*Plant Pathol. J. 19(6) : 294-300 (2003)*

# *Erwinia pyrifoliae*, a Causal Endemic Pathogen of Shoot Blight of Asian Pear Tree in Korea

**Rosemary Shrestha, Jun Hak Koo, Duck Hwan Park, Ingyu Hwang[1], Jang Hyun Hur and Chun Keun Lim***
*Division of Biological Environment, Kangwon National University, Chuncheon 200-701, Korea*
*[1]School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea*
*(Received on October 2, 2003; Accepted on November 30, 2003)*

**Bacterial strains were isolated from diseased samples of shoot blight collected from different pear growing orchards of Chuncheon, Korea from 1995 to 1998. Forty-nine strains showed their pathogenicity on immature fruit and shoot of pear. Microbiological, physiological, and biochemical tests were performed on these pathogenic strains. One strain, designated as WT3 in this study, was selected as a representative strain as it was collected from the first outbreak area in Jichonri, Chuncheon in 1995. Further detailed characterization of the strain WT3 was done by PCR amplification using specific primers described previously for distinguishing *Erwinia pyrifoliae* from its close pathogen *Erwinia amylovora*. Based on phenotypical, biochemical, and molecular analyses, strain WT3 was identified as a shoot blight pathogen which was the same as *E. pyrifoliae* Ep16 previously described by a German group in 1999.**

***Keywords*** : fire blight, Hwankum, necrogens, phytopathogens, plasmid profiles.

Pear (*Pyrus pyrifolia*) is one of the most important fruit crops in Korea. Major pear growing orchards are located in the southern parts, while a few are in the northern parts of the country. In 1995, necrotic disease in Asian pear tree (*P. pyrifolia* cv. Singo) appeared in the orchards of Chuncheon which is located in the northern part of Korea. Disease symptoms in pear tree were similar to those of *Erwinia amylovora* which causes fire blight in apple, pear, and other rosaceous plants (Van der Zwet and Keil, 1979). Diseased trees show symptoms such as dark brown stripes on the veins of the leaf which spread throughout the shoot affecting the whole plant including leaf stalks, flowers, and immature fruits resulting early fruit drop. Korean scientists initially observed these symptoms and collected diseased samples. Later, a German group worked on this disease and identified the causal microorganism as a new plant pathogen, *Erwinia pyrifoliae* (Kim et al., 1999; Rhim et al., 1999).

Infected shoots and leaves with necrotic disease were collected from different pear orchards from 1995 to 1998. Diseased samples were collected for the first time in 1995 from orchards located in Jichonri, Chuncheon. During the period 1996-1998, more diseased samples were collected from other orchards in Owolri and Duck Duwonri of the same region. After the year 1998, shoot blight could not be observed as the Korean government intensively controlled this disease by eradication so as to prevent its multiplication and dissemination to major pear growing orchards in the southern parts of the country.

To isolate the causal pathogen, approximately 1 g of infected leaf was crushed in sterilized 1.5 ml Eppendorf tube containing 1 ml distilled water. The crushed suspension was diluted in series, plated onto Mannitol Glutamic Yeast (MGY) agar supplemented with 0.25 g yeast extract per liter (Keane et al., 1970), and incubated at 28°C for the growth of isolated pathogens. Single, creamy white to white mucoid colonies were streaked separately and used for this study. Three different preservation methods were used: 1) storage of streaked plates at 4-10°C for their short-term preservation and routine work; 2) preservation at -70°C using nutrient broth containing 20% glycerol; and 3) freeze-drying method using 10% skimmed milk for long-term preservation as described by Perry (1995).

In this study, different reference strains of *E. pyrifoliae* [Ep1 (DSM 12162), Ep4 (DSM 12394), Ep8 (DSM 12393), and Ep16$^T$ (DSM 12163)], *E. amylovora* (ATCC 15580$^T$, LMG 1877, LMG 1946, and LMG 2068), *E. rhapontici* (ATCC 29283$^T$), *Pantoea agglomerans* (ATCC 33243$^T$), *Pectobacterium carotovorum* subsp. *carotovorum* (ATCC 15713$^T$), and *P. chrysanthemi* (ATCC 11663$^T$) were used for identification and comparative studies of physiological, biochemical, biolog, and pathogenic characteristics of all collected strains. WT3 was used as a representative strain of other pathogenic strains and characterized in detail because it was collected from the first outbreak area of Jichonri, Chuncheon in 1995. The WT3 culture was deposited in the Korean Culture Center of Microorganisms

*Corresponding author.
Phone) +82-33-250-6437, FAX) +82-33-256-8254
E-mail) chunkeun@kangwon.ac.kr

