Phylogenetic Analysis of *Erwinia* Species Based on 16S rRNA Gene Sequences†

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The phylogenetic relationships of the type strains of 16 *Erwinia* species were investigated by performing a comparative analysis of the sequences of the 16S rRNA genes of these organisms. The sequence data were analyzed by the neighbor-joining method, and each branch was supported by moderate bootstrap values. The phylogenetic tree and sequence analyses confirmed that the genus *Erwinia* is composed of species that exhibit considerable heterogeneity and form four clades that are intermixed with members of other genera, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Serratia marcescens*. Cluster I includes the type strains of *Erwinia herbicola*, *Erwinia milletiae*, *Erwinia ananas*, *Erwinia uredovora*, and *Erwinia stewartii* and corresponds to Dye's herbicola group. Cluster II consists of *Erwinia persicinus*, *Erwinia rhapontici*, *Erwinia amylovora*, and *Erwinia cypripedii*. Cluster III consists of *Erwinia carotovora* subspecies and *Erwinia chrysanthemi* and is characterized by the production of pectate lyases and cellulases. *Erwinia salicis*, *Erwinia rubrifaciens*, and *Erwinia nigrifluens* form the cluster that is most distantly related to other *Erwinia* species. The data from the sequence analyses are discussed in the context of biochemical and DNA-DNA hybridization data.

The genus *Erwinia* was proposed by Winslow et al. (51) for gram-negative, non-spore-forming, peritrichous, fermentative, rod-shaped bacteria, and it belongs to the family *Enterobacteriaceae*. This genus was proposed for plant-associated bacteria that are pathogens, saprophytes, and epiphytes.

Although the heterogeneous taxonomic structure of the genus *Erwinia* has been discussed by using phenotypic data (13, 15–19, 21, 22, 35, 42, 44) and genotypic data (3, 6–10), the taxonomic position of this genus remains problematic (5, 32, 40, 41). Previously, Dye (15–18) classified the members of the genus *Erwinia* into four natural clusters. The carotovora group is characterized by soft-rot-causing and biochemically active species. Although some authors (10, 45) have proposed that this group should be designated the genus *Pectobacterium* and differentiated from other *Erwinia* species on the basis of distinct pathogenic and biochemical properties and this view was partially supported by DNA-DNA hybridization studies (9, 10), it has not been generally accepted. The amylovora group consists of pathogens that cause dry necrosis or wilt in their specific host plants, and the taxonomic position of this group as a true *Erwinia* group has rarely been questioned. Furthermore, Dye (15) considered members of this group subspecies (or varieties) of *Erwinia amylovora*. However, each species belonging to the amylovora group forms a distinct phenon, as shown in the numerical analyses of Verdonck et al. (44) and Mergaert et al. (35). In addition, DNA-DNA hybridization studies (3, 6, 7, 23, 37) indicated that *Erwinia amylovora* had low levels of relatedness to other species of the amylovora group, as well as other *Erwinia* species, and the DNA-DNA relatedness values for *Erwinia salicis*, *Erwinia rubrifaciens*, and *Erwinia nigrifluens* are moderately high. The herbicola group of the genus *Erwinia* is taxonomically rather complex. Most members of this group produce a yellow pigment and are closely related to *Enter-*

obacter agglomerans, a taxon which Ewing and Fife (20, 21) proposed for clinical isolates. Recently, some strains belonging to the *Enterobacter agglomerans*-*Erwinia herbicola* complex, a heterogeneous group that includes strains of the herbicola group and *Enterobacter agglomerans*, were placed in a new genus, the genus *Pantoea* (23, 29, 36). Despite the various taxonomic reevaluations of the genus *Erwinia*, the taxonomic complexity of this taxon has not been completely resolved, and a dual system of nomenclature is in use (9, 10, 15, 23, 29, 36, 48).

Analysis of 16S rRNA sequences has been demonstrated to be one of the most powerful methods for investigating the natural relationships of microorganisms (52). In this study, we determined 16S rRNA gene (rDNA) sequences of 16 *Erwinia* species and obtained the sequences of members of representative genera of the family *Enterobacteriaceae* from the EMBL and GenBank databases in order to further clarify the taxonomy of the heterogeneous genus *Erwinia* on the intragenetic and intergeneric levels.

