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CHARACTERISATION OF AN ENDOSPORE POPULATION OF PASTEURIA THAT ADHERES TO THE PHYTONEMATODE HETERODERA SCHACHTII

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CHARACTERISATION OF AN ENDOSPORE POPULATION OF *PASTEURIA* THAT ADHERES TO THE PHYTONEMATODE *HETERODERA SCHACHTII*

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Keywords: biological control, host specificity, immunolocalization, collagen-like fibres, lectins

Abstract

Selected strains of *Pasteuria* species from the endospore forming Gram positive bacteria group have the potential to be developed into phytonematode control agents. If *Pasteuria* is to be deployed successfully as a control agent, endospores of the bacterium have to initially adhere to the cuticle of the infective juvenile. Studies of the bacteria isolated from root-knot nematodes have suggested that collagen-like fibres on the endospore surface interact with a cuticle receptor on the second-stage juvenile through a host specific *Velcro*-like mechanism. However, very little is known regarding the nature of the biochemical nature of the mechanism in *Pasteuria* strains isolated from cyst nematodes. Here using several polyclonal antibodies raised to whole endospores and to synthetic collagen-like peptides we compare two *Pasteuria* populations. One a strain from root-knot nematodes (*Meloidogyne javanica*) and another from cowpea cyst nematode (*Heterodera cajani*). Here we demonstrate that there is differential adhesion of endospores to the beet cyst nematode *Heterodera schachtii* and that the sugar moieties on the endospore surface may protect the collagen-like fibres on the endospore from proteolytic digestion and be involved in the endospore's specificity.

INTRODUCTION

Sugar is an important agricultural commodity throughout the world and is a plant product. Sugar beet (*Beta vulgaris*) is an economically important crop the root of which is swollen and has a high sucrose content and is grown for commercial sugar production. The plant-parasitic nematode *Heterodera schachtii* is a major obligate pest of sugar beet and has until recently been controlled by the use of nematicides. However, due to the toxicity of chemically synthesised nematicides and the increasing legislation in the United Kingdom, Europe and elsewhere across the globe, they are prohibited from use by growers and environmentally benign alternatives are being sought (Stirling, 2014).

The *Pasteuria* group of Gram-positive endospore forming bacteria are a group of different species of bacteria some of which are hyperparasites of plant-parasitic nematodes with potential to offer an environmentally benign method of nematode control as a replacement for nematicides (Davies, 2009). There are two major problems in the commercial development of *Pasteuria* as a biological control agent: 1) is the inability to easily mass produce endospores in sufficient quantities for application in the field; 2) the specificity of endospores, they adhere to and infect one population but not another and can therefore lead to highly mixed results of nematode control when deployed in the field (Davies, 2009)

Adhesion of endospores to the cuticle of the second-stage juvenile of the nematode is a key step in the infection process and recent research has focused on understanding the biochemical and molecular mechanism by which endospores adhere to the cuticle of second-stage juveniles. The use of transcriptome analysis combined with RNAi knockdown approaches has revealed several nematode genes, in particular Mi-FAR-1 and a mucin-like gene (Phani *et al*., 2017, 2018a and 2018b), that modulate endospore adhesion on the nematode side of the interaction; and on the *Pasteuria* side of the interaction it is thought there are an array of diverse collagens that form a *hair-like nap*

on the surface of the endospores that are responsible for endospore specificity through a *Velcro-like* attachment mechanism to the nematode cuticle (Srivastava *et al*., 2019 and 2022).

Studies on the interaction between a population of *Pasteuria* isolated from *Heterodera cajani* in India was found to adhere to populations of potato cyst nematodes from Scotland (Mohan *et al*., 2012). The current study was to broaden this knowledge and to characterise the interactions between selected endospore populations of *Pasteuria* and investigate their interaction with second-stage juvenile cuticle of *Heterodera schachtii* from Norfolk, UK.

MATERIALS AND METHODS

Pasteuria endospores

Pasteuria populations were selected from the Rothamsted Research isolate collection that had been transferred to the University of Hertfordshire. The two populations of *Pasteuria* had been mass produced on their host nematode species broadly following the method of Stirling and Wachtel (1980). Briefly, one population designated RES 147 (from a single spore isolate) had been produced on *Meloidogyne incognita* by allowing endospore encumbered second-stage juveniles to invade tomato roots (cv Money Maker) and infect developing females (Davies and Redden, 1997). Another population designated RES 007 (a field population) had been produced on *Heterodera cajani* growing on cowpea (cv Pusa Komal). Suspensions of the two populations of endospores were obtained by collecting hand-picked *Pasteuria* infected females and releasing the endospores by homogenising the females in 500 µL water using a tissue grinder (Mohan *et al*., 2012). The concentration of endospores was determined using a haemocytometer and stored frozen at -20°C.

