Detection and characterization of Pasteuria 16S rRNA gene sequences from nematodes and soils

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Various bacterial species in the genus Pasteuria have great potential as biocontrol agents against plant-parasitic nematodes, although study of this important genus is hampered by the current inability to cultivate Pasteuria species outside their host. To aid in the study of this genus, an extensive 16S rRNA gene sequence phylogeny was constructed and this information was used to develop cultivation-independent methods for detection of Pasteuria in soils and nematodes. Thirty new clones of Pasteuria 16S rRNA genes were obtained directly from nematodes and soil samples. These were sequenced and used to construct an extensive phylogeny of this genus. These sequences were divided into two deeply branching clades within the low-G+C, Gram-positive division; some sequences appear to represent novel species within the genus Pasteuria. In addition, a surprising degree of 16S rRNA gene sequence diversity was observed within what had previously been designated a single strain of Pasteuria penetrans (P-20). PCR primers specific to Pasteuria 16S rRNA for detection of Pasteuria in soils were also designed and evaluated. Detection limits for soil DNA were 100–10 000 Pasteuria endospores (g soil)−1.

INTRODUCTION

Plant-parasitic nematodes cause significant yield losses of crops and other plants of economic value each year. Losses due to nematodes in 1994 were estimated to exceed $1·5 billion for corn, soybean, wheat, cotton, peanut and vegetables combined (Koenning, 1999). There has been a steady decline in nematicides available for agronomic crops due to concern regarding environmental effects of these chemicals. As an example, methyl bromide is among the most effective means of controlling plant-parasitic nematodes. Methyl bromide is a potent degrader of ozone, however, and will be phased out of use in the United States by the year 2005 (Chitwood & Deshusses, 2001). In response to declining options for control of nematodes, environmentally safe alternatives to control nematodes are of great interest. Among the most promising of these alternatives are biocontrol agents that specifically target these pests.

Pasteuria represents a genus of Gram-positive, endospore-forming bacteria whose species have been observed parasitizing the majority of economically important plant-parasitic nematodes (Sayer & Starr, 1988). Members of the genus Pasteuria have been reported from 323 nematode species belonging to 116 genera, including plant-parasitic, entomopathogenic, predatory and free-living nematodes (Chen & Dickson, 1998). The potential of one species, Pasteuria penetrans, to be an effective biocontrol agent for Meloidogyne species (root-knot nematodes) has been demonstrated (Sayer & Starr, 1988). In addition to P. penetrans, Pasteuria thornei and Pasteuria nishizawai have been reported to infect other plant-parasitic nematodes (Chen & Dickson, 1998; Oostendorp et al., 1990). Variabilities in endospore size, endospore surface antigens and in the strength of endospore attachment to nematodes within the same population of Pasteuria species have been reported (Sharma & Davies, 1996). Very limited information on the molecular biology, phylogeny and ecology of Pasteuria species is available due to the current difficulty in cultivating these bacteria outside of the host nematode (Chen & Dickson, 1998). Development of a sensitive and specific tool for characterization of Pasteuria–nematode relationships, such as species-specific interactions between Pasteuria and nematodes, and for studying distribution of Pasteuria in soils would be of great benefit for evaluating the potential of this biocontrol agent as an alternative to nematicides (Stirling, 1991).

Analysis of 16S rRNA gene sequences is a well-established
and sensitive tool for detection and phylogenetic analysis of bacteria (Stahl, 1997). Use of 16S rRNA gene sequences to identify Pasteuria species and to assess the diversity of these bacteria within a nematode population or soil sample would be of great benefit in understanding nematode–parasite interactions and Pasteuria ecology. Development of 16S rRNA-based phylogeny and detection systems requires compilation of 16S rRNA gene sequences from a variety of representatives of the genus, yet only five 16S rRNA gene sequences for Pasteuria species could be retrieved from GenBank at the time of this study (Anderson et al., 1999; Atibalentja et al., 2000; Bekal et al., 2001; Ebert et al., 1996).

