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Genetic and Immunological Comparison of the Cladoceran Parasite *Pasteuria ramosa* with the Nematode Parasite *Pasteuria penetrans*▽

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***Pasteuria penetrans*, an obligate endospore-forming parasite of *Meloidogyne* spp. (root knot nematodes), has been identified as a promising agent for biocontrol of these destructive agricultural crop pests. *Pasteuria ramosa*, an obligate parasite of water fleas (*Daphnia* spp.), has been shown to modulate cladoceran populations in natural ecosystems. Selected sporulation genes and an epitope associated with the spore envelope of these related species were compared. The *sigE* and *spoIIAA*/*spoIIAB* genes differentiate the two species to a greater extent than 16S rRNA and may serve as probes to differentiate the species. Single-nucleotide variations were observed in several conserved genes of five distinct populations of *P. ramosa*, and while most of these variations are silent single-nucleotide polymorphisms, a few result in conservative amino acid substitutions. A monoclonal antibody directed against an adhesin epitope present on *P. penetrans* P20 endospores, previously determined to be specific for *Pasteuria* spp. associated with several phytopathogenic nematodes, also detects an epitope associated with *P. ramosa* endospores. Immunoblotting provided patterns that differentiate *P. ramosa* from other *Pasteuria* spp. This monoclonal antibody thus provides a probe with which to detect and discriminate endospores of different *Pasteuria* spp. The presence of a shared adhesin epitope in two species with such ecologically distant hosts suggests that there is an ancient and ecologically significant recognition process in these endospore-forming bacilli that contributes to the virulence of both species in their respective hosts.**

Pasteuria spp. are gram-positive endospore-forming obligate parasitic bacteria that have the unique distinction of being hosted by organisms in two distinct phyla, the Nematoda and the Arthropoda. These bacteria include parasites of phytopathogenic nematodes (4, 5, 13, 16, 19, 24, 26) and aquatic cladocerans (Moinidae and Daphinidae) (15) that suppress fecundity in populations occurring in natural environments. The ability of *Pasteuria penetrans* to suppress the growth of root knot nematodes supports its use as a benign alternative to chemical nematicides (6, 8, 11, 12, 18, 20). *Pasteuria ramosa*, first described by Metchnikoff in 1888 (22), is as the type species of the genus *Pasteuria*. The planktonic crustacean *Daphnia magna* is a vital component of the food chain in freshwater ecosystems, and fluctuations in populations have a profound effect on pond ecology. As one of several naturally occurring parasites of the Daphnidae (16), *P. ramosa* is thought to play a significant role in the temporal distribution of *Daphnia* spp. in natural ecosystems (29).

Species assignments for several phytopathogenic *Pasteuria* spp. and *P. ramosa* are based on 16S rRNA sequences, morphological properties of mature endospores, and host preferences (2, 4, 14, 16, 19, 24). The phylogenetic relationships based on highly conserved sporulation transcription factors (24, 28, 31) and multiple genetic loci (9) further define the position of *P. penetrans* in relation to genomically defined *Bacillus* spp. All of these characteristics indicate that *P. ramosa* is the most phylogenetically distinct species for which comparisons in this genus have been made. To determine evolutionary relationships, contiguous sequences of the *spoIIAA*/*spoIIAB* genes encoding highly conserved sporulation factors have been compared (3, 24). Significant sequence differences clearly distinguished *P. ramosa* and *P. penetrans* and also distinguished isolates of *P. ramosa* obtained from different locations based on the presence of single-nucleotide polymorphisms (SNPs). Isolates of *P. penetrans* have been shown to harbor silent SNPs in the *spoIIAA*/*spoIIAB* genes, and in some cases these SNPs may serve as markers that correlate with virulence for a specific host (23).

Endospore envelope peptides of *Pasteuria* spp. and biotypes associated with several species of phytopathogenic nematodes have been compared based on immunodetection with a monoclonal antibody (MAb) raised to *P. penetrans* biotype P20. This antibody is specific for an epitope shared by different polypeptides, resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and detected on immunoblots, and the "antigenic ladder" distinguishes *Pasteuria* spp. and biotypes exhibiting host preferences (24). This antibody was developed for environmental detection of *P. penetrans* and showed no cross-reactivity with endospore-forming bacteria outside the genus *Pasteuria* (27). The epitope recognized contains a putative β-1,4-linked *N*-acetyl-D-glucosamine carbohydrate residue (10) that is uniformly distributed on the surface of *P. penetrans* endospores (27) and is formed in the late stages of spore maturation (7). In the studies described here genetic and immunological approaches were employed to compare and define these obligate parasites of phylogenetically diverse hosts.

