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Characterization of a Ubiquitous Plasmid in *Erwinia amylovora*

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An approximately 30-kb plasmid was present in each of 66 strains of *Erwinia amylovora* isolated from various hosts and areas. These plasmids from different strains proved identical to pEA28, the unique plasmid from the virulent *E. amylovora* strain CFBP1430. The physical map of pEA28 was established by using six restriction enzymes. To determine a possible role in pathogenicity for this plasmid, CFBP1430 was cured of pEA28: The plasmid was first labeled by a Mu-derivative transposable element and was subsequently evicted in the presence of a fragment containing putative genes for plasmid incompatibility. A function necessary for thiamine independent growth was assigned to pEA28. When the cured strain was inoculated into sensitive hosts, delayed appearance and reduced severity of symptoms were observed.

Additional keyword: plasmid curing.

Erwinia amylovora causes fire blight, a necrotic disease affecting most species of the Pomoideae (Van der Zwet and Keil 1979). A cryptic plasmid of about 30 kb has been detected in several strains of *E. amylovora* (Kado and Liu 1981; Merckaert *et al.* 1982; Panopoulos *et al.* 1978). Diverse plasmid-associated traits have been described in plant pathogenic bacteria. They include chemical resistance, bacteriocin, and vitamin or pigment production, as well as virulence determinants (Panopoulos and Peet 1985). For example, it is well known that most of the pathogenicity genes of *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* are plasmid borne (Bevan and Chilton 1982; Comai and Kosuge 1982). Our findings in this paper confirm the ubiquity of this plasmid, called pEA28, in *E. amylovora*. This result then prompted us to study the role of pEA28 in the pathogenicity of *E. amylovora*. For that purpose, a plasmidless, or cured, strain was necessary. However, pEA28 appeared to be very stable because conventional curing methods were unsuccessful (Vanneste *et al.* 1985). A labeled derivative of pEA28 was constructed and subsequently eliminated in the presence of a cloned fragment. The cured strain was then characterized. Preliminary accounts of this work have been reported (Laurent *et al.* 1987a; Laurent and Barny, Fallen Leaf Lake Conference on the genus *Erwinia*, Fallen Leaf Lake, CA, U.S.A., September 17-20, 1987).

MATERIALS AND METHODS

Strains, plasmids, culture media, and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. Other strains of *E. amylovora* were obtained from various sources. The vector plasmid pTG10 was constructed by A. A. Gatenby (unpublished) by ligating the S1, blunt-ended, 433-bp *Hae*II fragment from pUC8 (Vieira and Messing 1982) to pACYC184 (Chang and Cohen 1978) cut with *Hin*dIII and *Bam*HI and polymerase I repaired. Due to α-complementation, pTG10 confers a Lac^+ phenotype to strain TB1, except when an insert has been introduced, 5′ to *lac′*Z in the polylinker containing three cloning sites: *Pst*I, *Hin*dIII, and *Sma*I.

Bacterial strains were grown in L broth (Miller 1972) at 30° C. The solid medium contained Bacto-Agar (15 g/L). When required, antibiotics were used at the following concentrations: 10 μg/ml of chloramphenicol (Cm) and 20 μg/ml of kanamycin (Km). Nicotinic acid (0.02%, w/v) was added to the minimal media, M9 or M63 (Miller 1972), for growth of *E. amylovora*.

Transformation of *E. amylovora* with plasmid DNA. *E. amylovora* cells could not be transformed by standard methods; therefore to prepare competent *E. amylovora* cells, a transforming procedure derived from Hanahan's (1983) was adapted. A fresh culture was incubated at 30° C in the specified medium until $A_{600} = 0.4$ was reached. The cells were then subjected to the specified treatments; the final volume was one-fiftieth of the original volume. The transforming DNA was added to the aliquot of competent cells and the mixture was treated as described by Hanahan (1983), except that the temperatures were lower: The heat pulse was at 37° C and incubation was at 25° C. The cells were pelleted and the whole sample was plated on a selective medium. This protocol gave transformation efficiencies of about 10^3 cells per microgram of plasmid DNA.

