Detection of *Pasteuria penetrans* infection in *Meloidogyne arenaria* race 1 in planta by polymerase chain reaction

L.M. Schmidt a, J.F. Preston a,*, G. Nong a, D.W. Dickson b, H.C. Aldrich a

a Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611, USA
b Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611, USA

Received 8 December 2003; received in revised form 3 March 2004; accepted 4 March 2004

First published online 12 April 2004

Abstract

We report on the development of a PCR-based assay to detect *Pasteuria penetrans* infection of *Meloidogyne arenaria* in planta using specific primers for recently sequenced *sigE*, *spoIIAB* and *atpF* genes of *P. penetrans* biotype P20. Amplification of these genes in crude DNA extracts of ground tomato root galls using real-time kinetic PCR distinguished infected from uninfected *M. arenaria* race 1 by analysis of consensus thresholds for single copy genes. Fluorescent in situ hybridization (FISH) using the *sigE* primer sequence as a probe shows hybridization to *P. penetrans* cells in various stages of vegetative (pre-endospore) development. Ratios of gene copies for *sigE* and 16S rDNA were obtained for *P. penetrans* and compared to *Bacillus subtilis* as a genomic paradigm of endospore-forming bacteria. Phylogenetic analysis of the *sigE* gene from Gram-positive, endospore-forming bacteria finds *P. penetrans* most closely related *Paenbacillus polymyxa*. The sporulation genes (*spo* genes), particularly *sigE*, have sequence diversity that recommends them for species and biotype differentiation of the numerous *Pasteuria* isolates that infect a large number of plant-parasitic nematodes.

© 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: *Pasteuria penetrans*; *Meloidogyne arenaria*; Biological control; PCR detection; *sigE*; Fluorescence in situ hybridization; Gram-positive endospore-forming bacteria

1. Introduction

First described by Metchnikoff in 1888 as a parasite of the Daphniidae [1], *Pasteuria* are Gram-positive, endospore-forming bacteria of which all described members are parasites of invertebrates, specifically, freshwater plankton phylum Arthropoda [2] and soil-borne phytopathogenic nematodes of the phylum Nematoda [3]. In all hosts, they proceed through a developmental growth phase that includes a mycelial-like stage followed by the formation of tetrad and diad clusters of cells, before differentiation into phases associated with endospore formation. While the vegetative stages are unique compared to other known bacteria, the mycelial-like growth has suggested an affinity with members of the endospore-forming genus, *Thermoactinomyces*. Phylogenetic analysis using the 16S rDNA gene finds *Pasteuria* spp. most closely related to Gram-positive, endospore-forming bacteria, including the *Alicyclobacillus* spp., *Thermoactinomyces* spp., and *Bacillus* spp. [2–7]. *Pasteuria penetrans* is an obligate parasite of root-knot nematodes, *Meloidogyne* spp., and has emerged as a promising biocontrol agent for this ubiquitous and destructive crop pest [8–12]. The effect of *P. penetrans* in reducing *Meloidogyne* spp. populations has been widely reported [13–19].

Presently two primary methods exist for quantification of *Pasteuria* in agronomic studies. Both of these are restricted to the detection of endospores. The traditional bioassay method requires cultivation of juvenile nematodes that are introduced into a sample of test soil, after which the number of endospores attached to the cuticles of the nematodes are observed by means of microscopic

* Corresponding author. Tel.: +1-352-3925923; fax: +1-352-3925922. E-mail address: jpreston@ufl.edu (J.F. Preston).
analysis and compared to a standard [20]. This method is laborious and often underestimates soil endospore levels [20]. More recently, immunological detection using polyclonal [21,22] and monoclonal antibodies [23] raised to endospore envelope proteins has been reported. An enzyme-linked immunosorbant assay (ELISA) using anti-Pasteuria monoclonal antibody (MAb 2A41D10) has been used to detect and quantify P. penetrans spores in soil and harvested root material [23]. Immuno-detection has been shown to be a robust technique for quantification of Pasteuria sp. endospores in soil and harvested root material [23].

