

Biocontrol potential of *Pasteuria* spp. for the management of plant parasitic nematodes

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Abstract

Plant parasitic nematodes represent a severe threat for agriculture, causing yield losses for several food and industrial crops, worldwide. Actually, increasing demand for organic food products and for sustainable management practices requires the development of biocontrol approaches based on suitable antagonists. In this review, some traits of members of the bacterial genus *Pasteuria* are discussed, focusing on their biology and taxonomy, host range, specificity and application for host nematode regulation. Given the high specificity of *Pasteuria* spp. and the biodiversity recognized within species, the exploitation of these parasites requires the collection of data on the isolates most suitable for practical use. Some traits of *Pasteuria* spp. biology appear favourable for biocontrol applications, such as the minimum impact on the other soil microorganisms and invertebrates, the durability of endospores, together with host specificity and regulation capability. Experimental evaluation of host–parasite compatibility for biocontrol purposes is a pre-requisite necessary for practical use. Isolates selection and evaluation may represent an additional cost in the development of commercial products and bioformulations based on mass cultivation of these bacteria. The *Pasteuria* spp. associated with nematode pests represent a valuable and widespread natural resource, whose benefits and application potential are not yet completely explored.

Keywords: Phytonematodes, Management, *Meloidogyne*, Rhizosphere, Soil bacteria

Review Methodology: The data examined proceed from articles published on the subject, identified through queries on internet search engines or using the following databases: CAB Helminthological Abstracts, EFSA, NCBI and Medline. The generic keyword search terms '*Pasteuria*' and/or 'nematode' were mostly used. References from the articles found were also used to get other data of interest, but available reviews were not discussed to avoid redundant comments. Further information and data proceed from previous research work of the author or were provided directly by colleagues, abstracts or upcoming studies not yet published.

Introduction

The genus *Pasteuria* (Firmicutes: Pasteuriaceae) includes G+ fastidious and aerobic endosymbionts of nematodes or crustaceans [1]. Members of this bacterial lineage are obligate parasites. They persist in soil or benthic microcosms as durable and resting endospores, dispersed by decaying hosts. Apart from *Pasteuria ramosa*, parasitic in water flies (*Daphnia* spp.), all the other species have been reported thus far from phytoparasitic or free-living host nematodes [2–7]. Nematode-parasitic *Pasteuria* spp. have been investigated in detail in the last decades, due to their potential as biocontrol agents of important pests [2, 4, 5].

Pasteuria penetrans, the first species described from nematodes, is an effective parasite of species within the genus *Meloidogyne* (root-knot nematodes, RKN).

The endospores produced by *Pasteuria* spp. are both a resting propagule, highly resistant to adverse conditions such as high temperature or desiccation, and an infective stage responsible for the parasite horizontal transmission. The parasite multiplies in the host body and eventually originates a sporulation phase. Sporulation is completed inside the host, usually after partial or total consumption of its body content, resulting in a dramatic reduction of its fecundity and reproductive capacity. Depending on the species, nematode parasitic *Pasteuria* spp. complete their

life cycles and sporulate within the adults (*P. penetrans*, *P. nishizawae*) [2, 4], juveniles [7–9] or both stages [7, 10].

Morphology and Life Cycle

The *Pasteuria* endospores are the stage most commonly encountered during surveys or when assessing prevalence in a host population. They may be identified due to their peculiar shape by using a stereoscope, or better by light microscopy examination at 100–250 \times . The nematode inspection may be performed on a temporary slide in a water drop, examining the inner body tissues and/or the cuticle surface to which the propagules adhere (Figure 1).

Detailed data on endospores morphology have been obtained from electron microscopy studies [2, 4, 5, 8]. In nematode-parasitic *Pasteuria*, the endospore is generally structured in a central, multilayered core (the true endospore) with surrounding layers of epicortical parasporal fibres and outer episporic coats. Details on terminology and structural components of *Pasteuria* spp. endospores are available in the literature [2, 4]. The endospore structure and shape appear relatively conserved among the nematode parasitic lineages, but differences may be observed among species, concerning the inner and outer core layers and the organization of the episporic fibres (Figure 2).

The endospores have a typically rounded or cup-like aspect. This ‘aerodynamic’ shape allows resistance to the forces produced on their surface by the moving host, when host adhering endospores match the soil particles. The forces acting on the endospore surface include in fact a component perpendicular to its adhesion plane, likely contributing to or reinforcing adhesion. Usually, host attachment is by the concave, basal endospore side. Adhesion by the convex or lateral sides, however, may also be observed [11]. This often occurs in nematodes highly encumbered with endospores, but the host movements usually facilitate the propagules re-positioning and their subsequent adhesion by the basal side.

The endospore cytoplasm is surrounded by a plasma membrane and a number of concentric walls with different electron densities (Figure 2). These include the cortex, an electron-dense layer directly in contact with the protoplasm membrane. The inner and outer layers are surrounded by a peripheral epicortical layer, and have taxonomic significance in relation to species [4]. Morphological differences in the layers surrounding the central core and cortex have been described. In some species, such as *P. penetrans*, an interruption in the basal layers facilitates the extrusion of the vegetative peg at germination. The cortex is responsible for properties such as durability and resistance. Analysis of *Pasteuria* endospore showed the presence of dipicolinic acid, a structural component found in several spores-forming bacteria, such as members of genera *Bacillus* and *Clostridium* [12].

