Accumulation of Copper and Other Metals by Copper-Resistant Plant-Pathogenic and Saprophytic Pseudomonads

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Copper-resistant strains of Pseudomonas syringae carrying the cop operon produce periplasmic copper-binding proteins, and this sequestration outside the cytoplasm has been proposed as a resistance mechanism. In this study, strain PS61 of P. syringae carrying the cloned cop operon accumulated more total cellular copper than without the operon. Several other copper-resistant pseudomonads with homology to cop were isolated from plants, and these bacteria also accumulated copper. Two highly resistant species accumulated up to 115 to 120 mg of copper per g (dry weight) of cells. P. putida 08891 was more resistant to several metals than P. syringae pv. tomato PT23, but this increased resistance was not correlated with an increased accumulation of metals other than copper. Several metals were accumulated by both PT23 and P. putida, but when copper was added to induce the cop operon, there was generally no increase of accumulation of the other metals, suggesting that the cop operon does not contribute to accumulation of these other metals. The exceptions were aluminum for PT23 and iron for P. putida, which accumulated to higher levels when copper was added to the cultures. The results of this study support the role of copper sequestration in the copper resistance mechanism of P. syringae and suggest that this mechanism is common to several copper-resistant Pseudomonas species found on plants to which antimicrobial copper compounds are applied for plant disease control.

Antimicrobial copper compounds have been successfully applied to agricultural crop plants for disease control for over 100 years. Recently, however, copper-resistant plant-pathogenic and saprophytic bacteria have been isolated from several crop plants (1, 2, 5, 10, 13, 19, 24, 25), and the presence of these resistant bacteria has resulted in a reduction of disease control with copper sprays in several instances (1, 9, 19).

Copper resistance in Pseudomonas syringae pv. tomato, the cause of bacterial speck disease of tomato, is determined by a highly conserved 35-kb plasmid, pPT23D (3, 4, 8). The resistant bacteria accumulate copper as part of the resistance mechanism encoded by the cop operon located on pPT23D (4, 7). The operon (20) encodes periplasmic and outer membrane proteins, two of which are known to bind copper. CopA is a 72-kDa protein that binds 11 copper atoms, and CopC is a 12-kDa protein that binds one copper atom. Both proteins are abundant in the periplasm and probably help to prevent the entry of toxic copper ions into the cytoplasm (7). The cop operon is specifically induced by copper (21) and is not thought to provide resistance to other metal ions. However, the specificity of metal accumulation by these bacteria has not yet been tested.

Several other copper-resistant Pseudomonas species have been isolated recently from tomato and other plants, and the cop operon was shown to hybridize with DNA from several of these species; in addition, immunoblot analyses indicated that protein products related to cop proteins were produced from these bacteria when induced with copper (12). In this study, we determined whether copper accumulation was a common function of copper-resistant strains of these different species, and the specificity of metal accumulation in two species was examined.

MATERIALS AND METHODS

Bacterial strains and MIC determination. The sources and MICs of the bacteria used are listed in Table 1. pCOP2 (4) is a recombinant plasmid containing the cop operon cloned in the vector pRK404 (14). MICs of cupric sulfate were determined on mannitol-glutamate agar (18) supplemented with yeast extract at 0.25 g/liter (MGY agar) and CuSO4·5H2O at appropriate concentrations as described previously (12). For MICs of other metals, bacteria were suspended in sterile water from cultures on MGY agar plates at a standardized concentration, and 10 µl of the bacterial suspension was inoculated into culture tubes containing 5 ml of MGY broth with different concentrations of metals added. The MIC of each metal was expressed as the concentration that inhibited growth after 7 days of incubation at 28°C with shaking at 200 rpm. MICs were confirmed in a second experiment performed in the same manner.

Metal accumulation measurements. Bacteria were grown in MGY broth containing appropriate concentrations of added metals for 48 h. The cells were harvested by centrifugation, suspended in 1 ml of distilled water, and freeze-dried. The metal content of bacterial cells was determined after acid dissolution of the bacterial cells according to the method of Ganji and Page (16). Metal concentrations were measured with an atomic absorption spectrophotometer (model 3000; Perkin-Elmer).

Different metal salts were added at subinhibitory concentrations for accumulation studies. For P. putida 08891, the metal concentrations added to MGY medium were 0.1 mM CuSO4·5H2O, 0.5 mM Al(NO3)3·9H2O, 0.25 mM Na2HAsO4·7H2O, 0.1 mM CdSO4, 0.5 mM CaCl2·2H2O, 0.4 mM CrCl3·6H2O, 0.1 mM CoCl2·6H2O, 0.3 mM Fe(NO3)3·9H2O, 0.1 mM Pb(NO3)2, 0.3 mM MnSO4, 0.01 mM HgCl2, 0.3 mM NiSO4·6H2O, 0.2 mM Na2SeO3, 0.003 mM AgNO3, 0.8 mM NaCl, and 0.3 mM ZnSO4·7H2O. Metals were added at the same concentrations for P. syringae pv. tomato PT23, except for CoCl2·6H2O (0.028 mM), HgCl2 (0.00075 mM), NiSO4·6H2O (0.15 mM), and AgNO3.