Plasmid-directed mutagenesis. The protocol for mini-muduction, described by Castilho *et al.* (1984), has been applied to specifically mutagenize plasmids. The plasmid pPV73 was introduced by a standard $CaCl_2$ transformation procedure (Maniatis *et al.* 1982) into *E. coli* strain POI1734 with selection for Cm^r. Lysates were prepared from the transformants and used to transduce *E. coli* strain M8820Mu to Cm^r and Km^r.

DNA isolation and analysis. Total DNA was isolated from *E. amylovora* cells by the method of Klötz and Zimm (1972). It was purified by density gradient centrifugation and precipitation in 70% ethanol. Plasmid preparations and DNA analysis were performed as previously described (Boccara *et al.* 1988). Southern blot hybridization was

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performed as described by Maniatis *et al.* (1982). Plasmid DNA, which was purified by density gradient centrifugation, was labeled with a nick translation kit from Bethesda Research Laboratories according to its recommendations, using [α-^{32}P]dCTP (specific activity = >800 Ci/mM).

Pathogenicity tests. Golden delicious apple seedlings, Passe crassane pear seedlings, and *Pyracantha rogersiana* shoots were inoculated as previously described for pear seedlings (Laurent *et al.* 1987b), except that the concentration of the bacterial suspension was 10^7 cells per milliliter.

RESULTS

Ubiquity of an approximately 30-kb plasmid among *E. amylovora* strains. By means of Eckhardt's method of plasmid detection (Eckhardt 1978), we confirmed the presence of an approximately 30-kb plasmid among 66 strains of *E. amylovora* from various sources (host plants and geographic areas). All of these strains contained at least the approximately 30-kb plasmid and most of them (49/66) contained only this plasmid (data not shown).

The plasmids from 10 *E. amylovora* isolates were studied in more detail. They appeared identical to pEA28, the plasmid from strain CFBP1430, used as a reference. This was shown by: 1) similar agarose gel electrophoresis patterns of their *Eco*RI digests (data not shown) and 2) Southern blot hybridization of their *Pst*I digests, with pEA28 as a probe (Fig. 1).

Physical map of pEA28. All of the fragments produced after *Pst*I digestion of pEA28 were cloned into the *Pst*I site of the vector plasmid pTG10. A restriction map of pEA28 (Fig. 2) was obtained by a combination of the following procedures: 1) analysis of DNA fragments obtained after single and double digestion of pEA28 with different enzymes, 2) analysis of the restriction products of pTG10 derivatives carrying *Pst*I fragments of pEA28, and 3) Southern blot hybridization of different endonuclease digests of pEA28, with a given *Pst*I fragment as a probe.

Genetic labeling of pEA28. The plasmid was labeled by marker exchange (Ruvkun and Ausubel 1981) so that its loss could be monitored easily. Plasmid-directed mutagenesis was used to introduce the transposable element MudII1734 (conferring resistance to Km^r) into pPV73, which carries the *Pst*I fragment A of pEA28. Several insertions of MudII1734 into the *Pst*I fragment A of pEA28 were thus obtained. One of the resulting mini-Mu-labeled plasmids, pPV97, was introduced by transformation into *E. amylovora* CFBP1430 in order to get genetic labeling of pEA28 by marker exchange. A population of transformants was screened for stable Cm^{s}Km^r clones. In such clones, MudII1734 had integrated into the indigenous pEA28 and

