Genetic Analysis of a Pathogenic *Erwinia* sp. Isolated from Pear in Japan

Kimberly Maxson-Stein, Gayle C. McGhee, James J. Smith, Alan L. Jones, and George W. Sundin

First, second, fourth, and fifth authors: Department of Plant Pathology, Michigan State University, East Lansing 48824; and third author: Department of Zoology, Michigan State University, East Lansing 48824.
Accepted for publication 2 June 2003.

ABSTRACT

Maxson-Stein, K., McGhee, G. C., Smith, J. J., Jones, A. L., and Sundin, G. W. 2003. Genetic analysis of a pathogenic *Erwinia* sp. from pear in Japan. *Phytopathology* 93:1393-1399.

Four *Erwinia* strains, originally isolated in Japan from pear trees with bacterial shoot blight symptoms, were analyzed to determine their genetic relationship with *Erwinia amylovora* and *E. pyrifoliae*. When genomes were characterized with amplified fragment length polymorphism markers and by comparative *groEL* sequence analysis, the Japanese *Erwinia* sp. and South Korean *E. pyrifoliae* strains were placed in the same group, which was phylogenetically distinct from a group of 15 strains of *E. amylovora*. Sequencing of the 29,593-bp plasmid pEJ30 from *Erwinia* strain Ejp556 revealed that this plasmid was nearly identical to plasmid pEP36 from *E. pyrifoliae* and was closely related to the nontransferable ubiquitous plasmid pEA29 from *E. amylovora*.

Twenty-one presumptive genes and their order in pEP36 were highly conserved in pEJ30; however, transposon Tn5394, which was present in pEP36, was not found in pEJ30. Short-sequence DNA repeats were conserved between pEJ30 and pEP36, and were different from short-sequence repeats in pEA29. Despite base-pair mismatches, primer pairs used in pEA29 polymerase chain reaction assays for *E. amylovora* amplified plasmid DNA from the Japanese *Erwinia* Ejp556 and Ejp562. Like *E. pyrifoliae* and a few strains of *E. amylovora*, Japanese *Erwinia* Ejp617 contained plasmids related to *E. pyrifoliae* ColE1-related plasmid pEP2.6. Based on these genetic analyses, we conclude that the *Erwinia* pathogen of pear in Japan is closely related to *E. pyrifoliae* and that both of these pathogens are demonstrably distinct from *E. amylovora*.

Additional keywords: Asian pear blight, fire blight.

The recent identification of *Erwinia pyrifoliae* as the cause of Asian pear blight of Asian pears (*Pyrus pyrifolia*) in South Korea has stimulated interest in reexamining the identification of *Erwinia* strains that cause bacterial shoot blight of pear in Japan (15). The symptoms produced by both pathogens on pear are virtually identical to those of fire blight caused by *E. amylovora*, but their host range is more restricted than the host range for typical *E. amylovora* (16,25). Prior to the description of *E. pyrifoliae* as a novel species (13), the Japanese *Erwinia* strains were designated as *E. amylovora* pv. *pyri* (8), *E. amylovora* biovar 4 (25), and *E. amylovora* (3,12). As a limited number of Japanese *Erwinia* strains have become available for study, the genetic makeup of these strains is being characterized in several laboratories. A recent investigation indicated that some molecular characteristics of the Japanese *Erwinia* strains were more closely related to *E. pyrifoliae* than to *E. amylovora* (15).

The genomes of *Erwinia* strains from the Japanese island of Hokkaido were subjected to random amplified polymorphic DNA (RAPD) analysis and were found to form a subgroup within *E. amylovora* (26), but a subsequent examination of *Xba*I and *Spe*I digested chromosomal DNA by pulsed-field gel electrophoresis (PFGE) and a phylogenetic analysis of the sequence preceding the *ams* region for exopolysaccharide synthesis indicated that the genomes of the Japanese strains were more closely related to those of *E. pyrifoliae* than to those of *E. amylovora* (15). *E. pyrifoliae* was distinguished from *E. amylovora* based on total DNA-DNA hybridization and thermal stability (13,21), a phylogenetic analysis of partial *groEL* nucleotide sequences (21), and

differences in the composition of their respective ubiquitous plasmids (21).

All naturally occurring strains of *E. amylovora* contain a non-transmissible plasmid called pEA29 or pEA28, and known as the ubiquitous plasmid (5,18). Strains cured of this plasmid exhibit reduced virulence, thiamine auxotrophy, and altered exopolysaccharide production on minimal medium without thiamine (5, 18). Our recent nucleotide sequence analysis of pEA29 revealed that the plasmid encoded a thiamine biosynthesis operon and several candidate genes which could affect virulence and survival in planta (20). *E. pyrifoliae* strains harbor a similar plasmid, pEP36; comparisons of the pEA29 and pEP36 sequences have shown that they have 12 conserved open reading frames (ORFs), many conserved in order, and closely related origins of replication (21). No plasmid DNA was amplified from *E. pyrifoliae* when a pEA29 polymerase chain reaction (PCR) assay commonly used for the detection of *E. amylovora* was applied to *E. pyrifoliae* (13,28); however, plasmid DNA was amplified when this assay was applied to the Japanese *Erwinia* strains (12,15,26), suggesting that the Japanese strains may contain a plasmid similar to *E. amylovora* pEA29.

