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A Simple Method for the Extraction, PCR-amplification, Cloning, and Sequencing of *Pasteuria* 16S rDNA from Small Numbers of Endospores

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Abstract: For many years the taxonomy of the genus *Pasteuria* has been marred with confusion because the bacterium could not be cultured in vitro and, therefore, descriptions were based solely on morphological, developmental, and pathological characteristics. The current study sought to devise a simple method for PCR-amplification, cloning, and sequencing of *Pasteuria* 16S rDNA from small numbers of endospores, with no need for prior DNA purification. Results show that DNA extracts from plain glass bead-beating of crude suspensions containing 10,000 endospores at 0.2×10^6 endospores ml^{-1} were sufficient for PCR-amplification of *Pasteuria* 16S rDNA, when used in conjunction with specific primers. These results imply that for *P. penetrans* and *P. nishizawae* only one parasitized female of *Meloidogyne* spp. and *Heterodera glycines,* respectively, should be sufficient, and as few as eight cadavers of *Belonolaimus longicaudatus* with an average number of 1,250 endospores of "*Candidatus* Pasteuria usgae" are needed for PCR-amplification of *Pasteuria* 16S rDNA. The method described in this paper should facilitate the sequencing of the 16S rDNA of the many *Pasteuria* isolates that have been reported on nematodes and, consequently, expedite the classification of those isolates through comparative sequence analysis.

Key words: DNA extraction method, *Pasteuria* spp., phylogeny, taxonomy, 16S rDNA sequence.

Bacteria of the genus *Pasteuria* are gram positive, mycelial, endospore-forming, and obligate parasites of invertebrates, including water fleas of the family *Daphnidae* and nematodes (Sayre and Starr, 1989). On nematodes, in particular, *Pasteuria* spp. are widespread, having been reported from more than 300 species distributed over more than 100 genera (Chen and Dickson, 1998; Ciancio et al., 1994; Sayre and Starr, 1988; Sturhan, 1988). In spite of the apparent ubiquity of *Pasteuria* spp., only five species have been described, namely *P. ramosa,* parasitic on *Daphnia* spp. (Metchnikoff, 1888); *P. penetrans* on root-knot nematodes, *Meloidogyne* spp. (Sayre and Starr, 1985); *P. thornei* on root-lesion nematodes, *Pratylenchus* spp. (Starr and Sayre, 1988); *P. nishizawae* on cyst nematodes of the genera *Heterodera* and *Globodera* (Sayre et al., 1991); and *Candidatus* Pasteuria usgae parasitic on the sting nematode, *Belonolaimus longicaudatus* (Giblin-Davis et al., 2003). The taxonomy of the genus *Pasteuria* has been in a state of confusion (Sayre and Starr, 1989) due to the fact that the bacterium could not be cultured in vitro (Bishop and Ellar, 1991; Williams et al., 1989). Consequently, descriptions were based solely on morphological, developmental, and pathological characteristics (Davies et al., 1990; Giblin-Davis et al., 1990; Metchnikoff, 1888; Noel and Stanger, 1994; Sayre and Starr, 1985; Sayre et al., 1991; Starr and Sayre, 1988; Sturhan et al., 1994). Recently, comparative analysis of 16S rDNA sequences was used to clarify the taxonomy and phylogeny of the genus by showing *Pasteuria* is more related to the *Alicyclobacillaceae* than to the *Actinomycetales* (Anderson et al., 1999; Atibalentja et al., 2000; Ebert et al., 1996).

