Phylogeny of *Photorhabdus* and *Xenorhabdus* Species and Strains as Determined by Comparison of Partial 16S rRNA Gene Sequences†

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Partial 16S rRNA gene sequences of 16 strains of the genera *Photorhabdus* and *Xenorhabdus* were determined by direct sequencing of PCR products. Aligned sequences were subjected to phylogenetic analysis by maximum-likelihood and maximum-parsimony methods. Distance matrix and phylogenetic analysis did not separate the genera unambiguously. Taxonomic grouping of the bacteria closely paralleled taxonomic grouping of their nematode associates and their geographic origins. We found at least two well-supported taxonomic groups in *Photorhabdus* species, which suggests that the genus *Photorhabdus* is coevolving with the nematodes and may be polyspecific.

The genera *Photorhabdus* and *Xenorhabdus* consist almost entirely of bacterial symbionts of entomopathogenic nematodes belonging to the families Heterorhabditidae and Steinernematidae (6). The bacterial symbionts are carried monoxenically in a special vesicle in the intestine in infective juveniles of species of the genus *Steinernema* in the Steinernematidae (4) and throughout the entire intestine and pharynx of infective juveniles of species of the genus *Heterorhabditis* in the Heterorhabditidae (12, 23). The nematodes provide protection and transport for their bacterial symbionts. The bacterial symbionts establish and maintain suitable conditions for nematode reproduction (21, 22) and provide nutrients and antimicrobial substances that inhibit the growth of a wide range of microorganisms (2, 6).

Since the original description of the genus *Xenorhabdus* by Thomas and Poinar (30), strains of this taxon have been isolated easily from entomopathogenic nematodes around the world (3, 5). *Xenorhabdus luminescens* (30) has been transferred to a new genus, the genus *Photorhabdus* (6). The genus *Xenorhabdus* contains four species: *Xenorhabdus nematophilus*, *Xenorhabdus bovienii*, *Xenorhabdus poinarii*, and *Xenorhabdus beddingii* (3). The taxonomy of the genera *Photorhabdus* and *Xenorhabdus* has been examined by several methods. Major studies have been based on both phenotypic (4, 5, 14, 30) and genotypic traits (6, 11, 14, 24). Few studies have been conducted to determine intergeneric relatedness; however, the branching of genus level clusters remains unclear (24). A number of isolates have been studied in some detail but have not been named, and none of the DNA relatedness groups have been described as separate species because too few strains have been studied (6). Phylogenetic studies are generally lacking at intergeneric and generic levels, and the taxonomy of the two genera is still incomplete.

Comparison of 16S rRNA gene sequences has proved to be extremely useful for determining phylogenetic relationship among eukaryotic and prokaryotic organisms (32) and has been used in determining the relatedness of the genera *Photorhabdus* and *Xenorhabdus* (24). Because only a few strains have been studied, the variation among strains may not be fully

evaluated. In this study, the partial 16S rRNA gene sequences of 16 *Photorhabdus* and *Xenorhabdus* strains were determined by direct sequencing of PCR products with an automated DNA sequencer, and the aligned sequences were subjected to phylogenetic analysis. Our studies were designed to (i) clarify the intergeneric structure of the genera *Photorhabdus* and *Xenorhabdus* and assess the congruence of sequencing results with genotypic and DNA relatedness results and (ii) investigate the potential of the 16S rRNA gene sequences for describing additional related strains that may be separate species.

MATERIALS AND METHODS

Bacterial strains, sources, isolation, and cultivation. The strains sequenced in this study are listed in Table 1. Nematode isolates from Oregon were collected by Liu and Berry (16) and Poinar (20a). Bacteria were obtained indirectly from nematodes by sampling the hemocoel of *Galleria mellonella* (L.) larvae within 48 h of infection by nematodes. The hemocoel samples were spread on nutrient agar (Difco Laboratories, Detroit, Mich.). All strains were purified by selecting a single colony from nutrient agar. Phase one bacteria were determined by examining colony characteristics on nutrient agar as described by Akhurst (1). Bacterial strains were cultured at room temperature for 48 h in brain heart infusion broth (Difco Laboratories). Cultures were harvested by centrifugation and were washed three times in sterilized deionized water.

