Molecular comparison of pathogenic bacteria from pear trees in Japan and the fire blight pathogen *Erwinia amylovora*

Won-Sik Kim, Maja Hildebrand, Susanne Jock and Klaus Geider

Author for correspondence: Klaus Geider. Tel: +49 6203 106 117. Fax: +49 6203 106 122.
e-mail: kgeider@zellbio.mpg.de

Max-Planck-Institut für
Zellbiologie, Rosenhof,
68526 Ladenburg,
Germany

Several strains of the genus *Erwinia*, which were isolated in Japan from pear trees with necrotic symptoms that resembled fire blight, and tentatively identified as *Erwinia amylovora*, were reinvestigated for their relationship to the fire blight pathogen. These isolates produced ooze on slices of immature pears and were mucoid on MM2Cu agar plates, but did not synthesize levan and did not give the expected PCR signals with several primer pairs specific for *Erwinia amylovora*. The isolates tested positive with PCR primers designed to detect the novel pear pathogen *Erwinia pyrifoliae*, which was isolated from Nashi pear trees in South Korea. The nucleotide sequence analysis of a DNA fragment preceding the gene cluster for exopolysaccharide synthesis revealed a closer relationship to *Erwinia pyrifoliae* than to *Erwinia amylovora*. Plasmid profiles, protein patterns and genomic DNA analysed by PFGE after *Xba*I and *Spe*I digestion were different than *Erwinia amylovora*. Experiments with strains of *Erwinia amylovora* isolated from raspberry (*Rubus* sp.), *Erwinia mallotivora* and *Enterobacter pyrinus* also did not reveal a relationship between these bacteria and the Japanese *Erwinia* strains. The latter are not identical to *Erwinia pyrifoliae*, but possess many similar features to this pathogen that causes Asian pear blight. It is concluded that pathogenic bacteria isolated in Japan from pear trees with symptoms resembling fire blight are possibly different from *Erwinia amylovora*.

Keywords: Asian pear blight, *Erwinia pyrifoliae*, nucleotide sequence alignment, PCR signals, PFGE

INTRODUCTION

Several years ago, shoot blight symptoms were observed on Asian pears (*Pyrus pyrifolia*) in orchards on the Japanese island of Hokkaido. The pathogen was tentatively designated in Japan as *Erwinia amylovora* pv. *pyri* (Goto, 1992). In two subsequent reports, isolates from necrotic tissues of Japanese Nashi pear trees were further investigated for their relationship to *Erwinia amylovora* (Beer *et al.*, 1996; Kim *et al.*, 1996). From microbiological tests, BIOLOG assays and DNA hybridization studies, a close resemblance with *Erwinia*

amylovora was suggested, confirming conclusions of a Japanese report from 1981 (cited in Beer *et al.*, 1996) about the occurrence of fire blight in Japan.

Recently, a bacterial pathogen, *Erwinia pyrifoliae*, was characterized as a novel *Erwinia* species that also causes necrotic symptoms on Asian pears (Kim *et al.*, 1999; Rhim *et al.*, 1999). The Asian pear pathogen was first detected in orchards near Chuncheon, South Korea, and has not been reported to occur in other countries. In several respects, this pathogen resembled *Erwinia amylovora*, the causative agent of fire blight of apple (*Malus domestica* L.) and European pear (*Pyrus communis*). The nucleotide sequence of the 16S rDNA from *Erwinia pyrifoliae* was almost identical with the corresponding sequence of *Erwinia amylovora*, and *Erwinia pyrifoliae* also produced ooze on slices of immature European and Asian pears. Biotype 100 and BIOLOG profiles, DNA–DNA hybridization kinetics,

Abbreviations: EPS, exopolysaccharide; ITS, intergenic transcribed spacer; SSR, short sequence DNA repeat.

The EMBL accession numbers for the sequences reported in this paper are AJ311829 (Ej546), AJ311830 (Ej617), AJ311831 (Ej557), AJ311832 (Ej562), AJ311833 (Ej556), AJ311834 (Ej546a), AJ311835 (Ep1/96), AJ311836 (Ea1/79) and AJ311837 (IL6).

as well as nucleotide sequence analysis of the intergenic transcribed spacer region between the 16S and 23S rDNA distinguished *Erwinia pyrifoliae* from *Erwinia amylovora*. Based on these features it was classified as a new species within the genus *Erwinia* (Kim *et al.*, 1999). Additionally, *Erwinia pyrifoliae* does not contain a plasmid identical to pEA29 of *Erwinia amylovora* (Rhim *et al.*, 1999) and its PFGE pattern after an *Xba*I digest of genomic DNA is distinct from *Erwinia amylovora* (S. Jock & K. Geider, unpublished). Other bacteria connected to necrotic symptoms of Asian pears include *Enterobacter pyrinus* in Korea (Chung *et al.*, 1993) and *Erwinia mallotivora* in Japan (Goto, 1992).

The claim of the existence of fire blight in Japan caused by *Erwinia amylovora* was reinvestigated in this study. Isolates from Japanese orchards described by Beer *et al.* (1996) and Kim *et al.* (1996) were characterized for molecular properties and were found to differ from *Erwinia amylovora*, but to be related to *Erwinia pyrifoliae*, the novel species causing Asian pear blight (Kim *et al.*, 1999; Rhim *et al.*, 1999).

