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CueO Is a Multi-copper Oxidase That Confers Copper Tolerance in *Escherichia coli*

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The putative multi-copper oxidase CueO had previously been implicated in intrinsic copper resistance in *Escherichia coli.* In this report we showed that the presence of CueO in the periplasm protected alkaline phosphatase from copper-induced damage. CueO contained four copper atoms per molecule and displayed spectroscopic properties typical of blue copper oxidases. CueO catalyzed the oxidation of *p*-phenylenediamine (*p*PD), 2,6-dimethoxyphenol (DMP) and exhibited ferroxidase activity *in vitro.*

Key Words: **copper; *Escherichia coli;* multi-copper oxidase; metal homeostasis; laccase; periplasm.**

Copper is a requirement for aerobic metabolism but can be toxic even at low concentrations. Therefore cells have developed homeostatic mechanisms to ensure proper handling of copper (1). In *Escherichia coli* copper induces the expression of *cueO*, encoding a putative multi-copper oxidase (2, 3). A disruption of *cueO* renders cells more copper sensitive (4). Other putative multi-copper oxidases involved in copper resistance include PcoA encoded by the plasmid-borne copper resistance operon *pco* in *E. coli* (5) and CopA of the *cop* operons in *Pseudomonas syringae* (6) and *Ralstonia metallidurans.* PcoA and CopA are largely identical to each other and probably have a similar function. The degree of similarity between CueO and PcoA or CopA is much lower, suggesting they are only distantly related. Multi-copper oxidases couple the one-electron oxidation of substrate(s) to full reduction of molecular oxygen to water by employing a functional unit formed by three types of copper binding sites with different spectroscopic and functional properties (7). Type 1 blue copper (T1) is the primary electron acceptor from the substrate, while a trinuclear cluster formed by type 2 copper and binuclear type 3 copper (T2/T3) is the oxygen binding and reduction site. Prominent members are mammalian ceruloplasmin, plant ascorbate oxidases and fungal laccases. Bacterial members include PcoA, CueO and CopA, which are involved in copper resistance, CumA, which is responsible for manganese oxidation in *Pseudomonas aeruginosa* and PpoA from *Marinomonas mediterranea* with unclear physiological function (5, 6, 8–10). While there have been extensive studies conducted with eukaryotic multi-copper oxidases, bacterial multi-copper oxidase have only recently been characterized (10) and none was purified prior to this report.

In this report we show that the purified multi-copper oxidase CueO contained four copper atoms per molecule and displayed spectroscopic properties typical of blue copper oxidases, suggesting the presence of one each of the different copper sites. CueO protected alkaline phosphatase from copper-induced toxic reactions. Thus, one possible biological function of CueO might be the periplasmic detoxification of copper. CueO not only catalyzed the oxidation of *para*-phenylenediamine (*p*PD), 2,6-dimethoxyphenol (DMP) and other common substrates of laccases but also exhibited ferroxidase activity. These properties make CueO an attractive model system to study bacterial multi-copper oxidases.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth media. Strains used in this work are *E. coli* DH5α, BL21, BL21 pCueO, GR1Δ*cueO*, GR1 Δ*cueO* (pCueO), GR10 Δ*cueO* Δ*cusCFBA*, GR10 Δ*cueO* Δ*cusCFBA* (pCueO), GR1 Δ*cueO* (pCueO $C_{500}S$, $H_{501}R$), GR10 Δ*cueO* Δ*cusCFBA* (pCueO $C_{500}S$, $H_{501}R$) and W3110 (3). *E. coli* was grown in Luria Bertani (LB) medium or Tris-buffered mineral salts medium (11) containing 2 ml glycerol and 1 g yeast extract per liter. Antibiotics [ampicillin (100 μg/ml), chloramphenicol (15–20 μg/ml), kanamycin (25 μg/ml)] or $CuCl_2$ were added where appropriate.

CueO cloning and expression. Genomic DNA from *E. coli* W3110 was employed to amplify *cueO* by PCR with the Expand High Fidelity PCR system (Roche) using the following primers: 5′-GAA-GAATTC$_{364}$ATGCAACGTCGTGATTTCTTAAAAT-3′ and 5′-GTC-CTGCAG$_{1887}$TACCGTAAACCCTAACATCATCCCC-3′. The PCR product was purified, cut and cloned into plasmid pASK-IBA3 (IBA, Göttingen, Germany) via the *Eco*RI and *Pst*I restriction sites and

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transformed into *E. coli* DH5α. The resulting plasmid pCueO expressed *cueO* as a C-terminal fusion protein with the Strep-TagII epitope (SAWSHPNFEK) which shows high binding affinity towards streptactin, plus eight additional residues at the N-terminus (GDRGPEF). *CueO* was expressed in *E. coli* strain BL21 (Stratagene). Overnight cultures were diluted 1:100 into fresh LB medium and grown at 37°C to an OD_{600} of approximately 0.7. Cells were allowed to cool down to RT and expression of *cueO* was then induced by addition of anhydrotetracycline (200 μg/liter, Sigma-Genosys) for 3 h. Cells were then chilled on ice, harvested (5000 rpm, 4°C, 10 min) and stored at −20°C until further use.

