Purification and Characterization of Cryptic Plasmids pLS1 and pLS2 from *Erwinia chrysanthemi*

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ABSTRACT

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Two cryptic plasmids were purified from cell lysates of *Erwinia chrysanthemi* NCPPB-377, an organism isolated from maize (*Zea mays* L.) with bacterial stalk rot. Electron microscopy of purified, supercoiled plasmid DNA revealed a large (pLS1, 50.4 ± 1.9 megadaltons) and a small (pLS2, 4.8 ± 0.3 megadaltons) plasmid. These plasmids were separated by centrifugation on linear-log sucrose gradients. Plasmids pLS1 and pLS2 were characterized by restriction endonucleases *Eco*RI, *Pst*I, *Sal*I, *Hind*III, and *Hin*cII.

Erwinia chrysanthemi (Burkholder, McFadden, and Dimock) causes soft-rot of diverse dicotyledonous and monocotyledonous plants (3). Although it belongs to the carotovora group of pectolytic erwinias, many of its strains exhibit an unusual degree of host specificity (3,13). Strains of *E. chrysanthemi* isolated from stalk rot-diseased maize (*Zea mays* L.) have an increased tendency to cause pathogenesis on maize compared to strains isolated from diseased, nonmaize hosts (9). The nature and genetic control mechanism of this specificity is unknown.

A survey of maize strains of *E. chrysanthemi* was undertaken to learn whether naturally occurring plasmids exist in them, and whether or not these plasmids may contribute to the pathogenic phenotype of their host. Extrachromosomal DNA of diverse molecular sizes was detected in a number of maize strains of *E. chrysanthemi* of different geographic origins (G. H. Lacy and R. B. Sparks, Jr., *unpublished*). This paper describes in greater detail two plasmids occurring in maize strain NCPPB-377.

MATERIALS AND METHODS

Organism. *E. chrysanthemi* strain NCPPG-377 (National Collection of Plant Pathogenic Bacteria, Harpenden, England) was isolated in Rhodesia from bacterial stalk rot of maize. The subculture of NCPPB-377, designated SR-145, which was used in this study was obtained from A. Kelman, department of Plant Pathology, University of Wisconsin, Madison, 53706.

Culture conditions and media. Strain NCPPB-377 was maintained on triphenyltetrazolium chloride agar (TZC) at 30 C (8). Plasmid DNA was purified from cells in the late exponential phase of growth in Luria broth (LB) modified by the exclusion of glucose (12). Flasks (125-ml) containing 10 ml of LB were inoculated from 48-hr TZC cultures and incubated with reciprocal shaking (140 rpm) for 6–8 hr. Fresh LB (1.5 L) was inoculated with 1.5 ml of the 48-hr culture and the incubation was continued at 30 C on a rotary shaker (100 rpm) for 14 hr.

Isolation of plasmid DNA. Two procedures were used most extensively to isolate supercoiled, closed, circular (CCC) DNA from NCPPG-377: The first method was essentially that of Guerry, et al (5). Cells grown in 1.5 L of LB were harvested by centrifugation for 10 min at 4,000 *g*. The pellet was resuspended in 50 ml of 25% sucrose in 0.05 M Tris (hydroxymethyl) aminomethane, pH 8, and 20 ml of 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8, was added. Sodium dodecylsulfate (SDS, 10% aqueous) was added to the cell suspension to a final concentration of 1.0%. The suspension was incubated at 50 C until the suspension became transparent (about 30 min). After cellular lysis was complete, 5 M NaCl was added with gentle mixing to a final concentration of 1 M. The lysates were stored at 4 C overnight and centrifuged for 20 min at 27,000 *g* (to pellet chromosomal DNA). The supernatant, which contained the plasmid DNA, was diluted with an equal volume of TEN buffer (0.05 M Tris, 0.005 M EDTA, and 0.05 M NaCl, pH 8). Solid CsCl (21.96 g) was gently dissolved in 22.68 g of the supernatant-TEN to give a density of about 1.55 g/cm^3. Aqueous ethidium bromide (5 mg/ml) was added to a concentration of 300 μg/ml. Density gradients were formed by centrifugation at 40,000 rpm in a Beckman 60 Ti rotor (Beckman Instruments, Inc., Fullerton, CA 92634) for 60 to 75 hr. Bands of supercoiled plasmid DNA were viewed with ultraviolet light and collected through the side of the centrifuge tubes with a 1.65-mm-diameter (16-gauge) syringe needle. Ethidium bromide was removed by partitioning against isopropanol saturated with 1.55 g/cm^2 CsCl in TEN. Dialysis against TEN was used to remove the CsCl. In the second method, cells were grown in 250 ml of LB, harvested by centrifugation, and resuspended in sucrose-Tris and EDTA in the same proportions described above. Lysozyme (10 mg/ml in 0.25 M Tris, pH 8) was added to a concentration of 1.4 mg/ml and the suspension was incubated at 0 C for 20 min. Sarkosyl (ICN, Plainview, NY 11803) was added to 0.33% and the cells were incubated at 50 C until lysis was complete. The lysate was diluted with TEN to a weight of 22.68 g, mixed with solid CsCl, and centrifuged as described above.