MATERIALS AND METHODS

Bacterial isolates. *P. penetrans* isolate P20 originating from *Meloidogyne arenaria* (Neal) Chitwood race 1 from Levy County, FL, was grown on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in greenhouses. *P. ramosa* isolates P1

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TABLE 1. DNA primers and fluorescent probe used in this study to amplify and detect selected genes

Organism	Gene(s)	Type	Direction[a]	Sequence (5′–3′)
Pasteuria spp.	*sigE*	Degenerate primer	F	AAGAAAATMAARCTWGCCACKTATGC
			R	MGCWACRTCCTTCTGTGTTTTCTC
Pasteuria spp.	*spoIIAA*/*spoIIAB*	Degenerate primer	F	GGKGARYTSGAYCAYCAT
			R	TCCATDATSGTRAARCCCAT
P. ramosa	*atpG*	Primer	F	GCGGATATAATAGCAGCCTTTTTCGAACA
			R	GCAAATTCACTAGCACGCGCCTCT
P. ramosa	*rpsA*	Primer	F	TGGCTTAGTGATCGATCTTGGGGTGAG
			R	TGCTGAGGCTAATGCGACCACTTTC
P. ramosa	*pheT*	Primer	F	GCCCTTTTTTGACATTTTTTGCACCA
			R	GGCTTTGCGATCGGAGTAGGTGTTG
P. ramosa	*pabA*	Primer	F	GTGGCTTTTGCTTCAGATGGGGTTAT
			R	TGGGAATCACAACCACAAATAAAGAGAAAT
P. ramosa	*polC*	Primer	F	CCCGCAAAACATCCAAAGGCTGA
			R	CGTGCGCTGCGATGATGGTGTC
P. ramosa	*sigE*	Probe		Fluorescein-CTACGAACAGCCCTATCTATG

[a] F, forward; R, reverse.

through P5 originated from the following locations: P1 originated from a single infected *D. magna* female in Gaarzerfeld, Germany; P2 was obtained from pond sediments in Kains in northern England; P3 was obtained from 10 hosts originating from a rock pool in southern Finland; P4 is a mixture of eight lines from eight *D. magna* females originating from Belgium; and P5 was obtained from sediments originating from a pond at the Moscow Zoo in Moscow, Russia. *P. penetrans* endospores were cultured and harvested as described previously (27).

Genomic DNA extraction. All chemicals and reagents used were reagent grade, enzyme grade, or molecular grade. *P. penetrans* vegetative cells were harvested from 14- to 21-day-old *M. arenaria* race 1-infected plants as described previously (28). Vegetative cells obtained from live 2-week-old *P. ramosa* P1-infected *D. magna* were processed as described previously (27) and placed in 50 μl of 0.01 M Tris-HCl (pH 7.0)–0.01 M EDTA–0.15 M NaCl (TNE buffer). To this preparation 10 μl of a 100-mg/ml lysozyme solution was added, and the resulting solution was incubated at 37°C for 1 h. DNA was extracted using a Qiagen stool DNA kit (Qiagen, Valencia, CA). The DNA was stored at −20°C until it was used. *P. penetrans* vegetative cells were washed in 0.01 M Tris-HCl (pH 8.0)–0.15 M NaCl (T-NaCl) and resuspended in 1 ml of T-NaCl with 5.0 mM $MgCl_2$. To this preparation 50 μl DNase I and 50 μl RNase were added. The solution was incubated at 37°C for 1 h and centrifuged at 10,000 × *g* for 5 min, and the pellet was resuspended in T-NaCl containing 10 mM EDTA. The cells were washed and resuspended in 50 μl TNE buffer, and to this preparation 20 μl of a 100-mg/ml lysozyme solution was added. The sample was incubated for 1 h at 37°C. DNA was extracted using a Qiagen DNeasy kit (Qiagen, Valencia, CA). Cell breakage was augmented by repeatedly freezing the sample (three times) in liquid N_2 and thawing it in a 65°C water bath. The genomic DNA was stored at −20°C prior to use.

Extraction of bacterial DNA for comparisons of *P. ramosa* isolate P1 to P5 nucleotide sequences was performed using an EZNA tissue DNA kit (Peqlab, Erlangen, Germany).

