The Heavy Metal Tolerant Soil Bacterium *Achromobacter* sp. AO22 Contains a Unique Copper Homeostasis Locus and Two *mer* Operons

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Copper-containing compounds are introduced into the environment through agricultural chemicals, mining, and metal industries and cause severe detrimental effects on ecosystems. Certain microorganisms exposed to these stressors exhibit molecular mechanisms to maintain intracellular copper homeostasis and avoid toxicity. We have previously reported that the soil bacterial isolate *Achromobacter* sp. AO22 is multi-heavy metal tolerant and exhibits a *mer* operon associated with a Tn21 type transposon. The present study reports that AO22 also hosts a unique *cop* locus encoding copper homeostasis determinants. The putative *cop* genes were amplified from the strain AO22 using degenerate primers based on reported *cop* and *pco* sequences, and a constructed 10,552 base pair contig (GenBank Accession No. GU929214). BLAST analyses of the sequence revealed a unique *cop* locus of 10 complete open reading frames, designated *copSRABGOFCDK*, with unusual separation of *copCD* from *copAB*. The promoter areas exhibit two putative *cop* boxes, and *copRS* appear to be transcribed divergently from other genes. The putative protein CopA may be a copper oxidase involved in export to the periplasm, CopB is likely extracytoplasmic, CopC may be periplasmic, CopD is cytoplasmic/inner membrane, CopF is a P-type ATPase, and CopG, CopO, and CopK are likely copper chaperones. CopA, B, C, and D exhibit several potential copper ligands and CopS and CopR exhibit features of two-component regulatory systems. Sequences flanking indicate the AO22 *cop* locus may be present within a genomic island. *Achromobacter* sp. strain AO22 is thus an ideal candidate for understanding copper homeostasis mechanisms and exploiting them for copper biosensor or biosorption systems.

Keywords: Heavy metals, copper, *mer*, *cop*, two-component signal transduction, genomic island

Copper-containing compounds are introduced into the environment through mining activities, use in agriculture as bactericides, fungicides, and growth promoters, as well as metal works. Copper is an essential trace element for cellular functions, mainly in electron transfer and dioxygen transport and activation. However, it is also highly toxic when in excess, owing to its high redox potential and ability to produce reactive oxygen species harmful to cellular components [15]. Thus intracellular copper concentrations need to be sensed and balanced carefully in all cell types.

Microorganisms occurring in environments contaminated with heavy metals often exhibit adaptation to such environments through having developed, and/or acquired by horizontal transfer, genetic systems that encode a variety of mechanisms of resistance to metal toxicity. For example, a number of genera and species of bacteria from natural environments (such as mercury deposits), as well as diverse aquatic and terrestrial sites contaminated by human activities, exhibit the mercury resistance-encoding *mer* operons (reviewed in Barkay et al., [3]). These operons are being researched intensely for development of mercury biosensors [6], and bioremediation/phytoremediation systems [9, 11]. In comparison, only a handful of bacteria have been reported as yet to encode copper tolerance. These include sequestration of copper by the *copABCDcopRS* operon of *Pseudomonas syringae* pv. *tomato* [8], the *pcoABCDcopRS* operon of *E. coli* strains isolated from piggeries [7], and *cueO* in *E. coli* [18], or P1-type ATPase-mediated efflux systems (e.g., by *copA* of *E. coli*) [42]. The multi-heavy metal resistant bacterium *Cupriavidus metallidurans* CH34,
originally isolated from a zinc-contaminated source, has been studied extensively and encodes a plasmid-borne cluster of 19 genes that include homologs of the cop system mentioned above, in addition to the chromosomally encoded copRS copABC copD [36]. Regulation of copper homeostasis systems commonly involves either a two-component signal transduction system, such as the copRS of *P. syringae* pv. *tomato* [8] and pcoRS and cusRS of *E. coli* [7, 38], or regulators similar to the merR of mer operons, such as cueR, which regulates copA transcription in *E. coli* [40]. In light of the environmental catastrophes in copper mining areas [21] or copper contamination in agricultural environments [21], as well as toxicity effects on human cells [15], it is highly desirable to identify biodiversity in copper homeostasis mechanisms, with a view to develop biosensors for detecting bioavailable and toxic amounts of copper in a given environment, as well as to develop detoxification systems.

