

Localization of transposon insertions in pathogenicity mutants of *Erwinia amylovora* and their biochemical characterization

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Transposon Tn5, on a mobilizable ColE1 plasmid, on a Ti plasmid derepressed for bacterial transfer, and on the bacteriophage fd genome, was used to construct pathogenicity mutants of the fire blight pathogen *Erwinia amylovora*. Eleven nonpathogenic mutants were isolated from 1600 independent mutants screened. These mutants were divided into three types: auxotrophs, exopolysaccharide (EPS)-deficient mutants and a mutant of the *dsp* phenotype. According to their insertion sites the Tn5 mutants were mapped into several classes. Some of the mutants could be complemented with cosmid clones from a genomic library of the parent strain for EPS production on minimal agar. EPS-deficient mutants and the *dsp* mutant could complement each other to produce virulence symptoms on pear slices.

Introduction

Fire blight is a disease of pome fruits and other species of the family Rosaceae and is caused by the Gram-negative bacterium *Erwinia amylovora* (Van der Zwet & Keil, 1979). The pathogen invades the vascular system of its host plants via natural openings like nectaries, lenticels or wound sites. Symptoms first appear on young succulent shoots, which turn black and may show droplets of ooze. The mechanisms of pathogenesis apparently differ considerably from that of soft-rot *Erwinias*, which secrete numerous pectolytic enzymes in order to degrade plant cell walls (Kotoujansky, 1987). The apparent absence of those enzymes in tissues infected by *E. amylovora* (Seemüller & Beer, 1976; R. Theiler, I. Schäfer & K. Geider, unpublished) has focused attention on other virulence factors like the involvement of extracellular polysaccharides (EPSs) (Sjulin & Beer, 1978; Ayers *et al.*, 1979; Bennett & Billing, 1980) and the influence of the hypersensitive reaction (HR) on pathogenicity. Solutions of EPS and other polymers cause a physical barrier in plant tissue (Van Alfen & McMillan, 1982) rather than producing host-specific wilting symptoms (Goodman *et al.*, 1974).

The acidic EPS of the fire blight pathogen (Smith *et al.*, 1990), formerly named amylovoran, may therefore be better termed amylovoran to highlight its resemblance to polysaccharides rather than to toxins. A gene involved in amylovoran synthesis may thus be named *ams*, whereas mutants of other organisms with deficiencies in exopolysaccharide synthesis were named *cps* or *exo* (Coplin & Cook, 1990). Another exopolysaccharide, levan (2,6- β -D-fructan), is produced by *E. amylovora* after secretion of the sugar-polymerizing enzyme levansucrase in the presence of sucrose (Gross *et al.*, 1990). The synthesis of both polysaccharides is affected by mutations in the *rcsA* gene (Bernhard *et al.*, 1990; Chatterjee *et al.*, 1990; Coleman *et al.*, 1990). The RcsA protein has homology to functionally similar activator proteins in other Gram-negative bacteria on the basis of its amino acid sequence and also by its complementation activity. On the other hand, the *rcsA* genes of *Erwinia stewartii* and *Escherichia coli* complemented an *E. amylovora rcsA* mutant for EPS synthesis on agar plates, but not for virulence on pear slices (Bernhard *et al.*, 1990).

Release of the phytotoxic L-2,5-dihydrophenylalanine (Feistner, 1988) is restricted to some *E. amylovora* strains (Schwartz *et al.*, 1991), suggesting several plant-cell-damaging principles in fire blight pathogenesis. A 29 kb plasmid, found in all *E. amylovora* isolates (Falkenstein *et al.*, 1988), modulates the development of fire blight symptoms (Falkenstein *et al.*, 1989). Its absence causes a thiamin requirement for bacterial cell growth (Laurent *et*

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Abbreviations: EPS, exopolysaccharide; HR, hypersensitive reaction; KDO, 2-keto-3-deoxyoctonic acid (3-deoxy-D-manno-2-octulosonic acid).

Table 1. *Bacteria, plasmids and bacteriophages used*

Bacterial strains	Characteristics	References
<i>A. tumefaciens</i> C58	Donor for Ti plasmid	Sprinzl & Geider (1988)
<i>E. amylovora</i> Ea1/79*	Permissive for ColE1 plasmids	Falkenstein <i>et al.</i> (1988)
<i>E. amylovora</i> Ea7/74*	No replication of ColE1 plasmids	Falkenstein <i>et al.</i> (1988)
<i>E. amylovora</i> Ea7/74 (F::Tn3)	Ap resistant†	This work
<i>E. amylovora</i> Ea11/88	Isolated 1988 from <i>Cotoneaster</i> spp. in Germany	This work
<i>Esch. coli</i> S17-1	RP4 derivative in chromosome	Simon <i>et al.</i> (1986)
Plasmids		
pBR322-mob	<i>mob</i> from pSUP201 in <i>Bam</i> HI site of pBR322	This work
pCA721	pVK100 with insertion complementing mutant A72	This work
pCA831	pVK100 with insertion complementing mutant A83	This work
pEA29	Common <i>E. amylovora</i> plasmid	Falkenstein <i>et al.</i> (1988)
pES2144	<i>cps</i> genes from <i>E. stewartii</i>	Dolph <i>et al.</i> (1988)
pfdA31	From pfdA3; Ap resistant	Geider <i>et al.</i> (1985)
pGV3100	pTi-C58 with constitutive transfer	Holsters <i>et al.</i> (1980)
pGV3100::Tn5	Tn5 in pTi-C58 <i>Eco</i> RI fragment 1	This work
pSS83	pBR325 with <i>Eco</i> RI fragment 1 from pTi-C58	Schweitzer <i>et al.</i> (1980)
pSUP201	pBR325 with <i>mob</i> from RP4	Simon <i>et al.</i> (1986)
pSUP201-Ω::Tn5	With pTiΩ-Tn5 fragment	Zahm <i>et al.</i> (1984); this work
pUC19	Ap, <i>lacZ'</i>	Yanisch-Perron <i>et al.</i> (1985)
pVK100	Km, Tc; cosmid with RP4 origin	Knauf <i>et al.</i> (1982)
Bacteriophages		
<i>E. amylovora</i> phage 4L	EPS-specific phage	Billing & Garrett (1980)
<i>Esch. coli</i> phage fd-Tn5	Tn5 in fd gene 2	M. Metzger & K. Geider, unpublished

