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Molecular Cloning of Copper Resistance Genes from *Pseudomonas syringae* pv. *tomato*

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A cosmid library of copper-resistant (Cu^r) *Pseudomonas syringae* pv. *tomato* PT23 plasmid DNA was constructed and mobilized into the copper-sensitive recipient *P. syringae* pv. *syringae* PS61. One resultant cosmid clone, pCOP1 (46 kilobases), conferred copper resistance. The PT23 Cu^r gene(s) was located on pCOP1 by subcloning *Pst*I restriction endonuclease fragments of pCOP1 in the broad-host-range vector pRK404. A subclone containing a 4.4-kilobase *Pst*I fragment conferred Cu^r on PS61. The Cu^r gene(s) was further located by insertional inactivation with Tn*5*. A subcloned fragment internal to the Cu^r determinant on pCOP2 was probed to plasmid and chromosomal DNA of four copper-resistant and three copper-sensitive strains of *P. syringae* pv. *tomato*. The probe hybridized to plasmids in resistant strains, but showed no detectable homology to copper-sensitive strains.

Copper sprays are frequently applied to plants for control of bacterial diseases. Therefore, microbial resistance to copper poses a threat to the continued successful use of copper sprays for disease control. We previously reported on plasmid-mediated resistance to copper in *Pseudomonas syringae* pv. *tomato* (2), which causes bacterial speck disease on tomato plants. Copper resistance is also a plasmid-coded trait in *Escherichia coli* (21) and in *Xanthomonas campestris* pv. *vesicatoria* (20), a pathogen of pepper and tomato plants. However, no detailed genetic analyses of the copper resistance (Cu^r) genes on these plasmids have been reported.

Twenty strains of *P. syringae* pv. *tomato* were previously surveyed, and 12 Cu^r strains were isolated (2). One resistant strain, PT23, functioned as a donor of Cu^r in matings with Cu^s strains of *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae*. PT23 was found to contain two conjugative Cu^r plasmids of 101 and 67 kilobases (kb), designated pPT23A and pPT23C, respectively. An additional plasmid of about 37 to 39 kb (pPT23D) was present in all Cu^r strains and was absent in Cu^s strains. This plasmid was not observed to be transmissible by conjugation (2).

In the present study, we describe the isolation of Cu^r genes from *P. syringae* pv. *tomato* PT23 by molecular cloning in *E. coli*. These genes were isolated on a cosmid clone designated pCOP1. The Cu^r determinant on pCOP1 was isolated and further characterized by restriction mapping, subcloning, and Tn*5* mutagenesis. Internal sequences of the Cu^r determinant were subcloned and hybridized to plasmids and chromosomal DNA from *P. syringae* pv. *tomato* strains to study the distribution of Cu^r genes in this phytopathogenic bacterium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used in this study are listed in Table 1. *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* were maintained on mannitol-glutamate (MG) medium (12) supplemented with 0.25 g of yeast extract per liter (MGY medium); antibiotics were added as necessary to maintain selection for resistance markers. Selective antibiotic concentrations were as follows: 50 μg of rifampin per ml, 50 μg of chloramphenicol per ml, 25 μg of kanamycin per ml, 20 μg of tetracycline per ml for *P. syringae* pv. *syringae* or 12.5 μg/ml for *E. coli*. Copper sulfate was added at 0.8 mM for *P. syringae* pv. *syringae* and 12 mM for *E. coli*. Copper sulfate at 12 mM was added to LB agar before autoclaving, and the pH was adjusted to 7.5; all other antibiotics were added to autoclaved media.

Bacterial conjugation between *P. syringae* pv. *syringae* and *E. coli* was performed on either King medium B (13) or *Pseudomonas* agar F (Difco Laboratories, Detroit, Mich.). The recombinant plasmids used in this study are listed in Table 2.

DNA isolation. Plasmid DNA was isolated essentially as described by Currier and Nester (5) for purification on cesium chloride gradients. Total DNA was isolated from *P. syringae* pv. *tomato* by omitting the alkaline denaturation step in this procedure (5). DNA was purified twice on CsCl-ethidium bromide gradients. When small amounts of plasmid DNA were to be isolated from *E. coli*, the method of Crosa and Falkow (3) was used with slight modifications.

