



## The polypeptide components of the parasporal fibres of *Pasteuria penetrans*

Alka Vaid<sup>1</sup>, Alistair H. Bishop<sup>1,\*</sup> and Keith G. Davies<sup>2</sup>

<sup>1</sup>School of Chemical and Life Sciences, University of Greenwich, Wellington Street, Woolwich, London SE18 6PF, UK

<sup>2</sup>Department of Entomology and Nematology, IACR-Rothamsted, Harpenden, Hertfordshire, UK

\*Author for correspondence: Tel.: +44-20-83318427, Fax: +44-20-83318305, E-mail: a.h.bishop@gre.ac.uk

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### Summary

A variety of treatments were tested for their ability to solubilize the parasporal fibres from *Pasteuria penetrans*, a parasite of some plant–parasitic nematodes. Selective solubilization of the parasporal fibres resulted from some of the extraction procedures tested. Subsequent acrylamide gel electrophoresis and Western blotting of the resolved polypeptides, using polyclonal sera against the spores, disclosed up to 15 distinct bands, ranging in size from 12 to 195 kDa. An N-terminal amino acid sequence was obtained from a 50 kDa polypeptide and an oligonucleotide primer deduced from it. A whole cell, fluorescent, primed *in situ* labelling (PRINS) technique was adapted to be applicable to spores of *P. penetrans* and *P. ramosa*, a parasite of water fleas. Positive responses were obtained using the parasporal fibre primer on spores of the former but not of the latter organism, implying that this 50 kDa polypeptide is produced by *P. penetrans* but not by *P. ramosa*.

### Introduction

Plant–parasitic nematodes are estimated to cause worldwide, annual crop losses of US\$100 billion (Sasser & Freckman 1987). Of the many genera involved, *Meloidogyne* spp., also called root-knot nematodes, are responsible for the majority of the damage and attack all of the world's major food staples. In the tropics, for example, they account for an estimated 11–25% reduction in crop productivity (Sasser 1979); much higher losses or total crop failure may be suffered by subsistence farmers (Plowright & Bridge 1990). Current approaches to control plant–parasitic nematodes include chemicals, cultural practices and resistant cultivars. These are often used in an integrated manner. Chemical control is expensive and involves the use of generally toxic and environmentally damaging substances. Cultural practices are widely used, particularly in tropical countries, but provide varying protection. The resistant varieties which are available for a limited number of crops may also suffer drawbacks (Atkinson *et al.* 1995).

*Pasteuria penetrans* is a parasite of certain species of plant–parasitic nematodes (Sayre & Starr 1985), including *Meloidogyne* spp. Different isolates of this spore-forming bacterium infect different species of plant–parasitic nematode, either preferentially or exclusively (Gowen & Ahmed 1990; Mendoza de Gives *et al.* 1999). The spores can lie dormant in the soil, possibly for years (Giannaku *et al.* 1997). When they come into contact

with a susceptible nematode an instantaneous, tenacious and highly specific attachment of the spore to the nematode cuticle occurs (Chen & Dickson 1998). Upon germination, a germ tube penetrates the cuticle and body wall. Multiplication of the vegetative form of *P. penetrans* within the host nematode ensues. Sporulation finally occurs and, upon the death of the nematode, about a million spores per female are released into the soil to repeat the cycle (Sayre 1980). This critical attachment stage is mediated by a characteristic 'skirt' of fibres that surround the spore (Sayre & Wergin 1977). The mechanism(s) by which these fibres mediate attachment to the cuticle of nematodes is unclear. It has been suggested that amino sugars on the spores play a role (Persidis *et al.* 1991; Davies & Danks 1992) and that hydrophobic (Davies *et al.* 1994) and electrostatic forces may be involved (Afolabi *et al.* 1995). Due to the great economic damage caused by plant–parasitic nematodes and problems with current methods of control there is considerable interest in the potential use of *P. penetrans* as a biological control agent (Chen & Dickson 1998). This goal is currently unrealistic since *P. penetrans* cannot be grown *in vitro* (Williams *et al.* 1989; Bishop & Ellar 1991). Two major problems in unravelling this mystery are the difficulty in producing adequate supplies of spores and the heterogeneous nature of populations derived from *in vivo* culture (Davies *et al.* 1992). It is likely that a full understanding of the spore–cuticle interaction will only be obtained by cloning and

expressing the genes encoding the components of the parasporal fibres.

