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The Pco proteins are involved in periplasmic copper handling in *Escherichia coli*

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Abstract

The interactions between the plasmid-borne copper resistance determinant, *pco*, and the main copper export system in *Escherichia coli* have been investigated and no direct interaction has been found. The PcoE and PcoC proteins are periplasmic and PcoC binds one Cu ion per protein molecule. PcoA is also periplasmic and can substitute for the chromosomally encoded CueO protein. The *pco* determinant is proposed to exert its effect through periplasmic handling of excess copper ions and to increase the level of resistance to copper ions above that conferred by *copA* alone. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Metal homeostasis; Copper resistance; TAT; Cu(II); *cop*

Copper is an essential element for all aerobic organisms but it is potentially very toxic due to its ability to produce reactive oxygen species in a Fenton-like reaction. Organisms have developed mechanisms to handle copper safely, and the most complete understanding of copper homeostasis is from the relatively simple system in *Enterococcus hirae* where uptake and export ATPases control intracellular copper concentrations under the control of the CopZ and CopY proteins [1]. Copper homeostasis has also been characterized in two classical model organisms, *Escherichia coli* and *Saccharomyces cerevisiae* [2–6]. Some strains of *E. coli* can survive in copper-rich environments that would normally overwhelm the chromosomally encoded copper homeostatic systems. Such strains possess additional plasmid-encoded genes that confer copper resistance. The conjugative plasmid pRJ1004 confers copper resistance and was isolated from *E. coli* in the gut flora of pigs fed a diet supplemented with copper sulphate as a growth promotant [7]. The copper resistance specified by this plasmid is encoded by the *pco* gene cluster, which contains seven genes, *pcoABCDRSE* [8]. Copper resistance in *Pseudomonas syringae* pv. *tomato* is specified by the *cop* determinant, which contains six genes, *copABCDRS*, arranged in a single operon and homologous to the equivalent *pco* genes [8–10]. In all cases copper resistance has been shown to be inducible [11–13]. The *pco* and the *cop* determinants are regulated by a plasmid-encoded and a chromosomally encoded two-component system [12,14–16]. Short-term radioactive ^{64}Cu uptake experiments showing reduced uptake suggested that an energy-dependent copper efflux mechanism is associated with the *pco* copper resistance genes from plasmid pRJ1004 [8,13]. Since these original findings the copper export protein, CopA, encoded by the *E. coli* chromosome has been identified [6], together with the regulator CueR which regulates CopA and CueO [4,17]. In this paper we further examine the function of the *pco* determinant and show that the *pco*-encoded gene products are involved in periplasmic copper handling.

Materials and methods

Bacterial strains and plasmids. The parental bacterial strains used were *E. coli* K12 strains ED8739 (*metB*, *gal*, *lac*, *supE*, *supF*, *hsdR*,

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hsdM) [18], TG2 (*lac-pro supE thi hsdD5* [F′*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15]) [19], and W3110 (*lam*$^-$, *in(rrnDE)1 rph*) [20]. *E. coli* strains BL21 (DE3) p*LysS* (Novagene) were used for overexpression of proteins and strains WK6 (Δ(*lac pro*), *galE*, *strA*, [F′ *proA*$^+$*B*$^+$, *lacI*^{q}, ZΔM15]), and WK6*mutS* [21] were used for mutagenesis. The *copA* disruptant of *E. coli* W3110 (*E. coli* DW3110 *copA*::K_m) was described previously [6]. Other *E. coli* strains were constructed as described in [3].

Cells were grown in LB medium [22] at 37 °C. Ampicillin (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (30 μg/ml), and anhydroxytetracycline (0.2 ng/ml) were added as required.

The *pco* copper resistance determinant was present on plasmid pPA173 [8] and was transformed into appropriate strains by standard methods as described elsewhere [8,22]. Manipulations of this plasmid and subcloning of the *pco* genes are described in Results and discussion. Plasmids pASK-IBA3 (IBA GmbH, Göttingen), pUC18 [23], and pT7-SC [24] were used for expression of proteins. Plasmid pACYC184 [25] was used for subcloning the *pco* determinant for mutagenesis.

