

Molecular Mechanism of Copper Resistance in *Pseudomonas syringae* pv. *tomato*.

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ABSTRACT

Copper resistance in *Pseudomonas syringae* pv. *tomato* is determined by copper-resistance operon (*cop*) on a highly conserved 35 kilobase plasmid. Copper-resistant strains of *Pseudomonas syringae* containing the *cop* operon accumulate copper and develop blue colonies on copper-containing media. The protein products of the copper-resistance operon 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. *P. syringae* cells containing *copCD* or *copBCD* cloned behind the *lac* promoter were hypersensitive to copper. The CopD (32 kDa), a probable inner membrane protein, function in copper uptake with CopC. The Cop proteins apparently mediate sequestration of copper outside of the cytoplasm as a copper-resistance mechanism.

INTRODUCTION

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 mechanism is available in general (9). Bitton and Freihofner (10) reported that *Klebsiella aerogenes* strains producing a polysaccharide capsule were more tolerant to copper than noncapsulated strains, and the isolated capsular polysaccharide 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-determinant on a conjugative plasmid, and the copper resistance was induced by copper. Induced cells accumulated less copper than uninduced cells, which suggested that an efflux mechanism is involved in the copper resistance (11, 12). Eradi *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 Condition. *P. syringae* pv. *tomato* strain PT23.2 [copper-resistant, Rifampicin-resistant (6)], PT23.3 [copper-sensitive, Rifampicin-resistant, cured of pPT23D (31)], and *P. syringae* PS61 were cultured in MGY medium (4) at 28°C with appropriate concentrations of copper adjusted by adding $\text{CuSO}_4 \cdot 6\text{H}_2\text{O}$.

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 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 spheroplast, the solution was centrifuged at 15,000 x 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 , pH8.0, sonicated for 20 sec at 100 W, and centrifuged at 77,600 x 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, pH8.4. The supernatant of the periplasmic fraction containing the outer membrane was diluted three-fold with water and centrifuged at 77,600 x g for 2.5 hr to separate periplasmic proteins into 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, pH8.4, 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 analysis 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 Na₂EDTA based on the method of Schnitman (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 CuSO₄ were harvested by centrifugation (7500 x g, 5 min), suspended in 50 mM Tris.HCl, pH8.0, and broken by sonication (1 min at 300 W). Cell debris and unbroken cells were removed by centrifugation (7500 x g, 5 min). CopA was precipitated from the supernatant by adding (NH₄)₂SO₄ to 30% saturation and pelleted by centrifugation (15,000 x 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 x 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 x 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)-Sephacryl CL-6B column (1.5 x 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 MgCl₂, and broken by a French press. Membranes were collected by ultracentrifugation (106,000 x 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 Na₂EDTA and loaded a DEAE-Sephadex A50 column (2.6 x 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 x g, 1.5 hr) to remove cellular debris. The supernatant was loaded onto a sulfopropyl (SP)-Sephadex C25 column (2.6 x 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 sonic digitizer (model GP-7, Science Accessories, Southport, CT). 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 x 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.

For determination of copper accumulation by PS61 containing different subcloned components of the *cop* operon, the bacteria were grown in 250 ml of MGY broth supplemented with tetracycline (10 ug/ml) to a density of 110 to 120 Klett units, and copper sulfate was then added to a final concentration of 0.2 mM. The cultures were incubated for an additional 20 min and then harvested by centrifugation. The cell pellets were washed once with 0.15 M NaCl and lyophilized. The copper content was determined by above method.

Minimum Inhibitory Concentration (MIC) Determination. Derivatives of *P. syringae* PS 61 containing different subcloned components of the *cop* operon were incubated on a rotary shaker at 200 rpm and 28°C overnight and subcultured (50 ul) in 5 ml of MGY broth supplemented with tetracycline (20 ug/ml) and various concentrations of copper sulfate and zinc sulfate in culture tubes to determine the MIC of copper sulfate or zinc sulfate. MICs were recorded after 24 hr, when cultures with subinhibitory levels of copper or zinc had reached stationary phase.