Cosmid cloning. *P. syringae* pv. *tomato* PT23 plasmid DNA was partially digested with *Sau*3A to produce a majority of fragments in the 20- to 30-kb size range. After the reaction was terminated at 70°C for 15 min, the insert DNA was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The solution was then extracted with phenol-chloroform and twice with chloroform. DNA was ethanol precipitated, washed with 70% ethanol, and suspended to 1.5 μg/μl in 10 mM Tris hydrochloride (pH 8.0) plus 1 mM EDTA.

Cosmid pLAFR3 DNA (5 μg) was digested with *Eco*RI, and an additional 5 μg was digested with *Hin*dIII and then treated with calf intestinal alkaline phosphatase. The reactions were terminated by heating at 70°C, and the samples were combined, extracted, and precipitated as described above. The *Eco*RI- and *Hin*dIII-cut vector DNA was then digested with *Bam*HI for 1 h at 37°C and extracted and precipitated. The vector DNA was suspended to 1.5 μg/μl in 10 mM Tris hydrochloride (pH 8.0) plus 1 mM EDTA.

Approximately 1.5 μg of vector DNA and 0.75 μg of insert DNA (*Sau*3A-cut plasmid from PT23) were ligated and

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TABLE 1. Bacterial strains

Strain	Relevant properties[a]	Plasmids	Source or reference
Pseudomonas syringae pv. *tomato*			
PT11	Cu^s	pPT11A, pPT11B	2
PT12	Cu^s	pPT12A, pPT12B	2
PT16	Cu^r	pPT16A, pPT16B, pPT16D	2
PT23	Cu^r	pPT23A, pPT23B, pPT23C, pPT23D	2
PT23.2	Spontaneous Rif^r mutant of PT23	pPT23A, pPT23B, pPT23D	This study
PT25	Cu^r	pPT25A, pPT25B, pPT25D	2
PT29	Cu^s	pPT29A, pPT29B	2
Pseudomonas syringae pv. *syringae*			
PS61	Cu^s Rif^r Cam^r	pPS6	2
PS51.7	Cu^r Rif^r Cam^r	100-kb plasmid	2
Escherichia coli			
CSH25a			17
HB101			14

[a] MICs of $CuSO_4$ for *P. syringae* pv. *tomato* were 0.4 to 0.6 mM for Cu^s strains and 1.2 to 2.0 mM for Cu^r strains (2). MICs for strains PS61 and PS51.7 were 0.1 and 1.8 mM, respectively (2).

packaged with a DNA packaging kit from Boehringer Mannheim. *E. coli* HB101 was grown to the late log phase in LB medium amended with 0.4% maltose. The transfection mixture consisted of 100 μl of HB101 cells, 100 μl of packaged DNA, and 100 μl of a solution containing 10 mM $MgCl_2$ and 10 mM $CaCl_2$. Transfections were conducted as described by Hohn (11) and plated on LB medium with tetracycline.

Mating experiments. The recombinant helper plasmid pRK2013 was used to mobilize pLAFR3 clones and pRK404 subclones into *P. syringae* pv. *syringae* PS61. HB101 (pRK2013) at 10^7 CFU/ml and HB101 recombinants containing the plasmid to be mobilized (10^7 CFU/ml) each were spotted (25 μl) on King medium B or *Pseudomonas* agar F plates. *P. syringae* pv. *syringae* PS61 (5×10^8 CFU/ml) was then spotted on top. Mating mixtures were incubated at 18°C for 8 h, refrigerated overnight at 4°C, and then plated on MG medium containing rifampin, chloramphenicol, and tetracycline to determine whether the conjugation was successful and on medium containing chloramphenicol, tetracycline, and copper sulfate to screen for Cu^r PS61 transconjugants. Plates were incubated at 28°C for 3 to 5 days.

Subcloning and restriction mapping. The cosmid pCOP1, which contained a Cu^r gene(s) from PT23 plasmid DNA inserted in pLAFR3, was identified as described above. To subclone the Cu^r gene(s) from pCOP1, plasmid pRK404 and pCOP1 DNA were digested with *Pst*I, and the pRK404 DNA was treated with calf intestinal alkaline phosphatase. DNA samples were electrophoresed separately on low-melting-point agarose gels, and the appropriate bands were cut from the gel and combined. The agarose was melted at 70°C in 10 mM Tris hydrochloride (pH 7.6), and the DNA was directly ligated (4). The ligated DNA was then transformed into competent *E. coli* HB101 cells. Transformants were then mobilized into PS61 and screened for copper resistance. Using this strategy, the subclone pCOP2, which contained the PT23 Cu^r gene(s), was identified. A restriction endonuclease map of pCOP2 was constructed by using several enzymes singly and in combinations and by analysis of restriction products by agarose gel electrophoresis (14).

