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Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins

(copper-binding proteins/blue copper proteins)

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ABSTRACT **Copper-resistant strains of *Pseudomonas syringae* pathovar *tomato* accumulate copper and develop blue colonies on copper-containing media. Three of the protein products of the copper-resistance operon (*cop*) were characterized to provide an understanding of the copper-resistance mechanism and its relationship to copper accumulation. The Cop proteins, CopA (72 kDa), CopB (39 kDa), and CopC (12 kDa), were produced only under copper induction. CopA and CopC were periplasmic proteins and CopB was an outer membrane protein. Leader peptide sequences of CopA, CopB, and CopC were confirmed by amino-terminal peptide sequencing. CopA, CopB, and CopC were purified from strain PT23.2, and their copper contents were determined. One molecule of CopA bound 10.9 ± 1.2 atoms of copper and one molecule of CopC bound 0.6 ± 0.1 atom of copper. The Cop proteins apparently mediate sequestration of copper outside of the cytoplasm as a copper-resistance mechanism.**

Copper is an essential trace element for many organisms. It usually occurs as part of the prosthetic group of oxidizing enzymes, which play a part in vital oxidation and reduction processes. Contrary to its role as an essential trace element, excess copper is toxic, especially to lower organisms. When the toxicities of 17 metals to soil bacteria, fungi, and actinomycetes were compared, copper ranked fifth highest, behind silver, mercury, chromium, and cadmium (1).

Copper compounds have been used extensively in agriculture to control plant diseases. Their relatively high toxicity to plant pathogens, low cost, and low toxicity to mammals have made them economically important. Copper compounds are the most common bactericides for control of plant bacterial diseases, especially since antibiotics are not registered for use on most edible crops.

The effectiveness of copper sprays for control of certain plant bacterial diseases is reduced by the appearance of copper-resistant bacterial strains. Copper-resistant strains of *Xanthomonas campestris* pathovar (pv.) *vesicatoria* were isolated from pepper and tomato plants in fields where copper compounds were frequently applied. The level of resistance was sufficient to reduce disease control with copper sprays (2). The copper-resistance determinant of those strains is on a large conjugative plasmid (3). Copper-resistant strains of *Pseudomonas syringae* pv. *tomato* have been found in tomato fields from California, and the copper-resistance determinant is on a highly conserved 35-kilobase (kb) plasmid, pPT23D (4, 5). The copper-resistance genes were cloned and sequenced; they are organized as an operon consisting of four genes, *copA*, *copB*, *copC*, and *copD*, under the control of a copper-inducible promoter (6–8).

The mechanisms of copper resistance in phytopathogenic bacteria are not known, and little information on bacterial copper resistance mechanisms is available in general (9). Bitton and Freihofer (10) reported that *Klebsiella aerogenes* strains producing a polysaccharide capsule were more tolerant to copper than noncapsulated strains, and the isolated capsular polysaccharides bound copper efficiently. A copper-resistant strain of *Escherichia coli*, isolated from pig effluent, where the pigs were fed a copper-supplemented diet, contained a copper-resistance determinant on a conjugative plasmid, and the copper resistance was induced by copper. Induced resistant cells accumulated less copper than uninduced cells, which suggested that an efflux mechanism is involved in the copper resistance (11, 12). Erardi *et al.* (13) reported that the copper-tolerant *Mycobacterium scrofulaceum*, which has a 173-kb plasmid carrying copper resistance, accumulated copper from the medium as a black intracellular precipitate of copper sulfide.

Cellular copper sequestration has been suggested as the copper-resistance mechanism in copper-resistant *P. syringae* pv. *tomato* (14), because the copper-resistant strains form bright blue colonies when grown on media containing high concentrations of copper.