METHODS

Bacterial strains and plasmids. Five *Erwinia* cultures, which originated from Nashi pear trees in Japanese orchards with symptoms resembling fire blight, were obtained from S. Beer,

Cornell University, Ithaca, NY, USA. The strains were previously classified as *Erwinia amylovora* (Beer *et al.*, 1996; Kim *et al.*, 1996). Two colony types were observed on LB agar from culture 'Ea546'; a small and a large colony were separately cultured and labelled Ejp546 and Ejp546a, respectively. All the strains used for comparison are listed in Table 1. Plasmids used in cloning experiments and molecular details of *Erwinia amylovora* strain Ea1/79, *Erwinia pyrifoliae* strain Ep1/96 and *Enterobacter pyrinus* 90.2 were described by Kim *et al.* (1999).

Microbiological characterization of strains. All strains were first assayed on semi-selective medium (Bereswill *et al.*, 1998). MM2Cu is a minimal medium with a high content of asparagine and causes the formation of yellow, mucoid colonies for *Erwinia amylovora*. MM1Cu with a low concentration of asparagine prevents growth of *Erwinia amylovora*. LBsuc is Luria-Bertani broth with 5% sucrose, used to determine levan production. The oxidative/fermentative reaction was determined according to Hugh & Leifson (1953).

PCR assays for identification of *Erwinia* strains from Japan.

Primers for detection and identification of *Erwinia amylovora* as well as *Erwinia pyrifoliae* are listed in Table 2 and their target positions are outlined in Fig. 1. The oligonucleotides were synthesized with a Beckman Oligo 1000M DNA Synthesizer. The PCR reactions were done in 50 µl with dilutions of bacterial cell cultures and 0.5 U *Tth* polymerase (Amersham Pharmacia) or 2 U *Taq* polymerase as described by Bereswill *et al.* (1992, 1995) for all primer pairs, and 30

Table 1. *Erwinia* strains isolated from necrotic tissue of Asian pear trees in Japan and other bacterial strains used for comparison

Cultures of the Japanese strains (a) were provided by Dr S. Beer, Cornell University, Ithaca, NY, USA. *, **, derived from a small and a large colony of culture Ea546, respectively. Ejp546a may not be an independent isolate. CFBP, Collection Française des Bactéries Phytopathogènes (French Collection of Phytopathogenic Bacteria); KCTC, Korean Collection of Type Cultures.

(a) <i>Erwinia</i> strains from Japan						
New name	Previous name	Other name	Year of isolation	Host	Location	Source
Ejp546*	Ea546	EA7971	1979	Asian pear	Hokkaido	A. Tanii
Ejp546a**	Ea546	EA7971	1979	Asian pear	Hokkaido	A. Tanii
Ejp556	Ea556	TP9405	1994	Asian pear	Hokkaido	A. Tanii
Ejp557	Ea557	EA9471	1994	Asian pear	Hokkaido	A. Tanii
Ejp562	Ea562	EA9476	1994	Asian pear	Hokkaido	A. Tanii
Ejp617	Ea617	144	1996	Asian pear	Japan	R. Roberts
(b) Other strains						
Strain	Origin			Reference		
Ea1/79	<i>Erwinia amylovora</i> , from <i>Cotoneaster</i> sp., Germany, 1979, applied as a strain from fruit trees/ornamentals			Falkenstein <i>et al.</i> (1988)		
IL6	<i>Erwinia amylovora</i> , from raspberry (<i>Rubus</i> sp.), USA			Kim & Geider (1999)		
Ep1/96	<i>Erwinia pyrifoliae</i> , from Nashi pear, Korea, 1996			Kim <i>et al.</i> (1999)		
90.2 ^T	<i>Enterobacter pyrinus</i> , from Nashi pear, Korea, KCTC 2520 ^T			KCTC		
CFBP2503 ^T	<i>Erwinia mallotivora</i> , ATCC 29573 ^T			CFBP		
DC283	<i>Pantoea stewartii</i> (formerly <i>Erwinia stewartii</i>), USA			Bereswill <i>et al.</i> (1998)		
DH5α	Mutated <i>Escherichia coli</i> K-12			BRL		

Table 2. Primers used for differentiation of *Erwinia* spp.

Positions of the primers can be deduced from Fig. 1. The expected sizes of the products are (kb): fd2/rP1, 1.4; EP16A/EP1G2c, 0.70; P29A/P29B, 1.0; RS1/RS2c, 0.15; AMS1/AMS2c, 1.2; AMS3/AMS4c, 0.83; AMSbL/AMsbR, 1.6; CPS1/CPS2c, 1.2.