* Virulent isolate from Germany.

† Transposon in F-episome transferred from *Esch. coli* by conjugation at 28 °C.

al., 1989), thus affecting EPS synthesis on minimal agar (Falkenstein *et al.*, 1989).

Insertion of transposons is an efficient way to generate nonpathogenic mutants of plant-pathogenic bacteria. This approach has been used for *Agrobacterium tumefaciens* (Thomashow *et al.*, 1987), *Pseudomonas syringae* (Lindgren *et al.*, 1986), *Xanthomonas campestris* (Shaw *et al.*, 1988), *Erwinia stewartii* (Dolph *et al.*, 1988), and also for *E. amylovora* (Steinberger & Beer, 1988; Geider *et al.*, 1989; Barny *et al.*, 1990). The vectors used to deliver a transposon were mostly suicide plasmids or a replication-defective phage λ. Thermolabile vectors cannot be used for *E. amylovora*, which is thermosensitive. A versatile transposon is Tn5, because it has no strong preference for sites of insertion and its resistance to kanamycin can be easily screened on plant-pathogenic bacteria.

In search of virulence mechanisms for the fire blight pathogen *E. amylovora*, deficiencies in EPS synthesis and loss of its ability to induce HR on non-host plants (Steinberger & Beer, 1988; Barny *et al.*, 1990; Walters *et al.*, 1990; Bellemann *et al.*, 1990) were found to be correlated with a loss of pathogenicity. We report here the genetic characterization of Tn5 mutants of *E. amylovora* and the effect of the mutations on bacterial EPS synthesis. Preliminary reports of these results have been published in the proceedings of several meetings (Geider *et al.*, 1990a, b, 1991; Bellemann *et al.*, 1990).

Methods

Bacteria, plasmids, and bacteriophages. These are listed in Table 1. Plasmid pSUP201-Ω::Tn5 was constructed by insertion of transposon Tn5 in an *Eco*RI fragment (Ω, approx. 0.8 kb) of the Ti plasmid (Zahm *et al.*, 1984) into the mobilizable pBR-based plasmid pSUP201. The Tn5-carrying plasmid was transformed into *Escherichia coli* strain S17-1, which carries a chromosomal insertion of an RP4 derivative expressing the *tra* genes for plasmid transfer. Plasmid pGV3100::Tn5 was formed by recombination of a cloned Ti-plasmid fragment in plasmid pSS83::Tn5, which was transferred into *Agrobacterium tumefaciens* strain C58(pGV3100). Creation and properties of phage fd-Tn5 will be described in more detail elsewhere (M. Metzger & K. Geider, unpublished). Briefly, a part of viral gene 2, which is absolutely required for phage propagation (Geider *et al.*, 1985), was replaced by a DNA fragment carrying transposon Tn5. The resulting defective phage fd-Tn5 was propagated in cells with cloned phage fd gene 2 (Geider *et al.*, 1985; Geider & Baldes, 1988). *Erwinia amylovora* phage 4L was propagated on strain Ea7/74.

Media and chemicals. *E. amylovora* strains were routinely cultured on St. I (Standard I) agar or St. I medium (Merck) at 28 °C and *Esch. coli* at 37 °C. Minimal medium (MM1) of Steinberger & Beer (1988) was slightly modified and sorbitol added as carbon source. Cas-medium for detection of siderophores was prepared according to Schwyn & Neilands (1987). Motility of bacteria was measured on 0.3% MM1 agar. Agar for propagation of *E. amylovora* phage 4L contained 3 g yeast extract, 5 g peptone, 1.8 ml glycerol (87%, v/v) and 10 g Bactoagar per litre (PP agar). The concentrations of antibiotics (Sigma or Boehringer-Mannheim) were (µg ml⁻¹): ampicillin (Ap), 100; carbenicillin (Cb), 100; chloramphenicol (Cm), 50; kanamycin (Km), 100; spectinomycin (Sp), 100; streptomycin (Sm), 500; tetracycline

Table 2. Transposition rate obtained with various vectors carrying Tn5 and characterization of mutants

The frequency of transposition was determined as Km^r cells of all recipient cells plated.

