Exploitation of immunofluorescence for the quantification and characterization of small numbers of *Pasteuria* endospores

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Abstract

The *Pasteuria* group of endospore-forming bacteria has been studied as a biocontrol agent of plant-parasitic nematodes. Techniques have been developed for its detection and quantification in soil samples, and these mainly focus on observations of endospore attachment to nematodes. Characterization of *Pasteuria* populations has recently been performed with DNA-based techniques, which usually require the extraction of large numbers of spores. We describe a simple immunological method for the quantification and characterization of *Pasteuria* populations. Bayesian statistics were used to determine an extraction efficiency of 43% and a threshold of detection of 210 endospores g^{-1} sand. This provided a robust means of estimating numbers of endospores in small-volume samples from a natural system. Based on visual assessment of endospore fluorescence, a quantitative method was developed to characterize endospore populations, which were shown to vary according to their host.

Introduction

The *Pasteuria* group of Gram-positive bacteria have been found to parasitise a wide range of plant-parasitic nematodes and the Cladoceran *Daphnia magna* (Chen & Dickson, 1998). Five different species have been described based on their morphology, development, host range and, more recently, 16S rRNA gene sequences (Ebert *et al.*, 1996): *Pasteuria ramosa* parasitises *Daphnia* (Metchnikoff, 1888), *P. penetrans* parasitises *Meloidogyne* (Sayre & Starr, 1985), *P. thornei* parasitises *Pratylenchus* (Sayre *et al.*, 1988), *P. nishizawae* parasitises *Heterodera* and *Globodera* (Sayre *et al.*, 1991) and *Candidatus P. usgae* parasitises *Belonolaimus longicaudatus* (Giblin-Davis *et al.*, 2003). Recent phylogenetic analysis based on 27 housekeeping genes found *P. penetrans* to be ancestral to *Bacillus* spp., both saprophytic (*B. subtilis* and *B. halodurans*) and pathogenic (*B. cereus* and *B. anthracis*) (Charles *et al.*, 2005).

The obligate nature of the *Pasteuria* parasite life-cycle and the inability to mass produce it *in vitro* has made it difficult to investigate traits of its biology and ecology that would elucidate its full potential as a biological control agent of plant-parasitic nematodes. *Pasteuria* endospores are resistant to heat, cold and chemical extremes, which hampers a straightforward DNA extraction that has only recently been successful for low concentrations of endospores (Duan *et al.*, 2003). This has complicated its detection and identification using DNA-based techniques.

Pasteuria penetrans has been studied most extensively as a potential biological control agent of root-knot nematodes (*Meloidogyne* spp.) (Stirling, 1984, 1991). The endospores produced by the bacterium attach to the cuticle of second-stage juveniles (J2) as they move through the soil. When they enter the root and start to feed, the endospores germinate and a germ tube penetrates the cuticle, and the bacterium proliferates inside the nematode. Up to two million *Pasteuria* endospores are released into the soil from an infected root-knot female cadaver (Chen & Dickson, 1998; Davies *et al.*, 1988).

The population dynamics of this interaction has been described as a typical predator–prey system, in which the number of *Pasteuria* endospores in soil is dependent on the number and activity of available hosts, resulting in a time-delayed density-dependent relationship (Ciancio, 1995a). Although attempts have been made to quantify endospores directly from soil, this has remained problematic and the quantification of endospores in soil has only been estimated indirectly by their attachment to J2.

Previous studies have shown that attachment of *P. penetrans* endospores to J2 varies both between and within populations of this bacterium (Espanol *et al.*, 1997; de Gives *et al.*, 1999; Wishart *et al.*, 2004), and that attachment is not related to the phylogeny of root-knot nematodes (Davies *et al.*, 2001). Although most *Meloidogyne* spp. reproduce parthenogenetically, J2 originating from the same egg mass can be phenotypically, if not genetically, different, and different numbers of endospores adhere to their cuticles accordingly (Davies & Danks, 1992; Fargette *et al.*, 1994; Davies & Redden, 1997). The interaction between this parasite and its host is, therefore, one with high specificity, which can occur at a subpopulation level. Therefore, the frequency of J2 with endospores attached and the number of endospores attached per J2 are not necessarily an indication of the number of endospores in soil.

