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International Journal for Parasitology 33 (2003) 1269–1276



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Invited review

Interactions between bacteria and plant-parasitic nematodes: now and then

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Received 24 January 2003; received in revised form 29 April 2003; accepted 19 May 2003

Abstract

Based on genome-to-genome analyses of gene sequences obtained from plant-parasitic, root-knot nematodes (*Meloidogyne* spp.), it seems likely that certain genes have been derived from bacteria by horizontal gene transfer. Strikingly, a common theme underpinning the function of these genes is their apparent direct relationship to the nematodes' parasitic lifestyle. Phylogenetic analyses implicate rhizobacteria as the predominant group of 'gene donor' bacteria. Root-knot nematodes and rhizobia occupy similar niches in the soil and in roots, and thus the opportunity for genetic exchange may be omnipresent. Further, both organisms establish intimate developmental interactions with host plants, and mounting evidence suggests that the mechanisms for these interactions are shared too. We propose that the origin of parasitism in *Meloidogyne* may have been facilitated by acquisition of genetic material from soil bacteria through horizontal transfer, and that such events represented key steps in speciation of plant-parasitic nematodes. To further understand the mechanisms of horizontal gene transfer, and also to provide experimental tools to manipulate this promising bio-control agent, we have initiated a genomic sequence of the bacterial hyper-parasite of plant parasitic nematodes, *Pasteuria penetrans*. Initial data have established that *P. penetrans* is closely related to *Bacillus* spp., to the extent that considerable genome synteny is apparent. Hence, *Bacillus* serves as a model for *Pasteuria*, and vice versa.

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Keywords: Horizontal gene transfer; *Meloidogyne*; *Pasteuria penetrans*; Rhizobia; Root-knot nematode; Synteny

1. Introduction

Nematodes are the most abundant and speciose metazoans, and account for up to 80% of the kingdom's members (Boucher and Lamshead, 1994). Not surprisingly, nematodes have evolved to occupy diverse ecological niches. Like the model organism *Caenorhabditis elegans*, most are free-living and graze on microbes or detritus, and as such, have no obvious direct impact on humans. Others however, are adapted as parasites and are responsible for such widespread problems as debilitation of livestock, human disease, and crop damage. Not surprisingly, *C. elegans* (Riddle et al., 1997) and animal- and human-parasitic species (Kennedy and Harnett, 2001) have been studied disproportionate to their numbers. However, the advent of relatively inexpensive genomic approaches, especially obtaining single-pass sequences from randomly selected, cDNA clones, termed expressed sequence tags (ESTs),

promises to expand studies to a broad range of nematodes occupying a broad range of habitats (McCarter et al., 2000).

In this short article, we will report on recent findings in plant-parasitic nematodes (McCarter et al., 2003), with a particular emphasis on the role that acquisition of bacterial genes by ancestral plant-parasitic forms might have played in evolution of plant-parasitism. Horizontal gene transfer (HGT) has previously been suggested for individual nematode genes (Smant et al., 1998; Yan et al., 1998; Lambert et al., 1999; Popeijus et al., 2000; Veronico et al., 2001; Jaubert et al., 2002), and indeed, an explicit model of HGT in nematode evolution has been proposed (Bird and Koltai, 2000; Bird and Bird, 2001). A recent comprehensive genomic analysis (Scholl et al., 2003) revealed a surprising number of nematode genes as being candidates for having arisen via HGT from bacteria. Because these data suggest that associations between nematodes and bacteria may have been extensive in the evolutionary past, we have become interested in extant interactions between bacteria and plant-parasitic nematodes (Bird and Koltai, 2000; Bird and Bird, 2001). In particular, a project to obtain the entire genomic sequence of *Pasteuria penetrans*, an obligate bacterial

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parasite of nematodes, has been initiated (Opperman C.H., Davies, K.G., unpublished), and in the second part of this article, we report initial findings from approximately 1.5 Mb of *P. penetrans* sequence. Collectively, genome-wide analyses of plant-parasitic nematodes and their bacterial pathogens will likely shed considerable light on the function and evolution of these pathogens, and further, will likely suggest new strategies for nematode control.