In comparative analysis of 16S rDNA sequences, the major challenge is to obtain *Pasteuria* DNA that is free from the DNA of other bacteria that usually contaminate *Pasteuria* endospore suspensions. When the body cavity of parasitized hosts can contain large quantities of endospores (e.g., *Daphnia* spp., *Meloidogyne* spp., and *Heterodera glycines*), an elaborate chemical extraction procedure that capitalizes on the fact that *Pasteuria* endospores are resistant to lysozyme can be used to obtain clean *Pasteuria* DNA (Atibalentja et al., 2000; Ebert et al., 1996). Briefly, lysozyme and nucleases are used sequentially to release and destroy nucleic acids from contaminating bacteria. The nucleases are then digested with proteinase K before *Pasteuria* endospores are disrupted, through glass bead-beating, in tris buffer (10 mM Tris-HCl, pH 8.0) equilibrated phenol-chloroform-isoamyl alcohol (25:24:1, v/v). *Pasteuria* DNA is subsequently purified, following standard procedures (Sambrook et al., 1989), and used in PCR reactions that involve universal primers for amplification of bacterial 16S rDNA (Lane, 1991). The chemical extraction method is laborious and time consuming and requires large quantities of endospores to compensate for losses incurred during the many purification cycles involved. For the *Pasteuria* isolates that parasitize ectoparasitic nematodes, such as the oat and pea cyst nematodes, *H. avenae* and *H. goettingiana,* respectively (Davies et al., 1990; Sturhan et al., 1994), and the sting nematode, *B. longicaudatus* (Giblin-Davis et al., 1990), endospore-filled nematode cadavers are rare and typically contain only a few hundred to a few thousand endospores. In those instances, obtaining the amounts of endospores that would be sufficient for the chemical extraction procedure is almost impossible. Bekal et al. (2001) resorted to gnotobiotic cultures of *B. longicau-*

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datus to produce endospores of *Pasteuria* strain S-1 from which DNA was extracted and purified using the FastDNA kit (BIO 101, Vista, CA). Gnotobiotic cultivation of *Pasteuria* spp. circumvents the problems of contaminating bacteria and loss of endospores associated with the chemical extraction method. However, the gnotobiotic method is too complex to be recommended as a routine procedure to produce endospores for sequencing of *Pasteuria* 16S rDNA. Furthermore, it is not obvious other nematode hosts of *Pasteuria* spp. will be as amenable to gnotobiotic cultivation as *B. longicaudatus*.

A significant breakthrough toward in vitro cultivation of *P. penetrans* was announced recently (Hewlett et al., 2002), although the details of the technique are still proprietary information. Even when in vitro cultivation becomes possible for every *Pasteuria* isolate found, comparative analysis of 16S rDNA sequences still will be the method of choice for the validation of species and the resolution of the phylogenetic relationships among those species and isolates of *Pasteuria*. Comparative sequence analysis, however, depends on the availability of reliable sequence data. Our objective, therefore, was to devise a simple method for PCR-amplification, cloning, and sequencing of *Pasteuria* 16S rDNA from small numbers of endospores, with no need for prior DNA purification.

Materials and Methods

Origin of endospores: Heterodera glycines females and cysts filled with endospores of the North American isolate of *Pasteuria* (Atibalentja et al., 2000) were washed off the rhizosphere of 3-month-old soybean plants (cv. Williams 82) grown in naturally infested soil in a greenhouse. The nematodes were transferred individually into 100 µl tap water in 1.6-ml microfuge tubes before they were crushed with a tissue grinder. Positive fractions, as determined by microscopic examination, were pooled in a 1.6-ml microfuge tube to constitute the crude stock suspension, which was adjusted to a concentration of 10^6 endospores ml^{-1} and stored at 4 °C until used.

DNA extraction methods: Three methods of DNA extraction were evaluated for their ability to yield sufficient template for PCR-amplification of *Pasteuria* 16S rDNA from small numbers of endospores. The three methods included:

i) Plain glass bead-beating: In this method, a 200-µl aliquot of the stock suspension was added to an equal volume of acid-washed, 0.150 to 0.212-mm diam. glass beads (Sigma-Aldrich, St. Louis, MO) in a screw-capped 2.0-ml microfuge vial, and the mixture was subjected to vibration in a Mini-BeadBeater (BiosPec Products, Bartlesville, OK) at 5,000 rpm for 1 minute. The slurry was centrifuged for 5 minutes, at maximum speed (approx. 16,000*g*), in an Eppendorf microcentrifuge before the supernatant was transferred into a fresh 1.6-ml microfuge tube and stored at −20 °C until used in PCR reactions (see below). The glass bead pellet was saved at −20 °C for future use, at which time the slurry was allowed to thaw before 50 µl sterile distilled water (sdH_2O) was added and the centrifugation repeated to collect additional supernatant.

ii) Phenol extraction: Pasteuria endospores in 200 µl stock suspension were disrupted as described above, except 200 µl tris buffer (10 mM Tris-HCl, pH 8.0) equilibrated phenol-chloroform-isoamyl alcohol (25:24:1, v/v) was added to the mixture prior to glass bead-beating. The aqueous phase obtained after 5-minute centrifugation at maximum speed was transferred into a fresh 1.6-ml microfuge tube, mixed with equal volume chloroform-isoamyl alcohol (24:1, v/v), and re-extracted after 5-minute-centrifugation at maximum speed. DNA was then precipitated with 2 volumes ice-cold ethanol (100%) and 0.1 volume 3 M sodium acetate (pH 5.2), washed with 70% ethanol, re-suspended with 20 µl sdH_2O, and stored at −20 °C until used in PCR reactions.

