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Creation and Complementation of Pathogenicity Mutants of *Erwinia amylovora*

Eva M. Steinberger and Steven V. Beer

Department of Plant Pathology, Cornell University, Ithaca, NY 14853, U.S.A.
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The transposon Tn*5* was used to create mutants of *Erwinia amylovora*, altered in their pathogenic capabilities on immature pear fruits or apple seedlings. Five mutant classes were established based on the following phenotypes: loss of pathogenicity, reduction in virulence, loss of the ability to cause the hypersensitive reaction (HR) in tobacco, reduction in the ability to cause the HR, and diminished production of extracellular polysaccharide (EPS). The genes responsible for these phenotypes were shown to be chromosomally inherited. A decrease in EPS production was correlated with low virulence, indicating that EPS acts as a virulence factor in *E. amylovora*, rather than as a pathogenicity factor. From two overlapping cosmid clones, a gene cluster of at least five genes was identified by genetic complementation. The cluster includes genes involved in the compatible interaction with a host and genes encoding the induction of the HR in a nonhost. This work provides evidence that genes encoding pathogenicity and hypersensitivity determinants are closely linked and that those involved in hypersensitivity are also required for pathogenicity.

Additional keywords: extracellular polysaccharide, fire blight, hypersensitivity, *Malus, Pyrus,* Tn*5*.

Erwinia amylovora (Burrill) Winslow *et al.* causes fire blight of many rosaceous plants; it is most destructive to apple and pear trees. The bacterium is apparently native to North America, but now it is present in Central and South America, Europe, the Middle East, and New Zealand (Aldwinckle and Beer 1979; Beer *et al.* 1986; Van der Zwet and Keil 1979). The continuing spread of *E. amylovora*, combined with the severity and economic importance of the disease, has elicited a vast amount of literature (Van der Zwet and Keil 1979). Unfortunately, despite numerous efforts, our knowledge of the mechanism of the plant-pathogen interaction during pathogenesis is still unclear.

Fire blight is a typical necrotic disease. Infected tissues first appear water-soaked, then dry and turn brown to black. When the bacteria progress in the plant, affected bark and cortex become red to brown to black. Blossoms, foliage, and fruitlets shrivel, turn dark, and cling to the twigs, giving the tree a scorched appearance. Under humid environmental conditions, bacterial ooze can be observed on recently affected plant parts. On the cellular level, the first evidence of pathogenesis is membrane leakage, followed by decompartmentalization that causes discoloration and collapse of the cell contents and subsequent cell death (Bachmann 1913; Burkowicz and Goodman 1969; Huang and Goodman 1976; Nixon 1927).

Evidence for and against the involvement of possible molecular determinants of pathogenicity (qualitative) or virulence (quantitative) has been presented. Cell-wall-degrading enzyme activities could not be detected in tissues infected by *E. amylovora* or in culture filtrates of the bacterium (Seemueller and Beer 1976). Extracellular polysaccharide (EPS) produced by *E. amylovora* causes wilting of excised cotoneaster shoots (Sjulin and Beer 1978) and apple shoots (Ayers *et al.* 1979; Goodman *et al.* 1974). There is circumstantial evidence for the involvement of additional substances in fire blight pathogenesis (Bennett and Billing 1980; Buchanan and Starr 1980; Feistner and Staub 1986; Hignett and Roberts 1985; Hsu and Goodman 1978; Lovrekovich *et al.* 1970; Seemueller and Beer 1977). However, unequivocal proof for the function of particular substances as pathogenicity or virulence determinants is lacking. Because we wanted to make a fresh start and assess pathogenesis in its entirety, we initiated a molecular genetic approach.

This report describes transposon mutagenesis and the genetic complementation of five transposon-induced mutants of *E. amylovora* as the beginning of a long-term project. Ultimately, we hope that this approach will explain the molecular mechanism(s) of the plant-bacterial interaction, serving to solve the mystery of how *E. amylovora* causes disease. Several preliminary reports have been made (Steinberger and Beer 1987a; Steinberger *et al.* 1987; Beer *et al.* 1984; Steinberger and Beer, 1984; Steinberger and Beer 1983).

MATERIALS AND METHODS

Media and growth conditions. Either Luria-Bertani (LB) medium (Silhavy *et al.* 1984), nutrient medium (Davis *et al.* 1980), nutrient-yeast-extract-glucose medium (Gantotti *et al.* 1981), or minimal medium (Miller 1972) was used. Minimal medium consisted of 3.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, 1.5 g of L-asparagine, 1 g of $(NH_4)_2SO_4$, 0.25 g of nicotinic acid, 2 g of glucose, 0.25 g of sodium citrate, and 5 mg of $MgSO_4 \cdot 7 H_2O$ per liter of H_2O.

Antibiotic concentrations (μg/ml) were as follows: kanamycin 50; gentamycin 40; ampicillin 100; tetracycline 10; spectinomycin 50. Bacteria, plasmids, and phages used are listed in Table 1.

Transposon insertion mutagenesis with pJB4JI. For quantitative studies, a filter mating technique was used. Donor and recipient strains were grown to late logarithmic phase in LB broth with appropriate antibiotics. One milliliter of each culture was mixed, and the cells were

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collected on a Gelman filter (47-mm diameter, 0.45-μm pore size), which was placed on fresh LB agar and incubated for 6 hr at 28° C. The cells were then washed off the filter and resuspended in 2 ml of 0.85% (w/v) NaCl. Portions of the undiluted cell suspension (100 μl) were plated on minimal medium with kanamycin. When quantitative data were not required, either of two spot-agar mating techniques were employed: 1) Donor and recipient (and helper strain, if appropriate) were grown in liquid culture with appropriate antibiotics to late logarithmic phase. Equal volumes of each strain were mixed, and 50 μl of the mixture was spotted onto LB agar. After incubation for 6 hr at 28° C, the cells were scraped from the agar surface with a sterile loop and suspended in 1 ml of 0.85% NaCl. Portions (100 μl) were spread on minimal medium selective for transconjugants. 2) Single colonies of donor, recipient, and helper strains were mixed together on LB agar with a sterile loop and then treated as described in the first method.

Transformation of Ea322 with pTROY11. A plasmid minipreparation (Tait *et al.* 1982) of pTROY11 was used to transform *E. amylovora* (Bauer and Beer 1983).

Transductional insertion mutagenesis with λ*b221c*I857. Cells of Ea322(pTROY11) were grown at 28° C to OD_{620} = 0.4 (4×10^8 colony-forming units [cfu] per milliliter) in LB broth containing ampicillin to ensure maintenance of the plasmid. The culture was centrifuged, washed 3× in 1 M NaCl, 1× in 0.85% (w/v) NaCl, and finally resuspended in the same volume of lambda dilution buffer containing 10 mM Tris-Cl, pH 7.5, and 10 mM $MgSO_4$. The cells were infected with lambda at a multiplicity of infection of 10 and incubated for 20 min at room temperature. After centrifugation, the pellet was resuspended in 0.5 volume LB broth and agitated at 28° C for 1 hr. Aliquots (0.1 ml) were spread on LB agar containing kanamycin.