In this study, the protein products of copper-resistance genes from *P. syringae* pv. *tomato* strain PT23.2 were characterized for investigations of the copper-resistance mechanism. The protein products CopA, CopB, and CopC of copper-resistance genes *copA*, *copB*, and *copC* were identified by immunoblotting analysis using antisera raised against β-galactosidase–Cop fusion proteins. Their amino-terminal amino acid sequence, subcellular locations, and copper-binding activities were determined. The results of this study suggest that Cop proteins mediate sequestration of copper outside of the cytoplasm as a copper-resistance mechanism of *P. syringae* pv. *tomato*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *P. syringae* pv. *tomato* strain PT23.2 [copper-resistant, rifampicin-resistant (6)] and PT23.3 [copper-sensitive, rifampicin-resistant, cured of pPT23D (C. A. Jasalavich and D.A.C., unpublished results)] were cultured in MGY medium (4) at 28°C with appropriate concentrations of copper adjusted by adding $CuSO_4 \cdot 6H_2O$.

Construction of β-Galactosidase–Cop Fusions and Antibodies. The translational fusions between β-galactosidase and Cop proteins were constructed by using the pUR series vectors (15). Blunt-ended 1858-base-pair (bp) *Stu* I–*Bsm* I, 1156-bp *Bsm* I–*Bst*EII, and 1320-bp *Hin*fI DNA fragments from pCOP4 (7) were ligated to the 3′ end of the *lacZ* gene for β-galactosidase–CopA, –CopB, and –CopC fusions, respectively. An 834-bp *Bal* I–*Pst* I fragment was cloned directionally into a blunt-end and a *Pst* I site in pUR290 to construct a β-galactosidase–CopD fusion. Rabbit polyclonal antibodies

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against the fusion proteins were raised according to previously described methods (16).

Cell Fractionation. A culture (200 ml) of the copper-resistant strain PT23.2 of *P. syringae* pv. *tomato* was grown in MGY broth containing 0.5 mM $CuSO_4$ for 20 hr (late logarithmic phase) and fractionated by Wood's method (17) with some modifications. After treatment to obtain spheroplasts, the solution was centrifuged at 15,000 × *g* for 20 min to give periplasmic proteins and much of the outer membrane in the supernatant and a pellet of spheroplasts. The spheroplasts were resuspended in 15 ml of 10 mM Tris·HCl/10 mM $MgCl_2$, pH 8.0, sonicated for 20 sec at 100 W, and centrifuged at 77,600 × *g* for 2.5 hr to separate cytoplasmic proteins in the supernatant from the pellet of cell membranes. The membrane pellet was resuspended in 15 ml of 10 mM Tris·HCl, pH 8.4. The supernatant of the periplasmic fraction containing the outer membrane was diluted three-fold with water and centrifuged at 77,600 × *g* for 2.5 hr to separate periplasmic proteins into the supernatant and the outer membrane into the pellet. Bacterial cells from an equal volume of culture were harvested, washed, resuspended in 15 ml of 10 mM Tris·HCl, pH 8.4, and sonicated at 150 W for 1.5 min for a total cell extract preparation. Sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE) and immunoblot analyses were performed as described previously (18, 19).

Cross-contamination of fractions was assessed by assays for 5′-nucleotidase (20), isocitrate dehydrogenase (21), lactate dehydrogenase (22), and lipopolysaccharide as periplasmic, cytoplasmic, inner membrane, and outer membrane markers, respectively. Lipopolysaccharides were detected on SDS/polyacrylamide gels by silver staining (23).

The outer membrane fraction of strain PT23.2 was prepared by the method of Hancock and Nikaido (24). CopB was solubilized from the outer membrane with 2% Triton X-100 and 10 mM Na_2EDTA based on the method of Schnaitman (25).

Amino-Terminal Sequencing. Amino acid sequences of the amino termini of CopA, CopB, and CopC were determined by a protein sequencer (Applied Biosystems model 475A) from Western-blotted protein samples at the Biotechnology Instrumentation Facility at the University of California, Riverside.

