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Identification of the Fire Blight Pathogen, *Erwinia amylovora*, by PCR Assays with Chromosomal DNA

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***Erwinia amylovora*, the causative agent of fire blight, was identified independently from the common plasmid pEA29 by three different PCR assays with chromosomal DNA. PCR with two primers was performed with isolated DNA and with whole cells, which were directly added to the assay mixture. The oligonucleotide primers were derived from the *ams* region, and the PCR product comprised the *amsB* gene, which is involved in exopolysaccharide synthesis. The amplified fragment of 1.6 kb was analyzed, and the sequence was found to be identical for two *E. amylovora* strains. The identity of the PCR products was further confirmed by restriction analysis. The 1.6-kb signal was also used for detection of the fire blight pathogen in the presence of other plant-associated bacteria and in infected plant tissue. For further identification of isolated strains, the 16S rRNA gene of *E. amylovora* and other plant-associated bacteria was amplified and the products were digested with the restriction enzyme *Hae*III. The pattern obtained for *E. amylovora* was different from that of other bacteria. The sequence of the 16S rRNA gene was determined from a cloned fragment and was found to be closely related to the sequences of *Escherichia coli* and other *Erwinia* species. Finally, arbitrarily primed PCR with a 17-mer oligonucleotide derived from the sequence of transposon Tn*5* produced a unique banding pattern for all *E. amylovora* strains investigated. These methods expand identification methods for *E. amylovora*, which include DNA hybridization and a PCR technique based on plasmid pEA29.**

Fire blight, a bacteriosis of pome fruit trees and other rosaceous plants, can be a disastrous disease in orchards and is therefore of great economic importance (25). Many assays have been developed for detection of the causative agent, the gram-negative bacterium *Erwinia amylovora*. Classical methods include plating on semiselective media (5, 13) or serological assays like enzyme-linked immunosorbent assay or immunofluorescence. Common surface antigens of gram-negative bacteria give rise to nonspecific antibodies, which can confuse serological detection of *E. amylovora* (16). Previously described monoclonal antibodies were too selective for specific strains and could therefore not be used for general detection of the pathogen (12).

Previous molecular methods for identification of the fire blight pathogen took advantage of the presence of a 29-kb plasmid in all strains from natural sources and were highly selective (9). Colony hybridization was used mainly for signal detection. The pathogen was also identified with nonradioactive probes, which were less sensitive than ^{32}P labeling in DNA hybridization experiments (22). The labor and time-consuming steps of colony or DNA hybridization of *E. amylovora* were circumvented by use of the specific and sensitive PCR analysis (3). Lysis of the bacterial sample directly in the reaction mixture by the mild detergent Tween 20 made isolation of DNA unnecessary. About 500 cells of *E. amylovora* could be detected in a large excess of other plant-associated bacteria. The oligonucleotides used for two-primer PCR (8) were derived from a 0.9-kb *Pst*I fragment of plasmid pEA29 common for *E. amylovora*. The plasmid carries information for the thiamine metabolism of the bacterium (11), which may render plasmid-free strains inferior to strains with the plasmid with respect to their biological fitness, and curing of pEA29 reduced the development of fire blight symptoms (10). Nevertheless, strains without the plasmid or those carrying variants of pEA29 occasionally occur in nature, or plasmid pEA29 may even be transferred to bacteria and maintained in foreign hosts. It was previously shown that the Ti plasmid of *Agrobacterium tumefaciens* can efficiently be transferred to *Escherichia coli*, although it was not replicated by the new host in this case (21). No conditions have been found for transfer or mobilization of pEA29, which still does not exclude the possibility of its transfer to other bacteria. Considering these ambiguities with pEA29, it appeared advantageous to develop additional molecular assays which are independent of the plasmid.

This report will describe two-primer PCR assays using information from the *ams* genes and the 16S rRNA genes (16S rDNAs) of *E. amylovora*, an arbitrarily primed PCR (AP-PCR) assay, and the sequence of the 16S rDNA.

