Virulence, growth, and surface characteristics of *Erwinia amylovora* mutants with altered pathogenicity

Michel Tharaud,¹ Mohammed Menggad,² Jean Pierre Paulin³ and Jacqueline Laurent²

Author for correspondence: Michel Tharaud. Tel: +33 16 41 22 54 54. Fax: +33 16 41 73 15 57.

¹ ENITHP, Laboratoire de Pathologie Végétale, rue Le Nôtre, F-49045 Angers, France

² Laboratoire de Pathologie Végétale, INRA, 16 rue Claude Bernard, F-75231 Paris Cedex 05, France

³ Station de Pathologie Végétale, INRA, Beaucouze, F-49070 Angers, France

Erwinia amylovora CFBP1430 was mutagenized by phage MudIIPR13 insertion. Thirty prototrophic pathogenicity mutants with a single insertion were isolated. Among these, 17 non-pathogenic (Path⁻) mutants were obtained; 11 of them were unable to induce a hypersensitive response (HR) on tobacco (Hrp⁻ mutants), whereas the remaining six were still able to do so (Dsp⁻ mutants). Thirteen other mutants showed reduced virulence (Rvi⁻) and were still able to induce an HR. One of them appeared to be Path⁻ on apple seedlings and Rvi⁻ on pear seedlings. All the Hrp⁻ mutants and all but three of the Dsp⁻ mutants mapped in the *hrp-dsp* gene cluster previously reported. Some Rvi⁻ mutants also proved to map in this *hrp-dsp* region; most of them, as well as two Dsp⁻ mutants map in an unknown genomic region. Cell-surface components thought to play a role in bacterial pathogenicity were examined, including exopolysaccharides (EPS), lipopolysaccharides (LPS), and outer-membrane proteins. One mutant only was found to be non-capsulated and unable to produce EPS. The insertion in this mutant mapped in a genomic cluster involved in amylovoran synthesis. Unlike the parental strain, some mutants exhibited sensitivity to the phage *Ffm* and this phenotype was associated with a modified LPS electrophoretic profile. Rvi⁻ mutants and some Path⁻ mutants were able to multiply *in planta* to some extent; other Path⁻ mutants reached only a low population level, except the non-capsulated one, which rapidly decreased to an undetectable level.

Keywords: *Erwinia amylovora*, plant pathogen, virulence, cell-surface components

INTRODUCTION

Erwinia amylovora causes fire blight, a bacterial necrotic disease which affects all plant species of the section *Pomoideae*. The bacterium is destructive to pear and apple trees, as well as to a range of ornamentals including *Crataegus*, *Cotoneaster* and *Pyracantha*. On non-host plants (e.g. tobacco) *E. amylovora* elicits a typical hypersensitive reaction (HR). The pathogenesis of fire blight, though investigated over a number of years, is still largely unknown. Physiological and genetical studies have shown that extracellular polysaccharides (EPS) play a role in virulence (Bennett & Billing, 1978; Steinberger & Beer,

1988; Belleman & Geider, 1992) but the existence of fully encapsulated avirulent strains suggested that at least one additional factor was essential for the expression of symptoms (Bennett, 1980; Steinberger & Beer, 1988; Vanneste *et al.*, 1990).

Molecular genetic tools have provided well-characterized pathogenicity mutants of *E. amylovora*, with single transposon insertions (Steinberger & Beer, 1988; Barny *et al.*, 1990; Walters *et al.*, 1990; Bauer & Beer, 1991). These insertions were localized, and most of them appeared to map in a gene cluster of approximately 40 kb on the chromosome. Several cosmids were isolated (Steinberger & Beer, 1988; Barny *et al.*, 1990; Walters *et al.*, 1990; Bauer & Beer, 1991), which were able to functionally complement these mutants. This large region was defined as an *hrp* region, i.e. essential for both the elicitation of the HR on non-host plants and pathogenicity on host plants

Abbreviations: DOC, sodium deoxycholate; EPS, exopolysaccharides; HR, hypersensitive reaction; OM-proteins, outer-membrane proteins; PCC, percentage of capsulated cells.

