Molecular Characterization of Natural *Erwinia pyrifoliae* Strains Deficient in Hypersensitive Response

Susanne Jock,¹ Won-Sik Kim,¹ Marie-Anne Barny,² and Klaus Geider^{1*}

Max-Planck-Institut für Zellbiologie, Rosenhof, 68526 Ladenburg, Germany,¹ and Laboratoire de Pathologie Végétale, UMR217 INRA/INA-PG/Université Paris VI, 75231 Paris Cedex 05, France²

Received 3 June 2002/Accepted 6 October 2002

From necrotic tissue of a Nashi pear tree, 24 *Erwinia pyrifoliae* strains, found to be identical by pulsed-field gel electrophoresis analysis, were isolated. Thirteen strains were not virulent on immature pears and did not induce a hypersensitive response in tobacco leaves. The defective gene *hrpL* was complemented with intact genes from *E. pyrifoliae* and *Erwinia amylovora*.

A bacterial disease similar to fire blight was observed on Nashi pear trees (*Pyrus pyrifolia*) in South Korea, caused by the novel species *Erwinia pyrifoliae* (16). The pathogen was consistently isolated from orchards in the area of Chuncheon from 1995 to 1998 and identified on semiselective agar plates and by PCR (15, 16). *E. pyrifoliae* is restricted mainly to pear and is highly related to pear-pathogenic *Erwinia* strains from Japan (14). It can be distinguished from the fire blight pathogen *Erwinia amylovora* by several microbiological and molecular features (13). Fire blight was first described for North America and has spread to New Zealand, Europe, and the Mediterranean region (6, 10). The disease has not been reported in countries of the Southern hemisphere other than New Zealand, except for a transient occurrence in 1997 in Australia (11). The pathogenicity of *E. amylovora* depends on both the synthesis of the acidic exopolysaccharide (EPS) amylovoran (2, 17) and a functional type III secretion system, involved in induction of a hypersensitive response (HR) (1, 5, 9, 17). The pear pathogen *E. pyrifoliae* shares substantial homologies to *ams* genes and also requires capsular EPS for pathogenicity (12).

Natural HR-deficient strains. In 1997, necrotic tissue from a Nashi pear tree was obtained from Korea and extracted for isolation of the causative pathogen. White colonies on Luria-Bertani (LB) agar (with 50 µg of cycloheximide/ml to avoid fungal growth) were assayed for the mucoid, yellowish colony morphology typical of *E. pyrifoliae* on MM2Cu agar as described before (13, 15, 16). The isolates were further assayed on LB plates with 5% sucrose and on MM1Cu agar as described for identification of *E. amylovora* (3). A lack of levan synthesis and no growth on MM1Cu agar were additional properties expected from *E. pyrifoliae*. The synthesis of capsular polysaccharide of the isolates on MM2Cu agar excluded being deficient in a gene associated with EPS synthesis. The 24 isolates were confirmed to be *E. pyrifoliae* by PCR analysis (4) with primers derived from the rRNA genes and the *cps* region (15). The strains were then assayed on pear slices for formation of an exudate (ooze) to assess their virulence and infiltrated

into tobacco leaves in order to determine their capacity to elicit an HR (Fig. 1, leaf sections 1 and 2). Fewer than one-half of these isolates produced exudate on pear slices and the typical necrotic symptoms at the infiltrations sites on tobacco leaves (Table 1). The ability to cause HR is a requirement for virulence of *E. amylovora* strains (1, 17). Harpin, encoded by *hrpN*, is transported to the surface of the bacteria and elicits HR in the plant tissue (20). The *dsp* locus, adjacent to the *hrp* gene cluster, encodes Avr-like proteins (5, 9) and is required for pathogenicity but does not affect HR. A deficiency of *dsp*-related genes of *E. pyrifoliae* was therefore not assumed for the nonpathogenic isolates.

