RESEARCH ARTICLE

Pasteuria endospores from *Heterodera cajani* (Nematoda: Heteroderidae) exhibit inverted attachment and altered germination in cross-infection studies with *Globodera pallida* (Nematoda: Heteroderidae)

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Abstract

The *Pasteuria* group of Gram-positive, endospore-forming bacteria are parasites of invertebrates and exhibit differences in host specificity. We describe a cross-infection study between an isolate of *Pasteuria* from pigeon pea cyst nematode, *Heterodera cajani*, which also infects the potato cyst nematode, *Globodera pallida*, from the United Kingdom. A proportion of the attached endospores, 13% on *H. cajani* and 22% on *G. pallida* adhere to the cuticle in an inverted orientation. Inverted and conventionally attached endospores germinated and produced bacillus-like rods that completed their life cycle in < 15 weeks within females of *G. pallida*. This is the first example in which the life cycle of a *Pasteuria* population was systematically followed in two different nematode genera. A 1430-base pair fragment of the 16S rRNA gene sequence of the *Pasteuria* isolate from *H. cajani* revealed 98.6% similarity to the orthologous gene in *Pasteuria nishizawae*. Additionally, their respective endospore sizes were not significantly different, in contrast their host ranges are. Potential reasons for this remain unclear and are discussed.

Introduction

The *Pasteuria* group of bacteria are Gram-positive, endospore-forming parasites of a wide range of invertebrate hosts. Originally observed as parasites of Cladocera (Water flea, *Daphnia* spp.) by Metchnikoff (1888), they are recently used as a model system to study coevolutionary trade-offs between hosts and parasites (Ebert *et al.*, 1996; Vale & Little, 2009; Little *et al.*, 2008; Decaestecker *et al.*, 2007). *Pasteuria* spp. are also of interest to nematologists because they have the potential to suppress plant-parasitic nematode populations (Davies *et al.*, 1990; Oostendorp *et al.*, 1991; Trudgill *et al.*, 2000) and research has focused on their development as biological control agents as an alternative to nematicides (Stirling, 1984; Davies *et al.*, 1991). To date, six of seven species of *Pasteuria* are parasites of nematodes namely *Pasteuria penetrans* (ex Thorne, 1940), on *Meloidogyne* spp. (Sayre & Starr, 1985); *Pasteuria thornei*, on *Pratylenchus brachyurus* (Sayre & Starr, 1985); *Pasteuria nishizawae*, on cyst nematodes of genera *Heterodera* (Noel *et al.*, 2005); '*Candidatus P. usage*', on *Belonolaimus longicaudatus* (Giblin-Davis *et al.*, 2003); *Pasteuria hartismeri* on *Meloidogyne ardenensis* (Bishop *et al.*, 2007); and '*Candidatus Pasteuria aldrichii*' sp. nov., on the bacterivorous nematodes of *Bursilla* sp. (Giblin-Davis *et al.*, 2010). Additionally, *Pasteuria* found to be parasites of free-living nematodes belonging to the Plectidae have been identified but as yet have not been assigned as a new species (Sturhan *et al.*, 2005).

Bacterial whole-genome sequencing and advances in comparative genomics have enhanced the resolution by which groups of bacteria can be defined, and in turn this has led to controversy surrounding the bacterial species concept, which typically has been based on the 16S rRNA gene (Achtman & Wagner, 2008). It is well known that host–parasite interactions are often highly specific, and this is also the case for *Pasteuria* species and their interactions with invertebrate hosts (Little *et al.*, 2007; Little *et al.*, 2008; Davies *et al.*, 2008; Davies, 2009) as outlined previously. Two sympatric populations of *Pasteuria*

infecting *Heterodera cajani* and *Meloidogyne javanica* have been found to adhere to different host nematodes from different geographical regions (Sharma & Davies, 1996), but whether or not they completed their life cycles was not investigated. The *Pasteuria* infection process involves a number of distinct stages, initiated by endospore attachment to the host, which is followed by germination, proliferation of vegetative material through exponential growth and culminates with sporogenesis (Davies *et al.*, 2011). Interruption of any of these stages prevents *Pasteuria* from completing its life cycle and limits the host range of a given species of *Pasteuria*. As yet the reasons for such stringent host specificity are poorly understood.

Recent work with *Pasteuria ramosa* infecting *Daphnia* has shown that there is host-type-independent spore activation whereby the endospore sheds its exosporium to reveal peripheral fibres that are involved in attachment to the *Daphnia* oesophageal wall, which was highly host dependent exhibiting extreme genotype–genotype interactions (Duneau *et al.*, 2011; Luijckx *et al.*, 2011). Earlier studies using monoclonal antibodies raised to the related bacteria *P. penetrans* have that shown attachment of the endospore to the cuticle of root-knot nematodes, *Meloidogyne* spp., is a determinant of host range (Davies *et al.*, 1994, 2008); host range is therefore likely to be an important criterion in the characterization of *Pasteuria* species, coupled with phenotypic traits such as spore size and genetic information based on the 16S rRNA gene. In this manuscript, we describe a *Pasteuria* from the pigeon pea cyst nematode, *H. cajani*, which also parasitizes the geographically distinct and economically important potato cyst nematode *Globodera pallida* from the United Kingdom.