Purified plasmid DNA was precipitated by adding 0.1 volume of 3 M sodium acetate, pH 6.0, 20 μg/ml yeast transfer RNA (R-9001 Sigma Chemical Co., Saint Louis, MO 63178) as carrier, and at least two volumes of cold (−20 C) 95% ethanol. The precipitated DNA was pelleted by centrifugation at 10,444 *g* at −10 C for 30 min in a Sorvall HB4 rotor (Ivan Sorvall, Inc., Newtown, CT 06470).

Electron microscopy of plasmid DNA. Preciptitated DNA was air-dried and redissolved in TEN. Ethidium bromide (200 μg/ml final concentration) was added and the solution was exposed for 30 min at 0.5 cm to a Sylvania F4Tf/D fluorescent lamp (Sylvania, Inc., Danvers, MA 01923) to nick the CCC molecules.

The spreading, staining, and rotary shadowing technique was the same as previously described (2). Plasmid DNA molecules were examined with a Zeiss EM9 S-2 electron microscope (Carl Zeiss, Inc., New York, NY 10018).

Separation of plasmid populations. Rate-zonal centrifugation in linear-log sucrose gradients was used to separate plasmid populations. The linear-log gradient developed by Brakke and Van Pelt (1) was modified by the use of TEN buffer and the addition of 10 μg/ml ethidium bromide. Up to 0.75 ml of DNA solution in TEN was layered on the gradients and centrifuged in a Beckman SW 27.1 rotor for 16 to 18 hr at 23,000 rpm. The location of DNA bands was visualized in ultraviolet light and they were removed from the tubes by side puncture with 1.65-mm-diameter

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needles. Ethidium bromide and sucrose were removed by dialysis in TEN and the separated plasmids were precipitated in ethanol.

Restriction of plasmid DNA. Plasmid DNA was redissolved at 4 C in a minimal volume of common restriction buffer (CRB) which consisted of 6 mM Tris (pH 7.5), 50 mM NaCl, and 1 mM EDTA (pH 8.0). Plasmid DNA was incubated with restriction endonucleases (New England Biolabs, Beverly, MA 01915) at 37 C for 90 min under the following conditions: *Eco*RI, 100 mM Tris pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$, 100 μg/ml gelatin; *Sal*I, 6 mM Tris pH 7.6, 100 mM NaCl, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol; *Pst*I, 6 mM Tris pH 7.6, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, 100 μg/ml gelatin; *Hinc*II, 10 mM Tris pH 7.6, 60 mM NaCl, 7 mM $MgCl_2$, 6 mM β-mercaptoethanol; *Hind*III, 7 mM Tris pH 7.6, 60 mM NaCl, 7 mM $MgCl_2$. The reactions were terminated by heating at 65 C for 5 min. The restricted DNA was electrophoresed as described previously (11) through 1% Agarose (Sea Kem) on a vertical slab gel apparatus.

RESULTS AND DISCUSSION

Supercoiled plasmid DNA was purified from *E. chrysanthemi* NCPPB-377 by the techniques described in Materials and Methods. In addition to cell lysis by the SDS and sarkosyl methods, we were able to purify supercoiled plasmid DNA after lysis with 0.2% Triton X-100, followed by a clearing centrifugation (7). Each of the lysis procedures yielded qualitatively similar DNA molecules.

