumulation of intracellular copper, suggesting the involvement of CueA in copper export. Insertion of the cloned *cueAR* operon into the copper-sensitive mutant strain fully restored its tolerance to copper. *cueA*::*gfp* expression studies confirmed that the *cueAR* operon was transcriptionally regulated by copper and CueR. Studies done on the mutant strain complemented with *cueR* and *cueA* revealed partial functional redundancy of *cueA* and *cueR*, respectively, in copper tolerance. Thus, the results of this study clearly suggest the involvement of *cueAR* in copper homeostasis in *P. putida*.

Keywords: copper tolerance, CPx-type ATPase, mini-Tn5::*gfp* mutagenesis

INTRODUCTION

Metals play a vital role in biological systems. It is estimated that over 50% of all proteins are metallo-proteins and that about a third of all structurally characterized proteins contain metals (Degtyarenko, 2000). Copper is one such element that is required for a number of important cellular enzymes such as cytochrome oxidase, superoxide dismutase, ascorbate oxidase, ceruloplasmin, laccase, lysyl oxidase and other oxygenases (Camakaris *et al.*, 1999; Buffoni & Ignesti, 2000; Dameron & Harrison, 1998). However, excess cellular copper can react non-specifically with cysteine and histidine residues in proteins and disrupt their activity. Copper is also known to produce reactive oxygen species, which can lead to cellular damage (Dameron & Harrison, 1998). Cells have to maintain

intracellular copper at subtoxic levels, as it is extremely toxic at supra-optimal concentrations.

Eukaryotic and prokaryotic systems possess several mechanisms for copper homeostasis, such as reduced influx, facilitated efflux, sequestration and modification (Camakaris *et al.*, 1999; Dameron & Harrison, 1998). In eukaryotes, many transition metals, including copper, are generally sequestered by small cysteine-rich proteins termed metallothioneins. Apart from metallothioneins, eukaryotes also possess transporters such as the P-type ATPases for the facilitated efflux of these metals. P-type ATPases form a phospho-aspartate intermediate in the ATP-driven cation-transport cycle and contain conserved domains for phosphorylation, ATP binding and phosphatase activity (Lutsenko & Kaplan, 1995). P-type ATPases involved in metal-ion transport contain additional motifs such as a conserved HP dipeptide, a transmembrane CPx motif and one or more amino-terminal metal-binding domains (Lutsenko & Kaplan, 1995; Solioz & Vulpe, 1996). These P-type ATPases are called P1-type ATPases or CPx-type ATPases. Examples of eukaryotic copper-transport P1-type ATPases include

Abbreviations: GFP, green fluorescent protein; MIC, minimal inhibitory concentration.

The GenBank accession number for the sequence reported in this paper is AF390440.

CaCRP1 of *Candida albicans* (Weissman *et al.*, 2000) and the Menkes disease and Wilson disease proteins of human origin (Bull & Cox, 1994).

Prokaryotic copper-transport systems generally use copper-binding proteins and/or P1-type ATPases that are encoded by plasmids or the chromosome. P1-type ATPases have been described for bacterial copper-transport systems in many genera, including *Enterococcus* (Odermatt *et al.*, 1993), *Helicobacter* (Bayle *et al.*, 1998) and *Escherichia* (Rensing *et al.*, 2000). Previous studies on *Pseudomonas* spp. have focused mainly on the high levels of copper resistance seen in strains recovered from agricultural soils with a history of copper fungicide applications. One of the best characterized determinants of copper resistance in prokaryotes is the *copABCD* operon system that resides on a 35 kb plasmid of the copper-resistant strain of *Pseudomonas syringae* pv. *tomato* PT23.2 isolated from Californian tomato fields (Mellano & Cooksey, 1988). This operon, encoding copper-binding periplasmic and membrane proteins, is similar in structure and function to the other known plasmid-encoded determinant of copper resistance, the *pco* operon identified on a plasmid from *Escherichia coli* (Tetaz & Luke, 1983). Similarly, chromosomal determinants of copper tolerance have been cloned from many *Pseudomonas* strains and these have been found to have similarity either to the plasmid-encoded *cop* operon or to the genes involved in cytochrome *c* biogenesis in *E. coli* (Cooksey *et al.*, 1990; Vargas *et al.*, 1995; Yang *et al.*, 1996). Recently, a copper-inducible gene was identified in the chromosome of *Pseudomonas fluorescens* and it was shown to affect the copper tolerance of this bacterium (Tom-Petersen *et al.*, 2001). A partial sequence of this chromosomal gene showed similarity to putative P-type ATPases.

Here, we show the involvement of a P1-type ATPase and a MerR-type regulatory protein encoded by a single operon in copper homeostasis in *Pseudomonas putida*. Further evidence shows that this chromosomally located operon, *cueAR*, is regulated by copper and the regulator, and that both members of this operon are partially functionally redundant.

METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. *Pseudomonas putida* PNL-MK25 is an antibiotic-resistant derivative of the plant-growth-promoting rhizobacterial strain ATCC 39169 and is referred to here as the wild-type strain. Strain PNL-MK25 was used as the recipient in conjugation experiments. The suicide delivery vector pAG408 carrying mini-Tn5::gfp was introduced into *E. coli* SM10 (*λpir*) and was used as a donor. Cloning vectors pUC18 and pBSISK⁺ were used for propagation in *E. coli* DH5α. The shuttle vector pGB1 was used for complementation studies.

