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Phylogenetic Analysis of *Pectobacterium* Species Using the 16S-23S rRNA Intergenic Spacer Regions

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For the taxonomic evaluation, 15 strains of the genus *Pectobacterium* and *Erwinia* were analyzed for 16S-23S rDNA intergenic spacer regions (ISRs). These species contained two types of ISRs, large and small ISRs. Large ISRs were on the range of 474-569 bp size, and coding transfer RNA^{Ile} (tRNA^{Ile}) and tRNA^{Ala}. Small ISRs were 354-459 bp in length and coding tRNA^{Glu}. The sequence variations of two ISRs among species and strains were very high as compared with 16S rRNA gene sequences. By phylogenetic trees on the basis of two ISRs, *Pectobacterium* were differentiated into *P. carotovorum-P. cacticidum* group and *P. chrysanthemi* group. However, the taxonomic position of *E. cypripedii* and *E. rhapontici*, which were not clear on taxonomic delineation between *Pectobacterium* and *Erwinia*, were not clearly resolved on the basis of ISRs.

Keywords : 16S-23S rRNA intergenic spacer region, phylogeny, *Pectobacterium*, *Erwinia*.

The genus *Erwinia* was proposed by Winslow et al. (1917) for Gram-negative, non-spore-forming, peritrichous, permentative, rod-shaped bacteria, and it belonged to the family *Enterobacteriaceae*. Recently, the genus *Erwinia* was reclassified into three genera *Erwinia*, *Pectobacterium* and *Brenneria* mainly on the basis of 16S rDNA sequence analysis (Hauben et al., 1998).

The genus *Pectobacterium* produces pectolytic enzymes and causes soft-rots, necrosis and wilts on food crops and ornamental plants. *Erwinia carotovora* subspecies, *E. cacticida*, *E. chrysanthemi* and *E. cypripedii* were reclassified into the genus *Pectobacterium*.

Although the genus *Pectobaterium* consisted of a small number of species and subspecies, the heterogenicity of these species was discussed on the light of physiological, biochemical and pathogenic characteristics (Dickey, 1979; Dye, 1969; Dye, 1981; Thomson, 1981). The *Pectobacterium carotovorum* has been divided into five subspecies *atrosepticum*, *carotovorum*, *betavasculorum*, *wasabiae* and *odoriferum* on the basis of physiological, biochemical and pathogenic features and sequences of 16S rRNA gene (De Boer et al., 1979; Goto and Matsumoto, 1987; Hauben et al., 1998; Lelliott and Dickey, 1984; Stanghellini, 1982). The pathogenic characteristics, serological test (De Boer et al., 1979), and RFLP analysis of *pel* gene (Darrasse et al., 1994) of *P. carotovorum* subsp. *carotovorum*, which is widely distributed as a soft-rotting pathogen attacking a wide variety of plants, showed a heterogeneity of this subspecies. *Pectobacterium chrysanthemum* also had a broad range of hosts and the serological study among strains revealed the heterogeneity of this species (Yakrus and Schaad, 1978).

The taxonomic delineations of *Erwinia cypripedii* and *Erwinia rhapontici* were problematic because these species were thought to be intermediate between the genus *Pectobacterium* and *Erwinia* on physiological, pathogenic and genetic characteristics (Dye, 1968; Dye, 1969; Hauben et al., 1998; Kwon et al., 1997).

Generally, the phylogenetic relationships among various taxa can be more clarified by the sequence analysis of ribosomal DNA. The intergenic spacer regions (ISRs) between the 16S and 23S rRNA genes have experienced relatively large nucleotide changes on evolutionary route as compared to 16S rRNA gene. Thus, the variability of ISR regions was used to analyze the taxonomic structure on the interspecific and intraspecific level (Chun et al., 1999; Kwon et al., 1998; Sawada et al., 1997).

In this study, we determined the structure of ISRs and examined the sequence variations for the genus *Pectobacterium*. And, the sequences of ISRs were applied for the taxonomic evaluation of the genus *Pectobacterium*.

