Copper Resistance in *Pseudomonas syringae* pv. *syringae* from Cherry Orchards and its Associated Transfer in Vitro and in Planta with a Plasmid

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Michigan State University Agricultural Experiment Station Article 13014.

This research was supported in part by USDA Agreement 88-34152-3380 and by Federal Hatch-Amended and North Central Regional Research (NC-135) funds.

Accepted for publication 3 April 1989 (submitted for electronic processing).

ABSTRACT

Sundin, G. W., Jones, A. L., and Fulbright, D. W. 1989. Copper resistance in *Pseudomonas syringae* pv. *syringae* from cherry orchards and its associated transfer in vitro and in planta with a plasmid. Phytopathology 79:861-865.

Copper-resistant *Pseudomonas syringae* pv. *syringae* was recovered from blossoms of sweet and of sour cherry from nine and 12 orchards in Michigan in 1987 and 1988, respectively. Isolates that did not grow on low nutrient CYE-glycerol medium amended with 40–120 μg of cupric sulfate per milliliter were considered sensitive. Isolates that grew on media amended with 160–240 μg of cupric sulfate per milliliter were considered to have low resistance and those that grew on media amended with 280–320 μg of cupric sulfate per milliliter were considered to have high resistance. Among 17 copper-resistant isolates collected in 1987, 14 with high resistance contained a single plasmid of 46–73 kilobase pairs (kb) and three with low resistance lacked a detectable plasmid. A 61-kb plasmid was present in all 29 copper-resistant isolates selected in 1988 on media containing 200 μg of cupric sulfate per milliliter. Among 15 copper-sensitive isolates, only one contained a plasmid. Copper resistance and a 61-kb plasmid were transferred in filter matings to three copper-sensitive recipient strains of *P. s. syringae* from each of six copper-resistant donor strains. A resulting exconjugant was successfully used as a donor strain in matings with two recipient strains. Copper resistance also was transferred when suspensions of donor and recipient strains were infiltrated into bean leaves. Populations of copper-sensitive strains of *P. s. syringae* were reduced significantly more than populations of copper-resistant strains on bean leaves sprayed with cupric hydroxide. All *P. s. morsprunorum* detected in blossom samples was copper sensitive, and copper resistance was not transferred from *P. s. syringae* in filter matings to *P. s. morsprunorum*.

Bacterial canker is a recent and important problem of sweet cherry (*Prunus avium* L.) and sour cherry (*P. cerasus* L.) in Michigan (6,10). The disease is caused by each of two bacteria, *Pseudomonas syringae* pv. *syringae* van Hall and *P. s. morsprunorum* (Wormald) Young et al (6). Growth and dissemination of bacterial populations are favored by cool, wet weather in spring and autumn. Control recommendations in Michigan include weekly sprays of tribasic copper sulfate starting with the bud burst stage of bud development to prevent infection of leaves, blossoms, and fruit of sour cherry and continuing for 6 wk (15). Because of problems with phytotoxicity, a spray of Bordeaux mixture or of fixed copper only is recommended in orchards of sweet cherry when the trees are dormant and only in orchards where bacterial canker has been a problem. Before the use of copper to control bacterial canker, copper fungicides were used in many sour cherry orchards to control cherry leaf spot, caused by *Coccomyces hiemalis* Higgins.

Resistance to copper has been reported in the bacterial plant pathogens *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye and *P. s. tomato* (Okabe) Young et al (1,13). Resistance to copper in both bacteria was associated with conjugative plasmids (1,18). Copper resistance also has been associated with conjugative plasmids in *Escherichia coli* (20) and was found to be plasmid encoded in *Mycobacterium scrofulaceum* Prissick & Masson (4). Selection of resistant strains in all cases was associated with prior exposures to copper.

Because of a long history of copper usage on cherries in Michigan, we undertook a study to determine if copper-resistant *P. s. syringae* and *P. s. morsprunorum* were present in cherry orchards and if copper-resistant strains were able to survive on plants sprayed with a copper bactericide. We also investigated whether the gene(s) involved in copper resistance were plasmid-borne.

