JOURNAL OF BACTERIOLOGY, Feb. 1986, p. 534–541
0021-9193/86/020534-08$02.00/0

Vol. 165, No. 2

Indigenous Plasmids in *Pseudomonas syringae* pv. *tomato*: Conjugative Transfer and Role in Copper Resistance

CAROL L. BENDER AND DONALD A. COOKSEY*

Department of Plant Pathology, University of California, Riverside, California 92521

Received 15 July 1985/Accepted 11 November 1985

Twenty strains of *Pseudomonas syringae* pv. *tomato* were examined for the presence of plasmid DNA. *P. syringae* pv. *tomato* plasmids were grouped into five size classes: class A ranged from 95 to 103 kilobases (kb); class B ranged from 71 to 83 kb; class C ranged from 59 to 67 kb; class D ranged from 37 to 39 kb; and class E was 29 kb. All strains contained at least two plasmids in classes A and B. The conjugative ability of *P. syringae* pv. *tomato* plasmids in three strains was demonstrated by mobilization of the nonconjugative plasmid RSF1010 into *Pseudomonas syringae* pv. *syringae* recipients. Plasmids from the three conjugative strains were labeled with Tn*5*. Four conjugative plasmids were identified by their repeated transfer to *P. syringae* pv. *syringae* recipients. *P. syringae* pv. *tomato* strains varied in sensitivity to copper sulfate ($CuSO_4$): MICs were 0.4 to 0.6 mM for sensitive strains, 1.2 mM for moderately resistant strains, and 1.6 to 2.0 mM for very resistant strains. One very resistant strain, PT23, functioned as a donor of copper resistance. Recipient *P. syringae* pv. *syringae* strains PS51 and PS61 were inhibited by 0.1 mM $CuSO_4$, whereas the $CuSO_4$ MICs for transconjugant strains PS51(pPT23A) and PS61(pPT23C) were 1.8 and 2.6 mM, respectively. *P. syringae* pv. *tomato* strains PT12.2 and PT17.2 were inhibited by 0.6 mM copper sulfate, but their copper sulfate MICs were 2.6 and 1.8 mM, respectively, when they acquired pPT23C. Therefore, copper resistance in PT23 was controlled by two conjugative plasmids, designated pPT23A (101 kb) and pPT23C (67 kb).

Many pathovars of *Pseudomonas syringae* contain indigenous plasmids (3, 6, 7, 26–28). Most of these plasmids are phenotypically cryptic; however, Comai and Kosuge (3) have demonstrated the involvement of an indigenous plasmid from *Pseudomonas syringae* pv. *savastanoi* in the production of indoleacetic acid, a compound essential for gall development in the olive plant.

Although conjugative plasmids are important mediators of genetic exchange, they have been identified in only two of the 41 pathovars of *P. syringae*. pBPW1 is a 30-megadalton conjugative plasmid isolated from *Pseudomonas syringae* pv. *tabaci* BR2 (B. J. Staskawicz, M. Sato, and N. J. Panopoulos, Phytopathology **71**:257, 1984), and pCG131 is a conjugative plasmid isolated from *Pseudomonas syringae* pv. *syringae* (14). Both of these plasmids are phenotypically cryptic. Since most *P. syringae* plasmids lack a selectable marker, they must be labeled with a drug resistance transposon or used to mobilize a nonconjugative drug resistance plasmid to demonstrate conjugative transfer.

Plasmid-determined resistance to toxic metal ions has been demonstrated for many bacterial species (13, 30) and is a useful selectable marker for these DNA molecules. Copper sprays are toxic to bacteria and are frequently applied for control of diseases caused by phytopathogenic species. Although copper resistance in bacteria is relatively rare, resistant species have been isolated from several diverse sources (2, 8, 9, 21, 32, 33). Copper resistance has recently been reported in *Xanthomonas campestris* pv. *vesicatoria*, which is pathogenic on tomato and pepper plants (24). Although the genetic basis of copper resistance is not well characterized, this phenotype has been associated with conjugative plasmids in several instances (16, 32; R. E. Stall, D. C. Loschke, and R. W. Rice, Phytopathology **74**:797, 1984). Copper resistance has not been reported in *Pseudomonas syringae* pv. *tomato*, the cause of bacterial speck disease of tomato. However, copper sprays are often only marginally effective in controlling the disease (10, 22). In a preliminary study (C. L. Bender and D. A. Cooksey, Phytopathology, **72**:1325, 1985), we found that *P. syringae* pv. *tomato* strains differed in sensitivity to copper sulfate. The objectives of the present study were to screen *P. syringae* pv. *tomato* strains for the presence of plasmid DNA, to determine whether indigenous plasmids in *P. syringae* pv. *tomato* were conjugative, and to assess the role of plasmid DNA in the resistance of the organism to copper.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in mating experiments are listed in Table 1. *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* strains were maintained on mannitol-glutamate (MG) medium (19) supplemented with yeast extract at 0.25 g/liter (MGY medium); antibiotics were added as necessary to maintain selection for resistance markers. *P. syringae* broth cultures were grown in MGY medium on a rotary shaker (200 rpm) at 28°C; *Escherichia coli* cultures were grown in LB broth at 37°C. Selective antibiotic concentrations were as follows: streptomycin sulfate, 25 μg/ml, rifampin, 50 μg/ml, chloramphenicol, 50 μg/ml, spectinomycin, 12.5 μg/ml; and kanamycin, 10 μg/ml for *P. syringae* or 25 μg/ml for *E. coli*.

Plasmids from 20 strains of *P. syringae* pv. *tomato* were isolated and sized in this study. These 20 strains were consecutively numbered PT11 through PT30. With the exception of PT29, which was provided by D. Fulbright, Michigan State University, East Lansing, Mich., all strains originated in this study. *P. syringae* pv. *tomato* plasmids were given the designation pPT, followed by a strain number and letter designation. *P. syringae* pv. *tomato* plasmids were sized as described by Crosa and Falkow (4).

Bacterial conjugations between *P. syringae* and *E. coli* were performed on either KMB (20) or YDC (34) agar medium. Conjugations between *P. syringae* pv. *tomato* and

* Corresponding author.

