Occurrence and Properties of Copper-Tolerant Strains of *Pseudomonas syringae*
Isolated from Fruit Trees in California

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**ABSTRACT**


Approximately 40% of the ice-nucleation-active strains of *Pseudomonas syringae* isolated from asymptomatic leaves and flowers from almond and navel orange trees from orchards with a history of copper usage were able to grow in a culture medium amended with 0.32 mM CuSO₄. While more than one-half of the strains were highly sensitive to cupric ions, some strains could tolerate as much as 1.12 mM CuSO₄ in a culture medium. Prior exposure of copper-tolerant strains to sublethal concentrations of copper in a culture medium increased the fraction of cells that could survive a higher concentration of copper by more than 1,000-fold compared with cells not receiving copper pretreatment. The mean LC₉₀ of copper-tolerant strains in aqueous copper solutions (23 ppb Cu²⁺) was about five times that of copper-sensitive strains (4.7 ppb) when cells were assayed without prior exposure to Cu²⁺ in growth medium. The LC₉₀ of copper-tolerant strains increased to approximately 160 ppb when cells were grown in medium containing sublethal concentrations of CuSO₄. Copper-tolerant strains of *P. syringae* grew as rapidly on Cu(OH)₂-treated leaves as on nontreated leaves, while sensitive strains showed little growth. The size of established epiphytic populations of copper-sensitive but not copper-tolerant strains was reduced significantly on treatment of bean and almond leaves with Cu(OH)₂ under greenhouse and field conditions.

Copper-containing compounds, including Bordeaux mixture, cupric hydroxide, various formulations of basic copper sulfate, ammoniacal copper, and copper salts of fatty acids, have been widely used for the control of phytopathogenic bacteria and fungi (22). These materials were the first biocides used for disease control and are the only bactericides registered for use on most crops. While these materials are chemically stable and are not readily washed from plants (33,40), control often has been incomplete when they were applied as protectants (1,15,34) or as eradicants (2,12,36) of bacterial diseases. Copper compounds are considered general biocides because of the many potential sites of action of copper ions in biological systems. Copper ions may act as respiratory poisons or react with many fungal and bacterial proteins and kill the cells after sufficient exposure (16,33). Until recently, it was considered unlikely that resistance to copper ions would develop among either fungal or bacterial populations because of the general mode of action of this heavy metal.

*Pseudomonas syringae* can reduce the productivity of many plant species, including tree fruits such as pear, almond, and citrus, in several ways. Pathovars (14) of this species cause diseases, including blossom and twig blast, bacterial canker, and fruit spots, of several fruit tree species (13,15,18,42). The incidence of diseases caused by *P. syringae* is correlated with the epiphytic population of this species on plants before infection (21,25). This bacterial species can also limit fruit tree production by causing ice formation, which is an important factor in frost damage to these trees (3,4,6,17,26,28,29). Ice-nucleation-active (Ice⁺) strains of *P. syringae* and other bacteria initiate ice formation at temperatures only slightly below 0°C (17,26,29,31,35) on surfaces of sensitive plants that cannot tolerate ice formation within their tissues. While nonbacterial sources of ice nuclei may exist in some fruit trees and may be important in inciting frost damage under some conditions (7,19), treatments, including copper compounds, that reduced the numbers and/or the ice-nucleation activity of bacteria reduce frost damage to tree crops (27,30). Populations of *P. syringae* are generally low on young vegetative tissues (17,27,30,39), and copper treatments can inhibit the potential increases of the epiphytic population that would otherwise occur on plant surfaces, thereby reducing the probability of plant frost injury during subsequent periods of low temperatures (29,30). Knowledge of the sensitivity of epiphytic bacteria to copper ions

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is therefore needed to determine the potential for sustained use of copper compounds for both disease and frost control.

Resistance to copper has been observed recently among both saprophytic and phytopathogenic bacteria (1,2,8,9,23,36,41,43). Some strains of Xanthomonas campestris pv. vesicatoria, Pseudomonas syringae pv. tomato, and P. s. syringae, pathogens of pepper, tomato, and cherry, respectively, have recently been found to be resistant to the concentrations of copper ions that are sufficient to kill sensitive strains of these species (1,9,36,41,43). Copper-tolerant strains are thought to be poorly controlled by standard applications of copper compounds in some areas (1,36), although the fungicide Maneb (manganese ethylene bis-dithiocarbamate) has improved disease control when mixed with copper compounds (12,36). Only one study has provided information on the population dynamics of copper-tolerant and copper-sensitive strains in the presence or absence of copper deposits on leaves (41).

