Inability of the Polyphasic Approach to Systematics To Determine the Relatedness of the Genera *Xenorhabdus* and *Photorhabdus*

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Comparative analysis of the genes coding for 16S rRNA of the type strains of *Xenorhabdus* and *Photorhabdus* species indicates the close phylogenetic relationship of these two genera. However, distance matrix analyses do not unambiguously separate the symbionts of entomopathogenic nematodes according to their assignment into different genera. When various 16S rRNA gene sequences from a selection of members of the gamma subclass of *Proteobacteria* and outgroup taxa were used, the intrageneric relationships of *Xenorhabdus* species and the positions of *Photorhabdus luminescens* and related species changed significantly.

The genus *Photorhabdus* has recently been described for a group of bioluminescent and facultatively anaerobic insect-pathogenic strains that are symbionts of entomopathogenic nematodes belonging to the family Heterorhabditidae (3). The exclusion of *Photorhabdus luminescens* from the genus *Xenorhabdus* was based on low interspecies DNA reassociation values (3, 6, 7, 16) and the presence of distinguishing phenotypic (2, 3) and chemotaxonomic (16) properties. Thus, the genus *Xenorhabdus* was emended to contain only strains isolated from different entomopathogenic nematode species of the family Heterorhabditidae. None of the studies, however, were able to determine the intergeneric relatedness of these two taxa. Similarly, 16S rRNA cataloging performed on two strains of *P. (Xenorhabdus) luminescens* and one *X. nematophilus* strain (5) was carried out not to determine the possible phylogenetic heterogeneity of the genus *Xenorhabdus*, but to investigate whether these organisms belong to the family *Enterobacteriaceae*. In this study, we determined the 16S rRNA gene (16S rDNA) sequences of all type strains of both genera, and we discuss the problem of unambiguous phylogenetic placement of organisms.

MATERIALS AND METHODS

Strains investigated. *Xenorhabdus nematophilus* DSM 3370^T, *X. beddingii* DSM 4764^T, *X. poinarii* DSM 4768^T, *X. bovienii* 4766^T, and *Photorhabdus luminescens* DSM 3368^T and DSM 3369 are deposited in the DSM-German Collection of Microorganisms and Cell Cultures. *P. luminescens* HSH2 was isolated by R.-U. Ehlers from the nematode *Heterorhabditis megidis* HSH2. All strains were cultivated on medium 423 as indicated in the DSM catalogue of strains.

Analysis of 16S rDNA. Extraction of genomic DNA and amplification of the 16S rDNA were carried out as described previously (11). PCR products were sequenced directly by using the *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems) according to the manufacturer's protocol. The sequence reaction mixtures were electrophoresed by using the Applied Biosystems 373A DNA sequencer.

Phylogenetic analysis. 16S rDNA sequences were compared with the existing 16S rDNA database of members of the family *Enterobacteriaceae* and other members of the gamma subclass of *Proteobacteria* (9). Dissimilarity values were transformed into phylogenetic distance values that compensate for multiple substitutions at any given site in the sequence (8). Phylogenetic trees were

reconstructed by applying additive treeing methods, such as neighbor joining (12) and the algorithm of De Soete (4), using the corrected dissimilarity values. One thousand bootstrap values for 920 polymorphic sites were calculated to test the stability of the neighbor-joining tree.

Nucleotide sequence accession numbers. The 16S rDNA sequences have been deposited at EMBL under accession no. X82248 to X82254.

RESULTS

The almost complete sequences of the seven strains of *Xenorhabdus* and *Photorhabdus* species were analyzed. Binary 16S rDNA similarities for the *Xenorhabdus* strains ranged between 96.0 and 97.7%, while the values for these organisms and *P. luminescens* ranged between 94.1 and 96.6%. With 92.3 to 95.2% 16S rDNA similarity, the membership of *Xenorhabdus* and *Photorhabdus* in the family *Enterobacteriaceae* is in accord with previous findings (5). *Proteus vulgaris* can be considered the nearest phylogenetic neighbor (between 93.5 and 95.1% similarity). Other members of this subclass, including *Vibrio parahaemolyticus*, selected as an out-group member of the gamma subclass of *Proteobacteria*, have significantly less similarity, mostly <90% (data not shown).

