Plant Pathol. J. 20(4) : 283-288 (2004)

Characterization of Korean *Erwinia carotovora* Strains from Potato and Chinese Cabbage

Sang-Tae Seo, Jun-Hak Koo[1], Jang-Hyun Hur[1] and Chun-Keun Lim[1]*

Division of Horticultural Environment, National Horticultural Research Institute, RDA, Suwon 441-440, Korea

[1]Division of Biological Environment, College of Agriculture and Life Sciences, Kangwon National University, Chunchon 200-701, Korea

(Received on May 21, 2004; Accepted on August 3, 2004)

Four *Erwinia carotovora* strains isolated from potatoes showing blackleg symptoms and rotted Chinese cabbage were analysed by biochemical tests and sequence analysis of 16S rDNA and 16S-23S rRNA intergenic spacer (IGS) regions, and the data were compared to related *E. carotovora* strains. Based on the results of the biochemical tests and sequence analysis, 2 of the 4 strains were identified as *E. carotovora* subsp. *carotovora* (Ecc), whereas the rest strains were distinct from Ecc. The last two strains, HCC3 and JEJU, were biochemically similar to *E. carotovora* subsp. *atroseptica* (Eca). However, the results of sequence analysis and Eca-specific PCR assays showed that the strains were distinct from Eca. On the basis of 16S rDNA sequence analysis, HCC3 and JEJU strains were placed in *E. carotovora* subsp. *odorifera* and *E. carotovora* subsp. *wasabiae*, respectively. The results of sequence analysis and specific PCR assay for Eca indicated that Asian Eca strains were distinct from European Eca strains, although they were phenotycally homogeneous.

Keywords : Chinese cabbage, *Erwinia carotovora*, PCR, potato

Erwinia carotovora, enteric bacterial species, has been particularly studied because of its pathogenicity to many different plant species, of which the potato is the most important. *E. carotovora* was divided into five subspecies including *carotovora* (Ecc), *atroseptica* (Eca), *betavasculorum* (Ecb), *wasabiae* (Ecw) and *odorifera* (Eco) on the basis of biochemical, pathological and molecular traits (De Boer and Kelman, 2000). Recently, one additional subspecies, namely, *E. carotovora* subsp. *brasiliensis* has been described as a new member of the *E. carotovora* (Duarte et al., 2004). The most important of the *E. carotovora* commercially are Ecc and Eca.

The identification of *E. carotovora* strains has been largely based on biochemical characters. Recently, DNA analysis techniques are now widely used for their identification (Darrasse et al., 1994; Toth et al., 2001). The phylogenetic relationships between the *Erwinia* species have been investigated by sequence analysis of their 16S rDNA (Hauben et al., 1998). Sequence polymorphisms in the 16S-23S rRNA intergenic spacer (IGS) regions can be also used to differentiate *E. carotovora* strains (Fessehaie et al., 2002). Primers were selected for detection of Eca using PCR (De Boer and Ward, 1995).

Four bacterial strains were isolated from potatoes showing blackleg symptoms and rotted Chinese cabbage and then subjected to identification on the basis of biochemical characteristics. Furthermore, the results are compared with those obtained using other methods including Eca-specific PCR and sequence analysis of 16S rDNA and 16S-23S IGS regions.

Materials and Methods

Bacterial strains. The strains investigated in this study are listed in Table 1. All strains were stored at -70°C. When required, each bacterial strain was cultured aerobically on YPDA (yeast extract 3 g, peptone 0.6 g, dextrose 3 g, agar 15 g, in 1 L distilled water, pH 7.2) for 2 days at 28°C.

Phenotypic characteristics. Twenty-one physiological and biochemical tests were performed according to the methods reported by De Boer and Kelman (2000). Tests included: potato soft rot; cavity formation on CVP medium at 27°C; growth on NA at 37°C; growth in 5% NaCl; sensitivity to erythromycin; production of reducing substances from sucrose; product of indole and phosphates; acid production from lactose, maltose, α-methyl glucoside, trehalose, cellobiose, sorbitol, inulin, raffinose, palatinose, melibiose and D-arabitol; utilization of organic acid citrate and malonate.