In this study, the relatedness of genomic and plasmid DNAs of *Erwinia* strains isolated from Asian pears in Japan were compared with those of *E. pyrifoliae* and *E. amylovora*. We utilized amplified fragment length polymorphism (AFLP) analysis (10,33) and comparative *groEL* sequence analysis as primary means for strain differentiation. The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of DNA. AFLP fingerprinting has been adapted to microbial strain differentiation at the species and subspecies level, including in *E. carotovora* and *E. chrysanthemi* (2,27). Comparative *groEL* sequence analysis also has been an increasingly utilized tool for bacterial species differentiation and phylogeny (9,21,32). We also deter-

Corresponding author: G. W. Sundin; E-mail address: sundin@msu.edu

Publication no. P-2003-0902-03R

© 2003 The American Phytopathological Society

mined whether an ≈30-kb plasmid found in the Japanese pathogen was more closely related to pEA29 or to pEP36, and whether the primers used in the pEA29-PCR assay for *E. amylovora* detect exact sequence matches or only highly similar sequences in plasmids from the Japanese *Erwinia* strains. Overall genetic relationships were determined based on an analysis of the sequence for pEA29-like plasmids found in each bacterium, evaluation of the bacteria for ColE1-related plasmids, and by analysis of AFLP markers and *groEL* sequence data from each species.

MATERIALS AND METHODS

DNA extraction and AFLP fingerprinting. Genomic DNA was extracted from the bacteria listed in Table 1 with a DNeasy tissue kit (Qiagen Inc., Valencia, CA) according to the manufacturer's directions. The DNA was digested, ligated to adaptor sequences, and pre-amplified with kit reagents according to the protocol provided in an AFLP microbial fingerprinting kit (PE Applied Biosystems, Foster City, CA). Unique fingerprints for each isolate were obtained by amplifying the diluted pre-amplification reaction with the selective primer pairs *EcoRI*-0/*MseI*-C, *EcoRI*-A/*MseI*-C, and *EcoRI*-G/*MseI*-A that were provided with the kit. The AFLP products were separated on an ABI Prism 3100 Genetic Analyzer and pattern analysis was performed using Genotyper software from PE Applied Biosystems. Peaks, representing products, in electropherograms were scored as present = 1 or absent = 0. The first set of selective primers yielded 54 products ranging in size from 50 to 305 bp, the second yielded 120 products ranging from 75 to 355 bp, and the third yielded 81 products ranging from 65 to 385 bp. A matrix of strains by presence or absence profiles for all three primer sets was created for analysis. The AFLP method was repeated for *E. amylovora* strains CA1R, Ea88, IL5, MR1, and OR6 to ensure consistency of the results.

Sequencing of *groEL*. Partial *groEL* sequences were PCR amplified from Japanese *Erwinia* strains Ejp556, Ejp557, Ejp562, Ejp617; *E. pyrifoliae* strain Ep8/95; and *E. amylovora* strains A1, B66, JL1196, and RJ2 with primers *groEL*-A (5'-GAAGTKGC-CTCTAAAGCGAAYGA) and *groEL*-B (5'-GCMACRCCAC-

CACCAGCAACC) as described previously (21). Four primers—*groEL*-A, *groEL*-B, and G4 (5'-CAGTACTGGAAGCGTTGC), and G3 (5'-CCSACGTTCTGRTCTTCG)—were used to determine the sequence of a 489-bp region. Sequencing reactions were conducted using ABI dye terminator technology at the Michigan State University Genomics Technology Support Facility (MSU-GTSF). Sequence fragments were analyzed and assembled using the Lasergene software of DNASTAR Inc. (Madison, WI). Sequences for other strains were obtained previously (21).

Phylogenetic analysis. The AFLP markers and *groEL* sequence data were each analyzed as pairwise genetic distances by neighbor-joining using the PAUP program package (version 4; D. L. Swofford, Sinauer Associates, Sunderland, MA). Branch support for groupings was assessed by bootstrapping using 1,000 pseudo-replicates (6). Trees were rooted using *E. rhapontici* strain ER102 as an outgroup.

