

New Host Plants of *Erwinia amylovora* in Bulgaria

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Nine strains of *Erwinia amylovora* were isolated from new host plants in Bulgaria – chokeberry and strawberry. The strains were characterized morphologically and biochemically using the API 20E and BIOLOG system. It was established that they showed three different API 20E metabolic profiles, not found by previous studies of *E. amylovora*. All strains were identified as *E. amylovora* due to their metabolic fingerprint patterns obtained by the BIOLOG system. The identification was confirmed by PCR amplification of a specific region of plasmid pEA29 and genome *ams*-region. This study is the first characterization and identification of *E. amylovora* strains isolated from chokeberry and strawberry by the API 20E and BIOLOG system and by polymerase chain reaction.

Key words: *Erwinia amylovora*, Chokeberry, Strawberry

Introduction

Fire blight is a disease of rosaceous plants that occurs in many countries around the world (Van der Zwet and Keil, 1979; Van der Zwet, 1996; Bonn and Van der Zwet, 2000). The causative agent is *Erwinia amylovora*. The bacterium affects mainly apple and pear, and other rosaceous plants of economic importance. Fire blight originated in North America but it has spread in many parts of the world, including Europe (Bonn and Van der Zwet, 2000). *E. amylovora* was reported in Bulgaria for the first time in 1990 (Bobeve, 1990). The pathogen was progressively detected in different regions in Bulgaria mainly on apples, pears and quince trees. The transmission and spread of *E. amylovora* in Bulgaria is a result from the import of plants from northern Europe in the country, the natural dissemination of the pathogen and the absence of an effective control of the disease. The study of the host range could improve our knowledge about dissemination origin of *E. amylovora* in Bulgaria.

Various methods have been described for detection of the pathogen such as screening on semi-selective media (Bereswill *et al.*, 1998) or by PCR based methods (Bereswill *et al.*, 1992, 1995). Many studies have provided information about phenotypic and genotypic characteristics of *E. amylovora* strains from several European countries (Bil-

ling *et al.*, 1961; Vantomme *et al.*, 1982, 1986; Mergaert *et al.*, 1984; Momol *et al.*, 1997; Zhang and Geider, 1997; Keck *et al.*, 2002), but only a few papers have treated the distribution and the characterization of this pathogen in Bulgaria (Bobeve, 1990; Bogatzevska and Kondakova, 1994; Garbeva *et al.*, 1996; Bobev *et al.*, 1999). This study is the first characterization and identification of *E. amylovora* strains isolated from chokeberry and strawberry by the API 20E and BIOLOG system and by polymerase chain reaction.

Material and Methods

Bacterial strains

Presumptive *E. amylovora* isolates from chokeberry and strawberry taken from different regions in Bulgaria and type strains of *E. amylovora* ATCC 15580 and *E. pyrifoliae* DSM 12163 were used in this study.

Isolation procedure

The strains were isolated from the shoots and leaves of chokeberry (*Aronia melanocarpa*) and different varieties of strawberry (*Fragaria ananassa*) with typical symptoms of fire blight (reddish to brown color of affected shoots and leaves) employing a standard procedure (Jones and Geider, 2001).

Media and culture conditions

King's medium B (King *et al.*, 1954) was used for the isolation of the strains. The morphology of colonies was examined on sucrose nutrient agar (Billing *et al.*, 1961), MM2Cu medium (Bereswill *et al.*, 1998). The mucoid growth and pigmentation was examined on YDC medium (Schaad, 2001). LB broth was used for DNA isolation (Sambrook *et al.*, 1989). The strains were maintained on potato dextrose agar (Oxoid Ltd, London, UK). The cultivation was done at 26 °C for 16 to 48 h depending on the analysis performed.

