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CHARACTERIZATION OF A NEW *ERWINIA* SPECIES AFFECTING ASIAN PEAR TREES

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Abstract

Bacteria causing symptoms which resembled fire blight were consistently isolated from necrotic branches of Asian pear fruit trees (*Pyrus pyrifolia* Nakai) in Korea. Isolated bacteria were studied in a series of microbiological, pathological and molecular assays. Serological and BIOLOG assays show a relationship of the novel pathogen to *E. amylovora* but differences to *Enterobacter pyrinus* also isolated from Asian pear trees in Korea. In general, colony morphology on MM2Cu agar was mucoid as for *E. amylovora* but only slightly yellow. Its EPS capsules were stained by lectin from *Abrus precatorius*, specific for amylovoran and degraded by the *E. amylovora*-specific phage ϕ-Ea1h, which cleaves amylovoran in a defined position of the sugar backbone. Sugar linkage analysis revealed identity with the repeating units of amylovoran. The new Asian pathogen produced ooze on pear slices and necrotic symptoms on pear seedlings. From a chromosomal region with high homology to *amsB* of *E. amylovora*, a DNA fragment was cloned by PCR and sequenced. The deduced amino acid of the ORF had 95% homology to AmsB. Site-specific mutants created in this gene were unable to form ooze on pear slices and lost virulence for pear seedlings. PCR assays with primers from plasmid pEa29 or from the *ams*-region of *E. amylovora* were negative for the novel pathogen due to different plasmids in both species. Dendrograms from Biotype 100 tests displayed a distance between the novel pathogen and *E. amylovora*. Moreover, the 16S/23S rRNA intergenic transcribed spacer regions (ITS) were divergent. Macrorestriction and subsequent PFGE analysis produced patterns entirely different from *E. amylovora*. Two sets of primers did not cross-react with *E. amylovora* and could be used for detection of the novel pathogen, which has been named *Erwinia pyrifoliae* according to its host *Pyrus pyrifolia*.

1. Introduction

Necrotic symptoms of aerial parts of European and Asian pear trees (*Pyrus communis* L. and *Pyrus pyrifolia* Nakai) are often related to a well known bacterial disease of *Maloideae*: fire blight caused by *E. amylovora* (Van der Zwet and Beer, 1995). Nevertheless, other bacteria can cause leaf spots and necrosis on shoots. This is the case

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for *Pseudomonas syringae* pv. *syringae* (pear blast), which seems to be widespread in almost all areas where pear trees are grown, and for *Enterobacter pyrinus*, which has only been described in Korea (Chung *et al.*, 1993).

A recently observed necrotic disease of Asian pear fruit trees (*Pyrus pyrifolia* cv. 'Shingo' and 'Mansamgil') near Chuncheon in Korea has resulted in isolation and partial characterization of a bacterial pathogen (S.-L. Rhim, B. Völksch, L. Gardan, J.-P. Paulin, C. Langlotz, W.-S. Kim and K. Geider, unpublished). Symptoms on pear trees in the Korean orchards remotely resembled those of fire blight, but microbiological assays and absence of the PCR signals expected for the presence of *E. amylovora* distinguished the novel Asian bacteria from the fire blight pathogen. The new pathogen was also different from the Korea pear pathogen *Ent. pyrinus* (Chung *et al.*, 1993) especially by its lack of nitrate reduction and for growth at high temperature as well as acid production in the presence of esculine or cellobiose (S.-L. Rhim, B. Völksch, L. Gardan, J.-P. Paulin, C. Langlotz, W.-S. Kim and K. Geider, unpublished). From genetical investigation, evidence will be presented that the new pathogenic bacteria are not *E. amylovora*, but closely related. Our findings resulted in the description of a new species: *Erwinia pyrifoliae*.

