Induction of Copper Resistance in Plant-Pathogenic Bacteria Exposed to Glutamate, Plant Extracts, Phosphate Buffer, and Some Antibiotics

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ABSTRACT

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In plant-pathogenic bacteria, minimum inhibitory concentrations of copper sulfate ($CuSO_4$) in aqueous solution ranged between 1 and 10 μM, 0.01 or less than those determined with potato-dextrose agar plates. The viability of bacteria in $CuSO_4$ solution greatly increased when glutamate was supplemented at concentrations of 10 μg/ml or more. Bacteria survived well in $CuSO_4$-glutamate mixtures in which concentrations of free cupric ions were much higher than the lethal doses, indicating that a novel copper resistance was induced in the presence of glutamate. Resistance was induced with other amino acids and amides but not with sugars, sugar alcohols, or organic acids. Copper resistance also was induced by phosphate buffer at 25 mM or more, tetracycline hydrochloride and oxytetracycline hydrochloride at 100 μg/ml, and plant extracts. The induction of copper resistance generally was more pronounced in copper-resistant bacteria than in copper-sensitive ones. The induction of copper resistance took place in water droplets placed on injured plant leaves carrying copper deposits but not on intact leaves. Copper resistance thus induced was transient, and elevated copper resistance was restored in the absence of these factors.

Additional keywords: complexation of cupric ions, copper bactericides, transitory copper resistance.

Copper resistance expressed at minimum inhibitory concentrations (MIC) of copper sulfate ($CuSO_4$) on potato-dextrose agar (PDA) was 100 times or greater than the MIC of $CuSO_4$ in aqueous solution. Such reduction of copper resistance in aqueous solution was independent of genetically controlled copper resistance (M. Goto, T. Hikota, Y. Fugita, M. Onohara, and M. Nakajima, *unpublished data*). Differentiation between copper-resistant and -sensitive bacterial strains as observed on copper-PDA plates could not be performed clearly in aqueous solution. Large reductions of copper toxicity on agar media may be explained by binding of free cupric ions (Cu^{2+}) by the media ingredients (10). A similar situation may be responsible for the limited effectiveness of copper bactericides in practical use resulting from binding Cu^{2+} with organic substances available on the plant surface (11). However, no experimental evidence has shown that this is the mechanism responsible for copper resistance.

Copper resistance in bacteria has been interpreted with mechanisms such as activated efflux, detoxification, and sequestration of Cu^{2+} (5,12,13). In addition, recent information has implied the involvement of osmolarity of bacterial cells (2–4,6). Elucidation of the discrepancies found in copper resistance in aqueous solutions and agar media and on plant surfaces, therefore, is significant not only for clarifying the in vitro mechanisms of copper resistance in plant-pathogenic bacteria but also for enhancing the effectiveness of copper bactericides in situ.

This paper describes the results of studies on the copper resistance of plant-pathogenic bacteria induced by various factors, including media components, plant extracts, and antibiotics.

MATERIALS AND METHODS

Bacteria. Bacteria used in the study are listed in Table 1. Bacteria were grown on slants of either yeast extract-peptone agar (YPA: 5 g of yeast extract, 10 g of peptone, 15 g of agar, 1,000 ml of water, pH 6.8) or Bacto PDA (Difco Laboratories, Detroit, MI; pH 6.8) and were stored for routine work at 4 C.

Copper resistance. To assay copper resistance in aqueous solution, bacteria were grown on PDA slants at 28 C for 24 h and suspended in sterile distilled water at about 10^8 cfu. Twenty microliters of bacterial suspension was mixed with 180 μl of $CuSO_4$ solution that was previously injected into polystyrene cell wells 6.4 mm in diameter (Corning Laboratory Science Company, New York) and was shaken at 20 C on rotary microplate mixer MPM-1 (Iwaki Glass Co., Ltd., Tokyo) for 2 h, 150–200 times per minute unless otherwise described. The viability of bacteria was determined by inoculating with 10 μl of the reaction mixture and spotting on PDA plates or by counting the number of colony-forming units on PDA plates with or without the addition of $CuSO_4$ by dilution plating. Resistance against $CuSO_4$ was assayed on PDA plates supplemented with the chemical at concentrations of 0–5 mM at 0.25 mM intervals.