MATERIALS AND METHODS

Organisms and culture conditions. Bacterial strains were obtained from the American Type Culture Collection. Table 1 shows the strains whose sequences were determined in this study and the reference strains used for comparison when phylogenetic trees were constructed. Table 1 includes the strain designations and the GenBank accession numbers for the 16S rDNA sequences. The culture media and conditions used were the media and conditions recommended in the *American Type Culture Collection Catalogue of Bacteria and Bacteriophages* (1).

DNA extraction. Chromosomal DNA was isolated by the method of Ausubel et al. (2), except that the lysates were extracted two times with chloroform to remove residual phenol.

PCR amplification of 16S rDNA. The 16S rDNAs were amplified by using universal primers fD1 and rP2 (47). Each PCR mixture (50 µl) contained primers (each at a concentration of 20 pmol), a mixture of deoxynucleoside triphosphates (Promega Co., Southampton, England) (each at a concentration of 200 µM), *Taq* polymerase buffer, and chromosomal DNA (ca. 100 ng). *Taq* polymerase (2.5 U) (Promega Co.) and 1 drop of mineral oil were added to each of the reaction solutions. The DNA thermal cycler (Perkin-Elmer Co., Norwalk, Conn.) used for amplification was programmed as follows: (i) an initial extensive denaturation step consisting of 94°C for 4 min; (ii) 35 cycles, with each cycle consisting of 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min; and (iii) a final extension step consisting of 72°C for 10 min.

Isolation and cloning of amplified 16S rDNA. The PCR solutions were electrophoresed on 0.8% agarose gels, and then 16S rDNAs were purified with a

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TABLE 1. Strains used and their nucleotide sequence accession numbers

Species or subspecies ^a	Strain ^b	Accession no.	Reference
<i>Erwinia amylovora</i>	ATCC 15580 ^T	U80195	This study
<i>Erwinia ananas</i> (<i>Pantoea ananas</i>)	ATCC 33244 ^{Tc}	U80196	This study
<i>Erwinia carotovora</i> subsp. <i>betavascularum</i>	ATCC 43762 ^T	U80198	This study
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	ATCC 15713 ^T	U80197	This study
<i>Erwinia carotovora</i> subsp. <i>wasabiae</i>	ATCC 43316 ^T	U80199	This study
<i>Erwinia chrysanthemi</i>	ATCC 11663 ^T	U80200	This study
<i>Erwinia cypripedii</i>	ATCC 29267 ^T	U80201	This study
<i>Erwinia herbicola</i> (<i>Pantoea agglomerans</i>)	ATCC 33243 ^T	U80202	This study
<i>Erwinia milletiae</i> (<i>Pantoea agglomerans</i>)	ATCC 33261 ^T	U80183	This study
<i>Erwinia nigrifluens</i>	ATCC 13028 ^T	U80203	This study
<i>Erwinia persicinus</i>	ATCC 35998 ^T	U80205	This study
<i>Erwinia rhapontici</i>	ATCC 29283 ^T	U80206	This study
<i>Erwinia rubrifaciens</i>	ATCC 29291 ^T	U80207	This study
<i>Erwinia salicis</i>	ATCC 15712 ^T	U80210	This study
<i>Erwinia stewartii</i> (<i>Pantoea stewartii</i> subsp. <i>stewartii</i>)	ATCC 8199 ^{Tc}	U80208	This study
<i>Erwinia uredovora</i> (<i>Pantoea ananas</i>)	ATCC 19321 ^T	U80209	This study
<i>Escherichia coli</i>		J01695	11
<i>Hafnia alvei</i>	ATCC 13337 ^T	M59155	Unpublished data
<i>Klebsiella pneumoniae</i>	DSM 30104	X87276	Unpublished data
<i>Proteus vulgaris</i>		J01874	12
<i>Serratia marcescens</i>	ATCC 13880 ^T	M59160	Unpublished data
<i>Yersinia intermedia</i>	ER 3854	X75279	27

^a The names in parentheses are subjective synonyms.

^b ATCC, American Type Culture Collection, Rockville, Md.; DSM, German Collection of Microorganisms, Braunschweig, Germany; ER, *Yersinia* Reference Center, Toronto, Canada.

