NOTE

The phylogenetic position of *Serratia*, *Buttiauxella* and some other genera of the family *Enterobacteriaceae*

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The phylogenetic relationships of the type strains of 38 species from 15 genera of the family *Enterobacteriaceae* were investigated by comparative 16S rDNA analysis. Several sequences of strains from the genera *Citrobacter*, *Erwinia*, *Pantoea*, *Proteus*, *Rahnella* and *Serratia*, analysed in this study, have been analysed previously. However, as the sequences of this study differ slightly from the published ones, they were included in the analysis. Of the 23 enterobacterial genera included in an overview dendrogram of relatedness, members of the genera *Xenorhabdus*, *Photorhabdus*, *Proteus* and *Plesiomonas* were used as a root. The other genera formed two groups which could be separated, although not exclusively, by signature nucleotides at positions 590–649 and 600–638. Group A contains species of *Brenneria*, *Buttiauxella*, *Citrobacter*, *Escherichia*, *Erwinia*, *Klebsiella*, *Pantoea*, *Pectobacterium* and *Salmonella*. All seven type strains of *Buttiauxella* share 16S rDNA similarities greater than 99%. Group B embraces two phylogenetically separate *Serratia* clusters, a lineage containing *Yersinia* species, *Rahnella aquatica*, *Ewingella americana*, and also the highly related pair *Hafnia alvei* and *Obesumbacterium proteus*.

Keywords: *Enterobacteriaceae*, *Buttiauxella*, *Serratia*, 16S rDNA analysis

In contrast to other taxon-rich families, such as the *Clostridiaceae*, the *Bacillaceae* and the *Pseudomonadaceae*, members of the *Enterobacteriaceae* have not been subjected to extensive analysis of 16S rDNA. While some genera have been investigated in detail, e.g. *Xenorhabdus* and *Photorhabdus* (Szallás *et al.*, 1997), *Yersinia* (Ibrahim *et al.*, 1994), *Salmonella* (Chang *et al.*, 1997; Christensen *et al.*, 1998), *Serratia* (Dauga *et al.*, 1990; Harada *et al.*, 1996), and *Erwinia* (Kwon *et al.*, 1997; Hauben *et al.*, 1998), other genera have been analysed less extensively, e.g. *Enterobacter* (Hauben *et al.*, 1998), *Proteus* (Nebel *et al.*, 1987), *Citrobacter* (Maidak *et al.*, 1997), and most of the monospecific genera. The reason for the dismissal of many members of the *Enterobacteriaceae* may stem from knowledge of the high intrafamily relatedness (Brenner, 1991), as measured by DNA–DNA hybridization. Because of the conserved primary struc-

ture of the 16S rDNA, this molecule was not thought to solve taxonomic problems concerning closely related species. Extensive phylogenetic analysis of the genera *Yersinia*, *Salmonella*, *Photorhabdus* and *Erwinia*, however, have demonstrated that the variable and highly variable regions of the 16S rDNA molecule have sufficient phylogenetic powers of discrimination to allow the recognition of the same sets of relatedness as those unravelled by DNA–DNA reassociation studies. Furthermore, the phylogenetic incoherence of *Erwinia* has been demonstrated in the studies of Kwon *et al.* (1997) and Hauben *et al.* (1998).

In this work, 16S rDNA-based analysis of the family *Enterobacteriaceae* is extended by adding sequences of 38 type strains to the database. Eleven type strains of *Erwinia* species investigated in the course of this study were published by Hauben *et al.* (1998), and several of these species were subsequently reclassified by these authors as species of *Pectobacterium* and *Brenneria*.

The organisms investigated in this study, their strain designation, and their 16S rDNA accession numbers

The EMBL accession numbers for the 16S rDNA sequences analysed in this paper are AJ233400–AJ233437.

