An Indigenous Virulent Strain of *Erwinia amylovora* Lacking the Ubiquitous Plasmid pEA29

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ABSTRACT

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An atypical strain of *Erwinia amylovora* was isolated near an outbreak of fire blight at a nursery in Spain in 1996. It was obtained from a *Crataegus* plant showing typical symptoms and was identified as *E. amylovora* by biochemical tests and enrichment-enzyme-linked immunosorbent assay, but not by polymerase chain reaction using primers based on the pEA29 sequence. Nevertheless, with primers from chromosomal regions, the isolate gave the expected amplification band. This strain

carries one plasmid of ≈ 70 kb, with no homology with the 29-kb plasmid common to all pathogenic strains, or with a large plasmid present in some *E. amylovora* strains. Growth of the strain in minimal medium without thiamine was slower compared with cultures in the same medium with thiamine, a characteristic typical of strains cured of the 29-kb plasmid. Nevertheless, aggressiveness assays on pear, apple, and *Pyracantha* plants and in immature pear fruit showed that this strain exhibited a virulence level similar to other strains containing pEA29. To the best of our knowledge, this is the first report of the isolation from naturally infected plant material of a pathogenic strain of *E. amylovora* without pEA29, but with a plasmid of ≈ 70 kb not previously described.

Erwinia amylovora is the causal agent of fire blight, a disease that causes severe losses on rosaceous crops as pear and apple and ornamental plants such as *Pyracantha*, *Crataegus*, *Sorbus*, and *Cotoneaster* spp. (8). The spread of the disease has been important all over the world and, particularly in recent years, in Europe (49). Consequently, there has been a growing concern about eradication measures and detection techniques to reduce the dissemination of the pathogen. Since the discovery of fire blight, intensive research has focused on the epidemiology and control of the disease (24,40,48) and the molecular biology of the genes involved in pathogenicity, virulence, and development of symptoms (2,11,15,28,44). Furthermore, other studies about the phenotypic and genotypic characteristics of the bacterium have shown that this species presents a considerable homogeneity at genetic level (25,42). This genetic homogeneity positively correlates with biological and biochemical similarity and with a broad host range within the family *Rosaceae*. The only exception is represented by the *E. amylovora* isolates from *Rubus* spp., which affect only this genus and not the subfamily *Maloideae* (46). In addition, all the pathogenic strains isolated from nature and analyzed so far share the presence of a 29-kb plasmid called pEA29. To date, no one has demonstrated conclusively the existence of indigenous isolates of *E. amylovora* without pEA29. Therefore, it has been assumed that all the pathogenic *E. amylovora* strains should carry the plasmid pEA29, due to its high stability and nontransmissibility. Because of these characteristics, some studies have focused on the putative role of pEA29 by curing this plasmid from different strains (17,29,37) and studying the subsequent mutant

strain behavior. Strains cured of the plasmid showed a decrease in the virulence properties (delayed appearance of symptoms, reduction in the severity of symptoms, and decreased amounts of ooze in artificial inoculations) compared with wild-type strains (17,29,37). Accordingly, involvement of pEA29 in fitness and virulence properties has been proposed.

Fire blight was detected in Spain in 1995 and, since then, our laboratory, which is the Reference Laboratory for plant-pathogenic bacteria, has analyzed most of the isolates representative of the different outbreaks that have occurred until now. More than 1,000 analyses from different fire blight hosts have been performed, and almost 300 isolates of *E. amylovora* with standard characteristics have been identified. In one survey performed in 1996 at a nursery and vicinities in Segovia, in central Spain, typical symptoms of fire blight were found in different ornamental plants from which *E. amylovora* was isolated. A detailed analysis of one strain (IVIA1614-2a) obtained from a *Crataegus* sp. 45 km from the nursery showed that it lacked the plasmid pEA29.

The objective of the present work was to perform an extensive phenotypic and molecular characterization of the strain, including plasmid profiles and DNA hybridization analyses. The results obtained are related to pathogenicity and virulence on several host plants, and were compared with those of strains of *E. amylovora* harboring the plasmid pEA29 and with a strain cured of it.