PCR amplification, cloning, and restriction digestion. Primers were designed and used to amplify partial coding sequences in *P. ramosa* (Table 1). PCR amplicons were purified using a GenElute gel extraction kit (Sigma, Buchs, Switzerland) and either sequenced directed or cloned into the TOPO TA pCR2.1 vector, transformed into chemically competent *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA), and prepared for DNA sequencing.

Double restriction digestion of the *spoIIAA*/*spoIIAB* amplicon sequence of *P. penetrans* and *P. ramosa* was performed using Sau3AI and RsaI with bovine serum albumin in NEbuffer I (New England Biolabs, Ipswitch, MA) at 37°C. The digest was resolved on a 1% agarose gel and visualized by staining in 0.1% ethidium bromide.

FISH. A protocol for performing fluorescence in situ hybridization (FISH) was developed from methods described by Amann et al. (1); this protocol has been described previously (28). A 5′-fluorescein-conjugated DNA probe, 5′-fluorescein-CTACGAACAGCCCTATCTATG corresponding to a segment of the *P. ramosa* *sigE* gene, was added to 20 μl of hybridization buffer (20 mm Tris-HCl [pH 7.2], 0.90 M NaCl, 0.01% SDS, 20% formamide) and applied to coverslips. Hybridization to fixed cells was performed at 48°C for 1.5 h in a moist chamber. Each coverslip was washed in 1.5 ml of distilled H_2O and air dried prior to mounting in Gel/Mount (Biomedia, Foster City, CA). Preparations were stored in the dark and visualized with a Nikon epifluorescent microscope fitted with an Episcopic fluorescence attachment that housed a 495-nm excitation filter under a ×40 fluorescent or ×100 differential interference contrast objective with oil.

Immunoblotting of endospore peptides. *P. ramosa* P1 endospores were recovered from *D. magna* cadavers provided by one of us (Dieter Ebert). *Pasteuria* endospore surface proteins used for SDS-PAGE immunoblotting were extracted as previously described (27). Host cadavers containing mature endospores were gently crushed (to limit maceration of host tissue) with a Teflon mortar in a sterile 1.5-ml microcentrifuge tube containing sterile deionized water. The homogenate was passed through a woven polyester 21-μm mesh filter (Spectra mesh) housed in a 13-mm Swinnex disk holder (Millipore) to separate the debris. The endospore cell pellet was then washed several times in sterile deionized water and stored at −20°C prior to use. *Pasteuria* endospores remain viable after freezing.

Endospore surface antigens were solubilized by incubation for 2 h at 37°C in a solution containing 6.0 M urea, 3.0 mM dithiothreitol, and 5.0 mM 2-[*N*-cyclohexylamino]-ethanesulfonic acid buffer (pH 10). Samples were prepared for gel loading in nonreducing treatment buffer (50 mM Tris-HCl [pH 6.8], 2.0% SDS, 10% glycerol, 0.10% bromophenol blue). Peptides were resolved on a 4 to 20% precast gradient polyacrylamide gel (Bio-Rad, Hercules, CA) and processed as previously described (27).

RESULTS

Morphological comparison and FISH. Figure 1 shows various life stage morphologies, visualized by differential interference contrast microscopy, for *P. ramosa* (Fig. 1A) and *P. penetrans* (Fig. 1B) which were recovered from infected cadavers. Early vegetative stage growth of *P. ramosa* is shown in Fig. 1A. Intermediate stages in the process of development leading to sporulation are represented by the formation of tetrads and dyads, which are developmental stages that define *Pasteuria* spp., including *P. ramosa* and *P. penetrans* (22, 25, 26). Prema-

