

The Copper-Inducible *cin* Operon Encodes an Unusual Methionine-Rich Azurin-Like Protein and a Pre-Q₀ Reductase in *Pseudomonas putida* KT2440[∇]

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The genome sequences of several pseudomonads have revealed a gene cluster containing genes for a two-component heavy metal histidine sensor kinase and response regulator upstream of *cinA* and *cinQ*, which we show herein to encode a copper-containing azurin-like protein and a pre-Q₀ reductase, respectively. In the presence of copper, *Pseudomonas putida* KT2440 produces the CinA and CinQ proteins from a bicistronic mRNA. UV-visible spectra of CinA show features at 439, 581, and 719 nm, which is typical of the plastocyanin family of proteins. The redox potential of the protein was shown to be 456 ± 4 mV by voltametric titrations. Surprisingly, CinQ is a pyridine nucleotide-dependent nitrile oxidoreductase that catalyzes the conversion of pre-Q₀ to pre-Q₁ in the nucleoside queuosine biosynthetic pathway. Gene disruptions of *cinA* and *cinQ* did not lead to a significant increase in the copper sensitivity of *P. putida* KT2440 under the conditions tested. Possible roles of CinA and CinQ to help pseudomonads adapt and survive under prolonged copper stress are discussed.

Copper is required as a cofactor for many enzymes, but in excess, it can have deleterious effects through metal-catalyzed protein oxidation and the generation of reactive oxygen species. Several mechanisms to handle and regulate intracellular levels of copper have been identified in bacteria (5, 10, 12, 31). In *Escherichia coli*, CopA is a P-type ATPase (8, 30) involved in ATP-dependent translocation of Cu(I) and Ag(I) from the cytoplasm to the periplasm, while CueO (12) is a multicopper oxidase that oxidizes Cu(I) to the less toxic Cu(II) in the periplasm. Both of the relevant genes, *copA* and *cueO*, are regulated by the MerR family-like activator CueR. In addition, *E. coli* contains the multicomponent Cus complex, which is responsible for expulsion of Cu(I) and Ag(I) from the periplasm across the outer membrane. CusA is localized in the cytoplasmic membrane; the essential energy-providing, proton-driven transporter CusB is also essential and belongs to the family of membrane fusion proteins; and CusC is an outer membrane factor. Another accessory protein, CusF, is believed to be a periplasmic copper chaperon delivering Cu(I) to the CusABC complex. In other prokaryotes, additional, often plasmid-encoded mechanisms have been shown to be involved in copper homeostasis. For *Pseudomonas syringae* *copABCD*RS (5) and its *E. coli* homolog, the plasmid-carried operon *pcoABCD*RSE (3), CopA is a multicopper oxidase that is thought to oxidize Cu(I) to Cu(II) in the periplasm, CopB is an outer membrane protein, CopC is a periplasmic protein, and CopD is probably an inner membrane protein.

Pseudomonas putida is a ubiquitous saprophytic bacterium that can be found in bulk soils, contaminated soils, waters, and the rhizosphere and has been studied extensively for its ability

to metabolize aromatic compounds (37). Genomic analysis revealed that *P. putida* is equipped with a variety of genes involved in metal homeostasis, including two *copA* (a gene homologous to those encoding the multicopper oxidase CopA from *P. syringae* pv. *syringae* and PcoA from *E. coli*) and two *copB* (a gene homologous to that encoding the *P. syringae* pv. *syringae* CopB outer membrane factor) genes, a *cusCBA* system similar to *E. coli* *cusCFBA*, and two genes encoding putative Cu(I)- and Ag(I)-transporting P-type ATPases (4).

Sequence comparison revealed another putative operon involved in copper homeostasis conserved in *P. putida* KT2440, *Pseudomonas fluorescens* PFO-1 and PF-05, *Pseudomonas aeruginosa* PAO1, and *Pseudomonas chlororaphis*. The *cinRS* operon encodes a histidine sensor kinase (*cinS*) and a response regulator (*cinR*) in the opposite direction of *cinAQ* (copper induced). Sequence analysis has annotated *cinA* as encoding a putative azurin-plastocyanin-like protein and *cinQ* as encoding a putative GTP cyclohydrolase/pre-Q₀ reductase (NCBI accession numbers NP_744308 and NP_744309, respectively). Known members of the azurin-plastocyanin family from bacteria are electron shuttles located in the periplasm.

CinA is similar to the *P. fluorescens* DF57 Cot protein, which is involved in copper tolerance (38). In addition, the *P. aeruginosa* homolog, *cinA* (PA2806), is also transcribed in copper-exposed cells, and its disruption leads to a slight reduction in copper tolerance (12-mm inhibition radius in a disk assay, compared to 8 mm for the wild type) (36).

A homolog of GTP cyclohydrolase I has been shown to catalyze the NADPH-dependent reduction of 7-cyano-7-deazaguanine (pre-Q₀) to 7-aminomethyl-7-deazaguanine (pre-Q₁) (39). The reductase in *Bacillus*, QueF, was found to be encoded in a cluster of genes that are thought to be involved in the biosynthesis of the hypermodified tRNA base queuosine (29).

In this paper, we report the analysis of the *cinAQ* operon in

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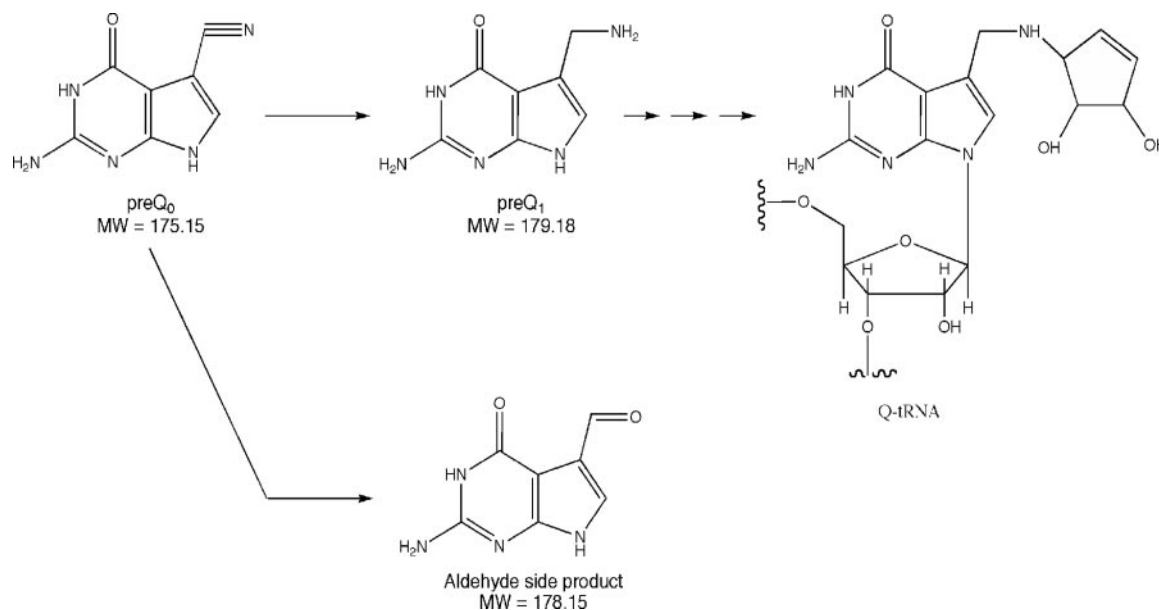


FIG. 1. Reaction catalyzed by CinQ. CinQ catalyzes the reduction of pre-Q₀ to pre-Q₁. The final product of the pathway is queuosine (Q-tRNA).