2. Nematodes are devastating parasites of plants

Nematodes are cosmopolitan parasites of plants, and exploit all parts of the host, and affect virtually every crop plant and every agricultural industry including subsistence farming, forestry, field and truck crops, and ornamental and turf production. On some crops, including soybean, nematodes are clearly recognised as the major pest (Wrather et al., 2001). On other crops nematodes contribute significantly to net reduction in yield, although assessing the true magnitude of the problem is difficult. Based on extensive survey data (Sasser and Freckman, 1987; Koening et al., 1999), it has been estimated that overall yield loss averages 12.3% annually; this figure approaches 20% for some crops (e.g., banana). In monetary terms the worldwide figure certainly exceeds \$US100 billion annually.

Another way to consider the impact of plant-parasitic nematodes is through the management strategies employed in their control. In 1982, 50,000 tonnes of nematocide active ingredient were applied to crops in the United States, at a cost exceeding \$US1 billion (Landels, 1989), and between 1986 and 1990 in The Netherlands, nematocide application was more than three times the combined total of chemicals needed to combat insects, fungi and weeds on experimental, sustainable farms (Lewis et al., 1997). However, in recent decades issues such as ground water contamination, toxicity to mammals and birds, and residues in food have caused much tighter restrictions on the use of agricultural chemicals, including suspension of nematocides in many countries (Thomason, 1987).

The most damaging family (the *Heteroderidae*) includes the root-knot (*Meloidogyne* spp.) and the cyst nematodes (*Globodera* and *Heterodera* spp.). Root-knot nematodes (RKN) penetrate plant hosts and migrate between the cells in roots, where they induce formation of large, multinucleate cells called ‘giant cells’. Galls form around the giant cells, and the roots become distorted, often leading to compromised root function and retardation of plant growth (reviewed by Bird and Koltai, 2000).

3. Origin of *Meloidogyne* parasitism genes

It is not clear which genetic differences between parasites and non-parasites are responsible for parasitic

ability. Based on phylum-wide phylogenetic analysis it appears that plant-parasitism arose independently multiple times over the course of nematode evolution (Blaxter et al., 1998). Consequently, one cannot be assured that any gene or set of genes which aid in the parasitic lifestyle in one nematode species will also exist or function similarly in another. Conceptually, several mechanisms affecting evolution to parasitism can be envisioned, including: adaptation of pre-existing genes to encode new functions; changes in genes regulating metabolic or developmental pathways; gene duplication; gene loss; acquisition of genes from other species via horizontal (or lateral) gene transfer. HGT has become a widely accepted mechanism of rapid evolution and diversification in prokaryotic populations (Jain et al., 1999; Lawrence, 1999; Ochman et al., 2000). In contrast, the extent of horizontal transfer involving eukaryotes remains controversial, with many hypothesised cases of horizontally transferred genes having been subsequently refuted (Stanhope et al., 2001; Brinkman et al., 2002).

3.1. A screen for HGT candidates in plant-parasitic nematodes

Claims of HGT frequently have pivoted on incongruencies between a specific gene tree and the assumed underlying species tree. Acquisition of new sequence data has often revealed that genes believed absent in a species were merely missing in the database rather than missing from the genome (Stanhope et al., 2001). Without full genomes for all plant and animal species, it obviously is not possible to make definitive statements about presence or absence of a specific gene in every organism. However, using the completed *C. elegans* genome as a reference, we comprehensively examined the emerging genetic resources for *Meloidogyne* (McCarter et al., 2003) to begin to address the question of evolution of parasitism and in particular a possible role for HGT (Scholl et al., 2003). The screening strategy was based on the notion that nematode genes encoding proteins with similarity to bacterial proteins but whose presence is incongruent with known nematode phylogeny pass the simplest criteria for an HGT candidate (Fig. 1), although presence of a gene in one nematode species (such as a *Meloidogyne* spp.) that is absent in another (such as *C. elegans*) might merely reflect a gene loss in the latter lineage. However, adding another complete genome to the analysis can help resolve this problem, and the completed *Drosophila melanogaster* genome was chosen (Scholl et al., 2003). Remarkably, the relationship between the invertebrate phyla Nematoda and Arthropoda (which includes *Drosophila*) is controversial. The traditional view is that nematodes are basal to arthropods, but recent molecular phylogenies place nematodes and arthropods together in a high-level taxon named Ecdysozoa (Aguinaldo et al., 1997; Blair et al., 2002), although other molecular studies give conflicting results (Hedges, 2002;