Table 1. Bacterial strains, plasmids, and phage

Strain, plasmid, or phage	Relevant properties[a]	Source or reference
Escherichia coli		
M8820Mu	M8820 with Mu	Castilho *et al.* 1984
POII1734	MC1040(Mu *cts*) with Mu dII1734 *lac*$^+$, Km^r	Castilho *et al.* 1984
TB1	JM83 + hsdr$^-$ hsdm$^+$ from MM294	Baldwin *et al.* 1984
Erwinia amylovora		
Wild-type isolates[b]		
CFBP1187	Isolated in U.S.A.	R. N. Goodman (39)
CFBP1196	*Pyrus communis*, United Kingdom, NCPPB595	J. E. Crosse (P 42)
CFBP1221	*Malus sylvestris*, Poland	A. Burkowicz (S9)
CFBP1227[c]	*Malus sylvestris*, New Zealand, 1963	W. Kemp
CFBP1228[d]	*Cotoneaster* sp., U.S.A., 1967	M. N. Schroth (FB 1)
CFBP1262	Isolated in Canada	R. Layne (FB 68 A)
CFBP1376[e]	*Cotoneaster* sp., Belgium, 1972 Naturally avirulent isolate	R. Veldeman (XIII)
CFBP1430	*Crataegus* sp., France	R. Samson (EI 25)
CFBP1986	*Mespilus germanica*, France, 1980	J.-P. Paulin (1586-2)
CFBP2045	*Pyrus communis*, France	J.-P. Paulin (1342-2)
Laboratory strains		
PMV6010	CFBP1430(pPV99), Km^r	This work
PMV6011	PMV6010(pPV99$^-$) (pPV77), Cm^r	This work
PMV6012	PMV6010(pPV99$^-$) (pPV76), Cm^r	This work
PMV6014	PMV6011(pPV77$^-$) = CFBP1430(pEA28$^-$)	This work
Plasmids		
pEA28	Unique plasmid of CFBP1430	This work
pPV73	*Pst*I-A fragment of pEA28 cloned in pTG10	This work
pPV76, pPV77	*Pst*I-B fragment of pEA28 cloned in pTG10	This work
pPV97	pTG10::pEA28(*Pst*I-A)::MudII1734[f]	This work
pPV99	pEA28(*Pst*I-A)::MudII1734[f]	This work
pTG10	pACYC184 derivative (see text) *cat, lac'Z*	A. A. Gatenby (unpublished)
Phage		
MudII1734	mini-Mu A$^-$B$^-$ Km^r *lac'Z*	Castilho *et al.* 1984

[a] Cm^r, Km^r: Resistant to chloramphenicol and kanamycin, respectively.
[b] Unless otherwise stated, the strain contained only one plasmid, pEA28.
[c] Three plasmids: pEA28 and two larger plasmids (about 60-kb and about 70-kb).
[d] Two plasmids: pEA28 and a smaller plasmid.
[e] Two plasmids: pEA28 and an about 60-kb plasmid.
[f] The mini-Mu insert maps at 19.4 kb.

the recombinant vector plasmid had been eliminated. The resulting mini-Mu-labeled pEA28 was named pPV99 and the corresponding *E. amylovora* strain, PMV6010.

Elimination of the labeled plasmid. Because a genetically labeled plasmid was now available, the loss of this plasmid could be monitored easily. It proved, however, to be impossible to eliminate pPV99 by growing PMV6010 at 37° C. Consequently, we worked out a strategy, which was intended to displace the plasmid, involving the introduction of cloned fragments of pEA28 carrying putative genes for plasmid incompatibility (Knauf and Nester 1982). Because the incompatibility functions of pEA28 have not been localized, pTG10 derivatives carrying different *Pst*I fragments of pEA28 were introduced by transformation into PMV6010. Transformants in which pPV99 had been eliminated were identified by Km sensitivity. Two pTG10 derivatives, pPV76 and pPV77, which contained the 4.3-kb *Pst*I fragment B of pEA28, eliminated pPV99 from PMV6010. The pTG10 derivatives carrying the other *Pst*I fragments of pEA28 did not interfere with the maintenance of pPV99 in PMV6010.

