Genetic Fingerprinting of *Erwinia amylovora* Strains Isolated from Tree-Fruit Crops and *Rubus* spp.

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ABSTRACT

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Genetic fingerprints were determined for 189 strains of *Erwinia amylovora* isolated from different hosts from fruit-producing regions in North America and New Zealand using two polymerase chain reaction (PCR)-based techniques. For repetitive element PCR (rep-PCR), outward-facing oligonucleotide primers complementary to the ends of highly conserved, repetitive, extragenic sequences directed amplification of DNA between adjacent repetitive elements. Identical rep-PCR fingerprints were detected among 87% of the tree-fruit strains, and similarity coefficients based on shared rep-PCR products ranged from 96 to 99% among tree-fruit strains that differed in at least one rep-PCR product. Strains isolated from *Rubus* spp. were genetically more heterogeneous, with no predominant rep-PCR fingerprints, and similarity coefficients based on shared rep-PCR products ranged from 89 to 97%. Several major rep-PCR products distinguished tree-fruit strains from *Rubus* strains. For PCR ribotyping, oligonucleotide primers directed amplification of the 16S-23S spacer region of the *rrn* operon(s). Four distinct PCR-ribotype fingerprints (PCR-ribotypes 1 through 4) were detected among strains of *E. amylovora*. PCR-ribotype 1 was common among 100, 97, and 27% of tree-fruit isolates from New Zealand, eastern North America, and the western United States, respectively. Strains of PCR-ribotype 2 were rare, accounting for only 4% of the strains from the western United States. Strains of PCR-ribotype 3 were found primarily in the western United States, and strains of PCR-ribotype 4 were all isolated from *Rubus* spp. Out of 11 strains of *E. herbicola* isolated from various hosts and locations, no 2 strains produced identical rep-PCR fingerprints using any of the three rep-PCR primer sets, but all strains of *E. herbicola* were PCR-ribotype 5. We conclude that strains of *E. amylovora* isolated from fruit trees are genetically homogeneous and can be distinguished from strains isolated from *Rubus* spp. using simple, rapid PCR techniques.

Fire blight, caused by the bacterium *Erwinia amylovora*, is an economically important disease that affects apple (*Malus* × *domestica* Borkh.) and pear (*Pyrus communis* L.) production worldwide. *Rubus* spp. and several other members of the Rosaceae also are susceptible to fire blight. Widespread trade of contaminated nursery stock and propagation material is believed to be responsible for the intercontinental dissemination of *E. amylovora*, although data supporting this hypothesis are lacking. The extent to which *E. amylovora* is spread over shorter distances, for example from orchard to orchard within a state, also is unknown. Cankers at the scion-rootstock union and a within-row pattern of trees with cankers have been cited as evidence that *E. amylovora* entered a commercial apple nursery in Michigan on contaminated budwood (32). These data were supported by the detection of *E. amylovora* in asymptomatic apple tissue, including budwood (32,33), but other sources of the pathogen, such as rootstock from the northwestern United States and Europe, were not investigated. The research reported here was prompted, in part, by the need to trace pathogen dispersal routes.

Polymerase chain reaction (PCR)-based methods have been used extensively to fingerprint clinical bacteria isolated in hospitals (5, 28,37,45) and trace regional outbreaks of disease (29). Repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and interspersed, repetitive BOX sequences are three unrelated families of repetitive elements common among eubacteria (18,19,30,40). In repetitive element PCR (rep-PCR), outward-facing oligonucleotide primers complementary to the ends of these highly conserved sequences direct amplification of the DNA that lies between adjacent elements (51). Agarose gel electrophoresis of the amplification products reveals banding patterns, or rep-DNA fingerprints, which vary among species and strains of bacteria. In addition to clinical applications, rep-PCR recently has been used to assess genetic diversity of and differentiate among plant-associated bacteria (13,26,27,46).

PCR ribotyping involves amplification of spacer regions between the 16S and 23S genes of prokaryotic *rrn* operons. In most bacterial species investigated, 2 to 11 copies of the *rrn* operon are present at noncontiguous sites on the chromosome (9,10). In contrast to the 16S and 23S rRNA genes, which are highly conserved among bacteria, the spacer regions between the rRNA genes, which include genes for tRNA, vary in size and nucleotide sequence among bacterial species. This variation is affected by the number and type of tRNA genes present (25). PCR ribotyping exploits size polymorphism of the 16S-23S spacer region among different bacteria and has been used to identify bacteria to the species level (20) and differentiate strains within a species (12, 23,34). The concordance of PCR ribotyping with standard ribotyping, in which rRNA was used to probe restriction enzyme-digested genomic DNA, was 98% for *Burkholderia cepacia*, but the PCR-based method was much simpler and quicker than the standard method (12).

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PCR-based methods for assigning unambiguous, strain-specific DNA fingerprints of limited complexity to *E. amylovora* might be valuable in elucidating the origins of fire blight epidemics and for differentiating among strains from different hosts and geographic regions. In this study, we test whether rep-PCR and PCR ribotyping can be used to distinguish strains of *E. amylovora* isolated from tree-fruit crops from strains isolated from *Rubus* spp. and to differentiate among strains isolated from different geographic regions.

MATERIALS AND METHODS

Bacterial strains, identification of *E. amylovora*, and growth conditions. Strains of bacteria and their relevant characteristics and sources are listed in Table 1. A total of 175 tree-fruit isolates and 14 *Rubus* isolates of *E. amylovora* and 11 strains of *E. herbicola* were analyzed by rep-PCR, PCR ribotyping, or both. All strains of *E. amylovora* were either identified previously or during this study by examining colony morphology on Crosse and Goodman (CG) medium (11), DNA hybridization to a 5-kb probe derived from pEA29, a plasmid unique to *E. amylovora* (16,31), and PCR amplification of a 1-kb fragment of pEA29 (1,33). Bacterial cultures were stored in Luria-Bertani medium (39) plus 20% glycerol at –70°C and cultured on King's medium B (KB [21]) at 27°C.

Determination of streptomycin phenotype. The streptomycin phenotype of each strain was determined by streaking bacteria on KB amended with streptomycin at 100 µg/ml (KBs); strains that did not grow on KBs were designated streptomycin sensitive (S). Strains that grew on KBs were streaked on KB amended with streptomycin at 2,000 µg/ml (KBhs); strains that grew on KBs but not KBhs were designated moderately resistant (MR). Strains that grew on KBhs were designated highly resistant (HR). Minimum inhibitory concentrations of streptomycin for some strains have been reported previously (8,31).