P. putida KT2440. The studies show that CinA is a copper-containing protein with a high redox potential and that CinQ catalyzes the conversion of pre-Q₀ to pre-Q₁ (Fig. 1). Disruption strains where *cinA* or *cinQ* was interrupted were examined to determine the role(s) of the encoded proteins in copper homeostasis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used for this study are listed in Table 1. *P. putida* strain KT2440 and all derived strains were grown either in Luria-Bertani broth (LB; EMD), on LB agar (Difco), or in minimal salts medium (MM) (22) with 5 mM benzoate and 1% agar (Bacto agar; Difco) at 27°C. *E. coli* strains were grown in LB at 37°C. *P. aeruginosa* PAO1 and its derivative transposon mutants PA2806::IS*phoA*/hah, PA2807::IS*lacZ*/hah, PA2807::IS*phoA*/hah, PA2809::IS*lacZ*/hah, and PA2810::IS*phoA*/hah, obtained from the University of Washington Genome Center, were grown in LB at 37°C. *E. coli* DH5 α was used to maintain engineered constructs. *E. coli* BL21(pLys) (Stratagene) was used for overexpression of recombinant proteins. For conjugative gene transfer, overnight cultures of donor strain *E. coli* S17-1 and recipient strain *P. putida* KT2440 were grown at 27°C in LB, mixed (3:1 and 1:3), and plated onto sterilized filters on LB agar plates. After overnight incubation, cells were suspended in saline solution (0.85% NaCl), diluted, and plated onto selective medium.

Antibiotics (kanamycin [25 mg/liter for *E. coli* and 50 mg/liter for *P. putida*], ampicillin [100 mg/liter], and chloramphenicol [25 mg/liter]) were added when appropriate.

Genetic techniques. Standard genetic techniques were used (33). Whole genomic DNA was extracted according to the method of Marmur (21). DNAs were amplified by PCR, using *Taq* polymerase (Fermentas). *P. putida* KT Δ *cinA* (*P. putida* KT2440 with an in-frame deletion of *cinA*) was constructed as follows. A 900-bp region upstream of the start codon and containing the first codons of the gene and a 500-bp region downstream of *cinA* were amplified using primers KTko1 and KTko2tag (PCR 1) and primers KTko3tag and KTko4 (PCR 2), respectively. These two flanking DNA fragments were joined together by overlap extension PCR (OE-PCR). For OE-PCR, 1 μ l each of the PCR 1 and PCR 2 products was used as a template in a PCR mixture containing a 0.2 mM concentration of each deoxynucleoside triphosphate, 2.25 mM of MgCl₂, 75 mM Tris-HCl (pH 8.8), 20 mM (NH₄)₂SO₄, and 2.5 U of *Taq* polymerase. The PCR program consisted of 55 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. The gene assembly reaction mixture (1 μ l) was diluted in 50 μ l of a PCR mixture containing a 0.2 mM concentration of each deoxynucleoside triphosphate, 2.25

mM of MgCl₂, 75 mM Tris-HCl (pH 8.8), 20 mM (NH₄)₂SO₄, and primers KTko1 and KTko4 and then amplified. The resulting PCR product (KTPCR4), containing a deletion of *cinA*, was first cloned into pGEM-T Easy (Promega) according to the manufacturer's protocol, and this construct was then digested using the *Sac*I restriction enzyme and ligated into pLO2 (18) to obtain pLO2KTPCR4. Plasmid pLO2 contains a kanamycin cassette and *sacB*.

A gene deletion was obtained by transfer of pLO2KTPCR4 from *E. coli* S17-1 to *P. putida* KT2440 via parental mating. Transformants were first selected on MM with 5 mM benzoate and 50 μ g/liter of kanamycin (single recombination event) and then counterselected on LB containing 10% sucrose to screen for the loss of *sacB* (double recombination event). Gene deletion was confirmed by a PCR using primers (KTDPD and KTDXU) external to the region used for the OE-PCR.

Strain KT $cinQ::kan$, containing a gene disruption of *cinQ*, was constructed as follows. The central part of the gene was amplified (using primers KTDXU and KTDXD) and cloned into plasmid pLO2, using the *Mlu*I and *Sac*I restriction sites, generating plasmid pLO2NCinQ. Gene disruption was obtained after parental mating with *E. coli* S17-1 (pLO2NCinQ) and selection of transformants growing on MM with 5 mM benzoate and 50 μ g/liter of kanamycin. Gene disruption was confirmed by Southern blotting.

Construction of expression vectors. To construct the *cinA* expression vector pASK-P12, *cinA* was amplified from the genomic DNA and cloned into the *Eco*RI and *Sac*II restriction sites of pASK-IBA3 (IBA). Plasmid pASK-P12 expressed CinA as a C-terminal fusion protein with Strep-TagII (amino acid sequence, VDLQGDHGLSAWSHPQFEK), with an MGDGPPEL peptide at the N terminus.

To construct the *cinQ* expression vector pASK-X4, *cinQ* was amplified from the genomic DNA and cloned into the *Eco*RI and *Sac*II restriction sites of pASK-IBA3. Plasmid pASK-X4 expressed CinQ as a C-terminal fusion protein with Strep-TagII (amino acid sequence, VDLQGDHGLSAWSHPQFEK), with an MGDGPPEL peptide at the N terminus.

The gene encoding QueF was amplified by PCR from *B. subtilis* genomic DNA (ATCC 23857), using forward primer VBD136 and reverse primer VBD137, with an Epicenter Technologies fail-safe PCR kit. The resulting fragment was cloned between the *Nde*I and *Hind*III sites of pET29 for expression of the native recombinant QueF protein. Constructs were introduced by electroporation into *E. coli* BL21(DE3) for protein expression (Novagen).

Protein expression and purification. CinA and CinQ were purified using Strep-TagII technology. For protein overexpression, pASK-P12 and pASK-X4 were always transformed fresh into *E. coli* BL21(pLys) (Stratagene).

Cells were cultivated overnight at 37°C in LB with ampicillin and chloramphenicol, diluted 1:50 into 0.5 liter of fresh medium, and cultivated with shaking at 37°C to an optical density at 600 nm (OD₆₀₀) of 0.5. Overexpression of

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or relevant phenotype	Reference or source
Strains		
<i>E. coli</i> strains		
DH5 α	F2 <i>end-1 hsdR17 supE44 thi-1 recA1 deoR gyrA96 relA1</i> Δ (<i>argF-lacZYA</i>)U169	New England Biolabs
S-17	<i>thi pro hsdR hsdM1 recA tra-1</i>	34
BL21	DE3(pLys) [<i>E. coli</i> B F ⁻ <i>dcm ompT hsdS_B(r_B⁻ m_B⁻)</i> <i>gal</i> λ (DE3) (pLysS Cam ^r)]	Stratagene, Novagen
<i>P. putida</i> strains		
KT2440	Wild type	ATCC 47054
KT Δ <i>cinA</i>	KT2440 with deletion of <i>cinA</i>	This work
KT <i>cinQ::kan</i>	KT2440 with gene disruption of <i>cinQ::kan</i>	This work
<i>P. aeruginosa</i> strains		
PAO1	Wild type	University of Washington Genome Center
PA2806:: <i>ISphoA/hah</i>	PAO with gene disruption of <i>cinQ::Tet^r</i>	University of Washington Genome Center
PA2807:: <i>ISlacZ/hah</i>	PAO with gene disruption of <i>cinA::Tet^r</i>	University of Washington Genome Center
PA2807:: <i>ISphoA/hah</i>	PAO with gene disruption of <i>cinA::Tet^r</i>	University of Washington Genome Center
PA2809:: <i>ISlacZ/hah</i>	PAO with gene disruption of <i>cinR::Tet^r</i>	University of Washington Genome Center
PA2810:: <i>ISphoA/hah</i>	PAO with gene disruption of <i>cinS::Tet^r</i>	University of Washington Genome Center
Plasmids		
PASK-IBA3	Overexpression vector (Strep-TagII); Amp ^r	IBA, GmbH
pGEM-T-Easy	Cloning vector; Amp ^r	Promega, Madison, WI
pLO2	Suicide vector; <i>sacB</i> Kan ^r	18
pET29	Overexpression vector (S tag); Kan ^r	Novagen
pLO2NCinQ	Contains nt 45 to 786 of <i>cinQ</i>	This work
pLO2KTPCR4	Contains deletion of <i>cinA</i>	This work
pASKP-12	CinA in pASK-IBA3 with C-terminal Strep-TagII	This work
pASK-X4	CinQ in pASK-IBA3 with C-terminal Strep-TagII	This work

recombinant protein was induced by the addition of anhydrotetracycline to a final concentration of 200 μ g/liter, and incubation was continued for 3 to 5 h at 30°C. Cells were harvested by centrifugation at 5,500 \times g for 12 min at 4°C. CinA was extracted from the periplasm by osmotic shock (12, 27). Briefly, cell pellets were suspended in buffer P (100 mM Tris-HCl, pH 8, 500 mM sucrose), kept on ice for 30 min, and then centrifuged at 15,000 \times g for 15 min at 4°C. CinQ was extracted from the cytoplasm. The pellet was resuspended in buffer W (100 mM Tris-HCl, pH 8) containing DNase I (10 mg/liter) and a proteinase inhibitor cocktail (Sigma), lysed with a French press (1,260 lb/in², cell type 20K; Amicon, SLM Instruments, Inc., Rochester, NY), and then centrifuged at 15,000 \times g for 15 min at 4°C.

