Identification of a Plasmid DNA Probe for Detection of Strains of *Erwinia herbicola* Pathogenic on *Gypsophila paniculata*

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ABSTRACT

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Pathogenic strains of *Erwinia herbicola* incite crown and root galls in *Gypsophila paniculata*. Two serotypic groups were detected in the population of strains isolated from *Gypsophila*, each of which was composed of pathogens and nonpathogens. An additional isolate of pathogenic *E. herbicola* did not react with serotype I or II. Galls caused by pathogenic serotype I strains varied in morphological appearance from galls of other pathogenic strains. All strains secreted indoleacetic acid (IAA) in culture. No correlation was observed between gall size and the amount of IAA produced in vitro. All strains also contained one to four plasmids with sizes ranging between 10 and 100 MDa. A 7.5-kilobase (kb) DNA fragment was subcloned from a library constructed from plasmid DNA of a pathogenic strain. This DNA fragment cross-hybridized with the sequences encoding the IAA biosynthetic pathway in *Pseudomonas savastanoi*. The cloned 7.5-kb fragment was used to distinguish among pathogenic and nonpathogenic strains of each serotypic group by blot hybridization experiments. The probe hybridized to 78-MDa plasmids present in pathogenic strains of serotypes I and III and to 100-MDa plasmids present in pathogenic strains of serotype II. The relationship between IAA production and the specificity of the probe is discussed.

Additional keyword: serology.

Erwinia herbicola (synonym *Enterobacter agglomerans*) (16) is widespread in nature as a saprophytic epiphytic bacterium (26). Strains of *E. herbicola* were identified as the causal organism of crown and root galls in *Gypsophila paniculata* L. (1,3,5,13). Gypsophila is an ornamental used in commercial cutflower production in Israel, California, Florida, and some European countries (5,23).

E. herbicola pv. *gypsophilae* (Brown) Miller, Quinn, and Graham is the most destructive pathogen of gypsophila in Israel and acts as the limiting factor in its propagation (28,29). The host range of *E. h. gypsophilae* is restricted to gypsophila (29). It induces gall formation at wound sites mainly in the crown region of the stem and can occur during the development of rooted cuttings in the nurseries. Gall formation weakens the plant and causes defoliation and, ultimately, death of the whole plant (29). Neither resistant clones nor effective chemical treatment are available. The only control measure is the production of pathogen-free cuttings through culture indexing, which requires a zero tolerance level rating, followed by strict sanitation practices. Identification of *E. h. gypsophilae* in Israel is currently based mainly on serological procedures (18). However, problems related to the specificity of the antiserum have been encountered because of the presence of more than one serotype. Moreover, saprophytic strains of *E. herbicola* cannot be distinguished from pathogenic strains by the immunoassays employed. Consequently, time-consuming pathogenicity tests are necessary to confirm the presence of *E. h. gypsophilae* whenever a positive identification is obtained through serology.