MATERIALS AND METHODS

Bacterial strains and cultural properties. The bacterial strains employed in the analyses are listed in Table 1. Strain IVIA1614-2a was isolated using a standard protocol (1) from a shoot of a *Crataegus* plant with fire blight symptoms. All the strains were grown on King's B medium (26), and incubated for 24 to 48 h at 26°C. Growth rate in King's B, sucrose nutrient agar

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(SNA) (7), and CCT media (cycloheximide [50 µg/liter], crystal violet [0.1%], and thallium nitrate [1%, wt/vol]) (22), as well as minimal medium M9 with glucose as carbon source (10 ml of a 20% solution per liter) and with and without thiamine (36), was compared with reference strains in two independent experiments using the Bioscreen C system (LabSystems Oy, Finland). Suspensions of 10 µl of 10⁴ CFU/ml of each strain added to 340 µl of each media were incubated at 26°C with 10 s of shaking every 10 min, and the optical density at 600 nm was recorded for 5 days at intervals of 1 h. Growth morphology on King's B, SNA, CCT, and M9 with and without thiamine and on the selective media MM1Cu and MM2Cu (4) and MS with mannitol (10 g/liter) as carbon source (41) were analyzed and compared among the reference strains.

Phenotypic characterization. Biochemical and serological characteristics of strain IVIA1614-2a were compared against some *E. amylovora* reference strains from different origins and hosts to confirm the identity of the isolate as belonging to such species. The strains were analyzed using biochemical tests, including commercial strips as API50CH, API20E, API20NE, and APIZYM, (BioMérieux, France) following the manufacturer's instructions with modifications (13), and also by their fatty acid profile (50). Enrichment-enzyme-linked immunosorbent assay (ELISA) with specific monoclonal antibodies according to Gorris et al. (19) also was used for identification of the isolate.

Resistance to antibiotics was tested on LB medium (36) supplemented with streptomycin (Str, 50 µg/ml), kanamycin, (Km, 50 µg/ml), kasugamycin (Ksm, 200 µg/ml), and chloramphenicol (Cm, 50 µg/ml), and incubated at 26°C for 48 h. Other analyses performed involved hypersensitivity reaction (HR) in tobacco leaves (*Nicotiana tabacum*). The test was performed by infiltrating leaves with bacterial suspensions at 10⁹ CFU/ml in sterile distilled water and monitoring the HR reaction after 24 h of incubation at room temperature.

Molecular characterization. Strain IVIA1614-2a was analyzed by different molecular techniques in order to find genetic differences in comparison to reference strains. These techniques included Rep-polymerase chain reaction (PCR) (REP, BOX, and ERIC) (33), pulsed-field gel electrophoresis (PFGE) on total DNA restricted with enzyme *Xba*I following the method described in Jock et al. (23), and PCR ribotyping with primers from 16S-23S rDNA intergenic spacer as described in McManus and Jones (39). PCR with three sets of chromosomal primers from the works of Bereswill et al. (*ams*BL and *ams*BR) (3), Guilford et al. (pEa71-pEa72) (21), and Maes et al. (EaF-EaR) (35) was performed in 1× reaction buffer, 1.5 mM MgCl₂, 100 µM dNTPs, and 5 pmols of each primer. The amplification conditions for each primer pair were as described by the authors.

Plasmid and hybridization analyses. The method of Zhou et al. (51) was employed to check the plasmid content of the different strains. The Maxiprep GFII reagent kit (Q-BIOgene) was used to extract the plasmids for subsequent restriction analyses. Restriction profiles with either *Bam*HI or *Eco*RI of plasmids from reference strains and IVIA1614-2a were compared (*Bam*HI has a unique restriction site in pEA29) (16). The plasmid size was calculated with the fragments obtained with enzyme *Bam*HI using the Kodak digital science 1D software (version 3.0.1; Kodak, New Haven, CT). Plasmids pEA29 from strain CFBP 1430 and the 70-kb plasmid from strain IVIA1614-2a were excised from gel, purified using the GeneClean Turbo kit (Qbiogene), and labeled using the DIG High Prime DNA labeling kit I (Roche, Mannheim, Germany) to be used as probes. The plasmid extractions and restriction digests were transferred onto nitrocellulose membranes and hybridized at 65°C. The 70-kb probe was stripped off the membrane according to manufacturer's instructions (Roche) to be further hybridized with the pEA29 probe. Plasmids of strain IVIA1614-2a and reference strains were hybridized against the probes before and after restriction with the two enzymes. In addition, total DNA was extracted following a simple protocol (31), digested with *Eco*RI, and hybridized against the pEA29 probe to detect any homology in the sequence of the chromosome of strain IVIA1614-2a with the pEA29. The detection was performed using the Dig Wash and Block buffer set (Roche) and the membranes developed using X-OMAT UV film (Kodak, France) or, alternatively, visualized using the LAS 3000 system (Fuji Film, Japan).