TABLE 1. Bacterial strains used in this study

Bacteria and strains	Source	Copper sensitivity or resistance
Agrobacterium		
tumefaciens A208	T. Yamada	Resistant; 2.25[a]
A. vitis GAg27	H. Sawada (grape)	Sensitive; 0.5
Pseudomonas		
cepacia ALQ8281	CCSU[b] (onion)	Resistant; 4.5
P. gladioli Akita	K. Ono (gladiolus)	Resistant; 4.5
P. glumae GM4	CCSU (rice grain)	Resistant; 4.5
P. syringae		
pv. *actinidiae* PA1	CCSU (kiwi)	Sensitive; 0.75
pv. *actinidiae* PA459	CCSU (kiwi)	Resistant; 3.0
pv. *maculicola* R1	CCSU (Japanese radish)	Resistant; 2.0
pv. *maculicola* R8427	CCSU (Japanese radish)	Sensitive; 0.2
pv. *tomato* PT23	D. A. Cooksey	Resistant; 2.5
Xanthomonas campestris		
pv. *campestris* campA	CCSU (cabbage)	Sensitive; 0.5
pv. *cucurbitae* 7714	CCSU (cucurbit)	Sensitive; 0.5
pv. *vesicatoria* 87-7	R. E. Stall	Resistant; 1.75

[a] Numbers indicate minimum inhibitory concentrations of $CuSO_4$ in millimolar concentrations determined on copper-PDA medium.
[b] Culture collection of phytopathogenic bacteria at the Laboratory of Plant Pathology, Shizuoka University, Shizuoka, Japan.

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The effect of amino acids, carbohydrates, and organic acids on copper toxicity was tested by mixing 20-μl aqueous solutions of the substances at final concentrations of 0, 1, 10, 100, and 1,000 μg/ml; 160-μl $CuSO_4$ solutions at final concentrations of 0, 1, 5, 10, 50, 100, 500, 1,000, and 5,000 μM; and 20-μl bacterial suspensions. The mixtures were shaken for 2 h, and the viability of bacteria was determined as described above. The organic substances tested included alanine, aspartic acid, cysteine, glycine, histidine, isoleucine, leucine methionine, phenylalanine, threonine, tryptophan, tyrosine, valine, asparagine, glutamine, glucose, fructose, sucrose, mannitol, sorbitol, citrate, fumarate, malate, succinate, and glutarate.

Determination of Cu^{2+}. Free Cu^{2+} was assayed with a copper ion electrode (DKK Corporation, Tokyo) and expressed as micrograms of Cu^{2+} per liter.

Chemicals. Unless otherwise described, all chemicals used in the study were analytical grade products obtained from Wako Pure Chemical Co., Ltd. (Osaka, Japan).

Preparation of leaf wash. Three grams of intact leaves of Japanese radish (cv. Tokinashi-Daikon) and tomato (cv. Ponte Roza) was sampled without injuring leaf surfaces and was immersed except for the cut end of petioles in 20 ml of distilled water at room temperature for 3 h. The water was then sterilized by filtering through a 0.22-μm membrane filter.

Preparation of leaf juice. Fresh young leaves of tomato (cv. Ponte Roza) were cut into small pieces with a scissors, placed in distilled water at 1 g/10 ml, and thoroughly homogenized. The preparations were centrifuged, and the supernatants were sterilized by filtering through a 0.22-μm membrane filter. The Lowry's method (8) was used to quantify the amount of protein contained in the preparation.

Effect of injuries inflicted to leaf surface on copper resistance. Two-month-old tomato plants were sprayed with copper hydroxide (Kocide, Kocide Chemical Corporation, Texas) at 500× dilution until the whole leaf surface became wet. The leaflets were sampled 24 h after the chemical spray, and the upper surfaces were injured with a razor blade. Three 3-mm-long incisions at a distance of 1 mm were made at each site. Each incision was shallow enough to reach only the parenchyma but not to cut through the leaf blade. Fifty microliters of bacterial suspension was dropped onto the injury sites and left at room temperature for 2 h. The number of surviving cells in a 10-μl water drop was assayed by counting colony-forming units on nutrient agar plates. Controls were prepared by dropping the same amount of bacterial suspension on uninjured portions of the same leaf and on the injured and uninjured portions of tomato leaves with no chemical spray. The Lowry's method (8) was used to determine protein content in the water drops placed on injury sites.