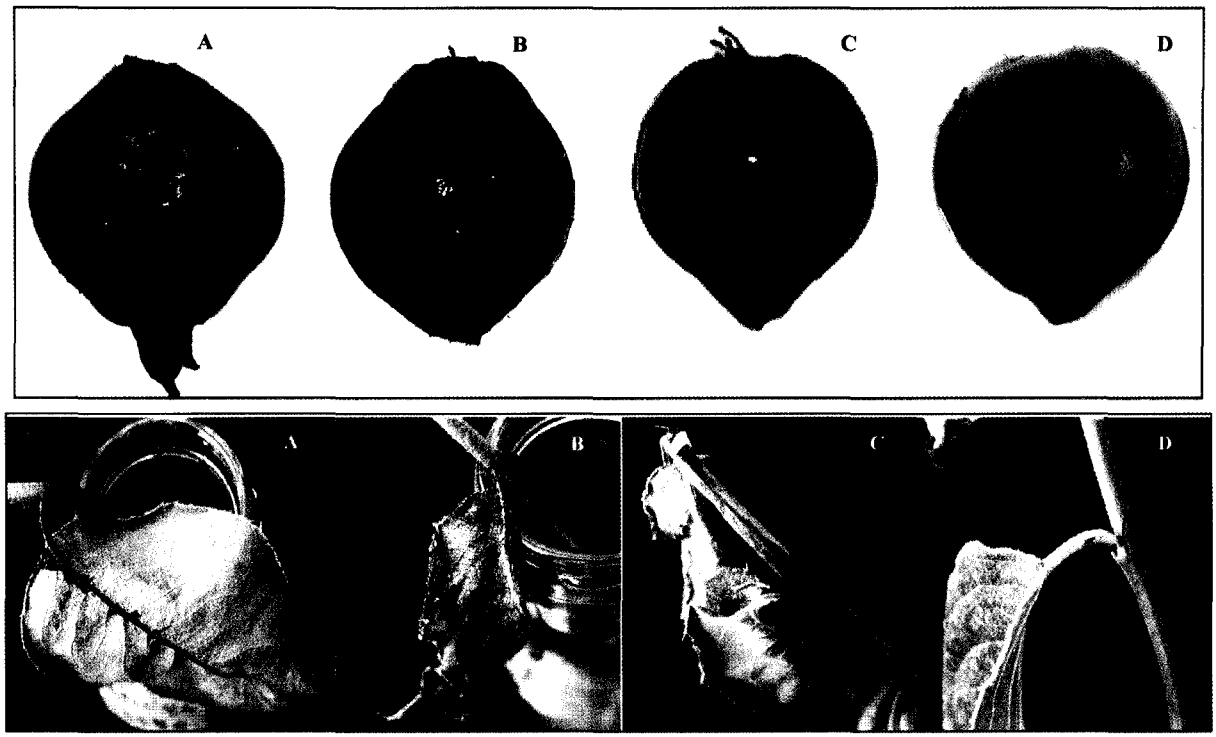


**Fig. 1.** Pathogenicity test for *E. pyrifoliae* and *E. amylovora* using their host pear (*P. pyrifolia* cv. Singo). Ooze formation after 4 days of inoculation on slices of immature pear (upper panel). Necrotic symptom on pear twigs showing its spread from stem to leaves through midrib (lower panel). (**A**) Strain WT3; (**B**) *E. pyrifoliae* Ep16; (**C**) *E. amylovora* ATCC15580; and (**D**) *E. coli*.

with accession number KCCM 10283. In this paper, comparative data of the strain WT3 and that of previously described strains *E. pyrifoliae* Ep16$^T$ and *E. amylovora* ATCC 15580$^T$ were shown.

Pathogenicity test was done both on immature pear fruits and shoots of two cultivars, *P. pyrifolia* cv. Singo and *P. pyrifolia* cv. Hwankum. Immature fruits were first rinsed with sterile water containing 0.5% sodium hypochlorate, then with sterile water, and cut into two halves. Sterilized fruits were either bored with sterile borer 4 mm in size or pinched with sterilized needle. Inoculum of approximately $2 \times 10^7$ colony forming unit (cfu)/ml was inoculated into immature pear and incubated at 28°C. To perform pathogenicity on pear shoot, 12-16 cm size of young shoots were selected, lower leaves trimmed, and dipped into a bottle containing water. The same quantity of inoculum was used for immature pear and injected into shoot with sterile syringe, and maintained at 28°C in the greenhouse. Results were recorded after 4 days of inoculation. Forty-nine strains showed their pathogenicity on immature pear by producing ooze and rotting on a slice of pear. Comparative data of pathogenicity test of the representative strain WT3, *E. pyrifoliae*, and *E. amylovora* are shown in Fig. 1. Similarly, the strain WT3 showed necrotic symptom on pear shoot along with *E. pyrifoliae* and *E. amylovora*. The symptom first appeared on the inoculated area of the shoot and then spread to the leaf through the midrib (Fig. 1).