Incompatibility of the 70-kb plasmid with pEA29 was studied by introduction by electroporation of a plasmid with resistance to kanamycin (27) with the origin of replication of pEA29 into the strain IVIA1614-2a and further analysis of the plasmid content. The vector (pBBR1MCS-2) and the plasmid pEA29 were digested with *Eco*RI and the fragments of the 29-kb plasmid ligated with the vector. The fragment containing the *repA* gene was employed to transform the IVIA1614-2a strain and to observe whether both plasmids co-existed after repeated culturing. To attempt to determine the incompatibility group of the 70-kb plasmid, several assays were performed by PCR using the primers described in Götz et al. (20) for the groups Inc N (*kikA* 1-2), Inc P (*trfA* 2 1-2 and *korA* 1-2), Inc Q (*oriV* 1-2), and Inc W (*oriT* 1-2), including appropriate positive controls, and following the authors' recommendations.

Aggressiveness assays. Aggressiveness assays were performed with strain IVIA1614-2a and reference strains on pear (cv. Conference) and apple (cv. Fuji) plants and on immature pear fruit (cv. Passe Crassane). Self-rooted plants obtained by micropropagation were inoculated by dipping scissors in a 10⁸ CFU/ml sus-

TABLE 1. Molecular characterization of strain IVIA1614-2a compared with *Erwinia amylovora* reference strains

Strain	Origin	Host	Polymerase chain reaction			PFGE ^x	Source ^y	Reference
			pEA29 ^u	Chromosome ^v	Ribotyping ^w			
CFBP1430	France	<i>Crataegus</i>	+	+	Type 1	Pt3A	CFBP	29
Ea273	United States	<i>Malus</i>	+	+	Type 1	Pt4	CUCM	45
NCPPB311	Canada	<i>Pyrus</i>	+	+	Type 1	Pt1	NCPPB	...
NCPPB3159	The Netherlands	<i>Malus</i>	+	+	Type 1	Pt1	NCPPB	...
EPS101	Spain	<i>Pyrus</i>	+	+	Type 1	Pt4	EPS	12
IVIA1614-2a	Spain	<i>Crataegus</i>	–	+	Type 1	Pt3	IVIA	This work
PMV6014 ^z	France	...	–	+	Type 1	ND	PMV	29

^u Using primers designed from the pEA29 (5,30,38).

^v Using primers designed from the chromosome (3,21,35).

^w Assay as described in McManus and Jones (39).

^x Pulsed-field gel electrophoresis (PFGE) profiles obtained according to Jock et al. (23); ND = not determined.

^y CFBP, Collection Française des Bactéries Phytopathogènes, INRA Angers (France); CUCM, Cornell University Collection of Microorganisms, Ithaca (USA); IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia (Spain); EPS, Escola Politécnica Superior-Universitat de Girona (Spain); NCPPB: National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, (UK); PMV, Laboratoire de Pathologie Moléculaire et Végétale, INRA-INA-PG, Paris (France).

^z Strain CFBP 1430 cured of plasmid pEA29.

pension of the bacteria and by cutting the three youngest leaves of the seedlings located at the tip. Plants were covered with plastic bags and maintained in a controlled environment chamber at 22°C with high humidity conditions (70 to 90% relative humidity) under a photoperiod of 16 h of light. Evaluation of blight symptoms was made after 10 days of incubation by means of a visual scale (from 0 to 4). The scale was based on necrosis progression as follows: 0 = no symptoms, 1 = necrosis located at the inoculation point and the midvein, 2 = necrosis affecting the midvein and the petiole, 3 = necrosis expanding through the shoot, and 4 = necrosis affecting the shoot and other leaves down in the shoot (14). Disease severity (S) was calculated for each plant according to the following formula:

$$S = \frac{\sum_{n=1}^N I_n}{N \times I_{\max}} \times 100$$

where I_n is the corresponding rating of infection in an inoculated leaf, N is the number of leaves inoculated per plant, and $I_{\max}$ is the maximum disease rate.