From indirect evidence, P. syringae strains in California appeared to be resistant to copper compounds. Complete control of strains of Ice" of P. syringae with copper compounds and significant improvement of control of Ice" strains of P. syringae on almond and citrus in California by copper-Maneb combinations, compared with copper alone, were observed (2,30). Therefore, a study to determine the susceptibility to copper bactericides of various Ice" strains of P. syringae isolated from various agricultural crops throughout California was initiated in this laboratory in 1983. Particular emphasis was placed on determining the variability of quantitative copper tolerance among strains, factors that influence the tolerance to copper ions, and the growth or survival of strains of P. syringae varying in tolerance to copper ions on greenhouse and field-grown plants treated with copper compounds.

**MATERIALS AND METHODS**

**Bacterial strains.** Strains of P. syringae were isolated from leaves and fruit spurs collected from trees in eight citrus and 14 almond orchards with a history of copper sprays and from four citrus and 12 almond orchards that had not been treated with copper bactericides within the last 3 yr. Leaf or fruit spur samples (20 g) were placed in Erlemeyner flasks containing 200 ml of sterile washing buffer (0.1 M potassium phosphate buffer, pH 7.0, containing 0.1% Bactopeptone), sonicated in an ultrasonic cleaner (Branson Sonic 52, Branson Sonic Power, Co., Danbury, CT) for 7 min, and appropriate 10-fold serial dilutions were plated onto King's medium B (KB) (24) containing 100 μg/ml of cycloheximide. Ice" bacteria were isolated by a replica-freezing technique from dilution plateings of leaf washings and subsequent purification by several single colony transfers in a manner similar to that described by Lindow et al. (28). Strains tentatively identified as P. syringae showed fluorescent pigment production on KB, negative arginine dihydrolase (45) and oxidase (44) reactions, and positive tobacco hypersensitivity and ice-nucleation (28) reactions, and were unable to rot potato. Strains were stored at −80°C in a solution of 15% (w/v) glycerol.

Mutant strains resistant to the antibiotic rifampicin were selected as single colonies found 4 days after 0.1 ml of a suspension of a parental strain containing 10^9 cells per milliliter was spread onto plates of KB containing 100 μg/ml of rifampicin (KBR) and incubated at 30°C. Individual mutant colonies were purified by streaking on KBR, were re-tested for the biochemical and physiological characteristics listed above, and were demonstrated to have growth rates similar to the corresponding parental strains on KB. Sources and characteristics of copper-sensitive (82-8, 80-5) and copper-tolerant (81-23, 75-3, 68-1, 83-6, E-3) strains of X. c. vesicatoria and copper-tolerant strain P1-23 of P. s. syringae as well as strain B728a of P. s. syringae have been reported (9,32,36).

**Culture medium.** A low-complexing mineral salts medium (CYE), similar to that described by Zevenuihen et al. (47), containing casitone, 1.7 g/L; yeast extract, 0.35 g/L; glycerol, 2.0 g/L; and purified agar (Difco Laboratories, Detroit, MI), 15 g/L, was used for assays of copper tolerance in culture. Copper from a filter-sterilized (0.45 μm) stock solution of 160 mM CuSO_4, was added by drops to autoclaved CYE medium cooled to 50°C immediately before pouring, to achieve the desired copper concentration. Copper gradient plates were prepared by dispensing 15 ml of CYE medium containing 1.12 mM CuSO_4 in a 100-×15-mm petri dish, then titling the dish at such an angle that the medium was less than 1 mm thick on the upper side of the raised portion of the dish. After the medium solidified, the dish was returned to a horizontal position, and an additional 15 ml of CYE medium containing no copper was added. Gradient plates were stored at 4°C for at least 8 h before use.

**In vitro copper sensitivity of bacterial strains.** Copper sensitivity of bacteria was determined in vitro by two different methods. In all assays, bacterial strains were recovered from storage at −80°C, streaked onto KB, and incubated for 24 h at 30°C. Bacteria were harvested with a loop, suspended in sterile distilled water, and adjusted by dilution in an optical density (OD) of approximately 0.2 at a wavelength of 600 nm measured with a Spectronic 20 spectrophotometer (Bausch and Lomb). A loopful of each of these bacterial suspensions (containing about 3 × 10^8 bacterial cells per milliliter) was streaked perpendicular to the gradient of copper ions in CYE-copper-gradient plates and was incubated at 30°C for approximately 72 h. Cells harvested from a loop of the portion of CYE-copper-gradient plates containing the highest concentration of copper at which the bacteria grew were transferred to sterile distilled water, and the suspension was adjusted to the desired cell density after spectrophotometric determination of cell concentration as described previously. To measure the minimum concentration of copper that prevented cell growth, bacterial suspensions were adjusted to approximately 10^7 cells per milliliter in distilled water, and a 10-μl drop of each suspension was placed on plates of CYE medium containing the following concentrations of CuSO_4: 0, 0.08, 0.16, 0.32, 0.48, 0.64, 0.80, 0.96, or 1.12 mM. Twelve strains, including one well-characterized copper-sensitive strain and one copper-tolerant strain, were tested on each plate of amended CYE medium. Cultures were incubated for 96 h at 30°C, and visible confluent growth of a bacterial strain on copper-amended CYE medium was interpreted as tolerance of the corresponding concentration of CuSO_4 in CYE medium. Each test was repeated three times. To determine the fraction of colonies of a given strain of P. syringae that was able to survive a given concentration of copper ions, one 10-μl drop of each of seven, 10-fold serial dilutions of a suspension of approximately 10^7 cells per milliliter was plated on each of three plates of CYE medium amended with a given concentration of CuSO_4 and incubated as described. Presence of individual colonies or a confluent aggregate of colonies was determined 96 h after inoculation. The fraction of surviving cells was estimated from the number of colonies found in samples from each dilution on CuSO_4-amended CYE medium divided by the number of colonies from corresponding samples found on unamended CYE medium. The mean fraction of surviving cells was derived from the average colony counts on the three plates of a given copper concentration in each of three replicate experiments.