A 610-base-pair (bp) *Kpn*I-*Sst*II fragment from pCOP2 was cloned into the vector pDSK509 by digesting pCOP2 DNA and plasmid pDSK509 with *Kpn*I and *Sst*II, separating the restriction fragments on a low-melting-point agarose gel (2%), and ligating excised fragments as described previously (4). The ligated DNA was transformed into competent cells of HB101, and transformants were screened for the 610-bp insert. One transformant, which contained the 610-bp fragment, was selected for further study and designated pCOP3.

Transposon mutagenesis. Bacteriophage stock of lambda::Tn*5* was prepared as described by De Bruijn and Lupski (6), and titers were determined on *E. coli* CSH25a as described by Miller (17). Approximately 50 μl of phage stock (2.8×10^{11} plaques per ml) was combined with 1 ml of HB101(pCOP2), and mutagenesis was performed as described previously (6). Tn*5* insertions in pCOP2 were selected by transforming competent HB101 cells with plasmid DNA isolated from kanamycin-resistant HB101(pCOP2) colonies and plating the transformation mixture on LB medium amended with kanamycin and tetracycline. Tn*5* mutants of pCOP2 were then mobilized into PS61 and screened for copper resistance.

DNA hybridizations. Biotinylated DNA probes were made by labeling DNA with photobiotin as described by the manufacturer (Vector Laboratories, Inc.). When specific restriction fragments of cloned DNA were to be labeled with photobiotin, they were separated on low-melting-point agarose gels, excised, and extracted from the agarose as

TABLE 2. Plasmids

Plasmid	Relevant characteristics	Source or reference
pLAFR3	Cosmid vector derived from pLAFR1 (10), Tc^r	B. J. Staskawicz
pRK2013	Mobilization helper, Km^r	9
pRK404	RK2-derived cloning vector, Tc^r	7
pDSK509	RSF1010-derived cloning vector, Km^r	N. T. Keen
pUC19	Ap^r	19
pXvCu116	4.8-kb fragment from *X. campestris* pv. *vesicatoria* Cu^r plasmid (20) coloned in pUC19	B. J. Staskawicz
pCOP1	pLAFR3 Cu^r clone, Tc^r	This study
pCOP2	4.4-kb *Pst*I fragment from pCOP1 subcloned in pRK404, Tc^r Cu^r	This study
pCOP3	610-bp *Kpn*I-*Sst*II fragment from pCOP2 subcloned in pDSK509, Km^r	This study
pDAC1	3.6-kb *Pst*I fragment from pCOP1 subcloned in pRK404, Tc^r	This study

described previously (2), but an additional purification by spun column chromatography (14) was used to remove residual agarose. Southern blotting was performed essentially as described by Maniatis et al. (14). 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, Inc. (Gaithersburg, Md.).

A ^{32}P-labeled DNA probe was made from the 610-bp *Kpn*I-*Sst*II insert in pCOP3. The 610-bp *Kpn*I-*Sst*II fragment was labeled with [α-^{32}P]dATP (Amersham Corp., Arlington Heights, Ill.) with a nick translation kit (Bethesda Research Laboratories) as recommended by the manufacturer. The specific activity of the probe was 4.0×10^7 cpm/μg. Prehybridization (4 h at 42°C) and hybridization (12 h at 42°C) were done in 50% formamide as described by Maniatis et al. (14). After hybridization, the filters were washed four times (15 min per wash) at 25°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate and twice at 50°C with 0.1× SSC–0.5% sodium dodecyl sulfate (1-h first wash; 30-min second wash).

RESULTS

Cosmid cloning. Infection of *E. coli* HB101 with packaged cosmids yielded approximately 8,000 Tet^r colonies per μg of PT23 plasmid DNA. To determine whether Cu^r was expressed in *E. coli*, 250 randomly chosen clones were isolated and replica plated to LB medium containing 12 mM copper sulfate. None of the clones grew, which suggested that the PT23 Cu^r gene(s) was not expressed in *E. coli* when cloned in pLAFR3. Fifty cosmid clones of PT23 plasmid DNA were mobilized into the Cu^s *P. syringae* pv. *syringae* PS61. One clone, which was designated pCOP1 and contained a 24-kb insert, conferred Cu^r on PS61. This clone enabled PS61 to grow on MGY medium containing 0.8 mM copper sulfate, whereas the wild-type PS61 was inhibited at 0.1 mM $CuSO_4$.