Table 1. Bacterial strains, bacteriophages and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
<i>Escherichia coli</i>		
ED8767	<i>supE supF hsdS recA56 met</i>	Murray <i>et al.</i> (1977)
<i>Erwinia amylovora</i>		
CFBP1376	Avirulent isolate from <i>Cotoneaster</i> sp.	Paulin & Samson (1973)
CFBP1430	Virulent isolate from <i>Crataegus</i> sp.	Paulin & Samson (1973)
PMV6025	Rvi ⁻ , ::MudIIPR13, Cm ^r	This work
PMV6028	Dsp ⁻ , ::MudIIPR13, Cm ^r	This work
PMV6031, PMV6034, PMV6036, PMV6037	Rvi ⁻ , ::MudIIPR13, Cm ^r	This work
PMV6041	<i>dsp-41</i> ::MudIIPR13, Cm ^r	Barny <i>et al.</i> (1990)
PMV6046	<i>hrp-46</i> ::MudIIPR13, Cm ^r	Barny <i>et al.</i> (1990)
PMV6047	Dsp ⁻ , ::MudIIPR13, Cm ^r	This work
PMV6067, PMV6068	Rvi ⁻ , ::MudIIPR13, Cm ^r	This work
PMV6073	<i>hrp-73</i> ::MudIIPR13, Cm ^r	Barny <i>et al.</i> (1990)
PMV6076	Δ (<i>hrp, dsp</i>), ::MudIIPR13, Cm ^r	Barny <i>et al.</i> (1990)
PMV6081, PMV6082, PMV6086–PMV6088	Hrp ⁻ , ::MudIIPR13, Cm ^r	This work
PMV6089	Dsp ⁻ , ::MudIIPR13, Cm ^r , non-capsulated	
PMV6096, PMV6099	Dsp ⁻ , ::MudIIPR13, Cm ^r	
PMV6100	Rvi ⁻ , ::MudIIPR13, Cm ^r	
PMV6101, PMV6102	Hrp ⁻ , ::MudIIPR13, Cm ^r	
PMV6103, PMV6105	Rvi ⁻ , ::MudIIPR13, Cm ^r	
PMV6109	Hrp ⁻ , ::MudIIPR13, Cm ^r	
PMV6110	Rvi ⁻ , ::MudIIPR13, Cm ^r	
PMV6112	*Rvi ⁻ , ::MudIIPR13, Cm ^r	
PMV6114	Hrp ⁻ , ::MudIIPR13, Cm ^r	
PMV6115	Dsp ⁻ , ::MudIIPR13, Cm ^r	
PMV6116	Rvi ⁻ , ::MudIIPR13, Cm ^r	
PMV6117, PMV6119	Hrp ⁻ , ::MudIIPR13, Cm ^r	
Bacteriophages		
Br60	Lyses most rough mutants of <i>Salmonella typhimurium</i>	Wilkinson <i>et al.</i> (1972)
C21	Lyses mutants of <i>E. coli</i> and <i>S. typhimurium</i>	Lindberg (1973)
Ffm	Lyses <i>rfa</i> and <i>rfb</i> mutants of <i>Salmonella</i>	Wilkinson <i>et al.</i> (1972)
P22	Mutant P22HT105	Schmieger & Backhaus (1973)
PhiEC2		Résibois <i>et al.</i> (1984)
Mu vir	Virulent mutant of Mu	
Plasmids		
pLA2917	<i>oriT</i> (RK2), <i>cos</i> (λ), Km ^r Tc ^r	Allen & Hanson (1984)
pPV130	pLA2917 with a 32 kb insert originating from CFBP1430, Tc ^r	Barny <i>et al.</i> (1990)
pPV132	pLA2917 with a 27 kb insert originating from CFBP1430, Tc ^r	Barny <i>et al.</i> (1990)
pPV133	pLA2917 with a 31 kb insert originating from CFBP1430, Tc ^r	Barny <i>et al.</i> (1990)
pPV152	pLA2917 with a 27 kb insert originating from CFBP1430, Tc ^r	This work

* See Table 2.

(Steinberger & Beer, 1988; Barny *et al.*, 1990; Bauer & Beer, 1991). Some mutations in a part of this large cluster induced a loss of pathogenicity but seemed not to be involved in the HR. Barny *et al.* (1990) considered this sub-region as being disease-specific (*dsp*, designation proposed by Boucher *et al.*, 1987, for genes involved in pathogenicity but no other phenotype); however, Bauer

& Beer (1991) found that the mutants in this sub-region exhibit a loss of pathogenicity but an attenuation of their ability to induce the HR, so they did not clearly differentiate two separate clusters. Another group of pathogenicity mutants appeared to be deficient in EPS production (Steinberger & Beer, 1988; Belleman & Geider, 1992). Transposon insertions in some of these

mutants mapped in a gene cluster of about 7 kb (Bernhard *et al.*, 1993) required for both EPS production and pathogenicity. These genes were designated *ams* (Belleman & Geider, 1992) for amylovoran synthesis (amylovoran is the main component of EPS from *E. amylovora*).

In this paper we present a further characterization of transposon-insertional mutants of *E. amylovora*, which share in common an alteration in pathogenicity. The features of mutants were chosen for their alleged or potential role in pathogenicity and/or in some step of the infection process: after a precise assessment of their residual pathogenicity (symptom expression on host plants, and HR on non-host plants), the mutants selected in this study were examined for properties associated with surface components (EPS, LPS), for *in vitro* and *in planta* growth rate, as well as for several physiological properties. The genomic localization of the corresponding mutations is also presented.

METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains, bacteriophages and plasmids used in this study are listed in Table 1. *E. amylovora* strains were routinely grown at 25 °C in either Luria broth (LB, Maniatis *et al.*, 1982) or King's medium B (KB, King *et al.*, 1954). *Escherichia coli* strains were grown in LB medium at 37 °C. Minimal media M63 or M9 (Miller, 1972) were supplemented with glucose as a carbon source (0.2%) and nicotinic acid (0.02%). YPA medium (Billing, 1984) supplemented with D-galactose (1%, w/v) was used for the capsulation studies. Assimilation of 49 amino acids, 49 carbohydrates and 49 organic acids was studied by using the API system (LRA 50). Media were supplemented with antibiotics, when indicated, at the following concentrations ($\mu\text{g ml}^{-1}$): chloramphenicol (Cm), 10; kanamycin (Km), 20; tetracycline (Tc), 10. For the screening of membrane mutants, LB or M63 agar medium was supplemented with either sodium deoxycholate (DOC, 1 mM for LB + DOC, 1.5 mM for M63 + DOC) or EDTA (400 mM for LB + EDTA, 20 mM for M63 + EDTA). *In vitro* growth of the mutants was compared to the growth of the virulent parent strain CFBP1430. For this purpose, shaken cultures in M9 + glucose medium were sampled by plating at intervals during 24 h.