Pasteuria penetrans has been shown to be a robust method for the detection and quantification of P. penetrans but has limited application for differentiating between Pasteuria spp. and biotypes, which is an important factor in determining soil suppressivity [9]. Moreover, immunological detection based on recognition of an epitope associated with endospore envelope is not useful for detection of bacterial cells in vegetative stages of development in which the epitope is not formed until endospore formation has occurred [24].

To expand the scope for detection and quantification of Pasteuria in environmental conditions, we have now developed a nucleic acid-based method to detect P. penetrans infection in Meloidogyne arenaria race 1 in planta using PCR. Several highly conserved genes specific to the endospore-forming, Gram-positive bacteria were obtained for P. penetrans biotype P20 genomic DNA (gDNA) by PCR using degenerate primers based upon established sequences of Gram-positive endospore-forming bacteria. P. penetrans gDNA PCR reaction products were purified, cloned into the Topo 2.1 vector (Invitrogen) and sequenced. Gene-specific primers for the P. penetrans P20 sigE, spoIIAB and atpF genes were then designed for the detection of P. penetrans infection of crude DNA extracts of M. arenaria race 1 infected root tissue in real-time PCR. Fluorescent in situ hybridization (FISH) using the sigE gene probe showed hybridization to P. penetrans cells in various stages of pre-endospore cellular development. This nucleic acid-based approach using Pasteuria-specific primers and real-time PCR is shown to distinguish infected versus uninfected M. arenaria in planta.

2. Materials and methods

2.1. Meloidogyne arenaria cultivation for P. penetrans vegetative DNA production

Pasteuria penetrans isolate P20 [16] originated from M. arenaria (Neal) Chitwood race 1, from Levy County, FL, was grown on tomato (Lycopersicon esculentum Mill. cv. Rutgers) in greenhouses. P20 spores were attached to the cuticles of 1–5-day-old Meloidogyne J2 via centrifugation at 1000 rpm for 5 min [20]. Infected J2 were transferred via pipet to steam-pasteurized soil in 6-in. clay pots containing Rutgers tomato. Each plant was inoculated with approximately 10,000 infected Meloidogyne J2 in approximately 15 ml of deionized water (dH2O) by adding the suspension to the topsoil using a bulb pipet. The plants were cultivated in an environmentally controlled growth room and exposed to 16 h of light and 8 h of dark and an average temperature of approximately 26 °C. Root systems were observed for the presence of gall formation and at 14 days the plants were harvested. Roots were harvested and washed free of debris and placed in a beaker containing 1.8 liters of 10% pectinase (Pomaliq; Gist-Brocades), 50 mM NaOAc, pH 5.0, 0.1% CaCl2 at approximately a 50:50 (v:v) ratio. The roots were digested for 1 day on an orbital shaker table (100 RPM) to free infected female Meloidogyne from the root material. Females were collected by decanting onto a 600-μm pore sieve nested on a 150-μm pore sieve under hard-water spray. Infected females, identified by a translucent cuticle, were hand-picked by pipet with the aid of a dissecting microscope.

2.2. Crude DNA extraction of M. arenaria-infected root galls

Tomato (L. esculentum Mill. cv. Rutgers) root systems containing 14-day-old M. arenaria race 1 (infected with P. penetrans P20 or uninfected) were harvested and rinsed free of debris by hard-water spray. Individual root galls (30–35) were excised from the root-system and ground using a ground glass mortar and pestle. The sample was transferred to a 1.5-ml microfuge tube and centrifuged briefly to separate larger pieces of material. The supernatant was transferred to a fresh tube and cell breakage was attained by boiling for 5 min at 95 °C followed by bead-beating using 0.13-mm glass beads for 1 min using a dental amalgamator. DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen) and purified on a silica gel mini-spin column.

2.3. Cloning and sequencing of genes from P. penetrans P20

Degenerate primers and PCR conditions for amplification of the partial P. penetrans sigE gene (GenBank Accession No. AY497315) were as described by Arcuri et al. [25]. Degenerate primers for the spoIIAB gene were designed by examining regions of homologous amino acid sequence in alignment profiles of highly conserved genes in the Gram-positive Bacillus sp. and Paenibacillus sp. These regions were further analyzed for nucleic acid sequence homology and primer suitability using GeneTool (Biotools software). Degenerate primers were used to amplify a portion of spoIIAB gene (GenBank Accession No. AF483656) from P. penetrans P20 endospore gDNA by touchdown PCR. PCR products generated from the paralogous gene in P. penetrans P20 were cloned and sequenced. The partial atpF gene
sequence (GenBank Accession No. AY497316) of *P. penetrans* was obtained by cloning and sequencing random PCR amplification products generated by *Bacillus subtilis* ORFmer primers on *P. penetrans* gDNA. Specific primers were then designed based on the gene sequences of the target organism and used as probes in this study (Table 1).

