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Hybridization and Functional Complementation of the *hrp* Gene Cluster from *Erwinia amylovora* Strain Ea321 with DNA of Other Bacteria

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The *hrp* gene cluster of *Erwinia amylovora* strain Ea321 was used as a probe for testing the presence of sequence variation in *E. amylovora* strains of diverse host and geographic origins. All *E. amylovora* strains tested yielded hybridization patterns identical to that of Ea321, except for those strains isolated from *Rubus* species, which yielded similar but distinct hybridization patterns. With lowered stringency hybridization conditions, portions of the *hrp* cluster of Ea321 contained in the cosmid pCPP430 also hybridized with genomic DNA from numerous other erwiniae and from *Pseudomonas syringae* pv. *syringae*. Restriction fragments of pHIR11, a cosmid containing a cloned *P. syringae hrp* gene cluster, and pES1044, a cosmid containing portions of the *wts* gene cluster of *E. stewartii*, hybridized colinearly with restriction fragments representing approximately 12 and 18 kb of pCPP430, respectively. Several Hrp$^-$ mutant strains of *E. amylovora* with transposon insertions within the region of hybridization were restored to the Hrp phenotype by the hybridizing DNA of the other two species.

Numerous necrogenic plant pathogenic bacteria produce grossly similar watersoaked and necrotic lesions on their hosts and elicit a hypersensitive response (HR) on nonhosts. From many of these bacteria, *hrp* genes have been identified (Willis *et al.* 1991). Bacteria in which such genes have been identified include numerous phytopathogens in the genera *Pseudomonas*, *Xanthomonas*, and *Erwinia* (Lindgren *et al.* 1986; Huang *et al.* 1988; Huang *et al.* 1990; Boucher *et al.* 1987; Kamoun and Kado 1990; Bonas *et al.* 1991; Bauer and Beer 1987, 1991; Steinberger and Beer 1988; Barny *et al.* 1990; Vanneste *et al.* 1990; Walters *et al.* 1990). Mutations in *hrp* genes result in the Hrp$^-$ phenotype, characterized by the dual inability to cause disease in host tissue and to elicit an HR in nonhost tissue. Alternatively, some transposon-induced *hrp*-like mutants exhibit a loss of pathogenicity on host plants but only an attenuation of their ability to elicit the HR on nonhosts (Beer 1991).

For plant pathogenic erwiniae or pseudomonads, cosmids have been identified containing multiple *hrp* genes or other determinants of pathogenicity. The cosmid pHIR11 apparently contains the entire *hrp* gene cluster from *P. syringae* van Hall strain 61, because it gives the nonplant pathogen *P. fluorescens* (Trevisan) Migula the ability to elicit the HR (Huang *et al.* 1988). The cosmid pCPP430 evidently contains the entire *hrp* gene cluster of *E. amylovora* (Burrill) Winslow *et al.* strain Ea321 (Beer *et al.* 1989b; Beer *et al.* 1991) (Fig. 1). Genes designated "*wts*", required for production of the "watersoaking" symptom by *E. stewartii* (Smith) Dye on corn, have been cloned on pES1044 (Coplin 1986) (Fig. 1) and pES411 (Coplin *et al.* 1992). This foliar pathogen does not elicit an HR as efficiently under typical experimental conditions as do most other phytopathogenic bacteria mentioned above. Thus, the relationship between functions encoded by *wts* genes and those encoded by *hrp* genes was not clear.

The entire *hrp* gene cluster of *E. amylovora* has been isolated only from strain Ea321. We compared the physical organization of the *hrp* gene cluster of this strain with that of other strains of the fire blight pathogen. We were also interested in determining if *hrp* genes or *hrp*-linked genes from *E. amylovora* share DNA sequence similarity with cloned *hrp* or *wts* genes from *P. syringae* and *E. stewartii*, respectively, and with DNA of other phytopathogenic bacteria.

This report describes DNA hybridization results that indicate DNA sequence similarities among the *hrp* gene cluster of *E. amylovora* Ea321, all other strains of *E. amylovora* tested, total DNA of numerous other phytopathogenic erwiniae, *wts* genes of *E. stewartii*, and the *hrp* gene cluster of *P. syringae*. Genetic complementation results that indicate functional homology between the *hrp* gene cluster of *E. amylovora*, the *wts* region of *E. stewartii*, and the *hrp* gene cluster of *P. s.* pv. *syringae* van Hall are also presented. Preliminary reports have been presented (Laby and Beer 1990; Beer *et al.* 1990).

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used are listed in Table 1. *Escherichia coli* (Migula) Castellani and Chalmers and *E. stewartii* strains were grown at 37° C and other erwiniae at 28° C in tryptone-yeast extract phosphate buffer (TYPB) (Sambrook *et al.* 1989).

DNA preparations and recombinant DNA techniques. Total DNA was isolated from bacteria in minipreps according to the procedure of Silhavy *et al.* (1984). Plasmid miniprep DNA was prepared according to standard protocols (Sambrook *et al.* 1989), with the following modification: bacteria were centrifuged in a microcentrifuge and the pellet was resuspended in 50 μl of SCT (20%

sucrose, 20 mM 1,2-cyclohexane diaminetetraacetic acid [CDTA], 50 mM Tris, pH 8.0). Restriction endonuclease digestions, ligations, and subcloning were performed according to standard protocols (Sambrook *et al.* 1989). Enzymes used were purchased from Boehringer-Mannheim (Indianapolis, IN), Promega (Madison, WI), or New England Biolabs (Beverly, MA) and used according to the suppliers' directions. DNA fragments for ^{32}P-labeling were purified from agarose with the GeneClean Kit (Bio 101 Inc., La Jolla, CA).