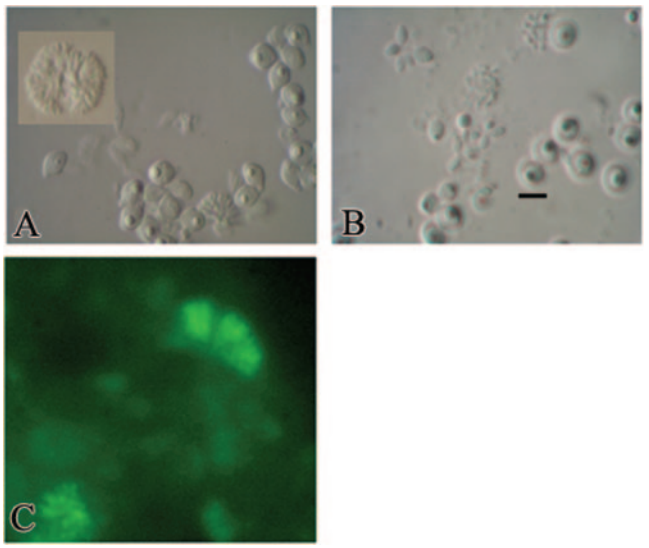

FIG. 1. (A and B) Phase-contrast micrographs comparing various life stage morphologies of (A) *P. ramosa* P1 and (B) *P. penetrans* P20. An early vegetative stage of *P. ramosa* P1 is shown in the inset in panel A. Intermediate stages of sporulation are represented by the formation of tetrads and dyads. Endospores are "grape-like" and disk-shaped cells in *P. ramosa* P1 and *P. penetrans* P20, respectively. The spore cortex is easily recognized as a dense spherical body at the apex of the cell. Micrographs were taken under oil (magnification, ×1,000). The line in panel B indicates 3 nm. (C) FISH of a *sigE*-specific gene probe hybridizing to *P. ramosa* P1 vegetative cells. The micrograph was taken with a Nikon epifluorescent microscope equipped with a 495-nm excitation filter under oil (magnification, ×1,000).

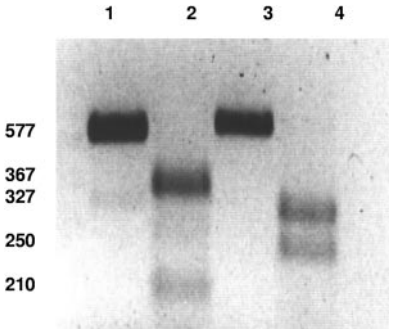

FIG. 3. Double restriction digestion of a PCR-amplified *spoIIAA*/*spoIIAB* partial coding sequence using Sau3AI and RsaI. Lane 1, undigested *spoIIAA*/*spoIIAB* amplicon from *P. penetrans* P20; lane 2, double digest of *P. penetrans* P20; lane 3, undigested *spoIIAA*/*spoIIAB* amplicon from *P. ramosa* P1; lane 4, double digest of *P. ramosa* P1. The numbers at left represent base pairs.

ture endospores of *P. ramosa* are "grape-like" cells, and mature spores of *P. ramosa* and *P. penetrans* are disk-shaped cells.

A probe designed using the unique sequence of the *P. ramosa sigE* gene allowed detection of this gene by FISH. The results show that this probe hybridized to chromosomal DNA present in the early stages of vegetative development, thus validating the origin of the gene sequence (Fig. 1C).

Genomic comparisons. Phylogenetic analysis of the partial coding sequence of *sigE*, *spoIIAA*/*spoIIAB*, and the 16S rRNA gene placed *P. ramosa* and *P. penetrans* together in a distinct clade within the gram-positive endospore-forming bacilli. Nucleic acid sequence alignment of *sigE* revealed 72.8% pairwise identity between *P. ramosa* and *P. penetrans*. The regions of the *sigE* gene corresponding to specific probe sequences are shown in a sequence alignment in Fig. 2. The partial coding sequences for *P. penetrans* and *P. ramosa spoIIAA*/*spoIIAB* exhibited 69.7% pairwise identity. Double restriction digestion of the partial coding sequences of the *spoIIAA*/*spoIIAB* genes using Sau3AI and RsaI enabled differentiation of the species (Fig. 3). Digestion of the *P. ramosa* P1 and *P. penetrans* P20 *spoIIAA*/*spoIIAB* PCR amplicons using Sau3AI and RsaI resulted in cleavage of the 577-bp parent amplicon that produced 367- and 210-bp fragments for *P. penetrans* P20 and 327- and 250-bp fragments for *P. ramosa* P1.

Nucleotide sequence variability between populations of *P. ramosa*. Partial coding sequences of nine housekeeping genes from five distinct populations of *P. ramosa* that infect *D. magna* were sequenced and compared to determine the level of conservation between populations (Table 2). The results indicated that SNPs were present in the partial coding sequences of five of the nine genes analyzed. The genes expressing SNPs included *spoIIAA*/*spoIIAB*, *atpG*, *rspA*, *pheT*, and *pabA*. The genes not displaying SNPs included *sigE*, *spo0A*, *polC*, and the 16S rRNA gene. Silent SNPs were observed in the *spoIIAA*/*spoIIAB* amplicon for all five *P. ramosa* populations in various locations. SNPs were observed to result in conservative amino

