

## Sensitive and Species-Specific Detection of *Erwinia amylovora* by Polymerase Chain Reaction Analysis

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Detection and identification of the fire blight pathogen, *Erwinia amylovora*, can be accurately done by polymerase chain reaction (PCR) analysis in less than 6 h. Two oligomers derived from a 29-kb plasmid which is common to all strains of *E. amylovora* were used to amplify a 0.9-kb fragment of the plasmid. By separation of the PCR products on agarose gel, this fragment was specifically detected when *E. amylovora* DNA was present in the amplification assay. It was not found when DNA from other plant-pathogenic bacteria was used for the assay. A visible band specific to the 0.9-kb fragment was produced with DNA from fewer than 100 *E. amylovora* cells. A signal of similar strength was also obtained from *E. amylovora* cell lysates in the presence of the mild detergent Tween 20. Signals were weaker when bacteria were added to the PCR mixture without the detergent. As with results obtained from hybridization experiments using pEA29 DNA, the PCR signal was obtained with *E. amylovora* isolates from various geographic regions. This technique could also be used for detection of the fire blight pathogen in extracts of tissue obtained from infected plant material.

The gram-negative bacterium *Erwinia amylovora* causes the disease fire blight on pome fruit trees and other rosaceous hosts (17). Historically, fire blight was earlier restricted to the northeast part of North America, with its occurrence first described more than 200 years ago. In this century, epidemic spread of fire blight has been reported from geographically widespread locations, including New Zealand (in 1916), England (in 1957), Egypt (in 1962), and various regions in western Europe (in 1966 and 1971). Annual surveys in the *Fire Blight Newsletter* describe recent outbreaks in Turkey, Israel, Lebanon, Cyprus, Scandinavia, Switzerland, and Italy. Although the long-distance spread of fire blight has not been unambiguously correlated with specific events, it is apparent that trade involving plants, fruits, and contaminated wood used for shipping containers may have been responsible for long-range distribution and that other possibilities cannot be excluded. Aerosols may give rise to atmospheric contamination with the pathogen or the disease may spread with migrating birds. However, plant material appears to be the most important source of fire blight epidemics, which may be confined by the monitoring of suspect plants lacking disease symptoms. Conventional diagnostic methods, including the use of semiselective media (13) or immunofluorescence techniques (14), cannot detect small quantities of *E. amylovora*, i.e., detection is correlated primarily with obvious disease symptoms. Therefore, we have developed DNA hybridization techniques with DNA from a 29-kb plasmid which is common to *E. amylovora* strains. The fire blight pathogen can thus be detected by colony hybridization with the whole-plasmid DNA or with its cloned fragments as probes (4). There is no known homology of plasmid pEA29 to the DNA of other plant-pathogenic bacteria. Its curing from *E. amylovora* causes a delay in the development of disease symptoms (5). The involvement of pEA29 in the bacterial metabolism of thia-

mine (11) emphasizes the importance of this plasmid for the pathogen. By applying colony hybridization, we were able to detect small numbers of *E. amylovora*, provided that it was plated on agar and formed colonies which were not overgrown by other microorganisms. This procedure requires numerous platings of a sample to obtain bacterial colonies suited for transfer to a membrane. To avoid this labor-intensive procedure of colony hybridization, we used DNA amplification by polymerase chain reaction (PCR) (3) for the detection of *E. amylovora* DNA. For this purpose, we cloned the 0.9-kb *Pst*I fragment from pEA29 into a pfd plasmid (6) and confirmed that the insert was specific for *E. amylovora* in colony hybridization. The fragment was sequenced at both ends, and two oligonucleotides were synthesized to effect its amplification by PCR. This procedure can be described as a fast, very sensitive, and specific detection method for the fire blight pathogen in culture and in samples of plant tissue.

### MATERIALS AND METHODS

**Bacterial strains.** The *E. amylovora* isolates and other bacterial species used for PCR analysis are listed in Table 1.

**Oligonucleotides for DNA amplification.** The 0.9-kb *Pst*I fragment from plasmid pEA29 was cloned into the plasmid pfdB14Z', which contains the origin of replication from bacteriophage fd, the chloramphenicol resistance gene, and the *lacZ'* gene (6a). Plasmid pfdB14Z'-0.9 carries the 0.9-kb pEA29 fragment inserted into the *Pst*I site of the *lacZ'* fragment. The insert was partially sequenced with the dideoxy method (15) by using forward and reverse *lacZ'* primers (Boehringer-Mannheim). Two 17-mer oligonucleotides from the borders of the pEA29 fragment with the sequences 5'-CG GTTTTTAACGCTGGG (primer A) and 5'-GGGCAAATAC TCGGATT (primer B) were chosen for amplification by PCR. They were synthesized with a DNA synthesizer, purified by high-performance liquid chromatography, and stored in aliquots at -20°C.

