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REVIEW

Phenotypic and genetic diversity of *Erwinia amylovora*: the causal agent of fire blight

Joanna Puławska · Piotr Sobiczewski

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Abstract *Erwinia amylovora* is a polyphagous bacterium causing fire blight on apple, pear and over 130 other plant species belonging mainly to the *Rosaceae* family. Although *E. amylovora* is regarded as a very homogenous species, the particular strains can differ in pathogenic ability as far as their host range is concerned (e.g. those originating from *Rubus* or *Maloidae* plants) as well as by the extent of the disease they cause. It was found that strains originating from North America are generally more genetically heterogeneous than those from Europe. Diversity of *E. amylovora* is also related to streptomycin resistance as a result of its application to control of fire blight. The level of genetic heterogeneity of *E. amylovora* is so low (comparative genome analysis revealed a similarity of over 99% for the two genomes tested) that standard DNA-based techniques fail in detection of intra-species variability. Amplified fragment length polymorphism was found to be most useful for differentiation of strains of fire blight causal agent as well as techniques ensuing release of pan-genome sequences of two *E. amylovora* strains: multi-locus variable number of tandem repeats analysis and clustered regularly interspaced short palindrome repeats.

Keywords Chromosomal DNA · *Erwinia amylovora* · Phenotypic characters · Plasmid DNA · Virulence

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J. Puławska (✉) · P. Sobiczewski
Pomology Division, Research Institute of Horticulture,
ul. Pomologiczna 18, 96-100 Skierniewice, Poland
e-mail: joanna.pulawska@inhort.pl

Introduction

Biodiversity of bacterial plant pathogens is a result of their variability. This variability is caused by both environmental conditions leading to not fully hereditary changes in phenotype, and variation in the genetic material (Agrios 2005). Changes in genotype can influence the pathogenicity of bacteria such as a reduction or increase of bacterial virulence including an acquired ability to infect plant species, which were originally resistant to disease caused by particular bacterium. This constitutes a major obstacle to the culturing of resistant cultivars. Variability of pathogens can also cause the induction of resistance to pesticides, especially antibiotics (reviewed by Jones and Schnabel 2000).

Assessment of the genetic diversity of pathogens is important in epidemiological studies on e.g.: pathogen spread, the resistance breeding of plants to diseases, plant protection and quarantine. The selection of groups of pathogens in order to measure the effectiveness of various factors in protecting plants against diseases is also necessary. Data from studies of genetic diversity can be used to monitor the prevalence of pathogenic strains, detection and identification of possible sources of primary infection, mapping genes of bacteria and plants, and identification of individual strains in the study on population genetics, phylogenetic studies, and biogeography. Techniques used in studies on genetic diversity provide information helpful in distinguishing the strains as well as the phylogenetic relationship between the strains.

The development of molecular techniques has allowed the variation in the genetic material of bacteria pathogenic to plants to be revealed, both in their chromosome and plasmid DNA. There has been intensive work in this area on *Erwinia amylovora* (reviewed by Vanneste 1995; reviewed by Momol and Aldwinckle 2000; Kim et al. 2001;

Gehring et al. 2011). This pathogen has caused great economic loss in many areas of apple and pear tree cultivation as well as areas where other host plants are cultivated.