Primer	Sequence (5'–3')/features	Reference/source
From rRNA region:		
fd2*	AGAGTTTGATCATGGCTCAG/16S rDNA consensus primer	Weisburg <i>et al.</i> (1991)
rP1*	ACGGTTACCTTGTTACGACTT/16S rDNA consensus primer	Weisburg <i>et al.</i> (1991)
EP16A	AGATGCGGAAGTGCTTCG/from 16S rDNA of <i>Erwinia pyrifoliae</i>	Kim <i>et al.</i> (2001)
EP1G2c	ACCGTTAAGGTGGAATC/from ITS of <i>Erwinia pyrifoliae</i>	Kim <i>et al.</i> (2001)
From plasmid pEA29:		
P29A†	CGGTTTTTAACGCTGGG/from <i>Erwinia amylovora</i> plasmid pEA29	Bereswill <i>et al.</i> (1992)
P29B†	GGGCAAATACTCGGATT/from <i>Erwinia amylovora</i> plasmid pEA29	Bereswill <i>et al.</i> (1992)
RS1	ACCTCAGTGCGATTACAG/used to analyse SSRs in pEA29	Kim & Geider (1999)
RS2c	GTCCCATTCTGTGTAAAG/used to analyse SSRs in pEA29	Kim & Geider (1999)
From region for EPS synthesis:		
AMS1‡	GGCAGAAGTTGTGAGCA/from <i>ams</i> of <i>Erwinia amylovora</i> corresponding to position of CPS1 in <i>Erwinia pyrifoliae</i>	Kim <i>et al.</i> (2001)
AMS2c‡	AAACAGGTGCGCCGAATA/from <i>ams</i> of <i>Erwinia amylovora</i> corresponding to position of CPS2c in <i>Erwinia pyrifoliae</i>	Kim <i>et al.</i> (2001)
AMS3	GACGGATCGGCAATCCATTC/from region preceding <i>amsG</i>	This work
AMS4c	CGCGCATAATTAGCTCT/from region preceding <i>amsG</i>	This work
AMsbL	GCTACCAGCAGGGTGAG/from <i>ams</i> region of <i>Erwinia amylovora</i>	Bereswill <i>et al.</i> (1995)
AMsbR	TCATCACGATGGTGTAG/from <i>ams</i> region of <i>Erwinia amylovora</i>	Bereswill <i>et al.</i> (1995)
CPS1§	CGCGGAAGTGTTGAGAA/ <i>cps</i> region of <i>Erwinia pyrifoliae</i>	Kim <i>et al.</i> (2001)
CPS2c§	GAACAGATGTGCCGAGTA/ <i>cps</i> region of <i>Erwinia pyrifoliae</i>	Kim <i>et al.</i> (2001)

* Expected fragments after a digest of the 1.4 kb PCR fragment from *Erwinia amylovora* with *Hae*III: 0.32, 0.22, 0.20, 0.17, 0.16, 0.12 kb. Expected fragments with *Sau*3A: 0.6, 0.42, 0.26, 0.15 kb.

† Expected fragments after a digest of the 1.0 kb PCR fragment from *Erwinia amylovora* strain Ea1/79 with *Sau*3A: 0.26, 0.17, 0.17, 0.11, 0.11, 0.1, 0.05 and 0.04 bp.

‡ Expected fragments after a digest of the 1.2 kb PCR fragment from *Erwinia amylovora* with *Hae*III: 0.57, 0.21, 0.14, 0.13, 0.09, 0.08 kb.

§ Expected fragments after a digest of the 1.2 kb PCR fragment from *Erwinia pyrifoliae* with *Hae*III: 0.65, 0.27, 0.21, 0.09 kb.

cycles at 94 °C for 15 s, 52 °C for 15 s and 72 °C for 30 s were used for denaturation, annealing and polymerization, respectively. The expected product sizes are given in Table 2.

Cloning and sequencing of DNA fragments from the 16S rRNA nucleotide sequence and a region preceding the gene cluster for exopolysaccharide (EPS) synthesis. For cloning of the 16S rDNA, primers fd2 and rP1 were mixed with 0.5 units *Tth* DNA polymerase (Pharmacia) and amplified as described by Kim *et al.* (1999) with approximately 10000 cells and 0.2 mM dATP, dCTP, dGTP and dTTP (Roche). Thirty cycles of amplification were carried out in an Eppendorf Mastercycler 5330. Denaturation was done at 94 °C for 45 s in the first cycle and for 30 s in subsequent cycles, followed by 45 s at 52 °C for annealing and 30 s at 72 °C for polymerization. The 1.4 kb DNA product was eluted from the gel, purified with a PCR purification kit (Qiagen) and the product inserted into pGEM-T (Promega). A DNA fragment upstream the gene cluster for EPS synthesis was amplified with primers AMS3 and AMS4c and inserted into plasmid pGEM-T. Nucleotide sequencing of the cloned fragments was performed with an automatic sequencer (ALFexpress; Amersham Pharmacia Biotech). The DNA sequences were

analysed with the programs Clone Manager v. 5 and Align v. 4 (Scientific & Educational Software, State Line, PA, USA). The Align program was also used for the dendrogram constructed with a distance-based tree building method using the neighbour-joining algorithm.

The *Erwinia amylovora* sequence overlaps with nt 234–1064 in the nucleotide sequence deposited in the EMBL sequence database under accession no. X77921 for strain CFBP1430.