Growth media. *P. putida* and *E. coli* were grown routinely in LB medium at 30 and 37 °C, respectively, with constant shaking at 240 r.p.m. Bacterial conjugations and selection of transconjugants were done on tryptic soy agar (TSA) plates, as described previously (Pillai & Swarup, 2002). Screening of the

mutants was performed on Stanier's mineral (SM) minimal medium (Stanier *et al.*, 1966) supplemented with 0.5% (v/v) glycerol and appropriate antibiotics. For gene-expression analyses, mannitol/glutamate/yeast extract (MGY) medium (Bender & Cooksey, 1986) was used. The antibiotics chloramphenicol, gentamicin, kanamycin, tetracycline and ampicillin were used at concentrations of 30, 20, 30, 60 and 100 µg ml⁻¹, respectively, in TSA medium, and at concentrations of 15, 10, 15, 40 and 75 µg ml⁻¹, respectively, in SM and MGY media.

Transposon mutagenesis and screening of mutants. For mini-Tn5::gfp mutagenesis, biparental mating was performed between the donor *E. coli* strain and the recipient *P. putida* strain. Patch-matings of 4 h duration were harvested in saline and spread onto TSA plates that contained chloramphenicol, gentamicin and kanamycin. A conjugation frequency of 2×10^{-3} (recipient cell)⁻¹ was obtained while the spontaneous mutation frequency was 1×10^{-7} (recipient cell)⁻¹. Insertion of mini-Tn5::gfp was checked by colony PCR, in which transconjugant colonies that had been grown for 36 h were used as templates together with primers for the amplification of *gfp* (forward, 5'-TAGATGGTGATGTTAATGGGC-3'; reverse, 5'-GCCATGTGTGTAATCCCAGCAG-3'). The PCR protocol used was: 30 cycles of denaturation at 95 °C for 40 s, annealing at 58 °C for 40 s and extension at 72 °C for 1 min. *Taq* DNA polymerase (Promega) was used to catalyse the reaction. Of the 120 randomly chosen colonies that were tested, all were positive for amplification of *gfp* (data not shown). Approximately 9000 transconjugants carrying *gfp* transcriptional fusions were screened by replica-plating for increased green fluorescence when grown in the presence of transition metals on SM medium (Stanier *et al.*, 1966). One of the eight putative mutants that showed the highest response to transition metals in our preliminary studies was chosen for further analyses. This mutant was designated CEM1 (copper export mutant), based on the results shown later.

Metal-sensitivity assays. The minimal inhibitory concentration (MIC) of each of the transition metals tested was estimated by streaking single colonies from MGY plates onto plates containing varying concentrations of transition metal ions, namely, Hg²⁺, Ag⁺, Zn²⁺, Fe²⁺, Cu²⁺, Ni²⁺, Co²⁺ and Cd²⁺. The lowest metal concentration that inhibited growth after incubation for 60 h at 30 °C was recorded as the MIC. Sensitivity of the cells to copper in MGY broth was checked as described below in 'Complementation studies'.

DNA manipulations and analyses. Routine recombinant DNA techniques were performed as described by Sambrook *et al.* (1989). Genomic DNA was isolated from the *Pseudomonas* cells as described previously (Syn & Swarup, 2000). The genomic DNA of CEM1 cells was digested with *Bgl*II and *Kpn*I, and Southern-blot analysis was done. Full-length DNA probes for the *gfp* and gentamicin-resistance genes were obtained from pAG408 (Suarez *et al.*, 1997). Digoxigenin-labelling was done according to the manufacturer's protocol (Boehringer Mannheim). *Bgl*II and *Kpn*I cut the transposon, leaving the kanamycin- and gentamicin-resistance genes intact (Suarez *et al.*, 1997). The transposon-flanking upstream genomic DNA from the mutant strain was cloned using *Bgl*III, and downstream genomic DNA was cloned using *Kpn*I. After cloning of these flanking DNA fragments into the vectors pUC18 and pBSISK⁺, further subcloning of the transposon-flanking DNA was done to sequence 3 kb of the upstream and 3.3 kb of the downstream region. Vector primers and gene-specific primers were used to sequence the cloned fragments. Automated DNA sequencing was performed with the BigDye Terminator Cycle Sequencing Ready Reaction DNA Se-

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics*	Source/reference
<i>E. coli</i>		
SM10(λ pir)	<i>thi1 thr1 leuB6 supE44 tonA21 lacY1 recA⁻::RP4-2-Tc::Mu</i> ; a derivative of <i>E. coli</i> strain C600; Km ^r	Suarez <i>et al.</i> (1997)
DH5 α	<i>supE44 Δ(lacU169) (ϕ80lacZAM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 F⁻</i>	Gibco-BRL
<i>P. putida</i>		
PNL-MK25	Derivative of plant-growth-promoting rhizobacterial strain ATCC 39169; referred to as the wild-type strain in this article; Cm ^r , Rif ^r	Mark Kingsley, PNNL, USA
CEM1	Mini-Tn5:: <i>gfp</i> cassette inserted into copper-transporting ATPase-encoding chromosomal locus in the wild-type strain; Km ^r , Gm ^r	This study
Plasmid		
pAG408	Suicide delivery promoter-probe vector carrying the mini-Tn5:: <i>gfp</i> cassette (5.75 kb); Ap ^r , Km ^r , Gm ^r	Suarez <i>et al.</i> (1997)
pUC18	Cloning vector (2.7 kb); Ap ^r	Vieira & Messing (1982)
pBSIISK +	Cloning vector (3.0 kb); Ap ^r	Stratagene
pGB1	Shuttle vector for <i>E. coli</i> and <i>Pseudomonas</i> spp. carrying a stabilizing fragment (6.3 kb); Ap ^r , Tc ^r	Bloemberg <i>et al.</i> (1997)
pGBcueAR	pGB1 carrying a 3.6 kb genomic fragment of the full-length <i>cueAR</i> locus; Ap ^r , Tc ^r	This study
pGBcueA	pGB1 carrying a 3.4 kb genomic fragment of full-length <i>cueA</i> and truncated <i>cueR</i> ; Ap ^r , Tc ^r	This study
pGBcueR	pGB1 carrying a 0.45 kb genomic fragment of <i>cueR</i> fused with <i>lacZ</i> promoter of the vector; Ap ^r , Tc ^r	This study

