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Phylogenetic Analysis of *Pasteuria penetrans* by 16S rRNA Gene Cloning and Sequencing[1]

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Abstract: *Pasteuria penetrans* is an endospore-forming bacterial parasite of *Meloidogyne* spp. This organism is among the most promising agents for the biological control of root-knot nematodes. In order to establish the phylogenetic position of this species relative to other endospore-forming bacteria, the 16S ribosomal genes from two isolates of *P. penetrans,* P-20, which preferentially infects *M. arenaria* race 1, and P-100, which preferentially infects *M. incognita* and *M. javanica,* were PCR-amplified from a purified endospore extraction. Universal primers for the 16S rRNA gene were used to amplify DNA which was cloned, and a nucleotide sequence was obtained for 92% of the gene (1,390 base pairs) encoding the 16S rDNA from each isolate. Comparison of both isolates showed identical sequences that were compared to 16S rDNA sequences of 30 other endospore-forming bacteria obtained from GenBank. Parsimony analyses indicated that *P. penetrans* is a species within a clade that includes *Alicyclobacillus acidocaldarius, A. cycloheptanicus, Sulfobacillus* sp., *Bacillus tusciae, B. schlegelii,* and *P. ramosa.* Its closest neighbor is *P. ramosa,* a parasite of *Daphnia* spp. (water fleas). This study provided a genomic basis for the relationship of species assigned to the genus *Pasteuria,* and for comparison of species that are parasites of different phytopathogenic nematodes.

Key words: bacterium, biological control, *Meloidogyne* spp., nematode, *Pasteuria penetrans, Pasteuria ramosa,* phylogeny, ribosomal RNA, root-knot nematode, 16S rRNA.

Pasteuria penetrans (Thorne) Sayre & Starr is a gram-positive, endospore-forming, bacterial parasite of root-knot nematodes (Sayre and Starr, 1985). This bacterial parasite has shown great potential as a biological control agent of root-knot nematodes (Brown and Kerry, 1987; Dickson et al., 1994; Oostendorp et al., 1990; Sayre and Starr, 1988; Stirling, 1991). Attempts at culturing the bacterium in vitro have been largely unsuccessful (Bishop and Ellar, 1991; Williams et al., 1989), and its mass production and exploitation thus have been limited. Nevertheless, *P. penetrans* population densities in soil have been linked to the suppression of plant infection by *Meloidogyne* spp. The managed amplification of this organism in fields heavily infested with root-knot nematodes provides protocols for its application in their biological control (Chen and Dickson, 1998).

Identification of this endospore-forming organism of nematodes has been changed several times since Cobb (1906) first identified highly refractile bodies in the pseudocoel of the nematode *Dorylaimus bulbiferus.* Cobb identified the bodies as part of a parasitic protozoan. This erroneous placement of what is now considered a bacterium continued for almost 70 years and was supported by numerous other investigators (see Chen and Dickson [1998] for review). The true bacterial characteristics of the nematode endoparasite were not recognized until 1975 when the organism was reexamined by electron microscopy (Mankau, 1975a) and subsequently renamed *Bacillus penetrans* (Thorne, 1940) Mankau 1975 (Mankau, 1975b). This classification remained for a decade until Sayre and Starr (1988) recognized the morphological similarities between *B. penetrans* and *P. ramosa* (Metchnikoff, 1888), an endoparasitic organism of water fleas, and correctly placed the organism in the genus *Pasteuria.* Members of the

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genus *Pasteuria* are gram-positive, dichotomously branching, endospore-forming bacteria with a septate mycelium (Williams et al., 1994). This genus is morphologically distinct from the closely related spore former, *Bacillus,* which is not a parasite. The morphology and the unique endoparasitic life cycle of the genus *Pasteuria* have been described numerous times (see Chen et al. [1997] for review). The current taxonomic arrangement of *P. penetrans* is based upon spore morphology and nematode host preference, which has been the basis for its distinction from *P. nishizawae* and *P. thornei* (Sayre and Starr, 1989). Of the four described species of *Pasteuria,* only *P. ramosa,* from water fleas, *Daphnia* spp., has been subjected to genotypic analysis (Ebert et al., 1996).

In this study, the 16S or small-subunit (SSU) rRNA gene was PCR-amplified from purified endospores of *P. penetrans*. The PCR product was cloned, the nucleotide sequence determined, and the phylogenetic relationship of this organism to *P. ramosa* and other spore-forming taxa was determined. This study represents the first report of the taxonomic position of *P. penetrans,* a parasitic bacterium of root-knot nematodes, based on DNA sequence analysis.