SDS-PAGE analysis. Proteins from cell extracts were separated by PAGE using a 5 % stacking gel and a 10 % resolving gel (Sambrook *et al.*, 1989). Whole-cell lysates were prepared for electrophoresis from 1.5 ml cultures, all grown overnight in LB medium. The cell pellets were resuspended in 100 µl SDS-PAGE sample buffer with β -mercaptoethanol and boiled for 5 min. Ten microlitres of supernatant was applied and the protein bands were stained with Coomassie blue R-250. Fructose-6-phosphate kinase (85 kDa), bovine serum albumin (67 kDa), pyruvate kinase (66 kDa) and carbonic anhydrase (30 kDa) were used as size markers.

Plasmids and PFGE analyses. Total plasmid DNA was isolated after alkaline lysis and purified with a Nucleobond AX column

Fig. 1. A schematic presentation of primers used for identification of the *Erwinia* strains from Japan. The nucleotide sequences designed for the detection of *Erwinia amylovora* and *Erwinia pyrifoliae* are listed in Table 2. (a) ITS region with parts of 16S and 23S rRNA; (b) region with EPS synthesis genes of *Erwinia amylovora* (*ams*) and the corresponding region of *Erwinia pyrifoliae* (*cps*); (c) a DNA fragment from pEA29 of *Erwinia amylovora* corresponding to fragment F of pEA29-Ea88 in the map of McGhee & Jones (2000) applied to detection of the pathogen (Bereswill *et al.*, 1992) and for SSR analysis (Kim & Geider, 1999).

(Macherey and Nagel) according to the manufacturer's instructions. Preparation of the plugs for PFGE, digestion with restriction enzymes and separation of the DNA fragments were as described by Zhang & Geider (1997) and Zhang *et al.* (1998).

Plant assays. Virulence tests with slices of immature pears and with apple seedlings, as well as hypersensitive response assays, were done as described by Rhim *et al.* (1999). Infiltration of three tobacco leaves (cultivar 'Samsun') was done with bacterial suspensions in water at 10^8 c.f.u. ml⁻¹. For the other inoculations, bacterial cultures were grown for 1 d in nutrient broth and cell suspensions transferred to several pear slices or wounded leaves of pear seedlings with a toothpick. Negative controls were done with an *Escherichia coli* strain.

RESULTS

Properties of the Japanese *Erwinia* strains in assays on agar

On MM2Cu agar plates (Bereswill *et al.*, 1998), the *Erwinia* strains from Japan formed white to pale-yellow mucoid colonies, typical for *Erwinia pyrifoliae* (Rhim *et al.*, 1999), in contrast to *Erwinia amylovora* colonies on MM2Cu agar (Bereswill *et al.*, 1998) which display an intermediate to bright-yellow colour. *Erwinia mallo-tivora* did not grow on MM2Cu agar. Neither the Japanese *Erwinia* strains nor the *Erwinia pyrifoliae* strains produced levan on LB-sucrose, whereas *Erwinia amylovora* did. As found for *Erwinia amylovora* and *Erwinia pyrifoliae* strains, the Japanese isolates did not

Fig. 2. Plasmid profiles of *Erwinia* strains from Japan, *Erwinia amylovora* and *Erwinia pyrifoliae*. (a) Undigested; (b) *Bam*HI-digested. Lanes: 1, *Erwinia amylovora* Ea1/79; 2, *Erwinia pyrifoliae* Ep1/96; 3, Ejp546a; 4, Ejp546; 5, Ejp556; 6, Ejp557; 7, Ejp562; 8, Ejp617; *, position of plasmid pEA29; M, marker DNA with 1 kb ladder (BRL). The agarose gel was run at 100 V for 90 min.

grow on MM1Cu agar (Bereswill *et al.*, 1998). They did not fluoresce on King's medium B and were facultatively anaerobic in Hugh-Leifson (Hugh & Leifson, 1953) agar, indicating that the isolates were not pseudo-monads.

Plant assays for confirmation as plant pathogens

The Japanese *Erwinia* strains produced a hypersensitive response on tobacco similar to *Erwinia amylovora* and *Erwinia pyrifoliae*. As with *Erwinia pyrifoliae*, they were not pathogenic on apple seedlings (cv. 'Golden Delicious'), but produced ooze on slices of immature pears as do strains of *Erwinia amylovora* and *Erwinia pyrifoliae* (Rhim *et al.*, 1999). In contrast, no ooze was produced after inoculation of pear slices with *Erwinia mallo-tivora* or *Enterobacter pyrinus*.