The only genetic analysis of members of the genus *Pasteuria* has concerned their taxonomy using 16S rRNA genes (Ebert *et al.* 1996; Anderson *et al.* 1999; Atibalentja *et al.* 2000). These studies indicated the taxonomical proximity of *P. penetrans* and *P. ramosa*, an obligate parasite of water fleas, to members of the genus *Bacillus*. Our aim was to further the molecular biological analysis of the attachment of the spore to nematode cuticle by resolving the polypeptide components of the parasporal fibres. By deriving an oligonucleotide primer, specific for one of the parasporal fibre components, the goal of cloning, analysing in detail and potentially exploiting such a polypeptide would be brought nearer.

## Materials and methods

### *Bacterial strains and growth conditions*

Spores of *P. penetrans* population PP1 were cultured as described by Davies & Danks (1992). Spores of *P. ramosa* (RM2-4) were obtained from Dr Dieter Ebert, Zoologisches Institute, Universität Basel, Switzerland and isolated from infected cultures of *Daphnia* spp., as described by Ebert *et al.* (1996). Contaminating material was removed from spores of both *Pasteuria* spp. by discontinuous density gradient centrifugation using sucrose solutions (Thomas & Ellar 1983) of 87, 82, 75 and 67% (w/v) or NaBr solutions (Ibarra & Federici 1986) of 56.3, 52.7, 48.9, 45.3, 41.9 and 38.5% (w/v). After centrifugation at  $20,000 \times g$  for 3 h at 10 °C the spore layer was removed by pipette. The sucrose or NaBr was removed by centrifuging the spores three times in a mini-centrifuge at  $11,600 \times g$  for 10 min in sterile distilled water at 5 °C.

*Alicyclobacillus acidocaldarius* was obtained from and cultured as recommended by NCIMB, Aberdeen, UK. Spores were harvested by scraping them from the surface of agar plates and washing them repeatedly in sterile, distilled water by centrifugation at  $17,520 \times g$  for 30 min. Spores of *Bacillus megaterium* (NCIMB 7581) and *Thermoactinomyces vulgaris* (NCIMB 11364) were produced as described by Vaid & Bishop (1998); spores of *B. thuringiensis* strain 13B were produced as described by Johnson *et al.* (1998). The spores of all species were washed as described above. The density of spores was determined with a haemocytometer before storing them at -20 °C. The spores of *P. penetrans* were sonicated, as described by Stirling *et al.* (1986) to remove the exo-sporium, before storing at -20 °C.

### *Solubilization of parasporal fibres*

Spores of *P. penetrans* ( $6.5 \times 10^5$ ) were suspended in 5  $\mu$ l of sterile distilled water before adding an equal volume

of gel sample buffer (GSB): 0.125 M Tris-HCl, pH 6.8; 4% (w/v) sodium dodecyl sulphate (SDS); 10% (w/v) glycerol; 0.02% (w/v) bromophenol blue; 4% (v/v) 2-mercaptoethanol (2-ME); 2 mM ethylenediamine tetraacetate (EDTA) and 2 mM phenylmethylsulphonyl fluoride). The samples were boiled for 3 min, followed by centrifugation ( $11,600 \times g$ , 2 min) prior to loading (total volume, 10  $\mu$ l) on a 10% SDS-polyacrylamide gel as described by Davies & Redden (1997). Similar aliquots of spore suspensions were also exposed to milder, sequential treatments in GSB: 5  $\mu$ l of GSB were added to the spore suspension at 20 °C for 4 h before mini-centrifugation ( $11,600 \times g$ , 15 min) and removal of the supernatants into fresh Eppendorf tubes. Pellets were re-suspended in fresh GSB (5  $\mu$ l) and sterile distilled water (5  $\mu$ l) before incubating the samples for 1 h at 37 °C. Once again the samples were centrifuged and the supernatant layers transferred into new tubes before adding fresh GSB (5  $\mu$ l) and water (5  $\mu$ l) to the pellet. The samples were then incubated at 60 °C for 10 min. This was repeated, each time centrifuging the sample, removing the supernatant layers and adding fresh GSB (5  $\mu$ l) and water (5  $\mu$ l) to the pellets. Fresh 2-ME (to a final concentration of 2%, v/v) was then added to all the extraction samples and the remaining spore pellet before preparing them for electrophoresis, as above. Extraction of the pellet in GSB was done to dissolve any unsolubilized proteins. The treated spores of *P. penetrans* were examined by light microscopy after each extraction step.