Independently of the number of generic affiliation of out-group reference organisms, two of the most widely used algorithms for generating phylogenetic trees from dissimilarity values, i.e., the distance matrix methods of Saitou and Nei (12) and De Soete (4), consistently placed strains of *Xenorhabdus* and *Photorhabdus* on a single line of descent, separate from other members of the family *Enterobacteriaceae*. The clustering is supported by 100% significance as determined by bootstrap analysis. The number of signature nucleotides in the 16S rDNAs of *Photorhabdus* and *Xenorhabdus* strains is small, i.e., at positions 19 to 916 (C-U versus A-U, found in other enterobacteria) and positions 154 to 167 (C-G versus U-A, found in most other enterobacteria). A few signature nucleotides are shared with *Proteus vulgaris*, such as those at positions 320 to 333 (G-C versus A-U), 576 to 647 (A-U versus U-A), 577 to 646 (A-U versus G-C), and 682 to 707 (Y-G versus G-C). The G+C contents of 16S rDNAs of both genera of the symbionts of entomopathogenic nematodes are about 2 to 3% higher (55.1 to 55.5%) than those of other members of the *Enterobacteriaceae* (52.8 to 53.4%).

With a selection of nine species of the family *Enterobacteriaceae* and *V. parahaemolyticus* used as reference organisms, a neighbor-joining tree was generated (Fig. 1). In contrast to the

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FIG. 1. Neighbor-joining tree showing the phylogenetic relationships among members of the genera *Xenorhabdus* and *Photorhabdus* and members of the family Enterobacteriaceae. *V. parahaemolyticus* served as a root. The region in which the order of branching points leading to species of *Xenorhabdus* and *Photorhabdus* cannot be determined with confidence is indicated (shaded box). The bar represents five nucleotide substitutions per 100 nucleotides.

distinctly lower 16S rDNA similarity of about 2%, the three strains of *P. luminescens* do not cluster apart from *Xenorhabdus* strains but, rather, branch off from within the radiation of strains of the latter genus. While *X. nematophilus* and *X. beddingii* form a pair and *X. bovienii* and *X. poinarii* form a pair of distantly related species, supported by bootstrap values of 37 and 93%, respectively, *X. poinarii* and *X. bovienii* branch more deeply than the three *P. luminescens* strains. This branching pattern is not in accord with the bootstrap analysis, which indicated that, to the exclusion of *P. luminescens*, the type strains of *Xenorhabdus* clustered together in 65% of simulated trees. The same topology was obtained when the tree was generated by using the algorithm of De Soete (4). To investigate whether the number and origin of homologous sequences from various taxa of the gamma subclass influence the branching point of the *Xenorhabdus* and *Photorhabdus* species, 15 different sequence sets were analyzed by the two distance matrix algorithms. The number of sequences ranged from 8 to 40, and the selection of sequences included those of the symbiotic bacteria of the genus *Buchnera* (10) as well as those of the most deeply branching taxa of this subclass, i.e., sequences of species of *Nitrosomonas*, *Coxiella*, *Xanthomonas*, *Chromatium*, and *Ectothiorhodospira*. With two exceptions, in which the two genera investigated form phylogenetic sister groups, *P. luminescens* grouped within the radiation of *Xenorhabdus* species. This was independent of whether only the type strain of *P. luminescens* or all three strains of this species were included in the analysis. In most of these cases, *P. luminescens* clustered with the pair *X.*

nematophilus-*X. beddingii*, and *X. bovienii* clustered most deeply. This situation is similar to the one depicted in Fig. 1. The exceptions are those cases in which, in addition to the sequences of *Xenorhabdus* and *Photorhabdus* strains, randomly selected sequences of either only *Escherichia coli* or of all enterobacteria, three sequences of *Buchnera* strains, and one representative each of the genera *Vibrio* and authentic *Pseudomonas* were used (not shown). In order to demonstrate the inability of the algorithms and reference sequences used to reliably depict the relatedness between members of the genera *Xenorhabdus* and *Photorhabdus*, the region of uncertain branching points is indicated in Fig. 1.