Materials and Methods

Organisms and culture conditions. The bacterial strains used in this study and the GenBank accession numbers for the ISR sequences are listed in Table 1. All bacterial strains were grown

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Table 1. Strains used in this study and their nucleotide sequence accession numbers

Speices or subspecies	Strains	Accession number		Reference
		Small ISR	Large ISR	
Pectobacterium carotovorum subsp. *carotovorum*^{T}	ATCC 15713	AF232684	AF234279	In this study
Pectobacterium carotovorum subsp. *betavasculorum*^{T}	ATCC 43762	AF232686	AF234280 AF234281	"
Pectobacterium carotovorum subsp. *wasabiae*^{T}	ATCC 43316	AF232679	AF234277	"
Pectobacterium carotovorum subsp. *oderiferum*	LMG 13009	AF232680	AF234278	"
Pectobacterium carotovorum subsp. *carotovorum*	KACC 10421	AF232677	AF234284	"
Pectobacterium carotovorum subsp. *carotovorum*	KACC 10401	–	–	"
Pectobacterium carotovorum	KACC 10422	AF234294	–	"
Pectobacterium chrysanthemi^T	ATCC 11663	AF232681	AF234287	"
Pectobacterium chrysanthemi	KACC 10163	AF232682	AF234285	"
Pectobacterium chrysanthemi	KACC 10165	AF232683	AF234286	"
Pectobacterium cacticida	LMG 2720	AF232685	AF234275	
Erwinia cypripedii^T	ATCC 29267	AF234288	AF234276	"
Erwinia rhapontici^T	ATCC 29283	AF232678	AF234283	"
Erwinia amylovora	Ea 1/79	AJ010485	–	(Kim et al, 1999)
Erwinia pyrifoliae	Ep 16/96	AJ132969	–	"

^{T}: *type strain*

ATCC, American Type Culture Collection, Rockville, Md., USA; LMG, Laboratorium voor Microbiologie, Gent, Belgium; KACC, Korean Agricultural Culture Collection, RDA, Suwon, Korea.

on Nutrient agar (1 g beef extract, 2 g yeast extract, 5 g peptone, 5 g NaCl, 15 g agar in 1,000 ml deionized water) at 28°C.

DNA preparation and PCR amplification of ISRs. Chromosomal DNAs were isolated by the method of Ausubel et al. (1987), except that the lysates were extracted twice with chloroform to remove residual phenol. The ISRs were amplified by using the primers, R16-1 (5'-CTTGTACACACCGCCCGTCA-3') and R23-3R (5'-GGTACTTAGATGTTTCAGTTC-3'), that were redesigned from the primers of Nakagawa et al. (1994). Each PCR mixture (50 μl) contained primers (each at a concentration of 20 pmol), a mixture of deoxynucleotide triphosphates (Promega Co., Southampton, England) (each at a concentration of 200 μM), and *Taq* polymerase buffer, chromosomal DNA (ca. 50ng), and *Taq* polymerase (2.5 Unit) (Promega Co.). The DNA thermal cycler (Perkin-Elmer Co., Norwalk, Conn.) used for PCR amplification was programmed as follows: (i) an initial extensive denaturation step consisting of 94°C for 5 min; (ii) 35 cycles, with each cycle consisting of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and (iii) a final extension step consisting of 72°C for 10 min.

Cloning and sequencing of ISRs. The PCR products were electrophoresed on 1.2% agarose gel, and then ISRs were purified with a QIAquick gel extraction kit (Qiagene GmbH, Hilden, Germany). Purified DNA were ligated into pGEM-T easy vector (Promega Co.), and ligated plasmids were then transformed into *Escherichia coli* DH5αF' cells (Sambrook et al., 1989). Plasmids containing ISRs were isolated by using QIAquick plasmid minikit (Qiagene GmbH), and nucleotides sequences of the ISRs were determined with an Applied Biosystems 377 sequencer. Primers R16-1, ILEF (5'-GTAGCTCAGTTGGTTAGAGC-3'), and R23-3R were used for the sequencing.