Origin of *E. amylovora* mutants. *E. amylovora* CFBP1430 was mutagenized by phage MudIIPR13 insertion as described by Barny *et al.* (1990). Among the 5800 chloramphenicol resistant (Cm^r) clones that were obtained, 49 appeared to be prototrophic, with a single transposon insertion and altered in pathogenicity. Out of these 49 mutants, 19 fully avirulent (Path⁻) ones have already been reported (Barny *et al.*, 1990) although not thoroughly characterized by the criteria of the present study. The 30 other mutants were specially studied in the present work: in addition to Path⁻ mutants they include mutants which were not completely avirulent (Rvi⁻). On the basis of specific characteristics noticed in the course of this study, four mutants out of the 19 already described by Barny *et al.* (1990) are also described in more detail here. All techniques used for obtaining, selecting and characterizing the mutants were as described before (Barny *et al.*, 1990).

Plant assays. The HR was tested on tobacco plants (*Nicotiana tabacum* L. cv 'Xanthi') infiltrated with a 10^9 c.f.u. ml^{-1} bacterial suspension in sterile distilled water. Appearance of the HR was scored after incubation at room temperature for 24 h. Patho-

genicity tests on young (six to eight leaves) pear (*Pyrus communis*) and apple (open-pollinated 'Golden Delicious') seedlings were performed in the greenhouse as described previously (Laurent *et al.*, 1987). Bacterial growth *in planta* was studied in the growth chamber. The youngest unfolded leaf of pear seedlings of 'Passe Crassane' (two to four leaves) was wounded by two parallel cuts through the midrib, and immediately inoculated with a drop of bacterial suspension (3×10^6 c.f.u. per leaf). At intervals (3, 8, 24, 48, 72 and 144 h) five seedlings were separately crushed in 10 ml phosphate buffer (0.2 M) and serial dilutions were plated on KB plates supplemented with chloramphenicol.

Serology. Bacteria were grown for 24 h on KB. The agglutination of the mutants in a specific antiserum was compared to that of the parental strain CFBP1430 by the slide agglutination test, using an antiserum raised against CFBP1430. Heat-killed bacteria (100 °C, 2 h) were used as antigens for the rabbit immunization (Samson, 1972). Control negative strains (*Erwinia herbicola*, *E. carotovora*, *Pseudomonas* sp.) were included in the test.

Capsulation. Bacterial cells were grown on YPA medium + galactose. The capsules were revealed by the wet India staining procedure of Duguid (1951). The percentage of capsulated cells (PCC) was determined using an interferential phase-contrast microscope (Polyvar) and 100 × magnification.

Sensitivity to bacteriophages. This was determined by spot assays, as described by Expert & Toussaint (1985).

Extraction and analysis of LPS and OM-proteins. LPS was extracted from *E. amylovora* cells by using the miniscale procedure of Schoonejans *et al.* (1987). Samples were analysed for LPS by SDS-PAGE on 13.5% (w/v) acrylamide gels, with silver-staining by the method of Hitchcock & Brown (1983). OM-proteins were prepared and analysed by SDS-PAGE as described by Vanneste *et al.* (1990).

General DNA manipulations. Genomic DNA was isolated from *E. amylovora* cells by the method of Klötz & Zimm (1972) and purified by CsCl/ethidium bromide density-gradient centrifugation. Plasmid DNA was isolated, and recombinant DNA was prepared and manipulated by using standard techniques (Maniatis *et al.*, 1982). Introduction of plasmids into bacterial cells (by transformation or by mobilization), Southern blot analyses, and colony hybridization were performed as described by Barny *et al.* (1990).

RESULTS

Pathogenicity of mutants

Two classes of non-pathogenic (Path⁻) mutants were obtained (Table 2): Hrp⁻ mutants, which were no longer able to induce an HR in tobacco leaves, and Dsp⁻ mutants, which were still able to induce the HR. Seventeen (11 Hrp⁻, 6 Dsp⁻) prototrophic Path⁻ mutants with a single MudIIPR13 insertion were obtained in addition to the 19 Path⁻ (13 Hrp⁻, 6 Dsp⁻) mutants already isolated by Barny *et al.* (1990).

Some mutants produced symptoms on host plants, but were less virulent than the parental strain CFBP1430 (fewer plants showed symptoms and the length of the stem necrosis was reduced); they were able to induce the HR. These less virulent mutants were designed Rvi⁻, for reduced virulence. Thirteen prototrophic Rvi⁻ mutants, with a single MudIIPR13 insertion, were added to the Path⁻ mutants. The complete set of mutants studied is

Table 2. Pathogenicity, mutated genomic region, and other studied characteristics of *E. amylovora* mutants