Subcloning. Various fragments of pCOP1 were cloned in the broad-host-range vector pRK404. pRK404 subclones were then mobilized into PS61 and screened for Cu^r. When pCOP1 was digested with *Pst*I, 11 fragments ranging in size from 22.8 to 0.4 kb were produced. Four of these fragments (6.6, 4.4, 3.6, and 2.5 kb) were subcloned into *Pst*I-cut pRK404. Tetracycline-resistant transformants were mobilized into PS61 and screened for Cu^r. pRK404 subclones of pCOP1 which contained the 4.4-kb *Pst*I fragment conferred Cu^r on PS61; subclones containing the other *Pst*I fragments did not yield Cu^r PS61 transconjugants. One subclone, which contained a 4.4-kb *Pst*I insert in pRK404, was selected for further study and designated pCOP2.

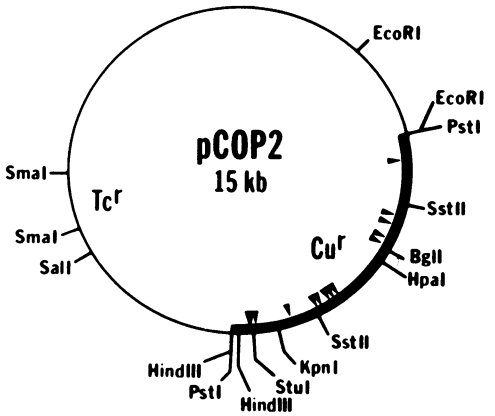

FIG. 1. Restriction endonuclease map of pCOP2, a recombinant plasmid containing *Pseudomonas syringae* pv. *tomato* PT23 Cu^r genes cloned on a 4.4-kb *Pst*I fragment. The locations of 14 Tn*5* insertions which inactivated Cu^r are indicated by arrowheads.

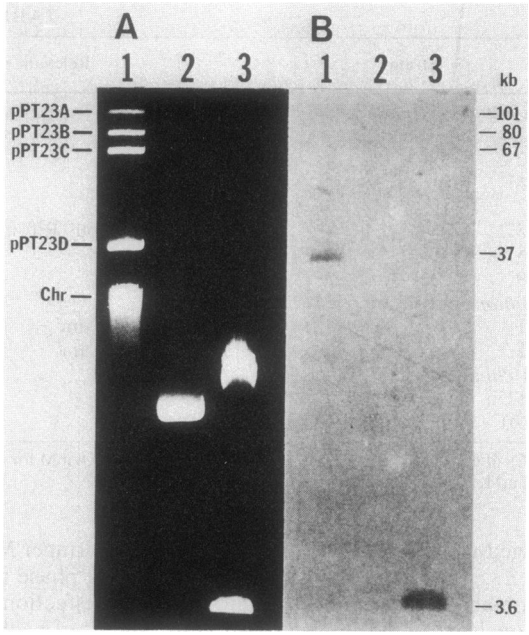

FIG. 2. Origin of the pCOP1 3.6-kb *Pst*I fragment. (A) Agarose gel electrophoresis of PT23 plasmid DNA (lane 1), uncut pRK404 (lane 2), and *Pst*I-restricted pDAC1 (lane 3). Electrophoresis proceeded for 12 h at 5 V/cm. (B) Hybridization of the biotin-labeled 3.6-kb *Pst*I fragment in pDAC1 to nitrocellulose filter containing DNA shown in panel A. Chr, Chromosomal DNA.

A restriction map of pCOP2 was constructed (Fig. 1). The following enzymes did not cut the 4.4-kb *Pst*I fragment: *Xho*I, *Eco*RI, *Bam*HI, *Apa*I, *Cla*I, *Bgl*II, *Eco*RV, *Mlu*I, *Sal*I, *Xba*I, *Sca*I, *Sma*I, and *Sac*I.