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TABLE 1. Strains used for PCR assays

Species	Strain	Year isolated	Origin	Reference
<i>E. amylovora</i>	Ea1/79	1979	Germany	4
	E5/84	1984	Egypt	4
	Ea11/88	1988	Germany	1
	EaX11/88		Ea11/88 without pEA29	For curing, see reference 5
	Ea273	1971	USA	S. Beer, Ithaca, N.Y.
	Ea1496/66	1966	New Zealand	J. Young, Auckland, New Zealand
	T91	1991	Turkey	T. Momol, Antalya, Turkey
<i>E. herbicola</i>	2035		England	4
	NZ	1989		— <sup>a</sup>
<i>A. tumefaciens</i>	C58			4
<i>E. carotovora</i> subsp. <i>atroseptica</i>	185	1986	Germany	4
<i>P. syringae</i> pv. <i>syringae</i>	2			4

<sup>a</sup> —, isolated from pears obtained from New Zealand and tentatively characterized in our laboratory as *E. herbicola*.

**PCR assays.** The PCR was carried out in a total volume of 50  $\mu$ l containing (final concentrations) 25 pmol of each primer, 0.5 U of *Tth* DNA polymerase (from *Thermus thermophilus*; Pharmacia) template DNA, 0.2 mM each of dATP, dCTP, dGTP, and TTP (Boehringer-Mannheim), 16 mM ammonium sulfate, 67 mM Tris-HCl (pH 8.8), 1.5 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, 160  $\mu$ g of bovine serum albumin per ml, 5% dimethyl sulfoxide, and 1% Tween 20, if indicated. The reaction was performed in a liquid temperature bath apparatus (designed by H.-P. Vosberg, Max-Planck-Institut, Heidelberg, Germany) in which the cycles were controlled by a computer program. Denaturation was done at 93°C (in the first cycle for 2 min and in subsequent cycles for 1 min), annealing of the primers to the template DNA was at 52°C for 2 min, and polymerization was at 72°C for 2 min. After 37 cycles (3.5 h), the PCR products were separated on a 1.5% agarose gel (1.5 to 2 h at 100 V), stained with ethidium bromide, and photographed under UV light (at 302 nm).

**Bacteria and DNA preparations for PCR.** For direct detection in the PCR assay, the strains were grown in 10 ml of Standard I nutrient broth (Merck), and the bacteria were centrifuged, resuspended, diluted in water, and applied to the reaction mixture. For DNA extraction, the bacteria were lysed by treatment with lysozyme and sodium dodecyl sulfate, and the nucleic acids were purified by repeated extraction with phenol and then with chloroform-isoamyl alcohol. Ten microliters of the diluted bacteria or 1  $\mu$ l of the diluted nucleic acids was used for the PCR.

To recover bacteria from plant tissue, small pieces of plant material were shaken for 2 h in a 0.9% NaCl solution. The supernatant was centrifuged, the pellet was resuspended in 0.1 ml of sterile water, and 10  $\mu$ l was used for the PCR.

## RESULTS

**Primers and specificity of PCR for detection of *E. amylovora* DNA.** Sequence analysis of plasmid pfdB14Z'-0.9 was done to determine oligonucleotide sequences for amplification of the 0.9-kb *Pst*I fragment of *E. amylovora* plasmid pEA29. The 17mer oligonucleotides A and B were used for PCR detection of *E. amylovora*. The application of 10 ng of DNA (corresponding to fewer than 10<sup>6</sup> bacteria) gave rise to an intense band at 0.9 kb, which decreased at higher substrate concentrations. After the DNA concentration was decreased to 1 pg, corresponding to approximately 100 bacteria, the signal could still be seen (Fig. 1). Hybridization of the band

to the labeled 0.9-kb *Pst*I fragment revealed homology, confirming the identity of the band with the cloned fragment (data not shown). Other plant-associated bacteria gave no signals at 0.9 kb or gave signals at positions often shorter than 0.9 kb. A strain of *Erwinia herbicola* from New Zealand produced a band above 0.9 kb, while DNAs from another *E. herbicola* strain and from *Agrobacterium tumefaciens*, *Erwinia carotovora* subsp. *atroseptica*, and *Pseudomonas syringae* mainly produced bands that were shorter than 0.9 kb (Fig. 1, lanes 7 to 10). Probing the DNA of *Escherichia coli*, *E. carotovora* subsp. *carotovora*, or *Erwinia chrysanthemi* also failed to give rise to PCR bands at the 0.9-kb position (data not shown). Lowering the temperature in the annealing steps of the primers from 52 to 46°C often produced multiple bands, whereas raising the temperature diminished the signal intensity to a complete loss at 60°C; an increase of the annealing temperature above 52°C also eliminated the production of nonspecific bands with DNA from bacteria other than *E. amylovora*. A similar effect was observed at the standard annealing temperature of 52°C when a concentration of 2 pmol for primers A and B was used instead of a concentration of 25 pmol. In this case, the signal strength