A density of *c.* 10^4 endospores g^{-1} soil of *P. penetrans* may be required to generate nematode-suppressive soils (Davies *et al.*, 1990; Stirling, 1991). A suppressive soil typically develops after continuous culture of crops susceptible to *Meloidogyne* (Oostendorp *et al.*, 1991; Chen *et al.*, 1997). One hundred endospores can be found attached to a single J2, but it only takes one endospore to initiate infection (Chen & Dickson, 1998). Nevertheless, it has been observed that J2 with over 20 endospores attached to their cuticle may not become infected (K.G. Davies, unpublished) and populations of the bacterium may differ in their pathogenicity. The relationship between the density of *P. penetrans* endospores required to regulate most populations and the attachment of spores to J2 is not clearly understood.

The ecology of *Pasteuria* in a natural sand dune system has been studied within the Ecotrain Project. Preliminary observations suggested that *Pasteuria* was widespread in dunes in northern and southern Europe and that the density of endospores of this bacterium is relatively high in sand, but they are patchily distributed. A method for extraction, detection and quantification of small numbers of *Pasteuria* endospores in soil has been developed using immunofluorescence, and exploits Bayesian statistics to analyse the data. We also present the potential of this method to characterize *Pasteuria* endospores using antibodies that recognize the surfaces of different endospore populations.

Material and methods

Pasteuria populations

Pasteuria penetrans isolates RES147, PPE and PP3 were selected from the collection of *Pasteuria* populations maintained at Rothamsted Research. They had been propagated *in vivo* in *Meloidogyne incognita* nematodes, using a standard procedure (Stirling & Wachtel, 1980), which was modified: about 2500 J2 with a mode of between five and seven endospores attached were inoculated into a 1-month-old tomato plant cultivar. Tiny Tim; plants were grown in a glasshouse with 16-h day 8-h^{-1} night at a constant temperature of 25 °C. Two months after inoculation, the plants were harvested, their roots were washed and observed using a stereomicroscope (×30 magnification). Infected females were picked with forceps into a micro-tube containing 0.5 mL of distilled water, then homogenized using a tissue grinder and the concentration of spores in the suspension was ascertained using a haemocytometer. A population of endospores (PPBSc) kindly provided by *Pasteuria* Biosciences LLC, Florida, was also used. The suspension of endospores collected for each *P. penetrans* population was stored at 4 °C until use.

A *Pasteuria ramosa* population was obtained from freeze-dried *Daphnia* tropical fish food (Interpet Ltd., Dorking, UK). One gram of this was ground in tap water with a pestle and mortar, and sieved through a 10-μm-pore sieve. The resulting stock suspension was stored at 4 °C until used.

Pasteuria populations from the Borth sand dunes (Ynyslas, Wales, UK), which had been detected as attaching to *Pratylenchus* sp., *Tylenchorhynchus* sp. and omnivorous Dorylaim nematodes, were collected in suspensions: sand (0.5 g) was placed in a 2-mL micro-tube containing 1 mL distilled water, mixed for 1 min using a vortex mixer and allowed to settle for 2 min. The supernatant, containing the *Pasteuria* endospores in suspension, was used immediately.

Immunodetection

A polyclonal antibody,∞PPORS pAB, previously raised in rabbit against *Pasteuria penetrans* endospores was used (Davies *et al.*, 1992; Fould *et al.*, 2001). The immunodetection technique was optimized through a series of preliminary experiments using different endospore suspensions, sand substrates, exposure-periods and incubation temperatures, from an existing method (Duponnois *et al.*, 2000).

Suspensions of *Pasteuria* endospores were diluted (1 : 1 v/v) from the original stock suspension with distilled water. Fifteen microlitres of the suspension were added to each of three wells in an eight-well multitest slide (MP Biomedicals Inc., Irvine, CA). The suspensions on the slides were allowed to dry completely before 15 μL of 1 : 500 ∞PPORS pAB in PBSTM (PBS, 10mM sodium phosphate buffer, pH 7.2%, 0.9% sodium chloride; T, 0.05% v/v Tween-20; M, 5% dried milk powder) were added. Slides were incubated at 37 °C for 1 h in a humid chamber and washed twice in PBST. They were then carefully dried between wells to prevent cross contamination, before adding 15 μL of goat antirabbit FITC-labelled antibody solution (F-0382, Sigma-Aldrich, St Louis, MO) at a 1 : 100 dilution in PBSTM to each well. Multitest slides were then placed in a dark humid chamber and incubated for 1 h at 37 °C. Following incubation, the multitest slides were again washed twice in PBST and dried between the wells using tissue paper. Five microlitres of the antifade reagent Citifluor (Agar Scientific, UK) was added to each well before covering with a 25 × 50 mm cover slip. All preparations were examined using a Zeiss Axioskop microscope fitted with epifluorescence illumination with a 455-nm excitation filter and a 520-nm barrier filter.