Plasmid Construction for MIC Determination. All of the plasmids used in the MIC determination except pCOP65 were described previously. Plasmid pCOP65 was constructed by subcloning an approximately 750-bp *EcoRI*-*Msc* I fragment containing *copC* from an exonuclease III deletion derivative of the *cop* operon that contained *copCD* (pCOP12). The fragment was treated with DNA polymerase I (Klenow fragment; New England Biolabs, Inc.) to fill in the 5' overhang and was ligated to pRK415, which had been cleaved with *Bam*HI and treated with the Klenow fragment.

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 CuSO₄. 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 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 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 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 Na₂EDTA 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.

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 Na₂EDTA 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 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 μ M CuSO₄ (Table 1). CopC production by the strain PT23.2 increased with increasing copper concentrations up to 200 μ M 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 μ M copper but only 7% at 500 μ M (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 μ M 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).

Copper Hypersensitivity and Uptake by *copCD*. While expression of the intact *cop* operon increased the resistance of *P. syringae* PS61 to copper sulfate about threefold, none of the individual *cop* genes or various combinations of one to three genes of the operon (except the combination *copAB* [7], which was not used in this study) increased the resistance of PS61 (Table 3). However, PS61 containing the combination *copCD* exhibited a 26-fold-greater sensitivity to copper. PS61 cultures containing either *copBCD* or *copACD* were also more sensitive to copper sulfate than wild-type strain PS61 containing the cloning vector pRK415 alone but were not as sensitive as PS61 containing *copCD*. *copC* or *copD* alone did not cause hypersensitivity to copper. No hypersensitivity or resistance to zinc sulfate was associated with *copCD* or the intact *cop* operon (Table 3).

Copper accumulation was 40% greater in hypersensitive strain PS61 (pCOP12A) containing *copCD* than in PS61 containing only the vector pRK415 (Table 3). Strains containing all of the other combinations of *cop* genes tested also showed greater copper accumulation than wild type ($P = 0.05$). However, copper accumulation by strains PS61

(pCOP13A) and PS61 (pCOP65), containing either *copC* or *copD* alone, was not significantly different from copper accumulation by the wild type at the 1% level.

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 crossreactivity 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 periplasmic 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 600nm. 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 hypersensitivity associated with specific combinations of cloned genes of the *cop* operon suggests that part of the operon (*copCD*) may function in cellular copper uptake. Similar observations led to the discovery of a mercury transport system (*merTP*) associated with the mercury resistance operon (34, 35). CopC is a periplasmic protein and CopD is probably an inner membrane protein. The predicted amino acid sequence determined from the *copD* nucleotide sequence (7) has several hydrophobic stretches that could be membrane-spanning regions. Previous work indicated that both *copC* and *copD* were required for full copper resistance in *P. syringae*, but subclones in which both of these genes were deleted still exhibited a low level of copper resistance from *copAB* (7). These observations are consistent with the hypothesis that the combination *copCD* functions in copper uptake, possibly as a mechanism to balance the periplasmic copper-sequestering activity of the abundant, multiple-copper-binding CopA and the activity of CopB and the outer membrane.

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 of a detoxification of the metal ions by reduction of 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.

