Transformation of *Erwinia herbicola* with Plasmid pBR322 Deoxyribonucleic Acid

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ABSTRACT

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Erwinia herbicola was transformed by a $CaCl_2$ technique with plasmid pBR322 deoxyribonucleic acid (DNA) at frequencies up to 1×10^{-6} transformants per recipient cell (TPR). Since pBR322 carries resistance to ampicillin (Ap^r) and tetracycline (Tc^r), transformants were selected on media containing either one or both of those antibiotics. Covalently closed circular (CCC) plasmid DNA was isolated from lysates of *E. herbicola* (pBR322) transformants by equilibrium centrifugation in cesium chloride-ethidium bromide gradients. The CCC DNA of *E. herbicola* (pBR322) was found by electron microscopy and gel electrophoresis to contain a DNA species the same size as that of pBR322. This plasmid had the same restriction pattern as pBR322. Transformation of *Escherichica coli* with CCC DNA from *E. herbicola*/ pBR322 yielded Ap^r Tc^r transformants at frequencies as high as 1×10^{-4} TPR. The CCC DNA isolated from all *E. coli* transformants tested had electrophoresis patterns identical to those of pBR322. Transformation of pBR322, a recombinant DNA cloning vehicle, into *E. herbicola* indicates that development of a gene cloning system in this species may be possible.

The genus *Erwinia* includes species pathogenic to plants and possibly animals (34). Recently, the genetics of this genus have been explored by several authors through genetic exchange mediated by introduced fertility plasmids (1,3,6-8,10,12,14,15,17, 21,22,25,29,30,35). There is also genetic or physical evidence for the presence of indigenous plasmids in strains of *Erwinia amylovora* (Burrill) Winslow et al. (29), *E. carotovora* var. *carotovora* Dye (18), *E. chrysanthemi* Burkholder, McFadden and Dimock (33), *E. herbicola* (Löhnis) Dye (5,9) and *E. stewartii* (Smith) Dye (16). Many of these plasmids are cryptic and phenotypic traits have not been associated unequivocably with them. Therefore, a system for transfer of specific plasmids from one genetic background to another *Erwinia* species would be advantageous. Furthermore, transfer of cryptic plasmids between members of the genus *Erwinia* may shed light on mechanisms of host specificity and pathogenicity. Methods of plasmid transfer could be by conjugation mediated by a conjugal helper plasmid (11,16,29), by transformation with purified plasmid DNA, or by transduction. However, transformation and transduction have not been confirmed among members of the genus *Erwinia*.

This paper describes the transformation of *E. herbicola* by purified plasmid DNA. *Erwinia herbicola,* is a common epiphyte that is occasionally isolated as a secondary nonpathogen in lesions caused by plant pathogens (2,23,32) and, rarely, as a plant pathogen (20). However, *E. herbicola* causes crop losses because

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many strains act as ice nuclei and incite "warm" temperature frost damage (26). Some strains isolated from clinical sources also have been identified either as *E. herbicola* or *Enterobacter agglomerans* (Beijerinck) Ewing and Fife (34).

MATERIALS AND METHODS

Origin of plasmids and bacterial strains. *Erwinia herbicola* strain L-244 was isolated in Connecticut from the surface of pear leaves (*Pyrus communis* L. 'Bartlett'). Strain L-321 is a spontaneous mutant of L-244 resistant to nalidixic acid (100 μg/ml). Strain L-322 is a white, thiamine-requiring variant (Pig Thi^-) of L-321 selected by the method of Chatterjee and Gibbins (5).

Escherichia coli Castellani and Chalmers strain C600 $r_K^- m_K^-$ was received from Richard Firtel (University of California, San Diego). The auxotrophic lesion at *leu* was transduced to prototropy with phage P1 and a spontaneous mutant (C600 *nal*) resistant to nalidixic acid (50 μg/ml) was selected.

Purified plasmid pBR322 (4) DNA was received from Herbert Boyer (University of California, San Francisco).