Materials and methods

Pasteuria endospores

The *Pasteuria* population isolated from the pigeon pea cyst nematode, *H. cajani* (HcP – *Heterodera cajani Pasteuria*), was routinely cultured (Davies *et al.*, 1988) in a temperature-controlled green house at 28 °C on *H. cajani*-infected cowpea, *Vigna unguiculata* cv Pusa Komal. Plants were grown in Rothamsted nematode compost (80% sterilized loam, 15% sand, 5% grit).

Endospore attachment assay

A 500-μl suspension of HcP spores (2×10^3 mL^{-1}) was separately mixed with 100 freshly hatched J2s from *G. pallida* or *H. cajani* and centrifuged at 6000 ***g*** for 3 min (Hewlett & Dickson, 1993). After 2 h, 25 J2s were randomly selected from each sample and observed using a microscope to ascertain the degree of spore attachment. The remainder of each sample was incubated at 15 °C (*G. pallida*) and 28 °C (*H. cajani*), for 12 days, and *Pasteuria* infection accessed. The differences in incubation temperature reflect the different soil temperatures during the growing season from where the nematodes were originally isolated.

Life cycle studies

Freshly hatched J2 (~ 200 per 10 cm pot) of *G. pallida* encumbered with 5–10 HcP endospores per second-stage juveniles (J2) were added around the root systems of 7-day-old seedlings of potato cultivar *Maris piper* grown in Rothamsted compost as described previously. Plants were destructively sampled after 5, 7, 10 or 15 weeks. This involved removing plants from pots and submerging each root system in water with gentle agitation to release nematode females from roots. Females were separated from the soil using 850-mm- and 250-mm-diameter pore sieves, females individually hand-picked under a stereoscopic binocular microscope and transferred to a cavity block containing tap water. Nematodes were washed several times in sterile water to remove adhering debris, developmental stages of *Pasteuria* were observed in individually squashed females using a light microscope (Olympus BH2; ×400).

Scanning electron microscopy (SEM) studies

A JEOL (UK) JSM 6700 FEG scanning electron microscope fitted with a GATAN (UK) Alto 2500 Cryo unit was used. The instrument was prepared by cooling with liquid nitrogen, and the temperature in both the preparation and microscope chambers was maintained at −160 °C throughout sample examination. Nematodes were pipetted onto 5 × 5-mm sections of Whatman™ filter paper attached to a cryo stub with OCT mountant (Sakura Finetek, Europe NL) and frozen by plunging into preslushed liquid nitrogen. The sample was transferred under vacuum to the GATAN cryo chamber stage and etched to remove contaminating ice by increasing the temperature to −95 °C for 1 min. Once the temperature of the stage had returned to −160 °C, the sample was coated with Au/Pd alloy for 1 min and transferred to the SEM chamber and mounted on the microscope stage for examination at −160 °C. Images were recorded using the JEOL on board system and software.

DNA from endospores

As previously described by Mauchline *et al.* (2010), up to 10^5 endospores were pelleted by centrifugation at 10 392 ***g***

for 5 min. Endospores were then resuspended in 20 μL microLYSIS®-PLUS (Microzone Ltd, Haywards Heath UK) and subjected to the following temperature conditions in a thermal cycler: 65 °C 15 min; 96 °C 2 min; 65 °C 4 min; 96 °C 1 min; 65 °C 1 min; and 96 °C for 30 s. Next, one volume of microCLEAN® (Microzone Ltd) solution was added to each sample, mixed and incubated at room temperature for 5 min. Samples were spun at 10 392 ***g*** for 7 min, and the supernatant was then removed before final resuspension in 20 μL water.

Multiple strand DNA amplification (MSDA)

Amplification was performed as described by Mauchline *et al.* (2010) using illustra Genome Phi V2 amplification kit (GE Healthcare, Little Chalfont, UK) was used on genomic DNA template as per manufacturer's instructions; 2 μL of template DNA was mixed with 9 μL sample buffer to ensure the required 10 ng of template DNA was added into the PCR reaction mixture. The isothermal amplification step was extended from 2 to 3 h. A 1-μL aliquot of each reaction was subjected to 1% gel electrophoresis with ethidium bromide staining (0.5 μg mL^{-1}) to ascertain whether genomic amplification had been successful.

Primer design

Pasteuria-specific primers for the 16S rRNA gene were designed using alignments of DNA with sequences from known *Pasteuria* spp. and those of close relatives including *Paenibacillus*, *Thermoactinoymces* and *Bacillus* spp. with the AlignX program in the Vector NTI suite. Regions of high sequence diversity were targeted for primer design.