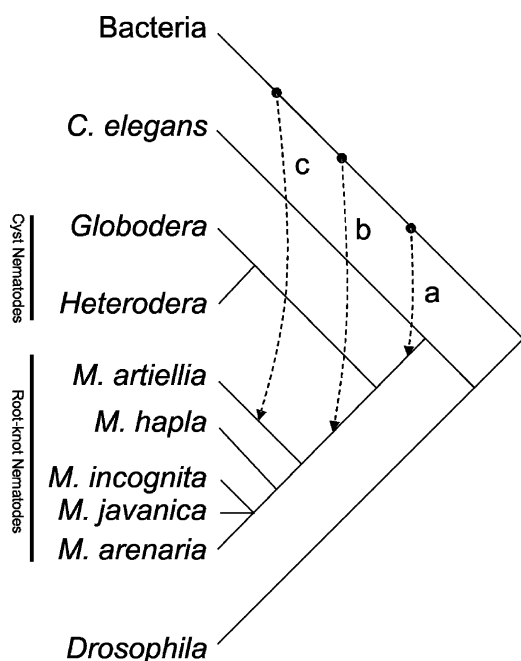


Fig. 1. Schematic species tree indicating relationships between bacteria, *Drosophila melanogaster*, *Caenorhabditis elegans* and plant parasitic nematodes in the family *Heteroderidae*. Location of three possible horizontal gene transfer events that would pass through our initial phylogenetic filter are indicated by dotted lines. Transfer 'a' occurs after divergence of the lineages leading to *C. elegans* and *Meloidogyne*, transfer 'b' after divergence of root-knot nematodes and cyst nematodes, and transfer 'c' to the lineage leading a specific *Meloidogyne* species. Figure adapted from Tandingan-De Ley et al. (2002) and Scholl et al. (2003).

Mallatt and Winchell, 2002). No matter what the evolutionary relationship between Nematoda and Arthropoda truly are, the relationships shown in Fig. 1 are consistent with both hypotheses. Thus, a bacteria-like gene present in *Meloidogyne* and *Drosophila*, but absent in *C. elegans*, is unlikely to have experienced HGT, but rather may reflect a gene loss in the *C. elegans* lineage. Genes that experienced

a transfer event from bacteria to nematodes pass through this phylogenetic filter if the transfer event occurred subsequent to the divergence of the *C. elegans* and *Meloidogyne* lineages (Fig. 1). Should a gene appear to be in other closely related plant parasites, such as the cyst nematodes, the transfer event likely affected a common ancestor of the two families of parasitic nematodes (event 'a' in Fig. 1). Alternatively, the transfer event may be more recent, such as to the progenitor of the *Meloidogyne* lineage since its divergence from the cyst nematodes (event 'b' in Fig. 1), or in a lineage leading to a single *Meloidogyne* species (event 'c'). A further criterion to for HGT candidates is absence of significant matches to any metazoan genes, i.e. that they arose via horizontal gene transfer from a non-metazoan pool, as opposed to multiple independent gene losses in the metazoan lineages.

3.2. HGT candidates in RKN

Applying these criteria to *Meloidogyne incognita*, *Meloidogyne hapla* and *Meloidogyne javanica* EST datasets revealed six of the seven genes postulated in the literature to have been horizontally acquired by these *Meloidogyne* species during evolution of plant parasitic nematodes (Smant et al., 1998; Yan et al., 1998; Popeijus et al., 2000; Jaubert et al., 2002). Also found were six new candidates, encoding glutamine synthetase, L-threonine aldolase, a putative *NodL* homologue, and three to which function could not be unequivocally ascribed (Table 1). The 'missing gene' is *Mj-CM*, which has been postulated to encode chorismate mutase in *M. javanica*, and is a good candidate for having been acquired by HGT (Lambert et al., 1999), but was simply absent from the EST datasets. Another RKN gene also postulated to have been acquired by HGT, and which encodes polyglutamate synthetase, was previously identified in *Meloidogyne artiellia* (Veronico et al., 2001). Significantly, hybridization data showed that

Table 1

List of horizontal gene transfer candidates from *Meloidogyne incognita*, showing best bacterial matches, their e-values from a BLASTX search, and percent identity as reported by BLAST