Plasmid pCueO $C_{500}S$, $H_{501}R$ coding for a CueO $C_{500}S$, $H_{501}R$ mutant protein was constructed in a PCR reaction using *cueO* cloned into pGEM T-Easy as a template. Mutagenesis was performed taking advantage of a *Xba*I-site introduced by the primers 5′-CACTCTAGACTGCTGGAGCATGAAGATACGGG-3′ and 5′-CAGTCTAGAGTGCGCCATATAAGCATGTTCTTT-3′. The resulting PCR product was digested with *Xba*I for religation and *Dpn*I destroying the template plasmid. The fragment containing the mutated *cueO* gene was subcloned into pASK-IBA3.

Alkaline phosphatase assays and immunoblotting of PhoA. To monitor alkaline phosphatase activity cells grown overnight in LB were diluted 1:500 into Tris minimal medium (11) supplemented with 0.2% glycerol, 0.1% yeast extract but not containing phosphate and grown overnight. A 3% inoculum of stationary cells were then diluted into fresh phosphate-deficient minimal medium with or without 5 μM $CuCl_2$, $ZnCl_2$ or both and excessively aerated for 6 h. Alkaline phosphatase (AP) activity was determined in a permeabilized whole-cell assay as previously described (12). In short, reactions were carried out at 22°C, initiated by the addition of *para*-nitro phenol phosphate (final concentration, 0.04%), to a reaction mixture of 100 μl of *E. coli* cultures and 900 μl of reaction buffer (1 M Tris–HCl [pH 8.0] at 25°C), permeabilized by addition of hexadecyl trimethylammonium bromide (CTAB) (final concentration, 0.005%), and vortexed for 5 s. The reaction was stopped with KH_2PO_4 (final concentration, 0.1 M). Specific activity of alkaline phosphatase was calculated using the formula $10^4 \times [OD_{420} - 1.75\ OD_{520}]/[\text{time} \times OD_{600}]$, where OD_{520} and OD_{420} are the absorbances of the reaction mixture at 420 and 520 nm, respectively (1 cm path length); and time is the duration (in minutes) of the reaction.

Aliquots of cells used for determination of PhoA activity were separated on polyacrylamid gel electrophoresis and blotted onto PVDF membrane. Alkaline phosphatase was detected with an anti-PhoA antibody conjugated with horseradish-peroxidase (Rockland, Gilbertsville, PA) and visualized using the chromogen 4-chloro-1-naphthol (Sigma).

β-Galactosidase assays and immunoblotting of LacZ. Cells were grown as described for alkaline phosphatase assays but minimal medium was supplemented with phosphate and IPTG (1 mM). β-Galactosidase activity was determined in a permeabilized whole-cell assay as previously described (13). 200 μl of cells were centrifuged and resuspended in 950 μl buffer Z (0.6 M Na_2HPO_4, 0.4 M NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.5 M mercaptoethanol). Cells were permeabilized by addition of 50 μl permeabilization solution (2.5 g/liter hexadecyl-triammonium bromide [CTAB], 5 g/liter sodium desoxycholate). Samples were vortexed for 10 s and incubated at RT for 10 min. The reaction was started by addition of *o*-nitrophenyl-β-D-galactopyranoside (ONPG, 100 μl of a 4 mg/ml stock solution in buffer Z). Reactions were carried out at 30°C for 5 min and stopped by addition of 500 μl of 1 M $NaCO_3$. Specific activity of β-galactosidase was calculated as Miller units (14). Western blotting was performed as described for alkaline phosphatase; however, peroxidase conjugated anti-β-galactosidase antibodies (Rockland, Gilbertsville, PA) were used.

CueO purification. Cell pellets were resuspended in 15 ml buffer W (100 mM Tris pH 8.0) and lysed by French press. After centrifugation (14000 rpm, 15 min, 4°C) the supernatant was applied to a strepactin column (1 ml/400 ml culture; Sigma-Genosys) equilibrated with the same buffer. The column was washed excessively with buffer W. CueO was eluted in aliquots of 1 ml buffer E (buffer W + 5 mM desthiobiotin, Sigma). To load CueO with copper, $CuCl_2$ (0.5 mM) was added to the crude extract and to the first 10 ml of wash buffer. Mutant protein CueO $C_{500}S$, $H_{501}R$ was purified accordingly.

