

## Relationship of Free Ionic Copper and Toxicity to Bacteria in Solutions of Organic Compounds

O. Menkissoglu and S. E. Lindow

Department of Agriculture, Aristotelian University of Thessaloniki, Thessaloniki, Greece, and Department of Plant Pathology, University of California, Berkeley 94720.

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### ABSTRACT

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The complexation of cupric ions added to solutions of different organic compounds and to culture media was determined using an ion-specific electrode to ascertain the toxic forms of copper. Toxicity of  $\text{Cu}^{2+}$  to copper-sensitive and copper-tolerant strains of *Pseudomonas syringae* was reduced in the presence of all organic compounds tested, including 100 mM solutions of glucose, fructose, sucrose, succinate, and particularly citrate. The apparent toxicity of copper solutions was reduced 30× or more in the presence of organic solutes. Copper-sensitive and copper-tolerant strains of *P. syringae* were killed in aqueous solutions of copper only when the molar concentration of added sodium citrate was less than that of the copper. Copper-citrate complexes had no detectable toxicity to either copper-sensitive or copper-tolerant strains of *P. syringae*. Concentration of free cupric ions in a complex culture medium (CYE: casitone-yeast extract-glycerol agar) increased nearly logarithmically as a function

of the amount of  $\text{Cu}^{2+}$  added to the medium. Copper-tolerant strains of *P. syringae* that grew in CYE medium amended with high (> 50  $\mu\text{g}/\text{ml}$ ) concentrations of  $\text{Cu}^{2+}$  exhibited much higher tolerance of free copper ions in distilled water relative to a sensitive strain that tolerated only about 10  $\mu\text{g}/\text{ml}$  of added  $\text{Cu}^{2+}$  in CYE medium. A direct relationship was observed between the  $\text{LC}_{99}$  of  $\text{Cu}^{2+}$  to copper-sensitive and copper-tolerant strains of *P. syringae* when assayed in copper-amended distilled water solutions and the maximum concentration of ionic copper remaining free in copper-amended CYE medium. These results indicate that a very high fraction (> 99%) of copper added to complex culture media is complexed, that such complexed forms of copper are not toxic to strains of *P. syringae*, and that these strains are sensitive only to the remaining free ionic copper.

Copper-containing compounds, including cupric hydroxide, Bordeaux mixture, and various formulations of basic copper sulfate, were among the first biocides used for disease control and remain the only registered bactericides for use on most agricultural crops (14,18). Although copper compounds are used widely for bacterial disease control in tree fruits, their efficacy is often low (8,9,19,20,24). Copper-resistant strains of some phytopathogenic bacteria recently have been discovered. Certain strains of *Xanthomonas campestris* pv. *vesicatoria*, a pathogen of pepper (1,20,26), *Pseudomonas syringae* pv. *tomato*, a pathogen of tomato (5), *P. s. syringae*, a pathogen of cherry (27), and some strains of *P. syringae* from tree fruits, particularly from orchards with a long history of copper usage (2), exhibited high levels of tolerance to copper. Resistance to copper by bacteria is manifested as a quantitative increase in tolerance to copper 10–50× that of comparable sensitive strains (6,20,28). This level of copper resistance is considered to be sufficient to prevent control of tolerant strains by standard applications of copper bactericides and has led to poor disease control in some areas (20).

Many reports have shown that binding of copper to environmental constituents, such as organic materials, has considerable effects on biological availability of copper and can reduce the toxicity of copper towards microorganisms (10,11,21,22,30). Copper can form soluble complexes with amino acids, hydroxyacids, and carbohydrates (12,16). A large number of organic substances have been described in leachates from leaves, including simple sugars, organic and amino acids, phenolics, and vitamins (4,7,21,29). The effect of such compounds has not been determined on either the chemical forms or the toxicity of copper toward phytopathogenic bacteria. Some or all of the organic compounds found on leaves could greatly affect the apparent toxicity of copper compounds toward *P. syringae* and other phytopathogens. Because these compounds also are constituents of common culture media, better understanding of their effects on the estimates of copper toxicity made in vitro also is needed. Greatly differing estimates of the tolerance of copper ions by phytopathogenic

bacteria have been reported (1,3,5,20). The estimate of copper tolerance of bacterial strains in culture media is often much greater than that in aqueous suspension. The effect of constituents of culture media on estimates of copper tolerance of phytopathogenic bacteria is not known.

To better understand the mechanisms of resistance of tree fruit strains of *P. syringae* to copper and to devise better means of control of bacterial diseases and frost injury, we studied the effects of various organic compounds on the toxicity of  $\text{CuSO}_4$  to sensitive and tolerant strains of *P. syringae*. The survival of different strains of *P. syringae* and the amount of free copper ions in copper-amended distilled water, in simple solutions of organic substances, and in complex growth media were examined to ascertain the toxicity of different chemical forms of copper to this phytopathogen and to determine the basis for methods-dependent differences in the estimation of copper toxicity to phytopathogenic bacteria.