DNA labeling. DNA fragments were labeled with [^{32}P]dGTP (Dupont-NEN, Boston, MA) (Feinberg and Vogelstein 1983), followed by probe purification on a prepacked Sephadex G50 spin column (Boehringer-Mannheim), according to the manufacturer's directions. The composite Ea-HRP probe used consisted of plasmids pCPP1003, pCPP1002, pCPP1006, pCPP1007, pCPP1005, and pCPP1008 (Fig. 1). These were digested, electrophoresed in 0.7% agarose with TAE buffer (Sambrook *et al.* 1989), and stained with ethidium bromide. The appropriate bands containing fragments of Ea321 DNA were cut from the gel, purified from agarose, precipitated with ethanol, resuspended in TE, and pooled for labeling. Radioactively labeled probes consisting of individual restriction fragments of DNA were prepared similarly.

Electrophoresis and Southern transfer. Genomic or cloned DNA to be probed was digested, electrophoresed in a 0.7% agarose gel, stained with ethidium bromide, depurinated in 0.25 M HCl, and transferred onto Gene Screen Plus (New England Nuclear); the manufacturer's suggested capillary blotting protocol was used (Anonymous 1987).

Hybridizations, washes, stripping of probes. Membranes were prehybridized in a solution of 1% sodium dodecyl sulfate (SDS), 1 M NaCl, and 10% dextran sulfate at 46–65° C for 1–3 hr in a sealed plastic pouch. Denatured salmon sperm DNA (Sigma, St. Louis, MO) (final concentration 100 μg/ml) was mixed with denatured ^{32}P-radiolabeled probe and added to the prehybridized blot. The membrane was hybridized overnight with agitation at 46°, 52°, 57° (lowered stringency), or at 65° C (high stringency). Membranes were washed in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) at 46–57° (lowered stringency) or at 65° C (high stringency) three times for 15–30 min each, or until no radioactivity was detectable in the last wash buffer. The filters were exposed to Kodak X-omat AR film at room temperature or at −80° C with an intensifier screen. Stripping of probes from membranes was performed according to manufacturer's directions (Anonymous 1987).

Interspecies complementation. pES1044, pHIR11, and subclones from them were introduced into *E. amylovora* Hrp$^-$ strains by triparental mating with pBW7 or pRK2013 as the helper plasmid or by electroporation (25 μF, 2.5 kV, 400Ω) with a Hoefer Gene Pulser (Hoefer Sci. Instr., San Francisco, CA).

Pathogenicity and HR tests. Pathogenicity tests on im-

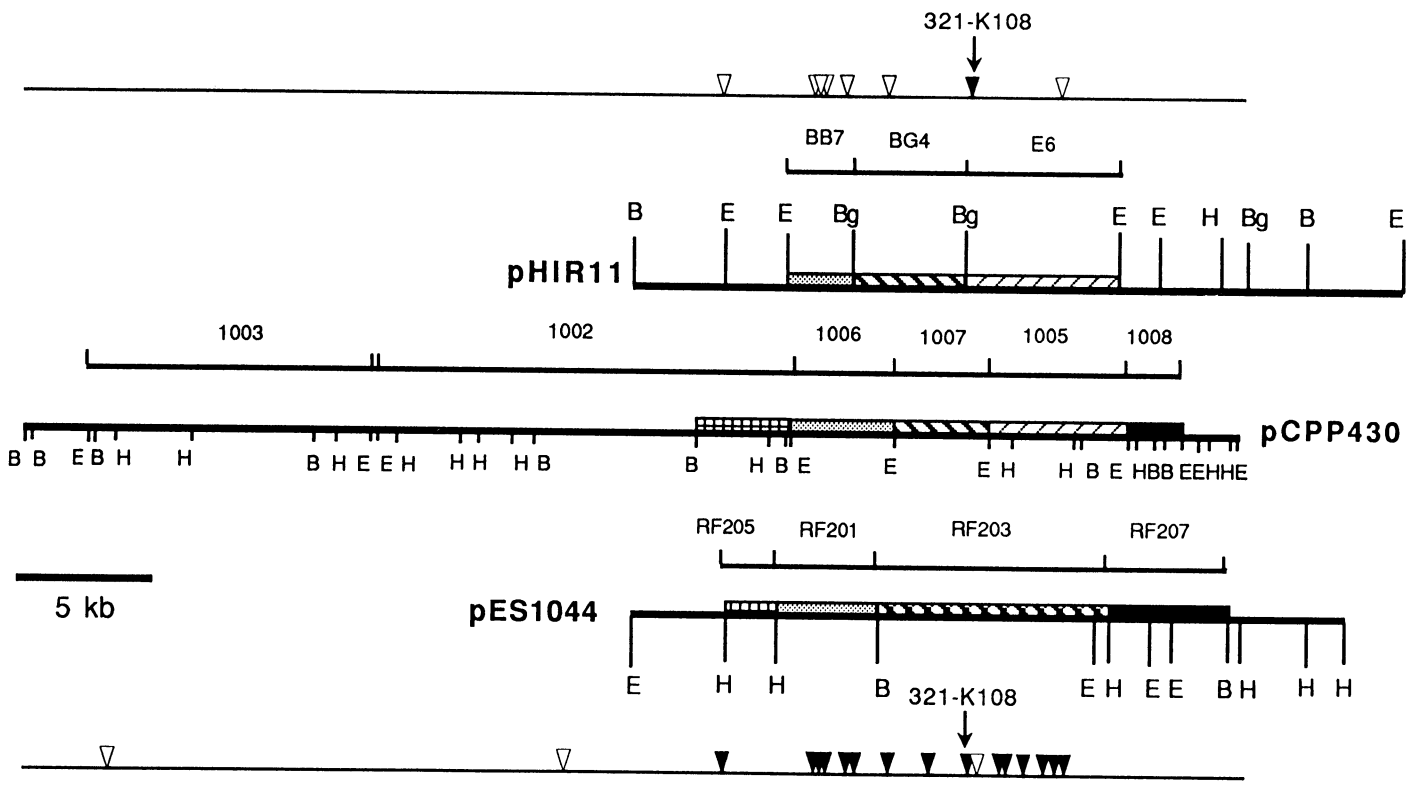


Fig. 1. DNA restriction map of cosmids pCPP430, pES1044, pHIR11, and subclones from them; filled patterns indicate cross-hybridizing restriction fragments of pHIR11, pES1044, and pCPP430. Top and bottom lines: Triangles indicate sites of transposon insertions in *Erwinia amylovora* Hrp$^-$ mutant strains. ▼ Indicates strains restored to Hrp$^+$ phenotype by DNA of *E. stewartii* or *Pseudomonas syringae*. ▽ Indicates strains not restored to Hrp$^+$ phenotype by DNA of *E. stewartii* or *P. syringae*. B = *Bam*HI, Bg = *Bgl*II, E = *Eco*RI, H = *Hin*dIII.