In both cases, after centrifugation (15,000 \times g, 15 min, 4°C) the supernatant was applied to a streptactin-agarose column (1 ml/400 ml of culture) equilibrated with buffer W. The column was washed with buffer W, and the protein was then eluted in aliquots of 2.5 ml of buffer E (buffer W containing 2.5 mM desthiobiotin).

E. coli BL21(DE3) transformants containing the QueF expression plasmid were plated on an LB agarose plate containing 34 μ g/ml kanamycin and incubated at 37°C. After 16 h, one colony from the plate was used to inoculate 50 ml LB containing 34 μ g/ml kanamycin. The culture was allowed to grow overnight at 37°C. To inoculate cultures for protein expression, 10 ml of the overnight culture was added to flasks containing 1 liter of LB and 34 μ g/ml kanamycin. Cells were grown to an OD₆₀₀ of ~0.5, and protein expression was induced by adding IPTG (isopropyl- β -D-thiogalactopyranoside) to a final concentration of 0.1 mM. After 6 h of growth at 37°C, cells were collected by centrifugation (20,500 \times g) and frozen in liquid N₂.

For protein purification, a cell paste (3 g) was suspended in a solution containing 0.05 M Tris-HCl (pH 8.0), 2 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride. Cell walls were disrupted using a Branson 450D sonifier at 50% power. Cell suspensions were then centrifuged at 26,500 \times g for 30 min. The soluble extract was loaded on an 80-ml (19.3 \times 2.3 cm) column of Q-Sepharose that had been equilibrated with buffer containing 0.05 M Tris-HCl

(pH 8.0) and 2 mM DTT. Protein was eluted using a linear gradient from 0 to 0.5 M KCl in a total volume of 0.9 liter. Fractions containing QueF were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pooled, and concentrated in an Amicon pressure cell (YM-10 membrane). The resulting protein was desalted on a Sephacryl S-300 size-exclusion column (Amersham) which had been equilibrated with a buffer containing 0.05 M Tris-HCl (pH 8.0) and 2 mM DTT. Fractions containing QueF were identified by gel electrophoresis, pooled, and concentrated as described above. The protein was quantified by a BCA assay (Pierce), with bovine serum albumin as the standard.

RNA-DNA hybridization. Regulation of transcription was examined by Northern blot analysis. *P. putida* KT2440 cells were grown on MM containing 5 mM benzoate to mid-log phase at 27°C, at which point either no metal (control), NiCl₂, CuCl₂, FeSO₄ (with 10 mM ascorbic acid), or ZnCl₂ was added to a final concentration of 100 μ M. Incubation was continued with shaking at 27°C for 30 min, after which cells were harvested and total RNA was extracted using a RNeasy Plant Mini kit (QIAGEN).

Equal amounts of total RNA were loaded in a 1.2% agarose-formaldehyde gel and electrophoresed for 3 h at 70 V/cm. RNAs were transferred to a nylon membrane by using a vacuum blotter (Boeckel Appligen) and then hybridized overnight with ³²P-labeled probes at 42°C. ³²P was purchased from Perkin-Elmer. The probes were prepared using the RadPrime DNA labeling system (Invitrogen). Probes for *cinA* and *cinQ* were obtained by PCR with the KTDPU-KTDPD and KTDXU-KTDXD primers, respectively, using *P. putida* KT2440 genomic DNA as the template.

RT-PCR analyses. Total RNA was isolated from CuCl₂-induced *P. putida* KT2440 as described above. The RNA sample was then digested with 1 U of DNase I (Sigma) per microgram of RNA for 2 h at 37°C. RNAs were then purified by phenol-chloroform extraction and resuspended in diethyl pyrocarbonate-treated water. Reverse transcription-PCR (RT-PCR) was performed using Superscript II reverse transcriptase (GIBCO BRL, Cambridge, MA) at 50°C for 1 h with primer RT-PX3'A (Table 2). The cDNA was PCR amplified using primers RT-PX3'A and RT-PX5'S and *Taq* DNA polymerase (Fermentas) for

TABLE 2. Primers used in this study

Primer	Sequence (5'-3') ^a	Use	Restriction site
KTDP	AAAACGCGTCATTACCGCCACCCCTTGCCCTG	NBA ^b	MluI
KTDPD	AAAGAGCTCCTGGTAATGCCCGGCACGTT	NBA	SacI
KTDXU	AAAACGCGTATACATCGCCACCTACTCCCG	Disruption of <i>cinQ</i> /NBA	MluI
KTDXD	AAAGAGCTCGTGTGCGATACGGGTTGATGTCC	Disruption of <i>cinQ</i> /NBA	SacI
KTko1	AAAGAGCTCAGCTTGTGGGGTGGCGGGTCC	<i>cinA</i> deletion	SacI
KTko2tag	cccatccactaaatttaaataGGTGGCGGTAATGAACAG	<i>cinA</i> deletion	
KTko3tag	tatttaaaatttagggattgagtgactggctctg	<i>cinA</i> deletion	
KTko4	GTGGAGCTCTTCTCCACCACCCGCTCC	<i>cinA</i> deletion	SacI
KTNCinQ3'	AGGGTCGACCTGCCGACCAGCCGTTTGTATCC	<i>cinQ</i> in pASK-IBA3	SalI
KTNCinQ5'	GTCCAATTGCATCCCGCTGCCGAACATTCC	<i>cinQ</i> in pASK-IBA3	MunI
KTcinA5'	GTCCAATTGAAACACCTGTTTATTACCGCCAC	<i>cinA</i> in pASK-IBA3	MunI
KTcinA3'	AGCGTCGACCTCATTGGTCAATGCCCGC	<i>cinA</i> in pASK-IBA3	SalI
KTDPOD	AGGGAATTCATCGTGTCTGCTGGCTCGGGC	Verification of <i>cinA</i> deletion	EcoRI
KTDXUU	CCTGAGCTCCGGCTTGTGGACGGTAGGC	Verification of <i>cinA</i> deletion	SacI
RT-PX5'S	GCGCGCCGAACCTGACCTGGAC	RT 5' sense	
RT-PX3'A	TGCTCCGGGGAGTAGGTGGCC	RT 3' antisense	
VBD136	AAAGGAAGATGCATATGACGACAAGAAAAGAATCAG	<i>queF</i> in pET29	NdeI
VBD137	CACACAACGGAAAGCTTATTAACGATTATCAATTGTC	<i>queF</i> in pET29	HindIII

^a Lowercase indicates DNA tag used for overlap extension PCR.

^b NBA, Northern blot analysis.

30 cycles with an annealing temperature of 63°C and an extension time of 30 seconds. RT-PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide.