### MATERIALS AND METHODS

**Bacterial strains and media.** The characteristics of 17 strains of *P. syringae* isolated from almond trees (3), strain B728a of *P. syringae* isolated from bean leaves (17), copper-tolerant *X. c. vesicatoria* (26), and strain PT-23 of *P. syringae* pv. *tomato* (5) have been reported previously. Spontaneous rifampicin-resistant mutants of strains of *P. syringae* were obtained as described by O'Brien and Lindow (23). All bacteria were routinely cultured on King's medium B (KB) (15) containing 100  $\mu\text{g}/\text{ml}$  of rifampicin (KBR) for 24 h at 30 C and then transferred onto casitone-yeast extract-glycerol agar (CYE) (30) plates. Bacterial cells not "induced" to high levels of copper tolerance were harvested from unamended CYE medium. "Induced" bacterial cells were cultured on CYE medium containing sublethal quantities of  $\text{Cu}^{2+}$  (0.016 mM  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$  [1  $\mu\text{g}/\text{ml}$  of  $\text{Cu}^{2+}$ ] for copper-sensitive strains, or 0.16 mM  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$  [10  $\mu\text{g}/\text{ml}$  of  $\text{Cu}^{2+}$ ] for copper-tolerant strains) to allow cells to increase the level of copper tolerance that they express. Copper-amended CYE medium was prepared by adding filter-sterilized solutions of  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$  to cooled (55–65 C) CYE medium. Copper-sensitive and copper-tolerant

strains then were transferred to CYE medium containing 0.16 mM CuSO<sub>4</sub>·5 H<sub>2</sub>O (10 µg/ml of Cu<sup>2+</sup>) or 0.80 mM CuSO<sub>4</sub>·5 H<sub>2</sub>O (50 µg/ml of Cu<sup>2+</sup>), respectively. Cells harvested after 24–48 h of growth on plates of copper-amended CYE medium were used in all assays.

**Measurements of copper ions with a copper-specific electrode.** Copper activity of aqueous solutions of CuSO<sub>4</sub> was measured using a Cu<sup>2+</sup>-specific electrode. Cupric ion measurements of all aqueous CuSO<sub>4</sub>·5 H<sub>2</sub>O solutions were conducted with an Orion model 94-29 copper-specific electrode (Orion Research, Inc., Boston) connected to an Orion model 701 pH/mV meter. An Orion model 90-01 electrode was used as the reference electrode. The ionic strength of test and stock solutions was adjusted to 0.1 M with 5 M NaNO<sub>3</sub>. Potentials of 100-ml samples that were constantly stirred were measured to ± 0.1 mV at 25 ± 1 C, under uniform lighting conditions. Cu(NO<sub>3</sub>)<sub>2</sub> solutions in glass-distilled deionized water yielding Cu<sup>2+</sup> concentrations in the range of 100 µg/ml down to 0.001 µg/ml (pCu 2.8–7.8) were used to relate millivolt potentials produced by copper electrodes with Cu<sup>2+</sup> ion concentrations. pCu units reported (pCu = -log<sub>10</sub>[Cu<sup>2+</sup>]) are a measure of the activity of copper ions in the solution and express the concentration of copper available in the ionic form and not the total amount of copper salt added to a sample. Cu<sup>2+</sup> ion concentrations in the range of pCu 7.8 to 18.0 were obtained with buffered solutions of Cu<sup>2+</sup> with different chelators yielding known pCu values (13). Curves relating millivolt potential and logarithm of molar Cu<sup>2+</sup> concentration were linear (*r* = 0.980 and *r* = 0.960) for Cu<sup>2+</sup> concentration ranges of pCu 2.8–7.8 and 7.8–18, respectively. The electrode slope was 29.5 mV and its response was rapid (0.5–2 min) when Cu<sup>2+</sup> concentrations ranged from pCu 2 to 7 and within 5–15 min at pCu 7 to 13. Electrode response time was improved substantially by frequent electrode surface polishing.