MATERIALS AND METHODS

Bacterial strains. The strains used and their sources are listed in Table 1.

DNA isolation and PCR analysis. Bacteria from 1-ml overnight cultures in the nutrient broth Standard I medium (Merck) were lysed by lysozyme-sodium dodecyl sulfate treatment, and the nucleic acids were purified by repeated extraction with phenol and then with chloroform-isoamyl alcohol. The oligonucleotides listed in Table 2 were synthesized with a DNA synthesizer and purified by high-pressure liquid chromatography.

All PCRs were carried out in a volume of 50 μl with 10 ng of template DNA and 0.5 U of *Tth* polymerase as described previously (3). When whole cells were added to the assay, the PCR mixture contained 1% Tween 20. The temperatures for primer annealing to the different chromosomal DNA fragments, including those of the unspecific amplification by AP-PCR, which was performed with 12.5 pmol of primer, are given in Table 2. After 37 cycles (3.5 h), the PCR products were separated on a 1.5% agarose gel for 1.5 to 2 h at 100 V, stained with ethidium bromide, and photographed under UV light (302 nm). For restriction analysis, the PCR products were precipitated with 3 volumes of ethanol. The pelleted DNA was then washed in 80% ethanol, dried in a SpeedVac evaporator for 5 min, and resuspended in 20 μl of distilled water. Restriction fragments of a *Hae*III digest were separated on a 2.5% agarose gel (MetaPhor agarose; FMC Corp.).

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TABLE 1. Strains used in this experiment

Species	Strain	Reference or source; year and country of isolation	Comments
E. amylovora	Ea7/74	9; 1974 in Germany	
	Ea1/79	9; 1979 in Germany	
	EaX1/79	10	Cured from common plasmid pEA29
	Ea7/84	9; 1984 in Egypt	
	T91	T. Mommol; 1991 in Turkey	
	Ea273	S. Beer; 1971 in United States	
	PD207	J. Janse; The Netherlands	Deficient in levan synthesis
E. carotovora			
subsp. *carotovora*	Ecc582	9	
	Ecc568	9	
	Ecc499	9	
subsp. *atroseptica*	185	9	
E. chrysanthemi	533	9	
	SR260	R. Montgomery	
A. tumefaciens	C58	9	
E. herbicola			
subsp. *herbicola*	NZ	—[a]	
subsp. *synvitivora*	A5	9	
E. stewartii	DC283	D. Coplin; in United States	
P. syringae			
pv. syringae	2U	W. Zeller; from pear in Germany	
	524	2	
	B1	2	
	dsm60062	W. Zeller; 1976 from apple in Germany	
E. coli K-12	1100	Laboratory collection	
B. subtilis	BD170	H. Matzura	

[a] Isolated from pears obtained from New Zealand.

Sequencing of PCR products. The PCR products were cloned by ligation with T4 DNA ligase into the thymidinylated *Eco*RV site of the plasmid vector pGEM5Z′f (pGEM-T; Promega) for 4 h at 16°C. Recombinant plasmids in *Escherichia coli* DH5α were screened by α-complementation and isolated with a cartridge system (Nucleobond; Macherey-Nagel). Free and cloned PCR products were sequenced by the chain termination method of Sanger et al. (17) with [α-^{35}S]dATP by the Sequenase 2.0 sequencing protocol (United States Biochemicals). For direct sequencing, PCR products were separated from residual primers with another cartridge system (QIAquick-spin; Qiagen). The relatedness of 16S rDNA sequences was calculated with the PC/Gene program CLUSTAL (release 6.7).