Table 1. Bacteria and plasmids used in this study

Bacterial strains or plasmids	Relevant characteristics, host, location, or source[a]
Agrobacterium radiobacter K84	T. Burr, NYSAES, Geneva, NY
A. tumefaciens	
C58	R. Dickey, Cornell Univ., Ithaca, NY
C649	Biovar 3; A. Collmer, Cornell Univ.
452	Biovar 1; J. Prunier, INRA, Angers, France
Enterobacter agglomerans	ATCC 12287
E. cloacae 1347/71	D. Brenner, Center for Disease Control, Atlanta, GA
E. gergoviae 604/77	D. Brenner
Erwinia amylovora	
Ea102	Pear, California, CUCPB 0003
Ea223	Pear, New York, CUCPB 0035
Ea227	Pear, New York, CUCPB 0039
Ea243	Apple, Illinois, CUCPB 0050
Ea246	Blackberry, Illinois; S. Reis, CUCPB 0053
Ea258	Cotoneaster, Netherlands, CUCPB 0064
Ea259	Pyracantha, Netherlands, CUCPB 0065
Ea261	Mountain ash, Netherlands, CUCPB 0067
Ea266	Apple, Canada, CUCPB 0071
Ea272	Serviceberry, Canada, CUCPB 0077
Ea273	Apple, New York, CUCPB 0273
Ea276	Apple, Michigan, CUCPB 0080
Ea286	Pear, U.K., CUCPB 0089
Ea303	Pear, U.K., ATCC 15580
Ea321	Hawthorn, France, CFBP 1367
Ea321-K108	Km^r; *hrp*::Tn*5*; Beer *et al.* 1989a
Ea338	Quince, New York, CUCPB 0285
Ea341	Apple, France, CUCPB 0383
Ea347	Cotoneaster, Germany, CUCPB 0467
Ea386	Pear, Poland, CUCPB 2016
Ea402	Pear, Egypt, CUCPB 2190
Ea416	Raspberry, CUCPB 2322
Ea483	Loquat, Turkey, CUCPB 3280
Ea491	Apple, New Zealand, CUCPB 3323
Ea492	Hawthorn, France, CFBP 1430
Ea505	Pear, Greece, CUCPB 3339
Ea510	Raspberry, Canada, CUCPB 3367
Ea511	Blackberry, Illinois, CUCPB 3373
Ea514	Blackberry, Illinois, CUCPB 3376
Ea528	Raspberry, Maine, CUCPB 3573
Ea530	Raspberry, Maine, CUCPB 3575
Ea531	Raspberry, Maine, CUCPB 3576
E. carotovora subsp. *atroseptica* Eca001	R. Dickey
E. carotovora subsp. *betavasculorum* Ecb101	CUCPB 0306
E. carotovora subsp. *carotovora* DB17	A. Chatterjee; Univ. of Missouri, Columbia
E. chrysanthemi	
EC16	A. Collmer
3937	A. Toussaint, Brussels, Belgium
E. herbicola	
Eh112Y	Apple, U.K., CUCPB 0119
Eh252	Apple, New York, CUCPB 2050
Eh282	Pineapple, Hawaii, CUCPB 2093
E. lupinicola W3L16	D. Gross; Regner and Gross 1988
E. mallotivora AM1	ATCC 29573; R. Dickey
E. nigrifluens 5D312	C. Kado, Univ. of California, Davis
E. quercina 11D3	C. Kado
E. rubrifaciens 6D3	C. Kado
E. stewartii DC283	D. Coplin, Ohio State Univ., Columbus
Escherichia coli DH5α	Bethesda Research Laboratories, Gaithersburg, MD
Klebsiella planticola 2529	R. Finn, Cornell Univ.
K. pneumoniae	ATCC 15574
Pseudomonas syringae pv. *syringae*	
B728a	A. Collmer
Y30	A. Collmer
P. syringae pv. *glycinea* race 0	N. Keen, Univ. of California, Riverside
Xanthomonas campestris pv. *malvacearum* 14	R. Dickey
X. c. pv. *manihotis* CIAT1134	W. Fry, Cornell Univ.
X. c. pv. *phaseoli* XPW	CUCPB 0378
X. c. pv. *vesicatoria* 21	R. Dickey
Serratia marcescens Ser101	R. Dickey
Plasmids	
pBW7	Tc^r; Achtman *et al.* 1971
pCPP9	Sp^r, Mob^+; D. Bauer 1990
pCPP25	Cm^r, Mob^+; D. Bauer 1990
pCPP430	46.1-kb Ea321 *Sau*3AI fragment in pCPP9; Beer *et al.* 1989
pCPP1002	15.8-kb *Eco*RI fragment of pCPP430 in pUC19; this work
pCPP1003	10.8-kb *Eco*RI fragment of pCPP430 in pUC19; this work
pCPP1005	5.2-kb *Eco*RI fragment of pCPP430 in pUC19; this work
pCPP1006	3.8-kb *Eco*RI fragment of pCPP430 in pUC19; this work
pCPP1007	3.8-kb *Eco*RI fragment of pCPP430 in pUC19; this work
pCPP1008	2.1-kb *Eco*RI fragment of pCPP430 in pUC19; this work
pCPP1050	9.3-kb *Bam*HI-*Hin*dIII fragment of pES1044 in pCPP25; this work
pCPP1063	3.6-kb *Bam*HI-*Hin*dIII fragment of pRF201 in pCPP25; this work
pHIR11BB7	2.4-kb *Bam*HI fragment of pHIR11 Huang *et al.* 1988
pHIR11BG4	4.1-kb *Bgl*II fragment of pHIR11; A. Collmer
pHIR11E6	6.0-kb *Eco*RI fragment of pHIR11; A. Collmer
pES1044	24-kb *E. stewartii* DC283 *Hin*dIII fragment; Coplin *et al.* 1986
pHIR11	36-kb *P. syringae Sau*3AI fragment in pLAFR3; Huang *et al.* 1988
pLAFR3	Tc^r; Mob^+; Staskawicz *et al.* 1987
pRF201	3.6-kb *Bam*HI-*Hin*dIII fragment of pES1044; D. Coplin
pRF203	9.3-kb *Bam*HI-*Hin*dIII fragment of pES1044; D. Coplin
pRF205	1.8-kb *Hin*dIII fragment of pES1044 in pLAFR3; D. Coplin
pRF207	4.9-kb *Bam*HI-*Hin*dIII fragment of pES1044; D. Coplin
pRK2013	Km^r; Figurski and Helinski 1979
pUC19	Ap^r; Messing 1983