TABLE 1. *P. syringae* and *E. coli* strains and plasmids used in conjugation experiments

Strain	Chromosomal phenotype	Plasmids	Construction (recipient × donor[s])
P. syringae pv. *tomato*			
PT11		pPT11A, pPT11B	
PT12		pPT12A, pPT12B	
PT15		pPT15A, pPT15B, pPT15D	
PT17		pPT17A, pPT17B	
PT23		pPT23A, pPT23B, pPT23C, pPT23D	
PT25		pPT25A, pPT25B, pPT25D	
PT29		pPT29A, pPT29B	
PT11.1		pPT11A, pPT11B, RSF1010	[PT11 × C600(RSF1010) × HB101(pRK2013)]
PT12.1		pPT12A, pPT12B, RSF1010	[PT12 × C600(RSF1010) × HB101(pRK2013)]
PT12.2	Rif^r Cm^r	pPT12B	Rif^r Cm^r PT12 cured of pPT12A
PT12.21	Cu^r	pPT12B	Cu^r PT12.2
PT12.22	Rif^r Cm^r	pPT12B, pPT23C	PT12.2 × PT23
PT15.1		pPT15A, pPT15B, pPT15D, RSF1010	[PT15 × C600(RSF1010) × HB101(pRK2013)]
PT17.1		pPT17A, pPT17B, RSF1010	[PT17 × C600(RSF1010) × HB101(pRK2013)]
PT17.2	Sp^r	pPT17A, pPT17B	Sp^r PT17
PT17.21	Sp^r	pPT17A, pPT17B, pPT23C	PT17.2 × PT12.22
PT17.3	Rif^r Cm^r	pPT17A, pPT17B	Rif^r Cm^r PT17
PT17.31	Rif^r Cm^r	pPT17A, pPT17B, pPT23C::Tn*5*	PT17.3 × PS52.3
PT23.1		pPT23A, pPT23B, pPT23C pPT23D, RSF1010	[PT23 × C600(RSF1010) × HB101(pRK2013)]
PT29.1		pPT29A, pPT29B, RSF1010	[PT29 × C600(RSF1010) × HB101(pRK2013)]
P. syringae pv. *syringae*			
PS5		No plasmids	
PS51	Rif^r Cm^r	No plasmids	Rif^r Cm^r PS5
PS51.1	Rif^r Cm^r	pPT11B, RSF1010	PT11.1 × PS51
PS51.2	Rif^r Cm^r	pPT23A::Tn*5*	PS51 × PT23::Tn*5* mutants
PS51.3	Rif^r Cm^r	pPT23C::Tn*5*	PS51 × PT23::Tn*5* mutants
PS51.4	Rif^r Cm^r	pPT11B::Tn*5*	PS51 × PS52.1::Tn*5* mutants
PS51.5	Rif^r Cm^r	pPT17A::Tn*5*	PS51 × PS62.1::Tn*5* mutants
PS51.6	Rif^r Cm^r	pPT17B::Tn*5*	PS51 × PS62.1::Tn*5* mutants
PT51.7	Rif^r Cm^r	pPT23A	PS51 × PT23
PS52	Sp^r	No plasmids	Sp^r PS5
PS52.1	Sp^r	pPT11B, RSF1010	PS52 × PT11.1
PS52.2	Sp^r	pPT23A	PS52 × PS51.7
PS52.3	Sp^r	pPT23C::Tn*5*	PS52 × PS51.3
PS6		pPS6	
PS61	Rif^r Cm^r	pPS6	Rif^r Cm^r PS6
PS61.1	Rif^r Cm^r	pPT17A, pPT17B, pPS6, RSF1010	PS61 × PT17.1
PS61.2	Rif^r Cm^r	pPT23C-pPS6 cointegrate	PS61 × PT23
PS61.3	Rif^r Cm^r	pPT23C::Tn*5*, pPS6	PS61 × PS52.3
PS62	Sp^r	pPS6	Sp^r PS6
PS62.1	Sp^r	pPT17A, pPT17B, pPS6, RSF1010	PS62 × PT17.1
PS62.2	Sp^r	pPT23C, pPS6	PS62 × PS61.2
E. coli			
HB101[a]		pRK2013	
C600[b]		RSF1010	
WA803[c]		pGS9::Tn*5*	

[a] The phenotype of *E. coli* HB101 is described by Maniatis et al. (23); plasmid pRK2013 is described by Figurski and Helinski (11).
[b] The phenotype of *E. coli* C600 is described by Maniatis et al. (23); plasmid RSF1010 is described by Guerry et al. (15).
[c] The phenotype of *E. coli* WA803 is described by Wood (35); plasmid pGS9::Tn*5* is described by Selvaraj and Iyer (29).

P. syringae pv. *syringae* were performed on MG or MGY agar plates.

Mating conditions. All matings were performed with overnight cultures of *E. coli* grown on LB plates and 36- to 48-h cultures of *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* grown on MGY or minimal A (25) agar plates. Two different agar surface matings were used in this study. Conjugations between the *E. coli* transposon donor C600(pGS9::Tn*5*) and *P. syringae* recipients were performed by a method similar to that described by Anderson and Mills (1). These matings were conducted on YDC agar plates by mixing loopfuls of approximately 10^{10} cells from both donor and recipient cultures in a 1:1 ratio. The plates were incubated at 28°C for 12 h, and cells were suspended in sterile distilled water, serially diluted, and plated onto MG agar plates containing kanamycin.

All other matings were performed by combining equal volumes of donor and recipient cultures containing approximately 5×10^8 cells per ml in sterile distilled water and plating 0.1 ml of the mixture to the mating medium. All matings were performed for 12 to 16 h at 18, 28, or 37°C as indicated.

RSF1010 mobilization. The nonconjugative plasmid RSF1010 was mobilized into *P. syringae* pv. *tomato* recipients by the recombinant helper plasmid pRK2013. *E. coli* C600(RSF1010), HB101(pRK2013), and various *P. syringae* pv. *tomato* recipients were combined in a 1:1:1 ratio and mated on KMB agar plates at 28°C. After mating, *P. syringae* pv. *tomato* (RSF1010) transconjugants were selected by plating on MG medium with streptomycin. Sm^r *P. syringae* pv. *tomato* colonies were purified to single colonies four times, and the presence of RSF1010 was confirmed by agarose gel electrophoresis. *P. syringae* pv. *tomato* (RSF1010) transconjugants were then mated with *P. syringae* pv. *syringae* recipients PS51 and PS61. After mating, mobilization of RSF1010 into the *P. syringae* pv. *syringae* recipients was selected on medium containing rifampin, chloramphenicol, and streptomycin. Sm^r recipients were purified and analyzed for the presence of plasmid DNA.

Plasmid-labeling experiments. The transposon suicide vector pGS9::Tn*5* was transferred to *E. coli* C600 by mating the recipient C600 (Sm^r) with *E. coli* WA803(pGS9::Tn*5*) on LB agar at 37°C. Transconjugants were selected on medium containing streptomycin, chloramphenicol, and kanamycin; the transfer of pGS9::Tn*5* to C600 was confirmed by agarose gel electrophoresis.