DNA isolation. Total bacterial genomic DNA was isolated by a cetyltrimethylammonium bromide (CTAB) miniprep procedure (52), quantified spectrophotometrically, and adjusted to a concentration of approximately 100 ng/µl. Plasmid pEa34, which harbors the streptomycin-resistance genes *strA* and *strB* on transposon Tn*5393*, was recovered from clone JMEa34-1 of *Escherichia coli* (7) by centrifugation on cesium chloride gradients (39).

Rep-PCR. Primers were synthesized with an automatic DNA synthesizer (model 380B, Applied Biosystems, Foster City, CA) at the Macromolecular Facility, Department of Biochemistry, Michigan State University, East Lansing. Primers REP1R-I (5′-IIIICGICGI CATCIGGC-3′), REP2-I (5′-ICGICTTATCIGGCCTAC-3′), ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′), ERIC2 (5′-AAGTAA GTGACTGGGGTGAGCG-3′), and BOXA1R (5′-CTACGGCAA GGCGACGCTGACG-3′) complement conserved repetitive sequences in bacteria (30,51). Amplification was performed in a total volume of 25 µl containing (final concentrations) 67 mM Tris-HCl (pH 8.8), 83 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, 30 mM 2-mercaptoethanol, 4 µg of bovine serum albumin, 10% dimethyl sulfoxide, 125 µM each of dATP, dCTP, dGTP, and dTTP, 2.0 units of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD), and 50 pmol each of opposing REP or ERIC primers or 100 pmol of BOXA1R. A 0.4-µl aliquot (approximately 40 ng) of genomic DNA, or water as a negative control, was added to reaction tubes. Alternatively, whole-cell PCR was performed by transferring 10^4 to 10^5 cells of *E. amylovora* from culture medium or glycerol stock to reaction tubes with a sterile, plastic pipette tip. Samples were overlaid with a drop of light mineral oil, and amplification was performed in a thermal controller (Model PTC 150, MJ Research, Watertown, MA). Initial denaturation was at 95°C for 7 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 44, 52, or 53°C for 1 min with REP, ERIC, or BOX primers, respectively, and extension at 65°C for 8 min, with a final extension at 65°C for 15 min and a soak at 4°C. PCR with each primer set (REP-, ERIC-, and BOX-PCR; rep-PCR collectively) was performed at least twice for each bacterial strain. PCR products (8 to 12 µl) were separated on 1.5 or 2.0% agarose gels, stained with ethidium bromide, and photographed under UV illumination with type 55 Polaroid film (Cambridge, MA).

Similarity coefficients. Calculation of similarity coefficients for pairwise comparisons between strains was done by combining REP-, ERIC-, and BOX-PCR data sets for each strain and applying the following formula, which was previously used to estimate genetic divergence based on the proportion of restriction fragments shared by two populations (35):

$$F = 2n_{xy}/(n_x + n_y)$$

where n_x and n_y are the numbers of fragments from populations x and y, respectively, and n_{xy} is the number of fragments shared by strains x and y. In our analysis, restriction fragments were replaced by rep-PCR bands, so $F \times 100\%$ was the percentage of rep-PCR products of the same size common to the two bacterial strains being compared. The relative intensity of bands was not considered in the calculation of similarity coefficients.

PCR ribotyping. Primers 16S (5′-TTGTACACACCGCCCGTC A-3′) and 23S (5′-GGTACCTTAGATGTTTCAGTTC-3′) are complementary to conserved regions of the rRNA operon of *B. cepacia* (12,23). Amplification was performed in a total volume of 50 µl containing (final concentrations) 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.1% Triton X-100, 200 µM each of dATP, dCTP, dGTP, and dTTP, 2.0 units of *Taq* polymerase, and 100 pmol each of primers 16S and 23S. Genomic DNA, whole cells, or water was added to reaction tubes as described above, and samples were overlaid with a drop of light mineral oil. Initial denaturation was at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min and a soak at 4°C. PCR products (3 to 5 µl) were visualized on 1.5% agarose gels as described above for rep-PCR.

Effect of plasmid pEa34 on rep-PCR and PCR-ribotype fingerprints. Strain CAll carries the self-transmissible, streptomycin-resistance plasmid pEa34 (6,7). Spontaneous mutants of streptomycin-sensitive strains P1, IL5, IL6, MR1, RKK2, and RBA4, resistant to nalidixic acid at 50 µg/ml, each were mated with strain CAll, and transconjugant strains were selected as described previously (6,31). Plasmid pEa34 was digested with *Pst*I and labeled with digoxigenin-11-dUTP as recommended by the manufacturer (Genius DNA labeling and detection kit, Boehringer Mannheim Corp., Indianapolis, IN). Southern analysis of rep-PCR products representative of the various fingerprints was performed by standard methods (39) using labeled pEa34 as a probe.

RESULTS

Rep-PCR. Several amplification products resulted from PCR with each primer set (Fig. 1). Comparisons between strains of *E. amylovora* were based on products in the range of 300 to 1,800 bp because products within this range were amplified consistently from one experiment to the next. Among the 154 strains of *E. amylovora* analyzed by rep-PCR, REP-, ERIC-, and BOX-PCR fingerprints comprised four (Fig. 1, A–D), six (Fig. 1, A–F), and three (Fig. 1, A–C) groups, respectively. Rep-PCR fingerprints indicated genetic homogeneity among tree-fruit strains of *E. amylovora* from diverse geographic locations (Fig. 1; Tables 1 and 2). Among the 140 tree-fruit strains analyzed by rep-PCR, including 54 strains collected over a 3-year period from four different fields at a commercial fruit-tree nursery in Michigan, 87% had identical (group A, A, A [Fig. 1]) REP-, ERIC-, and BOX-PCR fingerprints. An additional 9.3 and 2.2% of the strains, all from the western United States, differed from the predominant group only in having a 570-bp BOX-PCR product (BOX-PCR fingerprint B [Fig. 1]) or lacking a 360-bp ERIC-PCR product (ERIC-PCR fingerprint C [Fig.