Identity	Best prokaryote match	Identity	Citation
Cellulase	<i>Bacillus</i> sp. KSM-N252	($2.7e^{-24}$, 40%)	Yan et al., 1998
Cellulase	<i>Pseudomonas fluorescens</i>	($2.5e^{-75}$, 47%)	Uehara et al., 2001
Cellulase	<i>Streptomyces coelicolor</i>	($6.9e^{-13}$, 31%)	Uehara et al., 2001
Cellulase	<i>Pseudomonas fluorescens</i>	($1.2e^{-35}$, 44%)	Uehara et al., 2001
Exopolysaccharuronase	<i>Ralstonia solanacearum</i>	($8.8e^{-61}$, 50%)	Jaubert et al., 2002
Pectate lyase	<i>Streptomyces coelicolor</i>	($3.9e^{-12}$, 31%)	Popeijus et al., 2000
Chorismate mutase	<i>Pseudomonas syringae</i>	($1e^{-13}$, 31%)	Lambert et al., 1999
Polyglutamate synthase	<i>Azotobacter vinelandii</i>	($8e^{-92}$, 49%)	Veronico et al., 2001
NodL	<i>Rhizobium leguminosarum</i>	($8e^{-54}$, 58%)	Scholl et al., 2003
Glutamine synthetase	<i>Mesorhizobium loti</i>	($9e^{-45}$, 56%)	Scholl et al., 2003
L-Threonine aldolase	<i>Brucella melitensis</i>	($1e^{-23}$, 48%)	Scholl et al., 2003
Unknown function	<i>Sinorhizobium meliloti</i>	($9e^{-45}$, 51%)	Scholl et al., 2003
Unknown function	<i>Amycolatopsis mediterranei</i>	($4.9e^{-28}$, 53%)	Scholl et al., 2003
Unknown function	<i>Amycolatopsis mediterranei</i>	($3.0e^{-28}$, 58%)	Scholl et al., 2003

this particular gene is absent both from the *M. javanica* and *Globodera rostochiensis* genomes (Veronico et al., 2001). We speculate that acquisition of this gene by *M. artiellia* is a recent HGT event (event 'c', Fig. 1), and thus truly is absent from the *Meloidogyne* genomes from which our datasets were derived.

3.3. A rhizobial origin of *Meloidogyne* genes?

Of the six newly identified HGT candidates, four have highest similarity to genes in the class of nitrogen-fixing soil bacteria which have the ability to nodulate plant roots, and collectively are termed rhizobia. *Meloidogyne* and rhizobia are sympatric, i.e., they share an ecological niche in the soil (Bird and Koltai, 2000), and arguably in the plant too (Koltai et al., 2001), satisfying the minimal requirement for an HGT to occur, viz. physical proximity. Interestingly, models of bacterial evolution suggests HGT as a mechanism of adaptation into either symbiosis or parasitism (Ochman and Moran, 2001). This is specifically thought to be the case for divergent species of rhizobia, such as the symbiont *Sinorhizobium meliloti* and the pathogen *Rhizobium radiobacter* (formerly known as *Agrobacterium tumefaciens*), where differential selection and gene maintenance is likely responsible for different lifestyle strategies (Wood et al., 2001).

The rhizobial-like HGT candidate we identified as encoding NodL (Table 1) is worthy special mention. This protein encodes an *N*-acetyltransferase previously thought to be found only in rhizobia (Downie and Young, 2001), where it functions in the biosynthesis of Nod factor. Nod factors are a rhizobial species-specific family of lipochito-oligosaccharides which function in signal exchange between the bacterium and its symbiotic partner plant (Göttfert, 1993). Phylogenetic analysis revealed that the NodL candidates found in *M. incognita* and *M. javanica* both fall squarely within the rhizobial NodL clade (Scholl et al., 2003). Attempts to PCR amplify *NodL* from various nematodes confirmed its presence in each of the *Meloidogyne* species tested, but similar experiments do not yield amplification products from the cyst nematodes tested (Scholl et al., 2003). Although other interpretations can be made, these results are consistent with *NodL* being acquired by an 'event b' HGT (Fig. 1).

A question that arises in analysing eukaryotic sequences with strong and especially unique bacterial matches is whether the gene in question truly was isolated from a eukaryote, or whether it represents a prokaryotic contaminant. In the case of the *Meloidogyne NodL* genes, experimental evidence showed a SL1 trans-splice leader at the 5'-end of the message, and a poly(A) tail at the 3' end, as well as an intron in the genomic *Mi-NodL* sequences, confirming that this is a bona fide nematode gene (Scholl et al., 2003).