Tn5 vector plasmid	Recipient strain	Frequency of transposition	Transconjugants screened	Nonpathogenic mutants*
pSUP201-Ω::Tn5	Ea7/74Sm	1 × 10 ⁻⁴	500	2
pGV3100::Tn5	Ea7/74SmSp	1 × 10 ⁻⁷	850	9†
fd-Tn5	Ea7/74 (F::Tn3)	5 × 10 ⁻⁶	250	0

* Assays on immature pears and on pear seedlings.

† Including one auxotrophic mutant.

(Tc), 25. Restriction endonucleases, T4 DNA ligase, and the non-radioactive hybridization kit with digoxigenin were purchased from Boehringer-Mannheim, the Gene-clean kit from Bio 101, and hybridization membranes from Amersham-Buchler.

Manipulation of bacteria. For matings, the strains were grown overnight in St.I medium and 5 parts of a donor strain were mixed with 1 part of the recipient. About 0.2 ml was spread on top of a sterile nitrocellulose filter which had been placed on nutrient agar. After incubation overnight at 28 °C the filter was vortexed for about 3 min in 5 ml medium and 0.2 ml of the suspension, diluted or undiluted, was spread on selective agar. The plates were incubated for 2 d at 28 °C. In the case of pSUP201-Ω::Tn5, the conjugants were screened for ampicillin resistance of the Tn5-donor plasmid. Phage fd-Tn5 was added at a multiplicity of infection of 10 phages per bacterial cell at 28 °C and the cells plated on Km agar (St.I agar plus Km) 1 h after infection. Apparent mutants were also confirmed as *E. amylovora* by hybridization with plasmid pBGS18-5, which contains a fragment of plasmid pEA29 common to all isolates of the fire blight pathogen (Falkenstein *et al.*, 1988).

Isolation and analysis of EPS. *E. amylovora* strains were grown in MM1 medium for 2 d at 28 °C. The cells were removed by centrifugation, the supernatant was adjusted to 1 M-NaCl and the polysaccharides precipitated twice by addition of 3 vols cold ethanol and storage for 1 h at 4 °C. Precipitated EPS was dissolved in water, dialysed against water for 48 h and freeze-dried. Carbohydrate content was measured by the anthrone method of Spiro (1966). Uronic acid was determined according to Blumenkrantz & Asboe-Hansen (1973), and KDO according to Karkhanis *et al.* (1978). To measure viscosity, EPS was prepared as above except that ethanol precipitation was omitted, and dissolved in water to 5 mg ml⁻¹. The samples were monitored in a Cannon-Fenske viscosimeter at 22 °C.

Pathogenicity tests and in planta growth measurements. To test pathogenicity, young pear seedlings (*Pyrus communis* cv. 'Kirchensaller Mostbirne') were inoculated with bacterial suspensions in water (10⁸ bacteria ml⁻¹) about 1 cm below the shoot tip. The plants were kept in a growth chamber at 22 °C, with 12 h illumination per day and high humidity. Plants were assessed for the development of fire blight symptoms for 14 d. Slices of immature pears were inoculated on the surface with a sterile toothpick and kept in Petri dishes at 28 °C. For growth measurements of bacteria, the pear slices were homogenized and the bacteria plated on selective agar. HR was tested by infiltration of bacteria at a density of 10⁸ cells ml⁻¹ into tobacco leaves (Klement, 1982).

Other techniques. Cloning of DNA, analysis and formation of plasmids was done essentially as described by Sambrook *et al.* (1989). For construction of a genomic library, DNA of strain Ea7/74 was partially digested with *Eco*RI and ligated into the mobilizable cosmid

pVK100. After packaging *in vitro* and infection of strain S17-1, the recombinant plasmids were identified by colony hybridization with fragments carrying *Eco*RI-Tn5 fragments from the nonpathogenic mutants.

Results

Vectors for transposon mutagenesis of *E. amylovora*

For the creation of avirulent mutants, we used three suicide vectors to insert transposon Tn5 into the *Erwinia amylovora* genome, which were Tn5 derivatives of: (i) a pBR-based plasmid, (ii) a transfer-constitutive Ti-plasmid and (iii) bacteriophage fd (Table 1). Although most *Escherichia coli* plasmids can replicate in *E. amylovora*, we noticed a deficiency of the virulent strain Ea7/74 for propagation of ColE1 plasmids. *Esch. coli* S17-1(pSUP-Ω::Tn5) was an efficient donor strain to deliver the plasmid into *E. amylovora* strain Ea7/74 (Table 2). The second vector was based on the observation that the Ti plasmid can be transferred promiscuously from *Agrobacterium tumefaciens* into *Esch. coli* without being propagated in the recipient cells (Sprinzl & Geider, 1988). Selection against *A. tumefaciens* C58(pGV3100::Tn5) was difficult with one antibiotic due to a tendency to develop spontaneous resistances. We therefore made the recipient *E. amylovora* strain Ea7/74 resistant to streptomycin and spectinomycin (Ea7/74SmSp). The third transposition system was derived from bacteriophage fd. The *Esch. coli* F plasmid was labelled with transposon Tn3 and transferred to *E. amylovora* strain Ea7/74 with a low efficiency due to the thermosensitivity of *E. amylovora* and the requirement of elevated temperature for F-transfer. Preparations of fd-Tn5 contained a low amount of fd phage with an intact gene 2, derived from rare recombinations of fd-Tn5 with fd gene 2 in the bacterial chromosome of the permissive host. This contamination causes a low background for propagation of fd-Tn5 in cells doubly infected with mutant and wild-type phage.