Mutant	Pathogenicity*	HR†	Mutated genomic region‡	Growth <i>in vitro</i> §	Growth <i>in planta</i>	Colony morphology on KB¶	Cell capsulation#
PMV6081	Path ⁻	—	<i>brp</i>	+	—	+	=
PMV6082	Path ⁻	—	<i>brp</i>	+	+ / —	+	=
PMV6086	Path ⁻	—	<i>brp</i>	+	—	+	=
PMV6087	Path ⁻	—	<i>brp</i>	+ / —	—	+	<
PMV6088	Path ⁻	—	<i>brp</i>	+	—	+	=
PMV6101	Path ⁻	—	<i>brp</i>	+	+ / —	+	=
PMV6102	Path ⁻	—	<i>brp</i>	+	+ / —	+	=
PMV6109	Path ⁻	—	<i>brp</i>	+	—	+	<
PMV6114	Path ⁻	—	<i>brp</i>	+	+ / —	+	<
PMV6117	Path ⁻	—	<i>brp</i>	+	+ / —	+	=
PMV6119	Path ⁻	—	<i>brp</i>	+	—	+	<
PMV6028	Path ⁻	+	u	+ / —	ND	+	=
PMV6047	Path ⁻	+	<i>dsp</i>	+ / —	—	+	=
PMV6089	Path ⁻	+	<i>ams</i>	+	0	—	0
PMV6096	Path ⁻	+	<i>dsp</i>	+ / —	—	+	<
PMV6099	Path ⁻	+	u	+	+ / —	+	=
PMV6115	Path ⁻	+	<i>dsp</i>	+ / —	—	+	<
PMV6025	Rvi ⁻	+	u	+	ND	+	ND
PMV6031	Rvi ⁻	+	u	+	ND	+	=
PMV6034	Rvi ⁻	+	u	+	ND	+	ND
PMV6036	Rvi ⁻	+	u	+	ND	+	ND
PMV6037	Rvi ⁻	+	u	+	ND	+	ND
PMV6067	Rvi ⁻	+	<i>brp</i>	+	+	+	=
PMV6068	Rvi ⁻	+	<i>brp</i>	+	+	+	=
PMV6100	Rvi ⁻	+	ND	+	+	+	<
PMV6103	Rvi ⁻	+	u	+ / —	+	+	>
PMV6105	Rvi ⁻	+	ND	+ / —	ND	+	=
PMV6110	Rvi ⁻	+	u	+	+	+	<
PMV6112	*Rvi ⁻	+	<i>dsp</i>	+	+	+	=
PMV6116	Rvi ⁻	+	<i>brp</i>	+	+	+	=

ND, Not determined.

* Path⁻, non-pathogenic; Rvi⁻, reduced virulence on pear and apple seedlings; *Rvi⁻, reduced virulence on pear seedlings, non-pathogenic on apple seedlings.

† Hypersensitive reaction on tobacco plant: +, present; —, absent.

‡ *ams*, *dsp*, *brp*, see text; u, unknown, the insertion appeared not to be localized in the known regions *ams*, *dsp*, *brp*.§ Growth *in vitro*: +, identical to the parental strain; + / —, reduced as compared to the parental strain.

|| 0, mutant which failed to multiply and to survive; —, mutants which failed to multiply but were able to survive at low population level; + / —, mutants able to multiply but whose population did not regain the initial inoculum level; +, mutants able to multiply and to reach at best the initial inoculum level.

¶ +, Smooth; —, rough.

Percentage of capsulated cells: > higher, = identical, < lower than that of the parental strain; 0 non-capsulated.

given in Table 2. One of the Rvi⁻ mutants, PMV6112, exhibited a Rvi⁻ phenotype on pear seedlings but a Path⁻ phenotype on apple 'Golden Delicious'.

Physiological characteristics of the mutants

Utilization of amino acids, carbohydrates and organic acids as sole source of carbon and energy. This was tested for 19 prototrophic mutants (belonging to the three

defined classes Hrp⁻, Dsp⁻, Rvi⁻). No stable difference from the parental strain CFBP1430 was noticed (data not shown).

Bacterial growth *in vitro*. The results of *in vitro* bacterial growth studies are shown in Table 2. All the prototrophic mutants showed a growth curve identical to CFBP1430, with the exception of seven mutants which showed strongly reduced growth. In the latter mutants, the total

Fig. 1. Bacterial growth *in planta* of wild-type parental strain CFBP1430 and its mutants. Young pear seedlings were inoculated with 3×10^6 c.f.u. per leaf. At intervals, five seedlings were separately crushed and dilutions were plated on KB. Bars represent one standard error of the mean. ■, CFBP1430; △, PMV6089; ▲, PMV6086; ○, PMV6114; ●, PMV6116. Each mutant is representative of a type of growth (see Table 2).

or partial loss of pathogenicity, together with the reduced growth *in vitro*, might be due to an alteration in genes involved in housekeeping functions.

Bacterial growth *in planta*. The typical growth of mutants on pear seedlings is shown in Fig. 1. During the first 8 h after inoculation, the population level of all strains decreased. Six days (144 h) after inoculation, the wild-type strain CFBP1430 had multiplied to reach about 10^8 c.f.u. per plant (Fig. 1), while Path⁻ mutants showed three types of growth (Fig. 1, Table 2): (i) one mutant (PMV6089) failed to multiply and its concentration decreased to an undetectable level (less than 10^3 c.f.u. per plant) within 72 h; (ii) nine mutants represented by PMV6086 in Fig. 1, did not multiply, but their population level remained fairly stable, at a low level (about 10^4 c.f.u. per plant) during the 144 h of the experiment; (iii) six mutants, represented by PMV6114, were able to multiply for 48–72 h after inoculation and reached about 10^5 c.f.u. per plant, then their population level decreased sharply; in no case did their population regain the initial inoculum level. On the other hand, the Rvi⁻ mutants, represented by PMV6116, multiplied *in planta* and were able to reach at best the population levels of the initial inoculum (Fig. 1, Table 2).

Cell-surface characteristics of the mutants

The following approaches were used to determine whether some of our mutants were modified in their surface characteristics.

Serology. With suitable dilutions of the antiserum, the parental strain CFBP1430 was slide-agglutinated within 2–3 min. In the same conditions the mutant PMV6089 was agglutinated within 30 s; the other mutants were

agglutinated after more than 3 min only. Cells of the naturally avirulent CFBP1376 were agglutinated in the same time scale as CFBP1430. Agglutination was never observed with the other *Erwinia* spp. and *Pseudomonas* sp. tested.