Plasmid origin of pCOP1. We previously reported that two plasmids in strain PT23, pPT23A (101 kb) and pPT23C (67 kb), were able to confer the Cu^r phenotype (2). However, it was the 37- to 39-kb plasmid which was common to all Cu^r strains of *P. syringae* pv. *tomato* and absent in all Cu^s strains. Since the pPT23 plasmid DNA was pooled when the cosmid library was constructed, the origin of the Cu^r genes was unknown. To determine from which of the PT23 plasmids the Cu^r clone had originated, we hybridized sequences of pCOP1 outside the Cu^r gene(s) to the four PT23 plasmids. The probe used for this experiment was the 3.6-kb *Pst*I fragment. This fragment was subcloned in pRK404 (pDAC1), but did not confer Cu^r when mobilized into PS61. The probe hybridized to pPT23D, but did not hybridize to the other PT23 plasmids (Fig. 2), suggesting that the pCOP1 insert originated from pPT23D.

The 100-kb plasmid from the Cu^r transconjugant PS51.7 was labeled with biotin and hybridized to the indigenous plasmids in PT23 (Fig. 3). The probe hybridized to itself (lane 2) and to pPT23C and pPT23D (lane 1). Therefore, the 100-kb plasmid transferred from PT23 to PS51 in the previous study (2) was probably a cointegrate of the 67- and 37-kb plasmids, rather than pPT23A.

Isolation of Tn*5* insertions in pCOP2. pCOP2 was mutagenized with Tn*5* by infecting *E. coli* HB101(pCOP2) with lambda::Tn*5*, isolating plasmid DNA, and selecting HB101

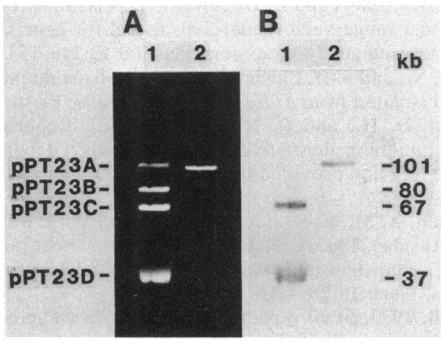

FIG. 3. Hybridization with the 100-kb copper resistance plasmid in PS51.7. (A) Agarose gel electrophoresis of plasmids from PT23 (lane 1) and PS51.7 (lane 2). Electrophoresis proceeded for 12 h at 5 V/cm. (B) Hybridization of the biotin-labeled 100-kb plasmid in PS51.7 to plasmid DNA shown in panel A.

transformants which were Tet^r (pCOP2) and Kan^r (Tn*5*). Transformants (600) were mobilized into PS61 and screened for Cu^r; 14 transposon mutants of pCOP2 failed to confer the Cu^r phenotype when mobilized into PS61; these were located by restriction endonuclease mapping as presented in Fig. 1.

Hybridization of Cu^r subclone to Cu^r and Cu^s *P. syringae* pv. *tomato* strains. A 610-bp *Kpn*I-*Sst*II fragment internal to the Cu^r determinant (Fig. 1) was chosen as a probe to study the distribution of Cu^r genes in *P. syringae* pv. *tomato* strains. The 610-bp fragment was hybridized to *P. syringae* pv. *tomato* plasmid DNA, *Pst*I-restricted plasmids, and *Pst*I-restricted total DNA of four Cu^r and three Cu^s *P. syringae* pv. *tomato* strains. The probe hybridized to the 37- to 39-kb plasmids in the Cu^r strains PT23, PT23.2 (a derivative of PT23 lacking pPT23C), PT25, and PT16, but did not show homology to plasmids in Cu^s strains (Fig. 4). The probe hybridized to a 4.4-kb band in the *Pst*I digest of plasmid and total DNA from strains PT23, PT23.2, PT25, and PT16 (data not shown), but did not hybridize to *Pst*I-digested plasmid or total DNA of the three Cu^s strains (PT11, PT12, and PT29). Hybridization of the entire 4.4-kb Cu^r determinant in pCOP2 to plasmid DNA from PT23 showed homology only to pPT23D (data not shown).

Lack of homology to *Xanthomonas* Cu^r clone. A cloned Cu^r determinant from another member of the family *Pseudomonadaceae*, *X. campestris* pv. *vesicatoria* (B. J. Staskawicz, unpublished data), showed no homology to pCOP1 in Southern hybridizations under stringent wash conditions (data not shown).