MATERIALS AND METHODS

Source and identification of copper-resistant bacteria. In 1987, epiphytic fluorescent pseudomonads were isolated from blossom samples collected from five sweet and 19 sour cherry orchards located in western Michigan (19). The sensitivity to copper of 154 such isolates was determined on CYE-glycerol medium (CYEG), a low nutrient medium with limited copper ion binding capacity (22). Cupric sulfate (Mallinckrodt Inc., St. Louis, MO) was added to the medium and the pH adjusted to 6.0 before autoclaving. Bacteria to be screened were grown for 48 hr at 23 C in petri plates containing King's medium B (KB) (8) amended with 50 μg of cycloheximide per milliliter (KBc), and then streaked onto CYEG amended with 160 μg of cupric sulfate per milliliter. Bacteria with confluent growth on the medium were considered copper-resistant, and those that failed to grow were considered copper-sensitive. The minimal inhibitory concentration (MIC) of copper necessary to inhibit confluent growth was determined by streaking copper-resistant and sensitive isolates on CYEG amended with 200, 240, 280, and 320 μg or 40, 80, and 180 μg of cupric sulfate per milliliter, respectively.

In 1988, blossom samples were collected from three sweet and 10 sour cherry orchards located in the western Michigan counties of Mason, Oceana, and Van Buren. The orchards that were sampled either contained copper-resistant bacteria in 1987 or had received applications of copper compounds in 1988. Two spurs with blossom clusters were collected from each of 100 trees per orchard. Samples were placed in plastic bags and kept in a cooler until processed. A sample of 200 blossoms, one blossom from each spur, was placed in a 2-L flask and washed with 100 ml of 0.01 M potassium phosphate buffer (pH 7.2) (K-buffer) by shaking the flask vigorously by hand for 2 min. The wash solution was centrifuged for 10 min at 5,858 *g* and the pellet resuspended in 10 ml of K-buffer. Aliquots (0.1 ml) from the original and from 10^{-2} to 10^{-5} dilutions of the original sample were plated (two replications) on KBc and on CYEG amended with 200 μg

of cupric sulfate per milliliter. Petri plates were incubated in an inverted position at 23 C for 3–4 days, and colonies of fluorescent pseudomonads present on the media were counted. Ten copper-resistant isolates from each of the 12 orchards were selected randomly, and the MIC of copper was determined for each isolate as described above.

Identification of copper-resistant bacteria. Isolates of fluorescent pseudomonads from both 1987 and 1988 were identified to species and pathovar based on ice nucleation-activity at −5 C as determined by the droplet freezing procedure of Lindow et al (12) and the gelatin liquefaction, aesculin hydrolysis, tyrosinase activity, and utilization of tartrate (GATTa) determinative tests (10). Isolates that were ice nucleation-active and GATTa positive were considered *P. s. syringae* and isolates that were ice nucleation-negative and GATTa negative were considered *P. s. morsprunorum*.

Plasmid characterization of copper-resistant strains. A total of 46 copper-resistant strains of *P. s. syringae* were screened for plasmids by a modification of the method of Kado and Liu (7). One milliliter of a 10-ml overnight culture grown in Luria broth (LB) (14) was centrifuged, and the pellet suspended in 0.15 ml of E buffer (0.04 M Tris, pH 7.9, 0.04 M Na-acetate, 0.02 M EDTA). The suspension was lysed with 0.30 ml of sodium dodecyl sulfate (SDS)-lysis buffer (0.05 M Tris, pH 12.6; 3% SDS), incubated for 45 min at 65 C, and extracted twice with two volumes phenol:chloroform:isoamyl alcohol (25:24:1), followed by a final extraction with two volumes chloroform:isoamyl alcohol (24:1). Plasmid DNA was separated by electrophoresis for 2.5 hr at 5 V/cm through a horizontal 0.7% agarose gel submersed in E buffer. Gels were stained for 30 min with ethidium bromide (0.5 μg/ml), destained for 5 min in 0.01 M $MgCl_2$, and photographed with a red Wratten filter under 302-nm UV light with type 55 Polaroid film. Plasmids isolated from strain SW2 of *Erwinia stewartii* (3) were used as molecular size markers in plasmid characterization.