2.4. Phylogenetic analysis of the sigE gene

The partial nucleotide sequence of *P. penetrans* sigE was evaluated by the Neighbor-Joining, ρ-distance, bootstrap consensus analysis (MEGA2) against other Gram-positive endospore-forming bacteria for which archived sigE sequences were attainable in GenBank.

2.5. Real-time kinetic PCR

DNA was amplified in a 50-µl PCR mixture containing 0.4-µl Amplitaq™ DNA polymerase, 5.0 µl of 10× Syber green, 4.0 µl of 12.5× MgCl2 (Syber Green PCR Core reagents PE Biosystems), 5.0 µl of 10× dNTPs (Boehringer Mannheim, GmbH, Germany), 5.0 µl of DNA template, and a primer concentration of 5 µM. PCR was performed in a BIO-RAD iCycler iQ™ (Bio-Rad). Product formation was detected by the Syber green 490 fluorophore (Life technologies Inc.) under the following thermal conditions: run 50 cycles starting with a hot start; hold 30 s at 95 °C; anneal at 55 °C for 30 s; extend at 72 °C for 40 s; hold 1 min at 95 °C. Oligonucleotides used for PCR amplification of specific gene targets are presented in Table 1. PCR products were analyzed by gel electrophoresis on 2% agarose gels stained with 0.1% ethidium bromide.

2.6. Fluorescence in situ hybridization

A protocol for performing FISH was developed from methods described by Amann et al. [26]. *M. arenaria* race 1 cadavers containing *P. penetrans* P20 in early stages of development were removed from 70 °C storage. Approximately 75 females were crushed using a plastic mortar and pestle and taken through a woven polyester 22-µm opening filter (Spectra/Mesh) in a 13-mm Swinnex disc filter (Millipore) to remove cuticle debris. The cells were washed in 50% ethanol in sterile nanopure water and placed on ice in 50% ethanol for 2 h. Cover slips (12 mm, #1 thickness) were washed in 70% ethanol and rinsed with excess water. The cover slips were prepared for cell mounting by adding a solution of 1% Alcian blue in water, heating to almost boiling and incubating for 10 min. The dye solution was poured off and the cover slips were rinsed in sterile water until becoming a uniform light blue in color. Cells were applied to cover slips and dried on a slide warmer for 5 min at 45 °C. The cells were dehydrated in a series of ethanol treatments consisting of 80% ethanol for 5 min followed by 96% ethanol for 5 min [27]. Slides were air-dried for 10 min and vacuum aerated to remove excess ethanol. Lysozyme (5 mg/ml) was prepared in 100 mM Tris–HCl, pH 8.0, containing 10 mM