Phylogenetic analysis. The sequences of ISRs were first aligned by using CLUSTAL W software (Thompson et al., 1994), and then the alignments were corrected by hand. The sequence homology values were calculated from the alignment. An evolutionary distance matrix was generated as described by Jukes and Cantor (1969). Evolutionary trees for the data sets were inferred by the neighbor-joining method of Saitou and Nei (1987) by using the neighbor-joining program of MEGA (Kumar et al., 1993). The stability of relationships was assessed by performing bootstrap analyses of the neighbor-joining data based on 1,000 resamplings.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank data library under the accession numbers shown in Table 1.

Results and Discussions

The total 15 strains of *Pectobacterium* and *Erwinia* spp. were analyzed for ISRs. The PCR profiles of ISRs which were amplified with the primers designed form conserved regions of 3' terminal sequences of 16S ribosomal DNA and 5' terminal sequences of 23S rDNA were very complex. PCR products had more than one bands and DNA lengths also were different from each other. We eluted the DNA bands as many as possible and cloned each bands into pGEM T vector. By sequence analysis, the ISRs from all *Pectobacterium* and *Erwinia* strains used in this study were differentiated into two types, small and large ISRs accord-

Table 2. Levels of similarity based on small ITS I region sequences for some strains of the genus *Pectobacterium* and *Erwinia* spp.

Organisms	% large ITS I region sequence homology														
	P. carotovorum KACC 10401	*P. carotovorum* subsp. *oderiferum* LMG 17566	*P. carotovorum* subsp. *carotovorum* ATCC 15713	*P. carotovorum* KACC 10421	*P. carotovorum* KACC 10422	*P. carotovorum* subsp. *betavasculorum* ATCC 43762	*P. carotovorum* subsp. *wasabiae* ATCC 43316	*P. cacticida* LMG 2720	*P. chrysanthemi* KACC 10163	*P. chrysanthemi* KACC 10165	*P. chrysanthemi* ATCC 11663	*Erwinia cypripedii* ATCC 29267	*Erwinia amylovora* Ea 1/79	*Erwinia rhapotici* ATCC 29283	*Erwinia pyrifoliae* Ep 16/96
P. carotovorum KACC 10401	100(453)														
P. carotovorum subsp. *oderiferum* LMG 17566	95.6	100(453)													
P. carotovorum subsp. *carotovorum* ATCC 15713	90.9	93.3	100(451)												
P. carotovorum KACC 10421	92.2	93.8	96.7	100(451)											
P. carotovorum KACC 10422	86.0	84.5	80.0	81.3	100(444)										
P. carotovorum subsp. *betavasculorum* ATCC 43762	82.7	87.4	83.4	83.4	91.7	100(445)									
P. carotovorum subsp. *wasabiae* ATCC 43316	81.2	84.6	79.7	81.0	89.9	94.4	100(448)								
P. cacticida LMG 2720	82.3	84.5	79.8	80.3	79.7	78.0	77.5	100(446)							
P. chrysanthemi KACC 10163	49.2	50.0	48.6	49.7	44.9	48.3	48.6	48.9	100(354)						
P. chrysanthemi KACC 10165	48.3	49.4	48.3	49.7	46.1	47.2	47.2	48.0	94.6	100(356)					
P. chrysanthemi ATCC 11663	48.3	48.9	48.3	50.0	45.5	48.0	48.6	48.9	92.7	89.9	100(356)				
Erwinia cypripedii ATCC 29267	42.7	42.4	43.5	43.0	42.2	42.2	42.2	43.0	47.2	46.3	46.3	100(377)			
Erwinia amylovora Ea 1/79	50.1	49.1	48.0	50.7	48.0	46.7	46.9	49.1	48.3	50.6	52.2	67.1	100(377)		
Erwinia rhapotici ATCC 29283	52.1	50.5	49.7	52.1	52.1	53.7	52.1	49.5	51.4	47.5	52.5	59.8	65.4	100(376)	
Erwinia pyrifoliae Ea 16/96	55.8	54.7	53.9	56.6	56.3	53.6	53.1	52.3	53.1	46.9	53.9	55.0	72.7	86.6	100(373)