Literature cited

1. Drucker, H., T. R. Garland, and R. E. Wildung. 1979. Metabolic response of microbiota to chromium and other metals, p. 1-25. in N. Kharash (ed.), Trace metals in health and disease. Reven Press, New York.
2. Marco, G. M., and R. E. Stall. 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. *Plant Disease* 67:779-781.
3. Stall, R. E., D. C. Loschke, and J. B. Jones. 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology* 76:240-243.
4. Bender, C. L., and D. A. Cooksey. 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato* : conjugative transfer and role in copper resistance. *J. Bacteriol.* 165:534-541.
5. Cooksey, D. A. 1987. Characterization of a copper resistance plasmid conserved in copper-resistant strains of *Pseudomonas syringae* pv. *tomato*. *Appl. Environ. Microbiol.* 53:454-456.
6. Bender, C. L., and D. A. Cooksey. 1987. Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. *J. Bacteriol.* 169:470-474.
7. Mellano, M. A., and D. A. Cooksey. 1988. Nucleotide sequence and organization of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. *J. Bacteriol.* 170:2879-2883.
8. Mellano, M. A. and D. A. Cooksey. 1988. Induction of the copper resistance operon from *Pseudomonas syringae*. *J. Bacteriol.* 170:4399-4401.
9. Trevors, J. T. 1987. Copper resistance in bacteria. *Microbiological sciences* 4:29-31.
10. Bitton, G., and V. Freihofer. 1978. Influence of extracellular polysaccharides on the toxicity of copper and cadmium toward *Klebsiella aerogenes*. *Microbiol. Ecol.* 4:119-125.
11. Tetaz, T. Z. and R. K. Luke. 1983. Plasmid controlled resistance to copper in *Escherichia coli*. *J. Bacteriol.* 154:1263-1268.
12. Rouch, D., J. Camakaris, B. T. O. Lee and R. K. Luke. 1985. Inducible plasmid-mediated copper resistance in *Escherichia coli*. *J. Gen. Microbiol.* 131:939-943.
13. Erardi, F. X., M. L. Failla, and J. O. Falkinham III. 1987. Plasmid-encoded copper resistance and precipitation by *Mycobacterium scrofulaceum*. *Appl. Environ. Microbiol.* 53:1951-1954.
14. Cooksey, D. A. 1990. Genetics of bactericide resistance in plant pathogenic bacteria. *Ann. Rev. Phytopathol.* 28:201-219.
15. Ruther, U., and B. Muller-Hill. 1983. Easy identification of cDNA clones. *EMBO J.* 2:1791-1794.

16. Rio, D. C., F. A. Laski, and G. M. Rubin. 1986. Identification and immunochemical analysis of biologically active *Drosophila* P element transposase. *Cell* 44:21-32.
17. Wood, P. M. 1978. Periplasmic location of the terminal reductase in nitrite respiration. *FEBS Letters* 92:214-218.
18. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. *Current Protocols in Molecular Biology* (Wiley, New York), Vol. 2, pp. 102.1-108.6.
19. Cooksey, D. A., H. R. Azad, J.-S. Cha, and C.-K. Lim. 1990. Copper resistance gene homologs in pathogenic and saprophytic bacterial species from tomato. *Appl. Environ. Microbiol.* 56:431-435.
20. Garber, N., and I. Nachshon. 1980. Localization of cholinesterase in *Pseudomonas aeruginosa* strain K. L. *Gen. Microbiol.* 117:279-283.
21. Goldberg, D. M. and G. Ellis. 1983. in *Methods of Enzymatic Analysis*, ed. Bergmeyer, H. U. (Verlag Chemie, Weinheim, F.R.G.), Vol. 3, pp. 183-190.
22. Vassault, A. 1983. in *Methods of Enzymatic Analysis*, ed. Bergmeyer, H. U. (Verlag Chemie, Weinheim, F.R.G.), Vol. 3, pp. 118-126.
23. Hitchcock, P. J. and T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* 154:269-277.
24. Hancock, R. E. W., and H. Nikaido. 1978. Outer membranes of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PA01 and use in reconstitution and definition of the permeability barrier. *J. Bacteriol.* 136:381-390.
25. Schnaitman, C. A. 1971. *J. Bacteriol.* 108:553-563.
26. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fusimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using Bicinchoninic acid. *Anal. Biochem.* 150:76-85.
27. Ganje, T. J. and A. L. Page. 1974. Rapid acid dissolution of plant tissue for cadmium determination by atomic absorption spectrophotometry. *Atomic Absorption Newsletter* 13:131-134.
28. Ouzounis, C., and C. Sander. 1991. A structure-derived sequence pattern for the detection of type I copper binding domains in distantly related proteins. *FEBS Lett.* 279:73-78.
29. Messerschmidt, A., A. Rossi, R. Ladenstein, R. Huber, M. Bolognesi, G. Gatti, A. Marchesine, R. Petruzzelli, and A. Finazzi-Agro. 1989. *J. Mol. Biol.* 206:513-529.
30. Silver, S., T. K. Misra, and R. A. Laddaga. 1989. in *Metal Ions and Bacteria*, eds. Beveridge, T. J., and Doyle, R. J. (Wiley, New York), pp. 121-139.
31. Mills, S., C. A. Jasalavich, and D. A. Cooksey. 1993. A two-component regulatory system required for copper-inducible expression of the copper resistance operon of *Pseudomonas syringae*. *J. Bacteriol.* 175:1656-1664.

32. Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X. Liang, D. Guiney, and D. Helinski. 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and monitoring gene expression. *Plasmid* 13:149-153.
33. Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* 70:191-197.
34. Foster, T. J., H. Nakahara, A. A. Weiss, and S. Silver. 1979. Transposon A-generated mutations in the mercuric resistance genes of plasmid R100-1. *J. Bacteriol.* 140:167-181.
35. Nakahara, H., S. Silver, T. Miki, and R. H. Rownd. 1979. Hypersensitivity of Hg²⁺ and hyperbinding activity associated with cloned fragments of the mercurial resistance operon of plasmid NR1. *J. Bacteriol.* 140:161-166.

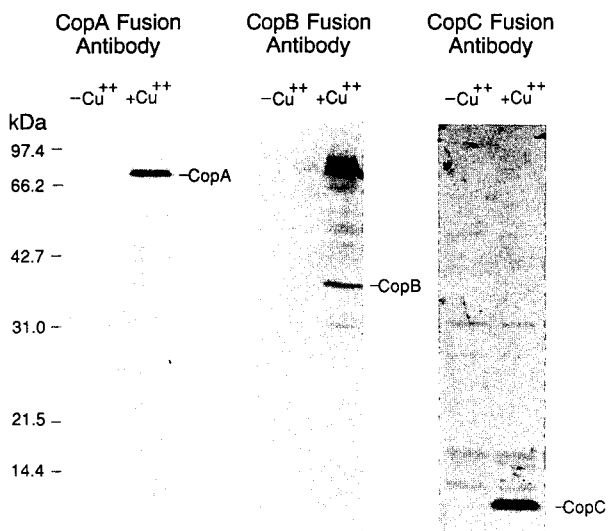


FIG. 1. Detection of Cop proteins from *P. syringae* pv. *tomato* strain PT23.2 by immunoblot analysis. Total proteins from strain PT23.2 grown without and with 0.2 mM CuSO_4 were separated by SDS/PAGE, transferred to nitrocellulose, and probed with antisera prepared against Cop fusion proteins.

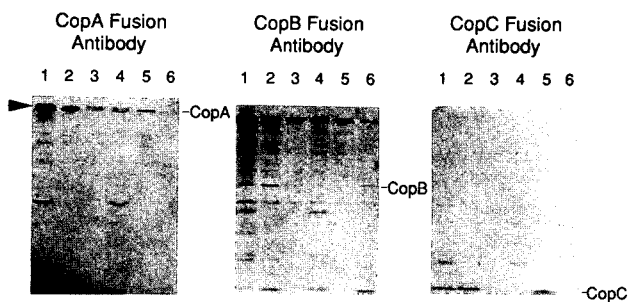


FIG. 2. Localization of Cop proteins in bacterial cells of strain PT23.2 by immunoblot analysis. Proteins of a whole cell extract (lanes 1), periplasmic fraction (lanes 2), cytoplasmic fraction (lanes 3), membrane fraction (lanes 4), supernatant of periplasmic fraction (lanes 5), and pellet of periplasmic 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.