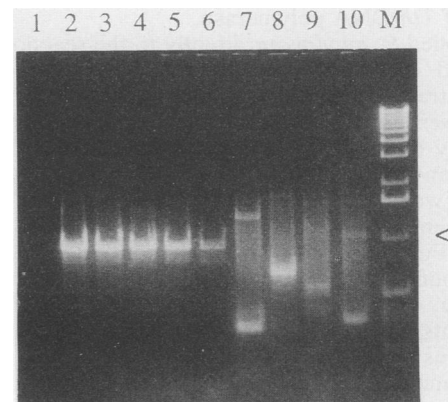


FIG. 1. PCR of DNA preparations from phyto-bacteria. Lanes: 1, control without DNA; 2 to 6, crude DNA from *E. amylovora* Ea1/79 (1 mg/ml) diluted 10<sup>-2</sup> to 10<sup>-6</sup> (a sample of 1  $\mu$ l applied); 7 to 10: ng of crude DNA from *E. herbicola* (strains NZ and 2035), *E. carotovora* subsp. *atroseptica* (strain 185), and *P. syringae* pv. *syringae* (strain 2), respectively. M, 1-kb ladder (Bethesda Research Laboratories) marker DNA (< indicates the size of 1 kb).

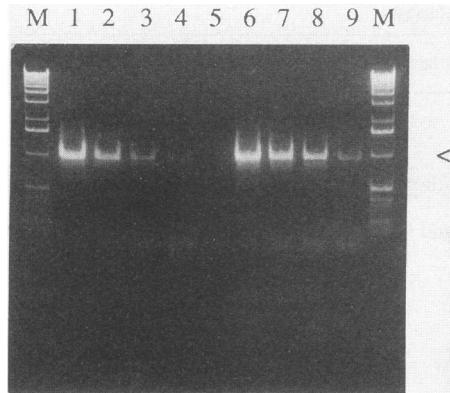


FIG. 2. PCR of *E. amylovora* cells applied directly to the amplification assay in the presence or absence of Tween 20. Cells from an overnight culture of *E. amylovora* Ea1/79 ( $5 \times 10^9$  bacteria per ml) were applied to the PCR assay. Lanes: 1 to 4 and 6 to 9, samples were diluted in water and 10  $\mu$ l was added to the PCR assay without (lanes 1 to 4) and with (lanes 6 to 9) Tween 20 in the reaction mixture; 5, control without bacteria. Numbers of bacteria added were as follows: lanes 1 and 6,  $5 \times 10^5$ ; lanes 2 and 7,  $5 \times 10^3$ ; lanes 3 and 8, 500; lanes 4 and 9, 50. M, 1-kb ladder marker DNA (< indicates the size of 1 kb).

was reduced in comparison to that at 25 pmol of primer. This temperature (52°C) and a primer concentration of 25 pmol were, therefore, optimal conditions for the sensitive detection of *E. amylovora* DNA. We avoided the production of false signals by minimizing laboratory contamination of equipment and the solutions used in the PCR assays.

**Signal detection after addition of whole cells to the PCR assay.** Phenol extraction of nucleic acids is a time-consuming procedure with the risk of a low yield. *E. amylovora* is a labile bacterium which has a tendency to spontaneously lyse in liquid culture or on solid media and which quickly dies at temperatures above 30°C. While this restricts the spread of the fire blight pathogen in nature, it causes the lysis of cells in the PCR assay without pretreatment. We have also tried to lyse the bacteria by applying the mild detergent Tween 20 to the PCR assay mixture (9). Only 1% of the detergent was added to the assay mixture, although 10% did not affect the activity of *Tth* DNA polymerase.