Heterodera schachtii second-stage juveniles

Soil containing cysts of *Heterodera schachtii* was kindly provided by Dr Alistair Wright (British Beet Research Organisation, Norwich Research Park, Coley Lane, Norwich, NR4 7UG) and stored in a cold

room at 4°C. Cysts were concentration by firstly washing out 200 g of soil sample using a Wye Washer to concentrate the cysts; the cysts were then hand-picked and placed on a hatching tray in water in a flat-bottomed evaporating dish (Pyrex Borosilicate, 140ml) covered with a petri dish, to reduce evaporation, at room temperature and allowed to hatch (Hooper, 1986). Freshly hatched second-stage juveniles for experimental purposes were collected from the water every 2 – 3 days.

Pasteuria attachment assays

Endospore attachment bioassays using second-stage juveniles of *H. schachtii* were performed using centrifugation as described by Hewlett and Dickson (1993). Small suspensions of endospores of each population (RES 007 and RES 147) were mixed with second-stage juveniles (approx. 50 J2s) in such proportions to result in a 30 µL total volume of a 1 percent solution of phosphate buffered saline containing 7.5 x 10^5 endospores in a micro-centrifuge tube and placed in a bench-top centrifuge and spun for 3 minutes at 6000g. Following centrifugation, the 30 µL sample of J2s and endospores were resuspended and placed on a microscope slide and the number of endospores adhering to 20 individual J2s was measured. The experiment was repeated several times.

Antibody and Lectin recognition of _Pasteuria_ endospores

Pasteuria endospores (RES 007 and RES 147) were allowed to adhere to multitest slides coated with poly-l-lysine (Harlow and Lane, 1988) by allowing 10 µL samples of endospores to dry on the slides at room temperature, and immuno-recognition undertaken using the antibodies and lectins described in Table 1, in a range of concentrations from 1:50 to 1:1000) in a humid chamber for 3 hours at room temperature. Primary polyclonal rabbit antibody recognition was detected with secondary anti-rabbit IgG antibodies conjugated to either FITC or TRITC (Sigma, UK) in a humid chamber maintained in the dark. To avoid photobleaching prior to the coverslip being placed over the sample *CitiFluor* (CitiFluor, EMS Acquisition Corp) was applied according to manufacturer's instructions. Visualization of the endospores was done using an epifluorescence microscope fitted with a fluorescence imaging camera (GT Vision; model G XML 3201) and assessment was done qualitatively on a minimum of four

randomly selected endospores per treatment and scored as no fluorescence (0); possible fluorescence (1); fluorescence (2); bright fluorescence (3) extremely bright fluorescence (4). The experiments were all repeated at least once and at different concentrations as described above.

Immunofluorescence following collagenase digestion

Collagenase C5138 (Sigma, UK) from *Clostridium histolyticum* was used in a concentration of 1000 units/ ml and diluted in 2% PBS. Each population of endospores (25 µL of RES 007 and 25 µL of RES 147) was mixed with 25µL collagenase in plastic tubes and were incubated for 2 hours at 37degrees Celsius. Following incubation, the endospore suspensions were washed in PBS (2%; x3). Resuspended endospores were then allowed to adhere to poly-l-lysine coated slides, as described above, and immunofluorescence done with primary antibodies PC 396 or Col 1981 as described above. Control endospores were probed with pre-immune sera and visualized using anti-rabbit IgG secondary antibodies conjugated to either TRITC or FITC (Sigma). Endospore fluorescence was measured as described above and the experiment repeated several times.

137 **RESULTS**

138 Endospore attachment to second-stage juveniles of *H. schachtii* was compared between RES 007, a
139 strain that had originated from an Indian population of *H. cajani* that infected cowpea, and RES 147,
140 a strain that originated from a population of *M. javanica* from Papua New Guinea that infected
141 sweet potato and had subsequently been mass cultured at Rothamsted Research on tomato plants.
142 The mean endospore attachment was 3.56 endospores per juvenile and 0.05 endospores per
143 juvenile respectively and was statistically highly significant (Figure 1; $P < 9.0 \times 10^{-10}$).

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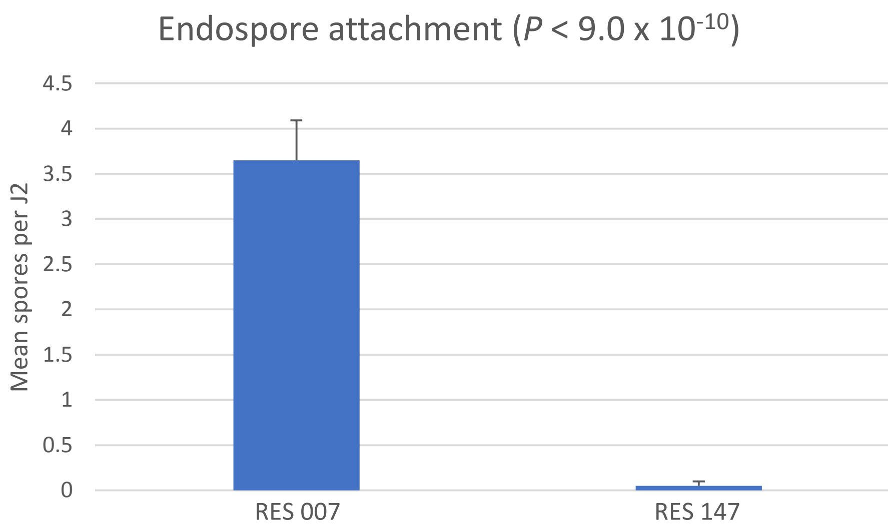