To prevent the adherence of endospores to the microtubes, they were all flooded with 5% dymethyl-dichlorosilane (D-3879, Sigma-Aldrich Biochemicals, St Louis, MO) in chloroform, rinsed in distilled water and dried completely before use (Sambrook *et al.*, 1989).

Quantification of endospores in a sand dune

The immunodetection method was used for the quantification of *Pasteuria* endospores in sand dunes; endospores were counted in each of three wells in the multitest slide. Numbers of endospores in the total suspension were estimated, and results expressed as the mean number of observed endospores per gram sand.

To elucidate the threshold of detection and efficiency of the immunodetection technique for quantification purposes, positive controls were made with *P. penetrans* isolate RES 147 endospores. An endospore suspension of 8000 endospores mL^{-1} was obtained from the stock suspension, and endospore concentration checked using a haemocytometer. Serial dilutions of this suspension were then prepared, to obtain concentrations 200, 400, 800, 2000 and 4000 endospores mL^{-1}. These were added to *Pasteuria*-free dune sand, quartz sand or used directly, and were subjected to exactly the same procedure as described above. Negative controls were also made using distilled water instead of the endospore suspensions. Results were Log_{10}-transformed to allow within-treatment homogeneity of variances, which was confirmed by plotting residuals in a fitted-value plot, and analysed using nested Analysis of Variance (Genstat for Windows v.7, Lawes Agricultural Trust, UK), with the treatment structure being the initial concentration of endospores nested within the substrate used (dune sand, quartz sand, water).

Data analysis and statistics

In the dune sand, results from the different initial concentration of endospores were analysed using a linear regression model in Bayesian Statistics software, Winbugs 1.4.1, available online (*http://www.mrc-bsu.cam.ac.uk/bugs/winbugs/contents.shtml*). The number of endospores recovered after the immunodetection process was assumed to be related by linear regression to the initial concentration of endospores, as revealed by frequentist statistical approaches, with the generic curve equation, for any pair *i*:

$$S_{\mathrm{R}}i = a \times S_{\mathrm{I}}i + b$$

where S_{R} is the number of endospores recovered, S_{I} is the initial number of endospores, a is the slope and b is the intercept.

The model was run to provide an estimate for S_{R} for each value of S_{I} that would be distributed normally within the distribution of endospores counted; and to generate an estimate of the slope for the regression curve. The precision values were taken from the actual variance found in the data. The value for b was 0, as determined from the negative control observations. The model was run for i consisting of seven different observations (0, 200, 400, 800, 2000, 4000 and 8000 endospores mL^{-1}), and a nonavailable data point (100 endospores mL^{-1}). The initial values for the two Markov Chain Monte Carlo (MCMC) samplings were generated by the software, and 10 000 iterations were performed; the first 5000 then being considered burn-in, and removed. The posterior confidence interval was analysed, and the MCMC error confirmed to be less than 5% of the standard deviation on each of the $\mathrm{S_R}i$. The estimated $\mathrm{S_R}i$ were plotted against the $\mathrm{S_I}i$ and a linear regression line and equation added using Microsoft Excel 2002 for Windows.

Characterization of endospores

To evaluate the heterogeneity of the *Pasteuria* populations in terms of the endospore surface properties, a quantitative means of assessing fluorescence after the immunodetection method was developed based on visual assessment. This consisted of evaluating the fluorescence of 20 randomly selected endospores. To minimize subjectivity in this assessment, each endospore was first sought and identified using brightfield illumination (×400 magnification), under which endospores do not fluoresce. Epifluorescence illumination was then applied and the endospore under study was scored on the following scale: 0, not fluorescent; 10, fluorescent; 100, strongly fluorescent. This method was used on endospores of the populations described above, namely *Pasteuria* from Borth sand dunes, *P. penetrans* populations PP3, PPE, RES 147 and PPBSc and the *P. ramosa* population, and using two different antibodies, ∞PP1 pAB (Persidis *et al.*, 1991) and ∞PPORS pAB. Controls consisted of replacing the primary antibody solution with the preimmune rabbit serum. Two replicate samples of each treatment were obtained and the data were transformed [$\mathrm{Log}_{10}(x+1)$]. Analysis of variance was then undertaken on the transformed data, and results further interpreted with LSD analysis at a 5% level, using Genstat for Windows v 7.0 (Lawes Agricultural Trust, UK). Images were obtained using a Xillix digital camera and processed using an image analysis system (Improvision, Coventry, UK).

