AN ABSTRACT OF THE THESIS OF

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Title: Physical Properties and Genetic Studies of Cryptic Plasmids Discovered in Plant Pathogenic Pseudomonads

Abstract approved: Redacted for Privacy
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Six plant pathogenic isolates of Pseudomonas syringae pv. glycinea, the causal agent of bacterial blight of Glycine max (L.) Merrill, and one isolate of P. syringae pv. phaseolicola, the causal agent of halo blight of Phaseolus vulgaris L., have been examined for the presence of circular duplex deoxyribonucleic acid (DNA). Extrachromosomal DNA from each strain was first observed by cesium chloride-ethidium bromide dye-buoyant density gradient centrifugation. The size and number of plasmids of four different isolates were initially determined by neutral sucrose gradient centrifugation. Subsequently, plasmid DNA from each of the seven isolates was obtained by a cleared lysate technique and analyzed by agarose gel electrophoresis. Each of the isolates of P. syringae pv. glycinea harbor a different complex array of plasmids. The plasmids ranged from 3 to 109 megadaltons (Mdal) in size and there were from two to seven discrete plasmids per

isolate. The isolate of P. syringae pv. phaseolicola contained a single plasmid which had a molecular weight of 98 Mdal.

Endonuclease-derived fingerprints of purified total plasmid DNA from each isolate of P. syringae pv. glycinea served as one criterion for measuring plasmid relatedness among these pathovars of varying geographical origin. Similar-sized fragments were present in the digests of some of the isolates, but no single fragment was observed to be present in the digests of all isolates. Evidence for relatedness among the plasmids of these strains was obtained by hybridization of nick-translated total plasmid DNA from one strain to membrane blots of fingerprints of plasmid DNA from other strains. Fifty-five to seventy-five percent of the plasmid fragments from five strains hybridized with radio-labeled plasmid probe prepared from a sixth strain. In reciprocal hybridizations, radio-labeled probe prepared from each of four strains hybridized to a specific fragment of pMC10 plasmid and to three fragments of pMC11 plasmid, which were present in the digests of the sixth strain. Other fragments of pMC10 and pMC11 showed hybridization to labeled probe from some of the strains, but not with others.

The plasmid, pMC7105, of a mutant of P. syringae pv. phaseolicola integrated into the bacterial chromosome. Biochemical evidence for integration was obtained

by demonstrating that all of the plasmid sequence was present in a plasmidless isolate when membrane blots of total restriction endonuclease-cleaved DNA was probed with radio-labeled pMC7105 DNA. Furthermore, new autonomous plasmids have been detected in three strains which have resulted from partial excision of pMC7105. The probable insertion locus of pMC7105 and regions of excision for one of the derived plasmids were surmised from fingerprint analysis of plasmid DNA.

The wide host-range antibiotic-resistance-plasmid RP4 was transferred by conjugation from Escherichia coli to strains of both pathovars. The RP4-containing strains of P. syringae pv. glycinea produced RP4-less colonies of which about 1.4% remained resistant to ampicillin, indicating transposition of Tn1 from RP4 onto DNA of the host. Ampicillin resistance was stably inherited by the Tn1 containing strains and could be mobilized in matings with nearly isogenic strains. However, the stability of Tn1 among the transconjugants was greatly reduced. RP4 was stably inherited by two strains of P. syringae pv. phaseolicola. Restriction endonuclease-cleavage patterns of plasmid DNA from an isolate of one of these strains reveal evidence for the insertion of a sequence into the pMC7105 plasmid. Its restriction pattern and molecular weight were consistant with results expected by insertion of Tn1. The competency of this strain to serve as a donor of RP4 or

the newly derived plasmid, pMC7105::Tn1, were greatly reduced when compared with identical strains which do not have Tn1 transposed onto pMC7105.

Physical Properties and Genetic Studies of Cryptic Plasmids Discovered in Plant Pathogenic Pseudomonads

by

Michael Steven Curiale

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed November 24, 1980

Commencement June 1981

APPROVED:

Redacted for Privacy

Associate Professor of Botany and Plant Pathology
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Date thesis is presented November 24, 1980

Typed by Lynn Ann Curiale for Michael Steven Curiale

ACKNOWLEDGEMENT

I wish to express my deep appreciation to Dr. Dallice Mills, my major professor, for his interest, patience and support during my course work and the preparation of this thesis.

I sincerely thank Dr. Lyle Brown, Dr. Robert Holton, Dr. George Pearson, and Dr. Henry Schaup for serving on my graduate committee and reviewing the manuscript.

I am deeply grateful to my wife, Lynn, whose endless patience, cooperation and love supported me throughout my graduate work.

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PHYSICAL PROPERTIES AND GENETIC STUDIES OF CRYPTIC PLASMIDS DISCOVERED IN PLANT PATHOGENIC PSEUDOMONADS

JOURNAL OF BACTERIOLOGY, July 1977, p. 224-228

Vol. 131, No. 1
Printed in U.S.A.

Detection and Characterization of Plasmids in *Pseudomonas glycinea*

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Received for publication 25 February 1977

Pathogenic strains of *Pseudomonas glycinea* were shown to possess plasmid deoxyribonucleic acid by dye-buoyant density gradient centrifugation. The size and number of plasmids of four different isolates were determined by neutral sucrose gradient centrifugation. Two isolates were found to harbor a single plasmid; however, they differed in size, having molecular weights of 43×10^6 and 54×10^6. Two other isolates each contained two different plasmids. Plasmids with molecular weights of 43×10^6 and 73×10^6 were observed in one isolate, and the other carried plasmids with molecular weights of 25×10^6 and 87×10^6. An auxotrophic mutant derived from the latter strain was found to contain plasmids of identical size. The plasmids were found to be under stringent control of replication, having plasmid copies of 1.0 to 2.7 per chromosome equivalent. By the dye-cesium chloride technique, the mutant showed twice as much covalently closed circular deoxyribonucleic acid as did the parental strain.

Pseudomonas glycinea (Coerper) is the causal organism of bacterial blight of soybean [*Glycine max* (L.) Merrill], a disease that is especially persistent in the upper midwestern regions of the United States. Pathogenic races can be distinguished by the degree to which disease symptoms are incited on several varieties of soybean (6, 11). However, little is known about the genetic control of virulence or the genetic organization of *P. glycinea* or other pathogenic *Pseudomonas* spp. Total deoxyribonucleic acid (DNA) from lysates of nine nomenspecies has been characterized by CsCl density gradient centrifugation. The DNA of each pathogenic strain studied appeared as a single band in all cases (9). That satellite DNA was not detected in numerous phytopathogenic *Pseudomonas* spp. by CsCl density centrifugation was suggestive of there being either (i) an absence of satellite DNA or (ii) a similar buoyant density for satellite DNA and chromosomal DNA.

Dye-buoyant density analysis (4) is a frequently used method for the identification and isolation of plasmid DNA, which has a buoyant density nearly identical to that of the bacterial chromosome. This technique was used in the present study to characterize the DNA of six isolates of *P. glycinea* obtained from different geographical regions of the midwest. Each isolate, without exception, carried at least one plasmid. The plasmids of these isolates were characterized with respect to size and copy number.

(This work was taken from a dissertation to be submitted by M. S. C. to Oregon State University, Corvallis, in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Organisms. Wild-type *P. glycinea* strains 10, 10a, and 11 were isolated from soybean field plots and supplied by D. W. Chamberlain, U.S. Department of Agriculture Soybean Research Laboratory, University of Illinois. Isolates R1 and R2, which were avirulent and virulent, respectively, on the soybean cultivar Chippewa (11), were provided by B. W. Kennedy. Strain PG5 (16) was provided by A. Vidaver. An auxotrophic mutant, designated LR229, derived from strain 11 after ethyl methane sulfonate mutagenesis was also used. Adenine and tryptophan satisfied the nutritional requirements of LR229. *Escherichia coli* K-12 W6 (F^+) was also used in this study.

Culture conditions and media. The phytopathogens were grown and maintained at 24°C on MaS medium, which contained: 1.05% K_2HPO_4, 0.45% KH_2PO_4, 0.10% $(NH_4)_2SO_4$, 0.05% trisodium citrate·$2H_2O$, 0.012% $MgSO_4$, 0.2% sucrose, and 1.5% agar (Difco) when required. Adenine (50 μg/ml) and DL-tryptophan (80 μg/ml) were added to sustain the auxotrophic mutant LR229. The complete medium, MaSNY, has 10 g of nutrient broth and 6 g of yeast extract added per liter of MaS.

Experiments were typically performed with overnight shake cultures grown to late logrithmic phase (about 10^9 cells/ml). The cells were harvested by centrifugation at $3,000 \times g$ for 5 min, and the cell pellet was typically suspended in fresh MaS medium at an optical density of 0.1 at 600 nm to initiate all experiments, unless otherwise indicated.

Mutagenesis: selection for adenine auxotrophy.

Adenine auxotrophs were desired to facilitate labeling of the chromosomal and plasmid DNA. Samples of strain 11 were suspended in MaS medium lacking sucrose and containing 3 to 5% ethyl methane sulfonate. After 30 min of vigorous shaking at 24°C, the cells were diluted and plated onto MaSNY complete medium to determine the percentage of surviving cells at the various ethyl methane sulfonate concentrations. The remaining cells were harvested by centrifugation, suspended in 22% glycerol, and stored at −20°C until they were analyzed. Ethyl methane sulfonate-treated cells having approximately 50% survival were thawed, suspended in MaSNY medium, and grown overnight. Cells were then diluted and plated at a density of about 100 cells per petri dish onto MaSNY complete-agar medium. After incubation (96 h) at 24°C, the colonies were replica plated onto MaS plates and incubated at 31°C. Colonies that failed to grow were tested for nutritional requirements and for temperature sensitivity on complete medium at 31°C. This procedure routinely yielded approximately 0.5% mutant cells among the survivors plated, all of which were auxotrophic.

Isolation of labeled plasmid DNA. The procedure for isolating labeled plasmid DNA was essentially according to Pemberton and Clark (14). Buoyant density gradient analysis was used to characterize DNA extracted from cells that had been growing for 6 h in the presence of 5 μCi of [^{3}H]adenine (15 Ci/mmol) or 1 μCi of [^{14}C]adenine (50 mCi/mmol) per ml. The cells were harvested by centrifugation and washed twice with ice-cold TES buffer [30 mM tris(hydroxymethyl)aminomethane, pH 7.6, 5 mM ethylenediaminetetraacetic acid, 50 mM NaCl] and suspended in 0.5 ml of 50 mM tris(hydroxymethyl)aminomethane buffer (pH 8.0) containing 25% (wt/vol) sucrose. One-tenth milliliter of lysozyme (5 mg/ml), which was dissolved in TES buffer immediately before each use, was added, and the suspension was incubated on ice for 5 min. Cell lysis occurred upon the addition of 0.9 ml of TES buffer containing 2% sodium lauryl sarcosine. Ribonuclease A (5 mg/ml), which was dissolved in TES buffer and heated for 10 min at 80°C, was then added (final concentration, 300 μg/ml), and the lysate was incubated for 20 min at 32°C. The lysate volume was subsequently increased to 2.0 ml with TES buffer or 2.5 ml with TES buffer containing 1.2 mg of ethidium bromide per ml. Solid CsCl (2.5 g) was added to the lysate and brought into solution. Centrifugation was subsequently carried out in a Spinco SW56 rotor at 88,500 × *g* for 62 h at 18°C. The contents of each tube were collected from the bottom in 4-drop fractions. When radioactivity of the CsCl gradient was determined, NaOH (0.3 N final concentration) was added to each fraction and the fractions were incubated at room temperature for 24 h. Cold 10% trichloroacetic acid was then added to each fraction, and the insoluble material was collected on Whatman GF/A filters and washed with 50 ml of cold trichloroacetic acid followed by 50 ml of ethanol. Dry filters were counted in 6 ml of toluene containing 0.05 g of 1,4-bis-[2]-(5-phenyloxazolyl)benzene and 4.2 g of 2,5-diphenyloxazole per liter.

Isolation of F factor DNA from *E. coli* was achieved by using a similar procedure. Cells were grown in the presence of [^{3}H]thymidine (420 mCi/mmol) (2) and prepared for lysis as described above. Efficient lysis was obtained by treating the cells for an additional 5 min in the presence of 80 mM ethylenediaminetetraacetic acid before the addition of lysozyme (1.5 mg/ml final concentration), and incubation was performed at room temperature.