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146 Figure 1. Mean number of two strains of endospore strains (RES 007 and RES 147) that adhered to
147 second-stage juveniles of *Heterodera schachtii* in a centrifugation attachment bioassay (bars = SEM)

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153 Recognition of endospores by PC 396 can be seen in Figure 2. The polyclonal antibody, PC 396, had
154 been raised in rabbit to whole endospores of a population of *P. penetrans* from Senegal (Fould *et al*.,
155 2001) and it recognised both RES 007 and RES 147 to a greater or lesser extent depending in which
156 secondary anti-rabbit conjugate had been used. Endospore population RES 007 fluoresced with a
157 stronger intensity, score 4, when the TRITC conjugate was used compared to score 2.3 with the FITC
158 conjugate. Conversely, in the RES 147 immunolocalization studies the FITC conjugate had the greater
159 fluorescence intensity, score 4, compared to the TRITC conjugate, score 1.7. Statistical analysis of the
160 differences in fluorescence intensity was highly significant (ANOVA $P < 0.001$).

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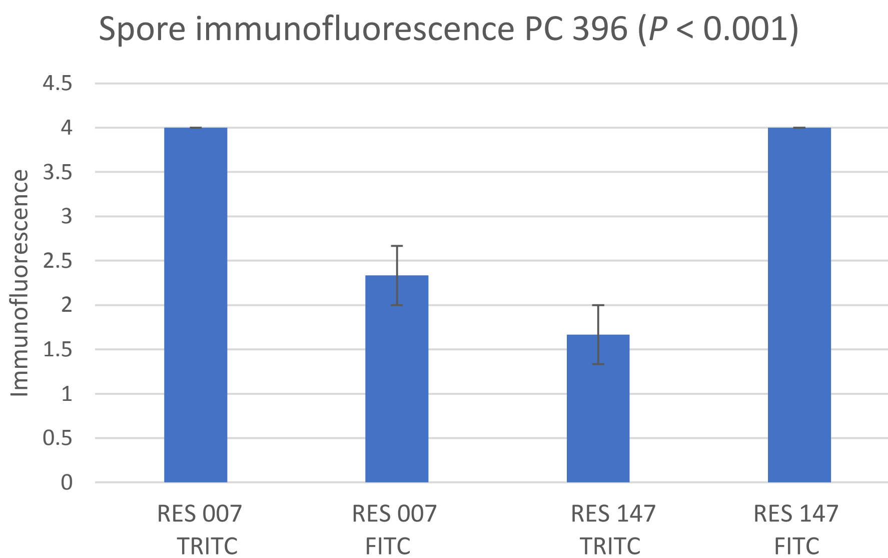

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163 Figure 2. Endospore recognition by immunofluorescence of two *Pasteuria* strains, RES 007 and RES
164 147, by the primary antibody PC 396 visualised using two anti-rabbit secondary antibodies, one
165 conjugated to FITC and another to TRITC (bars = SEM)

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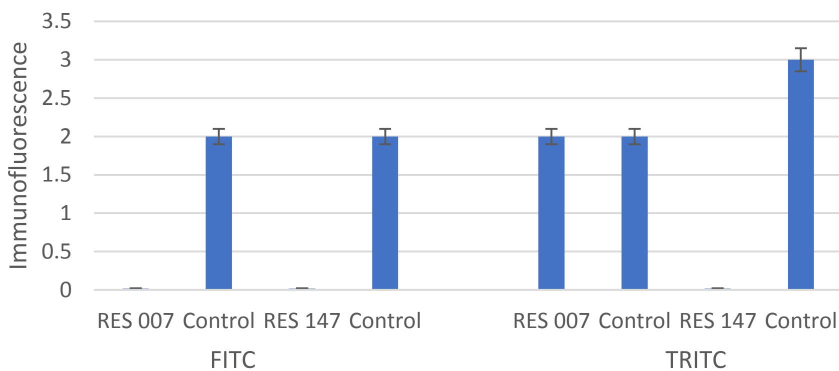
174 A comparison of immunofluorescence using PC 396 between endospores that had been pre-treated
175 with collagenase and untreated controls showed collagenase to reduce the fluorescence intensity to
176 nearly zero across all collagenase treatments apart from RES 007 where TRITC was used as the
177 secondary antibody and there was no difference in fluorescence intensity between treated and
178 untreated endospores (Figure 3A; ANOVA $P < 0.001$). However, when the experiment was repeated
179 but using a polyclonal antibody raised to a synthetic collagen-like peptide (GTPGTPGPAGPAGPA) the
180 reduction in fluorescence intensity was across all endospore types (RES 007 and RES 147)
181 irrespective of the secondary antibody used and was statistically significant (Figure 3B; ANOVA $P <$
182 0.0001).