Colony morphology was studied on LB, MGY, MM1Cu, and MM2Cu agar as described by Bereswill et al. (1998) and Rhim et al. (1999). White and mucoid colonies of strain WT3 along with *E. pyrifoliae* and *E. amylovora* were observed on LB agar. On MGY agar, all three strains showed white to transparent and mucoid colonies whereas, pale yellow mucoid colonies of the strain WT3, and *E. pyrifoliae*, and dark yellow mucoid colonies of *E. amylovora* were observed on MM2Cu agar. However, only *E. amylovora* grew on MM1Cu agar, not the strain WT3 and *E. pyrifoliae*, indicating a distinct cultural characteristic with that of *E. amylovora*.

Physiological and biochemical tests were done as described by Shaad et al. (1988). Most of the physiological and biochemical characteristics of strain WT3, *E. pyrifoliae*, and *E. amylovora* were similar to each other (Table 1). Major differences were observed in gelatin liquefaction and test for acetoin, in which only *E. amylovora* showed positive reaction. However, the strain WT3 produced acid from trehalose as *E. amylovora*, not by *E. pyrifoliae* indicating variation between WT3 and *E. pyrifoliae*. Biolog system [BIOLOG, Hayward, CA 94545, USA] was used to analyze the utilization of 96 different carbon and nitrogen sources by these phytopathogens. The strains were incubated in TSA (tryptic soy agar) at 28°C for 24 h. The culture

**Table 1.** Physiological and biochemical characteristics of *E. pyrifoliae* and *E. amylovora*

| Characteristic | WT3 | *E. pyrifoliae* Ep16$^T$ | *E. amylovora* ATCC 15580$^T$ |
|---|---|---|---|
| Growth in MS medium | + | + | + |
| Growth factor | + | + | + |
| Growth at 36°C | – | – | – |
| Growth of mucoid | + | + | + |
| Motility | + | + | + |
| Pink pigment on YDC | – | – | – |
| Yellow pigment | – | – | – |
| Decomposition of pectate | – | – | – |
| Hydrogen sulfide of cysteine | – | – | – |
| Urease | – | – | – |
| Indole test | – | – | – |
| Reduction of nitrate | – | – | – |
| Liquefaction of gelatine | – | – | + |
| Sensitivity to erythromycine (15 μg/disk) | + | + | + |
| Phosphatase | – | – | – |
| Lecithinase | – | – | – |
| Acetoin | – | – | + |
| Hypersensitivity to tobacco | + | + | + |
| Acid production from | | | |
| Arabinose | – | + | V |
| Cellobiose | – | – | – |
| Inositol | V | V | – |
| Melibiose | – | – | – |
| Methyl α-d glucoside | – | – | – |
| Salicin | – | – | – |
| Trehalose | + | – | + |

$^T$ = type strain; ATCC, American Type Culture Collection, MD., USA.
+ = positive reaction.
– = negative reaction.
V = variable.

strains were suspended to turbidity of 63% in a solution containing 0.4% sodium chloride, 0.03% pluronic F-68, and 0.01% gellan gum, and then inoculated onto the 96 wells. The strain WT3 did not utilize carbon and nitrogen sources such as gentiobiose, succinic acid, bromosuccinic acid, L-asparatic acid, and inosine as *E. pyrifoliae,* while *E. amylovora* utilized all these sources.

Plasmids were isolated from all three strains using the cesium chloride method as described by Sambrook and Russell (2001) (Fig. 2). The strain WT3 and *E. pyrifoliae* showed the same pattern of plasmid profiles. One large plasmid 36 kb was observed both in WT3 and *E. pyrifoliae*. The size and full sequence analysis of large plasmid of *E. pyrifoliae* was done by McGhee (2002) and designated as pEP36. Four additional small plasmids of size ranging from approximately 1 to 5 kb were observed both in strain WT3

