MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 5, pp. 493-499, 1991

Further Characterization of an *hrp* Gene Cluster of *Erwinia amylovora*

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Received 8 February 1991. Revised 9 May 1991. Accepted for publication 18 June 1991.

Two independent Tn*5*-induced mutants of *Erwinia amylovora*, Ea321T102 and Ea322T101, were identified that failed to elicit a hypersensitive response (HR) in a nonhost plant, tobacco. The two also were nonpathogenic on immature pear fruit. Two naturally occurring nonpathogenic strains, P66 and CFPB1376, also were found incapable of eliciting an HR. Three previously reported Tn*5*-induced nonpathogenic mutants (Steinberger and Beer, Mol. Plant-Microbe Interact. 1:135-144, 1988) were found to elicit a variable HR (Ea321T101 and Ea321T104) or a normal HR (Ea322T104). Two recombinant plasmids and a previously described cosmid containing wild-type *E. amylovora* DNA restored pathogenicity and the ability to elicit the HR to the seven strains. Restriction mapping and hybridization showed that the cosmid and plasmids overlap; thus, the mutated genes are clustered. Functional analysis of subclones from the two plasmids was used to determine the approximate region of DNA complementing each mutation. These results were combined with some results reported previously and the results of additional tests for complementation. The analysis revealed a cluster of at least six complementation regions involved in pathogenicity of host plants and elicitation of the HR in a nonhost plant.

Additional keywords: fire blight, *Pyrus communis*.

The introduction of bacterial plant pathogens into host plants under disease-conducive conditions results in a compatible interaction between plant and bacterium. Bacteria multiply dramatically and disease symptoms begin to appear after several days. In contrast, when a bacterial pathogen is introduced into a nonhost plant or a resistant cultivar, a resistant response (incompatible interaction) results. Many bacterial pathogens elicit a particular response associated with resistance, termed the hypersensitive response (HR) (Klement 1982; Sequeira 1984; Kiraly 1980). The HR is characterized by rapid (usually within 24 hr) collapse and death of the plant cells in contact with the pathogen. The pathogen is localized and further bacterial growth is restricted.

The genetics and biochemistry of plant-bacterial interactions are being studied using molecular biological techniques with a variety of bacterial disease models (Daniels *et al.* 1988; Hutcheson *et al.* 1989). Our laboratory has concentrated on *Erwinia amylovora* (Burrill) Winslow *et al.*, the causal agent of fire blight of apple, pear, and other rosaceous plants (Aldwinckle and Beer 1979; Van der Zwet and Keil 1979). Although *E. amylovora* was the first bacterium shown to cause disease in plants, the biochemical mechanisms by which it causes disease are still unknown. Extracellular polysaccharide has been shown by several studies to play a role in virulence (Bennett and Billing 1977; Hignett 1987; Steinberger and Beer 1988; Vanneste *et al.* 1990). However, the ability to produce extracellular polysaccharide is not sufficient to cause disease, as shown by the existence of extracellular-polysaccharide-producing, nonpathogenic strains (Bennett 1980; Steinberger and Beer 1988; Vanneste *et al.* 1990).

Previously in this laboratory transposon mutagenesis with Tn*5* was used to produce 22 mutants of *E. amylovora* that were nonpathogenic, or greatly reduced in virulence, on immature pear fruits and apple seedlings (Steinberger and Beer 1988). Several of the mutants also were incapable of eliciting an HR on tobacco (Steinberger and Beer 1988). Similar mutants have been described in a large number of plant pathogenic bacteria (Willis *et al.* 1991). Genes controlling this phenotype have been designated *hrp*, for *h*ypersensitive *r*esponse and *p*athogenicity (Lindgren *et al.* 1986). Several groups have described the production of mutants and the cloning of *hrp* genes from *E. amylovora* (Barny *et al.* 1990; Steinberger and Beer 1988; Vanneste *et al.* 1990; Walters *et al.* 1990).

This paper details the further characterization of a cluster of *hrp* genes of *E. amylovora*. Initial portions of this work were carried out simultaneously with work reported previously from this laboratory (Steinberger and Beer 1988). Several aspects of that work were reexamined and revised. The results were integrated with the main results of this study. Some preliminary accounts of this work have been published (Bauer and Beer 1987a; Bauer and Beer 1987b).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. All bacterial strains were maintained at −130° C in 15% glycerol. *Escherichia coli* was cultured routinely on L broth without glucose (LB) (Lennox 1955) or L broth with 1.8% agar (LA) at 37° C. *E. amylovora* was cultured at 28°-30° C on L broth medium, nutrient broth-yeast extract (NBY) medium (Schaad 1980), or minimal medium (Steinberger and Beer 1988). Antibiotics were added to media, when appropriate,

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at the following concentrations (μg/ml): ampicillin, 100; carbenicillin, 300; chloramphenicol, 20; kanamycin, 50; spectinomycin, 50; streptomycin, 25; and tetracycline, 10.