We applied *E. amylovora* directly to the reaction mixture and found a specific signal at 0.9 kb as observed for phenol-extracted DNA (Fig. 2, lanes 1 to 4). When the bacteria were lysed in Tween 20, which was present in the PCR assay mixture, the specific band at 0.9 kb was more intense than when the bacteria were added to assay mixtures without Tween 20 (Fig. 2, lanes 6 to 9). With detergent added, even as few as 50 cells of *E. amylovora* could be detected. Furthermore, the nonspecific bands (Fig. 1), occasionally found for DNA isolated from bacteria other than *E. amylovora*, were absent (Fig. 3, lanes 1 to 4). For these experiments,  $5 \times 10^4$  cells from two *E. herbicola* strains and from strains of *E. carotovora* subsp. *atroseptica*, *A. tumefaciens*, and *P. syringae* pv. *syringae* were added. We assume that partial lysis of the bacteria and an efficient release of the 29-kb plasmid of *E. amylovora* in the presence of Tween 20 occurred.

A clear signal of 0.9 kb was found for 500 *E. amylovora* cells in the presence of a 100-fold excess of the plant-associated bacteria when the cells were added to the PCR assay mixture containing Tween 20 (Fig. 3, lanes 5 to 8). This

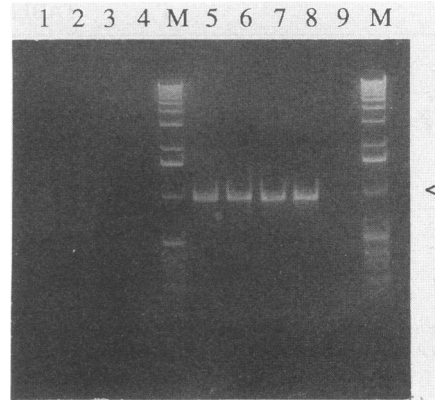


FIG. 3. PCR with cells from various plant-associated bacteria in the presence and absence of *E. amylovora*. Lanes: 1 to 4, no *E. amylovora* cells were added; 5 to 8, 500 cells diluted from an overnight culture of *E. amylovora* Ea1/79 were added. All reaction mixtures contained 1% Tween 20. From various species,  $5 \times 10^4$  bacteria were added. Lanes: 1 and 5, *E. herbicola* NZ; lanes 2 and 6, *E. carotovora* subsp. *atroseptica* strain 185; lanes 3 and 7, *A. tumefaciens* C58; lanes 4 and 8, *P. syringae* pv. *syringae* (strain 2); lane 9, control without bacteria. M, 1-kb ladder marker DNA (< indicates the size of 1 kb).

demonstrates that the specific signal for the fire blight pathogen is not altered in PCR assays in the presence of other bacteria which may occur in field samples.

**Identification of *E. amylovora* isolates from various geographic regions and from plant tissue.** In a previous study, we demonstrated the occurrence of the 29-kb *E. amylovora* plasmid in all strains tested (>75). Restriction fragment length polymorphism in the plasmid was rare (unpublished data). It can be expected that all naturally occurring isolates will contain the 0.9-kb sequence of pEA29. We have randomly selected five strains which originated in various parts of the world. All strains were positive by the PCR assay (Fig. 4, lanes 1 and 3 to 6). As expected, a strain cured of the common plasmid pEA29 (5) did not produce an amplification signal at 0.9 kb (Fig. 4, lane 2). Other isolates putatively characterized as *E. amylovora* by conventional methods were not all positive in the assay. The negative results of the PCR assays were confirmed by the characterization and identification of those isolates as pseudomonads by using additional criteria.

The fire blight pathogen is often eluted from plant material by extraction with salt buffers or water. Also, samples from orchards which were collected by an air sampler (2) require a sensitive method to show the presence or absence of *E. amylovora*. In a manner similar to the air sampler conditions, we suspended bacteria in sterile water. The samples were centrifuged, the pellet was suspended in 100  $\mu$ l of water, and an aliquot (10  $\mu$ l) of the solution was added to the PCR assay mixture. The specific signal was detected with approximately 100 *E. amylovora* cells, even when the bacteria were suspended in an NaCl solution. Furthermore, we cultivated in the laboratory an apple seedling which developed a stem canker after inoculation with *E. amylovora*. About 100 mg of canker tissue was extracted with 10 ml of water. Suspended material was concentrated by centrifugation, and an aliquot of 10  $\mu$ l was applied to the PCR. A signal at 0.9 kb on the agarose gel identified the presence of *E. amylovora* in the canker tissue (Fig. 4, lanes 8 and 9). From