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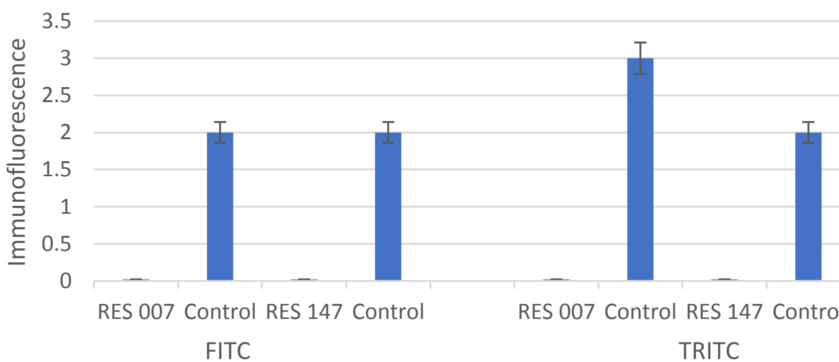

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185 Figure 3. A) Immunofluorescence using PC 396 of endospore populations RES 007 and RES 147
186 treated with collagenase and an untreated control and visualised using a secondary antibody
187 conjugated to either FITC or TRITC; B) Immunofluorescence using anti-col1981 of endospore
188 populations RES 007 and RES 147 treated with collagenase and an untreated control and visualised
189 (bars = SEM)

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In a panel of lectins that recognised different sugar moieties (Table 1) all the lectins recognised both RES 007 and RES 147 to a greater or lesser extent (Figure 4). However, differences could be seen in the intensities of fluorescence between different endospore types and the different lectins. For example, there was no difference in fluorescence between the two endospore types for the lectins, PNA, Con A and WGA. However, RCA and SBA showed reduced fluorescence intensity, down from 3 to 2, with RES 147. Conversely, with lectins DBA and UEA there was a reduction in fluorescence intensity, down from 3 to 2, with RES 007 (Figure 4).

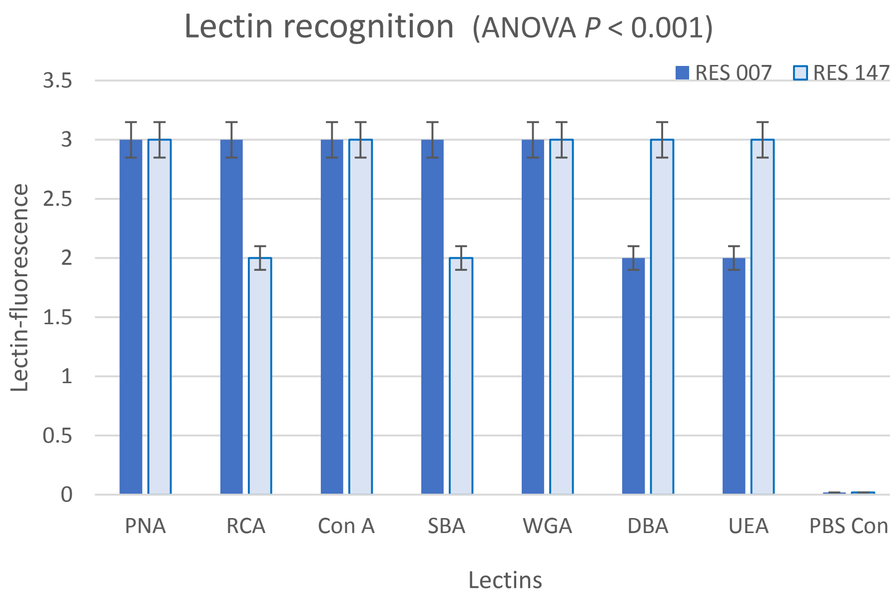

Figure 4. Lectin-fluorescence of endospores of *Pasteuria* strains RES 007 and RES147 when treated with a panel of FITC labelled lectins (PNA, RCA, Con A, SBA, WGA, DBA and UEA) compared with a phosphate buffered saline (PBS) control (bars = SEM)

DISCUSSION

The attachment of bacteria to the surface of phytonematodes has recently generated a resurgence of interest due to their functional role in nematode suppressive soils combined with the application of newly available molecular techniques (Davies and Spiegel, 2011; Topalovic *et al*., 2019; Mohan *et al*., 2020). The *Pasteuria* group of nematode hyperparasitic bacteria have long been associated with nematode suppressive soils and have been a focus of research due to their potential as biological control agents. However, the majority of this research has investigated the bacterium's interaction with the economically important root-knot nematode, *Meloidogyne* spp. in which endospores exhibit a high degree of host specific adhesion, where endospores are capable of attaching to one population of root-knot nematode but not another even from the same phylogenetic clad (Davies *et al*., 2001).