The elimination of the recombinant plasmid pPV77 was readily obtained due to the instability of pTG10 and its derivatives in *E. amylovora*. One antibiotic-sensitive clone, PMV6014, was purified and found to be devoid of plasmid. Southern blot hybridization of total DNA from PMV6014 with ^{32}P-labeled pEA28 showed that PMV6014 possessed no DNA sequences homologous to pEA28 (Fig. 3).

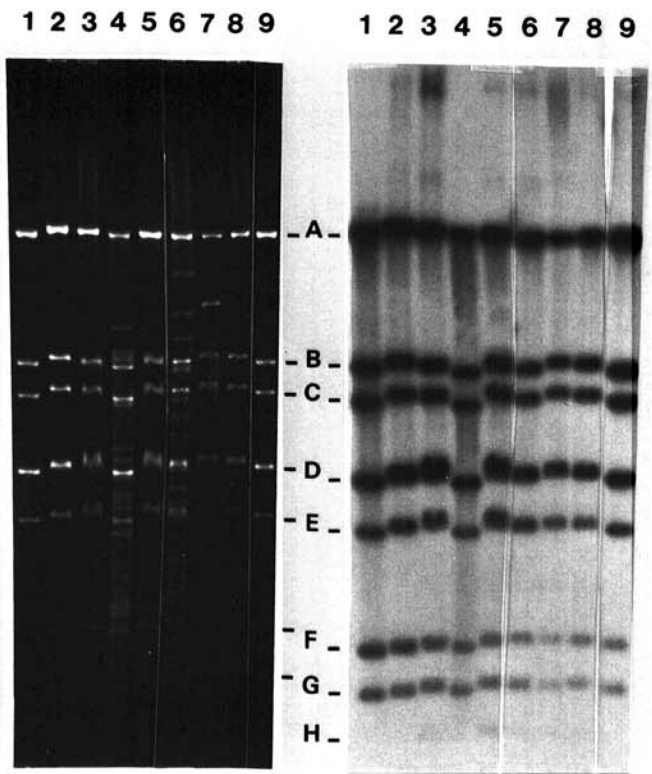

Fig. 1. Identical plasmids in different *E. amylovora* strains. Agarose gel electrophoresis of *Pst*I-digested plasmid DNA (left). Autoradiogram (right) of ^{32}P-labeled pEA28 DNA hybridized to a Southern blot of the gel shown on the left. Lane 1: pEA28, purified by density gradient centrifugation; lanes 2–9: plasmid DNA from the designed strains was prepared by miniscale alkali extraction (Birnboim and Doly 1979), lanes: 2, CFBP2045; 3, CFBP1430; 4, CFBP1376; 5, CFBP1986; 6, CFBP1227; 7, CFBP1228; 8, CFBP1262; 9, CFBP1221. Similar results were observed with plasmids from CFBP1187 and CFBP1196 (not shown).

Growth and virulence characteristics of the cured strain PMV6014. The growth rate of PMV6014, which presented a normal colony morphology, was determined in complete and minimal media. The only difference observed between strains with and without pEA28 was that the latter required thiamine for growth in minimal medium. Strain PMV6014 has been transformed with pPV99, which restored thiamine prototrophy. When introduced into TB1, a Thi$^-$ strain of *E. coli*, pPV77, holding the *Pst*I fragment B of pEA28, conferred a Thi$^+$ phenotype to the transformants. Therefore, the 4.3-kb *Pst*I fragment B of pEA28 carries a gene or genes necessary for thiamine synthesis.