PCR amplification. Bacterial DNA was extracted by the method Ausubel et al. (1987), except that the lysates were extracted twice with chloroform to remove residual phenol. PCR analysis was performed with a DNA thermal cycler (GeneAmp, Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The 16S rDNA fragment amplified with universal primers fD1 and rP2 (Weisburg

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

Table 1. List of bacterial strains used in this study

Strain[a]	Host plant	Origin and year of isolation
JEJU	*Solanum tuberosum*	Jeju, Korea, 1998
HCC3	*Brassica campestris*	Hongcheon, Korea, 1999
DAEPO4	*Solanum tuberosum*	Hoengseong, Korea, 1998
NWP	*Solanum tuberosum*	Namwon, Korea, 1998
E. carotovora subsp. *atroseptica*		
MAFF 301629	*Solanum tuberosum*	Japan
MAFF 301630	*Raphanus sativus*	Japan
LMG 2375	*Solanum tuberosum*	U.K.
LMG 6693	*Solanum tuberosum*	Sweden
ATCC 33260^T	*Solanum tuberosum*	U.K.
HCP1	*Solanum tuberosum*	Hongcheon, Korea, 1999
E. carotovora subsp. *carotovora*		
ATCC 15713^T	*Solanum tuberosum*	Denmark
E. carotovora subsp. *wasabiae*		
ATCC 43316^T	*Eutrema wasabi*	Japan
E. carotovora subsp. *betavasculorum*		
ATCC 43762^T	*Beta vulgaris*	U.K.
E. chrysanthemi		
ATCC 11663^T	*Chrysanthemum morifolium*	U.K.

[a]MAFF, Ministry of Agriculture, Forestry and Fisheries Genebank, Japan; ATCC, American Type Culture Collection; LMG, Laboratorium voor Microbiologie, Gent.

et al., 1991). Amplification was performed in a total volume of 50 μl containing 20 pmol each primer, 200 mM of mixture from dATP, dCTP, dGTP, dTTP (Promega, Southampton, England), 2 mM $MgCl_2$, 1× buffer, template DNA (ca. 20 ng) and 2.5 units of *Taq* polymerase (Promega), under the following reaction conditions; 94°C for 4 min for initial denaturation, 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 3 min, followed by a final elongation step of 72°C for 10 min. The 16S-23S rRNA intergenic spacer (IGS) regions were amplified by using primers R16-1F and R23-1R (Nakagawa et al., 1994). Reaction mixtures for PCR of IGS regions were prepared as described above. Amplification was performed under the following conditions; initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and final extension at 72°C for 3 min, and final extension at 72°C for 10 min.

Detection by a specific PCR assay of Eca was performed using primers ECA1f and ECA2r (De Boer and Ward, 1995) following a protocol described previously (Seo et al., 2002).

Cloning and sequencing. PCR products of 16S rDNA and IGS regions were analysed by electrophoresis in 1% (w/v) agarose gels using 0.5× Tris-borate- EDTA (pH 8.0), and then the products were purified with a QIA quick gel extraction kit (Qiagene GmbH, Hilden, Germany). Purified DNAs were ligated into pGEM-T easy vector (Promega). Plasmids containing the 16S rDNA and IGS regions were then sequenced directly by cycling sequencing using an ALFred autocycle sequencing kit with M13 forward and reverse primers.

Phylogenetic analysis. The software MegaAlign package (Window 3.88, Dnastar, Inc., Madison, WI, USA) was used for the alignment of nucleotides. The relationship between the strains was further analyzed by phylogenetic tree using Mega program (MEGA: molecular evolutionary genetic analysis, version 1.0, The Pennsylvania State University).

Results and Discussion

The results of 21 physiological and biochemical tests indicated that the four strains isolated from potato (JEJU, DAEPO4 and NWP) and Chinese cabbage (HCC3) were *E. carotovora* (Table 2). They were also confirmed as a soft rot pathogen by inoculation tests with potato and Chinese cabbage (data not shown). Eca can be distinguished from Ecc on the basis of acid production from α-methyl glucoside, production of reducing substances from sucrose, and ability to grow at 36°C (De Boer et al., 1978). Of the four strains, two strains (DAEPO4 and NWP) were identified by our tests as Ecc. However, the characteristics of the rest strains (JEJU and HCC3) were similar to those of Eca reference strains which did not grow at 36°C (Table 2). On the basis of Eca-specific PCR assays, the both strains produced no PCR product in contrast to the single 690 bp amplicon obtained with three European Eca strains tested (Fig. 1). In addition, two Japanese Eca strains (MAFF 301629 and MAFF 301630) and Korean Eca strain (HCP1) were not amplified under the conditions used. This lack of detection might be correlated with the geographical origin of these strains. Dellagi et al. (2000) reported that sequencing of the 690 bp product, generated by PCR using primers ECA1f and ECA2r revealed similarity to a sequence in *Escherichia coli* encoding formate acetyltransferase. We hypothesize that the sequence of its gene in Asian Eca

