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MULTIPLEX PCR ASSAY FOR IDENTIFICATION OF *ERWINIA AMYLOVORA* - THE CAUSATIVE AGENT OF FIRE BLIGHT

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ABSTRACT

Seven Bulgarian strains of Erwinia amylovora isolated from chokeberry and strawberry, previously identified by single PCR amplification of a specific region of plasmid pEA29 and genome ams-region were subjected to multiplex PCR using two pairs of primers – pEA29A/pEA29B and AJ245/AJ246. These primers allowed the identification of the strains in one-step PCR simultaneously in two genetic loci. The conditions of the multiplex PCR were optimized, and any nonspecific reactions with other related, and non-related species were avoided. The multiplex PCR proposed is a not time-consuming and reliable method for rapid identification and discrimination of E. amylovora from the closed E. pyrifoliae species.

Introduction

Fire blight, caused by *Erwinia amylovora*, is the most destructive bacterial disease affecting apple (*Malus*), pear (*Pyrus)* and several rosaceous hosts (ornamentals) and is therefore of great economic importance. The pathogen can survive as an endophyte (20), an epiphyte (17), or in latent infections. The movement of infected asymptomatic plants or plant part could serve as a means of introducing this serious disease into other geographic regions. *E. amylovora* was reported in Bulgaria for the first time in 1990 (6). Since that, the pathogen was progressively detected in different regions in Bulgaria (8, 9, 2).

Control of the disease relies on unambiguous identification of the pathogen. Furthermore, symptoms of other plant diseases might be confused for those of fire blight. The conventional diagnostic methods including the use of semi-selective media (3) or serological assays like enzyme-linked immunosorbent assay or immunofluorescence cannot detect small quantities of *E. amylovora,* i.e. detection is correlated primarily with obvious disease symptoms. Common surface antigens of gram-negative bacteria give rise to nonspecific antibodies, which can confuse serological detection of *E. amylovora* (18). A disadvantage of the use of semi-selective media is co-cultivation of other plant-associated bacteria. *Erwinia herbicola* (now *Panthoea agglomerans*) occasionally has colony morphology similar to that of *E. amylovora* on MS-medium, and *Pseudomonas syringae* and other pseudomonads may resemble *E. amylovora* on NSA medium. In order to overcome these problems molecular methods for identification have been developed. The advent of molecular biology in general and the polymerase chain reaction in particular have greatly facilitated genomic analyses of microorganisms, provide enhanced capability to characterized

TABLE

Origin of *E. amylovora* strains used

Strains (number)	Host-plant	Year of isolation	Region
1	Chokeberry	2004	Elena
2	Chokeberry	2004	Kostenets
2	Strawberry	2002	Lovech
2	Strawberry	2003	Suhindol

and classify strains, and facilitate research to assess the genetic diversity of population. Several molecular methods as PCR analysis, nested PCR, PCR-dot-blot and reverse-blot hybridizations (16), colony hybridization (10) used for identification of the fire blight pathogen took advantage of the presence of a 29-kb plasmid in all *E. amylovora* strains with very few exceptions from natural sources and were highly selective (21). Bereswill et al. (4) have developed a PCR method, which takes advantage of genes of the chromosomal *ams* region (5). These genes encode synthesis of the capsular polysaccharide amylovoran, which seems to be unique for *E. amylovora*. But a single-step PCR technique that would identify two specific genetic targets simultaneously (*ams* region and plasmid pEA29) has not reported yet.

In many cases for diagnosis of the pathogens it is important to use rapid, not time-consuming, economical and reliable techniques. In this study, we described the development of a simple and effective procedure, which consist of a one-step multiplex PCR for simultaneously identification of the both *ams*-genes and pEA29 in order to identify rapidly *E. amylovora*.

Materials and Methods

Bacterial strains. *E. amylovora* strains used in the present study are listed in **Table**. The strains *E. amylovora* 2331 NBIMCC (ATCC 15580), *Erwinia stewartii* 2323 NBIMCC (ATCC 8199), *Erwinia pyrifoliae* DSM 12163, *P. syringae* pv *syringae* 2420 NBIMCC were used as controls**.**

Media and culture conditions

The strains were cultivated in LB broth (19) and incubated for 24-36 h at 26 ºC

DNA preparation

Chromosome and plasmid DNA were isolated and purified by a DNA isolation kit (Scientific Technological Service (STS) Ltd., Sofia, Bulgaria) according to the manufacture's instruction.