CueO localization. CueO purified from periplasmic extract was isolated as previously described (15). Freshly prepared cells of *E. coli* BL21 (pCueO) (100 ml) were centrifuged and suspended in 1 ml of ice-cold buffer P (100 mM Tris/HCl, pH 8.0, 500 mM sucrose) at 4°C and incubated on ice for 30 min. Spheroplasts were removed by centrifugation at 14,000 rpm for 15 min at 4°C. The periplasmic extract was used to purify the CueO protein as described above.

Oxidase assays. For histochemical assays 10 μg of CueO were run onto 10% native PAGE gels. The gels were incubated in 3 mM *p*-phenylenediamine dihydrochloride (Sigma) dissolved in 100 mM sodium acetate buffer (pH 5.7) at room temperature (16) until bands of oxidized *p*PD were visible. Likewise a parallel gel was run, but 1 mM sodium azide was added to inhibit oxidase activity of CueO.

Ferroxidase assays were performed by altering the *p*PD oxidase assay protocol (17). 10 μg of CueO were run as described above in a native 10% PAGE gel, but the gel was then incubated for 1 h in freshly prepared 200 μM ferrous ammonium sulfate in 100 mM sodium acetate buffer (pH 5.7). The gel was then rinsed briefly with water to remove excess ferrous iron and overlaid with 15 mM ferrozine (Sigma).

For solution assays purified CueO (250 nM per assay) was diluted in 1 ml of 0.1 M sodium phosphate buffer (pH 6.5) with 250 μM $CuCl_2$. To start the reaction 2 mM substrate (*para*-phenylenediamine [*p*PD], 2,6-dimethoxyphenol [DMP] or 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) [ABTS]) was added. As controls heat inactivated CueO (2 min, 97°C) and buffer without protein were used. Likewise the mutant CueO $C_{500}S$, $H_{501}R$ protein was examined. Inhibition by azide was determined by supplementing the buffer with 1 mM or 10 mM (final concentration) sodium azide. Absorbance was measured at 468 nm for DMP, 570 nm for *p*PD and 436 nm for ABTS. Oxidation rates were determined using the extinction coefficients $\epsilon_{DMP} = 14800\ M^{-1}\ cm^{-1}$, $\epsilon_{ABTS} = 29300\ M^{-1}\ cm^{-1}$.

Analytical methods. CueO was loaded after purification by addition of 0.5 mM $CuCl_2$ and subsequent dialysis of unbound copper. Copper content of loaded CueO and pCueO $C_{500}S$, $H_{501}R$ was determined by atomic absorption spectroscopy on a T.J.A. ICP/AES inductively coupled plasma atomic emission spectrometer. Absorbance spectra were recorded on a Varian Cary 300 at room temperature.

Miscellaneous. Standard molecular genetic techniques were used (Sambrook *et al.*, 1989). PCR was performed in the presence of *Pwo* or *Taq* DNA polymerase (Roche, Qiagen). Native or SDS-PAGE was carried out as described (18). Protein bands were visualized either by Coomassie or silver staining. CueO was concentrated where applicable using Midi Centrifuge Filters (Nalgene). Protein concentration of purified CueO was determined at 280 nm ($\epsilon_{CueO} = 63063\ M^{-1}\ cm^{-1}$).

RESULTS

CueO protects alkaline phosphatase against copper-induced damage. Previously, CueO had been implicated in copper tolerance in *E. coli*. Sequence analysis suggested that CueO is a periplasmic protein possessing a leader sequence with a twin-arginine motif. To evaluate the physiological role of CueO in the periplasm we tested if CueO is able to protect periplasmic enzymes from copper stress. Activity of alkaline phosphatase was examined because it can be easily detected and assayed. Cells were challenged with a sublethal concentration of Cu(II) that did not signifi-