Minimum inhibitory concentration (MIC) assays. These were performed to determine the effect of metal salts on growth of cells. Cells were grown overnight in LB, incubated for 1 h with 0.5 mM $CuSO_4$, diluted 1:20 in isosaline and plated onto L-agar plates containing 0.5 mM differential concentrations of $CuSO_4$. The MIC was defined as the lowest concentration of metal ion at which no growth could be observed following overnight incubation at 37 °C.

Copper accumulation assays. The net accumulation of ^{64}Cu by *E. coli* cells was performed over 40 m incubation as described in [8]. Assays were performed in duplicate and mean values were taken; duplicate values were within 15% on each other.

DNA manipulations and mutagenesis. All routine DNA manipulations were performed by methods similar to those described in [22]. Disruption of the *pco* genes by introduction of frameshift mutations at restriction sites was as described in the Results and discussion. Mutagenesis of the *pcoC* gene to introduce specific amino acids changes in the PcoC protein sequence was by the gapped-duplex method [21].

Purification of the PcoC protein. A 450-bp PCR product containing the *pcoC* gene was cloned in plasmid pT7-SC [24] at the *Eco*RI and *Bam*HI sites and the spacing between the ribosome binding sequence of the vector and the start codon of *pcoC* was optimized by digesting with *Eco*RI and *Nde*I, treatment with Mung Bean exonuclease and intramolecular ligation. The ligation product was used to transform *E. coli* BL21 (DE3) p*LysS* (Novagene) and single colonies were grown in broth and induced in mid-exponential phase with 100 μM IPTG for 45 min. SDS–PAGE analysis of total protein identified a colony producing a protein of M_r ca. 12,000; the resident plasmid was sequenced and confirmed as containing the *pcoC* gene. PcoC was purified from 1 L of IPTG-induced cells by sonication and chromatographic separation of the soluble proteins on a Resource S column (Pharmacia, Piscataway, NJ), followed by concentration and size exclusion chromatography on Sepharose 4B. About 5 mg of protein was obtained at >95% purity. Atomic absorption spectroscopy showed that the molar ratio of copper bound to the purified protein was less than 0.006.

Results and discussion

The pco determinant has no influence on copper sensitivity of a copA-deleted strain

CopA from *E. coli* is a Cu(I)-translocating P-type ATPase responsible for efflux of excess copper across the cytoplasmic membrane [6]. Disruption has been shown to produce sensitivity to copper salts in *E. coli*. To determine if the *pco* determinant can protect *copA*-disrupted cells from copper-mediated toxicity, plasmid pPA173 [8] containing the complete *pco* determinant was used to transform *E. coli* DW3110 *copA*::K_m and the wild type strain *E. coli* W3110 as a control. *E. coli* W3110 (pPA173) was more copper resistant than *E. coli* W3110. However, the presence of plasmid pPA173 did not affect the copper tolerance in *E. coli* DW3110*copA*::K_m (Table 1). This might indicate that the *pco* gene products need CopA to confer copper resistance, as in the model originally proposed by Rouch et al. [13,26] or that the *pco* gene products are responsible for periplasmic copper handling whilst CopA is responsible for pumping excess copper out of the cytoplasm. A disruption in *copA* could give rise to an increase in cytoplasmic copper concentration and make cytoplasmic DNA and proteins vulnerable to copper-mediated toxicity regardless of the presence of the *pco* gene products.

The pco determinant reduces intracellular copper concentrations

The *copA* promoter is under the control of the regulator CueR and is inducible by increased copper concentrations [4,17], with a maximum induction in L broth when 200 μM $CuSO_4$ is added to the medium. If plasmid pPA173 is present in the same cell, added copper concentrations in excess of 800 μM are required for maximal induction of the *copA* promoter, indicating that the intracellular concentration of Cu ions has decreased. This is in agreement with the observation that *pco*-containing cells have a reduced accumulation of ^{64}Cu in the initial stages of exposure to 0.3 mM Cu ions compared with isogenic *pco*-free control cells (see Table 2 and [8]). This suggests that the Pco system is responsible for reduced uptake but CopA is required to pump out excess copper.