Hypersensitive response tests and pathogenicity tests. Tobacco plants (*Nicotiana tabacum* L. 'Xanthi') were grown in artificial soil mix to 90–100 cm in height under high light intensity (natural greenhouse lighting supplemented with artificial light in winter). A few hours to at most 1 day before inoculation, the plants were moved to a laboratory bench with relatively low light, temperature of 25 ±3° C and uncontrolled relative humidity. Bacterial suspensions containing 1×10^8 colony-forming units (cfu)/ml to 1×10^9 cfu/ml in 5 mM potassium phosphate buffer, pH 6.5, were infiltrated through a hole made in the leaf lamina with a simple dissecting needle. One side of the hole was covered with the outlet of a 1-ml syringe (without needle) containing a bacterial suspension; the other side was sealed with a finger and the suspension was forced into the intercellular spaces in the leaf. Pathogenicity tests on immature pear fruits and apple seedlings were done as described previously (Steinberger and Beer 1988).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Escherichia coli		
HB101	F⁻, *leuB6, proA2, recA13, thi-1, ara-14, lacY1, galK2, xyl-5, mtl-1, rpsL20, supE44, hsdS20*	Maniatis *et al.* 1982
DH1	F', *gyrA96, recA1, relA1, endA1, thi-1, hsdR17, supE44*, λ⁻	Hanahan 1983
DH5	Same as DH1	Bethesda Research Laboratories[a]
JC3272	F⁻, His, Trp, Lys, Sm^r, Gal, *lac*Δ×74, λ^r	Achtman *et al.* 1971
Erwinia amylovora		
Ea321	Isolated from hawthorn; CFPB1367	J.-P. Paulin[b]
Ea322	Isolated from pear; CFPB1368	J.-P. Paulin[b]
Ea321T101	Tn*5*-induced Hrp mutant of Ea321	Steinberger and Beer 1988
Ea321T102	Tn*5*-induced Hrp mutant of Ea321	Steinberger and Beer 1988
Ea321T104	Tn*5*-induced Hrp mutant of Ea321	Steinberger and Beer 1988
Ea322T101	Tn*5*-induced Hrp mutant of Ea322	Steinberger and Beer 1988
Ea322T104	Tn*5*-induced Dsp mutant of Ea322	Steinberger and Beer 1988
P66	Nonpathogenic strain	E. Billing[c]; Bennett 1980
CFPB1376	Nonpathogenic strain isolated from cotoneaster	J.-P. Paulin[b]
Plasmids		
pBR322	Ap^r, Tc^r, Cm^r, pMB1 *ori*V	Bolivar *et al.* 1978
pBR325	Ap^r, Tc^r, CM^r, pMB1 *ori*V	Bolivar *et al.* 1977
pBW7	Ap^r, Tc^r, Tra, RP1 derivative with deletion of Km^r and IS21	Rella *et al.* 1985
pGB2	Sp^r, Sm^r, pS101 *ori*V, par	Churchward *et al.* 1984
pCPP8	pGB2-mob	Bauer *et al.*, unpublished
pCPP101	pBR322 containing 21-kb *Eco*R1 fragment from Ea322	This work
pCPP102	pBR322 containing 21-kb *Eco*RI fragment with Tn*5* from Ea321T102	This work
pCPP103	pBR322 containing 18-kb *Eco*RI fragment with Tn*5* from Ea321T102	This work
pCPP114	pBR325 containing 11.4-kb *Sau*3A fragment from Ea322	This work
pCPP120	pBR322 containing 15.8-kb *Eco*RI fragment from Ea322	This work
pCPP410	pCPP9 with 38-kb Ea321 insert	Steinberger and Beer 1988

[a] Gaithersberg, MD.
[b] Institut National de la Recherche Agronomique, Angers, France.
[c] East Malling Research Station, Kent, England.

Bacterial transformations and conjugations. Strains of *E. coli* were transformed with plasmid DNA according to the procedure of V. Simanis as described by Hanahan (1985). Strains of *E. amylovora* were transformed according to the procedure of Bauer and Beer (1983).

Plasmids constructed with the vector pCPP8 (D. W. Bauer, A. B. Sprenkle, and S. V. Beer, unpublished) were conjugated from *E. coli* into *E. amylovora* by triparental mating (Ditta *et al.* 1980) using *E. coli* JC3272(pBW7) (Rella *et al.* 1985) as the helper strain and plasmid.

Recombinant DNA techniques. Genomic DNA was isolated as described by Silhavy *et al.* (1984), modified by eliminating the RNase treatment and the subsequent extraction and precipitation steps. Plasmid minipreparations were by an alkaline procedure (Birnboim 1983). Other recombinant DNA procedures were preformed essentially according to Maniatis *et al.* (1982). Southern blot analyses and restriction enzyme mapping were done as described previously (Steinberger and Beer 1988).