In this study, 16S rRNA gene sequences were amplified from Pasteuria endospores extracted from Meloidogyne nema-
todes or soil samples and their phylogenetic relationships were established using maximum-parsimony analysis. Two sets of primers specific for the Pasteuria 16S rRNA gene were designed for detection and characterization of Pasteuria species from nematodes and nematode-infested soils, and a phylogeny of these sequences was developed. This work greatly extends previous 16S rRNA phylogenies of Pasteuria, the most extensive of which include only two Pasteuria sequences (Anderson et al., 1999; Atibalentja et al., 1999).

**METHODS**

**Bacteria sources and soils.** P. penetrans P-20 endospores were collected from root-knot nematode Meloidogyne arenaria Race 1 as described by Anderson et al. (1999). In vitro cultures of Pasteuria originally collected from Pasteuria-infected M. arenaria Race 1 nematodes from peanut plants in North Central Florida were prepared as described by Gerber & White (2001). Soils 1–5 (sources of clones) are described in Table 1. Soil 6 was taken from a residential garden in Gainesville, FL, USA. Endospore-filled Meloidogyne females were collected from greenhouse-cultivated tomato roots and ruptured with a plant tissue grinder to form an endospore suspension (Oostendorp et al., 1990).

**DNA isolation.** Pasteuria endospore DNA was extracted as described by Atibalentja et al. (2000). Soil and nematode DNA was isolated using an UltraClean soil DNA kit (MoBio) according to the manufacturer’s instructions. For each sample, 0-25 g soil was used for extraction. For quantitative detection of endospores, P. penetrans endospores were spiked into soil 6 at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 endospores g⁻¹ and DNA was purified as described above.

**PCR amplification and RFLP.** 16S rRNA gene sequences were amplified with primers 27F or 39F, and 1388R (Anderson et al., 1999; Atibalentja et al., 2000; Lane, 1991). Primers 617F, 654R and

<table>
<thead>
<tr>
<th>Clone</th>
<th>Source</th>
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<tbody>
<tr>
<td>Np3, Np4, Np6, Np13, Np16, Np17</td>
<td>DNA extracted from endospores taken from female nematodes of <em>Meloidogyne arenaria</em> Race 1 (P-20) collected from peanut plants in North Central Florida and raised in tomato plants in a greenhouse</td>
</tr>
<tr>
<td>Nf3*, Nf7*, Nf9*, Nf21*</td>
<td>DNA extracted from endospores taken from female nematodes of <em>Meloidogyne arenaria</em> Race 1 (P-20) collected from peanut plants in North Central Florida and raised in tomato plants in a greenhouse</td>
</tr>
<tr>
<td>NCp2A5, NCp2B5, NCf4*, NCf8*, NCp9</td>
<td>P. <em>penetrans</em> in vitro cultures (Gerber &amp; White, 2001) originally from <em>Meloidogyne arenaria</em> Race 1 (P-20) collected from peanut plants in North Central Florida</td>
</tr>
<tr>
<td>SI1, SI6, SI10</td>
<td>Soil 1. Golf course at the Gainesville Golf and Country Club, Gainesville, FL, USA, infested with root-knot nematodes (<em>Belonolaimus longicaudatus</em>)</td>
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<tr>
<td>SIH4, SIH8, SI12</td>
<td>Soil 2. Nursery at the Gainesville Golf and Country Club, Gainesville, FL, USA, infested with root-knot nematodes (<em>Meloidogyne</em> species)</td>
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<tr>
<td>SIII6</td>
<td>Soil 3. Gainesville Country Club Golf Course, Gainesville, FL, USA, infested with <em>Tylenchus</em> species</td>
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<tr>
<td>SV7, SIV8</td>
<td>Soil 4. Golf course at Fort Lauderdale Research and Education Center, Fort Lauderdale, FL, USA, infested with root-knot nematodes (<em>Belonolaimus longicaudatus</em>)</td>
</tr>
<tr>
<td>SV24, SV25, SV26, SV27, SV35, SV36</td>
<td>Soil 5. Golf course at the Gainesville Golf and Country Club, Gainesville, FL, USA infested with lance nematode (<em>Hoplolaimus</em> species)</td>
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**Table 1. Clones of the Pasteuria 16S rRNA gene from nematodes and soils**