Multiplex PCR analysis

Seven *E. amylovora* strains and the type strains mentioned above were subjected to multiplex PCR analysis in which a simultaneous amplification of a fragment of the plasmid pEA29 and genome *ams*-region was achieved for the strains identification. Two pairs of primers were used: one pair was based on plasmid pEA29 [A (5`-CGGTTTTTAACGCTGGG-3`) and B (5`-GGGCAAATACTCGGATT-3`) (3)], and the other was based on genome *ams*-regions: [AJ245 (5`-AGCTGGCGGCACTTCACT-3`) and AJ246 (5`-CCCCGCACCGTTCAGTTTT-3`) (13)]. The multiplex PCR amplification was performed at the conditions of the single PCR amplification of *ams*-region and a fragment of the pEA29 plasmid (1) with modifications, which will be discussed below.

Electrophoresis

PCR products were separated on a 1.5% agarose gel in TBE (14) for 1 h at 100V, stained with ethidium bromide, and photographed under UV light.

Results and Discussion

E. amylovora possesses a low copy number plasmid pEA29 which seems to play a quantitative role in pathogenicity (11, 14). The presence of this plasmid in all the strains allowed primers specific to a DNA fragment of the plasmid for identification of the pathogen by PCR to be proposed - pE29A and pE29B (3). *E. amylovora* produces a polysaccharide complex named amylovoran, which is also a factor of pathogenicity. The genes coding for this