Results

Immunodetection

The immunodetection method permitted the clear identification of *Pasteuria* spp. endospores in soil suspensions of the Borth sand dunes, which typically contain particulate organic matter (Fig. 1). Different populations of these bacteria, which parasitise either endo- or ectoparasitic nematodes, and *Daphnia*, have been detected using the ∞PPORS pAB (Fig. 2).

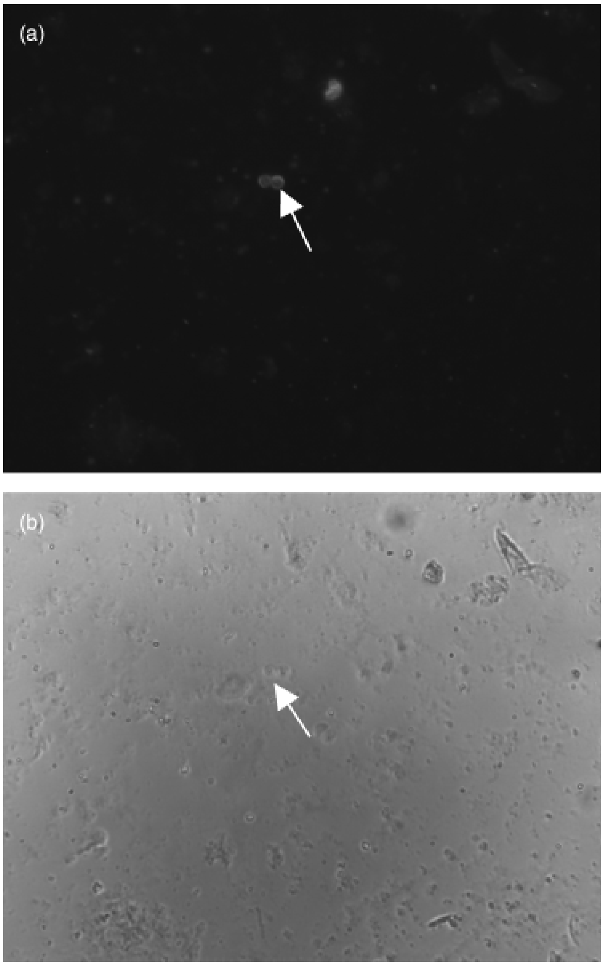

Fig. 1. Fluorescence (a) and brightfield illumination (b) micrographs of *Pasteuria* endospores in a sample from sand dunes in Borth, using an immunodetection method with the polyclonal antibody ∞PPORS pAB (× 400, arrows indicate endospores).

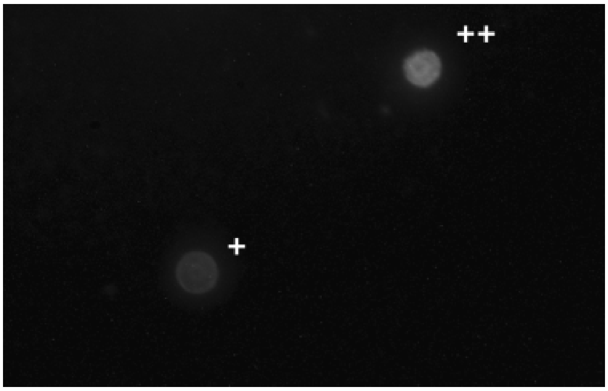

Fig. 2. Fluorescence evaluation of the *Pasteuria ramosa* population endospores, scored 10 (+) and 100 (++), using an immunodetection method with the polyclonal antibody ∞PPORS pAB (× 1000).

Quantification of endospores in a sand dune

The type of substrate in which the RES 147 endospores were incorporated did not significantly affect their detection, as recovered concentrations of endospores were not significantly different between substrate treatments ($P < 0.05$). However, there were significant differences in the number of endospores observed between the initial concentrations of endospores, within each of the substrates ($P < 0.01$). The R^2 value for the regression curve is close to 1 for the three substrates used, indicating a linear response (Fig. 3).

