Genetics

Indigenous Plasmids in *Pseudomonas solanacearum*

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ABSTRACT

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Twenty-two of 39 strains of *Pseudomonas solanacearum* harbor one or two plasmids with relative masses that range from 5.0 to more than 500 megadaltons. When plasmid DNA from strains 85, 26, and 10 was digested with Bam HI and Eco RI, very different fragment patterns were obtained, suggesting that each replicon represents a unique plasmid. The molecular weights of these plasmids, as calculated from the restriction patterns, are 84×10^6, 93×10^6, and 120×10^6, respectively. One strain (mps5) was found to harbor two small plasmids. A restriction enzyme map of the smaller (5-megadalton) plasmid was obtained. This plasmid, pWI5, may be useful in the construction of a stable cloning vector for this species. There was no apparent relationship between the presence of plasmids and host of origin, natural resistance to antibiotics, or geographic origin of the strains examined.

Plasmids code for a wide variety of physiological functions, including pathogenicity in plant and animal hosts (9). For example, the role of plasmids in tumor formation by *Agrobacterium tumefaciens* (14) and *Pseudomonas savastanoi* (1) and in nodulation of legume roots by *Rhizobium* (5) is well established. Plasmids are also useful for the study of microbial genetics, such as mapping by conjugation (4), and for genetic engineering.

Pseudomonas solanacearum E. F. Sm. is one of the most important plant pathogens in tropical and subtropical regions. It causes wilt in a wide range of plants, but species in the Solanaceae are especially susceptible. This bacterium produces avirulent derivatives under certain laboratory conditions, such as still culture. These derivatives show several changes in addition to avirulence; they usually have rough lipopolysaccharide, lack production of extracellular polysaccharide, and are motile. They also show reduced production of cellulase and pectinase, and different tryptophan and indoleacetic acid metabolisms (8).

The genetics of *P. solanacearum* is poorly understood because of the lack of efficient gene transfer systems. Plasmids that originated in other bacterial species are usually unstable in this plant pathogen. Native replicons offer a possible source of vectors for genetic manipulation in this species.

Rosenberg et al (12) detected a slow-migrating plasmid DNA band in eight of nine strains of *P. solanacearum*. This large plasmid was detected only by electrophoresis after direct lysis of the bacteria on the agarose gel. Currier (3) used four different techniques for cell lysis and found that six of 20 strains harbored plasmids. The molecular weights of these plasmids ranged from 3.5×10^6 to 250×10^6. He found that avirulent afluidal derivatives harbored the same plasmid(s) as the parental strain without detectable change in size. Thus, plasmids apparently do not encode virulence genes in *P. solanacearum*.

This study was undertaken to assess the plasmid content of strains of *P. solanacearum* from different geographic origins

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representing several host ranges. We confirm the frequent presence of plasmids in this species. Some of these replicons may be suitable for the development of gene transfer techniques in this important pathogen.

MATERIAL AND METHODS

The strains of *P. solanacearum* used were from the collection in the Department of Plant Pathology of the University of Wisconsin-Madison. Stocks were maintained in sterile water or lyophilized. For use, cultures were grown overnight in CPG medium (Bacto-peptone 10 g/L, Casamino acids 1 g/L, yeast extract 1 g/L and glucose 2.5 g/L) at 28 C.

Two procedures were used for plasmid extraction on a small scale. In one, the cells were lysed directly in wells formed in agarose gels before electrophoresis, as described by Taylor (13). The other technique involved alkaline lysis of the cells and phenol:chloroform extraction of the DNA, as described by Kado (4), except that in the lysing solution sodium dodecyl sulfate was replaced by 3% Sarcosyl (Sigma Chemical Co., St. Louis, MO). Electrophoresis was performed in 0.6% agarose gels at 20 mA for 16 hr in a vertical slab apparatus.