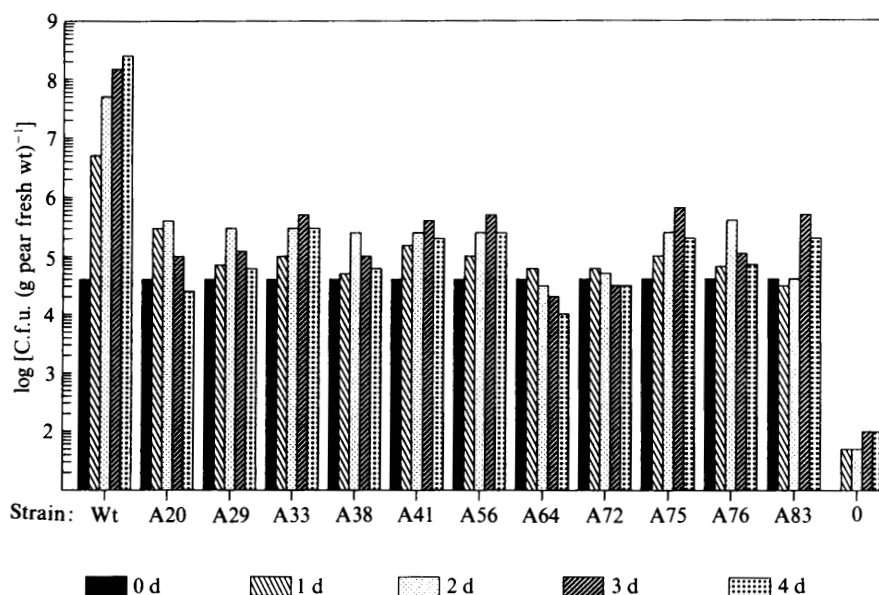


Fig. 1. Growth rates of *E. amylovora* mutants and their parent strain *in planta*. Wt, wild-type strain Ea7/74; A20, A29, A33, A38, A41, A56, A64, A72, A75, A76, A83, Ea7/74 mutants with Tn5; 0, background of pear tissue for colony-forming micro-organisms. The shading refers to various times after inoculation. Values are the mean of three repeated experiments.

Creation and phenotypic characterization of *E. amylovora* Tn5 mutants

The recipient strain for conjugation of *Esch. coli* S17-1(pSUP-Ω::Tn5) was *E. amylovora* Ea7/74Sm and that for conjugation of *A. tumefaciens* C58(pGV3100::Tn5) was Ea7/74SmSp. *E. amylovora* Ea7/74(F::Tn3) was used for infection with fd-Tn5. When the three donor systems for Tn5 were compared for Tn5 insertion efficiency, pSUP201-Ω::Tn5 gave the highest transposition rate and pGV3100::Tn5 the lowest rate (Table 2). Plasmid pSUP201-Ω::Tn5 persisted at a low level in Ea7/74Sm, because half of the Km-resistant cells carried the ampicillin resistance of the vector. Replica plating of these colonies on Km agar yielded a gradual loss of the vector part.

About 1600 Tn5 mutants of Ea7/74 were screened on slices of immature pears for development of virulence symptoms. Negative strains were replica-plated on Km agar and assayed again for virulence. Plating and virulence assay were repeated five times. Only mutants with complete lack of ooze production on pear slices were kept for further investigations. The 11 mutants so obtained were also inoculated on young shoots of pear seedlings, and they did not produce any symptoms.

The mutants were assayed for auxotrophy, for induction of HR on tobacco plants and for EPS synthesis. They induced HR like the parent strain and did not display a change in growth properties in rich medium or

in swarming on soft agar. Their growth on pear slices was measured at various times after inoculation (Fig. 1). Compared to the parent strain Ea7/74Sm, the mutants grew significantly more slowly within the first 2–3 d after inoculation. The number of colony-forming bacteria subsequently decreased in the case of the mutants, in contrast to a further increase for the parent strain. On MM1 with different carbon sources (galactose, glucose, fructose or sorbitol), one mutant did not form colonies (Table 2). This auxotrophic strain, Ea7/74-A64, grew in MM1 supplemented with leucine. Its inability to produce virulence symptoms on pear slices could be restored by soaking the slices in a solution of leucine. The lack of pathogenicity in strain Ea7/74-A64 is therefore due to a mutation in genes encoding leucine metabolism. The other mutants were assayed for a deficiency in iron uptake. On Cas-agar plates, a normal halo was produced around mutant colonies, indicating unchanged siderophore production.