Disk sensitivity assay. *P. putida* KT2440, *KTΔcinA*, and *KTcinQ::kan* were grown overnight at 27°C. *P. aeruginosa* PAO1 and its derivative transposon mutants PA2806::IS*phoA*/hah, PA2807::IS*lacZ*/hah, PA2807::IS*phoA*/hah, PA2809::IS*lacZ*/hah, and PA2810::IS*phoA*/hah were grown, and 100 μl of cell suspension was plated on an LB agar plate. Ten microliters of 1 M CuSO₄ or 1 M CuCl₂ was added to each filter disk. Disks were air dried and placed at the center of the agar plates. Plates were incubated at room temperature for 24 (*P. aeruginosa*) or 48 (*P. putida*) hours, and the zones of clearance were measured.

Spectroscopic and spectroelectrochemical analysis of CinA. UV-visible spectra of CinA were obtained with a Varian Cary 300 spectrophotometer. Before the spectrum was obtained, CinA was first loaded with 20 μM of CuCl₂, oxidized with one crystal of ferricyanide, and then dialyzed in buffer W.

The redox potential was investigated by spectroelectrochemical techniques as described by Ding et al. (6), carried out using the same optically transparent thin-layer electrode cell (OTTLE) and the same Ag/AgCl reference electrode (-205 mV versus a standard hydrogen electrode [SHE]; Bioanalytical Systems, Inc.) (6).

A 150 μM solution of CinA was prepared in 0.1 M sodium phosphate buffer (pH 7.5). The solution was degassed for 24 h at 4°C by dialysis against argon-bubbled buffer. After the addition of 0.2 mM of the electrochemical mediators ferrocene 1,1-dicarboxylic acid (Aldrich) ($E_0' = 644$ mV versus SHE) and hexaammineruthenium(II) chloride (Aldrich) ($E_0' = 51$ mV versus SHE), the sample was loaded into the OTTLE cell under argon. Electrochemical titrations were carried out by setting the potential at a chosen value, using a computer-controlled BAS CV-50W voltametric analyzer, for a period of 5 to 10 min until the optical spectrum did not change significantly with time. An optical spectrum was then recorded, the potential was changed in steps of 20 to 50 mV, and the process was repeated at the new applied potential. The temperature of the chamber was $27 \pm 1^\circ\text{C}$. The data were then fit to the Nernst equation, using the nonlinear least-squares fitting algorithm of Origin software. The Nernst equation describes the effect of applied potential on the ratio of the concentrations of oxidized and reduced forms of the complex and on the standard reduction potential, E_0 , as follows: $E_{\text{app}} = E_0 + 2.303(\text{RT}/nF) \cdot \log_{10}([\text{Ox}]/[\text{Red}])$, where E_{app} is the applied potential, E_0 is the reduction potential determined from these data, $[\text{Ox}]$ and $[\text{Red}]$ are the concentrations of the protein in the oxidized and reduced states, respectively, R is the universal gas constant, T is the temperature in kelvin units, n is the number of electrons transferred in half reaction, and F is the Faraday constant. $[\text{Ox}]$ and $[\text{Red}]$ were calculated from the optical spectra by using Beer's law.

Enzymatic characterization of CinQ. (i) **Synthesis of pre-Q₀.** Methyl formate (3.1 ml) and chloroacetaldehyde (3.2 ml) were added to anhydrous toluene (25 ml) at 0°C. Sodium methoxide (2.9 g) was added slowly over the course of 5 min. The reaction was allowed to proceed for 2 h. The precipitate was collected and

washed with 50 ml toluene and 25 ml anhydrous ethyl ether, yielding an off-white powder of chloro(formyl)acetone. The product was placed in 10 ml water containing 2.9 ml of acetic acid at <10°C. The pH of the solution was adjusted to ~4 to 5 prior to the solution being combined with 2,4-diamino-6-hydroxypyrimidine (6.3 g) in *N,N*-dimethylformamide (10 ml). The mixture was stirred for 2 h at room temperature. The solution was placed at 4°C overnight. The resulting precipitate was collected and washed with 0.4 liter of water and 0.2 liter of acetone. The resulting solid was refluxed for 1 h in 50 ml water. The precipitate that formed upon cooling was collected and washed with 0.4 liter water and 0.3 liter acetone. The nuclear magnetic resonance and mass spectra were consistent with those of the desired product (23).

(ii) **Assays for GTP cyclohydrolase I activity.** Assays to analyze the GTP cyclohydrolase I activity of *P. putida* KT2440 CinQ were carried out with the following reagents: 20 mM Tris-HCl (pH 8.0), 100 μM GTP, 1 mM MgCl₂, 1 mM DTT, and 10 μM CinQ protein. Reactions were carried out at 30°C and quenched by centrifugation through YM-10 Microcon centrifugal membranes to remove CinQ at 0, 30, 60, and 120 min. A control reaction mix lacking CinQ was also prepared in parallel. An aliquot (20 μl) obtained at each time point was loaded on a Zorbax SAX high-performance liquid chromatography (HPLC; Agilent) column (4.6 × 250 mm) that had been equilibrated with H₂O (solution A). The column was eluted at a flow rate of 0.75 ml/min with increasing proportions of 0.75 M NH₄H₂PO₄-2% acetonitrile (solution B) in water. The elution program was as follows: 5 min with 99% solution A, 25 min with 95% solution A, 30 min with 30% solution B, and 35 min with 30% solution B. An aliquot (5 μl) containing 0.8 mM (each) GTP, GDP, and GMP served as a standard. UV spectra were recorded from 190 to 500 nm on an Agilent 1100 series photodiode array detector.

(iii) **Assays for pre-Q₀ reductase activity.** The *P. putida* CinQ protein (18.2 nmol) was buffer exchanged into 0.05 M HEPES (pH 7.5) by repeated concentration and dilution in a Microcon centrifugal concentrator (YM-10 membrane). The assay mixtures contained 0.05 M HEPES (pH 7.5), 0.1 mM pre-Q₀, 0.1 M KCl, 1 mM NADPH, 10 mM DTT, and 10 μM CinQ protein. A positive control reaction was carried out as described above, but with recombinant *B. subtilis* QueF protein, which was shown previously to catalyze the conversion of pre-Q₀ to pre-Q₁ (37). Reactions were allowed to proceed for 60 min at 30°C and then quenched by centrifugation for 8 min at 12,000 × g through YM-10 Microcon centrifugal membranes to remove CinQ prior to being analyzed by reverse-phase HPLC as described below for liquid chromatography-mass spectrometry (LC-MS) experiments.

For LC-MS analysis of the reaction, an aliquot (20 μl) of each reaction mix was injected onto a 4.6- by 250-mm Eclipse XDB-C18 column (Agilent) which had been pre-equilibrated in 20 mM ammonium acetate at pH 6.0 (solution A) at a flow rate of 0.3 ml/min. The column was developed with a linear gradient of 0 to 40% methanol over 40 min, followed with a gradient of 40 to 50% methanol over 5 min. The elution was monitored by UV-visible and MS detection. UV-visible spectra were obtained from 220 to 500 nm, using a ThermoFinnigan Surveyor

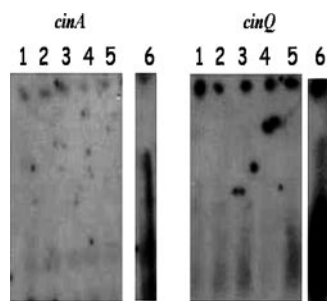


FIG. 2. Northern blot analysis of *cinA* and *cinQ*. Northern blot analysis was performed with *cinA* (left) and *cinQ* (right) from *P. putida* strain KT2440. RNAs were isolated from KT2440 grown on MM containing 5 mM sodium benzoate, supplemented with metal as follows: lanes 1, no metal; lanes 2, 100 μ M FeSO₄ and 10 mM ascorbic acid; lanes 3, 10 mM ascorbic acid; lanes 4, 100 μ M NiCl₂; lanes 5, 100 μ M ZnCl₂; and lanes 6, 100 μ M CuCl₂.

photodiode array detector. Mass spectra were obtained in positive mode, scanning the *m/z* range of 90 to 500 atomic mass units (amu), using an electrospray ionization-equipped LCO ThermoFinnigan Deca XP mass spectrometer. The instrument was set at a 42-V ionization energy and a 300°C ion source temperature.