Construction of genomic libraries. Genomic DNA of wild-type *E. amylovora* strain Ea322 was partially digested with *Eco*RI or *Sau*3A and fractionated on a 10–40% sucrose gradient (Maniatis *et al.* 1982). *Eco*RI fragments of about 10–25 kb were ligated to *Eco*RI-digested pBR322. *Sau*3A fragments were ligated to *Bam*HI-digested pBR325. The recombinant plasmids were transformed into *E. coli* HB101.

Marker exchange. Plasmids with cloned Tn*5*-containing fragments from each mutant were transformed into Ea321

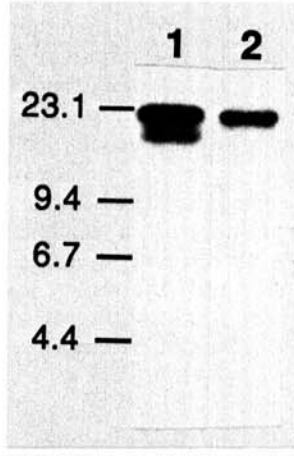

Fig. 1. Southern hybridization of *Eco*RI-digested genomic DNA from Tn*5*-induced mutants of *Erwinia amylovora* probed with ^{32}P-labeled pRZ102 to detect Tn*5*. Lane 1, Ea321T102; lane 2, Ea322T101. The sizes for λ *Hin*dIII size standards are given in kilobases.

or Ea322. The resulting strains were grown for 2 days in 100 ml of minimal medium with kanamycin. The culture was diluted and spread on LA containing kanamycin, and individual colonies were picked to LA, with and without tetracycline, to identify colonies that had lost the plasmid and were potential marker-exchange mutants.

Construction of subclones. Random fragments from pCPP114 (Table 1) were cloned by digesting the plasmid to completion with *Eco*RI, *Hin*dIII, or *Pst*I, ligating with appropriately digested plasmid pGB2 (Churchward *et al.* 1984) or pCPP8 (D. W. Bauer, A. B. Sprenkle, and S. V. Beer, unpublished), and transforming the DNA into *E. coli* DH1 or DH5. Random fragments from pCPP120 (Table 1) were similarly cloned using *Bam*HI, *Hin*dIII, *Sal*I, or a combination of *Bam*HI and *Bgl*II. The presence of particular inserts was confirmed by agarose gel electrophoresis of plasmid minipreparations, which had been digested with appropriate restriction enzymes.

RESULTS

Identification of Tn*5*-induced and naturally occurring HR$^-$ strains. Tn*5*-containing transconjugant derivatives of Ea321 and Ea322 were produced by E. M. Steinberger by conjugation with *E. coli* 1830(pJB4JI) (Beringer *et al.* 1978) as described (Steinberger and Beer 1988). Approximately 5,200 transconjugants were tested for elicitation of the HR in tobacco without regard to their reaction on pear. Two mutants, Ea321T102 and Ea322T101, failed to elicit the HR. Both mutants were nonpathogenic when tested on immature pear fruits (Steinberger and Beer 1988). No HR$^-$ mutants were found that were pathogenic. Several naturally occurring nonpathogenic strains were tested for elicitation of the HR. Two strains, P66 and CFPB1376, were HR$^-$.

In a previous report (Steinberger and Beer 1988), the nonpathogenic mutants, Ea321T101, Ea321T104, and Ea322T104, were listed as phenotypically HR positive. Reevaluation of the HR reactions elicited by the mutants revealed that Ea321T101 and Ea321T104 produced a sporadic and variable HR. At an inoculum density of 1×10^8 cfu/ml the wild-type strains, Ea321 and Ea322, elicited a normal HR on tobacco. However, the mutants, Ea321T101 and Ea321T104, elicited either no response, a weak HR characterized by partial collapse of the infiltrated area, or occasionally complete collapse within 24 hr. At high inoculum densities ($>1 \times 10^9$ cfu/ml) Ea321T101 and Ea321T104 usually elicited a normal HR, but sometimes elicited a weak HR or no HR. Strain Ea322T104 elicited a normal HR at both inoculum densities. In contrast, strains Ea321T102, Ea322T101, P66, and CFPB1376 did not elicit an HR even at high inoculum densities. In the initial experiments (Steinberger and Beer 1988), all tests had been conducted at inoculum densities of 1×10^9 cfu/ml or greater.