We have previously reported a lead, copper, and mercury tolerant soil bacterial strain, *Achromobacter* sp. AO22, from a disused battery-manufacturing site in Melbourne, Australia, which harbors a transposon of the Tn21 subgroup of the TnJ family, with the mer operon associated with it [39, 51]. This work reports the identification of an elaborate copper homeostasis locus and a second mer operon, suggesting the strong potential of AO22 for development of biosensor and metal detoxification systems.

**Materials and Methods**

**Amplification of cop Operon Genes, the Second mer Operon, and a Possible Genomic Island in Strain AO22**

In the absence of any molecular information on copper homeostasis genes of *Achromobacter* sp. AO22 (initially identified as *Achromobacter* sp. AO22; [51]), it was hypothesised that the reported sequence of two key genes, copA and copR, in *Cupriavidus metallidurans* CH34 (also first identified as *A. eutrophus* CH34; [33]) could be used initially to design primers to amplify any related genes in AO22. Degenerate primers COPA-F, COPA-R, COPR-F, and COPR-R were designed based on relatively conserved regions of the copA and copR genes of *C. metallidurans* CH34 pMOL30 (GenBank Accession No. NC_007971) and megaplasmid (MPL, NC_007974) (Table 1). As sequence data for AO22 genes were acquired progressively (see results), additional primers COPAB-F, COPBurf-R, COPA-R, 448-F, and 442-R were then designed based on the cop locus of *Delftia acidovorans* SPI-1 (NC_010002) [46], and additional primers were designed to test for the presence of related adjoining sequences of a genomic island (Table 1). The possibility of a second mer operon (designated mer2) within it was investigated using primers MER483-F and MER486-R based on a mer locus in *D. acidovorans* SPI-1. PCR amplifications were performed on genomic DNA (gDNA) of AO22 using the primer pairs and annealing temperatures indicated in Table 1. All reactions typically contained 1× BioMix (Bioline, Australia); contains dNTPs, *Taq* polymerase, MgCl\_2, 100–400 ng of template, and 0.1 µg of each primer in a final volume of 50 µl.

**Cloning and Sequencing of PCR Products**

The PCR products were cloned into pGEM-T Easy vectors (Promega, Australia) and colonies containing recombinant plasmids selected on Luria–Bertani (LB) agar plates containing ampicillin, IPTG and X-gal. Plasmids from these were isolated with the Wizard Plus SV Miniprep DNA Purification System (Promega, Australia) and sequenced using vector-based primers T7 (5′ATAACGACTCACTATAG3′) and SP6 (5′ATTAGGTACCTACATAATG3′), the gene-specific primers (Table 1), and additional internal primers designed progressively.

**Analysis of Sequence Data**

DNA sequence assemblies, restriction maps, translations into putative proteins, and other analyses were conducted using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The DNA sequences were used for BLASTN searches and aligned with corresponding reported copper homeostasis-related genes using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Each putative AO22 protein was analyzed for potential signal peptides using SignalP v.3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/) and subcellular locations by PSORTb v.2.0.4 (http://www.psort.org/psortb/index.html); a PSORTb score of at least 7.5 was considered significant. The characteristic features of putative proteins were identified by alignment with homologs from reported systems using ClustalW. DNA regions of interest were submitted in both directions to the BPROM server (http://linus1.sofberry.com/berry.html?topic=bprom&group=programs&subgroup=gfindb) for predicting potential promoter regions including the -10 and -35 hexamers and likely transcription initiation sites. The potential regulatory protein-binding sites, which are typically palindromic, were identified in the putative promoter regions using the Palindrome Search server (http://bioinfo.cs.technion.ac.il/projects/Engel-Freund/new.html).

**Homology Modeling of the Putative AO22 CopA Protein**

The three-dimensional structure of the putative AO22 CopA protein was predicted by homology modeling using SWISS-MODEL (http://swissmodel.expasy.org/workspace/). The automatic modeling tool was used, where a suitable template was automatically selected based on a BLAST result generated using the putative protein sequence of AO22 CopA as input data. Protein structures were visualized and images generated using DeepView/SWISS-PDB Viewer v.4.0.1 (http://spdbv.vital-it.ch/).
Molecular Characterization of the Achromobacter sp. Strain AO22 cop Locus