Phenotypic features

Host range and virulence

Erwinia amylovora is a pathogen of more than 130 plant species belonging to 40 genera, mainly from the family *Rosaceae* (Van der Zwet and Keil 1979). Disease can occur on pear (*Pyrus* spp.), apple (*Malus* spp.) and quince (*Cydonia* spp.) trees as well as on hawthorn (*Crataegus* spp.), sorb (*Sorbus* spp.), cotoneaster (*Cotoneaster* spp.), serviceberry (*Amelanchier* spp.), firethorn (*Pyracantha* spp.), loquat (*Eriobotrya japonica*), medlar (*Mespilus* spp.), *Stranvaesia* spp. and photinia (*Photinia* spp.). It is generally believed that *E. amylovora* is a homogeneous species and does not show pathogenic specialization. This means that each isolate of the pathogen is potentially able to infect any of the known host plants (Momol and Aldwinckle 2000). However, in an artificial inoculation experiment, De Ley et al. (1984) have shown that different isolates may exhibit some variations in host range. Isolates from plants of the genus *Rubus* are particularly noteworthy, because they are incapable of infecting apple and pear trees (Asselin et al. 2008; De Ley et al. 1984; Evans 1996; Ries and Otterbacher 1977; Starr et al. 1951). At the same time, the majority of isolates originating from pome fruit trees were not pathogenic for raspberries and blackberries (De Ley et al. 1984; Ries and Otterbacher 1977). Only Evans (1996) reported the successful inoculation of raspberry canes with *E. amylovora* isolate originating from apple trees. Because of these differences, Starr et al. (1951) suggested the creation of a distinct taxon—*E. amylovora* f.sp. *rubi* for isolates infecting *Rubus* shrubs.

Different strains of *E. amylovora*, however, differ greatly among themselves in relation to the virulence to the same plant genotype (Cabrefiga and Montesinos 2005; Hevesi et al. 2000; Puławska et al. 2006; Sholberg et al. 2001). The pathogenicity of the bacteria is determined mainly by their capacity for the biosynthesis of exopolysaccharide (EPS) and proteins, especially harpin. EPS is a major component of bacterial ooze, often accompanying necroses and cankers on plants and its production is correlated to virulence of the strain (Ayers et al. 1979; Maes et al. 2001). This compound complex may contain amylovoran, levan, and glucan (Geider 2000). Around the bacterial cell, EPS creates a capsule to protect it from adverse environmental effects as well as from recognition of the pathogen by the immune system of the attacked plant. The loss of this coating also affects the activity of harpin—a protein built mainly of glycine. Harpin is an inducer of hypersensitivity reaction. Most importantly, it acts as a signaling molecule inducing programmed cell death of the host plant (Greenberg 1996).

Norelli et al. (1986) examined *E. amylovora* strains and found that they showed different virulence for different cultivars of apple trees. On the other hand, Schwartz et al. (1991) showed that only some strains of this bacterium inhibited the growth of pear cells in agar medium. This inhibition was related to their ability to produce dihydrophenylalanine (DPH) which is toxic to plants. Individual isolates can also differ in pathogenicity on pear fruitlets and their ability to induce a hypersensitivity reaction on tobacco. However, for most of the isolates, obtained results are generally positive in both tests. In some cases, a negative results of one or both of tests were achieved (De Ley et al. 1984; Vantomme et al. 1982).

Biochemical tests, serology and susceptibility to phages

Isolates of *E. amylovora* create a very homogeneous group in terms of biochemical and physiological properties (Dye 1981; Holt et al. 1994; Mergaret et al. 1984; Paulin 2000). However, some differences may exist between different isolates in the ability to use certain carbohydrates as carbon sources. Numerical analysis of metabolic profiles of different isolates of *E. amylovora* based on the BIOLOG system was done. The analysis can be used to differentiate a group of isolates from plants belonging to the subfamily *Pomoideae,* from the isolates originating from plants belonging to the genus *Rubus*. Among the latter group, two subgroups were distinguished: *Rubus* I and *Rubus* II (Kim et al. 1996). In case of strains from Bulgaria, it was found that 41 out of 95 carbon sources were differentially used by the strains and numerical analysis of BIOLOG results allowed to distinguish five groups within tested strains. However, no relationship to geographic origin or host-plant was found (Atanasova et al. 2007). In contrast, analysis of phenotypic features with application of BIOLOG and API kits was found not useful for intraspecific discrimination of Spanish *E. amylovora* isolates (Donat et al. 2007). Our research included studying of the phenotypic characteristics of *E. amylovora* isolates originating from Poland using API 50CH, API ZYM and API 20NE. We found that out of the 87 examined traits, some of the tested isolates differed only in their capacity to utilize melibiose, cellobiose, sorbitol, D-glucose, and L-arabinose (Puławska et al. 2006). Analysis of serological divergence confirmed also high heterogeneity of *E. amylovora* isolates (Vantomme et al. 1982; Manulis et al. 1998).