Aggressiveness assays on immature pear fruit were performed as described previously (12). Immature pear fruit 2 to 3 cm in diameter of cv. Passe Crassane, collected after 6 weeks from fruit set, were washed once with a solution of sodium hypochlorite (1% active chlorine), rinsed twice with sterile distilled water for 5 min, and allowed to stand to remove excess wetness. Then, fruits were wounded in the equatorial zone (four wounds per fruit) with a flame-sterilized nail to a uniform depth of 5 mm and approximate width of 3 mm. Each wound was inoculated by deposition of 10 μ l of a suspension adjusted to 10³ CFU/ml. Then, fruits were placed in polystyrene tray packs, covered with plastic bags to maintain high humidity conditions, and incubated at 21°C. Wounds were considered as infected when either drops of bacterial exudates or necrosis appeared in and around the inoculation site.

The incidence of infected wounds (percent) for each repetition was assessed after 2, 3, 5, 6, 8, and 13 days of incubation.

The experimental design in plant assays consisted of three replicates of three plants with two to three shoots per treatment. In the fruit assay, three replicates of eight fruits with four wounds per fruit were performed, and each wound was analyzed independently, so that 96 evaluation points were made for each treatment. The effect of strain on severity and incidence of infections was determined by an analysis of variance (ANOVA) using the general linear model (GLM) procedure of the statistical analysis system (SAS) (version 8.2; SAS Institute Inc., Cary, NC). Means were separated by the Tukey's test. The aggressiveness assays were repeated twice.

RESULTS

Phenotypic and molecular characterization and growth rates. The strain IVIA1614-2a was identified as *E. amylovora* by characterization with API systems, fatty acid profile, and ELISA with specific monoclonal antibodies. It did not show resistance against any of the antibiotics assayed. Hypersensitivity reaction on tobacco leaves was positive for strain IVIA1614-2a as well as for the reference strains. The results obtained in the different assays performed for molecular characterization of the isolate are shown in Table 1. When analyzed by PCR with primers from the pEA29 plasmid, strain IVIA1614-2a and PMV6014 did not produce a signal. With primers designed for detection of chromosomal DNA, the same band size as the *E. amylovora* reference strains was obtained. Molecular analyses employed for characterization (REP, ERIC, and BOX) showed identical patterns between IVIA1614-2a and strains carrying plasmid pEA29 (data not shown). Strain IVIA1614-2a exhibited pattern Pt3 by PFGE analysis with restriction enzyme *Xba*I. This pattern corresponds to isolates obtained from Northern France and Belgium, whereas

pattern Pt1 is assigned to isolates from central Europe, pattern Pt2 to eastern Mediterranean regions, and Pt4 to western France and other areas. PCR ribotyping provided the same pattern band for strain IVIA1614-2a and strains corresponding to ribotype 1 as described by McManus and Jones (39).

All the analyses performed with strain IVIA1614-2a provided similar results to reference strains harboring the plasmid pEA29. The morphology of this isolate on King's B, SNA, and CCT media was identical to that of reference *E. amylovora* strains; however, on MS medium its colonies showed a rough instead of smooth surface. *E. amylovora* strains do not grow on MM1Cu medium, and the same was observed with the strain under study. On M9 medium without thiamine, the morphology of strain IVIA1614-2a was identical to that of the strain PMV6014 cured of pEA29, showing small colonies and without production of exopolysaccharide. Growth rate was the same for all the strains compared in all media except in M9 without thiamine. In this medium, PMV6014 and IVIA1614-2a had a similar growth rate, which was slower than that of reference strains which had pEA29 (Fig. 1).

Plasmid profiles and hybridization analyses. The plasmid content of strain IVIA1614-2a was compared with that of reference strains. Plasmid PJB4JI (90 kb) (6) and plasmids from *Escherichia coli* V517 strain (34) were employed as molecular size standards. Strain IVIA1614-2a harbors only one plasmid of larger size than pEA29, apparently similar to another plasmid present in some of the reference strains employed in this work (Fig. 2A). The band profiles after restriction with enzymes *Eco*RI and *Bam*HI were different with strain IVIA1614-2a compared with the reference strains (data not shown). It is named pEI70 because the calculation of the band sizes provided an estimate size of $\approx$ 70 kb (Fig. 2B). The restriction of the large plasmids present in some of the reference strains (Ea273 and NCPB311) also showed a band profile different than that of the plasmid from strain IVIA1614-2a (data not shown).