Bacteria also were assayed for tolerance to copper ions when suspended in distilled water. The highest levels of copper tolerance were induced by culturing strains on CYE medium containing sublethal concentrations of copper (0.1 mM CuSO_4 for copper-sensitve strains or 0.36 mM CuSO_4 for copper-tolerant strains) before assay. “Uninduced" cells were harvested from unamended CYE medium. Cells were suspended at a concentration of 10^8 cells per milliliter in tubes of sterile distilled water that contained various concentrations of copper ions (supplied as CuSO_4) placed on a rotary shaker for 1 h at 30°C, and then plated on KB. The probit of the percentage of cell survival was regressed against the logarithm of copper ion concentration for each strain, and mean lethal concentration (LC50) values were obtained by interpolation to probit = 5.0 (20). The mean of LC50 values was determined from three replications of each strain.

**Bacterial growth and survival on plants.** The growth and survival of different strains of P. syringae on plants treated with
Cu(OH)₂ (Kocide 101, Kocide Chemical Company, Houston, TX) and a manganese and zinc coordination production of ethylene bis-dithiocarbamate (Dithane M-45, Rohm & Haas, Philadelphia, PA) were determined in both greenhouse and field studies. In greenhouse studies, common bean (Phaseolus vulgaris L. 'Bush Blue Lake 274') was grown, eight plants per pot, in a sand/peat (1:1) mixture in 10-cm-diameter plastic pots. Growth of bacteria on copper-treated bean leaves was determined by applying Cu(OH)₂ at concentrations of either 0.6, 1.5, or 4.8 g/L with an air-pressurized handheld sprayer to runoff 1 h before inoculation of 2-wk-old plants (~30 cm high) with aqueous bacterial suspensions. Some plants also were treated with a mixture of 0.6 g/L of Cu(OH)₂ and 0.6 g/L of manganese-zinc ethylene bis-dithiocarbamate 1 h before inoculating plants with bacteria. Rifampicin-resistant bacterial strains were transferred from CYE-copper-gradient plates to KBR and incubated for 72 h at 30 C. Bacteria were suspended in sterile distilled water at a concentration of 1 x 10⁷ cells per milliliter and sprayed on untreated bean plants to runoff with an air-pressurized atomizer. Plants were then incubated in a moist chamber at about 21 C and misted intermittently to facilitate bacterial growth. To estimate bacterial population size, 25-30 leaves from each of four pots (replicates) of plants were harvested from each treatment and placed in washing buffer, and cells were removed by sonication as described. Appropriate serial dilutions of leaf washings were plated on KBR containing 100 μg/ml of cycloheximide. To determine the toxicity of Cu(OH)₂ on populations of P. syringae previously established on bean plants, cells of selected strains were harvested from KB and inoculated on tomato plants as described, incubated for 48 h in a moist chamber at 21 C, and the plants were then sprayed with a suspension of 1.2 g/L of Cu(OH)₂. Bacterial populations were determined on KBR (as described) immediately before and 3 h after treatment with Cu(OH)₂.

Survival of selected strains of P. syringae (differing in copper tolerance in culture) was determined on Cu(OH)₂-treated almond (Prunus dulcis 'Nonpareil') at the University of California, Westside Field Station, located near Five Points in the San Joaquin Valley of California. The experimental plot consisted of a randomized complete block design with four replications. Each replication consisted of one mature almond tree. At least one nontreated tree separated each treated tree within and between blocks. Strains of P. syringae grown for 3 days on KRB plates at 24 C were harvested by washing, suspended in tap water, and applied at a concentration of approximately 5 x 10⁶ cells per milliliter (~3 L/tree) to trees at approximately 50% bloom on 16 March 1986. The application was done within 30 min of harvest with a backpack mist-blower similar to that used in a previous study (30). Cupric hydroxide (1.2 g/L) or a mixture of Cu(OH)₂ and manganese-zinc ethylene bis-dithiocarbamate (0.6 g/L) was applied to a subset of trees at the rate of 26 L/tree with a piston-pressurized gun-sprayer within 4 h after inoculation with bacteria. Population sizes of rifampicin-resistant strains of P. syringae were determined from dilution-plating of washings from 20 bulked fruiting spurs for each replication on KBR containing 100 μg/ml of cycloheximide as described.