### Table 1

<table>
<thead>
<tr>
<th>Organism</th>
<th>Target gene</th>
<th>Oligonucleotide sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. penetrans</em> P20</td>
<td>16S rDNA</td>
<td>f-AGCGGTGGGAGATGGTGTATTTAAATTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r-CTACGGCTGGCAACCGGAGAT</td>
</tr>
<tr>
<td></td>
<td>sigE</td>
<td>f-TCGCCCTCCCAACAAACAAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r-GCCACGTATGTCTGCCAGAT</td>
</tr>
<tr>
<td><em>P. penetrans</em> P20</td>
<td>sigE</td>
<td>f-CACCTATTCCTCGAGATGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r-CACCTTCCTGAAACAAAC</td>
</tr>
<tr>
<td><em>P. penetrans</em> P20</td>
<td>spoIIAB</td>
<td>f-TGTGTTTCTGTGCGAGAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r-CAACCGGCCTACATTCTC</td>
</tr>
<tr>
<td><em>P. penetrans</em> P20</td>
<td>atpF</td>
<td>f-GGTGTCGAAAAATGTGGGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r-CAACTCCTGACCTTAGCAG</td>
</tr>
<tr>
<td><em>M. arenaria</em></td>
<td>18S rDNA</td>
<td>f-CGGCTTCTGACGTGCAAATCAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r-TCCGCAATCGGCGATTTGCT</td>
</tr>
<tr>
<td><em>L. esculentum</em></td>
<td>18S rDNA</td>
<td>f-GCACGCGCAGCGCCGAAATTACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r-CGATCGCAGAGCGCCAAATTACC</td>
</tr>
<tr>
<td><em>M. arenaria/L. esculentum</em></td>
<td>18S rDNA</td>
<td>f-GCGGCGGTTAATTCGCTGCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r-CGGCGCGGTTGAGTGTGCC</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>16S rDNA</td>
<td>f-CGGGGCGAGAGTGACAGGTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r-CGGGCTGCTGCGGTGTGCTG</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>sigE</td>
<td>f-AGCCCTGCGCGCTTCCAATATCTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r-GGGAGCCATAGGTGACAGCTGATTATTC</td>
</tr>
</tbody>
</table>
EDTA, added at a volume of 100 µl to the cover slips and incubated in a moist chamber at 37 °C for 40 min. The cover slips were rinsed with dH2O and 3 µl of a 57.2 ng/µl 5' fluorescein-conjugated DNA probe, 5'-[F]CCCCCCCTCCCAAACCAAAAC corresponding to a segment of the \( P. \) penetrans P20 \( \text{sigE} \) gene was added to 20 µl of hybridization buffer (20 mm Tris–HCl, pH 7.2, 0.9 M NaCl, 0.01% SDS and 20% formamide) and applied to the cover slip. Hybridization was performed at 48 °C for 1.5 h in a moist chamber. At 1.25 h its cover slip. Hybridization was performed at 48 °C for 1.5 h in a moist chamber. At 1.25 h its cover slip. Hybridization was performed at 48 °C for 1.5 h in a moist chamber. At 1.25 h 80

3. Results

3.1. Phylogeny of the \( \text{sigE} \) gene

The results generated by Neighbor-Joining, \( p \)-distance, bootstrap consensus analysis placed the \( P. \) penetrans \( \text{sigE} \) gene in a clade with \( \text{Paenibacillus polymyxa} \) and also shared significant sequence homology with \( \text{Bacillus} \) ssp. and \( \text{Clostridium} \) ssp. (Fig. 1).

3.2. Real-time kinetic PCR of crude DNA extracts of \( P. \) penetrans P20-infected and uninfected \( M. \) arenaria race 1

Real-time kinetic PCR was performed on a crude DNA preparation of excised tomato (\( L. \) esculentum) root galls (30–35 galls) containing \( P. \) penetrans-infected \( M. \) arenaria race 1. Primers specific for \( \text{atpF}, \text{sigE}, \text{spoIIAB} \) genes from \( P. \) penetrans P20, and \( \text{Meloidogyne} \) sp. 18S rDNA and \( L. \) esculentum 18S rDNA primers were used to amplify the template DNA. Product formation was observed using the Syber™ green 490 fluorophore. The results showed primer pairs amplified template DNA, producing cycle thresholds of 11.81 for \( L. \) esculentum 18S rDNA, 16.25 for \( \text{Meloidogyne} \) sp. 18S rDNA, 18.65 for \( P. \) penetrans \( \text{atpF}, \text{19.17 for } P. \) penetrans \( \text{sigE} \) and 19.23 for \( P. \) penetrans \( \text{spoIIAB} \) (Fig. 2(A)). Agarose gel electrophoresis of the PCR products produced a single band of the expected size for each primer set (data not shown). The parity observed for \( P. \) penetrans P20 \( \text{atpF}, \text{sigE} \) and \( \text{spoIIAB} \) cycle threshold values by kinetic PCR demonstrated the expected single copy number for these genes.