Table 1. Bacteria, plasmids, and phages

Strains, plasmids, phages	Description genotype, phenotype	Reference or source
Erwinia amylovora		
Ea321	Isolated from *Crataegus*	(CFPB1367) J.-P. Paulin, I.N.R.A., Angers, France
Ea322	Isolated from *Crataegus*	(CFPB1368) J.-P. Paulin, I.N.R.A., Angers, France
Escherichia coli		
1830	Pro⁻, Met⁻, Km^r, Gm^r, Sp^r/Sm^r	Beringer *et al.* 1987
DH1	F⁻, *gyrA96*, *recA1*, *relA1*, *endA1*, *thi-1*, *hsdR17*, *supE44* λ	D. Hanahan 1983
MD1	Phage Mu host	Toussaint and Resibois 1983
HB101	F⁻, *leuB6*, *proA2*, *recA13*, *thi-1*, *ara-14*, *lacY1*, *galK2*, *xyl-5*, *mtl-1*, *rpsL20*, *supE44*, *hsdS20*	Boyer and Roulland-Dussiox 1969
Plasmids, cosmids		
pJB4JI	pPH1JI::Mu::Tn*5*	Beringer *et al.* 1978
pTROY11	pBR322*malK*::IS-*3*, *lamB*	De Vries *et al.* 1984
pRZ102	ColEI::Tn*5*	Jorgensen *et al.* 1979
pCPP8	pGB2-mob, Sm^r/Sp^r	D. W. Bauer (this lab)
pCPP9	pGB2-mob, λcos, Sm$_r$/Sp$_r$	D. W. Bauer (this lab)
pCPP410	pCPP9 with 38-kb Ea321 insert	D. W. Bauer (this lab)
pCPP411	pCPP8 with 11-kb Ea321 insert	This work
pCPP412	pCPP8 with 20-kb Ea321 insert	This work
pCPP420	pCPP9 with 45-kb Ea321 insert	D. W. Bauer (this lab)
Bacteriophages		
Lambda	λ*b*221, *O*am29, *P*am80, *rex*::Tn*5*-132, *c*I857	de Bruijin and Lupski 1984
Mu	cts62	Toussaint and Resibois 1983

Quantification of EPS. Cells were grown with agitation in minimal medium for 3 days at 28° C. The numbers of viable bacteria were determined after incubating 1, 2, and 3 days by dilution-plating on minimal medium. The culture was centrifuged, and the remaining cells were removed from the supernatant by filtration through a 0.45 μm Millipore filter. The cell-free filtrates were brought to a final concentration of 1 M NaCl by adding 5 M NaCl. Crude EPS was precipitated by adding three volumes of 95% ethanol and holding for 1 hr at 4° C. The ethanol precipitation was repeated; the pellet was dried under vacuum and then resuspended in H_2O. EPS was estimated with anthrone using D-galactose as the reference standard (Updegraff 1969).

Pathogenicity test on apple seedlings. Seeds from domestic apple (Lawyer's Nurseries, Plains, MT) were surface-disinfested for 15 min in 0.6% sodium hypochlorite, rinsed with H_2O, coated with the broad-spectrum fungicide Captan 50-WP (Stauffer Chemical, Westport, CT), and embedded in moist vermiculite. After stratification at 10° C for 6–12 wk, germination was observed. The seedlings were used in pathogenicity tests when the hypocotyls were 3–5 cm in length. To ensure a moist environment, seedlings were arranged in plastic boxes on wet paper towels. For inoculation, the hypocotyl was stabbed once below the cotyledons with a toothpick carrying cells of *E. amylovora* taken from a fresh colony.

Pathogenicity test on immature pear fruit. Bartlett pears were picked when they were about 2 cm in diameter. They were stored with moderate aeration at 0–2° C until used. For pathogenicity tests, pears were surface-disinfested with 70% ethanol, halved or cut in slices, arranged in plastic boxes on wet paper towels, and stabbed once with a toothpick as described. For quantitative studies, a cavity approximately 5 mm deep was cut in the cheek of each pear half with a #1 cork borer to receive 40 μl of inoculum containing 5×10^6 cfu.

The inoculated plant material was incubated at 28° C or room temperature (25 ± 5° C). Strains considered positive for pathogenicity induced water-soaking, which was evident after 1–2 days, followed by the appearance of bacterial ooze and necrosis (blackening) of inoculated and adjacent tissues.

Determination of doubling times in rich medium. Shake cultures in LB broth, with or without kanamycin, were incubated at room temperature. Serial dilutions (50 μl aliquots) of three independent samples were spotted on LB agar three times during logarithmic growth. Colonies were counted after incubating for 1 day at 28° C.

Determination of bacterial growth in pear tissue. Immature pear fruits that had been in cold storage for 2 mon were surface-disinfested with 70% ethanol, halved, and placed in a moist chamber as described above. To hold 20 μl of inoculum, one well was cut in the cheek of each pear using an alcohol-flamed #1 cork borer. Two pear halves were inoculated with 5×10^6 cfu of *E. amylovora*. After 20 and 50 hr, separately, each pear half was removed from the moist chamber and homogenized for 25 sec in 0.05 M potassium phosphate buffer, pH 6.5, using a Waring Blendor. Dilutions (10^{-3}, 10^{-4}, and 10^{-5}) were spread on minimal medium amended with kanamycin, if appropriate. Colonies were counted after incubating for 2 days at 28° C.

Isolation of genomic and plasmid DNA. Crude preparations of *E. amylovora* plasmids were prepared by the method of Kado and Liu (1981). Special care was taken to maintain the pH of the lysing solution at 12.3 to minimize chromosomal contamination. Either of two plasmid preparation procedures was used to obtain plasmids of

restriction and transformation quality: a modified alkaline procedure (Birnboim 1983; D. W. Bauer, personal communication) and modified boiling procedure (Holmes and Quigley 1981; D. W. Bauer, personal communication). Rapid plasmid screening was done as described by Sekar (1987). Genomic DNA was isolated as described by Silhavy *et al.* (1984) and Maniatis *et al.* (1982).

Southern blots. Organismal DNA was digested with *Eco*RI and electrophoresed for 8–12 hr in 0.7% agarose at 2 v/cm. Plasmid DNA was separated for 7 hr in 0.7% agarose at 2.5 v/cm. The DNA was blotted onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) or on a nylon membrane (NEN Research Products, Boston, MA) as described (Southern 1975; Reed and Mann 1985). When used as probes, pRZ102 and phage Mu were nick translated as described (Maniatis *et al.* 1982). To obtain a hybridization probe without homology to $IS50_L$ or $IS50_R$, the central 2.7-kb fragment of Tn*5*, delineated by two *Bgl*II sites (Berg and Berg 1983), was purified from agarose using ultrapure glass powder (BIO101 Inc., La Jolla, CA). The radioactive probe was prepared by the random oligomer-primer method (Feinberg and Vogelstein 1983). [α-^{32}P]dGTP (New England Nuclear, Boston, MA) was used as the radioactive nucleotide in both procedures.

Genetic complementation for pathogenicity. A genomic library of *E. amylovora* strain Ea321, constructed in the mobilizable cosmid pCPP9 (Bauer *et al.* 1986) in *Escherichia coli* DH1, was diluted and spread on nutrient agar with spectinomycin to achieve about 500 colonies per plate. Plates were incubated at 37° C until the diameter of the colonies was about 1 mm. Recipient and helper strains were grown in LB broth with kanamycin or tetracycline, respectively. At late logarithmic phase, 0.5 ml of each culture was centrifuged and resuspended in one-tenth volume of LB broth without antibiotics. The cell suspensions were mixed, spread on LB agar, and incubated at 28° C until dry to create a bacterial lawn. The library clones were then replica-plated onto this lawn for mating. After incubation for 6 hr at 28° C, transconjugants were selected by replica-plating on minimal medium containing kanamycin and spectinomycin. Approximately 50 transconjugant colonies were recovered and allowed to grow to a diameter of about 1 mm. To select transconjugants that were pathogenic, five or six freshly cut slices of immature pear fruit were pressed onto the surface of each plate. The inoculated slices were inverted, arranged in a moist chamber, and incubated at 28° C. Transconjugants with cosmids containing inserts that restored pathogenicity were selected based on the development of typical fire blight symptoms in 2–3 days. The strains of interest were purified by streaking bacterial ooze on selective medium and isolating single colonies. The cosmid was mobilized back into *E. coli* by simple triparental spot mating as described.