1]), respectively. The remaining 1.4% of the strains (CA263 and CA264) lacked 1,600-bp REP-PCR and 360-bp ERIC-PCR products that were common among the other tree-fruit strains but had an additional 600-bp ERIC-PCR product that was lacking in all other strains (REP- and ERIC-PCR fingerprints B [Fig. 1]).

Several major rep-PCR products differentiated strains of *E. amylovora* isolated from *Rubus* spp. from each other and from tree-fruit strains (Fig. 1; Table 1). The ERIC- and BOX-PCR fingerprints for strains from Illinois were type A, the predominant ERIC- and BOX-PCR fingerprints of tree-fruit strains. However, all *Rubus*

TABLE 1. Bacterial strains studied and their relevant characteristics

Species Strain	Streptomycin phenotype[a]	Rep-PCR fingerprint[b]			PCR-ribotype	Origin; source, or reference[c]
		REP	ERIC	BOX		
Erwinia amylovora						
G12, K2, RN8, MK1, $P1_{nal}$, P1, PF1, PW5, BWS16, EL01	S	A	A	A	1	Michigan; apple, (6), this study
RBO1	S	A	A	A	3	Michigan; apple, this study
SCW17, DF9, WinR3, GH9, DR5, HOI33, AM10, Lyon14, HR25, JD20, RRP12, DA7, VHB1, SC25, RMFF17, RL3, TF6, BWN7a, DU3, Ma8-2, OK8, PF1, RBO7, RBO10, RBO18	S	–[d]	–	–	1	Michigan; apple, this study
RBO2, RBO3, RBO6	S	–	–	–	3	Michigan; apple, this study
CA11	MR[e]	A	A	A	1	Michigan; crabapple, (6)
RN21	MR[e]	A	A	A	1	Michigan; apple, this study
HN8, HD5, HS10, HO94-27, AM15, MJ7, Pug26	MR[e]	–	–	–	1	Michigan; apple, this study
BCN20, DM1, DM13	MR[f]	A	A	A	1	Michigan; apple, (31), this study
BCN77	MR[g]	A	A	A	1	Michigan; apple, (31), this study
BB5b, JR31b, SC30b, SC34b, S5, S11	HR	A	A	A	1	Michigan; apple, (8), this study
$P1_{nal}$(pEa34)	MR	A	A	A	1	Transconjugant of $P1_{nal}$ × CAll; this study
DP4, DP11	S	A	A	A	1	Michigan; pear, this study
Miscellaneous (54 strains)[h]	S	A	A	A	1	Michigan; apple nursery
Ea225	S	A	A	A	1	New York; apple, SB
Ea266 (E4001A)	S	A	A	A	1	Ontario; apple, (36), GB, SB, JN
FB93-5	HR	A	A	B	3	Idaho; pear, KM
FB93-9	S	A	A	A	1	Idaho; apple, KM
FB93-12	S	A	A	A	1	Idaho; crabapple, KM
PFB4	S	A	A	A	1	Idaho; plum, KM
87-70, 87-73, 87-74, WD2, WSDA1, WSDA14, LP100	S	A	A	A	1	Washington; apple, LP
87-80, LP101	S	A	A	A	3	Washington; apple, LP
88-38, WSDA34	HR	A	A	A	3	Washington; apple, LP
Ea88, LA004, LA011, LA022, LA033, LA092, LA097, JL1164, JL1189, JL1196	HR	A	A	A	3	Washington; pear, VS, JL, RR
LA029, LA077	HR	A	A	B	3	Washington; pear, VS
OR1, OR2	HR	A	A	A	3	Oregon; pear, VS
OR6, OR7, OR11, OR12, OR19, OR24, OR25, OR27, OR28, OR29	HR	A	A	B	3	Oregon; pear, VS
CA831, CA850	HR	A	A	A	3	California; tree-fruit host unkown, MS
CA263, CA264	HR	B	B	A	2	California; tree-fruit host unknown, MS
CA1R, CA6R	S	A	A	A	3	California; apple, BT
CA3R, CA4R, CA5R	MR[i]	A	C	A	1	California; apple, BT
NZR2, NZR3, NZR4, NZR5, NZR6, NZR10, NZR13, NZR25	HR	A	A	A	1	New Zealand; tree-fruit host unknown, JV
NZS4, NZS12, NZS24, NZS29	S	A	A	A	1	New Zealand; tree-fruit host unknown, JV
IL5, IL6	S	C	A	A	4	Illinois; *Rubus*, (38), SR
$IL5_{nal}$(pEa34)	MR	C	A	A	4	Transconjugant of $IL5_{nal}$ × CA11; this study
MR1, MR2, MR3, MR4	S	C	D	C	4	Michigan; site MR; *Rubus*, this study
RKK2, RKK3, RKK4, RKK5	S	D	E	C	4	Michigan; site KK; *Rubus*, this study
RBA4, RBA8, RBA10, RBAE	S	D	F	C	4	Michigan; site BA; *Rubus*, this study
Erwinia herbicola						
252, 112Y, 262, 312, 331, 351, 107	–	–	–	–	5	Various locations and hosts; SB, (4,53)
C9-1, 6a, 180, BC9	–	–	–	–	5	Michigan; apple, CI, (31,41)

[a] S = sensitive, minimum inhibitory concentration (MIC) < 100 μg/ml; MR = moderately resistant, 100 μg/ml < MIC < 2,000 μg/ml; HR = highly resistant, MIC > 2,000 μg/ml. Streptomycin phenotypes and MICs for some strains have been reported previously (8,31).

[b] Repetitive element-polymerase chain reaction (rep-PCR) fingerprints obtained using repetitive extragenic palindromic (REP)-PCR; enterobacterial repetitive intergeneric consensus (ERIC)-PCR; and BOX-PCR.

[c] GB = G. Bonn, Agriculture Canada, Harrow, Ontario; SB = S. Beer, Cornell University, Ithaca, NY; CI = C. Ishimaru, Colorado State University, Fort Collins; JL = J. Loper, USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR; KM = K. Mohan, University of Idaho, Moscow; JN = J. Norelli, New York State Agricultural Experiment Station, Cornell University, Geneva; LP = L. Pusey, USDA-ARS Tree Fruit Research Laboratory, Wenatchee, WA; SR = S. Ries, University of Illinois, Urbana; RR = R. Roberts, USDA-ARS Tree Fruit Research Laboratory, Wenatchee, WA; MS = M. Schroth, University of California-Berkeley; BT = B. Teviotdale, University of California Kearney Agricultural Center, Parlier; VS = V. Stockwell, USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR; JV = J. Vanneste, Hort Research Ruakura Research Centre, New Zealand.