In contrast to the parent strain, most of the mutants displayed non-mucoid colony growth on MM1 agar plates, indicating a deficiency of EPS synthesis (Table 3). Absence of plaque formation for the capsule-specific phage 4L on these mutants correlated with the non-mucoid colony type. Release of EPS into the medium was measured by the carbohydrate content in the culture supernatant after ethanol precipitation (Table 4). EPS production of a Sm-resistant and of a SmSp-resistant derivative of strain Ea7/74 was similar to that of the

Table 3. Characterization of the nonpathogenic Tn5 mutants

Nutrient requirements and colony morphology were examined on MM1 agar plates. Plaque formation was determined on PP agar. HR was measured 24 h after inoculation of tobacco leaves.

Strain	Prototrophy	HR on tobacco	Colony-type of strain*	Lysis by phage 4L	Viscosity of EPS fraction†	Colony-type with pES2144*
Ea7/74Sm‡	+	+	F	+	9.3	F
A7/74SmSp-A20§	+	+	B	—	1.8	F
Ea7/74SmSp-A29	+	+	B	—	1.6	(F)
Ea7/74Sm-A33	+	+	B	—	1.6	B
Ea7/74SmSp-A38	+	+	B	—	1.7	(F)
Ea7/74Sm-A41	+	+	B	—	1.7	B
Ea7/74SmSp-A56	+	+	B	—	ND	F
Ea7/74SmSp-A64	—	+	F	+	ND	ND
Ea7/74SmSp-A72	+	+	F	+	13.5	F
Ea7/74SmSp-A75	+	+	B	—	ND	(F)
Ea7/74SmSp-A76	+	+	B	—	ND	B
Ea7/74SmSp-A83	+	+	B	—	ND	F

ND, Not determined.

* B, nonmucoid colony type (butyrous); F, mucoid colony type (fluidal); (F), intermediate fluidity.

† Relative to water; EPS fraction means material prepared like EPS from the parent strain (see Methods).

‡ This strain and Ea7/74SmSp were the parent strains for Tn5 mutagenesis.

§ Originally with double insertion, which segregated into a single transposition event.

|| On MM1 supplemented with leucine.

Table 4. Analysis of the ethanol-precipitated culture supernatant for polysaccharides

The carbohydrate content was measured in galactose equivalents (one equivalent is defined as 1 ng of the sugar compound measured by the standard assay: see Methods), uronic acids in galacturonic acid equivalents and lipopolysaccharides in KDO equivalents. All the results are expressed per 10⁶ c.f.u., and are the means of five assays.

Strain	Carbohydrate content (Gal equiv.)	Uronic acid content (GalUA equiv.)	LPS content (KDO equiv.)
Ea11/88	120	17	2.5
Ea7/74Sm	73	12	1.9
Ea7/74SmSp	81	13	2.2
Ea7/74SmSp-A20	6	<1	1.2
Ea7/74SmSp-A29	6	<1	1.1
Ea7/74Sm-A33	4	<1	1.3
Ea7/74SmSp-A38	6	<1	1.4
Ea7/74Sm-A41	6	<1	1.1
Ea7/74SmSp-A56	7	<1	1.5
Ea7/74SmSp-A72	94	14	2.3
Ea7/74SmSp-A75	6	<1	1.1
Ea7/74SmSp-A76	10	<1	1.3
Ea7/74SmSp-A83	8	<1	1.2

recently isolated strain Ea11/88. Reduced synthesis of amylovoran measured on the basis of neutral sugars was confirmed by a corresponding decrease for glucuronic acid in 9 of the 10 prototrophic mutants (Table 4). Release of lipopolysaccharide (LPS) was not significantly affected in the mutants. Viscosity of the polysac-

charide preparations was low for all EPS-deficient mutants, whereas the EPS-positive mutant A72 produced EPS which was as viscous as the polysaccharide preparation from the parent strain (Table 3). Mutant A72 not only produced a high level of EPS in the culture supernatant (Table 4), but also induced HR on non-host plants (Table 3). Unknown functions required for pathogenicity have been named *disease-specific* (*dsp*) (Boucher *et al.*, 1987). By this definition strain A72 belongs in the class of *dsp* mutants distinct from *ams* and *hrp* mutants.

Localization of the Tn5 insertions in the *E. amylovora* genome

Plasmid pEA29 was isolated from all mutant strains and was found to be unchanged. Therefore, all Tn5 insertions were located in the bacterial chromosome. The pattern after *Eco*RI cleavage of genomic DNA of the mutants and subsequent hybridization with Tn5 DNA revealed insertions into *Eco*RI fragments of 5.2 kb for four of the mutants and of 1.5 kb for three of the mutants; the remaining four mutants carried Tn5 (5.7 kb) in a fragment of 10.5, 5.8, 3.8 and 2.0 kb, respectively (Fig. 2). The insertion into the 2 kb *Eco*RI fragment caused the leucine deficiency of mutant A64. The multiplicity of the insertions was assayed with two fragments of Tn5. First, the large *Hpa*I fragment of Tn5, which includes most of the IS elements, was eluted from an agarose gel and labelled. Second, the *Bgl*II fragment from Tn5 DNA