Table 1. Strains analysed in this study, and their 16S rDNA accession numbers

Species/subspecies	Strain	Accession no.
<i>Budvicia aquatica</i>	DSM 5075 ^T	AJ233407
<i>Buttiauxella agrestis</i>	DSM 4586 ^T	AJ233400
<i>Buttiauxella brennerae</i>	DSM 9396 ^T	AJ233401
<i>Buttiauxella ferruginae</i>	DSM 9390 ^T	AJ233402
<i>Buttiauxella gaviniae</i>	DSM 9393 ^T	AJ233403
<i>Buttiauxella izardii</i>	DSM 9397 ^T	AJ233404
<i>Buttiauxella noackiae</i>	DSM 9401 ^T	AJ233405
<i>Buttiauxella warmboldiae</i>	DSM 9404 ^T	AJ233406
<i>Brenneria alni</i>	DSM 11811 ^T	AJ233409
<i>Brenneria quercina</i>	DSM 4561 ^T	AJ233416
<i>Brenneria rubrifaciens</i>	DSM 4483 ^T	AJ233418
<i>Brenneria salicis</i>	DSM 30166 ^T	AJ233419
<i>Citrobacter freundii</i>	DSM 30039 ^T	AJ233408
<i>Erwinia amylovora</i>	DSM 30165 ^T	AJ233410
<i>Erwinia mallotivora</i>	DSM 4565 ^T	AJ233414
<i>Erwinia nigrifluens</i>	DSM 30175 ^T	AJ233415
<i>Erwinia rhapontici</i>	DSM 4484 ^T	AJ233417
<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i>	DSM 30104 ^T	AJ233420
<i>Leminorella grimontii</i>	DSM 5078 ^T	AJ233421
<i>Obesumbacterium proteus</i>	DSM 2777 ^T	AJ233422
<i>Pantoea agglomerans</i>	DSM 3493 ^T	AJ233423
<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	DSM 30168 ^T	AJ233411
<i>Pectobacterium chrysanthemi</i>	DSM 4610 ^T	AJ233412
<i>Pectobacterium cypripedii</i>	DSM 3873 ^T	AJ233413
<i>Pragia fontium</i>	DSM 5563 ^T	AJ233424
<i>Proteus vulgaris</i>	DSM 30118 ^T	AJ233425
<i>Rahnella aquatica</i>	DSM 4594 ^T	AJ233426
<i>Serratia entomophila</i>	DSM 12358 ^T	AJ233427
<i>Serratia ficaria</i>	DSM 4569 ^T	AJ233428
<i>Serratia fonticola</i>	DSM 4576 ^T	AJ233429
<i>Serratia grimesii</i>	DSM 30063 ^T	AJ233430
<i>Serratia marcescens</i>	DSM 30121 ^T	AJ233431
<i>Serratia odorifera</i>	DSM 4582 ^T	AJ233432
<i>Serratia plymuthica</i>	DSM 4540 ^T	AJ233433
<i>Serratia proteamaculans</i> subsp. <i>proteamaculans</i>	DSM 4543 ^T	AJ233434
<i>Serratia proteamaculans</i> subsp. <i>quinovora</i>	DSM 4597 ^T	AJ233435
<i>Serratia rubidaea</i>	DSM 4480 ^T	AJ233436
<i>Tatumella ptyseos</i>	DSM 5000 ^T	AJ233437

are listed in Table 1. Extraction of genomic DNA and the amplification of 16S rDNA were performed as described previously (Rainey *et al.*, 1996). The PCR products were purified by using the Prep-A-Gene kit (Bio-Rad), as described by the manufacturer. The DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) was used for direct sequencing of the PCR products, as described by the manufacturer. The sequence reactions were electrophoresed on an Applied Biosystems 373A DNA Sequencer. Sequences were

aligned manually against available sequences for members of the family *Enterobacteriaceae*. Evolutionary distances were computed from globally aligned sequences by using the correction of Jukes & Cantor (1969), omitting gaps and ambiguous positions. Dendrograms of relatedness were generated by the algorithms of De Soete (1983) and by neighbour-joining and maximum-likelihood analyses using the programs of the PHYLIP package (Felsenstein, 1993). Bootstrap values were determined as described elsewhere (Felsenstein, 1993). The following reference sequences were taken from the EMBL database: *Escherichia coli* (J01695, Brosius *et al.*, 1978); *Pectobacterium cacticidum* LMG 17936^T (AJ223409); *Erwinia persicinus* ATCC 35998^T (U80205); *Erwinia psidii* LMG 7034 (Z96085); *Erwinia tracheiphila* LMG 2906^T (Y13250); *Ewingella americana* NCPPB 3905 (X88848, entry cited unpublished); *Hafnia alvei* ATCC 13337^T (M59155, entry cited unpublished); *Plesiomonas shigelloides* ATCC 14029^T (M59159, entry cited unpublished); *Salmonella typhimurium* ATCC 19430^T (Z47544); *Yersinia pestis* D-28 (X75274); *Xenorhabdus nematophilus* DSM 3370^T (X82251); *Photorhabdus luminescens* DSM 3368^T (X82248) and *Plesiomonas shigelloides* ATCC 14029^T (X74688).