Physical characterization of Tn*5* insertions and cloning of Tn*5*-containing fragments. Southern blots of *Eco*RI-digested genomic DNA of Ea322T101 and Ea321T102 were probed with ^{32}P-labeled pRZ102 (Jorgensen *et al.* 1979) to detect Tn*5*. A single hybridizing band of about 21 kb was found in Ea322T101 and two bands of about 22 and 18 kb were found in Ea321T102 (Fig. 1). Previously, Ea322T101 was reported to have four insertions (Steinberger and Beer 1988). The reason for the discrepancy may be due to additional transpositions upon repeated transfer and growth of the strain analyzed by Steinberger and Beer (1988). The Ea322T101 stain analyzed here was taken from long-term frozen storage and used immediately.

The Tn*5*-containing *Eco*RI fragments from Ea322T101 and Ea321T102 were cloned in pBR322. The plasmid containing the insert from Ea322T101 was designated pCPP101. Two inserts of about 21 and 18 kb were obtained from Ea321T102 and the plasmids containing them were designated pCPP102 and pCPP103, respectively.

Marker exchange. To determine which insertion in Ea321T102 was responsible for the HR$^-$ phenotype, pCPP102 and pCPP103 were transformed into Ea321 and marker-exchange mutagenesis was attempted. Colonies in which marker exchange had taken place were obtained only with pCPP103. All marker-exchanged colonies tested

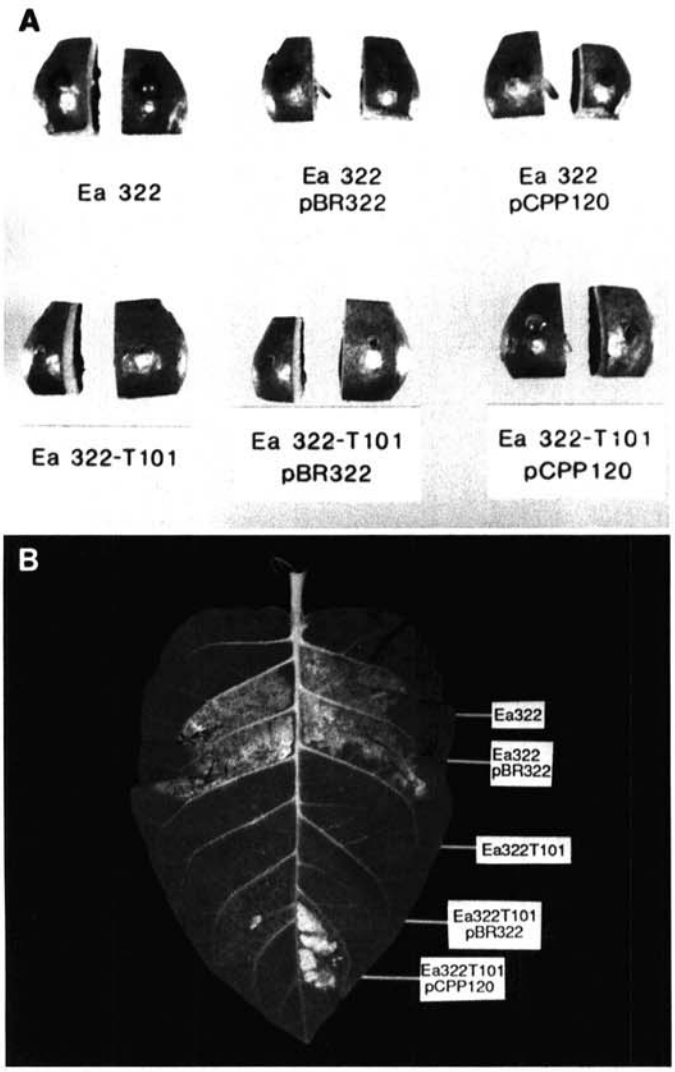

Fig. 2. A, Pathogenicity tests on immature pear fruit of the wild-type strain Ea322 and the Hrp$^-$ mutant, Ea322T101, containing pBR322 or pCPP120. **B,** HR tests on tobacco of Ea322 and Ea322T101 containing pBR322 or pCPP120. The same strains were infiltrated into panels on both sides of the leaf.

were HR^+ and pathogenic, indicating that the insertion cloned in pCPP103 was not the cause of the Hrp^- phenotype. Therefore, pCPP103 was not studied further.

The absence of marker exchange with pCPP102 indicated that the insertion may be Mu::Tn*5* rather than Tn*5*. Transposition of Mu::Tn*5* is common when using pJB4JI (Meade *et al.* 1982). Probing of Ea321T102 DNA with ^{32}P-labeled Mu DNA confirmed the presence of Mu in the mutant (Steinberger and Beer 1988). Cloning of an *Eco*RI fragment containing Tn*5* from a Mu::Tn*5* transpostion would result in one end of the fragment consisting of *E. amylovora* DNA and the other end consisting of Mu DNA. Thus, a double crossover by homologous recombination, required for marker exchange, would be impossible. Restriction digests of pCPP102 produced fragments corresponding in size to those expected from Tn*5* and part of Mu (data not shown).