The pcoABCD genes are required for copper resistance

The *cop* determinant from *P. syringae* pv. *tomato* [27] contains six genes (referred to here as *Ps-copABCDRS* to differentiate from the *E. coli* chromosomal *copA* gene) that are homologous to genes of the *pco* determinant

Table 1
Minimal inhibitory concentration (MIC) of different strains

E. coli strains	MIC Cu^{2+} (mM)	
	No plasmid	pPA173
W3110 (wild type)	4.0	7.0
GR1 *ΔcueO::cam*	3.5	7.0
GR10 *ΔcueO ΔcusCFBA::cam*	2.0	7.0
DW3110 *copA::*K_m	3.5	3.5
GR7 *ΔcusCFBA::cam*	4.0	7.0
	Without inducer	Inducer added[a]
GR10 *ΔcueO ΔcusCFBA::cam*	2.0	1.5
GR10 (pASK::*pcoA*)	2.0	3.0
GR10 (pASK::*pcoAB*)	4.0	3.5

[a] The inducer anhydroxytetracycline (AHT) was added at 0.2 ng/ml.

Table 2
Minimal inhibitory concentration (MIC) and ^{64}Cu accumulation of *pco* mutant strains

Plasmid	MIC Cu^{2+} (mM) in *E. coli* TG2	^{64}Cu accumulation (cpm$\times 10^{-3}$) in *E. coli* ED8739
–	6	28.8
pPA173	10	13.1
PPA173 (*pcoA*)	6	24.4
PPA173 (*pcoB*)	6	22.7
PPA173 (*pcoC*)	6	17.3
PPA173 (*pcoD*)	6	16.2
PPA173 (*pcoR*)	5	25.3
— (No preinduction)	6	37.7
pPA173 (no preinduction)	10	25.3

from plasmid pRJ1004. In addition, the *pco* determinant possesses another gene, *pcoE*, which shows similarity to the *silE* gene of the silver resistance determinant of *Salmonella* [28,29]. In *P. syringae* the presence of only two genes, *Ps-copA* and *Ps-copB*, was necessary to render the cells partially copper resistant; the *Ps-copC* and *Ps-copD* genes were required to confer full copper resistance [11,30]. *Ps-copC* and *Ps-copD* were suggested to be required for uptake of copper [30]. In contrast, frameshift mutations in each of the *pcoABCD* genes caused complete loss of plasmid-determined copper resistance in *E. coli* (Table 2).

The *pco* determinant from pPA173 was cloned as a 9-kb *Bgl*II–*Hin*dIII fragment in *Bam*HI–*Hin*dIII-cut plasmid pACYC184 [25]. Unique restriction sites in each of the *pcoABCD* genes, respectively, *Asp*718I, *Sph*I, *Bss*hII, and *Dra*III, were rendered blunt-ended and ligated with an excess of *Bgl*II linker oligonucleotide to generate a 14-bp frameshift mutation. The mutations were sequenced and restriction fragments containing the mutation were cloned to replace the wild-type sequence in pPA173 [8] in *E. coli* TG2. The recombinant strains were no more copper resistant than host strain lacking *pco* and ^{64}Cu-accumulation assays in the standard host *E. coli* ED8739 showed that the strains containing the *pco* mutations were less effective in reducing copper accumulation than the wild type *pco* determinant (Table 2). Mutations in *pcoA*, *pcoB*, and *pcoR* have a greater effect on copper accumulation than do those in *pcoC* and *pcoD*, but a reproducible difference in accumulation was noted between the *pcoC* and *pcoD* mutants and wild-type *pco*.