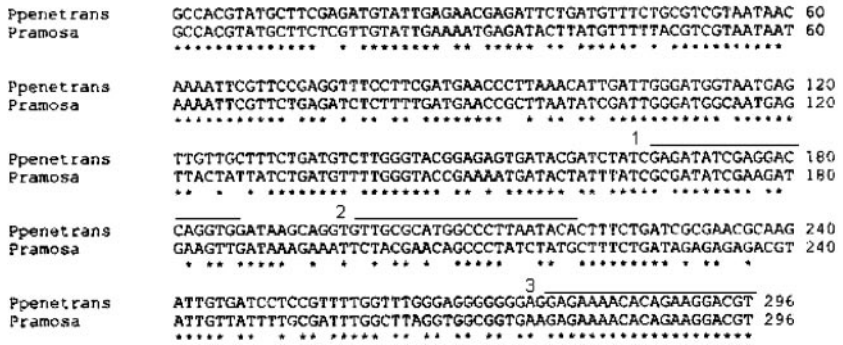

FIG. 2. Nucleic acid alignment of the *sigE* partial coding sequence using ClustalW, version 1.83. The top line shows the homologous region associated with the *P. penetrans* P20-specific fluorescein-conjugated probe; the middle line shows the homologous region for the *P. ramosa* P1-specific fluorescein-conjugated probe; and the bottom line shows the conserved region which is the target for the general *Pasteuria* probe.

TABLE 2. Comparison of selected partial coding sequences of conserved housekeeping genes for distinct populations of *P. ramosa* P1 through P5

Gene(s)	Accession no.	Protein function	Amplicon size (bp)	Nucleotide substitution(s)	Amino acid substitution	% Identity
sigE	AY713480	Sporulation sigma factor	278	None	None	100
spoIIAA/*spoIIAB*	AY569335	Antisigma regulatory factor	575	P1, T127→C; P2, A508→G; P3, A308→G; P4, A569→C; P5, C381→T	None	≥99.48
atpG	ABE19763	ATP synthase	393	P1, G192→T; P3, G385→A	Ser→Asn (neutral/polar)	>99.4
spo0A	CAD79641	Sporulation initiation	380	None	None	100
rspA	ABM55769	30S ribosomal protein S1	367	P1, C88→T; P2, G70→T and T94→A; P4, G70→T and T94→A	None	>99.1
pheT	ABM55771	Phenylalanyl-tRNA synthetase beta chain catalytic core domain	332	P2, G101→T	None	≥99.7
pabA	EF529447	*para*-Aminobenzoate synthase glutamine amidotransferase	259	P5, A230→C	Ile→Leu (neutral/non-polar)	≥99.6
polC	EF529446	DNA polymerase III catalytic subunit	227	None	None	100
16S rRNA	U34688	16S rRNA processing protein	277	None	None	100

acid substitutions in three instances or in 21.4% of the sequences analyzed, and these substitutions are not likely to contribute to significant biochemical changes in protein function. Percent identity for a given gene sequence in the five populations was shown to range from >99.1 to 100%.

Immunogenic comparison of endospore envelope proteins. Labile endospore coat polypeptides of *P. penetrans* and *P. ramosa* in endospore coat extracts were resolved by SDS-PAGE and detected on immunoblots with MAb 2A41D10 raised to whole spores of *P. penetrans* P20, which provided an immunological comparison of the two species of *Pasteuria* (Fig. 4). The blot demonstrates that there is a universally conserved epitope that is shared by *P. penetrans* and *P. ramosa* endospores. The antigenic ladder revealed distribution patterns that were significantly different in the two species. The phytopathogenic nematode parasite contained a more diverse molecular weight array of antigenically reactive peptides than the cladoceran parasite, with some peptides that overlapped and some peptides that were distinct in the two species.

DISCUSSION

This study revealed that *P. penetrans* and *P. ramosa*, which are obligate parasites that are associated with hosts belonging to two distant phyla, the Nematoda and the Arthropoda, are indeed closely related both genetically and immunogenically.

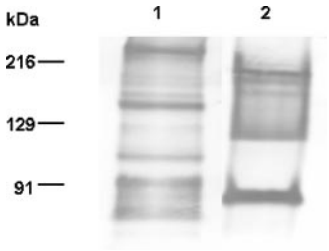

FIG. 4. SDS-PAGE Western blot showing the results of an immunological comparison of *P. penetrans* P20 and *P. ramosa* P1 using MAb 2A41D10. Lane 1, *P. penetrans* P20 endospore coat extract; lane 2, *P. ramosa* P1 endospore coat extract.