Pathogenicity test

The pathogenicity of the strains was examined by two methods. The first one is the hypersensitive reaction (HR) on tobacco cv. "Samsun NN" according to Klement (1963). The second method included vacuum infiltration of pear twigs with bacterial suspension (10^4 cfu/ml) of a 24 h-culture grown on potato dextrose agar (Bogatzevska and Kondakova, 1994).

Phenotypic characterization

The micromorphology of the strains (cell morphology, arrangement and motility) was investigated by light microscopy. Gram reaction, the presence of oxidase and catalase, growth at 36–39 °C, growth in 5% NaCl and nitrate reduction were examined according to the protocols described by Jones and Geider (2001). The fermentation of glucose with gas formation was determined using the medium of Board and Holding (Smibert and Krieg, 1981). The biochemical characterization of the strains was carried out by two miniaturized systems – API 20E (BioMerieux, Marcy – l'Etoile, France) and BIOLOG (BIOLOG Inc., Hayward, CA, USA). The API strips were inoculated with 48-h-old cultures grown on Standard I agar (Merck KGaA, Darmstadt, Germany), and then incubated at 26 °C for 48 h. Utilization of 95 carbohydrates was examined by BIOLOG GN2 microplates, following the manufacturer's instructions.

Isolation of DNA

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.

PCR

PCR amplification of fragments of the plasmid pEA29 and genome *ams*-region was used for the identification of the strains. Two pairs of primers (Invitrogen Life Technologies Ltd, Renfrew, UK) were used: one was based on plasmid pEA29 DNA [A (5'-CGGTTTTTAACGCTGGG-3') and B (5'-GGGCAAATACTCGGATT-3') (Bereswill *et al.*, 1992)] and the other was based on genome *ams*-region [AJ245 (5'-AGCTGGCGGGCACTTCACT-3') and AJ246 (5'-CCCCGCACCGTTCAGTTTT-3') (Jones and Geider, 2001)].

The PCR reaction mixture (25 µl) contained (final contents): 1 × PCR buffer (STS Ltd.); 5 mM MgCl₂; 0.125 mM each dNTP; 0.4 U *Taq* DNA polymerase (STS Ltd.); 10 pmol of each primer; 1 µl of template DNA. The reaction conditions were: a denaturation step at 94 °C for 4 min followed by 35 cycles at 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min. A final step at 72 °C for 5 min stopped the reaction.

Electrophoresis

PCR products were separated on a 1.5% agarose gel in TBE buffer (Maniatis *et al.*, 1982) (1 h at 100 V), stained with ethidium bromide, and photographed under UV light.

Results and Discussion

Isolation and initial characterization

The strains were isolated from infected plant material on King's medium B. Distinct colonies that possessed the morphology characteristic of *E. amylovora* (white, circular and mucoid) and which were oxidase-negative, catalase-positive and consisted of Gram-negative motile rods were picked after cultivation at 26 °C for 24 h. After the purification procedures, the isolates were screened for fermentation of glucose with gas production and nitrate reduction. Nine strains were selected on the base of this screening (4 from chokeberry and 5 from strawberry). All fermented glucose without gas formation and did not reduce nitrate. The strains did not grow at 36–39 °C and in the presence of 5% NaCl.

The strains plated on sucrose nutrient agar formed only one morphological type of colonies – typically white, domed, shiny, mucoid (levan type) with radial striations and a dense flocculent centre (Billing *et al.*, 1961). On MM2Cu medium they

formed the typical yellow, circular and smooth colonies (Bereswill *et al.*, 1997). The strains did not show a mucoid growth on YDC medium. All the isolated strains possessed the properties characteristic for *E. amylovora* (Holt *et al.*, 1994; Jones and Geider, 2001).

Pathogenicity tests

All isolates provoked hypersensitive reaction on tobacco leaves and produced the typical symptoms (brown to black color) of pear twigs.

Biochemical characteristics

The identification system API 20E was applied to all the isolates and to the authentic type species

Table I. Biochemical characterization of Bulgarian isolates by API 20E in comparison with the type species of *E. amylovora* ATCC 15580.