Differentiation by plasmid profiles and protein patterns

Plasmids from the Japanese *Erwinia* strains, *Erwinia amylovora* and *Erwinia pyrifoliae* were separated on 1% agarose gels (Fig. 2). At least one plasmid of the *Erwinia* strains from Japan was significantly larger than 29 kb, the size of plasmid pEA29, commonly found in *Erwinia amylovora*, and in all strains but Ejp556 at least one smaller plasmid was observed. Plasmid pEA29 (Fig. 2a, lane 1) carries a single *Bam*HI site (Falkenstein *et al.*, 1988) and was linearized after digestion (Fig. 2b, lane 1). Digestion with *Bam*HI cleaved most of the other large plasmids (Fig. 2a) into several DNA fragments (Fig. 2b). The plasmid patterns of four strains, Ejp546, Ejp546a, Ejp557 and Ejp562, were similar to each other, but Ejp556 did not carry the plasmid migrating as a supercoiled at the position of a 6 kb DNA fragment. Ejp617 produced different plasmid profiles than the other strains and resembled that of *Erwinia pyrifoliae* Ep1/96 except that it contained only one low-molecular-mass plasmid migrating at the position of a 2 kb DNA fragment or below. These small plasmids were not cleaved by

Fig. 3. Comparison of total protein patterns from cell lysates. Ten microlitre aliquots from 100 µl cell lysate were separated by 10% SDS-PAGE and stained with Coomassie blue. Lanes: 1, *Escherichia coli* DH5α; 2, *Pantoea stewartii* DC283; 3, *Erwinia amylovora* Ea1/79; 4, Ejp546; 5, Ejp546a; 6, Ejp556; 7, Ejp557; 8, Ejp562; 9, Ejp617; 10, *Erwinia pyrifoliae* Ep1/96; 11, *Enterobacter pyrinus*; M, molecular mass markers; *, position of proteins different to *Erwinia amylovora*.

*Bam*HI, similar to the small plasmids of *Erwinia pyrifoliae* (Rhim *et al.*, 1999). The complex plasmid pattern for the *Erwinia* strains from Japan supported the notion that they were not *Erwinia amylovora*.

Cells of the *Erwinia* strains from Japan and other bacteria were lysed and their protein patterns determined on an SDS-polyacrylamide gel (Fig. 3). All Japanese *Erwinia* strains gave essentially the same protein pattern, which was distinct from *Escherichia coli*, *Pantoea stewartii* and *Enterobacter pyrinus*, which is another micro-organism from necrotic Nashi pear trees in Korea (Chung *et al.*, 1993). The protein pattern of *Erwinia pyrifoliae* strain Ep1/96 was similar to that of the Japanese strains. Only a few differences in low-molecular-mass proteins were observed. Similarity coefficient values (Nei & Li, 1979) of six *Erwinia* strains from Japan and *Escherichia coli*, *Pantoea stewartii*, *Erwinia amylovora* and *Erwinia pyrifoliae* were 0.55, 0.58, 0.67 and 0.95, respectively. This result confirmed a closer relationship of the Japanese *Erwinia* strains to *Erwinia pyrifoliae* than to *Erwinia amylovora*.

PFGE patterns of *Spe*I and *Xba*I fragments

The genomes of the Japanese *Erwinia* strains Ejp546, Ejp546a, Ejp556, Ejp557, Ejp617 and Ejp562 were digested with restriction enzyme *Xba*I and the DNA fragments were analysed by PFGE. The band patterns were partially related to each other, but not to those of *Erwinia amylovora* (Fig. 4), which is highly homogeneous in its PFGE patterns after an *Xba*I digest (Zhang & Geider, 1997; Zhang *et al.*, 1998). The PFGE patterns of the Japanese strains were also different from *Erwinia pyrifoliae* Ep1/96, which shares the *Xba*I pattern with several other *Erwinia pyrifoliae* strains (unpublished). Similarities of *Xba*I DNA fragments were found for strains Ejp546a, Ejp557 and Ejp562 (Fig. 4). The *Xba*I DNA fragments of strains Ejp546 and Ejp546a differed from each other. Different pattern types were also observed for strains Ejp556 and Ejp617. Due to the complex patterns and overlapping bands in individual

Fig. 4. PFGE pattern of the Japanese *Erwinia* strains, *Erwinia amylovora* and *Erwinia pyrifoliae* after an *Xba*I digest. Lanes: M, size marker of λ DNA multimers; 1, Ea1/79; 2, Ejp546; 3, Ejp546a; 4, Ejp556; 5, Ejp557; 6, Ejp562; 7, Ejp617; 8, Ep1/96.

profiles, similarity coefficient values (Nei & Li, 1979) could only be roughly estimated for a close relationship of Ejp546a, Ejp557 and Ejp562, a less close relationship of Ejp546, Ejp617 and Ejp556, and even less with *Erwinia pyrifoliae* and *Erwinia amylovora*. PFGE analysis after an *Spe*I digest gave matching patterns for Ejp546a, Ejp557 and Ejp562, whereas Ejp546 differed from these strains in two bands (data not shown). As for *Xba*I digests, strains Ejp556 and Ejp617 were also divergent in their *Spe*I fragments. No strong relationship in their patterns was found with *Erwinia pyrifoliae* or *Erwinia amylovora*.