^c Type strains of proposed *Pantoea* species (23, 36).

QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). Purified rDNAs were ligated into pUC119 vectors. Ligated plasmids were then transformed into *Escherichia coli* DH5 α F' cells, and transformants were selected by the blue-white screening procedure (39).

Sequencing of 16S rDNAs. Plasmids containing the 16S rDNA fragments were isolated by using a QIAquick plasmid minikit (Qiagen GmbH). Purified plasmids were manually sequenced by using both a T7 sequencing kit (Pharmacia Biotech, Inc., Piscataway, N.J.) and the *fmoI* DNA sequencing system (Promega Co.) according to the suppliers' instructions. The ends of 16S rDNAs were sequenced by using forward sequencing primer pUC/M13 (5'-GTTTCCCGAGTCACGAC-3') and a reverse primer (5'-GCGGATAACAATTTCACACAGG-3'). The internal regions were sequenced by using a set of internal 16S rDNA primers which we designed. The primer sequences were as follows: 5'-GCCACACTGGAAGTACGACAC-3' (nucleotides 311 to 330), 5'-TGTAAGCGGTGAAATGCGTG-3' (nucleotides 684 to 703), 5'-GGAGCATGTGGTTTAAATTCG-3' (nucleotides 944 to 963), and 5'-CTACACACGTGCTACAATGG-3' (nucleotides 1228 to 1247).

Phylogenetic analysis. The 16S rDNA sequences which we determined and the sequences of the reference strains of members of the family *Enterobacteriaceae* obtained from the EMBL and GenBank databases were analyzed. The 16S rDNA sequences were first aligned by using the Clustal V program (26), and then the alignments were corrected by hand. An evolutionary distance matrix was generated as described by Jukes and Cantor (28). Evolutionary trees for the data set were inferred by the neighbor-joining method of Saitou and Nei (38) by using the neighbor-joining program of MEGA (30). 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 16S rDNA sequences which we determined have been deposited in the GenBank database under the accession numbers shown in Table 1.

RESULTS AND DISCUSSION

Almost complete 16S rDNA sequences (ca. 1,470 bp), corresponding to nucleotides 50 to 1518 of the *Escherichia coli* 16S rRNA sequence (11), were determined for the type strains of 16 *Erwinia* species. These sequences were compared with previously published sequences of *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Hafnia alvei*, *Yersinia intermedia*, and *Proteus vulgaris*, which are members of the family *Enterobacteriaceae*. The hypervariable regions of the 16S rDNA sequences of *Erwinia* strains were the regions at nucleotides 70 to 103, 458 to 478, and 1000 to 1023. The combination of these

regions can be useful in identifying *Erwinia* species. The variation in the sequence homology values (range, 92.5 to 99.9%) for different *Erwinia* species (Table 2) indicates that there is substantial intrageneric heterogeneity.

Interspecific relationships within clusters. One phyletic line, corresponding to the herbicola group of Dye (17), consists of the type strains of *Erwinia herbicola*, *Erwinia milletiae*, *Erwinia ananas*, *Erwinia uredovora*, and *Erwinia stewartii* (Fig. 1). The type strain of *Erwinia ananas*, a putative pathogen of pineapple rot, shows 99.9% sequence homology with the type strain of *Erwinia uredovora*, which attacks uredia of *Puccinia graminis* (Table 2). These organisms exhibited a high level of DNA-DNA relatedness (6, 36) and were phenotypically grouped in the same phenon (phenon 12) and the same subphenon (subphenon G1) by Verdonck et al. (44) and Mergaert et al. (35), respectively. Mergaert et al. (36) defined DNA hybridization group 2665, which included the type strains of *Erwinia ananas* and *Erwinia uredovora*, as a genomic species. Our 16S rDNA analysis supports the hypothesis that the strains belonging to hybridization group 2665 should be united in a single species. The sequence similarity between the type strains of *Erwinia herbicola* and *Erwinia milletiae* is very high (99.2%) (Table 2). The synonymy of some strains which were classified in the species *Erwinia herbicola*, *Erwinia milletiae*, and *Enterobacter agglomerans* was demonstrated on the basis of genomic data (4, 6, 34) and phenotypic data (35, 44). For example, Beji et al. (4) described hybridization group 27155, which included the type strains of *Erwinia herbicola*, *Erwinia milletiae*, and *Enterobacter agglomerans*, on the basis of DNA-DNA hybridization data, and Verdonck et al. (44) and Mergaert et al. (35) consistently grouped these strains in phenon 8 and subphenon F2, respectively, on the basis of numerical taxonomy results. The high sequence similarity of *Erwinia herbicola* ATCC 33243^T and *Erwinia milletiae* ATCC 33261^T confirmed that DNA hybridization group 27155 should be defined as a genomic species (23). To investigate the taxonomic position of another DNA hybridization group, group 14589, which in-