Reaction*	Chokeberry		Strawberry	<i>E. amylovora</i>
	2 strains	2 strains	5 strains	ATCC 15580
ONPG	–	–	–	–
ADH	–	–	–	–
LDH	–	–	–	–
ODC	–	–	–	–
CIT	–	–	–	–
H ₂ S	–	–	–	–
URE	–	–	–	–
TDA	–	–	–	–
IND	–	–	–	–
VP	+	+	+	+
GEL	+	+	+	+
Acid from:				
GLU	+	+	+	+
MAN	+	+	+	+
INO	–	+	+	–
SOR	+	+	+	+
RHA	–	–	+	–
SUC	+	+	+	+
MEL	+	+	+	+
AMY	–	–	–	–
ARA	+	+	+	+
OX	–	–	–	–
API 20E profile	0007562	007762	0007772	0007562

* ONPG, β -galactosidase; ADH, arginine dihydrolase; LDH, lysine decarboxylase; ODC, ornithine decarboxylase; CIT, citrate utilization; H₂S, formation of H₂S; URE, urease; TDA, tryptophan deaminase; IND, indole production; VP, acetoin formation; GEL, gelatin hydrolysis; GLU, glucose; MAN, mannitol; INO, inositol; SOR, sorbitol; RHA, rhamnose; SUC, sucrose; MEL, melibiose; AMY, amygdalin; ARA, arabinose; OX, oxidase.

of *E. amylovora*. The results obtained are presented in Table I.

After 48 h of incubation all Bulgarian isolates and the type culture of *E. amylovora* formed acetoin, hydrolyzed gelatin and produced acid from glucose, mannitol, sorbitol, sucrose, melibiose and arabinose. All of them were negative for β -galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H₂S formation, urease, tryptophan desaminase, and indole formation. Some variability was detected among the strains from chokeberry in acid production from inositol. They were separated on the base of this property into two groups – positive and negative (Table I). It should be noted that the strains from these two groups were isolated from different regions. All the strawberry strains produced acid from inositol. They also formed acid from rhamnose and differed in this property from the chokeberry strains and the type culture of *E. amylovora*. The type strain and two of the isolates from chokeberry showed an identical API 20E profile. The strawberry strains were homogeneous and displayed a different profile – 0007772. The comparison of our results with the description of *E. amylovora* strains given by Mergaert *et al.* (1984), Vantomme *et al.* (1986) and Donat *et al.* (2005) showed that our strains differed in their API 20E profiles. They showed three different profiles not found in previous studies, but all strains possessed the properties described for *E. amylovora* (Holt *et al.*, 1994; Jones and Geider, 2001).

The identification BIOLOG system is based on the utilization of 95 carbon sources in a microtiter plate. Cells were grown at 30 °C for 24 h on BUG agar (available from BIOLOG). The plates were read after 4 h and 24 h. The utilizable sources included in the BIOLOG system from the strains analyzed are presented in Table II.

The results from this analysis showed that the metabolic fingerprints of the strains from strawberry were closer to that of the type strain of *E. amylovora* and were divided into two groups. The isolates from chokeberry assimilated more compounds than the strawberry strains as well the type culture of *E. amylovora* (Table II). It is interesting to note that the strains isolated from one and the same region formed a separate group. The metabolic fingerprint patterns of the strains analyzed were compared using the MicroLog™ version 14.01B database software. All the strains were

Table II. Main biochemical characteristics of Bulgarian strains obtained by BIOLOG in comparison with the type species of *E. amylovora* ATCC 15580.