4. *Pasteuria penetrans*

Other than computational approaches to search for the possible bacterial origins of the HGT candidates in plant-parasitic nematodes, examining extant nematode-bacterial associations might provide valuable clues as to from where, and even how, gene flow occurred. We suspect that one of the most fruitful sources of information might be natural enemies of nematodes. Consequently, we have begun a detailed study of the endospore-producing, Gram-positive soil bacterium *Pasteuria penetrans*, which is an obligate parasite of nematodes, including the root-knot nematode and cyst nematodes. Intriguingly, *Pasteuria* appears to target the reproductive tissues in the host, potentially making it a powerful bacterial model in which to study the actual mechanisms of HGT. In addition, *Pasteuria* has been looked to as an economically- and environmentally-friendly approach for the bio-control of plant-parasitic nematodes (Akhtar and Malik, 2000). By sequencing the genome of *Pasteuria*, we hope to begin unravelling the complex interaction between parasite and nematode host. The complete genomic sequence of *Pasteuria* is an essential prelude to performing functional genomic experiments and is likely to result in an expansion of efforts to utilize this potentially excellent biological control agent.

Phylogenetic analysis using DNA sequences based on the 16S ribosomal sub-unit showed the genus *Pasteuria* to be a deeply rooted member of the *Clostridium-Bacillus-Streptococcus* branch of the Gram-positive eubacteria (Anderson et al., 1999; Atibalentja et al., 2000). Obtaining the genome of a new member of the *Bacillus* family will provide opportunities for insight into the basic biology of this important group of bacteria, and especially the manner in which *Bacilli* are able to function as parasites and pathogens. Further, because *P. penetrans* suppresses host immunity and eventually kills its nematode hosts, we postulate that the bacterium may contain nematode toxins and other molecules to modulate host functions, including host immunity.

4.1. Biology of *Pasteuria penetrans*

The genus *Pasteuria* was first described by Metchnikoff in 1888 as a parasite of *Daphnia*, and was observed in 1906 on a nematode (*Dorylaimus bulbiferous*) by Cobb. Subsequently, all plant-parasitic nematodes of major economic importance have been observed to be parasitised by either *P. penetrans* or closely related species (Sayre and Starr, 1988; Sturhan, 1988; Chen and Dickson, 1998). Based on host range, life cycle and morphology, three nominal species of *Pasteuria* able to parasitise plant parasitic nematodes have been proscribed, namely: (1) *P. penetrans*, which is a parasite of *Meloidogyne* spp., (2) *Pasteuria thornei*, which is a parasite of *Pratylenchus* spp., and (3) *Pasteuria nishizawae*, which is a parasite of *Heterodera* and *Globodera* spp. (Chen and Dickson, 1998). Historically,

there have been two factors limiting the widespread deployment of *P. penetrans* for practical nematode control: (1) absence of a robust in vitro culturing system for the bacterium, amenable to the mass-production of spores, and (2) the highly nematode-species-specific nature of the bacterium-host interaction, which means strains produced for one nematode species likely will not work for other species. Recently, however, major breakthroughs have been made in culturing *P. penetrans*. A company (Entomos LLC) has developed conditions for in vitro culture of *P. penetrans*. Nevertheless, it is clear that the culturing achievement made by Entomos is just a first step towards broad commercial use of this biocontrol agent. The ability to tightly regulate the vegetative growth-sporulation transition, for example, will be essential to large scale production. Further, the issues of host range remain to be solved. We anticipate that the genome sequence will suggest approaches to address these, and other aspects of *Pasteuria* biology, so we have begun to develop tools both for mutagenesis and transformation of this bacterial species.

4.2. The *Pasteuria*–nematode interaction

The *P. penetrans* bacterium produces highly durable endospores (Stirling and Wachtel, 1980; Sayre and Starr, 1985; Stirling, 1991). During migration in the soil in search of a host root, the nematode encounters *P. penetrans* endospores. Upon contact, the spores attach (by a poorly understood mechanism) and encumber the nematode (Fig. 2A). Although as many as 60 spores can be found

adhering randomly along the body of a single nematode, fewer than 10 spores is more common in a natural infestation. Specific strains of *Pasteuria* have highly defined attachment profiles on different nematode populations, indicative of an initial host recognition event between *Pasteuria* and its potential host. Some *Pasteuria* strains will only attach to relatively few nematode populations, whereas others have broader attachment profiles. Obviously, attachment is an absolute prerequisite for infection, though not all attached spores necessarily germinate. Endospore-encumbered nematodes often retain the ability to invade and migrate within the root and establish normal feeding sites.