Analysis of plasmid pEJ30 and ColE1-related plasmids in Japanese *Erwinia* strains. Strain Ejp556, which contains a single 30-kb plasmid, was selected for study to avoid the problem of cloning and sequencing unrelated fragments from other plasmids found in the remaining Japanese *Erwinia* strains (15, this study). Plasmid pEJ30 was isolated using the NucleoBond plasmid purification maxi kit (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's instructions for low-copy plasmids. *KpnI* and *ClaI* restriction digests of pEJ30 plasmid DNA were carried out as previously described for pEP36 (21). For high-throughput sequencing, 1 µg of plasmid DNA was partially digested with 2.8 U of *Sau3A1* for 15 min at 37°C followed by a heat-inactivation step. This procedure resulted in the generation of ≈1- to 2.5-kb fragments that were excised and purified using the Nucleotrap gel extraction kit (Clontech Laboratories) according to the manufacturer's instructions, except that the DNA was eluted with water at 80°C. The gel-purified fragments then were ligated into pGem7ZF (Promega Corp., Madison, WI) linearized with *Bam*HI and subsequently electroporated into *Escherichia coli* Gene Hog cells (Invitrogen, Carlsbad, CA). White colony transformants were processed for high-throughput sequencing using vector primers T7 and SP6 by ABI dye terminator technology at

TABLE 1. Strains of *Erwinia* examined in this study and GenBank entries for their *groEL* sequences

Species and strain(s)	Source; origin	Reference (citation no.)	Accession no. for <i>groEL</i>
<i>Erwinia amylovora</i>			
A1	Pear; Lebanon	(29)	AY245507
B66	Apple; Lebanon	(29)	AY245508
CA1R	Apple; California	(21)	AF258530
CA263	Host unknown; California	(21)	AF258531
CA3R	Apple; California	(21)	AF258532
Ea110	Apple; Michigan	(22)	AF258533
2-95	<i>Rubus</i> sp.; Canada	(23)	AF258534
Ea88	Pear; Washington	(21)	AF258535
IH3-1	Indian hawthorn; Louisiana	(23)	AF258539
IL5	<i>Rubus</i> sp.; Illinois	(21)	AF258540
JL1196	Pear; Washington	(21)	AY245509
MR1	<i>Rubus</i> sp.; Michigan	(21)	AF258541
NZR5	Host unknown; New Zealand	(21)	AF258542
OR6	Pear; Oregon	(21)	AF258543
RJ2	Apple; Utah	(31)	AY245510
<i>Erwinia pyrifoliae</i>			
Ep8/95	Asian pear; South Korea	K. Geider, (13)	AY245511
Ep1/96	Asian pear; South Korea	K. Geider, (13)	AF258537
Ep16/96 [†]	Asian pear; South Korea	K. Geider, (13)	AF258536
Ep4/97	Asian pear; South Korea	K. Geider, (13)	AF258538
<i>Erwinia</i> strains			
Ejp556	Asian pear; Japan ^a	S. V. Beer, (15)	AY245512
Ejp557	Asian pear; Japan	S. V. Beer, (15)	AY245513
Ejp562	Asian pear; Japan	S. V. Beer, (15)	AY245514
Ejp617	Asian pear; Japan	S. V. Beer, (15)	AY245515
<i>Erwinia rhapontici</i>			
ICPB ER 102	Rhubarb; England	ATCC 29283, (23)	AF464130

^a Previous designations for this isolate include Ea556 and TP9405; it was isolated on Hokkaido in 1994 by A. Tani (15).

the MSU-GTSF. Gaps in the sequence were closed by primer-walking from existing sequence using purified plasmid as template. This procedure resulted in the complete sequence of pEJ30 on both strands.

Sequences in ORF27 and ORF28 of pEP36 (GenBank entry AF449664) were examined to identify oligonucleotide primers for generating a probe for Tn5394. Hybridization and detection of the Tn5394 probe was performed with CDP-Star according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, Ind.). For analysis of short-sequence DNA repeats in pEJ30, primers AJ75 and AJ76 were used as previously described (22). Primers AJ1441 (5'-GTTTATCGTTATCGGGAGTTGGT) and AJ1443 (5'-TCTTGTCTATGGCGTTCTATGTT) were used to amplify the repeat region from *E. pyrifoliae* plasmid pEP36 using conditions previously described for AJ75 and AJ76 (22). PCR reactions (volume: 50 μ l) consisted of 20 mM Tris-HCl at pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM (each) dNTPs, 0.5 μ M concentrations of each primer, *Taq* DNA polymerase (Invitrogen) at 0.04 U/ μ l, and 10 to 100 ng of DNA as template. The PCR products were sequenced by the Genomics Technology Support Facility. The origin of replication from ColE1-related *E. pyrifoliae* plasmid pEP2.6 was used as a hybridization probe for

detecting ColE1-related plasmids in plasmid DNA from the Japanese *Erwinia* strains as described previously (21).