The quantification method has been assessed using Bayesian statistics, which point to the threshold of detection being 210 endospores g^{-1} sand, and an overall efficiency of 42.6%, as calculated from the linear regression equation of the model estimates ($S_R i = 0.426 \times S_I i$, $R^2 = 1$). The recovered endospore concentration model estimates are all situated within the standard deviation of the observed values (Fig. 4).

Endospores in samples taken from Borth dunes reached higher densities in one sample than those required to regulate nematode populations in an agricultural soil, although in 13 out of the 15 samples the endospore

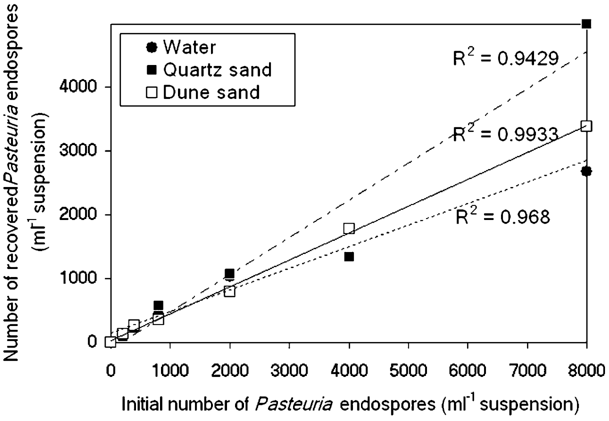

Fig. 3. Number of *Pasteuria penetrans* population RES 147 endospores recovered vs. the initial numbers per millilitre in suspension using an immunodetection method with the polyclonal antibody ∞PPORS pAB, in three different substrates: water, quartz sand and dune sand (values are means of three replicates; lines are linear regression curves).

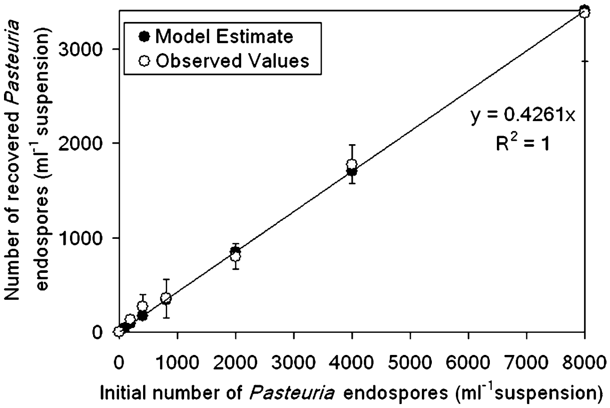

Fig. 4. Comparison of model estimates and observed values for number of *Pasteuria penetrans* population RES 147 endospores recovered vs. the initial numbers per millilitre in suspension using an immunodetection method with the polyclonal antibody ∞PPORS pAB, in dune sand substrate (points for model estimates are the mean result for 5000 iterations; the line is the linear regression curve of model estimates; points for observed values are means of three replicates; error bars are the standard deviation of observed values).

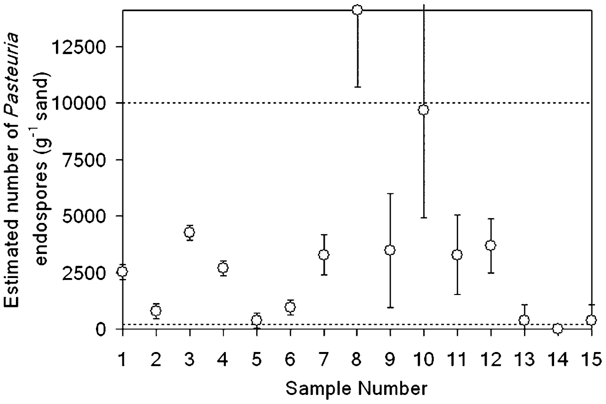

Fig. 5. Quantification of *Pasteuria* endospores per gram sand in 15 samples from sand dunes in Borth using immunodetection method with the polyclonal antibody ∞PPORS pAB [values are means of three replicates; error bars are the standard deviation; horizontal lines indicate the threshold of detection of the immunodetection method (a) and the estimated minimum number of endospores g^{-1} to generate a suppressive soil (b)].

population is below half this number. Of the 15 samples analysed, only one (sample 14) does not contain endospores at a detectable concentration (Fig. 5).