To fully assign the identities of the species that were observed in the course of the CinQ studies, reactions were carried out in 61% ¹⁸O-labeled water, or aliquots of the reaction mixtures (30 μ l) were treated with NaBH₄ and NaBD₄ at a 10 mM final concentration. The borohydride reduction was allowed to proceed for 1 h at room temperature prior to LC-MS analysis.

RESULTS

cinA and *cinQ* are induced by copper and cotranscribed.

Metal-dependent induction of transcription of *cinA* and *cinQ* was analyzed by RNA-DNA hybridization, using RNAs isolated from cells grown in the presence of no metal, nickel, copper, ferrous iron, or zinc at 0.1 mM. The RNA samples were probed with amplified DNAs corresponding to the *cinA* and *cinQ* genes. As shown in Fig. 2, induction was observed only in the presence of copper.

RT-PCR was performed to investigate if *cinA* and *cinQ* are transcribed from a bicistronic mRNA. The translational stop codon of *cinA* and the start codon of *cinQ* are separated by 68 nucleotides. Primers were designed to amplify the region between the last 100 nucleotides of *cinA* and the first 100 nucleotides of *cinQ*, spanning the intragenic space. DNase I-treated RNA from copper-induced cells was used as a template for RT. The obtained single-stranded DNA was amplified by PCR. Figure 3 shows that RT-PCR revealed a 250-bp band that contains the intragenic region between *cinA* and *cinQ*.

We not only demonstrated that *cinA* and *cinQ* are induced by the addition of copper (Fig. 2) but also detected the intragenic region (Fig. 3). These results, along with sequence analysis of the genomic region, strongly suggest that *cinA* and *cinQ* are transcribed as a bicistronic mRNA, not as two monocistronic mRNAs, in a copper-inducible manner.

Overexpression and purification of CinA and CinQ. CinA with a C-terminal Strep-TagII was overexpressed in *E. coli* BL21(pLys), extracted from the periplasm by osmotic shock, and then purified using a streptactin-agarose column. Samples from the eluates containing the overexpressed protein were

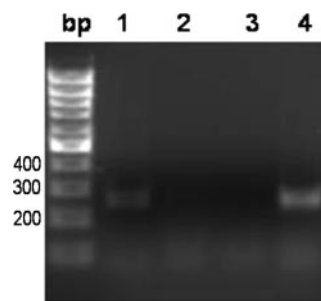


FIG. 3. RT-PCR analysis of expression of *cinA* and *cinQ* in *P. putida* KT2440. Electrophoresis was performed on a 2% agarose gel stained with ethidium bromide. Lane bp, 100-bp ladder; lane 1, RT-PCR product from RNAs extracted from CuCl₂-induced cells (after DNase I treatment), using RT 5' sense and RT 3' antisense primers; lane 2, negative control for RT, obtained by PCR amplification of the RT product obtained when the reverse transcriptase was omitted, to verify that no genomic contamination was present in the RNA extract; lane 3, PCR negative control obtained by omitting the genomic DNA from the PCR; lane 4, PCR positive control obtained using genomic DNA from *P. putida* KT2440 as the template.

analyzed in an SDS-polyacrylamide gel and stained with Coomassie brilliant blue 250 (Fig. 4).

The estimated molecular mass of CinA containing the C-terminal Strep-TagII, after cleavage of the N-terminal signal sequence, is 19.1 kDa. The purified protein preparation showed a band corresponding to the correct molecular mass (Fig. 4) in SDS-PAGE analysis. A band at 21.8 kDa would have been expected had the signal peptide not been processed. The UV-visible spectrum of the streptavidin-purified CinA protein revealed absorbance maxima at 439 nm and 581 nm and a shoulder at 719 nm; these spectral features are typical of members of the azurin-plastocyanin family (Fig. 5). The molar extinction coefficient at 581 nm (ϵ_{581}) was calculated to be 3,092 M⁻¹ cm⁻¹.

CinQ with a C-terminal Strep-TagII was overexpressed in *E. coli* BL21(DE3)pLys and purified by affinity chromatography.

CinA is an azurin-like protein of high electrochemical potential. The redox potential of CinA was determined by spectroelectrochemical titrations. The protein sample was placed in an OTTLE cell equipped with a Ag/AgCl reference electrode. By changing the potential applied to the OTTLE cell and analyzing the concentrations of the oxidized and reduced forms of the sample, it was possible to calculate the reduction

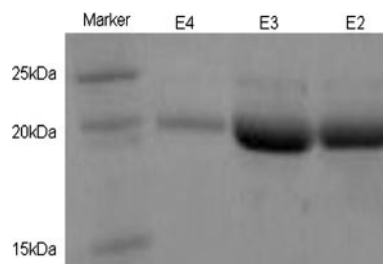


FIG. 4. SDS-PAGE analysis of CinA. Typical elution profiles of purified CinA are shown. Elution samples from the affinity column were loaded into a 12.5% SDS-PAGE gel and stained with Coomassie brilliant blue 250. Proteins usually eluted in the second and third fractions. Fermentas SM0661 was used as a molecular size ladder.

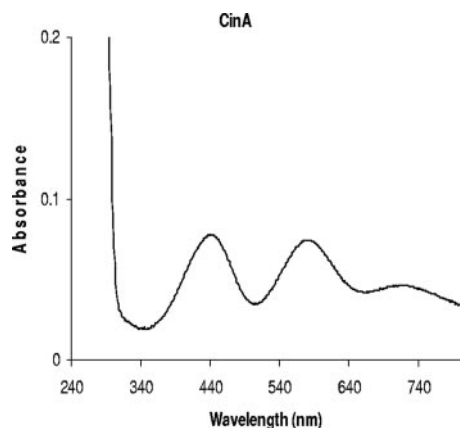


FIG. 5. UV-visible spectrum of CinA. The UV-visible spectrum of CinA was recorded with a Varian Cary 300 UV-visible spectrophotometer at room temperature. The CinA concentration was 0.5 mg/ml in 100 mM Tris-HCl, pH 8.

potential of the protein, using the Nernst equation. The potential in the cell was changed using a voltametric analyzer in steps of 20 to 50 mV; spectra were recorded after each change in the potential of the system. The data were fit to the Nernst equation, yielding a redox potential for CinA of 456 ± 4 mV (Fig. 6).

CinQ possesses queuosine reductase activity. To determine the enzymatic role of CinQ, the purified protein was assayed initially for turnover with GTP by HPLC. The traces (not shown) did not reveal conversion of GTP to dihydroneopterin triphosphate, as would have been expected. However, some GTP was converted to GDP in the presence of CinQ, to a greater extent than that in buffer solution alone (data not shown). Since PSI-BLAST analysis of the protein relative to

the protein database had also revealed similarity to a recently discovered class of proteins that are involved in the biosynthesis of queuosine (39) (49% similarity to *Bacillus subtilis* QueF), CinQ was assayed for pre- Q_0 reductase activity. In these experiments, 10 μ M CinQ was incubated with pre- Q_0 prior to being analyzed by reverse-phase HPLC. The chromatograms show that in the time course of the reaction (1 h), there was a virtually complete loss of the peak due to the substrate, pre- Q_0 (24 min) (Fig. 7A), and the appearance of two new species, eluting at 19 and 21 min (Fig. 7B). The retention time and UV-visible spectrum of the 19-min peak correlate with those for pre- Q_1 synthesized enzymatically from pre- Q_0 by recombinant QueF from *B. subtilis* (Fig. 7C). In control experiments, we showed that pre- Q_1 was not observed if pre- Q_0 , NADPH, or the enzyme was omitted from the incubation mixtures (data not shown).