Amplification of gDNA of Achromobacter sp. strain AO22 with COPA-F/COPA-R and COPR-F/COPR-R led to products of about 1.5 kilobase pairs (kb) and 600 base pairs (bp), respectively (Fig. S1). Amplification was then performed with the reverse primer pair COPR-R/COPA-R, to investigate whether the putative copR and copA genes might be transcribed divergently, and yielded a band of about 3 kb. Partial sequences (987 bp and 587 bp) from the ends of this 3 kb insert were used in a BLASTN search, and revealed >97% identity to the copRcopA region of D. acidovorans strain SPH-1 (Accession No. NC_010002). Further cop genes occur in SPH-1 flanking this region, prompting the design and use of primer pair COPR-F/COPS-R for amplifying sequences upstream of copRcopA, and pairs COPAB-F/COPBurf-R and DA448-F/DA442-R for downstream sections. Products of 2, 1.2, and 5 kb were noted, respectively (Fig. S1). The complete sequences of clones of these four fragments were determined and a 10,552 bp contig was constructed (reported as GU929214 in GenBank).

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R=A + G; Y = C + T; M = A + C; K = T + G; S = C + G

*Estimates for the first two primer pairs are as for the C. metallidurans CH34 pMOL30 cop locus (GenBank NC_007971) and megaplasmid (NC_007974) sequences, and estimates for the other cop operon sections, genomic island (GI) sections, and mer2 operon are as per the D. acidovorans SPH-1 sequence (NC_010002).
A six-frame translation of the 10,552 bp sequence led to identification of 10 complete open reading frames (ORFs). The locus was designated as cop owing to homology to reported loci (discussed below), and the genes were named copSRABGOFCDK (Fig. 1). The copR and copS appear transcribed divergently from other genes, with copR overlapping the putative start codon of copS. The spacer between copR and copA was 255 bp, and copB was 25 bp downstream of the putative copA stop codon. Unlike all known cop operons, wherein the ABCD genes are adjacent, AO22 copCD were separated from copAB by three ORFs (copG, copO, and copF). The last gene copK was 128 bp downstream of the stop codon of copD.

The genes were subjected to BLAST searches, and sequences of the genes and their putative proteins aligned to the reported systems; that is, copSRABGOFCD of C. metallidurans CH34 pMOL30 and MPL [36], P. syringae pv. tomato [8], P. putida [1], Xanthomonas campestris pv. juglandis [27], X. perforans pv. vesicatoria (X. axonopodis pv. vesicatoria) [53], cus from E. coli K-12 sub-strain MG1655 [38], pco from E. coli pRJ1004 [7], and E. coli cueO [18] (DNA alignments not shown). For copG, copO, copF, and copK, relevant genes from these systems (if applicable) as well as the top six BLAST hits were included. The analyses led to the following results.

Features of the Putative Structural Proteins of Strain AO22 cop Locus

The putative 619 amino acid (aa) long AO22 CopA was predicted to be periplasmic, and included an N-terminal 42 aa signal peptide with two conserved Arg residues, making it a potential target of the twin-arginine-translocation (tat) pathway for export to the periplasm (Fig. S2A). The three conserved domains (types 1, 2, 3) associated with the copper oxidase superfamily [34] and four His-rich clusters including a conserved C-terminal HCHXXXHM motif were also identified. The His-rich regions with 12 conserved ligands known to interact with type 1, 2, and 3 copper atoms, as revealed in the structures of plant ascorbate oxidases [34], correspond to His113, His115, His155, His157, His553, His556, His558, His601, Cys602, His603, His607, and Met612 in AO22 CopA. In order to visualize the potential role of these ligands, a 3D model of AO22 CopA was built using SWISS-MODEL (Fig. 2A), with zucchini ascorbate oxidase (PDB Accession No. 1ASQ) [34] selected by the server as template. This showed several regions in CopA (Phe220-Arg238, Met386-Met407, Asn419-Leu442, Pro494-Gly496, Met515-Gly524) where equivalent residues were absent in 1ASQ (Fig. 2B). However, superimposition of the two and closer inspection at the region within 4Å of the four copper atoms revealed the 12 ligands of the putative CopA to be very close to those in 1ASQ (Fig. 2C). In contrast to the relatively conserved N- and C-termini, there is a highly variable His- and Met-rich region in all CopA homologs; this region (Met379-His466 of AO22 CopA) also accounted for most of the residues that were absent in 1ASQ. AO22 CopA has six copies of the motif MXXM (3XMTGM, 1× each of MGSM, MGGM, MAGM) (Fig. S2A), shared with the related proteins (five in pMOL30 CopA, four in P. syringae CopA, five in E. coli PcoA). The main difference between CopA of AO22 and SPH-1 was also in this section; two more MTGM and an MSGM occur in SPH-1.