To discover the different effects of reducing agent and anionic detergent, normal GSB (5  $\mu$ l), and that from which either 2-ME (4% v/v) or SDS (4% w/v) had been omitted was added to one of three tubes, each containing  $6.5 \times 10^5$  spores in 5  $\mu$ l water. The suspensions were treated at 60 °C for 10 min. The supernatant layers were removed into fresh Eppendorf tubes by mini-centrifugation (5 min,  $11,600 \times g$ ) before adding water (5  $\mu$ l) and GSB (5  $\mu$ l) to the pellet in the same way (containing SDS, 2-ME or both). This was repeated two more times before adding fresh 2-ME to a final concentration of 2% (v/v) to all of the protein extraction tubes and the remaining pellets. The pellet was boiled in GSB for 3 min to dissolve any remaining unsolubilized proteins before resolution on a 10% polyacrylamide gel. To study further the nature of the detergent-mediated solubilization the above procedures were carried out using GSB which contained either SDS (0.2% w/v) or a cationic detergent, tetrapropylammonium hydrogen sulphate (0.2, 2 or 10%, w/v), in the absence of reducing agent.

To assess the effect of different pH values on solubility, two tubes of spores of *P. penetrans* ( $6.5 \times 10^5$ ) were mini-centrifuged for 15 min at  $11,600 \times g$ . The supernatant layers were removed and 5  $\mu$ l of  $K_2CO_3$ -HCl (50 mM), pH 9.5, were added to one tube and 5  $\mu$ l of 50 mM  $K_2CO_3$ -HCl, pH 11.5, were added to the other tube before incubation at 37 °C for

1 h. Once again the samples were mini-centrifuged (15 min,  $11,600 \times g$ ) and the supernatants removed. GSB (5  $\mu$ l) was added to all of the tubes, including the remaining spore pellet, before boiling for 3 min and resolution by SDS/PAGE.

#### *Polyacrylamide gel electrophoresis*

The method described by Davies & Redden (1997) was followed.

#### *N-terminal sequencing of 50 kDa polypeptide of the parasporal fibres of P. penetrans*

GSB (100  $\mu$ l) was added to the spores ( $2.26 \times 10^5$  in 100  $\mu$ l of water) before boiling for 3 min. The sample was then mini-centrifuged at  $11,600 \times g$  for 2 min before loading 25  $\mu$ l of the supernatant layer on a 10% SDS-PAGE gel. Electrophoresis was carried out as described above but using running buffer containing 2 mM thioglycollic acid to prevent N-terminal blocking. Western transfer was carried out as described by Davies & Redden (1997). N-terminal sequencing was carried out by Mr M. Weldon, Department of Biochemistry, University of Cambridge, UK.

#### *Immunological similarity between spores of P. penetrans and related bacteria*

Spores ( $5 \times 10^5$ ) of *B. megaterium*, *B. thuringiensis*, *T. vulgaris*, *A. acidocaldarius*, *P. penetrans* and *P. ramosa* were suspended in GSB (5  $\mu$ l) and 5  $\mu$ l of water added to the tubes. The samples were boiled for 3 min, centrifuged (2 min,  $11,600 \times g$ ) and the supernatants resolved by polyacrylamide electrophoresis and Western blotted with polyclonal antiserum against *P. penetrans* population PP1, as described by Davies & Redden (1997).

#### *Fluorescence and scanning electron microscopy*

Immunofluorescence and scanning electron microscopy were carried out on spores of *P. ramosa* and *P. penetrans* following the methodologies described by Davies & Redden (1997) and Vaid & Bishop (1998), respectively.

#### *Cycle primed in-situ labelling (PRINS)*

Spores of *P. penetrans* and *P. ramosa* and vegetative cells of *A. acidocaldarius* were treated by this DNA amplification technique, as described by Vaid & Bishop (2000), except that the permeabilization regime used for the spores was lysozyme (1 mg ml<sup>-1</sup>) and mutanolysin (750 units ml<sup>-1</sup>) in phosphate buffer, pH 7.5, for 30 min at 37 °C followed by 0.1 M HCl at 37 °C for 15 min. Vegetative cells of *A. acidocaldarius* were permeabilized by the enzymatic treatment alone. The amplification parameters were 35 cycles of 30 s at 98 °C, 30 s at 42 °C and 2 min at 76 °C using a primer (N-TER F1) 5'-GAR TGY TGY GGN GG-3'.