**Toxicity of copper in solutions of organic compounds.** Copper-tolerant Al489 and copper-sensitive Al487 of *P. syringae* were tested to determine the amount of copper they tolerated in the presence of 100 mM solutions of glucose, fructose, sucrose, succinic acid, or citric acid. The pH of the succinate and citrate solutions was adjusted to 6.5–7.0 using 0.1 M NaOH. A filter-sterilized stock solution of CuSO<sub>4</sub>·5 H<sub>2</sub>O was added to the sterile solutions of the organic compounds prepared in distilled deionized water to obtain the desired copper concentrations just before the addition of bacterial cells. All reagents used were of analytical grade. The copper-tolerant and copper-sensitive strains of *P. syringae*, cultured on copper-amended or unamended CYE medium for 24–48 h, were suspended at a final concentration of 10<sup>4</sup> cells per milliliter in 10 ml of distilled water or solutions of organic compounds containing various concentrations of cupric ions. The cell suspensions were shaken on a reciprocal shaker for 2 h at 30 C, and 0.1-ml aliquots of the cells were spread on the surface of plates of KB. Plates were incubated for 48–72 h at 21 C and the number of colony-forming units was counted. The viability test was repeated twice with three replications of each copper concentration in each solution containing an organic compound. The probit of percent cell survival, corrected for survival in solutions of the organic compound alone, was regressed against the logarithm of cupric ion concentration and LC<sub>50</sub> values obtained by interpolating to probit = 5.0. The survival of these two strains also was tested in two concentrations of copper in sodium citrate and fructose solutions ranging in concentration from 0.001 to 2,000 mM. Copper was added to final concentrations of 1 and 2 µg/ml of Cu<sup>2+</sup> (0.016 and 0.032 mM CuSO<sub>4</sub>·5 H<sub>2</sub>O) in sodium citrate solutions containing copper-sensitive Al487, while 10 and 20 µg/ml of Cu<sup>2+</sup> (0.16 and 0.32 mM CuSO<sub>4</sub>·5 H<sub>2</sub>O) were added to solutions containing copper-tolerant Al489. All tests were repeated three times.

**Toxicity of copper in CYE media.** Plates of CYE medium to which Cu<sup>2+</sup> (as CuSO<sub>4</sub>) was added at concentrations of 0, 5, 10, 15, 20, 30, 40, 50, 60, or 70 µg/ml were used to test for growth inhibition of all of the strains of *P. syringae* and *X. campestris* by CuSO<sub>4</sub>. Ten µl droplets of a 10<sup>7</sup> cells per milliliter bacterial suspension were spotted in triplicate onto CYE plates

amended with the concentrations of copper listed above. The plates were incubated at 21 C for 48–72 h and the presence or absence of bacterial growth in spots inoculated with bacteria was determined. The test was repeated three times.

**Statistical methods.** Statistical computations were made by using software provided by Statistical Analysis Systems (release 5.16; SAS Institute Inc., Cary, NC). The SAS means procedure was used to perform analysis of variance on cell survival. Linear regression was performed using the regression procedure.

## RESULTS

**Toxicity of copper to strains of *P. syringae* in solutions of organic compounds.** All of the organic compounds that were tested reduced the toxicity of aqueous solutions of CuSO<sub>4</sub> to *P. syringae*. Some cells of copper-sensitive Al487 survived in aqueous solutions of sodium citrate to which 0.16 mM CuSO<sub>4</sub> (10 µg/ml Cu<sup>2+</sup>) was added (Table 1). This amount of added copper is over 300× more than that tolerated in distilled water alone by this strain (Table 1, Fig. 1). Copper-tolerant Al489 survived in aqueous sodium citrate solutions containing up to 0.64 mM CuSO<sub>4</sub> (40 µg/ml Cu<sup>2+</sup>), exhibiting a copper tolerance ~500× higher than in distilled water alone (Table 1, Fig. 1). Copper-sensitive and copper-tolerant strains survived much higher concentrations of added CuSO<sub>4</sub> in solutions of sodium citrate than in solutions of sugars or other organic acids (Table 1, Fig. 1). Solutions of sucrose and succinate did not reduce the toxicity of added copper as much as the same concentrations of glucose and fructose. The amount of copper tolerated in the presence of a variety of organic compounds depended on the chemical structure of each compound, its concentration in the copper-containing aqueous solutions, and presumably the chemical nature of the complex formed in each case. When a constant amount of CuSO<sub>4</sub> (1 µg/ml and 10 µg/ml of Cu<sup>2+</sup> in the case of Al487 and Al489, respectively) was added to varying concentrations (5–100 mM) of fructose solutions, the fraction of surviving cells increased with increasing fructose concentration (data not shown). Measurements of copper activity of these solutions revealed that much (>95%) of the added copper existed in its ionic form and that little complexation occurred (data not shown).

Cells of copper-sensitive Al487 survived in citrate containing CuSO<sub>4</sub> solutions only when the amount of added copper ions occurred in less than an equimolar ratio with citrate anions. The free ionic copper concentrations of solutions in which at least a few cells survived was less than about pCu 5.5 (Table 2). More than 50% of the cells survived in citrate-amended CuSO<sub>4</sub> solutions when the copper activity was less than about pCu 7. The LC<sub>50</sub> and LC<sub>99</sub> of Cu<sup>2+</sup> for Al487 in distilled water was 6.99 and 5.2 pCu, respectively. At least a few cells of copper-tolerant Al489 survived in citrate-amended solutions of CuSO<sub>4</sub> when the measured free ionic copper concentration in the solutions was less than about pCu 4.2 (Table 2).