In order to test the specificity of primers designed to specifically amplify \( \text{Pasteuria} \) genes, DNA obtained from crude extracts of root galls containing \( M. \) arenaria race 1-uninfected with \( P. \) penetrans P20 were template for amplification using the \( P. \) penetrans-specific \( \text{atpF}, \text{sigE} \), and \( \text{Meloidogyne} \)-specific \( \text{atpF}, \text{sigE} \), and \( \text{Meloidogyne} \)-specific \( \text{spoIIAB} \) primers. The kinetic PCR chromatogram indicated similar cycle thresholds for \( \text{Meloidogyne} \) sp. 18S rDNA and \( L. \) esculentum 18S rDNA to those previously obtained, and incongruent late thresholds (>30 cycles) for \( P. \) penetrans-specific

Fig. 1. The phylogenetic relationship of the partial \( \text{sigE} \) nucleotide sequence from \( P. \) penetrans P20 and other Gram-positive endospore-forming \( \text{Eubacteria} \). The tree was created using Neighbor-joining, \( p \)-distance, bootstrap consensus analysis by MEGA2 biomolecular software.

Fig. 2. (A) Real-time PCR chromatogram of Syber™ green 490 fluorophore in response to product formation from DNA template obtained by crude DNA extraction of tomato (\( L. \) esculentum) root galls containing \( P. \) penetrans P20-infected \( M. \) arenaria race 1. Primers used from left to right (lower to higher threshold cycles): \( L. \) esculentum 18S rDNA; \( M. \) arenaria 18S rDNA; \( L. \) esculentum and \( M. \) arenaria 18S rDNA homologous sequence; \( P. \) penetrans \( \text{atpF}, P. \) penetrans \( \text{sigE} \) and \( P. \) penetrans \( \text{spoIIAB} \). (B) Real-time PCR chromatogram of Syber™ 490 fluorophore in response to product formation from DNA template obtained by extraction on root galls containing non-\( \text{Pasteuria} \) infected (uninfected) \( M. \) arenaria race 1. Primers used from left to right (lower to higher threshold cycle): \( \text{Lycopersicon} \) sp.18S rDNA; \( M. \) arenaria 18S rDNA; \( \text{Lycopersicon} \) and \( M. \) arenaria 18S rRNA; \( P. \) penetrans \( \text{atpF}, P. \) penetrans \( \text{spoIIAB} \) and \( P. \) penetrans \( \text{sigE} \).
primers atpF, sigE and spoIIAB (Fig. 2B). Gel electrophoresis analysis of PCR products showed no detectable PCR amplification products for P. penetrans specific sigE, atpF and spoIIAB primers on uninfected M. arenaria, indicating the late threshold cycle signals present in the chromatogram were not authentic PCR products (Fig. 3).

3.3. Ratio of 16S rDNA and sigE gene copies

Comparison of genome copy numbers for B. subtilis and P. penetrans genes were made using primers that were specifically designed to be similar in amplicon region, size and hybridization efficiency with respect to specific sequences in order to normalize PCR kinetics. Analysis of cycle thresholds for P. penetrans P20-specific 16S rDNA and sigE primers, defined a ratio for the two genes of approximately 3:1, respectively. This genome ratio differed from B. subtilis, which was found to have a corresponding ratio of 5:1 using species-specific primers for the 16S rDNA and sigE. Based upon complete genome sequencing, the genome of B. subtilis strain 168 is known to have 10 genes encoding 16S rRNA and one gene encoding sigE. The standard curve correlation coefficient for a series of 5, 10-fold dilutions of P. penetrans purified DNA template amplified by 16S rDNA and sigE primers was 0.96 and 0.99, respectively. P. penetrans starting DNA template concentration was estimated to be approximately 1.5 ng/\mu l based upon cycle thresholds for B. subtilis sigE for which an established

gDNA concentration was provided by spectrophotometric analysis.

3.4. Fluorescence in situ hybridization

Differential contrast microscopy (DIC) of material obtained from the body cavity of infected Meloidogyne female cadavers that was used for PCR experiments

![Fig. 3. Agarose gel electrophoresis of PCR products of DNA crude extract from ground tomato (L. esculentum) root galls containing M. arenaria race 1 uninfected with P. penetrans P20. Lanes 1, 5 and 9, 100 bp marker; Lane 2, P. penetrans sigE; Lane 3, P. penetrans spoIIAB; Lane 4, P. penetrans atpF; Lane 6, M. arenaria 18S rDNA; Lane 7, L. esculentum 18S rDNA; Lane 8, L. esculentum and M. arenaria 18S rDNA homologous sequence.](image)