[a] CUCPB, Cornell University collection of Phytopathogenic Bacteria; ATCC, American Type Culture Collection; CFBP, Collection Francaise Bacteria Phytopathogenique.

mature pear fruit and HR tests on tobacco cv. Samsun were carried out as previously described (Steinberger and Beer 1988; Bauer and Beer 1991).

RESULTS

DNA sequence similarities among *E. amylovora* strains. Total DNA isolated from 31 strains of *E. amylovora* of diverse host and geographic origins (Table 1) was immobilized on Southern-blotted membranes and hybridized with fragments of the *hrp* gene cluster of Ea321. Under high stringency conditions, the composite Ea-HRP probe (Fig. 1) hybridized only with DNA derived from *E. amylovora* and not with DNA from other organisms. Nearly identical hybridization patterns were observed among the *E. amylovora* strains (Fig. 2). All *E. amylovora* pomaceous strains yielded hybridization patterns identical to that of Ea321. DNA from strains isolated from *Rubus* spp. yielded two hybridization patterns distinct from the patterns obtained from *E. amylovora* strains that had been isolated from pomaceous hosts. Although the three groups of strains shared hybridizing bands of about 10.8, 5.2, and 3.8 kb (doublet), other hybridizing bands differed.

DNA sequence similarities among other erwiniae and phytopathogenic bacteria. Hybridization experiments performed at lowered stringency (hybridization and washes at 46–57° C) resulted in positive signals with total DNA of numerous *Erwinia* sp. (Fig. 3). Hybridization signals above background were not observed with genomic DNA of several other species, including *Agrobacterium radiobacter* (Beijerinck and van Delden) Conn, *A. tumefaciens* (Smith and Townsend) Conn, *Enterobacter agglomerans* (Beijerinck) Ewing and Fife, *E. cloacae* (Jordan) Hormaeche and Edwards, *E. gergoviae* Brenner *et al.*, *Erwinia herbicola* (Löhnis) Dye (nonphytopathogenic strains), *Klebsiella planticola* Bagley, Seidler and Brenner, *K. pneumoniae* (Schroeter) Trevisan, *P. syringae* pv. *glycinea* (Coerper) Young *et al.*, *Serratia marcescens* Bizio, *Xanthomonas campestris* pv. *manihotis* (Berthet and Bondar) Dye, *X. c.* pv. *phaseoli* (Smith) Dye, and *X. c.* pv. *vesicatoria* (Doidge) Dye. To define more precisely those portion(s) of the *E. amylovora hrp* gene cluster that hybridized to genomic DNA of other erwiniae, we prepared five identical Southern blots (a representative stained agarose gel is shown in Fig. 4). These blots contained *Eco*RI-digested pCPP430 (containing the *E. amylovora* Ea321 *hrp* gene cluster), seven subcloned portions of pCPP430, pES1044 (containing *wts* loci of *E. stewartii*;

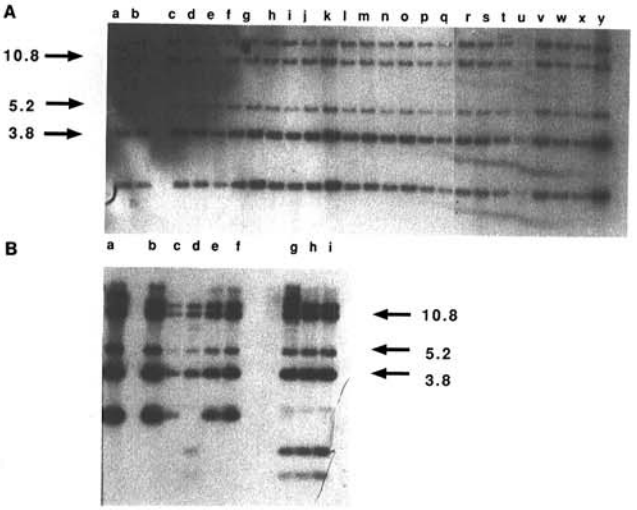


Fig. 2. Hybridization of the pooled Ea-Hrp probe with *Eco*RI-digested total DNA from strains of *Erwinia amylovora* of diverse origin. **A,** Total DNA from strains of *E. amylovora* isolated from pomaceous hosts (lanes b–x) and from *Rubus* (lane a, Ea246). Lane b, Ea321; c, Ea483; d, Ea347; e, Ea338; f, Ea272; g, Ea261; h, Ea259; i, Ea258; j, Ea276; k, Ea491; l, Ea341; m, Ea273; n, Ea266; o, Ea243; p, Ea492; q, Ea505; r, Ea402; s, Ea386; t, Ea303; u, Ea286; v, Ea227; w, Ea223; x, Ea102; y, pCPP430. **B,** Total DNA of strains of *E. amylovora* isolated from *Rubus* hosts (lanes b–i) and from a pomaceous host (lane a, Ea321). Lane b, Ea246; c, Ea416; d, Ea510; e, Ea511; f, Ea514; g, Ea528; h, Ea530; i, Ea531.