PCR

Pasteuria DNA from MSDA was used as template for PCR with various combinations of the primers listed in Table 1. The PCR mixtures contained 1 μL of template (diluted up to 20-fold in water), primers at a concentration of 0.1 μmol^{-1}, in PCR buffer (1.5 $mmol^{-1}$ Mg^{2+}; Bioline, London, UK), 1 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas Life Sciences, Burlington, ON, Canada) and 1 unit of Biotaq polymerase (Bioline). PCR thermo-cycling conditions were 95 °C for 1 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 5 min. Finally, 5–10 μL of each PCR sample was subjected to electrophoresis and ethidium bromide staining as described previously.

Table 1. 16S rRNA primer sequences used for the studies of relatedness between different isolates used in this study

Primer name	Sequence	Source
39F	GCGGCGTGCCTAATACA	Atibalentja *et al.* (2000)
Pspp16Sr1	TCGGCACAAAGATGCTGAGCA	This work
Pspp16Sf9	AGGGATGCTCAGCATCTTTG	This work
Pspp16Sr5	GCGAGCCCYACCTTCGGCRG	This work
T3	AATTAACCCTCACTAAAGGG	This work
T7	TAATACGACTCACTATAGGG	This work

Cloning and sequencing

PCR products were ligated into the pSC-A-amp/kan cloning vector, and the ligation products were cloned into StrataClone recombinant cells, as described by the manufacturer's instructions (Stratagene, La Jolla, CA). A proportion of colonies arising from transformations were screened by using a small sample of the colony as template for a PCR, along with T3 (AATTAACCCTCACTAAAGGG) and T7 (TAATACGACTCACTATA GGG) primers that anneal to sites flanking the insertion site. Clones were grown in Luria Bertani broth overnight at 37 °C in a shaking incubator with Ampicillin antibiotic selection. Plasmid DNA from cultures was extracted using the GeneJet tm Plasmid Miniprep kit (Fermentas). Sequencing of plasmid inserts was performed by Eurofins MWG/Operon (Ebersberg, Germany) using either the T3 or T7 primer. Sequences were then assembled using the contig Express program in the Vector Nti suite, and these were subjected to BLASTN analysis using default settings (Altschul *et al.*, 1990).

Phylogenetic tree construction

A maximum likelihood tree was constructed from 16S rRNA sequences aligned in MUSCLE (Edgar, 2004), and a transition/transversion ratio estimated as 1.7 from PUZZLE (Strimmer & von Haeseler, 1996) using DNAML (Felsenstein, 2004) with 1000 samplings providing bootstrap values.

Results

Endospore attachment and germination

The HcP endospores attached randomly to the entire body length of the juveniles. No significant difference in the number of spores adhering to the cuticle was observed between the original host *H. cajani* 7.3 ± 3.4 spores $J2^{-1}$ and *G. pallida* 6.7 ± 2.8 spores $J2^{-1}$ (ANOVA, $F = 0.46$; $P = 0.50$). Additionally, the average diameter of attached HcP spores attaching to *H. cajani* and *G. pallida*

was not significantly different, 5.04 ± 0.5 and 5.52 ± 0.4 μm, respectively (ANOVA, $F = 1.45$; $P = 0.27$; Fig. 1a and b). The majority of spores attached in the conventional manner, where parasporal fibres form a skirt-like structure around the central body, and the spore is orientated so the concave surface is in contact with the nematode cuticle (Fig. 2a and b). Some of these spores appear to have collapsed, and the central body no longer present (Fig. 3a). Endospores attached to the nematode cuticle by their convex surface (inverted; Figs 2c,d and 3b). Inverted endospores with holes at the position of the central body were originally seen at 6 days following spore attachment and again at 12 days, when their numbers recorded. The number of inverted endospores was significantly different between the two species of nematode (*t*-test; $P \leq 0.004$); 22 (21.9%) inverted spores were observed on *G. pallida*, compared with 7 (13.0%) inverted spores recorded on *H. cajani*. SEM micrographs showed that many of the inverted endospores contained a central hole. It was not possible to quantify those with a central hole because of the difficulty in seeing the hole because it was obscured from view. Observations using light microscopy showed evidence of germ tubes penetrating the nematode body from both 'cup-shaped' and inverted endospores.

Pasteuria development within *G. pallida*

All females were recovered from the root system at 5 weeks after inoculation of plants and were creamy white. They were kept at room temperature in water and observed daily. *Pasteuria*-infected cysts remained creamy white, while healthy females containing eggs tanned 10–15 days later. Females remained the same size after removal from root systems; infected females appeared slightly smaller than healthy females. Dissection of 5-week-old infected females revealed the presence of typical bacillus-like rods (Fig. 4a1, a2, b1 and b2). The rods were aggregated in large numbers, forming granular masses and chains of thalli. No rods, granular masses or chains of thalli were found in healthy females. Sporulation of the bacteria was evident in 7-week-old nematodes. The presence of clusters of sporangia, growth stages quartets, triplets and doublets at the same time point showed asynchronous development towards sporulation (Fig. 5). After 10 and 15 weeks of incubation, the creamy white females that had not begun to tan were collected and mature endospores were released demonstrating the completion of the HcP life cycle in *G. pallida* (Fig. 5). No eggs were observed in *Pasteuria*-infected females. After 15 weeks from inoculation *Pasteuria*, infected females remained creamy white and failed to tan indicating that no eggs were formed (Fig. 5).