E. coli C600(pGS9::Tn*5*) was used to mutagenize *P. syringae* pv. *tomato* strain PT23 and *P. syringae* pv. *syringae* strains PS52.1 and PS62.1. Between 400 and 450 Km^r mutants were recovered from each of these three matings after a 5-day incubation at 28°C. Mutants from each mating were removed and suspended separately in 4 ml of sterile distilled water. Two milliliters of the mutant mixture was then combined with an equal volume of the recipient PS51. The entire mixture was plated onto MG agar plates and incubated at 18°C for 16 h. The transfer of Tn*5*-labeled *P. syringae* pv. *tomato* plasmids was selected on medium containing rifampin, chloramphenicol, and kanamycin and confirmed by gel electrophoresis.

The transmissibility of Tn*5*-labeled *P. syringae* pv. *tomato* plasmids was further investigated by mating donor strains (Rif^r Cm^r Km^r) with the recipient strain PS52 (Sp^r). After mating, Sp^r Km^r colonies were screened for the donor markers (Rif^r Cm^r) and analyzed by gel electrophoresis.

Copper resistance experiments. All 20 *P. syringae* pv. *tomato* strains and *P. syringae* pv. *syringae* strains PS51 and PS61 were tested for sensitivity or resistance to copper sulfate. Cultures to be screened were grown for 36 to 48 h on MGY agar plates at 28°C. Strains were then streaked to MGY agar plates containing copper sulfate at concentrations ranging from 0.05 to 4.0 mM.

Potential donor (Cu^r) and recipient (Cu^s Rif^r Cm^r or Cu^s Sp^r) strains were mated on MGY agar plates for 12 to 16 h at 28°C. After mating, the transfer of Cu^r to recipient strains was selected on medium containing the appropriate antibiotics and 0.4 mM $CuSO_4$ for *P. syringae* pv. *syringae* recipients or 1.0 mM or 1.6 mM $CuSO_4$ for *P. syringae* pv. *tomato* recipients.

Plasmid DNA isolations. Plasmid DNA was isolated by a modification of the method described by Crosa and Falkow (4). Overnight cultures (5 ml each) were centrifuged, and each pellet was washed once in 1.0 ml of TE buffer (0.05 M Tris hydrochloride, 0.02 M EDTA; pH 8.5). After sodium dodecyl sulfate-alkaline lysis, the suspension was incubated over ice for 4 h. Plasmid DNA was separated by electrophoresis through a horizontal slab gel of 0.7% agarose submersed in recirculated Tris-borate running buffer at 4°C for the times indicated in the figures. The gels were stained with ethidium bromide and photographed with Polaroid type 55 Land film and Wratten yellow and red filters (no. 9 and 23A) under 302-nm UV light.

DNA hybridizations. Biotinylated DNA probes were made from pPT23C, pPS6, and an internal fragment of Tn*5*. Plasmid DNA from PT23 and PS6 was obtained as described above. Approximately 1 μg of pPT23C and pPS6 was obtained from gel electrophoresis of 5 and 2 μg of PT23 and PS6 plasmid DNA, respectively. The plasmids were separated at 5 V/cm for 12 h in a 0.7% low-melting-point agarose gel submersed in Tris-acetate buffer (5). Gel slices containing pPT23C and pPS6 were weighed in separate microfuge tubes, and the volume in each tube was brought to 500 μl by addition of TE buffer. Gel slices were then melted at 70°C for 15 min, 500 μl of warm phenol (50°C) and 200 μl of chloroform were added to each tube, and the mixture was chilled at −20°C for 10 min. The aqueous phase was obtained by a 15-min centrifugation in an Eppendorf microfuge; this was followed by two phenol-chloroform extractions and a final chloroform extraction. The two volumes of cold 95% ethanol were added, and the NaCl concentration was adjusted to 0.1 M. DNA was precipitated at −20°C for 30 min. Pellets were obtained by a 10-min centrifugation in an Eppendorf microfuge; these were washed once with 1 ml of 95% ethanol.

A 3.3-kilobase (kb) *Hin*dIII internal fragment of Tn*5* (18) was used as a probe to verify plasmid labeling. This fragment was obtained by digesting approximately 4 μg of ColEI::Tn*5* DNA with *Hin*dIII (purchased from Bethesda Research Laboratories and used as recommended). Linear fragments were separated by gel electrophoresis at 8.6 V/cm for 3 h in a 0.7% low-melting-point agarose gel submersed in Tris-acetate buffer. Nick translation of pPT23C, pPS6, and the 3.3-kb Tn*5* fragment was performed as specified in the nick translation reagent kit from Bethesda Research Laboratories. Unincorporated nucleotides were removed by spun-column chromatography (23).

DNA fragments from agarose gels were denatured and transferred to nitrocellulose filters as described by Maniatis et al. (23). Posthybridization washes, filter blocking, and detection of homologous sequences were performed as specified in the alkaline phosphatase DNA detection system manufactured by Bethesda Research Laboratories.

RESULTS

***P. syringae* pv. *tomato* plasmids.** *P. syringae* pv. *tomato* plasmids could be grouped into five size classes: class A, which ranged from 95 to 103 kb; size class B, which ranged from 71 to 83 kb; class C, which ranged from 59 to 67 kb; class D, which ranged from 37 to 39 kb; and class E, which was 29 kb. All 20 strains contained plasmids in classes A and B. Seven strains contained only the class A and B plasmids, 10 strains contained three plasmids (classes A, B, and D), two strains contained four plasmids (classes A, B, C, and D), and one strain contained class A, B, and E plasmids (Fig. 1).

RSF1010 mobilization. The nonconjugative plasmid RSF1010 was mobilized into *P. syringae* pv. *tomato* at frequencies ranging from 1.0×10^{-4} to 3.5×10^{-6}. The conjugative ability of *P. syringae* pv. *tomato* plasmids was investigated by determining whether they would mobilize RSF1010, which codes for streptomycin resistance, into *P. syringae* pv. *syringae* recipients. *P. syringae* pv. *syringae* strains PS51 and PS61 were chosen as recipients because they do not contain resident plasmids in the same size class as the *P. syringae* pv. *tomato* plasmids. The frequency of