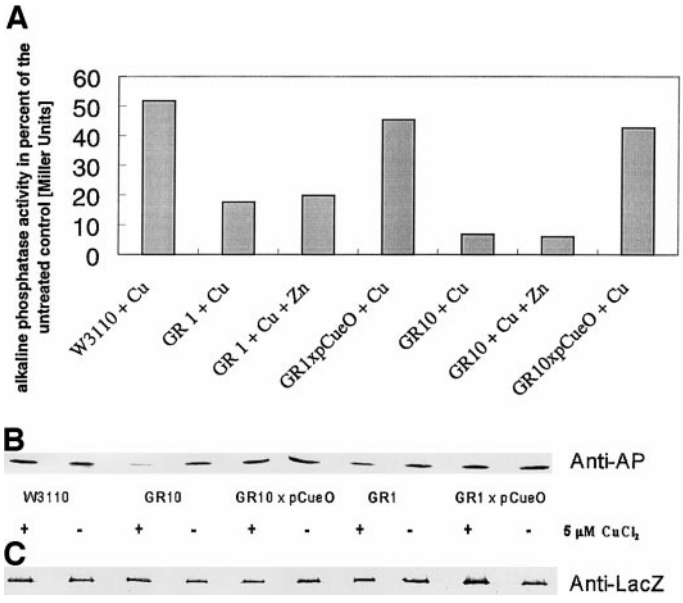

FIG. 1. AP activity and detection of AP and LacZ in the presence of copper. Overnight cultures of *E. coli* were diluted 1:500 into fresh minimal medium with 0.2% glycerol and 0.1% yeast extract without phosphate and grown overnight. To induce alkaline phosphatase under phosphate depleted conditions, cultures were diluted 1:500 into fresh medium without phosphate containing no added metal or 5 μM $CuCl_2$ with or without 5 μM $ZnCl_2$ and incubated at 37°C for 6 h with high aeration. (A) Alkaline phosphatase activity was determined (12). Shown are percentages of PhoA activity in cultures incubated with copper compared to the unchallenged control. Experiments were done in triplicate and an average calculated. (B) For PhoA immunoblotting samples (25 μg dry weight/lane) used for AP assays were separated on a 12.5% PAGE gel and blotted onto a PVDF membrane. Horseradish peroxidase-conjugated anti-PhoA antibody was used (diluted 1:1000) to detect PhoA and bands were visualized using the chromogenic substrate 4-chloro-1-naphthol. (C) For LacZ immunoblotting cells were grown as described for AP but the growth medium contained phosphate and 1 mM IPTG. Horseradish peroxidase-conjugated anti-β-galactosidase antibodies (diluted 1:1000) were employed to detect LacZ.

cantly affect growth in minimal medium. In all strains tested, growth in the presence of 5 μM Cu(II) led to a decrease in alkaline phosphatase activity when compared to growth with no added copper. However, compared to the wildtype the decrease in alkaline phosphatase activity was significantly higher in strain GR1 (Δ*cueO*) and even more pronounced in strain GR10 where both *cueO* and *cusCFBA* are deleted (Fig. 1A). The Cus system had previously been shown to be involved in copper extrusion (3, 4). Expression of *cueO* on a plasmid in both strains GR1 (pCueO) or GR10 (pCueO) led to a reduction of copper-mediated toxicity since alkaline phosphatase activity was almost restored back to wildtype level (Fig. 1A). This shows that the presence of CueO is linked to a higher AP activity in copper containing medium and indicated CueO can protect AP from copper-induced damage.

To test if this decline in AP activity is due to competition of Cu(II) and Zn(II) ions for the catalytic Zn(II) binding site in alkaline phosphatase, *E. coli* strains GR1 (Δ*cueO*) and GR10 (Δ*cueO, cusCFBA*) were grown in the presence of Cu(II) and Zn(II) at equimolar concentrations (5 μM). However, the addition of zinc did not result in an increased AP activity compared to cells grown in medium containing only copper (Fig. 1A). This indicated that the reduced AP activity is not due to displacement of zinc by copper ions. Additionally, increasing concentrations of $CuCl_2$ were added 15 min before measuring AP activity to determine if copper is toxic to the enzyme. However, addition of copper did not change AP activity in all strains tested, regardless if CueO was present or not (data not shown). This clearly indicated that the specific effect of copper did not influence AP activity per se but rather affected biogenesis or stability of AP. To test this hypothesis, immunoblotting of the strains used in AP assays was performed using a PhoA specific antibody. Figure 1B clearly shows that in the presence of sublethal concentrations of copper the amount of immunodetectable AP is dramatically decreased in strain GR10 and to a lesser extend in GR1 and this effect can only be reverted when CueO is expressed on a plasmid *in trans* (Fig. 1B). This decreased concentration of AP might be the result of improper folding of AP leading to an inactive enzyme that is readily degraded by periplasmic proteases such as DegP.

In contrast to the periplasmic enzyme alkaline phosphatase, addition of copper to the growth medium did not affect the amount of immunodetectable, cytoplasmic β-galactosidase (Fig. 3C). Additionally, copper did not reduce β-galactosidase activity in CueO-deficient strains when compared to wild-type strain *E. coli* W3110 (data not shown).