( ): number of base pair

ing to transfer RNA (tRNA)s coded within ISRs. The sequences of small ISRs contain putative transfer RNA glutamate ($tRNA^{Glu}$) whereas the sequences of large ISRs code two putative transfer RNAs, $tRNA^{Ile}$ and $tRNA^{Ala}$. ISRs of the genus *Pseudomonas* and *Eschericia coli* were most extensively studied among Gram negative bacteria. Pseudomonads showed only one type of ISRs coding $tRNA^{Ile}$ and $tRNA^{Glu}$ despite of their multiple ISR bands. *Escherichia coli* contains 7 ribosomal RNA (rrn) gene clusters and their ISRs were differentiated into large and small ISRs (Garcia-Martinez et al., 1996). The structure of ISRs of *Pectobacterium* and *Erwinia* spp. was similar to that of *E. coli*, and the heterogeneity of PCR band patterns of ISRs could be useful for the taxonomic differentiation of the genus *Pectobacterium*.

The large ISRs of the genus *Pecotobacterium* and *Erwinia* spp. were on the range of 474-569 bp in length (Table 2). The small ISRs were on the range of 354-459 bp size (Table 3). According to the phylogenetic tree on the basis of large ISRs with *E. rhapontici* as outgroup, the *Pectobacterium* and *Erwinia* species were grouped into four clades; *P. carotovorum* subspecies clade, *P. chrysanthemi* clade, and *E. cypripedii* clade and *E. rhapontici* clade (Fig. 1). In this tree, *P. chrysanthemi* clade was more closely related with *P. carotovorum-P. cacticidum* than other clades. The tree without outgroup showed that the *Pectobacterium* and *Erwinia* species were differentiated into three independent groups, *P. carotovorum* subspecies group, *P. chrysanthemi* group and *E. cypripedii-E. rhapontici* group (data not shown). The differences of phylogenetic relationships between the two trees were thought to come from the very low sequence homologies among these groups.

On the other hand, the phylogenetic tree using the small ISRs were produced with 15 sequences. ISR sequences of *E. amylovora* (accession no. AJ010485) and *E. pyrifoliae* (accession no. AJ132969) were borrowed from GenBank data base. The phylogenetic analysis using small ISR sequences grouped *Pectobacterium* and *Erwinia* species

Table 3. Levels of similarity based on large ITS I region sequences for some strains of the genus *Pectobacterium* and *Erwinia* spp.

Organisms	% small ITS I region sequence homology												
	P. carotovorum subsp. *oderiferum* LMG 17566	*P. carotovorum* KACC 10421	*P. carotovorum* subsp. *carotovorum* ATCC 15713	*P. carotovorum* KACC 10401	*P. carotovorum* subsp. *betavasculorum* ATCC 43762(subtype I)	*P. carotovorum* subsp. *wasabiae* ATCC 43316	*P. carotovorum* subsp. *betavasculorum* ATCC 43762(subtype II)	*P. cacticida* LMG 2720	*P. chrysanthemi* KACC 10163	*P. chrysanthemi* KACC 10165	*P. chrysanthemi* ATCC 11663	*Erwinia cypripedii* ATCC 29267	*Erwinia rhapotici* ATCC 29283
P. carotovorum subsp. *oderiferum* LMG 17566	100(489)												
P. carotovorum KACC 10421	90.3	100(487)											
P. carotovorum subsp. *carotovorum* ATCC 15713	93.3	90.1	100(494)										
P. carotovorum KACC 10401	93.3	92.4	95.1	100(493)									
P. carotovorum subsp. *betavasculorum* ATCC 43762 (subtype I)	86.2	82.7	83.5	85.2	100(486)								
P. carotovorum subsp. *wasabiae* ATCC 43316	81.3	78.4	77.8	81.1	89.4	100(482)							
P. carotovorum subsp. *betavasculorum* ATCC 43762 (subtype II)	83.5	80.6	79.7	80.2	90.7	83.5	100(474)						
P. cacticida LMG 2720	79.1	74.7	77.0	77.2	74.5	68.9	79.5	100(487)					
P. chrysanthemi KACC 10163	54.0	53.6	48.4	53.1	51.6	50.2	59.7	55.0	100(504)				
P. chrysanthemi KACC 10165	53.2	56.3	51.6	54.6	51.9	45.9	58.6	54.8	97.6	00(504)			
P. chrysanthemi ATCC 11663	53.0	54.4	49.3	53.4	51.4	45.9	57.8	54.8	85.3	85.7	100(491)		
Erwinia cypripedii ATCC 29267	51.7	52.6	50.0	50.7	50.4	48.8	54.6	51.5	57.1	56.3	56.8	100(538)	
Erwinia rhapotici ATCC 29283	56.6	61.0	55.5	56.8	54.5	55.4	63.5	57.7	61.9	61.1	60.3	64.1	100(569)

