Genotypic Characterisation of the *Erwinia* Genus by PCR-RFLP Analysis of *rpoS* Gene

*M. WALERON, K. WALERON and E. ŁOJKOWSKA**

Laboratory of Plant Protection & Biotechnology, Department of Biotechnology, Faculty of Biotechnology UG & AMG, 80-822 Gdańsk, Poland

**E-mail: lojkowsk@biotech.univ.gda.pl*

Abstract

Genotypic characterisation of the members of the genus *Erwinia*, based on the PCR-RFLP analysis of a fragment of the *rpoS* gene was done. PCR primers deduced from described *rpoS* gene sequences of *E. carotovora* allowed the amplification of about 880 bp DNA fragments from all tested *Erwinia* species. The *rpoS* fragments, amplified from 20 species of the studied *Erwinia* genus, were compared by RFLP analysis with 4 enzymes (*Alu*I, *Hin*6I, *Hinf*I, and *Tru1*I). Restriction analysis allowed drawing 63 common profiles of RFLP products for all tested *Erwinia*. From 1 to 3 specific RFLP profiles were identified among most of the species tested. However, in two cases: *E. chrysanthemi* and *E. c.* subsp. *carotovora* 15 and 20 specific RFLP groups were detected, respectively. High variability of genetic profiles of the *E. chrysanthemi* and *E. c.* subsp. *carotovora* can be explained by the wide spectrum of plants, which they infect. The results indicated that *rpoS* PCR-RFLP analysis is a useful tool for identification of species and subspecies belonging to the former *Erwinia* genus, as well as for differentiation of strains within *E. c.* subsp. *carotovora* and *E. chrysanthemi*.

Keywords: *Erwinia;* fingerprinting; PCR-RFLP; *rpoS*

INTRODUCTION

The genus *Erwinia* comprises Gram-negative bacteria. They have been grouped together mainly on the basis of their association with plants, either as pathogens, epiphytes or saprophytes. DYE (1981) classified the members of the genus *Erwinia* into three clusters. The *carotovora* group is characterised by the ability to produce pectinolytic enzymes. The *amylovora* group consists of pathogens that cause dry necrosis or wilt in their specific host plants. The *herbicola* group consists of epiphytes, plant pathogens, and saprophytes living in soil, water, air, and animal or human tissues.

The results of intensive studies of 16S rDNA, 16S-23S rDNA and *gapDH* sequences provide some evidence which can be used to resolve the taxonomic relationships of the genus *Erwinia* (HAUBEN *et al.* 1998; SPRÖER *et al.* 1999; BROWN *et al.* 2000; SUTRA *et al.* 2001; FESSEHAIE *et al.* 2002). The obtained results did not explain phylogenetic position of the *Erwinia* genus. Some of mentioned authors suggest dividing the former genus *Erwinia* into four new genera*: Erwinia, Pectobacterium, Pantoea,* and *Brenneria*, while others proposed only tree genera because the two genera: *Brenneria* and *Pectobacterium*, are not well separated from each other (SPRÖER *et al.* 1999; SUTRA *et al.* 2001).

Since the bacteria from the genus *Erwinia* cause economically important plant diseases, there are several methods available for their detection and identification. These include plating on selective CVP media, immunological methods and PCR-based methods. Most of them target individual species or subspecies.

In this study we present the usefulness of the method based on *rpoS* gene polymorphism for identification and differentiation and the bacteria belonging to the genus *Erwinia*.

Supported by Polish Scientific Committee (KBN), Grant No. 6 PO6A 033 21.

MATERIAL AND METHODS

Bacterial strains. 178 strains from the genus *Erwinia* were tested. For DNA preparation, *Erwinia* strains were grown overnight at 37°C (*E. chrysanthemi)*, 43°C (*E. cacticida)* or 28°C (other species) in tryptic soy broth. Cells were harvested by centrifugation and, resuspended in TE buffer. The bacterial DNA was extracted using the SDS/proteinase K method (AUSUBEL *et al.* 1992).

DNA amplification. Oligonucleotide primers were designed on the basis of the sequence of *rpoS* gene available in the GeneBank. The amplification was performed using a PCR Master Mix (MBI Fermentas) according to the manufacturer's instructions. The amplified products were electrophoretically separated in 1.5% agarose gel in TBE buffer, stained with ethidium bromide and visualised on an UV transilluminator.