**Table 2. Primers for amplification of Pasteuria 16S rRNA gene sequences**

<table>
<thead>
<tr>
<th>Name</th>
<th>5'→3' sequence</th>
<th>Reference</th>
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<tbody>
<tr>
<td>27F</td>
<td>AGAGTTTGATCMTGGCTCAG</td>
<td>Lane (1991)</td>
</tr>
<tr>
<td>39F</td>
<td>GCCGGCGTGCCTAATACA</td>
<td>Atibalentja et al. (2000)</td>
</tr>
<tr>
<td>617F</td>
<td>CGTGTAGGGGTCGAAACGGT</td>
<td>This study</td>
</tr>
<tr>
<td>654R</td>
<td>TACGCGACTCAAGATGCACCG</td>
<td>This study</td>
</tr>
<tr>
<td>1166R</td>
<td>CGCCGGCTGTCTCTCACA</td>
<td>This study</td>
</tr>
<tr>
<td>1388R</td>
<td>ACGGGCGGTGTGTACAAG</td>
<td>Atibalentja et al. (2000)</td>
</tr>
</tbody>
</table>
16S rRNA gene sequences among Pasteuria and related sequences. DNA was amplified using HotStarTag Master Mix (Qiagen) in a GeneAmp PCR system 2400 (Perkin-Elmer Applied Biosystems) under the following conditions: initial enzyme activation and DNA denaturation of 15 min at 95°C, followed by 35 cycles of 30 s at 94°C, 45 s at 61°C and 45 s extension at 72°C, and a final extension at 72°C for 7 min. PCR products amplified with primers 27F (or 39F) and 1388R were verified by digestion with BamHI and EcoRV, products amplified with 39F and 654R were verified by digestion with BamHI, and products amplified with primers 617F and 1166R were verified by digestion with Stul (Fig. 1). Amplification products were purified with the PCR Purification kit (Qiagen) and were cloned into the pCRII-TOPO cloning vector (Invitrogen). For RFLP assays, individual colonies were screened by direct PCR amplification of the target DNA fragment from Escherichia coli clones and the desired PCR products were subjected to PCR amplification analysis using the restriction enzyme HhaI and resolved by 2-0 % agarose gel electrophoresis.

**Sequencing and phylogenetic analysis.** 16S rRNA gene clones exhibiting different RFLP patterns were selected for sequencing. Selected amplification products were subjected to direct sequencing after cleaning with the QiAquick PCR Purification kit (Qiagen). Sequencing was carried out in the DNA Sequencing Core Laboratory at the University of Florida, Gainesville, Florida, USA. Six of the resulting sequences generated with universal primers were aligned using thePILEUP function of the GCG (Genetics Computer Group, 1999). Final alignments were constructed by visual inspection using PAUP 4.0b8 (Swofford, 2001). Representative sequences of Pasteuria were aligned with other low-G+C, Gram-positive bacterial species and maximum-parsimony analysis was conducted with Streptomyces griseus as the outgroup. The regions involving characters 1–30, 72–114 and 1463 to the end of the sequences were unalignable and therefore not included in the maximum-parsimony analysis. Phylogenetic trees were built by a maximum-parsimony method using PAUP 4.0b8. Trees were constructed using heuristic searches with 10 random stepwise additions of taxa and by tree-bisection reconnection branch-swapping. The characters were weighted to give more weight to characters with lower levels of homoplasy. Bootstrap supports were obtained by heuristic searches of the reweighted trees with 100 or 1000 replicates.

Maximum likelihood analyses were performed using the program TREEmaking version 5.0 obtained from http://www.tree-puzzle.de/Strimmer & von Haeseler, 1996). A sequence similarity matrix of all sequences described in this study was constructed using the distance matrix function in the RDP PHYLIP interface (Maidak et al., 2001).

**RESULTS AND DISCUSSION**

**PCR amplification and RFLP analysis of Pasteuria 16S rRNA genes**

Approximately 1400 bp of 16S rRNA genes was amplified from DNA preparations of *in vivo* and *in vitro* (Gerber & White, 2001) *P. penetrans* endospores resulting in clones NF3, NF7, NF9, NF21, NC64, NC9 (Table 1). These sequences shared 97.5–100.0 % homology with previously reported *P. penetrans* 16S rRNA gene sequences and were used to design PCR primers that would specifically amplify *Pasteuria* 16S rRNA genes. Based on consensus regions of these sequences, primer sets 617F/1166R and 39F/654R (Fig. 1) were thought to be *Pasteuria*-specific and were used in the subsequent amplification of *Pasteuria* sequences from soil and nematodes. A supplementary table showing sequence similarities of all clones described in this paper is available as supplementary material in IJSEM Online at http://ijse.sgmjournals.org/.