Erwinia amylovora isolates can also differ in susceptibility to bacteriophages, which were recognized as playing important role in epidemiology of fire blight (Erskine 1973). Several phages affecting *E. amylovora* strains were

found (Schnabel and Jones 2001; Gill et al. 2003). They differed in genome size and restriction patterns as well as in host range. Not every *E. amylovora* strain tested was susceptible for each phage infection. It can be explained by clustered regularly interspaced short palindrome repeats (CRISPR)/Cas system—a defense system against mobile genetic elements of phages or plasmid origins (Sorek et al. 2008).

Analysis of fatty acids and proteins

The cells of most isolates of *E. amylovora*, regardless of their geographical origin and host plants, are similar in fatty acid content. Only strains isolated from *Rubus* plants have slightly more cyclic acids. However, isolates resistant to streptomycin have fewer of these acids and more saturated fatty acids than isolates susceptible to this antibiotic (Van der Zwet and Wells 1993). According to Żarnowski et al. (2002), the differences observed in fatty acid profiles of individual isolates of *E. amylovora* are sufficient to distinguish them from one another. Garrett et al. (1987) even found a correlation between the obtained profiles, and virulence of tested isolates. Ivanović et al. (2011) found that based on FAME analysis, Serbian isolates were clustered into three groups: α, β, and γ. Cluster analysis revealed that group β and γ consisted only of strains isolated from northern Serbia, whereas all strains isolated from central or southern Serbia belonged to group α. Wider application of the analysis of fatty acids in epidemiologic and taxonomic studies, however, requires very strict compliance with the procedures. Such strictness is necessary because the profiles obtained may be affected, inter alia, by the age of the bacteria and composition of the growth medium (Casano 1986). However, the analyzed protein profiles indicate the high homogeneity of European isolates of *E. amylovora*, originated from plants belonging to the subfamily *Pomoideae*. The small differences observed among isolates did not correlate with their virulence, geographic origin or host plant (De Ley et al. 1984, Vantomme et al. 1982).

Streptomycin resistance

In 1952, just 8 years after its discovery by Schatz et al. (1944), streptomycin was registered in the U.S. as a plant protection compound (Murneek 1952). Its efficacy against fire blight has been confirmed in many studies on different continents. *E. amylovora* strains, resistant to streptomycin were detected for the first time in the U.S. The first report on this subject comes from California in 1971 (Miller and Schroth 1972), soon more reports appeared from Oregon and Washington (Coyier and Covey 1975) and in subsequent years from other parts of the USA where streptomycin was also used for control of fire blight (Chiou and Jones 1991). Recently, strains of *E. amylovora* resistant to this antibiotic have also been discovered outside of North America, for example, in Egypt (El-Goorani et al. 1989), New Zealand (Thomson et al. 1993), Israel (Manulis et al. 1996), and Lebanon (Saad et al. 2000). Among the resistant strains, two major phenotypes—high and medium-resistant were found (Chiou and Jones 1995; Coyier and Covey 1975; McManus and Jones 1994). Strains with low level of resistance rarely occur in nature (Schroth et al. 1979).

The resistance to streptomycin in bacteria may have a dual background: a point mutation in the *rps*L gene encoding the ribosomal protein S12 or by acquisition of genes associated with resistance, which are located on mobile genetic elements, such as plasmids or transposons. It was found that *E. amylovora* isolates with high resistance to streptomycin, possess chromosomal mutation in the gene *rps*L. This mutation prevents attachment of the antibiotic to the ribosome and thus inhibits protein synthesis. However, this kind of streptomycin resistance occurs rarely, e.g. it was found only in 3.4% streptomycin-resistant strains in Michigan (McGhee et al. 2011). More often, this feature is associated with the acquisition of strategic *str*A-*str*B genes located on plasmids or transposons. These genes allow the synthesis of aminoglycoside-modifying enzymes. Such enzymes alter the structure of streptomycin so that it cannot inhibit protein synthesis (Chiou and Jones 1991). *str*A-*str*B genes were found on the plasmid RSF1010, which occurs in different bacteria as well as in clinical strains of Gram-negative bacteria. Further studies showed that these genes were also found in Tn5393 transposon located on conjugal plasmid pEA34 occurring in *E. amylovora* (Chiou and Jones 1993). Until 1994, resistance to streptomycin associated with strategic *str*A-*str*B genes was observed only in Michigan, USA. Later strains of *E. amylovora* with plasmid pEa8.7 containing these genes, were discovered in California (Palmer et al. 1997).