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(0.0016 mM), which had to be used at lower concentrations to allow growth of this bacterium.

In one experiment, cells were treated with EDTA to remove metals bound to cell wall components. The cells were grown as described above, harvested by centrifugation, and resuspended in 2 ml of distilled water. Fifty milliliters of 20 mM EDTA was added to each cell suspension, and the mixture was vortexed for 2 min. The cell suspensions were centrifuged, and the cells were resuspended in 1 ml of distilled water and freeze-dried. Metal content was determined by atomic absorption spectrophotometry as described above.

RESULTS

Copper accumulation. When the cloned cop operon (pCOP2) was conjugated into a copper-sensitive strain of P. syringae (PS61), the resulting copper-resistant transconjugant accumulated more copper than the same strain with the cloning vector (pRK404) alone (Fig. 1). Copper accumulation by PS61(pCOP2) increased with higher levels of copper added to the medium, but PS61 without the cloned resistance genes would not grow at these higher levels.

Five other copper-resistant pseudomonads also accumulated copper (Fig. 2). P. putida 08891 and Pseudomonas sp. strain 02894 accumulated the highest levels of copper, and these strains were also highly copper resistant (Table 1). Copper accumulation increased with increasing concentrations of copper for most of the pseudomonads.

Accumulation of other metals. The copper-resistant strain PT23 of P. syringae pv. tomato accumulated several other metals when they were added at subinhibitory levels (Fig. 3A). For most metals, however, induction of the cop operon with 0.1 mM CuSO4 · 5H2O did not increase accumulation; the major exception was aluminum, which was accumulated at almost a threefold-higher level when copper was added to the medium in which PT23 was grown. Accumulation of cadmium and calcium increased to a lesser extent when copper was added. A decreased accumulation of chromium, lead, nickel, and selenium was observed when copper was added.

The copper-resistant strain P. putida 08891 had greater tolerance to cadmium, cobalt, mercury, nickel, and zinc than PT23 (Table 2), but it did not generally accumulate these metals to higher levels than PT23 (Fig. 3B). P. putida accumulated considerably less chromium than PT23. The addition of copper resulted in a large increase only in accumulation of iron for P. putida. Decreased accumulation of lead and, to a lesser extent, aluminum and selenium was observed with addition of copper to cultures of P. putida.

When cells of PT23 were harvested and washed with a solution of 20 mM EDTA, there was about a 50% loss of bound copper (Fig. 4A). Aluminum, calcium, lead, manganese, and nickel were mostly removed by the EDTA treatment, while cadmium, iron, selenium, sodium, and zinc were retained after the treatment. The copper-resistant strain P. putida 08891 also experienced large losses of calcium, lead, manganese, and nickel after the EDTA treatment but retained more aluminum than PT23 (Fig. 4B). This P. putida strain also retained copper, chromium, selenium, and sodium but lost more of its iron and zinc than PT23.

DISCUSSION

The cop operon is thought to confer copper resistance to P. syringae at least in part by sequestering copper in the periplasm with copper-binding proteins (7). In a previous study, strain PT23.3 of P. syringae pv. tomato, which is cured of the copper resistance plasmid pPT23D, accumulated less copper than the wild-type strain PT23 when subinhibitory levels of copper were added to the growth medium (7). In this study, we showed that P. syringae PS61 containing the cloned cop operon also accumulated more copper than PS61 lacking the operon, further supporting the direct role of the cop operon in accumulation as a part of the resistance mechanism of the bacterium.

Other copper-resistant pseudomonads were also shown to
FIG. 2. Accumulation of copper by several copper-resistant pseudomonads from tomato and other plants. Each value is the mean of three samples; error bars indicate one standard deviation.

accumulate copper, with two species accumulating up to 115 to 120 mg of copper per g (dry weight) of cells. Increasing copper accumulation with increasing exposure to copper was common to copper-resistant P. cichorii 07881, P. fluorescens 08908, P. putida 08891, Pseudomonas sp. strain 02894, and P. syringae pv. tomato PT23, and P. syringae pv. tomato PT23, suggesting that they have similar resistance mechanisms involving copper sequestration. A cop operon homolog has been detected in DNA from P. cichorii 07881, P. putida 08891, and Pseudomonas sp. strain 07887 (12), further suggesting that the copper resistance mechanisms of these bacteria and P. syringae are similar. While P. cichorii accumulated copper at levels similar to those of P. syringae, the other pseudomonads generally accumulated much higher levels of copper. Whether these different levels are due to variation in the efficiency of the cop system or due to the presence of other mechanisms of accumulation has not been determined.