In the case of the *pcoABC* genes, it could be argued that the loss of resistance is due to polarity effects on each of the downstream genes. In the case of the *pcoD* insertion, this is not the case, as this is the last structural gene of the transcript. Nor can lack of resistance be due to polarity effects on the regulatory genes *pcoRS*, as equivalent chromosomal genes, presumably *cusRS*, can also regulate the *pcoABCDRS* promoter [12]. This is a difference from the *P. syringae* pv. *tomato cop* system in which only the A and B genes are required to confer partial resistance [30]. However, as shown below, in a copper-sensitive strain PcoA and PcoB alone can also confer resistance (Table 1; differences in MIC values are due to the different strains, growth conditions, and batches media used in our separate laboratories). The remaining downstream gene, *pcoE*, is transcribed from its own promoter and is unlikely to be subject to polarity effects from upstream frameshift mutations.

PcoA can functionally substitute for CueO

CueO is a chromosomally encoded multi-copper oxidase involved in intrinsic copper tolerance in *E. coli* [3,31]. CueO was shown to be a periplasmic enzyme with laccase-like phenol-oxidase activity that protected periplasmic enzymes from copper-mediated toxicity [31–34]. PcoA is also a putative multi-copper oxidase and the presence of a twin arginine motif in both PcoA and CueO indicate that they are both translocated into the periplasm by the Tat pathway [35]. Plasmid-encoded proteins such as PcoA, CopA (*P. syringae*), and CopA (*R. metallidurans*) (EMBL/GenBank Accession No. AJ278983) are largely identical to each other and probably have a similar function. The degree of similarity between CueO and PcoA is much smaller suggesting they are distantly related. CueO has a methionine-rich region that is also observed in PcoA although at another position within the protein. To test if *pcoA* can substitute for *cueO* we expressed *pcoA* in the copper-hypersensitive double mutant *E. coli* GR10 where both *cueO* and *cusCFBA* are deleted (Table 1). The Cus system had previously been shown to be involved in copper extrusion [3]. The *pcoA* gene was cloned into pASK-IBA3 (IBA GmbH, Göttingen) under control of the inducible *tet* promoter. The expression of *pcoA* rendered the cells more copper resistant than the control *E. coli* GR10. Expression of *pcoA* had to be induced by anhydoxytetracycline (AHT) since cells were as copper sensitive as the control cells of *E. coli* GR10 if *pcoA* was not induced. Expression of *cueO* in the same strain led to copper resistance at wild type levels [32]. Interestingly, the presence of both *pcoA* and *pcoB* rendered *E. coli* strain GR10 more copper resistant without the inducer AHT. AHT induction of *pcoAB* in *E. coli* strain GR10 actually led to a slight decrease in copper resistance compared to cells without induction of *pcoAB*. Thus, PcoAB can confer partial copper resistance in *E. coli* GR10 but need PcoCDE to confer full resistance. This is very similar to what was observed with *Ps-copABCD* in *P. syringae*.

Expression of pcoE has no influence on copper resistance

The *pcoE* gene including its promoter was cloned into pUC18 to examine the function of PcoE. Although

overexpression of PcoE was observed, this had no detectable influence on copper resistance, as measured by minimum inhibitory concentration assays with $CuSO_4$, either in the absence of other *pco* genes or in the presence of pPA173 (data not shown). The PcoE protein was released from the cells by treatment with polymyxin B nonapeptide [36], indicating that it is located in the periplasmic compartment.