Identity of PFGE patterns. To confirm genomic similarity between the pathogenic, HR-positive strain Ep4/97 and the nonpathogenic, HR-negative strain Ep2/97, we applied pulsed-field gel electrophoresis (PFGE) analysis after digestion with restriction enzymes *Xba*I and *Spe*I. PFGE analysis of *E. pyrifoliae* strains can be used as a stringent method for identification of the pathogen. *E. amylovora* strains isolated at different times in various European and Mediterranean regions were closely related in their PFGE patterns after digestion with *Xba*I and *Spe*I, and a common origin for these strains was assumed (10, 21). For both *E. pyrifoliae* strains, the *Xba*I fragments obtained were identical, matching strains Ep8/95, Ep1/96, and Ep16/96 isolated in 1995 and 1996 in South Korea, but the DNA fragments were different for strain SLR21 (Fig. 2). This strain was isolated from necrotic Nashi pear tissue in 1996 and also differed from *E. pyrifoliae* by other molecular and microbiological properties (Table 1). Although the *Spe*I fragments of *E. pyrifoliae* strains have been more diverse than *Xba*I patterns (15), the digests of genomic DNA from strains Ep2/97 and Ep4/97 with *Spe*I also produced identical patterns in PFGE analysis (data not shown). Matching of the PFGE patterns supported the assumption that the HR-deficient mutant Ep2/97 was derived from Ep4/97 during development of the disease in the affected Nashi pear tree and excluded isolation of strains with microbiological and molecular properties that were merely similar to those of *E. pyrifoliae*. This coexistence of virulent and nonvirulent strains in the population is a rare example of a possible advantage for a nonpathogenic variant of a bacterial pathogen. The spontaneous *hrp* mutants seem to be well suited to support the HR-positive parent strain during

* Corresponding author. Mailing address: Max-Planck-Institut für Zellbiologie, Rosenhof, D-68526 Ladenburg, Germany. Phone: 49-6203-106-117. Fax: 49-6203-106-122. E-mail: kgeider@zellbio.mpg.de.

FIG. 1. HR assays on tobacco (cv. Samsun) with the HR-negative strain Ep2/97 (section 1), the coisolated positive strain Ep4/97 (section 2), and Ep2/97(pGEM-hrpL) with *hrpL* from strain Ep4/97 (section 3). The bacteria were grown in LB medium, incubated in inducing medium (7), suspended in water, and then infiltrated into tobacco leaves at 10^8 CFU/ml.

colonization by the pathogen and may also enhance pathogen survival in necrotic plant tissue.

Complementation. The properties of the 13 isolated defective *E. pyrifoliae* strains showed a correlation of virulence on pears and HR on tobacco (Table 1). An EPS-deficient mutant of *E. pyrifoliae* had previously been complemented with *ams* genes of *E. amylovora* (12). In a similar approach, we at-

FIG. 2. PFGE analysis to show the relatedness of *E. pyrifoliae* strains Ep2/97 and Ep4/97. Genomic DNA of agar-embedded cells was digested with restriction enzyme *Xba*I, and the PFGE was performed in a 1% agarose gel (Ultra Pure DNA grade agarose; Bio-Rad) with running buffer (3.5 mM HEPES, 3.5 mM sodium acetate, 0.35 mM EDTA [pH 8.3]) for 22 h at 14°C and a ramping time from 1 to 25 s. M, positions of marker DNA in kilobases. Multimeric λ genomes were run in a separate lane. Lanes: 1, *E. pyrifoliae* Ep8/95 (pattern identical with those of strains Ep1/96 and Ep16/96 in *Xba*I digests); 2, SLR21 (from Asian pear; not *E. pyrifoliae*); 3, Ep2/97; 4, Ep4/97; 5, *E. amylovora* Ea1/79.

tempted to complement the HR-deficient strain Ep2/97 with *hrp* genes of *E. amylovora*. Spontaneous mutants with streptomycin resistance were selected, and strain Ep2/97Sm was conjugated with *Escherichia coli* strain S17-1 carrying cosmids pPV130, pPV132, or pPV133 (1). The cosmids contain partially overlapping regions of the *hrp* cluster (Fig. 3). Ep2/97Sm(pPV130) and Ep2/97Sm(pPV133) gave a positive HR after incubation in inducing medium (7). To narrow down the

TABLE 1. Results of assays for identification and characterization of *E. pyrifoliae*