Bacterial conjugation. Antibiotic resistant strains to be used as recipients were selected by spreading approximately 10^8 cells on KB amended with 25 μg of streptomycin or 100 μg of rifampicin per milliliter. After 3 days of incubation at 23 C, colonies were selected that had growth rates similar to the parent strain, were ice nucleation-active, and GATTa positive.

Matings were conducted with overnight cultures grown in 20 ml of LB broth. The broth cultures were centrifuged, and the pellets resuspended in 1 ml of K-buffer. Matings were conducted on a medium containing 1.5 g of K_2HPO_4, 5 g of yeast extract, 5 g of glucose, 10 g of peptone, 2.5 g of NaCl, and 15 g of agar per liter by mixing approximately 10^8 donor cells with 5×10^8 recipient cells on 0.45-μm Millipore filters. The plates were incubated for 18 hr at 23 C. The filters were removed from the plates and placed in sterile centrifuge tubes. The cells were suspended by vortexing in 2 ml of K-buffer, serially diluted, and plated onto CYEG and KB media amended with the appropriate antibiotics to determine exconjugant and recipient populations, respectively. Colonies that grew on CYEG amended with 200 μg of cupric sulfate and 25 μg of streptomycin or 100 μg of rifampicin per milliliter were considered putative exconjugants. Controls consisting of donor and recipient cells alone were treated similarly to determine the frequency of spontaneous resistant mutants in the population. One to 10 exconjugant bacterial colonies were randomly selected per mating for plasmid characterization.

Some matings also were performed in bean leaves. Approximately 10^8 donor and 5×10^8 recipient cells in K-buffer were mixed and drawn into a sterile 10-ml plastic syringe. The end of the disposable tip of the syringe was cut off and placed inverted, with the large diameter opening facing outwards, on the end of the syringe. Approximately 0.2 ml of the cell suspension was infiltrated into the underside of individual bean leaves. The plants were incubated in plastic bags for 1 and 3 days at 25 C, after which the discolored areas of infiltration were diced in 1 ml of K-buffer. Aliquots (0.1 ml) were plated on CYEG and KB amended with 25 μg of streptomycin per milliliter as with filter matings. Controls consisted of donor and recipients cells infiltrated alone to datermine the frequency of spontaneous mutation.

Survival of *P. s. syringae* on copper sprayed bean plants. Populations of copper-resistant, copper-sensitive, and copper-resistant exconjugant strains were compared on bean leaves sprayed with cupric hydroxide. Bean plants were used instead of cherry because of their rapid growth and their ability to harbor high epiphytic populations of bacteria. All wild-type strains were isolated from cherry blossoms in 1987 (18). Inoculum was prepared by growing each strain overnight in 20 ml of LB broth. The cultures were centrifuged, and the pellets were resuspended in 1 ml of K-buffer, which was diluted to 150 ml with buffer, after which the populations were adjusted turbidimetrically to 10^7 cells per milliliter. Navy bean (*Phaseolus vulgaris* L. 'Seafarer') plants were grown in 473-ml plastic cups (three plants per cup) in the greenhouse to a height of approximately 15 cm. Groups of 15 plants were spray inoculated until the leaves were uniformly wet with an atomizer containing a bacterial suspension. The plants then were transferred to a dew chamber maintained at 19 C with a 12-hr photoperiod.

Three days after inoculation, both adaxial and abaxial leaf surfaces were sprayed to runoff with Kocide 101 (cupric hydroxide) at 4.8 mg/ml. Control plants were not sprayed. Samples of either leaf disks or excised leaf tissue consisting of approximately 0.3 g were taken from one leaf on each of three plants when leaf surfaces were dry. There were two replications of each sample. The plants were sampled 1, 2, 4, and 7 days after copper application. Plants inoculated with an exconjugant also were sampled immediately before copper application. Each sample was placed in a 250-ml flask and washed with 20 ml of K-buffer, a solution that inactivates the bactericidal activity of copper (15,21), for 30 min on a Burrell wrist-action shaker. Aliquots (0.1 ml) from the original and from 10^{-1} to 10^{-3} dilutions of the original washings were plated (two replications) on KBc. Colonies of fluorescent bacteria were counted after 3 days of incubation at 23 C.