DISCUSSION

A cosmid library of *P. syringae* pv. *tomato* PT23 plasmid DNA was constructed in *E. coli* and mobilized into the Cu^s recipient *P. syringae* pv. *syringae* PS61. The PT23 Cu^r gene(s) was identified by screening transconjugants for Cu^r. Several *Pst*I fragments of cosmid clone pCOP1 were subcloned in the broad-host-range vector pRK404 and mobilized into PS61 to screen for Cu^r. Subclones which contained a 4.4-kb *Pst*I fragment conferred Cu^r on PS61. The Cu^r genes were located on this subclone, which was designated pCOP2, by restriction mapping and insertional inactivation with Tn*5*. The Cu^r region was found to span a length of at least 3.9 kb. A 610-bp *Kpn*I-*Sst*II fragment, identified as an internal part of the Cu^r determinant, was used in hybridizations that indicated that the cloned Cu^r determinant originated from plasmid pPT23D. The cloned DNA showed homology to the 37- to 39-kb plasmids from three other Cu^r strains but showed no homology to DNA of Cu^s strains. Hybridization also occurred to a 4.4-kb *Pst*I fragment in total DNA of the Cu^r strains, but this may have been from pPT23D DNA present in the total DNA preparation and not representative of chromosomal homology.

We had previously reported that both pPT23A and pPT23C were involved in Cu^r (2). Evidence for the role of pPT23C in Cu^r was based on conjugative transfer and DNA-DNA hybridizations. However, the role of pPT23A (101 kb) was based only on mobility of a 100-kb plasmid and the conferral of Cu^r. The hybridization experiment conducted in the present study suggests that the 100-kb plasmid was actually a cointegrate of pPT23C and pPT23D. Therefore, there is no evidence for a role of pPT23A in Cu^r.

Neither the *Kpn*I-*Sst*II internal fragment of the Cu^r determinant (pCOP3) or the intact 4.4-kb Cu^r clone (pCOP2) showed homology to pPT23C. Therefore, the Cu^r determinant on pPT23C may be unrelated to the determinant on pPT23D, or perhaps these genes have diverged from each other. Less stringent hybridization conditions might reveal some homology.

The *X. campestris* pv. *vesicatoria* Cu^r DNA did not hybridize to the PT23 Cu^r DNA, which may indicate that the Cu^r genes from these two organisms share little or no sequence homology. However, hybridization under less stringent conditions may reveal homology which was not observed in the present study. Lack of homology does not necessarily indicate that the two Cu^r determinants have

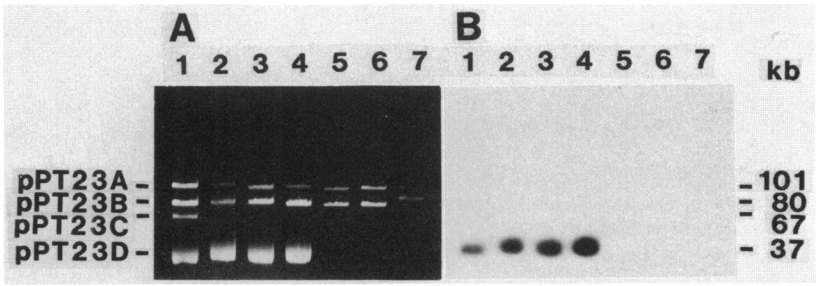

FIG. 4. Plasmid DNA isolated from Cu^r and Cu^s strains of *P. syringae* pv. *tomato* and hybridization with pCOP3. (A) Agarose gel electrophoresis of plasmids from seven strains of *P. syringae* pv. *tomato*. Electrophoresis proceeded for 12 h at 5 V/cm. (B) Hybridization of the ^{32}P-labeled 610-bp *Kpn*I-*Sst*II fragment to plasmid DNA shown in panel A. Plasmid bands in panel A are identified from top to bottom. Lanes: 1, strain PT23; pPT23A, pPT23B, pPT23C, pPT23D; 2, strain PT23.2; pPT23.2A, pPT23.2B, pPT23.2D; 3, strain PT25; pPT25A, pPT25B, pPT25D; 4, strain PT16; pPT16A, pPT16B, pPT16D; 5, strain PT11; pPT11A, pPT11B; 6, strain PT12; pPT12A, pPT12B; 7, strain PT29; pPT29A, pPT29B.

different mechanisms of resistance. Mendez et al. (16) have shown that there are four genetically distinct classes of Tet^r determinants which do not cross-hybridize. Yet all four produce inner membrane proteins with molecular weights of approximately 37,000 and code for the efflux of tetracycline (15).

DNA-DNA hybridization has also been used to study homology among mercury (1), arsenate (18), and cadmium resistance determinants (8). This methodology should also prove useful in comparing the PT23 Cu^r genes with the Cu^r determinants identified in other bacterial species, such as *E. coli* (21).

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