Molecular Relatedness Among Cryptic Plasmids in *Pseudomonas syringae* pv. *glycinea*

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ABSTRACT

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Molecular homology was detected among the plasmids harbored by six pathogenic strains of *Pseudomonas syringae* pv. *glycinea*. By using the Southern blot technique, radioactive plasmid DNA probe prepared from strain LR100 hybridized to 55–75% of the *Eco*RI-generated fragments of the other five strains. The reciprocal reaction was performed between membrane-bound *Eco*RI-digested LR100 plasmid DNA and plasmid probe prepared from four of the other strains. Each of these probes hybridized to three specific *Eco*RI fragments from plasmid pMC11 and one fragment from plasmid pMC10. From one to nine other bands showed homology with specific probes. This interrelationship among the plasmids was not detected by visual examination of the *Eco*RI fingerprints, although similarities between the restriction patterns of some plasmids were clearly evident.

Additional key words: bacterial blight, soybean.

Naturally occurring cryptic plasmids are frequently found among pathovars of *Pseudomonas syringae* (1,3,7,8,19–21). The plasmids vary in size, ranging from approximately 3 to over 100 megadaltons (Md), and many strains harbor more than a single plasmid (1,3,21). Plasmids of similar size can often be found among strains of a particular pathovar. In the absence of genetic markers and general procedures for curing cells of their plasmids, biochemical approaches have been used in attempts to identify sequences on plasmids of different origin that may define a common function. The relatedness of plasmids can be partially determined by restriction endonuclease analysis (26). Plasmids with similar fingerprints are regarded as being closely related. However, plasmids that have very different restriction patterns may have extensive homology when analyzed by DNA:DNA hybridization (12).

The present work was initiated to determine the extent of homology among plasmids from six strains of *P. syringae* pv. *glycinea* (29), the incitant of bacterial blight of soybean (*Glycine max* (L.) Merrill). No single plasmid or restriction fragment from the plasmids of these strains was common to all strains. In situ hybridization of endonuclease-generated fragments of plasmid DNA revealed sequence homology that was not apparent from the gel fingerprints of total plasmid DNA.

MATERIALS AND METHODS

Bacterial strains and media. Strains of *P. syringae* pv. *glycinea* used in this study are listed in Table 1. The designations of some strains have been changed to correspond with our laboratory stock numbers which conform with the recommendations for a uniform nomenclature for bacterial genetics (5). LR219 is an arginine auxotroph of strain LR200 which was previously described (3). All other strains are wild type.

Strain LR400 was grown on MaNY medium (3) supplemented with 0.2% sucrose to promote rapid growth. All other strains grew equally well on MaNY or MaNY with sucrose; consequently, they were grown and maintained on MaNY medium.

Escherichia coli strain CSH25 (18) was used as a source of bacteriophage λc1857S7. Growth of the lysogen, induction, isolation of phage particles, and extraction of phage DNA were performed as described by Miller (18).

Plasmid DNA isolation. Cells were harvested from stationary-

TABLE 1. Source of strains of *Pseudomonas syringae* pv. *glycinea* with approximate molecular weights of associated plasmids

Strain[a]	Genotype	Plasmid	Approx. size (Md)	Source
LR100	Wild type	pMC10	110	D. W. Chamberlain
		pMC11[b]	42	
LR219	*arg*	pMC21[b]	87	D. Mills
		pMC22[b]	46	
		pMC23	39	
		pMC24	4	
LR300	Wild type	pMC31	61	D. W. Chamberlain
		pMC32	46	
		pMC33	4	
LR400	Wild type	pMC41[b]	51	B. W. Kennedy
		pMC42	46	
		pMC43	3	
		pMC44	2	
LR500	Wild type	pMC51[b]	61	B. W. Kennedy
		pMC52[b]	46	
		pMC53	7	
		pMC54	6	
		pMC55	5	
		pMC56	4	
		pMC57	3	
LR600	Wild type	pMC61	62	A. K. Vidaver
		pMC62	42	
		pMC63	6	
		pMC64	5	

[a] Strains LR100, LR400, and LR500 were previously designated 10, R1, and R2 (3).
[b] Plasmids identified previously (3). All other plasmids were detected in this study.