## Results

### *Solubility of parasporal fibres*

Thirteen polypeptide bands with molecular weights of approximately 195, 188, 126, 105, 86, 58, 50, 42, 27, 24, 18, 13 and 12 kDa were observed on SDS/PAGE after boiling spores of *P. penetrans* in GSB for 3 min (Table 1). The minimum number of spores of *P. penetrans* required for good visualization was about  $2.0 \times 10^5$ . The component in GSB responsible for solubilization of the fibres was shown to be SDS: in 2-ME alone only partial solubilization of the parasporal polypeptides occurred. Treatment of parasporal fibres at 60 °C for 10 min in the presence of SDS, with or without reducing agent, extracted 15 different parasporal fibre polypeptides, i.e. two further bands appeared. Microscopic analysis showed that the fibres had been completely stripped from the spores (Figure 1). This method led to the resolution of the greatest number of components of the parasporal fibres, whereas boiling resulted in the detection of 13 of these bands (all except the 90 and 53 kDa). The reason for this could be that the cruder technique of boiling produced contamination that masked the two missing polypeptides. Support for this explanation came from light microscopy where, although it was evident that all of the parasporal fibres had been removed from the boiled spores, some of the cores had become phase dark, implying that (some of) the spore coat layer or even core contents had been extracted.

It should be noted that during some solubilization treatments some of the components apparently disappeared. In other words, a particular treatment, e.g. extraction with 2-ME alone (Table 1), appeared not to solubilize particular polypeptides; when the residue was finally boiled in GSB (a treatment that should solubilize all components) these polypeptides still did not appear. It is likely that these polypeptides did solubilize but leached out at undetectable levels or were masked contaminating polypeptides when resolved.

Selective solubilization of some polypeptides could be achieved under certain circumstances (Table 1). For example, in the absence of a reducing environment at both pH 11.5 and pH 9.5 no solubilization took place. When 2-ME was added, however, a markedly greater number of polypeptides were resolved in the less alkaline buffer. Similarly, extraction of parasporal fibres in GSB at 20 °C for 4 h gave rise to 10 polypeptide bands after electrophoresis. Subsequent extraction at 37 °C for 1 h released more of some of these bands and also two ( $M_r$  of 105 and 86 kDa) which had not appeared after the previous treatment. The remaining polypeptides (126, 86, 50 and 42 kDa), of the 10 visible after boiling in GSB, appeared on the acrylamide gel after treatment twice in GSB at 60 °C for 10 min. These results agreed with those seen by light microscopy as decreasing amounts of parasporal fibres were observed with successive rounds of extraction, leaving spores devoid of parasporal fibres. Up to this stage the spores remained

Table 1. Extraction of components of the parasporal fibres under different conditions after resolution by SDS/PAGE.

Treatment	Extracted polypeptides of the parasporal fibres (kDa)														
	195	188	126	105	90	86	58	53	50	42	27	24	18	13	12
Boil 3 min in GSB	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+
20 °C, 4 h in GSB	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+
37 °C, 1 h in GSB	-	+	-	+	-	+	-	-	+	+	+	+	-	+	-
60 °C, 10 min in GSB	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-
60 °C, 20 min in GSB	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-
Boil final pellet, 3 min	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SDS only, 60 °C, 10 min	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SDS only, 60 °C, 20 min	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Boil final pellet, 3 min	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-ME only, 60 °C, 10 min	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-
2-ME only, 60 °C, 20 min	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-
2-ME only, 60 °C, 30 min	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Boil final pellet, 3 min	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SDS and 2-ME, 60 °C, 10 min	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SDS and 2-ME, 60 °C, 20 min	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
Boil final pellet, 3 min	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50 mM K <sub>2</sub> CO <sub>3</sub> , pH 9.5, 1 h, 37 °C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50 mM K <sub>2</sub> CO <sub>3</sub> , pH 9.5 with 2-ME 1 h, 37 °C	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+
Boil final pellet, 3 min	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50 mM K <sub>2</sub> CO <sub>3</sub> , pH 11.5, 1 h, 37 °C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50 mM K <sub>2</sub> CO <sub>3</sub> , pH 11.5 with 2-ME 1 h, 37 °C	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Boil final pellet 3 min	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+

GSB – gel sample buffer; + – strong band; – – no band.