Materials and Methods

Collection of spores and DNA extraction: Isolates of *P. penetrans* designated P-20, originating from *M. arenaria* (Neal) Chitwood race 1, Levy County, Florida, and P-100 from *Meloidogyne* spp., Pasco County, Florida, were propagated in *M. arenaria* race 1 and *M. incognita,* respectively, growing on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in a greenhouse (Oostendorp et al., 1990). The root systems were harvested 45 to 60 days after inoculation, rinsed, and digested in a 12.5% cytolase PCL5 solution (Genencor International, Rochester, NY) overnight. The material was decanted onto a 600-µm-pore sieve nested over a 30-µm-pore sieve and subjected to high-pressure water spray to dislodge female nematodes (Hussey, 1971). Approximately 15 endospore-filled females were handpicked with forceps, placed in a sterile microfuge tube containing deionized water, and ground with a sterile pestle. Endospores were counted on a hemocytometer at ×40.

To remove external contaminating microorganisms, 2×10^6 spores were treated with lysozyme, sodium dodecyl sulfate solution, DNase, RNase, and proteinase K, as described by Ebert et al. (1996). Then, 160 µl of the spore suspension was subjected to lysis in 160 µl of phenol saturated with 10 mM Tris-HCl, pH 8.0, and 160-µl volume equivalents of Braun Glasperlen beads (0.1-mm diameter) by vigorous shaking in a beadmill homogenizing unit (Biospec Mini-Bead Beater, Bartlesville, OK) at 5,000 rpm for 1 minute. The spore suspension was microcentrifuged at 10,000*g,* and the aqueous layer was transferred to a new microfuge tube. To precipitate the released DNA, 16 µl 3 M sodium acetate (pH 5.6) was added, followed by two volumes of absolute ethanol. The mixture was placed on ice for 10 minutes. The DNA was collected by centrifugation, dried under vacuum, and resuspended in 20 µl TE (0.01 M Tris, 0.001 M EDTA) buffer (pH 8.0). The extracted spore DNA was then used as a template for PCR amplification.

PCR amplification: The 16S rDNA of the *P. penetrans* isolates was amplified by using the bacterial universal primers, 27f (5′-AGAGTTTGATCSTGGCTCAG) and 1392r (5′-ACGGGCGGTGTGTRC). These primers were designed to amplify almost the entire 16S ribosomal gene from most bacteria (Lane, 1991). A 50-µl reaction was prepared to include 2 µl DNA template solution, 5 µl 10× reaction buffer (10 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100); 10 µM (each) dATP, dCTP, dTTP, and dGTP; 5 pM primer 27f, 5 pM primer 1392r, and 0.5 units of the polymerase, PrimeZyme (Biometra, Tampa, FL). The template DNA was amplified with a programmable thermal controller (MJ Research, Watertown, MA) with the following temperature profile: denaturation for 1 minute at 94 °C; 30 cycles consisting of denaturation at 94 °C for 1 minute, annealing

for 1 minute at 58 °C, and extension for 1.5 minutes at 72 °C; and a final extension step of 72 °C for 15 minutes. PCR products were resolved in a 0.75% SeaKem LE agarose gel (FMC Bioproducts, Rockland, ME) with Tris-Acetate-EDTA (TAE) buffer (Sambrook et al., 1989) and visualized with 0.25 µg/ml ethidium bromide. The amplification products were purified with the use of QIAquick PCR Purification Spin Columns (Qiagen, Santa Clarita, CA) following the manufacturer's protocol and eluted with TE buffer (pH 8.0).