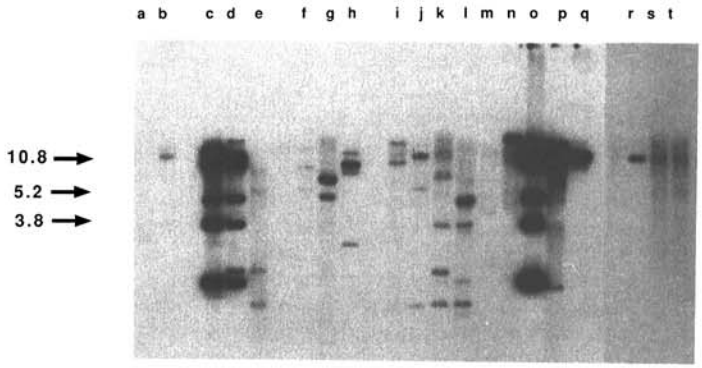


Fig. 3. Hybridization of the pooled Ea-Hrp probe with *Eco*RI-digested total bacterial DNA of selected strains and with cosmids containing DNA from three plant pathogenic species. Lane a, *Erwinia herbicola* Eh282; b, *E. stewartii* DC283; c, *E. amylovora* Ea321; d, *E. amylovora* Ea246; e, *E. nigrifluens* 5D312; f, *E. quercina* 11D3; g, *E. rubrifaciens* 6D3; h, *E. mallotivora* AM1; i, *E. lupinicola* W3L16; j, *E. carotovora* subsp. *atroseptica* Eca001; k, *E. c.* subsp. *betavasculorum* Ecb101; l, *E. c.* subsp. *carotovora* DB17; m, *E. chrysanthemi* 3937; n, *E. chrysanthemi* EC16; o, pCPP430 (*E. amylovora*); p, pES1044 (*E. stewartii*); q, pHIR11 (*P. syringae*); r, pHIR11; s, *P. syringae* pv. *syringae* B728a; t, *P. s.* pv. *syringae* Y30.

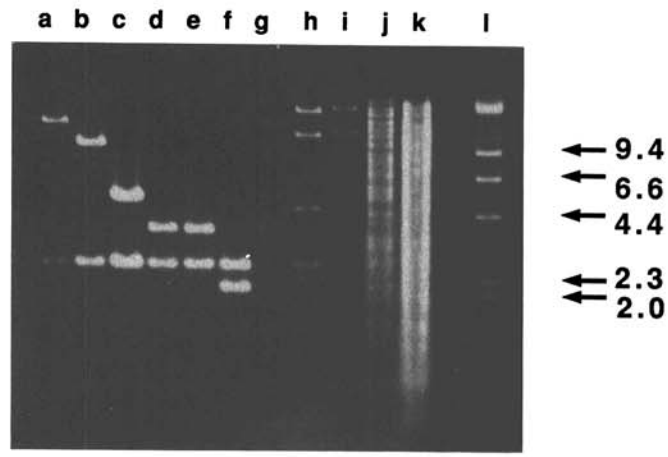


Fig. 4. Cross hybridization between cosmids pCPP430, pES1044, pHIR11 and total DNA from selected species of *Erwinia*. DNA digested with *Eco*RI, and subsequently transferred to membrane by Southern blotting. Lane a, pCPP1002; b, pCPP1003; c, pCPP1005; d, pCPP1006; e, pCPP1007; f, pCPP1008; g, pCPP430; h, *Hin*dIII-digested pES1044; i, pHIR11; j, total DNA of *E. rubrifaciens* 6D3; k, total DNA of *E. carotovora* subsp. *carotovora* DB17; l, *Hin*dIII-digested lambda DNA.

Fig. 1), pHIR11 (containing the *hrp* region of *P. syringae*; Fig. 1), and genomic DNA from *E. carotovora* subsp. *carotovora* (Jones) Bergey *et al.* and *E. rubrifaciens* Wilson, Zeitoun and Fredrickson. The five blots were probed individually with pCPP1002, pCPP1003, pCPP1005, pCPP1008, and a mixture of pCPP1006 and pCPP1007. The resulting patterns of hybridization are shown in Figure 5 and summarized in Table 2. Subsequent work showed that only the rightmost 3.6 kb of pCPP1002 hybridized with pES1044 (data not shown). These same five blots were stripped of hybridized probe and rehybridized with subclones pRF201, pRF203, pRF205, and pRF207 of pES1044 as probes (Fig. 6), and again with subclones pHIR11BB7, pHIR11BG4, and pHIR11E6 of pHIR11 as probes (Fig. 7).

E. stewartii DC283 possesses restriction fragments similar in DNA sequence to a portion of the Ea321 *hrp* region. The conserved region is defined by colinear adjacent cross-hybridizing restriction fragments extending along approximately 18.1 kb of pCPP430 and 18.5 kb of pES1044. These regions include part or all of the DNA inserts cloned in pCPP1002, pCPP1005, pCPP1006, pCPP1007, and pCPP1008 from *E. amylovora*, and part or all of the DNA inserts cloned in pRF201, pRF203, pRF205, and pRF207 from *E. stewartii* (Fig. 6).