The presence of a minor product in the CinQ incubation mixtures prompted a more careful LC-MS analysis of the reactions carried out by CinQ. Extracted ion chromatograms for the reactions are shown in Fig. 8. Pre- Q_0 (42 min) and pre- Q_1 (32 min) exhibited the appropriate masses, 176 and 180 amu, respectively, expected for the protonated molecular ion ($M + H^+$) (Fig. 8A and F). The pre- Q_1 standard (Fig. 8F) was obtained enzymatically as described above (Fig. 7C). When a sample of the CinQ reaction mix was treated with $NaBH_4$, we observed a peak at 181 amu, consistent with reduction of the minor product and incorporation of two protons (Fig. 8C). The small peak with the same retention time as that of pre- Q_1 , which is also observed in the chromatogram of the $NaBH_4$ -treated sample, is from the isotope envelope of pre- Q_1 . Consistent with the $NaBH_4$ -treated samples, when an aliquot of the CinQ reaction mix was treated with $NaBD_4$, the peak due to the minor product at 179 amu disappeared and a new peak at 182 amu appeared. This is consistent with the incorporation of

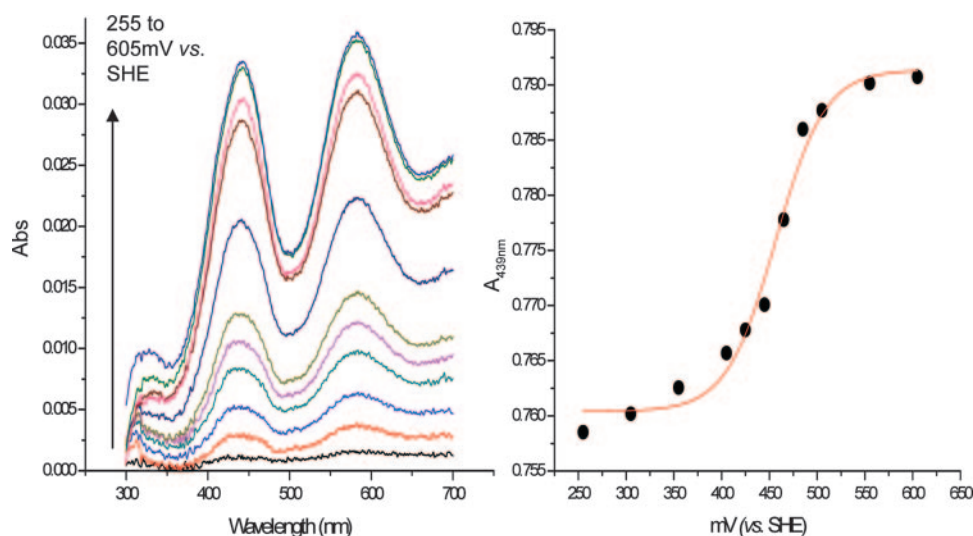


FIG. 6. Voltametric titrations for redox potential of CinA. A 150 μ M CinA solution was loaded into an OTTLE cell, and spectroelectrochemical titration was carried out by changing the potential in steps of 20 to 50 mV. Spectra were recorded 10 min after each change. Ferrocene 1,1-dicarboxylic acid (0.2 mM) and 0.2 mM hexaammineruthenium(III) chloride were used as mediators. (Left) Background-subtracted (205 mV) spectra taken at different potentials (255 to 605 mV versus SHE). (Right) Plot of absorbance at 439 nm versus equilibration potential. The red line shows the nonlinear least-squares fit with the Nernst equation ($n = 1$, with one electron transfer center), obtained using Origin ($R^2 = 0.98$). From the fit, the reduction potential of the protein is 456 ± 4 mV (versus SHE).

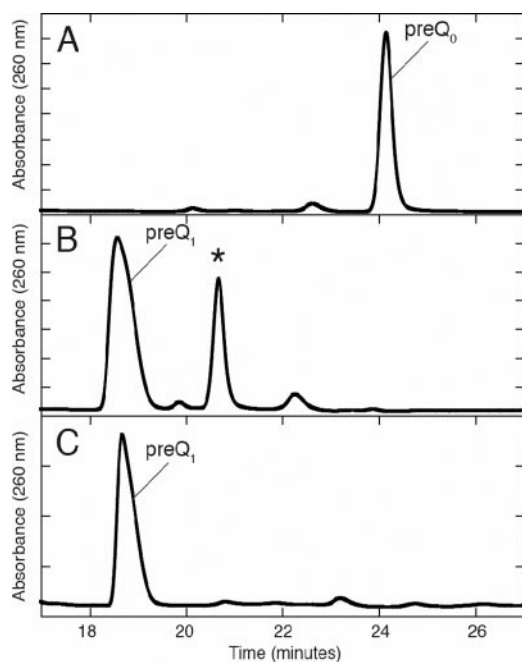


FIG. 7. UV chromatograms revealing the conversion of pre-Q₀ to pre-Q₁ by *P. putida* CinQ. The protein sampled (10 μM) was incubated with 50 mM HEPES (pH 7.5), 100 μM pre-Q₀, 100 mM KCl, 1 mM NADPH, and 10 mM DTT for 60 min at 30°C. An aliquot (20 μl) of each of the reaction mixtures was analyzed by HPLC. Trace A shows unreacted pre-Q₀ from a reaction mixture lacking CinQ and QueF. Trace B shows pre-Q₁ and an additional unknown product (*) from a reaction mixture containing CinQ. Trace C is a control reaction in which pre-Q₀ was converted to pre-Q₁ by the *B. subtilis* nitrile reductase, QueF.

one deuterium molecule and one proton into the starting material. Finally, when the reaction was carried out in the presence of 61% ¹⁸O-enriched water, both the ¹⁶O- and ¹⁸O-labeled forms of the unknown were observed in the chromatograms (Fig. 8E), at 179 and 181 amu, respectively. We infer from these results that the minor product is a 7-deazaguanine-7-aldehyde. This molecule is unlikely to be a physiologically relevant product of CinQ and was not observed in the published *B. subtilis* QueF studies (39), nor was it observed in the QueF reactions shown in this study. The unusual product may result from the release of the two-electron reduced pre-Q₀ from the active site of the protein prior to reduction with a second equivalent of NADPH. In solution, the imine would undergo spontaneous hydrolysis to the aldehyde.

Deletion of *cinA* or disruption of *cinQ* does not significantly influence copper sensitivity. To investigate the role of *cinA* and *cinQ* in copper homeostasis, strains where *cinA* or *cinQ* was inactivated by deletion (*cinA*) (Fig. 9) or insertion (*cinQ*) (not shown) were prepared as described in Materials and Methods.

The copper sensitivity of the mutated strains was investigated by exposing the strains to different concentrations of CuCl₂. First, wild-type or mutant *P. putida* strains were streaked on LB agar plates containing different concentrations (2 to 4.6 mM) of CuCl₂. *P. putida* KT*cinQ*::*kan* and KTΔ*cinA* exhibited slightly smaller colony sizes than did the isogenic *P. putida* KT2440 wild type, both on LB agar and on LB agar containing concentrations of copper between 2.5 and 3.75

mM CuCl₂. At concentrations above 4 mM CuCl₂, all three strains presented very small colonies. Disk assays were performed to measure the inhibition radii with CuSO₄ and CuCl₂, but no significant difference was observed between *P. putida* KT*cinQ*::*kan*, KTΔ*cinA*, and the wild type.

To analyze the possible roles of *cinA* and *cinQ* homologues in *P. aeruginosa* PAO1, disk inhibition assays were also carried out with *P. aeruginosa* PAO1 and strains carrying disruptions of the PA2806 (*cinQ*), PA2807 (*cinA*), PA2809 (*cinR*), and PA2810 (*cinS*) open reading frames (ORFs), obtained from the University of Washington Genome Center. The PA2807, PA2809, and PA2810 mutants presented larger inhibition radii than did the PAO1 wild type, as previously reported by Teitzel et al. (36), while the PA2806 (*cinQ*) mutant did not present any significant difference from the wild type in disk assays.