2. Results and discussion

2.1. Origin of the Asian pear pathogen

From necrotic plant tissue samples of diseased trees of pear orchards in Korea, bacterial populations were isolated on LB agar. The dominant colony type was white and smooth on nutrient agar. White colonies were screened on MM2Cu agar (Bereswill *et al.*, 1998), and displayed a slightly yellow color with a mucoid appearance. These strains, when inoculated on slices of immature pears (*Pyrus communis*), gave rise to typical ooze production. Interestingly, inoculation of strains of *Erwinia* sp. from Asian pear on seedlings from Asian pears (*P. pyrifolia*) and European pears (*P. communis*) induced typical fire blight symptoms with progressive necrosis along the midrib of leaves and blackening of petioles. On apple seedlings such symptoms were only occasionally induced by the Asian pear pathogen.

2.2. Classification of the pathogen

Preliminary classification was performed with API 20E tests and API 50CH strips. All pear strains tested were Gram-negative, rod-shaped, motile and facultatively anaerobic. According to the determinative scheme of Bergey´s Manual of systematic Bacteriology (Lelliott and Dickey, 1984) and Dye (Dye, 1983), these strains belong to the genus *Erwinia*. This was confirmed with the BIOLOG system for the identification of bacteria. The Asian *Erwinia* sp. resembled weakly *Erwinia amylovora* (Table 1), but differed in microbiological assays in the production of gelatinase and assimilation of melibiose, gentiobiose and succinamic acid. From the results of BIOLOG microtiter plates, additional biochemical tests, and the phenotypic characteristics the strains isolated from Asian pears were differentiated from *E. amylovora* and from 17 type strains of *Erwinia* and *Pantoea*. Including these characteristics, a new species name, *Erwinia pyrifoliae*, has been proposed (W.-S. Kim, L. Gardan, S.-L. Rhim, and K. Geider, unpublished).

2.3. Plasmids from *E. pyrifoliae*

Plasmids were isolated from the *E. pyrifoliae* strain Ep1/96 and the *E. amylovora* strain Ea1/79. On a 0.8% agarose gel, we observed the 29 kb plasmid from *E. amylovora* as a sharp band at a position of high molecular weight. In contrast, strain Ep1/96 did not contain a plasmid which migrated at the same position as pEa29. Instead, a larger plasmid than 29 kb was visible and three additional plasmids in the size range of 2 to 4 kb. When plasmid DNA was digested with restriction enzyme *Bam*HI, the single cleavage site was

confirmed for pEa29. The large plasmid from Ep1/96 was degraded into at least five DNA fragments, and the three small plasmids were not cleaved. The number, sizes and restriction patterns of the plasmids from both strains clearly differed and distinguished the two species from each other. In this context, no PCR signal was obtained when plasmid (Bereswill *et al.*, 1992) or chromosomal primers (Bereswill *et al.*, 1995) from *E. amylovora* were used for detection of *E. pyrifoliae*. Plasmid and PCR assays also confirm a difference of *E. pyrifoliae* and *E. amylovora* on the molecular level.

2.4. Properties of *E. pyrifoliae*

All strains from Asian pear trees gave a typical reaction with an antiserum raised against *E. amylovora* in the slide agglutination test. Similarly an antiserum prepared against an *E. pyrifoliae* strain agglutinated also other strains isolated from Asian pear trees and *E. amylovora* strains. However, in both cases, no reaction was obtained with *Ent. pyrinus*.

Its EPS capsules can be stained by lectin from *Abrus precatorius*, specific for amylovoran and degraded by *E. amylovora*-specific phage ϕ-Ea1h, which cleaves amylovoran. Sugar linkage analysis (with M.Nimtz, GBF, Braunschweig, Germany) revealed identity with the repeating units of amylovoran. From a chromosomal region, a high homology of the DNA sequence with *ams*B region of *E. amylovora* was found in the corresponding region of *E. pyrifoliae*. The fragment was amplified with primers derived from *ams*B region of *E. amylovora* and cloned into a sequencing vector. Homology of sequences was also 95% for the deduced amino acid sequence. Site-specific mutagenesis of the gene analogous to *amsB* produced a mutant unable to form ooze on pear slices or to cause necrotic symptoms on pear seedlings. This gene might have also a role for synthesis of EPS by *E. pyrifoliae*.