Subcloned portions of the *P. s.* pv. *syringae* strain 61 *hrp* region from pHIR11 were used as probes to the same five blots (Fig. 7). This *P. syringae* strain and *E. amylovora* Ea321 contain cross-hybridizing DNA restriction fragments including as much as 12.4 kb in each organism's *hrp* gene cluster. Hybridization results also indicate colinearity of DNA sequences between these conserved portions of the *hrp* regions of *E. amylovora* and *P. s.* pv. *syringae* strain 61. Restriction maps of pCPP430, pES1044, pHIR11, and

Figure 5

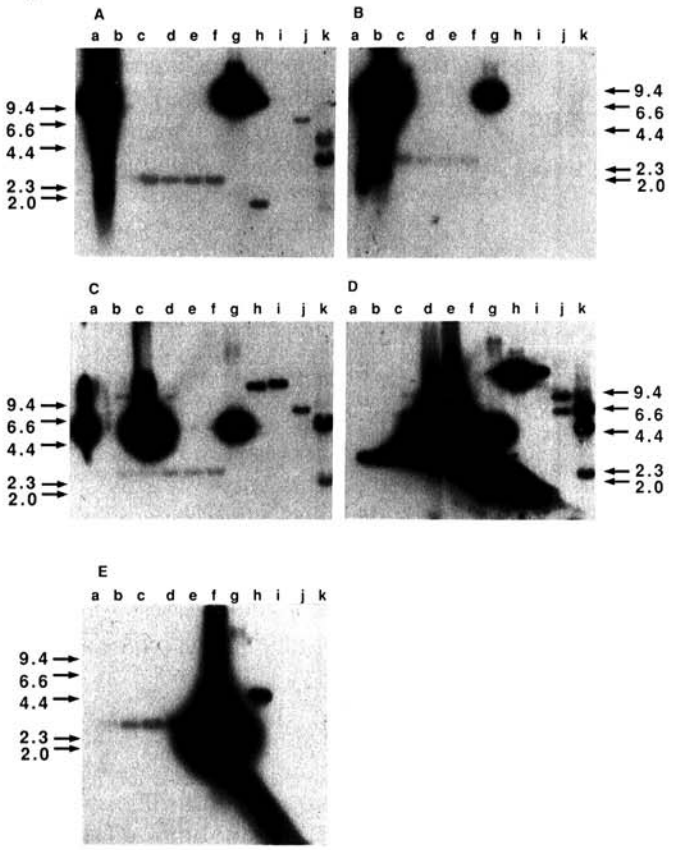


Figure 6

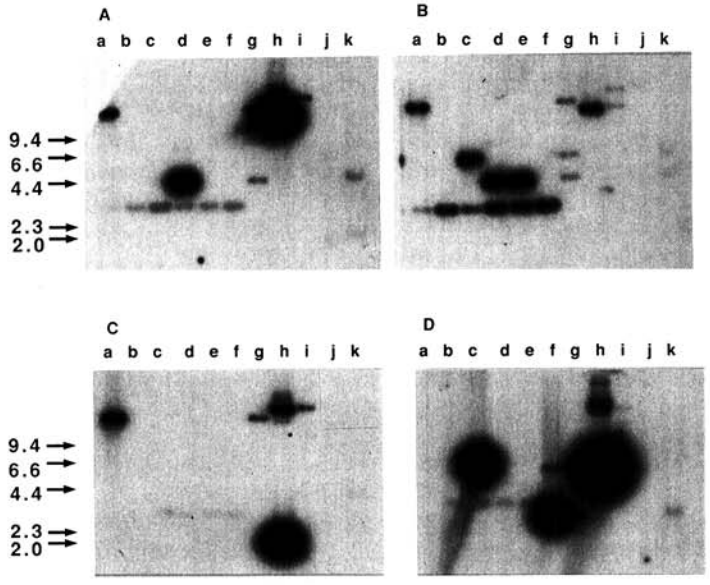
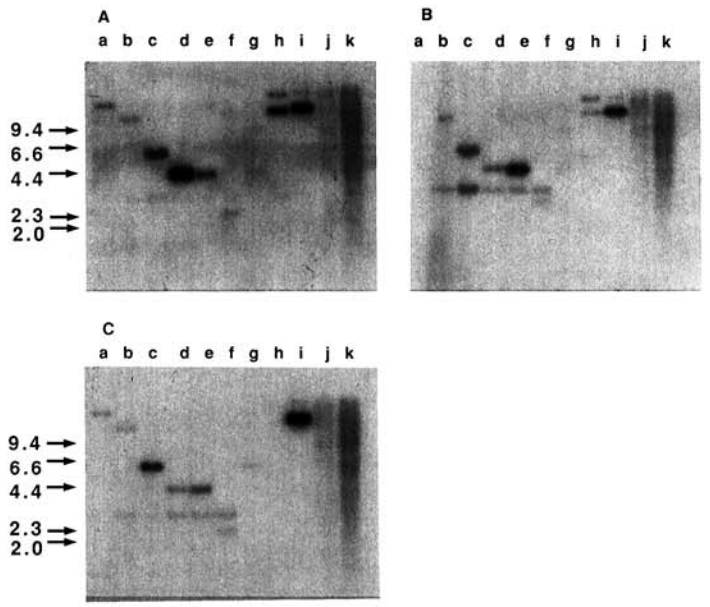


Figure 7



Figs. 5–7. Representative agarose gel containing cosmids pCPP430, pES1044, and pHIR11, and total DNA from selected species of *Erwinia*. Individual blots (as in Fig. 4) probed as follows: 5A, pCPP1002; 5B, pCPP1003; 5C, pCPP1005; 5D, pCPP1006 and pCPP1007; 5E, pCPP1008; 6A, pRF201; 6B, pRF203; 6C, pRF205; 6D, pRF207; 7A, pHIR11BB7; 7B, pHIR11BG4; 7C,pHIR11E6.