RESULTS

Source of copper resistant bacteria. Three levels of copper sensitivity in fluorescent bacteria were detected during testing of growth inhibition by cupric sulfate. Isolates were considered sensitive if they failed to grow on media amended with 40–120 μg of cupric sulfate per milliliter, as having low resistance if they grew on media amended with 160–240 μg of cupric sulfate per milliliter, and as having high resistance if they grew on media amended with 280–320 μg of cupric sulfate per milliliter. In 1987, 137 of 154 isolates (89.0%) were copper-sensitive. Of the remaining 17 isolates (11.0%), nine had low and eight high resistance.

In 1988, populations of fluorescent bacteria on blossoms in 13 orchards averaged 6.9 log colony-forming units per gram (cfu/g) fresh weight (Table 1). Recovery of bacteria on CYEG amended with 200 μg of cupric sulfate per milliliter and on KBc was high for 12 of 13 orchards. Ten isolates from those orchards were selected and 40 of them had low and 80 high resistance to copper. Four isolates from orchard M were copper-sensitive.

Identification of copper-resistant bacteria. All 137 copper-resistant isolates from both 1987 and 1988 were identified as *P. s. syringae* based on positive results for the GATTa tests and ice nucleation-activity. In 1987, 98 and 39 copper-sensitive isolates were identified as *P. s. syringae* and *P. s. morsprunorum*, respectively. In 1988, the four copper-sensitive isolates from orchard M were identified as *P. s. syringae*.

Plasmid characterization of copper-resistant strains. Fifteen of 102 randomly selected, copper-sensitive isolates from 1987 and 1988 were screened for plasmids and only one contained an indigenous, cryptic plasmid of approximately 58 kb. However, among the copper-resistant isolates identified in 1987, three isolates with low resistance lacked detectable plasmids, six isolates contained single plasmids of varied sizes (approximately 46, 55, 61, 67, and 73 kb), and eight isolates with high resistance all

contained a plasmid either 46 or 61 kb in size (Fig. 1). The three isolates lacking plasmids were from the same orchard and grew on CYEG with 160 μg of cupric sulfate per milliliter. Among 29 copper-resistant isolates screened in 1988, 11 with low and 18 with high resistance each contained a single plasmid of approximately 61 kb.

Copper-resistant isolates maintained their plasmids and resistance phenotypes after 50 transfers over a 1-yr-period on KB in the absence of copper ions.

Bacterial conjugation. Frequency of transfer of copper-resistance ranged from 3.4×10^{-3} to 3.4×10^{-8} exconjugants per recipient cell of *P. s. syringae* (Table 2). Repeated attempts to transfer copper resistance into two strains of *P. s. morsprunorum* were unsuccessful. Spontaneous mutants arose infrequently, and the observed rates of spontaneous mutation to copper or to antibiotic resistance were always less than 10^{-10} per cell. The frequency of transfer differed by as much as four orders of magnitude using the same donor but different recipients. Among the 20 matings, the highest frequency of transfer was between two strains (31B-4 and 31A-5 str^r) isolated from the same sour cherry orchard. The copper-resistant exconjugant 27A-4a, when used as a donor in subsequent matings, was able to transfer copper resistance to the two remaining recipients. Exconjugant colonies, randomly chosen from selection plates, always contained the 61-kb plasmid found in the donor strain (Fig. 2).

Frequency of transfer of copper-resistance between strains 31B-4 and 31A-5 str^r, infiltrated into bean leaves, averaged 3.6×10^{-6} per recipient cell among five 3-day matings. No exconjugants were detected among 12 1-day matings. The observed frequency of transfer was approximately 10^3 lower than that of the filter mating involving the two strains, but was higher than that of five other filter matings involving recipients 17A-5 str^r and 27A-4 rif^r. No spontaneous mutants were detected in the infiltration experiments.