The putative 326 aa CopB has a signal peptide and may be extra-cytoplasmic, similar to CopB of P. syringae [8], Orf2 of X. campestris [27], and PcoB of E. coli [7], which are all associated with the outer membrane. AO22 CopB and its homologs are more conserved at the C-terminal (Fig. S2B). The motif MDHXXXM occurs once at the N-terminus of AO22 CopB; it occurs 5 times in P. syringae CopB [8] and 10 times in pMOL30 CopB [36].

The putative CopC (128 aa) also had a signal peptide, may be periplasmic, and shares the Cu(I)- and Cu(II)-binding sites of E. coli PcoC [13, 23] and P. syringae CopC [2] (Fig. S2C). The predicted Cu(I) ligands are Met40, Met43, Met46, His49, and Met52 in the motif MXMXMX(X)HXXXM, also noted in PcoB, whereas the equivalent Cu(II)-binding site in AO22 CopC is likely constituted by His1, Glu27, Asp90, and His92.

The CopD (308 aa) was predicted to be a cytoplasmic (inner) membrane protein, with eight transmembrane helices (TMHs) (Fig. S2D), as noted in E. coli PcoD [7].
**Fig. 2.** Model of 3D protein structure of AO22 CopA. (A) Ribbon diagram of the predicted AO22 CopA structure. Elements of secondary structure (helices and beta sheets) are colored from N- to C-terminal in the order violet, blue, green, yellow, orange, red. (B) Superimposed image of the C\(^\alpha\) atoms of AO22 CopA (blue) and zucchini ascorbate oxidase (PDB accession 1ASQ) (pink). Light blue regions represent residues in AO22 CopA where the corresponding residues are missing in 1ASQ. Copper atoms bound to 1ASQ are shown as green spheres. (C) Superimposition of AO22 CopA residues onto those in zucchini ascorbate oxidase located within 4Å of the 4 copper atoms (blue spheres). AO22 CopA residues (labeled) are shown in CPk colors: white for carbon, red for oxygen, blue for nitrogen, yellow for sulfur. Zucchini ascorbate oxidase residues are shown in pink.

The **C. syringae** CopD [8], and pMOL30 and MPL CopD [36]. Three His (His141, His153, His160 of AO22 CopD) were conserved and could be the metal ligands, although the ligands of reported CopD proteins have not been confirmed as yet. The CopF (826 aa) was also predicted to be a cytoplasmic (inner) membrane protein with eight TMHs (Fig. S2E) and appears to be a homolog of copper-translocating P-type (P\(_{1\text{B}}\)-type) ATPases such as *E. coli* CopA [42]. It contained the motif DKTGT, characteristics of Cu P-type ATPases. The CPx/xPC motif, characteristic of heavy metal P-type ATPases, also occurred in the sixth TMH. The N-terminal 150 aa contains 17 His and 4 Cys, which may be the metal-binding sites common in heavy metal P-type ATPases.

The CopG is 161 aa long and also has a 33 aa signal peptide (Fig. S2F), hence it is possibly periplasmic, and likely a homolog of pMOL30 CopG [31]. It exhibited a number of potential metal-binding sites including 4 Cys, 4 His, and 2 Met, all of which except His160 were conserved in all sequences. The motif CGCC of pMOL30 [36] is constituted by Cys47, Cys49, and Cys50 in AO22 CopG.

The CopO (90 aa) had two predicted TMHs, and highest identity to SPH-1 CopO (98%) followed by HMPREF00004_1699 of *Achromobacter piechaudii* ATCC 43553 and SSKA14_1240 of *Stenotrophomonas* sp. SKA14, but very low identities (18% in protein, 30% in DNA) to pMOL30 CopO. The possible metal-binding sites of AO22 CopO include 10 His, three Met and 2 Cys (Fig. S2G). The Cys55, Met58, and His59 constitute a highly conserved CPLMH motif, as also His63. Interestingly, Met1, His5, His39, His42, Met62, His66, His68, and His71 were also conserved in all except for pMOL30 CopO.