Nucleic acid hybridization, which depends on a high degree of specificity inherent in the pairing of nucleotide base sequences, has been applied successfully for diagnostic purposes of human (20) and plant microbial pathogens (19). DNA probes offer an advantage over immunoassays because the DNA structure is relatively consistent, whereas antigens may differ among strains of the pathogen, in different stages of development, or their expression may be changed by environmental factors. Recently, DNA probes have been developed for identification of various pathovars of phytopathogenic bacteria, e.g., *Xanthomonas campestris* pv. *phaseoli* (11), *Pseudomonas syringae* pv. *phaseolicola* (22), *P. syringae pv.* tomato (7), and *Clavibacter michiganense* subsp. *michiganensi* Haywood and Waterston (27). In this paper, we describe the development of a DNA probe for identification of *E. h. gypsophilae*.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed Table 1. Strains PD128, PD459, and PD713 were from J. Janse (Plant Protection, Wageningen, Netherlands); strain Eh112Y from S. Beer (Cornell University, Ithica, NY); and strains K4 and B111 from J. Winkelman (Tubingen University, Germany). All strains of *E. herbicola* obtained in Israel were isolated from galls or the crown region of *Gypsophila paniculata* 'Perfecta' by H. Vigodsky-Haas or D. Zutra (ARO, Volcani Center). Isolation was carried out on a selective medium, which was developed by M. Sasser at the University of Delaware, and contained the following compounds (g/L of distilled water): D-trehalose, 2.0; 2,6-diaminopurine, 0.2; NaCl, 15; K_2HP0_4, 0.8; KH_2P0_4, 0.8; $MgS0_4 \cdot 7H_20$, 0.2; yeast extract, 0.01; and Bacto agar, 20. After autoclaving and cooling to 50–60 C, the pH was adjusted to 4.9 with 1 N HCl, then cycloheximide was added from a stock solution to a final concentration of 50 μg/ml. Each strain was grown from a single colony. Strains of other *Erwinia* spp., namely *E. carotovora* subsp. *chrysanthemi* 598; *E. carotovora* subsp. *carotovora* R1; *E. carotovora* subsp. *atroseptica* St8; *E. stewartii* 1090 and 638/1; and *E. amylovora* 1/88, were obtained from D. Zutra. *Escherichia coli* DH5α (BRL, Gaithersburg, MD) was used for transformations of cloned DNA. The *Erwinia* strains were maintained on nutrient agar (Difco) containing 1% glycerol (NAG), and *E. coli* was grown on LB medium (17). Liquid cultures were grown in LB broth (Difco).

Pathogenicity tests. Inoculum was prepared by growing the bacterial strain on NAG plates at 28 C for 1–2 days. The bacteria

were harvested from a plate in 5 ml of sterile saline. Pathogenicity tests were conducted by dipping cuttings of *G. paniculata* into the bacterial suspension before rooting. Four cuttings were inoculated with each bacterial strain. The cuttings were transferred into Jiffypots (Jiffy 9, Jiffy Products Ltd., Norway) and placed in the greenhouse at 20–25 C. The leaves were kept constantly moist by a sprinkler system. Galls could be observed in the crown region 7–10 days after inoculation. After 2 wk, the galls were removed from each cutting and fresh weight was determined.

TABLE 1. Summary of immunoassays, pathogenicity, indoleacetic acid production, and plasmid DNA probe hybridization of strains of *Erwinia herbicola*

Strain	Immunoassay[a]		Gall formation[b]	IAA production[b]	Hybridization with DNA probe[c]	
	SI	SII	(mg fresh wt)	(μg/ml)	Plasmid	Colony
6	+[d]	−	182	5.7	+[d]	+
6-2	+	−	148	7.7	+	+
6-3	+	−	136	9.1	+	+
24-2	+	−	97	15.0	+	+
13-2	+	−	80	3.8	+	+
29-3	+	−	192	9.4	+	+
350-1	−	+	123	17.1	+	+
350-2	−	+	97	6.2	+	+
350-4	−	+	113	3.5	+	+
420	+	−	198	13.6	+	+
446-2	+	−	174	6.7	+	+
479-2	+	−	115	4.9	+	+
53	+	−	150	4.1	+	+
824-1	+	−	240	8.3	+	+
824-2	+	−	142	4.6	+	+
824-3	+	−	176	11.8	+	+
29-2	+	−	179	14.0	+	+
615	−	+	167	3.3	+	+
611	−	+	24	10.0	+	+
52	+	−	225	3.9	+	+
13	+	−	256	3.0	+	+
441	−	+	125	7.9	+	+
30/88	+	−	230	14.0	+	+
24	+	−	129	10.0	+	+
102	−	−	136	14.5	+	+
300	+	−	164	10.0	+	+
N/2	+	−	145	9.0	+	+
227	+	−	132	9.5	+	+
PD128	+	−	172	12.0	+	+
PD459	+	−	190	11.0	+	+
PD713	+	−	182	15.3	+	+
1-10	+	−	···	7.2	−	−
135	+	−	···	4.6	−	−
23-9	+	−	···	6.5	−	−
24-8	+	−	···	3.4	−	−
717-2	+	−	···	15.0	−	−
717-4	+	−	···	4.8	−	−
163-5	−	+	···	5.3	−	−
163-6	−	+	···	0.5	−	−
27-3	+	−	···	10.1	−	−
40C	+	−	···	2.2	−	−
24-3	+	−	···	18.0	−	−
1-15	+	−	···	14.5	−	−
I4	−	−	···	2.0	ND	−
24-8FR	+	−	···	4.9	−	−
3-1	+	−	···	8.0	−	−
6-31	+	−	···	9.0	−	−
479-21	+	−	···	4.9	−	−
Eh112Y	−	−	···	9.0	−	−
K4	−	−	···	15.0	ND	−
B111	−	−	···	12.0	ND	−

