APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1990, p. 13–16
0099-2240/90/01013-04$02.00/0

Vol. 56, No. 1

Plasmid-Determined Copper Resistance in *Pseudomonas syringae* from Impatiens

DONALD A. COOKSEY

Department of Plant Pathology, University of California, Riverside, California 92521-0122

Received 31 July 1989/Accepted 10 September 1989

A strain of *Pseudomonas syringae* was recently identified as the cause of a new foliar blight of impatiens. The bacterium was resistant to copper compounds, which are used on a variety of crops for bacterial and fungal disease control. The bacterium contained a single 47-kilobase plasmid (pPSI1) that showed homology to a copper resistance operon previously cloned and characterized from *P. syringae* pv. *tomato* plasmid pPT23D (D. Cooksey, Appl. Environ. Microbiol. 53:454–456, 1987). pPSI1 was transformed by electroporation into a copper-sensitive *P. syringae* strain, and the resulting transformants were copper resistant. A physical map of pPSI1 was constructed, and the extent of homology to pPT23D outside the copper resistance operon was determined in Southern hybridizations. The two plasmids shared approximately 20 kilobases of homologous DNA, with the remainder of each plasmid showing no detectable homology. The homologous regions hybridized strongly, but there was little or no conservation of restriction enzyme recognition sites.

Copper compounds have been used for several decades in the control of bacterial and fungal plant pathogens, but copper-resistant pathogens have only recently been reported (1, 3, 17, 23). In the few pathogens in which copper resistance has been found, resistant strains are now common in field populations. In California a high proportion of strains of the tomato pathogen *Pseudomonas syringae* pv. *tomato* are copper resistant (3, 5), and in Florida copper-resistant strains of the tomato and pepper pathogen *Xanthomonas campestris* pv. *vesicatoria* are common (17, 22). Copper resistance in both pathogens is plasmid determined: *P. syringae* pv. *tomato* carries a highly conserved 35-kilobase (kb) copper resistance plasmid (4, 5), and *X. campestris* pv. *vesicatoria* carries larger plasmids with variable sizes (about 200 kb) that confer copper resistance and avirulence functions (15, 22). The copper resistance genes carried on these two plasmids are apparently not closely related (4). These copper resistance determinants are the only two in plant pathogens that have been characterized in genetic detail. With few alternatives to the use of copper compounds for chemical control of bacterial diseases of plants, the potential acquisition of these plasmids by other plant pathogens is of concern.

We recently described a new foliar disease of impatiens that was caused by a strain of *P. syringae* (D. A. Cooksey and S. T. Koike, Plant Dis., in press). The bacterium was resistant to copper at levels previously reported for strains of *P. syringae* pv. *tomato* carrying pPT23D (4). This paper describes the copper resistance plasmid (pPSI1) found in this pathogen and its relatedness to pPT23D of *P. syringae* pv. *tomato*.

MATERIALS AND METHODS

Bacterial strains. *P. syringae* PSI1 and PSI2 were isolated from diseased impatiens plants. *P. syringae* pv. *tomato* PT23, *P. syringae* pv. *syringae* PS61, and plasmids in these strains were described previously (3).

Plasmid isolation and electroporation. Bacteria were grown in mannitol-glutamate broth (14) supplemented with yeast extract at 0.25 g/liter (MGY medium). Plasmid DNA was isolated essentially as described by Currier and Nester (8) for purification on cesium chloride gradients. *P. syringae* pv. *syringae* PS61 was prepared for transformation by electroporation after growth to mid-log phase in MGY broth essentially as described for electroporation of *P. putida* (11). PS61 culture (1 ml) was harvested in a microcentrifuge, washed twice with 0.3 M sucrose, and resuspended in 40 μl of ice-cold 0.3 M sucrose. The suspension was placed on ice, and 0.5 μg of pPSI1 was added. Electroporation was performed at 7,500 V/cm with a single pulse by using a Gene Pulser apparatus (Bio-Rad Laboratories) with a 25-μF capacitor. MGY broth (1 ml) was added to the cells after electroporation, and the culture was incubated for 1.5 h at 28°C. The culture was then plated onto MGY agar supplemented with 0.6 mM cupric sulfate.