Previous studies suggested that the cloned cop operon provided no increased resistance to heavy metals other than copper when conjugated into P. syringae (11), but the specificity of metal accumulation by these bacteria has not been tested. We hypothesized that these bacteria would selectively accumulate copper over other metals when the cop operon was induced by copper. P. syringae pv. tomato PT23 accumulated metals other than copper, but for most metals, this accumulation did not increase when the cop operon was induced. The major exception was aluminum,

![Diagram](image1.png)

FIG. 3. Accumulation of different metals by P. syringae pv. tomato PT23 (A) and P. putida 08891 (B) with and without added CuSO₄·5H₂O (0.1 mM). Each value is the mean of three samples; error bars indicate one standard deviation.

<table>
<thead>
<tr>
<th>Metal</th>
<th>MIC (mM) for strain:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PT23</td>
</tr>
<tr>
<td>Al(NO₃)₃·9H₂O</td>
<td>2.0</td>
</tr>
<tr>
<td>Na₃AsO₃·7H₂O</td>
<td>&gt;24</td>
</tr>
<tr>
<td>CdSO₄</td>
<td>0.4</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>&gt;24</td>
</tr>
<tr>
<td>CoCl₂</td>
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</tr>
<tr>
<td>CrCl₃</td>
<td>0.035</td>
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<tr>
<td>Fe(NO₃)₃·9H₂O</td>
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</tr>
<tr>
<td>Pb(NO₃)₂</td>
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<tr>
<td>HgCl₂</td>
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<td>NiSO₄·6H₂O</td>
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<tr>
<td>Na₂SeO₃</td>
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<tr>
<td>AgNO₃</td>
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<td>NaCl</td>
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</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* Concentrations above 24 mM were not tested.

TABLE 2. MICs of different metals for P. syringae pv. tomato PT23 and P. putida 08891
and this metal will therefore be included in future studies of
the specificity of metal accumulation by purified Cop pro-
teins. *P. putida* 08891, which carries plasmid and chromo-
somal *cop* homologs, accumulated metals other than copper,
but this accumulation generally did not increase when cop-
per was added. *P. putida* 08891 was more tolerant of
cadmium, cobalt, mercury, nickel, silver, and zinc than
PT23, but this increased tolerance was not generally corre-
lated with higher accumulation of these metals, suggesting
that this strain of *P. putida* has other metal resistance
mechanisms not necessarily involving accumulation.

These results suggest specificity for copper in the cop-
directed metal uptake, but this needs to be confirmed with
purified Cop proteins that have been stripped of copper by
mild acid or EDTA treatment. The sequences of the Cop
proteins are known (7, 20); they are not cysteine-rich pro-
teins like metallothioneins (26) and phytochelatins (17) that
would be expected to bind several different metal ions.
Instead, they are histidine- and methionine-rich peptides
that generally lack cysteine residues. One cysteine is present in a
probable type I copper site at the carboxy terminus of CopA
(23), but the other 10 copper atoms in this protein must be
bound to other sites, presumably involving histidine or
methionine residues, that may show specificity for copper.

Washing cells of *P. syringae* pv. tomato PT23 with an
EDTA solution removed about 50% of the accumulated
copper. This result is consistent with the observation that
a large amount of accumulated copper in this bacterium is
associated with the outer membrane, possibly bound by the
outer membrane CopB protein (7). Accumulated copper was
not removed by the same EDTA treatment for the copper-
resistant strain of *P. putida*, suggesting that the copper
is accumulated more internally. Western blot (immunoblot)
analysis indicated that this strain contains an abundant
protein that is related to the periplasmic CopA copper-
binding protein (12). Accumulation of some other metals was
probably associated with outer membrane components in
both species, since an EDTA wash removed large amounts
of metals such as aluminum, calcium, lead, manganese, and
nickel; these metals were generally not lost when cells were
washed only with water (data not shown), suggesting that
they were not loosely associated with extracellular polysac-
charides or outer membrane proteins (6, 15). An unusual result
of washing cells with EDTA was an apparent increase in
selenium accumulation in *P. syringae* and *P. putida* and an
increased chromium accumulation in *P. putida*. Since no
additional selenium or chromium was added during the
EDTA wash, it appears that the treatment had an effect on
the detection of the metals by atomic absorption spectropho-
tometry, but the reason for that effect is not clear.

The results of this study support the role of copper
sequestration in the copper resistance mechanism of *P.
syringae* and suggest that this mechanism is common to
several copper-resistant *Pseudomonas* species found on
plants, to which antimicrobial copper compounds are ap-
plied. Some of these species are commonly found as epi-
phytic populations on the surfaces of plants, and it is
possible that they contribute significantly to the complexing
of copper ions applied to leaf surfaces. Since free copper
ions are required for the toxic effects of copper bactericides
applied to plants (22), the presence of copper-accumulating
epiphytes might influence the effectiveness of this control
method.

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Occurrence and properties of copper-tolerant strains of *Pseu-
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