RESULTS

Identification of *E. amylovora* by two-primer PCR with information from an area involved in exopolysaccharide synthesis. It was shown that *E. amylovora* cured of plasmid pEA29 can be created by introduction of a DNA fragment with replication functions of pEA29 (10, 11). Although plasmid-free strains have not been identified in nature, difficulties encountered in the isolation of pEA29 could be caused by loss of the plasmid or changes in its size. Furthermore, it could be transferred or mobilized into bacteria other than *E. amylovora*. For assays independent of plasmid pEA29, we have developed a PCR method, which takes advantage of genes of the chromosomal *ams* region (4). They encode synthesis of the capsular polysaccharide amylovoran, which seems to be unique for *E. amylovora*. From the nucleotide sequence of *ams* genes (6), primers AMSbL and AMSbR (Table 2) were designed for amplification of a defined area (Fig. 1). Primer AMSbL is located at the 3′ end of *amsA*, and primer AMSbR is located in the center of *amsC*. They include the borders of the genes and the complete sequence of *amsB*. The specificity of glycosyl

TABLE 2. DNA sequences used for identification of *E. amylovora* in PCR assays used

Primer	Sequence	Annealing temp (°C)	Target region and PCR product
AMSbL	GCTACCAGCAGGGTGAG	49	Specific 1.6-kb DNA fragment from the chromosomal *ams* region (*amsB*)
AMSbR	TCATCACGATGGTGTAG		
APT1	CAGGACGCTACTTGTGT	32	AP-PCR (primer sequence from Tn*5*)
fD2	AGAGTTTGATCATGGCTCAG	42	1.5-kb DNA fragment from 16S rDNA region and *Hae*III digest
rP1	ACGGTTACCTTGTTACGACTT		

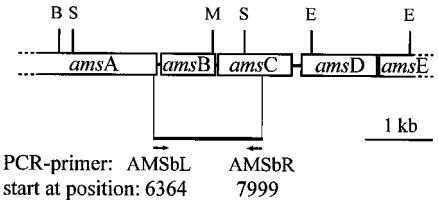

FIG. 1. Map of part of the *ams* region with the *amsB* gene. The position of the primers AMSbL and AMSbR in *amsA* and *amsC* and in the nucleotide sequence (5) (EMBL Nucleotide Sequence Database accession number X77921) is marked. Restriction sites are given for *Bam*HI (B), *Eco*RI (E), *Mlu*I (M), and *Sal*I (S).

transferases for biosynthesis of amylovoran suggests that this region is highly conserved for *E. amylovora* (6) and may not occur in other bacteria.

The reaction was optimal for the primer-annealing temperature of 49°C. All other conditions have been described previously (3). DNA was purified from the bacterial suspensions by phenol extraction and used at 10 ng for a 50-μl reaction mixture, or bacteria were directly added to the PCR assay. When whole bacteria were added to the reaction mixture, it was advantageous to add 1% Tween 20 to the PCR assay to efficiently release plasmid and chromosomal DNA from the cells. No difference in signal production was observed for addition of whole *E. amylovora* cells or of an equivalent of isolated DNA. Amplification was done in 37 cycles with 0.5 U of *Tth* DNA polymerase. The strongest signal was observed at 25 pmol of primer in assays with 3 to 25 pmol. The amplified 1.6-kb fragment was identical for *E. amylovora* strains isolated in various geographic regions at different times (Fig. 2; Table 3). Our standard strain Ea1/79 was isolated in Germany in 1979, T91 was isolated in Turkey in 1991, and Ea273 was isolated in the United States in 1971 (9, 20). When DNA or cells of other plant-associated bacteria were added, amplification was not observed. In rare cases, nonspecific fragments were found, mostly at positions different from 1.6 kb. As a powerful tool to confirm specific signals from *E. amylovora*, the PCR products were digested with a restriction enzyme. According to the nucleotide sequence of the *ams* region, the amplified DNA from strains Ea1/79, Ea273, and T91 was cleaved with *Mlu*I, which produced bands of 1.0 and 0.6 kb (Fig. 3). When *Sal*I was used with *Mlu*I in a double digest, bands of 1.0, 0.4, and 0.2 kb were obtained.