TABLE 2. Levels of similarity based on 16S rDNA sequences for several strains of the genus *Erwinia* and members of the family *Enterobacteriaceae*

	% 16S rDNA sequence similarity					
<i>Erwinia amylovora</i> ATCC 15580 ^T	100	96.4	100	100	100	<i>Erwinia amylovora</i> ATCC 15580 ^T
<i>Erwinia ananas</i> ATCC 33244 ^T	95.6	95.4	97.6	97.6	97.6	<i>Erwinia ananas</i> ATCC 33244 ^T
<i>Erwinia carotovora</i> subsp. <i>betavasculorum</i> ATCC 43762 ^T	96.0	95.9	98.0	98.3	98.3	<i>Erwinia carotovora</i> subsp. <i>betavasculorum</i> ATCC 43762 ^T
<i>Erwinia carotovora</i> subsp. <i>carotovora</i> ATCC 15713 ^T	95.4	95.1	96.5	96.2	96.9	<i>Erwinia carotovora</i> subsp. <i>carotovora</i> ATCC 15713 ^T
<i>Erwinia carotovora</i> subsp. <i>wasabiae</i> ATCC 43316 ^T	97.2	97.1	95.4	95.7	95.5	<i>Erwinia carotovora</i> subsp. <i>wasabiae</i> ATCC 43316 ^T
<i>Erwinia chrysanthemi</i> ATCC 11663 ^T	96.4	99.1	95.4	95.9	95.5	<i>Erwinia chrysanthemi</i> ATCC 11663 ^T
<i>Erwinia cypripedii</i> ATCC 29267 ^T	96.7	98.5	95.6	96.2	95.8	<i>Erwinia cypripedii</i> ATCC 29267 ^T
<i>Erwinia herbicola</i> ATCC 33243 ^T	95.0	94.6	95.2	96.4	96.0	<i>Erwinia herbicola</i> ATCC 33243 ^T
<i>Erwinia milletiae</i> ATCC 33261 ^T	97.8	95.9	95.6	95.9	96.4	<i>Erwinia milletiae</i> ATCC 33261 ^T
<i>Erwinia nigrifluens</i> ATCC 13028 ^T	98.2	96.1	95.8	96.2	96.7	<i>Erwinia nigrifluens</i> ATCC 13028 ^T
<i>Erwinia persicius</i> ATCC 35998 ^T	93.8	94.1	95.0	94.0	93.6	<i>Erwinia persicius</i> ATCC 35998 ^T
<i>Erwinia rhapontici</i> ATCC 29283 ^T	94.1	95.8	95.3	94.7	94.9	<i>Erwinia rhapontici</i> ATCC 29283 ^T
<i>Erwinia rubifaciens</i> ATCC 29291 ^T	96.8	98.2	95.5	95.8	95.4	<i>Erwinia rubifaciens</i> ATCC 29291 ^T
<i>Erwinia salicis</i> ATCC 15712 ^T	96.3	99.9	95.4	95.8	95.4	<i>Erwinia salicis</i> ATCC 15712 ^T
<i>Erwinia stewartii</i> ATCC 8199 ^T	96.1	95.6	95.2	95.0	95.2	<i>Erwinia stewartii</i> ATCC 8199 ^T
<i>Erwinia uredoovora</i> ATCC 19321 ^T	95.0	95.4	95.7	96.1	96.6	<i>Erwinia uredoovora</i> ATCC 19321 ^T
<i>Escherichia coli</i>	96.4	95.9	96.1	97.3	96.6	<i>Escherichia coli</i>
<i>Hafnia alvei</i> ATCC 13337 ^T	95.0	95.4	95.7	96.1	95.3	<i>Hafnia alvei</i> ATCC 13337 ^T
<i>Klebsiella pneumoniae</i> DSM 30104	92.9	92.7	92.8	93.1	93.1	<i>Klebsiella pneumoniae</i> DSM 30104
<i>Proteus vulgaris</i>	95.2	96.0	95.9	97.2	96.2	<i>Proteus vulgaris</i>
<i>Serratia marcescens</i> ATCC 13880 ^T	93.3	93.8	95.9	95.2	94.7	<i>Serratia marcescens</i> ATCC 13880 ^T
<i>Yersinia intermedia</i> ER 3854						<i>Yersinia intermedia</i> ER 3854