Nucleotide sequence accession numbers. The GenBank accession number for the sequence of plasmid pEJ30 from Japanese *Erwinia* strain Ejp556 is AY255829. The accession numbers for the *groEL* entries are listed in Table 1.

RESULTS

AFLP and *groEL* analysis. The phylogenetic analysis using a dataset of 255 AFLP characters revealed that the 15 strains of *E. amylovora* from tree fruit and *Rubus* spp. clustered together in all of the bootstrap replicates and that the Japanese *Erwinia* and *E. pyrifoliae* strains clustered together in all bootstrap replicates, indicating statistical support for consideration of the later strains as species separate from *E. amylovora* (Fig. 1A). Among the Asian *Erwinia* strains, the four strains of *E. pyrifoliae* clustered together in all of the bootstraps replicates and showed less genetic variability than the four *Erwinia* strains from Japan (Fig. 1A).

The analysis of the *groEL* sequence data indicated that all 15 strains of *E. amylovora* clustered together in all bootstrap replicates, supporting the results of the AFLP analysis that the *Erwinia* strains from Korea and Japan were not *E. amylovora* (Fig. 1B). The *E. pyrifoliae* strains and Japanese *Erwinia* strains clustered together in 96% of the bootstrap replicates and showed high genetic similarity to each other, indicating that they may be similar species. *Erwinia* strains Ejp557 and Ejp562 were more genetically distinct than the other *Erwinia* strains from Japan and South Korea.

Analysis of plasmid pEJ30 in *Erwinia* strain Ejp556. When the plasmid profile for Japanese *Erwinia* strain Ejp556 was compared with profiles for *E. amylovora* Ea110 and *E. pyrifoliae* Ep1/96, Ejp556 was found to contain a plasmid, designated pEJ30, that was slightly larger than pEA29 but smaller than pEP36 (Fig. 2). Except for the largest restriction fragment, all of the other fragments generated by single *Kpn*I and *Cla*I digests of pEJ30 from Ejp556 and pEP36 from Ep1/96 co-migrated in agarose gels (data not shown). The digests of pEJ30 yielded a 16-kb *Cla*I fragment and a 14-kb *Kpn*I fragment; both fragments

Fig. 1. Phylogenetic dendrograms for a set of strains of *Erwinia amylovora*, *E. pyrifoliae*, and an *Erwinia* sp. from Japan. **A**, Amplified fragment length polymorphism markers and **B**, *groEL* sequence data. Vertical positions of *E. amylovora*, *E. pyrifoliae*, and Japanese *Erwinia* sp. strains are indicated by hatched, open, and filled boxes, respectively. The percentages at the nodes indicate the level of bootstrap support (1,000 pseudoreplicates) based on a neighbor-joining analysis. *E. rhapontici* was used as an outgroup.

Fig. 2. Plasmid profiles for *Erwinia amylovora* Ea110 (lane 1), *E. pyrifoliae* Ep1/96 (lane 2), and Japanese *Erwinia* Ejp556 (lane 3). Arrows indicate plasmids investigated in this study.

were approximately 6.5 kb shorter than corresponding fragments from pEP36. The 6.5-kb difference in size suggested that a 6,339-bp transposon, Tn5394, previously detected in pEP36 (21), may not be present in pEJ30. The pEP36 OFR27/ORF28 PCR fragment used as a probe for Tn5394 hybridized with pEP36 DNA

from all four strains of *E. pyrifoliae* but not with plasmid DNA from any of the Japanese *Erwinia* strains or from two strains of *E. amylovora* (Table 2).

The complete nucleotide sequence of pEJ30 was determined to be 29,593 bp with a G+C content of 49.7%. The sequence was

TABLE 2. Examination of strains of *Erwinia amylovora*, *E. pyrifoliae*, and an *Erwinia* sp. isolated from pear in Japan with a pEA29 polymerase chain reaction (PCR) assay; sequence analysis for 8-bp repeats in the amplified fragment; and hybridization probes for ColE1-type plasmids and *E. pyrifoliae* transposon Tn5394^a

Species and strains	Amplicon size (bp) ^b	Repeats ^c	Number detected (size in kb) ^d	Strains containing Tn5394
<i>E. amylovora</i>				
Ea110	≈844	(GAATTACA) ⁷	nd	nd
Ea88	≈844	(GAATTACA) ³	nd	nd
IL5	≈844	(GAATTACA) ³	3 (9.0, 7.0, 5.2)	nd
<i>Erwinia</i> sp.				
Ejp556	≈1,100	(ATTCTGGG) ²¹	nd	nd
Ejp557	NT	NT	nd	nd
Ejp562	≈1,100	(ATTCTGGG) ²²	nd	nd
Ejp617	NT	NT	4 (7.0, 5.0, 3.0, 2.0)	nd
<i>E. pyrifoliae</i>				
Ep1/96	nd	(ATTCTGGG) ⁹	4 (4.7, 3.9, 2.6, 2.0)	+
Ep16/96	nd	(ATTCTGGG) ⁹	4 (4.7, 3.9, 2.6, 2.0)	+
Ep4/97	nd	(ATTCTGGG) ¹⁶	4 (4.7, 3.9, 2.6, 2.0)	+
Ep8/95	nd	(ATTCTGGG) ⁹	4 (4.7, 3.9, 2.6, 2.0)	+