DISCUSSION

Sequence comparison revealed a gene cluster, characterized by a heavy metal sensor kinase gene (*cinS*), a response regulator gene (*cinR*), a gene encoding a copper protein of the plastocyanin/azurin family (*cinA*), and a gene encoding a pre-Q₀ reductase (*cinQ*), conserved in several pseudomonads, including *P. putida* KT2440 and *P. fluorescens* Pf-5 and PfO-1. *P. aeruginosa* PAO1 contains a putative operon that is similar to the *cin* operon, but with an additional gene, the negative regulator of type III secretion gene *ptrA* (PA2808), preceding *cinA* (PA2807). CinA exhibits significant sequence similarity (67%) to the *P. fluorescens* DF57 Cot protein, which is involved in copper tolerance (38). Moreover, transcriptional analysis has shown that *cinA* from *P. aeruginosa* PAO1 (PA2807) is the most upregulated gene in copper-adapted cells (2,376-fold induction) (36). Transcriptional activation by copper was also demonstrated for PA2806, PA2809, and PA2810 (corresponding to the *P. putida* KT2440 *cinQ*, *cinR*, and *cinS* genes, respectively). Disruption of PA2807 resulted in a slight increase in copper sensitivity in disk assays, while disruption of PA2809 caused significant copper sensitivity (36). Our results showed that disruption of PA2806 (*cinQ*) did not result in any increase in copper sensitivity in disk assays.

In this paper, we show that *cinA* and *cinQ* from *P. putida* KT2440 are strongly induced by the presence of Cu²⁺ but not by Cd²⁺, Zn²⁺, or Fe²⁺. We also provide evidence that *cinA* and *cinQ* are transcribed as a bicistronic mRNA. In contrast to the analyses of copper sensitivity of a *cinA* disruptant in *P. aeruginosa* PAO1 performed by us and by Teitzel et al. (36), deletion of *cinA* in *P. putida* KT2440, although resulting in a smaller colony size for cells grown on LB, did not lead to an increase in copper sensitivity in disk assays (data not shown). Gene disruption of *cinQ* in either *P. aeruginosa* PAO1 or *P. putida* KT2440 did not result in an increase in copper sensitivity in disk assays (not shown). The different phenotypes in response to copper challenges for *P. putida* and *P. aeruginosa* could be the result of redundancy in copper resistance genes in *P. putida*. *P. putida* KT2440 possesses multiple genes thought to be involved in copper homeostasis that appear to be redundant. Genome analysis (4) revealed two *copA* genes, encoding multicopper oxidases, two copper heavy metal two-component systems, a putative *cus* system, and two putative copper-transporting P-type ATPases.

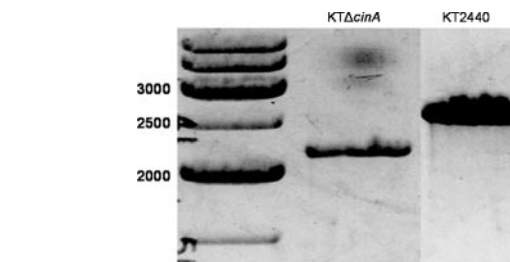
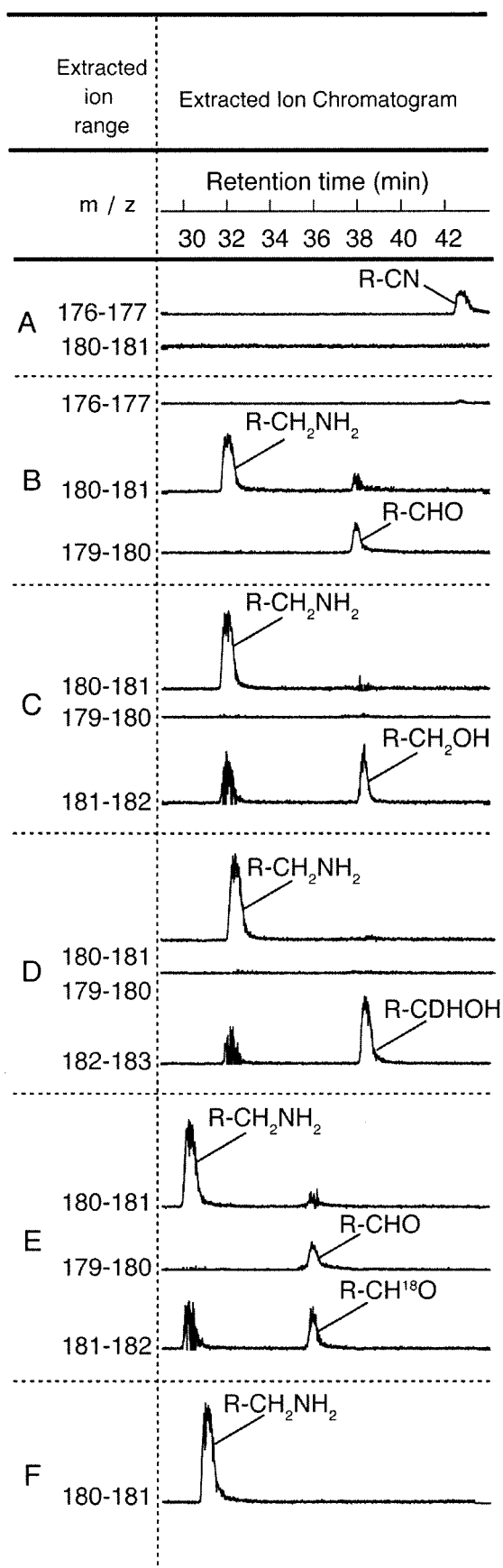


FIG. 9. Gene deletion of *cinA* in strain $KT\Delta cinA$. PCR amplification of genomic DNA was done using primers KTDPOD and KTDXUU, which are external to the DNA sequence used for the gene deletion of *cinA*. The expected size of the PCR product from the deletion strain was 2,200 bp, while the expected size of the PCR product from KT2440 (wild type) was 2,640 bp.

An indication of a possible role of CinA in conferring copper resistance comes from examining the promoter region of *cinA*. The promoter regions of proteins involved in copper homeostasis and resistance often contain a *cop* box, which is presumably the site of regulation of transcription in a copper-dependent fashion. *cop* boxes are found in promoters preceding *P. syringae copA* (24) and *E. coli cusC* (10) and *pcoA* (26). For *P. aeruginosa*, PA2808 (*ptrA*), which precedes *cinA*, has been reported to be induced by copper (14, 36) via a mechanism involving the two-component system *cinRS* (PA2809 and PA2810) and resulting in the repression of the type III secretion system (14).

cinA was upregulated by copper, and its gene product could be extracted from the periplasm after overexpression in *E. coli*. CinA contains a signal peptide, and CinA extracted from the periplasm had an estimated molecular mass of 19.1 kDa, corresponding to the cleaved mature peptide containing the C-terminal tag. CinA as a member of the plastocyanin/azurin family could function as an electron shuttle in the periplasm. The electrochemical potential was determined to be 456 ± 4 mV. However, members of this family of proteins usually have redox potentials that are lower than that found for CinA. For example, azurins and pseudoazurins have potentials of 260 to 310 and 260 to 275 mV, respectively, and the redox potentials of plastocyanins are ~ 375 mV. These proteins are believed to be involved in electron transfer to nitrous oxide reductase, another copper-containing enzyme (pseudoazurins), or in

FIG. 8. Extracted ion chromatograms for a set of six CinQ reactions. Reactions were conducted as described in Materials and Methods. The traces were obtained as follows: A, a control lacking CinQ; B, a reaction mix containing CinQ; C, the CinQ reaction treated with 10 mM $NaBH_4$; D, the CinQ reaction treated with 10 mM $NaBD_4$; E, a reaction mix containing CinQ run in 61% $H_2^{18}O$; and F, a *B. subtilis* QueF control reaction. Constituents resulting from chemistry on the cyano group of pre- Q_0 are indicated next to each representative peak. The R groups in the figure represent the 7-deazaguanine base (see Fig. 1). There is a slight inconsistency in retention times for products eluting in panels E and F, but UV spectra of the peaks (not shown) confirmed that each one represents expected compounds. The retention times in this experiment are internally consistent but different from those shown in Fig. 7 due to the presence of the MS detector, which substantially changes the backpressures that are observed, altering the retention times.

transfer between photosystems I and II (plastocyanin). Azurins are thought to be involved in anaerobic electron transfer in *P. aeruginosa* and are known to exchange electrons with cytochrome c_{551} . Rusticyanin, another member of this family, possesses a much higher redox potential (~680 mV) and is involved in iron oxidation (for reviews, see references 9, 13, 15, and 20).