Here, we further characterise a population of *Pasteuria* (RES 007) that originated from *Heterodera cajani* in India that had been shown to adhered to and infected potato cyst nematode *Globodera* spp. (Mohan *et* al., 2012). Although RES 007 did appear to adhere to root-knot nematodes at a very low level (mean 0.06 endospores per J2; Table 2) this was a single endospore and possibly mistaken due to nematode orientation during microscopic observation. However, although endospores did adhere to *H. schachtii* J2s (3.4 endospores per J2), we were unsuccessful in observing any evidence of subsequent germination and infection when endospore encumbered juveniles were placed on cabbage seedlings grown in CYG™ germination pouches; this may have been a problem of low numbers of *H. schachtii* J2s invading the cabbage roots (personal communication), but if perhaps not equally, may have been the result of the endospores not germinating and infecting the developing females (n = 2).

If *Pasteuria* is to be deployed successfully as a biological control agent an understanding of its host specificity is fundamental. Recent studies using antibodies made to synthetic collagen-like peptides, identified from genome survey sequences of a *Pasteuria* population that infects root-knot

nematodes, have suggested that collagen-like glycoproteins on the surface of the endospore are interacting with a cuticle receptor(/s) present on the surface of the J2 (Srivastava *et al*., 2022). Because similar collagen-like proteins have also been identified on the surface of endospores of *Bacillus thuringiensis,* which do not attach to J2s of phytonematodes but infect lepidopterous insects, a partial characterisation of the endospores of a *Pasteuria* deemed to be more closely related to *P. penetrans* and do not attach to *Meloidogyne* spp might aid our understanding on the molecular nature of the attachment mechanism.

The polyclonal antibody, PC 396, raised to whole endospores of a *Pasteuria* population that infected root-knot nematodes differentially recognised the endospores of the different *Pasteuria* strains (Fould *et al*., 2001). Interestingly, when detected using a secondary antibody conjugated to FITC it recognised the homologous population of *Pasteuria* to which the antibody was originally raised (RES 147) it produced more fluorescence than the non-homologous population (RES 007). This result is what we would have hypothetically expected. However, when we repeated this experiment and used a secondary antibody conjugated to TRITC we unexpectedly got exactly the opposite result. Repeating the experiment again but this time using antibodies raised to the synthetic collagen-like peptides, Col 1981 and Col 1982, the results obtained appeared hypothetically correct, i.e., greater fluorescence was observed with the homologous *Pasteuria* population, RES 147, than with the non-homologous *Pasteuria* population from *H. cajani*, RES 007. Intriguingly, pre-incubating the endospores in collagenase, a treatment previously shown to negatively affect fluorescence and endospore attachment to J2s (Srivastava *et al*., 2022) had no effect on the amount of fluorescence.

The counter intuitive results presented in this report require explanation. Why is there the discrepancy between the secondary FITC conjugate and the secondary TRITC conjugate when using the PC 396 primary antibody? And secondly, when using the antibodies raised to the synthetic collagen-like peptides, why does the collagenase treatment not reduce the observed fluorescence? We think the results using the FITC labelled lectins (Table 3) may provide some insights. Looking at

the results for RES 147 we can see there is a qualitative reduction in fluorescence of endospores when probed with the lectins DBA and UEA which recognise the sugar moieties N-acetyl-galactosamine and L-fucose respectively. Similarly, for RES 007 there is a qualitative reduction in fluorescence for RCA and SBA which recognise D-galactose and another variant of N-acetyl-galactosamine respectively. It is generally acknowledged that there are commonalities in the glycoconjugates expressed on the surfaces of bacteria (Tytgat and Lebeer, 1914), and therefore the discrepancy in the results between the FITC and the TRITC secondary antibodies may just be due to the immunological responses of the animals to the glycoconjugates they had been exposed to prior to rabbit IgG whole molecule immunisation. For example, the pre-immune rabbit may have been exposed to a bacterium that had a functional glycoconjugate similar to strain RES 007 and hence the increased fluorescence.

Hypothetically we envisaged that the collagenase treatment would reduce the fluorescence when probed with antibodies raised to the synthetic peptides. However, this was not the case and the level of fluorescence remained similar to the untreated controls (Table 2). The fact that each of the FITC labelled lectins bound to the surface of both populations of endospores (Table 3) might suggest that the collagen-like fibres were protected from the collagen treatments by their glycocongugates. This is in direct contrast to the result reported earlier on endospores of *Pasteuria* and *Bacillus* exposed to collagenase which affected both recognition by antibodies raised to collagen-like peptides, and also affected *Pasteuria* endospore attachment to J2s (Srivastava *et al*., 2022).