Almost complete 16S rDNA sequences were analysed for the type strains of 38 species from the genera *Brenneria*, *Budvicia*, *Buttiauxella*, *Citrobacter*, *Erwinia*, *Klebsiella*, *Leminorella*, *Obesumbacterium*, *Pantoea*, *Pectobacterium*, *Pragia*, *Rahnella*, *Serratia* and *Tatumella*. The lengths of the sequences ranged between 1493 and 1516 nucleotides, corresponding to 95.5 and 98.4%, respectively, of the *Escherichia coli* sequence. Some of the sequences of type strains of *Erwinia*, *Serratia*, *Citrobacter*, *Pantoea* and *Rahnella* species analysed in this study have been published recently (Dauga *et al.*, 1990; Harada *et al.*, 1996; Kwon *et al.*, 1997; Maidak *et al.*, 1997), but, as there are some nucleotide differences in sequences originating from the same strain (98.8–99.9% sequence similarity), sequences determined in this study were deposited in the EMBL database. Sequence similarities obtained for the sequences of the 11 type strains of *Erwinia*, *Brenneria* and *Pectobacterium*, published, during this study, by Hauben *et al.* (1998), ranged between 98.5 and 100%. A significant deviation from the data of Kwon *et al.* (1997) refers to *Brenneria salicis*. While the 16S rDNA sequences of strains DSM 30166^T (this study) and LMG 2698^T (Hauben *et al.*, 1998) are identical, they share only 94.5% sequence similarity with strain ATCC 15712^T. The branching point of *Pectobacterium cypripedii* DSM 3873^T within the radiation of authentic *Erwinia* species differs from the position of the type strain LMG 5657^T of the same species within the radiation of cluster III, which, consequently, has been reclassified as *Pectobacterium cypripedii* (Hauben *et al.*, 1998). Certainly, the authenticity of the type strains of these two species requires investigation.

Comparison of the sequences generated in this study

Fig. 1. Phylogenetic tree of the 16S rDNA of members of various genera of the family *Enterobacteriaceae*. The positions of *Erwinia* clusters I (*Pantoea*) to IV (Kwon et al., 1997) in group A and the *Serratia* clusters I and II in group B, defined in this study, are indicated. A detailed analysis of the *Buttiauxella agrestis* lineage is shown in Fig. 2. Numbers within the dendrogram indicate the occurrence (%) of the branching order in 200 bootstrapped trees (only values of 60 and above are shown). The scale bar represents 2 nucleotide substitutions per 100 nucleotides.

with those published previously for members of the family *Enterobacteriaceae* revealed a high degree of relatedness. The most unrelated members of the family were those of the rooting species, *Photorhabdus* and *Xenorhabdus* (90–94.5% similarity), *Proteus vulgaris* (92–95.6%) and *Plesiomonas shigelloides* (92.8–95.5%), but these values were not significantly higher than those separating members of different genera (94–97%). Phylogenetic trees obtained with two different additive treeing algorithms and by maximum-likelihood analysis showed only slight differences in the branching pattern. The few deviations were found for deeply rooting lineages, such as those of *Klebsiella pneumoniae* and *Citrobacter freundii*, and the lineage of *Leminorella*, *Pragia* and *Budvicia* species. Bootstrap values were low for almost all branching points. The decision to depict phylogenetic relatedness according to the algorithm of De Soete (1983) (Fig. 1) is based on experience with actinobacteria and many other phylogenetic groups of mainly Gram-positive bacteria in which patterns of chemotaxonomic markers and the distribution of signature nucleotides strongly supported the emergence of phylogenetic clusters (Stackebrandt *et al.*, 1997a, b).