Strain(s) tested	Result for indicated assay ^a					Reference(s) or source
	HR	Vir	PCR	MM2Cu	KB	
<i>E. amylovora</i>						
CFBP1367 (Ea321), CFBP1430, Ea1/79	+	+	—	+/+	—	1, 8, 21
Other bacterium						
SLR21	—	—	—	—	—	South Korea ^b
<i>E. pyrifoliae</i>						
Ep8/95, Ep1/96, Ep16/96, Ep28/96, Ep31/96, Ep4/97, Ep5/97, Ep42/97, Ep44/97, Ep45/97, Ep53/97, Ep43/97, Ep55/97, Ep56/97, Ep58/97, Ep60/97	+	+	+	+/+	—	16, this study ^c
Ep2/97, Ep3/97, Ep41/97, Ep46/97, Ep47/97, Ep48/97, Ep49/97, Ep50/97, Ep51/97, Ep52/97, Ep54/97, Ep57/97, Ep59/97	—	—	+	+/+	—	This study ^c

^a HR, assay on tobacco; Vir, assay for virulence on pear slices; PCR, assays were done as described before (3) with primers for specific detection of *E. pyrifoliae* (15); MM2Cu, colony morphology on MM2Cu agar; KB, fluorescence on King's medium B (16); +, positive for *E. pyrifoliae*; —, negative for *E. pyrifoliae*; +/+, yellow to slightly yellow color and mucoid.

^b Isolated from necrotic Nashi pear tissue in 1996 in South Korea; this strain is not *E. pyrifoliae*.

^c Strains ending with 97 were isolated from the bark of a branch from a diseased Nashi pear tree (*Pyrus pyrifolia*) in 1997 obtained from South Korea.

FIG. 3. Restriction map of cosmids and subclones used for complementation of the HR-deficient *E. pyrifoliae* strain Ep2/97. The dark central line indicates the *dsp-hrp* region of *E. amylovora* CFBP1430 (1). The plasmids named by numbers are insertions in vector pUC18, except for pMAB40 with an insertion in pRK767. Restriction sites are indicated for *Eco*RI (long lines pointing down, E), *Bam*HI (short lines down, B), *Hind*III (short lines up, H), and *Sal*I (long lines up, S). The *hrp* cluster and the *dsp* region (left) are indicated by broken lines.

area responsible for complementation, several smaller plasmids (9) covering DNA insertions of the positive fragment were introduced by electroporation: pMAB5, -31, -32, -38, -40, and -71 (Fig. 3). Only Ep2/97(pMAB71) induced a positive HR, indicating that the mutation affected either *hrpL* or *hrpJ*. To confirm the ability of pMAB71 for complementation of HR-deficient variants, this plasmid was also introduced into strains Ep41/97, Ep46/97, Ep49/97, Ep52/97, and Ep57/97 (Table 1), where HR was restored in each case. To dissect the involvement of *hrpL* or *hrpJ*, primers were designed from the nucleotide sequences of the *E. amylovora* genes (EMBL nucleotide sequence database accession numbers U36244 and L25828) as follows: for amplification of *hrpL*, HRPL1 (5'-GG CACAAGCCTTGCTAA) and HRPL2c (5'-CGGCAAGACA GGACACT), and for amplification of *hrpJ*, HRPJ1 (5'-TAT-GTCGCTGGCGACTT) and HRPJ2c (5'-CTGATGGCGAG GCGATT). In the case of the *hrpJ* primers, only PCR fragments from CFBP1430 and Ea321 could be cloned into pGEM-T (Promega). A faint PCR band but no cloning product was obtained with *E. pyrifoliae* strains as a template. Ep2/97 was not complemented with *E. amylovora hrpJ* clones, although a putative promoter region was part of the amplified DNA. In PCR amplifications of *hrpL*, the *E. amylovora* strains CFBP1430, Ea321, and Ea1/79 as well as the *E. pyrifoliae* strains Ep1/96, Ep16/96, Ep2/97, and Ep4/97 produced DNA fragments which were cloned into plasmid pGEM-T. The resulting plasmids were assayed with restriction enzyme *Pst*I for