Table 2. Summary of cross hybridization results depicted in Figure 4b–d

Immobilized DNA	Probes[a]				
	pCPP1003	pCPP1002	pCPP1006+ pCPP1007	pCPP1005	pCPP1008
pHIR11	−	−	+	+	−
pES1044	−	+	+	+	+
EccDB17	−	+	+	+	−
Eru6D3	−	+	+	+	−
	pRF205	pRF203	pRF203	pRF207	
pHIR11	+	+	+	−	
pCPP430	+	+	+	+	
pCPP1003	−	−	−	−	
pCPP1002	+	+	+	−	
pCPP1006	−	+	+	−	
pCPP1007	−	−	+	−	
pCPP1005	−	−	+	+	
pCPP1008	−	−	−	+	
EccDB17	+	+	+	−	
Eru6D3	+	+	+	−	
	pHIR11BB7	pHIR11BG4	pHIR11E6		
pES1044	+	+	−		
pCPP1003	−	−	−		
pCPP1002	−	−	−		
pCPP1006	+	−	−		
pCPP1007	+	+	+		
pCPP1005	+	+	+		
pCPP1008	−	−	−		
EccDB17	−	−	−		
Eru6D3	−	−	−		

[a] − = No hybridization signal (Figs. 5–7). + = Hybridization signal observed (Figs. 5–7).

subclones are shown in Figure 1; the cross-hybridizing regions (Figs. 5–7) are aligned vertically. Filled boxes with matching patterns illustrate cross-hybridizing restriction fragments.

The Ea-HRP probe also hybridized with sequences of genomic DNA isolated from *E. c.* subsp. *carotovora* DB17 and *E. rubrifaciens* 6D3. The hybridizing probes from *E. amylovora* (pCPP1002, pCPP1005, pCPP1006, and pCPP1007) are a subset of those hybridizing with DNA from *E. stewartii*, but because the hybridizing restriction fragments from *E. c.* subsp. *carotovora* and *E. rubrifaciens* have not been cloned or restriction-mapped, colinearity between these fragments and the *E. amylovora* restriction fragments could not be determined.

Interspecies complementation. *E. stewartii* DNA, cloned in pES1044, pRF201, pRF203, pRF205, pCPP1050, and pCPP1063 restored pathogenicity on pear (Fig. 8) and, in general, the ability to elicit a weak HR on tobacco (Fig. 8) to *E. amylovora* Hrp$^-$ strains with transposon insertions in the corresponding cross-hybridizing region of DNA. The function of *wts* on corn was restored to *E. stewartii wtsB* mutants by pCPP1005 (D. Coplin and R. Frederick, personal communication). A subclone of pHIR11, pHIR11BG4, also restored *E. amylovora* Hrp function to Ea321-K108 (Fig. 8).

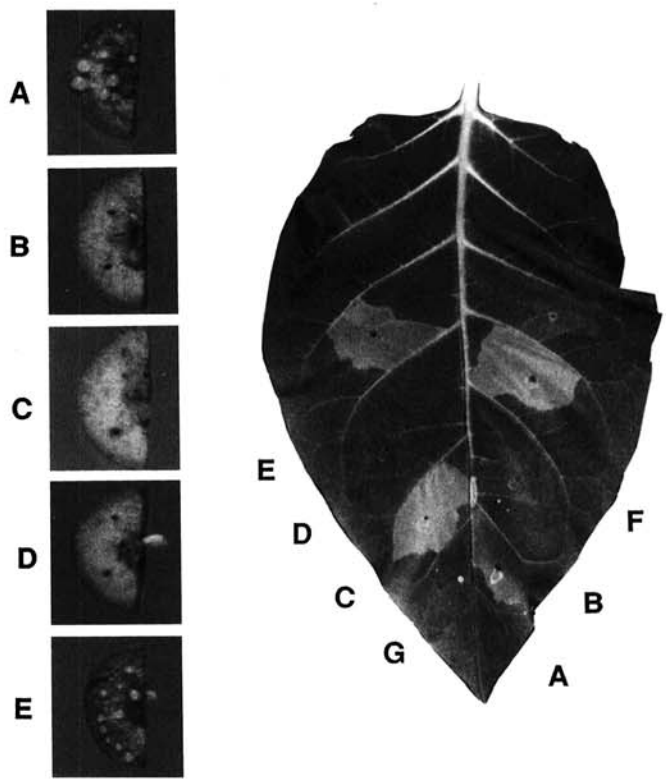


Fig. 8. Restoration of Hrp function to an *Erwinia amylovora* Hrp$^-$ mutant strain by DNA of *E. stewartii* and *Pseudomonas syringae*. Left, pathogenicity assays on immature pear fruit; right, hypersensitive response elicitation assays on tobacco leaf. **A,** *E. amylovora* Ea321; **B,** Ea321K108; **C,** Ea321K108(pES1044); **D,** Ea321K108(pHIR11); **E,** Ea321K108 (pHIR11BG4); **F,** *E. stewartii* DC283; **G,** buffer.

DISCUSSION

Hybridization of portions of the *hrp* gene cluster of one strain of *E. amylovora* indicated a remarkable degree of structural similarity with the DNA of other organisms. The validity of the relationship was strengthened by functional

complementation of certain *E. amylovora* Hrp$^-$ mutants by DNA of *E. stewartii* and *P. syringae*. Most striking was the apparent transfamilial homology, in a colinear manner along about 12 kb, with the *hrp* gene cluster of *P. syringae*. DNA of *E. stewartii* involved in pathogenic functions also hybridized, indicating that this organism may harbor *hrp* genes, although it does not elicit the HR under the usual experimental conditions. Hybridization of DNA of the *hrp* cluster of *E. amylovora* with genomic DNA of several seldom studied non-soft-rotting erwiniae as well as the two major soft-rotting species was unexpected for such diverse pathogens, but it confirms relationships within the genus. The similarity of the *hrp* cluster DNA among many strains of *E. amylovora* indicates apparent fitness of the organism with respect to DNA involved in pathogenesis.