Protein Purification. For purification of CopA, cells of PT23.2 grown in MGY with 0.5 mM $CuSO_4$ were harvested by centrifugation (7500 × *g*, 5 min), suspended in 50 mM Tris·HCl, pH 8.0, and broken by sonication (1 min at 300 W). Cell debris and unbroken cells were removed by centrifugation (7500 × *g*, 5 min). CopA was precipitated from the supernatant by adding $(NH_4)_2SO_4$ to 30% saturation and pelleted by centrifugation (15,000 × *g*, 10 min). The pellet was suspended and dialyzed against 50 mM Tris·HCl, pH 8.0. Insoluble material in the dialysate was removed by centrifugation (77,600 × *g*, 2.5 hr). Protein in the supernatant was concentrated in an ultrafiltration cell (Amicon) with a XM-50 membrane and loaded onto a Sephacryl S-200 column (1.5 × 90 cm; Pharmacia). Protein was eluted from the column with 50 mM Tris·HCl, pH 8.0, containing 0.2 M NaCl. Fractions containing CopA were combined, dialyzed against 30 mM Tris·HCl, pH 7.5, and applied to a diethylaminoethyl (DEAE)-Sepharose CL-6B column (1.5 × 20 cm; Pharmacia). Protein was eluted with linear gradient of 0–400 mM NaCl.

For CopB, cells were harvested, suspended in 30 mM Tris·HCl, pH 7.5, containing 10 mM $MgCl_2$, and broken by a French press. Membranes were collected by ultracentrifugation (106,000 × *g*, 1.5 hr) and washed with 30 mM Tris·HCl, pH 7.5, containing 2% Triton X-100. CopB was extracted from membranes with 2% Triton X-100/10 mM Na_2EDTA and loaded onto a DEAE-Sephadex A50 column (2.6 × 25 cm; Pharmacia). Protein was eluted with a linear gradient of 0–1 M NaCl. CopB was further purified by using a Sephacryl S-200 column.

For CopC, cells were harvested, suspended in Tricine buffer (30 mM Tricine, pH 8.0, containing 5 mM $MgCl_2$), and broken by a French press. The lysate was ultracentrifuged (106,000 × *g*, 1.5 hr) to remove cellular debris. The supernatant was loaded onto a sulfopropyl (SP)-Sephadex C25 column (2.6 × 24 cm; Pharmacia) and protein was eluted with a linear gradient of 0–1 M NaCl. Fractions containing CopC were pooled, dialyzed against 30 mM Tris·HCl, pH 8.0, concentrated in an ultrafiltration cell with a YM10 membrane, and loaded onto a Sephacryl S-200 column.

Protein Quantification. Protein content was determined with the Lowry method (18) and the bicinchoninic acid reagent (Sigma) according to Smith *et al.* (26) with bovine serum albumin as a standard. Protein content of whole bacterial cells was determined after solubilization of the bacteria with SDS. Concentrations of CopA and CopC were determined on polyacrylamide gels or immunoblots by using a densitometer (Ultrascan XL model 2222, LKB) and a sonic digitizer (model GP-7, Science Accessories, Southport, CT). Peak areas of CopA and CopC were compared with the peak area of bovine serum albumin as an internal standard.

Cellular Copper Accumulation and Copper Content of Proteins. Bacterial cells from PT23.2, which were grown in MGY broth containing appropriate concentrations of $CuSO_4$ for 20 hr, were harvested, washed with 0.15 M NaCl, and freeze-dried. Copper content of bacterial cells was determined after acid dissolution of the bacterial cells according to the method of Ganje and Page (27). For determination of the copper content of fractions of bacterial cells, the cells from PT23.2 and PT23.3 cultures (400 ml each), which were grown in MGY containing a subinhibitory concentration of $CuSO_4$ (100 μM) for 20 hr, were harvested, washed with 0.15 M NaCl, suspended in 10 ml of 30 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$ containing 0.2 mg of DNase I and 0.2 mg of RNase, and broken by a French press. The lysate was incubated with 1 mg of lysozyme for 30 min at room temperature and ultracentrifuged at 77,600 × *g* for 2.5 hr rpm in a Beckman 50 Ti rotor to separate the cytoplasm and periplasm as a supernatant from the pelleted membrane fraction. The pellet was suspended in 10 ml of water. Bacterial cells from the same cultures were freeze-dried for determination of dry weight and total copper content. About 50 mg of dried samples or 1 ml of liquid samples was digested with 2 ml of 11 M HNO_3 at 85°C for 3 hr. Copper content was determined with an atomic absorption spectrophotometer (Perkin–Elmer, model 5000) at 324.9 nm.