CueO is a periplasmic protein. CueO was expressed in *E. coli* under the highly inducible *tet*-promoter and subsequently purified on a streptactin column. The protein could be visualized in a denaturating PAGE gel (Fig. 2A). The amino acid sequence of CueO contains a leader sequence with a twin arginine consensus motif for export into the periplasm via the Tat-pathway (2). When CueO was isolated from the periplasmic extract following osmotic shock, the processed form became more predominant, consistent with a putative $A_{16}SA$ leader peptidase cleavage motif (A/GXA) at position 16 in CueO (Fig. 2B). $CuCl_2$ was added during the first steps of the purification process since the amount of copper in the medium was not sufficient to ensure complete loading of copper. After copper addition the elution process of CueO could be visually observed: a blue protein band eluted from the column. The protein solution was of blue color due to the type 1 "blue" copper center, as described for other multi-copper oxidases. However, for CueO a low 280/610 nm ratio was calculated (14.1) as compared to other multi-copper oxidases like Fet3 (21.5) and ceruloplasmin (22) (19,

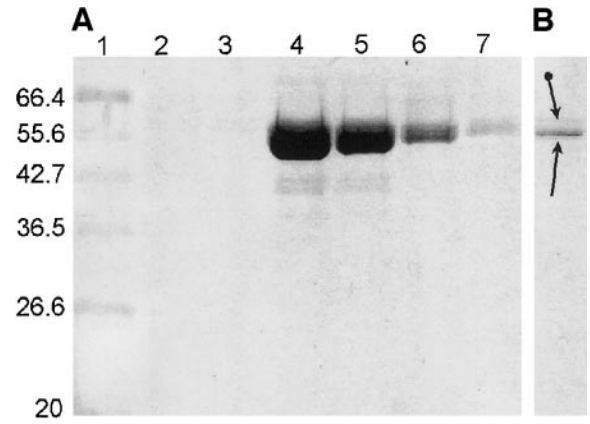

FIG. 2. SDS–PAGE analysis of CueO. Typical elution profile of purified CueO. CueO was size fractionated on a 12.5% acrylamide gel prior to staining with Coomassie brilliant blue 250. (A) Broad range marker (NEB), lane 1; CueO eluates 4 to 7 (10 μl each, lane 4 corresponds to 6 μg CueO protein). (B) Purified CueO protein from periplasmic extract. Arrow denotes the processed form of CueO, tailed arrow pre-CueO protein.

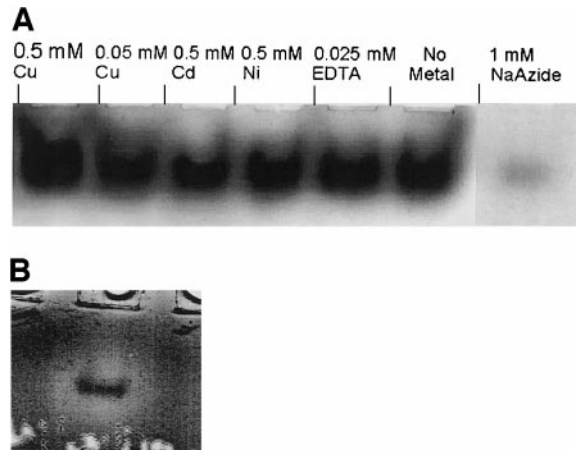

FIG. 4. Oxidase activity of CueO protein. 5 μg of CueO was run onto 10% native PAGE gels. (A) *p*PD oxidase assay. The gels were incubated in a *p*PD/sodium acetate solution without (A) or with 1 mM sodium azide and developed for 30 min. (B) Ferroxidase assay. The gel was soaked in a ferrous ammonium sulfate/sodium acetate solution and incubated for 4 h. The Fe(II) chelator Ferrozine was then applied on the gel. Ferroxidase activity leads to generation of Fe(III), which is not bound by ferrozine and thus not forming a dark purple complex.

20). CueO appeared to be a monomer, as judged from its size on a native PAGE gel (data not shown).

Copper content and spectroscopic properties. Typically multi-copper oxidases such as Fet3 from *Saccharomyces cerevisiae* or ascorbate oxidase from *Curcurbita pepo* contain four copper ions: two type 3 coppers, one type 2 and one type 1 ("blue") copper center. Based on the protein concentration of the samples used for AAS analysis and the molecular mass value, the ratio of copper atoms/molecule of CueO was calculated to be 3.94 $\pm$ 0.05. This ratio of approximately 4 copper atoms/molecule of CueO is consistent with the presumption that CueO belongs to the classical multi-copper oxidases. The UV/visible spectrum of CueO showed a maximum near 610 nm that is a common feature of multi-copper oxidases (Fig. 3). CueO contained the single type 1 copper site characteristic of these multinuclear copper proteins. A type 3 binuclear Cu(II) site in CueO was indicated by the transition at ~330 nm (Fig. 3). The additional peak at 410 nm may be due to a residual cytochrome present. The molar extinction coefficient at 610 nm was determined to be 4500 mol^{-1} cm^{-1}.