Sodium citrate was added to final concentrations varying from 1 to 100 µM to CuSO<sub>4</sub> solutions containing 1 or 2 µg/ml, or 10 or 20 µg/ml, of Cu<sup>2+</sup> to which Al487 and Al489, respectively, were exposed to ascertain the stoichiometry of interactions of

TABLE 1. Maximum concentrations of added Cu<sup>2+</sup> tolerated by strains of *Pseudomonas syringae* in aqueous solutions containing different organic compounds

Organic compound tested	Strain of <i>P. syringae</i>	
	Al489 (Cu <sup>1</sup> )	Al487 (Cu <sup>1</sup> )
H <sub>2</sub> O <sup>a</sup>	0.12 <sup>b</sup>	0.03
Sucrose	6.0	0.50
Glucose	16.0	1.60
Fructose	20.0	>2.00
Succinate	2.0	0.30
Citrate	>40.0	>10.0

<sup>a</sup>All solutions prepared in distilled deionized water at a concentration of 100 mM.

<sup>b</sup>Maximum copper tolerated expressed as µg/ml.

citrate and  $\text{Cu}^{2+}$  leading to detoxification. When the citrate concentration was varied independently of the amount of added  $\text{Cu}^{2+}$ , the percentage of surviving cells of A1487 and A1489 ranged between 70 and 100% when the copper activity was less than pCu 7.0 or 4.8, respectively, irrespective of the amount of  $\text{Cu}^{2+}$  added to citrate-containing solutions (Table 2). In solutions containing more than 100 mM citrate, less than 100% of the cells survived, but this appeared to be a result of high citrate concentrations alone and not of the presence of  $\text{Cu}^{2+}$  (Fig. 1).

**Comparison of survival of bacterial strains on copper-amended CYE medium and survival in aqueous  $\text{CuSO}_4$  solutions.** As reported elsewhere (3), copper-sensitive strains of *P. syringae* grew on CYE medium to which only 10  $\mu\text{g}/\text{ml}$  or less of  $\text{Cu}^{2+}$  was added, while copper-tolerant strains survived on CYE medium containing up to 50  $\mu\text{g}/\text{ml}$  of added  $\text{Cu}^{2+}$ . Some strains exhibited intermediate levels of tolerance of  $\text{Cu}^{2+}$  in this culture medium. The maximum concentration of  $\text{Cu}^{2+}$  added to CYE medium that allowed growth of different strains of *P. syringae* and the maximum concentration of free ionic copper tolerated by each strain in distilled water were not directly proportional (Fig. 2). Strains that were very sensitive to copper in CYE medium, exhibiting tolerance of less than 10  $\mu\text{g}/\text{ml}$  of added  $\text{Cu}^{2+}$ , were also killed by very low concentrations (less than about 15 ng/ml) of cupric ions in distilled water. In contrast, copper-tolerant strains that tolerated up to 50  $\mu\text{g}/\text{ml}$  of added  $\text{Cu}^{2+}$  in CYE medium survived proportionally much greater concentrations of cupric

ions in distilled water (up to about 1,500 ng/ml of  $\text{Cu}^{2+}$ ) (Fig. 2).

**Measurements of copper activity of copper-amended CYE medium.** The concentration of free ionic copper in copper-amended CYE medium did not increase proportionally with the amount of  $\text{Cu}^{2+}$  added (Fig. 3). Only a small portion of the total amount of  $\text{Cu}^{2+}$  added to CYE medium exists as free ionic copper, and most is bound with the medium constituents. The amount of available free ionic copper increased nearly exponentially with increasing amounts of  $\text{Cu}^{2+}$  added to CYE medium (Fig. 3). When 10  $\mu\text{g}/\text{ml}$  of  $\text{Cu}^{2+}$  was added to CYE medium, a concentration that the copper-sensitive bacterial strains could tolerate, practically all of the copper is bound; the concentration of the free  $\text{Cu}^{2+}$  ion was only 15 ng/ml (pCu 7.2). However, when 50  $\mu\text{g}/\text{ml}$   $\text{Cu}^{2+}$  was added to CYE medium, the highest copper concentration that most copper-tolerant strains could survive, the concentration of  $\text{Cu}^{2+}$  was 2.0  $\mu\text{g}/\text{ml}$  (pCu 4.50).