Pasteuria spp. are specific parasites characterized by a narrow host range. Host adhesion is mediated by the episporic fibres, attached to the central core (Figure 2). They are organized in the basal and surrounding layers whose fibres appear as collagen-like structures, distinguished by their electron densities [4, 9]. Polymorphic collagen-like proteins (PCL) have been characterized in *P. ramosa* and found to be involved in host specificity at the level of single bacterial clones. This mechanism is based on the recombination of repeated PCL gene variants, conferring a genotype-level match between host and parasite clones [13]. Similar collagen-like structures have been reported also in nematode-parasitic *Pasteuria* spp., and are considered as the main factor involved in host adhesion and specificity [14, 15].

The endospore diameters vary among species and populations. For small size *Pasteuria* spp., they range from 2.0–2.8 μm for a species parasitic in *Heterodera cajani* [16] to 3.0–3.5 μm , observed in an undescribed *Pasteuria* sp. parasitic in juveniles or males of the citrus nematode *Tylenchulus semipenetrans* (Figure 2a). Medium-size diameters are around 3.4–4.1 μm , reported for *Pasteuria thornei* parasitic in *Pratylenchus brachyurus* or 4.0–4.5 μm , reported for *P. penetrans* or other related taxa [2, 4, 6]. Larger propagules up to 6.0–7.5 μm or more can be found among species associated with large-size nematodes such as *Xiphinema* (Figure 2d), *Longidorus* spp. or other dorylaims [7]. Light microscopy observations on parasitized nematodes or specimens from permanent slide collections showed a link between the endospore dimensions and the cuticle and hypodermis thickness of the corresponding hosts [7]. This correlation fits Haldane’s law linking host and parasite sizes, indicative of an evolutive race of the Red Queen type, as also shown for *P. ramosa* and *Daphnia* spp. [17–19] and likely related to the parasite transmission mode. The Red Queen model considers the selective pressures exerted by the parasite on the host population, resulting in the selection of a few mutant individuals capable to escape parasitism. Similarly, the host population acts on the parasite mutants, favouring the insurgence of new virulent or more adaptive parasitism characters.

Pasteuria spp. transmission occurs after the exosporium-coated endospores are dispersed and activated in the nematodes environment (mostly soil and roots microcosms). The endospores are released following the rupture or decomposition of diseased hosts or carcasses. Once free from the exosporium, the non-motile endospores passively adhere to the cuticle of nearby susceptible hosts moving in soil [2–4]. At germination, the peg extruded from the central core penetrates the host cuticle and hypodermis. This is the first vegetative and infection stage. It originates further cells arranged in dichotomic, branched and septate mycelium-like thalli, which spread the infection inside the host by fragmentation. The factors triggering germination are not yet known, but they are putatively related to the biochemical changes occurring at the host–endospore interface, and likely involve one or more, specific signal

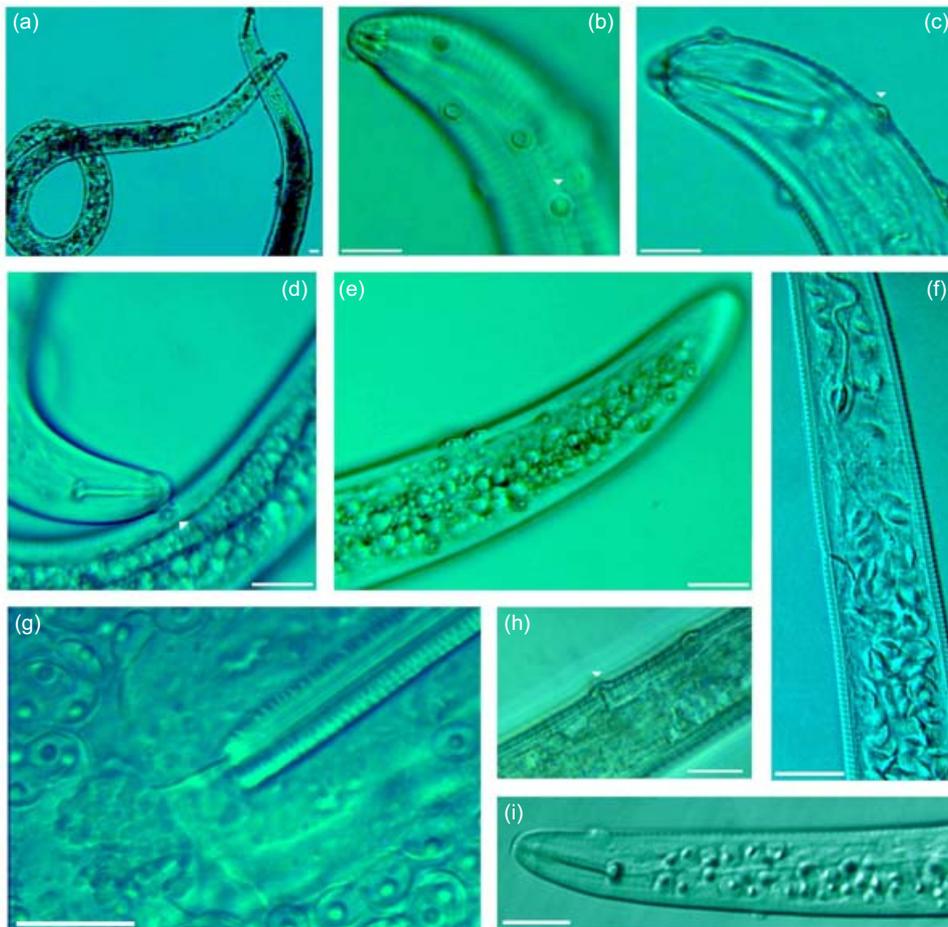


Figure 1 Endospores of *Pasteuria* spp. bacterial parasites adhering to or within host nematodes. Endospore-encumbered living *Helicotylenchus* sp. in water mounts (a). The cup-shaped aspect of endospores (arrowhead) is visible in top (b) or lateral views (c). Specimen of *Tylenchorhynchus ventrosignatus* scrubbing its cuticle to remove endospore attached at mid body (d) and tail (e). Endospore-filled *Dolichodorus* sp. (f), *Labronema* sp. (g), and juvenile *Tylenchulus semipenetrans* (i). Germinating endospore penetrating the cuticle of a juvenile *Heterodera goettingiana* (h). The nematodes proceeded from populations collected at Martina Franca (a–c), Valenzano (g), Pontecagnano (h), and Racale (i), Italy; Müncheberg, Germany (d, e), and Tarapoto, Peru (f). Scale bars = 10 μ m.