Whole cells of *Pseudomonas syringae* pv. syringae dsm60062 at various concentrations did not give rise to amplification products, unless *E. amylovora* cells were added to the mixture

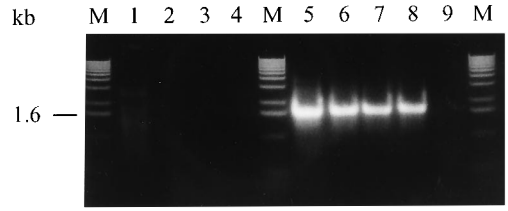

FIG. 2. PCR amplification with two primers derived from the chromosomal *ams* region encoding amylovoran synthesis. Lanes: 1 and 2, *P. syringae* pv. syringae dsm60062 was grown to the stationary phase, and 500,000 and 5,000 cells were added to the PCR assay, respectively; 3, *E. herbicola* NZ; 4, *E. carotovora* subsp. *atroseptica* 185; 5 and 6, 5,000 cells of *E. amylovora* Ea1/79 in the presence of 500,000 and 5,000 cells of *P. syringae* pv. syringae dsm60062, respectively; 7, *E. amylovora* Ea273; 8, *E. amylovora* T91; 9, control with addition of buffer; M, 1-kb ladder marker DNA (Bethesda Research Laboratories). The position of 1.6 kb is indicated.

(Fig. 2). The signal was also detected from plants which were previously inoculated with *E. amylovora*. Small pieces of leaf tissue (about 1 mg) adjacent to necrotic zones were homogenized in 100 μl of water, and 1 μl was analyzed in the PCR assay. Alternatively, the pieces were incubated in 100 μl of nutrient broth to propagate the bacteria overnight at 28°C, and the 1.6-kb signal was also obtained.

The results show that primers AMSbL and AMSbR can be used to identify *E. amylovora* by PCR on the basis of sequence information from the *ams* region. To determine the relative sensitivity of signal detection with primers AMSbL and AMSbR, the PCR assays were repeated with overnight cultures of *E. amylovora* Ea1/79. In parallel experiments, the same cells were assayed with the primers specific for pEA29, which results in an amplification product of 0.9 kb (3). Because of the low copy number of the chromosome, signal detection with information from pEA29 was about 10-fold more sensitive at a low concentration of bacteria (50 cells, compared with 500 cells for *ams* PCR).

Identity of the nucleotide sequence of a DNA fragment with the *amsB* gene from two different strains. On an amylovoran-inducing minimal medium, strains Ea7/74 and Ea1/79 differ with respect to the level of exopolysaccharide production (1), which could be due to nonidentical genes in the *ams* region. To find possible differences in the amplified DNA fragments of the two strains, the 1.6-kb amplification product with the *amsB* gene (Fig. 2) was cloned from strain Ea1/79 into plasmid pGEM-T (Promega) and the nucleotide sequence of the insertion was determined. For strain Ea7/74, a large fragment with the *ams* operon including the *amsB* gene was previously isolated from a cosmid library (4) and the complete DNA sequence of this region was determined (6) (EMBL Nucleotide Sequence Database accession number X77921). The 1,636-bp nucleotide sequence of the PCR fragment from strain Ea1/79 was identical to the corresponding sequence derived from strain Ea7/74.