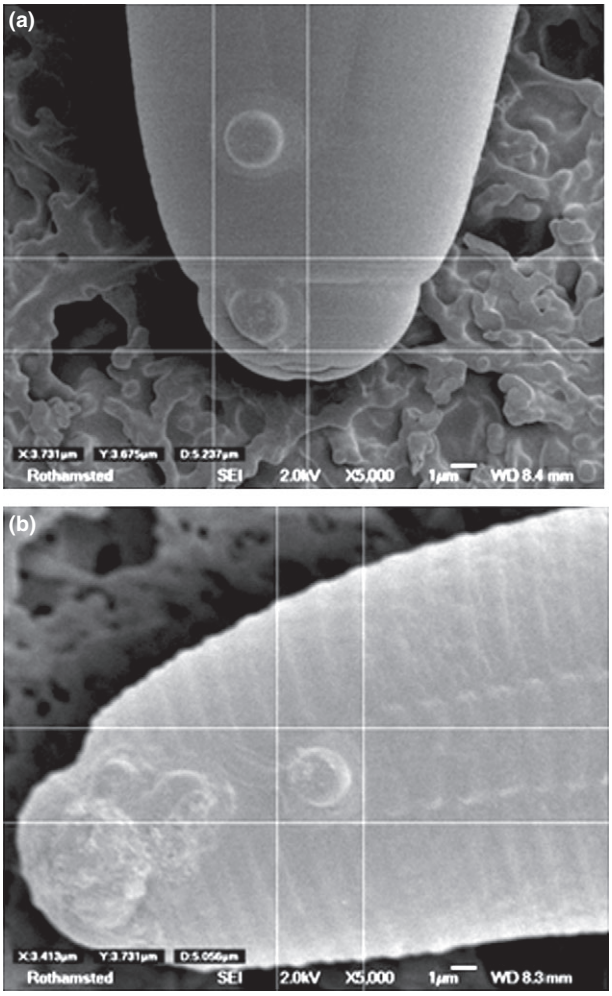

Fig. 1. Scanning electron micrographs of the anterior region of *Heterodera cajani* (a) and *Globodera pallida* (b) with endospores of *Pasteuria* from an Indian isolate of *H. cajani*; lines for estimation of the diameter (5.28 μm) of the endospores.

16S rRNA gene analysis

An almost complete fragment of the HcP 16S rRNA gene was amplified by PCR using primers 39F and Pspp16Sr1 as well as Pspp16Sf9 and Pspp16Sr5. The first primer pair yielded a product of 794 bp and the second primer pair, a product of 662 bp. These PCR products were cloned and sequenced, and these data were then used to construct a 1430-bp contig. BLASTN analysis of this fragment showed it to be most closely related to *P. nishizawae* with 98.6% identity. Additionally, a maximum likelihood phylogenetic tree revealed that HcP and *P. nishizawae* group together and separately from *P. penetrans* populations, which are also more similar to each other (Fig. 6). As such, it is not clear whether *P. nishizawae* and HcP belong to the same species or are different biotypes of the same species.

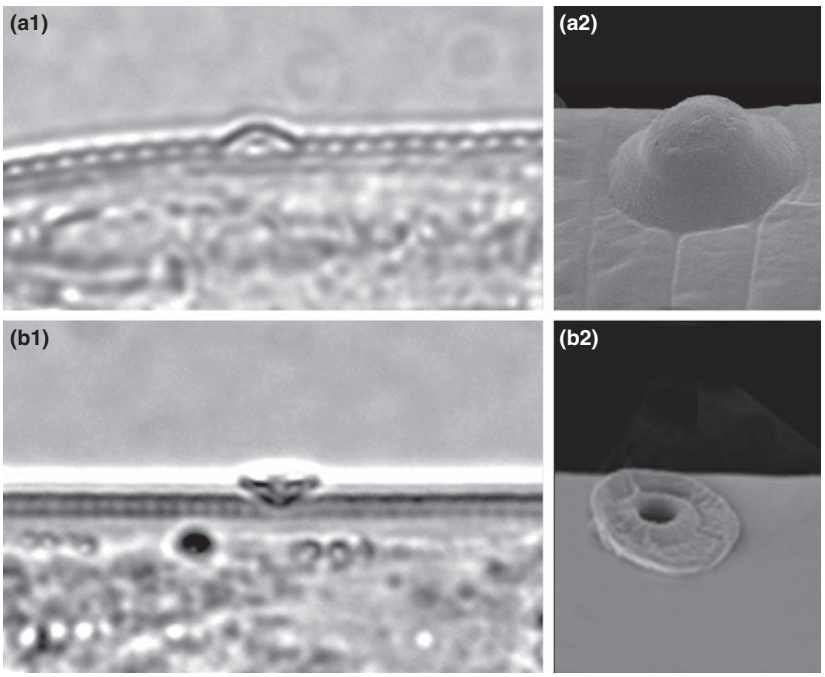

Fig. 2. Light micrograph (a1) and scanning electron micrograph (a2) of cup-shaped endospore of *Pasteuria* from *Heterodera cajani* attached in the usual orientation, concave face down, to the cuticle of *Globodera pallida* (endospore diameter; 5.28 μm) and similarly, a brightfield micrograph (b1) and scanning electron micrograph (b2) of an inverted endospore revealing a hole in the position of the central body.