Media. All incubations were carried out at 30 C. Bacterial strains were maintained on Plate Count Agar (Difco, Detroit, MI 48232) supplemented with 50 μg/ml nalidixic acid (PN). Transformants were isolated on either PN agar plus 30 μg/ml ampicillin (PNA) or PNA agar plus 10 μg/ml tetracycline HCl (PNAT). Recipient cells for transforming DNA were grown in H1 broth (31) modified by the addition of 2.5 mg/ml vitamin-free acid hydrolysate of casein (Difco) and 5 μg/ml thiamine HCl. Transformants, prior to CCC DNA isolation, were grown in Luria broth (27) without glucose (LB) supplemented with 5 μg/ml tetracycline HCl.

Preparation of competent cells. A modification of the method of Cohen, et al (13) was used to prepare competent cells. Cells were prepared by inoculation of 5 ml of modified H1 broth (H1M) with cells grown on PN. After 18 hr of incubation with shaking, the cells were suspended in 30 ml of fresh H1M at a density of 5×10^5 colony-forming units (cfu) per milliliter as determined turbidimetrically. Incubation was continued until the mid-exponential growth phase (10^7 cfu/ml). The cells were harvested by centrifugation at 4,600 *g* for 6 min and suspended in 10 ml of 10 mM NaCl. They were centrifuged a second time at 3,200 *g* for 5 min and suspended and held in 10 ml of 30 mM $CaCl_2$ for 60 min at 0 C. After a third centrifugation they were suspended in 0.8 ml of 30 mM $CaCl_2$ and stored at 4 C overnight.

Transformation. Plasmid pBR322 DNA, 1.6 μg DNA in 0.1 ml of 30 mM $CaCl_2$, was incubated 2 hr at 0 C with 0.2 ml of $CaCl_2$-treated cells. At the end of this period, the cells were heated in a water bath to 42 C for 60 sec. For expression of the plasmid phenotype (Ap^r Tc^r) in transformed recipient cells, the cell suspension was diluted with 3 ml of LB (without tetracycline HCl) and incubated 4 hr at 37 C. Decimal dilutions were spread on PN to estimate the total number of recipient cfu and on PNA and PNAT to estimate the cfu with transformant phenotypes. Controls with only DNA or only recipient cells were included in each experiment.

Isolation of plasmid DNA. Plasmid pBR322 DNA was prepared from *E. coli* C600*nal* transformants grown until the early stationary phase in LB plus 5 μg/ml tetracycline HCl. Chloramphenicol (200 μg/ml) was added and incubation was continued for 12 hr. Plasmid DNA was purified from cleared, Triton X-100 lysates (24) by two cycles of equilibrium centrifugation in cesium chloride-ethidium bromide gradients as described below.

Cells of putative pBR322 transformants were grown in the same manner as described above for *E. coli* (pBR322). They were harvested by centrifugation for 10 min at 4,000 *g*. The pellet was suspended in 8 ml of 25% sucrose in 50 mM tris-(hydroxymethyl)-aminomethane (Tris) and 25 ml of 500 mM disodium ethylenediaminetetraacetate (EDTA) (pH 8). Lysozyme (10 mg/ml in 250 mM Tris, pH 8) was added to a concentration of 1.2 mg/ml and the suspension was incubated at 0 C for 20 min. Sarkosyl (ICN, Plainview, NY 11803) was added to 0.33% (v/v) of the volume and the suspension was incubated at 50 C until it became transparent (about 30 min). After cellular lysis was complete, solid CsCl (21.96 g) was gently dissolved in 22.68 g of the lysate 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 38,000 rpm in a Beckman (Beckman Instruments, Fullerton, CA 92634) Ti 60 rotor for 60–72 hr. Bands of CCC plasmid DNA in the gradients were viewed with ultraviolet light and collected through the side of the centrifuge tubes with a 1.6-mm diameter (16-gauge) needle. Ethidium bromide was removed by partition into isopropanol saturated with CsCl. Dialysis against 50 mM Tris, 5 mM EDTA, and 5 mM NaCl, pH 8 (TEN buffer), removed CsCl and sarkosyl or Triton X-100.

Purified plasmid DNA was precipiated overnight by adding one-tenth volume of 3 M sodium acetate buffer (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 40 min in a Sorvall HB4 rotor (Newton, CT 06470).

Electron microscopy of plasmid DNA. Precipitated DNA was air dried and redissolved in TEN buffer (0.8 mg/ml). Ethidium bromide (200 μg/ml final concentration) was added and the solution was exposed 30 min at 0.5 cm beneath a fluorescent lamp (F4Tf/D Sylvania, Danvers, MA 01923) to nick the CCC molecules.