DNA extraction, amplification, and sequencing. Approximately 0.2 ml of pelleted frozen bacteria was pulverized with a motor-driven pestle. Genomic DNA was extracted from each strain with a DNA extraction kit (Stratagene, La Jolla, Calif.). The partial 16S rRNA gene was amplified by PCR in a 25- μ l reaction mixture containing 0.1 μ g of each primer, each deoxynucleoside triphosphate at a concentration of 100 μ M, 0.1 μ g of template DNA, 1.5 μ l of bovine serum albumin, and 1.4 U of *Taq* DNA polymerase in PCR buffer (10 mM Tris-HCl [pH 9.2], 1.5 mM MgCl₂, 75 mM KCl). The forward and reverse primers were 16S-F (5'-GAG TAA TGT CTG GGA AAC TGC C-3') and 16S-R (5'-GTT AGC CGG TGC TTC TTC TG-3'), respectively, corresponding to nucleotide positions 98 to 119 and 477 to 496, respectively, of the *Photorhabdus luminescens* 16S gene numbering system (24). The primer sequences were based on nucleotide positions 1632 to 1653 and 2028 to 2009 of the *Escherichia coli* numbering system (7). The reaction mixtures were heated to 94°C for 3 min; this was followed by 35 cycles consisting of 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 8 min. Twenty-five microliters of PCR products was loaded onto a 1.2% (wt/vol) agarose gel containing 0.5 μ l of ethidium bromide per ml and electrophoresed at 60 V for 1.5 h. Amplified DNA bands were excised from the gel and purified with GenElute Agarose spin columns (Supelco, Bellefonte, Pa.). Purified DNA was sequenced directly by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's protocol. The sequence reaction mixtures were electrophoresed with an Applied Biosystems model 373A DNA sequencer. In order to evaluate possible misincorporation of bases during the PCR, DNA sequences were determined from both strands and from multiple, independently amplified templates for selected species.

Sequence alignments and phylogenetic analysis. Clustal W (31) was used to obtain initial multiple alignments based on sequence similarity. Final alignments were improved manually by preferring gaps to transition differences (19). The

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TABLE 1. Origins of the *Photorhabdus* and *Xenorhabdus* species and strains and their nematode associates used in this study

Symbiotic bacteria		Nematode associates		Geographic origin
Species	Strain	Species	Strain	
<i>Photorhabdus</i> sp.	H.marB	<i>Heterorhabditis marelatus</i>	OH10	Seaside, Oreg.
<i>Photorhabdus</i> sp.	OH30B	<i>Heterorhabditis</i> sp.	OH30	Seaside, Oreg.
<i>Photorhabdus</i> sp.	OH31B	<i>Heterorhabditis</i> sp.	OH31	Seaside, Oreg.
<i>Photorhabdus</i> sp.	OS13HB	<i>Heterorhabditis</i> sp.	OS13HB	Gold Beach, Oreg.
<i>Photorhabdus</i> sp.	OHPAB	<i>Heterorhabditis</i> sp.	OHPA	Newport, Oreg.
<i>Photorhabdus</i> sp.	FL2122B	<i>Heterorhabditis indicus</i>	FL2122	Homestead, Fl.
<i>Photorhabdus</i> sp.	H.indB	<i>Heterorhabditis indicus</i>	Coimba	Coimbatore, Tamil Nadu, India
<i>Photorhabdus</i> sp.	HP88B	<i>Heterorhabditis</i> sp.	HP88	Logan, Utah
<i>Photorhabdus</i> sp.	OH23B	<i>Heterorhabditis</i> sp.	OH23	Hermiston, Oreg.
<i>Photorhabdus luminescens</i>	H1B	<i>Heterorhabditis bacteriophora</i>	Chino Hill	Chino Hill, Calif.
<i>Photorhabdus luminescens</i>	H7B	<i>Heterorhabditis bacteriophora</i>	Hercules	Hercules, Calif.
<i>Photorhabdus</i> sp.	NcbrB	<i>Heterorhabditis</i> sp.	Nebr	Nebraska
<i>Photorhabdus</i> sp.	OH25B	<i>Heterorhabditis</i> sp.	OH25	Redmond, Oreg.
<i>Xenorhabdus bovienii</i>	S.felB1	<i>Steinernema feltiae</i>	SN	France
<i>Xenorhabdus bovienii</i>	S.intB	<i>Steinernema intermedia</i>	SC	Charleston, S.C.
<i>Xenorhabdus</i> sp.	S.rioB	<i>Steinernema riobris</i>	TX	Weslaco, Tex.