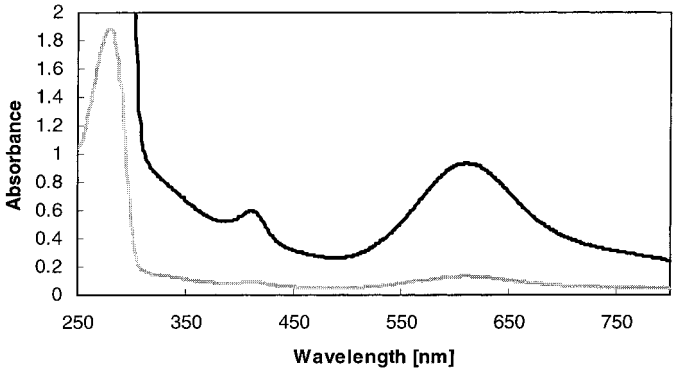

FIG. 3. UV/visible spectrum of CueO. The UV/visible spectrum of CueO was recorded on a Varian Cary 300 at room temperature. The CueO concentration was 500 μM (black line) or 30 μM (gray line) in Tris/HCl, pH 8.0, buffer.

CueO possesses p-phenylenediamine oxidase activity. *p*PD oxidase activity of purified CueO was examined to determine whether CueO has properties comparable to other related multi-copper oxidases such as Fet3 or laccases. We first tested if purified CueO oxidized *p*PD in a histochemical assay (Fig. 4A). Addition of $CuCl_2$ led to a significant increase of *p*PD oxidase activity while the UV/Vis spectrum of CueO was not altered (data not shown). Addition of EDTA or of other metals such as cadmium and nickel only led to an insignificant decrease in activity. However, the addition of sodium azide (an inhibitor of the electron transfer) to the assay led to a drastic decrease of *p*PD oxidase activity (Fig. 4A).

Ferroxidase activity of CueO. Related multi-copper oxidases such as Fet3 from *Saccharomyces cerevisiae* possess ferroxidase activity and are involved in high affinity iron uptake. To determine whether CueO also mediates oxidation of Fe(II) to Fe(III), ferroxidase assays were performed in a gel-based assay similar to the *p*PD oxidase activity assays. Whereas oxidase activity in *p*PD assays led to a darkening of the area where *p*PD is oxidized, the opposite can be observed in ferroxidase assays. The ferroxidase activity prevents formation of a dark purple Fe(II)–ferrozine complex by

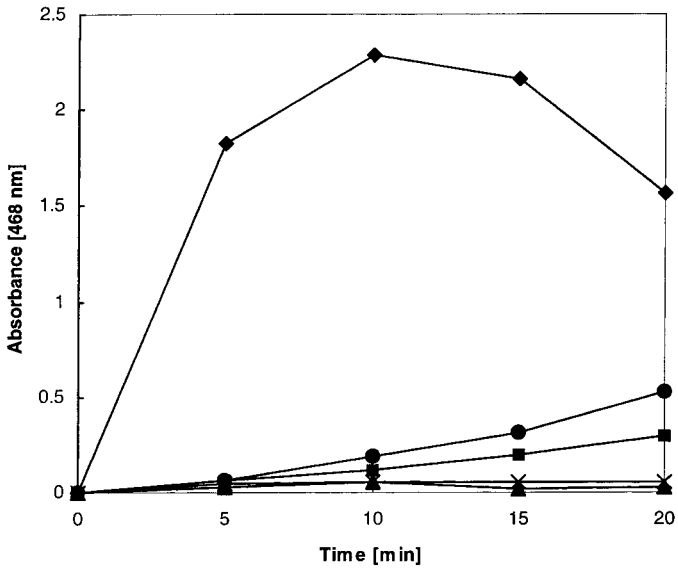

FIG. 5. Time-dependent oxidation of DMP by CueO and inhibition by azide. Oxidation of DMP in the presence and absence of sodium azide was examined over time. Each reaction was performed in 1 ml of 0.1 M sodium phosphate (pH 6.5) containing 250 nM CueO protein and 250 μM $CuCl_2$. Reactions were started by addition of DMP (2 mM final concentration) and the absorbance was recorded at 468 nm at the indicated times. (|) reaction was carried out without addition of sodium azide, (●) 1 mM or (■) 10 mM sodium azide, (▲) heat-inactivated CueO protein, and buffer only (×). A representative experiment is shown.

oxidation of Fe(II) to Fe(III), which is not chelated by ferrozine. We could show that purified CueO exhibited ferroxidase activity (Fig. 4B). In a native PAGE gel the area adjacent CueO remained transparent whereas all other areas of the gel turned purple. Thus, CueO is the first bacterial enzyme with this property to be identified.