To isolate plasmid DNA on a preparative scale, bacteria were grown in 1 L of CPG broth at 28 C on an orbital shaker to $OD_{600\ nm}$ = 0.6. Then the culture was centrifuged at 10,000 *g* for 30 min at 4 C. The pellet was washed once with cold E buffer (40 mM tris, 2 mM EDTA adjusted to pH 7.9 with glacial acetic acid) (7) and resuspended in 300 ml of cold E buffer. Cells were lysed after adding 600 ml of modified Kado's solution and incubating them at 60 C for 30–45 min. The lysate usually was clear and viscous. The pH was lowered to 8.0–8.5 by adding 2 M tris-HCl pH 7.0 (2) and the salt content was raised to 1 M by adding solid NaCl with slow stirring by hand. After incubation on ice for at least 1 hr, the lysate was centrifuged at 27,000 rpm for 15 min in a Beckman SW27 rotor at 4 C. The pellet was discarded, 0.25 volumes of 50% polyethylene glycol in water (average molecular weight 8,000) were added to the supernatant, and the mixture was incubated overnight at 4 C (6). Plasmid DNA was collected after centrifuging the mixture at 16,000 rpm for 5 min in a Beckman SW27 rotor at 4 C. The pellet was dissolved by gentle stirring in TES buffer (50 mM tris-HCl pH 8.0, 5 mM EDTA, and 50 mM NaCl) to a final volume of 8 ml. When the DNA was completely dissolved, 8.4 g of CsCl and 1 ml of ethidium bromide solution (4 mg/ml in TES buffer) were added, and the mixture was centrifuged at 38,000 rpm in a Beckman Ti50 rotor for 50 hr. The lower band was removed from the centrifuge tube by puncture with an 18-gauge needle, dialyzed, and concentrated against TE buffer (10 mM tris-HCl pH 8.0, 1 mM NaCl, 1 mM EDTA) in a Pro-Di-Con concentrator (Biomolecular Dynamics, Beaverton, OR). DNA digestions with restriction enzymes were performed as described by Maniatis (10). Intact and restriction nuclease-digested DNAs from plasmids pWI85, pWI26, and pWI10 were electrophoresed in 0.6% agarose gels in E buffer in a vertical slab apparatus at 50 mA. The gels were stained with ethidium bromide and visualized with UV light. Restriction endonuclease-digested DNA from plasmid pWI5 was electrophoresed in 5-mm-thick 0.6% agarose gels in E buffer with 60 μg/L of ethidium bromide in a minicell at 80V for 3 hr.

TABLE 1. Description and plasmid content of strains of *Pseudomonas solanacearum*

Name	Origin	Original host	Number	Molecular weight[a] (approx.) $\times 10^6$	Source
K60	North Carolina	Tomato	1	>500	Kelman
264	Taiwan	Tomato	1	120	Sequeira
26	Georgia	Tomato	1	93	Sequeira
84	Canada	Tomato	1	80	Sequeira
85	Georgia	Tomato	2	84,>300	Sequeira
143	Australia	Tomato	1	80	Sequeira
86	Georgia	Tomato	0		Sequeira
203	North Carolina	Tobacco	1	>500	Sequeira
278	Mexico	Tobacco	1	120	Sequeira
199	Philippines	Tobacco	0		Sequeira
210	North Carolina	Tobacco	0		Sequeira
256	Costa Rica	Potato	1	120	Sequeira
81	Colombia	Potato	2	80,>300	Sequeira
pouB	China	Potato	0		He
134	Kenya	Potato	0		Sequeira
152	Australia	Potato	0		Sequeira
198	Philippines	Potato	0		Sequeira
276	Mexico	Potato	0		Sequeira
273	Costa Rica	Potato	0		Sequeira
53	Honduras	Banana SFR	1	120	Sequeira
277	Mexico	Banana	1	120	Sequeira
J8306	Honduras	Banana	0		Sequeira
J8418F	Honduras	Banana	0		Sequeira
J8391	Honduras	Banana	0		Sequeira
J8375	Honduras	Banana	0		Sequeira
70	Colombia	Plantain	0		Sequeira
pps14	China	Peanut	0		He
pps13	China	Peanut	1	60	He
pps6	China	Peanut	1	60	He
pps7	China	Peanut	1	60	He
pps1	China	Peanut	1	90	He
pps9	China	Peanut	1	60	He
pps11	China	Peanut	2	60,120	He
268	Taiwan	Eggplant	0		Sequeira
10	Costa Rica	Heliconia	1	120	Sequeira
mps5	China	Mulberry	2	40,5	He
8	Costa Rica	Euparium	0		Sequeira
B1	Derived from K60	Avirulent	1	>500	Kelman
26RA	Strain 26 harboring R388		2	23,120	This paper