![Fig. 4. Differential interference contrast (DIC) micrograph (1000×) of the pseudocoelomic contents of M. arenaria race 1 cadavers used for obtaining P. penetrans DNA. The micrograph reveals the presence of P. penetrans in various stages of cellular development, including branching thalli (microcolonies), diads, tetrads and early-stage endospores (Bar = 10 μm).](image)

![Fig. 5. Epifluorescent micrograph of fluorescence in situ hybridization (FISH) with the P. penetrans sigE gene probe. (A) Oligomer hybridization to P. penetrans cells in various stages of development. (B) Enlarged view showing hybridization of the probe to a microcolony, illustrating the branched thalli characteristic of this early vegetative stage structure. Photos were taken using a Nikon microscope under epifluorescence using a 495-nm excitation filter at 1000× magnification under oil.](image)
shows Pasteuria in various stages of development (Fig. 4). These cells where fixed and used for fluorescent in situ hybridization (FISH) using a fluorescein-conjugated sigE gene sequence, 5’-[F]CCCCCTCCAAAA-CCAAAAC as a probe. The results of in situ hybridization revealed the sigE probe bound strongly to fixed preparations of vegetative cells of P. penetrans (Fig. 5(A)). The probe was also observed to hybridize to nuclear material of early stage endospores where it localizes in a polar region at the origin of the developing spore cortex. Target concentration was highest in early vegetative cells. A microcolony structure is shown to hybridize with the probe producing a distinct illumination of the mycelial microcolony structure (Fig. 5(B)).

4. Discussion

4.1. Gene-specific probes for detection of P. penetrans infection of M. arenaria in planta

Degenerate primer design based upon analysis of conserved regions of archived sequences for the sigE [25] and spoIIAB genes in other Gram-positive endospore-forming bacteria resulted in the identification of orthologous genes in P. penetrans P20 by PCR. The cloned partial sequence for these genes from P. penetrans P20 gDNA allowed the design of specific primers to be used as specific probes to detect P. penetrans in planta. Primers specific for sigE, atpF and spoIIAB successfully allowed detection of the bacterium within the Meloidogyne host. DNA extracts prepared from P. penetrans-infected Meloidogyne females that were RNase and DNase treated prior to bacterial DNA extraction, as well crude preparations of ground root galls containing P. penetrans-infected M. arenaria race 1 were evaluated. It was found that regardless of the purity of the DNA preparation, only those preparations containing P. penetrans-infected M. arenaria were amplified by these primers. Analysis of real-time PCR chromatograms showed similar cycle thresholds, which was the expected result for three single copy genes. Real-time PCR of crude extracts of root galls containing uninfected M. arenaria race 1 produced dissimilar and late cycle thresholds (>30 cycles) for P. penetrans genes and subsequent gel electrophoresis indicated an absence of products. Real-time PCR data generated under the conditions of this study showed dissimilar and late cycle thresholds for P. penetrans atpF, spoIIAB and sigE with spreads of greater than 12 cycles distant to the M. arenaria 18S rDNA threshold cycle in the absence of P. penetrans infection. Further study is needed to define the probable relationship between the developmental stage of the nematode and of the bacterium before real-time PCR can be used to ultimately assess infection in samples containing low genome copies. Many factors influence product formation in PCRs and the attainment of an initial gene copy number is difficult due to the stochastic behavior of PCR and variations in hybridization efficiency between primers. However, the results provided here indicate that these gene-specific primers can be used in a rapid assay of crude DNA extract to differentiate infected from uninfected Meloidogyne within root systems.