Because *E. amylovora* can attack a range of host species, the pathogenicity of the cured strain was compared to that of the parental strain on three different hosts. Both strains gave a systemic wilt symptom in most of the inoculated plants on *Pyrus* (CFBP1430: 28 diseased of 28 inoculated; PMV6014: 16 diseased of 18 inoculated) and on *Malus* (CFBP1430: 10 diseased of 10 inoculated; PMV6014: 17 diseased of 18 inoculated), while on *Pyracantha*, a less susceptible host, the ratio of diseased to inoculated plants at 19 days appeared to be significantly lower with the cured derivative (CFBP1430: 7 diseased of 10 inoculated; PMV6014: 7 diseased of 30 inoculated). On *Pyrus*, delayed symptom expression was observed with the cured strain: Five days after inoculation there were no symptoms on eight plants inoculated with PMV6014, although seven of eight plants showed symptoms when inoculated with CFBP1430. Moreover, with this strain, the lesions displayed on *Pyrus* as well as on *Malus* proved to be significantly shorter than with the parental strain (Fig. 4). On *Pyracantha*, the mean lesion length was similarly reduced (12.0 cm and 7.6 cm for CFBP1430 and PMV6014, respectively), although the difference was not significant due to large intrastrain standard deviations.

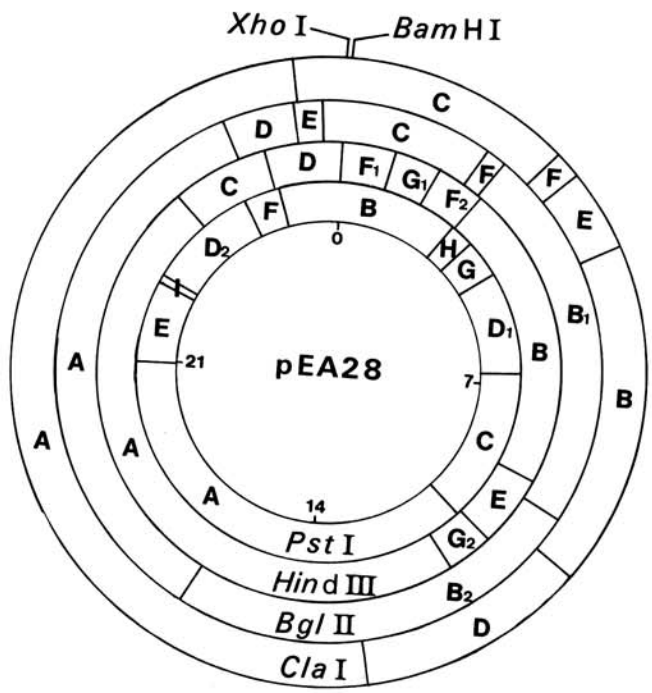

Fig. 2. Physical map of pEA28. Relative positions of the fragments obtained after digestion of pEA28 with the indicated endonucleases.

DISCUSSION

In this study we showed that a cryptic plasmid, pEA28, was ubiquitous for the phytopathogenic bacterium *E. amylovora*. In a recent paper, published after this manuscript was submitted, Falkenstein *et al.* (1988) described a plasmid isolated from strain Ea7/74, which has nearly the same restriction map as pEA28. With this plasmid as a probe, they obtained, in a colony hybridization experiment, a strong signal with all *E. amylovora* strains tested. We have shown by restriction pattern and Southern blot analysis that identical sequences were actually on the same plasmid in each strain. The availability of a strain cured of pEA28 was necessary in order to get information on the biological role of this plasmid. This report describes how we were able to obtain, first, a strain containing a genetically labeled pEA28 and second, a strain without this plasmid. Total DNA analysis proved that the cured strain contained no DNA sequence homologous to pEA28. The procedure of exclusion presented here proved simple and efficient. The results indicate that the *Pst*I fragment B, 4.3-kb, of pEA28 might carry the major incompatibility functions of pEA28, because it is the only fragment that was able to evict the labeled plasmid.