Electron microscopy of purified and ethidium-bromide-nicked plasmid DNA revealed two size classes of circular molecules (Fig. 1). Measurement of these molecules in relation to open-circular plasmid DNA (Col E1, 4.2 megadaltons), which was added to the sample before electron microscopy, gave a distribution as shown in Fig. 2. We have designated the large plasmid pLS1 and the small one pLS2. The average molecular weights determined by contour length were 50.4 ± 1.9 and 4.8 ± 0.3 megadaltons. To separate pLS1 from pLS2, modified linear-log sucrose gradients were used. Ethidium-bromide was added to the gradients to allow visualization of the fluorescent DNA bands. The gradients, in addition to separating pLS1 from pLS2, also resolved the supercoiled and open-circular components of each plasmid. (Table 1). It appeared, based on the intensity of fluorescent bands, that pLS2 DNA was present in greater amounts than pLS1 DNA. This may reflect a difference in copy number or recovery. Each purified plasmid was treated with restriction endonucleases *Eco*RI, *Pst*I, *Sal*I, *Hind*III, and *Hinc*II. Agarose gel electrophoresis of the restricted plasmids showed that plasmid pLS1 was restricted by *Eco*RI (11 sites), *Pst*I (about 24 sites), and *Hinc*II (about 28 sites) (Fig. 3). Plasmid pLS2 was restricted by *Pst*I (two sites) and *Hinc*II (11–15 sites, but not by *Eco*RI, *Sal*I, or *Hind*III (Fig. 4). The size of each *Eco*RI restriction fragment of pLS1 was determined in relation to known λ DNA restriction fragments (Fig. 5). The sum of these fragment sizes was 51.9 md. This value was within the standard deviation of the molecular weight determined by electron microscopy.

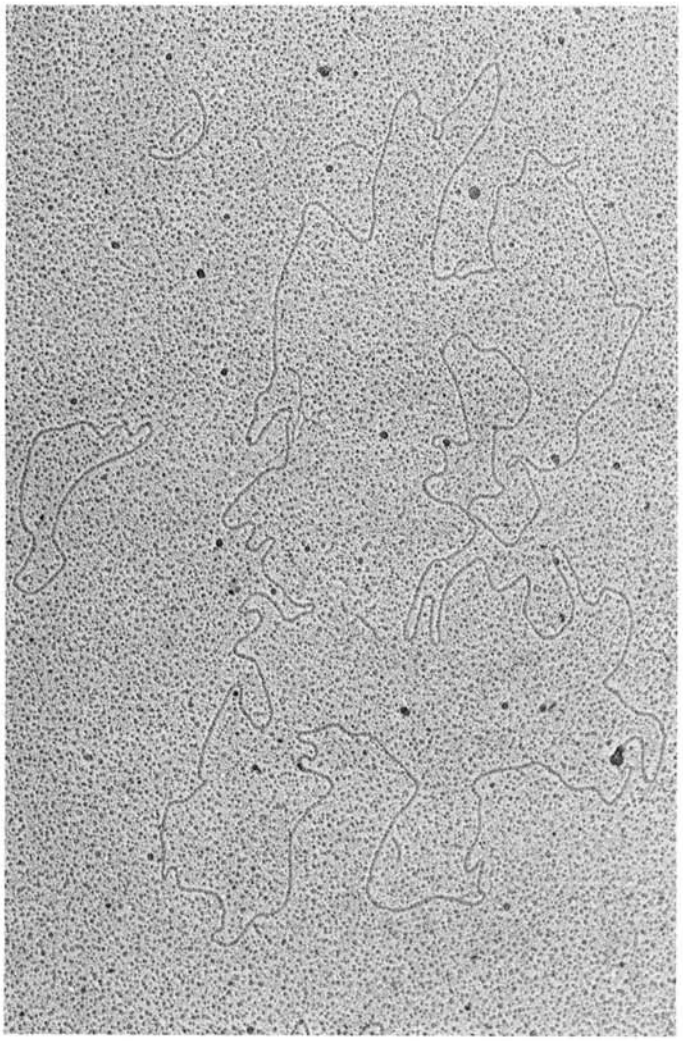

Fig. 1. Electron micrograph of circular DNA molecules recovered from plasmids of *Erwinia chrysanthemi* NCPP-377. Contour measurements (compared to ColE1, plasmid DNA [4.2 ± 0.05 megadaltons {md}] as a size standard) indicated that the larger molecule was representative of the plasmid pLS1 population (50.4 ± 1.9 md) and the smaller molecule was representative of the pLS2 population (4.8 ± 0.3 md).

TABLE 1. Relative sedimentation[a] of plasmid DNA in linear-log sucrose gradients

DNA forms	Relative sedimentation (R_f values) of DNA from:			
	λ phage	ColE1	pLS1	pLS2
Linear	0.41	0.23	0.50	0.32
Open-circular	0.50	0.27	0.58	0.37
Suspercoiled	···	0.35	0.85	0.40
Multimeric	0.58	···	···	···

[a]Gradients contained 10 μg/ml ethidium bromide and were centrifuged in a Beckman SW 27.1 rotor for 16 hr at 23,000 rpm and 7 C.