[a]The molecular weights were estimated using the following plasmids as standards: *Agrobacterium tumefaciens* Ti plasmid (130×10^6), R68.45 (36×10^6) and R388 (23×10^6).

Natural resistance of selected strains harboring plasmids to different antibiotics was tested with Dispens-O-Discs (Difco Laboratories, Detroit, MI) on CPG plate cultures started (10^5 cells per milliliter) with CPG broth cultures of the strains to be tested. The assay was repeated by subculturing in CPG plates containing one of the following antibiotics: streptomycin (100 μg/ml), ampicillin (400 μg/ml), chloramphenicol (25 μg/ml), nalidixic acid (75 μg/ml), erythromycin (25 μg/ml), penicillin G (100 units per milliliter) and neomycin (50 μg/ml).

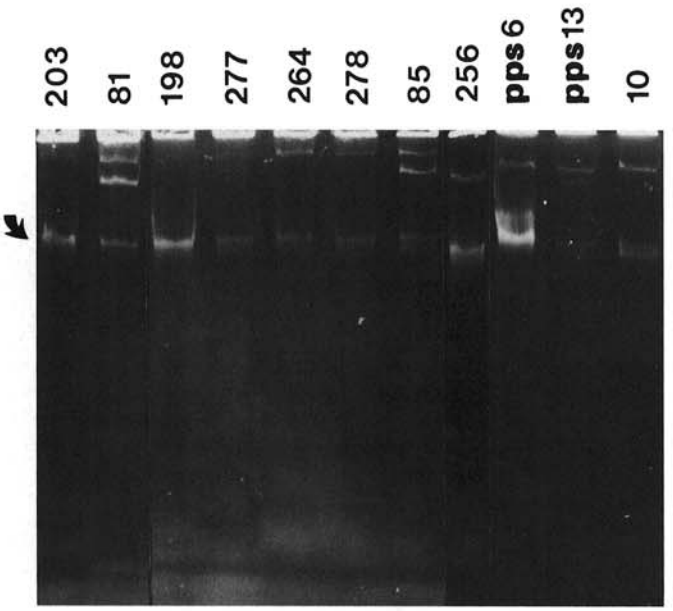

Fig. 1. Agarose electrophoresis of plasmid DNA from strains of *Pseudomonas solanacearum*. Bacterial cells were lysed directly on top of the agarose gel. The thick lower band (arrow) corresponds to chromosomal DNA which in some cases may appear as a doublet.