RESULTS

Detection of Cop Proteins. CopA, CopB, and CopC, the protein products of *copA*, *copB*, and *copC* of the copper-resistance operon, were detected in total protein preparations from *P. syringae* pv. *tomato* strain PT23.2 by immunoblotting analysis using antisera raised against β-galactosidase–CopA, –CopB, and –CopC fusion proteins (Fig. 1). Cop proteins were detected only from cells grown with $CuSO_4$. The Cop proteins were also detected in *P. syringae* pv. *syringae* PS61 carrying the cloned copper-resistance operon (pCOP2; ref. 6) only under copper selection (data not shown). Molecular masses of Cop proteins detected by immunoblot analyses were consistent with the predicted products of the sequenced genes (7). The molecular masses of CopA and CopB were 72 and 39 kDa, respectively, based on SDS/PAGE, and the molecular mass of CopC was 12 kDa, based on gel filtration chromatography analysis.

The β-galactosidase–CopD fusion protein did not appear as a single band on polyacrylamide/SDS gels, suggesting that the fusion protein was not stable in *E. coli*. A region of

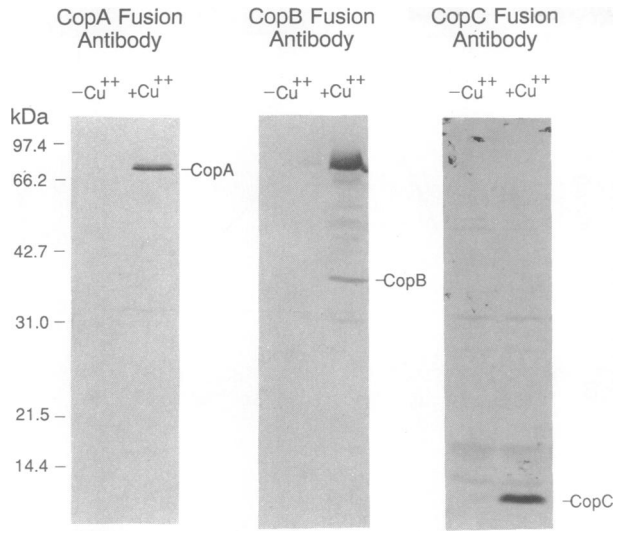

FIG. 1. Detection of Cop proteins from *P. syringae* pv. *tomato* strain PT23.2 by immunoblot analysis. Total proteins from strain PT23.2 grown without and with 0.2 mM $CuSO_4$ were separated by SDS/PAGE, transferred to nitrocellulose, and probed with antisera prepared against Cop fusion proteins.

multiple bands, apparently degradation products of the fusion protein, was excised from these gels for raising antibodies in rabbits, but no antiserum was obtained by this method that reacted with proteins from *P. syringae*.

Antiserum raised against the β-galactosidase–CopB fusion protein reacted not only with CopB but also with CopA (Fig. 1). Antiserum raised against the β-galactosidase–CopA fusion protein reacted with CopA and also cross-reacted with CopB at a low dilution of the antiserum (data not shown).

Cellular Location of Cop Proteins. Assays of marker enzyme activities in fractions of bacterial cells showed that 82%, 73%, and 80% of total activities of 5′-nucleotidase, isocitrate dehydrogenase, and lactate dehydrogenase were found in periplasmic, cytoplasmic, and membrane fractions, respectively. Lipopolysaccharide, which was the outer membrane marker, was detected in the periplasm, the pellet of the periplasmic fraction, and the membrane fraction (data not shown).