The AO22 CopK (94 aa) also had a predicted signal peptide. BLASTX search using AO22 *copK* DNA as query returned 12 unique hits (Fig. S2H). The highest hits (90% identity) contained two identical sequences, from *Achromobacter piechaudii* ATCC43553 and *Stenotrophomonas* sp. SKA14. Interestingly, three other top hits belonged to the plasmid pH101 of *Candidatus Accumulibacter phosphatis* clade IIA str. UW-1. Cu(I)-binding ligands (Met28, Met38, Met44, Met54) of pMOL30 CopK are absent in the mature...
AO22 CopK. Interestingly, of the potential metal-binding sites (Met31, Met48, His92) of AO22 CopK, Met48 was conserved in all 12 sequences and His92 in 9, including CopK of CH34 MPL, wherein it is proposed to be involved in Cu(II) binding [45].

Features of the Putative Two-Component Regulatory System of Strain AO22 cop Locus

The AO22 CopS (477 aa) may have a 41 aa signal peptide (predicted with a low probability of 0.23) and may be an inner-membrane protein, with two TMHs corresponding to E. coli PcoS [7]. Multiple alignment with the sensor histidine kinases (HK) of related two-component signal transduction (TCST) systems (Fig. S2I) showed it contained the three conserved regions; that is, a specific His [17] (His266), the dimerization domain [41] (256–309), and C-terminal ATP-binding domain [41] (367–469). It is noteworthy that although the reported CopS homologs are all HKs involved in responding to copper stimulation, their N-terminal regions shared little identity. The AO22 CopR (230 aa) was predicted to be cytoplasmic, and alignment with the response regulatory (RR) proteins of other copper TCST systems (Fig. S2J) showed conservation of the signal receiver domain [48], C-terminal effector domain [48], as well as the aspartate phosphorylation site (residues 4–116, 128–228, and Asp51 of AO22 CopR).

In order to evaluate the relatedness to RRs and HKs from other heavy metal TCST systems, alignments were performed with the Salmonella silver resistance determinants [19], the cadmium-zinc-cobalt (czc), and other heavy metal TCST systems from C. metallidurans CH34 [12, 32], P. syringae pv. tomato, Ralstonia solanacearum GMI1000, Fusobacterium sp., and Bacillus cereus ATCC 14579, as well as non-heavy metal TCST systems, composed of E. coli OmpR/EnvZ and those of unknown functions from C. metallidurans CH34 MPL, Corynebacterium diphtheriae NCTC 13129, and Streptococcus agalactiae. Proteins from two eukaryotic systems were also included: SKN7 (RR) and SLN1 (HK) from Saccharomyces cerevisiae and the Arabidopsis thaliana proteins ARR18 (RR) and ETR1 (HK) (alignments not shown). The phylogenetic tree showed three main clusters including one of copper/silver HKs wherein AO22 CopS clustered with CusS and SilS, whereas CopS from C. metallidurans CH34 and P. syringae and PcoS from E. coli form a separate branch (Fig. 3). Similarly, AO22 CopR grouped with the copper/silver RRs whereas other heavy metal and non-heavy metal RRs form two other clusters.

Putative AO22 copA and copR Promoters Have Signature “Copper Boxes”

Analysis of the 255 bp region between the proposed start codons of AO22 copR and copA using BPROM identified two potential promoters; one upstream of copA and the other in the complementary strand upstream of copR (Fig. 4A). The PcopA -35 (TTCAAC) and -10 (TAGCA[T]) and PcopR -35 (ATGAAT) and -10 (CAAAAT) elements in the respective directions differ in 3 or 2 positions from the consensus (TTTGACA[T]:TATAAT); however, such variations are not unusual [20]. The spacing between the -10 and -35 boxes of PcopA (17 bp) and PcopR (16 bp) agrees well with the 16–18 bp consensus for sigma-70 promoters [20]. A 7 bp perfect palindrome ATTACATGAATGTAAT was noted upstream of the -35 box of PcopA. Such palindromic cop boxes had been reported in other copper and silver responsive promoters [44]. A comparison with to these showed that in PcopR, the distance between the 3' end of the palindrome and 5' end of the -35 box was much longer (55 bp) than in others (~2 to 25 bp); hence PcopR was excluded in the final alignment (Fig. 4B). Interestingly, the putative PcopA cop box shared the 2 bp gap between the half-repeats and high identity with other cop boxes. The predicted AO22 PcopA transcription start site and -10 hexamer are also at the same distance from the palindrome as in PpcoE and PcusC. Based on these similarities, PcopA may also be copper (and silver?) inducible.