Colony morphology and cell capsulation. When grown on KB, all tested mutants but one (PMV6089) showed a smooth colony type (Table 2), indicating the production of EPS (Sijam *et al.*, 1983). PMV6089 gave rise to rough colonies. All smooth-type mutants showed a much more fluidal colony morphology than the parental strain CFBP1430. The naturally non-pathogenic strain CFBP1376 had the same colony type as CFBP1430. At least 400 cells of each mutant were observed under the microscope to determine the percentage of capsulated cells (PCC). This PCC was compared to the PCC of CFBP1430, grown in the same conditions (Table 2). Mutant PMV6089 was not capsulated. Most of the other tested mutants (16 out of 26) showed, like CFBP1430, a PCC of 12–15; eight mutants exhibited a lower PCC than CFBP1430 (1–5); one mutant (PMV6103) had a greater PCC than the parental strain (70).

Phage sensitivity and characteristics of the LPS. Some surface properties of the mutants were examined through their phage susceptibility. Phages used in this test are given in Table 1. None of the tested mutants appeared different from the parental strain CFBP1430, except for the reaction to phage *Ffm* (Table 3). The virulent phage *Ffm* infects *Escherichia coli* K12 and most rough mutants of *Salmonella typhimurium* or *Erwinia chrysanthemi* (Wilkinson *et al.*, 1972; E. D. Schoonejans, personal communication). For the two latter bacterial species phage *Ffm* appears to specifically attach to the core of LPS. Therefore, PAGE of the LPS from the *Ffm* sensitive mutants was performed.

The LPS from strain CFBP1430 showed a low molecular mass material corresponding to the core part, and a higher molecular mass heterogeneous material showing a ladder of equidistant bands representing the side-chain, an O-antigen-like structure. Similar profiles were observed for the LPS of mutants which were resistant to phage *Ffm*. All the LPS profiles from mutants sensitive to phage *Ffm* were modified (Fig. 2). For two mutants, PMV6028 and PMV6041, the side-chain appeared displaced towards lower molecular masses and there was a decrease in the number of bands. For the other *Ffm*-sensitive mutants, PMV6101, PMV6102 and PMV6112, there was no (or very little) side-chain material. The amount of side-chain material relative to the core part of LPS appeared to be lower in PMV6101 and PMV6102 than in PMV6112.

OM-proteins. DOC as detergent, and EDTA as chelator of divalent cations, are known to interfere with outer-membrane stability. For instance EDTA weakens LPS–LPS interactions and causes the release of LPS and proteins in the form of a LPS–protein complex (Gilleland, 1977; Nikaido & Vaara, 1985). It has also been shown that resistance to bacteriocins, corresponding to the loss or alteration of an OM-protein, sometimes results in increased sensitivity to detergents and chelating agents

Table 3. Characteristics of *E. amylovora* mutants exhibiting altered surface features

Strain	Aspect of the colonies on L-agar	Pathogenicity and mapping	Sensitivity to phage Ffm*	LPS profile†	Growth‡ on:			
					LB + DOC	LB + EDTA	M63 + DOC	M63 + EDTA
CFBP1430	Mucoid (+)	+	R	+	+	+	+	+
PMV6028	Slow growth rough	Dsp ⁻ ?	S	M	As on L-agar	+++	Very poor growth on M63	
PMV6076	+	Hrp ⁻ Δ hrp-brm	R	M	+	+	—	—
PMV6041	Rough	Dsp ⁻ brm	S	M	+	+	—	—
PMV6047	+	Dsp ⁻ brm	R	—	+	+	+ / —	+
PMV6101	+	Hrp ⁻ Δ brm	S	M	+	+	—	—
PMV6102	+	Hrp ⁻ Δ brm	S	M	+	+	—	—
PMV6112	+	Dsp ⁻ brm	S	M	+	+	—	—
					(more mucoid)			
PMV6089	Rough acapsular	Dsp ⁻ ams	R	+	+	+	+	—

* R, resistant; S, sensitive.

† +, normal; M, modified.

‡ Growth was tested on the agar media indicated. +, Growth as CFBP1430; + + +, better growth than CFBP1430; + / —, less growth than CFBP1430; —, no growth.

Fig. 2. LPS profiles of CFBP1430 and Ffm^s mutants of *E. amylovora*. LPS was obtained by the miniscale procedure and samples were analysed by silver-stained SDS-PAGE (13.5% acrylamide). Lane 1, standard proteins (Bio-Rad), from top to bottom: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. Lanes 2–8, LPS from CFBP1430 (5 µl), PMV6028 (5 µl), PMV6041 (5 µl), PMV6101 (5 µl), PMV6102 (5 µl), PMV6112 (4 µl), PMV6112 (8 µl), respectively.

(Expert & Toussaint, 1985). Thus the 49 pathogenicity mutants of *E. amylovora*, either Path⁻ or Rvi⁻, were screened for sensitivity to DOC or EDTA, either on LB agar medium or on M63 agar medium as described in Methods. Eight clones exhibited a modified sensitivity

when compared to CFBP1430 (Table 3). The OM-proteins from these mutants were analysed: no obvious difference was observed between the electrophoretic profiles of the OM-proteins from the mutants as compared to CFBP1430 (data not shown). Thus, the mutations

responsible for modified sensitivity to DOC and/or EDTA might affect a minor protein not easily detectable under the conditions used. Alternatively the effects of DOC and EDTA on the mutants studied might not be OM-protein specific and should be correlated to modifications in other outer-membrane components, especially LPS.

Localization of the MudII^{PR}13 insertions in a previously cloned chromosomal DNA region

We looked for MudII^{PR}13 insertions in the *hrp*-*dsp* region of the genomic DNA from the pathogenicity mutants. Three overlapping cosmids, pPV130, pPV132 and pPV133 (Table 1), covering the entire region considered, were used as probes for Southern blot hybridization with genomic DNA digests from each mutant.