Copper is essential for CueO-dependent oxidation of DMP and ABTS. To examine CueO-dependent oxidation of typical substrates of laccases, solution based assays were employed and the increase at absorbance at 468 nm for DMP and 436 nm for ABTS observed. CueO oxidized DMP and ABTS only in the presence of copper. The rate of oxidation was 324 mM min^{-1} mM $CueO^{-1}$ for DMP and 5.2 mM min^{-1} mM $CueO^{-1}$ for ABTS at RT. Without copper no detectable oxidation of DMP and a reduced rate of oxidation of ABTS (1.84 mM min^{-1} mM $CueO^{-1}$) were observed. To further characterize CueO-dependent enzymatic activity, oxidation of DMP was followed over time (Fig. 5). Azide inhibited DMP oxidation in a concentration dependent manner. The decrease in absorbance in the reaction containing DMP and copper was due to the formation of a dark insoluble "needle-like" polymerization product. This polymerization was also observed with 1,8-diaminonaphthaline (data not shown).

Copper centers in CueO are necessary for enzymatic activity and are indispensable in copper resistance. It was previously speculated that the function of related multi-copper oxidases such as PcoA involved in copper resistance is the binding of copper in the cytoplasm and subsequent transport of the copper bound protein into the periplasm (6). To determine if copper binding and subsequent transport is the resistance mechanism of CueO, a mutant CueO $C_{500}S$, $H_{501}R$ was generated where two copper centers were altered (Table 1). The properties of this mutant were examined. Growth experiments showed that the mutated CueO protein was not able to confer copper resistance in a *cueO* deletion strain (Fig. 6), indicating CueO has to be enzymatically active in order to protect cells from copper-mediated toxicity. Therefore, the function of CueO is not that of a "Cu-shuttle" from the cytoplasm to the periplasm. After overexpression and purification under the same conditions the yield was comparable for the unaltered and the mutated *cueO* ($C_{500}R$, $H_{501}R$) gene product. However, the mutant protein lacked the blue color due to the disruption of the "blue copper" C_{500} residue. CueO $C_{500}S$, $H_{501}R$ only had a residual DMP oxidase activity (3.68 mM min^{-1} mM $CueO^{-1}$) as compared to the wild-type CueO enzyme (324 mM min^{-1} mM $CueO^{-1}$). The mutated CueO protein had a residual copper content of 0.43 copper atoms/molecule of CueO $C_{500}S$, $H_{501}R$ as calculated by AAS.

DISCUSSION

In this report we demonstrated that CueO from *E. coli* is a periplasmic multi-copper oxidase that can protect periplasmic enzymes such as alkaline phosphatase from copper-induced damage. CueO is one of the first purified bacterial enzyme that showed typical spectroscopic properties and enzymatic activities of

TABLE 1

Sequence Alignment between CueO, PcoA, CopA (*Ralstonia metallidurans*), PpoA (*Marinomonas mediterranea*), Fet3 (yeast), and Lcc1 *Trametes versicolor*

Laccase	Sequence alignment[a]												
CueO	^{499}H	**C**	**H**	L	L	E	H	E	D	T	G	M	M^{511}
PcoA	^{586}H	C	H	L	L	Y	H	M	E	M	G	M	F^{598}
CopA	^{596}H	C	H	L	M	L	H	M	D	A	G	M	F^{608}
PpoA	^{656}H	C	H	I	L	D	H	E	D	Q	G	M	M^{667}
Fet3	^{483}H	C	H	I	E	W	H	L	L	Q	G	L	G^{495}
Lcc1	^{472}H	C	H	I	D	F	H	L	D	A	G	F	A^{484}
	[b]	[c]	[b]				[c]					[c]	

Note. Accession numbers: CueO (AAC73234), PcoA (Q47452), CopA (CAC07979), PpoA (AAF75831), Fet3 (P38993), Lcc1 (AAC49828).

[a] Bold letters represent the mutated residues of this study.

[b] Ligand to Type 3 Cu.

[c] Ligand to Type 1 Cu.

