Instability of a pEA29 Marker in *Erwinia amylovora* Previously Used for Strain Classification

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ABSTRACT

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We examined the use of previously observed restriction fragment length polymorphisms (RFLPs) of a polymerase chain reaction (PCR)-amplified fragment of plasmid pEA29 for differentiating strains of *Erwinia amylovora*. The PCR fragment from *E. amylovora* strain CA11 contains a region of 8-bp tandem repeats which is predicted to cause the RFLPs. Examination of a collection of 93 strains revealed the repeat sequence GATTACA(GAATTACA)_nGAATTATCA in pEA29 with *n* ranging from 3 to 14. Selected strains were examined after growth in liquid culture to establish the stability of this character. Four strains originally with *n* = 14, 13, 7, and 3 repeats were grown overnight in liquid culture and streaked onto agar plates to produce individual colonies. Respectively, 4, 10, 1, and 0 out of 17 colonies per strain had an altered copy number when retested. Considering the instability in the number of repeats, it is concluded that the polymorphism in this region of pEA29 is not useful as a marker for following the migration of *E. amylovora*.

The development of a system for characterizing and tracking *Erwinia amylovora* strains would be of great use in monitoring epidemics and tracing regional outbreaks of antibiotic-resistant strains. Replication extragenic palindromic-polymerase chain reaction (Rep-PCR), PCR ribotyping, nutrient-utilization assessment, fatty acid analysis, and random amplified polymorphic DNA fragment analysis have shown that strains of *E. amylovora* from apple and pear are genetically homogeneous (3,6,9,10,13). An examination of *E. amylovora* strains by pulsed-field gel electrophoresis (PFGE) after digestion of genomic DNA with rare cutting restriction enzymes indicated that it may be possible to trace outbreaks of fire blight on *Malus* and *Pyrus* spp. (14). However, the restriction fragment length polymorphism observed by Lecomte et al. (5) in plasmid pEA29, if reliable, would be a significantly easier method than PFGE for elucidating the

origin and migration of *E. amylovora* strains.

There has been widespread application of molecular assays based on a *Pst*I fragment (fragment F) of pEA29 to identify colonies of *E. amylovora* and to detect *E. amylovora* in extracts from symptomatic and asymptomatic plants (1,8). Size variation in the PCR product generated by primers for this region of pEA29 was observed by Lecomte et al. (5). Strains from a worldwide collection of *E. amylovora* were divided into three groups based on the size of a 250- to 340-bp *Sau*3A fragment and a 370- to 450-bp *Msp*I fragment derived from the pEA29 PCR product. They proposed that this length variation be used as a marker for tracing the movement of a group of *E. amylovora* currently found only in Austria and in southern Germany. Strains belonging to two additional groups were distributed worldwide.

The objective of our research was to determine the basis for the variability in the PCR product from plasmid pEA29 and to assess its potential usefulness for differentiating migrating populations of *E. amylovora*, including streptomycin-resistant strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 89 strains of *E. amylovora* from apple orchards in nine counties in Michigan were analyzed by PCR. Strain Ea110 was collected in 1975 (12), strains EL01 and CA11 in 1990 (2), and the remaining Michigan strains were collected from 1992 to 1994 (7,9) and had previously been characterized for resistance to

streptomycin. The group included 57 sensitive strains and 6 and 24 strains with resistance due to *rpsL* mutation or *strA-strB* streptomycin-modifying genes, respectively. Four additional strains from Illinois (raspberry, strain IL5), Oregon (pear, OR24), Washington (apple, WD2), and New Zealand (unknown host, NZR6) were also analyzed. Strains were cultured on Luria-Bertani agar (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g Bacto-Agar) and incubated for 48 h at 22°C. Liquid cultures were grown overnight at 28°C with shaking at 250 rpm.