TABLE 2. Concentration of free copper ions in aqueous citrate-amended  $\text{CuSO}_4$  solutions and the survival of copper-sensitive strain A1487 and copper-tolerant strain A1489 of *Pseudomonas syringae* in those solutions

Added citrate <sup>b</sup> ( $\mu\text{M}$ )	Strain A1487				Strain A1489	
	1 $\mu\text{g}/\text{ml}$ of $\text{Cu}^{2+}$ <sup>a</sup>		2 $\mu\text{g}/\text{ml}$ of $\text{Cu}^{2+}$		10 $\mu\text{g}/\text{ml}$ of $\text{Cu}^{2+}$	
	Cell survival (%)	Copper activity (pCu)	Cell survival (%)	Copper activity (pCu)	Cell survival (%)	Copper activity (pCu)
1	0	5.16	0	4.68	0	3.85
2	0.6	5.50	0	4.83	0	3.89
3	14.5	5.75	0	5.02	0	3.92
5	30.0	6.35	0.7	5.60	0	4.00
10	79.0	7.55	32.0	6.55	15.0	4.20
20	74.0	8.05	67.0	7.10	61.0	4.74
50	85.0	9.55	83.0	9.00	118.0	7.10
80	101.5	10.40	110.0	9.95	84.0	8.45
100	82.0	11.15	88.0	10.06	103.0	8.50

<sup>a</sup> $\text{Cu}^{2+}$  added in the form of  $\text{CuSO}_4$ . 1  $\mu\text{g}/\text{ml}$  of  $\text{Cu}^{2+}$  is equivalent to 16  $\mu\text{M}$   $\text{CuSO}_4$ .

<sup>b</sup>Citrate supplied as the sodium salt.

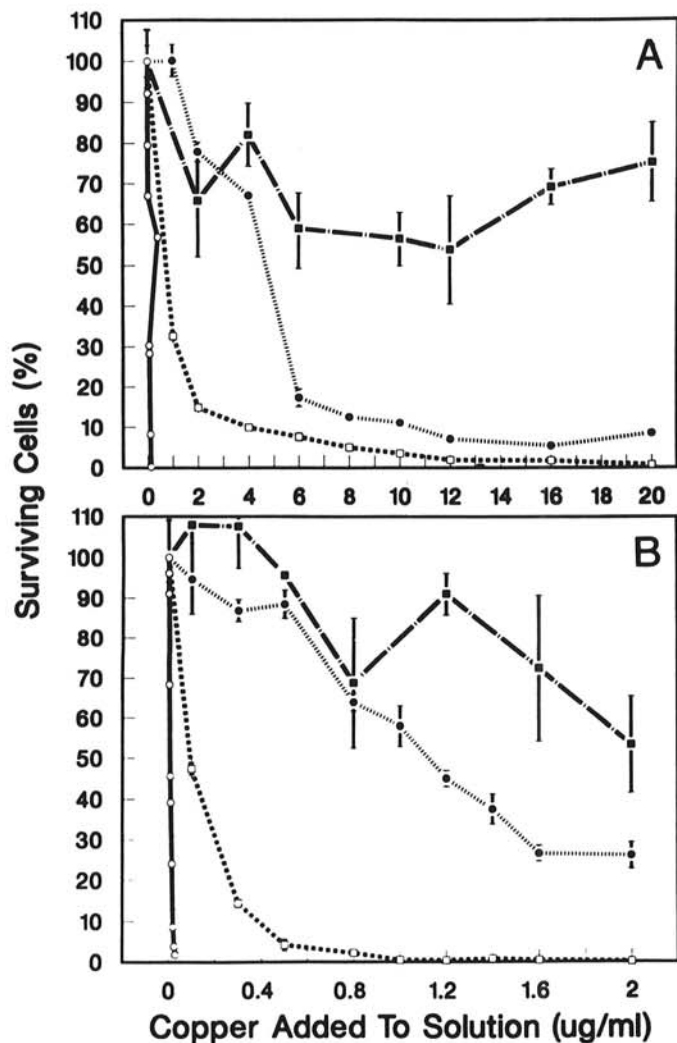


Fig. 1. Survival of cells of A, copper-tolerant strain A1489, and B, copper-sensitive strain A1487, of *Pseudomonas syringae* as a function of the amount of  $\text{Cu}^{2+}$  added to cell suspensions in water (O), 100 mM glucose ( $\square$ ), 100 mM fructose ( $\bullet$ ), and 100 mM sodium citrate ( $\blacksquare$ ). The vertical bars represent the standard error of mean cell survival.