Survival of *P. s. syringae* on copper sprayed bean leaves. Populations of copper-sensitive strains 18A-3, 19A-2, 47B-1, and 52A-2 were reduced more than populations of copper-resistant strains 18A-5, 22B-4, 31B-4, and 64B-3 at all sampling intervals except for day 2 after a spray application of Kocide 101 (Table 3). Throughout the four sampling intervals of the experiment, populations of copper-sensitive strains 18A-3, 19A-2, and 52A-2 averaged 3.3 log cfu/g and strain 47B-1 averaged 4.5 log cfu/g on copper sprayed bean leaves. Strains 18A-3, 19A-2, and 52A-2 were inhibited on CYEG amended with 40 μg of cupric sulfate per milliliter, while strain 47B-1 was inhibited with 120 μg of cupric sulfate per milliliter. Populations of copper-resistant strains averaged 5.6 log cfu/g on copper sprayed bean leaves and in some cases exceeded populations on nonsprayed controls.

Populations of exconjugant strain 27-17a remained high on bean leaves sprayed with copper through the 7-day sampling period. Populations of strain 27-17a and copper-resistance donor strain 31B-4 were consistently reduced less by sprays of copper than the populations of the other copper-resistant strains.

TABLE 1. Recovery of *Pseudomonas syringae* pv. *syringae* from cherry blossom washes on media with and without copper[a]

Orchard[b]	County	Bacterial populations[c] (log cfu/g fresh weight) KBc	CYEGc	Level of copper resistance[d] (no. isolates) Low	High
A	Mason	7.0	6.4	0	10
B	Mason	7.2	6.5	0	10
C	Mason	7.3	6.6	3	7
D	Oceana	7.6	7.1	1	9
E	Oceana	7.0	7.0	2	8
F	Oceana	7.1	6.4	6	4
G	Van Buren	6.9	6.9	5	5
H	Van Buren	6.9	6.9	5	5
I	Van Buren	6.9	6.9	5	5
J	Van Buren	6.8	6.8	4	6
K	Van Buren	6.9	6.9	4	6
L	Van Buren	6.8	6.8	5	5
M	Van Buren	4.9	0.0	0	0

[a] Blossom samples were collected from orchards that contained copper-resistant bacteria in 1987 or were sprayed with copper in 1988.
[b] Orchards C, D, and G were sweet cherry; the remaining orchards were sour cherry.
[c] Serial dilutions of washings of 200 blossoms per orchard were plated in duplicate on each medium and the number of colonies counted after 3 days of incubation at 23 C. KBc = King's medium B amended with 50 μg of cycloheximide per milliliter. CYEGc = CYE-glycerol medium amended with 200 μg of cupric sulfate per milliliter.
[d] Copper resistance level determined by confluent growth on CYEG medium amended with cupric sulfate. Low = inhibited by 160–240 μg of cupric sulfate per milliliter, high = inhibited by 280–320 μg of cupric sulfate per milliliter. A total of 10 isolates were tested per orchard.

TABLE 2. Frequency of conjugative transfer of copper resistance in *Pseudomonas syringae* pv. *syringae*[a]

Donor strains	Recipient strains[b] 17A-5 str^r	27A-4 rif^r	31A-5 str^r
31B-4	1.8×10^{-6}	3.5×10^{-6}	3.4×10^{-3}
70-7	5.8×10^{-4}	4.2×10^{-8}	2.7×10^{-5}
71-7	2.3×10^{-5}	3.2×10^{-4}	6.0×10^{-5}
72-9	1.3×10^{-4}	4.2×10^{-8}	4.8×10^{-5}
77-8	3.4×10^{-5}	7.0×10^{-7}	5.6×10^{-5}
79-2	8.5×10^{-6}	6.2×10^{-8}	3.2×10^{-4}
27A-4a[c]	2.0×10^{-4}	...	2.4×10^{-6}

[a] Frequency of spontaneous mutation of donors or recipients was always less than 10^{-10} per cell.
[b] Spontaneous antibiotic resistant copper-sensitive mutants, str = streptomycin and rif = rifampicin.
[c] Copper-resistant exconjugant constructed in mating of strains 31B-4 and 27A-4 rif^r.