iii) FastDNA extraction: The FastDNA kit (BIO 101, Vista, CA), including a "1/4 sphere + garnet + 1/4 sphere" lysing matrix and a "CLS-Y" cell lysis solution, was used to process 200 µl stock suspension according to the manufacturer's recommendations. The mixture was homogenized in the same Mini-BeadBeater used for the other two methods, and the final 100-µl eluate was evaporated in a vacuum microcentrifuge (Labconco, Kansas City, MO) before the pellet was re-suspended with 20 µl sdH_2O and stored at −20 °C until used in PCR reactions.

Minimum number of Pasteuria endospores: To determine the minimum number of endospores needed for PCR-amplification of *Pasteuria* 16S rDNA, 200, 100, 50, 25, 10, and 5 µl stock suspension were added to equal volumes glass beads, except for the last three treatments, which were adjusted to 50 µl with sdH_2O before they were mixed with 50 µl glass beads. The estimated numbers of endospores in the above suspensions were 200,000, 100,000, 50,000, 25,000, 10,000, and 5,000, respectively, corresponding to concentrations of 10^6 endospores ml^{-1} for the first three treatments, and 0.5×10^6, 0.2×10^6, and 0.1×10^6 endospores ml^{-1} for the last three treatments, respectively. The mixtures were subjected to plain glass bead-beating, and 20 µl of the supernatant was used in PCR reactions. When the supernatant was less than 20 µl, as was often the case with the last four treatments, an extra 20 µl sdH_2O was added to the slurry and the centrifugation repeated to collect more liquid.

PCR-amplification, cloning, and sequencing: PCR-amplification of *Pasteuria* 16S rDNA was achieved using two sets of primers. The first set combined the universal forward primer 27f: 5′-AGAGTTTGATCCTGGCTCAG-3′ (Lane, 1991) with the reverse primer 440r: 5′-

CATTTCTTCTTCCCGATG-3′, whereas the second set coupled the forward primer 440f: 5′-CATCGGGAAGAAGAAATG-3′ (notice that primers 440r and 440f are complementary to each other) with the universal reverse primer 1492r: 5′-TACGGTTACCTTGTTACGACTT-3′ (Lane, 1991). The primers were designed to specifically amplify 448- and 1063-bp *Pasteuria* 16S rDNA fragments with the 27f/440r and 440f/1492r pairs, respectively, for a total of 1493 bp. PCR reactions were performed in 50 µl comprising 20 µl sample or sdH_2O control, 5 µl 10× PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 µM each primer, 0.2 mM each dNTP, and 2.5 units *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA). The reactions were carried out in a GeneAmp® PCR System 9700 (PerkinElmer, Applied Biosystems, Foster City, CA) with the following thermal profile: 94 °C for 10 minutes; 45 cycles each of 94 °C for 1 minute, 52 °C for 1 minute and 72 °C for 2 minutes, final extension at 72 °C for 10 minutes, and incubation at 4 °C. PCR products were resolved in 2% agarose gel, visualized by ethidium bromide staining (0.3 µg/ml), and the DNA fragments of interest were gel-purified with the QIAEX® II gel extraction kit (Qiagen, Valencia, CA). The fragments were then ligated into pCR®2.1-TOPO® vector (Invitrogen, Carlsbad, CA) and the plasmids transformed into competent *Escherichia coli* cells, strain TOP10, according to the manufacturer's recommendations. The transfected cells were grown on Luria-Bertani (LB) agar supplemented with 100 µg ml^{-1} ampicillin and 40 µg ml^{-1} X-gal, at 37 °C overnight. Plasmid DNA was extracted with the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) from overnight cultures of selected white colonies grown at 37 °C (with 200-rpm shaking) on LB broth supplemented with 50 µg ml^{-1} ampicillin. Aliquots (10 µl) of individual clones were digested with *Eco*RI restriction enzyme for 2 hours at 37 °C, and the products were resolved in 2% agarose gel to confirm the presence of DNA inserts. Positive clones were sequenced using M13 primers with the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (PerkinElmer, Applied Biosystems, Foster City, CA). Sequence-similarity searches were performed with the BLAST algorithm (Altschul et al., 1990).