Statistical methods. Statistical computations were made by using software provided by Statistical Analysis Systems (SAS) (release 5.16; SAS Institute, Inc., Cary, NC). The SAS general linear models procedure was used to perform analysis of variance on LCB estimates and on log-transformed bacterial population sizes. Mean comparisons were made with Fisher's unprotected LSD test. This test controls the comparison-error rate.

RESULTS

In vitro copper tolerance of strains of P. syringae. Strains of P. syringae isolated as epiphytes from healthy leaves and flowers of almond and navel orange in California exhibited variable levels of tolerance to copper ions added to CYE medium, a culture medium that has limited cupric ion binding capacity. Thirty-three percent and 45% of the 126 strains of P. syringae, collected from navel orange and almond, respectively, were tolerant to more than 0.32 mM CuSO₄ in this medium (Fig. 1). Although a few strains were unable to tolerate the presence of even 0.16 mM CuSO₄ in CYE medium, no strains grew in CYE medium containing over 1.12 mM CuSO₄. Frequency of copper tolerance among the strains of P. syringae tested was distinctly bimodal. Whereas, over one-half of the tested strains were highly sensitive to cupric ions, the remaining strains had a high mean tolerance to cupric ions in CYE medium (Fig. 1). Qualitatively similar variations in copper tolerance also were observed in strains of P. syringae isolated from pear orchards in California (data not shown). While copper-tolerant strains of P. syringae were detected on both almond and citrus, a higher percentage of almond strains exhibited a high level of tolerance to CuSO₄ in CYE medium (>0.6 mM CuSO₄).

Cell populations of different strains of P. syringae showed distinct copper-concentration thresholds on which cell growth stopped with further addition of CuSO₄ to CYE medium. A large fraction of cells of most strains survived concentrations of CuSO₄ in CYE medium that approached the threshold concentration at which cell death began (Figs. 2 and 3). For example, while over 10% of the cells in populations from strains Al513R, Al489, Al529, and Al436 survived 0.8, 0.8, 0.48, and 0.08 mM CuSO₄.

![Fig. 1. Frequency distribution of maximum concentrations of CuSO₄ in CYE medium at which different strains of Pseudomonas syringae collected from almond (solid bars) or navel orange (hatched bars) grew.](image1.png)

![Fig. 2. Fraction of cells in populations of strains Al513R (○), Al529 (□), and Al436 (●) of Pseudomonas syringae that survived exposure to the increasing amounts of copper ions (as CuSO₄) added to CYE medium.](image2.png)
in CYE medium, respectively, only less than about \(10^{-4}-10^{-5}\) of the population of cells of each strain survived when an additional 0.16 mM CuSO\(_4\) was added to the medium (Figs. 2 and 3). In contrast, the proportion of the cells of strain B728a that tolerated increasing concentrations of CuSO\(_4\) in CYE medium decreased progressively with increasing concentration of CuSO\(_4\) above 0.24 mM and did not exhibit a distinct threshold of tolerance of CuSO\(_4\) as did most other strains (Fig. 3).

Prior exposure of several strains of *P. syringae* to sublethal concentrations of CuSO\(_4\) (0.2 mM) on CYE medium caused a proportion of the induced cells to survive concentrations of copper that killed nontreated (untreated) cells (Fig. 3). More than a 1,000-fold increase was observed in the fraction of induced cells of most copper-tolerant strains that grew on CYE medium containing a given concentration of CuSO\(_4\) compared with cells not receiving copper pretreatment (Fig. 3). However, previous exposure to CuSO\(_4\) had no effect on the ability of several copper-tolerant strains and all sensitive strains to grow in CYE medium containing near lethal amounts of CuSO\(_4\) (data not shown).

Strains of *P. syringae* differed greatly in their survival in aqueous solutions of CuSO\(_4\). Strains that grew only on CYE medium containing low concentrations of CuSO\(_4\) were highly sensitive to CuSO\(_4\) in distilled water. Conversely, the most tolerant strains on copper-amended CYE medium also survived at the highest concentrations of CuSO\(_4\) tested in distilled water. The mean LC\(_{50}\) of copper-tolerant strains of *P. syringae* (23 ppb) was about five times higher than that of copper-sensitive strains (4.7 ppb) when cells were assayed without prior exposure to Cu\(^{2+}\) in the growth medium (Table 1). However, the mean LC\(_{50}\) of copper-tolerant strains increased to about 160 ppb when cells were grown on a medium containing sublethal concentrations of CuSO\(_4\). In contrast, the LC\(_{50}\) of copper-sensitive strains treated in this way was no higher than cells not previously exposed to CuSO\(_4\) in growth media (Table 1). Exposure to CuSO\(_4\) in growth media therefore induced cells to express a sevenfold increase in the level of copper ions that could be tolerated compared to uninduced cells of copper-tolerant strains. Strain B728a of *P. syringae*, isolated from bean in Wisconsin, was significantly more tolerant of copper ions than sensitive fern tree strains when induced by exposure to sublethal doses of CuSO\(_4\) in CYE medium, but was much less tolerant of Cu\(^{2+}\) than other copper-tolerant strains (Table 1). The most copper-tolerant strains of *P. syringae* survived exposure to concentrations of copper ions in distilled water as high as those tolerated by most copper-tolerant strains of *X. c. vesicatoria* and higher than that of a copper-tolerant strain of *X. c. vesicatoria*.