The spreading, staining, and rotary shadowing techniques were the same as previously described (19). Plasmid DNA molecules

TABLE 1. Frequencies of transformant phenotypes recovered from recipient populations of *Erwinia herbicola* and *Escherichia coli* treated with purified DNA of plasmid pBR322 (Ap^r Tc^r)[a]

		Transformation[b]/Experiment number/Selection medium[c]						
		1	2		3		4	
Recipient	± DNA	PNA	PNA	PNAT	PNA	PNAT	PNA	PNAT
E. coli C600 nal	+	1.3×10^{-4}[d]	5.1×10^{-5}	9.2×10^{-5}	...[e]	...	...	...
	−	$<5.6 \times 10^{-9}$	$<1.2 \times 10^{-8}$	$<1.2 \times 10^{-8}$	...	...	...	...
E. herbicola L-322	+	2.9×10^{-6}	4.2×10^{-7}	2.1×10^{-7}	...	1.4×10^{-8}	5.2×10^{-7}	1.9×10^{-7}
	−	$<2.8 \times 10^{-9}$	$<7.7 \times 10^{-8}$	$<7.7 \times 10^{-8}$	$<7.0 \times 10^{-9}$	$<7.0 \times 10^{-9}$	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$
	+[f]	...	...	...	...	...	2.5×10^{-6}	1.2×10^{-6}
L-321	+	...	...	...	4.5×10^{-6}	3.4×10^{-9}	1.4×10^{-5}	2.4×10^{-8}
	−	...	...	...	2.4×10^{-6}	$<2.1 \times 10^{-9}$	5.0×10^{-5}	$<5.6 \times 10^{-9}$

[a] Ap^r Tc^r = ampicillin resistance and tetracycline resistance.
[b] See text for details of the transformation procedure.
[c] PNA = plate count agar plus 50 μg/ml nalidixic acid and 30 μg/ml ampicillin; PNAT = PNA plus 10 μg/ml tetracycline HCl.
[d] Frequency = transformants cells per recipient cell.
[e] ... = not performed.
[f] L-322 treated with pBR322 DNA and a similar amount (determined by electrophoresis) of plasmid DNA derived from L-244.

were examined with a Zeiss EM9 S-2 electron microscope (Carl Zeiss, Inc., New York, NY 10018).

Hydrolysis of plasmid DNA with restriction endonucleases. Plasmid DNA was dissolved at 4 C in a minimal volume of 6 mM Tris (pH 7.5), 50 mM NaCl, and 1 mM EDTA (pH 8.0). Plasmid DNA was incubated with restriction endonucleases *Eco*RI, *Hae*II, or *Hinc*II (New England Biolabs, Beverly, MA 01915) at 37 C for 90 min under the following conditions: *Eco*RI, 100 mM Tris (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 100 μg/ml gelatin; *Hae*II, 6 mM Tris (pH 7.4), 6 mM β-mercaptoethanol, 100 μg/ml gelatin and *Hinc*II, 10 mM Tris (pH 7.6), 60 mM NaCl, 7 mM $MgCl_2$, 6 mM β-mercaptoethanol. The reactions were terminated by heating at 65 C for 5 min. The hydrolyzed DNA was frozen (−20 C) until it was electrophoresed as described previously (28).

RESULTS

Phenotypic characters of plasmid pBR322 (Ap^r Tc^r) were detected among recipient populations of *Erwinia herbicola* (Table 1). The frequencies of transformant phenotypes ranged from 1.4×10^{-8} to 1.6×10^{-6} transformants per recipient cell (TPR). For *E. herbicola* strain L-322 (Nal^r Thi^- Pig^-), the TPR frequencies were twofold higher when ampicillin rather than ampicillin plus tetracycline HCl was used for selection. However, in strain L-321 (Nal^r Thi^+ Pig^+) a significant increase of 5.8×10^2 to 1.3×10^3-fold more Ap^r Tc^s clones than Ap^r Tc^r clones were observed. Similar increases in Ap^r Tc^s clones also were observed in controls without transforming DNA. This indicates that some degree of ampicillin resistance is a characteristic of strain L-321 and is possibly associated with the phenotype Thi^+ Pig^+.