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phase cultures or cultures that had attained a minimal optical density of 0.6 at 600 nm (~3×10^8 cells per milliliter). Plasmid-enriched lysates were prepared according to the protocol of Hansen and Olsen (11) with proportional adjustments to accommodate the quantity of cells. Residual polyethylene glycol 6000 (PEG), which was present in the lysate after PEG-partitioning of plasmid DNA, was removed by two or three extractions with cold octanol:chloroform (1:49, v/v) (24). The lysates were then either analyzed by agarose gel electrophoresis or further purified by isopycnic gradient centrifugation. Ethidium bromide (final concentration, 500 μg/ml) and CsCl (51% final concentration) were added to the solution of DNA, followed by centrifugation for 44 hr at 44,000 rpm in a Beckman type 65 rotor at 20 C. The plasmid band was removed through the side of the centrifuge tube by using a syringe equipped with a 22-gauge needle.

Restriction endonuclease digestion of plasmid DNA. Plasmid DNA was cleaved with restriction endonuclease *Eco*RI (Bethesda Research Laboratories, Inc., Gaithersburg, MD 20760) as described by McParland et al (15). The fragments were suspended in TEK (10 mM tris [hydroxymethyl]-aminomethane, 1 mM methylenediaminetetraacetic acid [EDTA], 100 mM KCl, pH 7.2) buffer for electrophoretic analysis or in 10 mM tris (pH 8.0) for nick translation.

Agarose gel electrophoresis. Agarose gel electrophoresis was performed with a vertical slab gel apparatus having slab dimensions of $20 \times 20 \times 0.3$ cm. Partially purified plasmid DNA was electrophoresed through 0.7% agarose at 5 V/cm for 6 hr with a running buffer containing 89 mM tris, 89 mM boric acid, and 2.5 mM EDTA (20). Plasmid DNA fragments were electrophoresed through 0.7% agarose at 2 V/cm for 21 hr in E buffer (16) containing 0.4 μg of ethidium bromide per milliliter. The DNA was visualized with 360-nm light and photographed with Kodak Tri-X film using a red (Vivitar No. 25) filter. The molecular masses of the cleavage fragments were determined from the comparative migration rates of restriction fragments of bacteriophage λc1857S7 DNA (10).

Nick translation of DNA. *Eco*RI-digested plasmid DNA was labeled to a specific activity of $1-10 \times 10^6$ cpm/μg by using the nick translation procedure described by Rigby et al (22) or to $10-30 \times 10^6$ cpm/μg using the nick translation kit obtained from New England Nuclear (Boston, MA 02118).

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 (14) version of the Southern (23) blotting technique. The filter blots were conditioned for hybridization by soaking for 4–6 hr at 60 C in Denhardt's preincubation solution (6) containing 0.2% Ficoll 400, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 20 μg of heat-denatured calf thymus DNA (CT-DNA) per milliliter in 2 × SSCP (SSCP is 120 mM NaCl, 15 mM sodium citrate, 13 mM KH_2PO_4, 1 mM EDTA, adjusted to pH 7.2 with NaOH). The filters were rinsed briefly with 2 × SSCP after conditioning and blotted dry with Whatman No. 1 filter paper.

The hybridization mixture consisted of 20 μg/ml CT-DNA, 0.2% SDS, and $0.5-2.0 \times 10^6$ cpm/ml ^{32}P-DNA in 2 × SSCP. The hybridization reactions were carried out at 71 C for 40 hr in plastic heat-sealable cooking pouches. These conditions (T_m–25 C) comply with those described by Wetmur and Davidson (27), for DNA of 58% GC (13). Following the reaction, the filters were washed at 71 C with five changes of wash buffer (500 ml of SSCP, 0.2% SDS) to remove some of the partial hybrids. Autoradiograms were exposed from 4 hr to 3 days on Kodak No-Screen medical X-ray film at room temperature.

RESULTS

Enumeration and molecular weight estimation of plasmids. Plasmid-enriched lysates were prepared from each of six *P. syringae* pv. *glycinea* strains and examined by agarose gel electrophoresis. High-molecular-weight plasmid bands were more clearly visible if residual PEG was removed from the lysates by octonal-chloroform extraction (Fig. 1a). When the residual PEG was not extracted, the plasmid bands became obscured by a smear of fluorescing material extending from the well to the chromosomal band (Fig. 1b). We have also observed that the extraction of PEG resulted in the selective removal of the small plasmids which migrated faster than the chromosomal fragments. The molecular masses of the plasmids of each isolate were initially estimated by comparative mobility with RSF2124 7.3 Md, RP4 (36 Md), and