FIG. 1. Phylogenetic dendrogram based on a comparison of nearly complete 16S rDNA sequences for *Erwinia* species and members of the family *Enterobacteriaceae*. The branching pattern was generated by the neighbor-joining method (38). The numbers at the nodes indicate the levels of bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets; only values that are more than 40% are given.

cludes some other strains of *Erwinia herbicola*, *Erwinia milletiae*, and *Enterobacter agglomerans* (23), the 16S rDNA sequences of the members of this group are needed.

Although the close relatedness of the type strains of *Erwinia herbicola* and *Erwinia milletiae* to the type strains of *Erwinia ananas* and *Erwinia uredovora* was demonstrated by relatively high DNA binding values (51 to 56%) (4) and relatively high levels of 16S rDNA sequence homology (>98.5%) (Table 2), the relationship of *Erwinia stewartii* to other species of the herbicola group is not clear. The type strain of *Erwinia stewartii* did not belong to any of the DNA hybridization groups in the *Erwinia herbicola*-*Enterobacter agglomerans* complex as defined by Brenner et al. (6), and phenotypically, strains of *Erwinia stewartii* showed relatedness to other biochemically inert *Erwinia* species, such as *Erwinia amylovora* (35, 44). On the other hand, the relatively high genomic relatedness of the type strain of *Erwinia stewartii* to other species of the *Erwinia herbicola*-*Enterobacter agglomerans* complex was demonstrated by Beji et al. (4) and Gavini et al. (23). The type strain of *Erwinia stewartii* exhibited 46% DNA relatedness to DNA hybridization group 27155 of Beji et al. (4) (defined to include the type strains of *Erwinia herbicola*, *Erwinia milletiae*, and *Enterobacter agglomerans*) and 51% relatedness to *Pantoea dispersa* as described by Gavini et al. (23) (a newly defined species identical to hybridization group 14589 of Brenner et al. [6]). These findings are supported by our 16S rDNA sequence analysis data (Fig. 1 and Table 2) and by the DNA hybridization data of Beji et al. (4)

and Gavini et al. (23), who found that *Erwinia stewartii* is a member of the *Erwinia herbicola*-*Enterobacter agglomerans* complex.

Cluster II was identified as a branch that contained the type strains of *Erwinia amylovora*, *Erwinia rhapontici*, *Erwinia persicinus*, and *Erwinia cyripedii* (Fig. 1). In view of the findings of Dye (15, 16), this clade is unexpected because *Erwinia amylovora* was a representative of the amylovora group, whereas *Erwinia rhapontici* and *Erwinia cyripedii* were classified in the carotovora group along with *Erwinia carotovora* and *Erwinia chrysanthemi*. *Erwinia persicinus* was recently created for water-soluble pink pigment-producing strains isolated from tomatoes and other sources and was formerly known as "*Serratia rubefaciens*" (25). The 16S rDNA sequence of the type strain of *Erwinia persicinus* is very similar (99.0%) (Table 2) to that of the type strain of *Erwinia rhapontici*, which is the causative agent of crown rot of rhubarb (*Rheum rhaponticum*) or pink grain of wheat strains and is another water-soluble pink pigment-producing organism. DNA-DNA hybridization data (25) revealed that the type strain of *Erwinia persicinus* showed high levels of DNA relatedness (68 to 72%) to strains of *Erwinia rhapontici*, levels on the borderline for defining species (a group of "strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m " [ΔT_m is the difference between the melting temperature of a homoduplex and the melting temperature of a heteroduplex]) (46). In addition, *Erwinia persicinus* can be differentiated from *Erwinia rhapontici* by only a few biochemical reactions, such as methyl red, glyco-