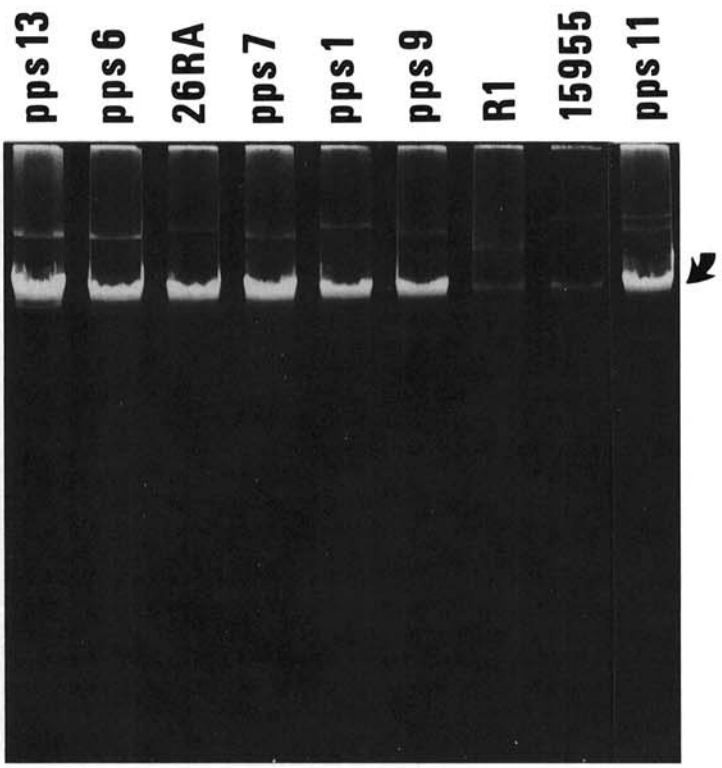

Fig. 2. Agarose gel electrophoresis of plasmid DNA extracted by Kado's procedure. R1 is an a strain of *Escherichia coli* K12 harboring plasmid R68.45 (36 Mdaltons). Strain 15955 is an *Agrobacterium tumefaciens* harboring a Ti plasmid (120 Md). All the others were strains of *Pseudomonas solanacearum*. The lower band indicated with an arrow corresponds to chromosomal DNA.

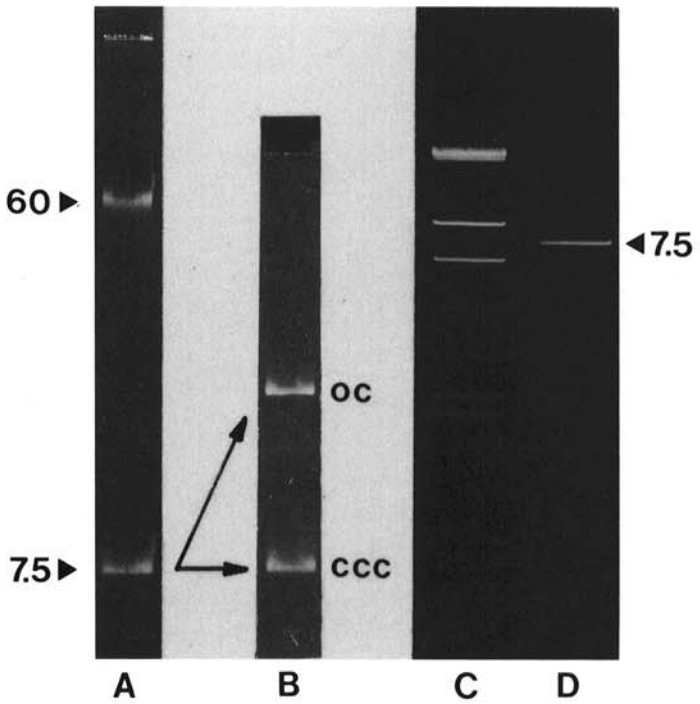

Fig. 3. Agarose gel electrophoresis of plasmid DNA from strain mps5 of *Pseudomonas solanacearum*. Lane A, DNA after CsCl gradient centrifugation. Lane B, DNA from a small plasmid (pWI5) after extraction from the low-melting-point agarose gel. The upper band (oc) corresponds to nicked circular form. The lower band (ccc) is the covalently closed circular form. Lane C, lambda Hind III fragments. Lane D, small-plasmid DNA (pWI5) after digestion with Hind III.