Induction of copper resistance by antibiotics. The effect of inhibitors of protein biosynthesis on induction of copper resistance was tested with actinomycin D, chloramphenicol, streptomycin sulfate, oxytetracycline hydrochloride, and tetracycline hydrochloride. Twenty microliters of bacterial suspension was mixed with 140 μl of water and 20 μl of antibiotic solution in cell wells and shaken on a microplate mixer for 10 min. Twenty microliters of $CuSO_4$ solution was subsequently added and shaken for 2 h. The antibiotics were tested at final concentrations of 0.5 and 5 μg/ml for actinomycin D, 10 and 100 μg/ml for the other antibiotics, and $CuSO_4$ was tested at final concentrations of 0, 5, 50, 100, 250, 500, 750, and 5,000 μM. The viability of bacteria was determined as described above.

Effect of antibiotics on the induction of copper resistance in the presence of glutamate. Twenty microliters of bacterial suspensions was mixed with 140 μl of glutamate solution and 20 μl of antibiotic solution in cell wells and shaken for 10 min. Twenty microliters of $CuSO_4$ solution was subsequently added to the mixtures and shaken for 2 h. Glutamate was added at 100 μg/ml, and antibiotics and $CuSO_4$ were added in the doses mentioned above. The viability of bacteria was determined as described above.

Assay of efflux of potassium ions (K^+) from bacterial cells exposed to glutamate or antibiotics. Bacteria were grown in 200 ml of YP broth shaken on a rotary shaker 80 times per minute at 28 C. Bacterial cells were harvested and washed two times with 200 ml of distilled water by centrifugation (12,000 *g*). Bacterial cells were suspended in 400 ml of water and divided into four 100-ml aliquot parts to which glutamate or antibiotic (tetracycline hydrochloride) was added at 100 μg/ml and shaken for 10 min. Subsequently, $CuSO_4$ solution was added at 50 μg/ml and shaken for 2 h. After centrifugation, the concentration of free K^+ in the supernatants was determined by a potassium ion electrode (DKK Corporation).

TABLE 2. Minimum inhibitory concentrations of $CuSO_4$ to plant-pathogenic bacteria in the presence of glutamate

Bacteria and strains	Glutamate (μg/ml) 0	1	10	100	1,000
Agrobacterium tumefaciens A208	5[a]	5	500	10,000	10,000
A. vitis GAg27	5	5	500	10,000	10,000
Pseudomonas cepacia ALQ8281	1	5	5	50	10,000
P. glumae GM4	5	5	10	10,000	10,000
P. syringae					
pv. *actinidiae* PA1	5	5	10	500	5,000
pv. *actinidiae* PA459	5	50	5,000	10,000	10,000
pv. *maculicola* R1	5	5	50	5,,000	10,000
pv. *maculicola* R8427	1	5	5	50	1,000
pv. *tomato* PT23	5	10	50	5,000	10,000
Xanthomonas campestris					
pv. *campestris* campA	1	5	5	50	500
pv. *cucurbitae* 7714	1	5	5	50	500
pv. *vesicatoria* 87-7	5	5	50	500	1,000

[a] Minimum inhibitory concentrations of $CuSO_4$ in micromolar concentrations determined in aqueous solution.

RESULTS

Survival of bacteria suspended in $CuSO_4$ solution. The MIC of $CuSO_4$ was between 1 and 5 μM, irrespective of bacterial species

TABLE 3. Residual amount of free cupric ions (Cu^{2+}) and pH in the mixture of $CuSO_4$ and glutamate[a]

Glutamate μg/ml	Glutamate μM	Copper sulfate (μM) 0	1	5	10	50	100	500	1,000
0	0	23 (6.14)	164 (6.14)	545 (6.14)	1,060 (6.14)	5,050 (6.14)	10,100 (6.17)	47,000 (6.15)	90,800 (6.13)
1	5	15 (6.14)	109 (6.14)	431 (6.14)	851 (6.14)	4,390 (6.14)	9,100 (6.17)	45,400 (6.15)	89,800 (6.13)
10	54	14 (6.14)	44 (6.14)	178 (6.14)	354 (6.13)	2,200 (6.13)	5,300 (6.12)	33,900 (6.12)	69,500 (6.10)
100	535	4 (6.16)	8 (6.16)	24 (6.15)	42 (6.15)	207 (6.13)	518 (6.12)	9,160 (5.98)	38,000 (5.68)
1,000	5,348	0.3 (6.22)	0.3 (6.22)	0.6 (6.21)	1.2 (6.20)	6.2 (6.17)	15.1 (6.15)	233 (5.90)	1,070 (5.72)