The AO22 cop Locus is Possibly Located on a Genomic Island

While this work was under way, a new report indicated that the cop locus of D. acidivorans SPH-1 was part of a 66 kb genomic island (GI), DAGI-2 [52]. Hence, to assess this possibility for the AO22 cop locus, primers based on selected sections of DAGI-2 were used in various combinations (Table 1) to amplify the AO22 gDNA. The results indicated that these sections, including flanking regions of the cop locus, were present in AO22 (Fig. 5), suggesting a DAGI-2-like element. However, confirmation of this possibility will need amplification of overlapping sections and complete sequencing.

Presence of a Second mer Operon in Strain AO22

The data obtained on copper homeostasis determinants (see above) prompted investigations into a possible second mer operon in AO22. PCR amplification using primer pair MER483-F/MER486-R yielded a product of approximately 3 kb (Fig. 6A). Its clone yielded a 2,821 bp sequence. However, confirmation of this possibility will need amplification of overlapping sections and complete sequencing.

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AO22 \textit{mer} Determinants Confer Additional Tolerance to \textit{E. coli} Whereas AO22 \textit{cop} Determinants Do Not

In order to test if the AO22 \textit{mer} and \textit{cop} genes confer additional mercury and copper tolerance, respectively, in \textit{E. coli}, the MICs of Hg(II) and Cu(II) for the respective strains were determined. The Hg(II) MIC of \textit{E. coli} CB454 strains containing TnAO22, \textit{E. coli} (pVS520::TnAO22) [39], was determined and compared with wild-type AO22 and \textit{E. coli} without the transposon. The Hg MICs were 0.04, 0.02, and 0.01 mM, respectively. It was interesting to note that in \textit{E. coli}, pVS520::TnAO22 seemed to have conferred increase in resistance to Hg(II). However, \textit{E. coli} (pVS520::TnAO22), though estimated to have multiple copies of the \textit{mer} operon on TnAO22 (which corresponds to the copy number of plasmid RP1 and its derivatives [16]), did not show higher resistance to Hg(II) compared

\textbf{Fig. 3.} Neighbor-joining distance dendogram of copper-homeostasis-related two-component signal transduction systems. (A) Histidine kinases; (B) response regulators. Bootstrap percentages (500 replicates) are shown to the left of the respective node. The three main clusters of copper/silver, other heavy metal, and non-heavy metal, two-component signal transduction systems are shaded in grey, dark grey, and light grey, respectively, with GenBank accession numbers given in parentheses. HMRR: heavy metal response regulator; RR: response regulator; HMHK: heavy metal histidine kinase; HK: histidine kinase.
with wild-type AO22. This could indicate that the TnAO22 mer requires host factors found in AO22 but not E. coli; for example, the presence of mer2, to confer full resistance.

To test the effect of the AO22 cop determinants in E. coli, E. coli JM109 strains carrying the cloned copSRAB [amplified using primer pair COPS-R/COPABurl-R (Table 1)]
or the copG to copK (copG-K) regions of the AO22 cop locus were tested for copper MIC. Strain AO22 and E. coli JM109 without plasmid were tested in parallel for comparison. The copper MICs for the three E. coli strains were 3 mM compared with 6 mM for strain AO22, suggesting that the cloned cop genes of AO22 did not confer additional copper tolerance to E. coli. However, it was not clear whether the genes from AO22 were not expressed or whether the AO22 mechanism of copper tolerance was not functional in a different bacterial genus.

**DISCUSSION**

In this work, a cop locus composed of 10 genes, copSRABGOFCDK (not necessarily representing a single operon), was isolated from the gDNA of Achromobacter sp. strain AO22. It is reasonable to assume that this locus is on the main chromosome of AO22, as no plasmid could be isolated after numerous attempts (data not shown). The putative proteins are not identical to known copper resistance proteins but show conservation of functionally important features. In addition, a putative copper-responsive promoter was also identified.