Analysis of nucleic acids

Analysis of plasmid DNA

Application of molecular techniques in studies on the diversity of *E. amylovora* has allowed for demonstrating the differences in size of pEA29 plasmid. It was found that the size of plasmid pEA29 ranges from about 27.6 to about 34.9 kb (McGhee and Jones 2000). Restriction analysis of this plasmid revealed a relatively high homogeneity of the isolates from fruit trees and their otherness from raspberry isolates and the *E. pyrifoliae* bacteria that cause symptoms similar to fire blight in Asian pear varieties (Kim et al.

1999). These bacteria differ from the typical strains in pathogenicity and host range of plants. Originally, there was a consensus among researchers that pEA29 plasmid is present in all isolates of *E. amylovora* (Bereswill et al. 1992; Falkenstein et al. 1988). No genes, responsible for the transfer and mobilization within the sequence of this plasmid in conjunction with its high stability, caused the belief that pEA29 should occur in all wild strains of *E. amylovora.* Therefore, the first attempts to use DNA analysis to identify and detect the pathogen was based on this plasmid (Bereswill et al. 1992; Llop et al. 2000; McManus and Jones 1995a). But, over time it appeared that *E. amylovora* can lose pEA29 without losing pathogenicity (Bereswill et al. 1995). Brown et al. (1996) did not receive any product from five isolates originating from raspberry, pear and apple trees after amplification with primers complementary to the plasmid. In their view, the negative result of PCRs was evidenced by the absence of pEA29. In addition, one of the acquired Gram-positive, unidentified isolates, also reacted with these primers. Such a reaction may indicate an earlier acquisition of pEA29 from *E. amylovora*. By contrast, a few isolates of *E. amylovora* without this plasmid were found in Egypt, Iran, Spain and the USA (Carey et al. 2011; Llop et al. 2006; Mohammadi et al. 2009). Also, it was found that another plasmid of approximately 65 kb (pEI70) present in one of these isolates, showed no homology with pEA29. Based on the analysis of the plasmid composition of European strains of *E. amylovora,* it was found that the plasmid pEI70 is quite common in *E. amylovora* on our continent. In some areas, for example in Poland, it was found in about 10% of the over 100 strains tested. In other areas such as in Belgium, this plasmid was found in almost all tested isolates. It is assumed that the plasmid may carry genes important for the virulence of *E. amylovora* (Llop et al. 2008).

Of the primers complementary to the pEA29 plasmid DNA, a few (Bereswill et al. 1992; Llop et al. 2000; McManus and Jones 1995a) allow the amplification of 0.9 kb plasmid fragment flanked by *Pst*I digestion sites. Several authors, however, obtained products of different length in amplification with some of these primer sets (Brown et al. 1996; Kim et al. 1996; Lecomte et al. 1997). Restriction analysis of these products showed that in this plasmid fragment, an insertion which has the size of 30–90 bp occurs (Lecomte et al. 1997). Sequencing of this amplified fragment revealed a 8-bp short sequence repeats (SSR), which may be found in different isolates in 3–15 copies (Kim and Geider 1999; Ruppittsch et al. 2004; Schnabel and Jones 1998). The described phenomenon was assessed as unstable, for example, in stress conditions (Jock et al. 2003). Therefore, the analysis of SSR is not recommended in epidemiological studies and diagnostics (Jock et al. 2003; Kim and Geider 1999; Schnabel and Jones 1998). Different observations were noted by Ruppittsch et al. (2004). They analyzed 104 strains of *E. amylovora* isolated in Austria. They checked for stability in the number of repetitions of the previously described 8-bp sequence after repeated passage, and under stress. The vast majority of studies showed the number of repetitions of the SSR as being unchanged. Depending on the area of Austria, isolated strains possessed different numbers of SSR. Based on these results, Ruppittsch et al. (2004) came to the conclusion that fire blight does not spread from one source. Instead, the sources of infection were introduced several times over the years, after the first appearance of the disease in 1993 in this country.