Size determination of plasmid DNA by sucrose gradient analysis. Labeled plasmid DNA that was to be analyzed by sucrose gradient centrifugation was initially purified by CsCl-ethidium bromide density centrifugation. The main band DNA and the heavier plasmid DNA band were observed to fluoresce in the presence of long-wavelength ultraviolet light, which aided in the collection of plasmid DNA. The CsCl and ethidium bromide were removed from the DNA by dialysis overnight against TES buffer at 4°C. Samples were subsequently centrifuged through linear 5 to 20% (wt/wt) neutral sucrose gradients made with TES buffer containing 0.5 M NaCl. Fractions (0.1 ml) were collected from the top using an ISCO gradient fractionator, and the radioactivity was determined as previously described (3). The molecular weights of the plasmids were calculated by use of formulas previously derived to fit the sedimentation properties of open circular (OC) and supertwisted DNA molecules (4).

RESULTS

Detection of plasmid DNA in CsCl-ethidium bromide gradients. The [^{3}H]adenine-labeled DNA of a whole-cell lysate of *P. glycinea* LR229 was detected as a single radioactive peak in fractions collected from a CsCl equilibrium gradient (Fig. 1A). Two distinct fluorescent bands were observed under long-wave ultraviolet illumination when the lysate was treated with ethidium bromide prior to equilibrium centrifugation in CsCl (Fig. 1B). The denser band, presumed to be covalently closed circular (CCC) DNA, contained 9.05 ± 0.39% of the label incorporated into the main band DNA.

Lysates of six wild-type isolates from three geographical locations of the United States were also analyzed by CsCl-ethidium bromide density gradient centrifugation, and each was found to harbor a plasmid band. The percentage of [^{3}H]adenine incorporated into plasmid DNA differed for each isolate, and it ranged from 3.76 to 7.06% of the chromosomal DNA (Table 1).

Sedimentation properties of plasmid DNA. The ^{14}C-labeled CCC DNA band of LR229 was isolated from a CsCl-ethidium bromide density gradient, dialyzed overnight to remove the CsCl, and centrifuged in a 5 to 20% neutral sucrose gradient. The F plasmid of *E. coli* labeled with [^{3}H]thymidine served as a reference molecule for the determination of the sedimen-

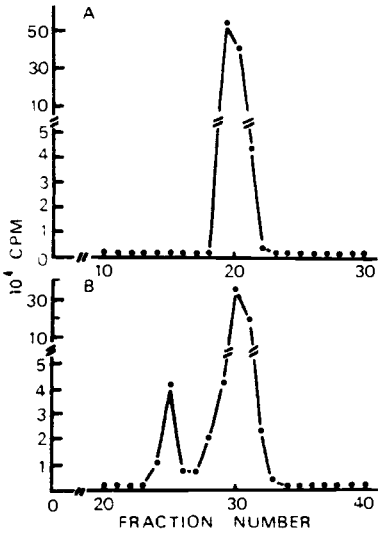

FIG. 1. *Equilibrium centrifugation of P. glycinea LR229 DNA in CsCl gradients in the presence and absence of ethidium bromide. Cells of LR229 were grown for 6 h in the presence of 5 μCi of [³H]adenine (1.5 Ci/mmol) per ml. Lysates were centrifuged in CsCl gradients, and fractions were collected and processed as described in the text. (A) Centrifugation in the absence of ethidium bromide; (B) in the presence of ethidium bromide.*

TABLE 1. *CsCl-ethidium bromide analysis of P. glycinea DNA*

Isolate	% Plasmid DNA[a] (no. of determinations)
10	4.63 ± 0.08 (2)
10a	5.24 ± 0.40 (2)
11	4.28 ± 0.07 (2)
LR229	9.05 ± 0.39 (4)
R1	3.76 ± 0.01 (2)
R2	5.32 (1)
PG5	7.06 (1)

[a] Percentage of plasmid DNA is expressed as (total counts per minute in plasmid peak/total counts per minute in the chromosomal peak) × 100. Values are expressed as the mean ± the deviation from the mean.

tation coefficients of *Pseudomonas* plasmid DNA. The *E. coli* plasmid sedimented as two peaks in a neutral sucrose gradient with sedimentation coefficients of 56.0*S* and 90.9*S*, representing the OC and CCC forms, respectively (Fig. 2). The size of the F plasmid has been previously determined to be 64 × 10^6 daltons (64 Mdal) (4). The plasmid DNA of LR229 sedimented as three peaks, with sedimentation coefficients of 54.2*S* (B1), 62.8*S* (A2), and 105*S* (A1) (Fig. 2). After limited incubation in the presence of deoxyribonuclease I (1), an additional peak (B2) with a sedimentation coefficient of 37.0*S* was observed (Fig. 3). The 105*S* form was found to be unstable and could not be detected after storage at −20°C for 5 to 7 days. The four peaks suggest that LR229 harbors two plasmids. Peaks A1 and A2 correspond to the respective CCC and OC tertiary structures of a plasmid calculated to be 87 Mdal, and peaks B1 and B2 are the similar forms of a 25-Mdal plasmid.

Wild-type isolate 11, the parent strain of LR229, contained plasmids identical to those found in mutant LR229. However, less plasmid DNA was observed in isolate 11 than in LR229 (Table 1).

Plasmid DNAs from isolates 10 and R1 sedimented as single peaks corresponding to 72.4*S* (D1) and 81.5*S* (C1), respectively, in neutral sucrose gradients (Fig. 4A). After storage of the plasmids for 7 to 10 days at −20°C, they sedimented as a mixture of two forms (Fig. 4B). The

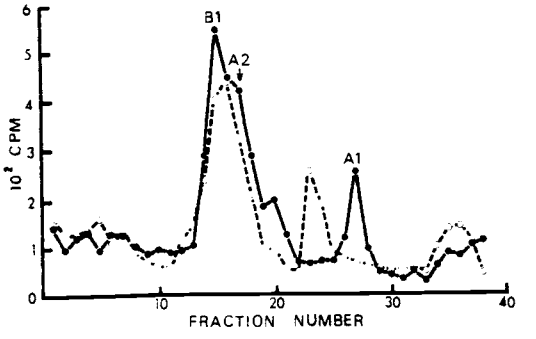

FIG. 2. *Neutral sucrose gradient analyses of E. coli plasmid F (○) and P. glycinea LR229 plasmid DNA (●). The CCC and OC forms of F plasmid peak at fractions 23 and 16, respectively. The CCC and OC forms of one plasmid are labeled A1 and A2, respectively. B1 is the CCC form of a second plasmid. Centrifugation was for 16 h at 11,500 rpm (13,000 × g) in a Spinco SW56 rotor at 18°C. Gradient top appears on the left (first fractions).*

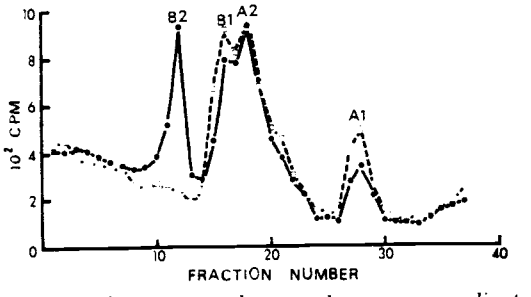

FIG. 3. *Superimposed neutral sucrose gradient analyses of LR229 plasmid DNA before (□) and after (●) treatment with DNase. Peaks labeled A1, A2, and B1 are as described in Fig. 2. The OC form of plasmid B1 is labeled B2. The gradients were centrifuged for 18 h at 11,500 rpm (13,000 × g).*

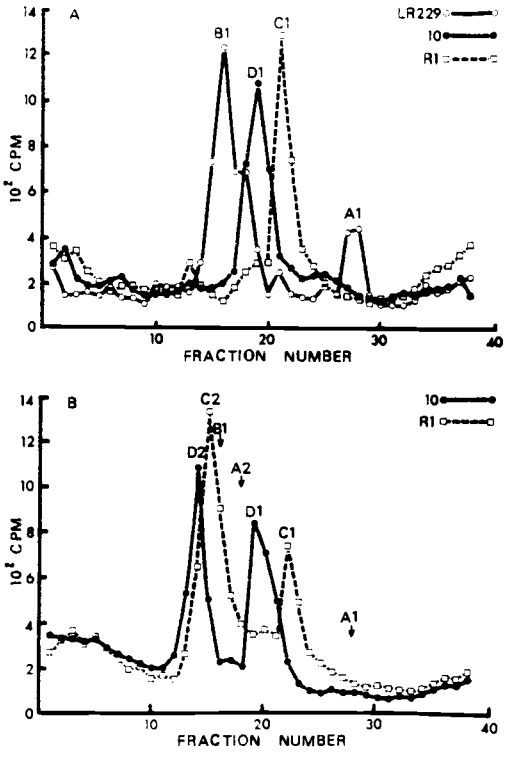

FIG. 4. *Analyses of plasmid DNA from isolates R1 and 10 by neutral sucrose gradient centrifugation. ^{3}H-labeled plasmid DNA from isolates R1 and 10 was centrifuged in the presence of ^{14}C-labeled plasmid DNA of LR229, which served as a reference marker. The ^{3}H-labeled DNA was characterized at days 2 (A) and 14 (B) after isolation from dye-buoyant density gradients. The descriptions of peaks A1, A2, and B1 are asdescribed in Fig. 2. Peaks C1 and C2 are the CCC and OC forms of the R1 plasmid; D1 and D2 are the corresponding tertiary forms of the plasmid obtained from isolate 10.*

46.7*S* (D2) and 51.3*S* (C2) peaks represent the OC configuration of the CCC forms of D1 of isolate 10 and C1 of isolate R1, respectively. The calculated size of the plasmid in isolate 10 is 43 Mdal, and R1 contains a plasmid calculated to be 54 Mdal.

Four peaks with sedimentation coefficients of 46.7*S* (E2), 58.5*S* (F2), 72.4*S* (E1), and 95.7*S* (F1) were resolved when plasmid DNA from isolate R2 was sedimented in a neutral sucrose gradient (Fig. 5). Cosedimentation of isolates 10 and R2 indicated that they each contained a 43-Mdal plasmid; the tertiary forms of the plasmid are identified for isolate 10 as described above and as F1 (CCC) and F2 (OC) for isolate R2. The remaining peaks, E1 and E2, correspond to the CCC and OC forms of a second plasmid in isolate R2 calculated to be 73 Mdal.

DISCUSSION

Six isolates of *P. glycinea* were examined for the occurrence of plasmid DNA. Each isolate was shown to contain a satellite band in a dye-buoyant density gradient that was not evident in the absence of ethidium bromide. The molecular weights of the plasmids of four isolates and an EMS-derived mutant were determined with neutral sucrose gradients.

Two plasmids, 25 and 87 Mdal, were found in mutant LR229 and isolate 11. Peaks B1 and B2 (Fig. 3) were interpreted as representing the CCC and OC forms, respectively, of a small plasmid rather than the linear form and fragments of a larger plasmid designated A1. The sharpness of these peaks and their unique sedimentation coefficients suggested distinct classes of DNA molecules, and the application of formulas derived to fit the sedimentation properties of the CCC and OC form of a small plasmid are consistent with the interpretation. That a more heterodisperse population of DNA molecules was never observed also substantiates the interpretation of there being two distinct plasmids in these strains.

However, it is interesting that LR229 incorporated 2.1 times more label into plasmid DNA than the parent strain. The disparity could be attributed to an actual difference in plasmid copy number. A number of mutants affecting plasmid copy number have been previously isolated from *E. coli* (5, 15). Certainly there could be other explanations advanced to explain our results. Although the intent in obtaining an

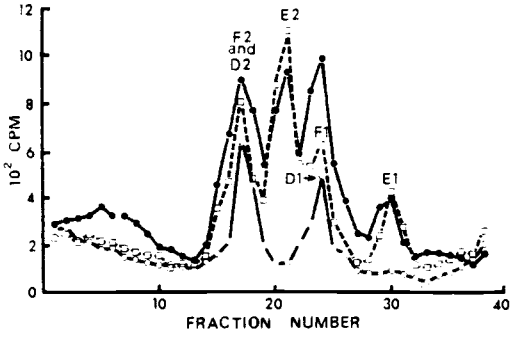

FIG. 5. *Comparison of isolates 10 and R2 plasmid DNA by sucrose gradient centrifugation. Plasmid DNAs of isolate 10 (○) and isolate R2 (□) and a mixture of the two (●) were run on sucrose gradients, and the results are plotted on a single graph. Designation of plasmid DNA from isolate 10 is as described in Fig. 4. The plasmid DNA of isolate R2 sediments as four peaks; E1 (CCC) and E2 (OC), the two tertiary forms of a large plasmid, and peaks F1 (CCC) and F2 (OC) correspond to a smaller plasmid. Centrifugation in the Spinco SW56 rotor was for 175 min at 27,000 rpm (71,600 × g).*

adenine auxotroph was simply to facilitate labeling of DNA, a thorough examination of this mutant is currently underway in an attempt to resolve the differences we observed, and the results will be published elsewhere.

If one makes the assumption that the plasmids occur in equimolar ratios, and that the molecular weight of the main chromosome is 2.5×10^9 (13), isolate 11 has a single copy of each plasmid per chromosome equivalent and LR229 has 2.0 copies (Table 2). The plasmids of the other strains also were under stringent control of replication, having plasmid copies of 1.2 to 2.7 per chromosome equivalent (Table 2).