Table 2. Physiological and biochemical characteristics of *E. carotovora* strains

Characteristic	HCC3	JEJU	DAEPO4	NWP	MAFF 301629	MAFF 301630	LMG 6693	LMG 2375	Ecc[a]
Potato soft-rot	+	+	+	+	+	+	+	+	+
Cavity formation on CVP	+	+	+	+	+	+	+	+	+
Growth at 36-37°C	−	−	+	+	−	−	−	−	+
Growth in 5% NaCl	+	+	+	+	+	+	+	+	+
Sensitivity to erythromycin	−	−	−	−	−	−	−	−	−
Reducing substance from sucrose	+	−	+	−	+	+	+	+	−
Indole porduction	−	−	−	−	−	−	−	−	−
Phosphatase activity	−	−	−	−	−	−	−	−	−
Acid production from									
lactose	+	+	+	+	+	+	+	+	+
maltose	+	+	−	−	+	+	+	+	−
α-methyl glucoside	−	−	−	+	+	−	−	−	−
trehalsoe	−	+	−	−	+	+	+	+	+
cellobiose	+	+	+	+	+	+	+	+	+
sorbitol	+	−	−	−	−	−	−	−	−
inulin	−	−	−	−	−	−	−	−	−
raffinose	+	+	+	+	+	+	+	+	+
palatinose	+	−	−	−	+	+	+	−	−
melibiose	+	+	+	+	+	+	+	+	+
d-arabitol	−	−	−	−	−	−	−	−	−
Utilization of organic acid									
citrate	+	+	+	+	+	+	+	+	+
malonate	−	−	−	−	−	−	−	−	−

[a] Data from De Boer and Kelman (2000) and Lelliot and Dickey (1984).

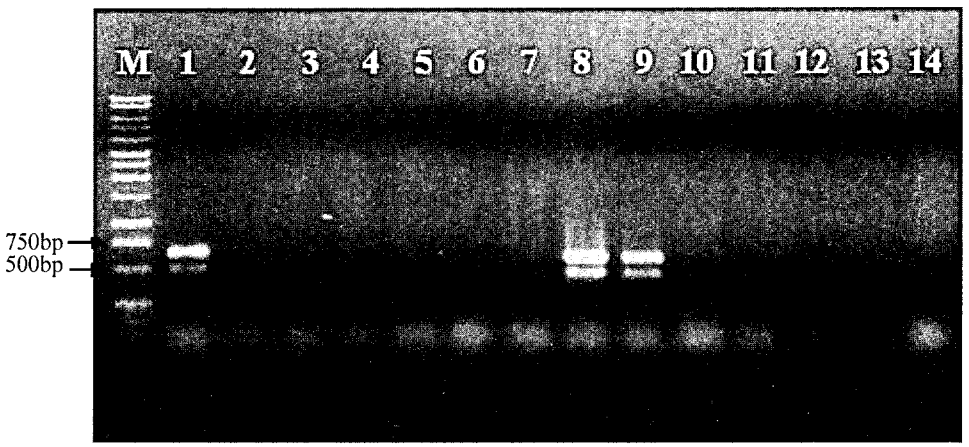

Fig. 1. Agarose gel electrophoresis of PCR-amplification products generated from Eca strains with primers ECA1f and ECA2r. Lanes 1-16 contain, respectively, ATCC 33260^T, ATCC 15713^T, ATCC 43316^T, ATCC 43762^T, ATCC 11663^T, MAFF 301629, MAFF 301630, LMG 2375, LMG 6693, HCP1, HCC3, JEJU, NWP, DAEPO4. Molecular size marker was run in lane M.

strains are different from those of European Eca strains.

The 16S rDNA sequence of the four strains was consistent with its identity as a member of the *E. carotovora* species (Fig. 2). Results of 16S rDNA sequence analysis showed high similarity between HCC3 and Eco reference strains. Similarly, two Japanese Eca strains (MAFF 301629 and MAFF 301630) and one Korean Eca strain (HCP1) grouped with the Eco strains rather than with the other *E. carotovora* subspecies. Strain JEJU was similar to Ecw with sequence identities of 98.4-99.1%. The identification and classification of many microorganisms now depend heavily on rRNA gene sequences (Asai et al., 1999). Focusing on the 16S rDNA sequence as major identification tool, two strains HCC3 and JEJU were identified as Eco and Ecw, respectively.

