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

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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 biochemical (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 cataloguing 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 33707, *X. beddingii* DSM 4765T, *X. pontarii* DSM 4766T, *X. bovienii* 4766T, and *Photorhabdus luminescens* DSM 3368T 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 A-U), 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
distinctly lower 16S rDNA similarity of about 2%, the three strains of *P. liminiscens* 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. beddingtonii* 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. liminiscens* strains. This branching pattern is not in accord with the bootstrap analysis, which indicated that, to the exclusion of *P. liminiscens*, 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*, *Coxella*, *Xanthomonas*, *Chromatium*, and *Ectothiorhodospira*. With two exceptions, in which the two genera investigated form phylogenetic sister groups, *P. liminiscens* grouped within the radiation of *Xenorhabdus* species. This was independent of whether only the type strain of *P. liminiscens* or all three strains of this species were included in the analysis. In most of these cases, *P. liminiscens* clustered with the pair *X. nematophilus*-*X. beddingtonii*, 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. liminiscens* 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 intrageneric 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. liminiscens* 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, nucleotide sequences 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. beddigi 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 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 1028 to 1039 and nucleotides 1254 to 1255. 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.

REFERENCES