The presence of plasmid DNA in some phytopathogenic bacteria may be an essential prerequisite for virulence. Tumorigenic isolates of *Agrobacterium tumefaciens* contained plasmid bands when analyzed by the dye-CsCl technique (18). Small (27 to 45 Mdal) and large (107 to 158 Mdal) plasmids were identified (7). A large plasmid was shown to be required for crown gall induction (7, 17, 18). A heat-cured derivative of one virulent isolate that lacked the large plasmid was rendered avirulent (17). However, large plasmids have been found in avirulent isolates of *A. radiobacter* (10).

It has been speculated that the genetic determinants for virulence in *Pseudomonas* spp. may be plasmid borne (8, 12). The possibility that virulence on soybeans will be linked to one or more of these plasmids has yet to be determined and will have to await the isolation of plasmidless derivatives of virulent isolates. It is also entirely possible that many other phytopathogenic pseudomonads will harbor plasmids, in view of our recent finding that virulent isolates of *P. phaseolicola* and *P. syringae* have been found to contain one or more plasmids per cell (M. S. Curiale and D. Mills, unpublished data).

TABLE 2. *Molecular weights and copy number of plasmids in P. glycinea isolates*

Isolate	Plasmid	Mol wt (Mdal)	Plasmid copies/chromosome equivalent
11	pMC21	87	1.0[a]
	pMC22	25	1.0[a]
LR229	pMC21	87	2.0[a]
	pMC22	25	2.0[a]
R1	pMC41	54	1.7
10	pMC11	43	2.7
R2	pMC51	73	1.2[a]
	pMC52	43	1.2[a]

[a] Plasmids were assumed to be in equimolar ratios.

ACKNOWLEDGMENTS

We wish to thank Lynn Peterson for her aid in preparing the figures.

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PHYSICAL PROPERTIES AND MOLECULAR RELATEDNESS OF CRYPTIC PLASMIDS OF SIX ISOLATES OF *PSEUDOMONAS SYRINGAE* PV. *GLYCINEA*

Running Title: PLASMID INTERRELATIONSHIPS

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ABSTRACT

Total plasmid DNA from six isolates of Pseudomonas syringae pv. glycinea was examined by agarose gel electrophoresis. Two to seven plasmids were detected in each strain and their molecular masses ranged from 3 to 109 megadaltons (Mdal). The electrophoretic banding patterns of EcoRI restriction fragments of total plasmid DNA from each strain served as one criterion for measuring relatedness among plasmids. Similar-sized fragments were present in several of the isolates, but no single fragment was observed to be present in the plasmid digests of all strains. Evidence for relatedness among the plasmids of these strains was also obtained from hybridization of nick-translated total plasmid DNA to membrane blots of the EcoRI restriction patterns. Each of five strains contained plasmid fragments that hybridized with plasmid probe prepared from a sixth strain. This probe contained a 1.7-Mdal fragment of pMC10 and three fragments of pMC11 which hybridized to plasmid sequences from the other strains. Some fragments of pMC10 and pMC11 failed to hybridize with labeled plasmid probe from some strains, but not with others.

INTRODUCTION

Plasmids occur naturally among plant pathogenic isolates of Pseudomones syringae pv. glycinea (Curiale and Mills, 1977), P. syringae pv. glycinea (Gonzalez and Vidaver, 1979) and P. syringae pv. phaseolicola (Gantotti et al., 1979). The plasmids in these pathovars range in molecular mass from 19 to 87 megadaltons and each isolate harbors one to three plasmid species. Interest in the plasmids of plant pathogens derives from works with other bacterial species which showed an association between virulent properties and extrachromosomal DNA (Crosa et al., 1977; Gyles et al., 1977; Stirm and Ørskov, 1966; Van Larebeke et al., 1974; Watson et al., 1975). Some recent evidence suggests that certain plasmids of the plant pathogenic pseudomonads also may be required for the production of phytotoxins (Gonzalez and Vidaver, 1979; Gantotti et al., 1979).

Syringomycin, a wide host range toxin, is a polypeptide of low molecular weight produced by isolates of P. syringae pv. syringae (Gross et al., 1977; Sinden et al., 1971). Gonzalez and Vidaver (1979) have identified a 35-Mdal plasmid in a syringomycin-producing strain which incites holcus spot disease on corn (Zea mays). The ability to produce syringomycin was lost in approximately 15% of the survivors following treatment

with acridine orange. Furthermore, the nontoxigenic derivatives displayed an altered profile of sensitivity to a bacteriocin and to two bacteriophages, and they do not incite disease. Agarose gel analysis revealed the absence of the 35-Mdal plasmid in these isolates. Attempts to reintroduce the plasmid have been unsuccessful.

Each of three syringomycin-producing isolates, obtained from millet (Panicum miliaceum), almond (Prunus amygdalus) and apricot (Prunus armenia), were also shown to contain a plasmid of approximately 35 Mdal (Gonzalez and Vidaver, 1980). However, the EcoRI and HindIII fingerprints of these plasmids were different. The only similarity among the patterns was a 1.3-Mdal fragment that was produced by EcoRI cleavage.

The halo-blight agent on common bean (Phaseolus vulgaris) is P. syringae pv. phaseolicola. It produces a toxin which induces chlorosis, and it is a potent inhibitor of the enzyme ornithine-L-transcarbamylase (Patil et al., 1970). A mutant unable to produce the toxin on bean was recovered after ultraviolet mutagenesis of a toxigenic isolate (Patil et al., 1974). Subsequently, it was shown that the mutant had lost a 22.5-Mdal plasmid (Gantotti et al., 1979). Moreover, a correlation between toxin production and the presence of a 22.5-Mdal plasmid is apparent for three of four natural isolates. Two or three additional plasmids which are phenotypically cryptic are also harbored by these iso-

lates.

P. syringae pv. glycinea, the causal organism of bacterial blight on soybean (Glycine max) makes a chlorosis-inducing toxin which inhibits the biosynthesis of chlorophyll (Gulya and Dunleavy, 1980). Pathogenic isolates also have been shown to harbor one or two plasmids (Curiale and Mills, 1977), but no correlation has been established between toxin production and the occurrence of extrachromosomal DNA. Neither toxin-negative mutants nor plasmid-cured strains have been described for this host-pathogen system.

This study extends our previous work on the detection and characterization of plasmids of P. syringae pv. glycinea (Curiale and Mills, 1977). Additional plasmids have been detected in the isolates previously characterized. A molecular relationship has been demonstrated among the plasmids of six isolates by plasmid DNA hybridization to membrane blots of restriction endonuclease-generated fingerprints. This relationship was not evident from the plasmid fingerprints alone.

MATERIALS AND METHODS

Bacterial strains and media

The isolates of P. syringae pv. glycinea (Young et al., 1978) used in this study are listed in Table 1. The designations of some isolates were changed to correspond with our laboratory stock numbers which conform with the recommendations for a uniform nomenclature for bacterial genetics (Demerec et al., 1966).

Strain LR219 was derived from strain LR200 by ethyl methane sulfonate mutagenesis as described previously (Curiale and Mills, 1977). Its phenotype facilitated strain identification.

The complex medium, MaNY (Curiale and Mills, 1977), was used for growth and maintenance of the cultures. Strain LR400 was grown on MaNY supplemented with 0.2% sucrose (MaSNY) to increase its growth rate.

Escherichia coli strain CSH25 (Miller, 1972) was used as a source of bacteriophage λcI857S7. Growth of the lysogen, induction, isolation of phage particles, and extraction of phage DNA were performed as described by Miller (1972).

Plasmid DNA isolation

Cells were grown in one liter batches in six liter Erlenmeyer flasks until the culture attained an optical density of 0.6 at 600 nm (ca. 3 x 10^8 cells/ml).

Plasmid-enriched lysates were prepared essentially according to the protocol of Hansen and Olsen (1978) with proportional adjustments to accomodate the larger quantity of cells. Residual polyethylene glycol 6000 (PEG), which was present in the lysate after PEG partitioning of plasmid DNA, interfered with buoyant density-gradient centrifugation. Therefore, it was necessary to remove the PEG by two or three successive extractions with a cold 2% octanol solution in chloroform (Stanisich et al., 1977) before addition of ethidium bromide (final concentration, 500 ug/ml) and CsCl (1:1 weight/volume) to the solution of DNA. The gradients were formed by centrifugation for 44 h at 44,000 rpm in a Beckman Type 65 rotor at 20°C. After careful removal of the chromosomal DNA band, the plasmid DNA was removed with a syringe equipped with a 22-gauge needle inserted through the side of the tube. The ethidium bromide was extracted from the DNA with cold isopropanol and dialyzed against TEK (10mM Tris, pH 7.2, 1 mM EDTA, 100 mM KCl) buffer and stored at -20°C.

Restriction endonuclease digestion

Plasmid DNA was cleaved with the restriction endonuclease, EcoRI, in the buffer system of McParland et al. (1976). The DNA fragments were precipitated at -20°C following the addition of NaCl (0.3 M final concentration) and 2.5 volumes of ethanol. The precipitate was collected by centrifugation at 10,000 rpm for 15 min at

4°C in a Sorvall SS34 rotor. The fragments were suspended in TEK buffer for electrophoretic analysis or in 10 mM Tris (pH 8.0) for nick translation.

Agarose gel electrophoresis

Agarose gel electrophoresis of partially purified plasmid DNA was performed as previously described by Meyers et al. (1976). The gel and running buffer contained 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA. Electrophoresis was carried out at 5 V/cm for 6 h. The DNA bands were stained with a solution of ethidium bromide (0.4 ug/ml).

Restriction endonuclease-generated fragments were separated in a 0.7% agarose gel system using E buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA: adjusted with glacial acidic acid to pH 7.6) described by Mertz et al. (1972). The gel and running buffer were prepared with ethidium bromide at a final concentration of 0.4 ug/ml. Electrophoresis was carried out at 2 V/cm for 21 h.

A vertical slab gel apparatus was used for all electrophoretic operations. The dimensions of the slab were 20 by 20 by 0.3 cm. The gels were illuminated with 360 nm light and photographed with Kodak Tri-X film. The camera was equipped with a red (no. 25) filter.

The molecular masses of the cleavage fragments were determined by comparison to the migration rates of

standard-sized fragments which were run parallel to the unknowns. The standard-sized fragments were derived from bacteriophage λcI857S7. An incomplete digest with EcoRI and a dual digest with EcoRI and BamHI provided fragments with masses between 0.69 and 13.7 Mdal (Haggerty and Schleif, 1976). Linear phage DNA provided a 30.8 Mdal fragment.

Nick translation of DNA

EcoRI-digested DNA was labeled to a specific activity of 1 to 10 x 10^6 cpm/ug using the nick translation procedure described by Rigby et al. (1976) or to 10 to 30 x 10^6 cpm/ug using the nick translation kit manufactured by New England Nuclear.

Transfer of DNA fragments to membrane filters and DNA-DNA hybridization conditions

Plasmid DNA fragments were transferred from the agarose gel to nitrocellulose membranes according to the Ketner and Kelley (1976) version of the Southern (1975) blotting technique.

The filter blots were conditioned for hybridization by soaking for 4 to 6 h at 60°C in Denhardt's preincubation solution (Denhardt, 1966). The solution ingredients were 0.2% Ficoll 400, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 20 ug/ml heat denatured calf thymus DNA (CT-DNA) in 2 x SSCP (SSCP is

120 mM NaCl, 15 mM sodium citrate, 13 mM KH_2PO_4, 1 mM EDTA: pH 7.2 with NaOH). After conditioning, the filters were rinsed briefly with 2 x SSCP and blotted dry with Whatmann no. 1 filter paper.

The hybridization mixture consisted of 20 ug/ml CT-DNA, 0.2% SDS, and from 0.5 to 2 x 10^6 cpm/ml ^{32}P-DNA in 2 x SSCP. The hybridization reactions were carried out at 71°C for 40 h in plastic heat-sealable cooking pouches. These conditions comply with those described by Wetmer and Davidson (1968), of T_m-25°C for a DNA of 58% GC (Jackson and Sands, 1970). Following the reaction, the filters were washed at 71°C with five changes of 500 ml SSCP, 0.2% SDS. This step removes some of the partial hybrids (Breathrach et al., 1977) leaving those with a maximum base mismatch of about 15%.

Autoradiographic exposures lasted 4 h to 3 days on Kodak No-Screen medical X-ray film.

Materials

Reagents were obtained from the following sources: CsCl, agarose type II, ethidium bromide, bovine serum albumin, and polyvinylpyrrolidone, Sigma Chemical Co.; DNA polymerase I, Worthington Biochemicals, ^{32}P-dCTP, Amersham; EcoRI and BamHI restricion endonucleases, Bethesda Research Laboratories; formamide, Eastman Kodak. A portion of the EcoRI used was a generous gift from Reginald McParland.