![CuSO\(_4\) concentration in CYE (mM)](image)

**Fig. 3.** Fraction of cells in populations of strains B728a (A), A1529 (B), and A1489 (C) of *Pseudomonas syringae* that survived exposure to the increasing amounts of copper ions added to CYE medium when inoculum was harvested from medium containing no added copper ions (O), or containing 0.2 mM CuSO\(_4\) (●).

<table>
<thead>
<tr>
<th>Strain tested</th>
<th>Copper ion tolerance* LC(_{50}) (ppb)</th>
<th>Uninduced*</th>
<th>Induced*</th>
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<td>6.8 e</td>
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<td>8.5 cd</td>
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<td>E-3</td>
<td>32.1 ab</td>
<td>408.9 a</td>
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*Means in each column followed by the same letter do not differ significantly by Fisher's unprotected LSD-means comparison test.

*Cells were harvested from unamended CYE medium.

*Cells of copper-sensitive strains (LC\(_{50}\) < 10 ppb) were harvested from CYE medium containing 0.1 mM Cu\(^{2+}\) (as CuSO\(_4\)). Cells of copper-tolerant strains were harvested from CYE medium containing 0.36 mM Cu\(^{2+}\) (as CuSO\(_4\)).
P. s. tomato (Table 1). The maximum concentration of CuSO₄ in aqueous solutions in which bacterial strains survived was not directly proportional to the maximum concentration of CuSO₄ in CYE medium in which growth occurred (Table 1; Figs. 2 and 3).

Growth of strains of P. syringae on copper-treated plants. Strains of P. syringae varied in their ability to grow on plants treated with a copper-containing bactericide. Copper-tolerant strains grew as rapidly on plant surfaces treated with 1.5 g/L of Cu(OH)₂ (a typical field rate of this bactericide) as on plants not treated with Cu(OH)₂ (Fig. 4). However, about 90-95% more of the cells of copper-tolerant strains died shortly after application to plants treated with Cu(OH)₂ compared to cell survival on nontreated leaves (Fig. 4). Population sizes of copper-tolerant strains were similar on Cu(OH)₂-treated and nontreated leaves within 3-4 days after inoculation. In contrast, copper-sensitive strains of P. syringae were unable to grow on plants treated with Cu(OH)₂ or exhibited growth rates much lower than those on untreated plants (Fig. 5). The population sizes of copper-tolerant strains were from 100 to more than 10³ times higher than those of copper-sensitive strains after 4 days of growth on Cu(OH)₂-treated plants (Figs. 4 and 5).

The ability of copper-tolerant strains to grow on Cu(OH)₂-treated plants could not be overcome simply by increasing the amount of Cu(OH)₂ applied to leaves (Fig. 6). Little difference was observed in either the growth rate of copper-tolerant strains of P. syringae on leaves or the population size of such strains after 3 days of growth on nontreated leaves and on leaves treated with 0.6 g/L or 4.8 g/L of Cu(OH)₂ (0.5 and four times the recommended disease control concentrations, respectively) (Fig. 6). The fraction of cells of copper-tolerant strains that survived initially on Cu(OH)₂-treated plants decreased with increasing amounts of Cu(OH)₂ applied, but surviving cells rapidly colonized treated plants (Fig. 6). In contrast, increasing the amount of Cu(OH)₂ applied to leaves decreased the maximum population size that copper-sensitive strains attained on treated plants (Fig. 7). Both copper-tolerant and copper-sensitive strains of P. syringae grew slowly and attained much lower population sizes on plants treated with a mixture of Cu(OH)₂ and manganese-zinc ethylene bis-dithiocarbamate than on plants treated with an equivalent rate of Cu(OH)₂ alone (Figs. 6 and 7).

Killing of strains of P. syringae previously established on plants by Cu(OH)₂. While large decreases in the population size of copper-sensitive P. syringae occurred when Cu(OH)₂ was applied to leaves before application of bacterial strains, Cu(OH)₂ was much less effective at killing established populations of such strains. Viable populations of copper-sensitive strains of P. syringae that were recovered from colonized bean leaves in a greenhouse decreased to about 80% of original levels within 3 h after treatment of leaves with a Cu(OH)₂ suspension (Table 2). No measurable death of copper-tolerant strains occurred on leaves after application of Cu(OH)₂.