erol, and D-xylose reactions (25). *Erwinia cypripedii* causes a brown rot on members of the genus *Cypripedium* and was considered a member of the former carotovora group along with *Erwinia rhapontici* on the basis of pathogenic symptoms and biochemical activity. However, neither of these organisms produces pectate lyases, and they have a fatty acid profile that is clearly distinct from the fatty acid profiles of *Erwinia carotovora* subspecies and *Erwinia chrysanthemi*; the former organisms have higher levels (49.56 to 48.28%) of saturated straight-chain fatty acids and lower levels (24.87 to 27.16%) of unsaturated acids than the latter organisms (35.79 to 40.86% and 46.37 to 50.95%, respectively) (48). As determined by the 16S rDNA sequence analysis, *Erwinia amylovora* is closely related to *Erwinia rhapontici* (98.2% sequence homology), *Erwinia persicinus* (97.8% sequence homology), and *Erwinia cypripedii* (97.2% sequence homology) (Table 2). The phenotypic differences between *Erwinia amylovora* and other members of clade II are apparent in biochemical properties; *Erwinia amylovora* utilizes a restricted range of carbon compounds and requires organic nitrogen compounds for growth (15, 16, 32). Although *Erwinia amylovora*, the type species of the genus *Erwinia*, exhibited no significant affinity with other *Erwinia* species on the basis of the genomic relatedness data of Brenner et al. (7). Hao et al. (25) showed that *Erwinia persicinus* exhibited a relatively high level of genomic relatedness with *Erwinia amylovora* (49%) and a moderate level of relatedness with *Erwinia cypripedii* (39%), suggesting that there is genomic relatedness among the species of cluster II. The reason for the inconsistency in the substantial phenotypic differences and the moderate level of genomic relatedness is not known, which hinders any further explanation of the taxonomic relationships of members of cluster II.

The *Erwinia carotovora* subspecies (*Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *wasabiae*, and *Erwinia carotovora* subsp. *betavascularum*) and *Erwinia chrysanthemi* formed a distinct cluster (Fig. 1). This cluster contains biochemically active and soft-rot-causing organisms that produce pectate lyases and cellulases. The phenotypic relatedness (16, 19, 24, 32, 35, 44, 48) and genotypic relatedness (9, 10, 37) of these species were supported by our results. A high sequence similarity (98.3%) was observed between *Erwinia carotovora* subsp. *carotovora* and *Erwinia carotovora* subsp. *wasabiae* (Table 2). Although the genomic relatedness between these two subspecies was not studied, *Erwinia carotovora* subsp. *carotovora* and *Erwinia carotovora* subsp. *wasabiae* had indistinguishable pathogenicities and the same G+C content range (51 to 53 mol%) (24). In addition, in physiological and biochemical properties, *Erwinia carotovora* subsp. *wasabiae* was more similar to *Erwinia carotovora* subspecies than to *Erwinia chrysanthemi* (24). *Erwinia carotovora* subsp. *betavascularum*, the sugarbeet pathogen, is readily distinguished from the other subspecies of *Erwinia carotovora* and *Erwinia chrysanthemi* by nutritional and physiological tests (13, 14, 42–44). Dickey (13) suggested that *Erwinia carotovora* subsp. *betavascularum* should have specific rank on the basis of several distinctive characteristics (acid is produced from inulin but not from α -D-galacturonic acid, sodium citrate is not utilized, and growth is inhibited by KCN). The levels of 16S rDNA sequence homology between *Erwinia carotovora* subsp. *betavascularum* and *Erwinia carotovora* subsp. *carotovora* (97.6%) and between *Erwinia carotovora* subsp. *betavascularum* and *Erwinia carotovora* subsp. *wasabiae* (98.0%) are less than the level of 16S rDNA sequence homology between *Erwinia carotovora* subsp. *carotovora* and *Erwinia carotovora* subsp. *wasabiae* (98.3%) (Table 2). To elucidate the taxonomic position of *Erwinia carotovora* subsp. *betavascularum* more precisely, a DNA-DNA hybridiza-