insertion of the gene in the direction of the *lac* promoter (pGEM-*hrpL*). Strain Ep2/97 was successfully complemented for HR on tobacco with pGEM-*hrpL* carrying inserts from the HR-positive *E. pyrifoliae* strains Ep4/97 (Fig. 1, leaf section 3), Ep1/96, and Ep16/96 and also from *E. amylovora* strains CFBP1430, Ea321, and Ea1/79. With the cloned *hrpL* fragment from the nonpathogenic strain Ep2/97 in pGEM-T, HR on tobacco leaves and virulence on pear tissue were not restored for Ep2/97, confirming the nonfunctional gene in this strain. On the other hand, Ep2/97 with pGEM-*hrpL* of virulent *E. amylovora* and *E. pyrifoliae* strains caused necrotic symptoms on pear seedlings, which were slightly retarded compared to those of the wild-type strain Ep4/97, possibly due to the instability of the complementing plasmid. Attempts to find spontaneous HR-negative mutants by passaging Ep4/97 on nutrient agar plates or by reisolation of cells from slime formed on pear slices were not successful. Mutation to HR deficiency is therefore not frequent for *E. pyrifoliae* wild-type strains.

Change of single base pair. The insertions obtained from strains Ep2/97 and Ep4/97 were sequenced with labeled SP6 and T7 primers in both strands by using an ALF sequencer, and the nucleotide sequences were independently confirmed by commercial sequencing. A comparison of the nucleotide sequences of the HR-positive and the HR-negative strains revealed a change of nucleotide C into T at position 277 (Fig. 4), converting the codon TCA (for serine) into TTA (for leucine), thereby changing a polar amino acid into an unipolar

```

1  GGCACAAGCCTTGCTAAAGAGATGACGCCGCTGAGGCATCACCTGATTGAGTAACGGAGCAAGCC ATG ACA GAA ATT
78  CAC CTG CAG TCA ACA GAA CCC ACA TCG GTC AAC GAA GGG CTA CCG CTG AAT ATT GAT TGG GAA
141 GGT ATC TTT CGT GAG CAT GGG CGA CGC GTG CAT CAT TTT ATT CGC AAG CGC GTT TCC CAT CGC
204 GAA GAT GCC GAG GAT CTG GAG CAA ATG ACC TGG CTG GAA GTT TTG CGT AAC CGT GAC AAA TTC
267 GCC GGT GCG TGA CGG CCA GAA ACC TGG GTG TTT GGT ATT GCC TTA AAT TTA GTA CGC AAC CAT
330 TTT CGC CTG CAG TCC GGA CGG CCG CGT TGT GAT GAG CTG GAC GAC GAT ATC ATT CTG ACT CAG
393 GGC GAC GAT CCA AGC CAT ATC ACC GAA TAT CAG CGC CTG AAC TCG ACG CTT AAT TCC ATT
456 GCC GTA TTG CCG GAA GAT GCC CGC CGT CTG ATG AAC ATG CTG GTC GAG AAA GAC GGC AGC TAT
519 CAG GAT ATC GCA GCA CAT TTA AAT ATC CCG ATT GGC ACC GTG CGT TCA CGT CTG TCG CGC GCG
582 CGC GTC ACG CTG AAG CAG TCA GTA TTT TCT TAA AGTTGGATAGACGGATAATTCGCTAAATCGTAAAGTGAA
656 TTGCACAGTGTCCTGTCTTGCCG

```

FIG. 4. Nucleotide sequence of the *hrpL* gene of *E. pyrifoliae* strain Ep4/97. The nucleotide change in Ep2/97 (from C to T) is indicated with dark shading. The PCR primers used for amplification are in italics and underlined at both ends of the nucleotide sequence, and a putative ribosome binding site is printed in bold. The triplets indicate the range of the structural gene.


```

Ea1/79 (1) MTEIHLQTTESTSVNDGLPLNIDWEGIFREHGRRVHHFIRKRVSHREDAEDLEQMTWLEVLNRDKFAGASRPETWVFGIALNLVRNHFRL
Ep4/97 (1) .....S..P...E.....
Ep2/97 (1) .....S..P...E.....

Ea1/79 (92) QSGRPRCDELEDDIILTQGGDPSHITEYQRLNLSNLSIANLPEDTRRLNMLVEKDGSYQAIHAHLNIPIGTVRSRLSRARVTLKQSVFS
Ep4/97 (92) .....D.....V...A...M.....D.....
Ep2/97 (92) .....D.....V...A...M.....D.....