^a NT = strain not tested; nd = no DNA detected by PCR or hybridization assay; + = hybridization with a probe for Tn5394.
^b Amplicon size with pEA29-PCR using primers AJ75 and AJ76. Primers developed by McManus and Jones (22) are internal to primers P29A and P29B (4).
^c Short sequence DNA repeats in the plasmid PCR amplicons.
^d Number of ColE1-related plasmids detected.

Fig. 3. Linear genetic maps for plasmid pEA29 from *Erwinia amylovora* Ea88 (accession no. AF264948), pEP36 from *E. pyrifoliae* Ep1/96 (AY123045), and pEJ30 from Japanese *Erwinia* Ejp556 (AY255829). A common *Bam*HI site was used as the origin for each map. Putative genes were inferred from sequence data in databases and arrows with similar color indicate similar genes and their order. Brackets indicate pseudogenes. A key to the genes is found in Table 2 of McGhee et al. (21). The bottom row of boxes indicate regions of pEA29 that were not detected in the other plasmids.

annotated, and the identification of ORFs larger than 300 bp was determined using searches of the Genbank nonredundant database using the suite of BLAST programs (1). The genetic map generated from the pEJ30 sequence then was compared with genetic maps that we had previously constructed for pEP36 (49.6% G+C) from *E. pyrifoliae* Ep1/96 and for pEA29 (50.2% G+C) from *E. amylovora* Ea88 (Fig. 3). A common *Bam*HI restriction site was used as the origin for each map. The annotated sequence of pEJ30 was highly similar to that of pEP36 (conservation of ORFs and gene order), with few exceptions. Major differences included the presence of an additional ORF in pEJ30 (ORF EJ8), the absence of Tn5394 (ORFs EP23 to EP28 in pEP36), and a slightly different insertion site and opposite insertional orientation of IS285 (Fig. 3). Plasmid pEP36 contained three ORFs (EP3, EP22, and EP30) that are retained as pseudogenes in pEJ30; these sequences are missing a start codon or contain frame shifts resulting in the generation of a premature stop codon. Among the 21 putative ORFs conserved between pEJ30 and pEP36, 12 of the ORFs were found also in pEA29 from *E. amylovora* Ea88 as previously reported for pEP36 (21). The ORFs common to the three plasmids were present in approximately the same order and orientation, but four regions in pEA29 did not match sequences found in pEJ30 or pEP36 (Fig. 3).

PCR assays with primers from pEA29 are commonly used for detection of *E. amylovora* (4,19,22,24), and two laboratories have reported amplification of plasmid DNA from the Japanese *Erwinia* strains with primers P29A and P29B (12,15). Primers AJ75 and AJ76, developed as nested primers for pEA29 (22), amplified a 1.1-kb fragment when applied in single-round PCR to strains of *Erwinia* from Japan, no fragments were amplified when applied to strains of *E. pyrifoliae*, and an 844-bp fragment was amplified when applied to strains of *E. amylovora* (Table 2; Fig. 4). The presence of 21 DNA repeats (ATTCTGGG) in the fragment amplified from pEJ30 accounted in part for its larger size. Fewer of these same 8-bp repeats were found in fragments amplified from pEP36 and a different 8-bp repeat (GAATTACA), as previously reported for *E. amylovora* (30), was found in fragments amplified from pEA29 (Table 2). Failure of the primer sets to amplify pEP36 DNA from *E. pyrifoliae* was associated with a greater number of mismatches between the oligonucleotide primers and the template DNA compared with pEJ30 DNA. Sequence analysis revealed that primers P29A and P29B had 2 and 6 mismatches, respectively, when the template was pEP36, and 0 and 2 when the template was pEJ30, whereas AJ76 and AJ75 had 5 and 14 mismatches in pEP36 and 4 and 2 in pEJ30 (Fig. 4).