Cloning and restriction analysis: PCR products were cloned with a pGEM-T cloning vector (Promega, Madison, WI). Competent *E. coli* cells, DH5α, (Life Technologies, Gaithersburg, MD) were transformed, plated, and grown according to Sambrook et al. (1989). The plasmid DNA was extracted according to Nicolett and Condorelli (1993). The cloned insert DNA was PCR-amplified using primers to the pGEM-T promoters, T7 (5′-TAATACGACTCACTATGGG) and SP6 (5′-GATTTAGGTGACACTATAG), with the following mix: 1 ng template DNA, 3.0 mM $MgCl_2$, reaction buffer mix (as before), 5 pM T7 primer, 5 pM SP6 primer; 10 µM (each) dATP, dCTP, dTTP, dGTP; and 0.5 units of PrimeZyme. The program described above was followed with the exception of an annealing temperature of 52 °C. PCR-amplified cloned DNA was visualized following electrophoresis in a 0.75% agarose gel stained with ethidium bromide. The PCR products were digested with restriction endonucleases Alu I and Rsa I (New England Biolabs, Beverly, MA) according to the manufacturer's recommendations. Digestion products were subjected to gel electrophoresis in TAE buffer on a 3% NuSieve agarose gel (FMC Bioproducts). Eight cloned PCR products from each isolate, P-100 and P-20, were subjected to analysis using both enzymes.

Sequencing and phylogenetic analysis: The cloned amplification products were sequenced by the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research. Sequencing was accomplished with the Taq DyeDeoxy Terminator and the DyePrimer Cycle Sequencing protocols (Applied Biosystems, Perkin-Elmer, Foster City, CA) with fluorescent-labeled dideoxynucleotides and primers, respectively. The labeled extension products were subjected to analysis on a DNA Sequencer (Applied Biosystems). Both strands of several rDNA clones of each isolate, P-20 and P-100, were fully sequenced with the use of vector primers, SP6 and T7, and two newly designed internal primers, 66r (5′-GGTGTTCCTTCTGATAT) and 66f (5′-GCGTAGATATCAGAAGGAAC). Sequence runs were aligned and assembled with the computer programs Sequence Navigator and AutoAssembler (Applied Biosystems) and then aligned with *E. coli* 16S rDNA with Clustal version 1.7 (Higgins and Sharp, 1988).

Phylogenetic analysis of the 16S rRNA gene was performed by comparing the sequences obtained from the cloned PCR product of *P. penetrans* isolates P-20 and P-100 with those of related spore-forming organisms (Berkeley and Ali, 1994) obtained from GenBank with BLAST (basic local alignment search tool) network service (Altschul et al., 1990) and aligned with Clustal version 1.7. Phylogenetic trees were derived by maximum parsimony analysis with version 4.0b2a of PAUP (Swofford, 1999). Characters were assumed to be unordered and tree evaluation was made using heuristic searches with 1,000 random, stepwise addition of taxa and by subtree pruning and regrafting (SPR) branch-swapping on shortest trees. Initially, the parsimony analysis was conducted with all characters assigned equal weights. The matrix was then successively weighted twice to give more weight to the characters with the least amount of homoplasy, thus emphasizing the sites that are more consistent (Lledó et al., 1998). Trees were rooted with *Mycobacterium tuberculosis* and *Propionibacterium acnes,* which are gram-positive bacteria that do not form spores. Gaps were treated as missing data; characters 1–35, 73–124, 210–270, 293–532, 891–911, 1,063–1,114, 1,207–1,219, and 1,500–1,625 were unalignable sections of the matrix and were excluded from the

analysis. The aligned data matrix is available from the corresponding author. The significance of phylogenetic affiliations between the isolates and other taxa selected from GenBank was estimated by nonparametric bootstrap analysis with 1,000 replications, each round with a single replication of random addition of taxa and SPR branch-swapping. Bootstrap support values were placed on the re-weighted trees. The robustness of the clades was further analyzed by determining decay value, which is the number of extra steps that would be needed to collapse a given parsimonious clade (Bremer, 1994). A decay analysis, which determines the value of the most parsimonious tree that does not contain the clade of interest, was performed using the Autodecay program, version 3.0 (Eriksson and Wilkstrom, 1995) and version 4.0b2a of PAUP.

Results and Discussion

PCR amplification, cloning, and sequence analysis: A single 1.4-kb product was PCR-amplified from endospore extractions obtained from P-20 and P-100 isolates. All the cloned PCR products produced the same digestion profiles for a particular restriction enzyme. The restriction profiles for both Alu I and Rsa I were the same as those predicted based upon analysis of the *P. ramosa* 16S rDNA sequence (GenBank U34688). The homogeneity among clones provides evidence that a single bacterial rDNA was obtained from *P. penetrans* endospores prepared from the nematodes. This identity was supported by restriction fragment analysis. Sequence identities supported assignments of both strains to a single genus and species (*P. penetrans*). The single consensus sequence for both *P. penetrans* isolates P-100 and P-20 without the primer sequences has been deposited in GenBank as AF077672.