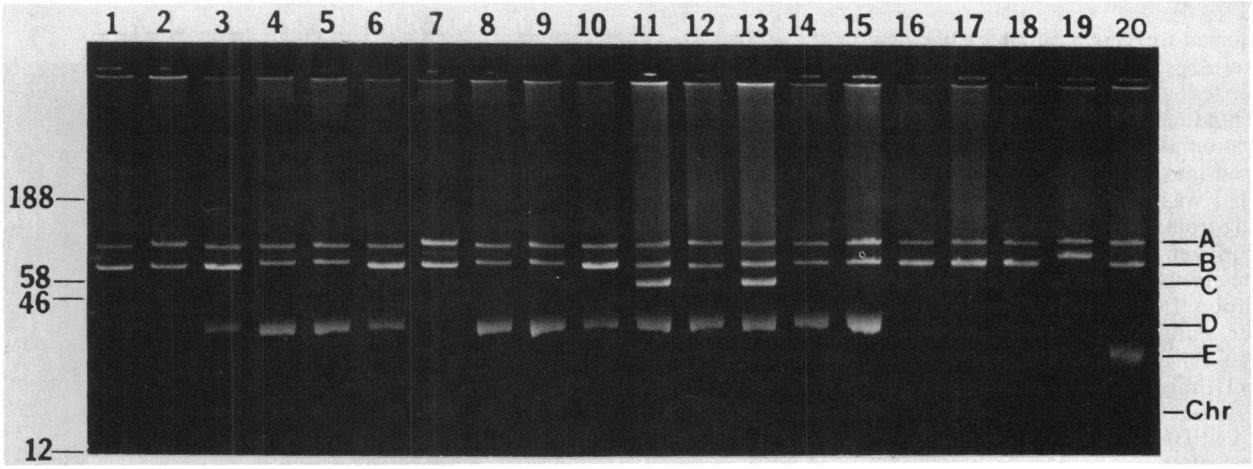

FIG. 1. Agarose gel electrophoresis of indigenous plasmid DNA from *P. syringae* pv. *tomato*. Electrophoresis proceeded for 12 h at 5 V/cm. Plasmid bands are identified from top to bottom. Lane 1, PT11; lane 2, PT12; lane 3, PT13; lane 4, PT14; lane 5, PT15; lane 6, PT16; lane 7, PT17; lane 8, PT18; lane 9, PT19; lane 10, PT20; lane 11, PT21; lane 12, PT22; lane 13, PT23; lane 14, PT24; lane 15, PT25; lane 16, PT26; lane 17, PT27; lane 18, PT28; lane 19, PT29; lane 20, PT30. The migrations of the molecular weight standards, ColE1::Tn*5* (12 kb), RP4 (58 kb), and the indigenous plasmids in *Agrobacterium tumefaciens* K84 (188 and 46 kb), are indicated to the left of the gel. The mobilities of plasmid size classes A through E are indicated to the right of the gel. Chr, Chromosomal DNA.

streptomycin resistance in PS51 and PS61 after mating with the *P. syringae* pv. *tomato* (RSF1010) donors ranged from 1.3×10^{-3} to $<1.6 \times 10^{-9}$. Control platings of PS51 and PS61 onto media containing streptomycin indicated that their spontaneous frequencies of streptomycin resistance were 6.2×10^{-9} and $<5.2 \times 10^{-10}$, respectively. The frequency of streptomycin resistance was higher than the spontaneous level in five matings. Streptomycin resistance was transferred to PS51 at frequencies of 2.0×10^{-4} and 3.7×10^{-5} when donor strains were PT11.1 and PT23.1, respectively. When PS61 was the recipient in matings with PT11.1, PT17.1, and PT23.1, streptomycin resistance was transferred at frequencies of 1.3×10^{-3}, 1.4×10^{-8}, and 5.0×10^{-4}, respectively. Ten Sm^r recipients from each of these five matings were purified, and plasmid DNA was isolated. When the donor PT11.1 was mated with PS51, Sm^r recipient colonies contained both RSF1010 and pPT11B. When PT17.1 was mated with PS61, Sm^r recipients contained RSF1010 and both pPT17A and pPT17B. In the other three matings (PT11.1 × PS61, PT23.1 × PS51, and PT23.1 × PS61), RSF1010 was present in the Sm^r recipient colonies, but *P. syringae* pv. *tomato* plasmids were absent.

Plasmid labeling with Tn*5*. The transposon Tn*5* was used to label several *P. syringae* pv. *tomato* plasmids with kanamycin resistance. This experiment was designed to determine which of the four plasmids in PT23 was conjugative and how pPT11B, pPT17A, and pPT17B were transferred to the *P. syringae* pv. *syringae* recipients in the RSF1010 mobilization experiment. Conjugative Tn*5*-labeled plasmids would be self-transmissible and selectable (Km^r), whereas Tn*5*-labeled, nonconjugative plasmids would not be transferred in the absence of a second conjugative plasmid.

The suicide vector pGS9::Tn*5* was used in *E. coli* C600 rather than the original *E. coli* strain, WA803, because preliminary work indicated a higher frequency of mutagenesis in *P. syringae* pv. *tomato* with C600(pGS9::Tn*5*). PT23 and two *P. syringae* pv. *syringae* transconjugants, PS52.1(pPT11B) and PS62.1(pPT17A, pPT17B), were mutagenized with C600(pGS9::Tn*5*). The frequencies of mutagenesis in PS52.1, PS62.1, and PT23 were approximately 5.0×10^{-5}, 5.2×10^{-8}, and 6.5×10^{-8}, respectively.

When transposon mutants of PS52.1, PS62.1, and PT23 were mated with PS51, kanamycin resistance was transferred to PS51 at frequencies ranging from 1.9×10^{-5} to 1.3×10^{-7}. No spontaneous Km^r mutants were recovered in control platings of recipient cells alone. Therefore, these frequencies suggested the transfer of Tn*5*-labeled *P. syringae* pv. *tomato* plasmids to the Km^r recipients. Ten Km^r recipient colonies from each of the three matings were purified, and plasmid DNA was obtained. Two different kinds of transconjugants were recovered when Tn*5* mutants of PT23 were mated with PS51. Three transconjugants contained pPT23A::Tn*5*, and seven received pPT23C::Tn*5* (Fig. 2A). All Km^r transconjugants contained pPT11B::Tn*5* when Tn*5* mutants of PS52.1 were mated with the recipient PS51. When Tn*5* mutants of PS62.1 were mated with PS51, eight transconjugants contained pPT17A::Tn*5*, and two transconjugants received pPT17B::Tn*5*. Plasmid labeling with Tn*5* was confirmed by hybridizing pPT23A::Tn*5*, pPT23C::Tn*5* (Fig. 2B), pPT17A::Tn*5*, pPT17B::Tn*5*, and pPT11B::Tn*5* (data not shown) with the biotinylated 3.3-kb *Hin*dIII fragment of Tn*5*.