( ): number of base pair

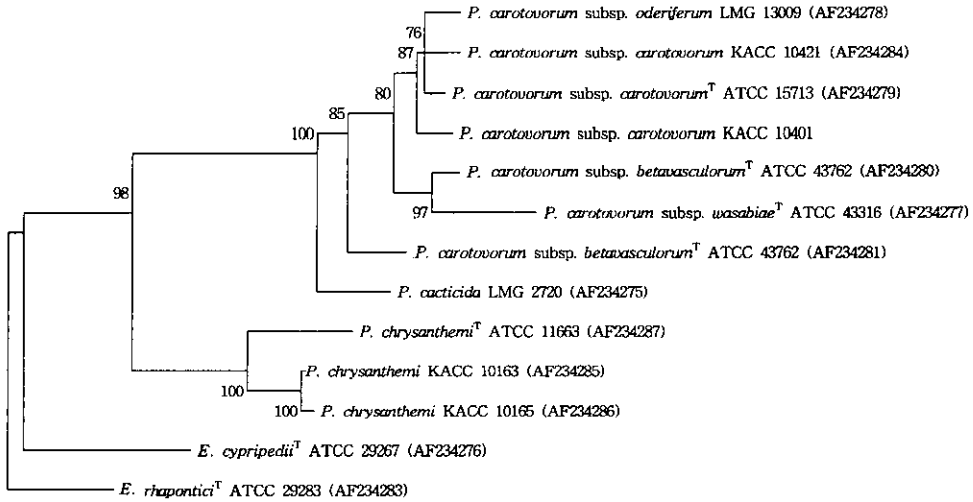

Fig. 1. Phylogenetic tree based on a comparison of large ISR sequences for *Pectobacterium* and *Erwinia* spp. The branching pattern was generated by the neighbor-joining method. The numbers at the nodes indicate the levels of bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets.

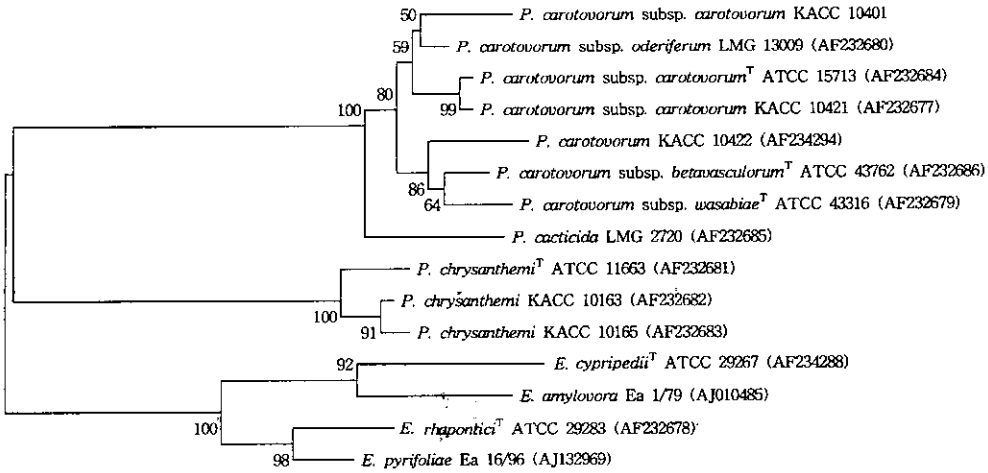

Fig. 2. Phylogenetic tree based on a comparison of smal ISR sequences for *Pectobacterium* and *Erwinia* spp. The generation of tree was conducted as Fig. 1.

into four groups (Fig. 2).