Polymerase chain reaction. Bacteria were suspended in 50 µl deionized H₂O. PCR mixtures (20 to 100 µl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Tween-20, 160 µM each dNTP, 0.5 to 1.0 µM each primer, 0.025 units/µl *Taq* DNA Polymerase (Gibco/BRL, Grand Island, NY), and 2 to 5 µl of bacterial suspension. Primer pairs were AJ75 (5'-CGTATTCACGGCTTC-GCAGAT-3') and AJ76 (5'-ACCCGC-CAGGATAGTCGCATA-3'), or 29RPT-F (5'-CTTACTTAGCCATAACGCAACAA-3') and 29RPT-R (5'-CAGAAGCCGGATGTCTTTC-3'). Primers were synthesized by the Michigan State University (MSU) Macromolecular Structure Facility (East Lansing). Reactions were carried out in an MJ Research PTC-100 thermocycler equipped with a heated lid. Cycling parameters for AJ75/AJ76 reactions were 95°C for 5 min followed by 40 cycles of 92°C for 1 min, 56°C for 1 min, and 72°C for 2 min. Cycling parameters for 29RPT-F/29RPT-R reactions were 94°C for 5 minutes followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. Reactions were then held at 4°C until processing. Reaction products were analyzed by electrophoresis through an acrylamide gel (8.7% acrylamide/0.3% bis-acrylamide in 1× Tris-borate EDTA buffer) at 250 V for 4 h and were then visualized by illumination of the ethidium bromide stained gel with UV light. A sizing ladder was constructed from the PCR products of strains containing 3 to 14 copies of the repeat, as verified by sequencing. The repeat number of each strain tested was determined by comparison to this sizing ladder.

DNA sequencing. Amplification products from some AJ75/AJ76 PCR reactions were purified with the PCR Preps Kit (Promega Corp., Madison, WI) and se-

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quenced at the MSU/Department of Energy (DOE)-Plant Research Laboratory Sequencing Facility (East Lansing). Primers used for sequencing include AJ75, AJ76, 29RPT-F, 29RPT-R, and AJ218 (5'-ATCCTCAGGGCAGCATCA-3').

RESULTS AND DISCUSSION

PCR primers AJ75 and AJ76 recognize a region of pEA29 internal to that recognized by the primers reported by Bereswill et al. (1) and amplify a band of 844 bp from strain CA11. The sequence of this region of pEA29 from *E. amylovora* strain CA11 was previously deposited in GenBank (accession #U19254). Analysis of the sequence revealed 11 identical tandem copies of an 8-bp sequence within the PCR fragment. This repetitive sequence lies within predicted 307-bp *Sau3A* and 395-bp *MspI* fragments which are likely to correspond to the variable length restriction fragments reported by Lecomte et al. (5).

PCR products from *E. amylovora* strains CA11, Ea110, and EL01, generated using primers AJ75 and AJ76, were digested with *Sau3A*. The resulting restriction fragment patterns were identical except that the size of the expected 307-bp band varied between the strains (*data not shown*). To test the hypothesis that a repeat number polymorphism was responsible for the observed variation, the sequences of the PCR products were obtained. Alignment of the sequences revealed sequence identity between all three strains except for a variation in the number of copies of the 8-bp repeat. A general structure of GAT-TACA(GAATTACA)_nGAATTATCA was found for the repeat region with $n = 11, 7,$ or 6 , respectively, for the three strains.

We next determined the extent of variation in 89 streptomycin-sensitive and -resistant strains of *E. amylovora* collected from various locations throughout Michigan. To facilitate the differentiation of PCR products which differ in size by as little as 8 bp, new primers (29RPT-F/29RPT-R) were developed which flank the repeat region, producing a fragment of 164 bp plus the length of the repeats. All strains, except two, yielded a single 188- to 276-bp fragment which could be assigned to 1 of 12 distinct size classes. The frequency distribution of the size classes found in the collection of Michigan strains is shown in Figure 1. Sequencing of representatives of each of the 12 size classes showed that n varied from 3 to 14 (Fig. 2, marker lanes). Strains from Illinois, Oregon, Washington, and New Zealand were tested and also showed variability ($n = 3, 3, 6,$ and 6 , respectively).