The insertion in every Hrp⁻ mutant appeared to be localized in this region (Fig. 3). In addition to the Hrp⁻ mutant PMV6076, three Hrp⁻ mutants showed a deletion in this region. In PMV6082 the entire region (about 52 kb) was deleted, as it was in PMV6076. The deletion in PMV6101 and PMV6102 was smaller (about 11 kb) and involved the *dsp* subregion (Barney *et al.*, 1990). The MudII^{PR}13 insertions in three additional Dsp⁻ mutants (PMV6047, PMV6096, PMV6115) proved to map in this subregion.

For all but four of the Rvi⁻ mutants, and for three Dsp⁻ mutants (PMV6028, PMV6089, PMV6099), the insertions did not map in the above-mentioned region. The insertions in three Rvi⁻ mutants (PMV6067, PMV6068 and PMV6116) mapped in the *hrp*-*dsp* region. The insertion of PMV6112, which was Hrp⁺ on tobacco leaves, Path⁻ on apple shoots and Rvi⁻ on pear shoots (Table 2), was localized at the left-end limit of the 3.9 kb *Sal*I fragment (Fig. 3).

Cloning of the genomic DNA mutagenized in the acapsular mutant PMV6089 and search for other insertions in the same region

Total DNA from the acapsular mutant PMV6089 was digested to completion with *Bgl*II and fractionated in a sucrose gradient. The fragments sizing at about 20 kb were ligated to the cosmid pLA2917 previously linearized with the same enzyme. The ligation mixture was packaged in the presence of λ phage encapsidation extracts and introduced into *Escherichia coli* ED8767 cells, selecting for resistance to both chloramphenicol and tetracycline.

A *Bgl*II-*Hind*III fragment was isolated from the PMV6089 insertion. This *Bgl*II-*Hind*III fragment was ³²P-labelled and used as a probe to screen a pLA2917 genomic library from CFBP1430 (Barney *et al.*, 1990). Five clones hybridized with the probe: as deduced from its physical map, the corresponding CFBP1430 genomic region was different from the *hrp* area (Fig. 4). The physical map of the *E. amylovora* insertion in the cosmid pPV152 was compared with that of the plasmid pEA109

Fig. 3. Location of MudII^{PR}13 insertions in the *hrp* gene cluster of *E. amylovora*. The restriction enzyme map shows the sites for S, *Sal*I; H, *Hind*III; E, *Eco*RI; and B, *Bam*HI. The locations of MudII^{PR}13 insertions are indicated above the physical map. Seventeen insertions were previously mapped; the names of the corresponding mutants are given in Barney *et al.* (1990). The insertions that were not previously mapped are highlighted by a vertical arrow; from left to right (in square brackets are mutations which could not be precisely localized in the indicated area and are not represented on the map) they are: PMV6117, [PMV6109], PMV6067 and PMV6068, PMV6086, PMV6088, [PMV6087, PMV6116], PMV6114, PMV6081, PMV6119, PMV6112, PMV6115, PMV6096, and PMV6047. The directions of the insertions are indicated by the directions of the pointers (I) or of the flags (P). Flags indicate insertions which could be used to monitor gene expression via β -galactosidase activity (Barney *et al.*, 1990; M.-A. Barney & J. Laurent, unpublished data); pointers indicate insertions which could not be used for that purpose. The asterisks (*) correspond to Rvi⁻ mutants. The deletion mutants are not represented: PMV6082 spans the whole region as previously shown for PMV6076 (Barney *et al.*, 1990); the deletions in PMV6101 and PMV6102 are restricted to the *dsp*-*hrm* sub-area.

Fig. 4. Location of a MudII^{PR}13 insertion in an *ams* gene cluster of *E. amylovora*. The restriction enzyme map shows the sites for H, *Hind*III; E, *Eco*RI; and B, *Bam*HI. The location of the MudII^{PR}13 insertion in PMV6089 is indicated above the physical map. The direction of the pointer (I) indicates the direction of the insertion. Extension of the insert present in pPV152 is indicated by the horizontal line (bottom).

obtained by others (Belleman & Geider, 1992; Bernhard *et al.*, 1993) and covering a large genomic cluster involved in amylovoran synthesis (*ams* genes). Part of both plasmid maps appeared similar and hybridization experiments proved that they shared homology (data not shown). This region has been studied in detail by M. Menggad (unpublished). The cosmids pPV151 and pPV152, covering a large part of this region, were mobilized into PMV6089 cells. As a control pLA2917 was also mobilized into PMV6089 cells. The resulting transconjugants were

assayed for pathogenicity on pear and apple seedlings. The transconjugants harbouring pPV151 or pPV152 were able to restore typical fire blight symptoms while those harbouring pLA2917 were not, indicating that the loss of pathogenicity was due to the MudIIPR13 insertion in the chromosome of strain PMV6089.

The cosmid pPV152 was used as a probe for Southern blot hybridization with genomic DNA digests from each pathogenicity mutant. None of the mutations appeared to map in the same genomic region as the insertion in PMV6089.