[a] SI and SII denote ELISA with antibodies obtained against serotype I cells and serotype II cells respectively, as described in Materials and Methods.

[b] Results are an average of at least three independent experiments. The maximum variability expressed as percentage of the mean was 42% for gall size and 50% for IAA production.

[c] A 7.5-kb *Eco*RI fragment of a plasmid from *E. herbicola* pv. *gypsophilae* PD713 was used as a probe. Hybridization was performed either after separation of plasmids by gel electrophoresis (plasmid) or with colonies (colony).

[d] + = positive response, − = negative response, ND = not determined.

Serology. Strains 3-1 (serotype I) and 350-1 (serotype II) of *E. h. gypsophilae* were cultured on nutrient agar for 24 hr at 27 C. Strain 3-1 was pathogenic when used for antiserum preparation but lost its pathogenicity during culturing. The cultures were harvested, washed twice in 0.01 M phosphate-buffered saline (PBS, pH 7.2), and adjusted to a concentration of 10^9 cells per milliliter. Prior to injection, the bacteria were incubated at 60 C for 30 min, then 1 ml of cells was mixed with 1 ml of Freund's incomplete adjuvant. Antiserum was prepared in New Zealand white rabbits by an intramuscular injection of the dead cells each week for 4 wk. A booster intravenous injection of 0.5-ml live cells without adjuvant was given during the fifth week, and bleedings were made a week later. A titer of 1:30,000 was obtained by enzyme linked immunosorbent assay (ELISA). Competitive indirect ELISA (18) was used for immunoassay.

Indole-3-acetic acid (IAA) determination. For IAA production, bacteria were grown in flasks containing 100 ml of minimal A medium (17) supplemented with 200 mg of L-tryptophan per liter. The cultures were grown on a rotary shaker in the dark at 28 C for 48 hr, and the cells were removed by centrifugation. The supernatant was acidified to pH 3.0 with 1 M HCl and then extracted three times with equal volumes of ethyl acetate. The ethyl acetate extracts were pooled and flash-evaporated at 30 C. The dry pellet was dissolved in 5 ml of 80% methanol containing 100 mM ammonium acetate and 45 mM butylated hydroxytoluene (BHT). The resultant solution was subjected to three open column liquid chromatography steps on polyvinylpolypyrrolidone (Polycar AT), DEAE-Sephadex, and C-18 Sep-Pak columns according to a procedure described by Sagee et al (21). Following the latter purification steps, IAA was the predominant indole compound. IAA was eluted from the last column with methanol and its chemical identity was confirmed by gas chromatography-mass spectrometry as previously described (30), except that a temperature program of 130–200 C at 3 C min^{-1} was started upon injection. IAA concentration was determined by Salkowski reagent (12).

Isolation and analysis of DNA. Plasmid DNA was isolated from *E. herbicola* according to the procedure described by Comai and Kosuge (4). For cloning purposes, the plasmid DNA was further purified by density gradient centrifugation in CsCl-ethidium bromide (17). Total genomic DNA was prepared from 50 ml of late logarithmic phase cultures as described by Silhavy et al (24).

Restriction enzyme digestions and transformation of *E. coli* were done by standard procedures (17). Plasmid DNA from *E. herbicola* was separated on horizontal agarose gels (0.5%) run at 5 V/cm in TAE electrophoresis buffer (40 mM Tris-acetate + 1 mM EDTA, pH 8.0) (17). Indigenous plasmids from *E. stewartii* SW-2 (6) were used as size standards for estimating the molecular size of the plasmids.