All *Pasteuria* sequences amplified with universal bacterial primers 27F (or 39F) and 1388R shared a few highly conserved restriction sites, including BamHI (nucleotide position 246 based on *E. coli* numbering), EcoRV (697) and Stul (1001) (Fig. 1). No related non-*Pasteuria* rRNA gene sequences included in the original alignment (Fig. 2) contained these digestion sites at these locations (as determined *in silico* data not shown), suggesting that these restriction sites may be useful in preliminary identification of *Pasteuria* clones. Digestion of clones produced from amplification products using these primers provides a presumptive test for *Pasteuria* sequences before the DNA sequence is determined.

Amplification with primer set 39F/654R or 617F/1166R resulted in amplification of 16S rRNA gene sequences from all soils and nematodes tested that were known to harbour *Pasteuria* (Fig. 3). All amplification products were initially verified by restriction digestion with either BamHI or Stul; 24 were sequenced (Table 1) and confirmed to be closely related to previously reported *Pasteuria* sequences (Anderson et al., 1999; Atibalentja et al., 2000).

The region amplified by primers 39F and 654R is more variable between different clones than the region amplified by 617F and 1166R, such that PCR products amplified with
these primers exhibited a greater diversity of RFLP patterns (Fig. 4). Although a variety of RFLP patterns was observed for clones from individual nematode and soil samples, the majority of clones from each sample exhibited a single dominant RFLP pattern. For example, only 5 out of 40 clones showed different RFLP patterns in the Np series (data not shown).

Phylogenetic relationships between P. penetrans and other bacterial species

To confirm the placement of Pasteuria within the low-G+C, Gram-positive division and its association with the family Alicyclobacillaceae, a phylogenetic tree was constructed with maximum-parsimony from sequences available through GenBank and those from this study amplified from endospores and culture (Fig. 2). A total of 208 characters was excluded from the maximum-parsimony analysis yielding a matrix of 1389 characters. The matrix used in the phylogenetic analysis included 714 constant characters, 535 parsimony informative characters and 140 parsimony uninformative characters. The most parsimonious tree obtained had a tree length of 842 changes, a consistency index of 0.65, a retention index of 0.81, a rescaled consistency index of 0.53 and a homoplasy index of 0.35. According to maximum-parsimony, Pasteuria sequences were placed in a clade that contained previously described Pasteuria, Alicyclobacillus, Sulfoabacillus and Bacillus species. This result is in general agreement with previously reported phylogenetic analyses (Anderson et al., 1999; Atibalentja et al., 2000; Ebert et al., 1996).

Pasteuria species have been characterized as belonging to the Alicyclobacillus group by GenBank and the

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**Fig. 2.** Phylogenetic placement of Pasteuria species within the low-G+C, Gram-positive division. Analysis based on 1349 nt sequences from positions 39–1388 (based on E. coli numbering) with Streptomyces griseus as the outgroup taxon. Numbers above nodes represent bootstrap values (100 replicates). GenBank accession numbers for sequences from ‘Sulfoabacillus yellowstonensis’, ‘Sulfoabacillus monserratensis’, ‘Sulfoabacillus ambivalens’ and ‘Bacillus caldolyticus’ are AY007665, AY007663, AY007664 and Z26924, respectively.
Alicyclobacillaceae family by the taxonomic outline of the Archaea and Bacteria of Bergey's Manual of Systematic Bacteriology (http://www.cme.msu.edu/bergeys/). However, inclusion of additional Pasteuria, Alicyclobacillus and Sulfovibrio sequences resulted in a clear delimitation of a distinctive clade for Pasteuria from the rest of the Alicyclobacillus group (bootstrap value of 100). This distinction suggests that a re-evaluation of the placement of Pasteuria within the family Alicyclobacillaceae may be in order.