Restriction enzyme cleavage map. The fragment sizes generated from single and double digests were calculated according to their electrophoretic mobility. Phage lambda DNAs digested with different restriction enzymes were used as size standards. Fragment sizes greater than 14 kb are only approximate, and the smallest fragment that could be detected was 0.1 kb. At least two independent measurements on different gels were used to calculate the average size of restriction fragments. The map was constructed from the best fit of averaged fragment sizes. To determine the order of the restriction fragments, the method of Fitch *et al.* (1983) was used.

Table 2. Transposon mutagenesis of *Erwinia amylovora*

Tn*5* vector	No. of transconjugants screened[a]	No. of pathogenicity mutants	No. of auxotrophic mutants[b]
pJB4JI	11,000	22	n. d.[c]
λ*b*221*c*I857	246	1	4

[a] Kanamycin-resistant transconjugants or transductants were tested for pathogenicity on immature pear fruits or apple seedlings.
[b] Auxotrophic mutants were observed on minimal medium.
[c] n. d. = not determined.

RESULTS

Production of Tn*5*-induced mutants. Tn*5*-carrying, kanamycin-resistant transconjugants were derived from crosses of *E. coli* 1830(pJB4JI) as the donor and 26 strains of *E. amylovora* as the recipient. pJB4JI is a conjugative plasmid that harbors bacteriophage Mu. The Mu sequence carries a Tn*5* insertion, making the phage defective in its lytic functions. The Mu sequence apparently makes this plasmid unstable in hosts other than *E. coli* (Boucher *et al.* 1977; Beringer *et al.* 1978; Hirsch and Beringer 1984; Van Vliet *et al.* 1978). The transposable element can rescue itself by insertion into the recipient's genome and is detected by expression of kanamycin resistance. Spontaneous resistance to kanamycin occurred at a frequency of $<10^{-8}$ per cell. Only three strains of *E. amylovora*—Ea321, Ea322, and Ea346—gave rise to kanamycin-resistant colonies at a frequency well above the spontaneous resistance rate. The frequencies were 2×10^{-7}, 8×10^{-7}, and 4×10^{-7} per recipient cell, respectively. Since Ea346 is a nonpathogenic mutant isolated from nature, only Ea321 and Ea322 were chosen for transposon mutagenesis on a large scale.

Using pJB4JI, 11,000 prototrophic transconjugants were selected on minimal medium on the basis of their kanamycin resistance (Table 2). To ensure the transposition of Tn*5* into the recipient genome, all transconjugants were tested for sensitivity to gentamycin, encoded by pJB4JI. Approximately 70% of these colonies were still resistant to gentamycin, indicating the presence of pJB4JI or a part of it. The plasmid profile of several randomly chosen gentamycin-resistant colonies confirmed the presence of pJB4JI. However, the resistance marker was frequently lost after several transfers on nonselective media (data not shown). Since the stability of pJB4JI in *E. amylovora* resulted in the screening of a large number of kanamycin-resistant transconjugants that were probably not insertion mutants, we used a different Tn*5* vector as soon as it became available.

Transduction of Ea322 with λ*b*221*c*I857 resulted in the recovery of 246 kanamycin-resistant strains. Approximately 2% of the kanamycin-resistant colonies failed to grow on minimal medium, indicating auxotrophy (Table 2). The frequency of auxotrophy was similar to that found in *E. coli* (Shaw and Berg 1979) and *Rhizobium leguminosarum* (Beringer *et al.* 1978). Because our primary interest was the characterization of mutants affected in their ability to be pathogenic, auxotrophic mutants were not further analyzed. One λ-transductant was not pathogenic on immature pear fruit.

Establishment of mutant phenotypes. Of the 11,000 kanamycin-resistant colonies tested for pathogenicity on immature pear fruits or apple seedlings, 22 mutants were affected in their pathogenic capability. Three of them were affected additionally in their ability to cause the hypersensitive reaction (HR) on tobacco (Sequeira 1979). Sixteen of the mutants (rough) had lost the fluidal

phenotype of the parent when grown on carbohydrate-rich medium like nutrient-yeast-extract-glucose medium or minimal medium. The fluidal (smooth) phenotype was caused by the presence of a large amount of EPS (Sijam *et al.* 1983). Five mutants retained the fluidal phenotype of the wild-type parent strain. Quantitative determinations of EPS from mutant and wild-type strains revealed that strains with no visible alteration in colony morphology produced EPS in amounts comparable to the wild-type parent strains. Rough mutants did not produce EPS at detectable levels (Table 3).

On the basis of pathogenicity, HR-inducing ability, and EPS production, several mutant classes were established (Table 4). Class A mutants retain the fluidal phenotype and are nonpathogenic. They do not cause symptoms of fire blight on any plant material tested (Fig. 1c). In addition,

Table 3. Extracellular polysaccharide (EPS) produced by wild-types and Tn*5*-induced mutants of *Erwinia amylovora*

Strain	EPS nanogram per 10^6 colony-forming units[a]
Ea321	7.5
Ea321-T101	4.3
Ea321-T102	1.5
Ea321-T103	<0.01
Ea321-T104	0.6
Ea322	0.6
Ea322-T101	0.6
Ea322-T102, T103	<0.01
Ea322-T104	0.6
Ea322-T105, T106,	<0.01
T107, T108,	<0.01
T109, T110,	<0.01
T112, T113,	<0.01
T115, T117,	<0.01
T118, T119	<0.01
Ea322-T120	1.0
Ea322-T121	<0.01

[a]The minimum level of EPS detectable was 10^{-2} ng per 10^6 colony-forming units.

Table 4. Classification and characteristics of Tn*5*-induced mutants of *Erwinia amylovora*

Class	Mutants	No. of mutants	EPS[a]	Path[b]	HR[c]
A	Ea321-T102 Ea322-T101	2	+	−	−
B	Ea321-T101 Ea321-T104 Ea322-T104 Ea322-T120	4	+	−	+
C	Ea322-T110 Ea322-T113 Ea322-T117 Ea322-T119	4	−	−	+
D	Ea321-T103 Ea322-T102 Ea322-T103 Ea322-T105 Ea322-T106 Ea322-T107 Ea322-T108 Ea322-T109 Ea322-T112 Ea322-T115 Ea322-T121	11	−	− (lv)	+
E	Ea322-T118	1	−	− (lv)	−+[d]

[a]EPS = extracellular polysaccharide; EPS + = fluidal colony morphology; EPS − = rough colony morphology.
[b]Path = pathogenicity on immature pear fruit; lv = low virulence.
[c]HR = ability to induce the hypersensitive reaction on tobacco.
[d]−+ = HR induced only by concentrations ⩾ 10 times higher than with wild-type strain.