The insertion cloned in pCPP101 was marker exchanged into Ea322 to determine whether it was the cause of the Hrp^- phenotype of Ea322T101. Several marker-exchanged colonies were tested on tobacco and immature pear fruits and found to be Hrp^-. The result indicated that the Tn*5* insertion in Ea322T101 was the cause of the Hrp^- phenotype.

Complementation of Ea322T101 with cloned wild-type DNA. The screening of about 2,000 colonies from each library by colony hybridization for homology to the Tn*5*-containing *Eco*RI fragment from pCPP101 resulted in the identification of eight plasmids with distinct restriction patterns. The plasmids were transformed into Ea322T101 and tested for restoration of the Hrp^+ phenotype. One plasmid, pCPP120, that weakly restored pathogenicity on pear fruits and the HR on tobacco (Fig. 2) was chosen for further study.

The weak complementation for pathogenicity by pCPP120 could be due to instability of the plasmid in the absence of antibiotic selection as has been reported by others (Niepold *et al.* 1985; Barny *et al.* 1990). Kanamycin-resistant *E. amylovora* cells were isolated from ooze that developed after 5 days on eight pear fruits inoculated with Ea322T101 (pCPP120). Only 1–8% of the colonies were still resistant to tetracycline, indicating rapid loss of pCPP120. Colonies that had lost pCPP120 were nonpathogenic and HR^-. It appears that a small percentage of pathogenic cells provide an environment in which nonpathogenic cells also can grow. Similar findings were reported with revertants of Mu-derived nonpathogenic mutants of *E. amylovora* (Vanneste *et al.* 1990).

Complementation of additional Hrp^- mutants. In a previous report a cluster of genes was identified in *E. amylovora* that was involved in pathogenicity and elicitation of the HR (Steinberger and Beer 1988). The plasmids that hybridized to pCPP101 were transformed into each of the Hrp^- mutants and tested for complementation. Plasmid pCPP120 weakly complemented Ea321T104 and P66 in addition to Ea322T101. Plasmid pCPP114 complemented