Identification of *E. amylovora* with PCR amplification of the 16S rDNA and the pattern of its digestion products. The 16S rDNA of different *Erwinia* species, other plant-associated bacteria, and *Escherichia coli* K-12 strain 1100 was amplified by PCR with primers fD2 and rP1 (Table 2). *Escherichia coli* was analyzed as an enterobacterial species closely related to *E. amylovora*. The primers used are homologous to conserved sequences of the 16S rRNA genes of many gram-negative bacteria such as *Escherichia coli*, *E. herbicola*, and *E. carotovora*. They are located 7 nucleotides downstream of the gene start and 34 nucleotides upstream of the stop of the *Escherichia coli* 16S rRNA sequence. Computer analysis revealed that the enzymes *Sau*3A, *Hpa*II, *Fnu*4HI, and *Hae*III are good candidates to distinguish *E. amylovora* by restriction fragment length polymorphisms in this region. A 1.5-kb 16S rDNA fragment was amplified from DNA of *E. amylovora* Ea7/74, Ea1/79, T91, Ea7/84, Ea273, and PD207; *E. herbicola* NZ and A5; *E. carotovora* subsp. *carotovora* 499 and 582; *E. carotovora* subsp. *atroseptica* 185; *E. chrysanthemi* 533; *E. stewartii* DC283; *P. syringae* pv. syringae 524; *Agrobacterium tumefaciens* C58; and *Escherichia coli* K-12 strain 1100 and digested with these four enzymes. Only *Hae*III produced a restriction fragment length polymorphism pattern suitable for differentiation of *E. amylovora* within the genus *Erwinia*. Fragments of 317, 222, and 204 bp and a triplet containing fragments of 170, 165, and 164 bp were obtained. The other plant-associated erwinias analyzed gave rise to a 278-bp *Hae*III fragment, which was not observed for *E. amylovora* (Fig. 4). In the *Hae*III digest, *Escherichia coli* could be distinguished by a band of 180 bp, which is positioned above the three close bands of *E. amylovora* at the bottom of

TABLE 3. Strains assayed by several PCR methods

Species	Strain	Result in PCR assay[a]			Sample analyzed
		amsB primers	16S rDNA (*Hae*III)[b]	Ea pattern by AP-PCR	
E. amylovora	Ea7/74	+	+	+	DNA
	Ea1/79	+	+	+	DNA
		+			Whole cells
	EaX1/79	+	+	+	DNA
	Ea7/84	+	+	+	DNA
	T91	+	+	+	DNA
		+			Whole cells
	Ea273	+	+	+	DNA
		+			Whole cells
	PD207	+	+		DNA
E. carotovora					
subsp. *carotovora*	Ecc582	−	−	−	DNA
	Ecc568		−	−	DNA
	Ecc499		−	−	DNA
subsp. *atroseptica*	185	−	−	−	DNA
		−			Whole cells
E. chrysanthemi	533	−	−	−	DNA
	SR260	−	−		Whole cells
A. tumefaciens	C58	−	−	−	DNA
		−			Whole cells
E. herbicola					
subsp. *herbicola*	NZ	−	−	−	DNA
		−			Whole cells
subsp. *synvitivora*	A5	−	−	−	DNA
E. stewartii	DC283	−	−	−	DNA
		−			Whole cells
P. syringae					
pv. syringae	2U	−			Whole cells
	524		−	−	DNA
	B1	−		−	DNA
	dsm60062	−			Whole cells
E. coli K-12	1100	−	−	−	DNA
B. subtilis	BD170		−	−	DNA

[a] +, positive signal or characteristic band pattern for *E. amylovora*; −, no signal or atypical for *E. amylovora*; blank, not determined.
[b] With subsequent *Hae*III digestion and pattern analysis of the PCR product.

lanes 1 to 3 in Fig. 4. The enzymes *Sau*3A, *Hpa*II, and *Fnu*4HI produced different restriction patterns of the PCR amplification products from *E. amylovora*, *P. syringae*, and *A. tumefaciens*, but they did not allow the differentiation of the fire blight pathogen from other *Erwinia* species or from *Escherichia coli*, which showed the same or a very similar pattern of restriction fragments. The restriction enzymes produced identical restriction patterns of the 16S rDNA fragment amplified from *E. amylovora* strains isolated from various geographic regions (Table 3).

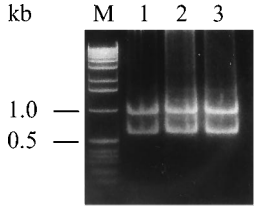

FIG. 3. Cleavage of the specific amplification products from *E. amylovora* with a restriction enzyme. The PCR products were ethanol precipitated and digested with *Mlu*I. Lanes: 1, strain Ea1/79; 2, strain Ea273; 3, strain T91; M, 1-kb ladder marker DNA. The positions of 1.0 and 0.5 kb are indicated.