tion study is needed. The average level of 16S rDNA sequence similarity between *Erwinia chrysanthemi* and *Erwinia carotovora* subspecies was 96.5% (Table 2). The closest relationship was the relationship between *Erwinia chrysanthemi* and *Erwinia carotovora* subsp. *wasabiae*, and the most distant relationship was the relationship between *Erwinia chrysanthemi* and *Erwinia carotovora* subsp. *carotovora* (Table 2 and Fig. 1). *Erwinia chrysanthemi*, which causes soft rot on a broad range of (sub)tropical plants and lacks the enterobacterial antigen common in the family *Enterobacteriaceae* (33), was thought to be more closely related to *Erwinia carotovora* than to other species of the genus *Erwinia* on the basis of physiological, pathological, serological, DNA-DNA hybridization, and 16S rDNA sequence analysis data (Fig. 1).

The last clade consists of *Erwinia salicis*, *Erwinia nigrifluens*, and *Erwinia rubrifaciens* (Fig. 1). *Erwinia nigrifluens* and *Erwinia rubrifaciens* cause bark necrosis and phloem necrosis of Persian walnut, respectively, and can be isolated from the same host (49, 50). *Erwinia salicis* is a pathogen for a vascular wilt of *Salix* species. The levels of sequence similarity for the type strains of *Erwinia salicis*, *Erwinia nigrifluens*, and *Erwinia rubrifaciens* are 95.1 to 95.4% (Table 2). Because of their similar pathogenic symptoms and relative biochemical inertness, these three species were classified in the amylovora group, which is represented by *Erwinia amylovora* and includes *Erwinia malloffora*, *Erwinia tracheiphila*, and *Erwinia quercina*. The fact that taxonomic resolution of the amylovora group by phenotypic tests is not adequate is consistent with DNA-DNA hybridization results (3, 8, 37). The levels of genomic relatedness between *Erwinia amylovora* and other members of this group are only 13 to 23%, but *Erwinia salicis*, *Erwinia nigrifluens*, and *Erwinia rubrifaciens* are about 50% related as determined by genomic relatedness studies (3, 8). Our data are consistent with the DNA-DNA hybridization data.

Phylogenetic relationships among clusters. The results of the analysis of 16S rDNA sequences support the heterogeneous taxonomic structure of the genus *Erwinia* (Table 2 and Fig. 1). The genus *Erwinia* consists of multiphyletic lines (at least four phyletic lines) which are closely related to other members of the *Enterobacteriaceae*, such as *Escherichia coli*, *K. pneumoniae*, *S. marcescens*, and *H. alvei* (Fig. 1). In particular, clusters I and II, along with *Escherichia coli*, *K. pneumoniae*, and *S. marcescens*, form a macrophyletic line (Fig. 1), whose members exhibit greater than 95.1% sequence homology (Table 2). The taxonomic significance of this line is supported by the bootstrap value (41% based on 1,000 samplings). The relationships of the genus *Erwinia* to other genera of the family *Enterobacteriaceae* were demonstrated by genomic DNA relatedness data (7, 8, 37). Murata and Starr (37) stated on the basis of DNA segmental homology test results that the genera *Erwinia*, *Escherichia*, *Klebsiella*, *Enterobacter*, and *Serratia* and possibly some other enterobacteria should be associated at a genetic homology level of about 10% and could be integrated into a rather broad genus. Although complete comparative studies of these genera have not been conducted, our data revealed the possibility that clusters I and II, *Escherichia coli*, *S. marcescens*, and *K. pneumoniae* may form a macrocluster and possibly a new genus.