Most of CopA and CopC were released into the medium when the bacterial cells became spheroplasts. Furthermore, when the periplasmic fraction containing the outer membrane was centrifuged to pellet the outer membrane, both proteins were in the supernatant (Fig. 2, lanes 5). Thus, CopA and CopC are periplasmic proteins. A protein with a molecular

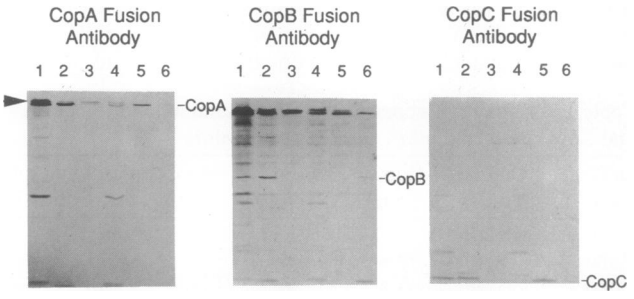

FIG. 2. Localization of Cop proteins in bacterial cells of strain PT23.2 by immunoblot analysis. Proteins of a whole cell extract (lanes 1), periplasmic fraction (lanes 2), cytoplasmic fraction (lanes 3), membrane fraction (lanes 4), supernatant of periplasmic fraction (lanes 5), and pellet of periplasmic fraction (lanes 6) were separated by SDS/PAGE, transferred to nitrocellulose, and probed with antisera prepared against Cop fusion proteins. A putative unprocessed form of CopA is indicated by the arrowhead.

mass slightly larger than that of CopA was detected in the whole cell extract (arrowhead in Fig. 2), cytoplasmic, and total membrane fractions. This suggested the presence of some unprocessed CopA protein that still contained a signal sequence (Fig. 2). CopB was considered initially to be a periplasmic protein, because most of CopB was released into the periplasmic fraction during spheroplast formation of the bacterial cells (Fig. 2). However, when CopB was obtained from the periplasmic fraction by ammonium sulfate precipitation and loaded onto a gel filtration column (Sephacryl S-300, theoretical molecular mass exclusion, 1.5 megadaltons), CopB failed to pass through the matrix, while CopA and CopC passed through. The CopB protein present in the periplasmic fraction was apparently not in a soluble form. CopB was detected in the pellet when the periplasmic fraction was centrifuged at a force sufficient to pellet the outer membrane component (Fig. 2). In another experiment, CopB was solubilized only with a Triton X-100 and Na_2EDTA treatment from an outer membrane preparation of strain PT23.2 (data not shown).

The results of localization of the Cop proteins in this study, which showed that the Cop proteins were secreted into the periplasm and outer membrane of the cell, strongly suggested the presence of signal peptides. To investigate the presence of the signal peptides, the amino-terminal sequences of CopA, CopB, and CopC were determined. The first five amino acids of CopA (Ala-Ser-Gly-Ser-Pro-), CopB (Ala-Glu-Ala-Ala-Met-), and CopC (His-Pro-Lys-Leu-Val-), as determined by amino-terminal peptide sequencing, matched amino acids 33–37, 28–32, and 25–29 of the predicted amino acid sequence (7) of CopA, CopB, and CopC, respectively. Thus, the first 32, 27, and 24 amino acids are leader peptides of CopA, CopB, and CopC, respectively.

Copper Content of Cop Proteins. CopA was released by sonication of the bacterial cells, and most of CopA was precipitated from the supernatant fluids by 0–30% ammonium sulfate saturation. CopA was eluted from a DEAE-Sepharose column at about 300 mM NaCl and was accompanied by minor proteins (Fig. 3). The copper content of the fractions was determined with an atomic absorption spectrophotometer, and the copper content of each fraction was correlated with its CopA concentration (Fig. 3). This detection of copper binding by CopA was reproduced in another independent CopA purification. The copper concentration of CopA was estimated as 10.9 ± 1.2 atoms of copper per molecule of CopA.