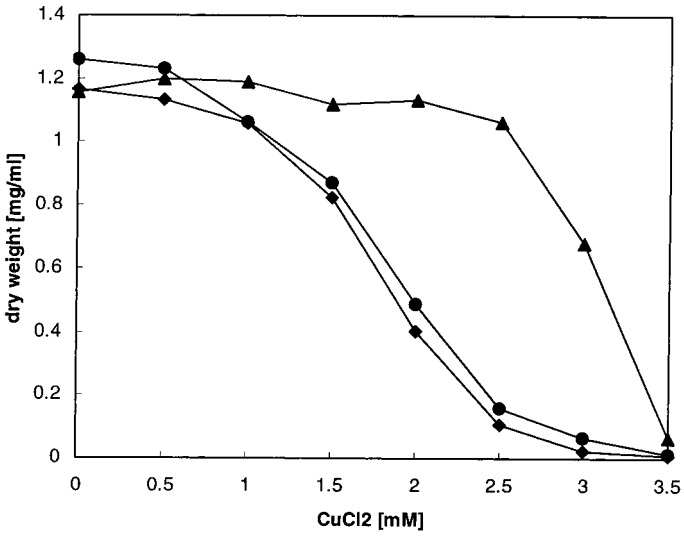

FIG. 6. Growth of different strains with increasing $CuCl_2$ concentrations. Overnight cultures were diluted 1:500 into fresh LB medium with indicated concentrations of $CuCl_2$. Cell growth was monitored as mg/ml dry weight after 15 h incubation at 37°C with shaking. *E. coli* GR10 (Δ*cueO* Δ*cusCFBA*) (●), *E. coli* GR10 (pCueO) (▲), *E. coli* GR10 (pCueO $C_{500}S$, $H_{501}R$) (|). The averages of three independent experiments are shown.

classical multi-copper oxidases. CueO appears to be a monomer containing 4 copper atoms per molecule. Recently, Dean and colleagues also purified and characterized CueO (YacK) (21). Their enzymatic characterization is in qualitative agreement with our findings. However, they determined that CueO contained 6 or 4.5 copper atoms per molecule of CueO depending on the protein assay employed (21). Another bacterial putative multi-copper oxidase from *Marinomonas mediterranea* has been cloned and expressed in *E. coli.* Recombinant expression indicated that this enzyme possesses both laccase-like and tyrosinase-like activities (10, 22). It could be demonstrated that CueO not only possesses laccase-like (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) activity and oxidized substrates such as *p*PD, DMP and ABTS but also exhibited ferroxidase activity. Multi-copper oxidases that have been extensively characterized include Fet3 from *S. cerevisiae,* mammalian ceruloplasmin, plant ascorbate oxidases and fungal laccases (7, 16). Multi-copper oxidases were shown to oxidize a variety of substrates but so far only Fet3 and ceruloplasmin exhibited ferroxidase activity and both have been implicated in iron transport. However, CueO does not appear to be involved in iron uptake. Recently, we demonstrated involvement of CueO in copper tolerance in *E. coli* (4). The expression of *cueO* is regulated by CueR, a copper-activated regulator belonging to the MerR family of metal-responsive DNA-binding activators (2). CueR also regulates expression of the Cu(I)-translocating P-type ATPase CopA (2, 23, 24). In analogy to Fet3, CueO might confer copper tolerance by oxidizing Cu(I) to Cu(II). This might prevent the Fenton-like reaction causing oxidative damage in the periplasm. The P-type ATPase CopA was shown to extrude only Cu(I) not Cu(II) (22). Additionally, Cu(II) might be reduced to Cu(I) by proteins in the periplasm. Alternatively, CueO could be involved in the production of polyphenolic compounds in the periplasm. Polyphenols have been shown to be able to protect cells from oxidative damage by scavenging radicals (25). Laccases in the fungus *Pleurotus ostreatus* maybe have a similar function in protection against copper-mediated toxicity (26).

Other putative multi-copper oxidases implicated in copper resistance include PcoA, CopA (*P. syringae*) and CopA (*R. metallidurans*), which are encoded by genes present in the plasmid-borne copper resistance operons *pco* in *E. coli* and the *cop* operons of *R. metallidurans* and *P. syringae.* The gene for PcoA or CopA (*R. metallidurans*) functionally complemented a CueO deletion (4). These bacterial multi-copper oxidases possess an extensive methionine rich region proximal to the copper centers that might be involved in copper binding. CopA from *Pseudomonas syringae* was shown to bind approximately 11 copper atoms per molecule (27). However, purified CueO was shown to bind only 4 copper atoms per molecule. This discrepancy might be the result of different binding affinities of these proteins for copper. Possibly, if CueO is a copper-oxidizing enzyme, the methionine-rich region may be involved in binding of Cu(I) but then releases copper after oxidation to Cu(II).

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