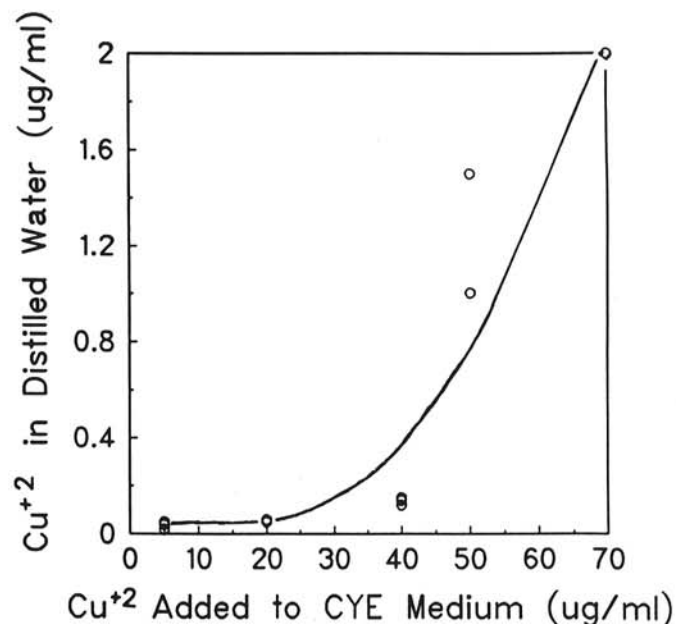


Fig. 2. Relationship between maximum concentration of  $\text{Cu}^{2+}$  added to CYE medium at which different strains of *Pseudomonas syringae* survived (abscissa), and the maximum concentration of cupric ions that each strain tolerated in distilled water (ordinate). Each point represents a different strain of *P. syringae* tested by both methods for copper sensitivity. Linear regression of log-transformed values for the amount of  $\text{Cu}^{2+}$  tolerated in distilled water with the amount of  $\text{Cu}^{2+}$  tolerated in CYE medium yields the equation  $y = 0.027x - 1.66$  ( $r^2 = 0.85$ ).



**Relationship between maximum concentration of cupric ions tolerated by strains of *P. syringae* in CYE medium and in distilled water.** The maximum concentration of  $\text{Cu}^{2+}$  added to CYE medium that supported the growth of different strains of *P. syringae* was determined by Andersen et al (3). The concentration of free ionic copper in CYE medium amended with different amounts of  $\text{Cu}^{2+}$  was determined here (Fig. 3). The maximum concentration of cupric ions tolerated by at least 1% of the cells of these strains in distilled water containing  $\text{CuSO}_4$  ( $\text{LC}_{99}$ ) was obtained from the regression of the probit of percent cell survival against the logarithm of molar  $\text{Cu}^{2+}$  concentration for probit = 1.91. The maximum copper activity (free  $\text{Cu}^{2+}$  concentration) at which cell growth ceased in a complex growth medium was very similar to the maximum  $\text{Cu}^{2+}$  concentration tolerated by a strain in distilled water (Fig. 4).

## DISCUSSION

The toxicity of cupric ions toward both copper-sensitive and copper-tolerant strains of *P. syringae* is low in the presence of all of the organic compounds tested here. Substantial differences in the ability of different organic compounds to negate the toxicity of cupric ions in aqueous solutions, however, was noted (Table 1). For example, 50–150 $\times$  higher concentrations of cupric ions had to be added to solutions of the sugars sucrose, glucose, and fructose when compared to distilled water alone to achieve the same level of toxicity (Table 1). In contrast, it was necessary to add over 300 $\times$  more cupric ions to sodium citrate solutions to achieve the toxicity seen in distilled water. It is therefore clear that the amount of  $\text{Cu}^{2+}$  that bacteria can tolerate in the presence of a variety of organic compounds depends on the chemical structure of the compound, its concentration in aqueous copper-containing solutions, and the chemical nature of complexes formed with copper in each case. The inactivation of copper by complexation is most easily explained in the case of copper-citrate coordination products.  $\text{Cu}^{2+}$  forms complexes with citrate. The nature of the complex formed between citrate and copper depends both on pH and the ratio of copper to citrate in aqueous solutions. When both copper and citrate solutions are very dilute, the formation of the  $[\text{citrate-Cu}]^{1-}$  complex takes place. However, in an excess of citrate, the complex  $[(\text{citrate})_2\text{-Cu}]^{-4}$  is formed (16). Because the pH of the aqueous solutions in our experiment was approximately 6–7, and the solutions were very dilute, the formation of the  $[\text{citrate-Cu}]^{1-}$  complex was favored. The ultraviolet absorption spectrum of the copper-citrate solutions used

in our experiments showed a maximum absorbance at 740 nm, indicative of the formation of the  $[\text{citrate-Cu}]^{1-}$  complex (16).