[d] – = not tested.

[e] *strA-strB* carried on plasmid pEa34 (6,7).

[f] *strA-strB* carried on plasmid pEA29 (31, P. S. McManus, *unpublished data*).

[g] *strA-strB* inserted on the chromosome (31).

[h] Collected from four nursery plantings during 1992 to 1994.

[i] *strA-strB* carried on a plasmid <20 kb in size (P. S. McManus, *unpublished data*).

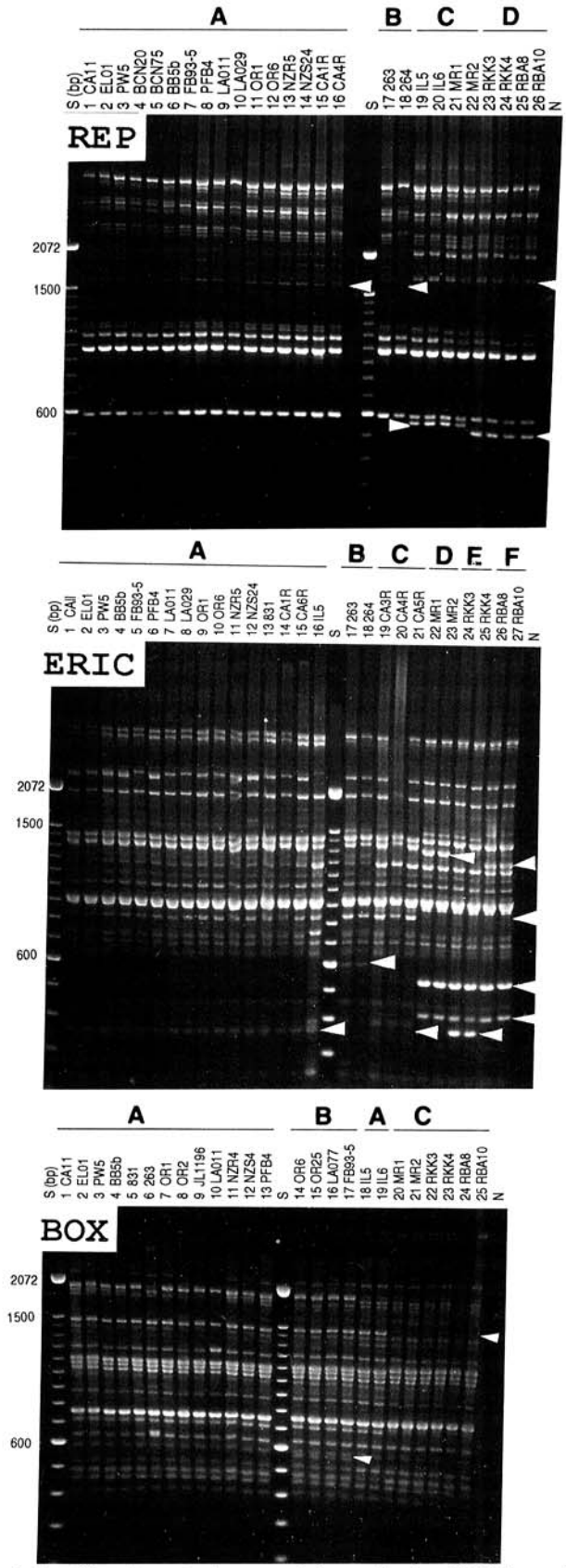

Fig. 1. Repetitive element-polymerase chain reaction (rep-PCR) fingerprints of representative strains of *Erwinia amylovora*. Rep-PCR products (8 to 12 µl) were separated on 2.0% agarose gels, stained with ethidium bromide, and photographed under UV illumination. Strains representing repetitive extragenic palindromic (REP)-PCR types **A–D**; enterobacterial repetitive intergeneric consensus (ERIC)-PCR types **A–F**; and BOX-PCR types **A-C** are indicated above panels. White arrow heads denote polymorphisms among products, i.e., the presence or absence of bands used to discriminate among rep-PCR types. Lanes S, 100-bp ladder; lanes N, negative control.

strains, including those from Illinois, were distinguished by a unique 1,700-bp REP-PCR product (Fig. 1, lanes 19–26). *Rubus* strains from Michigan were distinguished by having major 420- and 520-bp ERIC-PCR products and lacking 820-bp ERIC-PCR (Fig. 1, lanes 22–27) and 1,480-bp BOX-PCR (Fig. 1, lanes 20–25) products that were common among all other strains. Several other REP- and ERIC-PCR products were unique to *Rubus* strains from different sites in Michigan.

The formula used to calculate similarity coefficients is based on the premise that the degree of genetic relatedness between two species or populations is expected to be correlated with the proportion of DNA restriction fragments shared by the two groups (35). Although the bands in restriction fragment and rep-PCR analyses are not equivalent, polymorphisms by either method reflect nucleotide divergence between the two groups analyzed. Thus, the formula was useful for pairwise comparisons between representative strains that differed in at least one rep-PCR product (Table 2). Similarity coefficients ranged from 96 to 99% among tree-fruit strains and from 89 to 97% among *Rubus* strains. Similarity coefficients were slightly lower between *Rubus* strains from Illinois and Michigan (89 to 92%) than among Michigan strains (95 to 97%). *Rubus* strains from Illinois were more similar to tree-fruit strains (similarity coefficients of 95 to 99%) than they were to other *Rubus* strains (similarity coefficients of 89 to 92%). Similarity coefficients were lowest between *Rubus* strains from Michigan and tree-fruit strains, ranging from 86 to 92%.

When rep-PCR fingerprints of bacterial strains were compared on the same agarose gel, species other than *E. amylovora* and strains incorrectly identified as *E. amylovora* were easily recognized when run with a series of true *E. amylovora* strains (data not shown). Also, rep-PCR fingerprints of *E. herbicola*, an epiphytic bacterium with a wide host range that includes apple, were clearly different from those of *E. amylovora* (Fig. 2). Several rep-PCR products were shared by some of the 11 strains of *E. herbicola* tested, but no 2 strains had identical REP-, ERIC-, or BOX-PCR fingerprints. A major 480-bp BOX-PCR product was the only rep-PCR product common among all strains of *E. herbicola*.