* Cm^r, Chloramphenicol-resistant; Gm^r, gentamicin-resistant; Km^r, kanamycin-resistant; Rif^r, rifampicin-resistant; Tc^r, tetracycline-resistant; Ap^r, ampicillin-resistant.

quencing Kit (Applied Biosystems). Primers containing an *Xba*I site (*cueAR*-F, 5'-CGTCTAGAGCGATGGCAAGGT-CAAGTTC-3', and *cueAR*-R, 5'-CCTCTAGAGCTGTTCC-ACGGCATTCCC-3'; restriction site shown in bold) were designed to amplify a 3.6 kb fragment that encoded the entire *cueAR* operon along with its promoter. A proof-reading-type Vent DNA polymerase (New England Biolabs) was used to catalyse the reaction. The PCR protocol used was: 30 cycles of denaturation at 95 °C for 40 s, annealing at 60 °C for 40 s and extension at 76 °C for 3 min 40 s. Plasmid pGBcueAR was constructed by subcloning the 3.6 kb fragment into the *Xba*I site of the shuttle vector pGB1 (Bloemberg *et al.*, 1997). Using this construct, pGBcueA was generated by excising a 0.2 kb DNA fragment from the 3' end of *cueR* by using *Sac*I, thereby truncating *cueR*. The second gene of the *cueAR* operon, *cueR*, was amplified with an upstream primer containing an *Eco*RI site (*CueR*-F, 5'-AGAATTCCTGatGCGCTGTGCGAG-3'; restriction site in bold, ATG of *cueR* in lower-case) and the downstream primer *cueAR*-R that was used for amplification of *cueAR*. The *cueR* gene fragment was cloned into the *Eco*RI and *Xba*I sites of pGB1 to construct pGBcueR, resulting in *cueR* expression from the *lacZ* promoter. In *Pseudomonas*, the shuttle vector pGB3 (a derivative of pGB1) allows the expression of genes under the control of the *lac* promoter without the need for any exogenous inducer (Bloemberg *et al.*, 1997). Hence, no inducer was added in the expression studies with cells containing pGBcueR. The secondary structure of a 50 bp RNA sequence of the *cueA*–*cueR* junction was predicted by using the Vienna RNA package (Hofacker *et al.*, 1994).

Complementation studies. The pGBcueAR, pGBcueA and pGBcueR constructs were introduced into cells of the copper-sensitive *P. putida* CEM1 strain by electroporation, as

described by Bloemberg *et al.* (1997). Cells of the wild-type strain, the CEM1 strain and the CEM1(pGBcueAR) strain were grown in MGY broth to an OD₆₀₀ of 0.32, before the addition of the appropriate amounts of copper sulphate from a 100 mM stock solution to the medium. Functional complementation was studied by monitoring the growth of the cells for another 12 h at 30 °C with constant shaking (240 r.p.m.). For spectrophotometric measurements, the cells that had been grown in MGY broth were pelleted by centrifugation and resuspended in PBS (Sambrook *et al.*, 1989). For each experiment four replicates were maintained. For the CEM1 cells carrying pGBcueA and pGBcueR, complementation was checked by MIC analysis on MGY agar plates, as described in 'Metal-sensitivity assays'.

Gene-expression analysis. The *cueAR* transcriptional fusion with *gfp* (*cueA*::*gfp*) was used to study gene expression. Two methods were employed to study the production of green fluorescent protein (GFP). In the first method, the CEM1 cells were grown in MGY broth supplemented with varying levels of copper; GFP production by these cells was visualized under a confocal laser microscope (Olympus Fluoview 300 LSM). In the second method, the CEM1 strain and the CEM1(pGBcueR) strain were grown in MGY broth to an OD₆₀₀ of about 0.32, before the addition of various amounts of copper sulphate to the medium. Based on the results from our preliminary studies, the cells were incubated for 4 h at 30 °C with constant shaking (240 r.p.m.). The cells were pelleted by centrifugation and resuspended in filter-sterilized PBS for measurement of their GFP fluorescence by flow cytometry (Coulter Epics Elite; Beckman Coulter). For each experiment, data from 30000 events were collected and three replicates were maintained.

Copper-accumulation analysis. Copper sulphate was added at different concentrations to the bacterial cultures when they had reached an OD₆₀₀ value of 0.32 in MGY medium. The cells were then incubated again and samples were taken from the cultures at various time intervals (0, 2, 4, 6 and 8 h). These samples were pelleted, and the copper content of the pelleted cells was analysed using an Atomic Absorption Spectrophotometer (model A6800F; Shimadzu) as described by Beard *et al.* (1997). The dry weights of the cells were obtained after the pellets had been freeze-dried.