PCR analysis with primers from plasmid pEA29 and from EPS- and rDNA-encoding regions

To further confirm differences between the Japanese *Erwinia* strains and *Erwinia amylovora*, primers P29A and P29B, which were derived from plasmid pEA29 and have been used for specific detection of the fire blight pathogen, were applied. They also produced a PCR product of 1 kb with *Erwinia amylovora* strain IL6, isolated from *Rubus* sp. As reported by Beer *et al.* (1996) for the *Erwinia* strains from Japan, the plasmid primers derived from pEA29 produced a band slightly larger than the band specific for detection of *Erwinia amylovora*. A subsequent *Sau*3A digest of the amplified DNA produced bands from the *Erwinia amylovora* plasmid of the expected sizes (Table 3). *Sau*3A digested the 1.2 kb band, amplified from chromosomal or plasmid DNA of the Japanese *Erwinia* strains with primers P29A and P29B, into fragments of 0.60, 0.17, 0.17, 0.11, 0.05 and 0.035 kb. In the case of strain Ejp546, the large DNA fragment of the digest was slightly smaller than the corresponding fragment of the other strains. Comparison of the two sets indicated different PCR products for the *Erwinia* strains from Japan and

Table 3. Summary and comparison of molecular properties of the *Erwinia* strains from Japan, *Erwinia pyrifoliae* strain Ep1/96 and *Erwinia amylovora* strain Ea1/79

The standard strain used for *Erwinia amylovora* was Ea1/79 with the PFGE pattern (*Xba*I) of the type strain S59/5, and the standard for *Erwinia pyrifoliae* was Ep1/96 with the PFGE pattern (*Spe*I) of the type strain Ep16/96. The data for Ea1/79 and Ep1/96 are representative for many strains of the species. db, Double band; tb, triple band.

Molecular property	<i>Erwinia</i> strains from Japan	Ep1/96	Ea1/79
Plasmids and PCR products:			
Plasmid preparations:			
Undigested	Several	Several	One (pEA29)
<i>Bam</i> HI digest	Multiple cuts	Multiple cuts	Single cut
PCR signal with primers:			
P29A,B	+ (1.2 kb)	Weak bands	+ (1.0 kb)
<i>Sau</i> 3A digest of band	0.60, 0.17(db), 0.11, 0.05, 0.035 kb		0.26, 0.17(db), 0.11(tb), 0.05, 0.035 kb
RS1, RS2	No signal	No signal	+ (~ 0.15 kb)
PCR products from <i>ams/cps</i> regions:			
CPS1, CPS2c (Ep)	+ (1.2 kb)	+ (1.2 kb)	No signal
<i>Hae</i> III digest of band (kb)	0.65, 0.27, 0.21, 0.09	0.65, 0.27, 0.21, 0.09	—
AMSbL, AMSbR	No signal	No signal	1.6 kb
AMS1, AMS2c	No signal	No signal	1.2 kb
Nucleotide sequences of region in front of EPS-synthesis-encoding genes:			
Identity to Ejp546	99–96 % (for others)	95 %	83 %
Protein pattern:			
Bands on gel	Similar to Ep1/96	Reference	Distinct from Ep1/96

Erwinia amylovora. Primers used previously to determine the size of the short sequence DNA repeats (SSR) in plasmid pEA29 (Kim & Geider, 1999) did not produce a PCR signal with the *Erwinia* strains from Japan or with *Erwinia pyrifoliae* (data not shown).

With another primer pair specific for the detection of *Erwinia amylovora*, derived from the region for amylovoran synthesis (*ams*) (Bereswill *et al.*, 1995), a product was obtained for *Erwinia amylovora* from fruit trees/ornamentals and from raspberry (*Rubus* sp.), but not for the Japanese *Erwinia* strains or for *Erwinia pyrifoliae* (data not shown). The *cps* region of *Erwinia pyrifoliae* encodes an EPS similar to amylovoran synthesized by proteins homologous to Ams proteins (unpublished). On the other hand, the nucleotide sequence of the *cps* region is not identical to the *ams* region of *Erwinia amylovora* and PCR primers were designed to discriminate *Erwinia pyrifoliae* from *Erwinia amylovora* (W.-S. Kim, S. Jock, J.-P. Paulin, S.-L. Rhim & K. Geider, unpublished). With the Japanese *Erwinia* strains, these primers amplified a DNA fragment of the same size as for *Erwinia pyrifoliae* (Fig. 5a). In a *Hae*III digest of the PCR band, strains Ejp557 and Ejp617 produced DNA fragments identical to each other and with fragments of *Erwinia pyrifoliae* strain Ep1/96

Fig. 5. PCR assay with (a) *cps* primers (CPS1/CPS2c) and (b) 16S ITS primers (EP16A/EPIG2c) from *Erwinia pyrifoliae*. Lanes: 1, *Erwinia amylovora* Ea1/79; 2, Ejp546; 3, Ejp546a; 4, Ejp556; 5, Ejp557; 6, Ejp562; 7, Ejp617; 8, *Erwinia pyrifoliae* Ep1/96; 9, water control; M, 1 kb ladder marker DNA.

Ep1/96	gttatggatgcaaaagctatgttgacactacttatcaggcataccgcatttcataatt-t
6 Ejp strains	t.....t.....tg.g....g....aa.a...c..c.a.t.....a.
Ea1/79	...ccct...-.....t.....tg.g....g....aa.a...c..c.a.t.....a.
IL6	...ccct...-.....t.....tg.g....g....aa...t.c..c.a.t.....a.