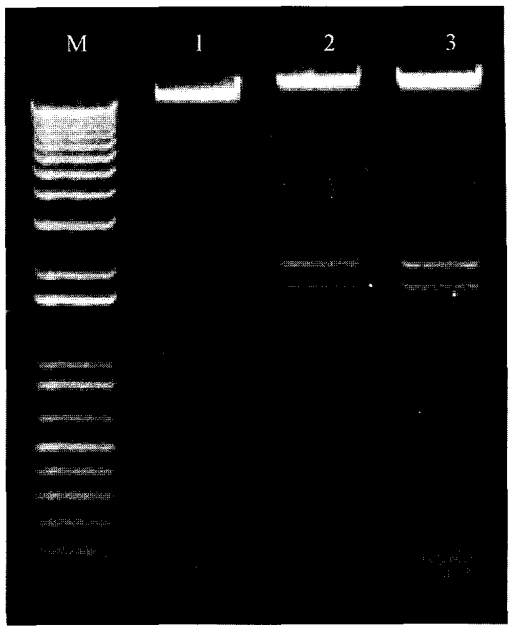


**Fig. 2.** Plasmid profiles of *E. pyrifoliae* and *E. amylovora* in 0.7% agarose gel. M, 1 kb DNA ladder (Gibco, BRL), 1, Strain WT3, 2, *E. pyrifoliae* Ep16, and 3, *E. amylovora* ATCC15580.

and *E. pyrifoliae*. However, *E. amylovora* had only one plasmid of 29 kb size as previously determined by Falkenstein et al. (1989).

Phylogenetic analysis of the strain WT3 was done by nucleotide sequence analysis of its 16S rRNA gene and 16S-23S ITS (intergenic transcribed spacer) region. The software MegAlign package (Window 3.88, Dnastar, Inc., Madison, WI, USA) was used for the alignment of nucleotides of different pathogens as listed in Table 2 along with their accession numbers. The relationship between the pathogens was further analyzed by phylogenetic tree using mega program (Kumar, S., Tamura, K. and Nei, M. 1993. MEGA: molecular evolutionary genetic analysis, version 1.0. The Pennsylvania State University, University Park, PA 16802). The 16S rRNA gene of 1500 bp was amplified by PCR using fD1 and rP2 primers (Weisburg et al., 1991) and cloned into pGEM-T Easy vector for its nucleotide sequence analysis. The GenBank accession number for the 16S rRNA sequence of the strain WT3 is AY509610. The alignment of 16S rRNA gene of the strain WT3 with that of other pathogens showed 98.9%, 97.5%, 95.1%, 94.3%, 93.5%, and 92.7% identities to *E. pyrifoliae*, *E. amylovora*, *E. rhapontici*, *Pantoea agglomerans*, *Pectobacterium carotovorum* subsp. *carotovorum*, and *P. chrysanthemi*, respectively. Phylogenetic tree developed for this comparative study also revealed that the strain WT3 is closer to *E. pyrifoliae* than to *E. amylovora* and other pathogens (Fig. 3).

The 16S-23S ITS region of WT3 was amplified by PCR

**Table 2.** List of strains with their accession numbers used in this study for their nucleotide sequences alignment and phylogenetic relationship analysis

| Species | Accession No. | | |
|---|---|---|---|
| | 16S rRNA gene | Large 16S-23S ITS | Small 16S-23S ITS |
| *E. pyrifoliae* Epl | AJ009930 | AF449669 | AF449664 |
| *E. pyrifoliae* Ep16$^T$ | – | – | AJ132969 |
| *E. amylovora* | – | AF290418 | AF290419 |
| *E. amylovora* ATCC15580$^T$ | U80195 | – | – |
| *E. amylovora* Eal | – | – | AJ010485 |
| *E. rhapontici* ATCC29283$^T$ | U80206 | AF234283 | AF232678 |
| *Pantoea agglomerans* ATCC33243$^T$ | U80202 | – | – |
| *Pectobacterium carotovorum* subsp. *carotovorum* ATCC 15713$^T$ | U80197 | AF234279 | AF232684 |
| *P. chrysanthemi* ATCC11663$^T$ | U80200 | AF234287 | AF232681 |

$^T$ = type strain; ATCC, American Type Culture Collection, MD., USA
– = Sequences not available in GenBank database.

