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Detection and Characterization of Plasmids in *Pseudomonas glycinea*

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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-

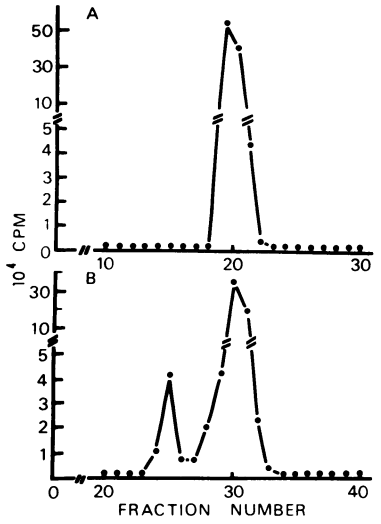

FIG. 1. *Equilibrium centrifugation of P. glycinea LR229 DNA in CsCl gradients in the presence and absence of ethidium bromide. Cells of LR229 were grown for 6 h in the presence of 5 μCi of [^{3}H]adenine (1.5 Ci/mmol) per ml. Lysates were centrifuged in CsCl gradients, and fractions were collected and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

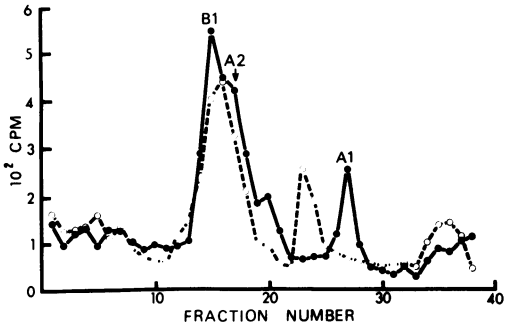

FIG. 2. *Neutral sucrose gradient analyses of E. coli plasmid F (○) and P. glycinea LR229 plasmid DNA (●). The CCC and OC forms of F plasmid peak at fractions 23 and 16, respectively. The CCC and OC forms of one plasmid are labeled A1 and A2, respectively. B1 is the CCC form of a second plasmid. Centrifugation 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).*

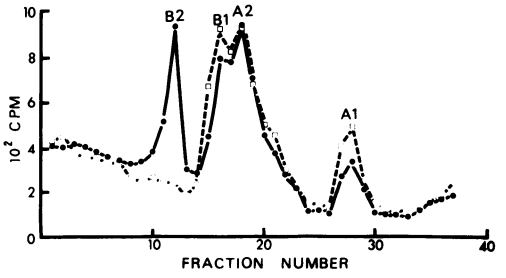

FIG. 3. *Superimposed neutral sucrose gradient analyses of LR229 plasmid DNA before (□) and after (●) treatment with DNase. Peaks labeled A1, A2, and B1 are as described in Fig. 2. The OC form of plasmid B1 is labeled B2. The gradients were centrifuged for 18 h at 11,500 rpm (13,000 × g).*

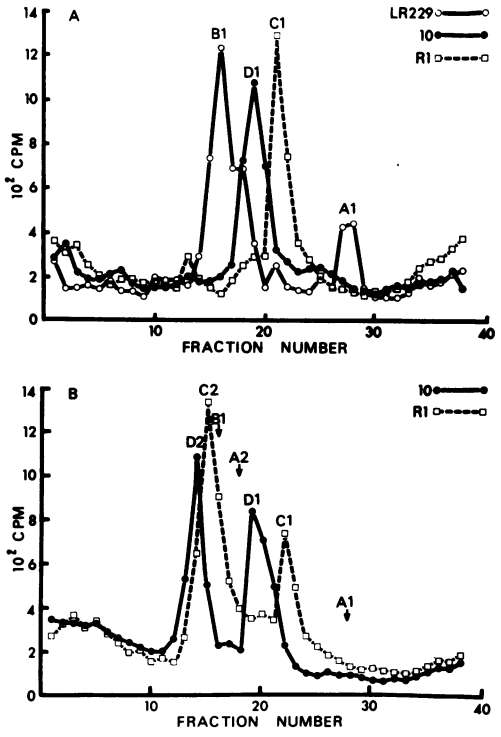

FIG. 4. *Analyses of plasmid DNA from isolates R1 and 10 by neutral sucrose gradient centrifugation. ^{3}H-labeled plasmid DNA from isolates R1 and 10 was centrifuged in the presence of ^{14}C-labeled plasmid DNA of LR229, which served as a reference marker. The ^{3}H-labeled DNA was characterized at 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

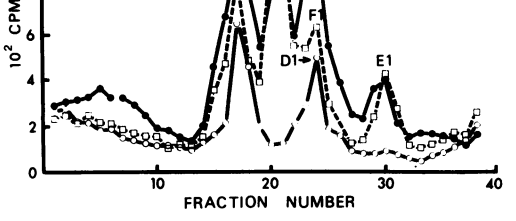

FIG. 5. *Comparison of isolates 10 and R2 plasmid DNA by sucrose gradient centrifugation. Plasmid DNAs of isolate 10 (○) and isolate R2 (□) and a mixture of the two (●) were run on sucrose gradients, and the results are plotted on a single graph. Designation of plasmid DNA from isolate 10 is as described 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

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