Analysis of sequences according to the occurrence of cluster-specific signature nucleotides reveals no strong pattern for any of the clusters. The most interesting two pairs of nucleotides are located at positions 590 and 649 and at positions 600 and 638 (*Escherichia coli* nomenclature; Brosius *et al.*, 1978). These two pairs enable separation of the organisms investigated into two groups. Group A ranges from the top (in Fig. 1) to *Brenneria rubrifaciens*, while all species between *Budvicia aquatica* and *Plesiomonas shigelloides* form cluster B. Members of group A are defined by the nucleotide composition C/u–G (small letters indicate minority composition) at positions 590–649 (the exceptions are *Escherichia coli*, its close relatives, and *Buttiauxella gaviniae* DSM 9393, all of which have a U–A base pair) and the pair G/a–C/u at positions 600–638. Members of this group which possess a A–U pair at the latter positions are *Escherichia coli* and close relatives, all members of *Erwinia* cluster I (*Pantoea*), and *Erwinia mallotivora*. All members of group B possess the nucleotide pairs U–A and A–U at positions 590–649 and 600–638, respectively. *Brenneria quercina*, which branches deeply within group A, exhibits the base pairs U–G and A–U at the relevant positions.

The genera *Erwinia*, *Brenneria* and *Pectobacterium*

Four clusters of *Erwinia* species have recently been defined and their structure discussed in detail, and in the light of DNA–DNA reassociation data, by Kwon *et al.* (1997). Cluster I contains former *Erwinia* species that are now classified as *Pantoea* species or united with *Pantoea* species, such as *Erwinia herbicola* and *Erwinia milletiae* with *Pantoea agglomerans*, and *Erwinia uredovora* with *Pantoea ananatis* (Mergaert *et*

Fig. 2. Phylogenetic tree of 16S rDNA of type strains of *Buttiauxella* species. Sequences of members of group A served as a root. The scale bar represents 1 nucleotide substitution per 100 nucleotides.

al., 1993). In a recent extensive study, Hauben *et al.* (1998) analysed additional type strains of *Erwinia* and reclassified four and six species as members of *Pectobacterium* and *Brenneria*, respectively, which correspond to the clusters III and IV of Kwon *et al.* (1997). Sequences of strains originally received in this study as *Erwinia* strains do not change the internal structure of clusters, now defined by the genera *Erwinia*, *Brenneria* and *Pectobacterium*. All of these genera are members of group A. Four species sequenced in the course of the present study were members of cluster II [*Erwinia amylovora*, *Erwinia mallotivora*, *Erwinia rhapontici* and *Erwinia cypripedii* (see above)], one was a member of cluster IV (*Erwinia alni*, reclassified as *Brenneria alni*), *Erwinia tracheiphila* branched slightly outside the *Erwinia* cluster, and *Brenneria quercina* branched at the root of clusters containing the genera *Brenneria* and *Pectobacterium*. In contrast to the data of Kwon *et al.* (1997) and Hauben *et al.* (1998), in which the latter two clusters and genera, respectively, are phylogenetically well separated, all treeing algorithms used in this study depict the origin of *Brenneria* species as being within the radiation of *Pectobacterium* species.

The genus *Buttiauxella*

The type species of this genus, *Buttiauxella agrestis*, has been described for strains of a group (Ferragut *et al.*, 1981) that in some phenotypic respects resembled members of *Citrobacter*, but differed from *Citrobacter* strains in terms of certain metabolic characteristics and the base ratio of the DNA. Low DNA–DNA reassociation values generally less than 40% were obtained with *Citrobacter* species and a large range of representatives of the *Enterobacteriaceae* (Ferragut *et al.*, 1981). Recently, six new species (isolated from soil, snails, and human sputum) were added to the genus *Buttiauxella* (Müller *et al.*, 1996). Analysis of 16S rDNA (Fig. 2) reveals a very high degree of phylogenetic relatedness among the type strains of the seven *Buttiauxella* species (99.1–99.7% similarity). The

dendrogram of relatedness (Fig. 2) does not permit a detailed intrageneric clustering of type strains, as the bootstrap value is low at any branching point. Both the high degree of relatedness among the species and the low resolution of the relatedness are in accordance with the results of DNA–DNA reassociation studies (Müller *et al.*, 1996). These data indicated mean intrageneric DNA–DNA relatedness values of about 53%, while interspecies similarity values ranged between 50 and 56%.