RESULTS

Enumeration and molecular weight estimation of plasmids

A plasmid-enriched lysate (Hansen and Olsen, 1978) was prepared from a 10 ml stationary-phase culture for each of the six strains of *P. syringae* pv. *glycinea*. Electrophoretic analysis of the lysates in agarose gels showed distinct plasmid bands and a wide band of chromosomal DNA fragments (Fig. 1). Typically, the plasmid bands were obscurred by a path of fluorescence which extended from the sample well to the chromosomal band (not shown). The visualization of the plasmids bands depended on an octanol-chloroform extraction of the plasmid lysate prior to electrophoresis. This step removes residual PEG (Stanisich, 1977) which also disrupts buoyant density gradients. However, the extraction also appeared to remove the smaller plasmids (compare Fig. 1a and b).

A total of 24 nonchromosomal bands were cataloged among the six isolates and each was assumed to represent a unique plasmid. The molecular mass of each plasmid was estimated by comparison of its mobility to the mobilities of plasmids of known mass (not shown). These results are presented in Table 2. Each isolate contained two or three plasmids larger than 39-Mdal, and each strain except LR100 had one or more small plasmids, approximately 3 to 6.8 Mdal in mass.

Restriction endonuclease analysis of plasmid DNA

If two plasmids have similar restriction endonuclease-generated fingerprints, they are considered related (Thompson et al., 1974). Using this rationale, we sought to compare the restriction patterns of the P. syringae pv. glycinea plasmids. Total plasmid DNA was purified from each of the isolates by buoyant density-gradient centrifugation. Following treatment with EcoRI endonuclease, the cleavage products were separated electrophoretically (Fig. 2a). No set of bands was common among all of the isolates. Hence, relatedness among the isolates on the basis of a common plasmid or even a common fragment was not perceived.

Many similarities were apparent between various fingerprints. The fingerprints of LR300 and LR500 contained 26 bands with identical electrophoretic mobilities. It was shown in Figure 1 and Table 2 that both of these strains have 46- and 61-Mdal plasmids. The cumulative mass of these two plasmids and the 26 bands was 107 and 105 Mdal, respectively. It seems likely that these strains harbor similar plasmids.

In addition to strains LR300 and LR500, strains LR219 and LR400 harbored a 46-Mdal plasmid. However, we observed only six common fragments among the fingerprints of these four strains and the total mass of 17.1 Mdal was well below the expected value, if each strain had an identical plasmid. LR219 and LR400 had 15

similar-sized fragments with a combined mass of 52.6 Mdal. This mass cannot be equated with the presence of one or more of the plasmids harbored by these strains.

Although we found a 42-Mdal plasmid in LR100 and PG5, the similarity was not supported by the fragment patterns.

Strain LR100 has two plasmids with masses of 42 and 109 Mdal (Fig. 1 and Table 2). However, a comparable amount of DNA was not found in the LR100 fingerprint. Instead, 14 stoichiometric bands were superimposed over a faint banding pattern. A BamHI fingerprint of LR100 plasmid DNA gave a similar result (not shown). Molecular masses of 41.5 and 42.7 Mdal were computed from 14 EcoRI bands and the BamHI fragments, respectively. Apparently these bands are derived from pMC11, the 42-Mdal plasmid described in Tables 1 and 2. If the background pattern consisted of chromosomal fragments, a similar pattern would be expected in the digest patterns of the other strains. This was never observed and, consequently, these bands were attributed to a second plasmid which is 109-Mdal in size.

Plasmid relatedness detected by DNA/DNA hybridization

Molecular relatedness among the plasmids of the six isolates was detected by DNA/DNA hybridization on nitrocellulose blots. The gel pattern shown in Figure

2a was transferred to nitrocellulose paper and allowed to hybridize with nick translated plasmid DNA from LR100 (Fig. 2b). Fifty-five to 75 percent of the EcoRI-generated bands of each isolate showed some homology with the LR100 plasmid probe. These results are summarized in Table 3.

Hybridizations between filter-bound fingerprints of LR100 plasmid DNA and labeled-probes prepared from LR219, LR300, LR500, and PG5 were also carried out (Table 4). As was expected from the previous hybridization each of these probes hybridized to LR100 plasmids. Each of the probes hybridized to a 1.7-Mdal fragment of pMC10 and 5.67-, 6.29-, and 7.39-Mdal fragments of pMC11. Of the remaining pMC11 fragments that are larger than 1.2 Mdal, all hybridized to LR300 plasmid DNA, and all but one fragment hybridized to LR219 plasmid DNA. However, PG5 and LR300 hybridized, respectively, to only one and two of the remaining fragments. Similarly, various fragments of pMC10 hybridized to the different probes. Here again, LR219 and LR400 hybridized to more fragments than LR300 or PG5.

DISCUSSION

In a previous study (Curiale and Mills, 1977) four isolates of P. syringae pv. glycinea were shown to harbor a total of six plasmids. The characterization was based on data obtained from sucrose velocity centrifugation analysis of plasmids that were labeled radioisotopically in vivo and purified by density gradient centrifugation. In this work, we described eleven additional plasmids among those four isolates and seven new plasmids harbored by two other isolates. A combination of the plasmid purification scheme of Hansen and Olsen (1978) and the agarose gel system of Meyers et al. (1976) enabled the discovery of the additional plasmids. These methods were especially sensitive to the number and molecular mass range of the plasmids indigenous to these bacterial isolates. Since a procedure that is developed for one plasmid does not necessarily give identical results when it is applied to another plasmid, or strain (Broda, 1979), it is entirely possible that additional plasmids remain undiscovered in these isolates.

Using site-specific endonuclease analysis, Gantotti et al. (1979) and Gonzalez and Vidaver (1980) attempted to show relatedness among the plasmids in P. syringae pv. phaseolicola and P. syringae pv. syringae, respectively. Neither group was able to demonstrate the presence of similar plasmids among all isolates that expressed the

toxigenic phenotype. However, certain combinations of strains did harbor identical plasmids or plasmid fragments of similar size. We have shown here that no single plasmid or plasmid fragment size occurs among these six isolates of P. syringae pv. glycinea.

We have shown by molecular hybridization that the plasmid species of the six isolates were partially related. Similar blot-hybridization experiments have established relationships among plasmids in other bacterial species. The degradative plasmids of P. putida have homologous sequences which are not apparent by perusal of the fingerprints (Heinaru et al., 1978). Likewise, the oncogenic plasmids of Agrobacterium tumefaciens exhibit two major regions of homology, but their banding patterns are dissimilar (Hepburn and Hindley, 1979; Depicker et al., 1978; Chilton et al., 1978). These homologous regions correlate with the plasmid-determined properties of octopine or nopaline utilization by the bacterium and octopine or nopaline synthesis by plasmid-transformed plant cells.

The homology among the P. syringae pv. glycinea plasmids correlated with four EcoRI fragments belonging to strain LR100. Plasmid probes prepared from each isolate hybridized to three fragments of pMC11 and to one fragment of pMC10 (Table 4). The fragments comprised 19.4 Mdal of DNA, and this value represents the maximum level of homology. The actual degree of

homology cannot be assessed because neither the size of the hybridizing probe fragment nor the homologous portion of it are known. An accurate assessment of homology can be computed from hybridization kinetic experiments. In contrast, the results derived from blot hybridization are more descriptive than quantitative and deal with the location of the reacting fragments. This permits a rapid determination of relatedness among diverse plasmids.

The biological significance of the homologous segments is not known but several possibilities can be advanced. Transposable elements are discrete segments of DNA that occur in large bacterial plasmids, such as the *E. coli* sex factor F and the R plasmids (Kleckner, 1977; Starlinger, 1980). DNA homologous with insertion sequences have also been located in *P. aeruginosa* and *P. putida* (Nisen *et al.*, 1979). Several strategically located insertion sequences may explain the homology observed among the plasmid contents of the *P. syringae* pv. *glycinea* isolates described in this communication.

Incompatibility is commonly used as a measurement of the degree of relatedness between natural isolates (Datta and Hedges, 1977). Although the converse does not always follow, it is feasible that the molecular homology, which we have reported, defines an incompatibility group. This could be determined as soon as suitable genetic markers become available.

Finally, it is possible that these plasmids may carry genes which are essential for virulence. These genes may specify the production of phytotoxin, host range, immunity to host defenses, or the utilization of plant products. Further investigation is required to determine the biological role, if any, of these plasmids.

Figure 1. Agarose gel electrophoresis of plasmid DNA from six isolates of P. syringae pv. glycinea. Residual PEG was extracted (a) or allowed to remain (b) in the cell lysate prior to analysis. The location of the chromosomal fragments is marked (CHR). The tracks contain plasmids from (1) LR100, (2) LR219, (3) LR300, (4) LR400, (5) LR500, and (6) PG5.

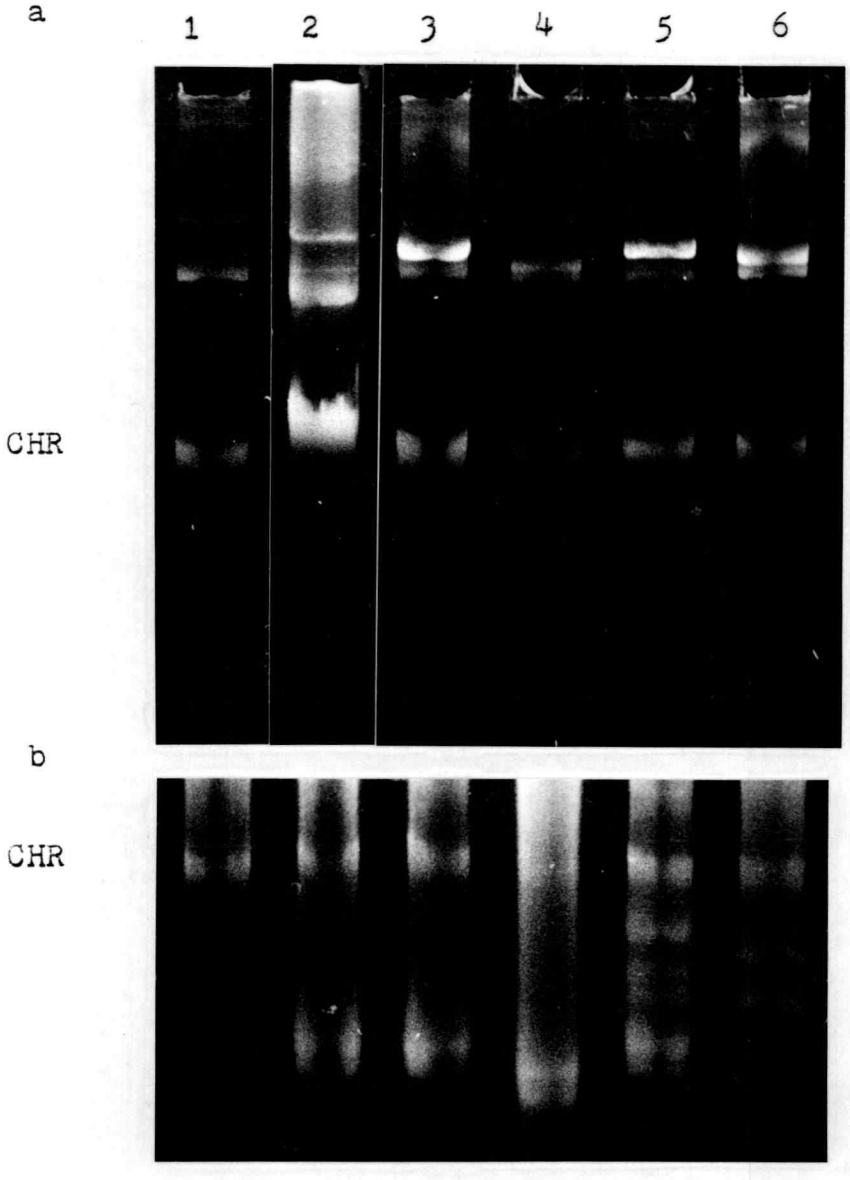

Figure 1

Figure 2. Homology among plasmids of six isolates of P. syringae pv. glycinea. (a) EcoRI-restriction endonuclease fingerprints of total plasmid DNA. (b) Hybridization of ^{32}P-plasmid DNA from strain LR100 to DNA fragments shown in (a). The tracks on the gel contain plasmid DNA from (1) LR100, (2) LR219, (3) LR300, (4) LR400, (5) LR500, (6) PG5.