[a] Free Cu^{2+}, micrograms per liter; numbers in parentheses indicate pH values.

and strains (Table 2). No significant differences were detected in aqueous solution between copper-resistant and -sensitive strains, which were clearly differentiated on copper-PDA medium. A small fraction of the bacterial population often showed higher copper resistance than did the bulk, forming scattered colonies on agar plates where a drop of the mixture was inoculated.

Effect of glutamate on the viability of bacteria in $CuSO_4$ solution. The survival of a population sharply increased as glutamate concentrations exceeded 1 μg/ml (Table 2). The effect was particularly pronounced at 100 μg/ml or more, although it varied depending on the bacteria tested. In general, the enhancement of copper resistance was more conspicuous in copper-resistant strains than in copper-sensitive ones, although exceptions were found with strain ALQ8281 of *Pseudomonas cepacia* and strain GAg27 of *Agrobacterium vitis*. In the copper-sensitive strains of *Xanthomonas campestris* pv. *campestris* and pv. *cucurbitae*, the protective effect of glutamate was insufficient. Small, live fractions of bacterial population could be detected even after 24 h in the mixture of 50 μM $CuSO_4$ and glutamate at 100 μg/ml. Such enhancement of copper resistance was not demonstrated on Bacto PDA supplemented with $CuSO_4$ and glutamate at different doses.

Complexation of Cu^{2+} with glutamate molecules. The amount of free Cu^{2+} in the $CuSO_4$ solutions supplemented with glutamate is shown in Table 3. The mixtures were in the pH range of 5.72 to 6.22, which was not inhibitory to bacteria. Lethal concentrations of Cu^{2+} were between approximately 100 and 500 μg/L depending on bacterial species and/or strains (Tables 2 and 3). The residual Cu^{2+} concentrations sharply decreased with increases in glutamate contents, implying the occurrence of complexation between Cu^{2+} and glutamate molecules. The free Cu^{2+} level in the mixture of 100 μM $CuSO_4$ and glutamate at 100 μg/ml roughly corresponded to that of 5 μM $CuSO_4$ solution. The results indicated that bacteria exposed for 2 h to glutamate at 100 μg/ml tolerated concentrations of Cu^{2+} that were about 10 to 1,000 times greater than the lethal dose. The improved viability of bacteria in copper-glutamate mixtures was considered to be due to increased copper resistance provided by glutamate molecules in addition to the consumption of free Cu^{2+} by complexation.

Effect of amino acids and amides on the viability of bacteria in $CuSO_4$ solution. Fifteen tested amino acids and amides increased copper resistance at 100 μg/ml. The degrees of acquired resistance were equivalent to that induced by glutamate. A few amino acids such as L-lysine and L-proline were somewhat less effective in comparison to glutamate. The concentration of free Cu^{2+} in the mixture was not necessarily equivalent depending on amino acids; for example, the residual Cu^{2+} in the presence of aspartate was about one-half of that in the presence of glutamate.

Effect of carbohydrates and organic acids on the viability of bacteria in $CuSO_4$ solution. None of the carbohydrates and organic acids tested increased copper resistance at 1,000 μg/ml except citrate. All bacteria were killed within 2 h in the mixture, whereas their viability was retained in the presence of citrate.

Effect of phosphate buffer (PB) on the viability of bacteria in $CuSO_4$ solution. The addition of PB (pH 7.0) at 25 mM in $CuSO_4$ solution resulted in an increase in copper resistance equivalent to that found in the presence of glutamate at 100 μg/ml. The amount of free Cu^{2+} in the presence of 25 mM PB was 572, 896, and 1,180 μg/L for 10, 50, and 100 μM $CuSO_4$ solutions, respectively. The levels of residual Cu^{2+} were greater than the lethal dose. No effect was observed in the mixture supplemented with PB at a 2.5 mM concentration.