using R16-1F and R23-1R primers (Nakagawa et al., 1994). Two fragments in strain WT3 and *E. pyrifoliae,* and three fragments in *E. amylovora* were amplified. Two amplified fragments of strain WT3 were cloned into pGEM-T Easy vector and then sequenced. The large fragment was 737 bp encoding tRNA$^{Ile}$ and tRNA$^{Ala}$, while the small fragment was 598 bp encoding region tRNA$^{Glu}$. The GenBank accession numbers are AY509611 and AY509612 for the sequence of large and small fragments of 16S-23S ITS region of the strain WT3, respectively. Alignment of nucleotides of large 16S-23S ITS region coding tRNA$^{Ile}$ and tRNA$^{Ala}$ of the strain WT3 with that of other pathogens showed 99.8%, 87.5%, 80.3%, 62.9%, and 57.3% identities to *E. pyrifoliae*, *E. rhapontici*, *E. amylovora*, *Pectobacterium chrysanthemi*, and *P. c.* subsp. *carotovorum*, respectively. Phylogenetic analysis based on nucleotide sequences of this region also revealed that the strain WT3 is closer to *E. pyrifoliae* than to *E. amylovora* and other pathogens (Fig. 4). Moreover, alignment of nucleotides of small 16S-23S ITS region coding tRNA$^{Glu}$ of the strain WT3 with that of other pathogens showed 92.7%, 92.5%, 86.4% 72.8%, 64.8%, 54.2%, and 45.2% identities to *E. pyrifoliae*, *E. rhapontici*, *E. amylovora*, *Pectobacterium chrysanthemi*, and *P. c.* subsp. *carotovorum*, respectively. Similar to the large 16S-23S ITS region, phylogenetic analysis of the small 16S-23S ITS region of the strain WT3 also showed that it is closer to *E. pyrifoliae* than to other pathogens (Fig. 5). Nucleotide sequences of both large and small fragments of 16S-23S ITS region of the strain WT3 and *E. pyrifoliae* had comparatively higher homology to those of *E. rhapontici*

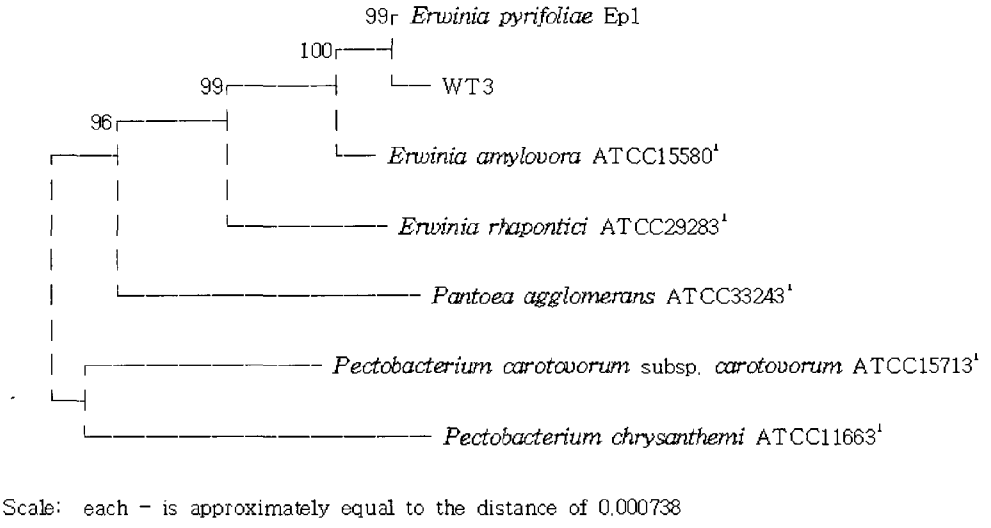


**Fig. 3.** Phylogenetic tree based on the16S rRNA gene sequences of different members of Enterobacteriaceae. The branching pattern was generated by the neighbor-joining method. The numbers at the nodes indicate the levels of bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets.

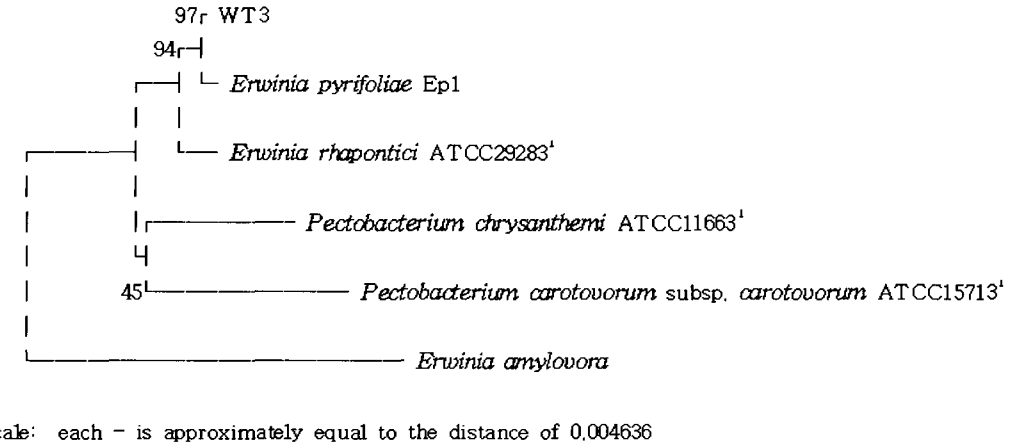


**Fig. 4.** Phylogenetic tree based on the sequences of 16S-23S ITS region coding $tRNA^{Ile}$ and $tRNA^{Ala}$ of different members of Enterobacteriaceae obtained by the neighbor-joining method as in Fig. 3.