Putative transformants were purified on PNAT. In all cases, whether the primary isolation medium was PNA or PNAT, C600 *nal* and L-322 transformants grew on PNAT. However, 50 clones of L-321 treated with pBR322 DNA selected on PNA, were Tc^s, but all 50 clones selected on PNAT were also Tc^r. The spontaneous mutation rates of L-322 to resistance to 30 μg/ml ampicillin and 10 μg/ml tetracycline HCl were less than 2.8×10^{-8} and less than $1.0 \times$

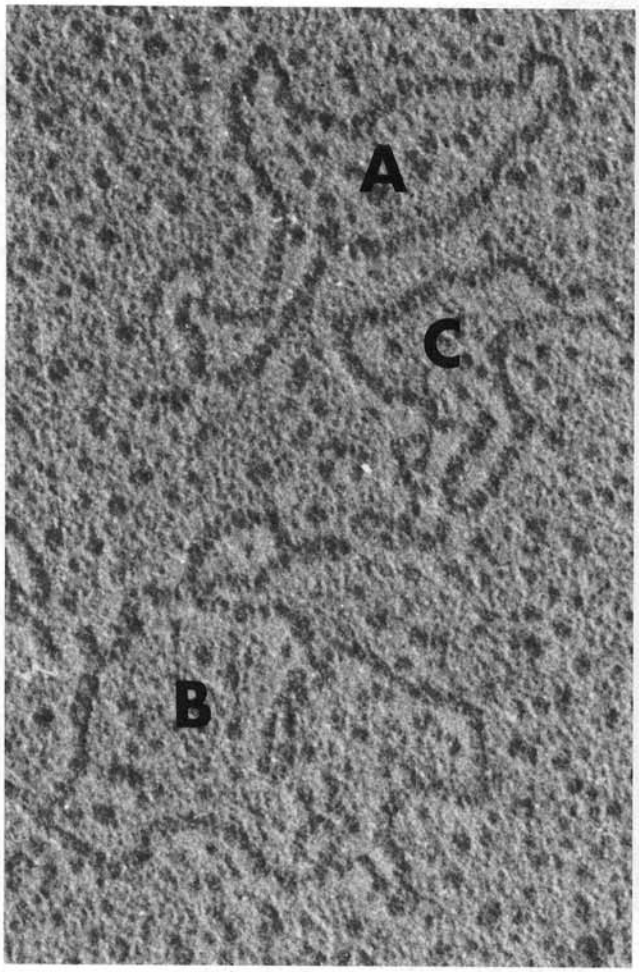

Fig. 1. Electron micrograph of plasmids (A) similar in contour length to pBR322 (2.6 Md) found in CCC DNA isolated from *E. herbicola* L-322 transformed with pBR322. Plasmid Col E1 (B;4.2 Md) was added just prior to spreading and shadowing to the DNA preparation as an internal standard. An indigenous L-322 plasmid (C) is also present.