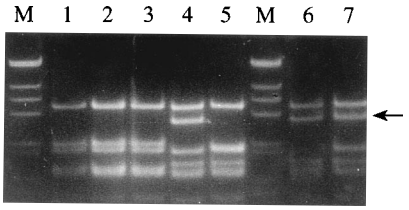

FIG. 4. PCR amplification of a DNA fragment from the 16S rDNA region of various bacteria and the cleavage pattern of a *Hae*III digest. Lanes: 1, *E. amylovora* Ea7/74; 2, *E. amylovora* Ea7/84; 3, *E. amylovora* Ea273; 4, *E. carotovora* subsp. *carotovora* 499; 5, *Escherichia coli* 1100; 6, *E. stewartii* DC283; 7, *E. chrysanthemi* 533; M, marker DNA in descending order (517 and 506, 398, 344, 298, 220, and 201 bp). The discriminating band at 278 bp is indicated by an arrow.

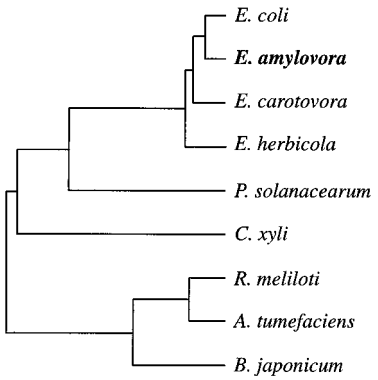

FIG. 5. Phylogenetic tree based on 16S rDNA sequences from various gram-negative bacteria. The sequence of the *E. amylovora* 16S rDNA can be obtained from the EMBL Nucleotide Sequence Database under the accession number X83265.

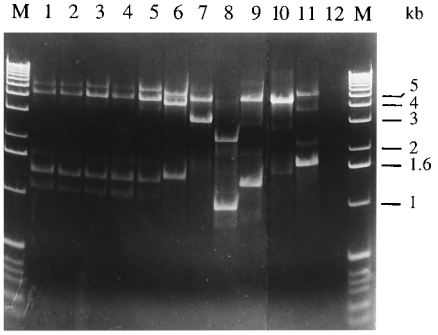

FIG. 6. AP-PCR analysis of *E. amylovora* and other plant-associated bacteria with the primer RPT1 derived from transposon Tn5. A 10-ng portion of DNA was applied in the assay. Lanes: 1 to 5, *E. amylovora* strains (1, Ea1/79; 2, EaX1/79 without plasmid pEA29; 3, T91; 4, Ea7/84; 5, Ea273); 6, *E. herbicola* SA5; 7, *E. chrysanthemi* 533; 8, *E. carotovora* subsp. *carotovora* 568; 9, *E. carotovora* subsp. *atroseptica* 185; 10, *A. tumefaciens* C58; 11, *B. subtilis* BD170; 12, control without DNA; M, 1-kb ladder marker DNA.

Nucleotide sequence analysis of the amplified 16S rDNA fragment of *E. amylovora*. DNA of *E. amylovora* Ea1/79 (10 ng) was added to a standard PCR assay mixture (3), and a 1.5-kb fragment was amplified with the 16S rDNA primers fD2 and rP1, as described above. The amplification product of 1.5 kb was cloned into vector pGEM-T and subsequently sequenced. The 1,507 nucleotides were compared with the corresponding sequences from *Escherichia coli*, *E. carotovora* subsp. *atroseptica*, and *E. herbicola* and showed homologies of 96, 94, and 94%, respectively. The sequence data correlated with the fragment sizes obtained in the *Hae*III digest and with the cleavage products obtained with other restriction enzymes. By comparison of the 16S rDNA sequence of *E. amylovora* with that of other bacteria, a phylogenetic tree was constructed (Fig. 5). By these criteria, *E. amylovora* and *Escherichia coli* are closely related, followed by *E. carotovora* subsp. *atroseptica* and *E. herbicola. P. solanacearum*, *Clavibacter xyli*, *Rhizobium meliloti*, *A. tumefaciens*, and *Bradyrhizobium japonicum* are more distantly related to *E. amylovora* than are the members of the genera *Erwinia* and *Escherichia.*