Discussion

The population of *Pasteuria* described in this article was originally isolated from the pigeon pea cyst nematode, *H. cajani*, in India. We report here that it is capable of attaching to second-stage juveniles of, and completing its life cycle in a population of the potato cyst nematode, *G. pallida*. Although isolates of *Pasteuria* have previously been shown to demonstrate attachment to second-stage juveniles of different nematode genera, there was no reported evidence of further infection in the females of these nematodes (Davies *et al.*, 1990; Atibalentja *et al.*, 2004; Noel *et al.*, 2005). The host range of *P. nishizawae* is however confusing, the first description of *P. nishizawae* suggested that it parasitized both *Heterodera* and *Globodera* (Sayre *et al.*, 1991), but this has since been revised stating that although *P. nishizawae* attached to second-stage juvenile cuticle, it did not completed its life cycle in any other species of nematode except *Heterodera glycines* (Noel *et al.*, 2005). A quantitative infection test between *P. ramosa* and *Daphnia magna* showed that nongenetic host heterogeneity played an important role (Ben-Ami *et al.*, 2008), but more recent evidence using clonal lineages suggests that there are extreme genotype–genotype interactions (Luijckx *et al.*, 2011). This confirms earlier work where the attachment of *P. penetrans* endospores to clonal lines of *Meloidogyne hapla* appeared to segregate (Davies *et al.*, 2008), suggesting the cuticle receptor/s to which endospores were binding was under genetic control possibly involving mucins (Davies, 2009; Davies & Curtis, 2011). Earlier studies within genera showed no relationship between endospore attachment and the phylogeny of the species of *Meloidogyne* tested (Davies *et al.*, 2001). The investigations reported here are therefore in line with Sayre *et al.* (1991) and will be discussed further later.

Overall, the number of spores attaching to *H. cajani* and *G. pallida* were not significantly different, but there were considerably more inverted endospores on *G. pallida* than on *H. cajani*. This suggests that the receptor(s) involved in attachment to the cuticle of the two species of nematode are different. Collagen-like fibres that produce a nap-like structure as part of the surface of the endospore have been implicated in the attachment process, and there is a greater density of these collagen-like fibres on the concave surface of the endospore than on the convex surface (Davies, 2009). The difference in the orientation of the attached endospores may suggest several possibilities: (1) a difference in the density and or spatial distribution of the collagen-like fibres, (2) that there are two or more possible molecular receptor-binding mechanisms involved or (3) a combination of both 1 and 2.

All stages of the HcP life cycle were completed in *G. pallida* nematodes, including the vegetative rods that

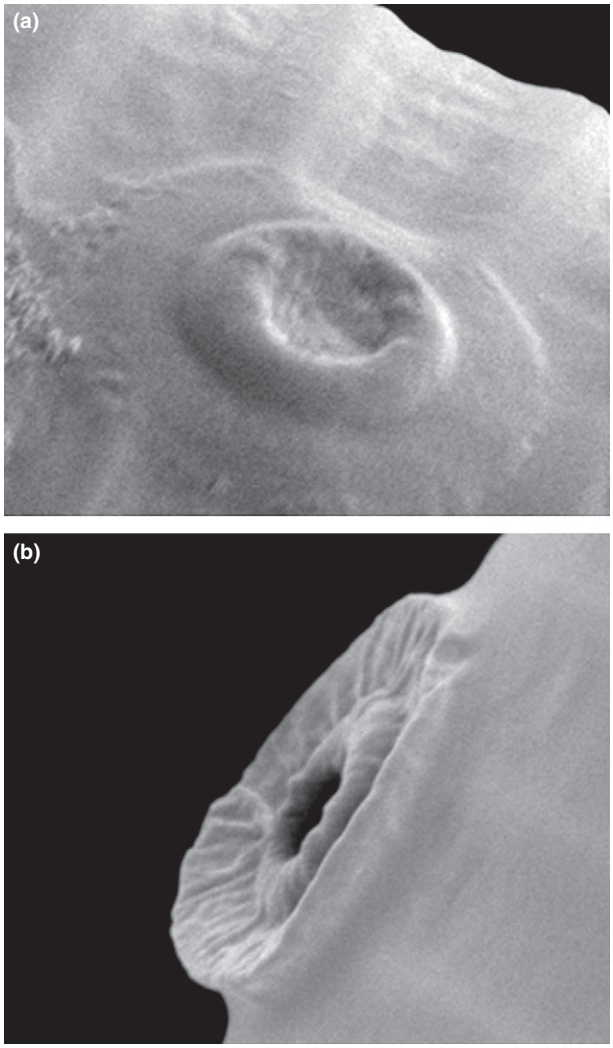

Fig. 3. Electron micrographs of endospores of *Pasteuria* from *Heterodera cajani* adhering to *Globodera pallida* in usual orientation; collapsed area in the position of the central body (a) and an inverted endospore (b) 12 days after attachment.