DISCUSSION

This study of insertional mutants of *E. amylovora* focused on pathogenicity, growth *in vitro* and *in planta*, and some membrane characteristics. After mutagenesis with MudIIPR13, stable potential pathogenicity mutants of *E. amylovora* were selected as having lost the ability to induce exudate formation on apple calli according to Paulin & Duron (1986) (Cal^- mutants, as opposed to Cal^+ , which are able to do so). Thirty prototrophic pathogenicity mutants with a single MudIIPR13 insertion were identified. Thirteen Rvi^- mutants showed reduced virulence *in planta*, and 17 Path^- mutants were avirulent on susceptible hosts. Among the Path^- mutants, 11 Hrp^- mutants were no longer able to induce HR in tobacco whereas six Dsp^- mutants were. All the Hrp^- mutants map in the large *hrp* gene cluster previously reported (Steinberger & Beer, 1988; Barny *et al.*, 1990; Bauer & Beer, 1991). Most Dsp^- mutants map in a sub-area of this large gene cluster that we previously defined as a *dsp* region (Barny *et al.*, 1990). Since Wei *et al.* (1992a) mentioned that mutants in the so-called *dsp* area showed a variable *Hrp* phenotype, and since we found that a deletion in this region (PMV6101, PMV6102) produced a clear-cut Hrp^- phenotype, we propose to consider this genomic region as a *brm* (hypersensitive response modulator: Huang *et al.*, 1991) region rather than a *dsp* one, although the observed phenotypes are not exactly similar to those described by these authors for *Pseudomonas syringae* pv. *syringae* 61. The discrepancy between the observations of Wei *et al.* (1992a) and our results might be due mainly to different experimental conditions, particularly the size of inoculum used in tobacco plant inoculation, which was higher in our experiments. However, since we did not obtain any other mutant with an insertion localized in the approximately 4 kb space between the location of the insertions in PMV6112 and in PMV6115, we have no indication of the phenotype that would be shown by such a mutant. Some Rvi^- mutants at least, PMV6067, PMV6068, PMV6112 and PMV6116, also proved to map in the *hrp* region. These data suggest that, in these mutants, the insertion is near the end of a gene with the resulting gene product shorter than the normal gene product, thus exhibiting a lower efficiency than the normal one. A similar alteration in the gene product might also explain the Rvi^- phenotype of PMV6112 on pear seedlings. The difference in virulence of PMV6112 on apple and pear seedlings might be due to a higher resistance level in our apple seedlings than in our

pear seedlings. In the hypothesis of an interaction between a bacterial and host product, the altered bacterial product in PMV6112 might still be capable of some interaction with a pear product, while unable to interact with an apple one. Interestingly PMV6112 seems to be located in the *hrpN* locus described by Wei *et al.* (1992b). Further studies are currently in progress to elucidate the different behaviour of this mutant on apple and pear and the role of the *hrpN* product in *E. amylovora*.

The reason why only one mutant without a capsule was found in this study is not known. It is noteworthy that among 49 pathogenicity mutants, either Path^- or Rvi^- , we found only one EPS^- mutant, while others (Steinberger & Beer, 1988; Belleman & Geider, 1992) found far more EPS^- mutants as compared to other pathogenicity mutants. Both groups used Tn5 insertion mutagenesis, with different parental strains. Our parental strain CFBP1430 appears much more fluidal than those of the other workers. It might have genetic characteristics such as gene duplication so that an EPS^- phenotype would rarely appear. Alternatively, although MudIIPR13 and Tn5 are supposed to insert randomly into the target genome, they might have preferential insertional sites so that the Mu derivative would rarely be inserted into EPS -encoding genes of *E. amylovora*.

Thus most Path^- mutations map in already known genomic regions. However, two Dsp^- (PMV6028 and PMV6099) and most Rvi^- mutations do not map either in the *hrp-dsp*, or in the *ams* cluster. It was also shown that none of the corresponding insertions was in the ubiquitous plasmid pEA28 (Falkenstein *et al.*, 1988; Laurent *et al.*, 1989) of *E. amylovora* (data not shown). At least for Dsp^- mutants this is consistent with the fact that *E. amylovora* strains which are cured of this plasmid are still able to induce fire blight symptoms on host plants (Laurent *et al.*, 1989; Falkenstein *et al.*, 1989). Because at least three restriction enzymes, *Bam*HI, *Eco*RI and *Hind*III, were used for the localization experiments, it is possible that pathogenicity genes might be located in other genomic regions. To identify the affected genomic region in these mutants, it would be necessary to clone the border of the transposon insertion. Since PMV6028 and PMV6099 show different growth and surface characteristics their mutated genes should govern different functions.

Some pathogenicity mutants exhibit sensitivity to the phage *Ffm*. This phenotype is associated with a modified LPS electrophoretic profile. Two types of modifications were observed: PMV6101, PMV6102 and PMV6112 had no or very few large LPS molecules with normal side-chain length, while PMV6028 and PMV6041 showed mainly molecules with short side-chains. Several clones from our collection of insertional mutants were screened for sensitivity to the phage *Ffm*. It is noteworthy that nine Cal^- mutants (unable to induce exudate formation on apple calli) out of 70 were Ffm^S , whereas no Ffm^S clone was found among 100 Cal^+ clones (data not shown). This might reflect a relation between the Cal^- phenotype and a modified LPS content, composition, and/or structure.

However, the Cal⁻ Ffm^S mutants showed diverse pathogenicity phenotypes, including full virulence. Thus there was no direct relationship between a modified LPS and pathogenicity. Two Dsp⁻ mutants, PMV6079, with a normal LPS profile (data not shown), and PMV6041, with a modified LPS profile, map very close to each other. This indicates that the LPS defect in PMV6041, which is not restored by the cosmid pPV132 (M.-A. Barny, personal communication), might be due to a spontaneous mutation in a genomic region different from the *hrp* gene cluster. The cloning and mapping of the mutation in PMV6028, as well as complementation assays with a cosmid harbouring the wild-type corresponding region, should help to test this hypothesis.