In our analysis, Pasteuria ramosa branched deeply within the Pasteuria clade and two additional major clades of Pasteuria could be distinguished (Fig. 2). One clade (P. penetrans clade 1; bootstrap support of 99%) includes Pasteuria that parasitize root-knot nematodes, including the type species for P. penetrans (GenBank no. AF077672; Anderson et al., 1999) and our isolates that parasitize root-knot nematodes. The second clade (clade 2; bootstrap support of 75%) includes Pasteuria that infect the cyst nematode Heterodera glycines (AF134868; Atibalentja et al., 2000; Sayre et al., 1991) and a strain that infects the sting nematode Belonolaimus longicaudatus (AF254387; Bekal et al., 2001).

Using primers 39F and 654R designed for specific amplification of Pasteuria 16S rRNA gene sequences, 25 partial sequences of approximately 600 bp each of Pasteuria from different sources were included in a more comprehensive phylogenetic analysis of this bacterial group (Fig. 5). Thermaactinomyces dichotomus was used as the outgroup. In this analysis, the alignment was edited to exclude the incomplete beginnings and ends of the sequences and yielded a matrix of 611 characters. The matrix used in phylogenetic analysis included 181 constant characters, 85 parsimony informative characters and 345 parsimony uninformative characters. The most parsimonious tree had a tree length of 476 changes, a consistency index of 0.94, a retention index of 0.84, a rescaled consistency index of 0.80 and a homoplasy index of 0.05.

This analysis revealed diversity among the different Pasteuria sequences (Fig. 5). As in Fig. 2, P. ramosa was positioned on a deep branch of the phylogenetic tree and the two additional Pasteuria clades were separated. Bootstrap support for the P. penetrans clade (clade 1) was 99%; however, support for clade 2 was quite low (67%).

Clade 1 consists of sequences from strains (including the type strain of P. penetrans) known to infect root-knot nematodes and clones predominantly from soils infested with root-knot nematodes. It is not known at this time whether all clade 1 strains belong to the species P. penetrans. Barring 16S rRNA gene sequence similarity of less than 97% (Stackebrandt & Goebel, 1994), physiological and genetic studies are required to confirm that two strains are from different species. Due to the current lack of appropriate laboratory cultivation systems for Pasteuria, these tests are difficult at best. Possible differences in infectivity in nematodes are also difficult to assess at this time. These uncertainties will be greatly reduced when efficient systems for Pasteuria cultivation are developed (Gerber & White, 2001).

Five subclades can be distinguished within clade 1. Subclades 1a, 1b and 1d have good bootstrap support values of 98, 94 and 93% respectively, suggesting that each is a distinctive clade within clade 1. Subclades 1c and 1e are supported by lower bootstrap values, 78 and 80%, respectively, which may compromise their phylogenetic position as individual clades within clade 1. Ten sequences (Np and Nf series; Table 1) generated from endospores from a single race of root-knot nematode (Meloidogyne species) were originally thought to belong to a single strain previously designated P. penetrans P-20 (Oostendorp et al., 1990; Anderson et al., 1999). In our analysis, ten P-20 sequences fell into three different subclades (1a, 1b and 1e).
Most sequences from soils 2 and 5 separated into a distinct subclade (1c).

Clade 2 includes sequences from clones taken from soils infested with a variety of nematodes, including root-knot, sting and cyst nematodes. This clade also includes previously described strains that infect cyst, lance and sting nematodes. It is not known at this time if clade 2 *Pasteuria* strains exclusively attack nematodes other than root-knot nematodes. A more complete understanding of relationships between phylogenetic groups of *Pasteuria* and the nematodes they infect would be very important in the accurate design and implementation of *Pasteuria*-based systems to control nematodes.

Clade 2 includes SIV7 and SIV8, clones obtained from soil 4 (which harboured sting nematodes), and SV36, obtained from soil 5 (known to harbour lance nematodes). Also included in clade 2 are sequences from a previously described cyst nematode-infecting *Pasteuria* (GenBank no. AF134868) (Atibalentija *et al.*, 2000) and a sting nematode-infecting *Pasteuria* (AF254387) (Bekal *et al.*, 2001). Bootstrap support for this clade is low (67 %), such that confidence that clade 2 is a single clade is not high. It is possible that clade 2 should be divided into two individual clades and additional full sequences from this clade would help clarify the topology of the tree and define the clade. Clade 2 may be separated into at least two subclades (Fig. 5).