PFGE. To ascertain the location of the *cueAR* region, Southern-blot analysis with the transposon-specific probe was performed on the mutant strain DNA after analysis of total DNA by PFGE. Alkaline lysis (Sambrook *et al.*, 1989) and a megaplasmid preparation (Casse *et al.*, 1979) were done to check for the presence of any indigenous plasmid(s). Later, PFGE was carried out to resolve the intact total DNA. Sample preparation was done based on the method described by Sambrook *et al.* (1989). In brief, agarose plugs were prepared by mixing 1 ml of *Pseudomonas* cells suspended in TE (10 mM Tris/HCl, 1 mM EDTA) with 1 ml of 1% low-gelling-temperature agarose prepared in TE containing 50 µg lysozyme ml⁻¹. The plugs were treated with proteinase K before they were loaded into the wells of the PFGE gel prepared with ultra-pure DNA-grade agarose (Bio-Rad). Gel electrophoresis was carried out in 0.5 × TBE at 140 V for 36 h at 1/25 with a 1:1 pulse ratio on a CHEF-DR-II apparatus (Bio-Rad). Commercially available yeast chromosomal DNA plugs (Sigma) were used for sizing the DNA fragments. Following electrophoresis, the gel was stained with ethidium bromide, visualized under a UV light and photographed. The gel was then used for Southern-blot analysis. A probe for *gfp* was prepared by using the DIG High-Prime Labelling and Detection Kit (Boehringer Mannheim), as per the manufacturer's protocol.

RESULTS

Sensitivity of the CEM1 strain to copper

A mutant strain of *P. putida* PNL-MK25, designated CEM1, which carried a transposon insertion in a gene responsive to transition metals was identified by genetic screening. To test the sensitivity and specificity of this strain towards a number of soft metal ions, the MICs of a number of different metal ions were determined by growing the strain on MGY agar plates containing varying concentrations of these ions. The *P. putida* wild-type and CEM1 strain had similar MIC values for the seven transition metals tested (mercury, silver, zinc, iron, nickel, cobalt and cadmium; data not shown). Differences in their MIC values were only seen for copper. The MIC of copper sulphate for the wild-type strain was 400 µM, whereas the MIC of copper sulphate for the CEM1 strain was 65 µM. Hence, it was inferred that the copper tolerance of the *P. putida* CEM1 strain was altered when compared to the wild-type strain, PNL-MK25.

Characterization of the *cueAR* region

Southern-blot analysis of CEM1 genomic DNA that had been digested with *Bgl*II and *Kpn*I showed single bands of 13.0 and 12.5 kb (data not shown) in size, respectively,

indicating a single insertion of the transposon. Sequencing of the 6.3 kb DNA region surrounding the transposon insertion site revealed six ORFs and also showed that the transposon had inserted at nucleotide position 79 of a 2.4 kb ORF (Fig. 1a). The insertion site had a signature 9 bp duplication of the sequence 5'-GGCATG-ACC-3'. Results of a BLAST search of the 2.4 kb ORF product against the GenBank database showed that the predicted protein of 797 aa had a high degree of similarity to several functionally characterized copper-transport ATPases, including CopA of *E. coli* (Rensing *et al.*, 2000), CopA of *Enterococcus hirae* (Odermatt *et al.*, 1993) and the Wilson and Menkes disease proteins of human origin (Bull & Cox, 1994). Based on sequence similarity and functional characteristics (shown later) similar to previously characterized copper-transport proteins, the 2.4 kb ORF of *P. putida* was named *cueA* (for *Cu* export ATPase *A*). The percent identities of the *P. putida* CueA amino-acid sequence with CopA of *Escherichia coli*, CopA of *Enterococcus hirae*, the human Wilson disease protein ATP7B, PA3920 of *P. aeruginosa* (accession no. AE004809), plasmid pPaCu1-OrfG from *P. syringae* (accession no. AB044355) and ActP of *Sinorhizobium meliloti* (accession no. AF129004) were 36, 39, 32, 68, 60 and 38, respectively. *P. putida* CueA is predicted to contain at least eight potential transmembrane regions, based on its hydropathy plot and its similarity to other characterized bacterial copper ATPases (Fig. 2). The CPx motif (CPC) and other domains conserved in all P-type ATPases are present in CueA (Fig. 2).

cueA is the first gene of an operon that contains a downstream gene that encodes a helix-turn-helix-type DNA-binding regulatory protein (Fig. 1a); this regulatory gene is designated *cueR* (for *Cu* export regulator). The predicted CueR protein consists of 137 aa and shows a high degree of similarity to the MerR family of proteins, including the recently published *Escherichia coli* copper-transport regulator CueR (Fig. 3a; Outten *et al.*, 2000; Stoyanov *et al.*, 2001).