Effect of leaf wash on the viability of bacteria in $CuSO_4$ solution. The $CuSO_4$ solution was added to leaf wash of Japanese radish and tomato at final concentrations of 1, 10, and 100 μM, and bacterial cells were exposed to the mixtures for 2 h. The addition of leaf washes had no effect on copper sensitivity of bacteria, because the MIC of $CuSO_4$ was similar to those in the aqueous solutions.

Effect of leaf juice on the viability of bacteria in $CuSO_4$ solution. An increase in copper resistance was observed with all bacteria tested in the mixtures that contained leaf juice at a strength of 0.05 and in some bacteria at 0.02 and lesser strengths (Table 4). The enhancement of copper resistance by plant extract was more pronounced in the copper-resistant strains than in the copper-sensitive ones. The amount of free Cu^{2+} in the mixture of 50 μM $CuSO_4$ and leaf juice at a strength of 0.01 was approximately $\geq$900 μg/L . It was greater than the lethal dose of Cu^{2+} in aqueous solution and indicated that the copper resistance of bacteria was enhanced by the components of leaf juice.

Effect of injuries on the viability of bacteria on leaf surfaces treated with a copper bactericide. The number of viable cells of bacteria on leaf surfaces with deposits of copper hydroxide was significantly greater at the injury sites than on intact leaf surfaces, regardless of the copper-resistant or -sensitive bacteria and the compatible or incompatible host–bacteria combinations (Table 5). The protein content in the water drops placed on the injury sites of tomato leaves was about 83 μg/ml. Such an increase in the viability of bacteria on injured sites may have resulted

TABLE 4. Effect of juice prepared from tomato leaves on copper resistance of plant-pathogenic bacteria

	Plant extract, protein, and free Cu^{2+} mixtures[a]				
Bacteria and strains	1	2	3	4	5
Agrobacterium tumefaciens A208	3^{+}[b]	3^{+}	3^{+}	3^{+}	...
A. vitis GAg27	3^{+}	3^{+}	3^{+}	3^{+}	...
Pseudomonas cepacia ALQ8281	3^{+}	3^{+}	3^{+}	3^{+}s	...
P. gladioli Akita	3^{+}	3^{+}	3^{+}	3^{+}	...
P. glumae GM4	3^{+}	3^{+}	3^{+}	3^{+}	...
P. syringae					
pv. *actinidiae* PA1	3^{+}	3^{+}	2^{+}s	...	...
pv. *actinidiae* PA459	3^{+}	3^{+}	3^{+}	3^{+}	...
pv. *maculicola* R1	3^{+}	3^{+}	3^{+}	2^{+}s	...
pv. *maculicola* R8427	2^{+}s	...	...	...	...
pv. *tomato* PT23	3^{+}	3^{+}	3^{+}	3^{+}s	...
Xanthomonas campestris					
pv. *campestris* campA	3^{+}	3^{+}	...	...	...
pv. *cucurbitae* 7714	3^{+}	3^{+}s	...	...	...
pv. *vesicatoria* 87-7	3^{+}	3^{+}	3^{+}s	...	...

[a] 1 = 1/20, 260, 187; 2 = 1/50, 106, 467; 3 = 1/100, 53, 933; 4 = 1/200, 27, 1,831; and 5 = 0, 0, 3,310 leaf-juice strength in 50 μM $CuSO_4$ (original juice protein content was 53 mg/ml), protein at micrograms per milliliter, and free cupric ions (Cu^{2+}) at micrograms per liter in aqueous solution of 50 μM $CuSO_4$ mixed with plant extract, respectively, in each mixture.

[b] Degree of viability in the reaction mixtures in terms of colonial growth on agar plates. s = scattered colonies, no growth.

TABLE 5. Effect of injuries on survival of bacteria in water drops placed on the surface of tomato leaves with or without copper deposits

Bacteria and strains	Chemical spray[a]	Bacteria recovered[b] Injured	Bacteria recovered[b] Uninjured
Agrobacterium			
tumefaciens A208	+	259 (±43.5)[c]	0
	−	374 (±15.5)	366 (±55.6)
Pseudomonas gladioli Akita	+	57 (±2.0)	0
	−	1,500 (±300)	1,650 (±150)
P. syringae			
pv. *tomato* PT23	+	97 (±31.6)	0
	−	108 (±19.7)	110 (±26.5)
Xanthomonas campestris			
pv. *cucurbitae* 7714	+	132 (±2.3)	0
	−	1,003 (±27.8)	1,232 (±35.7)
pv. *campestris* campA	+	141 (±27.4)	0
	−	748 (±54.0)	666 (±78.0)
pv. *vesicatoria* 87-7	+	107 (±20.2)	0
	−	137 (±14.2)	119 (±7.1)

[a] Leaves were sampled 24 h after being sprayed with copper hydroxide (Kocide) at 500× dilution.