Southern hybridizations were performed as described by Maniatis et al (17), with Hybond N nylon membranes (Amersham Inc., Arlington Heights, IL). The plasmid DNA to be used as a probe was purified from agarose gels by employing the Geneclean kit (Bio 101 Inc., San Diego, CA). Hybridization probes were labeled with P with either a nick translation kit (Amersham Inc.) or a random primer kit (Promega Inc., Madison, WI) according to the manufacturers' recommendations. Prehybridizations were performed at 65 C for 2 hr in a solution of 5× SSC (1× SSC is 0.15 M NaCl and 15 mM sodium citrate), 0.5% SDS (sodium dodecyl sulfate), 5× Denhardt's solution, and 100 μg of denatured salmon sperm DNA per milliliter. Hybridizations were performed by adding the probe directly to the prehybridization solution followed by gentle rocking at 65 C for 16 hr (17). The probed filters were washed twice at room temperature for 10 min in 2× SSC, 0.1% SDS solution, and once at 65 C for 10 min in 0.1× SSC, 0.1% SDS solutions. Filters were dried, and autoradiography was carried out at −70 C with Kodak XAR-5 film.

For colony hybridization, bacteria were grown on a Hybond

N nylon filter overlaid on a NAG plate and incubated overnight at 28 C. The filter then was lifted from the plate and laid for 2 min on Whatman No. 1 filter paper soaked in 2× SSC, 5% SDS. The procedure for denaturation, fixation, and hybridization of DNA was according to Buluwela et al (2).

CsCl-purified plasmid DNA from *E. h. gypsophilae* strain PD713 was used to construct a library in λ EMBL replacement vector (9). The DNA was partially digested with *Sau*3A, ligated into the *Bam*H1 site of EMBL3 (Stratagene, La Jolla, CA), and packaged with Gigapack Gold packaging kit of Stratagene according to the manufacturer's recommendations. The lambda library was screened with pCP3, which contains genes for IAA biosynthesis of *Pseudomonas savastanoi* (31). Plaques that cross-hybridized to pCP3 were selected with the ^{32}P-labeled probe under low stringency conditions (10,17). A 7.5-kb *Eco*RI fragment was subcloned from a λ clone that hybridized with pCP3 into pUC118. This fragment was used as a probe to distinguish between pathogenic and nonpathogenic strains of *E. herbicola*.

RESULTS

Biochemical and immunological characteristics of strains of *E. herbicola*. Israeli strains of *E. herbicola* were homogenous with respect to biochemical characteristics (8) including acid production from L-arabinose, D-adonitol, D-mannose, inositol, L-rhamnose, citrate, dulcitol, and arginine. Immunological characterization revealed three serotypic groups within the strains of *E. herbicola* examined which included strains from foreign countries. Serotype I constituted 78% of the strains, whereas 18% were serotype II (Table 1). One pathogenic strain, 102, could not be detected by either of the antisera, suggesting the presence of a third serotype. There was no cross reactivity among serotypic groups.

Pathogenicity and IAA production. IAA production was detected in pathogenic and nonpathogenic strains of *E. herbicola* (Table 1). For *E. h. gypsophilae*, no correlation was established between IAA secretion and gall size. Galls of serotype I were generally larger, softer, and darker than those of the other two serotypes.

Characterization of plasmids and DNA hybridizations. All 48 strains tested contained plasmids (Table 1). The plasmid DNA from these strains were subjected to gel electrophoresis, as shown for 14 representatives (Fig. 1A). The approximate size of the plasmids ranged from 100 MDa to less than 10 MDa. The number of plasmids varied among different strains with one to four major bands. The 100-MDa plasmid was characteristic of serotype II strains, whereas the 78-MDa plasmid was detected in serotype I and serotype III strains.