Fig. 6. Alignment of part of the nucleotide sequence preceding the gene clusters for EPS synthesis to compare *Erwinia pyrifoliae* Ep1/96 and the six Japanese *Erwinia* strains used in this study with *Erwinia amylovora* strains Ea1/79 and IL6.

(data not shown). When primers from the *ams* operon of *Erwinia amylovora* (AMS1 and AMS2c) were applied, these did not produce a signal with the *Erwinia* strains from Japan nor with *Erwinia pyrifoliae*. In addition, the *Hae*III digestion pattern from the DNA fragments obtained from *Erwinia amylovora* with primers AMS1 and AMS2c was different from the *Hae*III pattern obtained with primers CPS1 and CPS2c from *Erwinia pyrifoliae* and the Japanese strains (Tables 2, 3). Accordingly, the region encoding EPS synthesis in the Japanese *Erwinia* strains is closely related to *Erwinia pyrifoliae*, but less related to *Erwinia amylovora*.

Another PCR assay for *Erwinia pyrifoliae* was done with primers EP16A and EPIG2c from the 16S rRNA intergenic transcribed spacer (ITS) region of *Erwinia pyrifoliae* (Figs 1a and 5b). When these primers were used to assay the Japanese *Erwinia* strains Ejp546, Ejp546a, Ejp557 and Ejp652, a DNA fragment of 0.7 kb was produced, similar to the signal from the *Erwinia pyrifoliae* strains (eight different strains). The product was not obtained with strains Ejp556 and Ejp617. No signals were obtained for *Erwinia amylovora* with primers EP16A and EPIG2c or CPS1 and CPS2c.

The 16S rDNA of the Japanese *Erwinia* strains was amplified with consensus primers (Weisburg *et al.*, 1991) and the PCR product digested with *Hae*III. For the Japanese *Erwinia* strains, *Erwinia pyrifoliae* and *Erwinia amylovora* an identical pattern of DNA fragments was found. Sequence analysis of the 16S rDNA from the three *erwinias* revealed 99% identity (data not shown). This region is apparently highly conserved for the Japanese *Erwinia* strains, *Erwinia pyrifoliae* and *Erwinia amylovora*, and cannot be used for their differentiation.

Sequence analysis of a DNA fragment in front of the chromosomal region encoding EPS synthesis

Biosynthesis of amylovoran depends on sugar transferases and transport proteins encoded in the *ams* region of *Erwinia amylovora* (Bugert & Geider, 1995). No major ORFs were detected for the nucleotide sequence up to 1 kb upstream of *amsG*. Promoter signals can be assumed for the region adjacent to the first gene, *amsG*. From this region (nt 234–1064 in the sequence deposited in the EMBL nucleotide sequence database under accession no. X77921) 831 bp were subjected to sequence comparison for the *Erwinia* strains from Japan, *Erwinia amylovora* strains Ea1/79 (fruit trees/ornamentals) and IL6 (*Rubus* sp.), as well as *Erwinia*

Fig. 7. Dendrogram derived from the nucleotide sequence in front of the EPS-encoding regions for the investigated *Erwinia* strains from Japan, Ep1/96 (from Nashi pear trees, Korea), and *Erwinia amylovora* Ea1/79 (fruit tree strain) and IL6 (*Rubus* strain).

pyrifoliae strain Ep1/96, and a region with significant differences, presented in Fig. 6, was identified. Alignment of the sequence revealed several features for the nine strains investigated: (i) the *Erwinia* strains from Japan were highly related to each other (96–99% identity); (ii) Ejp546, Ejp546a, Ejp557 and Ejp562 can be distinguished from Ejp556 and Ejp617 by several mismatches; (iii) *Erwinia* strains from Japan are more closely related to *Erwinia pyrifoliae* (95%) than to *Erwinia amylovora* strains (fruit tree, 83%; *Rubus* sp., 84%); (iv) *Erwinia amylovora* strains from different hosts (fruit trees/ornamentals or *Rubus* sp.) carry an almost identical nucleotide sequence in this region. A dendrogram based on these nucleotide sequences placed the *Erwinia* strains from Japan close to *Erwinia pyrifoliae* and more distant from *Erwinia amylovora* (Fig. 7).

DISCUSSION

Although *Erwinia* strains from Japan, associated with fire-blight-like symptoms in Japanese pear orchards, have been reported to be *Erwinia amylovora* (Beer *et al.*, 1996; Kim *et al.*, 1996), a series of molecular data contradicts this claim. Recently, the novel species *Erwinia pyrifoliae* was described (Kim *et al.*, 1999), which causes necrotic symptoms on shoots of Asian pear trees in Korea (Rhim *et al.*, 1999). Strains of *Erwinia* sp. from necrotic tissue of Nashi pears, isolated in Japan, were reported to be only weakly virulent on apple seedlings (Beer *et al.*, 1996). This property was confirmed for the strains investigated in this report. On semi-selective plates, by symptoms on immature pears and with molecular tools, these strains were not found to be related to *Enterobacter pyrinus*, also isolated from

Nashi pear fruit trees (Chung *et al.*, 1993) or to *Erwinia mallotivora*, which causes necrotic leaf spots on Nashi pear (Goto, 1992). The nucleotide sequences of the 16S rDNA were almost identical for the Japanese pear pathogen, *Erwinia pyrifoliae* and *Erwinia amylovora* (Table 3). When the ITS region, which is often indicative for species (Kim *et al.*, 1999), was amplified, multiple DNA fragments were obtained, differing in size as well as the type of the embedded tRNA gene and could therefore not be used for classification of the three *erwinias* (M. Hildebrand, W.-S. Kim & K. Geider, unpublished).