previously published 16S rRNA gene sequences (Table 2) were also aligned with the region sequenced in this study. Absolute and mean distances among bacterial strains were obtained with PAUP, version 3.1.1 (29). To determine optimal outgroups for phylogenetic analysis, we tested the partial 16S rRNA gene sequences of three bacterial species (*E. coli*, *Proteus vulgaris*, and *Vibrio parahaemolyticus*) and their combinations by optimal outgroup analysis (18). The aligned sequence data were analyzed with *Proteus vulgaris* as the outgroup by two different methods: maximum likelihood by DNAML (in the PHYLIP, version 3.572, package) (13) and maximum parsimony by PAUP, version 3.1.1. For the maximum-likelihood analysis, global search was used. The transition/transversion ratio option was set to its default value of 2.0. For the maximum-parsimony analysis, all data were entered unordered, and gaps and ambiguities were treated as missing data. The most-parsimonious trees were constructed by using branch and bound searches. Support for nodes of the trees was evaluated by bootstrap analysis (100 replications) with heuristic search and by examination of all trees 1% longer than the shortest trees. When several most-parsimonious trees had been obtained, a 60% consensus tree was formed and treated as the most-parsimonious tree for constructing figures.

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequences determined in this study have been deposited in the GenBank under accession numbers U70318 to U70320 and U70546 to U70558.

RESULTS

The alignments of partial 16S rRNA gene sequences comprise approximately 356 bp. The nucleotide differences between the 16S rRNA gene sequences of members of the genera *Photorhabdus* and *Xenorhabdus* were confined to seven regions. Sixty-four of the 356 bp in the aligned sequences varied in at least one strain or species. Forty-four of the 64 bp that varied were phylogenetically informative in the most-parsimonious analysis. *Photorhabdus* species showed more sequencing variability than *Xenorhabdus* species. Absolute distances

ranged from 0 to 24 among *Photorhabdus* species or strains and from 1 to 20 among *Xenorhabdus* species or strains. The absolute distance between two strains of *X. bovienii* was 1. The absolute distances among five strains of a *Photorhabdus* species ranged from 0 to 2. The described species had absolute distances of at least 13 from each other. The distance analysis did not unambiguously separate the genera; the absolute distances between the genera ranged from 23 to 35.

The phylogenetic relationships among *Photorhabdus* and *Xenorhabdus* species changed significantly when different outgroup combinations were used (data not shown). The optimal outgroup combination was represented by the species *Proteus vulgaris* alone. Maximum-likelihood analysis generated a tree (Fig. 1A) with two monophyletic groups. Three strains of *X. bovienii* formed a monophyletic group. *X. poinarii*, *X. nematophilus*, and *Photorhabdus* spp. formed another monophyletic group. Five of the most-parsimonious trees were identified from the maximum-parsimony analysis, and each had a length of 129 steps and a consistency index (CI) of 0.74 (excluding uninformative sites). A phylogram of a 60% majority rule consensus tree had a topology similar to that of the maximum-likelihood tree (Fig. 1A), differing in that the node between OH23B and HP88B was supported in the maximum-parsimony tree. Both the maximum-likelihood tree and maximum-parsimony trees supported the monophyletic group of the three strains of *X. bovienii*. Seventeen strains of *X. poinarii*, *X. nematophilus*, and *Photorhabdus* spp. belonged to another monophyletic group.

PAUP yielded 85 trees with one step longer than the most-parsimonious trees. A 60% majority rule consensus tree with 127 steps and a CI of 0.75 (excluding uninformative sites) did not differ substantially from the most-parsimonious trees. The relationships among *Xenorhabdus* species and strains were the same as those generated from the most-parsimonious tree and the maximum-likelihood tree. Most relationships among *Photorhabdus* species and strains were supported. A bootstrap analysis with 100 replications, performed to check the robustness of the most-parsimonious tree, yielded a tree (Fig. 1B) with 132 steps and a CI of 0.72 (excluding uninformative sites). Different monophyletic groups of three strains of *X. bovienii* and other species and strains were supported by 88 and 72% of the bootstrapped trees. The clade between *X. poinarii* and *Photorhabdus* spp. was not supported.