Bootstrap support for separation of clade 2 into these two subclades is very strong and the sequence similarities between representatives of the two subclades are low (less than 93 %). As currently defined, 16S rRNA gene sequence similarity may not be used to define species, but may be used to determine whether two strains do not belong to the same species if they share less than 97 % similarity (Stackebrandt & Goebel, 1994). On this basis, it is likely that subclades 2a and 2b represent different species.

The number of characters used in this analysis was not high (85 parsimony informative characters) due to the relatively small amplification products and conserved regions within the amplicon. The robustness of branches formed in this analysis is therefore tenuous, although the topology of the tree did not change significantly when the near-full-length

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**Fig. 5.** Phylogenetic relationships among *Pasteuria* species. Phylogenetic reconstruction based on maximum-parsimony using heuristic search of 16S rRNA gene sequences (PAUP, version 4.0b8). Analysis based on ca. 600 nt sequences from positions 39–654 with *Thermoactinomyces dichotomicus* as the outgroup taxon. Numbers above nodes represent bootstrap values (1000 replicates).
sequences presented in Fig. 2 were subjected to a similar analysis using the same region of sequence as those in Fig. 5 (analysis not shown). Robustness of the subclades discussed above will be tested as more sequences become available.

Maximum-likelihood analysis obtained by the quartet-puzzling method revealed a similar topology for the phylogenetic tree presented in Fig. 5, although some changes in the major clades were observed and supports for the main clades were lower (data not shown). Using this method, clade 1 has a support of 59%, greater than the 50% recommended by the developers of the quartet-puzzling method. Clade 2 was not supported and subclades 2a and 2b became individual clades. Inside clade 1, each of the subclades exhibited frequencies of puzzling steps in the range 85–93, confirming the subclades achieved by maximum-parsimony. Moreover, subclades 1c and 1e received supports of 89 and 93%, respectively, possibly reaffirming their compromised identities as subclades in the maximum-parsimony analysis.

PCR detection of Pasteuria in soils and nematodes with Pasteuria-specific primers and their use in detection of Pasteuria in soils

The Pasteuria-specific PCR primers developed as part of this study were also evaluated for specific detection of Pasteuria in soils and nematodes. Detection and enumeration of Pasteuria will facilitate studies to determine the efficacy of their commercial use as biocontrol agents as new systems for laboratory cultivation of Pasteuria are developed (Gerber & White, 2001). Detection of sequences in serial dilutions of endospores added to soil 6 was consistently several orders of magnitude lower with primer set 617F/1166R than with 39F/654R (data not shown). The lowest detection limit for P. penetrans 16S rRNA genes in soil 6 using primer set 617F and 1166R was 100 endospores (g soil)−1 (Fig. 6). The primer sets proved to be specific for Pasteuria strains, as determined by sequence analysis (data not shown).

For initial screening (presumptive confirmation) of 16S rRNA gene clones prior to sequencing, PCR products can be verified by digestion with the restriction enzymes BamHI or Stul, depending on the primer set used. All Pasteuria sequences reported to date, including those found in this study, have a single BamHI site in the 39F/654R amplification product and a single Stul site in the 617F/1166R amplification product. For differentiation between different Pasteuria clones, digestion of individual 39F/654R products with HhaI is more informative than digestion of 617F/1166R due to greater sequence variability in this region between different clones. Evaluation of PCR primers for specific amplification and detection of these individual clades and subclades is currently being carried out.

In addition to providing information on the phylogeny of this potentially important biocontrol agent, the work presented here should form the basis for further studies that will elucidate the distribution and concentration of Pasteuria in soils and nematodes, and assist in defining host ranges of various strains and species of Pasteuria. Previous studies have shown that broader groups of Pasteuria exhibit preferences with regard to host nematodes (Davies et al., 1994). Additional studies are now needed to define host ranges of the various clades and subclades and to define potentially different species within these clades. Ultimately, however, a complete understanding of the relationship between Pasteuria and their nematode hosts will require an efficient system for laboratory cultivation.

ACKNOWLEDGEMENTS

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REFERENCES