PS51 transconjugants containing Tn*5*-labeled *P. syringae* pv. *tomato* plasmids were mated with the recipient PS52 (Sp^r) to determine whether these plasmids were conjugative. Control platings of the recipient PS52 indicated that the spontaneous frequency to Km^r was less than 10^{-10}. Control platings of the donor PS51 (Rif^r Cm^r) indicated that the spontaneous frequency to Sp^r was 5.0×10^{-7}. When PS51.2, PS51.3, PS51.4, and PS51.6 were used as donors, Km^r was transferred at frequencies of 3.1×10^{-3}, 1.1×10^{-3}, 1.5×10^{-2}, and 1.0×10^{-4}, respectively. Ten transconjugants from each of these four matings were purified, checked for Rif^s Cm^s, and analyzed for plasmid DNA. Transconjugants from these four matings were Rif^s Cm^s Sp^r; plasmid DNA isolations indicated the conjugative transfer of pPT23A::Tn*5*, pPT23C::Tn*5*, pPT11B::Tn*5*, and pPT17B::Tn*5* when the donor strains were PS51.2, PS51.3, PS51.4, and PS51.6, respectively. When PS51.5 was used as a donor, the transfer frequency of Km^r was 3.5×10^{-7}. This was similar to the spontaneous mutation frequency of PS51 to Sp^r. The Sp^r Km^r colonies obtained in this mating were

found to be Rif^r Cm^r; therefore, pPT17A::Tn*5* was not transferred to PS52 at a detectable level.

Screening for copper resistance. The $CuSO_4$ MIC was designated as the concentration of copper sulfate which prevented confluent growth of the culture after a 48-h incubation at 28°C. *P. syringae* pv. *tomato* strains could be grouped into three sensitivity classes based on their MICs. Group I was sensitive to concentrations of copper sulfate ranging from 0.4 to 0.6 mM. There were eight strains in this group; seven strains contained two plasmids in classes A and B; one strain contained three plasmids in classes A, B, and E. Group II contained two strains which could tolerate copper sulfate up to 1.2 mM; these had three plasmids (classes A, B, and D). Copper sulfate MICs for strains in group III ranged from 1.6 to 2.0 mM. There were 10 strains in this group containing either three (A, B, and D) or four (A, B, C, and D) plasmids. Group I was classifed as sensitive to copper sulfate, group II was classified as moderately resistant, and group III was classified as very resistant. Copper sulfate MICs for *P. syringae* pv. *syringae* strains PS51 and PS61 were both 0.1 mM.

To determine whether the copper resistance determinant of group III strains PT15, PT23, and PT25 was borne on a transmissible plasmid, these strains were used as Cu^r donors in mating experiments with a Cu^s group I strain, PT12.2. Resistance to 1.0 mM $CuSO_4$ was transferred to PT12.2 at frequencies ranging from 1.9×10^{-6} to 6.6×10^{-7}. These values were similar to the spontaneous mutation frequency of PT12.2 to copper resistance, which was 1.8×10^{-6}. However, two types of Cu^r colony morphology were observed when PT23 was mated with PT12.2. Most colonies were very small and resembled the spontaneous Cu^r mutants obtained in control platings of PT12.2; however, a few large fluorescent colonies were obtained. Plasmid DNA was isolated from both colony morphology types to determine whether a PT23 plasmid was transferred to PT12.2. The gel patterns indicated that the large fluorescent Cu^r colonies of PT12.2 contained an extra plasmid with a mobilty identical to that of pPT23C. The small, restricted Cu^r colonies contained only pPT12B, the single indigenous plasmid present in PT12.2. The Cu^r transconjugant PT12.22 (pPT23C) was mated with the group I strain PT17.2 to determine whether it could serve as a Cu^r donor in subsequent conjugations. Resistance to 1.6 mM $CuSO_4$ was transferred to PT17.2 at a frequency of 7.8×10^{-6}. Plasmid isolations indicated that Cu^r colonies of PT17.2 contained the pPT23C plasmid.

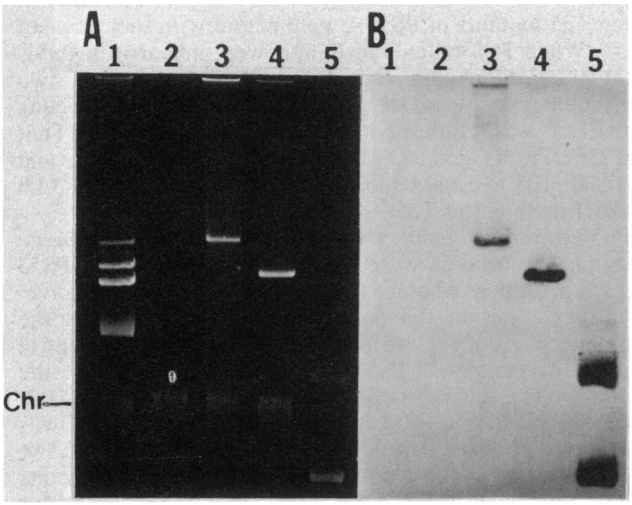

FIG. 2. Tn*5*-labeling of indigenous plasmids in *P. syringae* pv. *tomato* PT23. (A) Agarose gel electrophoresis of plasmid DNA in the donor PT23 and recipient PS51. Electrophoresis was at 5 V/cm for 12 h. (B) Hybridization of a biotin-labeled 3.3-kb *Hin*dIII internal fragment of Tn*5* to nitrocellulose filters containing plasmid DNAs shown in panel A. Bands are identified from top to bottom. Lane 1, PT23, pPT23A, pPT23B, pPT23C, and pPT23D; lane 2, PS51; lane 3, PS51.2 and pPT23A::Tn*5*; lane 4, PS51.3 and pPT23C::Tn*5*; lane 5, ColE1::Tn*5*, open circular, and covalent closed. Chr, Chromosomal DNA.