The intragenic classification of *Erwinia* species is rather complex. *Erwinia carotovora* and *Erwinia chrysanthemi*, defined as carotovora group by Dye (16), form an unstable phylogenetic line on the basis of 16S rDNA sequence data, which is supported by low bootstrap values (<40%) (Fig. 1). When a phylogenetic analysis is conducted only with *Erwinia* species, this cluster forms a distinct line supported by a moderate bootstrap value (71%) and can be separated from *Erwinia*

rhapontici and *Erwinia cypripedii*, as well as other *Erwinia* species (data not shown). Waldee (45) proposed a separate genus, the genus *Pectobacterium*, for the biochemically active soft-rot bacteria, such as *Erwinia carotovora* and *Erwinia chrysanthemi*. This proposal was supported by Brenner et al. (9), who contended that the genus *Pectobacterium* includes five species that are related by genomic and phenotypic properties. However, it was not generally accepted because there were intermediate species, such as *Erwinia rhapontici* and *Erwinia cypripedii*, whose characteristics were not fully compatible with those of *Erwinia carotovora* and *Erwinia chrysanthemi*. Considering the phenotypic heterogeneity (13, 31, 35, 44, 53) of the strains of these two species, along with 16S rDNA sequence data and genomic relatedness data, a taxonomic reevaluation of these organisms at the genus level is needed.

Cluster I is composed of species classified in the herbicola group by Dye (17) (Fig. 1). These species are associated with plants as saprophytes, epiphytes, and pathogens. Later, Ewing and Fife (20, 21) proposed the name *Enterobacter agglomerans* for the clinical isolates. The biochemical (20, 21, 35, 44) and genotypic (4, 6, 23, 34) characteristics of *Enterobacter agglomerans* are similar to those of the herbicola group. The separation of these strains at the genus level resulted in taxonomic complexity, and the name *Erwinia herbicola-Enterobacter agglomerans* complex, an invalid taxonomic name, was used for these strains. Recently, the genus *Pantoea* (23, 29, 36) was proposed, and this genus included some strains of the *Erwinia herbicola-Enterobacter agglomerans* complex and the tentatively defined organisms "*Erwinia citreus*," "*Erwinia punctata*," and "*Erwinia terreus*." The type strains of *Erwinia herbicola*, *Erwinia milletiae*, and *Enterobacter agglomerans* were assigned to *Pantoea agglomerans*, the type strains of *Erwinia ananas* and *Erwinia uredovora* were assigned to *Pantoea ananas*, and the type strain of *Erwinia stewartii* was assigned to *Pantoea stewartii* subsp. *stewartii*. It was observed during the 16S rDNA sequence analysis that among *Erwinia* species, strains reclassified as *Pantoea* strains form a homogeneous taxon supported by a moderate bootstrap value (Fig. 1) and a relatively high level of 16S rDNA sequence similarity (Table 2). Nevertheless, the separation of cluster I organisms as members of the genus *Pantoea* is not fully supported by the 16S rDNA sequence analysis since clusters I and II form a stable macrophyletic line which is not influenced by the different algorithms and exhibit greater than 95.9% sequence similarity (Table 2).

The coherence of cluster IV as a distinct taxon is weakened by a low bootstrap value (Fig. 1). However, it is apparent that the type strains of *Erwinia salicis*, *Erwinia rubrifaciens*, and *Erwinia nigrifluens* are the organisms that are least closely related to other *Erwinia* strains, as these strains did not exhibit significant affinity with any other *Erwinia* taxon with any of the analytical methods used in our study (data not shown). Murata and Starr (37) supposed that in the course of evolution, specific host-pathogen relationships exerted profound effects on the differentiation of the DNA sequences of organisms. At present, it is not clear whether the large sequence deviations found in cluster IV compared with other *Erwinia* species are affected by species-specific host-parasite relationships.

The results of our analysis of 16S rDNA sequences provide some evidence which can be used to resolve the taxonomic relationships of various species and groups stemming from the heterogeneity of the genus *Erwinia*. According to our analyses, the genus *Erwinia* is composed of four phyletic lines, and four clusters are phylogenetically intermixed with other taxa of the *Enterobacteriaceae*, such as *Escherichia coli* and the genera *Klebsiella* and *Serratia* (Fig. 1). Clusters I and II each is a well-separated cluster and these clusters are most closely re-

lated to each other (Fig. 1). Clear taxonomic positions for clusters III and IV cannot be determined on the basis of present data, but we recognize that to some degree these clusters can be separated from other members of the genus *Erwinia* and genera of the family *Enterobacteriaceae*. To determine the positions of these taxa, it will be necessary to carry out a comprehensive analysis in which more chemotaxonomic and molecular approaches are used.

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