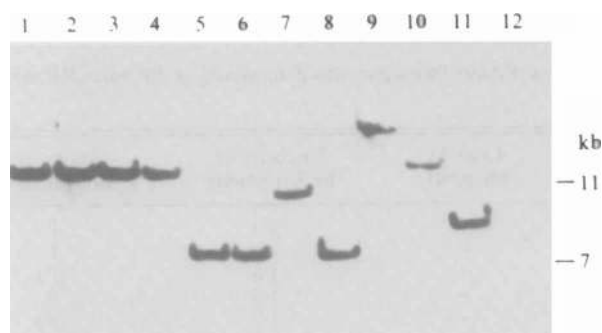


Fig. 2. Southern blot analysis of chromosomal DNA from the Tn5 mutants of *E. amylovora*. The DNA was digested with *EcoRI* and the fragments transferred from an 0.8% agarose gel to a nylon membrane. The probe was a digoxigenin-labelled *HpaI* fragment from Tn5. The signals were detected using a luminescence kit (Boehringer-Mannheim). Lane 1, Ea7/74-A33; lane 2, A41; lane 3, A56; lane 4, A83; lane 5, A38; lane 6, A75; lane 7, A20; lane 8, A29; lane 9, A72; lane 10, A76; lane 11, A64; lane 12, parent strain Ea7/74.

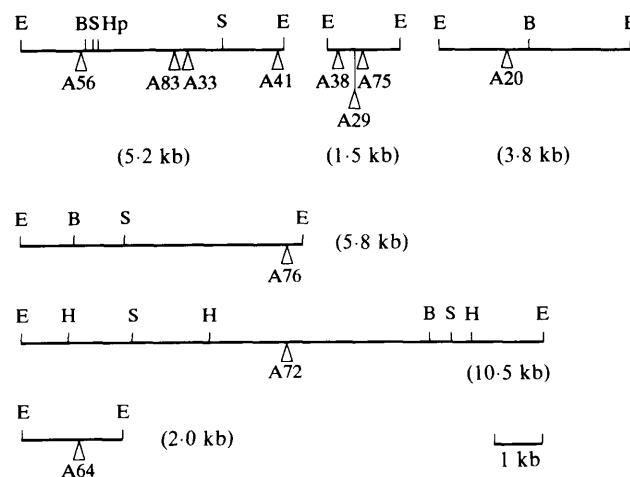


Fig. 3. Localization of the Tn5 insertion sites in the genome of the mutant strains. *EcoRI* fragments from pH79 cosmid DNA were subcloned in pUC19 and mapped with various restriction enzymes. Transposon Tn5 was localized within the fragments for each mutant (A20 to A83). The label Δ marks separate Tn5 insertions on an *EcoRI* fragment with the indicated size. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; S, *Sal*I. The first line represents fragments contiguous in the chromosome.

without the IS elements was labelled. The two probes gave identical hybridization patterns, indicating insertions of the whole transposon without additional IS50 insertions. The *EcoRI* fragments with Tn5 were cloned into cosmid pH79 and subcloned into the mobilizable plasmids pSUP201 or pBR322-mob. The precise sites of the transposon insertion were mapped with the restriction enzymes *Bam*HI, *Hind*III and *Sal*I (Fig. 3). All insertions were found at different sites within the fragments, i.e. they were derived from independent transposition events without a hot-spot in an area involved in EPS synthesis. The contiguous alignment of the *EcoRI* fragments of 5.2, 1.5, and 3.8 kb was recently detected in a large cosmid clone (F. Bernhard, D. Coplin & K. Geider, unpublished).

The fragment with the Tn5 insertion of mutant A33 was subcloned into plasmid pfdA31 and transferred into *E. amylovora* strain Ea1/79 by electroporation (Metzger *et al.*, 1992). Tn5 was rescued by recombination into a site with homology to the adjacent DNA of the transposon. Km-resistant colonies obtained were screened for loss of Ap resistance to eliminate unresolved cointegrates. The resulting mutant Ea1/79-A33 was then assayed for pathogenicity and EPS production. The strain was nonpathogenic and deficient in amylovoran synthesis like the original mutant Ea7/74-A33. Furthermore, recloning of Tn5 from Ea1/79-A33 revealed the transposon insertion in a 5.2 kb *EcoRI* fragment. The transposon insertion is therefore the causative event for the properties of both mutants.

Complementation of the nonpathogenic *E. amylovora* mutants

Genomic DNA from *E. amylovora* strain Ea7/74 was cleaved with *EcoRI* and the digest was ligated into the vector pVK100. This plasmid can be propagated in strain Ea7/74, in contrast to ColE1 plasmids. The DNA was treated with a phage λ packaging extract and transfected into *Esch. coli* strain S17-1. Plasmid pVK100 can be mobilized by the transfer functions of an RP4 derivative integrated in the S17-1 chromosome. Subcloned *EcoRI*-Tn5 fragments from mutants A83 and A72 were labelled and used as probes to identify inserts in the genomic library which were homologous to DNA sequences adjacent to the transposon insertion. About 1000 colonies were probed with these plasmids. In this way, cosmid pCA831 was identified. Its 5.2 kb *EcoRI* insertion was homologous to the *EcoRI* fragments with Tn5 from mutants A33, A41, A56 and A83. Transfer of pCA831 into these mutant strains gave complementation in the case of A33 and A83 and restored both EPS synthesis on MM1 agar and symptom formation on pear slices. Mutants A41 and A56 were not complemented by pCA831. This suggested that the sites of Tn5 insertions in these mutants were located in genes different from the genes mutated in A33 and A83 and that the genes affecting A41 and A56 were not completely cloned on the 5.2 kb *EcoRI* fragment (Fig. 3). The *EcoRI* fragment of

Table 5. Complementation of avirulent *E. amylovora* mutants on pear slices

Rating of virulence: 0 (strain by itself, no symptoms) or —, no exudate visible; +, ooze later than 10 d; ++, ooze at 7–10 d; +++, ooze earlier than 7 d.