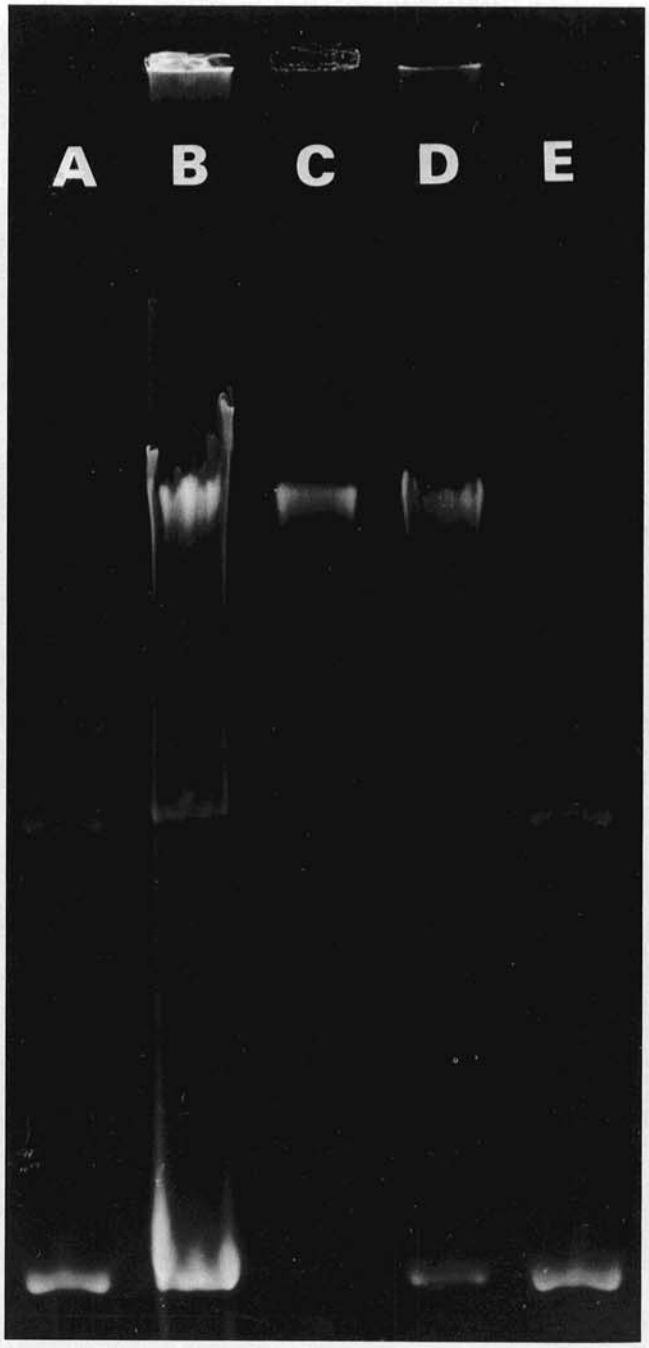

Fig. 2. Electrophoresis of purified supercoiled plasmid DNA from *Erwinia herbicola* L-322 (C) compared with plasmid DNA from two clones of the putative transformants L-322 (pBR322) (B and D) and authentic pBR322 DNA (A and E). Multiple pBR322 bands represent open circular, or supercoiled forms of unit length and multimeric forms of the plasmid. Restriction of pBR322 multimers with *Eco*RI produced only fragments of unit length. (Results not shown). The DNA was electrophoresed at 4.0 V/cm for 2.5 hr in 0.7% ME agarose prepared in 178 mM Tris, 5.5 mM EDTA, and 617 mM *ortho*-boric acid buffer (pH 8.0).

10^{-8}, respectively. Thus, the probability of recovery of a double mutant was less than 2.8×10^{-16} when primary selection was on PNAT.

Electron microscopy of open circular plasmid DNA from putative pBR322 transformants of L-322 revealed several size classes of molecules. Measurement of these molecules in relation to nicked Col E1 plasmid DNA (4.2 megadaltons [Md]), which was added to the sample just before electron microscopy, showed that a large plasmid of about 26.8 ± 0.6 (n = 26) Md predominated in both L-322 and L-322 (pBR322) as well as in L-244 and L-321. Several rare species of plasmid molecules also were observed. Two of these were 40.6 ± 11.1 (n = 6) and 6.8 ± 0.1 (n = 3) Md. However, only DNA from L-322 (pBR322) contained plasmids identical in contour length with pBR322 (2.6 Md) (Fig. 1). No plasmid species was clearly associated with the traits Pig^+ and Thi^+ in L-321.

Authentic pBR322 DNA was compared by electrophoresis with purified CCC DNA from L-322 and two clones of L-322 (pBR322) (Fig. 2). A dense band corresponding to the 26.8 Md plasmid present in L-322 was observed in both clones of L-322 (pBR322). In addition, both L-322 (pBR322) clones had two bands corresponding to the bands generated by authentic pBR322 DNA.

Plasmid DNA from L-322 (pBR322) was used to transform *E. coli* C600 *nal*. The frequency of recovery of the plasmid phenotype (Ap^r Tc^r) was 8.6×10^{-6} TPR on PNA and 3.0×10^{-6} TPR on PNAT. Two clones of putative transformants, C600 *nal*/L-322 (pBR322), were purified on PNAT.

Purified CCC DNA from the C600 *nal*/L-322 (pBR322) was prepared and compared by electrophoresis with authentic pBR322 DNA and plasmid DNA from L-322 (pBR322), C600 *nal* and L-322 (Fig. 3). Bands of DNA corresponding to pBR322 were observed in L-322 (pBR322) and C600 *nal*/L-322 (pBR322), but not in C600 *nal* or L-322. The large band corresponding to the 26.8 Md plasmid observed in L-322 and L-322 (pBR322) also appeared in DNA preparations from both C600 *nal*/L-322 (pBR322) clones examined.