The *cueA-cueR* junction carries a -1 shift in the reading frame for the initiation of CueR translation with respect to its *cueA* reading frame (Fig. 1a). The putative translational termination signal (TGA) of *cueA* is part of the translational start (ATG) signal of *cueR*. The GAGGA Shine-Dalgarno consensus sequence of *cueR* is located in the 8 bp preceding the ATG of *cueR*. The predicted RNA secondary structure of the *cueA-cueR* junction shows a stable hairpin loop (Fig. 4). In such a structure, the Shine-Dalgarno sequence and the overlapping translational start codon occur at locations that are inaccessible to free ribosomes for translation. The *cueAR* operon has a promoter of approximately 200 bp in size and possesses many putative DNA-binding sites for regulatory proteins. A *cop* box consisting of an 8 bp inverted repeat is found at the same location (between -10 and -35) as that described for the *E. coli* *copA* promoter, P_{*copA*} (Fig. 1c; Outten *et al.*, 2000; Stoyanov *et al.*, 2001). Six nucleotides within the consensus *cop* box that are known to be the binding sites for the *E. coli*

Fig. 1. The *cueAR* locus. (a) Organization of the genes and ORFs in the *cueAR* region is indicated by arrows. The insertion site of the mini-Tn5::gfp fusion, near the 5' end of *cueA*, is indicated by a triangle. The DNA and amino-acid sequence of the *cueA*-*cueR* junction is shown at the top of (a). The putative Shine-Dalgarno sequence (underlined) and the overlapping DNA sequences (bold) are also shown within the DNA sequence. (b) Regions of the *cueAR* operon included in the complementation constructs are shown as straight lines. The arrows indicate the orientation of the gene with respect to the promoter of the vector. The MICs of copper for the strains carrying the respective constructs are given at the right-hand side of the figure. (c) Sequence comparison of the promoter region of *E. coli* *copA* (PcopA_E) with the promoter regions of *P. putida* *copP* (PcopP_P) and *cueAR* (PcueA_P). The inverted repeats are indicated by straight arrows; the transcriptional start site is indicated by a bent arrow. The *E. coli* CueR binding site in PcopA_E, and its corresponding site in the *P. putida* promoters, is indicated by the *cop* box. The predicted translational start points are in bold. Asterisks below the alignment indicate nucleotides conserved among the three sequences.

copA transcriptional activator, CueR, are also present in *P. putida* P_{cueA}. However, in comparison to *E. coli* P_{copA}, the *P. putida* promoter carries additional inverted repeats both upstream and downstream of the *cop* box (Fig. 1c).

Among the other four ORFs found around the *cueAR* region, the most notable is the gene designated as *copP*, with its direction of transcription away from the *cueAR* operon (Fig. 1a). The *copP* gene encodes a putative

homologue of the periplasmic metal-binding protein MerP of the mercury-transport system (Lund & Brown, 1987), the CopZ chaperone of the *Enterococcus hirae* copper-transport system (Odermatt & Solioz, 1995) and the CopP protein of *Helicobacter pylori* (Bayle *et al.*, 1998). *P. putida* CopP contains a conserved heavy-metal-associated domain at its N terminus (Fig. 3b), and the promoter of *copP* carries a *cop* box (Fig. 1c). The other ORFs flanking the *cueAR* region – *orfX*, *mfsA* and *imeA* (Fig. 1a) – showed similarities to a hypothetical

Fig. 2. Kyte and Doolittle hydropathy plot for CueA. Transmembrane regions are numbered below the traces. Two metal-binding domains, GMTCASC, are indicated by arrows. Conserved sequence motifs of P1-type ATPases found in CueA are: SGES, phosphatase domain; CPC, ion-translocation domain; DKTGT, aspartyl kinase domain; SEHPL, unknown function (conserved HP dipeptide); GDGINDA, ATP-binding domain.

Fig. 3. Sequence alignments of the predicted CueR and CopP proteins of *P. putida* with similar proteins. The CLUSTAL W and BOXSHADE programs were used to generate the alignments. (a) Alignment of CueR of *P. putida* (CueR_P) with other members of the MerR family of heavy-metal-regulator proteins. The conserved helix-turn-helix (H-T-H) DNA-binding motif is shown. The mercury-binding cysteines of MerR are indicated by asterisks. SWISS-PROT accession numbers for the sequences are: CueR_E (of *E. coli*), P77565; ZntR_E (of *E. coli*), P36676; MerR_A (of *P. aeruginosa* Tn501), P06688. (b) Similarity of predicted CopP sequence to related proteins. The conserved heavy-metal-associated (HMA) domain is shown. The GenBank accession numbers for the proteins are: PA3520 (of *P. aeruginosa*), AE004772; CopZ (of *Enterococcus hirae*), Q47840; MerP (of *Shigella flexneri*), P04129.

ORF, PA3576, of *P. aeruginosa* (<http://www.pseudomonas.com>), sequences for major facilitator superfamily proteins and integral membrane efflux proteins.

Chromosomal localization of the *cueAR* region

Plasmid analysis was performed on the wild-type strain and on the CEM1 strain, to check for the presence of any indigenous plasmid(s) that could harbour the *cueAR* locus. Neither the alkaline lysis nor the mega-plasmid preparations yielded any plasmid DNA that was detectable in ethidium-bromide-stained agarose gels (data not shown). Intact genomic DNA from the wild-type strain and from the CEM1 strain was resolved by PFGE. The ethidium-bromide-stained gel showed the intact

chromosomal DNA (2.2 Mb in size) for both strains; no signs of detectable plasmid were observed even when a higher amount of total DNA was added to the wells (Fig. 5a). Southern-blot analysis using the transposon-specific probe revealed a strong hybridizing signal only for the chromosomal DNA of *P. putida* CEM1 (Fig. 5b). Thus, the transposon-tagged *cueAR* operon was identified as being located within the chromosome.