Strain	A20	A29	A33	A38	A41	A56	A72	A75	A76	A83
A20	0									
A29	—	0								
A33	—	—	0							
A38	—	—	—	0						
A41	—	—	—	—	0					
A56	—	—	—	—	—	0				
A72	+	+	++	+	++	++	0			
A75	—	—	—	—	—	—	+	0		
A76	—	—	—	—	—	—	+	—	0	
A83	—	—	—	—	—	—	++	—	—	0
Ea7/74SmSp	+++									

pCA831 reisolated after complementation of mutants A33 and A83 often differed from the expected size of 5.2 kb, suggesting that this region may contain an unfavourable DNA sequence, which is subject to deletions.

Screening of the genomic library with the subcloned Tn5-containing fragment from mutant A72 yielded cosmid pCA721. Transferred into strain A72, pCA721 restored pathogenicity. In contrast to the insert of pCA831, the 10.5 kb *Eco*RI insertion was stable in *E. amylovora*. The intact plasmid could be isolated from *Esch. coli* as well as from *E. amylovora*.

Some of the EPS-deficient mutants could be complemented with plasmid pES2144. The insert on this plasmid was derived from the *E. stewartii* genome and comprises five operons involved in biosynthesis of capsular polysaccharides of the maize pathogen (Dolph *et al.*, 1988). EPS synthesis on MM1 agar was restored by plasmid pES2144 to a mucoid colony type for *E. amylovora* mutants A20, A56 and A83, but not for mutants A41 and A76. Partially mucoid colonies were observed for mutants A29, A38 and A75. Complementation of the mutants with subclones of plasmid pES2144 was unsuccessful (F. Bernhard, D. Coplin & K. Geider, unpublished). The fluidal colony morphology of mutant A72 was not changed by plasmid pES2144. Hybridization with pES2144 did not produce signals with cloned fragments of the *ams* gene cluster (data not shown), indicating a limited amount of homology despite functional complementation of the mutants by the plasmid.

Mutant complementation

Attempts to complement mutants *in planta* by cocultivation with other prototrophic mutants obtained were

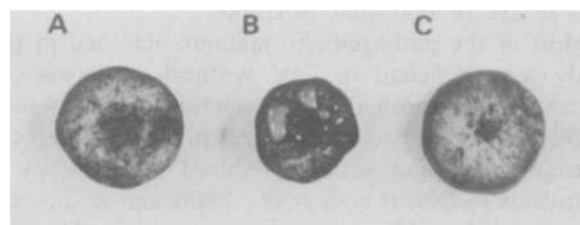


Fig. 4. Complementation of two nonpathogenic mutants to virulence on pear slices. Mutants Ea7/74-A83 and A72 were grown in liquid medium and used for inoculation of pear slices. A, mutant A83; B, 1:1 mixture of mutants A72 and A83; C, mutant A72. The photograph was taken 10 d after inoculation.

successful in the case of combining A72 with the EPS-deficient mutants (Fig. 4, Table 5). No complementation was observed among any mutants of the latter type. Mutant A83 was additionally labelled with the low-copy-number plasmid pVK100 (Tc, Km) to allow its convenient distinction from mutant A72. Cocultivation of Ea7/74-A83(pVK100) and A72 on pear slices and plating on MM1 agar resulted in a 1:1 ratio of mucoid and non-mucoid Km-resistant colonies. The latter bacteria were also identified as strain A83(pVK100) by the Tc resistance of the plasmid. Bacteria from both colony types were further screened on pear slices for virulence and found to display a nonpathogenic phenotype. Furthermore, the amount of EPS synthesized by A72 was sufficient to mask additionally EPS-deficient bacteria against recognition by plant defence mechanisms.

Discussion

In search of the molecular basis of pathogenicity of *E. amylovora* we mutated strain Ea7/74 with three systems

delivering Tn5. These methods circumvented complex vector systems like plasmids with phage Mu (Steinberger & Beer, 1988; Barny *et al.*, 1990) or phage λ as Tn5 donor, which was also barely efficient in our hands for Tn5 mutagenesis in *E. amylovora* strains containing a plasmid with the *lamB* gene. Most recently, electroporation with small Tn5-carrying suicide plasmids has provided another approach for efficient transposon mutagenesis of *E. amylovora* (Metzger *et al.*, 1992). Many of the Tn5 mutants obtained were initially variable on successive screenings for the production of virulence symptoms and could revert to pathogenic strains after passaging them on plates. Similar to the situation with mutations in the *cps* genes of *E. stewartii* (Dolph *et al.*, 1988), the interruption of steps for EPS synthesis of *E. amylovora* could lead to the accumulation of products that interfere with bacterial cell metabolism. Spontaneous deletions in cloned fragments from the *E. amylovora* *ams* cluster may thus reflect an analogous situation.