PCR ribotyping. From *E. amylovora*, primers 16S and 23S generated major PCR products that ranged in size from approximately 700 to 1,100 bp. Four distinct PCR product profiles resulted from tree-fruit and *Rubus* strains of *E. amylovora* and were designated PCR-ribotypes 1 through 4 (Table 1; Fig. 3). PCR-ribotype 1 was common among 100, 97, and 27% of tree-fruit isolates of *E. amylovora* from New Zealand, eastern North America, and the western United States, respectively (Table 3). PCR-ribotype 2, which differed from PCR-ribotypes 1 and 3 by lacking 920- and 1,100-bp products, was unique to strains CA263 and CA264 from California. PCR-ribotype 3, which differed from PCR-

TABLE 2. Similarity coefficients of the combined data from REP-, ERIC-, and BOX-PCR, expressed as a percentage of shared rep-PCR products from *Erwinia amylovora*[a]

E. amylovora strain	*E. amylovora* strain						
	Tree-fruit strains			*Rubus* strains			
	FB93-5	CA264	CA3R	IL5	MR1	RKK2	RBA4
CA11	99	96	97	96	88	90	88
FB93-5	...	97	96	95	86	89	86
CA264		...	96	97	89	92	89
CA3R			...	99	88	88	88
IL5				...	92	92	89
MR1					...	95	95
RKK2						...	97

[a] Strains listed are representative of eight fingerprints obtained from 154 strains of *E. amylovora* by combining data from repetitive element-polymerase chain reaction (rep-PCR) fingerprints obtained using repetitive extragenic palindromic (REP)-PCR; enterobacterial repetitive intergeneric consensus (ERIC)-PCR; and BOX-PCR (Fig. 1).

ribotype 1 by lacking an approximately 800-bp product, was found in 69 and 3% of the strains from the western United States and eastern North America, respectively. PCR-ribotype 4, characteristic of *Rubus* strains, differed from PCR-ribotypes 1 through 3 by lacking 880-, 1,050-, and 1,100-bp products but having an additional broad band at approximately 980 bp. All strains of *E. herbicola* were PCR-ribotype 5, which was distinct from PCR-ribotypes 1 through 3 but similar to PCR-ribotype 4.

PCR ribotype was not strictly correlated with streptomycin phenotype or geographic origin (Table 3). Strains with S and HR streptomycin phenotypes varied in PCR ribotype, whereas all MR strains were PCR-ribotype 1. Strains from the western United States were PCR-ribotype 1, 2, or 3; strains from eastern North America were PCR-ribotype 1 or 3; and strains from New Zealand were PCR-ribotype 1.

Effect of plasmids on rep-PCR and PCR ribotyping. Frequency of transfer of plasmid pEa34 from strain CA11 to nalidixic acid-resistant mutant strains $P1_{nal}$, $IL5_{nal}$, and $IL6_{nal}$ was 5.0, 7.7, and 6.2×10^{-2}, respectively. Frequency of transfer of pEa34 to strains $MR1_{nal}$, $RKK2_{nal}$, and $RBA4_{nal}$ was less than 1×10^{-9} in all cases. Rep-PCR and PCR ribotyping were unaffected by plasmid pEa34 because transconjugant strains $P1_{nal}$(pEa34) and $IL5_{nal}$ (pEa34) had fingerprints identical to parental strains $P1_{nal}$ and $IL5_{nal}$, respectively. Plasmid pEa34 did not hybridize to any rep-PCR products of representative isolates.

DISCUSSION

The rep-PCR analyses performed in this study indicated that fire blight on tree-fruit crops in the United States and New Zealand is caused by *E. amylovora* strains with very closely related chromosomal genotypes. The limited genetic diversity detected using rep-PCR is consistent with the finding of about 100% DNA-DNA hybridization between strains of *E. amylovora* (3,17). Physiological and serological homogeneity within *E. amylovora* also is well documented (2,15,22,43,49). The apparent clonal population structure of *E. amylovora* may be due to limited genetic recombination in this species, a high degree of specialization of the pathogen to a narrow ecological niche, or the relatively recent adaptation of this bacterium to fruit crops. The limited genetic variability described using rep-PCR provides a baseline for future characterization of strains of *E. amylovora* from other hosts and geographic regions.

Rep-PCR and PCR-ribotype fingerprints differentiated strains of *E. amylovora* isolated from fruit trees from strains isolated from *Rubus* spp. Several major polymorphisms among rep-PCR products of *Rubus* strains are evidence that these strains are genetically more variable than strains from fruit trees. Other workers have found that strains from *Rubus* spp. do not infect pome fruits, strains from pome fruits do not infect *Rubus* spp., and strains from apple and pear are pathogenic on all other known fire blight hosts (14,38,42,48). Starr et al. (42) proposed a forma specialis designation for strains pathogenic on *Rubus* spp. to reflect this host specificity. Furthermore, tree-fruit strains can be distinguished from