The two trees based on large and small ISRs showed consistent genealogical relationships among *Pectobacterium* and *Erwinia* spp. although the number of strains analyzed in each tree a little changed the structures of two trees. Thus, it was supposed that two types of ISRs might experience nearly the same evolutionary route in the view of evolutionary rates.

The sequence homologies among *P. carotovorum* subspecies were in the range of above 77.8% on large ISRs and above 79.7% on small ISRs (Table 2 and 3). The trees shown on Fig. 2 and 3 grouped *P. c.* subsp. *carotovorum* (ATCC 15713, KACC 10401 and KACC 10421) and *P. c.* subsp. *oderiferum* (LMG 17566) into one clade. The strains of *P. c.* subsp. *carotovorum* (ATCC 15713, KACC 10401 and KACC 10421) and subsp. *oderiferum* (LMG 17566) were closely related on the homology level of above 90.1% (large ISRs) and above 90.9% (small ISRs) (Table 2 and 3). *P. c.* subsp. *oderiferum*, isolates mainly from witloof (broad-leaf chicory, *Cichorium intybus* L.), were previously designated "atypical" *P. c.* subsp. *atrosepticum* on the basis of the biochemical and pathogenic properties (Samson et al., 1980). However, DNA-DNA hybridization (Gallois et al., 1992) and 16S rDNA sequence data (Hauben et al., 1998) demonstrated that *P. c.* subsp. *oderiferum* was more closely related with *P. c.* subsp. *carotovorum*. Genomic DNA of *P. c.* subsp. *oderiferum* was hybridized with those of *P. c.* subsp. *carotovorum* strains on the level of 58-88% relatedness, which were as high as hybridization values shown among strains of *P. c. oderiferum* (Gallois et al., 1992). The sequence analysis of two types of ISRs clearly supported DNA-DNA hybridization data and 16S rDNA sequence analysis of these species.

P. c. subsp. *betavasculorum*, *P. c.* subsp. *wasabiae* and *P. carotovorum* KACC 10422, were identified to be closely related subspecies on sequence homologies of 89.9-94.4% of large ISRs and 83.5-90.7% on small ISRs. *P. carotovorum* KACC 10422, an isolate from the soft-rot potato, was identified as *P. c.* subsp. *carotovorum* on Biolog test. However, the sequences of small ISRs were shown to be more closely related with *P. c.* subsp. *betavasculorum* and *P. c.* subsp. *wasabiae*. The soft rots of potato plant are caused by *P. c.* subsp. *carotovorum* and *P. c.* subsp. *atrosepticum* (Perombelon, 1987). Although KACC 10422 were clearly separable from *P. c.* subsp. *carotovorum*, the exact taxonomic position of this strain could not be defined because we did not obtain the ISRs of *P. c.* subsp. *atrosepticum*.

The two subtypes of large ISR of *P. c.* subsp. *betavasculorum*, subtype I and II (AF234280 and AF234281, respectively), were recovered. Two sequences of subtypes showed relatively small sequence variations. The sequence variations which were shown on ISRs of the same type were reported from ISRs of other species (Chun et al., 1999; Garcia-Martinez et al., 1996). Although the reason for the sequence variations of ISRs of the same type is not clear, the differences of evolutionary rate among ribosomal RNA gene (rrn) clusters might generate their sequence variation on ISRs. *P. c.* subsp. *wasabiae* and *P. c.* subsp. *betavasculorum*, isolated from Japanese horseradish and sugarbeet, respectively, showed higher reciprocal sequence homology on 16S rDNA sequence than those of *P. c.* subsp. *carotovorum* (Hauben et al., 1998). The results of phylogenetic analysis of ISR region were consistent to data from 16S rDNA sequences, and furthermore, the high variation of ISRs will be more powerful for taxonomic analysis of *P. carotovorum* subspecies.