```

FIG. 5. Alignment of the HrpL proteins from *E. amylovora* strain Ea1/79 and *E. pyrifoliae* strains Ep4/97 and Ep2/97. Identical amino acids are indicated by dots, and the amino acid change (to L) in Ep2/97 is shaded in black.

amino acid. In the amino acid sequences of HrpL of *E. amylovora* (SWISS-PROT accession number Q46616) and of *Pseudomonas syringae* (P37929), this amino acid is also serine, which may be needed for the function of the protein. The amino acid sequences for HrpL of *E. pyrifoliae* and *E. amylovora* were 95% identical to each other and differed at eight positions, which were located mainly at both ends of the protein (Fig. 5). The nucleotide sequence of *hrpL* from Ep16/96 corresponded with the nucleotide sequence from Ep4/97 with C at position 277. To reconfirm the *hrpL* sequences, DNA from strains Ep2/97, Ep4/97, and Ep16/96 was also amplified with a proofreading DNA polymerase (AccuTaq; Sigma). The same sequence as in Fig. 4 was obtained for the three wild-type strains, and the change from C to T at position 277 for strain Ep2/97 was observed.

Growth properties. HrpL of *E. amylovora* has been associated with the function of a σ -factor, controlling expression of the *hrp* gene cluster (19). The mutation in the investigated population of *E. pyrifoliae* from the necrotic tissue of Nashi pears can be explained by a spontaneous base change. The mutants lacking recognition by plant defense mechanisms could derive an advantage in some stages of the pathogen's life cycle. We coinoculated the HR-deficient strain Ep2/97Sm and wild-type Ep4/97 mixed at different ratios (10:1, 1:1, and 1:10) into slices of immature pears. In most cases, approximately equal amounts of cells from each strain were recovered in the exudate developed after 1 week. These data indicate a helper function of the HR-positive strain for efficient multiplication of the HR-negative variant in host plant tissue. After inoculation of *E. amylovora* *hrp* mutants, which had been complemented with cosmids carrying parts of the *hrp* cluster, a large proportion of the recovered cells had lost the cosmid, indicating feeding by the other bacteria (1), similar to in vivo complementation between *E. amylovora* *ams* and *dsp* mutants (2). Although Ep2/97 was not virulent due to its inability to damage plant cells, the wild-type strain relieved the restriction, allowing both strains to grow. In the presence of wild-type cells, a spontaneous HR-deficient variant may be advantageous in pear tissue, because it does not challenge the plant defense mechanism (18). In addition, the variant could also have a better chance than the HR-positive parent strain for survival during decline of the population in a late stage of plant colonization.

Nucleotide sequence accession number. The nucleotide sequence of *hrpL* of *E. pyrifoliae* strain Ep4/97 has been deposited in the EMBL nucleotide sequence database with the accession number AJ438881.