Loss of *cueAR* function and copper sensitivity

The growth of the wild-type strain and of the CEM1 strain in the presence of varying concentrations of copper was compared. As shown in Fig. 6, both strains had comparable growth rates up until the 0.5 μ M copper level, but the CEM1 strain started showing increasingly

Fig. 4. Secondary structure of RNA. The stem-loop structure of the RNA sequence of the 50 bp *cueA-cueR* junction is shown. The putative Shine-Dalgarno sequence (GAGGA) and the overlapping of the AUG and UGA sequences are indicated in bold.

Fig. 5. Localization of *cueAR* by Southern hybridization. (a) Photograph of an ethidium-bromide-stained gel showing the total DNA resolved by PFGE. (b) Autoradiogram of a Southern blot done with the gel shown in (a). The gel was probed with digoxigenin-labelled *gfp* DNA. Lanes: M, DNA ladder (Sigma-Aldrich; Pulse marker 0.23–2.2 Mb); 1, *P. putida* wild-type strain, with 0.5 µg DNA added to the well; 2, *P. putida* wild-type strain, with 15 µg DNA added to the well; 3, *P. putida* CEM1, with 0.5 µg DNA added to the well; 4, *P. putida* CEM1, with 13 µg DNA added to the well.

slower growth in the presence of 3–30 µM extracellular copper. When strain CEM1 was grown in the presence of 100 µM extracellular copper there was no detectable increase in its growth (Fig. 6). The wild-type strain

exhibited comparable growth rates in all of the sub-MIC copper concentrations tested. The above results indicate that the *cueAR* mutation in strain CEM1 led to decreased copper tolerance.

The effect of the *cueAR* mutation on the rate of intracellular copper accumulation was studied in growth medium supplemented with 3 µM copper (Fig. 7). This concentration of copper was chosen as it was the lowest level at which the growth of CEM1 showed significant retardation (Fig. 6). At 0 h, the CEM1 cells contained 38 µg Cu (g dry weight cells)⁻¹, whereas the wild-type cells had 24.5 µg Cu (g dry weight cells)⁻¹. Over the time course of the study, the CEM1 cells consistently accumulated higher levels of intracellular copper than the wild-type cells (Fig. 7). After 4 h of growth in copper-supplemented medium, a maximum mean difference of 55 µg Cu (g dry weight cells)⁻¹ between the wild-type and the CEM1 strain was observed. This time period with its highest level of copper accumulation for CEM1 is in agreement with the results of the growth studies done at 3 µM copper shown in Fig. 6, where the sensitivity of the CEM1 strain became apparent from 4 h onwards. At 6 h, the copper contents in the two strains showed less difference (Fig. 7). Mean intracellular copper levels after 8 h of growth in the presence of copper were comparable in the two strains studied (Fig. 7), although their growth rates were significantly different (Fig. 6). Therefore, increased intracellular copper accumulation in CEM1 strongly suggests that the *cueA*-encoded P-type ATPase is involved in the copper export process.

Complementation of the *cueAR* mutation

The transposon insertion within *cueA* of the *cueAR* operon would have also affected the function of *cueR*. Therefore, the MICs of copper were determined for the CEM1 strain containing the constructs pGB*cueAR*, pGB*cueA* or pGB*cueR* (Fig. 1b). When inserted into CEM1, the pGB*cueAR* construct fully restored copper tolerance to CEM1 to the wild-type level (MIC = 400 µM). Similarly, when pGB*cueA* was inserted into CEM1 it also restored copper tolerance in this strain to a level comparable to that of the wild-type (MIC = 375 µM). Interestingly, pGB*cueR* restored the MIC of copper to 275 µM when it was inserted into CEM1. It is noteworthy that unlike the pGB*cueAR* and pGB*cueA* constructs that included the native promoter, the expression of CueR from the pGB*cueR* construct was through the *lac* promoter. This makes the transcription of *cueR* from pGB*cueR* independent of any effects caused by varying copper concentrations. CEM1 (pGB*cueAR*) was tested in more detail for its ability to grow in varying concentrations of extracellular copper (0–100 µM) that had been added to liquid MGY medium (Fig. 6). At all of the copper concentrations used in the experiments, the growth of the wild-type strain and of the CEM1(pGB*cueAR*) strain was comparable. In summary, the pGB*cueAR* construct fully restored the wild-type phenotype in strain CEM1, and the pGB*cueR* construct partially restored the wild-type phenotype.

Fig. 6. Copper sensitivity of *P. putida* strains. The *P. putida* wild-type (□), mutant CEM1 (○) and CEM1(pGBcueAR) (▲) strains were grown in MGY broth. When an OD₆₀₀ value of 0.32 was reached, 0–100 µM copper sulphate was added to the growth medium and bacterial growth was monitored over a 12 h time period. Each data point is based on the mean of four replicates. The standard error was too small to be seen in most cases.

Fig. 7. Intracellular accumulation of copper in *P. putida* cells. The wild-type (□) and CEM1 (■) strains were grown in MGY medium to an OD₆₀₀ value of 0.32. The amount of intracellular copper was then estimated by atomic absorption spectrophotometry at different time points after the addition of 3 µM copper sulphate to the medium.