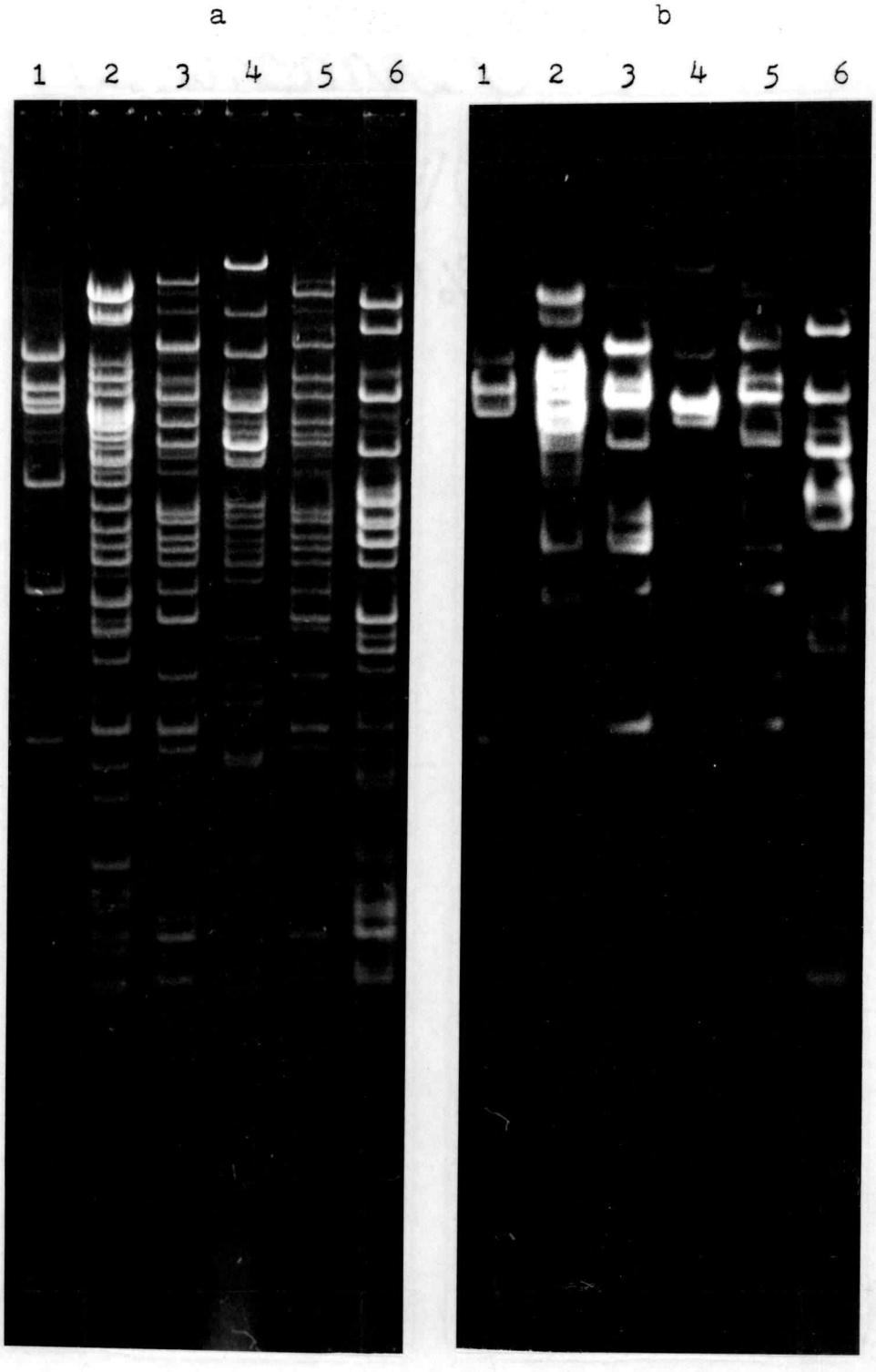

Figure 2

TABLE 1

P. syringae pv. *glycinea* strains used in this study.

Strain	Known plasmid[a]	Mol. mass (Mdal)	Former strain designation and source
LR100	pMC11	43	PG10; D.W. Chamberlain
LR219	pMC21 pMC22	87 25	PG11; D.W. Chamberlain
LR300	pMC31 pMC32		PG10a; D.W. Chamberlain
LR400	pMC41	54	PGR1; B.W. Kennedy
LR500	pMC51 pMC52	73 43	PGR2; B.W. Kennedy
PG5	pMC61 pMC62		PG5; A.K. Vidaver

[a]Determined previously (Curiale and Mills, 1977).

TABLE 2

The number and molecular mass of plasmids detected in six virulent strains of P. syringae pv. glycinea by agarose gel electrophoresis.

Strain	Plasmid designation	Molecular mass (Mdal)
LR100	pMC10	109
	pMC11	42
LR219	pMC21	84
	pMC22	46
	pMC23	39
	pMC24	3.8
LR300	pMC31	61
	pMC32	46
	pMC33	3.7
LR400	pMC41	51
	pMC42	46
	pMC43	3.3
	pMC44	3.0
LR500	pMC51	61
	pMC52	46
	pMC53	6.8
	pMC54	5.5
	pMC55	4.9
	pMC56	3.8
	pMC57	3.3
PG5	pMC61	52
	pMC62	42
	pMC63	6.0
	pMC64	4.8

TABLE 3

Hybridization of ^{32}P-labeled plasmid DNA from LR100 to *Eco*RI fingerprints of total plasmid DNA from five strains of *P. syringae* pv. *glycinea*.

	Source of unlabeled plasmids				
	LR219	LR300	LR400	LR500	PG5
Number of *Eco*RI bands[a]	33	31	29	29	28
Number of bands having homology with labeled probe	20	17	16	18	21

[a]Some bands may contain two or more similarly sized DNA fragments. Hybridization to fragments smaller than 1.2 Mdal was irreproducible and not included.

TABLE 4

An empirical determination of the extent of hybridization of total ^{32}P-plasmid DNA from four strains of *P. syringae* pv. *glycinea* to *Eco*RI fingerprints of LR100 plasmids pMC10 and pMC11.

Plasmid	Fragment mass (mdal)[a]	Source of labeled plasmid probe and extent of hybridization			
		LR219	LR300	LR500	PG5
pMC10	10.4	-[b]	-	+	-
	5.3	+	-	+	-
	5.0	++	-	-	-
	2.9	+	+	-	-
	2.4	+	-	+	-
	1.7	+	++	+	++
pMC11	7.39	++	+	+++	+
	6.29	+++	+++	+++	+++
	5.91	++	-	+++	-
	5.67	+++	+++	+++	+++
	4.33	+	-	+	-
	3.15	+	+	+++	-
	2.16	-	+	++	++
	1.37	+	-	+	-
	1.26	+	-	+	-

[a]Hybridization of probe DNA to fragments smaller than 1.2 Mdal was irreproducible and omitted from this table. Restriction fragments which failed to hybridize to at least one of the four DNA probes were not included in the table.

[b]An empirical measure of the autoradiographic spot density as a measure of the extent of hybridization: -, no hybridization; +, ++, +++, indicate increasing degrees of density.

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INSERTION AND PARTIAL EXCISION OF A NATURALLY OCCURRING PLASMID IN *PSEUDOMONAS SYRINGAE* PV. *PHASEOLICOLA*

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Pseudomonas syringae pv. phaseolicola is a pathogen of the common bean, Phaseolus vulgaris. At the site of infection, a water-soaked lesion with a chlorotic halo is formed. The chlorosis results from the production of halo blight toxin (HBT) by the bacterium[1,2]. HBT inhibits the activity of the enzyme ornithine-L-transcarbamylase, which is required for both arginine biosynthesis and the urea cycle[3,4,5]. However, toxin production is not essential for pathogenicity because nontoxigenic strains are virulent[6,7,8,9]. Recent work has shown that all virulent isolates tested to date contain one or more plasmids[10]. Evidence has been presented that suggests a correlation between the presence of a 22.5 x 10^6 molecular weight (MW) plasmid and the ability to produce HBT[10]. We have been analyzing the extrachromosomal DNA of a virulent, toxin-producing isolate which contains a single plasmid which has a MW of 98 x 10^6. During the course of our analysis, a "plasmidless", toxin-producing isolate was obtained. Evidence is provided here which indicates that the 98 x 10^6 MW plasmid, pMC7105, has integrated into the bacterial chromosome. Furthermore, partial excision can occur, thereby generating autonomous plasmids that are smaller in size.

Strain LR715 was derived from the toxigenic wild type strain LR700 following mutagenesis with ethyl

methane sulfonate[11]. The colonies formed by the mutant were flat and grainy; wild type colonies were smooth and convex. When LR715 was grown above 26°C or on a medium supplemented with $CoCl_2$ (50 uM), the mutant phenotype was suppressed. The phenotypic symbol Lco^{cs} was devised to describe these characteristics. LR715 is virulent and it causes chlorosis on plants. However, unlike the parent, it was unable to cause systemic infection.

If the Lco^{cs} phenotype were linked to pMC7105, it is conceivable that a colony of a plasmidless derivative would express a different morphology. Hence, a spontaneous streptomycin-resistant segregant of LR715 (Str^{R}, 100 ug per ml) was selected for a plasmid-curing experiment and designated LR716. A 1 ml aliquot containing 10^3 cells of LR716 was incubated in the presence of mitomycin C (1 ug per ml) for 16 h without illumination. Forty colony-forming survivors were recovered and one expressed a slightly altered phenotype. This isolate was designated LR719 and retained for further analysis. However, upon storage at 4°C, the mitomycin C-induced phenotype was lost. This reversion to the Lco^{cs} phenotype contradicted the assumption that LR719 was derived by the curing of pMC7105.

The plasmid purification scheme developed by Hansen and Olsen for the characterization of large plasmids[12] was used to detect plasmids in the strains derived from LR700. A single plasmid, pMC7105, which was known to be

in LR700 was also present in LR715 and LR716, but absent from LR719 (Fig. 1). However, upon repeated subculturing of LR719 an isolate was recovered that yielded three plasmid bands upon electrophoresis. The isolate was a mixture of three cell types which were subsequently purified and designated LR743, LR744, and LR745 on the basis that each contained a single plasmid; pMC7113, pMC7114, and pMC7115, respectively (Fig. 1). The presence of extrachromosomal DNA was the only feature which distinguished these strains from LR719.

In order to assess the relatedness between the new smaller plasmids and pMC7105, purified plasmid DNA was digested with BamHI and EcoRI endonucleases and the products were separated by agarose gel electrophoresis (Fig. 2). A comparison of the fingerprints revealed two BamHI and 14 EcoRI fragment sizes that were common to all four plasmids. The third BamHI band and first EcoRI band of pMC7115 represented the only bands of the three small plasmids that did not occur in pMC7105. The molecular weight of each plasmid was computed from the fragment mobility[13] and determined to be 35, 50, 58, and 98 x 10^6 for pMC7113, pMC7114, pMC7115, and pMC7105, respectively. It was apparent from these data that the smaller plasmids resembled deletion products of pMC7105. Since they were derived from LR719, the "plasmidless" strain, it was reasoned that LR719 must contain all or a portion of pMC7105.

If pMC7105 inserted into the chromosome of LR719 by reciprical recombination as bacteriophage lambda enters the Escherichia coli genome[14], it will be cut at a single site and its ends will be joined to chromosomal DNA at the site of integration. When total DNA is isolated from this strain and digested with a restriction enzyme which repeatedly cleaves pMC7105 DNA, all of the plasmid fragments except the fragment containing the integration site will be present, and, in addition, there should be two new fragments containing plasmid DNA adjacent to chromosomal DNA[15,16,17,18]. Reversing the integration process regenerates the original plasmid and chromosome. However, if excision involves recombination between sites other than the plasmid-chromosome junction, the newly formed plasmid and chromosome will contain sequences that were not previously adjacent. When total DNA is treated with a restriction endonuclease, the two fragments that contained the sites of excision will be absent. They will be replaced by two new fragments which were formed by recombination during excision. One of the new fragments will be cleaved from chromosomal DNA and the other will come from the new plasmid.

Total DNA was isolated from the mutant strains, digested with BamHI nuclease, and the fragments were separated electrophoretically on an agarose gel. The patterns were transferred to cellulose nitrate paper and hybridized with ^{32}P-pMC7105 DNA that had been labeled

by nick translation. Endonuclease BamHI was chosen because it produced 18 discrete fragments from pMC7105. The hybridization of pMC7105 to BamHI-restricted total DNA from the "plasmidless" strain LR719, and the strains derived from it are presented in Figure 3. A control containing purified pMC7105 DNA extracted from LR716 showing bands 1 through 16 is shown in lane a. Bands 17 and 18, with estimated MWs of 8.2 and 7.6 x 10^5, respectively, did not transfer. Lane b contains BamHI restriction fragments of total DNA isolated from LR716. In addition to the 16 plasmid bands of pMC7105, six new bands are visible due to hybridization of pMC7105 with chromosomal DNA. These bands are present in all strains and show hybridization to pMC7105. Strain LR719 (lane c) and strains LR743, LR744, and LR745 (lanes d, e, f), which contain the smaller plasmids, displayed similar but not identical patterns.