Little restriction fragment length polymorphism (RFLP) in the *hrp* gene cluster of *E. amylovora* was observed among numerous strains of diverse host and geographic origins after *Eco*RI-digested total DNA from these strains was probed. The data of others are consistent with this notion of little variation in *hrp* genes of *E. amylovora*. The *Eco*RI and *Hin*dIII restriction map of the *hrp* gene cluster of strain CFBP1430 (our Ea492) (Barny *et al.* 1990) is identical to that of Ea321 (CFBP1367), and DNA of Ea492 yielded a hybridizing band pattern identical to that of Ea321 and the other non-*Rubus* strains of *E. amylovora* (Fig. 2A). In addition, the map of a portion of the *hrp* cluster reported by Walters *et al.* (1990) is consistent with our data.

The only RFLP seen among *E. amylovora* strains was observed in the genomes of strains isolated from raspberry or blackberry (*Rubus* sp.) (Fig. 2A,B). The RFLP correlates with limitations in the host ranges of strains of *E. amylovora* isolated from *Rubus* or non-*Rubus* sp. (Starr *et al.* 1951). Although most strains of *E. amylovora* isolated from one host are pathogenic on many other hosts, strains isolated from *Rubus* spp. cause symptoms only on plants of that genus. Similarly, non-*Rubus*-derived strains do not cause symptoms on *Rubus* spp. Introduction of pCPP430 into *Rubus*-derived strains did not confer on them the ability to produce ooze on immature pear fruit (data not shown).

Most *Erwinia* species tested yielded some hybridization signal in lowered stringency hybridization experiments (Fig. 3). On the basis of the temperature at which the hybridization signal was lost (Meinkoth and Wahl 1984; Bonner *et al.* 1973), the DNA sequence similarity between cross-hybridizing *E. amylovora* sequences and those of other phytopathogenic erwiniae is approximately 80% or greater. Whether the cross-hybridizing sequences in other erwiniae are also in pathogenicity-associated genes has been confirmed only with *E. stewartii*. In that case, the single hybridizing fragment from *Eco*RI-digested genomic DNA is identical in size to the hybridizing fragment from *Eco*RI-digested pES1044 DNA.

Hrp function was restored to *E. amylovora* Ea321 Hrp$^-$ strains by DNA of both *E. stewartii* and *P. syringae*. These results indicate common pathogenicity-related functions between the *hrp* gene clusters of *E. amylovora* and *P. syringae*, as well as the *wts* gene cluster of *E. stewartii*. The colinearity of cross-hybridizing DNA restriction fragments in these *hrp* and *wts* gene regions, as well as (partial) restoration of function to appropriate mutants within the cross-hybridizing regions of DNA, indicates substantial conservation of genetic organization and pathogenic mechanism.

If the hybridizing restriction fragments from the various erwiniae examined represent functional *hrp* genes, functions encoded by *hrp* genes are implicated in the disease-causing ability of all plant pathogenic erwiniae. The pectolytic *Erwinia* do not cause typical necrotic or watersoaked lesions or elicit a typical HR. However, the action of pectic enzymes may mask these indications of *hrp* gene function in those species.

Precisely which subcloned fragment(s) of pCPP430 was responsible for the hybridization signal (Fig. 3) was determined only with respect to *E. stewartii*, *E. rubrifaciens*, and *E. c.* subsp. *carotovora* DNA. In the case of *E. stewartii*, adjacent colinear restriction fragments of pES1044, 18.1 kb in total length, hybridized to adjacent colinear restriction fragments of pCPP430, 18.5 kb in length (Table 2). Some, but not all, of these individual restriction fragments of pCPP430 hybridized with DNA from *E. rubrifaciens* and *E. carotovora*. Hybridization of pRF203 with pCPP1002 also was seen, however. This may reflect the presence of repeated highly similiar DNA sequences within the gene cluster or may indicate DNA rearrangements.

Lindgren *et al.* (1988) reported that *hrp* genes of several *P. syringae* pathovars are conserved and interchangeable among these pathovars. DNA hybridization and genetic complementation data supported this claim. The strength of the hybridization signals seen generally corresponded to the taxonomic distance between the pathovars as measured by DNA-DNA hybridization in solution. In that study, no DNA hybridization was observed between the probe derived from *P. s.* pv. *phaseolicola* (Burkholder) Young *et al.* and DNA from organisms other than *P. syringae*.

In contrast, under the low stringency conditions used in this study, genomic DNA of *P. s.* pv. *syringae* strains Y30 and B728a yielded a hybridization signal with probes derived from *E. amylovora* (Fig. 3). When cloned DNA containing *hrp* genes of *P. syringae* 61 was tested for cross hybridization with portions of pCPP430, adjacent colinear restriction fragments of pHIR11, approximately 12 kb in length, hybridized with adjacent colinear restriction fragments of pCPP430. With the same methods, genomic DNA from other gram-negative bacteria gave no detectable hybridization signal.

Although Barny *et al.* (1990) used cloned plasmid DNA from *P. s.* pv. *phaseolicola*, *P. s.* pv. *tomato* (Okabe) Young *et al.*, and *P. solanacearum* (Smith) Smith in cross-hybridization studies with pPV130 from *E. amylovora* CFBP1430, they did not detect interspecific DNA sequence similarity. Differences in their Southern blot, probe preparation, hybridization, and washing procedures may account for the different results obtained.

The DNA sequence similarity and interspecies genetic complementation observed between the *hrp* gene cluster of *E. amylovora* on pCPP430, the *wts* gene cluster of *E. stewartii* on pES1044, and the *hrp* gene cluster of *P. syringae* on pHIR11, as well as sequence similarity in total DNA from numerous other erwiniae, suggest common patho-

genicity-related functions among these bacteria. Further work, including DNA sequencing, may suggest if the ultimate origin of these genes was within the Enterobacteriaceae or Pseudomonadaceae.

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