16S-23S IGS regions exhibit a greater sequence and length variation. Thus, the IGS regions are suitable for differentiating below the species level (Toth et al., 2001).

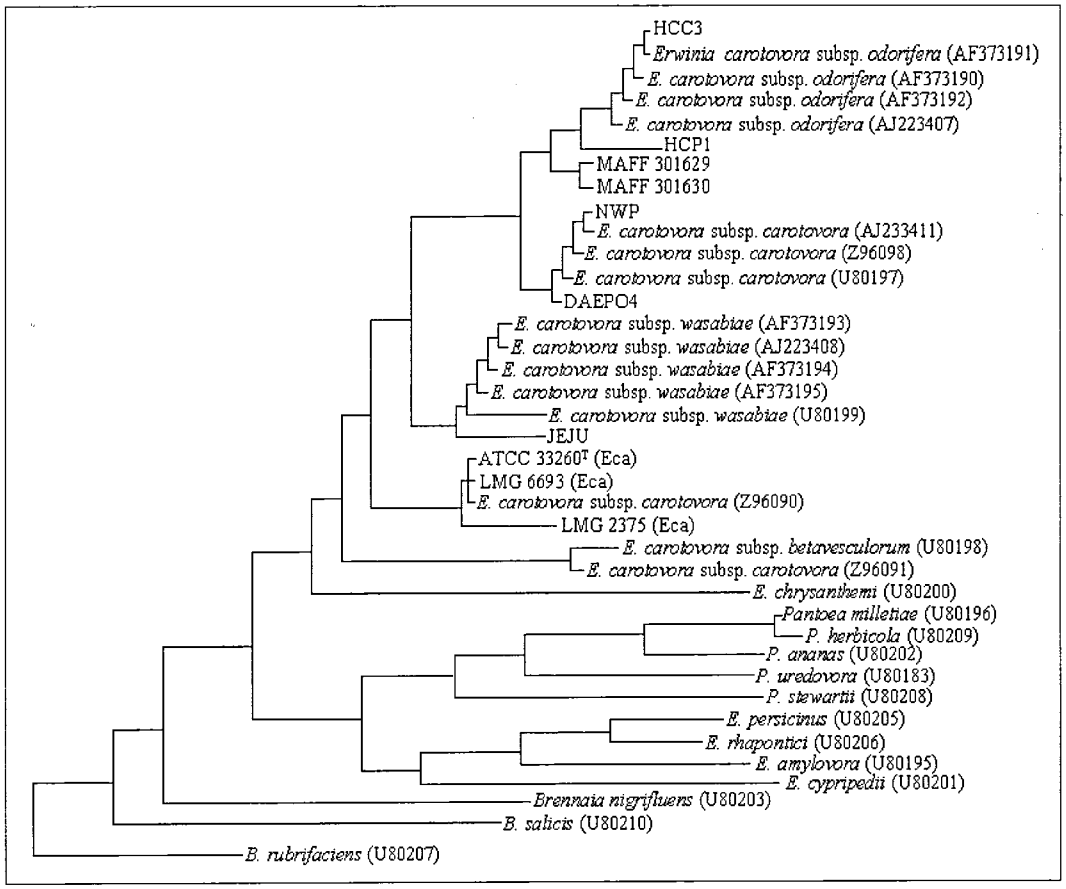

Fig. 2. Phylogenetic tree based on the 16S rDNA sequences showing the evolutionary relationships among *Erwinia* spp. The branching patterns were generated by the neighbor-joining method.

Many bacteria have multiple IGSs, differentiated on the basis of size and restriction site polymorphisms, reflecting the 1 to 11 alleles of the rRNA genetic locus (Condon et al., 1995). In our study, PCR amplification of the IGS regions generated two fragments (data not shown). The sequence analysis of the small region showed the presence of a single copy of the $tRNA^{Glu}$ gene and a single copy of the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes in the large region. Alignment of HCC3 and JEJU showed high homology of 98.0% to LMG 13009 (Eco) and 90.1% to ATCC 43762^T (Ecb) in $tRNA^{Glu}$ gene, respectively. In the case of $tRNA^{Ile}$ and $tRNA^{Ala}$ genes, the both strains showed high homology to MAFF 301629 (98.2%) and ATCC 43762^T (87.7%), respectively. As expected, the dendrogram of distances indicated that European Eca strains were genotypically distant from Asian Eca strains based on comparisons of the small and large 16S-23S rDNA sequences (Fig. 3).