Detection of homology between pPSI1 and pPT23D. The 4.5-kb *Pst*I-*Pst*I fragment from pCOP2 (4), containing the entire copper resistance operon (19, 20) was gel purified with DEAE-cellulose paper (2) and labeled by random-primed labeling with digoxigenin-11-dUTP and the Genius DNA Labeling and Detection Kit (Boehringer Mannheim Biochemicals). Southern blotting of plasmids separated on 0.7% agarose gels to nylon membranes, hybridization conditions, stringent washes, and detection of labeled DNA were essentially as specified by the manufacturer of the detection kit. Regions of homology between pPSI1 and pPT23D outside the copper resistance operon were detected by using digoxigenin-labeled pPT23D as a probe in Southern blots of digested pPSI1 and by using pPSI1 as a probe to digests of pPT23D. More specific detection of the borders of homology between the two plasmids involved double digests of gel-purified fragments of pPSI1 containing the border regions and Southern hybridizations with pPT23D as a probe.

Physical characterization of pPSI1. A circular map of pPSI1 was constructed by using a series of partial and double digests with *Bam*HI and *Xba*I. For double digests, specific *Bam*HI or *Xba*I fragments were purified from low-melting-temperature agarose gels (7) and digested with the second enzyme. *Eco*RI fragments overlapping *Bam*HI and *Xba*I sites were used as probes in Southern blot experiments to confirm the *Bam*HI and *Xba*I fragment order. Fragments to be used as probes were purified from low-melting-temperature agarose gels and labeled with digoxigenin.

MIC determination. Bacteria were grown on MGY agar,

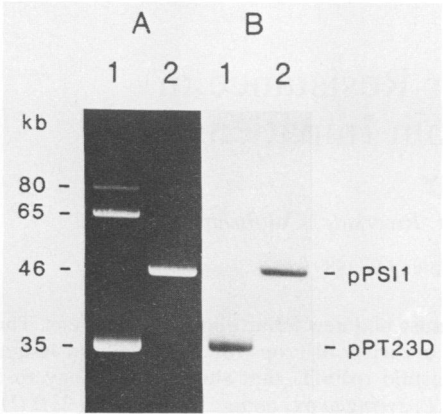

FIG. 1. Southern hybridization of the copper resistance operon cloned from pPT23D of *P. syringae* pv. *tomato* to plasmid DNA from the impatiens pathogen *P. syringae* PSI1. Lanes: 1, PT23 plasmids; 2, PSI1 plasmid. (A) Ethidium bromide-stained gel; (B) Southern blot hybridization with digoxigenin-labeled copper resistance operon cloned from pPT23D. In addition to the three plasmids shown in lanes 1, strain PT23 contains a larger (100-kb) plasmid that was only faintly visible in this particular plasmid DNA preparation, which involved some breakage of larger plasmids.

suspended in sterile water to about 5×10^8 cells per ml, and spotted in triplicate (10 μl per spot) onto MGY agar containing different levels of cupric sulfate from 0 to 3.2 mM. The MIC of cupric sulfate was expressed as the concentration that inhibited confluent growth of the culture after 3 days at 28°C.

RESULTS

Plasmid-encoded copper resistance. *P. syringae* PSI1 contained a single 47-kb plasmid (pPSI1) that showed homology to the copper resistance operon of pPT23D from *P. syringae* pv. *tomato* in Southern hybridizations (Fig. 1). Hybridization of the pPT23D copper resistance operon to pPSI1 appeared to be as strong as hybridization to pPT23D, under stringent wash conditions. Preliminary attempts to conjugatively transfer pPSI1 to a copper-sensitive *P. syringae* recipient were not successful (data not shown). Therefore, purified pPSI1 DNA was transformed into the recipient PS61 by electroporation. Transformants were selected on MGY agar with cupric sulfate (0.6 mM); the transformation efficiency was about 2×10^4 transformants per μg of DNA. No copper-resistant colonies appeared in control platings of bacterial suspensions at the same concentration that were not electroporated with pPSI1 DNA. Transformants contained pPSI1 and the smaller indigenous plasmid (pPS6) of PS61 (Fig. 2) and grew at about an eightfold-higher copper concentration than the PS61 recipient strain did (Table 1).