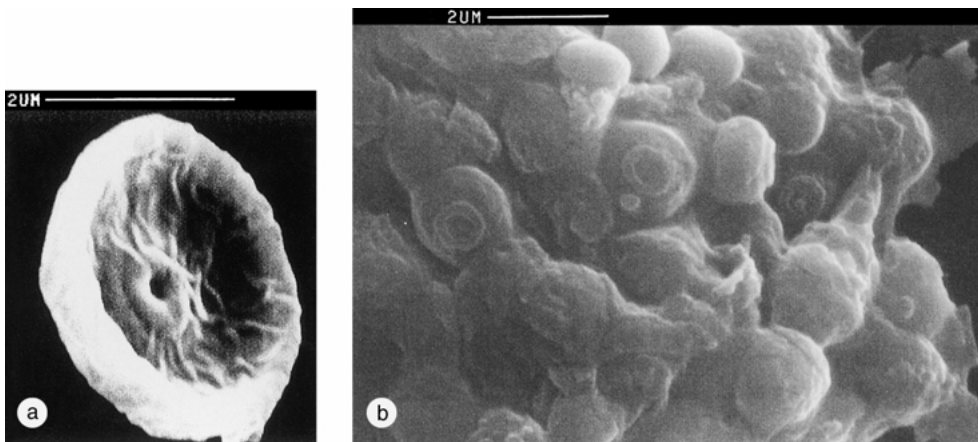


Figure 1. Electron micrographs of spores of *P. penetrans* (a) untreated (b) after treatment in SDS (2%, v/v) at 60 °C for 10 min.

phase bright. Final boiling of the remaining pellet resulted in the appearance of no further polypeptides. No discernible release of polypeptides, as shown by Coomassie Blue staining of acrylamide gels, and no visible damage to the parasporal fibres, as seen by light microscopy or SEM, was found using a 10-fold lower concentration of SDS (0.2% (w/v), final concentration) than normal at 60 °C for 10 min. An equal lack of effect was found under similar conditions at all concentrations tested (0.2, 2 and 10%, w/v) of the cationic agent, tetrapropylammonium hydrogen sulphate. The same polypeptide profiles from all of the above treatments were obtained regardless of whether the spores had been isolated by sucrose or NaBr density gradients. Centri-

fugation in high salt solutions such as NaBr density gradients or simply 10–20% (w/v) NaCl removes the endogenous proteases that cause partial degradation of the parasporal proteins of *B. thuringiensis* (Carroll *et al.* 1989). It appears that such proteases do not become associated with the parasporal fibres of *P. penetrans* during sporulation.

#### Resolution and N-terminal sequencing of parasporal fibres of *P. penetrans*

As efficient a method as possible was required for solubilizing the components of the parasporal fibres due to the limited number of spores available. Boiling in

GSB resulted in quite diffuse bands after gel electrophoresis with an unacceptable level of background contamination. Although treatment with SDS at 60 °C for 10 min (Table 1) solubilized the greatest number of polypeptides this method was unsuccessful for N-terminal sequencing: interference was experienced from background contamination which was not apparent on the acrylamide gel. A more successful approach involved three periods of heating in SDS and 2-ME at 60 °C. The presence of 2-ME, while not improving upon the solubilizing power of SDS alone, allowed the parasporal fibre polypeptides to be separated better from any background contamination. Solubilization at pH 9.5 in 50 mM K<sub>2</sub>CO<sub>3</sub> for 1 h in the presence of 2-ME produced very little background and solubilized the majority of the polypeptides (Table 1). Loss of material occurred, however, during Western transfer making this an unsuitable method for N-terminal sequencing.

During the course of these experiments the 50 kDa polypeptide was seen to be one of the most abundant species present and was generally solubilized by the mildest conditions used. Attention was, therefore, focused on this polypeptide and the following N-terminal amino acid sequence was obtained: AASTSGGECCGGG(uncertain)VSP. From part of this sequence an oligonucleotide primer (N-TER F1) was deduced: GAR TGY TGY GGN GG (where N = A, G, C or T; R = A or G and Y = C or T).