Hybridizations performed after restriction of plasmid content with *Eco*RI and *Bam*HI enzymes with the 70-kb plasmid from strain IVIA1614-2a as probe were negative with the reference strains, and only such strain produced a hybridization signal (Fig. 3A). With pEA29 as probe, all the reference strains tested hybridized; however, strain IVIA1614-2a did not provide any signal (Fig. 3B). The results obtained with total DNA showed that no homologous sequence belonging to pEA29 was present in the chromosome of strain IVIA1614-2a (data not shown). All these analyses demonstrated, along with the negative results of the PCR assays with primers specific for pEA29 and the analysis of the plasmid content, that this isolate does not harbor the plasmid pEA29 characteristic of all wild *Erwinia amylovora* strains described. In addition, these results showed that sequences from pEA29 were found in neither the chromosome nor the 70-kb plasmid.

The analysis of the plasmid content of the strain IVIA1614-2a transformed with the plasmid carrying the replication origin of pEA29 showed that both plasmids co-exist and are maintained after repeated culturing on King's B medium supplemented with Km, which demonstrates that pEI70 is compatible with pEA29. The analyses performed to identify the incompatibility group of the large plasmid did not provide positive results with the primers assayed (data not shown).

Aggressiveness assays. Severity of infections and incidence of infected wounds developed by isolate IVIA1614-2a on apple and pear plants and immature pear fruit were compared with those of strains with or without pEA29 (Table 2). Strain IVIA1614-2a exhibited aggressiveness similar to most of the reference strains with pEA29. The values of severity and incidence of infections of strain IVIA1614-2a were similar or below that of Ea273, EPS101, NCPB3159, and CFBP1430, depending on the host and assay performed. Additionally, strain IVIA1614-2a exhibited similar

(apple plant assays) or higher aggressiveness (pear plant and immature fruit assays) than the pEA29-cured strain, PMV6014. Ooze production in immature fruit was observed at the same time for all the strains, with exception only of strain NCPPB311, which produced no symptoms or very small necrosis, without ooze production (data not shown). Significant differences in aggressiveness were observed in all assays between this last strain and all the others analyzed.

The time course of incidence of infected wounds in immature pear fruit is shown in Figure 4. Disease development of strain IVIA1614-2a was produced at intermediate levels when compared with the other strains. However, after the thirteenth day from inoculation, no differences in disease levels were observed among

strains, with the exception of strain NCPPB311 that showed a much lower level. The time required to observe lesions in wounds was shorter for strain IVIA1614-2a (3 days) than for strains NCPPB3159 and PMV6014 (5 days), and NCPPB311 (8 days).

DISCUSSION

Several works have demonstrated the presence of plasmid pEA29 in pathogenic strains of *E. amylovora* (16,25,29,30,47). The lack of both transfer and mobilization genes in pEA29 as judged from its sequence, along with its high stability, has prompted the general idea that all natural virulent strains of *E. amylovora* should harbor this plasmid.

Fig. 1. Growth of strain IVIA1614-2a compared with reference strains in M9 minimal medium without thiamine at 26°C. The experiment was performed three times. The figure shows the results from one representative experiment. The behavior of strain IVIA1614-2a was similar to the cured strain PMV6014. O.D. = optical density.

Fig. 2. A, Plasmid content of strain IVIA1614-2a compared with reference *Erwinia amylovora* strains. Lane 1, *Escherichia coli* strain V517 (plasmid marker); lane 2, strain EPS101; lane 3, strain Ea273; lane 4, strain NCPPB311; lane 5, plasmid PJB4JI (plasmid marker); lane 6, strain CFBP1430; lane 7, strain IVIA1614-2a. Analysis performed with 0.8% agarose gel in Tris-acetate EDTA buffer. **B,** Restriction analysis of pEI70 from strain IVIA1614-2a with *Bam*HI enzyme and size estimation using the Kodak digital science software (v. 3.0.1). The size of the bands obtained is shown in kilobases. The size of the plasmid was approx 70 kb.