Cu(OH)₂ sprays were an effective eradicant of copper-sensitive but not copper-tolerant strains of P. syringae on plants under field conditions. The population size of copper-sensitive P. syringae on almond trees was generally reduced over 90% after treatment with Cu(OH)₂ under field conditions (Table 3). In contrast, little, if any, reduction of population size of copper-tolerant strains occurred after application of Cu(OH)₂ unless manganese-zinc ethylene bis-dithiocarbamate was also applied (Table 3). Similar results were observed in other field trials on almond, navel orange, and pear trees (data not shown).

DISCUSSION

A bimodal distribution of copper tolerance was discovered among strains of P. syringae tested for copper tolerance on CYE
medium, indicating that at least two separate classes of resistance exist. Although a majority of the strains were tolerant only of low concentrations of CuSO₄ (<0.16 mM) in CYE medium, a number of strains, especially those isolated from almond fruiting spurs, were tolerant to much higher concentrations of added copper. Up to 40% of the strains of _P. syringae_ isolated from almond were able to tolerate concentrations of CuSO₄ in CYE medium that were inhibitory to the most tolerant strains isolated from citrus leaves (Fig. 1), even though higher amounts of copper compounds were applied to citrus compared with almond trees. Differences in copper tolerance may, therefore, reflect ecological variations in the strains selected by the host plant. Substantial differences in the growth and survival characteristics of various epiphytic strains of _P. syringae_ have been documented (17,18).

The wide range of distinct, reproducible tolerances to CuSO₄ on CYE medium exhibited by the different strains suggests either that more than one gene is responsible for this phenotype, as observed in _P. s. tomato_ (38), or that other properties of the strains conditioned the tolerance to copper conferred by such genes. For example, the strains could differ in the amount and type of extracellular polysaccharide, which has been shown to confer partial copper tolerance (11).

The level of CuSO₄ tolerated by a given strain was highly reproducible under a given set of conditions, but was greatly influenced by culture conditions and by the assay procedure itself. A more quantitative assessment of variations in copper tolerance among cells of a given genotype was obtained in this study, because the probit survival response of a population of cells exposed to different concentrations of CuSO₄ was measured. The LC₅₀ of other

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**Fig. 6.** Population size of copper-tolerant strains AI489 (A), AI513R (B), and B728a (C) of _Pseudomonas syringae_ on nontreated bean leaves (○), leaves treated with 0.6 g/L of Cu(OH)₂ (▲), 4.8 g/L of Cu(OH)₂ (●), or with 0.6 g/L Cu(OH)₂ plus 0.6 g/L of Maneb (■) at the times after inoculation shown on the abscissa. The vertical bars represent the standard deviation of the determination of mean log bacterial population size.

**Fig. 7.** Population size of copper-sensitive strains AI423 (A), and AI487 (B) of _Pseudomonas syringae_ on nontreated bean leaves (○) leaves treated with 0.6 g/L of Cu(OH)₂ (▲), 4.8 g/L of Cu(OH)₂ (●), or with 0.6 g/L Cu(OH)₂ plus 0.6 g/L of Maneb (■) at the times after inoculation shown on the abscissa. The vertical bar represents the standard error of the determination of mean log bacterial population size.
copper-tolerant bacterial species that were measured in this study in distilled water were significantly lower (up to 80-fold) than reported (9,36). Methodological differences may account for the discrepancy in the reported tolerances of Cu^{2+}. Increasing the concentration of \textit{X. c. vesicatoria} cells to the level tested by Marco and Stall (5 × 10^{13} cells ml^{-1}) (36) in aqueous solutions resulted in threshold copper tolerances (concentrations at which at least one cell survived) similar to the reported values. The cupric ion has been reported to be the toxic form of copper (5,16,47). The highly reactive cupric ion can bind with a multitude of chemical species present in whole and lysed cells, and in tap water, to form non-toxic copper complexes. With higher numbers of bacterial cells in an aqueous suspension, a higher concentration of CuSO_{4} is necessary to overcome the complexing ability of the bacteria. The apparent tolerance of strains of \textit{P. syringae} to more than 100 times the concentration of Cu^{2+} in amended CYE medium compared to that in distilled water is probably due to the complexing of the toxic cupric ion by components of the medium itself into copper species that are not toxic to the bacterium. Increasing the concentration of CuSO_{4} added to this medium probably causes the amount of toxic-free cupric ions present to increase in a more than proportional manner (47). While the true sensitivity of a bacterial strain to cupric ions is best measured in distilled water, a rapid estimate of the relative sensitivity of different bacterial strains to copper may be obtained by scoring for growth on CYE medium amended with various concentrations of CuSO_{4}. Accurate measurements of the amount of free Cu^{2+} in the CYE medium have been made and should permit the rapid quantitative estimation of the copper tolerance of test strains by testing growth on amended CYE medium (O. Menkissoglio and S. E. Lindow, unpublished data).