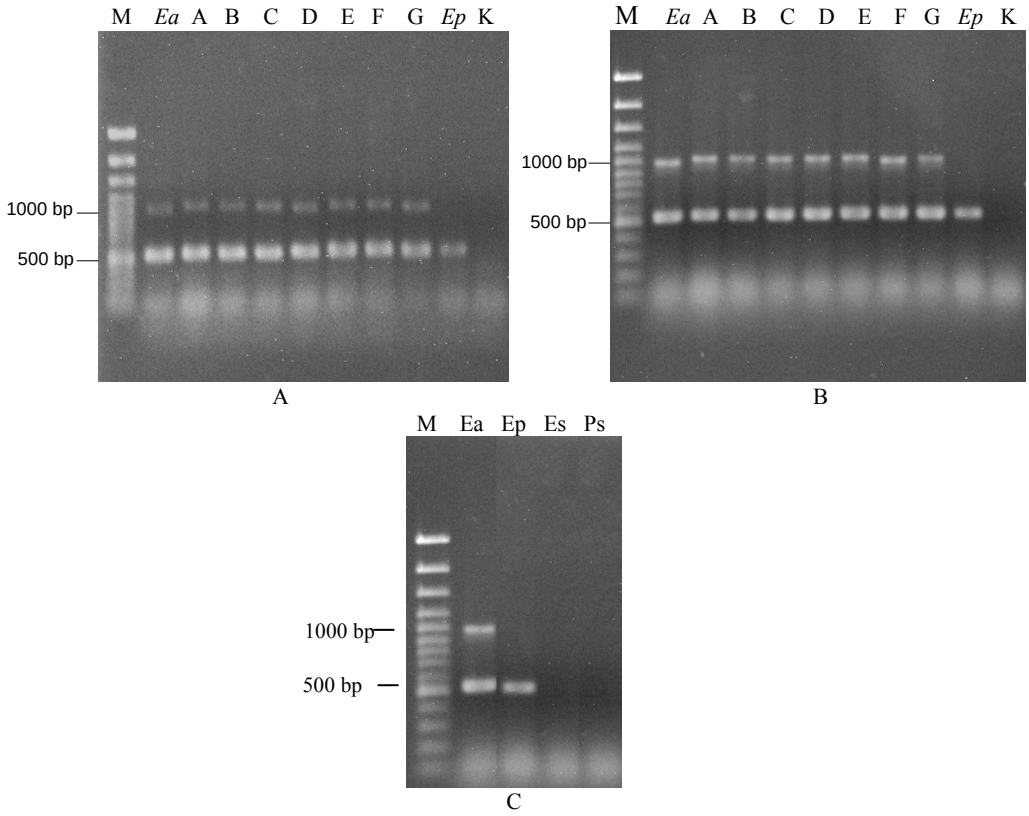

Fig. 1. Multiplex PCR of the *ams*-region and the pEA29 plasmid of the *E. amylovora* strains. Lanes A – C, strains from chokeberry; lanes D – G, strains from strawberry; *Ea*, type strain of *E. amylovora* ATCC 15580; *Ep*, type strain of *E. pyrifoliae* DSM 12163; Ps, type strain of *E. stewartii* ATCC 8199; *Ps*, type strain of *P. syringae* pv *syringae* 2420 NBIMCC; K, control (PCR-mixture without DNA без); M, 100 bp DNA marker (Amersham Biosciences).

low molecular weight polysaccharide are located in the chromosome in so called *ams*-region (22, 5, 9) and consisted of 13 genes (from *ams*A to *ams*M). The specificity of glycosyl transferases for biosynthesis of amylovoran suggests that this region is highly conserved for *E. amylovora* (9) and may not occur in other bacteria. The sequence of the *ams*A- and *ams*B-genes serves for design of species-specific primers, which can be used for an identification of *E. amylovora*. In the present study two pairs of primers (AJ245/AJ246 and pE29A/pE29B) for PCR identification of *E. amylovora* were used.

Seven strains of *E. amylovora* preliminary identified to species level (1) were used for development of this one-step multiplex PCR procedure. The initial experiments were performed at conditions and concentration of the primers identical to the single PCR amplification of *ams*-regions and a fragment of the pEA29 plasmid (1). This leaded to the preferential amplification of the fragment from *ams*-region (**Fig. 1A**). To avoid this, the conditions of the reaction were optimized in the following points: a temperature of hybridization of the primers with mDNA, a concentration of the primers and quantity of the template

DNA. The best results were obtained when the hybridization temperature and the concentration of the primers pEA29A и pEA29B was increased with 2 ºC and 20 pmol, respectively, and the concentration of primers AJ245 and AJ246 was reduced with 10 pmol in comparison with the single PCR amplification (**Fig. 1B**). The quantity of the template DNA was 100 ng. The increase of the annealing temperature eliminated the production of nonspecific bands with DNA from bacteria other than *E. amylovora*. The conditions of the multiplex PCR procedure were determined as followed: the PCR reaction mixture (25 µl) contained (finale contents) - 1 x PCR buffer (STS Ltd.); 5 mM $MgCl_2$; 1.1 mM each dNTP; 0.5 U *Taq* polymerase (STS Ltd.); 40 pmol of primers pEA29A и pEA29B; 10 pmol of primers AJ245 and AJ246; 100 ng of template DNA. The reaction conditions were: a denaturation step at 94 ºC for 4 min followed by 30 cycles at 94 ºC for 45 s, 60 ºC for 45 s and 72 ºC for 1 min. A final step at 72 ºC for 5 min stopped the reaction. At these conditions a generation of two expected PCR amplicons – one about 1100 bp and the second of 519 bp was achieved. The presence of amplification in all our isolates with the primer pairs indicated that they belonged to the species *E. amylovora*. The specifity of one-step multiplex PCR was tested in other plant-associated bacteria as *P. syringae, E. pyrifoliae* DSM 12163, and *E. stewartii* (**Fig. 1C**). No PCR products were obtained with any of the type strains, except *E. piryfoliae,* which formed a band characteristic of *ams*-region. However, *E. pyrifoliae* did not generate a positive band corresponding to the amplified locus of pEA29 plasmid. *E. pyrifoliae* is closely related to *E. amylovora*. It was shown that the species possessed genes for exopolysaccharide production having substantial homology with the *ams*-genes of *E. amylovora* (12). The use of single PCR amplification of *ams*-region by only the primers AJ245 and AJ246 for the identification of *E. amylovora* is not enough reliable, but their use in the multiplex PCR proposed could be adequate to differentiate *E. amylovora* and *E. pyrifoliae.* Bereswill et al. (3) reported that a strain of *E. herbicola* from New Zealand reacted with primers pEA29A and pEA29B and produced a band above 0.9–kb, while DNAs from another *E. herbicola* strains and from *Agrobacterium tumefaciens, Erwinia carotovora* subsp. *atroseptica* and *P. syringae* mainly produced bands that were shorter than 0.9-kb.