ColE1-related plasmids in Japanese *Erwinia* strains and *E. pyrifoliae*. Plasmids with sequences that hybridized with a pEP2.6 ColE1-related origin of replication were detected by Southern analysis in *E. pyrifoliae* Ep1/96 (source of probe) and *E. amylovora* IL5 as reported previously (21). The probe also hybridized with plasmid DNA in *E. pyrifoliae* Ep8/95, Ep16/96, and Ep4/97, and *Erwinia* Ejp617, but not with DNA in the other Japanese strains (Table 2). Three ColE1-related plasmids were detected in *E. amylovora* IL5, and four were detected in *Erwinia* Ejp617 and in each strain of *E. pyrifoliae*. One plasmid detected in Ejp617 was similar in size to a plasmid detected in each strain of *E. pyrifoliae* (Table 2). No correlation between ColE1 plasmid content and identification of the bacteria was observed.

DISCUSSION

The results of our phylogenetic analyses based on AFLP markers and *groEL* nucleotide sequences were in close agreement, and in agreement with a previous report based on a phylogenetic analysis of nucleotide sequences preceding the gene cluster for exopolysaccharide synthesis (15); all three analyses indicated that the Japanese *Erwinia* strains were more closely related to *E. pyrifoliae* than to *E. amylovora* (including strains of *E. amylovora*

from *Rubus* spp.). *Erwinia* strains from Japan were classified previously as a distinct subgroup from the *Maloideae* and *Rubus* subgroups of *E. amylovora* based on RAPD analysis (26), but *E. pyrifoliae* was not included in that analysis. The results from the three phylogenetic studies that included strains of *E. pyrifoliae* were consistent with the observation that PFGE banding patterns for the Japanese *Erwinia* sp. and for *E. pyrifoliae* were distinct from patterns for strains of *E. amylovora* (15). Strains of *E. amylovora* from *Rubus* spp. do not show cross pathogenicity with tree fruit strains and differ from tree fruit strains in a number of molecular characteristics (17,20,21,23,26); however, strains from *Rubus* spp. are classified as *E. amylovora* based on their high DNA-DNA homology with tree fruit strains (7,21). Strains of the Japanese *Erwinia* sp. differ in DNA-DNA homology from *E. amylovora* (25), indicating that grouping these strains with *E. amylovora*, as was done with strains from *Rubus* spp., would not be appropriate based on current evidence. The phylogenetic differences exhibited by the Japanese *Erwinia* strains support the view that the Japanese *Erwinia* sp. are demonstrably distinct from *E. amylovora*.

The discovery that nontransferable plasmid pEA29 was ubiquitous in all natural strains of *E. amylovora* led to the development of a pEA29-PCR assay for the detection and identification of *E. amylovora* (4); this pEA29-PCR method was further adapted for use in other PCR formats (19,22,24). No amplification products were detected when the pEA29-PCR assay was applied to the causal pathogen of Asian pear blight in South Korea (28); the negative test suggested that the bacteria were not *E. amylovora* and eventually led to the characterization and description of a new species *E. pyrifoliae* (13). Unlike *E. pyrifoliae*, a larger than normal fragment of 1.1 to 1.2 kb was amplified when the pEA29-PCR assay was applied to the pathogen of bacterial shoot blight of

Fig. 4. A, Schematic representation of the region of plasmid pEA29 in *Erwinia amylovora* and pEJ30 in Japanese *Erwinia* Ejp556 amplified by primer sets P29A/P29B and AJ75/AJ76; no amplification was detected when the primers were applied to *E. pyrifoliae*. Vertical lines within the boxed area indicate the presence of a series of GAATTACA repeats in pEA29 (30) and of ATTCTGGG repeats in pEJ30. **B**, Sequence alignment for plasmids pEA29, pEP36, and pEJ30 template DNA in the binding sites for the two oligonucleotide primer sets used for pEA29-PCR assays. Asterisks indicate conserved nucleotides in comparison with pEA29 sequence.

pear in Japan, leading researchers to suggest that the bacterium was *E. amylovora* (12,26). Our sequencing data indicate that this amplification result is likely a cross amplification; plasmid pEJ30 contains target sequences that are slightly different from the target sequences in pEA29 and some features of the internal sequence for pEJ30 are quite different from those for pEA29. These sequence differences are reflected in different *Sau*3A digest patterns for fragments amplified by pEA29-PCR from the Japanese *Erwinia* sp. compared with the fragments amplified from *E. amylovora* (15). The short repeat sequences found in pEJ30 were similar to those found previously in *E. pyrifoliae* (21), but not to those found previously in *E. amylovora* (Table 2) (14,30). The nonidentical but similar structure of the sequences across the pEA29-PCR fragments amplified from *E. amylovora* and the Japanese *Erwinia* sp. also was found recently by Jock and colleagues (11).