Sequencing and phylogenetic analysis: Comparison of 16S rDNA sequences, using the BESTFIT alignment program (Wisconsin Sequence Analysis Package, Version 8), of *P. penetrans* isolate P-20 with P-100 indicated that they shared complete base identity for the amplified region, which comprised 92% of the gene encoding 16S rDNA. Further comparison of this region from both isolates indicated a 93% base similarity of each with *P. ramosa* 16S rDNA, which strongly supports assignment to the same genus (Strackebrandt and Liesack, 1993). These results strengthen the taxonomic placement of *Pasteuria,* which up to this time was placed according to morphological characters and host preference (Mankau and Prasad, 1975).

A 1,390-bp fragment was used for phylogenetic analysis. A consensus tree derived from the SSU ribosomal gene sequence of 32 bacterial taxa is shown in Fig. 1. The analysis excluded 600 characters and included 1,025 characters from the matrix. All characters were evaluated with equal weights, and 550 characters were invariant. One hundred seven variable characters were parsimony-uninformative while 338 characters were parsimony-informative. The final tree length was 1,920 changes with a retention index of 0.456 and a consistency index of 0.397. The *P. penetrans* sequence belongs in a clade containing *Alicyclobacillus acidocaldarius, A. cycloheptanicus, Sulfobacillus sp., Bacillus tusciae, B. schegeltii,* and *P. ramosa.* The results of maximum parsimony analysis were consistent with the findings of Ebert et al. (1996), who found that *P. ramosa* was most related to *Alicyclobacillus* sp. and *Bacillus tusciae.* Support for the positioning of *P. penetrans* isolates P-20 and P-100 was high (100) as judged by bootstrap proportions in maximum parsimony analysis. High decay values for *P. ramosa* (33) and *P. penetrans* (19) indicate that the clade support was strong. The sequence analysis of both isolates supports the placement of *P. penetrans* in a clade with other spore-forming organisms, with the closest neighbor being *P. ramosa,* the type species for *Pasteuria.*

This study is the first presentation of a phylogenetic analysis of any of the *Pasteuria* species that are parasites of phytopathogenic nematodes. Before Sayre and Starr (1985) reevaluated *P. penetrans,* the bacterium was named *Bacillus penetrans,* and its taxonomic position was in doubt. This report confirms the present placement of this