The process of sporulation is tightly regulated in the endospore-forming bacilli (30), and the sigma factor genes principally regulating this pathway (17, 21) are highly conserved between *Clostridium* spp. and *Bacillus* spp. (3). Phylogenetic analysis of *P. penetrans* and *P. ramosa* based on the sporulation factors *sigE* and *spoIIAB* showed that these organisms are both members of a distinct clade within the gram-positive endospore-forming bacilli, supporting previous findings for *spo0A* (31). Sporulation genes are desirable targets for detection and biotyping or ecotyping of *Pasteuria* spp. based on their level of conservation and the fact that there is a greater degree of nucleotide sequence variability between and within *Pasteuria* populations in these genes than in the 16S rRNA gene. To investigate these genes as targets for detection, a specific probe for *sigE* was designed and used to detect *P. ramosa* by in situ hybridization. The fluorescein-conjugated gene probe was shown to specifically recognize the *sigE* sequence in *P. ramosa* in vegetative cells, thus providing qualitative evidence that the sequence obtained indeed originated from the target organism and was not derived from other host- or culture-associated microparasites or epibionts (29).

The ability to assign a phenotype based on host preference with a particular genotype is a critical factor in further understanding the influence of *Pasteuria* spp. on soil and water ecology. Nucleic acid sequence alignment analysis of the *spoIIAA*/*spoIIAB* gene cluster with only 69.7% identity, along with the presence of SNPs, suggested that this genomic region is a good candidate for genetic isotyping of *Pasteuria* isolates. Different restriction digestion fragments of the partial coding sequences of the *P. penetrans* and *P. ramosa spoIIAA*/*spoIIAB* amplicons illustrated this capability. A comparison of partial coding sequences of nine housekeeping genes from five distinct populations of *P. ramosa* infecting *D. magna* showed the presence of SNPs in five of the nine housekeeping genes investigated, indicating the existence of genetic ecotypes within a species infecting a common host. These data support previous findings

showing that there are SNPs in *spoIIAB* in two biotypes of *P. penetrans* infecting different *Meloidogyne* sp. hosts (23). Variations in the nucleotide sequence resulting in silent SNPs and conservative amino acid substitutions observed in isolated populations of *P. ramosa* may reflect a correlation with host preference predicated by more substantive variations in genes associated with virulence. Comparisons of sequences of sporulation genes from additional isolates of *Pasteuria* spp. obtained from members of the Nematoda and Arthropoda could provide an opportunity to further evaluate these genes as candidates for genetic markers to distinguish *Pasteuria* ecotypes and biotypes.

Of considerable interest was the finding that a conserved epitope was present on the endospore envelope of two ecologically divergent *Pasteuria* spp. The antigenic ladders shown in an SDS-PAGE immunoblot, while distinctly different for the two species, provided evidence that a universally conserved chemistry is associated with endospore surface or coat proteins. The MAb used to probe the immunogenic profile of the endospore envelope was previously shown to be specific for *Pasteuria* based on challenges with a number of soilborne gram-positive endospore-forming bacteria (27) and was also shown to detect epitopes on a variety of *Pasteuria* isolates obtained from other phytopathogenic nematodes (24). The antigenic ladders have been shown to differ even at the biotype level for all isolates examined so far when comparisons between isolates from different hosts and host strains have been made. These results demonstrate that *Pasteuria* spp. harbor a unique spore envelope epitope, presented late in sporulation (7), that is universal and distinct from the epitopes of other closely related organisms. Because *Pasteuria*, like most pathogenic bacteria, must first recognize and bind to a host ligand as a prelude to infection, the common endospore epitope found here may well represent a virulence determinant critical to the infection process for both species that warrants further evaluation.

The evolution of the obligate host-parasite relationships of *P. ramosa* with *Daphnia* spp. and of *P. penetrans* with *Meloidogyne* spp. suggests that there are common developmental requirements in both the cladoceran and nematode hosts. All *Pasteuria* spp. suppress or eliminate the fecundity of their hosts (16). Both the cladoceran and nematode hosts reproduce by cyclic parthenogenesis (i.e., alteration of asexual and sexual reproduction) and produce eggs having a chitinous shell. Based on lectin blotting with wheat germ agglutinin, the epitope recognized by MAb 2A41D10 contains β-1,4-linked *N*-acetyl-D-glucosamine residues (10, 24). It may be that a common glycochemistry and the clonal population dynamics of the host population contribute to the interaction between the host and the parasite and thereby confer virulence to a *Pasteuria* sp. or biotype for a particular host.

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