Within the *E. pyrifoliae* and Japanese *Erwinia* group, the extensive conservation of pEP36 genes in pEJ30 appears to correlate broadly with phylogeny based on genome analysis using AFLP and *groEL* analysis. Although ubiquitous plasmids found in *E. amylovora*, *E. pyrifoliae*, and the *Erwinia* pathogen of pear in Japan were different based on size and restriction patterns, sequence alignments and assignment of putative functions based on similarity to genes in databases revealed remarkable conservation among these plasmids. Several putative genes found in pEA29 also were present, often in the same order, in pEJ30 and pEP36; plasmids pEJ30 and pEP36 exhibited extensive sequence homology whereas pEA29 exhibited less homology and therefore was more distant. Transposon Tn5394, previously detected in pEP36 (Ep1/96) (21), was found in other *E. pyrifoliae* strains from South Korea but not in the strains of *Erwinia* from Japan. Except for Tn5394, the content of pEP36 and pEJ30 was nearly identical, including the presence of insertion element IS285. Although the timing of IS285 and Tn5394 insertions into pEJ30 and pEP36 is unknown, it is clear that pEJ30 and pEP36 share a common ancestor, and that pEJ30 has diverged through loss of function of three ORFs now retained as pseudogenes. Because these nontransferable plasmids are inherited vertically within a bacterial cell line, the evolution of this family of pEA29 plasmids is an indication of the evolution of the three pathogens. Based on the genetic makeup of their ubiquitous plasmids, the bacterium isolated from pear in Japan was most closely related to *E. pyrifoliae* and more distant from *E. amylovora*. Collectively, the molecular analyses presented in this article and those published previously (15) support the conclusion that the Japanese *Erwinia* strains are different from *E. amylovora*. However, a wider array of strains must be examined with techniques such as total DNA-DNA homology to establish whether the pathogen is *E. pyrifoliae* or a novel species.

ACKNOWLEDGMENTS

This research was supported in part by the Michigan Apple Research Committee, the Michigan Agricultural Experiment Station, and by grants from USDA/CREES. The first and second authors contributed equally to this study. We thank M. Cameron for assistance with the graphics, K. Geider for cultures of *E. pyrifoliae*, and S. V. Beer for cultures of the Japanese *Erwinia* strains.

