Symposium Session III : Taxonomy and Molecular Diagnostics

SIII-1

Taxonomy and molecular diagnostics of *Erwinia* species

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Introduction

Several Gram-negative, non-spore-forming, facultatively anaerobic, rod-shaped bacteria have been classified traditionally into the genus *Erwinia*, mainly based on their association with plants as pathogens, epiphytes or saprophytes. The *Erwinia* species, which cause diseases on different plants, are categorized into two groups: 1) the 'amylovora' group and 2) the 'soft-rot' group (Dye, 1968, 1969). The 'amylovora' group includes two major pathogens, *E. amylovora* and *E. pyrifoliae*, which cause necrotic or wilting symptoms on their host plants. In Korea, however, only *E. pyrifoliae*, which causes shoot blight in Asian pear, has been reported. The 'soft-rot' group involves the pathogens, which produce pectolytic enzymes and cause rot diseases on various agricultural crops such as potato, sugar beet, chicory, etc. *E. carotovora*, *E. chrysanthemi*, *E. cypripedii*, and *E. rhapontici* are included in this group. Among these *Erwinia* species, *E. carotovora* is the major causal agent of soft-rot disease of different crops. This paper describes diagnostic methods for *E. pyrifoliae* and *E. carotovora* based on their pathogenic, biochemical, physiological, and genetic characteristics.

Erwinia pyrifoliae, a phytopathogen of 'amylovora' group:

The endemic phytopathogen, *E. pyrifoliae*, was first reported in Asian pear orchards of Chuncheon, Korea in 1995 (Rhim et al., 1999; Kim et al., 1999; Shrestha et al., 2003). The disease symptoms were similar to 'fire blight' caused by *E. amylovora*. However, the host range of *E. pyrifoliae* differs from that of *E. amylovora*, being restricted to pear and not infecting apple or other rosaceous plants (Kim et al., 2001a). Also, *E. pyrifoliae* produces acid when grown on arbutin, esculin and cellobiose, produces gelatinase, and assimilates melibiose, gentiobiose, and succinamic acid as distinct to *E. amylovora*. Furthermore, the *in vitro* growth study showed that *E. pyrifoliae* had favorable growth at lower temperatures than at higher temperatures when compared to that of *E. amylovora* (Shrestha et al., 2001).

Recent molecular approaches have further distinguished and characterized *E. pyrifoliae*. All known strains of *E. pyrifoliae* contain one large plasmid of 36 kb (pEP36) and four additional small plasmids of different sizes ranging from 1-5 kb (Kim et al., 1999; McGhee et al., 2002; Shrestha et al., 2003). In contrast, *E. amylovora* has only one plasmid (pEA29) of 29 kb (Falkenstein et al., 1989). Sequence analyses of the 16S rRNA gene and the 16S-23S ITS (intergenic transcribed spacer) region show high homology between *E. pyrifoliae* and *E. amylovora*. However, a 0.73 kb fragment of 16S rRNA gene and 16S-23S ITS region is amplified only from *E. pyrifoliae* by PCR using specific primers Ep16A-Ep16G2c, but not from *E. amylovora* (Kim et al., 2001b). Similarly, a 1.2 kb fragment of the capsular polysaccharide (*cps*) region is amplified from *E. pyrifoliae*, but not from *E. amylovora* using specific primers CPS1-CPS2c (Kim et al., 2001b). It is noteworthy that *cps* region of *E. pyrifoliae* is highly homologous to amylovoran synthesis (*ams*) region of *E. amylovora*. The specific primers (AMSbL-AMSbR) prepared from *ams* region, were also useful to distinguish these two pathogens since the fragment of 1.6 kb was amplified only in *E. amylovora* but not in *E. pyrifoliae* (Kim et al., 2001b). These results suggested that PCR amplification using specific primers would be a rapid tool for distinguishing these two close pathogens. Differentiate of *E. pyrifoliae* from *E. amylovora* is also demonstrated by the DNA-DNA hybridization with *E. pyrifoliae* showing only 50-60% relatedness to *E. amylovora* in comparison to 102-110% of DNA relatedness amongst *E. pyrifoliae* strains (Shrestha et al., 2003). Genomic DNA analyzed by pulsed-field gel electrophoresis (PFGE) after *Xba*I and *Spe*I digestion also distinguished these two pathogens (Kim et al., 2001b). Furthermore, *groEL* analysis, which encodes a heat shock protein essential for cellular growth in *Escherichia coli*, showed sufficient resolving power for discriminating these two species (Maxson-Stein et al., 2003).