molecules. The endospore activation and induction to germinate, however, may be independent from the host metabolism. Germination has been also observed in endospores adhering to already parasitized nematodes, such as juveniles of *Heterodera goettingiana* or specimens of *Tylenchorhynchus cylindricus*, that were already filled with propagules, originated by a previous parasitic cycle [9, 10]. Other hypotheses consider a direct effect of root sap on the germination of *Pasteuria* spp. from endoparasitic and sedentary hosts. There are no detailed experimental data on this subject indeed, as these assays are difficult to set up. However, it is worth to note that many *Pasteuria* spp. attack free-living hosts such as plectid, bacteriovorous or predatory nematodes, which do not feed on roots [6, 7]. A further possibility is that different germination factors may be active among the bacterium lineages. In any case, germination has been always observed on endospores adhering to their hosts' cuticle.

After germination, the vegetative stages fill, at various extents, the host body (Figure 1). Sporulation is the last stage, and originates in cell pairs arising after lysis of intermediate cells in thalli. The endospore precursors are arranged in tetrads and then pairs or in clusters, such as in *Pasteuria hartismerii* [4, 6, 20, 21]. They show an asymmetric cell division, in which the endospore matures inside the enlarged terminal cell, whose envelope forms the exosporium, maintained until release from the host [4, 6, 8, 9]. Several genes, including some active in sporulation, have been sequenced in *Pasteuria* spp. using available sequences from *Bacillus* spp. (Table 1) [22]. Sporulation is likely induced by the exhaustion of some nutrients provided by the host or by other metabolic processes such as metalloregulation, which trigger sporulation through an increase in bivalent ions concentration [22]. A single parasitized female of RKN may produce up to $2.0\text{--}2.5 \times 10^6$ endospores of *P. penetrans*, a number

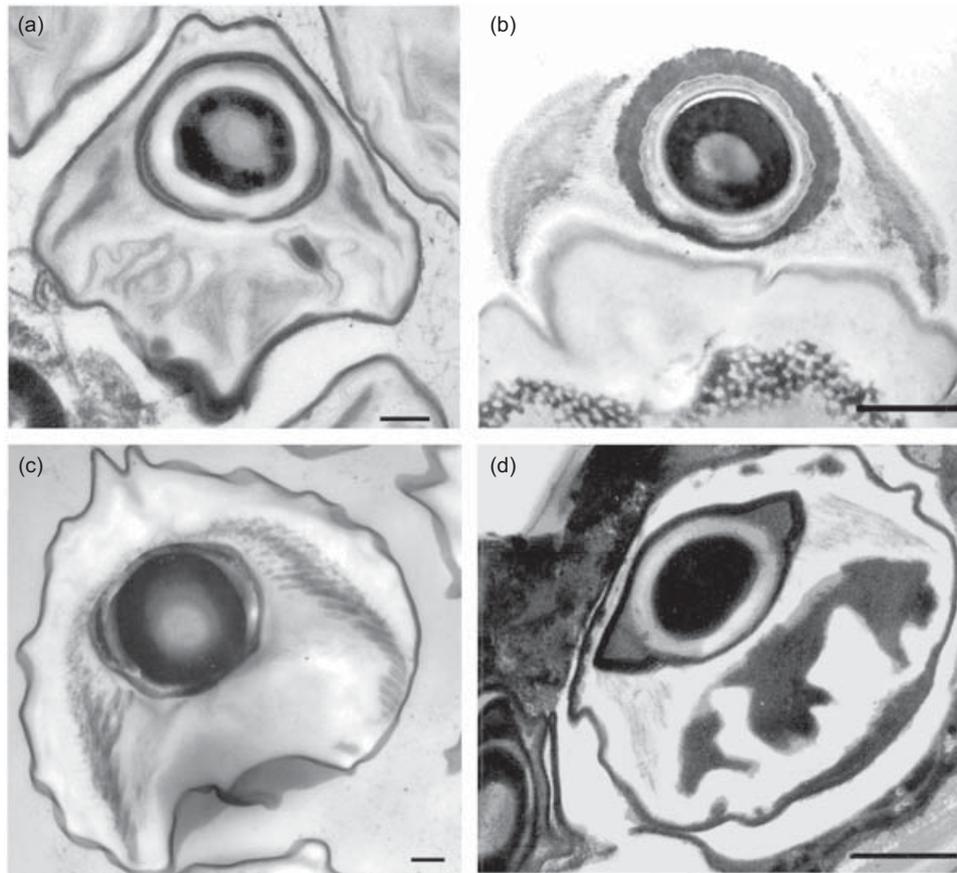


Figure 2 Transmission electron microscopy images of transversal sections of undescribed *Pasteuria* spp. endospores showing central core layers and episporal fibres. The images were produced from: juveniles of *Tylenchulus semipenetrans* (a), *Heterodera goettingiana* (b), specimens of *Discolaimoides bulbiferus* (c) and *Xiphinema pachtaicum* (d). The nematodes proceeded from populations collected in Italy at Racale (a), Pontecagnano (b, c) and Turi (d). Scale bars = 300 nm (a, c), 1 μ m (b, d).

sufficient to infect thousands of nematode juvenile stages [3]. A few hundred endospores are produced by species parasitic in juveniles or vermiform nematodes.