The results of Southern analyses with the 7.5-kb DNA fragment are summarized in Table 1 and illustrated for 14 representative strains in Figure 1B. Hybridization of the probe occurred only with plasmid DNA from pathogenic strains of *E. herbicola* and not with that from nonpathogenic strains. Moreover, the plasmid DNA probe hybridized only with the 78-MDa plasmid in serotype I and III strains (with one exception, 52, where the hybridized plasmid was smaller), and only with the 100-MDa plasmid in serotype II strains (Fig. 1B). When the total DNA of strains 824-1 (serotype I) and 350-1 (serotype II) was digested with *Eco*RI, subjected to gel electrophoresis, and hybridized with the 7.5-kb plasmid DNA probe, a single band corresponding to 7.5 kb was detected (*data not shown*).

To further test the specificity of the probe, colony hybridization was conducted with all 51 strains of *E. herbicola*. Results summarized in Table 1 and demonstrated in Figure 2 indicate that the probe distinguishes between pathogenic and nonpathogenic strains. The plasmid DNA probe did not hybridize with DNA from colonies of other species of *Erwinia*.

DISCUSSION

Strains of *E. herbicola* isolated from gypsophila appear to constitute different serotypic groups. Two of the serotypes

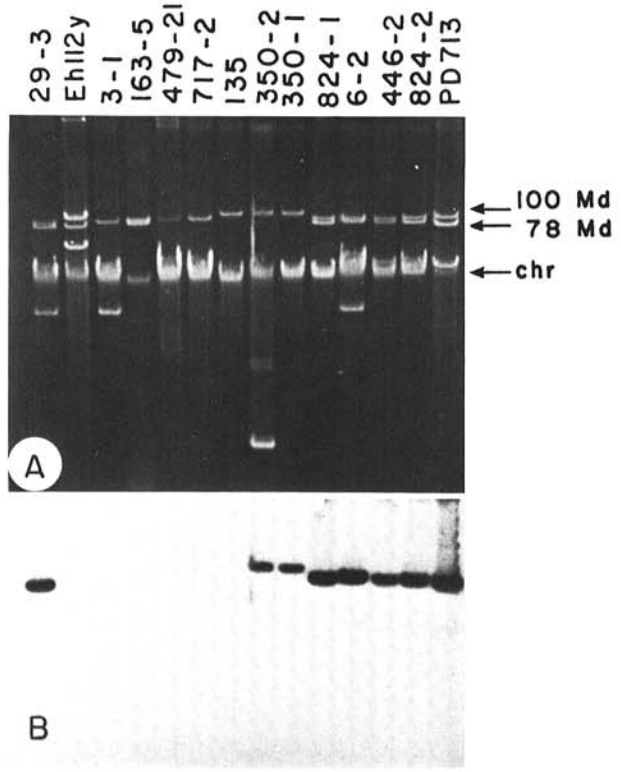

Fig. 1. Gel electrophoretic and Southern analysis of plasmid DNA of pathogenic and nonpathogenic strains of *Erwinia herbicola*. DNA was fractionated on a 0.5% agarose gel as described. **A,** Gel stained with ethidium bromide. **B,** Southern transfer of DNA probed with the 7.5-kb plasmid *Eco*RI fragment of *E. herbicola* pv. *gypsophilae* strain PD713. The numbers PD713, 824-2, 446-2, 6-2, 824-1, 350-1, 350-2, and 29-3 designated pathogenic strains, whereas 135, 717-2, 479-21, 163-5, 3-1, and Eh112Y are nonpathogenic strains (Table 1).