Electrophoresis of plasmid DNA from L-322 (pBR322) following hydrolysis with *Hae*II, *Hinc*II, and *Eco*RI was compared with authentic pBR322 restricted with the same enzymes (Fig. 4).

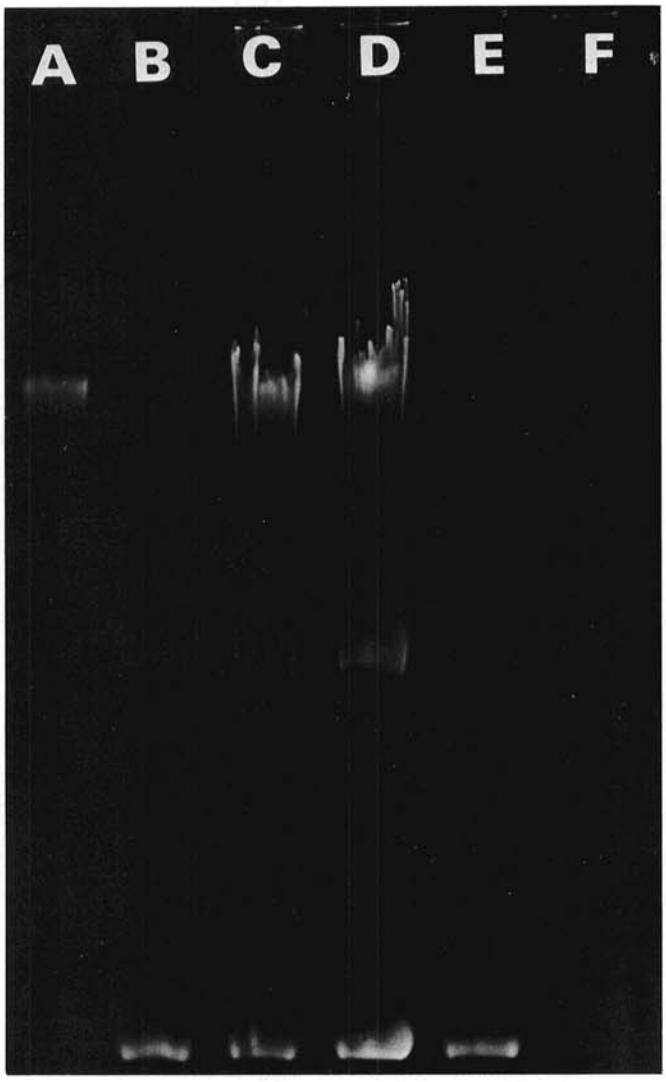

Fig. 3. Electrophoresis of purified plasmid DNA from *Escherichia coli* C600 *nal* transformed with covalently closed circular (CCC) DNA of L-322 (D) compared with plasmid DNA from L-322 (pBR322) (C) and L-322 (A) and authentic pBR322 DNA (B and E) plus the plasmid region from dye-bouyant density centrifugation of C600 *nal* (F). The C600 *nal* sample was eight times more concentrated than the corresponding C600 *nal*/L-322 (pBR322) DNA preparation. Multiple pBR322 bands represent open circular or supercoiled forms of unit length and multimeric forms of the plasmid. Restriction of pBR322 multimers with *Eco*RI produces only fragments of unit length. (Results not shown). The DNA was electrophoresed at 4.0 V/cm for 2.5 hr in 0.7% ME agarose prepared in 178 mM Tris, 5.5 mM EDTA, and 617 mM *ortho*-boric acid buffer (pH 8.0).