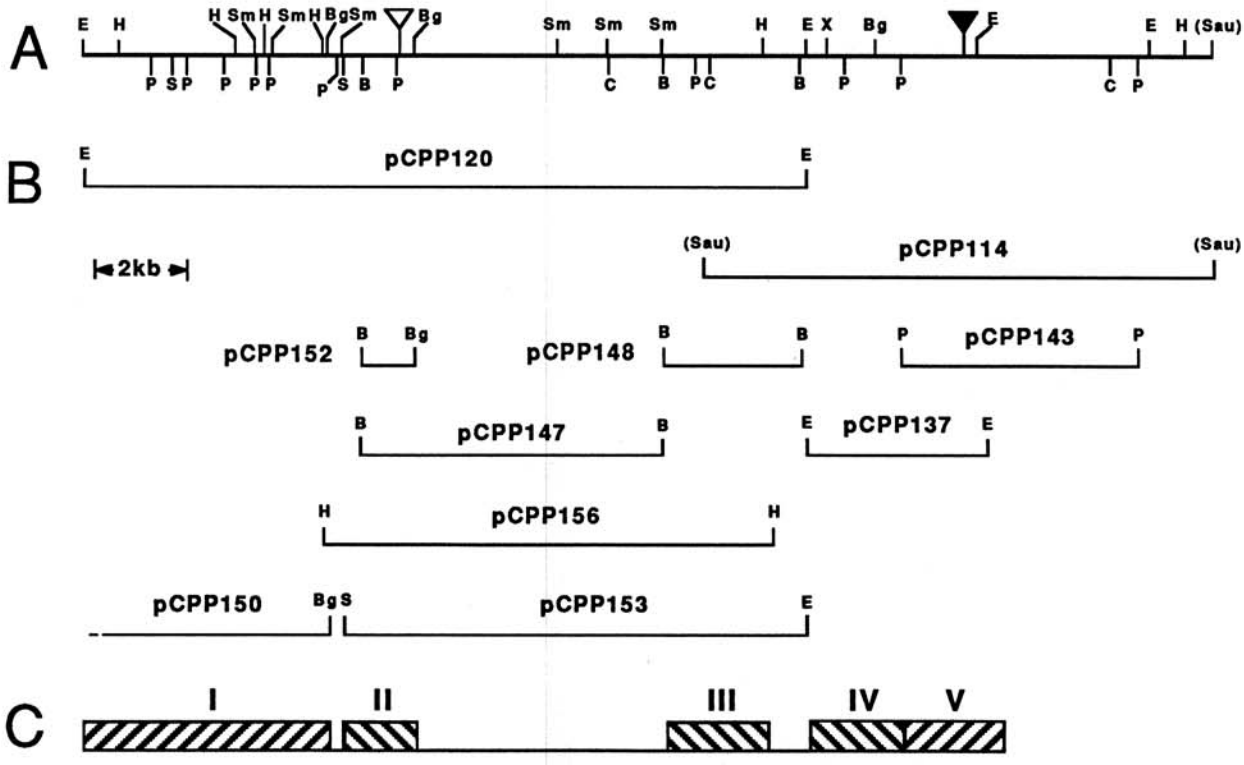

Fig. 3. A, Combined restriction map of the Ea322 DNA contained in pCPP114 and pCPP120. B, *Bam*HI; Bg, *Bgl*II, C, *Cla*I; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sau, *Sau*3A; Sm, *Sma*I; X, *Xho*I. Open triangle indicates site of insertion of Tn*5* in Ea322T101; filled triangle indicates site of insertion of Mu::Tn*5* in Ea321T102. **B,** Regions of Ea322 DNA contained in various subclones of pCPP114 and pCPP120. Dashed lines indicates that the subclone contains some DNA from the original vector. Only plasmids that complemented one or more mutants are shown. **C,** Regions complementing each mutant as determined from overlapping subclones.

Ea321T102 and CFPB1376. Mapping of plasmids pCPP114 and pCPP120 with several restriction enzymes indicated an overlap of about 2.1 kb (Fig. 3), which was confirmed by Southern blot hybridization (data not shown). The approximate locations of the Tn*5* insertion in Ea322T101 and the Mu::Tn*5* insertion in Ea321T102 were determined by mapping pCPP101 and pCPP102, respectively (Fig. 3). Subsequent experiments showed a lack of restriction fragment length polymorphism in the *hrp* region between Ea321 and Ea322 (Fig. 4; R. Laby, personal communication). Thus, the site of insertion in Ea321T102 on the map generated from Ea322 DNA is justified.

Delineation of regions complementing specific mutants. Subclones of pCPP114 and pCPP120 were used to delineate further the regions of DNA required for complemention of the mutants. Based on the results, the region complementing each mutant was determined (Fig. 3). Five separate regions, I through V, were found that complemented the mutations in strains Ea321T104, Ea322T101, P66, CFPB1376, and Ea321T102, respectively. The mutations in Ea322T101 and P66 probably belong to the same transcriptional unit, because complementation of Ea322T101 for HR elicitation never occurred without complementation of P66.

In several cases (pCPP150 in Ea321T104 and pCPP147 or pCPP152 in Ea322T101) (Fig. 3) the plasmids complemented weakly for pathogenicity in 10–40% of the pears tested and did not complement for HR elicitation. It is possible that such subclones do not contain the entire transcriptional unit required for complementation. The sporadic positive results may have been due to homologous recombination between the plasmid and the chromosome that restored the transcriptional unit. The pear fruit responded to the few recombinants, but the tobacco leaf did not. When *E. amylovora* was isolated from ooze from the occasionally infected pear, up to 50% of the colonies were no longer resistant to spectinomycin (vector) or kanamycin (Tn*5*). These bacteria were Hrp$^+$, suggesting that double recombination resulting in exchange of the mutated region for the wild-type region occurred. Control inoculations with the mutant alone, or the mutant containing the vector, never resulted in a pathogenic reaction on pears. Similar results were reported by Barny *et al.* (1990) and Steinberger and Beer (1988).

Map of *E. amylovora* DNA region containing *hrp* genes. While the experiments reported here were in progress, similar studies were under way using a cosmid library of Ea321 DNA (Steinberger and Beer 1988). Cosmid pCPP410 was reported to complement Ea321T102 and Ea322T101. However, pCPP410 did not complement strains P66 and CFPB1376 (E. Steinberger, personal communication). In Ea322, the region complementing P66 and CFPB1376 lies between the regions complementing Ea321T102 and Ea322T101 (Fig. 3). To determine the possibility that a difference in the organization of the genes existed between Ea321 and Ea322, a Southern blot containing *Eco*RI and *Hin*dIII-digested genomic DNA from Ea321 and Ea322, and plasmids pCPP114, pCPP120, and pCPP410 was probed with ^{32}P-labeled pCPP410 (Fig. 4). The result showed that the hybridization patterns obtained with Ea321 and Ea322 were identical. However, the Southern blot showed that pCPP410 hybridization with pCPP120, but not with pCPP114. Therefore, we reexamined all of the complementation results for pCPP410 reported by Stein-