Restriction fragment length analysis. The amplified DNA fragments were digested with four restriction endonucleases (*Alu*I, *Hin*6I, *Hinf*I and *Tru*1I). Enzymes were chosen on the basis of the nucleotide sequence of the *rpoS* gene available in the GeneBank by using Vector NTI software. Restriction fragments were separated in 12% polyacrylamide gel at 120 V for 10 h in TBE buffer and visualised with UV light after staining in ethidium bromide (0.5 μg/ml).

Sequencing of rpoS gene. Nucleotide sequences of *rpoS* gene were determined directly from PCR fragments amplified using PCR primers. Sequencing was carried out using an ABI PRISM Dye Terminator Cycle Sequencing Kit and 373A DNA Sequencer (Perkin-Elmer) according to the manufacturer's instructions.

Sequence alignment and phylogenetic reconstruction. Sequence alignment and phylogenetic analysis were performed using CLUSTAL X 1.8.

RESULTS AND DISCUSSION

The designed primers generated amplification products of 880 bp with target DNA isolated from all 177 strains of the tested *Erwinia*. Only in case of one strain of *E. pyrifoliae* Ep 16/96 the obtained product was about 780 bp. The obtained PCR products were sequenced directly in both directions. While alignment of obtained sequences, the deletion of about 140 bp in the fragment of *rpoS* gene amplified from *E. pyrifoliae* Ep 16/96 was observed.

The results of the *rpoS* RFLP analysis of PCR product based on number of bands and their position revealed the presence of 63 different *rpoS* combined RFLP groups. All RFLP groups were species specific. Strains belonging to 11 species (*E. amylovora, E. cypripedii, E. mallotivora, E. nigrifluens, E. psidii, E. quercina, E. rubrifaciens, E. salicis, E. stewartii, E. tracheiphila)* and 2 subspecies of *E. carotovora* (subsp. *betavasculorum* and *wasabiae*) occupied single RFLP groups based on *rpoS* PCR-RFLP. Strains of *E. cacticida*, *E. c.* subsp. *odorifera*, *E. herbicola, E. persicinus* and *E. pyrifoliae* occupied 2 different RFLP groups and those of *E. ananas*, *E. c.* subsp. *atroseptica,* three groups.

The *rpoS* PCR-RFLP method allow for differentiation of strains from the same species. The highest diversity of the *rpoS* gene was observed among 68 strains of *E. c.* subsp. *carotovora* (20 RFLP groups) and 21 strains of *E. chrysanthemi* (15 RFLP groups).

The same high level of differentiation for species *E. carotovora* and *E. chrysanthemi* was observed when polymorphism of the genes encoding recombinase (WALERON *et al.* 2002), 16S-23S rDNA spacer (TOTH *et al.* 2001; FESSEHAIE *et al.* 2002), and pectate lyases (DARRASSE *et al.* 1994; NASSAR *et al.* 1996) were examined. The low variability among *E. amylovora* strains was also observed earlier in biochemical (DYE 1968; VERDONCK *et al.* 1987), serological (VANTOMME *et al.* 1982), DNA hybridisation tests (BRENNER *et al.* 1974) Pulsed-Field Gel Electrophoresis studies (ZHANG & GEIDER 1997) and in *recA* PCR-RFLP analysis (WALERON *et al.* 2002).

The amplified fragments of the *rpoS* gene from 2 species: *E. amylovora, E. pyrifoliae* (two strains Ep1/96, Ep16/96) and 5 subspecies of *E. carotovora* were sequenced. The comparison of the obtained sequences with the sequence of *rpoS E. amylovora, E. carotovora* and *E. chrysanthemi* from the GeneBank was done and the % of identities between sequences of different *Erwinia* species was decreased.

The results of phylogenetic analysis based on *rpoS* sequences amplified from several *Erwinia* species are in agreement with phylogeny based on 16S rDNA. The species belonging into genera *Pectobacterium* and new *Erwinia* are grouped in different clusters.

The work presented indicated that PCR-RFLP analysis of the fragment of *rpoS* gene is a useful tool for identification of bacteria from the *Erwinia* genus and differentiation of *Erwinia c.* subsp. *carotovora* and *Erwinia chrysanthemi.*

Our results indicated the usefulness of *rpoS* PCR-RFLP analysis and sequencing of the *rpoS* gene for study the phylogenetic relationships between different species belonging into former *Erwinia* genus.