Two new bands, which presumably contain the plasmid-chromosome junction, are found in LR719. One is located between the first and second BamHI fragments of pMC7105 and the second is half-way between fragments 13 and 14 (Fig. 3). Plasmid band 8 of LR716 which exhibits strong hybridization with labeled pMC7105 shows only weak hybridization with LR719 DNA at this position. If the integration site occurred within fragment 8 of pMC7105, this band should be absent from LR719 DNA. If this interpretation is correct, the faint band which is

observed at this position represents a chromosomal fragment which shows limited homology with pMC7105.

The probable excision sites of pMC7115 occur in fragment 12 of pMC7105 since it is absent, and in the large fragment containing the plasmid-chromosome junction since it is also absent from these digests (Fig. 3). The sum of the molecular weights of these two fragments (approximately, 18 x 10^6 MW) should equal the sum of the molecular weights of the newly formed fragments. One of the new fragments is visible as the third BamHI fragment of pMC7115 and its MW is estimated to be 6.0 x 10^6 (Fig. 2, lane d). The computed molecular weight of the other new fragment (approximately, 12 x 10^6 MW) indicates that it should comigrate with BamHI-fragment 2 of pMC7105.

The excision sites of pMC7113 and pMC7114 could not be determined. The only detectable change in the hybridization fingerprints was the absence of the larger fragment containing the plasmid-chromosome junction in LR743 (Fig. 3, lane d). Also, no new bands were observed in the restriction patterns of these plasmids (Fig. 2). This result may occur if the newly formed fragments comigrate with existing fragments, or if the fragments are so small that they migrate off the gel during electrophoresis.

A series of experiments which were designed to localize the Lco^{cs} phenotype to either the chromosome or

plasmid were inconclusive. Instead, they lead us to the fortuitous discovery of the "plasmidless" strain LR719. It is not known whether the insertion of pMC7105 into the bacterial chromosome resulted from natural events or as the result of the curing treatment with mitomycin C. Additional studies must be carried out to determine whether pMC7105 is a true episome analogous to the sex factor F[19] and the phages P1 and P7[18] of Escherichia coli. The sex factors FP2, FP39, and FP5 of P. aeruginosa might integrate, however, no physical evidence or F'-like plasmids have been recovered[19].

Insertion sequences provide homologous regions between F factor and the E. coli chromosome which are believed to be essential for the formation of Hfr strains[20,21]. The six chromosomal bands which show sequence homology with pMC7105 may represent integration sites analogous to those found in E. coli.

The possibility that integration and excision occurs naturally in this plant pathogen may lead to an understanding of general mechanisms of plasmid evolution and recombination between plasmid and chromosomal sequences in these bacteria. The exchange of genetic material may place essential chromosomal genes on newly derived plasmids thereby ensuring that these plasmids will be stably inherited, since their loss from the cell would be lethal.

The derived strains may prove to be very useful

for studying the genetics of P. syringae pv. phaseolicola. If the plasmid promotes conjugation, a genetic map of the chromosome can be developed with the aid of Hfr-like strains. This may lead to a better understanding of the organization of the genes for virulence in this organism and other closely related plant pathogenic pseudomonads.

Figure 1. Plasmid species in isolates of P. syringae pv. phaseolicola having normal and altered plasmid content. Plasmid DNA was isolated by a modified version of the Hansen and Olsen[12] technique as described by Curiale and Mills[13]. Samples were subjected to electrophoresis through a 0.7% agarose gel as previously described[13]. The bands were visualized by staining with ethidium bromide (0.4 ug per ml). Strain and plasmid designation: a, LR716 pMC7105; b, LR719 "plasmidless"; c, LR743 pMC7113; d, LR744 pMC7114; e, LR745 pMC7115.

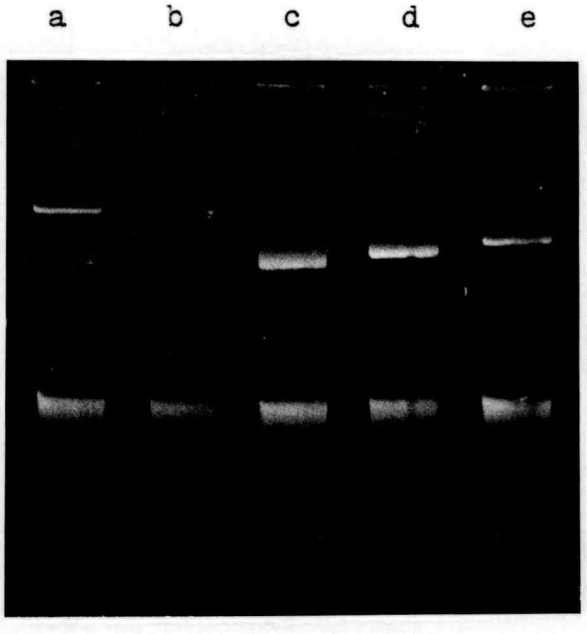

Figure 1

Figure 2. Restriction endonuclease-cleavage patterns of purified plasmid DNA. Plasmid DNA (1 to 2.5 ug) purified from buoyant density gradients[13] was digested with either BamHI or EcoRI at 37°C in a buffer consisting of 10 mM Tris (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl and 1 mM ethylenediaminetetraacetic acid. The products were separated by electrophoresis through a 0.7% agarose gel as previously described[13]. The plasmid samples are: lanes a and e, pMC7105; lanes b and f, pMC7113; lanes c and g, pMC7114; lanes d and h, pMC7115. DNA digested by BamHI, lanes a-d; by EcoRI, lanes e-h.

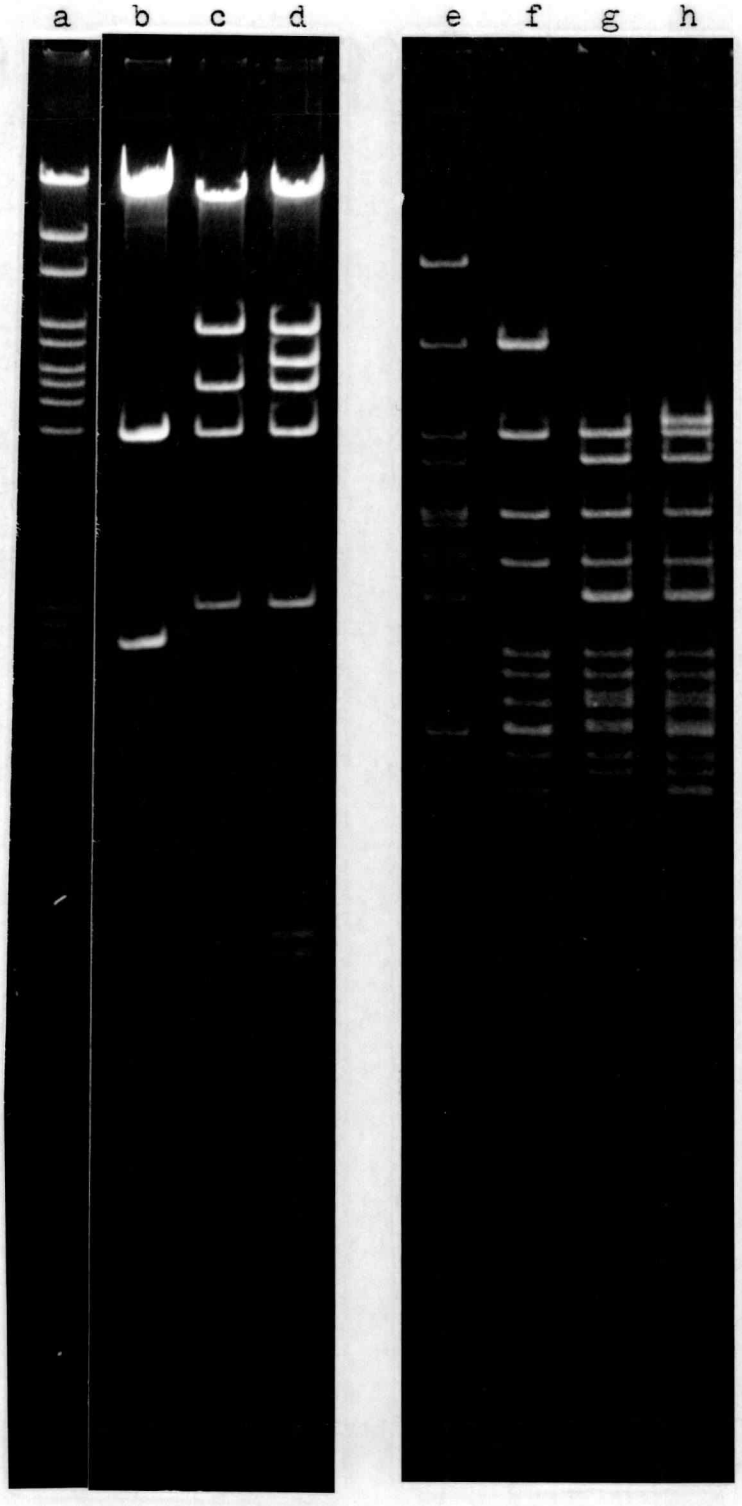

Figure 2

Figure 3. Homology between pMC7105 and total cell DNA from strains with altered plasmid content. Total cell DNA was purified using the procedure of Chesney et al[18]. Two ug of DNA was digested with BamHI and the fragments were separated by electrophoresis as described in Fig. 2. The fragments were transferred to a nitrocellulose membrane using the Ketner and Kelley[16] blotting technique. The probe DNA was labeled with ^{32}P to specific activities of 10 to 30 x 10^6 cpm per ug using the Nick Translation Kit, New England Nuclear. After preincubation of the filter for 6 h at 65°C in Denhardt's solution containing 20 ug per ml denatured calf thymus DNA, the filter was rinsed with 2 x SSC (SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0) at 71°C. The hybridization solution contained 20 ug per ml denatured calf thymus DNA, 0.2% sodium dodecylsulfate, 2.25 x SSCP (SSCP: 120 mM NaCl, 15 mM sodium citrate, 13 mM KH_2PO_4, 1 mM ethylenediaminetetraacetic acid, pH 7.2 with NaOH), 44% formamide, and 7.5 x 10^5 cpm per ml denatured radioactive probe. The filters were incubated for 40 h at 44°C, given 4 or 5 serial washes (SSCP, 0.1% sodium dodecylsulfate, 70°C), dried and exposed to Kodak No-Screen X-Ray film. Lane a contains pMC7105 DNA; the total DNA samples are: lane b, LR716; lane c, LR719; lane d, LR743; lane e, LR744; lane f, LR745.

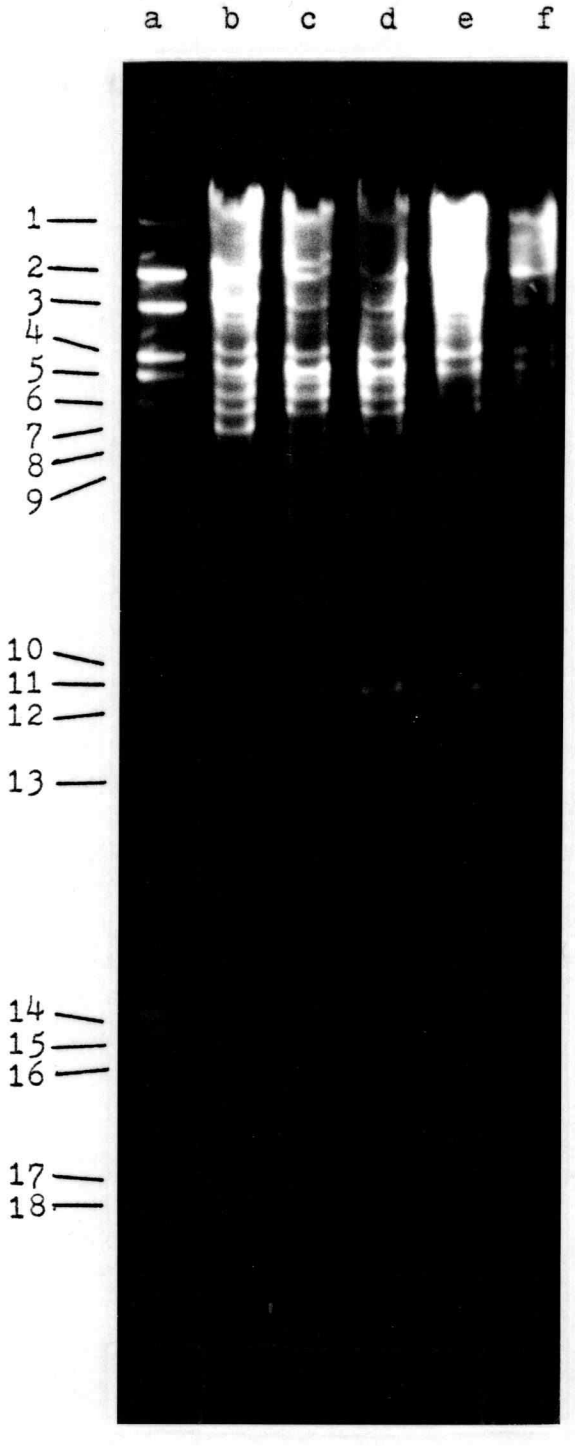

Figure 3

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STABILITY OF RP4 AND INDIGENOUS PLASMIDS AND EVIDENCE OF TRANSPOSITION OF TN1 IN *PSEUDOMONAS SYRINGAE* PV. *GLYCINEA* AND *PSEUDOMONAS SYRINGAE* PV. *PHASEOLICOLA*

Running Title: TRANSPOSITION AND PLASMID STABILITY

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ABSTRACT

The feasibility of several approaches to establish a plasmid-phenotype association for plasmids of two pathovars of Pseudomonas syringae were examined. Plasmid-cured strains were sought by dye-buoyant density gradient centrifugation or gel electrophoresis following treatment with the curing agents, acridine orange and mitomycin C. Among a relatively small number of colonies that were examined for plasmid content, neither chemical completely cured isolates of their plasmids. Many more colonies were examined by colony-blot hybridization with radio-labeled plasmid DNA. No cured strains were detected among over 5000 colonies that had been treated with the mutagen ethyl methane sulfonate for plasmid elimination. The sensitivity of this method was possibly reduced by homology between plasmid and chromosome.