Regulation of *cueAR* expression

Accumulation of GFP was used to study the expression of the *cueAR* operon, which was facilitated by the transcriptional fusion created by the fusion of mini-Tn5::gfp with *cueA* (*cueA*::gfp). Visualization of GFP expression in strain CEM1 under confocal laser microscopy showed increasing amounts of GFP fluorescence produced when the strain was grown in medium supplemented with 0–10 µM copper (Fig. 8a). At higher concentrations of copper (up until 50 µM), no further increase in fluorescence was visible (data not shown). No auto-fluorescence of the wild-type cells was observed (Fig. 8a). It is noteworthy that a basal level of fluorescence was seen even when copper was not added to the growth medium. The relative amounts of fluorescence of GFP from whole cells of CEM1 and CEM1(pGBcueR) grown in the presence of varying levels of copper (0–60 µM) was measured by flow cytometry. Accumulation of GFP in CEM1 was dose-dependent from 0 to 30 µM copper (Fig. 8b). At the point of maximal induction the mean level of *cueA*::gfp expression was twofold higher (11.59) than the background level when

the cells were grown without copper (5.28). In comparison to the CEM1 cells, GFP production in the CEM1(pGBcueR) cells showed a higher response to copper. The above results clearly indicate that the *cueAR* operon is regulated by copper and that CueR is a positive regulator of this operon.

DISCUSSION

In the present work, we have characterized an operon, *cueAR*, in *P. putida* which consists of two genes, one of which encodes a P1-type ATPase (*cueA*) and one of which encodes the *cueA* regulator (*cueR*) that belongs to the MerR family of proteins. To the best of our knowledge, none of the previously characterized genes encoding copper-transport proteins are organized in this manner. For example, other characterized copper-transport ATPase operons that consist of two genes, such as the *copAP* operons of *Helicobacter* spp. (Bayle *et al.*, 1998) and the *atkYB* operon of *Lactobacillus sakei* (Stentz *et al.*, 2000), do not have MerR-type helix–turn–helix regulators. In the *cueAR* region, a homologue of the recently published *Streptococcus* *copZ* regulator (Vats & Lee, 2001), *copP*, is present. However, the three-gene operon in *Streptococcus*, namely *copYAZ*, encodes a regulator similar to that encoded by the *copYZAB* copper-transport system of *Enterococcus* – this regulator is not of the MerR type. The copper-transport genes most similar to those of *cueAR* of *P. putida* are *copA* and *cueR* of *Escherichia coli* (Rensing *et al.*, 2000; Outten *et al.*, 2000; Stoyanov *et al.*, 2001). However, the organization of these genes in *E. coli* is once again different as the two genes are separated by two unrelated genes. Two contiguous genes encoding a MerR-type regulator and a P-type ATPase involved in cadmium resistance were recently reported for *P. putida*, but these genes were divergently transcribed (Lee *et al.*, 2001). Therefore, to the best of our knowledge, the organization of the genes in the copper-transport system of the *P. putida* strain studied here is unique among the ATPase-encoding copper-transport systems characterized so far.

Many *Pseudomonas* strains have been reported to be tolerant to varying levels of copper (Cooksey *et al.*, 1990; Vargas *et al.*, 1995; Bender & Cooksey, 1986). In one report, based on the screening of 20 *Pseudomonas* strains for copper resistance, eight strains of *P. syringae* pv. *tomato* were placed into a copper-sensitive group because of their lower tolerance to copper sulphate when grown in MGY medium supplemented with this compound (MICs of 0.4–0.6 mM). All eight of these strains contained plasmids (Bender & Cooksey, 1986). Although the genes responsible for copper tolerance, and their localization, were not characterized for the eight strains, the range of their copper tolerance would place the wild-type strain studied here in the copper-sensitive group (MIC = 0.4 mM). Furthermore, in those strains that are resistant to higher levels of copper, the determinants of copper tolerance have been shown to encode either copper-binding and sequestering proteins or copper oxidases (Mellano & Cooksey, 1988; Cooksey *et al.*, 1990; Cervantes & Gutierrez-Corona, 1994). Therefore, the present study is a report of the involvement of P1-type ATPase in conferring copper tolerance to a copper-sensitive group of *Pseudomonas* strains. Additionally, the transporter-encoding *cueAR* operon proved to be located in the chromosome, in contrast to the plasmid localization of many of the copper tolerance determinants of other pseudomonads (Mellano & Cooksey, 1988; Stall *et al.*, 1986; Bender & Cooksey, 1986).

cueAR is likely to play a significant role in copper homeostasis. Firstly, the wild-type strain has a relatively low level of tolerance (MIC = 0.4 mM) in the context of copper resistance in bacteria. Secondly, as shown by growth studies, the loss of *cueAR* leads to reduced tolerance that is apparent at levels as low as 3 μM extracellular copper (Fig. 6). This observation is in agreement with the earlier understanding that micro-organisms usually satisfy their copper requirement in the 1–10 μM range (Cervantes & Gutierrez-Corona, 1994); thus, *cueAR* putatively functions even within the range of copper levels required by the cells. Therefore, we argue that the *cueAR* operon in *P. putida* is likely to be involved in copper homeostasis, rather than conferring resistance to high levels of copper. Furthermore, the increase followed by a decline in the intracellular copper contents observed for both the wild-type and the CEM1 strain at their sub-MIC (3 μM) level (Fig. 7)