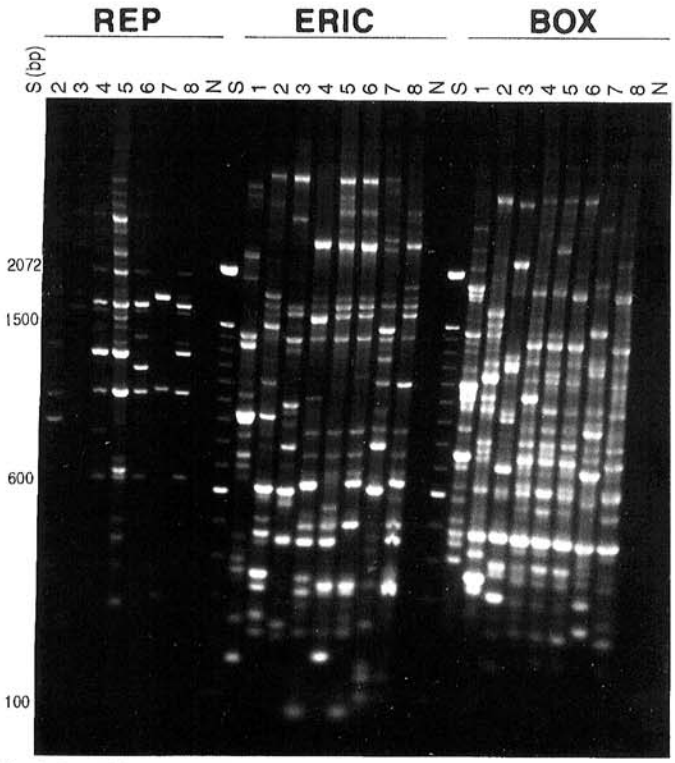

Fig. 2. Repetitive extragenic palindromic (REP)-, enterobacterial repetitive intergeneric consensus (ERIC)-, and BOX-polymerase chain reaction (PCR) fingerprints of *Erwinia herbicola* (lanes 2–8), and ERIC- and BOX-PCR fingerprints of *E. amylovora* (lane 1). PCR products (8 to 12 µl) were separated on a 2.0% agarose gel, stained with ethidium bromide, and photographed under UV illumination. Lane 1, strain CA11; lane 2, strain 112Y; lane 3, strain 262; lane 4, strain 312; lane 5, strain 351; lane 6, strain 107; lane 7, strains C9-1; lane 8, strain 6a; lanes S, 100-bp ladder; lane N, negative control.

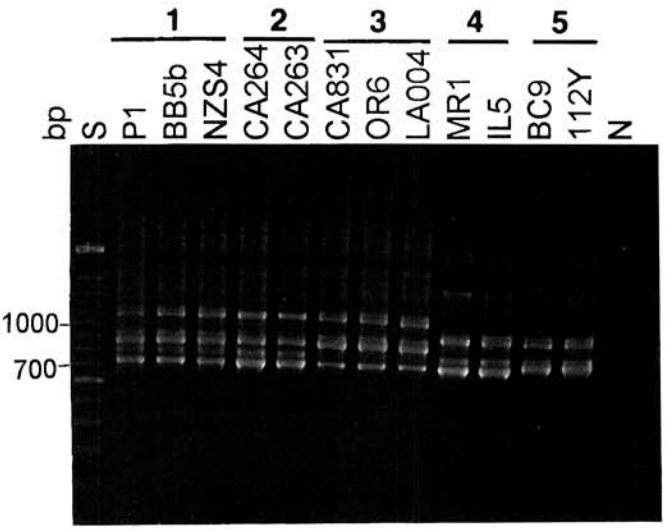

Fig. 3. Representative polymerase chain reaction (PCR)-ribotype fingerprints. PCR products (3 to 5 µl) were separated on 2.0% agarose gel, stained with ethidium bromide, and photographed under UV illumination. Strains P1, BB5b, NZS4, CA264, CA263, CA831, OR6, LA004 of *Erwinia amylovora* were isolated from fruit trees; strains MR1 and IL5 were isolated from *Rubus* spp. Strains BC9 and Eh112Y are *E. herbicola*. Strains representing PCR-ribotypes 1–5 are indicated by numbers above the panels. Lane S, 100-bp ladder.

TABLE 3. Polymerase chain reaction (PCR)-ribotype frequencies for tree-fruit strains of *Erwinia amylovora*

Level of resistance and origin	Total no. of strains	PCR-ribotype frequency (%)		
		1	2	3
Streptomycin phenotype[a]				
S	114	93	0	7
MR	16	100	0	0
HR	45	30	4	66
Geographic origin				
Eastern North America	115	97	0	3
Western United States	48	27	4	69
New Zealand	12	100	0	0

[a] Minimum inhibitory concentration (MIC) determined by plating strains on King's B medium (KB [21]) and KB amended with streptomycin at 100 or 2,000 µg/ml. Sensitive (S), MIC < 100 µg/ml; moderately resistant (MR), 100 µg/ml < MIC < 2,000 µg/ml; highly resistant (HR), MIC > 2,000 µg/ml.

Rubus strains based on restriction fragment length polymorphism in the *hrp* gene cluster (24). The results of rep-PCR and PCR-ribotype fingerprinting corroborate previous conclusions based on pathogenicity tests that *E. amylovora* consists of at least two distinct subgroups. Although differences in host specificity and genomic fingerprints readily differentiated tree-fruit strains from *Rubus* strains, rep-PCR fingerprints of *Rubus* strains IL5 and IL6 from Illinois were more similar to the fingerprints of tree-fruit strains than to *Rubus* strains from Michigan.

We evaluated the use of PCR-based methods to trace the possible origins of *E. amylovora* in a commercial apple nursery in Michigan by comparing genetic fingerprints of nursery isolates with fingerprints of isolates from local orchards, including budwood sources, and isolates from the states in the Pacific Northwest of the United States that supply rootstocks. Unfortunately, we could not distinguish among nursery isolates, budwood isolates, and strains isolated from orchards in the western United States using rep-PCR. The results of PCR ribotyping also were insufficient to pinpoint the probable source of pathogen entry into the nursery. However, all isolates from the nursery plantings, budwood sources, and orchards near the nursery were PCR-ribotype 1, while all but seven isolates from orchards in Oregon and Washington were PCR-ribotype 3. Although these data do not exclude the possibility that *E. amylovora* entered the nursery on rootstocks from western states, they bolster previous evidence that local orchards were the source of the pathogen (32).

Streptomycin is the most effective chemical used for controlling fire blight. High-level resistance to streptomycin in *E. amylovora* has likely evolved independently by mutation at least twice because the HR strains from Michigan and New Zealand were PCR-ribotype 1, whereas HR strains from the western United States were either PCR-ribotype 2 or 3. All MR strains were PCR-ribotype 1, which would be expected for MR strains from Michigan, since 97% of the strains from eastern North America were PCR-ribotype 1. Although MR strains from California were also PCR-ribotype 1, there is no basis to expect that the MR phenotype is restricted to *E. amylovora* of PCR-ribotype 1. Rather, the occurrence of the MR phenotype in *E. amylovora* of PCR-ribotype 1 is probably due to the relatively recent acquisition of *strA-strB* in *E. amylovora*.