Eca strains constitute a homogeneous group, whatever the original country and the year of isolation (Darrasse et al., 1994; Toth et al., 2001). However, they did not investigated Asian strains, offering no information about the variation within each continental. In the present study, Asian Eca strains and European Eca strains were found to be clearly distant by sequence analysis of 16S rDNA and 16S-23S IGS regions but to be closely related by biochemical tests. It would be interesting to investigate further the possible existence of a particular population in Asia.

Overall, two strains (NWP and DAEPO4) out of the four strains investigated were identified as Ecc. However, the other two strains JEJU and HCC3 were not clearly defined in this study, because they showed a mixture of Eca, Eco and Ecw phenotypic and genotypic characteristics. We believe that a more intensive survey would have revealed a greater occurrence of these bacteria. Perombelon and Salmond (1995) reported that any one of a number of soft rot erwinias can lead to the development of similar disease symptoms on a common host, for example, Eca, Ecc, Ecw and *E. chrysanthemi* all cause similar disease on potato. Our results suggest that there is more than one *E. carotovora* subspecies involved in potato blackleg disease in Korea.

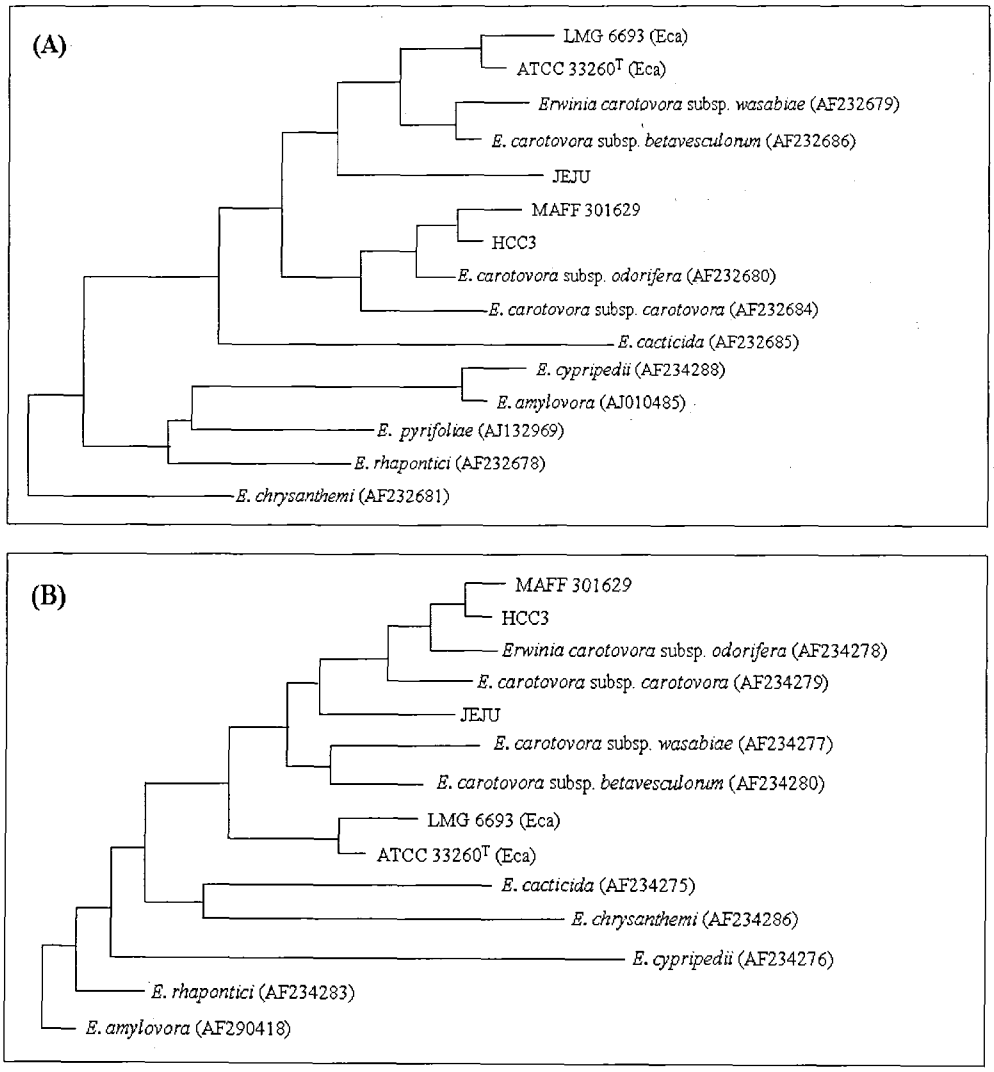

Fig. 3. Phylogenetic tree of *Erwinia* spp. based on a comparison of 16S-23S intergenic spacer (IGS) region involving $tRNA^{Glu}$ (A) and $tRNA^{Ile}$ and $tRNA^{Ala}$ (B). The branching patterns were generated by the neighbor-joining method.

Acknowledgement

This work was supported by Agriculture and Forestry Special Research Funds from the Ministry of Agriculture and Forestry of Korea in 1999 to 2002.

References

Asai, T., Zaporojets, D., Squires, C. and Squires, C. L. 1999. An *Escherichia coli* strain with all chromosomal rRNA operons inactivated: complete exchange of rRNA genes between bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 96:1971-1976.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidmann, J. G., Smith, J. A. and Struhl. K. 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley Interscience, N. Y.

Condon, C., Squires, C. and Squires, C. L. 1995. Control of rRNA transcription in *Escherichia coli. Microbiol. Rev.* 59:623-645.

Dallagi, A., Birch, P. R. J., Heilbronn, J., Lyon, G. and Toth, I. K. 2000. cDNA-AFLP analysis of differential gene expression in the bacterial plant pathogen *Erwinia carotovora. Microbiology* 146:165-171.