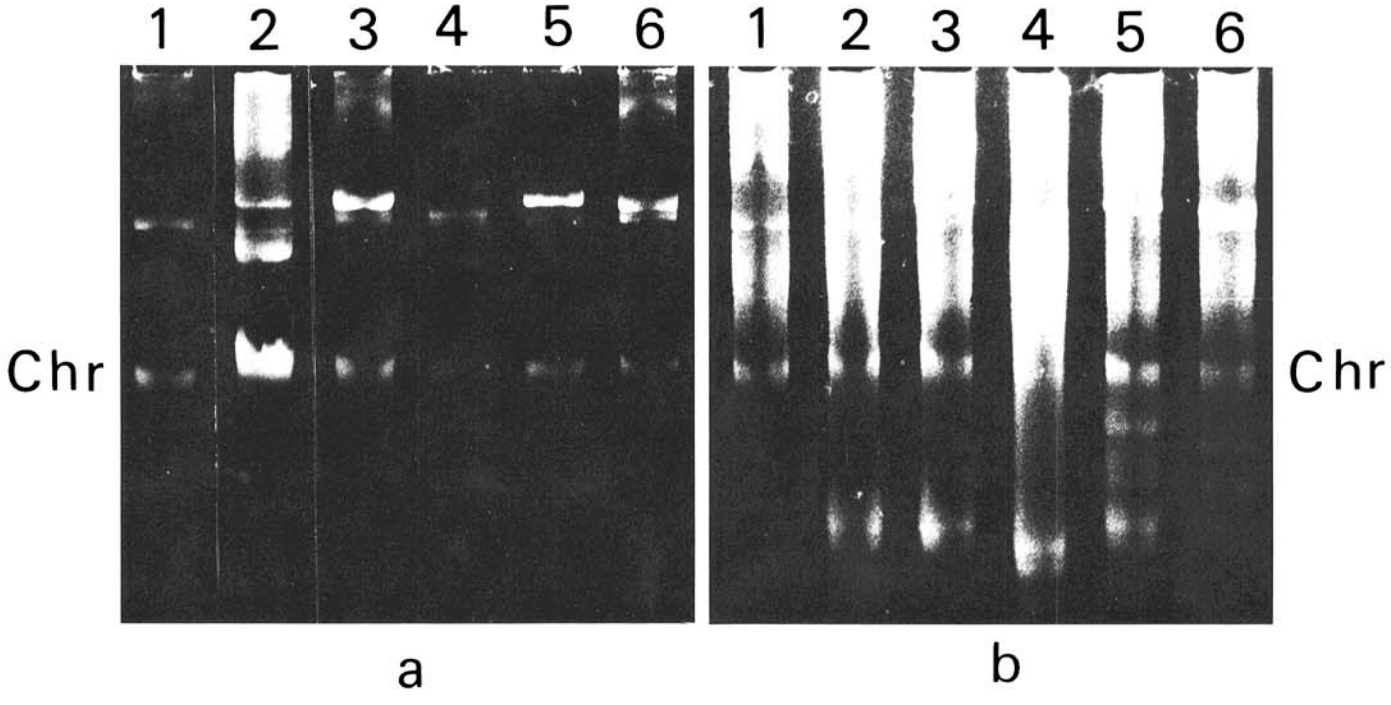

Fig. 1. Agarose gel electrophoresis of plasmid DNA from six isolates of *Pseudomonas syringae* pv. *glycinea*. Residual PEG was extracted **(a)** or allowed to remain **(b)** in the cell lysate prior to analysis. The location of the chromosomal fragments is marked (Chr). The tracks contain plasmids from strain: LR100 (lane 1), LR219 (lane 2), LR300 (lane 3), LR400 (lane 4), LR500 (lane 5), and LR600 (lane 6).

pMC7105 (98 Md). Plasmid sizes ranged from 2 to 110 Md (Table 1). The very high molecular weight material seen near the wells in Fig. 1a is thought to be either the open circular form of large-plasmid DNA or aggregated DNA molecules.