Test	Chokeberry		Strawberry		<i>E. amylovora</i>
	2 strains	2 strains	3 strains	2 strains	ATCC 15580
Dextrin	±	±	—	±	—
N-Acetyl-D-glucosamine	—	+	+	+	+
D-Fructose	+	+	+	+	+
D-Galactose	+	+	+	+	+
Gentiobiose	+	+	+	+	+
α-D-Glucose	+	+	+	+	+
m-Inositol	—	—	±	±	±
D-Mannitol	+	+	+	+	+
β-Methyl-D-glucoside	+	+	+	±	±
D-Psicose	±	+	+	+	+
D-Raffinose	±	—	—	—	—
D-Sorbitol	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
Pyruvic acid methyl ester	+	+	—	+	—
Succinic acid mono-methyl-ester	±	±	—	+	±
D-Gluconic acid	+	+	+	+	+
α-Hydroxybutyric acid	—	±	—	—	—
α-Ketoglutaric acid	±	±	—	—	±
D,L-Lactic acid	—	—	—	±	—
Succinic acid	+	+	+	+	+
Bromosuccinic acid	+	+	±	+	±
Succinamic acid	±	±	—	—	—
L-Alaninamide	±	±	—	—	—
L-Alanine	±	±	—	±	—
L-Alanyl-glycine	—	±	—	—	—
L-Asparagine	—	—	—	±	—
L-Aspartic acid	+	+	+	±	+
L-Glutamic acid	+	+	+	+	+
Glycyl-L-glutamic acid	—	±	—	±	—
L-Proline	—	±	—	±	—
L-Serine	±	±	—	—	—
Inosine	+	+	+	+	+
Uridine	±	±	±	±	—
Thymidine	—	±	—	—	—
Glycerol	±	±	+	+	+
α-D-Glucose-1-phosphate	+	+	+	+	+
D-Glucose-6-phosphate	+	+	+	+	+

identified as *E. amylovora* (with similarity between 0.782 and 0.932).

PCR amplification of genome *ams*-region

E. amylovora produces a complex exopolysaccharide with high molecular weight named amylovoran. It is a factor of pathogenicity. The genes coding for this polysaccharide are located in the chromosome and are arranged in clusters of one or more transcriptional units (Whitfield and Valvano, 1993). Bernhard *et al.* (1993) and Bugert and Geider (1995) described a cluster of 12 genes coding for amylovoran of *E. amylovora* (*amsG*, *amsH*,

amsI, *amsA* to *amsF*, *amsJ*, *amsK* and *amsL*). The sequence of the *amsA*-gene serves for the construction of species-specific primers, which can confirm the species belonging of isolates preliminarily identified by classical methods as *E. amylovora* (Jones and Geider, 2001). Using one pair of such primers (AJ245 and AJ246) all our isolates were subjected to PCR amplification on the base of which could be proved the presence of the *ams*-region in the chromosome. *E. amylovora* ATCC 15580 and *E. pyrifoliae* DSM 12163 were used as controls. The results are presented in Fig. 1. All isolates gave a positive reaction for the presence of the *ams*-region like the type culture of *E. amy-*

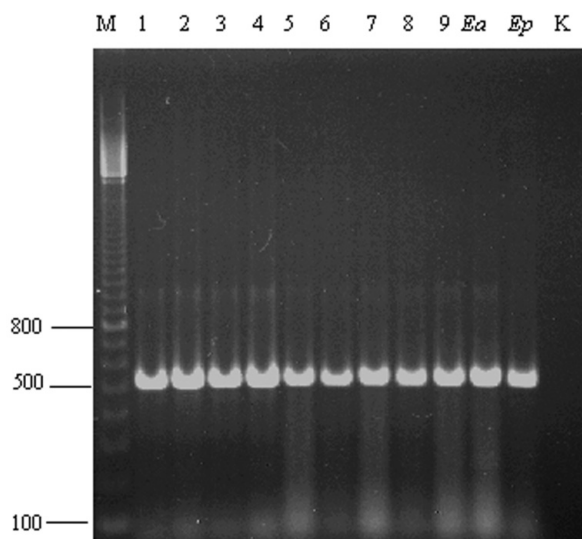


Fig. 1. PCR amplification with two primers (AJ245 and AJ246) of Bulgarian *E. amylovora* strains isolated from chokeberry and strawberry. Lanes 1–4, number of the strains from chokeberry; lanes 5–9, number of the strains from strawberry; *Ea*, type strain of *E. amylovora* ATCC 15580; *Ep*, type strain of *E. pyrifoliae* DSM 12163; K, control (PCR mixture without DNA); M, 100 bp DNA marker (Amersham Biosciences, Vienna, Austria).