DISCUSSION

Justification for the exclusion of *X. luminescens* from the genus *Xenorhabdus* and the description of *Photorhabdus* to accommodate this species was based on results of DNA-DNA pairing experiments, as well as chemotaxonomic and phenotypic characteristics. The intragenetic relatedness of *Xenorhabdus* species has been determined previously by different DNA-DNA reassociation methods (3, 6, 7, 16). In all of these studies, DNA relatedness determined for the type strains of *X. nematophilus* and *X. luminescens* was <20%, while the similarity between the type strains of the other *Xenorhabdus* species ranged between 20 and 40%. As DNA reassociation values for use in genus delineation are lacking, and low similarity values alone do not justify the dissection of a genus, further evidence

for the exclusion of *X. luminescens* was needed. While the ubiquinone composition and the DNA G+C content were not able to discriminate between these two taxa, differences in the major cellular fatty acids had been reported (16). However, the strains of *X. luminescens* differed from the other members of the genus only in having a slightly higher content of i-15 branched fatty acids. Results of a numerical taxonomic study showed that the symbionts of *Heterorhabditis* spp., *X. luminescens* strains, form a discrete cluster regardless of the datum set and the clustering strategy used. Thus, the phenetic data are in accordance with the DNA pairing study, the fatty acid analysis, and the 16S rDNA analysis. Strains of *X. luminescens* were linked most closely to the *Xenorhabdus* strains isolated from *Steinernema* organisms of Australian origin (group V as defined by Akhurst and Boemare [1]) which were described as *X. beddingii*. In three of four numerical analyses *X. luminescens* and *X. beddingii* formed a sister taxon to strains of *X. bovienii*, and in only one analysis strains of *X. luminescens* branched earlier than strains of the other species of *Xenorhabdus*. It thus appears that the exclusion of *X. luminescens* from the genus *Xenorhabdus* and the description of *P. luminescens* were done mainly on the basis of some phenotypic data in which strains of this species differed exclusively from strains of *Xenorhabdus*, such as presence of bioluminescence, positive catalase reaction, assimilation of arbutin, lack of DL-lactate production, and the host nematode. However, not all strains of *P. luminescens* are luminescent (1), and some strains closely related to *X. bovienii* are also catalase positive.

We do not want to question the validity of the transfer of *X. luminescens* to a new genus. The 16S rDNA analysis reflects the situation of the numerical phenetic analysis closely in that the relationship of *P. luminescens* to the species of *Xenorhabdus* depends heavily on the database used. Thus, if one agrees to this taxonomic conclusion, the two genera can nevertheless be considered very closely related, to the extent that the degree of genomic differences observed between the type species of the two genera is not significantly higher than that found between the species of *Xenorhabdus*.

The finding that the selection of reference organisms influences tree topologies quite significantly has been observed previously (14, 17). In general, every new sequence which is different from those in the existing datum set provides additional data on the information content of individual sequence positions as well as on the characteristics defining phylogenetic groups. The inclusion of this additional information may change the tree locally or even globally. This effect is recognized by those who construct phylogenetic trees from sequence data. Of the most obvious parameters, we can exclude a bias introduced by differences in the base composition of rDNA, as all *Xenorhabdus* and *Photorhabdus* species have very similar G+C values. Bias introduced by partial sequences (13) can be excluded, as almost complete sequences have been compared. However, we have noted that *Photorhabdus* species may be subjected to a slightly higher evolutionary rate ("fast clock" or tachytelic behavior). Support for this hypothesis originates from the finding that the level of similarity between these strains and the reference organism is about 2% lower than that found for *Xenorhabdus* species and the reference organisms (which should be very similar in comparison with out-group sequences) (15). At the nucleotide level, this hypothesis is supported by the lack of several absolutely unique positions in

the sequences of *P. luminescens* which are conserved for members of the gamma subclass of *Proteobacteria* (nucleotides 1001 to 1002 pairing with nucleotides 1038 to 1039 and nucleotides 1006 to 1009 pairing with nucleotides 100 to 1023; bp 1254 to 1283). Furthermore, strains of *P. luminescens* show greater variation in the nucleotide composition of hypervariable regions than is found among the type strains of the *Xenorhabdus* species.

The strategy that should be considered next is to increase the stability of the tree by adding more sequences of members of the two species, especially more strains of *P. luminescens*. If, as expected, *P. luminescens* strains are slightly more rapidly evolving, then this should be reflected and detectable in a greater variation of those nucleotide positions considered to be conserved in neighboring taxa.

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