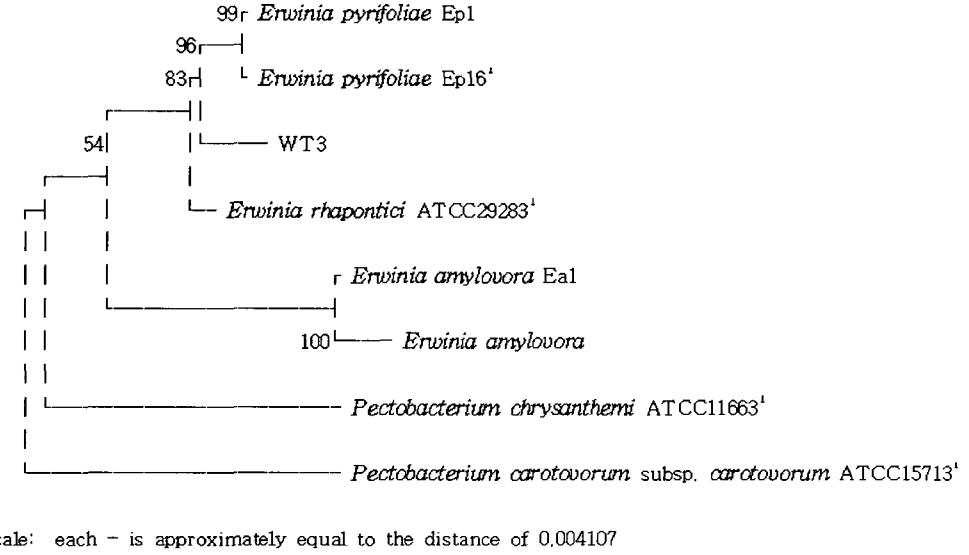


**Fig. 5.** Phylogenetic tree based on the sequences of 16S-23S ITS region coding $tRNA^{Glu}$ of different members of Enterobacteriaceae obtained by the neighbor-joining method as in Fig. 3.

than to *E. amylovora.*

To understand the DNA relatedness of strain WT3, *E. pyrifoliae* and *E. amylovora*, total chromosomal DNA was isolated by cesium chloride method as described by Sambrook and Russell (2001). One hundred of TE buffer, 4 μl of 10N NaOH, and 35 μl of 20 x SSC were added to 100 μl of unlabeled total DNA (1 μg/μl) and denaturated by boiling at 80°C for 10 min. Denatured DNA was applied to Hybond-$N^+$ nylon membranes by slot blot apparatus. Total chromosomes of these three strains, WT3, *E. pyrifoliae* and *E. amylovora* DNA, were used for probe labeling. Each native DNA was labeled with DIG11-dUTP by DIG-High Prime (Roche Molecular Biochemicals, Germany), prehybridized at 49°C for 3 h, and then hybridized for 16 h at the same temperature. Hybridization signal was detected by DIG Luminescent Detection Kit (Roche Molecular Biochemicals, Germany). Hybridized signals were quantified by Densitometer (Bio-Rad, Laboratories, Hercules, CA). The strain WT3 was 102-110% related to *E. pyrifoliae,* whereas, low hybridized signal was observed with 50-60% relatedness to *E. amylovora.*

Specific primers CPS1, CPS2c, Ep16A, Ep16G2c, AMSbL, AMSbR, AMS1, and AMS2c, previously reported by Bereswill et al. (1998) and Kim et al. (2001a, 2001b), were used for further characterization of the strain WT3. The fragments of 1.2 kb and 0.73 kb were amplified both from the strain WT3 and *E. pyrifoliae* by specific primers CPS1-CPS2c and Ep16A-Ep16G2c, respectively, but not from *E. amylovora* (Fig. 6). However, fragment of 1.6 kb was amplified only from *E. amylovora* by specific primers AMSbL-