Known pathogenicity or virulence genes have not been found in the sequence of this plasmid and a number of aggressiveness experiments have shown a loss in the virulence properties of *E. amylovora* strains cured of pEA29 with respect to the same strains harboring the plasmid. This observation led to the conclusion that pEA29 may play a role in aggressiveness and fitness (17,29,37). Nevertheless, pEA29 has been implicated only in the metabolism of thiamine biosynthesis and it has been suggested that this capacity may play a role in fitness of the pathogen in plant tissues. This rationale was used to explain the delay in the development of symptoms in the cured strains and the failure in finding strains without pEA29 in nature. However, some studies have suggested the possible existence of naturally occurring strains that did not carry pEA29. For example, Brown et al. (10) reported that 5 of 100 strains analyzed by PCR for the presence of the plasmid failed to amplify with primers of pEA29. More recently, Brennan et al. (9) analyzed 75 *E. amylovora* strains with

primers for the pEA29 and amplicons were not detected from four isolates. Nevertheless, additional molecular or biochemical analyses to verify the lack of pEA29 in such samples were not performed. Thus, the existence in nature of pathogenic strains without the pEA29 has been suggested but not demonstrated prior to our study.

The isolate IVIA1614-2a is identified as an *E. amylovora* strain with regard to its biochemical, serological, and molecular characteristics. Concerning its rough morphology on MS medium (41), when 130 *E. amylovora* strains were assayed for growth on this medium, only 6 of them (5%) showed rough colonies (13). However, all strains were able to produce levan on SNA and CCT media and showed normal mucoid colony morphology on King's B medium. In addition, these strains were able to produce ooze in inoculated green pear fruit (13). Thus, the rough morphology on MS does not seem to be associated with the absence of pEA29 in *E. amylovora* or to a lack of exopolysaccharide production.

Fig. 3. Hybridization analyses performed with the plasmids of the *Erwinia amylovora* strains after restriction with *Eco*RI **A**, with the 70-kb plasmid from strain IVIA1614-2a and **B**, with pEA29 after stripping the membrane as probes. Lane 1, strain EPS101; lane 2, Ea273; lane 3, marker VI (Boehringer Mannheim); lane 4, NCPPB311; lane 5, CFBP1430; lane 6, IVIA1614-2a. No hybridization signal was obtained between the two plasmids with any of the probes assayed.

TABLE 2. Virulence of strain IVIA 1614-2a in plants and immature pear fruit compared with reference *Erwinia amylovora* strains^x

Strain	Severity of infections in plants (%) ^y				Incidence of infected wounds (%) ^z	
	Pear		Apple			
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
CFBP1430	86.8 a	87.9 ab	36.8 ab	42.2 a	78.3 ab	74.4 b
Ea273	60.4 ab	63.5 cd	48.6 ab	56.1 a	100.0 a	100.0 a
NCPPB311	0.0 d	1.0 e	1.4 c	0.0 b	0.0 d	0.0 d
NCPPB3159	62.9 ab	77.4 abc	53.1 ab	47.2 a	56.7 bc	86.7 ab
EPS101	81.6 a	91.7 a	58.3 a	65.6 a	100.0 a	100.0 a
IVIA1614-2a	66.7 ab	74.0 bcd	31.4 b	51.0 a	93.3 a	94.4 ab
PMV6014	46.5 c	58.0 d	34.8 ab	46.6 a	41.7 c	25.6 c

^x Means within the same column followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test.
^y Plants of Conference pear and Fuji apple were inoculated by cutting youngest leaves with scissors dipped into a bacterial suspension of 10^8 CFU/ml. Severity was assessed after 10 days of incubation at 22°C and high relative humidity. Severity was calculated as percentage of the maximum observed according to the formula described in Materials and methods. Results are means of three replicates of three plants with two to three shoots per plant.
^z Incidence of infected wounds in immature pear fruit. Immature pear fruit of Passe Crassane were inoculated by deposition of 10 µl of a bacterial suspension of 10^3 CFU/ml in each wound. Incidence was assessed after 6 days of incubation at 21°C and high relative humidity. Results are means of three replicates of eight fruit with four wounds per fruit.

Nevertheless, strain IVIA1614-2a showed a rough phenotype, as the mutant PMV6014, whereas the wild strain with the 29-kb plasmid (CFBP1430) did not. Whether this characteristic is related to pEA29 or is specific to the strain is unknown, and analysis with additional strains would be necessary to establish a possible relationship. The growth rate of the strain IVIA1614-2a in several media was similar to that of other *E. amylovora* strains. IVIA1614-2a presented a slower growth rate only on minimal broth medium without thiamine strain comparable to that shown by PMV6014, the mutant cured of pEA29. Both IVIA1614-2a and PMV6014 had the same colony morphology on M9 medium without thiamine.