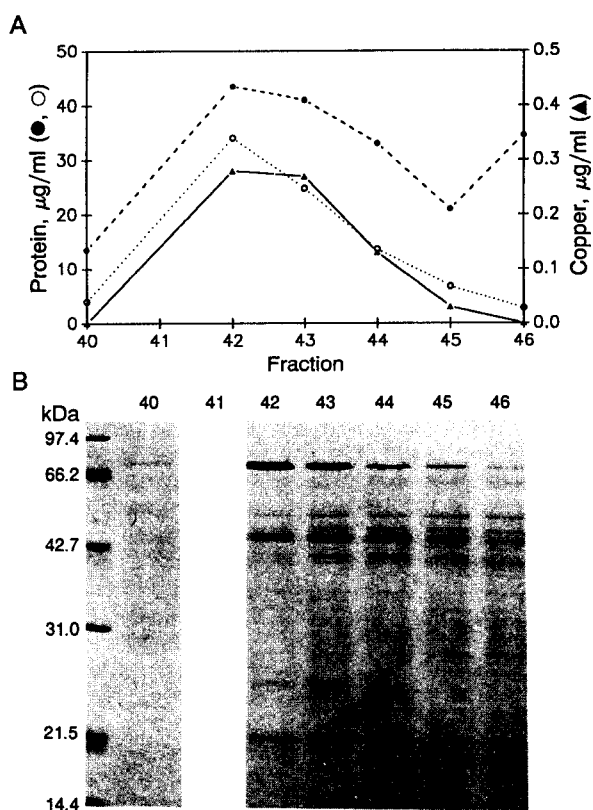


FIG. 3. Copper content of the CopA protein. (A) Concentrations of copper (▲), total protein (●), and CopA (○) in fractions eluted from a DEAE-Sepharose column. (B) Proteins of the fractions were separated by SDS/PAGE and stained with Coomassie brilliant blue. Fraction 41 was lost.

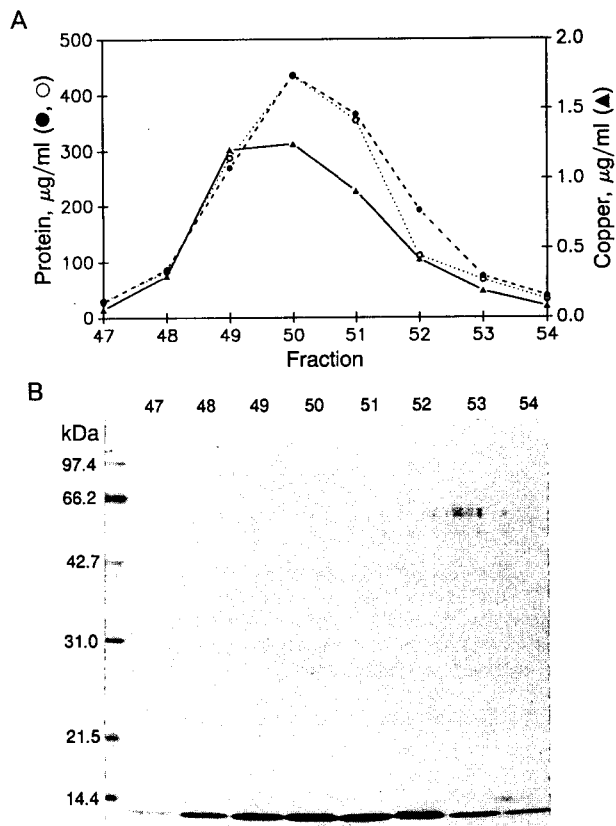


FIG. 4. Copper content of the CopC protein. (A) Concentrations of copper (▲), total protein (●), and CopC (○) in the fractions eluted from a Sephacryl S-200 column. (B) Proteins of the fractions were separated by SDS/PAGE and stained with Coomassie brilliant blue.