The RP4 plasmid was used as a source of the transposable element Tn1 in attempts to genetically tag naturally occuring plasmids with the gene for ampicillin resistance. Transposition was detected among some of the strains of P. syringae pv. glycinea that had spontaneously lost RP4. Ampicillin resistance was mobilized by the Tn1-containing strains in matings with strains that harbored a similar complement of plasmids. No physical evidence was apparent in these strains for the

localization of the Tn1 sequence on the plasmids of the donors or transconjugants. However, an analysis of fingerprints of plasmid DNA from a strain of P. syringae pv. phaseolicola revealed physical evidence for the insertion of the Tn1 sequence into the indigenous plasmid pMC7105. The restriction properties and size of the inserted DNA are consistant with the expected results of insertion of Tn1. The competency of this strain to serve as a donor of RP4 or pMC7105::Tn1 in matings with related pseudomonads is greatly reduced.

INTRODUCTION

The discovery of plasmids in plant pathogenic pseudomonads was a relatively recent occurrence (7, 8, 12, 15). Attempts to ascertain a role for these plasmids have been directed toward certain aspects of the pathogen-host interaction. Indeed, there has been evidence to suggest that the production of syringomycin and phaseolotoxin, by Pseudomonas syringae and P. syringae pv. phaseolicola, respectively, may be associated with the presence of specific plasmids (12, 15). Although these toxins cause chlorosis in diseased plants, their synthesis is not essential for expression of virulence by the bacteria (13). The definitive genetic evidence which would link the production of toxin or other phenotypic expression to the presence of a specific plasmid is lacking. Without the definitive linkage of a specific phenotype to a particular plasmid, a determination of a biological role, if any, for these cryptic plasmids will be difficult.

Several approaches can be used to establish a plasmid-phenotype association in these pseudomonads. First, chemical agents (6, 11, 15, 23) and abnormal growth conditions (19, 20) which have been successfully used to cure other organisms of their plasmids, may be exploited. Unfortunately, many of these agents induce mutations and, therefore, a change in phenotype after

treatment may not be due to the loss of plasmid sequences. Second, biochemical evidence can be sought for the presence or absence of plasmid DNA by using the technique of colony hybridization to radio-labeled plasmid DNA (16). Colonies which do not have the plasmid sequences should not hybridize to the plasmid probe. Third, transposable elements which carry genes for drug resistance can be inserted into a cryptic plasmid, thereby providing the required plasmid-phenotype association (14, 24). Once inserted, the presence of the plasmid will confer upon the host cell, the drug-resistance property.

This paper presents evidence for the feasability of each approach in attempts to either isolate cured strains or genetically tag cryptic plasmids in isolates of P. syringae pv. glycinea, the causal organism of bacterial blight of soybean (Glycine max [L.] Merrill) and P. syringae pv. phaseolicola, the causal organism of halo blight of common bean (Phaseolus vulgaris L.).

MATERIALS AND METHODS

Strains and media. The Pseudomonas strains used in this study are described in Table 1. Escherichia coli strain J53 (1) was used to propagate the antibiotic resistance plasmid RP4 (10). All strains were grown and maintained on MaSNY medium (7).

Curing procedures. Overnight bacterial cultures were diluted with fresh medium to a density of about 50 cells per ml. Acridine orange (final conc. 10 to 50 ug per ml) or mitomycin C (final conc. 0.01 to 1 ug per ml) was added and incubation continued for 4 to 24 h at 25°C in the dark. The cells were washed and plated onto fresh medium and incubated at 25°C until colonies formed. Single colonies were transferred into MaNY and overnight cultures were examined for the presence of plasmid DNA by dye-buoyant density gradient analysis (7) or by agarose gel electrophoresis of cleared lysates (8).

Colony blot hybridization. The poor replica-plating property of these pseudomonads necessitated the transfer of individual colonies to establish a grid of 60 well-spaced colonies per petri plate. After incubation for 18 h at 25°C, the colonies of LR731 were absorbed onto Whatman 541 filter circles by placing the

paper on top of the colonies for about 30 min. The filters were transferred to a solution of 0.5 N NaOH, shaken gently for 10 min and washed with five changes of SSCP (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The air-dryed filters were baked overnight at 60°C and then stored at 4°C before use. The indigenous plasmid pMC7105 was labeled to a specific activity of about 10 x 10^7 cpm per ug with [^{32}P]-dCTP by nick translation. The hybridization mixture included 2 x SSCP, 1% Sarkosyl, and 4 x 10^5 cpm per ml of heat-denatured probe. One ml of the hybridization mixture was added per filter in a heat sealable cooking pouch. The sealed bag was placed in a 73°C water bath for hybridization and incubated 16 h. After incubation, the filters were washed with several changes of 2 x SSCP that had been heated to 73°C. The filters were air dryed and placed in contact with Kodak No-Screen medical X-ray film for autoradiography.

Transposition of Tn1. The P1-incompatibility group plasmid RP4, was used as the source of the Tn1 element (5, 17). E. coli J53 (RP4) was used to transfer RP4 into both Pseudomonas pathovars. Donor and recipient cells were mixed on very dry MaSNY medium plates and incubated at 20°C for 18 h. The cells were scraped off the plates, washed, and spread onto MaSNY-agar plates containing ampicillin (500 ug per ml) and streptomycin

(100 ug per ml) or rifampicin (100 ug per ml) when appropriate to counter select against donor and recipient cells. The presence of RP4 among transconjugants was determined by growth on medium containing ampicillin (Ap^r), kanamycin (Km^r), and tetracycline (Tc^r). Transposition of Tn1 was detected by sensitivity to kanamycin (Km^s) and tetracycline (Tc^s) but Ap^r among exconjugants. The electrophoretic mobility of restriction fragments of purified plasmid DNA was also used to detect transposition of Tn1 to indigenous plasmids.

Gel electrophoresis of plasmid restriction fragments. The isolation of purified plasmid DNA and fingerprint analysis by agarose gel electrophoresis has been described elsewhere (8).

Mutagenesis. The method of mutagenesis with ethylmethane sulfonate was previously described (7).

RESULTS

Curing of plasmids from LR219 and LR229. The effectiveness of the plasmid curing agent mitomycin C and acridine orange was tested on two strains of P. syringae pv. glycinea. Since the plasmids carried by these strains are phenotypically cryptic, it was necessary to use physical means to record the presence or absence of the indigenous plasmids.

Nineteen isolates of LR229, which survived treatment with mitomycin C, were examined for the presence of a satellite DNA band by dye-buoyant density gradient centrifugation. In each instance, a fluorescent plasmid band was observed when the gradient was illuminated with long wavelength ultraviolet light. Since the parent strain was known to harbor more than one plasmid species (7, 8), any condition other than complete curing will not be readily detected by this procedure.

In contrast to the gradient method, the plasmid species contained by an isolate can be discriminated on agarose gels. Using this method, four plasmids have been observed in cleared lysates of strain LR219 (8). This strain was treated with the curing agent acridine orange and survivors were subjected to gel analysis. Of the 42 isolates examined, none had lost any of the four plasmid bands.

Colony filter hybridization with labeled pMC7105.

P. syringae pv. phaseolicola LR731 was chosen for this study because it contains a single indigenous 98 megadalton (Mdal) plasmid, pMC7105 (9), and RP4 which served as an internal standard for assisting in the detection of cured colonies. In control experiments, labeled RP4 plasmid DNA does not hybridize to colony blots of these pathovars which do not contain RP4 and, therefore, its presence does not effect the results of colony blot hybridization described here. Prior to picking colonies for blotting to Whatman 541 paper, the cells were subjected to EMS mutagenesis as described in Materials and Methods. The EMS treatment was invoked to possibly enhance the frequency of events which may result in defective segregation of pMC7105 among the survivors. A total of 5,398 randomly picked colonies have been analyzed after hybridization to labeled pMC7105 probe DNA. Several colonies showed a reduced level of hybridization but gel electrophoresis of total DNA from each isolate confirmed the presence of pMC7105.

The apparent low frequency of defective segregation of pMC7105 suggested that this plasmid is either very stable in LR731 or that because of sequence homology between the chromosome and pMC7105, this procedure fails to detect a plasmidless derivative. That the latter assumption may prove to be correct is suggested

by results of hybridization of pMC7105 to restriction fragments of total DNA (9). The probe hybridized to all of the fragments of pMC7105 and to 6 chromosomal fragments.

Transposition of Tn1. Crosses were made between E. coli J53 (RP4) and two strains of each pathovar. RP4 was transferred at frequencies of 10^{-3} to 10^{-5} per donor cell in the crosses. Since the ultimate goal of these experiments was to detect transposition of Tn1 to an indigenous plasmid, crosses were made between isogenic strains of the pathovar phaseolicola. Over 7500 randomly selected Ap^r transconjugants were initially selected from four crosses of donor strains, LR731 and LR702, with appropriately marked recipients. All transconjugants were subsequently shown to be Km^r and Tc^r indicating that the entire RP4 plasmid was mobilized by the donor and maintained in the transconjugants.

If transposition of Tn1 occurs in the pseudomonads that harbor RP4, and a subsequent event results in the defective segregation of RP4, the cells should retain Ap^r but exhibit Tc^s and Km^s. Since the degree of stability of RP4 was not known for these pseudomonads, two isolates of each pathovar were analyzed for Tn1 transposition and stability of RP4. Approximately 11% (251/2198) of the colonies derived from LR220

showed defective segregation of RP4 and about 1.4% (31/2198) had undergone a transposition event (Table 2). However, less than 0.5 percent of the colonies derived from LR253 showed defective segregation for RP4 and none was observed to have undergone Tn1 transposition. RP4 is very stable in both isolates of P. syringae pv. phaseolicola since defective segregants and transposition events were not detected in 6,954 colonies.

The stability of Tn1 after transposition was determined by streaking an Ap^r segregant of LR220 onto medium lacking ampicillin to obtain individual colonies, and subsequently testing these colonies for Ap^r. None of 819 colonies tested was Ap^s (Table 3).

The transfer of the transposable element from two Ap^r segregants of LR220 to LR252 was demonstrated. The crosses were performed as previously described, but the frequency of transfer of Tn1 was not determined. Transconjugants were selected on medium containing streptomycin and ampicillin and subsequently shown to have the auxotrophic markers of LR252. The stability of the Tn1 element in LR252 recipients was substantially reduced relative to LR220 (Table 3). Approximately 77% of the colonies from a transconjugant of one cross lost the Tn1 element after growth on medium lacking ampicillin, as did 4% of the colonies from a transconjugant of the other mating. The reason for the instability is not clear since LR220 and LR252 were derived from a

common parent strain after EMS mutagenesis.

If the Tn1 element was inserted onto a plasmid of LR220, and mobilized into LR252, it would not be stable in the transconjugants, since both strains were derived from a common parent, and the mating would bring together two plasmids of the same incompatibility group. However, a preliminary screening of the EcoRI fingerprints of the parent strain, the Tn1 donor and a transconjugant revealed no apparent differences in the fingerprints, except for the presence of RP4 in the parent strain (data not shown).