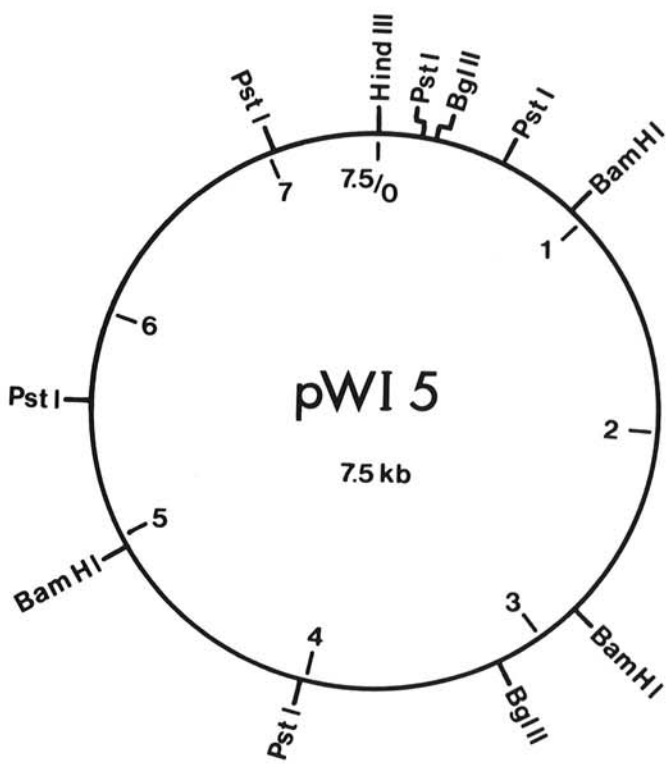

Fig. 4. Restriction map of plasmid pWI5 from strain mps5 of *Pseudomonas solanacearum*.

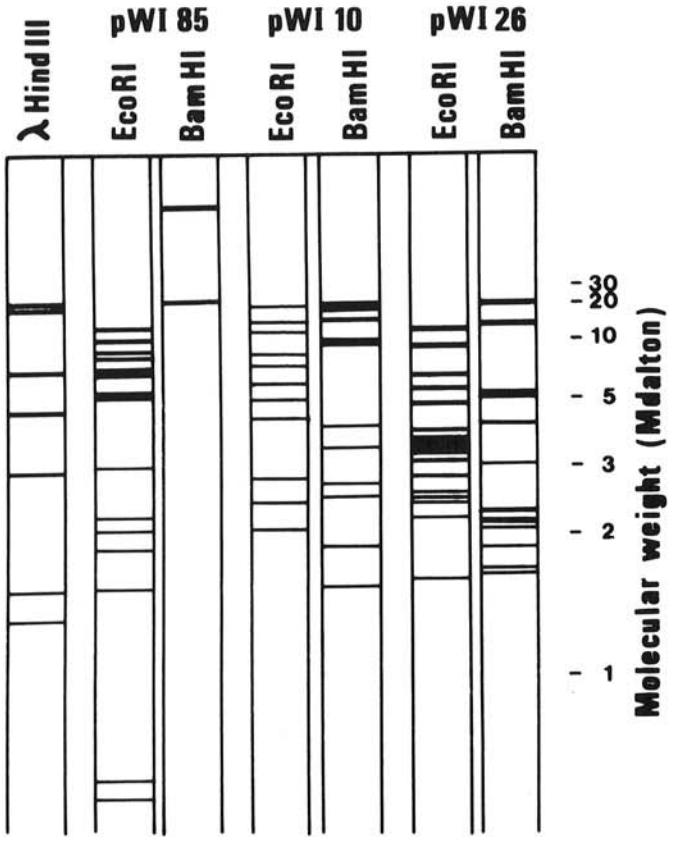

Fig. 5. Restriction fragment patterns of plasmids from strains 85, 10, and 26 of *Pseudomonas solanacearum*. Lambda *Hind* III fragments were used as molecular weight markers.

RESULTS AND DISCUSSION

Plasmids were commonly found in *P. solanacearum*. Twenty two of 39 strains tested harbored one or two replicons. After electrophoresis of bacteria lysed directly in the wells, gels showed DNA bands corresponding to plasmids ranging from 40 to several hundred megadaltons (Fig 1). The technique gave clear and consistent results for plasmids in the lower size range (40–300 Mdaltons) although band duplication was observed in a few cases (see strain pps13 in Fig. 1). The bands corresponding to the larger plasmids (larger than 300 Mdaltons), however, were not always resolved. The age of the culture and the number of cells per well were critical for visualization of the large plasmids by this technique.