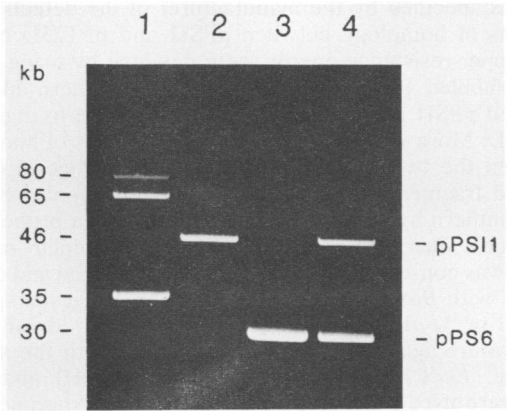

FIG. 2. Transformation of pPSI1 into a copper-sensitive *P. syringae* strain by electroporation. Lanes: 1, plasmid DNA from PT23; 2, plasmid DNA from PSI1; 3, plasmid DNA from the recipient PS61; 4, plasmid DNA from the transformant PS61(pPSI1).

TABLE 1. MICs of cupric sulfate for *P. syringae* PS61 transformed with pPSI1

Strain	Plasmid(s)	$CuSO_4$ MIC (mM)
PT23	pPT23A, pPT23B, pPT23C, pPT23D	1.6
PSI1	pPSI1	1.8
PS61	pPS6	0.2
PS61 transformant	pPS6, pPSI1	1.6

Physical characterization of pPSI1 and extent of pPT23D homology. Several common restriction endonucleases, including *Hin*dIII, *Pst*I, *Hpa*I, and *Eag*I, did not cut pPSI1. *Xba*I and *Bam*HI cut it infrequently, and a physical map of pPSI1 was constructed with these two enzymes (Fig. 3). *Eco*RI had at least 13 recognition sites, including many small fragments; however, three of the larger *Eco*RI fragments contained *Bam*HI sites, and these fragments were used in hybridization experiments as overlapping fragments to confirm the positioning of the *Bam*HI fragments on the map. An apparently identical plasmid from an independently isolated impatiens strain of *P. syringae* (PSI2) showed the same restriction digest patterns as pPSI1 (data not shown).

The copper resistance operon cloned from pPT23D hybridized to a 7.0-kb *Eco*RI fragment in *Bam*HI fragment B of pPSI1 (Fig. 3). When the entire pPT23D plasmid was hybridized to digests of pPSI1, homology was detected with all three *Xba*I fragments of pPSI1 but only with *Bam*HI frag-

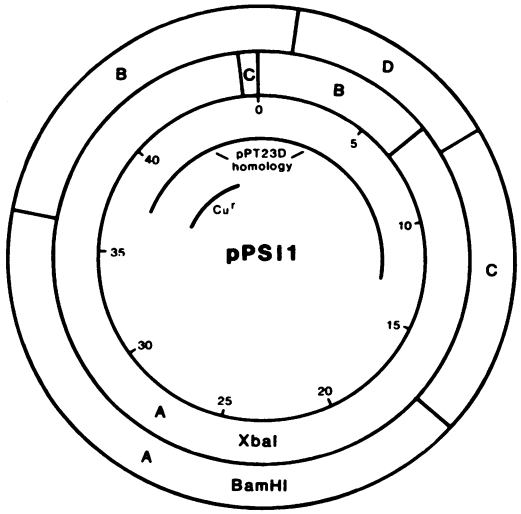

FIG. 3. Restriction enzyme map of the 47-kb copper resistance plasmid pPSI1. The region of homology between pPSI1 and pPT23D and the area specifically related to the copper resistance operon of pPT23D are indicated. Letters indicate restriction fragments in descending order of size. Map units are in kilobases.