Restriction endonuclease analysis of plasmid DNA. Using the rationale that two plasmids having similar restriction endonuclease-generated fingerprints are related (26), we sought to compare the restriction patterns of the plasmids in the six strains. Total purified plasmid DNA from each strain was digested with *Eco*RI and the cleavage products were fractionated by agarose gel electrophoresis (Fig. 2a). Upon close examination, none of the plasmid preparations contained a single fragment that could be unequivocally identified in all other plasmid digests. However, similar patterns were apparent in select comparisons of plasmids from some strains. Strains LR300 and LR500 each have two large plasmids of identical size (46 and 61 Md) (Fig. 1a, lanes 3 and 5). The fingerprint analysis of total plasmid DNA from these strains revealed 26 bands with identical electrophoretic mobilities (Fig. 2a, lanes 3 and 5), with a combined molecular mass of approximately 105 Md. This value corresponds to greater than 80 and 90%, respectively, of the total plasmid sequences in LR500 and LR300. However, since PEG extraction selectively eliminates the small plasmids (3–7 Md) from these extracts (Fig. 1a, lanes 3 and 5), the 26 common bands would comprise 98% of the sequences of two identical plasmids having a combined mass of 107 Md. These results indicate that the two large plasmids in LR300 and LR500 are closely related even though the strains have diverse origins (5, and Table 1).

Although strains LR219 and LR400 contain multiple-sized plasmids, each has a plasmid similar in size to the 46-Md plasmids of LR300 and LR500 (Fig. 1a). If these plasmids share extensive homology, a digest of total plasmid DNA from these strains should produce a series of common fragments with a combined mass of 47 Md. Regions of homology among the other plasmids in these strains, or co-migration of similar-sized fragments that have no homology would increase the apparent extent of homology among these plasmids. The fingerprints of total plasmid DNA of LR219 and LR400 have 15 similar-sized fragments with a combined mass of approximately 53 Md. However, only six fragments of identical size were detected among the fingerprints of total plasmid DNA from strains LR219, LR300, LR400, and LR500. These results indicate that the restriction pattern of at least one of the 46-Md plasmids is substantially different from the others. The total mass of these six fragments is only 17 Md, well below the expected value, if each strain had an identical 46-Md plasmid. Finally, a comparison of the fingerprint patterns of plasmid DNA from strains LR100 and LR600 (Fig. 2a, lanes 1 and 6) revealed no extensive homology even though each contains a similar-sized (~42 Md) plasmid (Fig. 1a and Table 2).

The *Eco*RI (Fig. 2a, lane 1) and *Bam*HI (not shown) restriction patterns of LR100 plasmid DNA always contained a prominent and weak set of fingerprints. Combined molecular masses of 41.5 and 42.7 Md were computed for the intense *Eco*RI and the *Bam*HI bands, respectively. Those bands are presumed to be derived from the digestion of pMC11, the 42-Md plasmid that is readily extracted by using this procedure. The faint set of bands was determined to be due to digestion of a second plasmid, pMC10 (110 Md), which is seen as a faint band above pMC11 in the extracts of total DNA (Fig. 1a, lane 1).