Exposure of cells of copper-tolerant strains to sublethal concentrations of CuSO_{4} in growth medium resulted in an increase in tolerance to copper ions in distilled water. This could be because of amplification of a conjugal plasmid in response to copper stress, as has been reported by Bender and Cooksey (9) for \textit{P. s. tomato} and by Stall et at (43) for \textit{X. c. vesicatoria}. Mellano and Cooksey (38) recently reported that a copper-resistant operon from \textit{P. s. tomato} is inducible by copper ions. We, however, found that, while copper-tolerant strains of \textit{P. syringae} and of \textit{X. c. vesicatoria} showed significantly higher tolerance to copper ions than sensitive strains even in the absence of prior exposure to copper ions, strain PT-23 of \textit{P. s. tomato} did not (Table 1). Genes involved in copper tolerance and/or their manner of regulation appear to differ between these species and pathovars (10; S. E. Lindow, unpublished data). A low constitutive level of tolerance of Cu^{2+} ions shown by cells of \textit{P. syringae} may be important in the process by which cells are induced to express higher levels of copper tolerance upon sensing higher concentrations of copper ions. It will be necessary to study this induction phenomenon further in culture and in leaves to elucidate the basis for survival of at least a part of the cell population after the sudden deposition of large amounts of copper on leaves.

Threshold levels of copper tolerated by strains of \textit{P. syringae} on copper-amended CYE medium and their mean tolerance of copper in distilled water correlated well with their ability to survive and grow on plants treated with various copper compounds. Only copper-tolerant strains of \textit{P. syringae} (LC_{50} > 15 ppb Cu^{2+}) grew after inoculation onto Cu(OH)_{2}-treated leaves (Figs. 4–7) or survived when treated with Cu(OH)_{2} after colonization of plants had occurred (Tables 2 and 3). Strains of copper-tolerant \textit{P. s. syringae} from cherry were recently shown to be less effectively eradicated from Cu(OH)_{2}-sprayed leaves than sensitive strains (41), but growth of these strains after application to copper-treated leaves has not been previously demonstrated.

A transient reduction in the viable population size of copper-tolerant strains of \textit{P. syringae} occurred immediately after inoculation onto copper-treated bean leaves with high concentrations of copper (Figs. 4 and 5). The Cu(OH)_{2} deposits on such leaves were probably not uniform. Small areas of the leaf may have had little or no Cu(OH)_{2} deposited, and the amount of Cu(OH)_{2} found in leaf openings such as stomata, which may harbor bacteria, is likely to be low. A small percentage of inoculant cells may have survived on areas of the leaf where the copper deposit was low, and the maximum concentration of free copper ions was less than the maximum level tolerated by the bacteria. Cells inoculated onto plants in our experiments were not induced to maximum levels of copper tolerance before inoculation. The surviving cells, however, were probably induced to express a high level of copper tolerance by the sublethal concentrations of copper to which they were exposed. These induced cells probably then multiplied at an equivalent rate and reached similar maximum populations as cells on bean leaves not treated with copper. Because the concentration of cupric ions, and not the amount of Cu(OH)_{2}, determines toxicity to the bacterial cell, increasing the amount of Cu(OH)_{2} applied to leaves to four times the recommended rate did not inhibit the growth of copper-tolerant strains of \textit{P. syringae} inoculated onto treated bean leaves (Fig. 6). The concentration of cupric ions on leaves is not directly

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Copper tolerance</th>
<th>Cu(OH)_{2} treatment</th>
<th>Bacteria recovered (log [cells per gram])</th>
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<tr>
<td>A1436</td>
<td>–</td>
<td>–</td>
<td>4.75 a 4.32 abc</td>
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<td>A1423</td>
<td>–</td>
<td>+</td>
<td>2.04 c 2.54 cfe</td>
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<td>–</td>
<td>2.57 a 4.69 a</td>
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<td>+</td>
<td>+</td>
<td>1.49 b 2.08 fg</td>
</tr>
<tr>
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<td>–</td>
<td>–</td>
<td>3.77 a 4.37 abc</td>
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<tr>
<td>A1448</td>
<td>+</td>
<td>–</td>
<td>2.06 bc 3.54 bdef</td>
</tr>
<tr>
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<tr>
<td>A1448</td>
<td>+</td>
<td>–</td>
<td>3.05 d 4.06 abcde</td>
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<tr>
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<td>–</td>
<td>–</td>
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<td>+</td>
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<tr>
<td>A1513</td>
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<td>–</td>
<td>3.33 ab 2.67 defg</td>
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<td>–</td>
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<td>A1513</td>
<td>+</td>
<td>+</td>
<td>3.33 ab 2.67 defg</td>
</tr>
</tbody>
</table>

* Rifampicin-resistant \textit{P. syringae} strain applied to trees.
* Copper-tolerant strains grown on CYE medium containing >0.36 mM Cu^{2+} (as CuSO_{4}) and had an LC_{50} for Cu^{2+} of >50 ppb when assayed in distilled water containing CuSO_{4}.
* Cu(OH)_{2} (1.2 g/L) was applied to a subset of the trees 4 h after inoculation with bacterial strains on 23 March and again on 10 April 1986.
* Numbers in the same column followed by the same letter are not significantly different (P = 0.05), according to Duncan’s multiple range test.
* Manganese-zinc ethylene bis-dithiocarbamate was applied (0.6 g/L) 1 h after application of Cu(OH)_{2}.