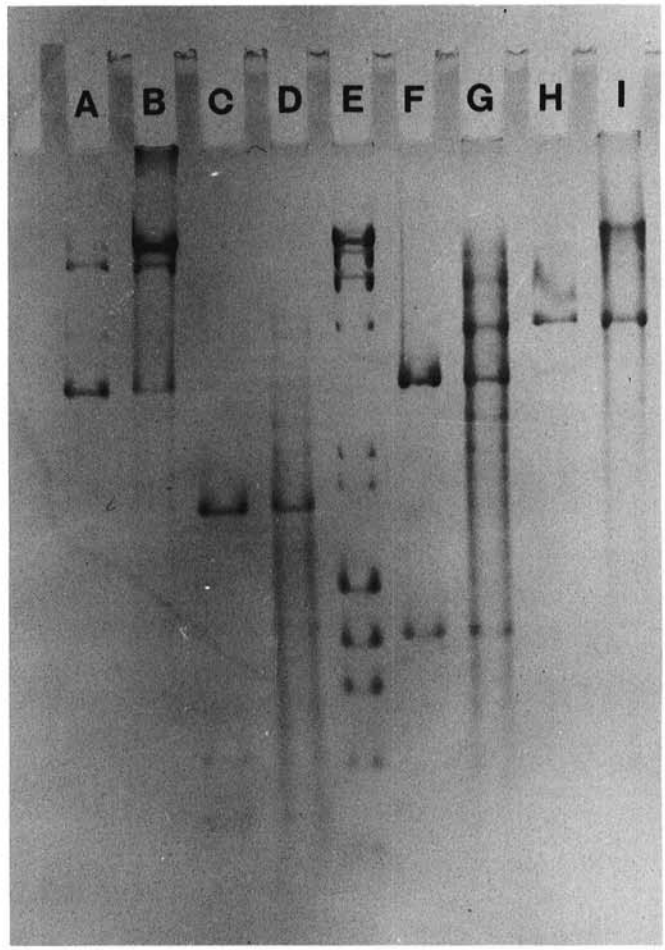

Fig. 4. Electrophoresis of unrestricted pBR322 DNA (A) and purified covalently closed circular DNA from L-322 (pBR322) (B) compared with pBR322 restricted by *Hae*II (C), *Hinc*II (F), and *Eco*RI (H) and L-322/pBR322 DNA restricted with *Hae*II (D), *Hinc*II (G), and *Eco*RI (I). The molecular weight markers (E) are a mixture of restriction digests of phage λ-DNA by *Hind*III and ØX174 by *Hae*II. The molecular masses (Md) of the fragments (from slowest to the fastest migrating fragment) are: 14.6, 6.1, 4.0, 2.8, 1.4, 1.2, 0.9, 0.7, 0.6, 0.4, and 0.2. Multiple pBR322 bands represent the unit length and multimeric forms of the plasmid. Bands representing fragments of indigenous L-322 plasmids are also present in D, G, and I. The DNA was electrophoresed at 0.42 V/cm for 16 hr in 1.0% ME agarose prepared with 178 mM Tris, 5.5 mM EDTA, and 617 mM *ortho*-boric acid buffer (pH 8.0).

Major band similarities were clearly indicated between restricted and unrestricted pBR322 and L-322 (pBR322) DNA. Major identifiable pBR322 restriction fragments in the L-322 (pBR322) plasmid DNA digests were: the 1.9 and 0.7 Md *Hin*cII fragments: the 1.1, 0.4, and 0.2 Md *Hae*II fragments; and the 2.6 Md *Eco*RI fragment. Bands representing fragments of indigenous L-322 plasmids generated by the restriction enzymes appear in addition to the pBR322 fragments in the L-322 (pBR322) CCC DNA digest.

DISCUSSION

Transformation of pBR322 plasmid DNA into *Erwinia herbicola* using the $CaCl_2$ method (13) was demonstrated by the stable and simultaneous inheritance of the plasmid phenotype (Ap^r Tc^r) at frequencies exceeding the probability of spontaneous double chromosomal mutations with resistance to both antibiotics. Physical confirmation of the presence of pBR322 DNA in *E. herbicola* was demonstrated by electron microscopy, electrophoresis, and restriction endonuclease analysis. It also was possible to transform purified pBR322 DNA from *E. herbicola* into *Escherichia coli.*

Transformation of plasmid DNA between *Erwinia* and *Escherichia* may provide a means to study the function of cryptic plasmids from plant epiphytic and pathogenic bacteria in the better known genetic background of *E. coli.* This technique will be helpful for reintroducing plasmids into plasmid-cured or plasmid-less strains of plant inhabiting bacteria in order to understand the function of cryptic plasmids in governing host specificity and phytopathogenicity. In addition, introduction of pBR322, a vehicle for recombinant DNA (4), into *E. herbicola* establishes a basis for development of a gene cloning system in this species.

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