have been reported in *P. penetrans* infecting root-knot nematodes (Davies *et al.*, 2011) as they developed on potato roots. Whether or not endospores that germinated in second-stage juveniles would have completed their life cycle in that life stage had they not invaded the root system and developed into mature nematodes was not ascertained. However, it is likely that if they did infect the second-stage juvenile, they would have produced far fewer endospores because of fewer available resources. This describes a situation similar to the situation in *Heterodera avenae* infected by *Pasteuria*, which led to the production of < 1000 endospores in the second-stage juvenile (Davies *et al.*, 1990). In comparison with *P. penetrans*, which infect *Meloidogyne* spp., germination usually occurs only when the nematode has set up a feeding site and leads to the production of over 10^6 spores per individual female (Davies *et al.*, 1988).

It is interesting that the number of inverted endospores germinating on juveniles of the original host, *H. cajani*, were statistically significantly less, 13%, as opposed to 22% on second-stage juveniles of *G. pallida*. This begs the question as to why the geographically distant host, *G. pallida*, which presumably has not had time to coevolve with the parasite, has a higher level of germination than the presumably more coevolved interaction of this *Pasteuria* with *H. cajani*. It might be speculated that the signalling processes involved in triggering germination, as yet unidentified, have not yet optimally coevolved. Similarly, the life cycles of the *Pasteuria* on the different nematodes were conducted at different temperatures, reflecting the different optimal temperature environments for the different nematodes. As the different nematode species have different optimal temperatures, it is likely that there is a complex temperature-dependent interaction in the progression of *Pastueria* infection, although this was not tested.

The HcP 16S rRNA gene fragment was found to be most closely related to *P. nishizawae* (with 98.6% similarity), and further investigation is needed to establish if they belong to the same species. Endospores of *P. nishizawae* have been shown to attach to *H. glycines* (soya bean cyst nematode), *Globodera rostochiensis* (potato cyst nematode), *Heterodera lespedezaei* (Lespedezae cyst nematode), *Heterodera schachtii* (sugar beet cyst nematode) and *Heterodera trifolii* (clover cyst nematode). However, neither infection nor the completion of the life cycle was observed in any nematode other than *H. glycines* (Sayre *et al.*, 1991; Atibalentja *et al.*, 2004; Noel *et al.*, 2005). In this study, we have shown that HcP is able to complete its life cycle in *H. cajani* as well as *G. pallida*, the latter being a very close relative to *G. rostochiensis*. We report the average endospore diameter of HcP spores as 5.28 μm, which is not significantly different from the endospore diameter described for *P. nishizawae* (Atibalentja *et al.*, 2004). However, as described previously, HcP has a very different biology in regard to its host range, as well as demonstrating inverted attachment of endospores, a unique feature in *Pasteuria* biology.

Several publications describing new species of *Pasteuria* have been based on the host nematode from which the *Pasteuria* was originally isolated, endospore encumbrance assays and 16S rRNA gene sequence (Giblin-Davis *et al.*, 2003; Atibalentja *et al.*, 2004; Bishop *et al.*, 2007). Other authors have been more circumspect and unwilling to ascribe new species status (Sturhan *et al.*, 2005). However, the use of the 16S rRNA gene as a phylogenetic marker has been criticized as not containing sufficient genetic resolution (Achtman & Wagner, 2008). Within