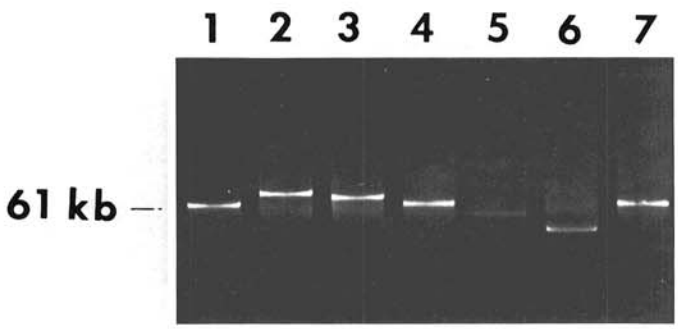

Fig. 1. Agarose gel electrophoresis of cleared lysates of copper-resistant strains of *Pseudomonas syringae* pv. *syringae* isolated from cherry orchards in Michigan in 1987. Lanes 1–7 are strains 31B-4, 43A-1 45A-3, 22B-4, 47B-4, 39B-1, and 18A-5, respectively.

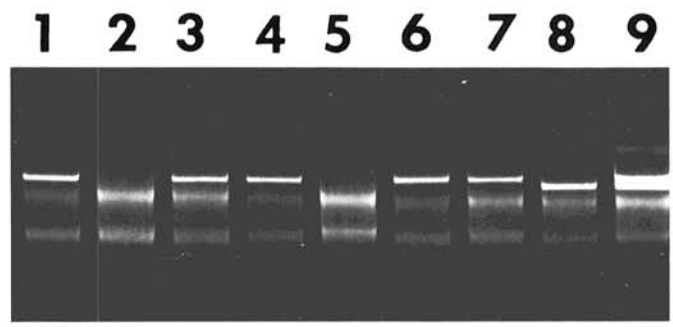

Fig. 2. Agarose gel electrophoresis of cleared lysates of donor, recipient, and exconjugant strains of *Pseudomonas syringae* pv. *syringae*. Lane 1, donor strain 31B-4; lane 2, recipient strain 27A-4 rif^r; lane 3, exconjugant 27A-4a; lane 4, donor strain 70-7; lane 5, recipient strain 31A-5 str^r; lane 6, exconjugant 31A-5a; lane 7, exconjugant donor strain 27A-4a; lane 8, recipient strain 17A-5 str^r; lane 9, exconjugant 27-17a.

TABLE 3. Recovery of copper-resistant and copper-sensitive strains of *Pseudomonas syringae* pv. *syringae* from bean leaves sprayed with a single copper treatment of 4.8 mg/ml of Kocide 101 (cupric hydroxide)

Bacterial strain[x]	Bacteria recovered from nonsprayed/copper sprayed leaves (log cfu/g leaf tissue)				Reduction in population on copper sprayed leaves (log cfu/g leaf tissue)			
	Day 1	Day 2	Day 4	Day 7	Day 1	Day 2	Day 4	Day 7
18A-3[s]	6.9/3.3	5.7/3.9	6.7/3.7	6.5/3.2	3.3 b[y]	1.7 b	3.0 a	3.3 a
19A-2[s]	6.5/0.0	6.0/2.7	6.4/3.7	6.4/3.2	6.5 a	3.3 a	2.7 a	3.2 a
47B-1[s]	6.9/4.3	6.4/5.0	6.6/4.1	6.9/4.5	2.6 c	1.4 bc	2.5 a	2.4 b
52A-2[s]	6.4/4.2	6.5/3.2	6.3/3.8	6.0/4.0	2.2 d	3.3 a	2.5 a	2.0 b
18A-5[r]	5.8/5.0	5.9/4.7	6.5/5.3	6.7/5.9	0.8 e	1.2 bc	1.2 b	0.8 c
22B-4[r]	6.7/5.7	5.9/5.4	5.5/6.7	6.4/5.8	1.0 e	0.5 cd	0.0 c	0.6 c
31B-4[r]	7.1/7.0	5.9/6.2	6.7/6.5	6.6/6.3	0.1 f	0.0 d	0.2 c	0.3 cd
64B-3[r]	6.6/5.5	6.8/4.8	6.2/4.9	6.8/6.0	1.1 e	2.0 b	1.3 b	0.8 c
27-17a[rz]	5.6/6.4	5.7/5.6	5.7/5.6	5.2/5.2	0.0 f	0.0 d	0.2 c	0.1 d

[x] s = copper-sensitive, r = copper-resistant.
[y] Means within a column followed by the same letter do not differ by Duncan's multiple range test ($P = 0.05$).
[z] Copper-resistant exconjugant constructed in mating between strains 27A-4a and 17A-5 str[r].