Our results show there is differential binding of antibodies and several of the lectins to the endospores of *Pasteuria* strains RES 007 and RES 147. Genome sequencing of *Pasteuria* and other closely related *Bacillus* spp. suggests that the hair-like nap expressed on the surface of endospores comes from a diversity of collagen-like genes that differ between different strains (Orr *et* al., 2018; Srivastava *et* al., 2019; 2022) and has result in the hypothesis that the diversity of these collagen-like fibres is responsible for the specificity of endospore adhesion to J2s. Here we suggest that sugar

moieties on the surface of the endospores may be responsible for protecting endospores from extracellular proteolytic digestion and therefore play a part in endospore survival in the soil. Earlier work (Davies and Danks, 1993) suggests they may also have a functional role in binding specificity.

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We would like to thank Dr Alistair Wright of British Beet Research Organisation for providing the *Heterodera schachtii* population.

Ethics and conflicts of interest

This research was based on a project conducted by RH in part fulfilment of a Master of Science Degree in Applied Biotechnology at the University of Hertfordshire. KGD is the founding Director of KG Davies Ltd (Company Registration Number 7850939). This does not alter the authors' adherence to *Nematology*'s policies on sharing data and materials.

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Table 1. Code designation of polyclonal antibodies and FITC labelled lectins used in the study together with their primary specificity recognition, source and reference or manufacturer

Code Designation	Antigen/ Specificity*	Antibody/Plant Source	Reference/manufacturer
PC 396	Pasteuria whole endospore	IgG Rabbit	Fould *et al*., 2001
Col 1981	Synthetic collagen GTPGTPGPAGPAGPA	IgG Rabbit	Srivastava *et al*., 2022
Col 1982	Synthetic collagen GPQGPQGTQGIQGIQ	IgG Rabbit	Srivastava *et al*., 2022
Con A	D-Man/Glc	Jack Bean	Vector Laboratories, FLK-2100
DBA	GalNAc	Horse Gram	Vector Laboratories, FLK-2100
PNA	D-Gal	Peanut	Vector Laboratories, FLK-2100
RCA120	D-Gal	Castor Bean	Vector Laboratories, FLK-2100
SBA	GalNAc	Soya Bean	Vector Laboratories, FLK-2100
UEA	L-Fuc	Gorse plant	Vector Laboratories, FLK-2100
WGA	GlcNAc	Wheat	Vector Laboratories, FLK-2100

*Further details of lectin specificities see Table V (Davies and Danks, 1993).

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Table 1. Code designation of polyclonal antibodies and FITC labelled lectins used in the study together with their primary specificity recognition, source and reference or manufacturer

Code Designation	**Antigen/ Specificity***	**Antibody/Plant Source**	**Reference/manufacturer**
PC 396	Pasteuria whole endospore	IgG Rabbit	Fould *et al.*, 2001
Col 1981	Synthetic collagen GTPGTPGPAGPAGPA	IgG Rabbit	Srivastava *et al.*, 2022
Col 1982	Synthetic collagen GPQGPQGTQGIQGIQ	IgG Rabbit	Srivastava *et al.*, 2022
Con A	D-Man/Glc	Jack Bean	Vector Laboratories, FLK-2100
DBA	GalNAc	Horse Gram	Vector Laboratories, FLK-2100
PNA	D-Gal	Peanut	Vector Laboratories, FLK-2100
RCA120	D-Gal	Castor Bean	Vector Laboratories, FLK-2100
SBA	GalNAc	Soya Bean	Vector Laboratories, FLK-2100
UEA	L-Fuc	Gorse plant	Vector Laboratories, FLK-2100
WGA	GlcNAc	Wheat	Vector Laboratories, FLK-2100

*Further details of lectin specificities see Table V (Davies and Danks, 1993).