[b] Population of epiphytic bacteria, determined in the same way with leaves without chemical spray, was 2.2 ± 1.9 cfu/10 μl, and the bacteria were all copper sensitive.

[c] Colony-forming units per 10 μl.

from the acquisition of copper resistance by plant components released from wounds (Tables 4 and 5).

Induction of copper resistance by antibiotics. Oxyteracycline hydrochloride and tetracycline hydrochloride induced copper resistance in all bacteria tested when added to bacterial cells suspended in water at about 10^7 cfu/ml (Table 6). The level of acquired resistance corresponded to that induced by glutamate at 100 μg/ml. Increases in copper resistance by actinomycin D, chloramphenicol, and streptomycin sulfate were insignificant, although bacteria survived in the mixtures with no supplement of $CuSO_4$. The amount of residual Cu^{2+} in the mixtures containing tetracycline hydrochloride or oxytetracycline hydrochloride at 100 μg/ml and $CuSO_4$ at 100 μM was only 10–30 μg/L. A notable reduction of Cu^{2+} was caused by the formation of chelate complex. However, the concentration of Cu^{2+} in mixtures of tetracycline hydrochloride or oxytetracycline hydrochloride at 100 μg/ml and $CuSO_4$ at 250 and 500 μM was in the order of 10^3 and 10^4 μg/L, respectively. The mixtures containing tetracycline hydrochloride or oxytetracycline hydrochloride at 100 μg/ml were acidic; pH ranged between 3.8 and 3.5 depending on the doses of $CuSO_4$ at 0 to 1,000 μM. However, induction of copper resistance by tetracycline hydrochloride or oxytetracycline hydrochloride also occurred to a similar extent at pH 6.8.

Effect of antibiotics on the induction of copper resistance in the presence of glutamate. The experiment was conducted with the bacteria listed in Table 6. No significant change in copper resistance was demonstrated in the presence of glutamate by adding antibiotics, although the number of viable cells was considerably reduced depending on bacteria. The degree of copper resistance of the survivors was similar to that induced by glutamate (Table 2).

Effect of glutamate and antibiotics on the efflux of K^+. The amount of free K^+ in the reaction mixtures containing $CuSO_4$ was consistently greater than that in the absence of Cu^{2+}. Furthermore, K^+ concentrations in the presence of glutamate were significantly higher than those in the absence of glutamate. The concentrations of K^+ in the mixtures containing $CuSO_4$ were considerably reduced by adding tetracycline hydrochloride at 100 μg/ml (Table 7).

DISCUSSION

Complexation of Cu^{2+} added either in complex culture media or in solutions of different organic compounds have been extensively discussed with respect to copper resistance in *P. syringae* (10,11). Copper complexes with the ingredients of culture media or organic substances are not toxic to bacteria and only the remaining free ionic copper is responsible for the toxicity. Thus, there are substantial differences between the MIC of copper compounds incorporated in culture media and those in aqueous solutions.

As far as copper resistance in aqueous solution is concerned, no appreciable differences were detected between copper-resistant strains and copper-sensitive ones that could be differentiated on agar media. Consequently, copper resistance was less visible in aqueous solution, unless bacteria were previously exposed to sublethal doses of copper ions (1). This was true not only for the copper-resistant bacteria isolated in Japan but also for those found in the United States (1,9).

The present study revealed that glutamate was complexed with ionic copper, and copper toxicity to bacteria was reduced. The toxicity of $CuSO_4$ solution decreased in reverse proportion to the amount of glutamate added. The residual Cu^{2+} in the mixture of equimolar solutions of $CuSO_4$ and glutamate at 50 μM was approximately 1,000 μg/L. This was distinct from the mixture of equimolar solutions of $CuSO_4$ and citrate in which little Cu^{2+} remained unbound. The involvement of pH in the copper resistance induced by glutamate can be eliminated because pH values ranged from 6.22 in low concentrations of 1 μM $CuSO_4$ and glutamate at 1 μg/ml to 5.72 in high concentrations of 1,000 μM $CuSO_4$ and glutamate at 1,000 μg/ml.