The PcoC protein contains a single copper binding site

The PcoC protein is predicted from the DNA sequence of the *pcoC* gene to be a periplasmic protein [8], and it was purified as described in Materials and methods. Triplicate 250 μl aliquots of 2 μM PcoC protein in 50 mM HEPES, 150 mM NaCl, and pH 7.2 were dialysed for 48 h at 4 °C against 250 μl buffer containing ^{67}Cu–histidine complex at different concentrations between 0.01 and 20 μM. The radioactivity present in 100 μl samples of the protein and dialysate, and on the dried dialysis membrane were determined and used to determine the molar ratio of copper bound to the protein (B) and the amount of free Cu–histidine complex, allowing for the amount of copper bound to the dialysis membrane. Klotz–Hughes plots ($1/B$ vs 1/[free Cu–His]) gave an apparent binding constant for Cu of $9.6 \times 10^{-8}\,M^{-1}$. The maximum directly measured value of $B = 0.7$ moles Cu bound per mole PcoC protein at 9-fold molar excess free Cu–histidine, and a value of 0.86 moles Cu per mole PcoC from the intercept on Scatchard Plots (B/[free Cu–His] vs B) indicated that there is one Cu binding site on PcoC. A ratio of one copper per molecule was also observed with Ps-CopC from *P. syringae* [37].

The sequence MKGMSSH in the PcoC protein was postulated to be a copper-binding site. Separate mutagenesis experiments (using the gapped duplex method [21]) converted each methionine residue to isoleucine and the histidine to alanine. The three mutant proteins were purified and their copper-binding capacities were determined (Table 3). None of the mutations abolished copper-binding indicating that this motif does not directly constitute the copper-binding site of PcoC, but the binding constant did change, as measured from Hughes–Klotz plots (which had r^2 values > 0.999).

Table 3
Binding of copper to wild type and mutated PcoC

PcoC mutation[a]	Bound Cu:protein ratio[b]	K (nM^{-1})[b]
Wild type	0.86	96.1
M66I mutant	0.89	47.7
M69I mutant	0.83	171.7
H72A mutant	0.91	117.4

[a] The mutation is in the amino acid sequence Met-Lys-Gly-Met-Ser-Ser-His at positions 66–72 in the PcoC sequence; methionines were mutated to isoleucine and the histidine to alanine.

[b] The ratio was obtained from Scatchard plots and the apparent binding constant from Klotz–Hughes plots.

Conclusion

In this report we showed that there is no direct interaction between CopA, the main copper efflux system, and the *pco*-encoded gene products, but that CopA is required for maximal copper resistance. Several lines of evidence suggest that PcoABCDE are involved in periplasmic copper handling. First, PcoA is periplasmic and can functionally substitute for CueO, a multi-copper oxidase involved in copper tolerance in *E. coli* [32]. CopA is not required for the function of CueO and this probably is also true for PcoA [3]. Recent results suggest that CueO and possibly PcoA and Ps-CopA can protect periplasmic enzymes from copper induced damage and ensure proper iron uptake while preventing copper uptake [32,33]. Second, PcoC is periplasmic and can bind one equivalent of copper. Third, PcoE is also periplasmic and is strongly induced by cupric salts under the control of *cusRS*, whereas PcoABCD appears to be primarily controlled by *pcoRS* [12,16]. PcoE may provide initial sequestration of copper in the periplasm before the remaining genes of the *pco* system are fully induced, thus minimizing the effects of copper stress but not contributing to maximal resistance levels. PcoB appears to be an outer membrane protein, possibly preventing copper uptake. The functions of PcoC and PcoD are not known, but they might be involved in copper uptake across the cytoplasmic membrane. Possibly, copper is required for insertion into PcoA which is then transported out by the Tat pathway. In the absence of PcoA (or the equivalent Ps-CopA) this might lead to copper hypersensitivity as reported in [30]. In *P. syringae* both Ps-CopC and Ps-CopD had to be present to make cells hypersensitive for copper [30]. Expression of Ps-CopC or Ps-CopD alone showed slight copper accumulation, but did not make cells hypersensitive. These data suggest that Ps-CopC binds Cu in the periplasm which is then transported across the cytoplasmic membrane by Ps-CopD and incorporated into Ps-CopA. Ps-CopA is then transported into the periplasm via the Tat pathway. The functions of *pcoABCD* and *Ps-copABCD* are probably very similar. However, *P. syringae* and many other pseudomonads have homologues of *pcoA* and *pcoB* on the chromosome and it is not evident why expression of Ps-CopCD should lead to copper sensitivity and not resistance.

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