Fig. 8. Expression of *cueA::gfp* when *P. putida* cells were grown in the presence of different concentrations of copper. (a) Fluorescence of whole cells was observed under a confocal laser microscope. The cells were excited with a 488 nm laser and fluorescence was detected at 510 nm. (A) Wild-type cells; (B) CEM1 grown in the presence of 0 μM copper; (C) CEM1 grown in the presence of 3 μM copper; (D) CEM1 grown in the presence of 10 μM copper. (b) GFP production in whole cells of CEM1 and CEM1(pGBcueR), as measured by flow cytometry. The strains were grown in MGY for 4 h to an OD_{600} value of 0.32. Different concentrations of copper were then added to the cultures and the level of fluorescence was measured. Each histogram was obtained with 30000 events. The geometric mean value for the histograms is given at the top of each plot.

suggests more than one mechanism to be involved in copper homeostasis in *P. putida*. Although CueA-mediated copper transport is one mechanism involved in copper homeostasis in *P. putida*, the nature of other copper-homeostasis mechanisms operating in this strain is as yet unknown. In the genus *Pseudomonas*, and its related genus *Xanthomonas*, *cop* genes and their homologues encoded by the chromosome have been reported (Cooksey *et al.*, 1990; Lim & Cooksey, 1993; Lee *et al.*, 1994; Vargas *et al.*, 1995). These genes impart relatively low levels of copper tolerance upon strains when compared to those in a *P. syringae* strain carrying plasmid-borne *cop* genes (Mellano & Cooksey, 1988). It is noteworthy that unlike the *cueA* gene reported here, these plasmid-borne *cop* genes do not encode ATPases. In the wild-type strain studied here, which has a low level of copper tolerance, one or more of the above chromosomal genes could, therefore, possibly be present. In our study, the MIC results confirm that the loss of *cueAR* results in a sixfold reduction in copper tolerance; this intolerance is only to copper and not to the other metals tested (namely mercury, silver, zinc, iron, nickel, cobalt and cadmium). Moreover, it is evident that the sensitivity of strain CEM1 to copper is due to increased accumulation of this metal. Therefore, these results suggest that *cueAR* is specific to copper transport and CueA functions as an exporter of copper.

Gene-expression analyses, based on the *cueA::gfp* transcriptional fusion, indicate that the *cueAR* operon is autoregulated, as well as being regulated by copper. The *cueAR* promoter carries *cis*-signals (a *cop* box and other inverted repeats) known to bind MerR-like helix–turn–helix proteins such as CueR. Moreover, CueR contains putative metal-binding residues at its C terminus. When *cueR* is expressed in a plasmid with the *lacZ* promoter that is non-responsive to copper, the expression of the native chromosomal *cueAR* promoter in strain CEM1 gets upregulated in a copper-dependent manner. CueR is highly similar to the Tn501 mercury-operon regulator MerR, and proteins of this class are known to become competent transcriptional activators upon metal binding (Lund & Brown, 1987; Outten *et al.*, 2000; Stoyanov *et al.*, 2001). Therefore, it is clear that CueR positively regulates the function of its own promoter. Additionally, as was shown in the *cueA::gfp* expression studies with the CEM1 strain and the *cueR*-complemented strain, the expression of GFP varies with respect to the extracellular concentrations of copper. Thus, it is evident that the *cueAR* operon is regulated by extracellular copper levels via CueR.

There may be partial functional redundancy of both *cueR* and *cueA*. The increased copper tolerance of the CEM1(pGB*cueR*) strain indicates that CueR alone is able to partially alleviate copper toxicity. In *E. coli*, it is known that the *copA* regulator CueR regulates the expression of another gene, *cueO*, which is also involved in copper tolerance (Outten *et al.*, 2000; Grass & Rensing, 2001). Perhaps an, as yet, undiscovered orthologue of CueO might exist in *P. putida* that may explain the partial redundancy of *cueA* function. With regard to

P. putida CueR, the increasing GFP fluorescence obtained when CEM1 was grown in the presence of increasing levels of copper shows that the *cueAR* promoter responds to other transcriptional activators in the absence of CueR. Additionally, pGB*cueA* introduced into strain CEM1, in the absence of intact CueR, is able to restore copper tolerance levels to nearly equal those of the wild-type strain. To our knowledge, such regulation of a P1-type ATPase operon by multiple activators needs to be studied in more detail.

In addition to being under transcriptional control, *cueAR* function may also be under post-transcriptional control. The *cueA*–*cueR* junction shows features characteristic of translational coupling (McCarthy & Gualerzi, 1990), with a –1 frameshift at the *cueA*–*cueR* junction and the placement of a Shine–Dalgarno sequence as part of a stable RNA hairpin loop structure (Fig. 4) that could prevent independent translation. Examples of tightly controlled systems created by the formation of a secondary RNA structure in the inter-cistronic boundary have been demonstrated in many cases, including that of the *nifLA* genes of *Klebsiella pneumoniae*, where inappropriate levels of NifA synthesis are avoided (Govantes *et al.*, 1996). It is, therefore, tempting to speculate that translational coupling could be involved in *cueAR* regulation, which makes this operon an interesting candidate for future studies.

ACKNOWLEDGEMENTS

We thank the Environmental Engineering Laboratory, Department of Civil Engineering, and the Analytical Chemistry Laboratory, Department of Chemistry, of the National University of Singapore for providing their atomic absorption spectrophotometry facility. We thank Mark Kingsley, PNNL, USA, for providing the wild-type *Pseudomonas* strain. V. A. wishes to thank the National University of Singapore for the award of his research scholarship.

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Received 15 February 2002; revised 4 May 2002; accepted 24 May 2002.