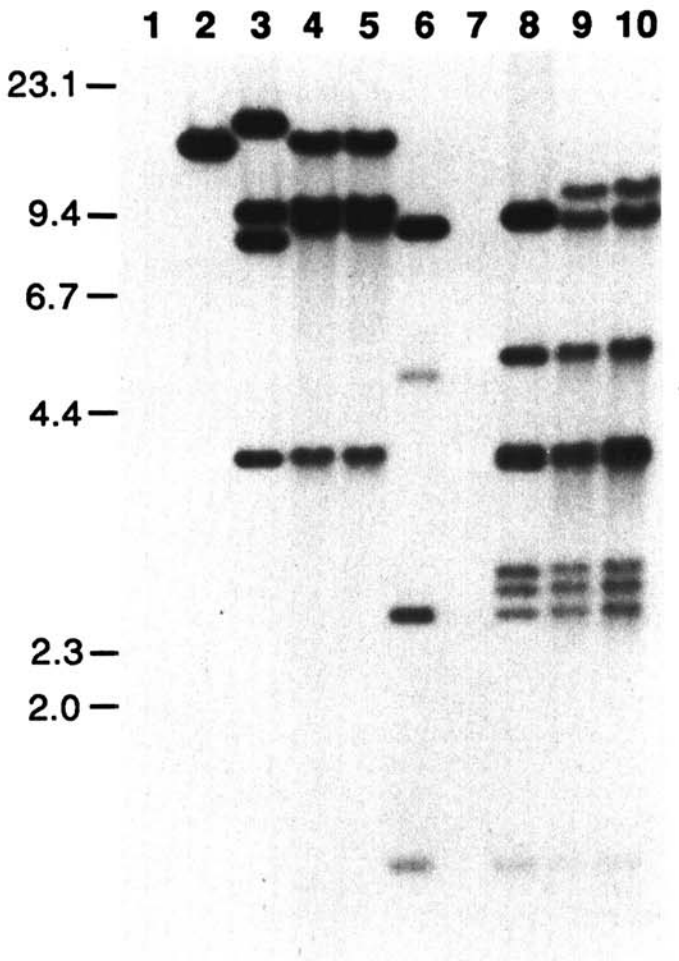

Fig. 4. Autoradiograph of Southern blot of pCPP114, pCPP120, pCPP410, Ea321 genomic DNA, and Ea322 genomic DNA probed with ^{32}P-labeled pCPP410. Lane 1, pCPP114; lane 2, pCPP120; lane 3, pCPP410; lane 4, Ea321; lane 5, Ea322; lane 6, pCPP120; lane 7, pCPP114; lane 8, pCPP410; lane 9, Ea321; lane 10, Ea322. Lanes 1–5 contain *Eco*RI digests. Lanes 6–10 contain *Hin*dIII digests.

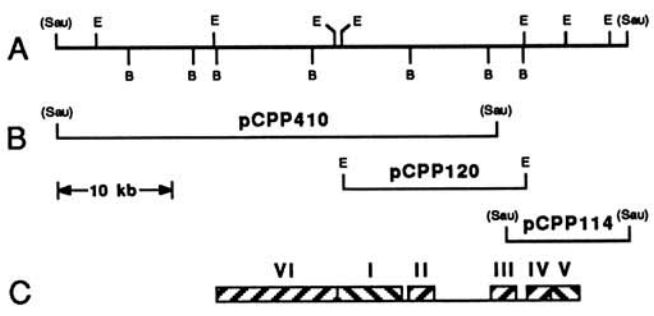

Fig. 5. A, Combined restriction map of *Erwinia amylovora* DNA contained in pCPP114, pCPP120, and pCPP410. B, *Bam*HI; E, *Eco*RI; Sau, *Sau*3A. **B,** Regions of Ea321 and Ea322 DNA contained in the cosmid and plasmids. **C,** Regions complementing various mutants as determined from overlapping clones. The mutations in Ea321T101 and Ea322T104 map into the same region.

berger and Beer (1988). We found that pCPP410 was able to complement Ea322T101 but not Ea321T102. The most likely explanation for the descrepancy is that Ea322T101 had been inadvertently used for Ea321T102 in the experiments described by Steinberger and Beer (1988). In addition, Ea322T104 was complemented by pCPP410, in contrast to the previously published results (Steinberger and Beer 1988).

A composite restriction map (Fig. 5) was constructed based on the maps of pCPP114, pCPP120, and pCPP410. The restriction sites for the region covering pCPP410 have been modified based on more recent data (R. Laby, personal communication). The diagram shows the regions covered by the plasmids as well as the approximate region that complemented each of the mutants. When this analysis was used, the mutations in Ea321T101 and Ea322T104 could not be separated but were complemented by a sixth region (Fig. 5). The mapping and complementation results revealed a cluster of genes in *E. amylovora* involved in disease and elicitation of the HR as was described previously (Steinberger and Beer 1988). The cluster consists of at least six complementation regions based on complementation for pathogenicity.

DISCUSSION

A cluster of genes involved in pathogenesis and elicitation of the HR has been identified in *E. amylovora*. Most of the genes are required for both pathogenicity on the host plants, pear and apple, and for elicitation of the HR on a nonhost plant, tobacco. These mutants have the Hrp^- phenotype that has been described for a large number of plant pathogenic bacteria (Willis *et al.* 1991).