Performance test: For comparison with the chemical extraction procedure, 200 µl each of the crude stock suspension and a clean suspension of endospores obtained after the proteinase K step of the chemical extraction method were subjected to glass bead-beating (Atibalentja et al., 200; Ebert et al., 1996). A 20-µl aliquot of either suspension was then added to PCR reactions that included either 27f/440r or 440f/1492r primer combinations.

Results

DNA extracts from three different extraction methods including "plain glass bead-beating," "phenol extraction," and "FastDNA kit" were compared for their ability to direct PCR-amplification of *Pasteuria* 16S rDNA from small numbers of endospores. The extracts (20 µl for each method) were added individually into PCR reactions that included either the 440f/1492r or the 27f/1492r primer combinations. Amplification products were obtained only from reactions that included extracts from the plain glass bead-beating method (Fig. 1). With extracts from the plain glass bead-beating method, the 440f/1492r primer combination produced a discrete bright band of the expected 1063-bp size, the nucleotide sequence of which matched the *Pasteuria* 16S rDNA, following sequence-similarity searches with the BLAST program. The 27f/1492r primer combination also produced the expected 1493-bp fragment, but with abundant smears indicating unspecific amplification of DNA from contaminating bacteria. A BLAST search with the DNA nucleotide sequence from the 1493-bp fragment returned the 16S rRNA gene of *Pentoea* sp. strain P102 (GenBank accession number AF394539) as the best match. In performance tests comparing a crude and a clean suspension of endospores subjected to plain glass bead-beating, amplification products of the 448- and 1063-bp size ex-

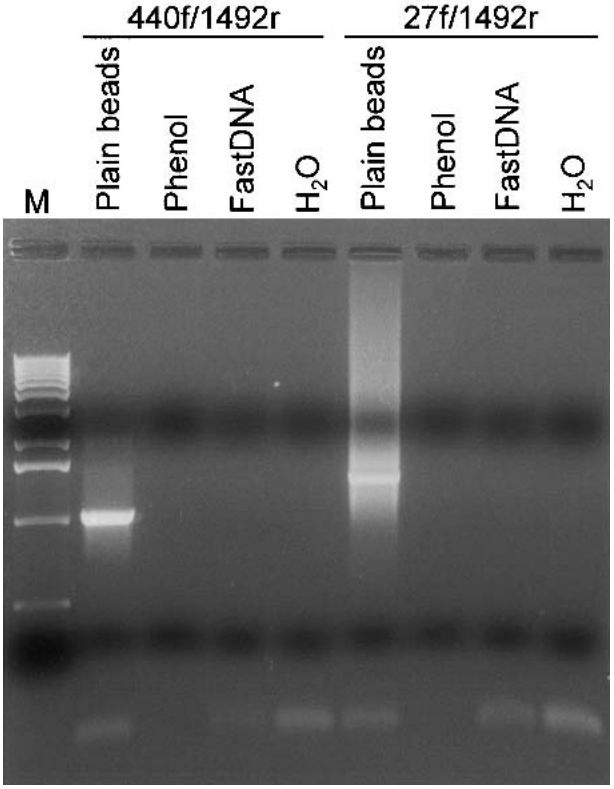

FIG. 1. Comparison of DNA extracts from three different extraction methods including plain glass bead-beating, phenol extraction, and FastDNA kit for their ability to direct PCR-amplification of *Pasteuria* 16S rDNA from small numbers of endospores, using the 440f/1492r and the 27f/1492r primer combinations (control reactions included H_2O instead of DNA extracts), with expected fragment sizes of 1063 and 1492 bp, respectively. PCR products were resolved in 2% agarose gel alongside a 1-kb DNA marker (M).