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**TABLE 3. Survival of copper-tolerant and copper-sensitive \textit{Pseudomonas syringae} strains on almond trees in the field**

- Copper-tolerant strains grown on CYE medium containing >0.36 mM Cu^{2+} (as CuSO_{4}) and had an LC_{50} for Cu^{2+} of >50 ppb when assayed in distilled water containing CuSO_{4}.
- Cu(OH)_{2} (1.2 g/L) was applied to a subset of the trees 4 h after inoculation with bacterial strains on 23 March and again on 10 April 1986.
- Numbers in the same column followed by the same letter are not significantly different (P = 0.05), according to Duncan’s multiple range test.
- Manganese-zinc ethylene bis-dithiocarbamate was applied (0.6 g/L) 1 h after application of Cu(OH)_{2}.
proportional to the amount of Cu(OH)₂ present (O. Menkissoglu and S. E. Lindow, unpublished data). In contrast, there was a concentration-dependent increase in death of copper-sensitive strains with increasing amounts of Cu(OH)₂ applied to leaves (Fig. 7). The cupric ion concentration in most habitats on treated leaves where P. syringae exists appears to be higher than the concentrations tolerated by these sensitive strains. Between 0.1 and 1% of the cells of copper-sensitive strains appear to occur at sites where the concentration of cupric ions, after uniform application of Cu(OH)₂ at the standard rate, is less than the LC₅₀ (Fig. 7). The LC₅₀ of cupric ions at about 90–95% of such protected sites appears to be exceeded when the rate of Cu(OH)₂ applied is increased fourfold (Fig. 7).

P. syringae cells that have colonized leaves (even in the absence of applied copper) appear to be less sensitive to copper ions than laboratory-cultured cells. While, on average, about 90% of laboratory-cultured cells of copper-tolerant strains of P. syringae were killed when applied to Cu(OH)₂-treated plants (Figs 4–6), no significant death occurred when Cu(OH)₂ was applied to plants previously colonized by these strains (Tables 2 and 3). Similarly, over 99.5% of laboratory-cultured cells of copper-sensitive strains were killed when applied to Cu(OH)₂-treated leaves (Figs 5 and 7), while only about 90% of cells of these strains were killed when Cu(OH)₂ was applied to plants previously colonized by these strains (Table 2). Some, but probably not all, of the cells of copper-sensitive and tolerant strains that survived eradicative applications of Cu(OH)₂ may have colonized portions of leaves not readily accessible to chemical applications. Cells on colonized plants also may survive better than laboratory-cultured cells if they are physiologically different, such as by accumulating extracellular polysaccharides in a slime layer around the cells. Such a slime layer may have reduced the concentration of cupric ion to which plant-grown cells are exposed. The exposure of cells to copper may be sufficiently low that all cells of copper-tolerant strains could survive to express induced levels of copper tolerance, and sensitive strains in as many as 10% of all the microsites on a leaf could escape death.

The application of manganese-zinc ethylene bisdithiocarbamate in mixtures with Cu(OH)₂, either as a protectant or an eradication was effective in the control of copper-tolerant strains of P. syringae in both greenhouse and field trials. The toxicity of ethylene bisdithiocarbamate (EBDC) compounds appears to be associated with the ability of the dithiocarbamate anion to chelate copper and transport the cation to a copper-susceptible site within the cell (37). In addition, EBDC has been reported to alter the permeability of the cell membrane, resulting in the disruption of cellular transport mechanisms (46). Thus, the synergistic effect between the copper compounds and EBDC in controlling copper-resistant strains is most likely attributable to the circumvention of resistance mechanisms to allow lethal amounts of cupric ion into the bacterial cell. While this material shows promise in the control of copper-tolerant strains of P. syringae, it is no longer registered for use on tree fruits.

The large number of copper-tolerant strains of P. syringae present in agricultural areas where copper bactericides have been used is probably responsible for the incomplete control of bacteria on crops with copper compounds in such areas. It is unlikely that simply increasing the amounts of copper bactericides alone will lead to effective control of such strains. Further studies designed to measure the concentrations of free cupric ions on leaves should determine whether or not copper-tolerant strains of P. syringae can be controlled by copper-containing bactericides under any environmental conditions.

**LITERATURE CITED**