REFERENCES

1. Barny, M.-A., M. H. Guinebretiere, B. Marçais, E. Coissac, J.-P. Paulin, and J. Laurent. 1990. Cloning of a large gene cluster involved in *Erwinia amylovora* CFBP1430 virulence. *Mol. Microbiol.* **4**:777–786.
2. Bellemann, P., and K. Geider. 1992. Localization of transposon insertions in pathogenicity mutants of *Erwinia amylovora* and their biochemical characterization. *J. Gen. Microbiol.* **138**:931–940.
3. Bereswill, S., S. Jock, P. Bellemann, and K. Geider. 1998. Identification of *Erwinia amylovora* by growth morphology in the presence of copper sulfate and by capsule staining with lectin. *Plant Dis.* **82**:158–164.
4. Bereswill, S., A. Pahl, P. Bellemann, W. Zeller, and K. Geider. 1992. Sensitive and species-specific detection of *Erwinia amylovora* by PCR-analysis. *Appl. Environ. Microbiol.* **58**:3522–3526.
5. Bogdanove, A. J., J. F. Kim, Z. Wei, P. Kolchinsky, A. O. Charkowski, A. K. Conlin, A. Collmer, and S. V. Beer. 1998. Homology and functional similarity of an *hrp*-linked pathogenicity locus, *dspEF*, of *Erwinia amylovora* and the avirulence locus *avrE* of *Pseudomonas syringae* pathovar tomato. *Proc. Natl. Acad. Sci. USA* **95**:1325–1330.
6. Bonn, W. G., and T. Van der Zwet. 2000. Distribution and economic importance of fire blight, p. 37–54. In J. Vanneste (ed.), *Fire blight, the disease and its causative agent Erwinia amylovora*. CABI Publishing, Wallingford, United Kingdom.
7. Coplin, D. L., and C. I. Kado. 2001. Gram-negative bacteria. B-3 *Pantoea*, p. 73–83. In N. W. Schaad, J. B. Jones, and W. Chun, (ed.), *Laboratory guide of identification of plant pathogenic bacteria*. APS Press, St. Paul, Minn.
8. Falkenstein, H., P. Bellemann, S. Walter, W. Zeller, and K. Geider. 1988. Identification of *Erwinia amylovora*, the fire blight pathogen, by colony hybridization with DNA from plasmid pEA29. *Appl. Environ. Microbiol.* **54**:2798–2802.
9. Gaudriault, S., L. Malandrin, J. P. Paulin, and M. A. Barny. 1997. DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with *AvrE* of *Pseudomonas syringae*, is secreted via Hrp secretion pathway in a DspB-dependent way. *Mol. Microbiol.* **26**:1057–1069.
10. Jock, S., V. Donat, M. M. Lopez, C. Bazzi, and K. Geider. 2002. Following spread of fire blight in Western, Central and Southern Europe by molecular differentiation of *Erwinia amylovora* strains with PFGE analysis. *Environ. Microbiol.* **4**:106–114.
11. Jock, S., B. Rodoni, M. Gillings, W.-S. Kim, C. Copes, P. Merriman, and K. Geider. 2000. Screening of ornamental plants from the Botanic Gardens of Melbourne and Adelaide for the occurrence of *Erwinia amylovora*. *Australasian Plant Pathol.* **29**:120–128.
12. Kim, W.-S., M. Schollmeyer, M. Nimtz, V. Wray, and K. Geider. Genetics of biosynthesis and structure of the capsular exopolysaccharide from the Asian pear pathogen *Erwinia pyrifoliae*. *Microbiology* **148**:4015–4024.
13. Kim, W.-S., L. Gardan, S.-L. Rhim, and K. Geider. 1999. *Erwinia pyrifoliae* sp. nov., a novel pathogen that affects Asian pear trees (*Pyrus pyrifolia* Nakai). *Int. J. Syst. Bacteriol.* **49**:899–906.
14. Kim, W.-S., M. Hildebrand, S. Jock, and K. Geider. 2001. Molecular comparison of pathogenic bacteria from pear trees in Japan and the fire blight pathogen *Erwinia amylovora*. *Microbiology* **147**:2951–2959.
15. Kim, W.-S., S. Jock, J.-P. Paulin, S. L. Rhim, and K. Geider. 2001. Molecular detection and differentiation of *Erwinia pyrifoliae* and host range analysis of the Asian pear pathogen. *Plant Dis.* **85**:1183–1188.
16. Rhim, S.-L., B. Völksch, L. Gardan, J.-P. Paulin, C. Langlotz, W.-S. Kim, and K. Geider. 1999. An *Erwinia* species, different from *Erwinia amylovora*, causes a necrotic disease of Asian pear trees. *Plant Pathol.* **48**:514–520.
17. Steinberger, E. M., and S. V. Beer. 1988. Creation and complementation of pathogenicity mutants of *Erwinia amylovora*. *Mol. Plant-Microbe Interact.* **1**:135–144.
18. Venisse, J. S., G. Gullner, M. N. Brisset. 2001. Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora*. *Plant Physiol.* **125**:2164–2172.
19. Wei, Z., and S. V. Beer. 1995. *hrpL* activates *Erwinia amylovora* *hrp* gene transcription and is a member of the ECT subfamily of σ factors. *J. Bacteriol.* **177**:6201–6210.
20. Wei, Z. M., R. J. Laby, C. H. Zumoff, D. W. Bauer, S. Y. He, A. Collmer, and S. V. Beer. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* **257**:85–88.
21. Zhang, Y., and K. Geider. 1997. Differentiation of *Erwinia amylovora* strains by pulsed-field gel electrophoresis. *Appl. Environ. Microbiol.* **63**:4421–4426.