Identification of *E. amylovora* with AP-PCR. AP-PCR can be used for genomic analysis without information about specific sequences of an organism (27). We have assayed several oligonucleotides in the 17-mer range for the resulting band pattern in AP-PCR amplification. Primer APT1 (Table 2) was found to produce reliable fragments for amplification of *E. amylovora* DNA and was previously applied for amplification of DNA from *P. syringae* pathovars to detect coronatine producers (24). A related primer was recently used to analyze *P. syringae* pathovars (23). In AP-PCR analysis of *E. amylovora*, four intermediate to strong bands at 5.5, 4.4, 1.4, and 1.1 kb, as well as three weak bands, were typical for *E. amylovora*. Strains from Germany, Turkey, Egypt, and the United States produced identical signals (Fig. 6). Other plant-associated bacteria could be distinguished from the fire blight pathogen by this procedure; i.e., primer APT1 allowed an unambiguous identification of *E. amylovora*. When DNA from strain EaX1/79, which was cured of plasmid pEA29 (10), was used as the template, the pattern of bands was identical to that obtained with DNA from the parent strain Ea1/79 and other strains with plasmid pEA29 (Fig. 6). We thus assume that pEA29 does not contribute to the AP-PCR band pattern typical for *E. amylovora* DNA and primer APT1. Different patterns were produced with DNA from *E. herbicola*, *E. chrysanthemi*, *E. carotovora* subspecies, *A. tumefaciens*, and *Bacillus subtilis* (Table 3; Fig. 6). DNA of *Pseudomonas* spp. also produced banding patterns (24) that differed from those obtained with *E. amylovora*. The identical AP-PCR patterns of the *E. amylovora* strains indicate a high degree of similarity among strains derived from various geographic regions and at different times.

DISCUSSION

In addition to a PCR assay based on information from plasmid pEA29 (3), in the present study we extended the identification of *E. amylovora* by using three PCR methods based on target DNA localized on the bacterial chromosome. A specific signal of 1.6 kb was obtained by amplification of a region in the *ams* operon that encodes synthesis of the capsular exopolysaccharide amylovoran, which is unique in its structure for *E. amylovora* compared with other bacterial capsules. Consequently, the nucleotide sequence of the *ams* region shares only low homology with other sequences presently available in data libraries (6). Also, the *cps* region of *E. stewartii*, which can be complemented by *ams* genes (4), did not produce amplification products with primers designed from the *E. amylovora amsB* region. It seems unlikely that homologous sequences occur in other bacteria which give rise to the signal obtained with *E. amylovora* DNA. Some phytopathogenic bacteria have been identified by PCR assays. Coronatine-producing *P. syringae* pathovars were detected by amplification of a 0.65-kb fragment from a gene involved in biosynthesis of the phytotoxin (2). A specific 1.9-kb fragment was obtained after amplification of the phaseolotoxin gene region of *P. syringae* pv. phaseolicola strains (15). Restriction fragment length polymorphisms from amplification of a *pel* gene sequence were used to identify and differentiate *E. carotovora* strains which cause potato diseases (7).

Analysis of 16S rDNA sequences is a common tool for research in evolutionary development of organisms (14). The complex structure of ribosomes does not allow significant sequence variation of rRNAs. By applying primers derived from consensus sequences (26), the 16S rDNA of bacteria is a suitable target for PCR amplification with these primers and for detection of nucleotide exchanges in the variable region within the genera. Consequently, for identification of isolated strains, the method requires additional analysis of the amplification product. Restriction digests with enzymes which recognize 4 bp and thus frequently cleave DNA is a promising approach for subsequent differentiation. The *Hae*III digest of amplified 16S