Taxonomy and Phylogeny

Anteriorly to its definitive affiliation to the genus *Pasteuria* [2], *P. penetrans* was moved from *Dubosqia* (a microsporidian genus in which it was originally placed by G. Thorne) to the genus *Bacillus* [23]. Subsequent analyses based on 16S rRNA ribosomal gene sequence data showed the proximity of *P. penetrans* to the family Bacillaceae [24–26]. However, other observations showed a closer proximity of *Pasteuria* spp. to members of the genus *Thermoactinomyces* [6]. Subsequent studies based on the RAxML algorithm analysis using thousands of bacterial taxa showed a positioning within the family Pasteuriaceae, with closest proximity for members of family Thermoactinomycetaceae (genera *Seinonella*, *Laceyella* and *Thermoactinomyces*), followed by Bacillaceae, Paenibacillaceae and Clostridiaceae at a higher distance [1].

Due to their specificity and biodiversity, the radiation of *Pasteuria* spp. can be considered of the co-evolutive type and likely congruent with the hosts phylogeny. However, differences in virulence and specificity have been observed among endospore populations or when considering host species. The *P. ramosa* and *Daphnia* interactions actually represent a model for the study of genetic recombinations and host genotypes interactions, including evolutionary mechanisms underlying the host and parasites persistence, and related genetic races [17–19].

Actually, the identification of *Pasteuria* spp. relies on host inspection and/or on sequencing the 16S rRNA ribosomal gene [27]. Sequenced isolates or species show a 20 nt motif (5' CATTCTTCTTCCCGATG 3') in the V3 region of the 16S rRNA ribosomal gene [28] that favours PCR amplification from a reduced number of endospores or a few nematodes, by offering forward and reverse regions for primers [27].

Apart from *P. penetrans*, the other *Pasteuria* spp. with a potential in nematode biocontrol are: *P. thornei* described from *P. brachyurus* [5]; *P. nishizawae*, parasitic in females of the soybean cyst nematode *Heterodera glycines* [4];

Table 1 A summary of *Pasteuria* spp. sequences available in GenBank (NCBI) related to sporulation and metabolism

Gene data and sequence accession number	Length (bp)	Product	Pathway	Activity/annotations reported
<i>Pasteuria penetrans</i> strain P20 coproporphyrinogen III oxidase (hemN) gene, partial cds AY570914.1	341	Coproporphyrinogen III oxidase	Haeme biosynthesis	One of the last three pathway enzymes, catalyses in two sequential steps the oxidative decarboxylation of two of the four propionate sides chains on coproporphyrinogen-III, to yield protoporphyrinogen-IX Scavengers of Fe ³⁺ ions
<i>Pasteuria penetrans</i> strain P20 putative siderophore biosynthesis protein (frgA) gene, partial cds AY570910.1	472	Siderophore biosynthesis protein	Iron metabolism	
<i>Pasteuria penetrans</i> RNA polymerase sigma-G factor (sigG) gene, partial cds AY580166.1	351	RNA polymerase sigma-G factor	Sporulation	Sigma factors are initiation factors promoting the attachment of RNA polymerase to specific initiation sites and are then released. This sigma factor is responsible for the expression of sporulation-specific genes in the forespore
<i>Pasteuria penetrans</i> strain PP3 sporulation sigma factor SigE (sigE) gene, partial cds HQ849303.1	260	RNA polymerase sporulation-specific sigma-E factor	Sporulation	–
<i>Pasteuria penetrans</i> strain P20 putative D-alanyl-D-alanine-carboxypeptidase (dacF) gene, partial cds AY570909.1	237	D-alanyl-D-alanine-carboxypeptidase	Peptidoglycan chains	Cross-links chains to form rigid cell wall
<i>Pasteuria ramosa</i> SpoIIAA (spolIIAA) and SpoIIAB (spolIAB) genes, partial cds AY569335.1	574	Sporulation protein	Sporulation	Produced in the mother cell chamber of sporangium during sporulation
<i>Pasteuria penetrans</i> spo0A gene, strain Pp1 AJ550852.1	783	Sporulation protein	Sporulation	Involved in the initiation of sporulation
<i>Pasteuria penetrans</i> SpoIIAA gene, partial cds; SpoIIAB gene, complete cds; and SigF gene, partial cds AF483656.1	802	Sporulation protein	Sporulation	Expressed early during sporulation
<i>Pasteuria hartismeri</i> strain PH1 sporulation protein SpoIIAB (spolIAB) and sporulation protein SpoIIAA (spolIIAA) genes, partial cds HQ849347.1	602	Sporulation protein	Sporulation	Expressed early during sporulation, regulatory protein
<i>Pasteuria ramosa</i> SigE gene, partial cds AY713480.1	296	Sigma E sporulation factor	Sporulation	–
<i>Pasteuria penetrans</i> strain PP3 carboxy-terminal processing protease (yvjB) gene, partial cds HQ849339.1	202	PD Zn metalloprotease, carboxy-terminal processing protease	Protein-binding site	Involved in signalling and regulatory mechanisms
Gene		Product	Pathway	Activity/annotations reported
<i>Pasteuria penetrans</i> strain Thies carboxy-terminal processing proteinase (ctpA) gene, partial cds HQ849331.1	367	C-terminal processing peptidase family S41	A carboxypeptidase, involved in degradation of proteasomal products	In tricorn core protease: the active site is a tetrad (serine, histidine, serine, glutamate)

Table 1 (Continued)