This approach represents a powerful tool for assessing Pasteuria infection levels in root-knot nematode infected crop systems. PCR detection provides the capability to monitor “in-time” host–parasite dynamics and these data can be analyzed along with a variety of crop and nematode indices for evaluation of biocontrol. Assays for Pasteuria based either upon endospore attachment or immunodetection are dependent upon the presence of adhesins or adhesin epitopes that are not present until the endospores have matured [23,24]. While the inaccessibility of DNA that is compartmentalized in the mature endospore limits the application of PCR-based detection of genes, it is clear that PCR is able to amplify these genes into the early stages of endospore formation. Several treatment approaches to increase extraction efficiency and DNA recovery from P. penetrans mature endospores were attempted in this study. They included repeated rapid freeze/thaw, lysozyme and proteinase K pretreatment, hot phenol extraction, sonication, lyophilization followed by bead-beating and microwave irradiation. None of these treatments were found to significantly increase DNA extraction efficiency for mature endospores. It is suggested that preferential selection of galls nearer plant root-tips (representing the most recent infection sites) may provide the highest level of analytical sensitivity for PCR. The combined use of PCR-based detection for vegetative and early endospore stages and immunodetection for mature endospores will allow quantitative monitoring of Pasteuria in all stages of development.

4.2. Sporulation in Gram-positive endospore-forming bacteria and the phylogenetic relationship of P. penetrans sigE

The process of sporulation in B. subtilis is a highly coordinated process involving some 125 genes [28]. An important feature is the sequential activation of a variety of α subunits of RNA polymerase which direct transcription of genes, the products of which direct the cell through morphological transitions, ultimately resulting in the formation of a mature endospore [29]. Following replication and condensation of DNA to each of the two cell poles, sporulation of B. subtilis involves the formation of a polar septum, which divides the mother cell from the developing endospore. The sigma E transcription factor encoded by the spoIIIG operon is a cell-specific regulatory protein that directs gene expression in the mother cell [30]. It is produced initially as an inactive
propoprotein (pro-sigma E) and is proteolytically cleaved into an active form by a spoIIIGA-mediated processing event [30]. Sigma E activity is confined to the mother cell where it directs transcription of several genes including spoIIID [30]. Evidence suggests that regulatory processes in the sporulation pathways of Bacillus spp. and Clostridium spp. are conserved [30]. The P. penetrans σE sequence showed a high degree of homology and aligned in clade with P. polymyxa. These data confirm a common origin of sporulation sigma factors for the species of Bacillus, Clostridium and Pasteuria [7] and are supported by recent findings with regard to phylogenetic analysis of the P. penetrans spoOA gene [31].

4.3. Use of highly conserved, group-specific genes as probes and their potential as biotype indicators

In contrast to the immunodetection methods, which detect one or more epitopes that are shared on different Pasteuria species and biotypes, the PCR method may have the important consequence of detecting species and biotypes that target different species and races of root-knot nematodes, thereby allowing the assessment of the extent to which a soil may be considered suppressive for a particular phytopathogenic nematode. Moreover the use of probes designed to target unique sequence strings within highly conserved genes specific to the Gram-positive endospore-forming bacteria narrows the target window to a particular group within the Eubacteria, effectively reducing the probability of obtaining false positives when analyzing complex environmental samples.

Molecular phylogenetic markers including the sigE gene should prove useful in distinguishing species and biotypes of endospore-forming Gram-positive bacteria [25]. Sequence heterogeneity of sigE is shown to be greater than for 16S rDNA, suggesting a potential to develop species and biotype-specific probes to discriminate between P. penetrans having different host specificities. The sporulation genes including sigE may well provide a more discriminating means to speciate Pasteuria once gene sequences are obtained from other isolates. DNA isolation and sequencing of additional Pasteuria isolates should allow for expanded use of this approach to differentiate Pasteuria. This capability will have a substantial impact on our understanding Pasteuria phylogeny and ecology. More practically, it should permit an accurate assessment of the levels of different species and biotypes of Pasteuria in agronomic environments, and thereby allow the use of Pasteuria sp. for the effective biocontrol of their respective nematode hosts.

Acknowledgements

The authors thank Dr. Peter Kima and Donna Williams, Department of Microbiology and Cell Science, University of Florida, for their assistance with the fluorescence in situ hybridization work. We thank Dr. Madeline Rasche, Department of Microbiology and Cell Science, University of Florida for critically reviewing the manuscript. This work was supported by USDA-CSREES, T-STAR (CRIS project FLA-MCS-04080), Regional USDA-CSREES NE-171 (CRIS project FLA-MCS-03798) and as the University of Florida IFAS Agricultural Experiment Station (CRIS project FLA-MCS-03703). This manuscript represents Journal Series No. R-10023 of the University of Florida Agricultural Experiment Station.

References