The three mutants (PMV6101, PMV6102, PMV6112) which exhibit the other type of modified LPS profile map in the *brm* sub-area of the large *hrp* gene cluster. Since there is a deficit of side-chain material in the LPS of these mutants, which include two deletion mutants, one can postulate that at least one gene in the deleted fragment has regulatory functions on LPS biosynthetic genes. Alternatively, one of these *brm* genes at least encodes an outer-membrane component so that the outer membrane of the mutants PMV6101, PMV6102 and PMV6112 might have an altered structure, thus preventing the large LPS molecules with long side-chains remaining tightly attached to the membrane. It is worth pointing out that the mutant PMV6028, with the other modified LPS profile, shows in addition a specific EDTA-dependent phenotype suggesting an altered membrane structure. Since the mutants showing a modified LPS profile are agglutinated in the CFBP1430-specific antiserum, one might assume that the corresponding antigenic sites are conserved. Taken together these data suggest that several outer-membrane components, in relation with LPS molecules, are involved in plant-bacteria interactions.

The presence of a capsule around bacterial cells was another approach to determine whether our mutants were altered in their surface characteristics, as compared to the parental strain CFBP1430. One mutant (PMV6089) appeared to be non-capsulated, and unable to produce EPS, even if grown on YPA medium supplemented with D-galactose, which is known to stimulate capsule synthesis (Billing, 1984). Most of the other mutants studied exhibited a PCC similar to, or lower than that of CFBP1430; their colony morphology was smooth but much more fluidal than that of CFBP1430. The mutation in PMV6103, which led to a higher PCC, might affect a gene encoding a repressor of capsular polysaccharide biosynthesis. Since there is no correlation (with the exception of PMV6089) between PCC and colony morphology, one cannot infer from our results that regulators of EPS biosynthesis such as *rcs*-like or *lon*-like genes were altered in our mutants (for reviews see Coplin & Cook, 1990; Roberts & Coleman, 1991).

The slide agglutination test used, though simple, gave interesting results because it applies to somatic-O antigens (Samson, 1972). With this method all mutants tested were agglutinated but differences were noticed: PMV6089 was

much more rapidly agglutinated than CFBP1430, while the other mutants were more slowly agglutinated. This could be related to a modification of the quantity and/or the quality of the EPS produced by the mutants. Modified EPS, altered in its chemical and/or physical properties might thus hamper the accessibility to somatic-O antigenic sites and slow down the reaction in the agglutination test. This is consistent with the observed fast agglutination of PMV6089, whose cells are not protected by EPS. Among the Path⁻ mutants described by Barny *et al.* (1990) all but two (PMV6046, PMV6073) were slowly agglutinated like most of the mutants described in this paper. PMV6046 and PMV6073, in contrast, were never agglutinated in the conditions described here, agglutination only being observed from 3-d-old cultures; these particular mutants, which most probably map in the same gene, are currently under study.

The population levels of the mutants able to multiply in the host plant never reached the level of the initial inoculum (3×10^6 c.f.u. per plant), except for the Rvi⁻ mutants, while in the same time period the fully pathogenic parental strain reached about 5×10^7 c.f.u. per plant. The other mutants were able to survive only at a low population level, but could not multiply in the host plant. This ability to survive and/or to multiply in the host plant appeared to be linked with EPS production, since only the EPS⁻ PMV6089 rapidly decreased to an undetectable level. Norelli *et al.* (1990) reported similar observations with two EPS⁻ mutants of *E. amylovora*, and so did Ayers *et al.* (1979) with a naturally acapsular strain of *E. amylovora*. All EPS-producing mutants, even those exhibiting a low PCC, were able to survive in the host plant. It seems therefore that (i) the presence of EPS is a requisite to ensure the survival of bacteria, and (ii) a low PCC is sufficient to allow such survival. The question of how much EPS and what kind of EPS (capsule and/or slime) is required for bacterial survival in the host plant remains open.

Each of the three classes of mutants defined here (Hrp⁻, Dsp⁻, Rvi⁻) includes strains which are modified in their PCC as well as strains with a normal PCC. Insertions in several *hrp* or *dsp* genes appear to affect the PCC and the agglutinating properties of the mutants. Thus, mutations in the *hrp*-*dsp* cluster might modify EPS production. More studies are needed to correlate a modified PCC with mutations in particular genes before investigating the nature of the changes in EPS (quality and/or quantity) reported in this work.

Assuming that avirulent strains of *E. amylovora*, when able to multiply *in planta*, might be of interest in biological control against fire blight, the ability of our Path⁻ mutants to protect apple seedlings against infection by a virulent challenger strain was tested. In our conditions all Path⁻ mutants described in this paper failed to protect apple seedlings, in contrast with our positive check CFBP1376 (a naturally avirulent isolate), which had a high protective ability (data not shown). However, among the Path⁻ mutants described by Barny *et al.* (1990) we found three which exhibited the ability to protect (Tharaud *et al.*,

1992). The transposon insertions in the chromosome of these three mutants might be located in the same transcription unit in the *hrp* genes cluster.

Our results are consistent with the current hypothesis on the role of *hrp* genes in plant-pathogenic bacteria. Some *hrp* genes are clearly regulatory, responding to plant signals to induce transcription of several other *hrp* genes (Clarke *et al.*, 1992; Genin *et al.*, 1992). Other *hrp* genes code for the synthesis and export of plant and animal pathogenicity factors (Clarke *et al.*, 1992; Gough *et al.*, 1992). Experiments are in progress aimed at understanding the interactions between avirulent mutants, virulent strains, and the host plant.

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