P. cacticida, an isolate from cacti (Alcorn et al., 1991), was closely related with *P. carotovorum* subspecies rather than *P. chrysanthemi* on the analysis of ISR sequences

(Table 2, 3 and Fig. 1, 2). While the sequence homology values of *P. cacticida* with *P. carotovorum* subspecies were 68.9-79.5% on large ISRs and 77.5-84.5% on small ISRs, the sequence homology values with *P. chrysanthemi* strains were 48.0-48.9% on small ISRs and 54.8-55.0% on large ISRs. *P. cacticida* was related to *P. chrysanthemi* on numerical analysis of physiological and fatty acid characteristics (Alcorn et al., 1991), but DNA-DNA hybridization and 16S rDNA sequences showed that *P. cacticida* were most closely related with *P. carotovorum* subspecies on 25-38% DNA hybridization values and 97-97.9% 16S rDNA homologies (Alcorn et al., 1991; Hauben et al., 1998).

The sequence analyses of two ISRs of *P. chrysanthemi* showed that these strains (ATCC 11663, KACC 10163 and KACC 10165) were remotely related with other species including *P. carotovorum* strains on about 50% sequence homology levels. Actually, *P. chrysanthemi* was closely related with *P. carotovorum* on pathogenic characteristics and had common pectate lyase genes, but genomic DNA of *P. chrysanthemi* was hybridized to those of *P. carotovorum* on relatively low relatedness of about 40% (Brenner et al., 1973). These molecular data suggest that *P. chrysanthemi* was clearly differentiated from *P. carotovorum* on evolutionary view. The three strains of *P. chrysanthemi* were related on 85.3-97.6% sequence homology of large ISRs and 89.9-94.6% sequence homology of small ISRs. Especially, two strains (KACC 10163 and KACC 10165) isolated from orchid (*Cymbidium* sp.) were separable from type strain (ATCC 11663) isolated from chrysanthemum. It is meaningful to analyze the taxonomic structure of the strains within *P. chrysanthemi*, considering that these species had been classified into subspecies and pathovars (Dickey, 1979; Lelliott, 1974). The high sequence variations of ISRs could be used for the taxonomic evaluations among *P. chrysanthemi* strains.

Erwinia cypripedii and *Erwinia rhapontici* was thought to be intermediate between the genus *Pectobacterium* and *Erwinia* on physiological, pathogenic and genetic characteristics (Alcorn et al., 1991; Dye, 1968; Dye, 1969; Gallois et al., 1992; Hauben et al., 1998; Kwon et al., 1997). Recently, Hauben et al. (1998) proposed that *E. cypripedii* should be classified into the genus *Pectobacterium* and *E. rhapontici* into the genus *Erwinia* on 16S rDNA sequence analysis. However, '*Pectobacterium cypripedii*' was not recognized by IJSB (Validation list no. 68., 1999). The analysis of large ISRs for *E. cypripedii* and *E. rhapontici* showed that these two species had low relatedness (48.8-64.1%) with *Pectobacterium* species, and the sequence comparison of small ISRs revealed that *E. cypripedii* and *E. rhapontici* were more closely related to the genus *Erwinia* species (55.0-67.1% homologies) rather than the genus *Pectobacterium* species (42.2-53.7%). The inaccuracy of analytic methods due to the low sequence homologies (<67.1%) shown on *E. cypripedii* and *E. rhapontici* to other species made difficult to evaluate the exact taxonomic structure of these two speies.

In conclusion, the structures of ISRs for the genus *Pectobacterium* and *Erwinia* were very complex and the sequence variations were on higher range than those of 16S rDNA sequences. Thus, the accumulation of sequence data of ISRs will be helpful for the exact taxonomic analyses of these genera.

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