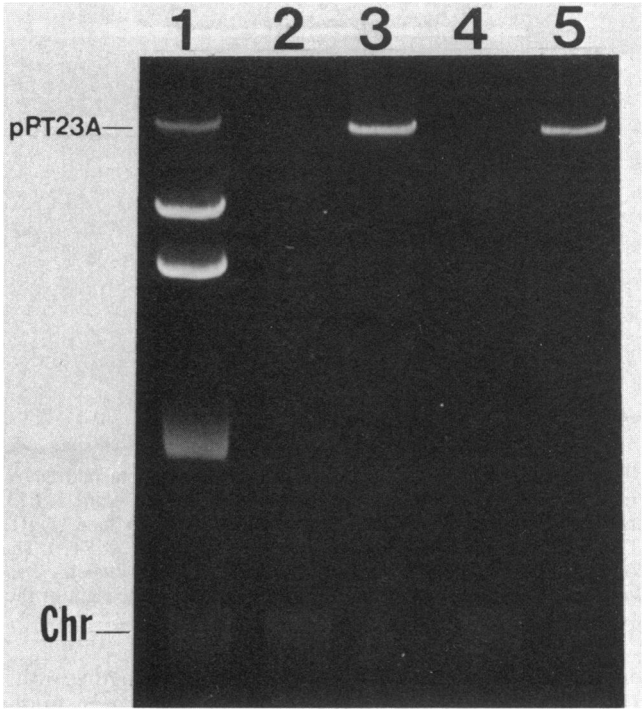

FIG. 3. Agarose gel electrophoresis of plasmid DNA from PT23 and Cu^r *P. syringae* pv. *syringae* transconjugants. Electrophoresis was at 5 V/cm for 24 h. lane 1, PT23, pPT23A, pPT23B, pPT23C, and pPT23D; lane 2, PS51; lane 3, PS51.7 and pPT23A; lane 4, PS52; lane 5, PS52.2 and pPT23A. Chr, Chromosomal DNA.

Mating experiments were also conducted between the donor strain, PT23, and PS51 and PS61 to determine whether copper resistance could be transferred to *P. syringae* pv. *syringae*. Cu^r was transferred from PT23 to PS51 at a frequency of 2.9×10^{-5}. This was significantly higher than the PS51 frequency of spontaneous mutation to copper resistance, which was 4.0×10^{-8}. To confirm that a plasmid was transferred from PT23 to PS51, plasmid DNA was isolated from 10 Cu^r PS51 colonies (Fig. 3, lanes 1 through 3). The gel patterns showed that the recipient strain PS51 contained no plasmids (Fig. 3, lane 2), but all Cu^r PS51 transconjugants contained a single plasmid with a mobility identical to that of pPT23A (lanes 1 and 3). The PS51 transconjugant, PS51.7, was used as a Cu^r donor in a subsequent mating with the Cu^s recipient, PS52. Copper resistance was transferred to PS52 at a frequency of 2.0×10^{-1}. Copper-resistant PS52 colonies contained the pPT23A plasmid from PS51 (Fig. 3, lanes 3 through 5).

Copper resistance was transferred from PT23 to PS61 at a frequency of approximately 3.8×10^{-5} per recipient cell; the PS61 frequency of spontaneous mutation to copper resistance was less than 1.6×10^{-10}. When plasmid DNA was

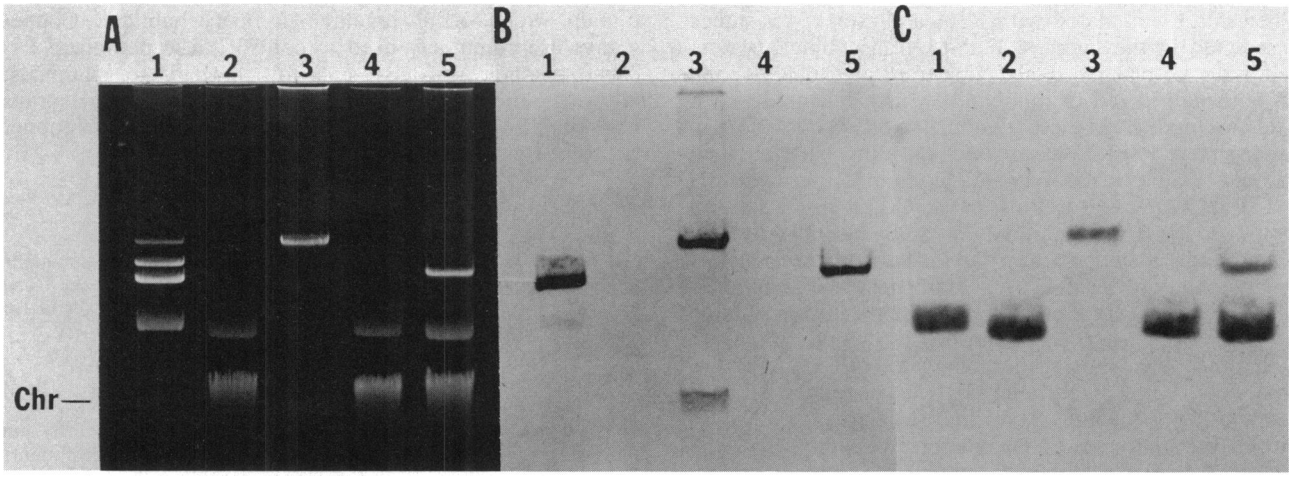

FIG. 4. Transfer of Cu^r from PT23 to Cu^s *P. syringae* recipients. (A) Agarose gel electrophoresis of plasmid DNA from PT23, Cu^s *P. syringae* recipients, and Cu^r *P. syringae* transconjugants. Electrophoresis was at 5 V/cm for 8 h. (B) Hybridization of biotin-labeled pPT23C to nitrocellulose filter containing plasmid DNA shown in panel A. (C) Hybridization of biotin-labeled pPS6 to nitrocellulose filter containing plasmid DNA shown in panel A. Lane 1, PT23, pPT23A, pPT23B, pPT23C, and pPT23D; lane 2, PS61 and pPS6; lane 3, PS61.2 and a pPT23C-pPS6 cointegrate; lane 4, PS62 and pPS6; lane 5, PS62.2, pPT23C, and pPS6. Chr, Chromosomal DNA.

isolated from Cu^r PS61 colonies, they were found to contain a single band of approximately 101 ± 9 kb; the indigenous pPS6 plasmid band was absent (Fig. 4A, lanes 1 through 3). When the Cu^r transconjugant, PS61.2, was used as a Cu^r donor in a mating with PS62 (Sp^r Cu^s), Cu^r was transferred to PS62 at a frequency of approximately 1.7×10^{-3} per recipient cell. The Cu^r PS62 recipients contained two plasmid bands with mobilities approximating that of pPT23C and the indigenous plasmid pPS6 (Fig. 4A, lanes 3 through 5). This suggested that the 101-kb plasmid present in the PS61 Cu^r transconjugant was actually a cointegrate of pPT23C and pPS6. This was demonstrated when pPT23C and pPS6 were nick translated and hybridized to the plasmid patterns shown in Fig. 4A. Both pPT23C and pPS6 hybridized to the 101-kb band in lane 3 (Fig. 4B and C). pPT23C also hybridized to itself and pPT23D (Fig. 4B, lane 1) and to the larger plasmid band in lane 5. pPS6 hybridized to pPT23D (Fig. 4C, lane 1), itself (lanes 2 and 4), and both plasmid bands in lane 5.