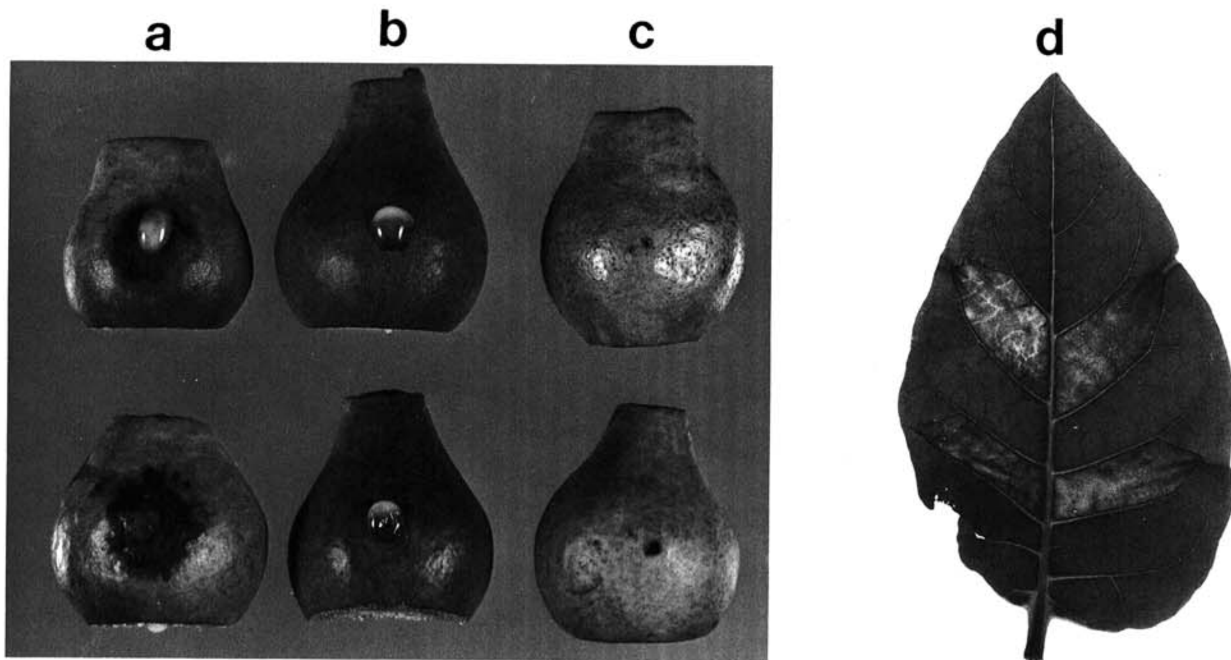

Fig. 1. Representative examples of symptoms on immature pear fruit and tobacco leaf tissue caused by a wild-type strain of *Erwinia amylovora* or Tn*5*-induced mutants of different phenotypes. Halves of immature pears were stabbed with toothpicks carrying bacterial cells taken from fresh colonies. Panels of the tobacco leaf were infiltrated with a bacterial suspension, as described by Sequeira (1979). The photographs were taken 4 days (pears) and 18 hr (tobacco) after inoculation. (a), Wild-type reaction: black necrosis spreading from the point of inoculation; white exudate is present at the point of inoculation and cut surfaces. (b), Reaction caused by Class D mutants: no necrosis; a drop of watery exudate is present at the point of inoculation. (c), Reaction caused by mutants from Classes A, B, and C: no necrosis or exudate. (d), Dry necrosis of leaf tissue in the four panels infiltrated with bacterial suspensions of mutants from Classes B, C, D, and E.

mutants in this class have lost the ability to cause the HR on tobacco. Class B mutants differ from Class A only in that they are still capable of inducing the HR (Fig. 1d). Mutants of Class C and Class D do not produce detectable amounts of EPS and were nonpathogenic when tested on moderately susceptible immature pear fruits. When highly susceptible pear fruits were used, mutants of Class D produced a watery exudate, relative to ooze produced by the wild-type strain (Fig. 1b). Often, and especially when pear slices rather than halves were used, a localized water-soaked zone was observed (data not shown). These mutants are fully capable of inducing the HR (Fig. 1d). One mutant (Class E) is difficult to classify, since it induces the HR only when its concentration is 10× higher than that required of the wild-type strain or other Tn*5*-induced mutants not affected in HR (D. W. Bauer, personal communication).

Multiplication *in vitro* and *in planta*. To assure that the alterations in pathogenic capabilities are not the result of a mutation in a trivial "housekeeping" gene, the growth rates of Tn*5*-induced mutants and wild-type strains were compared. All strains, except Ea322-T119, had similar doubling times in LB broth.

To determine whether the Tn*5*-induced mutants that were altered in their pathogenic capabilities were still capable of using plant nutrients, we examined their ability to grow in the host plant. Twenty hr after inoculation, the wild-type strain Ea322 had multiplied about 200-fold (Fig. 2:1). Its Tn*5*-induced mutants had multiplied between eight- and 100-fold (Fig. 2:2-19). The wild-type strain Ea321 had multiplied 50-fold, and its Tn*5*-induced mutants had multiplied between 16- and 50-fold (Fig. 2:21-24). Fifty hr after inoculation, Ea321 and Ea322 had multiplied to about 700 and 500 times their initial levels, respectively (Fig. 2:1, 20). The populations of eight mutants had still increased, whereas the populations of 12 mutants had remained the same or decreased (Fig. 2:2-19, 21-24).

Physical characterization of Tn*5* insertions. To verify that Tn*5* was present in the genome of the mutants, Southern blot hybridization was performed. Strains of *E. amylovora* digested with *Eco*RI were hybridized with a Tn*5*-containing plasmid pRZ102. Because Tn*5* does not have an *Eco*RI site, the restriction pattern of Figure 3 indicated that eight mutants with altered pathogenicity functions had single Tn*5* insertions (Fig. 3A: h, j, l, m, o, p, q; Fig. 3C: a). In the case of 14 mutants, several fragments hybridized with Tn*5*, indicating multiple insertions (Fig. 3A: d, f, k, n, r, s, t; Fig. 3B: b, c, d, e, f, g, h). Tn*5* is flanked by two inverted repeats (IS*50*$_R$ and IS*50*$_L$), which were shown to have the ability to rise as independent insertions through intermolecular transposition (Hartl *et al.* 1983). We therefore probed *Eco*RI-digested genomic DNA with the central 2.7-kb

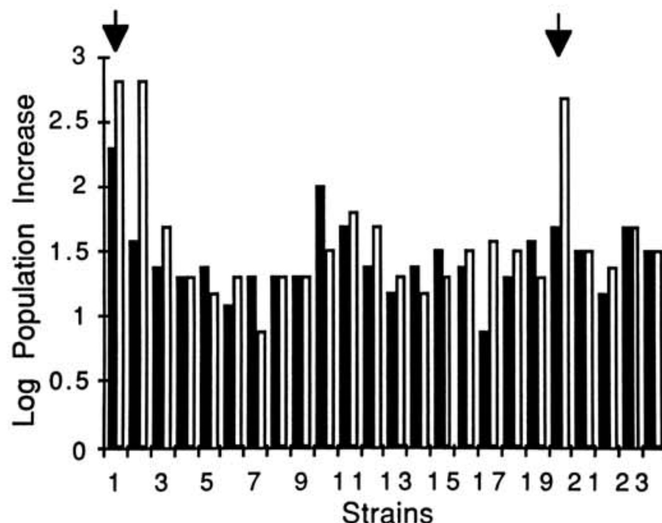

Fig. 2. Bacterial growth in pear tissue of wild-type and Tn*5*-induced mutant strains of *Erwinia amylovora*. Immature pear fruits were inoculated with 5 × 10^6 colony-forming units (cfu). After 20 and 50 hr, pears were homogenized, and dilutions were spread on minimal medium. Colonies were counted after 2 days at 28° C. Black bars represent the population increase over 5 × 10^6 cfu after 20 hr; open bars represent population increase over 5 × 10^6 cfu after 50 hr. Arrows indicate wild-type strains. (1) Ea322; (2) Ea322-T101; (3) Ea322-T102; (4) Ea322-T103; (5) Ea322-T104; (6) Ea322-T105; (7) Ea322-T106; (8) Ea322-T107; (9) Ea322-T108; (10) Ea322-T109; (11) Ea322-T110; (12) Ea322-T112; (13) Ea322-T113; (14) Ea322-T115; (15) Ea322-T117; (16) Ea322-T118; (17) Ea322-T119; (18) Ea322-T120; (19) Ea322-T121; (20) Ea321; (21) Ea321-T101; (22) Ea321-T102; (23) Ea321-T103; (24) Ea321-T104.