pected from the 27f/440r and 440f/1492r primer combinations, respectively, resulted from both the crude and clean endospore suspensions (Fig. 2). Furthermore, the DNA nucleotide sequence of each of the four fragments matched the *Pasteuria* 16S rDNA when submitted to sequence-similarity searches using the BLAST program. Of the six treatments that were assayed to determine the minimum number of endospores needed for PCR-amplification of *Pasteuria* 16S rDNA using the plain glass bead-beating method and the 440f/1492r primer combination, unequivocal amplification products of the expected 1063-bp size were obtained from all the five reactions that included 10,000 or more endospores, at concentrations of at least 0.2×10^6 endospores ml^{-1} (Fig. 3). Based on a threshold of 10,000 endospores and published data on the numbers of endospores per parasitized individual, the estimated numbers of endospore-filled cadavers of some nematode hosts of *Pasteuria* needed for PCR-amplification of *Pasteuria* 16S rDNA, using the plain glass bead-beating method with specific primers, are shown in Table 1. The plain glass bead-beating method has been used to sequence the 16S rDNA of *P. nishizawae* and that of the *Pasteuria* isolate that parasitizes *H. goettingiana* (GenBank accession numbers AF516396 and AF515699, respectively).

Discussion

The results from this study clearly indicate that DNA extracts from plain glass bead-beating of crude suspensions containing 10,000 endospores at concentrations of 0.2×10^6 endospores ml^{-1} are sufficient to direct PCR-amplification of *Pasteuria* 16S rDNA, when used in conjunction with primer pairs that include one of the 440 (r/f) specific primers. This finding is significant and surprising in view of the great length of precautions observed in DNA extraction protocols to protect against the action of nucleases following cell disruption (Sambrook et al., 1989). Thus, extraction buffers containing detergents (e.g., SDS), chelating agents (e.g., EDTA), and (or) organic solvents (e.g., phenol:chloroform:isoamyl alcohol) are commonly used to inactivate nucleases in DNA preparations. Then, DNA purification steps are necessary to remove those protein denaturing agents, which would otherwise inhibit subsequent enzymatic reactions. Unfortunately, purification steps are often associated with losses of DNA that can become an important factor when small numbers of endospores are involved. Such losses of DNA may account for the lack of amplification product observed with the "phenol" and "FastDNA" extraction methods. With plain glass bead-beating, however, loss of DNA is minimized and nucleases are more likely inactivated by the heat generated by the frictional forces involved. The pertinence of our results stems from the simplicity of the plain glass bead-beating method and the fact that with nematode hosts such as *Meloidogyne* spp. or *H. glycines* it takes only one parasitized female to PCR-amplify *Pasteuria* 16S rDNA. With vermiform nematode hosts such as *B. longicaudatus* as few as eight endospore-filled cadavers are sufficient, which is far less than the 100 previously required (Bekal et al., 2001).

Specific amplification of *Pasteuria* 16S rDNA using extracts from the plain glass bead-beating method depends on the nucleotide sequence of the 440 (r/f) primers. This specific oligonucleotide sequence was first identified in *P. ramosa* (Ebert et al., 1996) and then in *P. penetrans* (Anderson et al., 1999) and in the North American isolate of *Pasteuria* that parasitizes *H. glycines* (Atibalentja et al., 2000). It is present in all other *Pasteuria* 16S rDNA sequences that have been subsequently

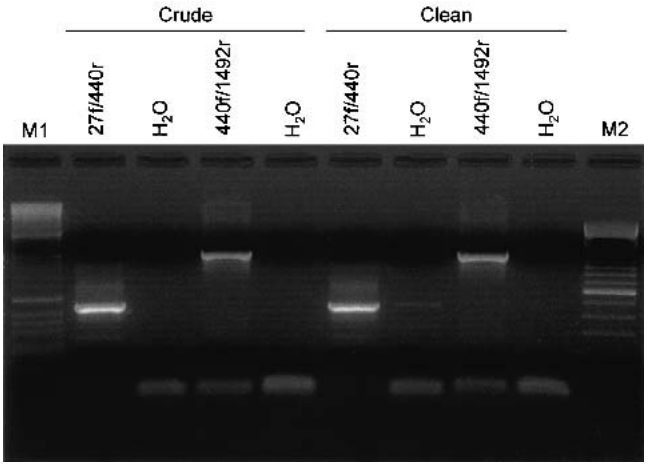

Fig. 2. A crude stock suspension and a clean suspension of endospores collected after the proteinase K step of the chemical extraction procedure were both subjected to plain glass bead-beating and the extracts used for PCR-amplification of 448-bp and 1063-bp fragments of *Pasteuria* 16S rDNA, respectively, with the 27f/440r and the 440f/1492r primer combinations (control reactions included H_2O instead of DNA extracts). PCR products were resolved in 2% agarose gel alongside 1-kb (M1) and 100-bp (M2) DNA markers.