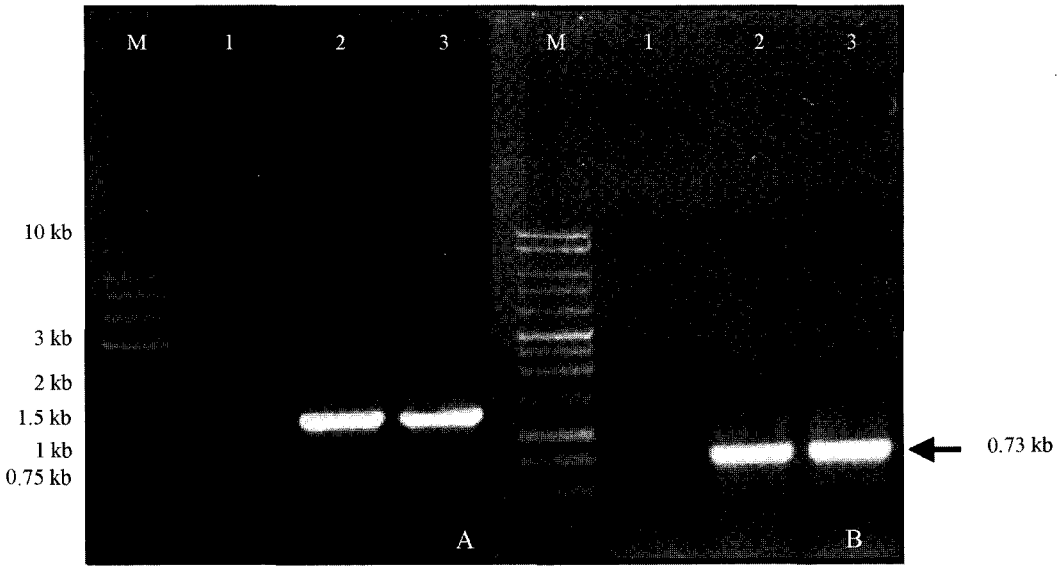


**Fig. 6.** PCR assay for *E. pyrifoliae* and *E. amylovora* using specific primers of *E. pyrifoliae* in 0.7% agarose gel. (**A**) Amplification of 1.2 kb fragment using CPS1 and CPS2c primers. B) Amplification of 0.73 kb by Ep16A and Ep16G2c primers. **M**, 1 kb DNA ladder (Promega), **1**, Strain WT3, 2, *E. pyrifoliae* Ep16, and **3**, *E. amylovora* ATCC15580.

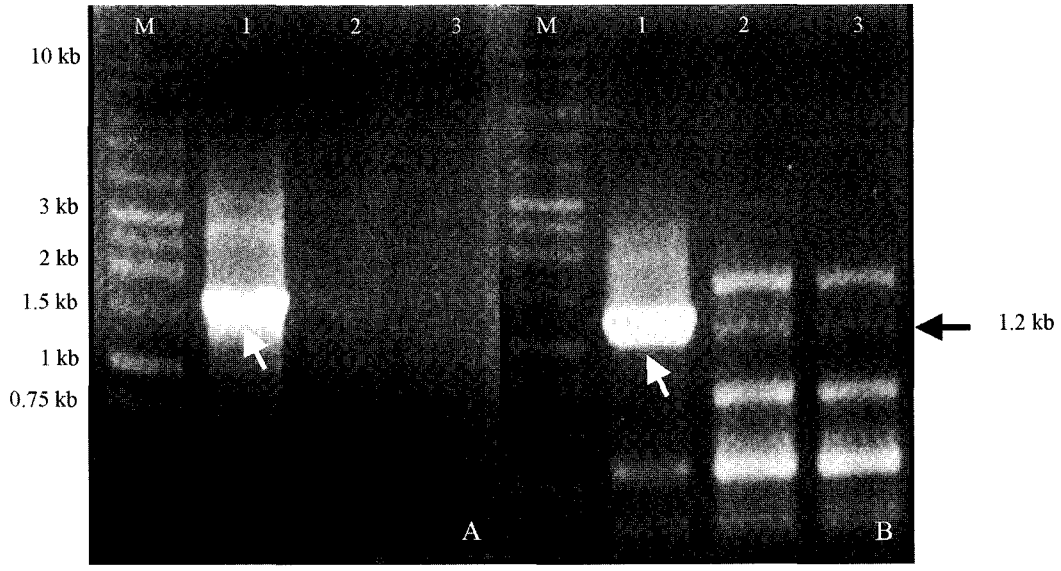


**Fig. 7.** PCR assay for *E. pyrifoliae* and *E. amylovora* using specific primers of *E. amylovora* in 0.7% agarose gel. (**A**) Amplification of 1.6 kb fragment using AMSbL and AMSbR primers. (**B**) Amplification of 1.2 kb by AMS1 and AMS2c primers. M, 1 kb DNA ladder (Promega), 1, Strain WT3, 2, *E. pyrifoliae* Ep16, and 3, *E. amylovora* ATCC15580.