Elevation in the MIC of $CuSO_4$ in the presence of glutamate, therefore, could not be explained by the inactivation of Cu^{2+} through complexation alone. In the presence of glutamate, most bacteria survived in the mixtures containing free Cu^{2+} at much higher concentrations than the lethal doses, which were about 100 μg/L in copper-sensitive bacteria and about 500 μg/L in copper-resistant ones. This phenomenon could be explained by the induction by glutamate of a novel copper resistance; a similar explanation may be applied to copper resistance in the presence of other amino acids and amides. The induction occurred in both copper-resistant and -sensitive bacteria reaching levels that were similar to those exhibited on copper-PDA plates. The strains of *X. campestris* pathovars were distinct from other bacteria because copper resistance was insufficient at higher concentrations of glutamate. The great sensitivity of *X. campestris* pathovars to $CuSO_4$ and fixed copper compounds on copper-PDA plates may be responsible for this effect (M. Goto, T. Hikota, Y. Fugita, M. Onohara, and M. Nakajima, *unpublished data*).

Copper resistance increased even at a relatively low concentration of glutamate at 1 μg/ml in strain PA459 of *P. s.* pv. *actinidiae*. In general, however, resistance was expressed at glutamate concentrations of 10 μg/ml and was very high at concentrations ≥100 μg/ml. The higher the glutamate concentration, the greater the resistance level to free Cu^{2+}. In contrast, the increased viability exhibited in the mixture of 100 μM $CuSO_4$

TABLE 6. Induction of copper resistance by antibiotics in plant-pathogenic bacteria

Bacteria and strains	Antibiotics ATCD[a]	CP	TC	OTC	SM
Agrobacterium tumefaciens A208	50[b]	50	10,000	5,000	50
Pseudomonas gladioli Akita	50	50	10,000[c]	10,000[c]	50
P. glumae GM4	50	50	10,000[c]	10,000[c]	50
P. syringae					
pv. *actinidiae* PA1	5	5	10,000	10,000	50
pv. *actinidiae* PA459	50	50	10,000	10,000	50
pv. *tomato* PT23	50	50	10,000	10,000	50
Xanthomonas campestris					
pv. *campestris* campA	5	50	5,000	5,000	5
pv. *vesicatoria* 87-7	50	5	10,000	10,000	50

[a] Antibiotics and their concentrations: ACTD = actinomycin D at 5 μg/ml, CP = chloramphenicol at 100 μg/ml, TC = tetracycline hydrochloride at 100 μg/ml, OTC = oxytracycline hydrochloride at 100 μg/ml, and SM = streptomycin sulfate at 10 μg/ml, respectively.
[b] Minimum inhibitory concentrations of $CuSO_4$ determined at 0, 5, 50, 500, and 5,000 μM concentrations.
[c] Bacterial growth at 5,000 μM $CuSO_4$ was confluent and suggested that the minimum inhibitory concentration is higher than this level.

TABLE 7. Effect of glutamate (Glu) and tetracycline hydrochloride (TC) on the efflux of potassium ions (K^+) from bacterial cells

Treatments	Bacteria[a] At(A208)	Pg(Akita)	Xcc(campA)
Glu^+, $CuSO_4^{+b}$	18.1[c]	39.2	6.0
Glu^+, $CuSO_4^-$	3.9	18.6	4.4
Glu^-, $CuSO_4^+$	10.5	21.7	5.7
Glu^-, $CuSO_4^-$	3.7	3.9	1.8
TC^+, $CuSO_4^+$	7.8	1.0	1.8
TC^+, $CuSO_4^-$	1.1	0.9	1.1
TC^-, $CuSO_4^+$	4.2	1.2	3.1
TC^-, $CuSO_4^-$	1.2	0.6	1.2

[a] At(A208) = *Agrobacterium tumefaciens* strain A208; Pg(Akita) = *Pseudomonas gladioli* strain Akita; Xcc (campH) = *Xanthomonas campestris* pv. *campestris* strain campA.
[b] The presence (+) or absence (−) of these substances in the reaction mixtures.
[c] Concentrations of K^+ in wet bacterial cells at micrograms per liters per milligrams.

and glutamate at $\geq$1,000 μg/ml was attributed to complexation of all free Cu^{2+}.