The discriminating power of the gel system that we used in this study allowed us to identify 12 groups rather than the 3 groups reported by Lecomte et al. (5). Our results indicated that this trait had the level of polymorphism needed to characterize the relatively homogeneous population of

E. amylovora found in Michigan apple orchards (9). However, a couple of observations led us to believe that repeat number may not be a stable character of pEA29.

First, two Michigan strains yielded two PCR fragments each instead of just one. Second, when some strains were retested, there was an indication that the number of

Fig. 1. Frequency distribution in the number of 8-bp repeats found in a fragment of plasmid pEA29 amplified from each of 87 *Erwinia amylovora* strains from Michigan apple orchards.

Fig. 2. Stability of the plasmid pEA29 repeat polymorphism during culturing of *Erwinia amylovora* strains. (A) Strain AM19 with 14 copies; (B) strain BB924b with 13 copies; (C) strain Ea110 with 7 copies; and (D) strain HOR17 with 3 copies of an 8-bp repeat. All strains were cultured overnight in liquid media, streaked onto solid media, and 17 resulting colonies (lanes 1 to 17) were tested for repeat number following polymerase chain reaction (PCR) using primers 29RPT-F and 29RPT-R. Markers (M) are a sizing ladder with bands representing from 3 to 14 copies (189 to 277 bp) as determined by sequencing the PCR fragments generated from the strains included in the ladder (IL5, HR16, DWW6, EL01, Ea110, D71-1, RN5, CA11b, C3-3, HO63, BB924b, and AM19 with 3 to 14 copies, respectively).

repeats had changed. For example, a variant of strain CA11 turned up that contained 10 repeats rather than the 11 identified when it was initially sequenced by McManus and Jones (8) and when it was sequenced again at the beginning of this study.

To determine if the rate of change of the repeat number is high enough to effect reliable monitoring of strains over several years, representative strains were retested after passage in liquid culture. Single colonies of four strains with $n = 3, 7, 13$, and 14 were grown overnight in liquid culture; aliquots were then streaked out onto plates and 17 of the resulting colonies from each strain were tested for repeat number (Fig. 2). Each of the colonies from the $n = 3$ strain displayed a 188-bp band, indicating that all had three repeats. However, many of the colonies (respectively, 1, 10, and 4 out of 17 colonies) from the other three strains displayed different size bands or multiple bands, indicating a change in their repeat copy number after a limited number of generations. Interestingly, strains which contained higher numbers of the repeat appeared to produce altered colonies at a higher rate than strains which contained fewer copies. Data was obtained for two additional strains with $n = 11$ and $n = 3$ (data not shown); many of the retested colonies from the $n = 11$ strain had altered copy number, but none of the colonies from the $n = 3$ strain had changed.

Bereswill et al. (1) concluded, based on unpublished data, that restriction fragment length polymorphism in pEA29 was rare. Our results support the conclusion of Le-

conte et al. (5) that length variation in at least part of the plasmid is common. We show that the underlying basis for the fragment length variation is the presence of a variable repeat region. Unfortunately, this fragment length polymorphism was found to be unstable, presumably due to homologous recombination between repeats within the plasmid or between two copies of the plasmid (11), and although the rate of change in repeat number in planta has not been established, this marker is presumably not useful for tracking the regional movement of *E. amylovora*. However, *Pst*I restriction digest data (4) showed size variation in at least 8 of the 9 *Pst*I fragments obtained when pEA29 plasmids purified from several *E. amylovora* strains were examined. It would therefore be interesting to assess if any of the other polymorphisms could be reliably utilized for differentiating subgroups within those strains of *E. amylovora* that commonly infect apple and pear.

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