The thiamine auxotrophy displayed by PMV6014 indicates that *E. amylovora* carries plasmid-borne gene(s) for thiamine synthesis. We located thiamine-synthesizing ability on a 4.3-kb *Pst*I fragment of pEA28 and showed that this function was expressed in *E. coli*. Plasmid-associated thiamine synthesis has been described with other *Erwinia* species; namely, *E. uredovora* (Thiry 1982) and *E. herbicola* (Gantotti and Beer 1982), a bacterium having common hosts with *E. amylovora*. This situation might be indicative of a selective advantage of thiamine prototrophic strains in natural conditions. In addition, these data could explain why Falkenstein *et al.* (1988) observed nonspecific hybridization of some *E. herbicola* strains with the whole *E. amylovora* plasmid, but not when a 5-kb *Sal*I fragment from this plasmid was used as a probe. We suggest that the weak signal obtained with *E. herbicola* strains might be due to homology between plasmid-borne thiamine encoding sequences.

The inoculation data showed that the cured strain presented delayed expression and reduced severity of

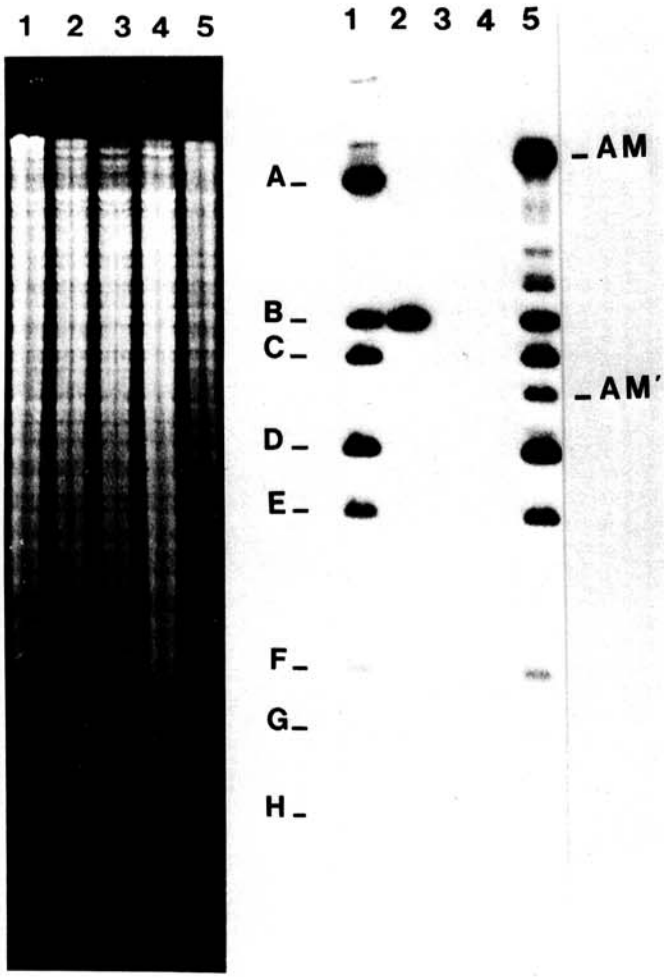

Fig. 3. Total DNA analysis of *E. amylovora* strains. Agarose gel electrophoresis of *Pst*I-digested DNA (left). Autoradiogram (right) of ^{32}P-labeled pEA28 DNA hybridized to a southern blot of the gel shown on the left. Lanes: 1, CFBP1430; 2, PMV6011; 3, PMV6014; 4, independent antibiotic-sensitive *E. amylovora* clone, similarly cured of plasmid; and 5, PMV6010. AM and AM′ are boundary fragments resulting from insertion of MudII1734 into the *Pst*I fragment A of pEA28.