Gene data and sequence accession number	Length (bp)	Product	Pathway	Activity/annotations reported
<i>Pasteuria hartismeri</i> strain PH1 DNA gyrase subunit B (gyrB) gene, partial cds HQ849355.1	1183	DNA gyrase subunit B	TopoIIA_Trans_DNA_gyrase: transducer domain, having a ribosomal S5 domain 2-like fold; found in proteins of the type IIA family of DNA topoisomerases, similar to the B subunits of <i>Escherichia coli</i> DNA gyrase	Topoisomerase-primase domain, a nucleotidyl transferase/hydrolase domain found in type IA, type IIA and type IIB topoisomerases, bacterial DnaG-type primases, small primase-like proteins from bacteria and archaea
<i>Pasteuria penetrans</i> strain P20 YacL (yacL) and 4-diphosphocytidyl-2C-methyl-D-erythritol synthase (yacM) genes, partial cds AY570908.1	521	Operon	Hypothetical proteins	–
<i>Pasteuria penetrans</i> strain RES148 phenylalanyl-tRNA synthetase β subunit (pheT) gene, partial cds HQ849315.1	473	Phenylalanyl-tRNA synthetase β subunit	Anticodon binding domain found in phenylalanyl tRNA synthetases, has a ferredoxin fold, with an $\alpha + \beta$ sandwich with anti-parallel β -sheets ($\beta - \alpha - \beta x^2$)	Ferredoxin-fold anticodon binding domain
<i>Pasteuria penetrans</i> strain PP3 cytochrome c oxidase subunit II (ctaC) gene, partial cds HQ849311.1	267	Cytochrome c oxidase subunit II	Component of the respiratory chain	Involved in the transfer of electrons from cytochrome c to oxygen
<i>Pasteuria penetrans</i> strain Thies β -ketoacyl-acyl-carrier protein synthase II (fabF) gene, partial cds HQ849295.1	490	β -ketoacyl-acyl-carrier protein synthase II	Fatty acid biosynthesis	Responsible for elongation steps in fatty acid biosynthesis
<i>Pasteuria penetrans</i> ATP synthase C subunit (atpE) gene, partial cds; ATP synthase B subunit (atpF) gene, complete cds; and ATP synthase δ subunit (atpH) gene, partial cds AY497316.1	758	ATP synthase C,B, δ subunits	ATP synthesis	Membrane-bound enzyme complexes/ion transporters that combine ATP synthesis and/or hydrolysis with the transport of protons across a membrane
Gene		Product	Pathway	Activity/annotations reported
<i>Pasteuria penetrans</i> strain PP3 ATP synthase subunit b (atpF) gene, partial cds HQ849289.1	245	ATP synthase subunit b	Part of the CF(0) (base unit) of the ATP synthase.	Translocates protons through membrane
<i>Pasteuria ramosa</i> DNA polymerase III PolC-type (polC) gene, partial cds EF529446.1	227	DNA polymerase III PolC-type	DNA synthesis	Required for replicative DNA synthesis
<i>Pasteuria ramosa</i> para-aminobenzoate synthase-like (pabA) gene, partial sequence EF529447.1	259	Para-aminobenzoate synthase-like	Cofactor biosynthesis; tetrahydrofolate biosynthesis; 4-aminobenzoate from chorismate	In <i>Bacillus</i> it catalyses the biosynthesis of 4-amino-4-deoxychorismate (ADC) from chorismate and glutamine
<i>Pasteuria ramosa</i> PclA gene, complete cds EU309708.1	1878	Collagen-like protein A	Polymorphic collagen-like surface proteins	–

<i>Pasteuria ramosa</i> isolate P1 tRNA-Ser gene, complete sequence EF203481.1	500	Transfer RNA (tRNA)	Transfer RNA (tRNA) show a secondary structure made up of three hairpin loops and a terminal helical stem (cloverleaf) with L-shaped tertiary structure	The main functional regions in tRNA are the anticodon triplets which read the messenger RNA (mRNA) codons and the 3' CCA nucleotides where an amino acid cognate to the tRNA is attached
<i>Pasteuria ramosa</i> isolate P1 RpsA (rpsA) gene, complete cds EF203477.1	1179	30S ribosomal protein S1	Component of the small ribosomal subunit	Thought to be involved in the recognition and binding of mRNAs during translation initiation
<i>Pasteuria ramosa</i> isolate P1 PlsC (plsC) gene, partial cds EF203475.1	396	1-acyl-sn-glycerol-3-phosphate acyltransferase	Glycerophospholipid biosynthesis	Lysophospholipid acyltransferases (LPLATs)
<i>Pasteuria ramosa</i> isolate P1 PheS (pheS) gene, partial cds; and PheT (pheT) gene, complete cds EF203478.1	3290	Phenylalanyl-tRNA synthetases α and β subunits	Aminoacyl-tRNA synthetases	Phenylalanyl-tRNA synthetase (PheRS) α chain catalytic core domain. PheRS belongs to class II aminoacyl-tRNA synthetases (aaRS), based upon its structure and the presence of three characteristic sequence motifs
<i>Pasteuria ramosa</i> isolate P1 MiaB (miaB) gene, complete cds EF203476.1	1086	2-methylthioadenine synthetase	Radical SAM superfamily	Enzymes of this family generate radicals by combining a 4Fe-4S cluster and S-adenosylmethionine (SAM) in close proximity

Candidatus Pasteuria usgae, parasitic in the sting nematode *Belonolaimus longicaudatus* [29]. Other taxa have been also described from RKN such as *Candidatus P. hartismeri* [21] or *Candidatus Pasteuria aldrichii* from a bacteriovorous *Bursilla* sp. [26]. The 16S rRNA ribosomal gene sequence data also showed the occurrence of further taxa not yet described, including a '*Pasteuria goettingiana*' from a population of *H. goettingiana* found on faba bean at Pontecagnano, Italy (position: 40°39'14"N, 14°53'15"E) (NCBI accession AF515699) (Figure 2b). Sequencing indicated that it was a further species parasitic in cyst nematodes [27], differing from *P. nishizawae* and the *Pasteuria* sp. parasitic in *H. cajani* by completing its life cycle and sporulating inside the juveniles.