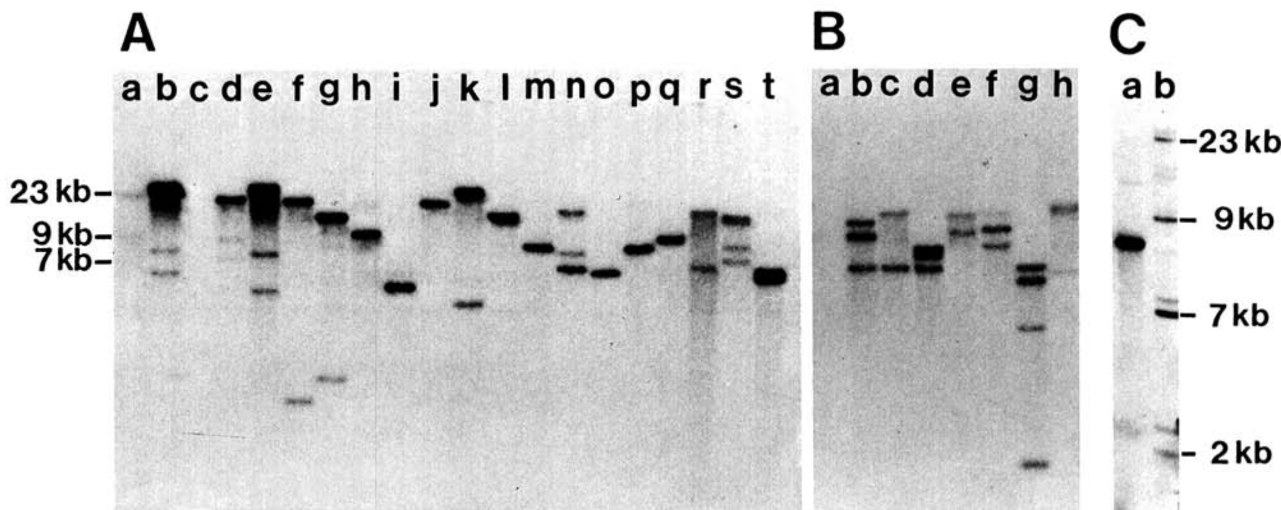

Fig. 3. Hybridization with pRZ102 of EcoRI-digested genomic DNA of wild-type and Tn*5*-induced mutants of *Erwinia amylovora*. Panel A: (a) λ *Hind*III, size standard; (b) *E. coli* 1830(pJB4JI); (c) Ea322; (d) Ea322-T101; (e) Tn*5*-containing pathogenic, derivative of Ea322; (f) Ea322-T110; (g) Tn*5*-containing pathogenic, derivative of Ea322; (h) Ea322-T113; (i) Tn*5*-containing pathogenic, derivative of Ea322; (j) Ea322-T120; (k) Ea322-T118; (l) Ea322-T115; (m) Ea322-T112; (n) Ea322-T119; (o) Ea322-T109; (p) Ea322-T117; (q) Ea322-T103; (r) Ea322-T108; (s) Ea322-T104; (t) Ea322-T105. Panel B: (a) Ea321; (b) Ea322-T107; (c) Ea322-T106; (d) Ea322-T102; (e) Ea321-T101; (f) Ea321-T102; (g) Ea321-T103; (h) Ea321-T104. Panel C: (a) Ea322-T121; (b) λ *Hind*III, size standard. A: b, e, g, i are positive controls. A:c and B:a are negative controls. A: d, f, h, j, k, l, m, n, o, p, q, s, t; B: b, c, d, e, f, g, h and C:a are kanamycin-resistant pathogenicity mutants.

*Bgl*II fragment of Tn*5*, which does not have homology to IS50. The hybridization pattern in Figure 4 (A: a, c, h, k, n, p; B: d, e, f, g) when compared to Figure 3 (A: d, f, k, n, r, s; B: e, f, g, h) revealed the presence of insertion sequences in 10 mutants. The vector plasmid pJB4JI carries Mu sequences. The intact Mu is a transposon itself (Toussaint and Resibois 1983). To detect possible insertion of Mu sequences, we hybridized the genomic blots with Mu DNA. Indeed, Mu sequences were associated with six mutants (Fig. 5A: e, i, j; Fig. 5B: d, e, g;). In the case of mutant Ea322-T110, one