Figure 1

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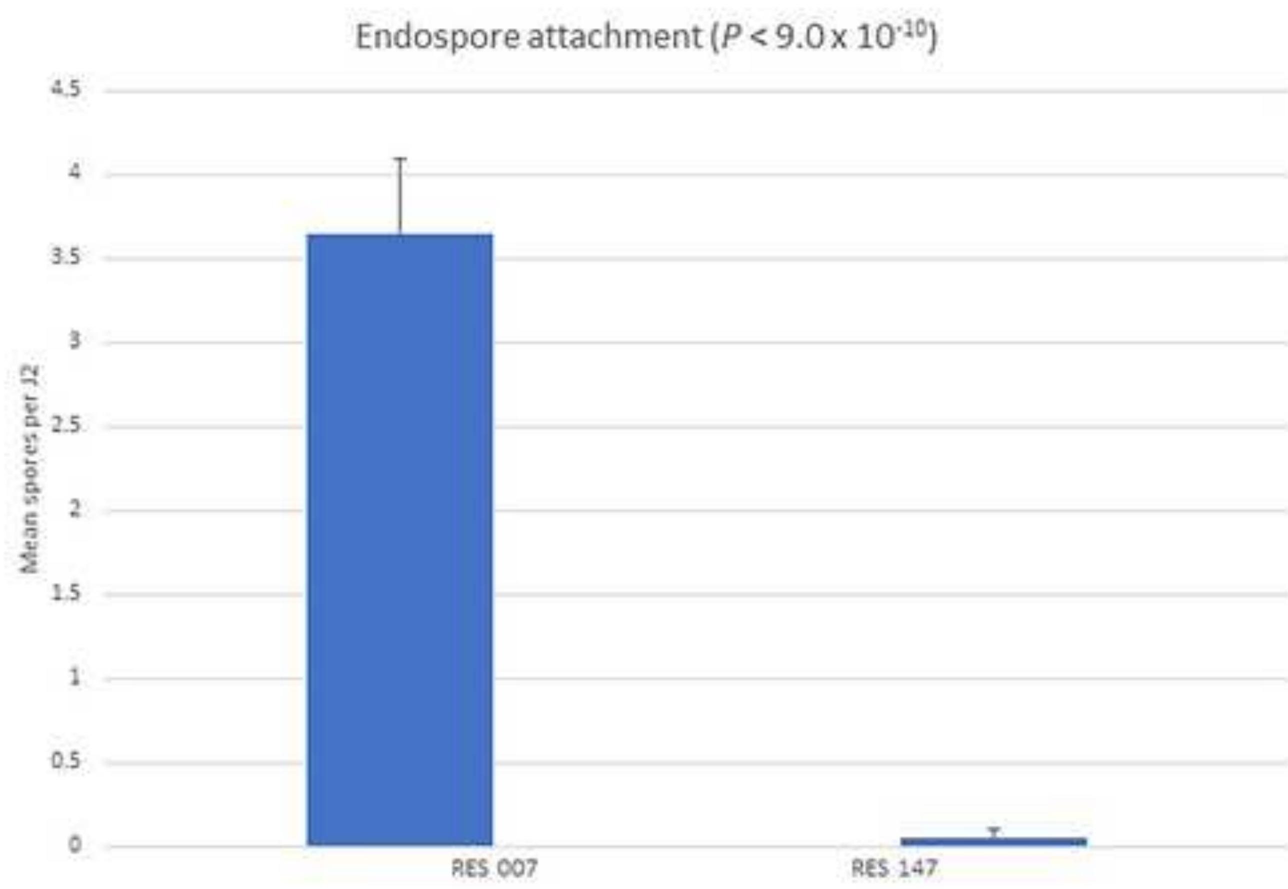

Figure 2

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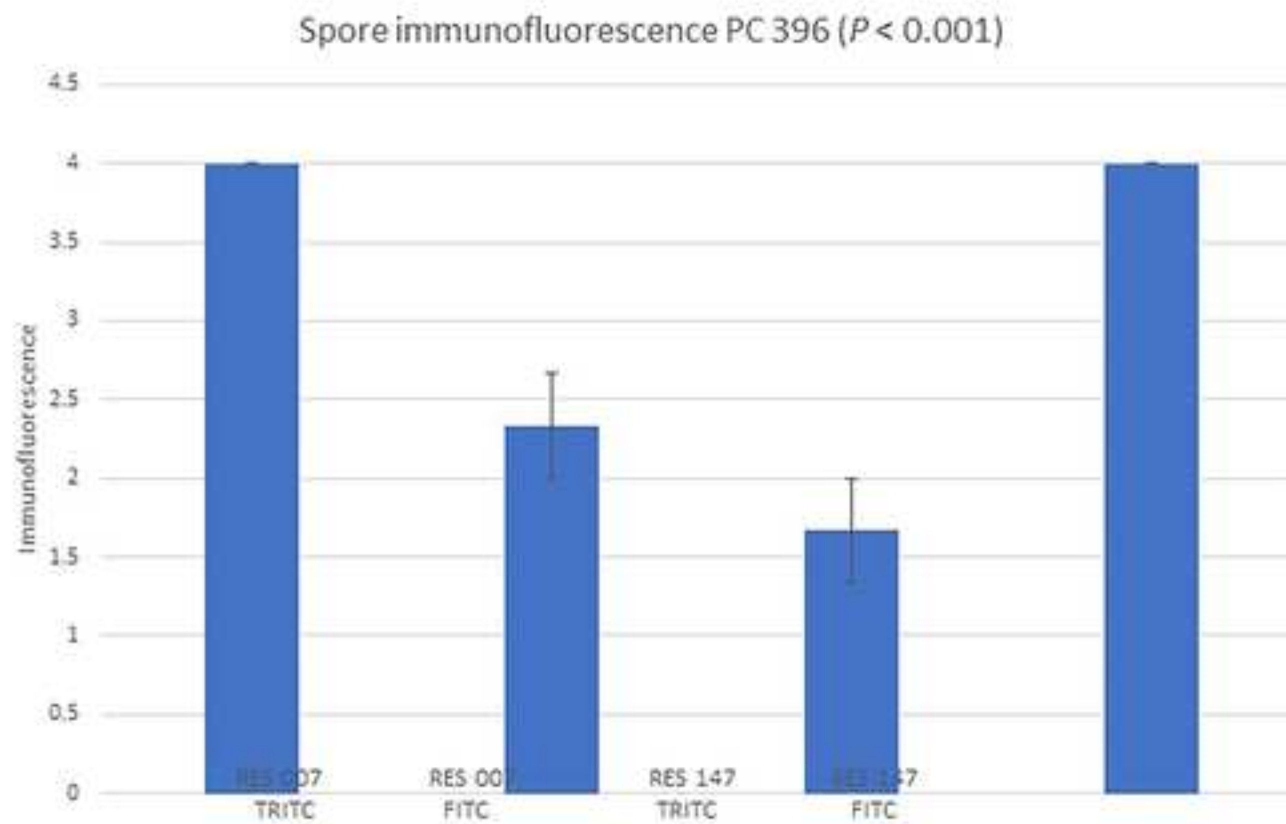