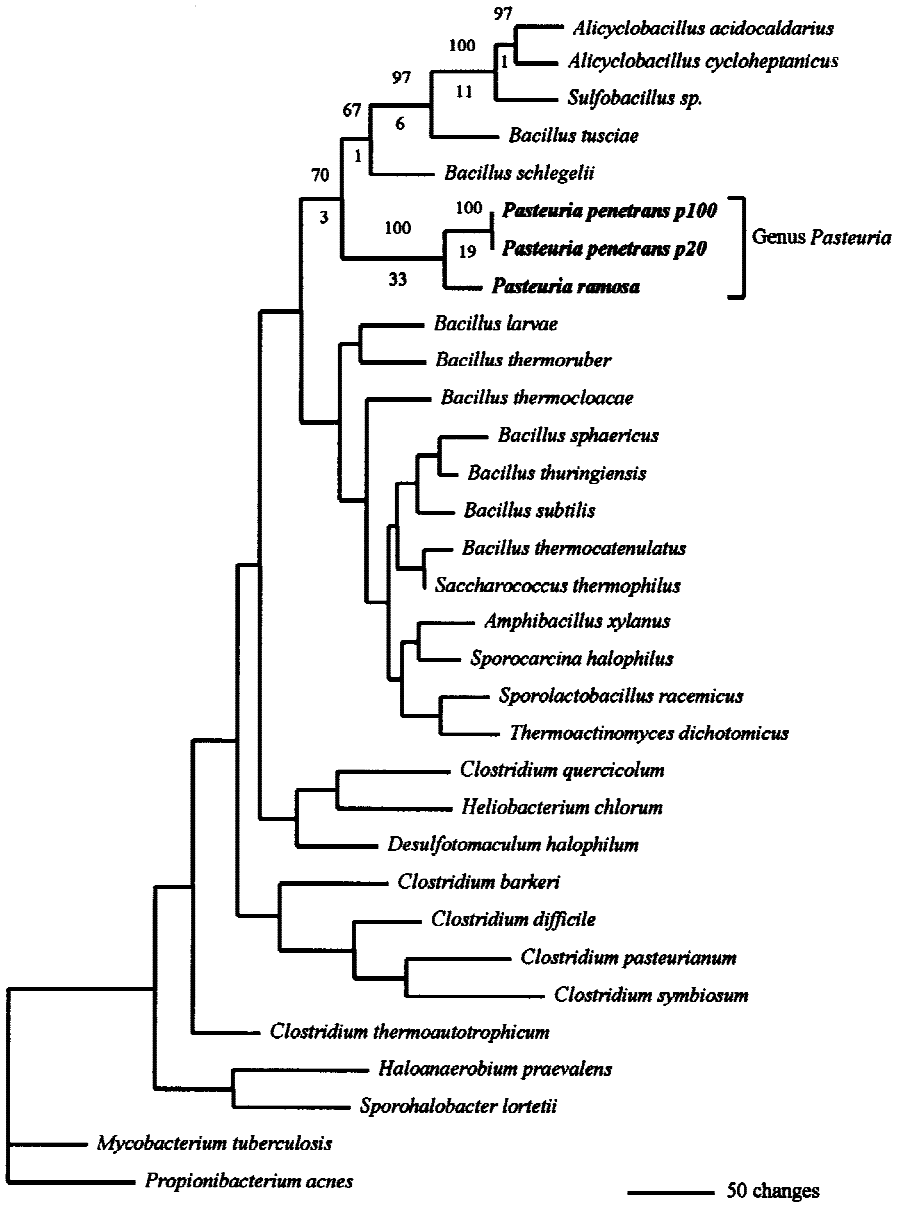

FIG. 1. Phylogenetic relationship of *Pasteuria penetrans* isolates P-20 and P-100. The tree was constructed with 1,390 bp alignable sequences from position 31 to 1,408 (based on *E. coli*) with *Mycobacterium tuberculosis* and *Propionibacterium acnes* as the outgroup. A heuristic search was conducted with PAUP. Numbers over the branch indicate bootstrap proportions (500 replicates), which indicate branches that are significantly supported (>75%). Numbers under the branch are decay indices (100 replicates). The bar indicates proportional branch-length values based on the number of informative characters that supports each clade.

organism in the genus *Pasteuria* and its genetic relationship with *P. ramosa.*

Pasteuria-like organisms are distributed worldwide and have been reported from 325 species belonging to 117 genera of free-living, predatory, plant-parasitic, and entomopathogenic nematodes (Chen and Dickson, 1998). *Pasteuria ramosa* is the only described species in the genus that does not infect nematodes. Therefore, the 16S rDNA sequence of *P. penetrans* will serve as a standard for comparing nematode-parasitic iso-

lates. Comparison of the 16S rDNA sequences of *P. thornei,* a parasite of *Pratylenchus spp.* (lesion nematodes), and *P. nishizawae,* a parasite of *Heterodera* and *Globodera* spp. (cyst nematodes), should help to define evolutionary constraints on host preference. With the 16S rDNA sequence, primers specific for the genus *Pasteuria* could be designed, which would reduce the possibility of contamination associated with the use of universal 16S rRNA primers and could be used to screen field soil samples as well as confirm culture attempts. Three *Pasteuria*-like isolates from *Heterodera goettingiana* Liebscher in Munster, Germany (Sturhan et al., 1994), *Belonolaimus longicaudatus* Rau in Florida (Giblin-Davis et al., 1995), and *H. glycines* in Illinois (Atibalentja et al., 1998) have been proposed as new species based on morphological characters. Specific primers for the genus *Pasteuria* would be helpful to determine if these isolates are, in fact, new species or if they belong to one of the previously described species.

Ribosomal DNA analysis is just one tool for phylogenetic analysis and has been the predominant gene used in bacterial studies (Woese, 1987). There are current questions as to how well species can be identified solely on 16S rRNA analysis (Fox et al., 1992), and it is still important to consider morphological characteristics as well as other gene regions. One such region that may further identify the genus *Pasteuria* is the small acid-soluble spore protein gene found in several genera of spore-forming bacteria (Setlow, 1988). The 16S rRNA gene data presented in this paper provide a starting point from which continued genetic analysis of *P. penetrans* could begin. It is hoped that this current work will stimulate continued genetic research of *P. penetrans,* and so lead to elucidation of a technique to culture and exploit this potentially important biological control agent.

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