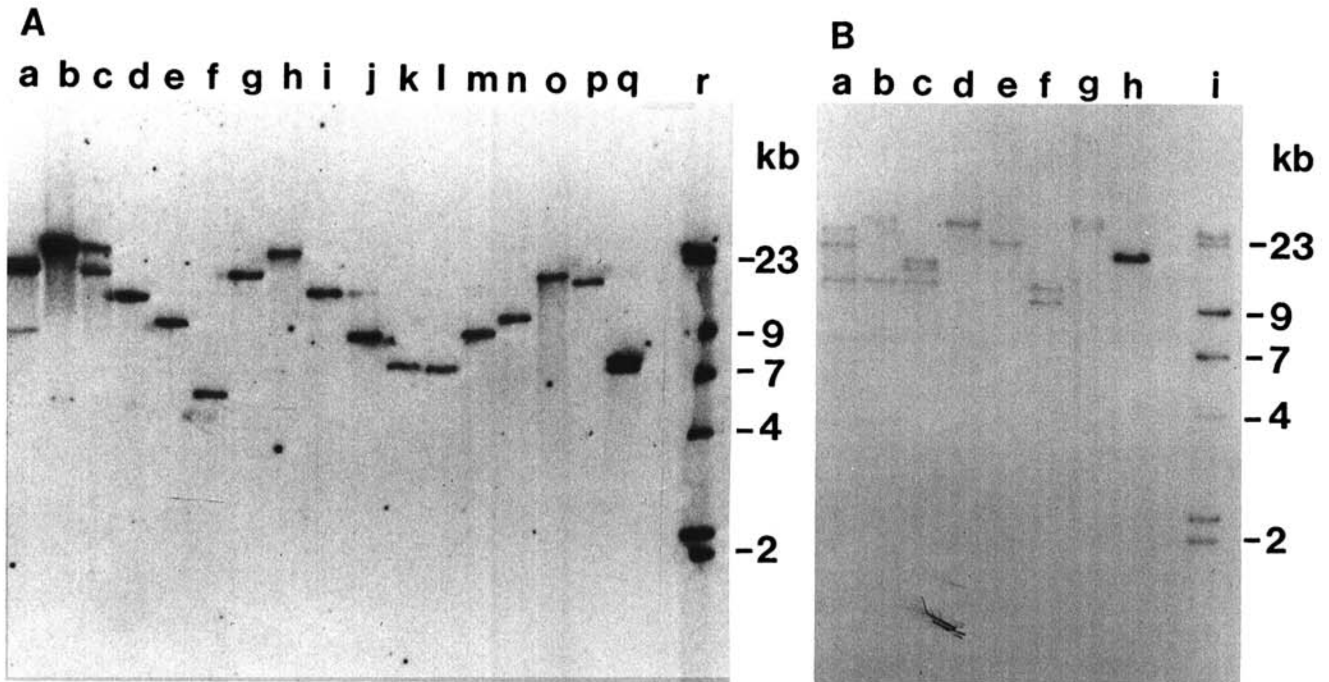

Fig. 4. Hybridization with the *Bgl*II fragment of Tn*5* of *Eco*RI-digested genomic DNA of wild-type and Tn*5*-induced mutants of *Erwinia amylovora*. Panel A: (a) Ea322-T101; (b) Tn*5*-containing, pathogenic, derivative of Ea322; (c) Ea322-110; (d) Tn*5*-containing, pathogenic, derivative of Ea322; (e) Ea322-T113; (f) Tn*5*-containing, pathogenic, derivative of Ea322; (g) Ea322-T120; (h) Ea322-T118; (i) Ea322-T115; (j) Ea322-T112; (k) Ea322-T119; (l) Ea322-T109; (m) Ea322-T117; (n) Ea322-T108; (o) Ea322-T103; (p) Ea322-T104; (q) Ea322-T105; (r) λ *Hind*III, size standard. Panel B: (a) Ea322-T107; (b) Ea322-T106; (c) Ea322-T102; (d) Ea321-T101; (e) Ea321-T102; (f) Ea321-T103; (g) Ea321-T104; (h) Tn*5*-containing, *E. coli* HB101. A: b, d, f and B:h are positive controls. A: a, c, e, g, h, i, j, k, l, m, n, o, p, q and B: a, b, c, d, e, f, g are kanamycin-resistant pathogenicity mutants.

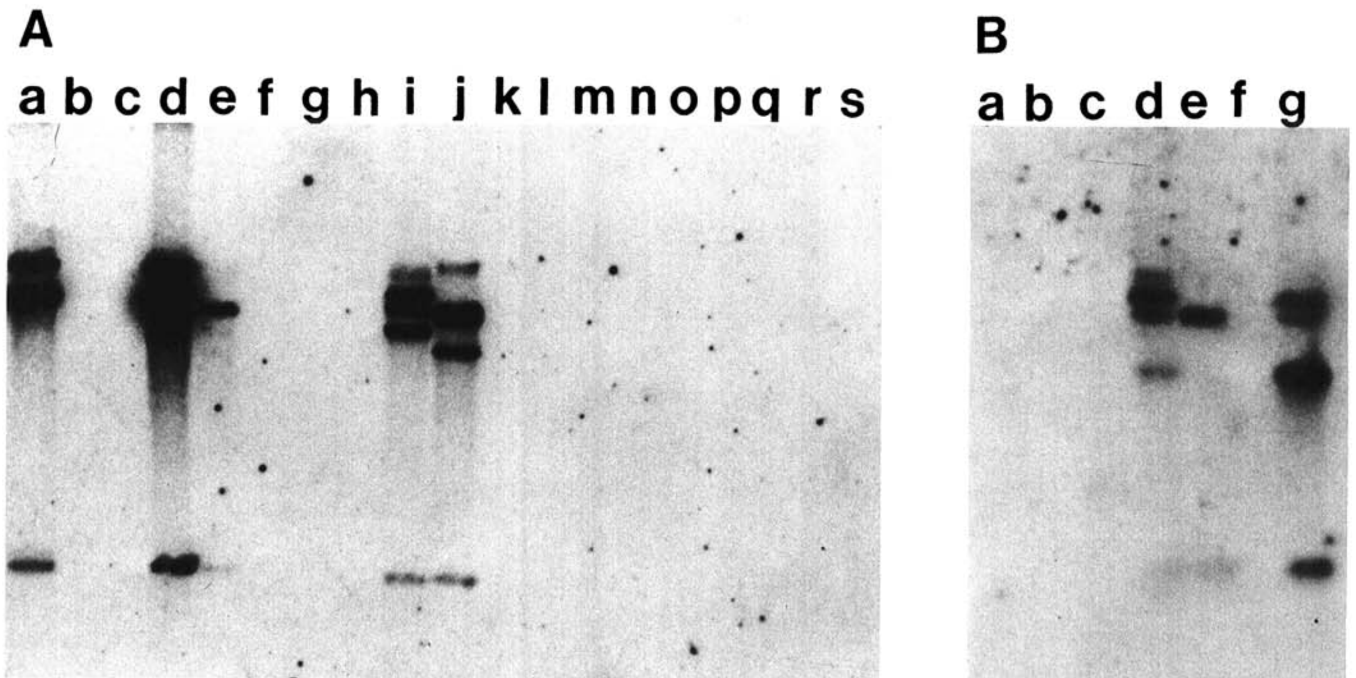

Fig. 5. Hybridization with phage Mu DNA of *Eco* RI-digested genomic DNA of wild-type and Tn*5*-induced mutant strains of *Erwinia amylovora*. Panel A: (a) *E. coli* 1830(pJB4JI); (b) Ea322; (c) Ea322-T101; (d) Mu-containing, pathogenic, derivative of Ea322; (e) Ea322-T110; (f) Tn*5*-containing, pathogenic, derivative of Ea322; (g) Ea322-T113; (h) Tn*5*-containing, pathogenic, derivative of Ea322; (i) Ea322-T120; (j) Ea322-T118; (k) Ea322-T115; (l) Ea322-T112; (m) Ea322-T119; (n) Ea322-T109; (o) Ea322-T117; (p) Ea322-T108; (q) Ea322-T103; (r) Ea322-T104; (s) Ea322-T105. Panel B: (a) Ea322-T107; (b) Ea322-T106; (c) Ea322-T102; (d) Ea321-T101; (e) Ea321-T102; (f) Ea321-T103; (g) Ea322-T104. A: a, d are the positive controls. A: b, f, h are the negative controls. A: c, e, g, i, j, k, l, m, n, o, p, q, r, s and B: a, b, c, d, e, f, g are kanamycin-resistant pathogenicity mutants.

additional fragment is visible in Figure 4 (A:c) that was not visible in Figure 3 (A:f). Four mutants experienced multiple Tn*5* transpositions (Fig. 3A: t; Fig. 3B: b, c, d; Fig. 4A: q; Fig. 4B: a, c, f;). Two mutants experienced multiple Tn*5* transpositions in additon to insertions of IS*50* (Fig. 3A: f,g; Fig. 4A: g, h). The complex information from Figures 3, 4, and 5 is summarized in Table 5.

E. amylovora strains Ea321 and Ea322 harbor two plasmids of approximately 30 kb and 56 kb. When plasmid DNA preparations of the 22 mutants were probed with pRZ102, Tn*5* was not detected, indicating that the disrupted pathogenicity functions are chromosomally inherited (data not shown).

Complementation of nonpathogenic mutants with cosmids pCPP410 and pCPP420. Two cosmids carrying *E. amylovora* pathogenicity genes were identified among approximately 1,500 members of a genomic library. The sizes of the *E. amylovora* DNA inserts were measured as 38 kb (in pCPP410) and 45 kb (in pCPP420). The cosmid pCPP410 restored pathogenic capability to the wild-type level in four mutants, representing Class A (path$^-$ HR$^-$) and Class B (path$^-$ HR$^+$). The cosmid pCPP420 restored pathogenic capability to the wild-type level in two mutants representing Class B (path$^-$ HR$^+$) (Fig. 6). Concomitant with pathogenicity, ability to induce the HR was restored as well (D. W. Bauer, personal communication).