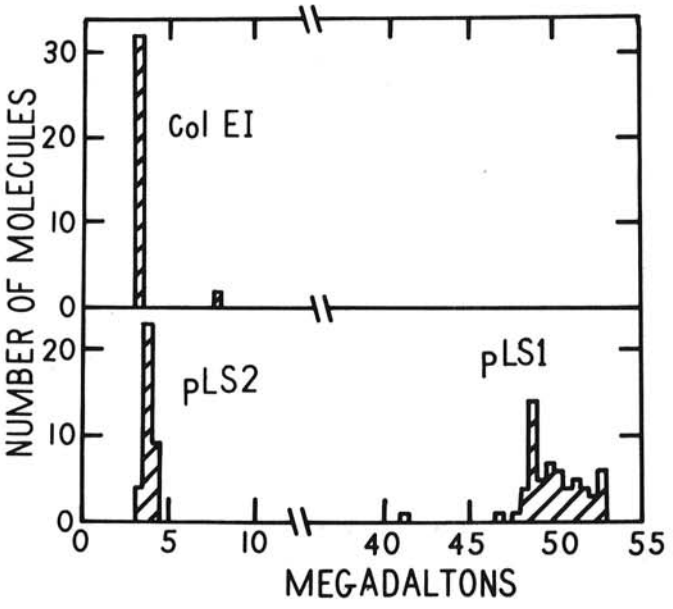

Fig. 2. Frequency distribution of molecular contour measurements of electron micrographs of pLS1 (50.4 ± 1.9 megadaltons [md]) and pLS2 (4.8 ± 0.3 md) relative to marker ColE1 plasmid DNA (4.2 ± 0.05 md) observed on the same grid.

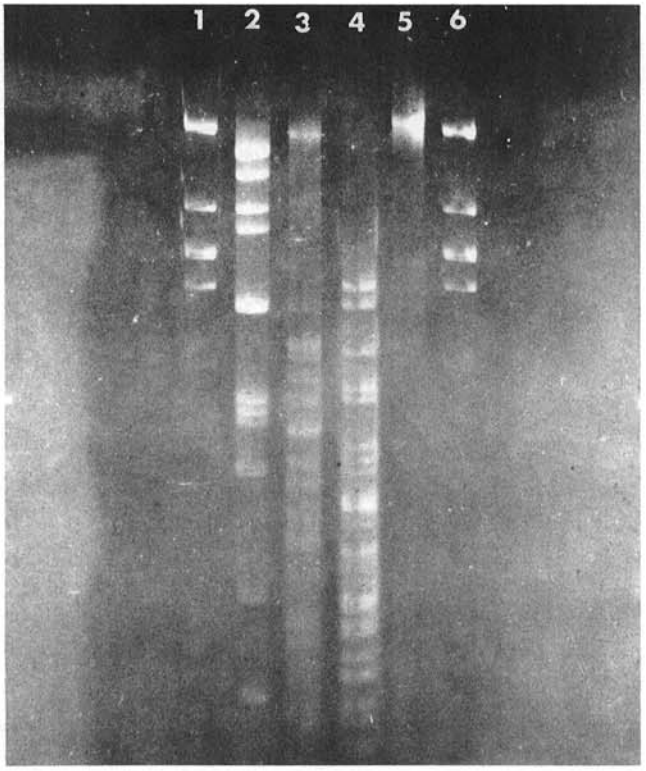

Fig. 3. Electrophoretic resolution of restriction endonuclease fragments of pLS1 DNA on 1.0% agarose in Tris-borate buffer. *Eco*RI, *Pst*I, and *Hind*III digests appear in columns two through four. Unrestricted, open-circular, pLS1 DNA appears in column five. *Hind*III-digested λ DNA appears in columns one and six.