CinA contains higher percentages of methionine (8%) and histidine (6%) residues than those of azurins and pseudoazurins. A single T1 “blue copper” metal binding site is predicted to be formed by the side chains of H131, C158, H163, and M168. Furthermore, CinA presents a series of methionine- and histidine-rich motifs similar to *P. syringae* CopA’s MXXMX HXXM motif. Methionine-rich regions can be found in many proteins involved in copper homeostasis, and it has been proposed that they might be involved in copper binding (5). Roberts et al. (32) suggested that although the methionine-rich region in CueO binds a labile Cu(II) molecule, this might not be the main role of this region, while Wernimont et al. (40) proposed that the role of the similar methionine-rich region in PcoC may be in protein recognition with PcoA.

Homologs of CinA can be found in other genomes, located in clusters containing other genes involved in copper homeostasis. Most CinA homologs are associated with a CopA multicopper oxidase, suggesting a possible role in electron transfer (Table 3). Genes encoding CinA can be found associated with *copA*, encoding putative multicopper oxidases; with *copB*, encoding putative outer membrane factors; and with two-component systems (*Roseovarius nubinhibens* ISM 334 and *Alcanivorax borkumensis* SK2) or without a two-component system (*Sulfitobacter* sp. strain NAS-14.1). In other cases, *cinA* can be found with *copA* and an ORF encoding a putative outer membrane protein of the TolC family (*Fulvamarina pelagi* HTCC2506, *Agrobacterium tumefaciens*, and *Sinorhizobium meliloti* 1021). In *Hahella chejuensis* KCTC 2396, *cinA* is found associated with a putative *cus* system. Finally, in *Xanthomonas campestris* pv. vesicatoria, *cinA* is associated with the multicopper oxidase, a TolC-like outer membrane protein, and a two-component heavy metal sensor kinase and response regulator. Basim et al. (1) reported that for *X. campestris* pv. vesicatoria, disruption of *cinA* (ORF5) resulted in a partial loss of copper resistance, while disruption of the sensor kinase gene (ORF1), a multicopper oxidase gene (ORF4), or the TolC family outer membrane factor gene (ORF3) had a much greater effect. These results suggest an as yet undefined accessory role for CinA homologs in copper homeostasis. However, the finding that CinA homologs are most often associated with a CopA multicopper oxidase suggests a possible role in electron transfer.

In this report, we demonstrate that contrary to its database annotation, *P. putida* CinQ is an NADPH-dependent pre- Q_0 reductase, not a GTP cyclohydrolase I. The activity of this protein is similar to that of the *B. subtilis* QueF protein, whose gene is colocalized in a cluster of genes involved in the biosynthesis of the hypermodified tRNA base queuosine (29, 39). Queuosine is found in the wobble position of several tRNAs and is known to prevent the readthrough of the stop codon UAG by tRNA^{Tyr} (2, 11), but it does not seem to have a major influence on the translational efficiency of tRNA (7). A lack of queuosine was also found to strongly reduce the virulence of *Shigella flexneri*, possibly altering the efficiency of translation

TABLE 3. CinA homologues in selected genomes

Bacterium	ORF ^a	NCBI accession no.
<i>Roseovarius nubinhibens</i> ISM 334	CinA	ZP_00958990
	CopA	ZP_00958992.1
	CopB	ZP_00958993.1
	CopR	ZP_00958995.1
	CopS	ZP_00958994.1
<i>Alcanivorax borkumensis</i> SK2	CinA	YP_693084
	CopA	YP_693083.1
	CopB	YP_693082.1
	CopR	YP_693085.1
	CopS	YP_693086.1
<i>Sulfitobacter</i> sp. strain NAS-14.1	CinA	ZP_00964732
	CopA	ZP_00964730.1
	CopB	ZP_00964731.1
	ORF4	ZP_00964733.1
	CinA	ZP_00963604
	CopA	ZP_00963605.1
<i>Fulvamarina pelagi</i> HTCC2506	TolC	ZP_00963606.1
	CinA	ZP_01440306
	CopA	ZP_01440305.1
	TolC	ZP_01440304.1
	CinA	ZP_01440770
	CopA	ZP_01440771.1
	TolC	ZP_01440772.1
<i>Agrobacterium tumefaciens</i> strain C58	CinA	NP_534476.1
	CopA	NP_534477.1
	TolC	NP_534478.1
<i>Sinorhizobium meliloti</i> 1021	CinA	NP_384693
	CopA	NP_384692.1
	TolC	NP_384691
	CinA	NP_435810.1
	CopA	NP_435809.1
	TolC	NP_435808.1
<i>Hahella chejuensis</i> KCTC 2396	CinA	YP_434213
	CusA	YP_434212.1
	CusB	YP_434211.1
	CusC	YP_434210.1
<i>Xanthomonas campestris</i> pv. vesicatoria	CinA	AAP42068.1
	CopA	AAP42069
	TolC	AAP42070
	CopR	AAP42072
	CopS	AAP42071

^a CinA homologues and adjacent ORFs potentially involved in copper homeostasis in selected genomes.

of the *virF*-encoded transcription activator of the AraC family (7). In contrast, *E. coli* mutants lacking queuosine exhibit reduced survival in stationary phase when competing with the isogenic wild-type strain (28) but are otherwise indistinguishable from the wild type.

While conversion of pre- Q_0 to pre- Q_1 was readily observed with CinQ, analysis of the reactions revealed the formation of a second product as well. Using LC-MS analysis and carrying out the reaction in ¹⁸O-labeled water or reducing the product mixture with NaBH₄ or NaBD₄, the product was assigned tentatively as a 7-deazaguanine-7-aldehyde. The pyridine nucleotide-dependent reduction of pre- Q_0 presumably occurs in two half-reactions, one where the nitrile is reduced to an imine

and the second where the imine is reduced to the amine of pre-Q₁. Escape of the imine from the active site into bulk solvent could lead to conversion to the observed aldehyde. Recent homology modeling (35) suggested that *B. subtilis* QueF is a homodimeric protein with active sites located at intersubunit interfaces. It is possible that the affinity tag that was used in our studies to facilitate purification of CinQ interferes with the native quaternary structure of this protein and leads to the diffusion of intermediates from the active site.

A link between copper and tRNA modification is novel. The presence of CinQ in a copper-induced system was found only for pseudomonads. CinQ is upregulated sevenfold in *P. aeruginosa* PAO1 in copper-adapted cells (36). Several lines of evidence prompted us to hypothesize a possible role for CinQ as a regulator of biofilm formation. Environmental stresses are known to induce the expression of resistance factors. Copper enhances the production of the exopolysaccharide (EPS) alginate in *P. syringae* (17), and EPSs are known to contribute to metal resistance by a mechanism involving chelation (25). Kazy et al. (16) also reported that a copper-resistant strain of *P. aeruginosa* was found to adsorb more copper in its EPS than a copper-sensitive strain did. Furthermore, a homolog of pre-Q₀ reductase (*cinQ*) from *P. chlororaphis* PCL1391 (*ippA*; NCBI accession number DQ339483) was reported to induce phenazine production. Phenazines were recently shown to be involved in biofilm formation by *Pseudomonas chlororaphis* (*aureofaciens*) strain 30-84 (19). The expression of a pre-Q₀ reductase gene during copper stress could therefore be linked to copper tolerance of the organism; however, a more precise link between tRNA modification and copper must await more detailed analysis of the physiological role(s) of queuosine.

Further studies regarding the roles of *cinA* and *cinQ* in biofilm formation, copper resistance, and copper accumulation in the biofilm matrix are currently in progress in our laboratory.

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