Compared with other copABCD systems, the AO22 cop locus has an unusual organisation in that copAB and copCD are separated. The conserved twin-Arg motif in the CopA signal peptide suggests its translocation to the periplasm via the TAT pathway. The E. coli PcoA and P. syringae CopA have a role in periplasmic handling of copper [26, 30], and CueO, a multicopper oxidase with a role in E. coli copper tolerance [40], is distantly related (17–21% identity) to CopA homologs and shares these features. The AO22 CopA also shares the conserved HCHXXXHM motif with its homologs, the first two residues being responsible for copper binding and cuprous oxidase activity of CueO proposed to protect the cells by converting Cu(I) to its less toxic form Cu(II) in a cytoplasmic reaction [18]. The P. syringae CopA binds 11 Cu atoms per molecule [8] whereas CueO binds 4 [18], and the Met-rich region is thought to determine the copper-binding capacities of multi-copper oxidases. However, the crystal structure of CueO showed that this region includes a helix that blocks access of bulky organic substrate to the Cu-binding site, thus providing CueO the specificity as cuprous oxidase [24]. Furthermore, PcoA catalyses the Cu(I) in Cu(I)Cu(II)-bound PcoC [14], likely through direct interaction of the Met-rich region of PcoA with the Met-rich Cu(I) site in PcoC [23]. Based on the conservation of the twin-Arg, HCHXXXHM, and the Met-rich region in AO22 CopA, AO22 CopA appears to be a copper oxidase and may function in protecting the periplasmic space from Cu toxicity by oxidizing Cu(I) to Cu(II).

Comparatively limited information is available on CopB proteins. P. syringae CopB and PcoB are predicted to be outer membrane proteins, and together with P. syringae CopA and PcoA, respectively, confer low-level resistance [8, 26]. Similarly, X. axonopodis pv. citri CopA and CopB are sufficient for low-level resistance, and inactivation of copAB leads to copper sensitivity [50]. The consensus HXXXXXLM, likely involved in Cu(I) fixation [26, 36], occurs only once in the putative AO22 CopB. The pMOL30 CopB has more metal-binding motifs compared with its chromosomal counterpart. Adaikkalam and Swarup [1] propose that the number of metal-binding domains, such as the Met-rich regions of CopB-like proteins, is positively correlated to levels of copper tolerance. The outer membrane location of CopB-like proteins has led to proposals that, in combination with CopA and possibly CopC, they facilitate the export of copper from the periplasm [8, 14, 26, 43]; thus, AO22 CopB may play a role in detoxification of periplasmic copper.

The conservation of Cu(I)- and Cu(II)-binding sites in the putative AO22 CopC suggests it may have similar functions
in copper handling as its homologs. Both P. syringae CopC and E. coli PcoC effect intermolecular transfer of Cu ions regardless of their oxidation states [13, 55]. As mentioned above, the Met-rich Cu(I) site of E. coli PcoC seems to interact with PcoA. CopC is also proposed to interact with CopD. In P. syringae, expression of CopC and CopD together (but not alone) increases sensitivity to copper [8, 10]. Because of the likely inner membrane location of P. syringae CopD, together with CopC, they are suggested to be required for copper uptake into the cytoplasm [10]. This suggests that CopAB and CopCD may have evolved separately, possibly explaining their non-adjacent positions at the AO22 cop locus.

The AO22 CopF exhibits features of efflux pumps of the P-type ATPase family and is phylogenetically related to the subgroup that transports Cu(I). In E. coli, disruption of copA, which encodes a P-type ATPase CopA, results in sensitivity of cells to Cu salts [42]. Located in the cytoplasmic (inner) membrane, these may confer tolerance by facilitating ATP-dependent transport of Cu ions out of the cytoplasm, as seen through accumulation of copper ions catalyzed by CopB in an inside-out membrane vesicle of E. hirae [47]. Based on relatedness to Cu ATPases, AO22 CopF may have a role in detoxifying cytoplasmic copper.

The copG, copO, and copK genes were first described within the 21-gene cop cluster of pMOL30 of C. metallidurans CH34 [36]. The genes are highly up-regulated under copper, but the physiological functions of their proteins are unconfirmed as yet. AO22 CopG and its homologs harbor a number of potential copper ligands (i.e., certain His, Met, and Cys residues). The CGCC motif suggests they may function as chaperones that deliver copper ions to/from partner P-type ATPases, such as CopZ of E. hirae [37]. On the other hand, although a conserved CPLMHxxxH is noted in CopO, its role is unknown. Based on pMOL30 CopO [31], these proteins may be located in the inner membrane and also serve as copper chaperones. The structure and metal-binding properties of pMOL30 CopK have shed some light on their roles. This CopK is periplasmic and binds up to 2 Cu(I) per molecule [5], and binding of Cu(I) through four Met causes a conformational change, probably leading to formation of the Cu(II)-binding site involving a His [45]. However, equivalent properties of the AO22 CopK could not be predicted from alignments alone, as the Cu(I)-binding ligands were not conserved; further work is required in this regard.