lovora. The amplified product was of 519 bp and corresponded to preliminary sequential analysis on GenBank data. It was shown that *E. pyrifoliae* gave also a positive reaction. The molecular characterization of *E. pyrifoliae* demonstrated substantial homology with the *ams*-genes of *E. amylovora*. The pathogenicity of these bacteria is known to require the presence of a capsular exopolysaccharide, with a structure similar to amylovoran (Jock *et al.*, 2003). The results obtained showed that the use only of this pair of primers could give a cross-reaction similar to *E. amylovora* species.

PCR amplification of specific region of plasmid pEA29

Many phytopathogenic bacteria carry plasmids. In the case of *E. amylovora*, it was established that all the strains studied possessed a low copy number plasmid pEA29. This plasmid seems to play a quantitative role in pathogenicity (Falkenstein *et al.*, 1989; Laurent *et al.*, 1989). The presence of this plasmid in all the strains allowed primers specific to a DNA fragment of pEA29 for detection

of *E. amylovora* by PCR to be proposed (Bereswill *et al.*, 1992), which were used in this study. The PCR amplification was carried out with all the strains studied. The type cultures of *E. amylovora* ATCC 15580 and *E. pyrifoliae* DSM 12163 were used as controls. The results are presented in Fig. 2. All strains, except *E. pyrifoliae* gave a single DNA fragment after PCR amplification. In electrophoretical conditions the size of the amplified segment was about of 1,100 kb. Bereswill *et al.* (1993) and McManus and Jones (1995) have already obtained an amplified fragment of the same length.

It may be concluded on the base of morphological, biochemical and PCR analyses that the nine strains isolated from *A. melanocarpa* and *F. ananassa* belong to the species *E. amylovora*. This paper presents the first characterization of *E. amylovora* strains isolated from these new host plants in Bulgaria.

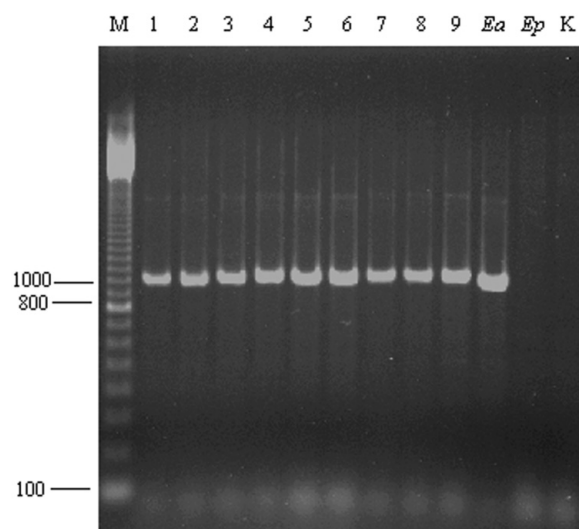


Fig. 2. PCR amplification with two primers (pEA29 A and pEA29 B) of Bulgarian *E. amylovora* strains isolated from chokeberry and strawberry. Lanes 1–4, number of the strains from chokeberry; lanes 5–9, number of the strains from strawberry; *Ea*, type strain of *E. amylovora* ATCC 15580; *Ep*, type strain of *E. pyrifoliae* DSM 12163; K, control (PCR mixture without DNA); M, 100 bp DNA marker (Amersham Biosciences, Vienna, Austria).

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