CopB was eluted from a DEAE-Sephadex column at approximately 400 mM NaCl. A discrete CopB band was obtained on polyacrylamide gels from the fractions eluted from the Sephacryl S-300 column, but other proteins were still in the fractions with CopB (data not shown). Without further purification, the fractions were used to determine copper content, and no significant copper was detected in the fractions. If CopB initially contained copper, the Na_2EDTA treatment, which was essential to extract CopB from the membrane, may have removed copper from CopB.

CopC was obtained pure as determined by Coomassie blue staining after separation on a Sephacryl S-200 column. The copper content of the fractions from the Sephacryl S-200 column was determined (Fig. 4). The copper concentration was estimated at 0.6 ± 0.1 atom of copper per molecule of CopC. The CopC fractions from larger-scale purifications were blue.

Copper Accumulation and CopA and CopC Production. Colonies of copper-resistant strains of *P. syringae* pv. *tomato* become blue on media with high levels of copper, which suggests that the bacteria accumulate copper. The amount of copper accumulated and the amount of CopA and CopC produced by the bacteria were determined to estimate the percentage of copper bound by CopA and CopC from the

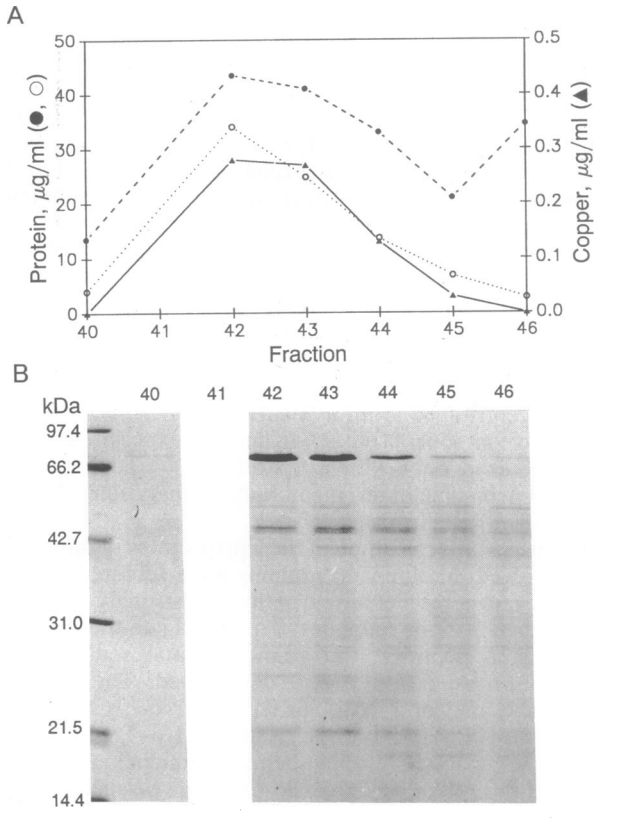

FIG. 3. Copper content of the CopA protein. (*A*) Concentrations of copper (▲), total protein (●), and CopA (○) in fractions eluted from a DEAE-Sepharose column. (*B*) Proteins of the fractions were separated by SDS/PAGE and stained with Coomassie brilliant blue. Fraction 41 was lost.