On the base of the results obtained it may be concluded that the conditions of the multiplex PCR developed in this study eliminated the possibility of nonspecific reactions and were very specific and selective for *E. amylovora* strains. This method is rapid, not time-consuming and discriminates the two closely related species *E. amylovora* and *E. pyrifoliae*. This method is useful for rapid molecular identification of *E. amylovora* simultaneously on two genetic loci.

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REFERENCES

1. **Atanasova I., Kabadjova P., Bogatzevska N., Moncheva P.** (2005) Z. Naturforsch., **60C,** 893-898.

2. **Atanasova I., Moncheva P., Tishkov S., Bogatzevska N.** (2003) Biotechnol & Biotechnol. Eq., **17(2),** 65-72.

3. **Bereswill S., Pahl A., Bellemann P., Berger F., Zeller W., Geider K.** (1992) Appl. Environ. Microbiol., **58,** 3522-3526.

4. **Bereswill S., Bugert P., Bruchmüller I., Geider K.** (1995) Appl. Environ. Microbiol., **61,** 2336-2642.

5. **Bernhard F., Coplin D.L., Geider K.** (1993) Mol. Gen. Genet., **239**, 158-168.

6. **Bobev S.** (1990) Higher Inst. of Agric., Plovdiv, Scientific Works, **35(4)**, 227-231.

7. **Bobev S., Garbeva P., Hauben L., Crepel C., Maes M.** (1999) Acta Hortic., **489,** 121-127.

8. **Bogatzevska N. Kondakova V.** (1994) In: Plant Pathogenic Bacteria, Versailles (France), June 9 - 12, 1992. Ed. INRA, Paris (Les Colloques no 66), 835-840.

9. **Bugert P., Geider K.** (1995) Mol. Microbiol., **15,** 917-933.

10. **Falkenstein H., Bellemann P., Walter S., Zeller W., Geider K.** (1988) Appl. Environ. Microbiol., **54,** 2798-2802.

11. **Falkenstein H., Zeller W., Geider K.** (1989) J. Gen. Microbiol., **135,** 2643-2650.

12. **Jock S., Kim Won-Sik, Barny M.-A., Geider K.** (2003) Appl. Environ. Microbiol., **69,** 679-682.

13. **Jones A.L., Geider K.** (2001) In: Laboratory Guide for Identification of Plant Pathogenic Bacteria (N.W. Schaad, J.B. Jones, W. Chun, Eds.), APS Press, The American Phytopathological Society, Minnesota, USA, 40-55.

14. **Laurent J., Barny M.-A., Kotoujansky A., Dufriche P., Vanneste J.L.** (1989) Mol. Plant Microbe Interact., **2,** 160-164.

15. **Maniatis T., Fritsch E.F., Sambrook J.** (1982) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

16. **McManus P.S., Jones A.L.** (1995) Phytopathology, **85,** 618-623.

17. **Miller T.D., Schroth M.N.** (1972) Phytopathology, **62,** 1175-1182.

18. **Roberts P.** (1980) Plant Pathol., **29,** 965-970.

19. **Sambrook J., Fritish E.F., Maniatis T.E.** (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

20. **Van der Zwet T, Beer S.V.** (1991) Fire blight – its nature, prevention, and control: A practical guide to integrated pest management. USDA Agric. Inf. Bull., p. 631.

21. **Vanneste J.L.** (1995) *Erwinia amylovora*. In: Pathogenesis and Host Specifity in Plant Diseases: Histopathological, Biochemical, genetic and Molecular Bases (U.S. Singh, R.P. Singh, K. Kohmoto, Eds.), Oxford and London: Pergammon Press, 21-41.

22. **Whitfield C., Valvano M.A.** (1993) Adv. Microbiol. Physiol., **35,** 135-246.