The difficulties encountered in culturing and/or the lack of deposited material limited the extensive study of *Pasteuria* taxonomy and phylogeny [1], and no public collection is available for this particular clade. The numerous isolates/populations are usually referred to the records from institutional laboratories and/or nematode collections and a few have been deposited, i.e. *Pasteuria* sp. culture ATCC PTA-9643 from a reniform nematode (Table 2). A public repository is helpful for the deposit of standards as taxonomic references, or when isolates are used for commercial purposes. Of the many *Pasteuria* lineages/species reported thus far in association with nematodes, only a few are geo-localized [30, 31], whereas whole-genome sequencing attempts have been reported only for a few isolates.

Distribution and Host Range

Members of *Pasteuria* are widespread and have been reported from all continents, including extreme environments such as hydrothermal vent sediments off the West Antarctic Peninsula [32]. Several species have been found in relation to nematological surveys, or when examining field nematode populations and collections. Surveys have been carried out in Australia, Florida, Hawaii, Spain, Russia and many other countries [3, 33–37]. Sampling in host speciation zones may be helpful when searching for new isolates or populations for practical purposes. The RKN-parasitic *P. penetrans* has been frequently reported from tropical areas, ranging from Senegal and other West Africa regions to the Caribbean, including Puerto Rico, Martinica, Cuba, Southern USA or South America [2, 8, 36, 37]. Tropical areas are considered as speciation zones for *Meloidogyne incognita* and other RKN, and were connected in the Pangean pre-oceanic division, around 180 million years ago. Similarly, a *Pasteuria* sp. parasitic in juveniles of the potato cyst nematode (PCN) *Globodera pallida* was found to be endemic in the central Andean region in Peru, a speciation area for potato and PCN [38], whereas a further *Pasteuria* sp. is associated to the pigeon pea cyst nematode *H. cajani*, endemic in India [11, 16].

Table 2 A summary of international patents concerning *Pasteuria* spp¹

Patent title	Code	Content description
Novel <i>Pasteuria</i> strain and uses thereof	US2012114606	Novel <i>Pasteuria</i> with nematicidal activity against lance nematodes
Materials and methods for controlling nematodes with <i>Pasteuria</i> spores in seed coatings	CA2950287	Control of nematodes by attaching an effective amount of <i>Pasteuria</i> spores to a seed delivered to nematodes in site
Novel <i>Pasteuria</i> strain	US2010189693	Novel <i>Pasteuria</i> with activity against reniform nematodes; culture referred to as ATCC PTA-9643
Materials and methods for controlling nematodes with <i>Pasteuria</i> spores in seed coatings	AU2009335725	Materials and methods for control of nematodes by attaching an effective amount of <i>Pasteuria</i> spores to seeds delivered to nematodes
Method for detecting <i>Pasteuria penetrans</i> by virtue of fluorogenic quantitative PCR	CN104745682	Method for detecting <i>P. penetrans</i> by fluorogenic quantitative PCR, using the single copy spoIIAB gene as a target
Combined composition, which comprises a pesticidal agent for controlling pests, and methods to use thereof	UA105163	Composition combining a pesticide, at least one nematicide, wherein at least one nematicide is an avermectin, and at least one biological agent selected from <i>Pasteuria</i> spp
Pesticidal mixtures comprising isoxazoline derivatives	US2013261069	A mixture comprising some pesticides and biocontrol agents including, among others, <i>Pasteuria</i> spp
Nematicidal agent combinations comprising Fluopyram and <i>Bacillus firmus</i>	EP2564698	A mixture comprising some pesticides and biocontrol agents including, among others, <i>Pasteuria</i> spp
Slow-release biodegradable granules of <i>P. penetrans</i>	ZA9110063	na
Materials and methods for the efficient production of <i>Pasteuria</i>	US2004137600	Method for growing parasitic bacteria <i>in vitro</i> , without the presence of host tissue. <i>Pasteuria</i> spores, such as those infecting <i>Meloidogyne arenaria</i> or other host nematodes, are grown <i>in vitro</i> , in the absence of nematode tissue
Monoclonal antibody probe for the detection of adhesins associated with mature endospores of <i>Pasteuria</i> spp	US6569632	Method for the identification of <i>Pasteuria</i> endospores in soil and soil extracts
Materials and methods for <i>in vitro</i> production of bacteria	US2003232422	Method for growing parasitic bacteria <i>in vitro</i> , without host tissue, such as <i>Pasteuria</i> endospores, infecting <i>M. arenaria</i> or other host nematodes, grown <i>in vitro</i> under acidic conditions in the absence of nematode tissue
Controlling a plant parasitic nematode with <i>Pasteuria</i>	US5593668	Formulation for controlling a plant parasitic nematode, with <i>Pasteuria</i> spp. suspended in water

¹Source: EPO; na = not available.

Several plant parasitic or free-living nematodes have been listed as hosts of one or more *Pasteuria* spp. Data have mostly been produced when examining permanent nematode collections or resulted from taxonomy studies or survey activities, highlighting endospore diversity and morphometric variations, as well as host ranges [7, 8]. Actually, several host associations are reported, but the number of *Pasteuria* species or isolates is expected to increase, largely depending on the frequency of nematological studies, in particular from unexplored environments.