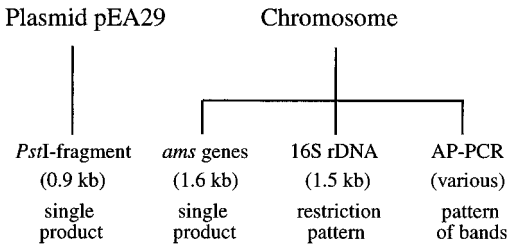

FIG. 7. Scheme for various approaches to identify *E. amylovora* by PCR analysis. The type of the specific signal or pattern is listed in the bottom row.

rDNA produced specific bands for *E. amylovora* and provided a tool for its discrimination from other plant-associated bacteria. A similar approach was reported for differentiation of phytopathogenic mycoplasmas (19). We fitted the sequence of the 16S rDNA of *E. amylovora* into a phylogenetic tree, which revealed the close relatedness of the fire blight pathogen to *Escherichia coli.*

E. amylovora originated from northeastern North America and was spread in this century to other parts of the world (25). Genomic differences may therefore be rare in independently isolated strains. Divergences in *E. amylovora* strains were found for strain E9, isolated in the United States, which lacks a *Pst*I site in pEA29; for strains E9 and Ea273, which produce the phytotoxin dihydrophenylalanine (20), in contrast to many other *E. amylovora* strains; and for strain Ea7/74, isolated in Germany, which cannot efficiently propagate plasmids with the ColE1 replication origin. Amplification of genomic DNA with a single arbitrary oligonucleotide primer gave characteristic bands for *E. amylovora*. This principle of random primer DNA amplification has been used for characterization of many organisms, including mammalian cells and fungi. For example, AP-PCR was used to discriminate between aggressive and nonaggressive strains of the fungus *Phoma lingam* pathogenic for rape (18). *P. syringae* pv. glycinea could be ordered by AP-PCR into coronatine-producing and -nonproducing strains (24). The appearance of an additional band was indicative of coronatine-positive strains. Furthermore, a single primer, which was also derived from the terminal repeats of transposon Tn*5*, was applied to differentiate *P. syringae* pathovars (23). On the other hand, AP-PCR is not suitable for general classification of bacteria, because a variety of different signals may be produced for strains within species, subspecies, or pathovars. AP-PCR relies on DNA from homogeneous populations. AP-PCR is also sensitive to changes in the amplification conditions and might produce altered patterns in different machines or with different batches of reagents. For instance, the addition of whole bacteria and lysis in a PCR assay diminished signal strength for *E. amylovora* (data not shown). The method is empirical for the primer used and very sensitive to its sequence and the annealing temperature.

A hierarchy of molecular identification methods for *E. amylovora* is depicted in Fig. 7. A sensitive PCR assay is based on sequence information from pEA29 (3), and primers derived from the *ams* region are a complementary choice for PCR analysis of samples containing *E. amylovora*. The loss of plasmid pEA29 in strains isolated in nature can now be detected by the independent assay with the *ams* region. The *ams*-derived-primers method can also be used to identify *E. amylovora* in the presence of other plant-associated bacteria and in plant tissue without preceding DNA isolation. Amplification of 16S rDNA relies on homogeneous DNA preparations or homogeneous populations of bacteria. The addition of Tween 20 to the PCR assay (3) is recommended to increase sensitivity for signal detection from whole cells. AP-PCR is the third of the novel methods described and depends on homogeneous DNA preparations; application of whole cells reduced the signal strength for this method. In contrast to amplification of DNA fragments from the *ams* region, these two approaches are therefore not suited for analysis of field samples. For analysis of DNA preparations in the laboratory or for complementary methods to identify *E. amylovora* with molecular tools, 16S rDNA amplification and AP-PCR are valid assays for classification of bacteria, especially for the uniform species *E. amylovora*. For identification of *E. amylovora* colony hybridization, Southern hybridization and PCR assays based on plasmid pEA29 were thus replaced by amplification of a fragment with the *amsB* gene, by AP-PCR, and by analysis of amplified 16S rDNA.

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