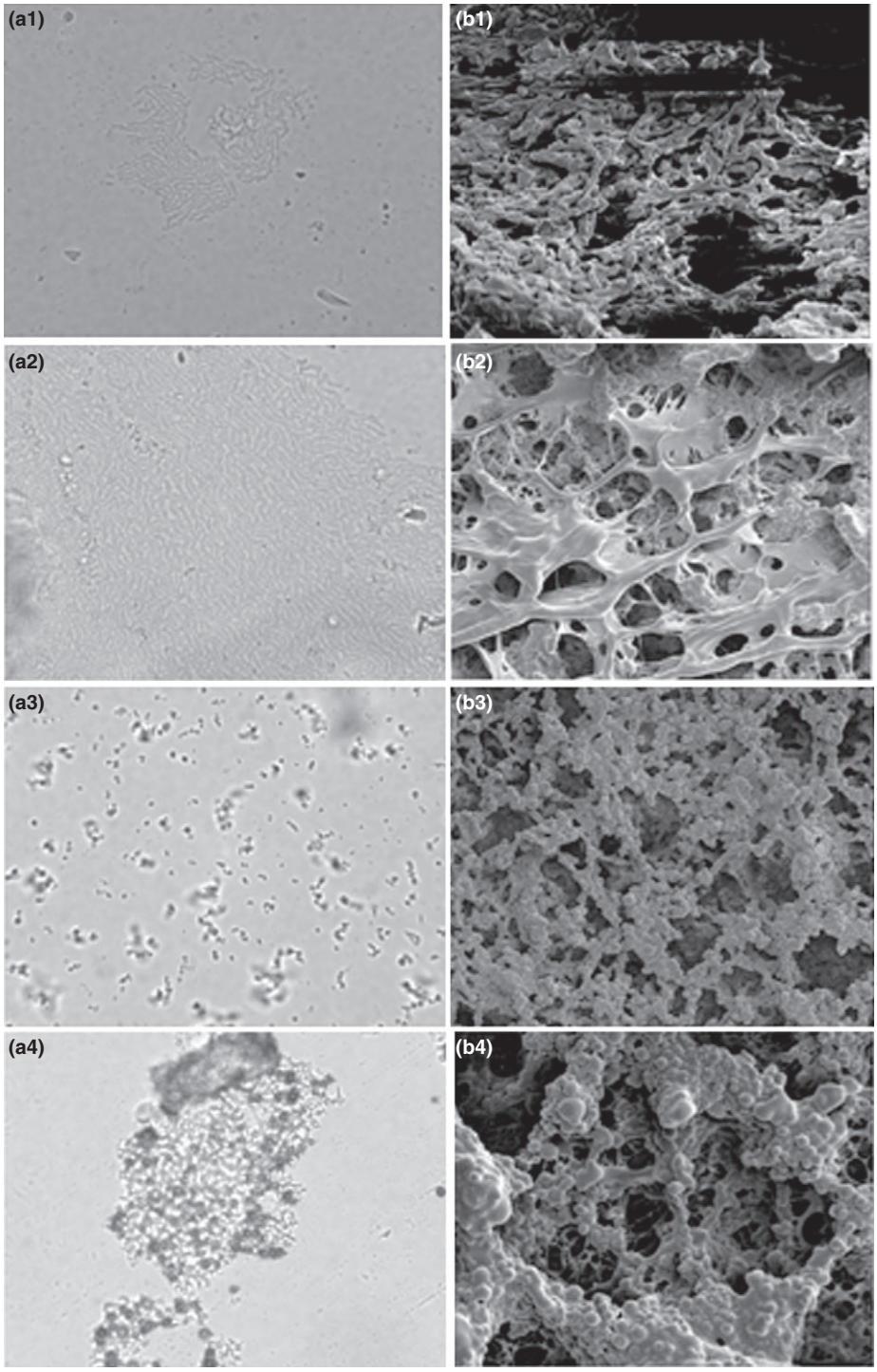

Fig. 4. Light micrographs (a1–4) and scanning electron micrographs (b1–4) of *Bacillus*-like rods (a1 and b1) forming chains (a2) and rhizoids (b2) at 5 weeks postinoculation; clusters of sporangia showing various growth stages (a3 and b3) forming granular masses of sporulating immature endospores (a4 and b4).

the *P. penetrans* group, protein-encoding genes involved in sporulation appear to have sufficient polymorphism to be used for species differentiation (Schmidt *et al.*, 2004; Nong *et al.*, 2007), although they appear to be insufficient to resolve populations at the intraspecies level (Mauchline *et al.*, 2011). In addition to these, genes encoding

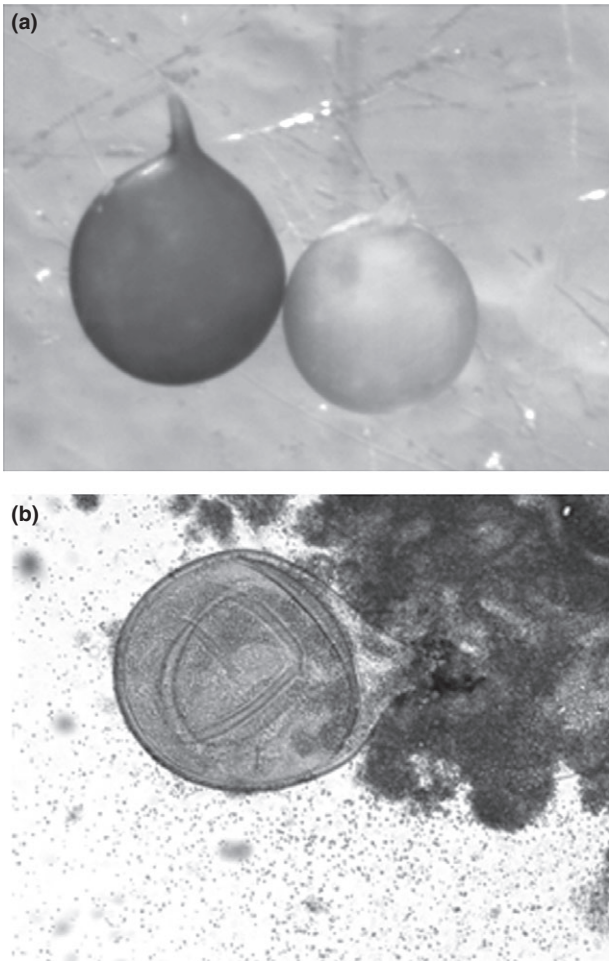

Fig. 5. Cyst visibly tanned (left) and infected female (right) untanned at 15 weeks (a); crushed untanned infected female with released endospores at 15 weeks (b).