The free  $\text{Cu}^{2+}$  ion is apparently the only toxic form of copper to copper-sensitive and copper-tolerant strains of *P. syringae*. When the concentration of  $\text{Cu}^{2+}$  that was added to aqueous solutions was greater than the equimolar concentration of citrate needed to form the  $[\text{citrate-Cu}]^{1-}$  complex, part of the added  $\text{Cu}^{2+}$  remained unbound. When equimolar concentrations of  $\text{Cu}^{2+}$  and citrate are present in solution, the  $[\text{citrate-Cu}]^{1-}$  complex is formed, and little  $\text{Cu}^{2+}$  remains unbound. In equimolar solutions of  $\text{Cu}^{2+}$  and citrate, almost 100% of the cells of both copper-sensitive and copper-tolerant strains survived (Table 2). Copper-sensitive Al487 survived in copper-containing sodium citrate solutions only when the amount of free cupric ions was lower than about pCu 5.5, which is close to the  $\text{LC}_{99}$  value for this strain ( $\text{LC}_{99} = \text{pCu } 5.21$ ). Similarly, some cells of copper-tolerant Al489 survived in copper-containing sodium citrate solutions with a free copper ion concentration of less than pCu 4.2. Since the  $\text{LC}_{99}$  for this strain determined in distilled water containing copper sulfate was pCu 5.1, these results strongly indicate that the large amounts of copper-citrate complex that were formed in these copper-amended citrate solutions were not toxic to the cells, and that only a small fraction of cells that would have survived free copper ion concentrations of pCu 4.2 in water did so in citrate solutions.

The survival of strains of *P. syringae* in copper-amended complex media was determined by the free  $\text{Cu}^{2+}$  ion concentration in the medium and not by the amount of cupric ion added to the medium. For example, copper-tolerant Al489 survived in CYE medium amended with up to 50  $\mu\text{g/ml}$  of  $\text{Cu}^{2+}$ . The concentration of free copper ions in this medium amended with this amount of copper was only about 2  $\mu\text{g/ml}$  (pCu 4.5), close to the  $\text{LC}_{99}$  for this strain. Indeed, there was a nearly direct relationship between the concentration of free copper ions tolerated by strains differing in copper tolerance in aqueous solutions and the concentrations of free copper ions tolerated in a complex medium (Fig. 4). Since between 96 and 99.8% of the total amount of  $\text{Cu}^{2+}$  added to a complex medium such as CYE was bound and no longer in a free ionic form, the toxicity of such complexed forms of copper must be low or nonexistent.

The toxicity of copper to strains of *P. syringae* in solutions of neutral sugars at high concentrations was not directly related to the concentration of free cupric ions. Neutral sugars form weak

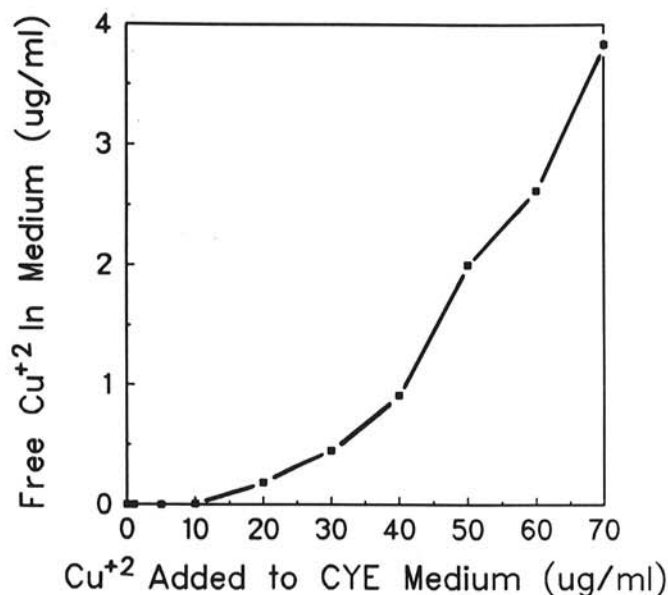


Fig. 3. Concentration of free cupric ions in CYE medium amended with different amounts of  $\text{Cu}^{2+}$  (as  $\text{CuSO}_4$ ).

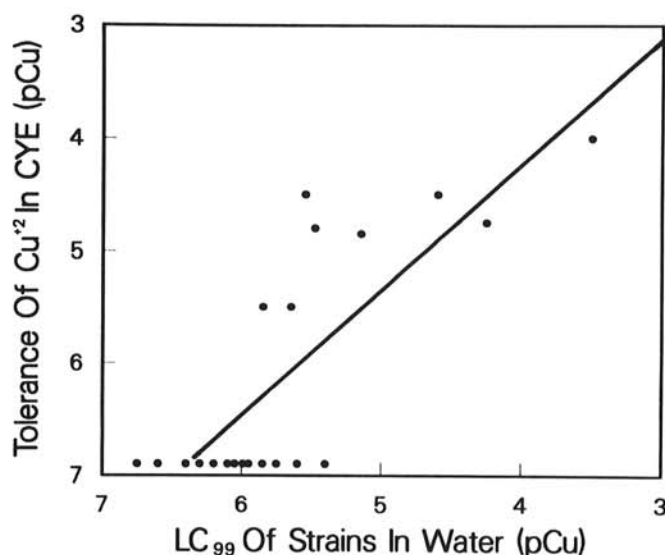


Fig. 4. Relationship between maximum concentration of free cupric ions in CYE medium in which different strains of *Pseudomonas syringae* would grow and the maximum concentration of cupric ions tolerated by these strains in distilled water. Each point represents a different strain of *P. syringae*. The line represents the regression equation  $y = 1.105x - 0.16$  ( $r^2 = 0.62$ ).