There were six major clades in the bootstrap 60% majority

TABLE 2. Selected species whose 16S rRNA gene sequences were aligned and analyzed with the sequences of *Photorhabdus* and *Xenorhabdus* species

Species	Accession no. ^a	Reference
<i>Photorhabdus luminescens</i>	X82250	24
<i>Xenorhabdus nematophilus</i>	X82251	24
<i>Xenorhabdus bovienii</i>	X8852	24
<i>Xenorhabdus poinarii</i>	X82253	24
<i>Proteus vulgaris</i>	J01874	8
<i>Escherichia coli</i>	J01695	7
<i>Vibrio parahaemolyticus</i>	X56580	10

^a The 16S rRNA gene sequences for these species are available for electronic retrieval from GenBank by using the accession numbers.

FIG. 1. Phylogenetic relationships of nematodes inferred from the partial 16S rRNA gene sequences. (A) Phylogram derived from maximum-likelihood analysis by DNAML. (B) Phylogram of 60% majority rule consensus tree obtained from bootstrap analysis with 100 replications by PAUP. Abbreviations: H.megB, *Photorhabdus luminescens* (accession no. X82250); S.carB, *X. nematophilus* (accession no. X82251); S.felB2, *X. bovienii* (accession no. X82252); S.glaB, *X. poinarii* (accession no. X82253); P.vul, *Proteus vulgaris* (accession no. J01874).

rule consensus tree (Fig. 1B). These clades correlated with host nematode species as follows: clade 1, for symbiotic bacteria of *Steinernema feltiae* and *Steinernema intermedia*, equivalent to *X. bovienii*; clade 2, for symbiotic bacteria of *Steinernema glaseri*, equivalent to *X. poinarii*; clade 3, for symbiotic bacteria of *Steinernema carpocapsae* and *Steinernema riobravii*, equivalent to *X. nematophilus*; clade 4, for symbiotic bacteria *Heterorhabditis marelatus* and four other isolates from the Oregon coast region, equivalent to an undescribed *Photorhabdus* sp.; clade 5, for symbiotic bacteria of *Heterorhabditis megidis*, equivalent to *Photorhabdus luminescens* (?); and clade 6, for symbiotic bacteria of two isolates of *Heterorhabditis indicus*, two isolates of *Heterorhabditis bacteriophora*, and four isolates of *Heterorhabditis* spp. from noncoastal regions, equivalent to *Photorhabdus luminescens* (?).

Clade 6 was supported by 71% of the bootstrapped trees. This clade was split further in the maximum-likelihood tree (Fig. 1A) as follows: two strains isolated from *H. indicus*, two strains isolated from *H. bacteriophora*, and four strains isolated from *Heterorhabditis* species collected from noncoastal areas.

There was a close correspondence between the taxonomic grouping of the bacteria and the taxonomic grouping of their nematode associates. There was an apparent correlation between the clades and their geographic origins; for example, clade 4 (five isolates from the Oregon coast) was supported by all analyses.

DISCUSSION

The choice of multiple candidate outgroups can dramatically alter the topology of a phylogeny (9, 24, 27). The intrageneric relationships of *Xenorhabdus* species and the positions of *Photorhabdus luminescens* and related species changed significantly when various 16S rRNA gene sequences from a selection of members of the gamma subclass of the class *Proteobacteria* and an outgroup were used (24). There have been numerous approaches to outgroup analysis (20, 28). Optimal outgroup analysis (18) takes advantage of the fact that when character states that are not plesiomorphic are constrained, less covariation is generally found between the characters in the ingroup. The advantage can be used to determine which of a set of candidate outgroups maximizes the character covariation in the group under study by comparing the test statistics between analyses rooted with different sets of outgroup taxa (18). *Proteus vulgaris* was the nearest phylogenetic neighbor of *Photorhabdus* and *Xenorhabdus* spp. when *V. parahaemolyticus* was used as an outgroup (24). In our analyses, *Proteus vulgaris* was considered a sister group of *Photorhabdus* and *Xenorhabdus* spp. because these species do not share their common ancestor with any other group (24). It is generally assumed that the sister group renders the most accurate estimate of the ancestral states assessed for the ingroup node and, therefore, the most accurate placement of the root (20). Our outgroup selection was also supported by optimal outgroup analysis (18).