TABLE 2. Hybridization of pPT23D to fragments produced by digesting pPSI1 *Bam*HI fragments B and C with *Eco*RI, *Kpn*I, or *Sal*I

Fragment no.	Sizes (kb) for *Bam*HI fragment B digests with:			Sizes (kb) for *Bam*HI fragment C digests with:		
	*Eco*RI	*Kpn*I	*Sal*I	*Eco*RI	*Kpn*I	*Sal*I
1	7.0[a]	8.0[a]	7.8[a]	9.5[a]	5.4[a]	7.0[a]
2	1.5	2.4[a]	1.4		2.6	1.2
3	1.0[a]	1.1[a]	1.2[a]		1.4	1.0[a]
4	1.0[a]		1.2[a]			
5	0.7[a]					

[a] Fragments which hybridized with pPT23D.

ments B, C, and D. When pPSI1 was used as a probe to an *Eco*RI digest of pPT23D, homology was detected with pPT23D *Eco*RI fragments A, B, and D but not fragments C, E, and F (data not shown). These data suggested that a maximum of 27 kb was shared between pPT23D and pPSI1.

To further define the extent of homology between pPT23D and pPSI1, pPSI1 *Bam*HI fragments B and C were gel purified and digested with additional enzymes for Southern hybridizations with pPT23D as a probe (Table 2). The largest such double-digest product of *Bam*HI fragment B that did not show homology with pPT23D was 1.5 kb (Table 2), and this measurement was used to define the approximate left border of pPT23D homology (Fig. 3). Similarly, 4.0 kb of DNA from a *Kpn*I digest of *Bam*HI fragment C failed to hybridize with pPT23D, and this was used to define the approximate right border of pPT23D homology.

*Bam*HI fragment A of pPSI1, which showed no detectable homology with pPT23D, was used as a probe in a hybridization experiment with other plasmids contained in PT23. No homology was detected between this fragment of pPSI1 and pPT23A, pPT23B, or pPT23C (data not shown).

DISCUSSION

This is the first detection of copper resistance genes with homology to the copper resistance operon of *P. syringae* pv. *tomato* in another *P. syringae* pathovar. The *P. syringae* from impatiens does not infect tomato, and of the plants tested, it infected only impatiens (Cooksey and Koike, in press). It is likely that the impatiens strain is another pathovar of *P. syringae* and is not directly descended from *P. syringae* pv. *tomato*. The presence of homologous copper resistance genes in these two bacteria therefore suggests that plasmid DNA may have been exchanged between them. Although *P. syringae* pv. *tomato* and *P. syringae* PSI1 are pathogens of different hosts, *P. syringae* pv. *tomato* is known to reside as an epiphyte on nonhost plants (18, 21), as do certain other *P. syringae* pathovars (13). Since I have observed tomato plants infested with *P. syringae* pv. *tomato* adjacent to impatiens and other bedding plants in commercial nurseries, the possibility of contact and genetic exchange between these two bacteria on either tomato or impatiens plant surfaces seems plausible.

pPT23D has not been shown to be self-transmissible, but it can be mobilized by another indigenous plasmid (pPT23C) from *P. syringae* pv. *tomato* PT23 (3, 4). The highly conserved nature of pPT23D in field populations of *P. syringae* pv. *tomato* in California at first suggested that the plasmid may have spread recently in that species by conjugation (5). However, a more recent study of genomic relatedness in this species showed that copper-resistant strains had nearly identical genomic restriction profiles, whereas copper-sensitive strains were more diverse; this suggested the recent introduction of a single copper-resistant strain that may have spread to diverse locations in California on infested tomato seeds or transplants, rather than the spread of copper resistance by conjugation (6).

As an alternative to the direct acquisition of copper resistance genes from *P. syringae* pv. *tomato*, the recent detection, in saprophytic bacterial species from tomato, of DNA homologous to the pPT23D copper resistance operon (D. A. Cooksey, H. R. Azad, J.-S. Cha, and C.-K. Lim, submitted for publication) suggests that these copper resistance genes are more widespread than was previously thought and that PSI1 could have acquired the genes from another species. When pPT23D was mobilized by pPT23C in mating experiments between *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae*, the two plasmids fused to form a cointegrate plasmid (4). However, the lack of detectable homology between pPSI1 and pPT23C indicates that pPSI1 is not simply a derivative of a pPT23C::pPT23D cointegrate, and this might argue against a direct or recent acquisition of the copper resistance genes from *P. syringae* pv. *tomato*. In addition, there was little or no conservation of restriction enzyme recognition sites between the homologous regions of pPSI1 and pPT23D, indicating some divergence between the sequences.