2.5. Characterization of rRNA genes

The 16S rRNA sequence were amplified with consensus primers and were cleaved with *Hae*III producing identical DNA fragments for *E. pyrifoliae* and *E. amylovora*, but different fragments in comparison to *Ent. pyrinus* and *Pantoea stewartii* subsp. *stewartii*. In the sequence analysis of 16S rRNA, *E. pyrifoliae* was more than 99% homologous to *E. amylovora* and 97% homologous to *P. stewartii* and less to other Erwinias. The DNA sequence of the intergenic transcribed spacer region (ITS) can be quite divergent among bacteria and has widely been used for differentiation of species (Zavaleta *et al.*, 1996). The ITS regions of *E. pyrifoliae*, *E. amylovora*, *Ent. pyrinus* and *P. stewartii* subsp. *stewartii* contained a single copy of the $tRNA^{Glu}$ gene. The $tRNA^{Glu}$ sequence of *E. pyrifoliae* was most divergent to the other species. A dendrogram for ITS-sequences of related bacteria determined by us and from data libraries showed a remarkable evolutionary distance of *E. pyrifoliae* to *E. amylovora* and other bacteria.

2.6. Additional taxonomic studies with the novel pear pathogen

E. pyrifoliae strain Ep1/96 constituted a discrete DNA genomic group (genomospecies) with the type strain E16/96(CFBP 4172), since they were 89% to 100% related to each other. *E. amylovora* strains including the type strain CFBP 1232 were 40% to 52% related to the type strain of *E. pyrifoliae* with a ΔTm above 5°C (5.2 to 6.8°C). Presently, the molecular definition of bacterial species is applied to strains which are about 70% related, with ΔTm values that do not exceed 5°C. Thus, the bacteria associated with necrosis of Asian pear trees (Nashi) are members of a new species. A numerical taxonomy study of *E. pyrifoliae* was related to 25 phytopathogenic *Erwinia*, *Pantoea*, and *Enterobacter* strains including type strains of each species and the four strains pathogenic to *Pyrus pyrifolia* that were characterized by using Biotype 100 strips and 22 additional tests. Since these strains constitute a close DNA group which can be identified by using

phenotypic tests, this new phytopathogenic organism was thus confirmed as a new species.

E. pyrifoliae can be specifically detected by two set of primers from the ITS region and the EPS-encoding region without cross-reaction to *E. amylovora*. Macrorestriction and subsequent PFGE analysis produced patterns entirely different from *E. amylovora*, but revealed at least three related but distinguishable variants for isolated *E. pyrifoliae* strains. The pattern of pathogenicity of this novel

3. Concluding remarks

A novel pathogenic bacterium was isolated from necrotic Nashi pear fruit trees in Korea and produced similar symptoms as *E. amylovora*. The novel pathogen differed from *E. amylovora* in many criteria and has been described as a new species within the genus Erwinia. Its impact on the production of Asian pears has still to be determined. The pattern of pathogenicity of *E. pyrifoliae* is currently under investigation.

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Table 1. Similarity and differences between *E. pyrifoliae* and *E. amylovora*

	Properties
Similarities	- Mucoid, yellow colonies on MM2Cu agar - Ooze production on pear slices - Typical "fire blight" symptoms produced on pear seedlings - EPS capsule stained by FITC-lectin from *A. precatorius* - Degradation EPS by *E. amylovora*- specific phage - Same repeating units of amylovoran in sugar linkage analysis - High homology of a protein analogous AmsB - Closely related 16S rRNA sequence (99%) - Some biochemical characteristics
Differences	- Low levan production on LB agar containing sucrose - Utilization of some carbon sources used for typing - Dendrogram from Biotype 100 assays - No PCR signal with primers from plasmid pEa29 and the *ams* region of *E. amylovora* - Different size of a large plasmid and the number of plasmids - Different pattern in PFGE analysis - Low homology of the DNA sequence in the 16S-23S rRNA intergenic transcribed spacer region(ITS) and $tRNA^{Glu}$ - DNA/DNA hybridization kinetics and thermal stability of genomic DNA