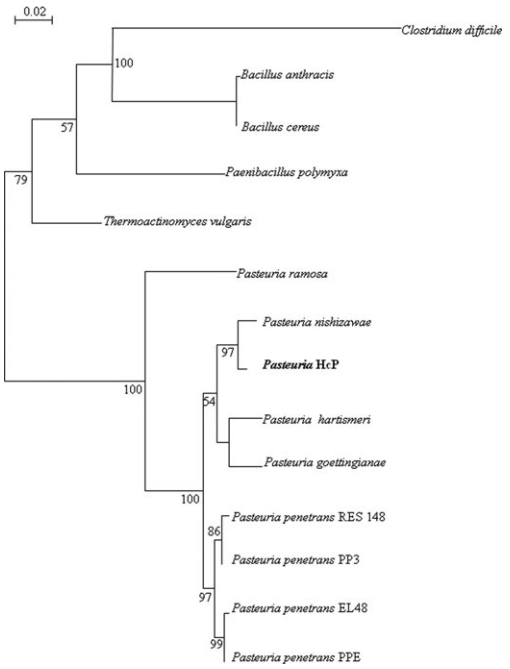

Fig. 6. Maximum likelihood tree based on 16S rRNA gene sequences, accession numbers within parentheses, showing relationship between HcP (JN592479), other *Pasteuria* spp. (*P. hartismeri*, HQ849363; *Pasteuria nishizawae*, AF134868; *Pasteuria penetrans* strains EL48, HQ849357; PP3, HQ849362; PPE, HQ849358; RES148, HQ849359; *P. goettingianae*, AF515699; *Pasteuria ramosa*, U34688) and other *Firmicutes* (*Bacillus anthracis*, AE017334; *Bacillus cereus*, JF705198; *Clostridium difficile*, FN668941; *Paenibacillus polymyxa*, CP000154; *Thermoactinomyces vulgaris*, EU430570). Bootstrap values > 50% are indicated; bar indicates 0.02 substitutions.

proteins such as heat shock proteins Hsp60 (GroEL) and Hsp70/DnaK, alanyl-t-RNA, succinyl-CoA synthetase, pyrophosphatase, Lon protease, biotin synthase, DNA gyrase B, UDP-glucose epimerase, PAC-transformylase, RecA and RNA polymerase subunit B are now also being considered for phylogenies (Gupta, 2000; Adekambi *et al.*, 2009). DNA–DNA reassociation remains a robust method for bacterial species delineation (Mulet *et al.*, 2010), but this technique is not appropriate for obligate and fastidious bacteria such as *Pasteuria* where obtaining sufficient pure genomic DNA is not practical. However, multilocus SNP based typing has been suggested as an alternative to DNA–DNA reassociation (Mulet *et al.*, 2010).

In addition to the housekeeping genes described previously, more specialized genes are sometimes used for phylogenetic analyses. For example, strains of *Bacillus cereus and Bacillus anthracis*, which are closely related to *Pasteuria*, demonstrate variability in the *bclA* gene that encodes a collagen-like fibrous nap that is present in the exosporium (Jackson *et al.*, 1997; Keim *et al.*, 2000; Sylvestre *et al.*, 2002, 2003; Henriques & Moran, 2007). Strains of *B. anthracis* have been shown to exhibit polymorphism in the collagen-like region of this gene, which is correlated with differing filament length in the exosporium (Sylvestre *et al.*, 2002, 2003). Although BclA is not required for *B. anthracis* pathogenesis, mutations in this gene alter the ability of endospores to adhere to epithelial, fibroblast and endothelial cells (Bozue *et al.*, 2007). Collagen-like sequences have also been observed in *P. penetrans* (Davies & Opperman, 2006) and *P. ramosa* (Mouton *et al.*, 2009), and recently, it has been suggested that they are important in a *Velcro*-like attachment process of endospores to the nematode cuticle (Davies, 2009). Indeed, the collagen-binding domain of fibronectin can inhibit endospore attachment to nematode cuticle, suggesting their importance in cuticle attachment (Mohan *et al.*, 2001). Polymorphisms in this gene could therefore be taxonomically useful in species and biotype differentiation, and

they may relate to the endospore attachment process that is important in the determination of host range. However, as important as the endospore attachment process may be, the work in this manuscript suggests that a combination of phenotypic characteristics with molecular phylogeny is likely to provide the most complete picture of *Pasteuria* taxonomic status.

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