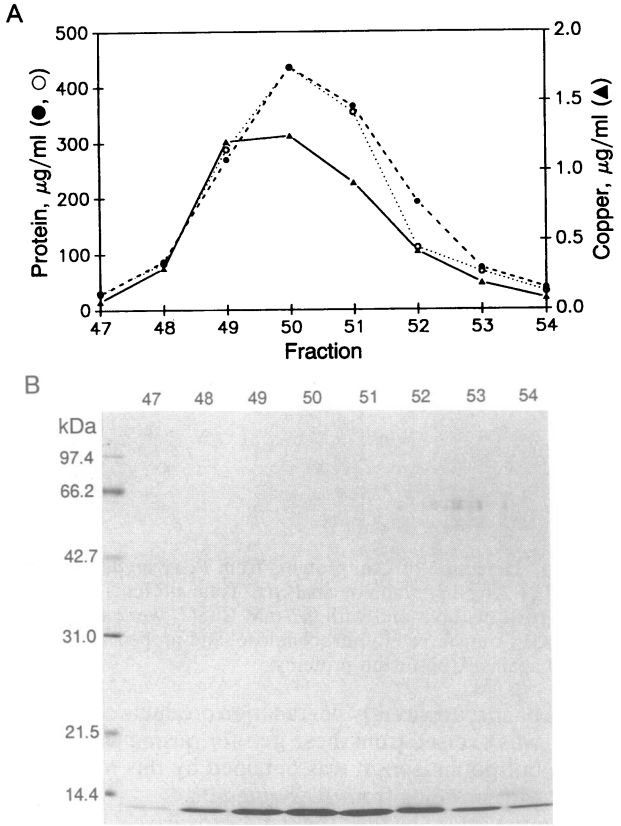

FIG. 4. Copper content of the CopC protein. (*A*) Concentrations of copper (▲), total protein (●), and CopC (○) in the fractions eluted from a Sephacryl S-200 column. (*B*) Proteins of the fractions were separated by SDS/PAGE and stained with Coomassie brilliant blue.

total copper accumulated by the bacteria. Copper accumulation by strain PT23.2 increased with increasing concentration of copper in the medium up to 1.8 mg/g dry weight of cells when cells were grown in medium containing 500 μM $CuSO_4$ (Table 1). CopA and CopC production by the strain PT23.2 increased with increasing copper concentrations up to 200 μM and then leveled off. The percentage of copper bound by CopA and CopC, which was calculated based on 11 atoms of copper bound per molecule of CopA and 1 atom of copper bound per molecule of CopC, was about 20% at 100 and 200 μM copper but only 7% at 500 μM (Table 1). Strain PT23.2 produced 11.7 mg of CopA and 3.3 mg of CopC per gram dry weight of cells at 200 μM copper, which was equivalent to about 3% and 0.8% of the total protein of the bacterial cells.

The copper-resistant strain PT23.2 accumulated more copper than a copper-sensitive strain, PT23.3, at subinhibitory concentrations of copper, although this difference was significant only for the soluble fraction (periplasmic and cytoplasmic) and not for whole cells (Table 2). The periplasm and cytoplasm contained about 35–42% of the total copper accumulated by the bacteria. In previous experiments, whole cells of two strains of *P. syringae* carrying the cloned *cop* operon consistently accumulated more copper at subinhibitory levels than the same strains without the cloned genes (unpublished data).

DISCUSSION

CopA, CopB, and CopC were identified as the protein products of *copA*, *copB*, and *copC* of the copper-resistance operon from plasmid pPT23D of *P. syringae* pv. *tomato* strain PT23.2. The Cop proteins were detected in the copper-resistant strain PT23.2 and in strain PS61 containing the cloned copper-resistance operon only under copper selection. Specific copper inducibility of the *cop* operon was previously shown at the mRNA level (8).

Antisera to β-galactosidase–CopA and –CopB fusions cross-reacted with both the CopA and CopB proteins. Both CopA and CopB contain conserved eight-amino acid peptide repeats according to the nucleotide sequences of the respective genes (7). These repeated peptide sequences were included in the construction of both the β-galactosidase–CopA and –CopB fusions and probably account for the cross-reactivity of the antisera.

Several results suggested that CopB is tightly associated with the outer membrane: (*i*) CopB was released into the medium when spheroplasts were made; (*ii*) CopB did not pass through a gel filtration column unless solubilized with Triton X-100 and EDTA; (*iii*) CopB was present in the pellet along with lipopolysaccharide after centrifugation of the periplas-

Table 1. Amount of copper accumulated and amount of CopA and CopC produced by *P. syringae* pv. *tomato*

$CuSO_4$, μM	CopA, mg	CopC, mg	Copper, μg	Copper bound by CopA and CopC, %*
0	ND	ND	17	ND
100	8.4	2.8	453	21
200	11.7	3.3	672	19
500	10.8	2.8	1823	7

Results represent average values from duplicate experiments; values were calculated per gram of dry weight of bacterial cells. ND, not determined.