We also found that the two genera were not separated unambiguously based on distance matrix analyses (24). Two *Xenorhabdus* species (*X. nematophilus* and *X. poinarii*) formed a monophyly with *Photorhabdus* species in all of our analyses. The proposal to transfer *X. luminescens* to the new genus *Photorhabdus* was based on DNA relatedness, as well as chemotaxonomic and phenotypic characteristics (3, 5, 6). In all of these studies, the DNA relatedness determined for the type strains of *X. nematophilus* and *X. luminescens* was less than 20%, while the relatedness between the type strain of *X. nematophilus* and other *Xenorhabdus* species ranged from 20 to 40%. As DNA relatedness values for genus delineation are lacking, low relatedness may not justify the dissection of a genus (24). A numerical taxonomic study (3) showed that strains of *X. beddingii* (group V) were more closely related to *Photorhabdus* species than to other *Xenorhabdus* species. Further evidence for the exclusion of *X. luminescens* may be needed.

Some of the relationships derived from the partial 16S rRNA gene sequence data differed from the results of chemotaxonomic studies (3). For example, the 16S rRNA gene sequence comparison linked *X. poinarii* more closely to *Photorhabdus* species than to *Xenorhabdus* species. Such a result is not unexpected; phenotypic markers do not necessarily provide phylogenetic conclusions. Because of the increasing use of 16S rRNA gene sequences in bacterial systematics, it is essential that the congruence between the phylogeny derived from sequencing data and the phylogeny derived from chemotaxonomic data be investigated comprehensively for a wide range of bacterial taxa.

The validity of the species *X. bovienii*, *X. nematophilus*, and *X. poinarii* was confirmed from 16S rRNA gene sequence com-

parison. The symbiotic bacterial strain isolated from *S. intermedia* was closely related to *X. bovienii*. Similar results were also obtained from DNA relatedness studies (6). These described species had absolute distances of at least 13 from each other in this study. We found that there may be two bacterial species distinct from *Photorhabdus luminescens* in the symbiotic bacterial strains associated with nematodes in the genus *Heterorhabditis*, one comprising the five symbiotic bacterial strains isolated from *H. marelatus* (17) and four nematode isolates obtained from the Oregon coast and the other comprising two symbiotic bacterial strains isolated from *H. indicus*. Phylogenetic analyses also supported more than one species in the genus *Photorhabdus*. Maximum-likelihood analysis showed four major clades that represented one of the currently recognized nematode species. These clades were well-supported by bootstrap analysis. For example, the clade of five symbiotic bacterial strains isolated from *H. marelatus* and four other nematode isolates obtained from the Oregon coast was supported by 100% of the bootstrapped trees. We believe that these clades may be recognized as species. Other evidence also supports the existence of more than one species in the genus *Photorhabdus*. For example, several DNA homology groups have been found, and these bacterial groups may be considered separate species (26). There also was an apparent geographical correlation within the clades. For example, five bacterial strains from the Oregon coast formed a significantly supported clade, and four bacterial strains from noncoastal regions also formed a clade. Because these clades are associated with different nematode species, we believe that this is initial evidence of coevolution between the nematodes and their symbiotic bacteria. The close correspondence between the taxonomic grouping of the bacteria and the taxonomic grouping of their nematode associates has been confirmed by phenotypic studies (2, 3, 5).

It has been confirmed that sequencing only a part of the 16S rRNA gene can be sufficient to establish phylogenetic relationships (15, 33). However, phylogeny estimated from a single gene should be treated with caution (25). We are sequencing more genes and will combine phenotypic, molecular, and DNA sequence data to provide a definitive taxonomy of the genera *Photorhabdus* and *Xenorhabdus*.

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