LITERATURE CITED

- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
- Avrova, A. O., Hyman, L. J., Toth, R. L., and Toth, I. K. 2002. Application of amplified fragment length polymorphism fingerprinting for taxonomy and identification of the soft rot bacteria *Erwinia carotovora* and *Erwinia chrysanthemi*. *Appl. Environ. Microbiol.* 68:1499-1508.
- Beer, S. V., Kim, J.-H., Zumoff, C. H., Bogdanove, A. J., Laby, R. J., Gustafson, H. L., Aldwinckle, H. S., Tani, A., and Tamura, O. 1996. Characterization of bacteria that cause 'bacterial shoot blight of pear' in Japan. *Acta Hort.* 411:179-181.
- Bereswill, S., Pahl, A., Bellemann, P., Zeller, W., and Geider, K. 1992. Sensitive and species-specific detection of *Erwinia amylovora* by PCR-analysis. *Appl. Environ. Microbiol.* 58:3522-3526.
- Falkenstein, H., Zeller, W., and Geider, W. 1989. The 29 kb plasmid common in strains of *Erwinia amylovora*, modulates development of fire blight symptoms. *J. Gen. Microbiol.* 135:2643-2650.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
- Gardner, J. M., and Kado, C. I. 1972. Comparative base sequence homologies of the deoxyribonucleic acids of *Erwinia* species and other *Enterobacteriaceae*. *Int. J. Syst. Bacteriol.* 22:201-209.
- Goto, M. 1992. Fundamentals of Bacterial Plant Pathology. Academic Press, San Diego, CA.
- Harada, H., and Ishikawa, H. 1997. Phylogenetical relationship based on *groE* genes among phenotypically related *Enterobacter*, *Pantoea*, *Klebsiella*, *Serratia*, and *Erwinia* species. *J. Gen. Appl. Microbiol.* 43:355-361.
- Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M., and Kersters, K. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* 142:1881-1893.
- Jock, S., Jacob, T., Kim, W.-S., Hildebrand, M., Vosberg, H.-P., and Geider, K. 2003. Instability of short-sequence DNA repeats of pear pathogenic *Erwinia* strains from Japan and *Erwinia amylovora* fruit tree and raspberry strains. *Mol. Genet. Genom.* 268:739-749.
- Kim, J.-H., Beer, S. V., Zumoff, C. H., Laby, R. J., Gustafson, H. L., Aldwinckle, H. S., and Tani, A. 1996. Characterization of *Erwinia amylovora* strains from different hosts and geographical areas. *Acta Hort.* 411:183-186.
- Kim, W.-S., Gardan, L., Rhim, S.-L., and Geider, K. 1999. *Erwinia pyrifoliae* sp. nov., a novel pathogen that affects Asian pear trees (*Pyrus pyrifolia* Nakai). *Int. J. Syst. Bacteriol.* 49:899-906.
- Kim, W.-S., and Geider, K. 1999. Analysis of variable short-sequence DNA repeats on the 29 kb plasmid of *Erwinia amylovora* strains. *Eur. J. Plant Pathol.* 105:703-713.
- Kim, W.-S., Hildebrand, M., Jock, S., and Geider, K. 2001. Molecular comparison of pathogenic bacteria from pear trees in Japan and the fire blight pathogen *Erwinia amylovora*. *Microbiology* 147:2951-2959.
- Kim, W.-S., Jock, S., Paulin, J.-P., Rhim, S.-L., and Geider, K. 2001. Molecular detection and differentiation of *Erwinia pyrifoliae* and host range analysis of the Asian pear pathogen. *Plant Dis.* 85:1183-1188.
- Laby, R. J., and Beer, S. V. 1992. Hybridization and functional complementation of the *hrp* gene cluster from *Erwinia amylovora* strain Ea321 with DNA of the other bacteria. *Mol. Plant-Microbe Interact.* 5:412-419.
- Laurent, J., Barny, M. A., Kotoujansky, A., Dufriche, P., and Vanneste, J. L. 1989. Characterization of an ubiquitous plasmid in *Erwinia amylovora*. *Mol. Plant-Microbe Interact.* 2:160-164.
- Llop, P., Bonaterra, A., Penalver, J., and Lopez, M. M. 2000. Development of a highly sensitive nested-PCR procedure using a single closed tube for detection of *Erwinia amylovora* in asymptomatic plant material. *Appl. Environ. Microbiol.* 66:2071-2078.
- McGhee, G. C., and Jones, A. L. 2000. Complete nucleotide sequence of ubiquitous plasmid pEA29 from *Erwinia amylovora* strain Ea88: Gene organization and intraspecies variation. *Appl. Environ. Microbiol.* 66:4897-4907.
- McGhee, G. C., Schnabel, E. L., Maxson-Stein, K., Jones, B., Stromberg, V. K., Lacy, G. H., and Jones, A. L. 2002. Relatedness of chromosomal and plasmid DNAs of *Erwinia pyrifoliae* and *Erwinia amylovora*. *Appl. Environ. Microbiol.* 68:6182-6192.
- McManus, P. S., and Jones, A. L. 1995. Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse-blot hybridizations. *Phytopathology* 85:618-623.
- McManus, P. S., and Jones, A. L. 1995. Genetic fingerprinting of *Erwinia amylovora* strains isolated from tree-fruit crops and *Rubus* spp. *Phytopathology* 85:1547-1553.
- Merighi, M., Sandrini, A., Landini, S., Ghini, S., Girotti, S., Malaguti, S., and Bazzi, C. 2000. Chemiluminescent and colorimetric detection of *Erwinia amylovora* by immunoenzymatic determination of PCR amplicons from plasmid pEA29. *Plant Dis.* 84:49-54.
- Mizuno, A., Sato, S., Kawai, A., and Nishiyama, K. 2000. Taxonomic position of the causal pathogen of bacterial shoot blight of pear. *J. Gen. Plant Pathol.* 66:48-58.
- Momol, M. T., Momol, E. A., Lamboy, W. F., Norelli, J. L., Beer, S. V., and Aldwinckle, H. S. 1997. Characterization of *Erwinia amylovora* strains using random amplified polymorphic DNA fragments (RAPDs). *J. Appl. Microbiol.* 82:389-398.

27. Mueller, U. G., and Wolfenbarger, L. L. 1999. AFLP genotyping and fingerprinting. *Trends Ecol. Evol.* 14:389-394.
28. Rhim, S.-L., Völksch, B., Gardan, L., Paulin, J.-P., Langlotz, C., Kim W.-S., and Geider, K. 1999. An *Erwinia* species, different from *Erwinia amylovora*, causes a necrotic disease of Asian pear trees. *Plant Pathol.* 48:514-520.
29. Saad, A. T., Hanna, L., and Choueiri, E. 2000. Evaluation of Streptomycin and Oxytetracycline resistance of *Erwinia amylovora* populations in Lebanon. (Abstr.) *Phytopathology* 90(suppl.):S68.
30. Schnabel, E. L., and Jones, A. L. 1998. Instability of a pEA29 marker in *Erwinia amylovora* previously used for strain classification. *Plant Dis.* 82:1334-1336.
31. Thomson, S. V., and Ockey, S. C. 2001. Sudden widespread appearance of streptomycin resistance in *Erwinia amylovora* in Utah. (Abstr.) *Phytopathology* 91(suppl.):S88.
32. Viale, A. M., Arakaki, A. K., Soncini, F. C., and Ferreyra, R. G. 1994. Evolutionary relationships among eubacterial groups as inferred from GroEL (chaperonin) sequence comparisons. *Int. J. Syst. Bacteriol.* 44:527-533.
33. Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* 23:4407-4414.