At least one gene in the cluster is required for pathogenicity, but not the HR. Boucher *et al.* (1987) have proposed the designation *dsp* (*d*isease-*sp*ecific) for genes involved in pathogenicity, but no other phenotype. The association of *hrp* and *dsp* genes in *E. amylovora* has been reported previously (Steinberger and Beer 1988; Barny *et al.* 1990). The Dsp mutant described here (Ea322T104) is distinct from the Hrp mutants and most other nonpathogenic mutants of *E. amylovora* that appear to be altered in production of extracellular polysaccharide (Steinberger and Beer 1988). Similar mutants were produced by Vanneste *et al.* (1990) using phage Mu as the mutagen.

Based on mapping and complementation results, the cluster of *hrp* and *dsp* genes described here spans at least 30 kb of *E. amylovora* chromosomal DNA. However, the boundaries of the region cannot be determined from the present data. Our results are very similar to those described by Barny *et al.* (1990). Their nonpathogenic mutants mapped into a single region of nearly 30 kb. A comparison of the restriction maps reveals that they are identical or nearly so.

Barny *et al.* (1990) divided their virulence region into an *hrp* cluster and a *dsp* cluster. Based on our present data we are unable to clearly differentiate two separate clusters. Their *dsp* cluster maps into the same region as the mutation in our strains Ea321T101 and Ea322T104. The mutation in Ea321T104 maps in the region between their *hrp* and *dsp* clusters. We found that Ea321T101 and Ea321T104 were variable in their ability to elicit the HR in tobacco; thus, we consider them to be a subset of Hrp mutants. The differences between our results and those of Barny *et al.* (1990) may be due to the way the assays were performed or in interpretation of the results. Barny *et al.* (1990) described using 10^9 bacteria per milliliter in the HR assays. We found that the clearest differences between the wild-types and Ea321T101 or Ea321T104 occurred when the inoculum contained 1×10^8 bacteria per milliliter, or less. Under these conditions the mutants usually elicited no HR or a weak HR. Occasionally, however, the HR reactions were normal. Variation in sensitivity of individual tobacco plants accounts for a least some of the variability observed in the reaction elicited by strains. The basis for variation in sensitivity may be genetic or environmental. In any case, intensity of the HR elicited varies among plants and among leaves on individual plants.

Earlier, we identified strain P66 as unable to elicit the HR and reported its complementation by DNA from the pathogenic strain, Ea322 (Bauer and Beer 1987). We have shown that the lesion in P66 that results in the Hrp phenotype maps in the *hrp* cluster. Independently, Walters *et al.* (1990) reported the cloning of the gene that complemented the strain P66. An examination of the restriction map of the clone isolated by Walters *et al.* (1990) shows that it is identical or nearly identical to the region we have mapped. Previously, Walters *et al.* (1990) reported that there was no clear similarity between their map and the map published by Steinberger and Beer (1988). Revision of the restriction map of Steinberger and Beer (1988) has eliminated this discrepancy.

Clusters of *hrp* genes have been described in *Pseudomonas syringae* van Hall pv. *phaseolicola* (Lindgren *et al.* 1986), *P. s.* pv. *syringae* (Huang *et al.* 1988), *P. solanacearum* (Smith) Smith (Boucher *et al.* 1987), and *Xanthomonas campestris* (Pammel) Dowson pv. *vesicatoria* (Bonas *et al.* 1991), in addition to *E. amylovora*. The cluster from *P. s.* pv. *syringae* cloned by Huang *et al.* (1988) spans 31 kb and contains all of the genes necessary for the elicitation of the HR by *P. fluorescens* and *E. coli*. None of the cosmids or plasmids of *E. amylovora* described here enables *E. coli* to elicit the HR. This is not surprising, because none of the cosmids or plasmids contains the entire gene cluster, based on their individual inability to complement all Hrp^- mutants. Neither Barny *et al.* (1990) nor Walters *et al.* (1990) reported that their clones enabled *E. coli* to elicit the HR.

The cosmids and plasmids described here provide the basis for further characterization of the *E. amylovora hrp* cluster, which is in progress. Identification of additional cosmids that flank the present clones, along with mutagenesis of the clones, should result in the identification of the borders of the cluster and a determination of the number of transcriptional units therein.

ACKNOWLEDGMENTS

We are grateful to E. M. Steinberger for creation of mutants and helpful discussions. We thank C. H. Zumoff for assistance with certain pathogenicity and HR elicitation tests. The research reported here was supported in part by grants 89-37263-4692, 86-CRCR-1-2225, and 83-CRCR-1-1211 from the Competitive Research Grants Office of the U. S. Department

of Agriculture and the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, the U. S. Army Research Office, and the National Science Foundation.

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