At intrafamily level, the genus *Buttiauxella* is clearly separated from other members of other genera in the family (>97% 16S rDNA similarity), which supports earlier reports of low intrafamily DNA–DNA relatedness values (Gavini *et al.*, 1983). The *Buttiauxella* lineage (represented by *Buttiauxella agrestis* in Fig. 1) branches at a position that is intermediate to *Erwinia* clusters II and III/IV and their respective relatives, but bootstrap values are low.

The genus *Serratia*

The type strains of all nine species have been analysed; the phylogenetic branching pattern is very similar to that described by Dauga *et al.* (1990). As the descriptions of the *Serratia* species are supported by low values for DNA–DNA reassociation with their nearest neighbours (Gavini *et al.*, 1979; Grimont *et al.*, 1978, 1979, 1988), it is not surprising to see most of them well separated in the phylogenetic tree. The species form two phylogenetic clusters – abbreviated *Serratia* clusters I and II (Fig. 1) – both of which are members of group B. The presence of two clusters could not be unravelled in the study of Dauga *et al.* (1990), as *Serratia* species were analysed exclusively. All treeing algorithms place the species pair *H. alvei* and *Obesumbacterium proteus* at the root of cluster II. *Serratia odorifera*, *Serratia marcescens* and *Serratia rubidaea* are members of cluster I (97–98% sequence similarity). Under stringent reassociation conditions, members of the three species show less than 37% relative binding (similarity), which characterizes them as genomically well-defined species (Steigerwalt *et al.*, 1976; Grimont *et al.*, 1978). Members of cluster II contain the other species, of which *Serratia proteamaculans* subsp. *quinovora* and *Serratia grimesii* have almost identical 16S rDNA sequences (>99.7% similarity). These taxa share strong biochemical similarities (Grimont *et al.*, 1982b) but the separation of *S. grimesii* from *S. proteamaculans* is supported by moderate DNA–DNA reassociation values (Grimont *et al.*, 1982a). Another pair of closely related species is *Serratia entomophila* and *Serratia ficaria* (99.5% similarity) which, under stringent DNA reassociation conditions, also share a high degree of DNA relatedness. While the type strains share 72% DNA similarity, other strains of *S. ficaria* are somewhat less closely related with the type strain of *S. entomophila* (Grimont *et al.*, 1988).

The genera *Tatumella*, *Budvicia*, *Pragia*, *Leminorella* and *Obesumbacterium*

These five genera have been described and verified, respectively, on the basis of a combination of low values for DNA–DNA reassociation with other members of the *Enterobacteriaceae* and a unique pattern of biochemical reactions. Phylogenetic analysis indicates that, except for *Obesumbacterium*, these genera form lineages that are well separated from other genera in the family (the 16S rDNA similarity values ranging between 94 and 97%). *Tatumella ptyseos* (Hollis *et al.*, 1981) is a member of family-cluster A, branching close to members of *Escherichia*, *Salmonella* and *Erwinia* clusters I (*Pantoea*) and II. The three genera *Budvicia* (Aldová *et al.*, 1984), *Pragia* (Aldová *et al.*, 1988) and *Leminorella* (Hickman-Brenner *et al.*, 1985) form a separate cluster that is phylogenetically placed at the base of family-cluster B. The three species are well separated within this cluster, confirming their affiliation to different genera. *Obesumbacterium proteus* shares 99.5% 16S rDNA sequence similarity with *H. alvei*. According to Brenner (1991), a type strain of *O. proteus* does not exist, as the deposited type strain is a strain of *H. alvei*. On the basis of DNA–DNA reassociation values of 70% obtained for the two type strains, Priest *et al.* (1973) suggested the reclassification of *O. proteus* as *Hafnia protea*. The data presented here confirm the strong degree of relatedness between the type strains of the two species, but decisions about changes in taxonomy must await additional characterization.

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