Since RP4 was stably maintained in both strains of P. syringae pv. phaseolicola, the occurrence of transposition of Tn1 would not be detected on drug-containing medium. However, physical evidence for transposition to the indigenous plasmid would be detected by fingerprint analysis. Indeed, the transposition of Tn1 to pMC7105 was detected, upon routine analysis of plasmid DNA of LR731. The EcoRI and BamHI fingerprints of purified pMC7105 DNA are presented in Figure 1, lanes a and d. For comparison, the fingerprints of plasmid DNA from LR731 are presented in Figure 1, lanes c and e. RP4 which is present in LR731 contains single EcoRI and BamHI sites (2) and, therefore, a single large fragment is generated upon digestion with either enzyme. Evidence for insertion of Tn1 into pMC7105 in a derivative, LR791, is presented in Figure 1, lanes b and f.

EcoRI fragment 3 of pMC7105 is absent and it is replaced by a fragment which migrates between fragments 1 and 2 of pMC7105. The mobility of the new fragment indicates that its molecular weight is approximately 3.4 Mdal larger than fragment 3 of pMC7105. Since the Tn1 transposon does not contain an EcoRI site, its insertion into pMC7105 is expected to result in the loss of one fragment and the appearance of a new fragment which is larger by 3.2 Mdal. A BamHI fingerprint further substantiates the evidence for Tn1 insertion into pMC7105. BamHI fragment 1 of pMC7105 migrates slightly faster than the linear fragment of RP4 (Fig. 1, lanes d and e), and its position is somewhat obscurred when the two fragments are present together (Fig. 2, lane e). Upon close inspection, the insertion of Tn1 resulted in a loss of pMC7105 fragment 1 and the appearance of two new fragments which migrate between fragments 1 and 2 of pMC7105 (compare lanes d and f of Fig. 1). The sum of the molecular weights of these two fragments is estimated to be 4-5 Mdal larger than BamHI fragment 1 of pMC7105. Since the BamHI site of RP4 maps within the Tn1 transposon, its insertion into pMC7105 is expected to result in the loss of one fragment and the appearance of two new fragments, upon digestion with BamHI. The sum of the molecular weights of these two fragments should be approximately 3.2 Mdal larger than the fragment which is lost. The difference between the

computed size of the inserted DNA (4-5 Mdal) and that expected for Tn1 (3.2 Mdal) is assumed to result from the error inherent in measuring small increases in molecular weights of the two new, large fragments.

Plasmid transfer properties of LR791. Strain LR731, the parent of LR791, donates RP4 to other strains of this pathovar with transfer frequencies of 10^{-5} to 10^{-7} per donor cell. However, in five experiments with LR791 as the donor of RP4, no transconjugants were recovered at transfer frequencies as low as 10^{-9} per donor cell. Attempts to recover an RP4 defective segregant of LR791 have also been unsuccessful. None of 1280 colonies examined had lost Km^r. Also, 200 survivors of novobiocin treatment (21) and 160 survivors of tetracycline-cycloserine selection (4) were examined and all retained the drug resistance complement of RP4.

DISCUSSION

The chemical curing agents acridine orange and mitomycin C have been used successfully to obtain plasmid-cured isolates of P. syringae pv. syringae (15) and P. putida (6, 23), respectively. About 15% of the survivors of acridine orange treatment were plasmidless (15) whereas the effectiveness of mitomycin C varied according to the host-plasmid combination (6, 23). In these instances of curing, the loss of the plasmid always correlated with the loss of a selectable phenotype such as the production of toxin or the utilization of a metabolite. Since there was no evidence for a plasmid-linked trait among these pathovars, we examined random colonies that survived chemical treatment for the presence or absence of the indigenous plasmids. None of the 42 survivors of acridine orange treatment or 19 survivors of mitomycin-C treatment appeared to have been cured of their plasmids. Since relatively high frequencies of curing were observed with P. syringae pv. syringae, it seemed possible that direct examination of plasmid content in related pathovars may provide evidence for a cured strain.

Colony blot hybridization is a sensitive technique for ascertaining the presence or absence of probe sequences among bacterial populations (16). Using this technique, over 5000 colonies were examined in this

study and all showed hybridization to the labeled plasmid probe. The possibility that a cured isolate went undetected is very real in light of recent evidence which indicates that pMC7105 and the bacterial chromosome share regions of homology (18). At least 6 regions on the chromosome may exhibit homology to this plasmid. Although the nature and extent of the homology is unknown, sufficient sequence homology between pMC7105 and the bacterial chromosome could have resulted in a failure to detect a cured isolate.

The stability of RP4 and the transposition of Tn1 was examined in two pathovars of P. syringae. Defective segregation of RP4 was detected in P. syringae pv. glycinea, but not in P. syringae pv. phaseolicola. The degree to which RP4 is maintained in these strains is consistant with the stability of RP1, which is essentially identical to RP4 (4a, 18), in other organisms (21, 22). Transposition was detected phenotypically among 1.4% of the colonies that had lost RP4. None of these isolates exhibited an altered phenotype which could have resulted from insertion mutagenesis (2, 3). That ampicillin resistance could be transferred in matings with related strains suggests that at least one of the indigenous plasmids may be a conjugative plasmid which is capable of mobilizing the Tn1 sequence during matings.

However, physical evidence indicates that a DNA

sequence has inserted into pMC7105, an indigenous plasmid of P. syringae pv. phaseolicola (Fig. 1). Restriction analysis with BamHI and EcoRI indicates that the size and restriction properties of the insert are consistant with the known physical properties of Tn1 (2, 17). That RP4 is stably maintained in this strain makes it difficult to select for the Tn1-tagged pMC7105 in the presence of a similarly marked RP4 plasmid. Unexpectedly, this strain does not transfer RP4, unlike its parent which transfers RP4 with frequencies between 10^{-5} and 10^{-7} per donor cell. The loss of conjugal ability must have resulted from a spontaneous mutation to one of its transfer genes. That pMC7105::Tn1 failed to transfer in these crosses was also noted. If pMC7105 is indeed a conjugative plasmid, there are several possible explanations for the absence of Ap^r transconjugants. Since the crosses were between isogenic strains, the expression of superinfection inhibition (21a) may have prevented the establishment of the plasmid. It is possible that the plasmid has become transfer defective by insertion of the transposon into a transfer gene (2, 3). Lastly, the pMC7105 copy of the penicillinase gene may have mutated to Ap^s.

One of the greatest deterrents to establishing a biological role for the cryptic plasmids of the plant pathogenic pseudomonads has been the inability to positively associate a phenotype with the presence of

a plasmid. The insertion of Tn1 into pMC7105 should confer Ap^r upon cells which acquire this plasmid. Although the plasmid was not mobilized in matings with related strains, it should be possible to use transformation as a means for obtaining Ap^r in the absence of RP4. The detection of cured isolates by selection on medium containing ampicillin or by colony-blot hybridization with Tn1 probe DNA is now possible.

Figure 1. Restriction analysis of plasmid DNA showing the insertion of Tn1 into the plasmid pMC7105. Plasmid DNA was isolated from three strains, treated with either EcoRI or BamHI, and subjected to electrophoresis on a 0.7% agarose gel. The location of the RP4-plasmid fragment is designated (RP4). The sources of plasmid DNA were: lanes a and d, LR721; lanes b and f, LR731; lanes c and e, LR791. DNA digested by EcoRI, lanes a-c; BamHI, lanes d-f.

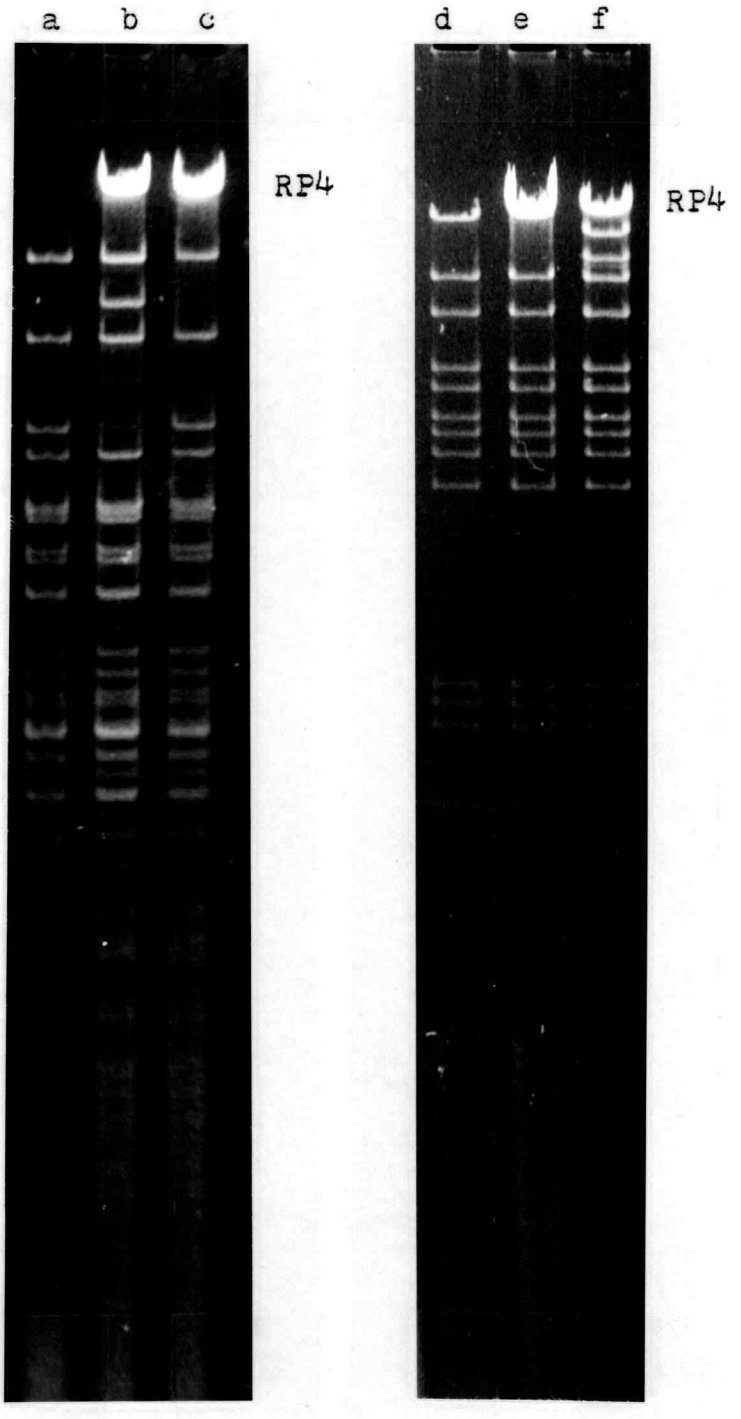

Figure 1

TABLE 1

Pedigree and source of strains of *Pseudomonas* used in this study

Strain	Genotype	Source or origin
P. syringae pv. *glycinea*		
LR219	*arg-1*	(8)
LR220	Like LR219, RP4	RP4 transfer: this work
LR220-1	Like LR220, Tn1	Tn1 transposition and loss of RP4: this work
LR220-2	Like LR220, Tn1	Tn1 transposition and loss of RP4: this work
LR229	*ade-1 trp-1*	(7)
LR252	*str-1* derivative of LR229	Streptomycin selection
LR252-1	Like LR252, Tn1	Tn1 transfer: this work
LR252-2	Like LR252, Tn1	Tn1 transfer: this work
LR253	Like LR252, RP4	RP4 transfer: this work
P. syringae pv. *phaseolicola*		
LR700	Wild type	(9)
LR701	*str-1* derivative of LR700	Streptomycin selection
LR702	Like LR701, RP4	RP4 transfer: this work
LR712	*ser-1* derivative of LR700	EMS mutagenesis
LR721	*rif-1* derivative of LR712	Rifampicin selection
LR731	Like LR721, RP4	RP4 transfer: this work
LR791	Like LR731, pMC7105::Tn1	Tn1 transposition: this work

TABLE 2

Segregation of RP4 and transposition of Tn1 among colonies derived from RP4-containing strains of *P. syringae* pv. *glycinea* and *P. syringae* pv. *phaseolicola*.

Strain	No. colonies tested	No. colonies retaining RP4[a]	No. colonies without RP4[b]	No. colonies with Tn1 transposition[c]
P. syringae pv. *glycinea*				
LR220	2198	1916	251	31
LR253	2498	2487	11	0
P. syringae pv. *phaseolicola*				
LR702	1815	1815	0	0
LR731	5139	5139	0	0

[a]Resistant to Km, Tc, and Ap.

[b]Sensitive to Km, Tc, and Ap.

[c]Resistant to Ap, sensitive to Km and Tc

TABLE 3

The apparent stability of Tn1 in defective segregants of LR220 and in Ap-resistant transconjugants of LR252.

Strain	Source of Ap^r	experiment		No. colonies examined	No. Ap^s colonies
LR220-1	RP4	1		240	0
		2		360	0
		3		20	0
		4		159	0
		5		40	0
			total	819	0
LR220-2	RP4	1		40	0
LR252-1	LR220-1	1		39	1
		2		119	7
		3		80	2
			total	238	10
LR252-2	LR220-2	1		24	24
		2		16	1
		3		28	28
			total	68	53

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