Intensive research on the genome of *E. amylovora* revealed the presence of other plasmids in the cells of some isolates of this species. Foster et al. (2004) determined the nucleotide sequence and distribution of pEU30 plasmid (size 30.314 kb) and the pEL60 plasmid (size 60.145 kb). The presence of pEU30 plasmid was found in isolates from western USA, while pEL60 in isolates from Lebanon. Sequences of plasmids and genes located on them suggest that the smaller one is similar to other plasmids isolated from various bacteria associated with plants. The bigger one showed high similarity to plasmids occurring in the human intestinal bacteria. Analysis of genome sequence of the strain Ea273 (well-known and widely used in research) revealed the existence of another new plasmid called AMYP2 (size 71,487 bp) (Sebaihia et al. 2010). Recently, a new plasmid of about 60 kbp has also been found in the *E. amylovora* strain isolated from *Sorbus* in Poland. Its partial sequence analysis shows no similarity to any sequence deposited in GenBank (J. Puławska, unpublished).

Analysis of chromosomal DNA

Analysis of repetitive sequences-Rep-PCR

Amplification of DNA regions located between repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC), and BOX was one of the first techniques applied for estimation of *E. amylovora* genetic diversity (McManus and Jones 1995b). This technique was used both in diagnostics and in epidemiological studies (Louws et al. 1999; Versalovic et al. 1991). The application of this technique allowed McManus and Jones (1995b) to easily distinguish isolates originating from *Rubus* and *Pomoideae* plants. The latter proved to be very homogenous. DNA amplification of more than 170 isolates, mostly from the North American continent, allowed only 2–3 profiles to be obtained, depending on the primers used (the largest variation was obtained using ERIC primers). It should be noted, that irrespective of the primers used, one of the profiles obtained was always

dominant. For the majority of isolates from *Rubus* plants, different profiles were obtained than for those isolates from fruit trees. Rico et al. (2008) also found little interest for the rep-PCR technique when determining the phylogenetic relationships between strains of *E. amylovora*. There was little diversity of the obtained amplification products, but very few reported polymorphic products allowed for the development of primers for amplification of marker containing DNA fragments. These primers can serve as a tool in epidemiological studies - for example, to track the spread of pathogen from a specific disease outbreak.

Ribotyping

McManus and Jones (1995b) amplified the DNA region located in the rrn operon, between genes encoding 16S rRNA and 23S rRNA (Gürtler and Stanisich 1996). Amplification of this region of the *E. amylovora* isolates allowed for easy distinction of *Rubus* isolates forming a homogeneous group. Also, isolates from fruit trees proved to be rather homogenous—amplification of DNA from about 170 isolates showed only three different profiles (McManus and Jones 1995b). Jeng et al. (1999) reported similar results of ribotyping where strains isolated from *Rubus* shrubs, and isolates derived from plants of the subfamily *Pomoideae* formed two homogeneous groups. Momol et al. (1999) used a modification of ribotyping. This modification consisted of additional digestion products obtained by using restriction enzymes (amplified ribosomal DNA restriction enzyme analysis, ARDREA). This procedure only allowed raspberry and blackberry isolates to be distinguished from isolates originating from other host plants. Garbeva et al. (1997), using the same technique, did manage to show variation among the isolates from fruit trees. They actually obtained distinct profiles for only 2 isolates, out of 14 tested.