**Gene Regulation by CopRS**

The AO22 CopS and CopR exhibit features of the histidine kinases and response regulators, respectively, of TCST systems. In E. coli, pcoRS were shown to be essential for maximal copper-inducible expression from PpcoA [7], and cusRS essential for expression from PpcuE and PpcuC. By analogy with other copper TCST systems, AO22 CopS may sense the copper in the external environment and relay the signal to CopR, which may in turn regulate the expression of the adjacent structural genes copA and copB. The promoter elements in the spacer between copR and copA represent possible binding sites for CopR. Cop boxes have been reported so far in phytopathogens or enteric isolates (e.g., promoters of P. syringae copABCDRS [35], X. campestris pv. vesicatoria [4], E. coli pcoABCDRS [44], and E. coli cusSRCFBA [38, 54]). In P. syringae and E. coli, purified CopR and CusR bind at the copper box of the copper-inducible promoters PcoA and PcusC, respectively [35, 54]. Our results indicate that TCST systems for copper homeostasis is a common theme in diverse bacteria.

**Functionality and Possible Location of the Second mer Operon**

The fact that the mer2 of AO22 may be functional can be inferred from the Hg(II) MIC experiments, wherein the wild-type AO22 had higher Hg resistance compared with TnAO22-harboring E. coli. Genes encoding the Zn/Cd/Pb transporters of Pseudomonas putida KT2440 [28] localize on different parts of the genome, but their products are shown to interact synergistically and compensate each other. Studies are required to assess a similar interplay between the two mer operons in strain AO22.

With exceptions such as Tn503 from Xanthomonas sp. and TnMER11 from Bacillus megaterium [22, 25], most mercury resistance determinants of environmental isolates are located on plasmids. Despite numerous attempts to isolate plasmids, none was found in AO22. However, the possibility of a megaplasmid in AO22 that harbors TnAO22 and mer2 cannot be ruled out, as it is often difficult to isolate such plasmids owing to being very similar in size and physical properties to the main chromosome. For example, pMOL28 and pMOL30 of C. metallidurans CH34 are conjugative, but their transfers were limited to close taxonomic groups [33]. Thus, further efforts to identify conjugative plasmids in AO22 should include strains of beta-proteobacteria (to which Achromobacter sp. AO22 belongs) as recipients.

**A Possible Genomic Island in AO22**

Amplifications of key regions and sequencing showed that the cop locus of strain AO22 is on a possible genomic island, similar to the D. acidovorans SPH-1 DAGI-2 [52]. This is highly interesting, considering the different geographic origins of the two strains. However, genes in GIs are generally of foreign origin, indicating acquisition through horizontal transfer, and identical GIs are often identified in unrelated bacteria (e.g., CMGI-1 of C. metallidurans CH34 is almost identical to the PAGI2-C island of a P. aeruginosa strain isolated from cystic fibrosis patients [32, 52]). The presence of a Tn21-related transposon in AO22 reported by us earlier [39] supports such a possibility. The present results indicate the DAGI-2-like GIs may be ancient, more
widespread than expected, and may play a role in survival of the host in contaminated environments. Besides the cop and mer loci, the Dagi-2 of SPH-1 encodes ars (arsenic resistance), cd/pbr (cadmium/lead resistance) and sil (silver resistance) [52]; thus, it needs to be investigated whether such loci also occur in strain AO22. Exploring these will provide better understanding as to how Achromobacter sp. strain AO22 has adapted to its heavy metal rich microenvironment.

In conclusion, the present data provide insights into how environmental bacteria may balance intracellular copper levels. The AO22 cop locus appears to encode systems for periplasmic handling as well as cytoplasmic detoxification, under the regulation of a classical two-component signal transduction system, and exhibits a potential copper-responsive promoter. Achromobacter sp. AO22 thus has high potential as a copper biosensor as well as for a biosorption system for environmental samples. The presence of two mer operons provides similar opportunities for mercury biosensor and detoxification systems.

REFERENCES