Recently, comparative study on HR related genes $hrpN_{Ep}$ and $hrpN_{Ea}$ of *E. pyrifoliae* and *E. amylovora*, respectively, were investigated (Shrestha et al., unpublished). Intergenic nucleotides insertion fragments (INFIs) were observed in $hrpN_{Ep}$ gene when aligned with $hrpN_{Ea}$ gene. Specific primers, which amplify $hrpN_{Ep}$ from *E. pyrifoliae* but not $hrpN_{Ea}$ from *E. amylovora*, have been developed (Shrestha et al., unpublished). In addition, *Sma*I cleavage site exist in $hrpN_{Ea}$ but not in $hrpN_{Ep}$ allowing restriction enzyme analysis to further distinguish the species. Lastly, $hrpN_{Ep}$ is cloned in a 8.4 kb *Hin*dIII-*Hin*dIII fragment from *E. pyrifoliae* whilst $hrpN_{Ea}$ resides in a 1.3 kb *Hin*dIII-*Hin*dIII fragment (Wei et al., 1992), demonstrating additional differences in generic background between these two species. These results show *E. pyrifoliae* and *E. amylovora* possess distinct molecular characteristics, which may be used to distinguish these two pathogens and indicate that they might have evolved independently.

Erwinia carotovora, a phytopathogen of the 'soft-rot' group:

E. carotovora, which causes severe diseases on various economically important crops, is a complex pathogen since the strains are diverse at various levels. The diversity within the population might be due to several factors, such as genetic change,

host plants, migration from other geographic areas, etc. *E. carotovora* is divided into five subspecies: *atroseptica* (Eca), *carotovora* (Ecc), *betavasculorum* (Ecb), *wasabiae* (Ecw) and *odorifera* (Eco) based on their physiological and biochemical features, and pathogenesis (Thomson et al., 1981; Goto and Matsumoto, 1987; Annie et al., 1992). Recently, one additional taxa, *E. carotovora* subsp. *brasiliensis* has been described as a member of *E. carotovora* (Duarte et al., 2004). Ecc strains have a wide distribution in both temperate and tropical zones with wider host ranges than those of other subspecies (Perombelon and Kelman, 1980; Wells and Moline, 1991). Eca, a close phytopathogen of Ecc, causes serious disease on potato called potato black leg, which is responsible for severe economic losses in potato cultivation, especially in North America and Europe. Generally, Ecc and Eca strains can be differentiated based on acid production from α-methyl glucoside, production of reducing substances from sucrose, and ability to grow at 36°C (De Boer et al., 1978). However, variations of Ecc strains in these properties were also reported (Tanii, 1984; Karnjanarat et al., 1987). In addition, serological tests (De Boer et al., 1987) and RFLP analysis of pectate lyase encoding gene (*pel*) of Ecc strains showed a heterogenecity of this subspecies (Darrasse et al., 1994; Helias et al., 1998). These results indicated that Ecc strains were more variable than Eca strains. Since most of the studies concerning genetic and phenotypic diversity of the pathogen have not included Asian strains, and little is known about the diversity of Ecc strains in Asian areas, this paper describes the phenotypic and pathogenic characteristics of 87 Ecc strains isolated from Japan, Korea and Thailand.