AMSbR but not from the strain WT3 and *E. pyrifoliae* (Fig. 7). Another sharp 1.2 kb fragment was amplified by primers AMS1-AMS2c *E. amylovora,* but only a smear fragment of the same size was amplified from the strain WT3 and *E. pyrifoliae*. These results suggest that the strain WT3 is closer to *E. pyrifoliae* than to *E. amylovora.*

Based on the results of this study, the representative strain was identified as phytopathogen *E. pyrifoliae* WT3, which produced disease symptoms as those of previously described *E. pyrifoliae* Ep16 (Kim et al., 1999; Rhim et al., 1999). This is the first report on the endemic phytopathogen, *E. pyrifoliae* WT3, carried out by the Korean group. Although phenotypical and biochemical characters of *E. pyrifoliae* are similar to those of *E. amylovora*, detailed study supported that these two pathogens are different from each other in their distribution, plasmid profiles, low sequence homology of their total genomes, and 16S-23S ITS region as previously reported by Kim et al. (1999, 2001a, b) and

McGhee et al. (2002). Besides these characteristics, comparative *in vitro* growth studies at different temperatures and pH levels showed that these two pathogens are different from each other as *E. pyrifoliae* had cold tolerance than *E. amylovora* (Shrestha et al., 2001). It is important to note that Chuncheon is comparatively colder than the other pear growing orchards in the southern part of the country from where *E. pyrifoliae* has not been previously reported. The outbreak of *E. pyrifoliae* in the restricted areas of Chuncheon could be related to its cold-tolerant character.

## References


Bereswill, S., Pahl, A., Bellemann, P., Zeller, W. and Geider, K. 1992. Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis. *Appl. Environ. Microbiol.* 58:3522-3526.

Bereswill, S., Jock, S., Bellemann, P. and Geider, K. 1998. Identification of *Erwinia amylovora* by growth in the presence of copper sulfate and by capsule staining with lectin. *Plant Dis.* 82:158-164.

Falkenstein, H., Zeller, W. and Geider, W. 1989. The 29 kb plasmid common in strains of *Erwinia amylovora*, modulates development of fire blight symptoms. *J. Gen. Microbiol.* 135:2643-2650.

Keane, P. J., Kerr, A. and New, P. B. 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agribacterium* isolates. *Aust. J. Biol. Sci.* 23:85-595.

Kim, W.-S., Hildebrand, M., Jock, S. and Geider, K. 2001a. Molecular comparison of pathogenic bacteria from pear trees in Japan and the fire blight pathogen *Erwinia amylovora*. *Microbiology* 147:2951-2959.

Kim, W.-S., Jock, S., Paulin, J.-P., Rhim, S.-L. and Geider, K. 2001b. Molecular detection and differentiation of *Erwinia pyrifoliae* and host range analysis of the Asian pear pathogen. *Plant Dis.* 85:1183-1188.

Kim, W.-S., Garden, L., Rhim, S.-L. and Geider, K. 1999. *Erwinia pyrifoliae* sp. nov., a novel pathogen that affects Asian pear trees (*Pyrus pyrifolia* Nakai). Intl. J. Sys. Bacteriol. 49:899-906.

McGhee, G. C., Schnabel, E. L., Maxson-Stein, K., Jones, B., Stromberg, V. K., Lacy, G. H. and Jones, A. L. 2002. Relatedness of chromosomal and plasmid DNAs of *Erwinia pyrifoliae* and *Erwinia amylovora*. *Appl. Environ. Microbiol.* 63:4421-4426.

Nakagawa, T., Shimada, M., Mukai, H., Asada, K., Kato, I., Fujino, K. and Sato, T. 1994. Detection of alcohol-tolerant Hiochi bacteria by PCR. *Appl. Environ. Microbiol.* 60:637-640.

Perry, S. F. 1995. Freeze drying and cryopreservation of bacteria. pp. 21-30. In: Methods in molecular biology. J. G. Day and M. R. McLellan (eds.) Humana Press Inc.

Rhim, S.-L., Voelkisch, B., Gardan, L., Paulin, J.-P., Langlotz, C., Kim, W.-S. and Geider, K. 1999. An *Erwinia* species, different from *E. amylovora* cause a necrotic disease of Asian pear trees. *Plant Pathol.* 48:514-520.

Sambrook, J. and Russell, D. W. 2001. Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor, NY, U.S.A.

Schaad, N. W., Jones, J. B. and Chun, W. 1988. Laboratory guide for identification of plant pathogenic bacteria. 2nd ed. Amer. Phytopath. Soc., MN, USA.

Shrestha, R., Hur, J. H. and Lim, C. K. 2001. The effects of temperature and pH on the growth of Asian pear pathogen, *Erwinia pyrifoliae*. (abstr.) *Phytopathology.* 91(suppl.):S82.

Van der Zwet T. and Keil, H. L. 1979. Fire blight: a bacterial disease of rosaceous plants. Agriculture Handbook No. 510, US Government printing office, Washington, DC.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173:697-703.