Table 2 summarizes the $CuSO_4$ MICs for donors, recipients, and various transconjugants obtained in this study. The $CuSO_4$ MICs for the donor strain PT23 and transconjugants PT17.21, PS51.2, and PS51.7 were all 1.8 mM. The wild-type recipients PT17.2 and PS51, however, were inhibited at 0.6 and 0.1 mM $CuSO_4$, respectively. The $CuSO_4$ MICs for the transconjugants PT12.22 and PS61.2 were both 2.6 mM; the wild-type recipient strains PT12.2 and PS61, however, were inhibited at 0.6 and 0.1 mM $CuSO_4$, respectively.

Three pPT23C-containing transconjugants, PT12.22, PT17.21, and PS61.2, were more resistant to copper than were the original recipient strains (PT12.2, PT17.2, and PS61, respectively). However, PS51.4, which contained pPT23C::Tn*5*, was Cu^s. Transconjugants PT17.31 and PS61.3, which contained this same plasmid (pPT23C::Tn*5*), were also Cu^s. The transfer of pPT23A and pPT23A::Tn*5* to PS51 resulted in the Cu^r transconjugants, PS51.7 and PS51.2. All transconjugants containing pPT11B, pPT17A, and pPT17B were Cu^s.

TABLE 2. MICs of copper sulfate for *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae*

Strain	Plasmids	$CuSO_4$ MIC (mM)
PT23	pPT23A, pPT23B, pPT23C, pPT23D	1.8
PT12.2	pPT12B	0.6
PT12.22	pPT12B, pPT23C	2.6
PT17.2	pPT17A, pPT17B	0.6
PT17.21	pPT17A, pPT17B, pPT23C	1.8
PT17.22	pPT17A, pPT17B, pPT23C::Tn*5*	0.6
PS51	None	0.1
PS51.1	pPT11B, RSF1010	0.1
PS51.2	pPT23A::Tn*5*	1.8
PS51.3	pPT23C::Tn*5*	0.1
PS51.4	pPT11B::Tn*5*	0.1
PS51.7	pPT23A	1.8
PS61	pPS6	0.1
PS61.1	pPT17A, pPT17B, pPS6, RSF1010	0.1
PS61.2	pPS6::pPT23C	2.6
PS61.3	pPT23C::Tn*5*, pPS6	0.1
PS62	pPS6	0.1

DISCUSSION

All strains of *P. syringae* pv. *tomato* were found to contain at least two indigenous plasmids in size classes A and B. DNA restriction patterns from strains containing plasmids in the same size class indicate that class A, B, C, and D plasmids from different strains are highly homologous (unpublished data). Four conjugative plasmids were identified in this study: pPT11B, pPT17B, pPT23A, and pPT23C. One other plasmid, pPT17A::Tn*5*, could not be transferred from PS51 to PS52 after Tn*5*-labeling. Therefore, this plasmid was probably originally mobilized into PS51 by pPT17B, the conjugative plasmid present in the donor strain, PT17.

Two conjugative plasmids in *P. syringae* pv. *tomato* PT23 were involved in the copper resistance phenotype, pPT23A and pPT23C. When PS51 was mated with PT23, Cu^r transconjugants contained pPT23A. PS51.2, a transconjugant containing pPT23A::Tn*5*, was also Cu^r. The recipients PT12.2, PT17.2, and PS61 became Cu^r when they acquired pPT23C. However, PS51.3, a PS51 transconjugant containing pPT23C::Tn*5*, was Cu^s. This phenotype might be attributed to Tn*5* inactivation of some function involved in Cu^r because the transfer of pPT23C::Tn*5* to PT17.2 and PS61 also resulted in Cu^s.

When PT23 was mated with PS61 and copper resistance was selected in the latter, Cu^r PS61 transconjugants contained a 101-kb plasmid band. Nick-translated pPT23C and pPS6 both hybridized to this band, which indicated that it was a cointegrate of these two plasmids. When the Cu^r transconjugant PS61.2 was mated with the Cu^s recipient PS62, Cu^r PS62 transconjugants contained two plasmid bands (Fig. 4A, lane 5). Both pPT23C and pPS6 showed homology to the larger band, but only pPS6 hybridized to the smaller band. Therefore, the larger band in lane 5 was probably mainly pPT23C DNA with a short stretch of pPS6 DNA sequences. This band migrated slightly slower than the wild-type pPT23C, which supports this hypothesis. Both pPT23C and pPS6 showed homology to pPT23D. The homology between pPS6 and pPT23D was surprising because PS6 and PT23 originated from diverse sources; PT23 was isolated from southern California tomato plants, and PS6 originated from woody hosts in the Pacific Northwest. Both pPT23D and pPS6 are cryptic plasmids, so the significance of this homology is unknown.

Transconjugants PT12.22 and PS61.2, which contained pPT23C, were more resistant to copper than were the donor PT23 and the transconjugants PS51.7 (pPT23A) and PT17.21 (pPT23C). The reason for the difference in sensitivity is not clear, but it may involve regulation of plasmid copy number. pPT23C is smaller than pPT23A and might be maintained at higher copy numbers in a cell with one or no plasmids. In strain PT17.21, which contains two large indigenous plasmids in addition to pPT23C, Cu^r was expressed at the same level as the wild-type PT23.

To our knowledge, this is the first report of plasmid-controlled resistance to copper in a wild-type *Pseudomonas* strain. pPT23A is similar in size to the conjugative copper plasmid identified in *E. coli* (32). However, it is smaller than the 125-megadalton plasmid identified in *X. campestris* pv. *vesicatoria* (Stall et al., Phytopathology **74**:797, 1984) and Rts1, the 140-megadalton Cu^r plasmid obtained from *Proteus* (16, 31). pPT23C is smaller than any previously reported Cu^r plasmid. Possible homologies between Cu^r genes on PT23 plasmids and other Cu^r plasmids are being investigated.

The presence of two distinct plasmid species encoding heavy metal resistance within a single bacterial strain has not been previously reported. Acquisition of multiple copies of a resistance gene is one way in which an organism can adapt to elevated levels of a toxic material. For example, spontaneous Cu^r in yeast cells has been associated with tandem gene amplification of the $CUP1^r$ locus (12). The possession of multiple copies of a transposon is another way in which bacteria can adapt to elevated levels of a toxic material. For example, cultivation of *Haemophilus influenzae* cells harboring a plasmid with a Tc^r transposon in subinhibitory concentrations of tetracycline results in the emergence of variants harboring plasmids with two, three, or four copies of the transposon (17). A similar event may explain why PT23 contains two plasmids with Cu^r determinants. We have cloned the Cu^r genes from PT23 plasmid DNA and plan to use fragments of the clone as probes to detect homologous sequences on the class A and C plasmids.