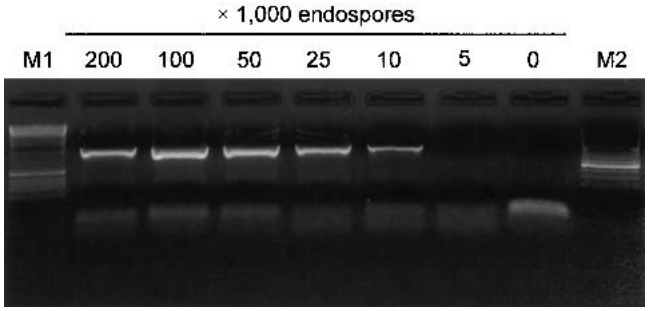

Fig. 3. Crude suspensions containing 200,000, 100,000, 50,000, 25,000, 10,000, and 5,000 endospores (at concentrations of 10^6 endospores ml^{-1} for the first three treatments, and 0.5×10^6, 0.2×10^6, and 0.1×10^6 endospores ml^{-1} for the last three treatments, respectively) were subjected to plain glass bead-beating and the extracts used for PCR-amplification of a 1063-bp fragment of *Pasteuria* 16S rDNA with the 440f/1492r primer combination (control reactions with zero endospores included H_2O instead of DNA extracts). PCR products were resolved in 2% agarose gel alongside 1-kb (M1) and 100-bp (M2) DNA markers.

TABLE 1. Estimated numbers of endospore-filled nematode cadavers needed for PCR-amplification of *Pasteuria* 16S rDNA using the plain glass bead-beating method in conjunction with specific primers.

Pasteuria nematode host	Parasitized stage	Endospores per nematode	Need[a]	Reference
Meloidogyne incognita	Female	2.0×10^6	1	Sayre et al. (1991)
M. javanica	Female	2.1×10^6	1	Mankau (1975)
M. javanica	Female	2.2×10^6	1	Stirling (1981)
Heterodera glycines	Female	0.5×10^6	1	Sayre et al. (1991)
Heterodera glycines	Female	0.4×10^6	1	Nishizawa (1986)
Heterodera glycines	Female	0.3×10^6	1	Atibalentja et al. (1998)
H. goettingianae	J2	500	20	Sturhan et al. (1994)
H. avenae	J2	800	13	Davies et al. (1990)
Belonolaimus longicaudatus	J2 & adult	364–2,128	8[b]	Giblin-Davis (1990)

[a] The minimum number of endospore-filled cadavers of the parasitized stage of each *Pasteuria* nematode host is based on the requirement of at least 10,000 endospores at a concentration of 0.2×10^6 endospores ml^{-1} for PCR-amplification of *Pasteuria* 16S rDNA.
[b] Based on a median value of 1,246 endospores per cadaver.

deposited in GenBank, but it has not yet been found in any other bacterial species, as shown by sequence-similarity searches of the Ribosomal Database Project (Maidak et al., 1999). In our experience with the 440 (r/f) specific primers in combination with the 27f and 1492r primers, we have never obtained anything other than the *Pasteuria* 16S rDNA sequence despite the fact that extracts from the plain glass bead-beating method include mixtures of DNA from *Pasteuria* and other contaminating bacteria. On the other hand, use of the 27f/1492r primer combination has always resulted in a 16S rDNA sequence of a bacterium other than *Pasteuria* sp., except when the extracts originated from plain glass bead-beating of clean post-proteinase K endopore suspensions from the chemical extraction method.

Ciancio et al. (2000) described a procedure similar to the plain glass bead-beating method to obtain DNA extracts that were used, with Scorpion fluorescent probes (Whitcombe et al., 1999) involving the *Pasteuria*-specific oligonucleotide sequence, to amplify a 139-bp fragment of the 16S rDNA of the *Pasteuria* isolate that parasitizes *H. goettingiana*. The Scorpion fluorescent probe technique aimed to detect the bacterium in soil and did not include any strategy for amplification, cloning, and sequencing of the (almost) full length of *Pasteuria* 16S rDNA, which is required for comparative sequence analysis. The plain glass bead-beating method presented here is meant to facilitate the sequencing of the 16S rDNA of the many *Pasteuria* isolates that have been reported on nematodes. Accumulation of 16S rDNA sequence data should, in turn, expedite the validation of species and the resolution of the phylogenetic relationships within the genus *Pasteuria*.

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