Characterization of endospores

Scoring the endospore fluorescence for *Pasteuria* populations characterization revealed differences between populations, depending on the antibody used ($P < 0.05$; Table 1). Using the ∞PPORS pAB led to greater fluorescence score than using the ∞PP1 pAB. Fluorescent endospores were observed for the *P. ramosa* and for the PP3 populations after exposure to the preimmune rabbit serum, but the PBSTM negative control also resulted in one fluorescent *P. ramosa* endospore, out of the 40 endospores scored (data not shown). The *Pasteuria* endospores from the Borth sand dunes were not recognized by the ∞PP1 pAB, while both antibodies recognized the *P. ramosa* and the *P. penetrans* populations. The fluorescence scores obtained were significantly different for each of the *Pasteuria* species ($P < 0.01$) but not for different populations within each species.

Discussion

The immunofluorescence method described here permitted the detection of endospores in suspensions, even when these contained organic matter and other debris; it also facilitated

Table 1. Fluorescence scores of *Pasteuria* spp. endospores after staining for immunofluorescence, with antibodiesPP1 pAB orPPORS pAB

		Pasteuria populations					
			P. penetrans				
		P. ramosa	RES147	PPE	PP3	PPBsc	*Pasteuria* sp.[‡]
Antibody	Antibody dilution*	Fluorescence score[†]					
(Preimmune serum)	1/500	1.27 ± 0.32 f	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
	1/1000	0.66 ± 0.94 e	0.00 ± 0.00 a	0.00 ± 0.00 a	0.521 ± 0.76 e	0.00 ± 0.00 a	0.00 ± 0.00 a
PP1 pAB	1/500	2.16 ± 0.02 d	1.91 ± 0.08 c	1.81 ± 0.14 cf	1.49 ± 0.00 cf	1.49 ± 0.00 cf	0.00 ± 0.00 a
	1/1000	2.06 ± 0.03 d	1.88 ± 0.04 c	1.59 ± 0.37 cf	0.00 ± 0.00 a	1.18 ± 0.20 f	0.00 ± 0.00 a
PPORS pAB	1/500	2.80 ± 0.14 b	2.79 ± 0.08 b	3.04 ± 0.10 b	3.22 ± 0.02 b	3.24 ± 0.03 b	3.11 ± 0.04 b
	1/1000	2.65 ± 0.08 bd	2.27 ± 0.17 d	2.49 ± 0.26 bd	3.00 ± 0.03 b	3.12 ± 0.07 b	2.92 ± 0.03 b
Grand mean		1.93 a	1.48 b	1.49 b	1.37 b	1.50 b	1.01 c

*Volume of antibody serum : volume of PBSTM.

[†] Mean ± standard deviation of two replicates, each consisting of 20 observations; values followed by the same letter in both columns and rows are not significantly different after ANOVA and according to LSD values at a 5% level.

[‡] *Pasteuria* sp. indigenous to Borth.

the identification of *Pasteuria* endospores in natural populations. The threshold of detection for this method was lower than for other methods, which varied from a minimum of an estimated 100 endospores g^{-1} in soil using PCR with specific primers (Duan *et al.*, 2003), to about 300 endospores g^{-1} for antibody-based ELISA techniques (Schmidt *et al.*, 2003), and up to 1000 endospores g^{-1} (Fould *et al.*, 2001) and above. Most authors do not present an overall efficiency estimate, which for our work was key as it enables quantification; using a similar method but at very large spore densities (Fould *et al.*, 2001), 94% of all spores were extracted from soil, but the immunodetection method is comparable with the 59% estimate obtained after a complex differential centrifugation extraction (Davies *et al.*, 1990). Bayesian statistics was particularly useful in determining these parameters, as the linear regression curve calculated with usual frequentist statistics was inappropriate for their estimation. This was due to higher than expected concentrations being detected for low initial concentrations of endospores in the standard curve, and therefore correspondingly lower than expected concentrations of endospores for higher initial concentrations. The same effect has been reported before (Fould *et al.*, 2001), however in this case we believe it to be due to and inherent counting error, as Bayesian estimates within the same normal distribution of observed values provided a corrected and workable curve. The observed number of endospores in the three different substrates used to optimise the quantification method did not differ significantly.