Sequence analysis of housekeeping and pathogenicity-related genes

Kim et al. (1996) analyzed genomic DNA of selected isolates of *E. amylovora* using the enzyme *Eco*RI, and then conducted a hybridization with a probe including a group of *hrp* genes. For all isolates from fruit trees, one profile was obtained. For isolates from the shrubs belonging to the genus *Rubus* two different profiles have been obtained. Sequence analysis of the gene *hrp*N encoding harpin and amino acid sequence of the protein deduced from the nucleotide sequences, confirmed the greater diversity of American strains compared to European strains. The sequences of the gene were almost identical in European strains (Jock and Geider 2004). A study by Giorgi and Scortichini (2005) on *E. amylovora* isolates originating from different continents confirmed that there was a greater variation in the gene *hrp*N of bacteria isolated in North America where fire blight was discovered and described for the first time in the world. RFLP analysis of genes *hrp*N and *dsp*A/E revealed significant differences between the strains isolated from plants of *Rubus* and *Amelanchier*, and those from the plants belonging to the family *Maloidae* (Giorgi and Scortichini 2005). Restriction fragment analysis of *ams*B gene (involved in the production of specific polysaccharide of *E. amylovora*-amylovoran) showed no differences between strains isolated in Poland (Puławska et al. 2006).

Analysis of housekeeping genes also confirmed the high homogeneity of *E. amylovora*. Waleron et al. (2002) amplified the *rec*A gene encoding recombinase A of nine isolates of *E. amylovora*, originated from fruit trees in various parts of the world. The amplification products were then digested with four restriction enzymes. For all isolates, however, the same profiles were obtained. Just a few nucleotide differences were found in *groEL* sequences of *E. amylovora* strains isolated in apple and pear orchards in Michigan and strains from the western United States or from *Rubus* spp (McGhee et al. 2011).

Random amplified polymorphic DNA

For the study on genetic variation of *E. amylovora*, Momol et al. (1997) used the Random amplified polymorphic DNA (RAPD) technique (Williams et al. 1990). Profiles were obtained by amplification of DNA from 16 *E. amylovora* isolates from different geographical regions, with 6 random primers. This procedure allowed each of the isolates to be distinguished. At the same time isolates from *Rubus* plants and trees of the subfamily *Pomoideae* formed two groups. The similarity between these isolates, as determined by UPGMA and expressed by the Nei-Li coefficient, was approximately 0.7 (Momol et al. 1997). Brennan et al. (2002) applied the same primers to study isolates originating mainly from Ireland. Although most of the profiles obtained a similarity above 0.9 (Nei-Li coefficient, UPGMA method), some of the profiles differed significantly from the others. The RAPD technique also revealed the high homogeneity of *E. amylovora* isolates from other geographical regions (Keck et al. 2002; Manulis et al. 1998; Taylor and Hale 1998).

The RAPD was also used to examine the diversity of *E. amylovora* isolates from Poland. The study included 64 isolates, of which 50 were isolated during 1983–2002 from different host plants (apple, pear, quince, firethorn, hawthorn, and cotoneaster), from different regions of the country. The remaining 12 isolates originated from other European countries, including two from the Middle East.

The studies confirmed the high homogeneity of the tested isolates. No correlation between the genetic diversity of isolates and their geographical origin or the plant from which they were isolated, was found (Puławska et al. 2006).

Amplified fragment length polymorphism

In an epidemiological study performed in Italy the amplified fragment length polymorphism (AFLP) technique was used. The use of AFLP led to the conclusion that the occurrence of fire blight in the provinces of Modena and Ferrara, was caused by a single introduction of *E. amylovora* (Minardi et al. 2000). Similar analysis of Austrian and Hungarian isolates of this bacterium did not show any differences between the isolates (Keck et al. 2002).

In Spain, an attempt was made to differentiate isolates of *E. amylovora* from different countries, using the rep-PCR (ERIC, BOX, IS50, and M13 primer) and the AFLP technique. Of the 23 tested isolates, 18 were not resolvable with the use of rep-PCR. The use of AFLP with 6 primers allowed for differentiation of the remaining 18. For all, except two strains, a unique combination of AFLP profiles was obtained (Rico et al. 2004). Extended study of both phenotypic and genotypic characteristics of 63 strains isolated in different regions of Spain, confirmed that from among all the techniques, the greatest distinction was achieved by using AFLP. This technique allowed the differentiation of bacteria according to their geographical origin. The results indicate that new outbreaks of fire blight in Spain are the result of multiple introductions of infected plant material or other sources of inoculum from different European countries (Donat et al. 2007).