The induction of copper resistance was not observed with sugars and sugar alcohols. Sugars such as glucose, fructose, and sucrose significantly reduced toxicity of Cu^{2+} at a 100 mM concentration (10). In this study, however, these sugars showed no effect on the toxicity of $CuSO_4$. This discrepancy may be attributed to the lower concentration of sugars used in the present study, which was 1,000 μg/ml or about 5.6 mM. Among organic acids tested, complete reduction of copper toxicity was observed with citrate at a 1,000 μg/ml concentration or 3.9 mM but not with other organic acids tested. As has been reported (10), the remarkable reduction of copper toxicity in the mixture with citrate should be attributed to the formation of chelate compounds.

The viability of bacteria also was increased significantly in the presence of 25 mM PB (pH 7.0) in spite of residual Cu^{2+} that was greater than the lethal dose. The concentrations of residual Cu^{2+} in the mixture of 50 μM $CuSO_4$ and 50 mM PB, however, was below or close to the lethal dose. Because the pH of the mixture of 500 μM $CuSO_4$ and 25 mM PB was 6.31, pH changes in PB seemed to have an insignificant effect on bacterial viability. The phosphate transport system might play a role in this phenomenon (12).

The antibiotics (inhibitors of protein biosynthesis) caused no significant alterations in the MIC of $CuSO_4$ in the presence of glutamate. On the other hand, tetracycline hydrochloride and oxytetracycline hydrochloride induced notable enhancement of copper-resistance when these antibiotics were added to bacterial cell suspensions at sublethal doses that were about 100 times greater than those in culture media. Copper resistance was selectively induced by tetracycline hydrochloride and oxytetracycline hydrochloride but not by actinomycin D, chloramphenicol, or streptomycin sulfate. The degree of resistance was equivalent to that induced by glutamate. Tetracycline hydrochloride and oxytetracycline hydrochloride formed chelate complexes with Cu^{2+}. The concentration of free Cu^{2+} in the mixture of 100 μM $CuSO_4$ and tetracycline hydrochloride at 100 μg/ml was only 10 μg/L. At 250 μM $CuSO_4$, however, the amount of Cu^{2+} was 3.6×10^3 μg/L, which was about seven times greater than the MIC for all bacteria tested. Survival of bacteria at such a high dose of Cu^{2+} indicated the substantial increase in copper resistance. The mechanisms underlying such phenomena are unknown.

An increase in the K^+ efflux was observed whenever bacterial cells were exposed to Cu^{2+}. It was particularly notable in copper-resistant strains in the presence of glutamate. Because the K^+ efflux seemed to take place in exchange with the glutamate influx, osmolarity may play a role in the induction of copper resistance by glutamate (7). The activation of protein biosynthesis for sequestration or detoxification of Cu^{2+} may not be excluded, however (12). The significance of increased K^+ efflux accompanied by elevation of copper resistance in the antibiotic-treated cells is unknown.

By examining residual Cu^{2+} on plant leaves sprayed with copper hydroxide, Menkissoglu and Lindow (11) concluded that the concentration of Cu^{2+} that remained on leaf surfaces was sufficient to kill copper-sensitive cells. In citrus leaves previously sprayed with copper hydroxide at a 500$\times$ dilution, we also confirmed that a fully loaded water film (0.23 ml) on 1 g of leaves contained 174 mg of Cu^{2+} per liter. This concentration of free Cu^{2+} was high enough to destroy not only copper-sensitive bacteria but also copper-resistant ones.

Survival of bacteria was enhanced by the addition of diluted leaf juice to $CuSO_4$ solution but not by liquids from whole leaves. On leaves carrying copper deposits, bacterial cells surviving on the injury sites were significantly more numerous than those on uninjured sites of the same leaf. It is likely that these survivors acquired copper resistance through uptake of the organic substances, especially amino acids, secreted from injuries. These observations suggest that induction of copper resistance generated in the injury sites of leaves enables plant-pathogenic bacteria to survive until they successfully infect through wounds or stomata. Wounds may enhance copper resistance of plant-pathogenic bacteria at infection sites and eliminate the bactericidal effect of copper compounds. Consequently, the temporary copper resistance of plant-pathogenic bacteria incited at injury sites may be responsible for the relatively low protective values of copper bactericides in the field.

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