#### Whole cell cycle PRINS

Amplification of DNA by cycle PRINS using the N-terminal primer, N-TER F1, gave rise to specific fluorescence in about 60% of the spores of *P. penitans* visualized (Figure 2). It might be that the non-reacting spores were insufficiently permeabilized to allow entry of the reagents. Alternatively, genetic heterogeneity might be the explanation in that our deduced primer sequence did not have sufficient homology with the non-reacting spores to allow amplification. Single cycle PRINS never gave positive results. Fluorescence was never seen after cycle PRINS with vegetative cells of *A. acidocaldarius* nor with spores of *P. ramosa*, implying that they do not possess a DNA sequence corresponding to the N-terminal sequence deduced for *P. penitans*. We attempted PRINS and cycle PRINS with the vegetative stages of both *Pasteuria* species. No recognizable structures were ever seen after processing, indicating that they were too fragile to remain intact during the permeabilization and heating processes.

#### Immunological similarity between spores of the genus *Pasteuria* and those of other species

Polyclonal antibodies raised against whole spores of *P. penitans* recognized the surface of endospores of *P. ramosa*, producing a halo of fluorescence around them (data not shown). Western blot analysis using the same polyclonal antibodies also showed significant homology

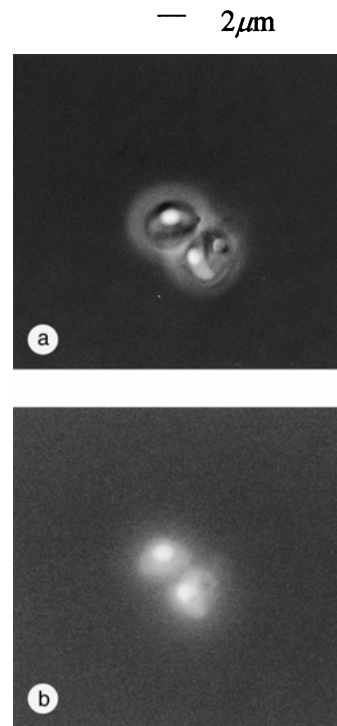


Figure 2. Spores of *P. penitans* after cycle PRINS using an oligonucleotide, derived from the N-terminal sequence of the 50 kDa parasporal fibre polypeptide, viewed (a) by phase contrast and (b) epifluorescent microscopy.

between *P. penitans* and *P. ramosa* (Figure 3). A faint signal was also seen from spores of *A. acidocaldarius* which was identified by 16S rRNA analysis (Ebert *et al.* 1996; Anderson *et al.* 1999) to be one of the most closely related organisms to those of the genus *Pasteuria*. Similarities with species of the genus *Bacillus* or with *T. vulgaris* were not seen. This suggests that the two

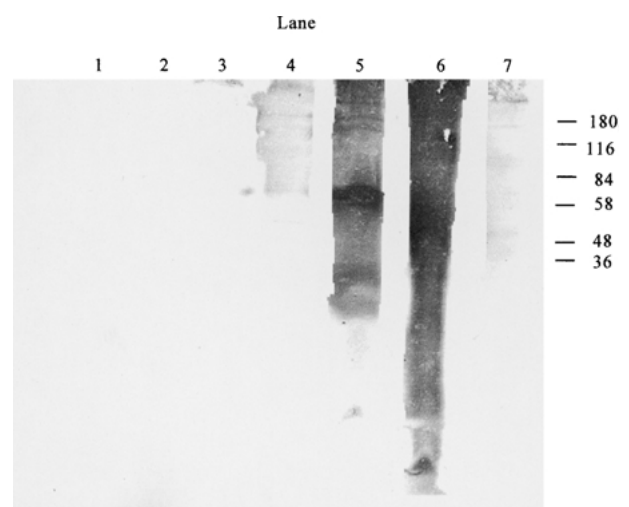


Figure 3. Western blot analysis of extracts of  $5 \times 10^5$  spores per species using a polyclonal antibody raised against whole spores of *P. penitans*. Lanes: (1) *T. vulgaris*; (2) *B. megaterium*; (3) *B. thuringiensis*; (4) *Al. acidocaldarius*; (5) *P. ramosa*; (6) *P. penitans*; (7) M<sub>r</sub> markers (kDa). (The signal seen in lane (7) is due to pre-staining of the molecular weight marker and not reaction with the antibody).

*Pasteuria* species are more related to the *Alicyclobacillus* group than to the other organisms tested.