A preliminary restriction map was constructed using four restriction enzymes (Fig. 7). The inserts represent overlapping chromosomal sequences. The position of the vector was located in a 5.7-kb fragment bounded by *Bam*HI and *Bgl*II sites. The exact location could not be determined with this method, because the single *Bam*HI site in pCPP9 was destroyed by ligation with a fragment having *Sau*3A

Table 5. Insertions in pathogenicity mutants of *Erwinia amylovora*

Mutant	Tn*5*[a]	IS50$_R$[a]	Mu[b]
Ea322-T103	1	0	–
Ea322-T109	1	0	–
Ea322-T112	1	0	–
Ea322-T113	1	0	–
Ea322-T115	1	0	–
Ea322-T117	1	0	–
Ea322-T121	1	0	–
Ea322-T105	2	0	–
Ea322-T106	2	0	–
Ea322-T107	3	0	–
Ea322-T101	3	1	–
Ea322-T104	1	2	–
Ea322-T108	1	1	–
Ea322-T118	1	1	–
Ea322-T119	1	2	–
Ea321-T103	2	2	–
Ea322-T102	3	0	+
Ea322-T120	1	0	+
Ea322-T110	2	1	+
Ea321-T101	1	1	+
Ea321-T102	1	1	+
Ea321-T104	2	1	+

[a]Determined by probing *Eco*RI digested genomic DNA with pRZ102 (Fig. 3) or with the *Bgl* II fragment of Tn*5* (Fig. 4).
[b]Determined by probing with phage Mu (Fig. 5).

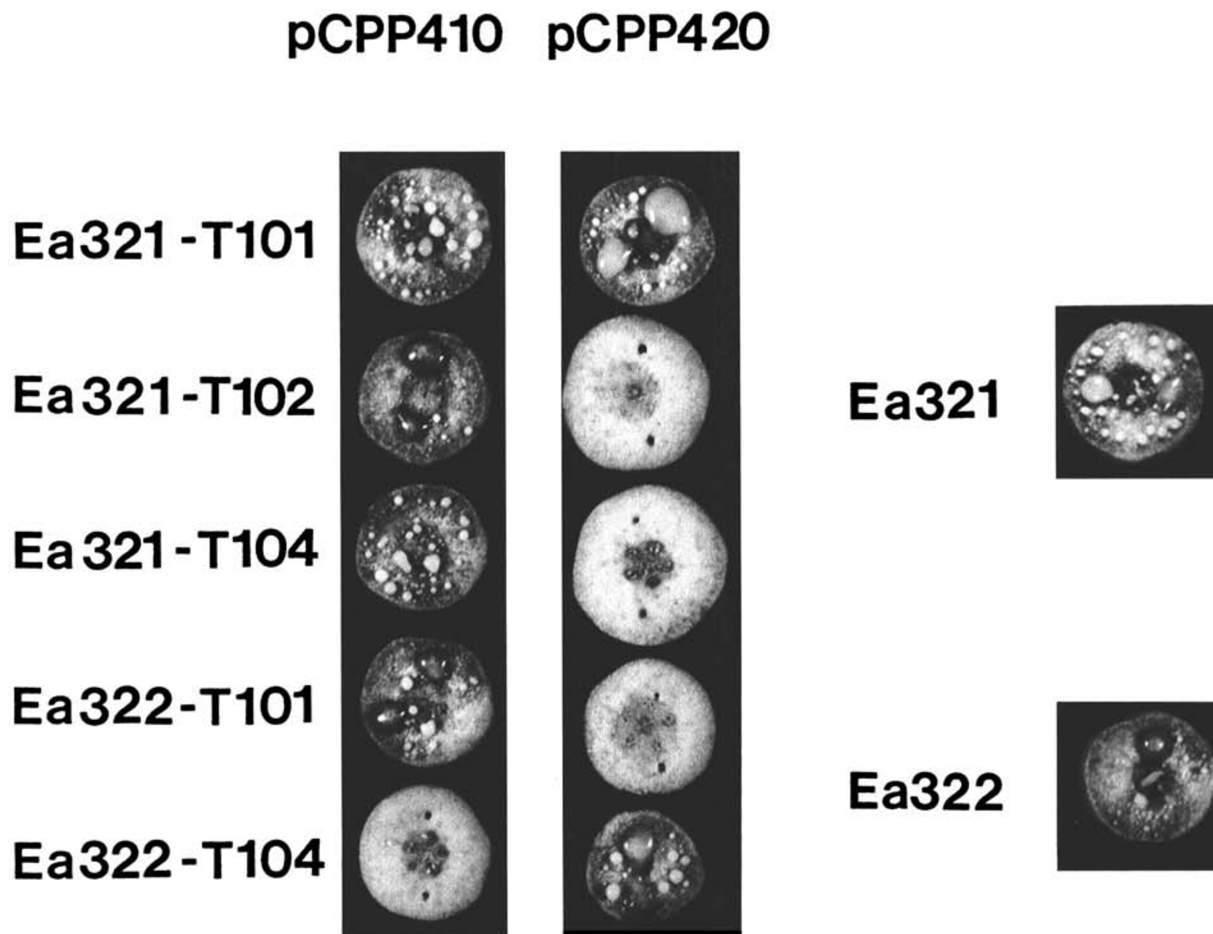

Fig. 6. Genetic complementation for pathogenicity of Tn*5*-induced mutants of *Erwinia amylovora*. Freshly cut slices of immature pear fruits were stab-inoculated with *E. amylovora* and incubated in a moist chamber at 28° C. The photograph was taken 5 days after inoculation.

ends. The overlapping region was mapped to an approximately 18-kb region between an *Eco*RI site and a *Bgl*II site. The exact boundaries are not known.

To narrow the region carrying mutated pathogenicity genes further, subclones of pCPP410 were constructed in the mobilizable, low-copy-number plasmid pCPP8 (Bauer *et al.* 1986). Subclone pCPP411, carrying an 11-kb *Eco*RI fragment from the region overlapping with pCPP420, restored pathogenicity in two mutants from Class B (path$^-$ HR$^+$) (Fig. 7). However, the onset of fire blight symptoms was greatly delayed as compared with the wild-type strain. Once the first symptoms appeared, the disease developed to the same degree as that induced by the parent strain. This result perhaps indicates that pCPP411 does not carry the complete transcriptional unit necessary for complementation. For transcription to occur, pCPP411 likely requires integration into the chromosome through homologous recombination. Indeed, the plasmid was not detected extrachromosomally in 18 randomly chosen colonies isolated from ooze (data not shown). Subclone pCPP412, carrying a neighboring 20-kb *Eco*RI fragment, fully complemented one mutant from Class B (path$^-$ HR$^+$) and two mutants from Class A (path$^-$ HR$^-$) (Fig. 7).