Biocontrol and Nematode Regulation

The potential of *P. penetrans* for biocontrol of RKN has been recognized since the mid-1970s. Several studies, carried out worldwide, in controlled or field conditions, consistently showed the efficacy of one or more isolates/

species on RKN or other host pests [2, 3, 23, 30, 36]. Controlled assays also highlighted the effect of host specificity on biocontrol efficacy. Isolates of *P. penetrans* proceeding from different locations showed varying efficacy levels depending on host populations tested, indicating parasitic adaptations reflecting either the bacterium geographic origins and/or the host genetics [15, 39–42]. In a long-term study in Florida, the specificity of a *P. penetrans* population induced a shift in the nematode species composition in a field with different RKN species. The bacterium was found to suppress a susceptible *Meloidogyne arenaria* race 1 population, exerting a selective pressure favouring a second, less susceptible *M. javanica* population, in which it could not develop [36].

Host specificity cannot indeed be considered as a negative trait in biocontrol, because the narrow host range and trophic specialization of *Pasteuria* spp. spare other beneficial nematodes present in soil. It may have, however, a practical consequence with limitations in the

application of biocontrol products based on a single isolate of such specific bacteria. To be effective, *P. penetrans* and/or other species should be applied in numbers exceeding their natural field densities, using a combination of isolates suitable to cover the natural genetic variability in the target pest(s) to control. This implies a detailed knowledge about the nematodes susceptibility. A range of different isolates applied for biocontrol may in fact reduce the risk of selecting a less susceptible, or even resistant, host population. Host resistance mechanisms may be active at transmission and/or during infection. At transmission, depending on the cuticular determinants that allow the bacterium adhesion and subsequent host penetration. During infection, a number of defence mechanisms are known among nematodes, including the production of antimicrobial peptides and other innate defence responses [43]. Endospore attachment also appeared affected in soil by root exudates, proceeding either from nematode host and non-host plants [44, 45].

Laboratory assay showed a high degree of *Pasteuria* endospores persistence and durability. These properties allow conservation of the endospores in a lyophilized root powder containing parasitized RKN females, a method developed also to consistently apply the bacterium in biocontrol trials [3]. *Pasteuria* spp. populations persist for long time in their environment. This is a positive trait for biocontrol, since persistence may be required to keep a long-term biocontrol capacity. A population parasitic on the citrus nematode *T. semipenetrans* was detected in a citrus grove in Southern Apulia, more than 20 years after its initial discovery [31]. In a long-term study carried out in Piedmont (Italy) on a *Xiphinema diversicaudatum* population attacking peach and associated to an undescribed *Pasteuria* sp. [46], the bacterium population persisted for at least 20 years. The bacterium was also isolated from adjacent peach fields after the original tree crop was removed. Further examples confirm the stability of the host–parasite association, such as the re-discovery of a *Pasteuria* sp. parasitic in a population of *Robustodorus megadorus*, originally found in Utah in 1941. The bacterium was recovered on the same host by sampling the same area, more than 70 years after its first report [47].

Studies on *Pasteuria* parasitism ecology have been carried out in perennial crops infested by different nematode populations, by applying replicated temporal and spatial samplings. Population dynamics data have been produced for *Meloidogyne* spp. on kiwi in Spain and grapevine in India, *H. glycines* in the USA, the virus-vector *X. diversicaudatum* on peach and *T. semipenetrans* on citrus in Italy, each naturally regulated or suppressed by the *Pasteuria* spp. examined [31, 44, 48–52]. The basic hypothesis of these studies was that, due to specificity and obligate parasitism, the *Pasteuria* spp. could regulate host numbers at non-damaging levels. Data allowed evaluation of their suppression potential in natural conditions and the efficacy of regulation, including the

successful transfer of the antagonist in a previously uninfested field [51].

Detailed information on the mechanisms of regulation and on the factors affecting the *Pasteuria* efficacy could also be derived by applying non-linear, descriptive models [31, 49]. Field data from different studies confirmed the density-dependent relationship of *Pasteuria* spp. with their hosts. In naturally infested fields, however, the species studied could regulate host densities only after their populations peaked to high densities (in the order of $12\text{--}13 \times 10^3$ nematodes/100 cc of soil in the case of *T. semipenetrans*). The prevalence levels observed in natural conditions vary from 1 to 18% found for *M. incognita* on kiwi [48], to around 98% reported from other RKN by Mankau in Senegal [23]. For other hosts, the prevalence ranged from 1.5 to 21% for *X. diversicaudatum* [46], and from 0.8 to 14.0% for *T. semipenetrans* [31]. High prevalence levels, up to 80–90%, were also observed for juveniles of *H. goettingiana* parasitized by the undescribed *Pasteuria* sp. (data not shown).

Data on the density of *Pasteuria* spp. may provide useful indications about its regulation potential and multiplication. Direct methods based on monoclonal antibodies have been developed to determine the number of endospores in soil [53]. By applying Anderson and May model G (based on three equations accounting for the numbers of healthy or parasitized hosts and of parasitic propagules), the density of *Pasteuria* sp. from *T. semipenetrans* was estimated to be in the order of 1–10 propagules/cc of soil [31]. Using field data, a simple estimate of the endospore density in soil may be obtained considering the average number of host-adhering propagules (around 1–3 endospores per individual in the case of *T. semipenetrans*) and the average fraction of the host population with attached endospores [31]. A host population around 1500 nematodes/100 cc of soil moving towards roots may hence remove, from the same soil volume, an amount of spores in the order of 75–900 propagules. Considering that each infected *T. semipenetrans* juvenile or male produces around 200–250 propagules, the density of endospores released in the microcosm by infected nematodes in the previous cycle appears affected by removal processes other than host adhesion. These mechanisms likely include water percolation in soil pores and dispersal in deeper layers [54, 55]. A direct feeding by other microbiovorous soil invertebrates cannot also be excluded.