Rep-PCR and PCR ribotyping should prove valuable in ecological and evolutionary studies aimed at investigating genetic diversity in populations of *E. amylovora* worldwide. For example, *E. amylovora* may have been associated with host plants indigenous to North America prior to the introduction of apple from Europe. That apple is not the original host of *E. amylovora* is supported by the fact that fire blight was not reported in eastern Europe, the probable center of origin of apple, until the late 1980s (47). Genetic fingerprints of isolates from additional host species, especially those indigenous to eastern North America and eastern Europe, might be useful in analyzing evolutionary aspects of host specificity. If *E. amylovora* originated on species native to eastern North America, then one would expect greater strain-to-strain variation among *E. amylovora* from these hosts in North America than among strains from native hosts in Europe where fire blight has been known to occur for less than 40 years.

The primary advantages of rep-PCR and PCR ribotyping over typing methods that entail DNA purification, probe labeling, and Southern blotting, are their ease of application and rapidity. Rep-PCR and PCR-ribotype fingerprints were obtained in approximately 12 and 6 h, respectively. An additional advantage is that differences in fingerprints apparently reflect genetic variation of the bacterial chromosome and not extrachromosomal DNA. Although plasmid profiles were not established for all isolates, some strains of *E. amylovora* contained the self-transmissible plasmid pEa34 (e.g., strain CA11 [6,7,31]) or pCPP60 (e.g., strain BCN20 [31,44]) yet had rep-PCR and PCR-ribotype fingerprints identical to strains lacking these plasmids (e.g., strain P1). The absence of hybridization of rep-PCR products to plasmid pEa34 is further evidence that rep-PCR products were not amplified from pEa34. Also, MR strains that varied in the genomic location of *strA-strB* (31) had identical fingerprints. Thus, no correlation was obvious between fingerprints and plasmid content or presence and genomic location of *strA-strB*. In addition, mutations in the chromosomal *rpsL* gene, which confer a high level of resistance to streptomycin (8), had no apparent effect on rep-PCR or PCR-ribotype fingerprints.

We included strains of *E. herbicola* in our study to demonstrate the use of rep-PCR and PCR ribotyping on an epiphytic bacterium reported to be genetically heterogeneous (3,43,50). While rep-PCR fingerprints varied greatly among strains of *E. herbicola*, PCR-ribotype fingerprints were identical for all strains. Identification of *E. herbicola* has traditionally involved a battery of physiological and biochemical tests. Methods to rapidly and accurately identify members of this ecologically significant epiphyte might be developed based on the major 480-bp BOX-PCR product or PCR-ribotype fingerprint common to all strains tested.

LITERATURE CITED

1. 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.
2. Billing, E., Baker, L. A. E., Crosse, J. E., and Garret, C. M. E. 1961. Characteristics of English isolates of *Erwinia amylovora* (Burrill) Winslow *et al.* J. Appl. Bacteriol. 24:195-211.
3. Brenner, D. J., Fanning, G. R., and Steigerwalt, A. G. 1974. Deoxyribonucleic acid relatedness among Erwinieae and other Enterobacteriaceae: The gall, wilt, and dry-necrosis organisms (Genus *Erwinia* Winslow et al., *sensu stricto*). Int. J. Syst. Bacteriol. 24:197-204.
4. Burr, T. J., Norelli, J. L., Reid, C. L., Capron, L. K., Nelson, L. S., Aldwinckle, H. S., and Wilcox, W. F. 1993. Streptomycin-resistant bacteria associated with fire blight infections. Plant Dis. 77:63-66.
5. Chatulet, M., Dournes, J. L., Chabanon, G., and Marty, N. 1995. Epidemiological typing of *Stenotrohomonas* (*Xanthomonas*) *maltophilia* by PCR. J. Clin. Microbiol. 33:912-914.
6. Chiou, C.-S., and Jones, A. L. 1991. The analysis of plasmid-mediated streptomycin resistance in *Erwinia amylovora*. Phytopathology 81:710-714.
7. Chiou, C.-S., and Jones, A. L. 1993. Nucleotide sequence analysis of a transposon (Tn*5393*) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram-negative bacteria. J. Bacteriol. 175:732-740.
8. Chiou, C.-S., and Jones, A. L. 1995. Molecular analysis of high-level streptomycin resistance in *Erwinia amylovora*. Phytopathology 85:324-328.
9. Cole, S. T., and Saint Girons, I. 1994. Bacterial genomics. FEMS Microbiol. Rev. 14:139-160.
10. Condon, C., Philips, J., Fu, Z.-Y., Squires, C., and Squires, C. L. 1992. Comparison of the expression of the seven ribosomal RNA operons in *Escherichia coli*. EMBO J. 11:4175-4185.
11. Crosse, J. E., and Goodman, R. N. 1973. A selective medium for and a definitive colony characteristic of *Erwinia amylovora*. Phytopathology 63: 1425-1426.
12. Dasen, S. E., LiPuma, J. J., Kostman, J. R., and Stull, T. L. 1994. Characterization of PCR-ribotyping for *Burkholderia* (*Pseudomonas*) *cepacia*. J. Clin. Microbiol. 32:2422-2424.
13. de Bruijn, F. J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58:2180-2187.
14. de Ley, J., Vantomme, R., Swings, J., Kersters, K., Rijckaert, M. G., Mergaert, J., and Geenen, J. 1984. Research report on *Erwinia amylovora*. Acta Hortic. 151:241-248.
15. Elrod, R. P. 1941. Serological studies of the Erwinieae. I. *Erwinia amylovora*. Bot. Gaz. 103:123-131.
16. Falkenstein, H., Bellemann, P., Walter, S., Zeller, W., and Geider, K. 1988. Identification of *Erwinia amylovora*, the fire blight pathogen, by colony hybridization with DNA from plasmid pEA29. Appl. Environ. Microbiol. 54:2798-2802.
17. 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.
18. Gilson, E., Clément, J. M., Brutlag, D., and Hofnung, M. 1987. A family of dispersed repetitive extragenic palindromic DNA sequences in *E. coli*.