DISCUSSION

Copper-resistant *P. s. syringae* were recovered from blossoms from nine and 12 cherry orchards in Michigan in 1987 and 1988, respectively. Orchards harboring the resistant strains were located in four counties and were previously sprayed with copper. In 1988, populations of copper-resistant bacteria on blossoms from seven orchards exceeded 10^7 cfu/g of blossom tissue, and at this level the populations were near the carrying capacity of cherry trees for *P. s. syringae* (16). The wide distribution of copper-resistant *P. s. syringae* on cherry and its ability to survive and multiply on the surfaces of bean plants sprayed with copper is evidence that copper bactericides are no longer effective for reducing populations of *P. s. syringae* in the Michigan orchards surveyed.

The detection of copper-resistant strains for two consecutive years in eight orchards is evidence that these strains survive from season-to-season in the presence of copper applications. Copper-resistant *P. s. syringae* under laboratory conditions remained resistant to copper and retained a plasmid after 1 yr of growth on media without copper ions. The stability of metal resistance in bacteria has been reported by Griffiths et al (5). It is possible that copper-resistant *P. s. syringae* may persist in orchards for long periods even in the absence of copper applications, although the proportion of resistant strains in the population may decline. Once populations of resistant strains have developed in an orchard, it may no longer be practical to use copper as resistant populations will redevelop quickly.

The spread of copper resistance within field populations of *P. s. syringae* likely is enhanced by conjugation. Copper-sensitive strains of *P. s. syringae* isolated from three cherry growing areas of Michigan were able to acquire the 61-kb plasmid from all six donors used in mating experiments. The frequency of transfer of copper resistance, however, was highest in filter matings between isolates from the same orchard. The demonstration of conjugation in bean leaves indicates that transfer of the 61-kb plasmid and associated gene(s) for copper resistance is not limited by the host. Plasmid transfer of antibiotic resistance in plants previously was demonstrated for *P. s. glycinea* (9). Recent reports of conjugative transfer of copper-resistance in *P. s. tomato* (1) and of streptomycin resistance in *P. s. papulans* (2) are further evidence that pathovars of *P. syringae* have a high potential for developing resistance to antibiotics and copper under selection pressure in the field.

Copper-resistant strains of *P. s. syringae* harboring a 61-kb plasmid were detected in orchards separated from one another by up to 160 km. An explanation for the presence of copper-resistant *P. s. syringae* in a number of widely separated Michigan cherry orchards may be that the copper-resistant bacteria are disseminated on nursery trees. In 1988, high populations of copper-resistant *P. s. syringae* were recovered as epiphytes on blossoms of sweet cherry taken from trees used as a source for budwood. The retention of resistance once it is acquired and the ability of *P. s. syringae* to grow epiphytically on a wide variety of plant hosts (11) would contribute to the buildup and survival of disseminated strains harboring plasmids. It is also likely, because the size of the plasmid in copper-resistant strains varied between orchards, that resistant strains were selected independently in some orchards due to frequent use of copper sprays.

The recovery of only copper-sensitive *P. s. morsprunorum* from orchards containing copper-resistant *P. s. syringae* and the unsuccessful transfer of the 61-kb plasmid from *P. s. syringae* to *P. s. morsprunorum* in repeated attempts at conjugation is evidence that these pathovars are incompatible at the genetic level. Although the pathovars exist together as epiphytes and as pathogens in cherry orchards, they have mingled very little at the phenotypic level (17). Failure of natural conjugation would help to maintain a high level of genetic diversity between the pathovars. If this is true, there should be no problem in differentiating between *P. s. syringae* and *P. s. morsprunorum* at the genetic level.

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