Attempts to conjugate pPSI1 into a copper-sensitive recipient were not successful, but electroporation was shown to be a useful alternative for testing the phenotype of this plasmid. Transformation of pPSI1 into *P. syringae* PS61 by electroporation was achieved at about the same efficiency as the reported electroporation of *P. putida* with plasmid DNA (11). Although this efficiency was not as high as that reported for *Escherichia coli* (10), it may be an attractive alternative to the conjugative introduction of both wild-type and recombinant plasmid constructions (9, 12, 16) into plant-pathogenic bacteria which are not readily transformed by other methods.

ACKNOWLEDGMENT

This work was supported by National Science Foundation grant BSR-8717421.

LITERATURE CITED

1. **Adaskaveg, J. E., and R. B. Hine.** 1985. Copper tolerance and zinc sensitivity of Mexican strains of *Xanthomonas campestris* pv. *vesicatoria*, causal agent of bacterial spot of pepper. Plant Dis. **69**:993–996.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
3. **Bender, C. L., and D. A. Cooksey.** 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. J. Bacteriol. **165**:534–541.
4. **Bender, C. L., and D. A. Cooksey.** 1987. Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. **169**:470–474.
5. **Cooksey, D. A.** 1987. Characterization of a copper resistance plasmid conserved in copper-resistant strains of *Pseudomonas syringae* pv. *tomato*. Appl. Environ. Microbiol. **53**:454–456.
6. **Cooksey, D. A., and J. H. Graham.** 1989. Genomic fingerprinting of two pathovars by phytopathogenic bacteria by rare-cutting restriction enzymes and field inversion gel electrophoresis. Phytopathology **79**:745–750.
7. **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.
8. **Currier, T. C., and E. W. Nester.** 1976. Isolation of covalently

closed circular DNA of high molecular weight from bacteria. Anal. Biochem. **76**:431–441.
9. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X. Liang, D. Guiney, and D. Helinski.** 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and monitoring gene expression. Plasmid **13**:149–153.
10. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16**:6127–6145.
11. **Fiedler, S., and R. Wirth.** 1988. Transformation of bacteria with plasmid DNA by electroporation. Anal. Biochem. **170**:38–44.
12. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18**:289–296.
13. **Hirano, S. S., and C. D. Upper.** 1983. Ecology and epidemiology of foliar bacterial plant pathogens. Annu. Rev. Phytopathol. **21**:243–269.
14. **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.
15. **Kearney, B., P. C. Ronald, D. Dahlbeck, and B. J. Staskawicz.** 1988. Molecular basis for evasion of plant host defense in bacterial spot disease of pepper. Nature (London) **332**:541–543.
16. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70**:191–197.
17. **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.
18. **McCarter, S. M., J. B. Jones, R. D. Gitaitis, and D. R. Smitley.** 1983. Survival of *Pseudomonas syringae* pv. *tomato* in association with tomato seed, soil, host tissue and epiphytic weed hosts in Georgia. Phytopathology **73**:1393–1398.
19. **Mellano, M. A., and D. A. Cooksey.** 1988. Nucleotide sequence and organization of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. **170**:2879–2883.
20. **Mellano, M. A., and D. A. Cooksey.** 1988. Induction of the copper resistance operon from *Pseudomonas syringae*. J. Bacteriol. **170**:4399–4401.
21. **Schneider, R. W., and R. G. Grogan.** 1977. Bacterial speck of tomato: sources of inoculum and establishment of a resident population. Phytopathology **67**:388–394.
22. **Stall, R. E., D. C. Loschke, and J. B. Jones.** 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology **76**:240–243.
23. **Sundin, G. W., A. L. Jones, and D. W. Fulbright.** 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.