Figure 3

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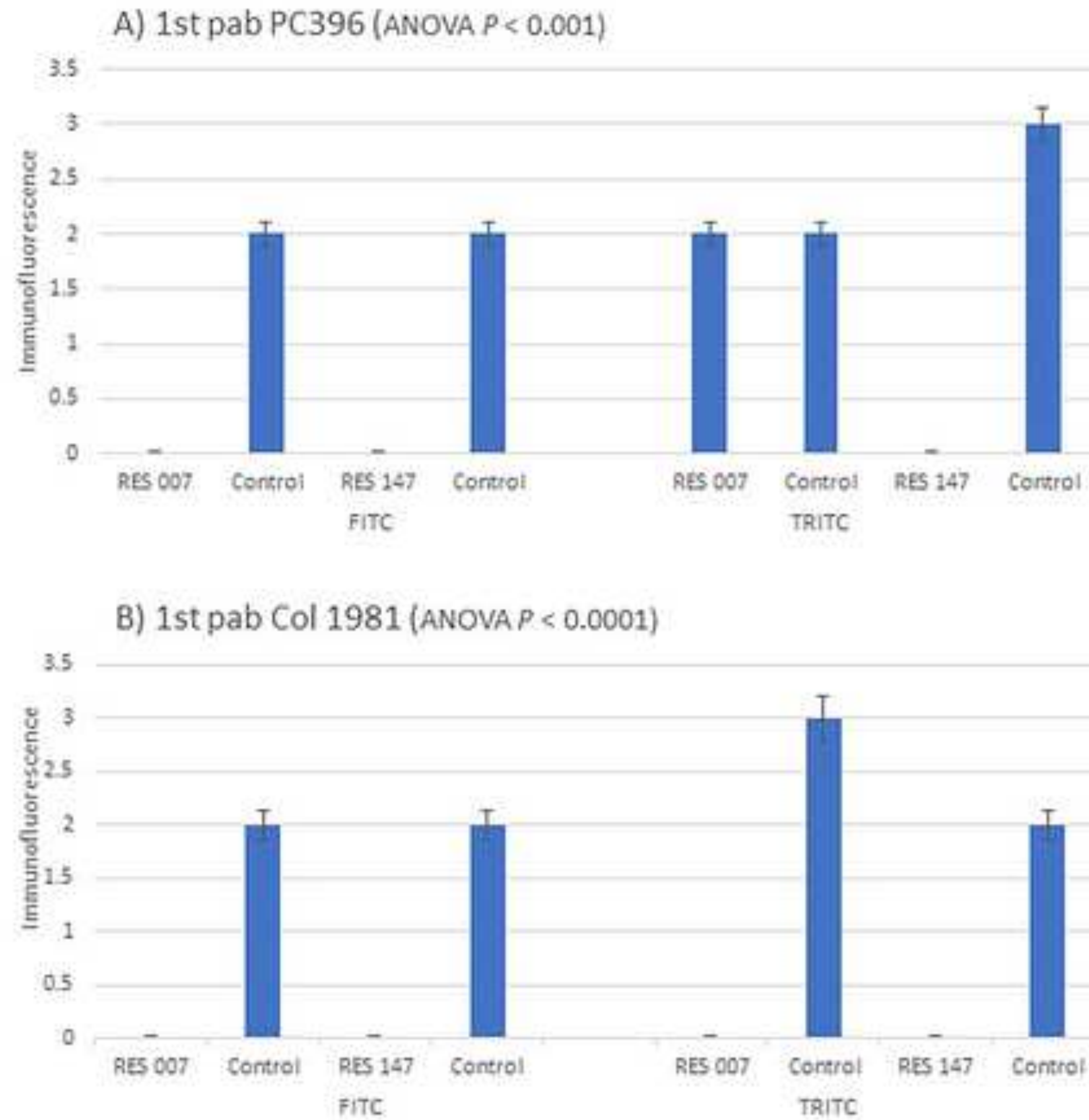

Figure 4

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