EMBO J. 3:1417-1421.
19. Hulton, C. S. J., Higgins, C. F., and Sharp, P. M. 1991. ERIC sequences: A novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. Mol. Microbiol. 59: 925-934.
20. Jensen, M. A., Webster, J. A., and Strauss, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol. 59:945-952.
21. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44: 301-307.
22. Komagata, K., Tamagawa, Y., and Kocur, M. 1968. Differentiation of *Erwinia amylovora*, *Erwinia carotovora*, and *Erwinia herbicola*. J. Gen. Microbiol. 14:39-45.
23. Kostman, J. R., Edlind, T. D., LiPuma, J. J., and Stull, T. L. 1992. Molecular epidemiology of *Pseudomonas cepacia* determined by polymerase chain reaction ribotyping. J. Clin. Microbiol. 30:2084-2087.
24. Laby, R. J., and Beer, S. V. 1992. Hybridization and functional complementation of the *hrp* gene cluster from *Erwinia amylovora* strain Ea321 and DNA of other bacteria. Mol. Plant-Microbe Interact. 5:412-419.
25. Loughney, K., Lund, E., and Dahlberg, J. E. 1982. tRNA genes are found between the 16S and 23S genes in *Bacillus subtilis*. Nucleic Acids Res. 10:1607-1624.
26. Louws, F. J., Fulbright, D. W., Stephens, C. T., and DeBruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Appl. Environ. Microbiol. 60:2286-2295.
27. Louws, F. J., Fulbright, D. W., Stephens, C. T., and DeBruijn, F. J. 1995. Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology 85: 528-536.
28. Lupski, J. R. 1993. Molecular epidemiology and its clinical application. J. Am. Med. Assoc. 270:1363-1364.
29. Makino, S.-I., Kurazono, T., Okuyama, Y., Shimada, T., Okada, Y., and Sasakawa, C. 1995. Diversity of DNA sequences among *Vibrio cholerae* O139 Bengal detected by PCR-based DNA fingerprinting. FEMS Microbiol. Lett. 126:43-48.
30. Martin, B. O., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenbeck, R., Morrison, D. A., Boulnois, G. J., and Claverys, J.-P. 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Res. 20:3479-3483.
31. McManus, P. S., and Jones, A. L. 1994. Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. Phytopathology 84:627-633.
32. McManus, P. S., and Jones, A. L. 1994. Role of wind-driven rain, aerosols, and contaminated budwood in incidence and spatial pattern of fire blight in an apple nursery. Plant Dis. 78:1059-1066.
33. McManus, P. S., and Jones, A. L. 1995. Detection of *Erwinia amylovora* by nested PCR, PCR-dot blot and reverse-blot hybridization. Phytopathology 85:618-623.
34. Nastasi, A., and Mammina, C. 1995. Epidemiological evaluation by PCR ribotyping of sporadic and outbreak-associated strains of *Salmonella enterica* serotype Typhimurium. Res. Microbiol. 146:99-106.
35. Nei, M., and Li, W.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 82:5078-5082.
36. Norelli, J. L., Aldwinckle, H. S., and Beer, S. V. 1988. Virulence of *Erwinia amylovora* strains to *Malus* sp. Novole plants grown in vitro and in the greenhouse. Phytopathology 78:1292-1297.
37. Reboli, A. C., Houston, E. D., Monteforte, J. S., Wood, C. A., and Hamill, R. J. 1994. Discrimination of epidemic and sporadic isolates of *Acinetobacter baumannii* by repetitive element PCR-mediated DNA fingerprinting. J. Clin. Microbiol. 32:2635-2640.
38. Ries, S. M., and Otterbacher, A. G. 1977. Occurrence of fire blight on thornless blackberry in Illinois. Plant Dis. Rep. 61:232-235.
39. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
40. Sharples, G. J., and Lloyd, R. G. 1990. A novel repeated DNA sequence located in the intergenic regions of bacterial chromosomes. Nucleic Acids Res. 18:6503-6508.
41. Sobiczewski, P., Chiou, C.-S., and Jones, A. L. 1991. Streptomycin-resistant epiphytic bacteria with homologous DNA for streptomycin resistance in Michigan apple orchards. Plant Dis. 75:1110-1113.
42. Starr, M. P., Cardona, C., and Folsom, D. 1951. Bacterial fire blight of raspberry. Phytopathology 41:915-919.
43. Starr, M. P., and Chatterjee, A. K. 1972. The genus *Erwinia*: Enterobacteria pathogenic to plants and animals. Annu. Rev. Microbiol. 26:389-426.
44. Steinberger, E. M., Cheng, G.-Y., and Beer, S. V. 1990. Characterization of a 56-kb plasmid of *Erwinia amylovora* Ea322: Its noninvolvement in pathogenicity. Plasmid 24:12-24.
45. Struelens, M. J., Bax, R., Deplano, A., Quint, W. G. V., and van Belkum, A. 1993. Concordant clonal delineation of methicillin-resistant *Staphylococcus aureus* by macrorestriction analysis and polymerase chain reaction genome fingerprinting. J. Clin. Microbiol. 31:1964-1970.
46. Sundin, G. W., Demezas, D. H., and Bender, C. L. 1994. Genetic and plasmid diversity within natural populations of *Pseudomonas syringae* with various exposures to copper and streptomycin bactericides. Appl. Environ. Microbiol. 60:4421-4431.
47. van der Zwet, T. 1993. Worldwide spread and present distribution of fire blight—An update. Acta Hortic. 338:29-31.
48. Vanneste, J. L. 1995. *Erwinia amylovora*. Pages 21-41 in: Pathogenesis and Host Specificity in Plant Diseases: Histopathological, Biochemical, Genetic and Molecular Bases. Vol. 1, Prokaryotes. U. S. Singh and K. Kohmoto, eds. Pergamon Press, Oxford.
49. Vantomme, R., Swings, J., Goor, M., Kersters, K., and De Ley, J. 1982. Phytopathological, serological, biochemical and protein electrophoretic characterization of *Erwinia amylovora* strains isolated in Belgium. Phytopathol. Z. 103:349-360.
50. Verdonck, L., Mergaert, J., Rijckaeert, C., Swings, J., Kersters, K., and De Ley, J. 1987. Genus *Erwinia*: Numerical analysis of phenotypic features. Int. J. Syst. Bacteriol. 37:4-18.
51. Versalovic, J., Koeuth, T., and Lupski, J. R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19:6823-6831.
52. Wilson, K. 1989. Preparation of genomic DNA from bacteria. Pages 2.4.1-2.4.5 in: Current Protocols in Molecular Biology. Vol. 1. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds. Greene Publishing Associates and Wiley-Interscience, New York.
53. Wilson, W. J., Weidmann, M., Dillard, H. R., and Batt, C. A. 1994. Identification of *Erwinia stewartii* by a ligase chain reaction assay. Appl. Environ. Microbiol. 60:278-284.