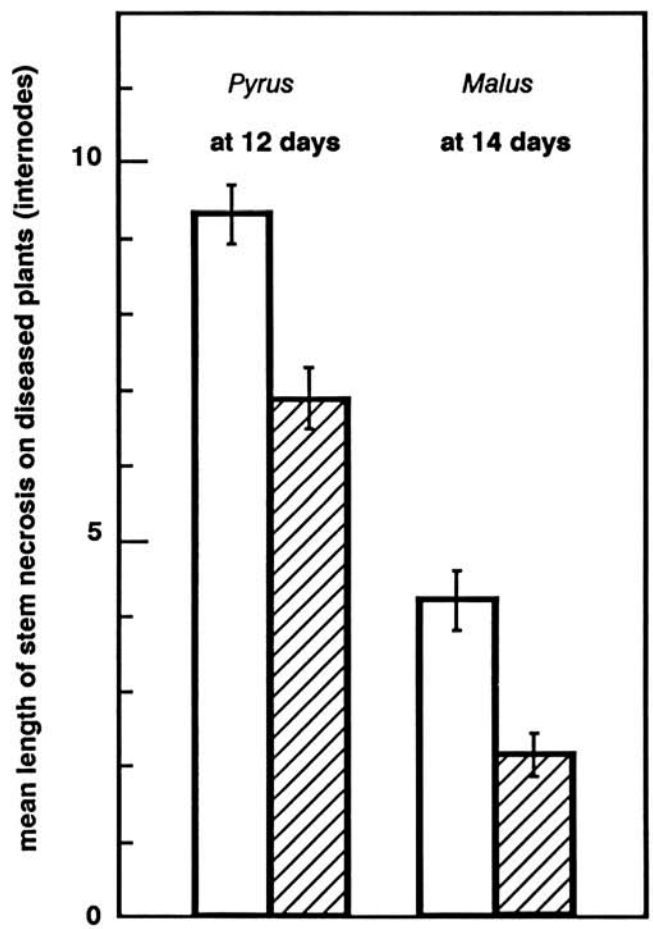

Fig. 4. Reduced severity of symptoms induced on pear or apple seedlings inoculated with PMV6014 (▨) or CFBP1430 (□). At specified times after inoculation, the mean length of necrosis on diseased plants (internodes) was calculated (vertical bar: standard error). Values are averages of one to three independent series of inoculation (8 or 10 replicates per experiment). Comparison between means indicated that there was a significant difference between the two strains on *Pyrus* [t = 4.08 (df = 42); critical *t* value: *t* ($\alpha = 0.01$)(df = 42) = 2.58] and on *Malus* [t = 4.45 (df = 25); critical *t* value: *t* ($\alpha = 0.01$)(df = 25) = 2.79].

symptoms. These observations led us to conclude that the loss of pEA28 conferred to the bacterial cell an attenuated phenotype with regard to virulence. Thus, pEA28 could enhance the virulence of *E. amylovora* by a still unknown mechanism. This might explain why the plasmid is present in all *E. amylovora* field isolates. It is noteworthy that the more resistant the host is, the less frequent is the occurrence of symptoms induced by the cured strain compared to the parental strain. Thus, pEA28 would be more useful to the bacterium when the host is more resistant. Finally, pEA28 does not carry essential genes for multiplication *in planta* and for symptom production because the cured strain is able to cause disease. It should be mentioned that *E. amylovora* mutants altered in these characteristics all proved to have chromosomal mutations (Steinberger and Beer 1988; our unpublished data). Instead, pEA28 could have a role in other aspects of the disease cycle of *E. amylovora*. One should remember that the inoculation method used in this study implied a wounding of the plant tissue, thus bypassing the first steps of a large part of natural infections, most of which occur via the flower (Van der Zwet and Keil 1979). Therefore, we cannot exclude a role of pEA28 during the early steps of infection.

The plasmid-borne nature of differential virulence has recently been demonstrated (Norelli *et al.* 1987). Differential virulence is the virulence that is expressed by some strains of *E. amylovora* for some *Malus* cultivars, which are resistant to most *E. amylovora* strains (Norelli and Aldwinckle 1986). Because CFBP1430 has a certain degree of differential virulence, experiments are in progress to test the isogenic cured strain PMV6014 for that characteristic.

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