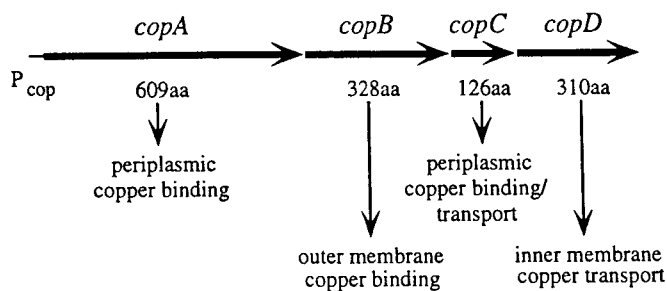


Fig. 5. The *cop* operon of *P. syringae* pv. *tomato* and probable locations and functions of its protein products. P_{cop} is the copper-inducible promoter that drives *copA-D*.

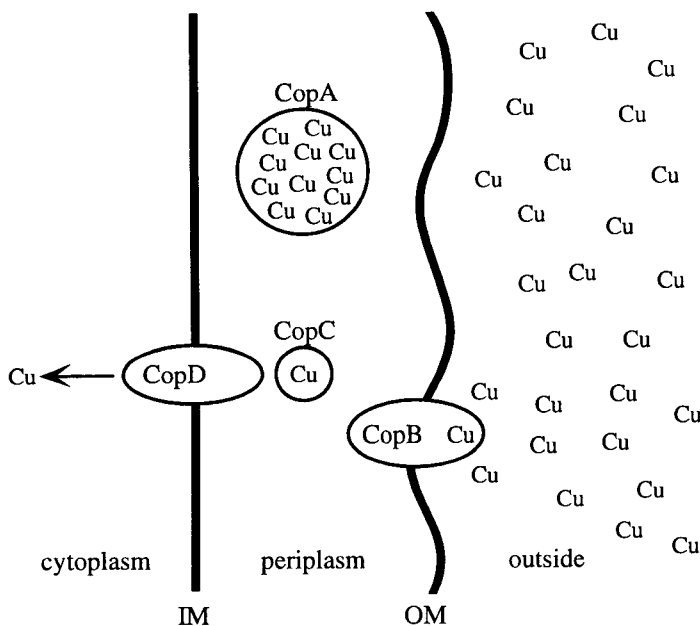


Fig. 6. Model for the mechanism of copper resistance in *P. syringae* pv. *tomato*. Copper is sequestered by the periplasmic proteins CopA and CopC and probably by the outer membrane protein CopB. CopC and CopD may also function in copper transport into the cytoplasm. IM=inner membrane. OM=outer membrane.

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 ($P < 0.05$).

Table 3. Copper sensitivity and accumulation in *P. syringae* obtained with cloned components of the *cop* operon.

Strain (plasmid) ^a	Plasmid characteristics and source ^b	CuSO ₄ MIC (mM)	ZnSo ₄ MIC (mM) ^c	Amt of copper accumulated (pg/g[dry wt])
PS61(pRK415)	Broad-host-range cloning vector derived from pRK404, Tc rd	0.4	0.08	9.2 ± 0.3
PS61 (pCOP2)	<i>copABCD</i> cloned in pRK404, Tc ^{re}	1.2	0.08	14.0 ± 1.2
PS61 (pCOP12A)	<i>copCD</i> cloned in pRK415, Tc ^{rf}	0.015	0.08	12.8 ± 0.9
PS61 (pCOP13A)	<i>copD</i> cloned in pRK415, Tc ^{rf}	0.4	ND	11.1 ± 1.1
PS61 (pCOP27A)	<i>copBCD</i> cloned in pRK415, Tc ^{rf}	0.1	ND	14.4 ± 0.4
PS61 (pCOP29A)	<i>copACD</i> cloned in pRK415, Tc ^{rf}	0.15	ND	13.1 ± 0.4
PS61 (pCOP65)	<i>copC</i> cloned in pRK415, Tc ^f	0.4	ND	11.0 ± 0.8

^a Each pCOP plasmid used in this study had *cop* genes cloned in the orientation expressed from the *lac* promoter of the vector.

^b Tc^r, tetracycline resistance.

^c ND, not determined.

^d Data from references 32 and 33.

^e Data from reference 6.

^f Data from reference 7.