Most of the plasmids that could be visualized by Kado's procedure had molecular weights ranging from 40×10^6 to 140×10^6 (Fig 2). The larger plasmids did not survive this extraction procedure, even though shearing was kept to a minimum. Although the procedure has been utilized successfully for isolation of large plasmids (300 Md)(C. I. Kado, *personal communication*) from other bacterial species, the failure with plasmids of *P. solanacearum* might be due to the harsher conditions needed to lyse this bacterium. Small plasmids may not be visualized by this technique either because they band in the same position as the chromosomal DNA (as in the case of R388 in Fig. 2) or there is not enough plasmid DNA to form a clearly defined band (as in the case of the small plasmid pWI5 [*unpublished*]). Excepting these problems, this technique gave very reproducible results even though there were small differences in age of the culture and in number of cells per sample. Two strains, 81 and 85, consistently showed two bands of plasmids after direct lysis in the wells, but not after preparation according to Kado's procedure. The upper band may correspond to a large plasmid that does not survive Kado's extraction procedure or may represent the linear form of the same plasmid.

Plasmid DNA from each of four strains (85, 10, 26, and mps5) was purified on a preparative scale. Electrophoresis of all the preparations showed a single DNA band except that for strain mps5, which showed an extra band corresponding to a small plasmid (Fig. 3). This small plasmid was separated by electrophoresis in a low-melting-point agarose gel. The DNA was extracted from the gel with phenol and then used to construct a restriction map (Fig. 4).

This small plasmid should be very useful because it could be easily tagged by ligating a suitable marker, such as antibiotic resistance, into the single Hind III site and then reintroduced into *P. solanacearum* by transformation. A procedure for transformation is now available for this species (*unpublished*). In addition, the origin of replication of this plasmid could be cloned into an *E. coli* plasmid, producing a shuttle vector that may replicate in both species.

The plasmids extracted from strains 85, 10, and 26 had very different fragment patterns after digestion with *Eco* RI or *Bam* HI (Fig. 5). From calculations based on the lengths of the fragments, their approximate relative molecular masses were 84, 93, and 120 Mdaltons, respectively.

There was no correlation between the presence of plasmids and antibiotic resistance, host of origin, geographical origin, or colony morphology of the different strains. When resistance to antibiotics was tested in 13 strains, only some of them harboring plasmids, all were resistant to vancomycin, polymyxin, bacitracin, and low levels of ampicillin and penicillin G. All strains were sensitive to kanamycin, erythromycin, rifampin, carbenicillin, streptomycin, gentamycin, novobiocin, tetracycline, and nalidixic acid. There were differences among strains in resistance to neomycin, chloramphenicol, and high levels of ampicillin and penicillin G. However, all sensitive strains had a high rate of spontaneous mutation to resistance and this hindered attempts to observe transfer of the resistance trait.

Plasmids pWI85 and pWI26 were not easily lost from strains harboring them; cured derivatives were not found after treatment with acridine orange (11). Because of the lack of an appropriate selective marker, however, the probability of finding a cured derivative was relatively low. Avirulent spontaneous variants from strains 26, 85, and pps 11, isolated from still cultures (5), showed no detectable change in plasmid number or size compared to the parental strains.

We found that most of the plasmids commonly used for genetic manipulation in *E. coli* or *P. aeruginosa* are not stable in *P. solanacearum*. Cloning the origins of replication of some of the plasmids of *P. solanacearum* should allow construction of cloning vectors for this and probably for other related species. The introduction of markers will permit evaluation of the conjugation ability of these plasmids. We are currently labeling these replicons with transposon Tn5 introduced by means of a suicide plasmid developed in our laboratory. This suicide vector will be described in a separate publication.

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