Based on pathogenicity on potato, tomato, onion, and cucumber, the strains were divided into four groups. All tested strains were pathogenic to potato slices. Cluster analysis based on 26 phenotypic characters of Ecc strains produced two groups, A (typical Ecc) and B (atypical Ecc). Ecc strains of group A did not produce reducing substances from sucrose and acids from α-methyl glucoside whilst group B did. All Thai strains and most of Korean strains belonged to group A, whereas Ecc strains of group B were isolated in Japan and Korea. The properties of this group were similar to those of Eca. Analysis of 16S rDNA RFLP generated by *HinfI*, Ecc strains were again differentiated into two groups, with most strains from Korea and Japan belonged to the same group. The *MboI* restriction of amplified ISRs genes showed two patterns among Ecc strains. All Thai strains showed the same pattern. In the analysis of the *pel* gene RFLP with *Sau*3AI, all strains were separated into two independent patterns except for one strain. The strain isolated from mulberry showed a unique RFLP pattern of the *pel* gene. When the combined RFLP groups of 16S rDNA, ISRs and *pel* gene were compared with the two phenotypic groups, a close relationship was detected between genetic groups and phenotypic ones. We also performed ERIC-PCR to differentiate the Eca and Ecc. Similarity of ERIC-PCR patterns within the Eca strains was very high, whereas within the Ecc group extensive genetic diversity was found. An ERIC-PCR fragment from Eca type strain was isolated, and it was used as probe for Southern hybridization. The probe hybridized only with Eca strains. We have shown the potential for rapid identification of Ecc and Eca strains by ERIC-PCR analysis.

In a previous study, one mulberry strain differed in physiological characteristics of Ecc in both physiological and biochemical properties. The strain differed from Ecc especially in production of acid from α-methyl glucoside and reducing substances from sucrose. Moreover, the strain showed an unique RFLP pattern from amplified *pel* gene sequence. Thus, we tested several additional strains from mulberry and reference strains for their differentiation among soft rot *Erwinia* species. Based on the results of biochemical tests, the mulberry strains were divided into two different types, type 1 and type 2. Two strains of type 1 were similar to Ecc, whereas seven strains of type 2 were distinct from Ecc. The type 2 strains were assessed by performing a polyphasic study, and the data were compared to those of related *E. carotovora* subspecies. The results of specific PCR assay for Eca showed that the type 2 strains were distinct from Eca. SDS-protein profiles of type 2 strains were highly similar to each other and easily distinguished from those of Ecc as well as other soft rot *Erwinia* species, and they had the specific peptide band for type 2 strains at ca. 28 kDa. In the RFLP analysis of the *pel* gene using *Sau*3AI, the type 2 strains showed an unique RFLP pattern. Based on RAPD analysis, similarity of RAPD patterns within the type 2 strains was very high. An unique RAPD fragment from the type 2 strains was isolated and it was used as a probe for Southern hybridization. This probe hybridized only with PCR products from the type 2 strains. This was not a definitive conclusion but a strong indication that type 2 strains of *E. carotovora* could indeed belong to different subspecies.

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SIII-2

Plant quarantine: another way to control plant diseases

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Plant pathology ultimately tries to develop control methods for plant diseases. The various control methods are classified as regulatory, cultural, biological, and chemical, depending on the agents employed (Agrios, 1997). Plant diseases can be more effectively controlled when these methods are integrated. However, the importance of the regulatory methods has been overlooked rather than that of other methods. Regulatory methods aim at excluding pathogens from hosts or from certain areas by means of legal procedures including plant quarantines.

When foreign pests are carelessly introduced to new areas of the world, serious losses can result (Waterworth and White, 1982). The Dutch elm fungus, the chestnut blight fungus, the grape downy fungus and the citrus canker bacterium are examples of pathogens that caused severe damage after introduction in Europe and USA. The examples are also found in Korea. Introduced pathogens from overseas are listed up to 22 species since 1910. Among the pathogens, the potato blight fungus, the rice blight bacterium and the carnation leaf spot fungus cause severe damage (Lee, 1996). Recently the diseases caused by the tomato Verticillium wilt fungus and the gourd root rot fungus have been severely occurred over the country.

Plant quarantine aims to prevent the introduction and spread of harmful pests to new areas. The first evidence of the plant quarantine was in France in 1660 when a barberry suppression edict was issued in order to prevent the spread of wheat stem rust (Palm, 1999). Many countries began to enact plant quarantine legislation from the end of 19C. In Korea, quarantine legislation was first enacted on imported cherry trees and other fruit trees in 1912, but the real quarantine practices had not been conducted until promulgation of the Plant Protection Act in 1961.

In order to develop more precise requirement for international phytosanitary measures, the Agreement on Sanitary and Phytosanitary Measures (SPS) under the World Trade Organization was adopted in 1994 (WTO, 1994). The main goal of