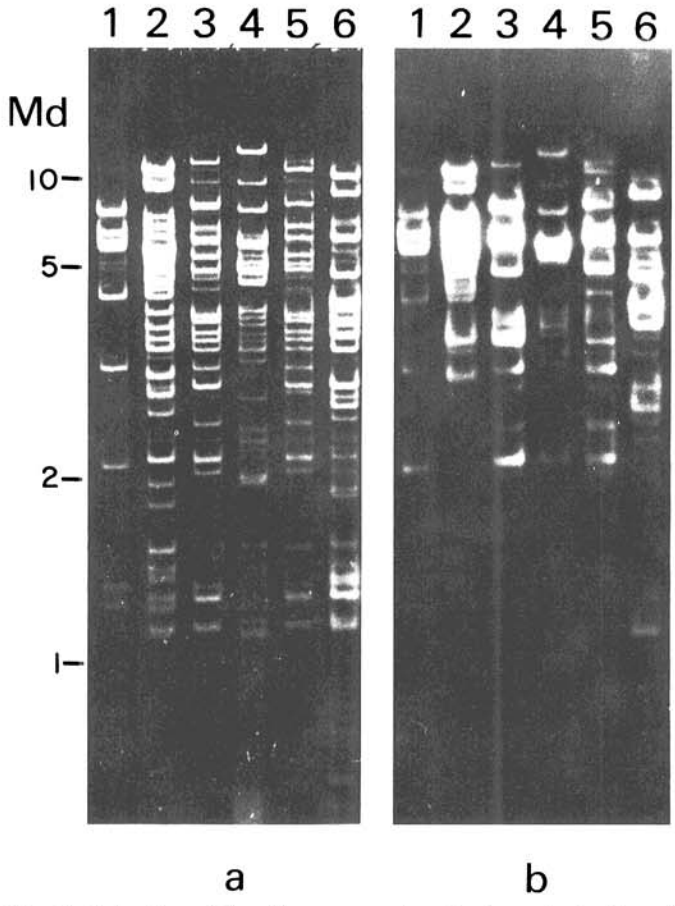

Fig. 2. Molecular relationships among plasmids from six isolates of *Pseudomonas syringae* pv. *glycinea*. **a,** The *Eco*RI-restriction endonuclease fingerprints of total plasmid DNA. **b,** Hybridization of ^{32}P-labeled plasmid DNA from strain LR100 to nitrocellulose filters containing DNA fragments shown in (a). The tracks on the gel contain plasmid DNAs from strain: LR100 (lane 1), LR219 (lane 2), LR300 (lane 3), LR400 (lane 4), LR500 (lane 5), and LR600 (lane 6).

Plasmid relatedness detected by DNA:DNA hybridization. Molecular relatedness among the plasmid fragments of the six isolates was also revealed by DNA:DNA hybridization. The *Eco*RI restriction patterns shown in Fig. 2a were transferred to nitrocellulose paper and allowed to hybridize with nick-translated total plasmid DNA from LR100 (Fig. 2b). Fifty-five to 75% of the plasmid bands from each strain showed some homology with the LR100 plasmid probe, whereas in a control experiment (*unpublished*), hybridization was not observed between the probe and bacteriophage lambda fragments. These results are summarized in Table 2.

In reciprocal hybridization experiments, filter-bound *Eco*RI fragments of pMC10 and pMC11 plasmid DNA from strain LR100 were probed with ^{32}P-labeled total plasmid DNA from LR219, LR300, LR500, and LR600. Each of these probes exhibited a different amount of homology with pMC10 and pMC11. Three fragments (5.7, 6.3, and 7.4 Md) from pMC11 and a 1.7-Md fragment from pMC10 hybridized with labeled plasmid probe from each strain (Table 3). The 42-Md plasmid, pMC11, appears to share extensive homology with plasmid sequences from strains LR219 and LR500. All of pMC11 fragments hybridized to labeled-plasmid probe from LR500, and all except one hybridized to the

TABLE 2. Number of bands from *Eco*RI-digested total plasmid DNA of *Pseudomonas syringae* pv. *glycinea* (Psg) that show some homology with pMC10 and pMC11 probe from strain LR100

	Psg strain and number of plasmids[a]				
Band characteristics	LR219 4	LR30 3	LR400 4	LR500 7	LR600 4
Number of bands detected by fluorescence[b]	33	31	29	29	28
Number of bands with some homology[c]	20	17	16	18	21

[a] The PEG was extracted from the lysates which selectively removes the smaller (3–7 megadaltons [Md]) plasmids present in these strains (Table 1).
[b] Some bands may contain more than one fragment.
[c] Hybridization of probe to fragments smaller than 1.2 Md was irreproducible and not included in this table.

probe derived from LR219. Strains LR219 and LR500 each contain plasmids with masses similar to pMC11, but the fingerprints of total plasmid DNA from these strains suggested that these plasmids were not closely related to pMC11 (compare lanes 1, 2 and 5 in Fig. 2a). In contrast, plasmid probe from LR600 and LR300 hybridized, respectively, to only four and five of the nine fragments of pMC11 (Table 3). These results indicate that pMC11 (42 Md) and a plasmid of similar size in LR600 are not homologous plasmids. Each of the plasmid probes also shows some homology with various fragments from pMC10, the 110-Md plasmid in LR100.

DISCUSSION

In a previous study (3), four strains 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 radio-isotopically labeled in vivo and purified by density gradient centrifugation. In work presented here, the plasmid purification scheme of Hansen and Olsen (11) and the agarose gel system of Meyers et al (17) revealed numerous additional plasmids in these strains. Although these procedures have been effective for isolating large plasmids, at least one small plasmid (2–7 Md) was also detected in five of the strains when the extraction of PEG was omitted from the procedure (Fig. 1).