*Percent of copper bound by CopA and CopC from total copper accumulated was calculated based on 11 atoms of copper bound per molecule of CopA and 1 atom of copper bound per molecule of CopC.

Table 2. Accumulation of copper by a copper-sensitive and a copper-resistant strain of *P. syringae* pv. *tomato*

Strain	Copper, μg/g		
	Periplasm and cytoplasm	Total membrane	Whole cell
PT23.3	48 ± 20*	209 ± 26	280 ± 20
PT23.2	117 ± 26*	252 ± 56	358 ± 60

Results are mean ± SD from four replicated experiments; values were calculated per gram dry weight of bacterial cells. Dry weights of bacterial cells from 1-liter cultures were 394 ± 21 mg for PT23.3 and 397 ± 16 mg for PT23.2. *, Significantly different from one another ($P < 0.05$).

mic fraction containing outer membrane material obtained by spheroplasting; and (*iv*) CopB was also present in outer membrane fractions purified on sucrose gradients after the cells had been ruptured with a French press (data not shown).

CopC is a blue copper protein with an absorption maximum at 600 nm. CopC binds 1 atom of copper per protein molecule, while CopA binds about 11 atoms of copper per protein molecule. CopA may also be a blue protein, but we have not purified it in sufficient concentrations to observe the color.

The copper-binding sites in CopA and CopC have not been determined, but Ouzounis and Sander (28) have noted that CopA contains a probable type-1 copper site near its carboxyl terminus with strong similarities to the single type-1 copper site in multicopper oxidases. The predicted copper ligands at this single copper site are His-542, Cys-591, His-596, and Met-601. There are no other cysteines in CopA and none in CopC, suggesting that the other 10 copper atoms of CopA and the single copper atom of CopC are bound in a different manner. CopA contains 18 additional histidine residues, several of which are contained in a multiply repeated sequence that is also found once in CopC (15). This sequence (Met-Xaa-Xaa-Met-Xaa-His-Xaa-Xaa-Met), together with other histidine ligands, could probably account for the large number of copper atoms bound by CopA. Multinuclear copper clusters with copper ligated only to histidine residues have been described, such as the trinuclear copper site of ascorbate oxidase, with eight histidine ligands (29).

The copper-binding capacity of periplasmic CopA and CopC, and their abundance in copper-induced cells, suggests that their function in copper resistance could be the simple sequestration of copper ions in the periplasm, which could prevent the entry of the toxic copper ions into the cytoplasm. However, these proteins accounted for only about 20% of the copper accumulated by strain PT23.2, and the concentration of these proteins did not continue to increase at higher levels of copper, while total accumulated copper did increase. Other cellular components, such as CopB or lipopolysaccharide, must be involved in this further accumulation of copper. Since all of the *cop*-encoded proteins are required for growth at higher levels of copper (7), their role at these higher levels, when their binding capacity would seem to be saturated, might be in the delivery of copper ions to other binding components of the cell wall.

This protein-mediated sequestration of copper is an unusual mechanism of resistance to a heavy metal for a bacterium. Most other metal-resistance systems involve either an active efflux or a detoxification of the metal ions by reduction or other transformations (30). Unlike most other heavy metals for which resistance mechanisms are well defined, such as mercury, cadmium, and arsenate, copper is an essential element for the bacterial cell. Therefore, resistance by sequestration of this ion, rather than by its complete removal, could be a more efficient mechanism to allow further growth of the bacteria in the presence of copper. The presence of a copper-inducible chromosomal homolog to the plasmid-borne *cop* operon in several species of *Pseudomonas* (14, 19) suggests that this resistance mechanism may have evolved from indigenous genes with a copper-related function.

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