DISCUSSION

Our studies have established that Tn*5* can be employed to create mutants in *E. amylovora*, as it has been in many other Gram-negative bacteria (Beringer *et al.* 1978; Lindgren *et al.* 1986; Merrick *et al.* 1978; Shaw and Berg 1979; Biek and Roth 1980; Niepold *et al.* 1984; Pischl and Farrand 1984; Downie *et al.* 1983). Tn*5* insertion occurred randomly, as reflected by the number of different insertion sites and the frequency of auxotrophic mutations. The ability to obtain Tn*5* mutants of *E. amylovora* with the plasmid vector pJB4JI varied greatly among strains; those with less fluidal colony morphology were more amenable to this system. Fluidal colony morphology is caused by the production of copious amounts of EPS. It is not clear whether differences in mutation frequencies are due to reduced transposition into the genome of *E. amylovora* from the Tn*5* vector plasmid or to reduced transfer of the vector plasmid into fluidal strains. The latter possibility seems more likely, because transformation frequencies and lambda transduction frequencies also are lower in fluidal bacterial phenotypes (D. W. Bauer and C. H. Zumoff, personal communication).

Although mutants of *E. amylovora* with alterations in their pathogenic capability were created with pJB4JI, we were not satisfied with this Mu-harboring plasmid as a vehicle to deliver the transposon. Plasmids containing Mu

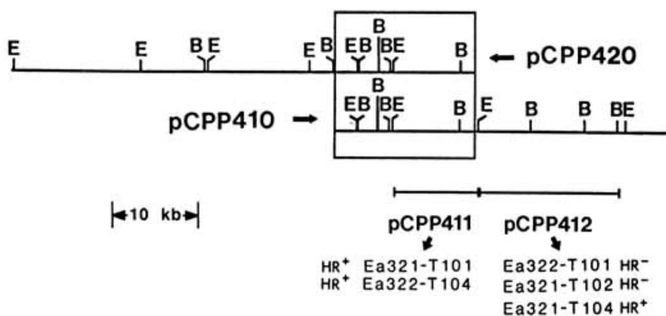

Fig. 7. Characterization of cosmid clones pCPP410 and pCPP420. E = *Eco*RI; B = *Bam*HI. The framed area represents overlapping sequences. pCPP411 and pCPP412 are subclones of pCPP410. The mutants and phenotypes complemented by pCPP411 or pCPP412, respectively, are indicated.

sequences reportedly have reduced ability to maintain themselves in Gram-negative hosts other than *E. coli* (Boucher *et al.* 1977; Beringer *et al.* 1978; Hirsch and Beringer 1984; Van Vliet *et al.* 1978). Unfortunately, pJB4JI was maintained in *E. amylovora* for several generations in 70% of the transconjugants. Thus, we could not calculate the frequency of pathogenicity mutations or auxotrophic mutations per Tn*5* insertion. More importantly, the prolonged presence of pJB4JI presumably allowed intermolecular transposition to occur within the chromosome, which already contained Tn*5* and the Tn*5*-containing pJB4JI (Hartl *et al.* 1983). In this event, DNA rearrangements of target and transposon sequences are known to occur. The transposed sequence can be the transposon bordered by one or two IS elements, or the IS only (Berg and Berg 1987; Berg *et al.* 1982). The prolonged presence of pJB4JI also allowed the transposition of Mu into the genome of *E. amylovora*, carrying Tn*5* with it. However, pJB4JI was the only suitable Tn*5* vector available until De Vries *et al.* (1984) constructed the narrow-host-range plasmid, pTROY11, with the *lamB* gene of *E. coli*. It constitutively synthesizes the lambda receptor and makes *E. amylovora* susceptible to infection with λ*b*221*c*I857. Intramolecular recombination of Tn*5* is indicated with mutant Ea322-T110, because the hybridization pattern changed in the absence of the Tn*5* vector. Ea322-T102, Ea322-T105, Ea322-T106, and Ea322-T107 must have experienced the rare event of multiple Tn*5* transposition.

The insertions of all 22 pathogenicity mutants are chromosomal and did not take place into either of the two indigenous plasmids (30 and 56 kb). However, we have isolated one mutant, not discussed in detail in this communication, that has Tn*5* inserted in the 56-kb plasmid. Subsequent studies demonstrated that this plasmid is not involved in pathogenicity (Steinberger and Beer 1987b). Other workers have evidence that the 30-kb plasmid of *E. amylovora* also is not involved in pathogenicity (J. Laurent, personal communication). The available molecular evidence thus indicates that in *E. amylovora*, genes affecting pathogenicity are chromosomal, which is consistent with the classical genetic data of Pughasetti and Starr (1975).

There is considerable circumstantial evidence that the EPS of *E. amylovora* plays a role in pathogenesis (Ayers *et al.* 1970; Bennett 1980; Bennett and Billing 1980; Goodman *et al.* 1974; Hsu and Goodman 1978). Indeed, most of our mutants are altered in the amount of EPS they produce. Colonies of these appear "rough" on minimal medium or other carbohydrate-rich media. With the methods employed, no EPS was detected in these mutants. However, small amounts of EPS may be produced in culture, and more importantly, in association with the host plant. One Class D mutant, Ea322-T103, appeared fluidal when grown on minimal medium with 1% glucose, instead of 0.2%, as was usually used (data not shown).

Classification of Class D mutants according to their pathogenic capabilities was difficult. They tested nonpathogenic on apple seedlings and immature pears that had been stored for several months. When freshly harvested pear fruits were used for pathogenicity tests, 80% of Class D mutants produced localized water-soaked lesions and some watery exudate at the point of inoculation. Kanamycin-resistant cells of *E. amylovora* were isolated from this exudate. Although the interpretation of the significance of the rough phenotypes should be considered with caution, the role of EPS in the interaction of *E. amylovora* with a host plant has again been indicated. The virulence of strains

of *E. amylovora* seems to be related to the amount of EPS produced. Whether EPS is required for pathogenicity remains in question.

All mutants, except for one, appear as fit as the parent strains, judged by their growth rates in culture. Ea322-T119 grew in LB broth at a rate approximately 0.6 times that of its wild-type parent strain. Because this mutant strain has multiple insertions, its general metabolism may be affected in addition to its pathogenicity.

Based on their growth *in planta*, all 22 mutants apparently were not affected in their ability to use plant nutrients. But once readily available nutrients were depleted, the mutants apparently lacked the ability to destroy plant cells to obtain more nutrients necessary for multiplication. Mutants from Class D, which can be of low virulence on very susceptible tissue, experienced the same fate as others in these experiments. This result was expected because the study was done with pear fruits that had been in cold storage for more than 2 mon and therefore were less susceptible to infection by *E. amylovora*.

Our genetic analysis of the different pathogenic phenotypes, concentrated initially on mutants proficient in EPS but deficient in pathogenicity (Classes A and B). Because all mutants in these classes carry multiple insertions, we considered marker-exchange experiments to identify the insertion responsible for the nonpathogenic phenotype. Insertions of IS*50*, as well as Mu, rendered these experiments unsuccessful. To circumvent this problem, we used a technique to identify pathogenicity genes affected by mutation similar to that used by Long *et al.* (1982). Wild-type genes involved in pathogenicity were identified directly on the basis of the pathogenicity-restoring property of a particular library construct in one or several mutants.

This work demonstrates clearly that the pathogenic capability of *E. amylovora* is based on several chromosomally inherited genes specifically responsible for this function. However, we cannot estimate the number of pathogenicity genes mutated because 14 of our 22 mutants had multiple bands hybridizing with Tn*5* or Mu. A gene cluster has been cloned that encodes factors involved in pathogenicity and/or induction of the HR. These genes are not likely to be involved in the production or activity of EPS. They are intimately connected on a genetic and functional basis, and some genes might be involved in both pathogenicity and HR. These genes now provide the groundwork for studies on the mechanism of pathogenesis. Knowledge of gene regulation, expression, and function will lead to a better understanding of pathogenesis by *E. amylovora*.

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