References

AUSUBEL F.M., BRENT R., KINGSTON R.E., MOORE D.D., SEIDMAN J.G., SMITH J.A., STRUHL K. (eds) (1992): Short Protocols in Molecular Biology. 2nd ed. Green Publ. Ass. and John Wiley & Sons, New York.

BRENNER D.J., FANNING G.R., STEIGERWALT A.G. (1974): Deoxyribonucleic acid relatedness among *Erwiniae* and other *Enterobacteriaceae*: the gall, wilt, and drynecrosis organisms (Genus *Erwiniae* Winslow et al., sensu stricto). Int. J. Syst. Bacteriol., **24**: 197–204.

BROWN E.W., DAVIS R.M., GOUK C., VAN DER ZWET T. (2000): Phylogenetic relationships of necrogenic *Erwinia* and *Brenneria* species as revealed by glyceraldehyde-3-phosphate dehydrogenase gene sequences. Int. J. Syst. Evol. Microbiol., **50**: 2057–2068.

DARRASSE A., PRIOU S., KOTOUJANSKY A., BERTHEAU Y. (1994): PCR and Restriction Fragment Length Polymorphism of a *pel* gene as a tool to identify *Erwinia carotovora* in relation to potato diseases. Appl. Environ. Microbiol., **60**: 1437–1443.

DYE D.W. (1968): A taxonomic study of the genus *Erwinia*. I. The"Amylovora" group. N.Z. J. Sci., **11**: 590–607.

DYE D.W. (1981): A taxonomic study of the genus *Erwinia*. N.Z. J. Agric. Res., **24**: 223–231.

FESSEHAIE A., DE BOER S.H., LÉVESQUE 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., VERDONCK L., SWINGS J. (1998): Phylogenetic position of phytopathogens within the *Enterobacteriacae*. System. Appl. Microbiol., **21**: 384–397.

NASSAR A., DARRASSE A., LEMATTRE M., KOTOUJANSKY A., DERVIN C., VEDEL R., BERTHEAU Y. (1996): Characterisation of *Erwinia chrysanthemi* by pectynolitic isozyme polymorphism and Restriction Fragment Length Polymorphism analysis of PCR-amplified fragments of pel genes. Appl. Environ. Microbiol., **62**: 2228–2235.

SPRÖER C., MENDROCK U., SWIDERSKI J., LANG E., STACKEBRANDT E. (1999): The phylogenetic position of *Serratia*, *Buttiauxella* and some other genera of the family *Enterobacteriaceae*. Int. J. Syst. Bacteriol., **49**: 1433–1438.

SUTRA L., CHRISTEN R., BOLLET C., SIMONEAU P., GARDAN L. (2001): *Samsonia erythrinae* gen. nov., sp. Nov., isolated from bark necrotic lesions of *Erythrina* sp., and discrimination of plant pathogenic *Enterobacteriaceae* by phenotypic features. Int. J. Syst. Evol. Microbiol., **51**: 1291–1304.

TOTH I.K., AVOROVA A.O., HYMAN L.J. (2001): Rapid identification and differentiation of the soft rot erwinias by 16S-23S Intergenic Transcribed Space-PCR and restriction fragment length polymorphism analyses. Appl. Environ. Microbiol., **67**: 4070–4076.

VANTOMME R., SWINGS J., GOOR M., KERSTERS K., DE LEY J. (1982): Phytopathological, serological, biochemical and protein electrophoretic characterization of *Erwinia amylovora* strains isolated in Belgium. Phytopathol. Z., **103**: 349–360.

VERDONCK L., MERGAER J. RIJCKAERT C., SWINGS J., KERSTERS K., DE LEY J. (1987): The genus *Erwinia*: a numerical analysis of phenotypic features. Int. J. Syst. Bacteriol., **37**: 4–18.

WALERON M., WALERON K., PODHAJSKA A.J., ŁOJKOWSKA E. (2002): Genotyping of bacteria belonging to the former *Erwinia* genus by PCR-RFLP analysis of a *recA* gene fragment. Microbiology, **148**: 583–595.

ZHANG Y., GEIDER K. (1997): Differentiation of *Erwinia amylovora* strains by pulsed-field gel electrophoresis. Appl. Environ. Microbiol., **63**: 4421–4426.

ZHANG Y., MERIGHI M., BAZZI C., GEIDER K. (1998): Genomic analysis by pulsed-field gel electrophoresis of *Erwinia amylovora* strains from the Mediterranean region including Italy. J. Appl. Plant. Pathol., **80**: 225–232.