This is the second report of plasmid-coded Cu^r in a phytopathogenic bacterium. Copper resistance in *X. campestris* pv. *vesicatoria* was associated with failure to control race 2 of the bacterium with fixed-copper sprays (24). Subsequent studies have indicated that the Cu^r phenotype was associated with a conjugative plasmid (Stall et al., Phytopathology **74**:797, 1984). *X. campestris* pv. *vesicatoria* and *P. syringae* pv. *tomato* are both seed-borne pathogens of tomato which occur together in many habitats. Copper sprays are commonly used to control these pathogens because of their low cost and low toxicity to mammals. However, the prophylactic administration of copper sprays may increase the selective pressure for acquisition of copper resistance plasmids.

ACKNOWLEDGMENTS

We thank N. T. Keen and B. Hyman for their helpful comments and critical review of this manuscript. We are grateful to H. E. Stone for his help with bacterial cultures, and to BettyAnn Merrill for secretarial assistance.

LITERATURE CITED

1. **Anderson, D. M., and D. Mills.** 1985. The use of transposon mutagenesis in the isolation of nutritional and virulence mutants in two pathovars of *Pseudomonas syringae*. Phytopathology **75**:104–108.
2. **Calomiris, J. J., J. L. Armstrong, and R. J. Seidler.** 1984. Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. Appl. Environ. Microbiol. **47**:1238–1242.
3. **Comai, L., and T. Kosuge.** 1980. Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in *Pseudomonas savastanoi*. J. Bacteriol. **143**:950–957.
4. **Crosa, J. H., and S. Falkow.** 1981. Plasmids, p. 266–282. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D. C.
5. **Crouse, G. F., A. Frischauf, and H. Lehrach.** 1983. An integrated and simplified approach to cloning into plasmids and single-stranded phages. Methods Enzymol. **101**:78–89.
6. **Curiale, M. S., and D. Mills.** 1983. Molecular relatedness among cryptic plasmids in *Pseudomonas syringae* pv. *glycinea*. Phytopathology **73**:1440–1444.
7. **Currier, T. C., and M. K. Morgan.** 1983. Plasmids of *Pseudomonas syringae*: no evidence of a role in toxin production or pathogenicity. Can. J. Microbiol. **29**:84–89.
8. **Dutta, G. N., and L. A. Devriese.** 1981. Sensitivity and resistance to growth promoting agents in animal lactobacilli. J. Appl. Bacteriol. **51**:283–288.
9. **Elek, S. D., and L. Higney.** 1970. Resistogram typing—a new epidemiological tool: application to *Escherichia coli*. J. Med. Microbiol. **3**:103–110.
10. **Farley, J. D., and G. Oakes.** 1979. Evaluation of copper treatments for control of bacterial speck. Fungic. Nematic. Tests **34**:83.
11. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76**:1648–1652.
12. **Fogel, S., and J. W. Welch.** 1982. Tandem gene amplification mediates copper resistance in yeast. Proc. Natl. Acad. Sci. USA **79**:5342–5346.
13. **Foster, T. J.** 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. **47**:361–409.
14. **Gonzalez, C. F., S. K. Layher, A. K. Vidaver, and R. H. Olsen.** 1984. Transfer, mapping, and cloning of *P. syringae* pv. *syringae* plasmid pCG131 and assessment of its role in virulence. Phytopathology **74**:1245–1250.
15. **Guerry, P., J. Van Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117**:619–630.
16. **Ishihara, M., Y. Kamio, and Y. Terawaki.** 1978. Cupric ion resistance as a new genetic marker of a temperature sensitive R plasmid, Rtsl in *Escherichia coli*. Biochem. Biophys. Res. Commun. **82**:74–80.
17. **Jahn, G., R. Laufs, P.-M. Kaulfers, and H. Kolenda.** 1979.

Molecular nature of two *Haemophilus influenzae* R factors containing resistances and the multiple integration of drug resistance transposons. J. Bacteriol. **138**:584–597.
18. **Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff.** 1979. A restriction enzyme cleavage map of Tn*5* and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177**:65–72.
19. **Keane, P. J., A. Kerr, and P. B. New.** 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. **23**:585–595.
20. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44**:301–307.
21. **Kovalenko, T. V., and G. I. Karavaiko.** 1981. Effect of temperature on the resistance of *Thiobacillus ferrooxidans* to divalent copper ions. Mikrobiologiya **50**:690–695.
22. **MacNab, A. A.** 1979. Compatabilty and control of early blight with Kocide 101 and Bravo 500, 1978. Fungic. Nematic. Tests **34**:85.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Marco, G. M., and R. E. Stall.** 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. Plant Dis. **67**:779–781.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Piwowarski, J. M., and P. D. Shaw.** 1982. Characterization of plasmids from plant pathogenic pseudomonads. Plasmid **7**:85–94.
27. **Quant, R. L., and D. Mills.** 1984. An integrative plasmid and multiple-sized plasmids of *Pseudomonas syringae* pv. *phaseolicola* have extensive homology. Mol. Gen. Genet. **193**:459–466.
28. **Sato, M., B. J. Staskawicz, and N. J. Panopoulos.** 1982. Indigenous plasmids of *Pseudomonas syringae* pv. *mori*, the causal agent of bacterial blight of mulberry. Ann. Phytopathol. Soc. Jpn. **48**:27–33.
29. **Selvaraj, G., and V. N. Iyer.** 1983. Suicide plasmid vehicles for insertion mutagenesis in *Rhizobium meliloti* and related bacteria. J. Bacteriol. **156**:1292–1300.
30. **Summers, A. O., and S. Silver.** 1978. Microbial transformations of metals. Annu. Rev. Microbiol. **32**:637–672.
31. **Terawaki, Y., H. Takayasu, and T. Akiba.** 1967. Thermosensitive replication of a kanamycin resistance factor. J. Bacteriol. **94**:687–690.
32. **Tetaz, T. J., and R. K. J. Luke.** 1983. Plasmid-controlled resistance to copper in *Escherichia coli*. J. Bacteriol. **154**: 1263–1268.
33. **Varma, M. M., W. A. Thomas, and C. Prasad.** 1976. Resistance to inorganic salts and antibiotics among sewage-borne *Enterobacteriaceae* and *Achromobacteriaceae*. J. Appl. Bacteriol. **41**:347–349.
34. **Wilson, E. E., F. M. Zeitoun, and D. L. Fredrickson.** 1967. Bacterial phloem canker, a new disease of Persian walnut trees. Phytopathology **57**:618–621.
35. **Wood, W. B.** 1966. Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affecting the restriction and modification of DNA. J. Mol. Biol. **16**:118–133.