The immunodetection method combined a low threshold of detection with quantification using relatively small sample volumes (0.5 g sand in 1 mL suspension) and maintained versatility in terms of *Pasteuria* populations detected. The suspension preparation was simplified and standardised and the overall quantification procedure took a maximum of 2 h of active working time for each population being analysed. The dune sand substrate does not affect endospore extraction or their surface properties, and does not interfere with the activity of the antibodies used, as revealed by the absence of significant differences between the uses of the different substrates. The method was, therefore, considered valuable for screening and quantification of *Pasteuria* endospores in sand dunes.

This method revealed the unexpected high concentrations of endospores in the Borth sand dunes, a natural system with presumably low primary production, and in which numbers of nematodes are lower than in agricultural systems. The *Pasteuria* showed great variability in numbers between and within samples. This variability is thought not to be related to the methodology, but to the spatial patchiness of the population in dune systems.

Various populations of *Pasteuria* from different geographical and host origins were characterized using the two polyclonal antibodies, ∞PP1 pAB and ∞PPORS pAB. The ∞PP1 pAB did not react to the *Pasteuria* from Borth, allegedly a parasite of *Pratylenchus*, *Tylenchorhynchus* and omnivorous Dorylaims. As we could not identify individual endospores in the debris-filled suspension for fluorescence scoring, we base this result on the lack of observed fluorescence in the suspension, and so we cannot exclude the possibility that this may be a false negative result. However, a similar result has been obtained for *Pasteuria* parasites of ectoparasitic nematodes (Fould *et al.*, 2000). The *P. penetrans* population PP1, to which the antibody was raised originally, has been described as having a highly heterogeneous surface (Davies *et al.*, 1994). The fact that ∞PP1 pAB recognized endospores from a *P. ramosa* population, with a completely different host is puzzling, and we speculate that *Pasteuria* parasites of vermiform plant-parasitic nematodes may be more differentiated from the core of the *Pasteuria* group than previously acknowledged. This could not be confirmed as we have performed this method with a limited set of *Pasteuria* populations. Literature on phylogeny within the *Pasteuria* group is lacking. It is a common aspect of a few 16S rRNA gene studies that *P. ramosa* branches from the *Pasteuria* group of nematode parasites and that this group is divided in two clades, one of each containing mainly *P. penetrans* that are separated from *Pasteuria* parasites with other nematode hosts (Anderson *et al.*, 1999; Bekal *et al.*, 2001; Duan *et al.*, 2003; Preston *et al.*, 2003; Trotter & Bishop, 2003). We have only found one reference to 16S rRNA gene sequence analysis incorporating *Pasteuria* sp. parasitic on free-living nematodes, and these populations branched out of those parasitic on plant-parasitic nematodes (Sturhan *et al.*, 2005). Although all these phylogeny analyses were performed with a limited number of populations, and most of the populations analysed belonged to *P. penetrans*, their results show the same trend.

The characterization method provided different quantitative fluorescence scores for different *Pasteuria* species according to their hosts, but did not vary significantly between different populations within the same species. *Pasteuria* endospores will show differences in their outer coat depending on the nematodes they parasitise (Ciancio, 1995b). The antibodies used recognized the *P. penetrans* endospore surface and they are expected to react differentially to the parasporal fibres of different populations. The fact that they consistently did so for the populations tested as analysed by the fluorescence score, indicates that this method can be useful for the characterization of *Pasteuria* populations. To date there is no evidence that the 16S rRNA gene sequences show sufficient variation to relate to endospore host specificity whereas antibodies have this capability.

Other studies have focused on immunofluorescence methods of evaluating virulence in *Pasteuria* populations

(Davies *et al.*, 1994; Davies & Redden, 1997; Schmidt *et al.*, 2003). The method we describe is not sensitive enough for this, but the fluorescence score provides an indication of heterogeneity of endospore populations. Other authors have qualitatively classified endospore fluorescence within *Pasteuria* populations, in an attempt to characterize not only the heterogeneity of the populations but also the discriminatory ability of the antibodies used (Davies & Redden, 1997; Fould *et al.*, 2001). The fluorescence score seems to be a good tool to quantify these variations, but care should be taken in selecting the particular polyclonal antibodies. The ∞PP1 pAB did not react at all with Borth endospores, but successfully differentiated populations, whereas the ∞PPORS pAB showed a constant result across populations and also elicited a higher fluorescence score. Such an antibody is recommended for use in endospore detection and quantification, particularly at the antibody concentration 1/500, as there was no significant difference between the reactions to different populations at this dilution.

In summary, we have shown that immunological techniques can be exploited to quantify scarce endospores in soil, and to characterize different endospore populations from different hosts.

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