Darrasse, A., Priou, S., Kotoujansky, A. and Bertheau, Y. 1994. PCR and restriction length polymorphism of a *pel* gene as a tool to identify *Erwinia carotovora* in relation to potato diseases. *Appl. Environ. Microbiol.* 60:1437-1443.

De Boer, S. H. and Kelman, A. 2000. Gram-negative bacteria: *Erwinia* soft rot group. In Laboratory Guide for Identification of Plant Pathogenic Bacteria, 3rd edn. ed. Schaad, N. W., Jones, J. B. and Chun, W. pp. 56-72. St Paul, MN: American Phytopathological Society.

De Boer, S. H. and Ward, L. J. 1995. PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue.

Phytopathology 85:854-858.

De Boer, S. H., Cuppels, D. A. and Kelman, A. 1978. Pectolytic *Erwinia* spp. in the root zone of potato plants in relation to infestation of daughter tubers. *Phytopathology* 68:1784-1790.

Duarte, V., De Boer, S. H., Ward, L. J. and De Oliveira, A. M. R. 2004. Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil. *J. Appl. Microbiol.* 96:535-545.

Fessehaie, A., De Boer, S. H. and Levesque, C. A. 2002. Molecular characterization of DNA encoding 16S-23S rRNA intergenic spacer regions and 16S rRNA of pectolytic *Erwinia* species. *Can. J. Microbiol.* 48:387-398.

Hauben, L., Moore, E. R. B., Vauterin, L., Steenackers, M., Mergaert, J., Verdonck, L. and Swings, J. 1998. Phylogenetic position of phytopathogens within the *Enterobacteriaceae*. *System. Appl. Microbiol.* 21:384-397.

Lelliot, R. A. and Dickey, R. S. 1984. Genus VII. Erwinia Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith 1920, 209^{AL}. *In* Krieg, N. R., Holt, J. G. eds. Bergey's manual of systematic bacteriology, vol. 1. Baltimore, USA.

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

Perombelon, M. C. M. and Salmond, G. P. C. 1995. Bacterial soft rots, p. 1-17. *In* ingh, U. S., Singh, R. P. and Kohmoto, K. (ed.), Pathogenesis and host specificity in plant diseases, vol. 1. Pergamon Press, Oxford, England.

Seo, S. T., Furuya, N., Lim, C. K. Takanami, Y. and Tsuchiya, K. 2002. Phenotypic and genetic diversity of *Erwinia carotovora* ssp. *carotovora* strains from Asia. *J. Phytopathol.* 150:120-127.

Toth, I. K., Avrova, A. O. and Hyman, L. J. 2001. Rapid identification and differentiation of the soft rot Erwinias by 16S-23S intergenic transcribed spacer-PCR and restriction fragment length polymorphism analyses. *Appl. Environ. Microbiol.* 67:4070-4076.