Most of the pathogenicity mutants obtained in this study were deficient in EPS synthesis, whereas two mutants showed normal EPS production. Mutagenesis of EPS-synthesizing bacteria in genes involved in polysaccharide production strongly reduced the capacity of *Rhizobium meliloti* (Leigh *et al.*, 1985) and *E. stewartii* (Dolph *et al.*, 1988) to colonize host plants. The EPS mutants described here may be affected in many genes concerned with the formation of polysaccharides. Some of the mutants could be complemented by an *E. stewartii* DNA fragment carrying a cluster of *cps* genes. In addition to complementation of regulatory genes (Bernhard *et al.*, 1990), other common functions exist in *E. amylovora* and *E. stewartii* for EPS synthesis. Most recently, a large *Hind*III fragment was identified to carry three (in the order 5.2, 1.5, 3.8 kb) of the four *Eco*RI fragments found here to be mutated in *ams* genes (F. Bernhard, D. Coplin & K. Geider, unpublished). EPS formation should involve synthesis of the repeating unit of amylovoran, which seems to contain four galactose residues and one glucuronic acid residue (Smith *et al.*, 1990). Further steps of biosynthesis comprise transport via a lipid carrier through two cell membranes and its polymerization outside the bacterium (Coplin & Cook, 1990). Gene clusters involved in the biosynthesis of EPSs from *Neisseria meningitidis* (Frosch *et al.*, 1990) and *Esch. coli* (Kröncke *et al.*, 1990) have been functionally dissected into individual steps comprising the formation of EPS precursors in the cytoplasm and their transport to the cell surface. Our mutations in *ams* genes have yet to be assigned to distinct processes in amylovoran production, and to be characterized for enzymic functions encoded by the *ams* genes. We cannot rule out that at least some of our mutants may be affected in regulatory rather than structural genes for amylovoran synthesis.

Regulation of *cps* (*ams*) genes depends on several genes (Coplin & Cook, 1990). The *rcaA* gene from *E. amylovora* was recently cloned, sequenced and compared to corresponding genes from *Esch. coli* and *Klebsiella pneumoniae* (*aeruginosa*) (Bernhard *et al.*, 1990). The significant homology revealed common regulator proteins for polysaccharide synthesis in Gram-negative bacteria. Additional proteins have been implied for regulation of the *cps* (*ams*) genes, which may also be affected by signals from the host plants in the case of phytopathogenic bacteria. Further elements affecting EPS synthesis comprise precursors like UDP-activated sugars or lipid and membrane components, which lead to the synthesis and transport of EPS precursors.

The lack of pathogenicity of the leucine-dependent mutant indicated that a low and insufficient level of this amino acid was available for the fire blight pathogen in immature pears. Tn5 in the prototrophic EPS-positive mutant A72 was localized in an *Eco*RI fragment different from the insertion sites characterized for the other mutants. The gene product missing in strain A72 could be complemented for appearance of virulence symptoms *in planta* by co-inoculation of mutant A72 with an EPS-deficient mutant. It is also possible that growth of A72 on pear slices was due to nutrients leaking from cells damaged by the action of the EPS-deficient mutants which were able to be pathogenic due to the presence of EPS from A72. A similar phenomenon was described for complementation *in planta* of the spontaneous mutants P66 and 4S2 (Bennett, 1980). *E. amylovora* strain P66 has a defect for induction of HR on non-host plants (Walters *et al.*, 1990), whereas strain 4S2 is deficient in EPS synthesis. By reisolation of the unchanged mutants from ooze on pear tissue we conclude that no exchange of DNA occurred among the *E. amylovora* mutants *in planta*, in contrast to the DNA uptake observed in cultures of *Haemophilus influenzae* or *Neisseria gonorrhoeae* (Goodman & Scocca, 1988). The ability of mutant A72 to induce HR implies that not only compounds involved in HR, but also other components in the interaction of *E. amylovora* with its host plants can be complemented *in planta* by EPS-deficient strains. A possible mutation in genes for synthesis of siderophores (Vanneste *et al.*, 1990) was excluded for mutant A72 by the growth pattern of this strain on an indicator agar for iron uptake (data not shown). The restriction map of this fragment suggested that the mutation in the *dsp* genes was adjacent to the *hrp* gene cluster (Barny *et al.*, 1990). The inability of some nonpathogenic mutants to induce HR on non-host plants defines another major requirement for establishment of the pathogen *in planta*. It appears that EPS masks recognition of the bacteria by plant defence mechanisms whereas the inability to induce HR could deplete *E. amylovora* of nutrients

released by the damaged plant cells (Hignett, 1987). The poorly defined and enigmatic *dsp* region could also be involved in plant cell destruction by coding for the biosynthesis of toxin-like compounds. Some virulent *E. amylovora* strains produce the phytotoxin dihydrophenylalanine (Feistner, 1988; Schwartz *et al.*, 1991), which is an inhibitor of the shikimate pathway in both bacteria and plants. The lack of dihydrophenylalanine production in many *E. amylovora* strains thus implies the existence of additional pathways for the fire blight pathogen to damage its host plants, which could include functions coded for by *hrp* and *dsp* genes.

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