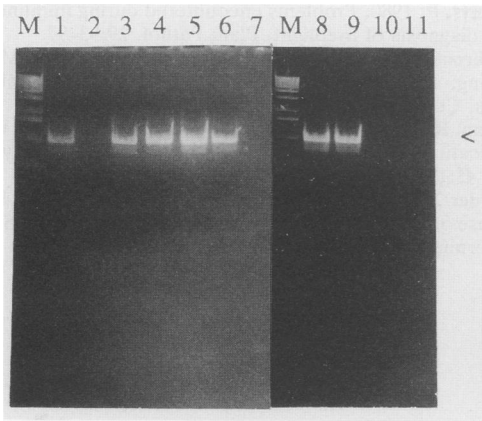


FIG. 4. Detection of the specific PCR signal for cells of *E. amylovora* isolates from various geographic regions and of *E. amylovora* from plant tissue. Lanes: 1 to 6,  $5 \times 10^3$  cells were added to the reaction mixture with Tween 20; 1, strain Ea11/88; 2, strain EaX11/88 (without plasmid pEA29); 3, strain Ea273; 4, strain Ea5/84; 5, strain Ea1496/66; 6, strain T91; 7, control without bacteria; 8 and 9, 10- $\mu$ l sample from tree canker, undiluted and  $10^{-2}$  dilution, respectively; 10 and 11, 10- $\mu$ l sample from a healthy leaf of an apple seedling, undiluted and  $10^{-2}$  dilution, respectively. M, 1-kb ladder marker DNA (< indicates the size of 1 kb).

the same seedling, a leaf without symptoms did not give a signal (Fig. 4, lanes 10 and 11). This demonstrates the possibility of recovery of the fire blight pathogen under field conditions and the usefulness of the method for environmental screening.

## DISCUSSION

Long-distance as well as local spread of fire blight, caused by movement of *E. amylovora*-contaminated plant material, could be considerably minimized by monitoring such material for the pathogen by using sensitive detection techniques. Serological techniques (14), including the use of monoclonal antibodies (12) or semiselective media (13), can be applied in the case of massive contaminations, but latent infections with low concentrations of the pathogen (below  $10^5$  CFU/ml) require a more sensitive and reliable method to detect the bacterium in a field sample. Molecular biology has provided a powerful tool to identify *E. amylovora*. The whole genome of *E. amylovora* has been used as a probe to detect the pathogen (7). However, considering the homology among the genomes of gram-negative bacteria, including repetitive sequences, the signal might be ambiguous and, especially when of low intensity, not indicative of the occurrence of fire blight. We have screened *E. amylovora* isolates for a 29-kb plasmid, which was recognized to be common to the fire blight pathogen (4). Plasmid pEA29 has a function in the thiamine (vitamin B<sub>1</sub>) metabolism of the bacterium (11). The strains Ea7/74, Ea1/79, Ea11/88, all isolated in Germany, were cured of the common plasmid (5); this resulted in an observed dependency of these isolates on exogenous thiamine at vitamin concentrations of 1 ng/ml and lower (unpublished data). It appears likely that thiamine is barely available for *E. amylovora* in planta and therefore is a limiting factor for bacteria deficient in the cellular synthesis of thiamine. Consequently, *E. amylovora* strains without plasmid pEA29 seem to be less efficiently suited for causing the disease in planta than strains carrying the plasmid. Plasmid-free strains are therefore unlikely to occur in nature because

their pathogenic potential is low (5). The plasmid is therefore a reliable marker for *E. amylovora*. It exists in low copy numbers in the cell and is therefore difficult to be isolated as a pure DNA preparation. We have cloned fragments of pEA29 in multicopy plasmids and used specifically the 5-kb *Sa*I fragment as a probe to identify *E. amylovora* by DNA hybridization (4). A positive signal for all strains investigated indicated a strict correlation of the pEA29 plasmid in isolates from diseased plants.

The use of oligonucleotides derived from the 0.9-kb fragment of pEA29 in the PCR provides a sensitive and specific tool for detection of the fire blight pathogen. The limit of detection of an amplifiable DNA sequence is estimated to be as low as five molecules in the reaction mixture (3). We found an easily detectable signal for as few as 50 bacteria, representing approximately 150 plasmid copies. We consider this a practical detection limit for the PCR assay described here. As is often found in PCR assays, there was only a coarse correlation between the signal strength and the amount of template added to the reaction mixture. PCR applications for the detection of bacteria in water or food samples (10, 16) and of pathogenic viruses have been described (8). We circumvented analysis of the amplification product by DNA hybridization by using the specific signal recognized in electrophoresis of the amplified sample on a gel. The pathogen was efficiently detected by lysing bacteria in a reaction mixture containing detergent, thus avoiding isolation of nucleic acids.

The PCR analysis outlined may be applied for practical purposes to monitor the presence of *E. amylovora* in latent infections of pome fruit orchards, in nurseries, or in exported fruits and other host plant material.

## ACKNOWLEDGMENTS

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