## Discussion

The visible structure of the parasporal fibres is composed, predominantly at least, of protein. Under normal conditions the fibres retain a durable and insoluble structure even after years of storage and after heating (Giannakou *et al.* 1997) and even boiling in water (Williams *et al.* 1989). We have shown that they can be dissociated into 15 polypeptide components by specific chemical treatments. *B. thuringiensis* is a related spore-forming bacterium, which also synthesizes parasporal proteins (Schnepf *et al.* 1998). These crystalline, insecticidal proteins are also produced during sporulation and remain insoluble under normal physiological conditions. They may, however, be solubilized selectively by incubation in alkaline solution: some classes of these proteins require a reducing environment to achieve this while others do not (Nicholls *et al.* 1989). We found some selectivity of solubility of the parasporal proteins of *P. penetrans* (Table 1) in that most of the polypeptides dissolved at pH 9.5 but only in the presence of a reducing agent; only two polypeptides were soluble at pH 11.5, however, even in the presence of 2-ME. The lack of solubility in a non-reducing environment at pH 9.5 indicates that the weak disulphide bonds, characteristic of the parasporal crystals of *B. thuringiensis* (Du *et al.* 1994), are not used to maintain the integrity of the parasporal fibres of *B. thuringiensis*. It is of interest that the more alkaline buffer was less able to dissolve the parasporal fibres. Although not as clear-cut a finding as that of Nicholls *et al.* (1989) on the insecticidal proteins of *B. thuringiensis*, this observation would facilitate the separation of some of the components of the parasporal fibres.

The pattern of solubility of the parasporal fibres in surfactants was very marked in that 2% SDS at 60 °C for 10 min was completely effective, whereas using 0.2% SDS was ineffective. This is in spite of the fact that the latter concentration is close to the critical micellar concentration for SDS. The anionic agent, tetrapropylammonium hydrogen sulphate, even at high concentrations, had no discernible effect.

The highly specific nature of the attachment of spores to nematode cuticle has been well documented (Davies *et al.* 1994; Davies & Redden 1997; Mendoza de Gives *et al.* 1999). It is hard to imagine, however, how the crystalline proteins of the parasporal fibres could mediate this interaction themselves. An involvement in attachment to nematode cuticle of several factors, including carbohydrate components on the surface of spores has been indicated by several authors (Bird *et al.* 1989; Persidis *et al.* 1991; Davies & Danks 1992). It seems plausible, therefore, that the proteins of the parasporal fibres could act as scaffolding on which carbohydrate ligands are displayed. Several precedents

for the production of glycosylated proteins by bacteria have been reported (Messner 1997). Since *P. penetrans* does not appear to produce true endospores, a process involving the engulfment of the fore-spore, such glycosylation may be all the more feasible.

It is particularly interesting to observe that the basal ring of the spore, which becomes tightly clamped to the nematode cuticle (Mankau & Prasad 1977; Sayre & Wergin 1977) remains intact (Figure 1) after treatments which completely remove the parasporal proteins. This indicates that it is a component of the spore coats and not part of the parasporal fibres. It could, nevertheless, be coated with a thin layer of the adhesins necessary to form a tight seal, thus preventing the escape of the lytic enzymes, which, presumably, aid in the penetration of the germ tube into the cuticle and hypodermis of the nematode. Were this to be the case it would be in keeping with the speculation that the proteins of the parasporal fibres themselves are not directly involved in binding to the cuticle.

The cross-reactivity of spores of *P. ramosa* with anti-serum against spores of PP1 is explicable due to the relatedness of these organisms. Although spores of *P. ramosa* do not possess obvious parasporal fibres, antigenically related molecules appear to exist, as revealed by immunofluorescence. Since the anti-serum was raised against whole spores, other common surface molecules could also be recognised. The slight cross-reactivity with spores of *A. acidocaldarius* implies that surface similarities of this organism to *P. penetrans* and *P. ramosa* exist, enhancing the findings of Ebert *et al.* (1996) and Anderson *et al.* (1999). In view of the different growth environment and life cycle of this organism compared to the invertebrate parasites it is surprising that similarities remain.

Elucidating the exact nature of attachment of spores of *P. penetrans* to plant-parasitic nematodes is greatly hampered by the difficulty in obtaining appreciable numbers of spores and by the heterogeneity of the populations produced. The cloning and expression of the genes encoding the proteins of the parasporal fibres, although challenging, might be an alternative solution. Unfortunately, we currently do not have access to sufficient spores to use the probe that we have derived for Southern blotting analysis. We hope that the information reported here will, nevertheless, assist in the achievement of this goal. The responses in the cycle PRINS reaction provided strong evidence that the probe, N-TER F1, was specific for a component of the parasporal fibres of *P. penetrans* and not for a protein shared with *P. ramosa*.

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