On the other hand, several properties, such as the lack of a PCR product with primer pairs of the *ams* region and primers RS1 and RS2 from pEA29 (Table 3), allowed differentiation of strains Ejp546, Ejp546a, Ejp557, Ejp562, Ejp556 and Ejp617 from *Erwinia amylovora*. With the *Erwinia* strains from Japan, the plasmid primers P29A and P29B gave rise to a DNA fragment around 1 kb (Beer *et al.*, 1996). Digestion with a restriction enzyme showed differences in the cleavage products in comparison to *Erwinia amylovora* (Table 3), confirming that the signal with primers P29A and P29B was derived from a sequence not identical to the 1 kb fragment of pEA29 amplified from *Erwinia amylovora*. In contrast, the Japanese *erwinias* resembled *Erwinia pyrifoliae* in several molecular properties (Table 3). All Japanese *Erwinia* strains gave a positive PCR signal with the *cps* primers from *Erwinia pyrifoliae* and produced identical DNA fragments after a restriction enzyme digest, confirming that the genes for EPS synthesis of the Japanese *erwinias* are related to the *cps* genes of *Erwinia pyrifoliae*. When the corresponding chromosomal regions preceding the gene clusters for EPS synthesis of *Erwinia pyrifoliae*, the *Erwinia* strains from Japan and *Erwinia amylovora* strains isolated from fruit trees/ornamentals and from *Rubus* sp. were aligned, the Japanese strains had a closer relationship to *Erwinia pyrifoliae* than to *Erwinia amylovora*. The similarity to *Erwinia pyrifoliae* was confirmed by PAGE of total cellular proteins and by the plasmid profiles. Plasmid pEA29 was found in all *Erwinia amylovora* strains investigated by DNA hybridization and by PCR assays with plasmid-derived primers (Falkenstein *et al.*, 1988; Bereswill *et al.*, 1992). In contrast to *Erwinia amylovora*, *Erwinia pyrifoliae* strains (Rhim *et al.*, 1999) and the *Erwinia* strains from Japan displayed a complex plasmid pattern. The *Rubus* sp. strains share a simple plasmid pattern with the *Erwinia amylovora* strains from fruit trees and ornamentals, and the pEA29 derivatives are largely identical for strains from different hosts (McGhee & Jones, 2000). Cross-hybridization was observed with labelled DNA from plasmid pEA29 and DNA from *Erwinia amylovora* fruit tree and *Rubus* sp. strains, *Erwinia pyrifoliae* and the *Erwinia* strains from Japan (S. Jock & K. Geider, unpublished), limiting the use of total plasmid DNA to distinguish these sets of strains.

The PFGE patterns of *Erwinia* strains from Japan appeared unrelated to *Erwinia amylovora*, whereas they

were highly conserved for all European strains assayed (Zhang & Geider, 1997; Zhang *et al.*, 1998). A relationship to *Erwinia amylovora* strains isolated from *Rubus* sp. could also be excluded by additional molecular data obtained. *Rubus* sp. strains differ from fruit tree strains in their PFGE pattern (S. Jock & K. Geider, unpublished), but they share an almost identical nucleotide sequence in a region upstream of the EPS-synthesis-encoding gene clusters and do not only produce PCR signals with pEA29 targeted primers, but also with *ams* primers used to identify strains from fruit trees and ornamentals. Similar to *Erwinia pyrifoliae* found in Korea, which have variable PFGE patterns, the Japanese *erwinias* appear to be also more variable than *Erwinia amylovora*. BIOLOG profiles and DNA hybridizations with the *hrp* region of *Erwinia amylovora* placed the Japanese strains close to the fire blight pathogen (Beer *et al.*, 1996). Other criteria, such as molecular features, including the nucleotide sequences of a DNA fragment from a chromosomal region preceding the gene cluster for EPS synthesis, seem to be quite powerful for differentiation of the Japanese *Erwinia* strains from *Erwinia amylovora*, although they are not classical taxonomic criteria. After the molecular differentiation of the *Erwinia* strains from Japan, molecular criteria proposed for the detection and identification of fire blight such as specific PCR primers (Bereswill *et al.*, 1992, 1995; Kim & Geider, 1999) are still valid for diagnosis of the disease and were recently successfully applied to describe a temporary infection of plants with *Erwinia amylovora* in the Melbourne Botanic Gardens (Jock *et al.*, 2000). Consequently, previous reports about fire blight in Japan on the island of Hokkaido (Beer *et al.*, 1996; Kim *et al.*, 1996) have to be reconsidered in the light of the new findings that the pear-pathogenic bacteria from Japan are more closely related to the Korean pear pathogen *Erwinia pyrifoliae* than to the fire blight pathogen *Erwinia amylovora*.

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