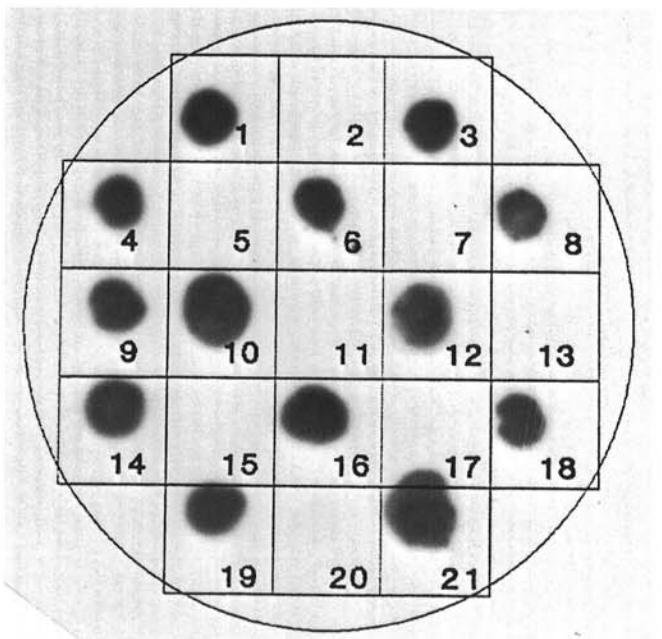

Fig. 2. Colony hybridization analysis of DNA from pathogenic and nonpathogenic strains of *E. herbicola*. Colonies were blotted on Hybond N nylon filter and probed with the 7.5-kb plasmid *Eco*RI fragment as described in Materials and Methods. Colonies showing signals are of pathogenic strains. Numbers from 1 to 21 signify the following strains: 824-1, 135, 350-1, 611, 717-2, 102, 3-1, PD459, 52, PD713, 1-10, 300, 23-9, PD128, 6-3, 29-2, 24-8, N/2, 350-2, 24-3, and 24-2, respectively.

characterized in this study contained pathogenic as well as saprophytic strains (Table 1), thereby indicating no association between serotypes and pathogenicity. However, in contrast to immunoassays, which could distinguish between serotypic groups but not between pathogenic and nonpathogenic strains within each group, the plasmid DNA probe hybridized only with DNA from pathogenic strains of all serotypic groups. Strain 102 did not react with either of the two antisera, but DNA of this strain hybridized with the probe (Table 1). Thus, the plasmid DNA probe may offer an advantage over serological methods for detection of *E. h. gypsophilae*. The antigenic determinants in this study were not related to pathogenicity.

The role of IAA in gall formation by *Agrobacterium tumefaciens* and *Pseudomonas syringae savastanoi* has been established (15). Although direct proof of the role of IAA in gall development by *E. h. gypsophilae* has yet to be provided, previous studies indicate a close similarity between the mode of hyperplasia production by this pathogen and *P. s. savastanoi* (3). The accumulation of IAA in culture by *P. s. savastanoi* was correlated with virulence on oleander as determined by gall size (25). In contrast to *P. s. savastanoi*, we did not detect a correlation between in vitro IAA production and gall size in *E. h. gypsophilae* (Table 1). Moreover, the nonpathogenic strains, including those that lost pathogenicity in culture (e.g., 3-1, 6-31), produced IAA. IAA biosynthesis in *P. s. savastanoi* is carried out from tryptophan via the indoleacetamide pathway (15). Results recently obtained in our laboratory indicate the existence of two pathways for IAA production in *E. h. gypsophilae*: one involved the indoleacetamide route, and another in which indolepyruvate and indoleacetaldehyde serve as intermediates (*unpublished results*). One of the pathways (the indoleacetamide route) may be necessary for pathogenicity whereas the other, which has also been detected in higher plants (14), may be adapted for saprophytic survival. In the latter case, this additional pathway might mask the correlation between IAA production and virulence in *E. h. gypsophilae*.

The nature of the specificity of the plasmid DNA probe for pathogenic strains has yet to be clarified. This DNA probe was selected by hybridization with pCP3, which contains the genes for the indoleacetamide pathway of *P. s. savastanoi* (i.e., *iaa*M and *iaa*H) (4). It is possible that the 7.5-kb *Eco*RI fragment contains genes of the indoleacetamide pathway in *E. h. gypsophilae*. If the indoleacetamide pathway for IAA production is mandatory for gall formation, then the specificity of the probe may be caused by the presence of these genes. Preliminary experiments suggest that the indoleacetamide route is indeed present only in pathogenic strains of *E. herbicola*. A molecular genetic approach is being taken to prove this hypothesis. Studies of the application of the probe for practical diagnosis of *E. h. gypsophilae* are currently being conducted.

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