Pulse field gel electrophoresis

Promising results were obtained using the pulse field gel electrophoresis (PFGE) technique (Jock et al. 2002; Zhang and Geider 1997; Zhang et al. 1998). Application of this technique after digestion of genomic DNA with *Xba*I endonuclease allowed six pattern types to be distinguished from among European isolates of *E. amylovora*. Use of the PFGE technique also showed a hypothetical spread of the disease: from the British Isles to Central Europe, separately to France and then to the Iberian Peninsula and to northern Italy; from western France to north-eastern Spain; from Egypt via the Middle East to Asia Minor, and then to the Balkans (Jock et al. 2002; Zhang and Geider 1997; Zhang et al. 1998). More detailed analysis of *E. amylovora* strains isolated in Serbia and Montenegro revealed three new patterns and indicated spread of *E. amylovora* across the Balkan Peninsula from the south to the north (Ivanović et al. 2010).

Unlike the European strains, PFGE profiles of strains from North America were much more diverse. European isolates can be classified into six different groups based on restriction profiles. Among North American isolates, only part were of the same profile as the isolates of *E. amylovora* from northern Spain, western France and England. Another group was identical to isolates from the Central Europe. The remaining isolates were characterized by a high degree of profile diversification, unprecedented in Europe. This observation led to the hypothesis that fire blight had spread throughout the world, by escaping from North America only a few times (Jock et al. 2002).

Genome analysis and derivative techniques

Comparative genome analysis of two *E. amylovora* strains: European—CFBP 1430 and American—Ea273, confirmed high genetic similarity within this species. The similarity of both genomes was estimated as 99.99% with only a low number of nucleotide differences (Smits et al. 2010). For differentiation of such a monomorphic pathogens, analysis of single nucleotide polymorphisms (SNPs) has been found suitable as well as multi-locus variable number of tandem repeats analysis (MLVA) and CRISPR analysis.

Single nucleotide differences in the genes: *gal*E, *acr*B, and *hrp*A were found between individual *E. amylovora* strains from North America and from Europe, the Mediterranean region, Australia, and New Zealand. This single nucleotide polymorphic site allowed for the designing of primers differentiating these two groups of strains. Regarding the raspberry strains, which have only been isolated in North America, the nucleotide sequences in *gal*E, *acr*B, and *hrp*A were consistent with the sequences of Ea273 strain isolated from *Malus* in the primer regions, in the USA, while other base changes were present in other parts of the genes (Gehring et al. 2011).

Tandem repeat structures have been recognized as a highly polymorphic loci in all organisms. In bacteria, they usually have a length of one to several hundred nucleotides and can be present in coding and intergenic regions. Silico analysis of *E. amylovora* chromosome for the presence of variable number of tandem repeats (VNTR) confirmed their presence at a density slightly lower than in other analyzed plant pathogenic bacteria. MLVA applied to 600 *E. amylovora* strains of different geographical origins revealed relatively high diversity. Moreover analysis of isolates from two fire blight outbreaks in Slovenia (in 2003 and 2007), showed just a few high-frequency genotypes mixed with a variety of low-frequency genotypes (Dreo et al. 2010).

Clustered regularly interspaced short palindrome repeats consist of identical repeated DNA sequences, interspaced by highly variable sequences called spacers. This newly recognized genetic structure plays the role of a defense