Aggressiveness experiments exhibited differential levels among the *E. amylovora* strains studied, including IVIA1614-2a. Differential virulence has been reported in *E. amylovora* (43,47) and the effect of strain, cultivar, and plant material has been verified (12). Taking into account the range of aggressiveness among reference *E. amylovora* strains, strain IVIA1614-2a was intermediate in this characteristic. Mutant PMV6014, a strain cured of pEA29, showed a loss of virulence in the different assays compared with the parental strain, CFBP1430, and our results confirmed those previously reported (17,29,37). In contrast, strain IVIA1614-2a, also lacking the 29-kb plasmid, presented virulence levels similar to wild strain CFBP1430 (Table 2). Mutant PMV6014 without pEA29 was similar to the strain IVIA1614-2a only in apple experiments (Table 2). This may indicate that pEA29 is not essential for aggressiveness in IVIA1614-2a.

Strain IVIA1614-2a does not harbor pEA29, which is characteristic of all pathogenic strains. Furthermore, chromosomal preparations of IVIA1614-2a did not hybridize with pEA29 used as a probe. Nonetheless, strain IVIA1614-2a does have a plasmid of ≈ 70 kb. This plasmid has a size similar to a large plasmid of 56 kb described in strain Ea273 (47), but with no relationship between them, as demonstrated by the restriction and hybridization analyses. Other large plasmids with sizes similar to the pEI70 have been reported in other *E. amylovora* strains (18,29); however, the possible association with the plasmid of strain IVIA1614-2a has not been determined. The large plasmid of IVIA1614-2a was compatible with the origin of replication of

pEA29 and, therefore, both plasmids should be maintained in *E. amylovora* strains. However, the PCR assays performed did not allow us to assign the new plasmid to a known incompatibility group. Nevertheless, it is noteworthy that some of the primers used do not recognize all plasmids from a specific group, and further analyses are necessary to identify the plasmid incompatibility group (20).

Regarding all of the studies carried out on *E. amylovora* strains, the lack of the 29-kb plasmid represents an uncommon characteristic, and the analyses performed until now have not found, at least in Spain, more strains with this attribute. However, strains without pEA29 able to infect susceptible plants and cause the disease may exist in nature. A wider screening of isolates not analyzed previously by PCR based on the 29-kb plasmid should be performed to evaluate the incidence of strains with no pEA29.

The fact that such strains have not been found previously could be related to (i) the stability of this plasmid, making it very difficult to get rid of; (ii) a reduced fitness for surviving and competing against other strains; or (iii) the low number of studies analyzing large collections of strains by PCR with primers specific to pEA29. In two studies (9,10), several strains that might lack the 29-kb plasmid were reported, but additional research is needed to determine whether they harbor pEA29 or integrated fragments of the plasmid in the chromosomal DNA.

The PCR using primers designed from the 0.9-kb *Pst*I fragment of the pEA29 kb plasmid is considered one of the most sensitive techniques for the detection of *E. amylovora* (5,30,38). Therefore, the existence of strains without pEA29 compromises these detection systems, an aspect of great concern in epidemiological studies and eradication programs. Furthermore, the detection of these strains strongly supports the need to use a combination of several techniques in an integrated approach for detection of *E. amylovora* in plant material (1,32).

The existence of a strain of *E. amylovora* in nature without pEA29 represents a novelty, but probably is not an exception. The origin of this strain remains unclear. Is its 70-kb plasmid involved in virulence features such as those observed in strains with the pEA29? Further studies on the pEI70 and chromosomal content

Fig. 4. Time course of appearance of fire blight symptoms in wounded immature pear fruit (cv. Passe Crassane) inoculated with strains IVIA1614-2a (Δ), NCPPB 3159 (▲), NCPPB 311 (■), CFBP1430 (◆), Ea 273 (□), EPS 101 (●), and PMV 6014 (○). Three replicates of eight fruits were inoculated by deposition of 10 μ l of a bacterial suspension of 10^3 CFU/ml in each of the four wounds per fruit. The incidence of fire blight symptoms (spreading, necrosis, or ooze) in each wound was calculated over time for each strain. The mean confidence interval (CI) for all data points is indicated.

of strain IVIA1614-2a, compared with those of other *E. amylovora* strains, will be of great interest to clarify this aspect.

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