one-to-one complexes with  $\text{Cu}^{2+}$  ions. Formation constants at pH 6.1 for  $\text{Cu}^{2+}$  complexes with glucose and fructose are 0.15 and 0.26, respectively (12). In our experiments, 100 mM solutions of glucose and fructose amended with  $\text{CuSO}_4$  contained only a small quantity of complexed copper. Even though the free copper ion concentration in copper-amended glucose and fructose solutions was higher than the  $\text{LC}_{99}$  for our strains, a substantial fraction of the cells survived (Fig. 1). It is possible that the presence of carbohydrates in high concentrations affects the penetration of cupric ions into bacterial cells. Changes in internal cell solutes could have occurred because of alterations in the osmotic environment of the cell that affected internal complexation of  $\text{Cu}^{2+}$ . It should be noted that glucose, sucrose, and fructose at equimolar concentrations each provided similar protection against the toxic effects of copper ions in aqueous solutions and that all such solutions have similar osmotic potentials, while the osmotic potential of CYE medium is approximately 5 $\times$  less than the sugar solutions tested here. It is possible that increases in the ionic strength of media might enhance the ability of the cell to exclude copper ions, particularly in copper-tolerant strains, although the mechanism of tolerance in such strains is unknown.

The complexation of copper by constituents of complex culture media can lead to errors and inconsistencies in the reported estimates of copper tolerance among bacteria. The addition of  $\text{Cu}^{2+}$  to complex culture media such as CYE results in a titration of the most reactive copper-binding constituents of the media upon addition of copper (Fig. 3). A high capacity of the constituents of complex culture media to bind copper is indicated. For example, when only 10  $\mu\text{g}/\text{ml}$  of  $\text{Cu}^{2+}$  is added to CYE medium, over 99.8% of that copper is complexed. However, if the concentration of copper added to CYE medium is increased to 50  $\mu\text{g}/\text{ml}$ , only about 96% of that copper is complexed. Thus as more copper is added to culture media, only those less reactive copper-chelating agents remain available to bind the added copper, leaving higher concentrations of residual copper free to bind to bacterial cells when they are added to the medium. This phenomenon gives rise to the strongly nonlinear relationship between the concentration of  $\text{Cu}^{2+}$  that must be added to a complex medium to inhibit cell growth, and the maximum concentration of  $\text{Cu}^{2+}$  in distilled water that different strains of *P. syringae* can tolerate (Fig. 2). Thus, while simple observation of the growth of bacterial cells on culture media containing different concentrations of added copper is a rapid test of their sensitivity to copper, such tests underestimate the difference in the true level of copper tolerance exhibited by different strains. For example, copper-sensitive strains of *P. syringae* do not grow when more than 5 or 10  $\mu\text{g}/\text{ml}$  of  $\text{Cu}^{2+}$  is added to CYE medium, while copper-tolerant strains can grow when up to 70  $\mu\text{g}/\text{ml}$  is added, an apparent 14-fold difference in copper tolerance. However, copper-sensitive strains tolerate only approximately 3 ng/ml of free copper ions in aqueous solutions while tolerant strains can endure up to 180 ng/ml (3), a 60-fold difference in tolerance of copper ions. The very high levels of copper ions that some copper-tolerant strains of *P. s. tomato* and *X. campestris* have been reported to tolerate in complex culture media (1,5,27) are greatly overestimated due to the complexing ability of culture media (3). Different culture media will complex different quantities of copper ions, depending on the composition and quantity of the different organic constituents of that medium (25). However, rapid tests to compare the sensitivity of bacterial strains and to simultaneously elucidate the true copper tolerance of bacterial strains easily can be performed with the CYE culture medium reported here (Fig. 3).

It is likely that the free copper ion is the only form of copper that is toxic to strains of *P. syringae* in natural environments. CYE culture medium contains a diversity of organic compounds, although at low individual concentrations. Leaf surfaces probably also contain a diversity of organic compounds in low concentration (7,29). Since no evidence for the toxicity of copper complexed with any component of CYE medium was observed, it is unlikely that complexes would show toxicity to strains in the low concentrations they might be found on leaf surfaces. Leaf

surfaces might, however, contain organic compounds not found in CYE medium that might complex copper and yet be toxic to the bacteria. The chemical forms of copper on leaf surfaces and their toxicity to bacteria is the topic of the companion paper.

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