system against mobile genetic elements of phages or plasmid origins (Sorek et al. 2008) and it is found to be very diverse among *E. amylovora* strains. McGhee and Sundin (2011) found that the CRISPR repeat sequence among *E. amylovora* strains consists of 29 bp and is universal despite host range, geographic distribution, or array number. To date, a total of 357 unique spacers have been identified in CRISPR arrays present in *E. amylovora*. Spacer patterns from Michigan strains were mainly distinct from strains isolated in western USA. But strains from France and Lebanon included in the study, shared the same patterns as some strains from Michigan. Spacer sequence identities to foreign genetic elements included plasmids from *E. tasmaniensis* and *Salmonella enterica*. Rezzonico et al. (2011) found 18 different CRISPR genotypes within a collection of 37 cosmopolitan strains. Within them, a total of 454 distinctive CRISPR spacers were identified. The strains isolated from *Spiraeoideae*, clustered in three major CRISPR groups. Both group II and group III were composed exclusively from bacteria originating from the United States. Group I generally contained strains from Europe, New Zealand, and the Middle East. The authors concluded that these results suggest that the genotype of the causative agent of fire blight in *Pomaceae* was selectively enriched from the broader genetic pool, which is present on wild host plants in North America.

Conclusion

Erwinia amylovora strains belong to one of the most homogeneous species of plant pathogenic bacteria in terms of biochemical and genetic characters, but differ in terms of virulence. Clear differences were observed only between isolates derived from raspberries and blackberries, and isolates originating from apple and pear trees and other host plants. In areas where the measures on the basis of streptomycin against fire blight are applied, some strains show resistance to this antibiotic. Depending on the type of mechanism responsible for the resistance, the bacteria strains were divided into high and medium resistance. Studies on the genetics of *E. amylovora* showed greater diversity among the strains from North America, where fire blight was first described, than among European strains. Recent studies show that *E. amylovora*'s content of plasmid DNA may vary. Furthermore, some plasmids, e.g. that discovered in Spain-pEI70, may involve genes important for pathogenicity of bacteria other than those already known to be responsible for the synthesis of: exopolysaccharides, siderophores, and proteins such as harpins. Despite considerable progress in the research of the functions of the genes, their overall importance has not yet been identified. Out of all molecular techniques used, AFLP and those developed on the basis of released pan-genome

Table 1 Differentiation of *E. amylovora* isolates on the basis of their genomic DNA

Method	Result	Reference
Rep-PCR	Differentiation between isolates from *Rubus* spp. and isolates from *Pomoidae*	McManus and Jones (1995b)
	Polymorphic products were used as DNA markers	Rico et al. (2008)
Ribotyping	Differentiation between isolates from *Rubus* spp. and from *Pomoidae*	Gürtler and Stanisich (1996), Jeng et al. (1999), McManus and Jones (1995b), Momol et al. (1999)
	Slight differentiation of isolates from fruit trees	Garbeva et al. (1997)
RFLP of genomic DNA + PFGE	Distinction of different types among European isolates and presentation of hypothetical ways of spreading of the pathogen	Jock et al. (2002), Ivanović et al. (2010), Zhang and Geider (1997), Zhang et al. (1998)
	Diversity among isolates from North America	Jock et al. (2002)
Housekeeping genes	High homogeneity between tested strains	McGhee et al. (2011), Waleron et al. (2002)
Pathogenicity-related genes	Greater diversity of American strains compared to European strains. Differences between the strains isolated from plants of *Rubus* and *Amelanchier*, and bacteria from the plants belonging to the family *Maloidae*	Kim et al. (1996), Giorgi and Scortichini (2005), Jock and Geider (2004), Puławska et al. (2006)
RAPD	Distinction between isolates from *Rubus* spp. and from *Pomoidae*	Momol et al. (1997)
	High homogeneity among isolates tested	Brennan et al. (2002), Keck et al. (2002), Manulis et al. (1998), Puławska et al. (2006), Taylor and Hale (1998)
AFLP	Lack of differences between isolates from Italy, Austria and Hungary	Keck et al. (2002), Minardi et al. (2000)
	Differentiation of isolates according to geographic origin in Spain	Donat et al. (2007), Rico et al. (2004)
MLVA	High diversity revealed among isolates tested	Dreo et al. (2010)
CRISPR	High diversity revealed among isolates tested	Rezzonico et al. (2011)

E. amylovora sequences: multi- MLVA and CRISPR—seem to be the most promising in differentiation of strains of fire blight pathogen (Table 1).

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