Models fitting population dynamics data showed that artificially increasing the number of endospores in soil may yield a delayed, dramatic effect on the host nematode density, with a significant decline expected a few months after application [31, 46]. Models also indicate the likelihood for a local extinction of the host nematode population, after a high-density increase. This factor is worth further investigations, since it may account for the evolutive pressure inducing *Pasteuria* spp. to develop long-lasting endospores for local persistence, a factor indirectly indicating its suppressive capacity.

Application and Formulation

A number of factors may affect the successful application of *Pasteuria* spp. and/or endospore-based formulations, apart from the compatibility between host and parasite populations. These factors include: soil inoculation modes, i.e. adding the endospores as a bioformulation, as a lyophilized powder of roots [3], by the introduction of *Pasteuria*-filled nematodes in soil or inoculation with pasteurized soil containing endospores; the effect of soil properties such as texture, salts content, irrigation and water percolation [54, 55]; the integration with chemical nematocides and the related tolerance level of the isolates used.

In the USA, the application of 1,3-dichloropropene (1,3-D) at 28 litres/ha for the control of RKN on cotton reduced the density of *P. penetrans* endospores in treated soils. Using a soil bioassay, however, the variation appeared less pronounced than the natural annual fluctuation in the endospore soil numbers [55]. Chloropicrin, but not 1,3-D, had a negative effect on *P. penetrans* when applied for the management of RKN on peanut [55]. In a greenhouse trial in Crete, endospores of *P. penetrans* from mixed populations of different origins were added at 25×10^3 propagules/g of soil for RKN management on tomato and cucumber. The treatments showed a synergy with applications of oxamyl and soil solarization, and additive effects [56].

Successful control of RKN through artificial inoculation of *P. penetrans* endospores elicited interest in commercial exploitation of some species. Industrial mass cultivation was a significant achievement allowing the development of *Pasteuria*-based products for field management of some economically important nematode pests [57]. A first progress in the comprehension of *P. penetrans* metabolism was achieved by the observation that sterilization of the nematodes (from which the *P. penetrans* vegetative forms were obtained) could interfere with the viability of the bacterium during first *in vitro* culture attempts. Fresh culture filtrates of *Enterobacter cloacae*, a bacterium often found in association with the cuticle of *M. arenaria*, also allowed a sustained growth of *P. penetrans* vegetative forms for approximately 3 months at low pH. However, the filtrates lost this capacity if stored at 4°C for a few days, likely due to the degradation or loss of a fundamental, but labile, component. Scientists of Pasteuria Bioscience Inc. (Alachua, FL, USA) eventually improved the system, cultivating the bacterium for a longer time in bioreactors with acidic submerged cultures. Further studies on industrial media led to mass culturing conditions inducing sporulation, thus allowing the first bioformulations based on the endospores produced on artificial media, that were viable and infective [57].

A bioformulation produced with a *Pasteuria* sp. parasitizing *Rotylenchulus reniformis* on cotton appeared as effective as an applied nematocide, in pot tests. The bacterium inoculum, however, had to be applied for seed

coating at a concentration of 10^8 endospores per seed [58]. Granular or liquid endospore suspensions with *P. usgae* showed effective biocontrol of *B. longicaudatus* in controlled conditions, with best performance obtained by a liquid formulation applied at 1.38×10^6 endospores/cm² of treated soil [59]. However, field treatments with a commercial product did not show sufficient control capacity of *B. longicaudatus*, in turf grass trials [60]. *Pasteuria nishizawae* has passed the EFSA evaluation for qualified presumption of safety. The decision was based on the obligate nature of nematode parasitism and the widespread occurrence of *Pasteuria* spp. in nature, none arising any safety concern [61].

The dimension of the world market of users promoted efforts and applied research because of the commercial potential initially considered for *Pasteuria*-based bioformulations. The initial enthusiasm for the progress in culturing has been eventually balanced by the observation that the high degree of diversity and host specificity, observed at the nematode and *P. penetrans* population levels [62], likely act as a limiting factor for the widespread exploitation of isolates, in particular from the big industry standpoint. The investments required for the bacterium cultivation, however, may still be worth for small-scale industries or local producers, focalized on niche sectors such as a given nematode pest and/or a circumscribed market. Some properties of these bacteria favour indeed their industrial exploitation as efficient biocontrol agents. They are: density dependence, host specificity, abundance of forms and biodiversity richness within species, together with the possibility of long-term storage in dry conditions and field persistence. The widespread occurrence of nematode pests, the high yield losses they induce and the need for a substantial improvement in management by applying safer and/or environment-friendly products represent further factors promoting the practical or commercial exploitation of *Pasteuria* spp. as biocontrol agents. This consideration is supported by the number of patents related to nematode biocontrol agents, of which those for *Pasteuria* spp. represent a significant share (Table 2).

Conclusion

Future directions for research should consider the identification and availability of new *Pasteuria* species or isolates, and their characterization through genomic and -omics approaches. Biocontrol trials will be required for the selection and evaluation of the most promising isolates and for eventual commercial exploitation. In spite of the efforts deployed thus far, the ecology of *Pasteuria* spp. is still poorly investigated. In particular, data should be produced on the endospores environmental fate and on their natural regulation and interactions with other soil organisms. If suitable and economically convenient on a local scale, cultivation processes and bioformulations based on *Pasteuria* spp. will become progressively available.

It is hence plausible to expect that these bacteria will progressively integrate other antagonists or sustainable practices and tools, applied worldwide for the management of most important nematode pests.

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