Site-specific endonuclease analyses have been previously used to measure relatedness of plasmids in strains of several pathovars of *P. syringae*. In the *P. syringae* pv. *syringae*, three similar-sized plasmids were shown to have very different fingerprints and, therefore, not homologous plasmids (9). In contrast, two plasmids of approximately 30 and 70 Md in the *P. syringae* pv. *phaseolicola* have extensive homology with a large (98 Md) integrative plasmid, pMC7105 (21). In another analysis, single plasmids identified in two strains of the *P. syringae* pv. *tabaci* were shown to be identical, whereas a third plasmid of similar size from another strain showed little homology (20). This latter plasmid, however, was closely related to plasmids detected in three strains of the *P. syringae* pv. *angulata*. In the present analysis, the fingerprints of plasmids in some strains were essentially identical. The 46- and 61-Md plasmids of LR300 and LR500 have nearly identical restriction patterns, but similar-sized plasmids in other strains have different fingerprints.

TABLE 3. Hybridization of total ^{32}P-plasmid DNA from four strains of *Pseudomonas syringae* pv. *glycinea* to *Eco*RI fragments from pMC10 and pMC11

Plasmid	Fragment size Md[a]	Source of labeled plasmid probe and extent of hybridization to pMC10 and pMC11[b]			
		LR219	LR300	LR500	LR600
pMC10	10.4	−	−	+	−
	5.3	+	−	+	−
	5.0	++	−	−	−
	2.9	+	+	−	−
	2.4	+	−	+	−
	1.7	+	++	+	++
pMC11	7.4	++	+	+++	+
	6.3	+++	+++	+++	+++
	5.9	++	−	+++	−
	5.7	+++	+++	+++	+++
	4.3	+	−	+	−
	3.2	+	+	+++	−
	2.2	−	+	++	++
	1.4	+	−	+	−
	1.3	+	−	+	−

[a] Restriction fragments which failed to hybridize to at least one of the four DNA probes were not included in the table. MD = megadaltons.

[b] An empirical measure of the autoradiographic spot density as a measure of the extent of hybridization: −, no hybridization; +, ++, and +++ indicate increasing degrees of density. Hybridization of plasmid probe to fragments smaller than 1.3 megadaltons was irreproducible and the data were not included in the table.

Consequently, no single plasmid was common to all strains and, indeed, nor was even a single *Eco*RI restriction fragment. The results of restriction analysis alone suggested that certain similar-sized plasmids should have extensive sequence homology, whereas others would not. This type of analysis will not reveal whether a plasmid such as pMC10, which is very different from any of the other plasmids detected in this study (Table 1), has homology with any other plasmid.

It is known from previous studies that plasmids with dissimilar restriction fingerprints may share extensive sequence homology. The degradative plasmids of *P. putida* have homologous sequences which are not apparent by fingerprint analysis (12). Likewise, the oncogenic plasmids of *Agrobacterium tumefaciens* (25) and *A. rhizogenes* (28) exhibit very different fingerprints, but share regions of homology. We have determined that the plasmids of *P. syringae* pv. *glycinea*, which differ both in size and fingerprint pattern, nevertheless have sequence homology when analyzed by DNA:DNA hybridization. For example, pMC10 in strain LR100 is substantially larger than all other plasmids in these strains, but it has a 1.7-Md fragment which has homology with plasmid sequences in five other strains. Furthermore, pMC11, which resides with pMC10, has three fragments that also show homology with plasmid sequences in the other strains. The combined mass of these four (19.4 Md) represents the maximum degree of homology among all of the plasmids. However, since the size of probe is variable and the extent of homology on each fragment is unknown, the precise amount of homology among these plasmids cannot be determined from Southern blot hybridization.

Incompatability is commonly used as a measure of the degree of relatedness between natural indigenous plasmids (4). Although the converse does not always follow, it is feasible that some of the molecular homology we detected among these plasmids defines a particular incompatibility group. This could be determined as soon as selectable genetic markers become available. Homology between diverse plasmids may also result from the occurrence of common repetitive sequences. Finally, some homologous sequences may encode products essential either for growth or pathogenicity.

Further investigation is required to determine a biological role for these plasmids. This task is complicated by the presence of multiple plasmid species in each of these strains. However, if the homology detected between the *Eco*RI restriction fragments from pMC10 and pMC11 and sequences on other plasmids in these strains (Table 3) is not repetitive sequence homology, they may provide the most promising genetic material for these investigations. Indeed, molecular homology has been successfully used to identify the nitrogen fixation genes in *Rhizobium meliloti* by probing the genomic library of *R. meliloti* with the nitrogenase structural genes of *Klebsiella pneumoniae* (2).

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