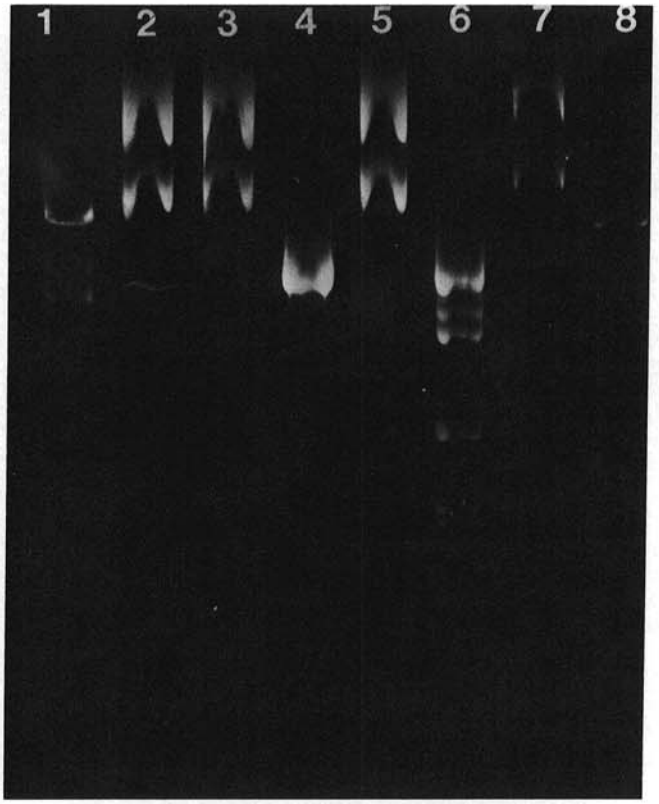

Fig. 4. Electrophoretic resolution of restriction endonuclease fragments of pLS2 DNA resolved by electrophoresis on 1.0% agarose in Tris-borate buffer. λ phage fragments generated by *Eco*RI cleavage appear in columns one and eight. Unrestricted pLS1 DNA appears in column seven. Fragments generated by *Pst*I and *Hinc*II are shown in columns four and six, respectively. *Eco*RI, *Sal*I, and *Hind*III digests of pLS1 DNA, appear in columns two, three, and five, respectively.

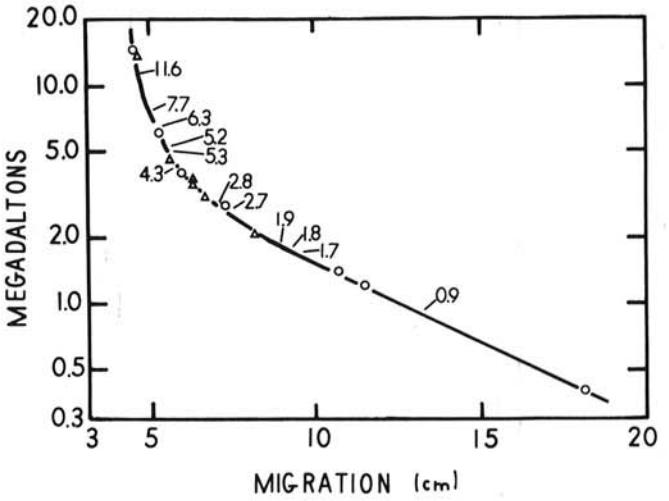

Fig. 5. Distribution and approximate molecular weight (megadaltons [md]) of *Eco*RI-generated fragments of pLS1 DNA among fragments of λ phage DNA digested by *Eco*RI (Δ) and *Hind*III (o). The sum of the fragments of pLS1 was 51.9 megadaltons.

Plasmid pLS2 was determined by identical procedures to produce 3.8 and 1.0 md fragments after restriction with *Pst*I. Again, the sum of the fragments (4.8 md) correlated well with the measurements obtained by contour measurement (4.8 ± 0.3 md) and electrophoresis of linear forms of the plasmid (4.8 md).

At present, phenotypic traits for either plasmid have not been identified. However, strain NCPPB-377 exhibits a bacteriocinlike activity against several strains of *E. chrysanthemi* and *E. carotovora* (G. H. Lacy and R. B Sparks, Jr., *unpublished*). Experiments are in progress to correlate this activity with one or both plasmids.

Examination of other *E. chrysanthemi* strains that are pathogens on maize also has revealed the presence of plasmid DNA (G. H. Lacy and R. B. Sparks, Jr., *unpublished*). Comparisons of pLS1 and pLS2 with these plasmids are in progress. Among other bacterial plant pathogens reported to have plasmid DNA are *Agrobacterium tumefaciens* (14) and *Pseudomonas syringae* (4). The plasmids of these pathogens have been shown to have a direct role in the pathogenicity. Also, plasmids found in *Rhizobium* spp. may be involved in host specificity (6). A review of plasmid DNA molecules found in phytopathogenic bacteria and their role in pathogenesis has been published (10). What roles, if any, plasmids pLS1 and pLS2 have in pathogenicity or host specificity of *E. chrysanthemi* on maize awaits definition by the results of further experiments.

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