The life cycle of the bacterium on RKN is initiated when endospores adhere to the nematode cuticle (Fig. 2A,B). Germination occurs with the production of an infection peg that penetrates the nematode cuticle after the nematode has entered the root (Fig. 2C), and in the case of RKN, germination and infection by the bacterium appear to be tied to the initiation of feeding by the nematode. The terminal region of the infection peg undergoes differentiation by dichotomous branching and produces a mycelial ball, or microcolony (Fig. 2D). Infected female nematodes continue to develop but microcolonies of the bacteria proliferate and prohibit infected females from producing eggs (Sayre and Starr, 1985). The bacterium undergoes sporogenesis and the females eventually die. Each infected female can produce up to 2×10^6 endospores that are eventually released into the soil (Fig. 2E) when the roots decay and the female cadavers break open (Sayre and Starr, 1985; Davies et al., 1988; Chen and Dickson, 1998).

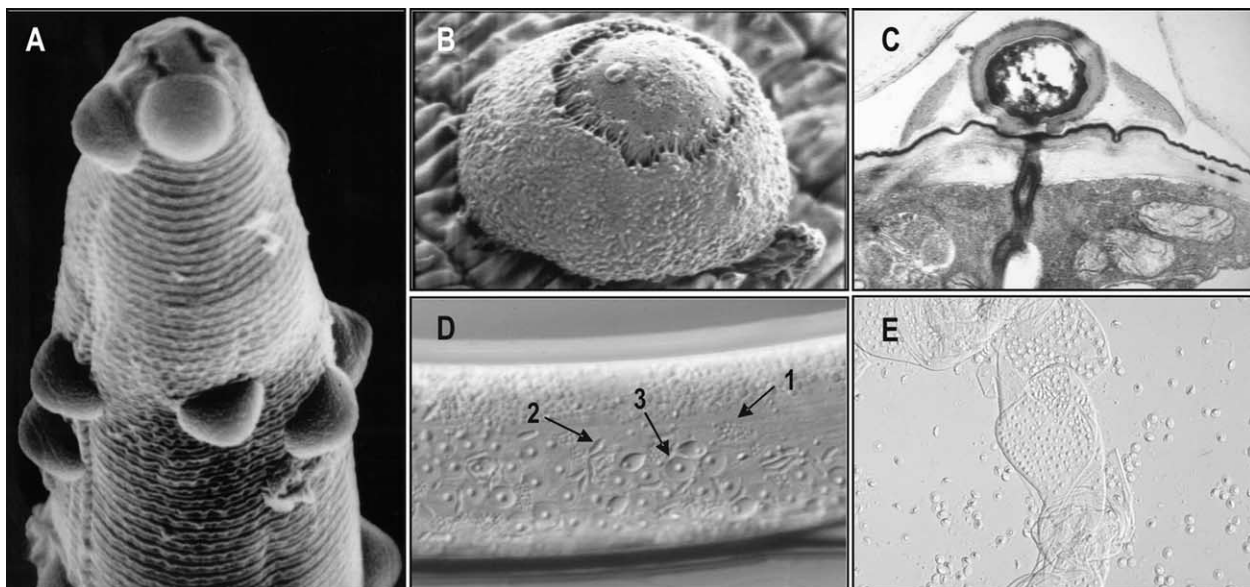


Fig. 2. (A) Head of a root-knot nematode covered with endospores of the Gram-positive hyper-parasite *Pasteuria penetrans*. (B) Spore of *Pasteuria nishizwae* attached to an *Heterodera glycines* (soybean cyst nematode) larva. The exosporium (a membrane that covers the spore during sporogenesis) has been retained. (C) Germinating endospore of *Pasteuria penetrans*. Courtesy: Bert Endo, USDA. (D) *Pasteuria* spp. in the body cavity of *Xiphinema americanum*. At least three stages ((1) microcolonies, (2) separated quartets or doublets and (3) single sporangia) of the life cycle are visible. (E) Female cadaver breaks open and releases endospores back into the soil.

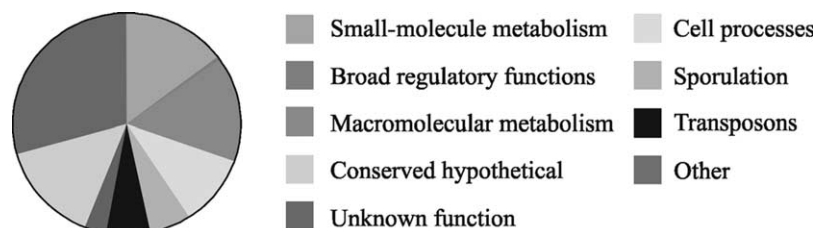


Fig. 3. Gene repertoire of *Pasteuria penetrans* by functional category (%).

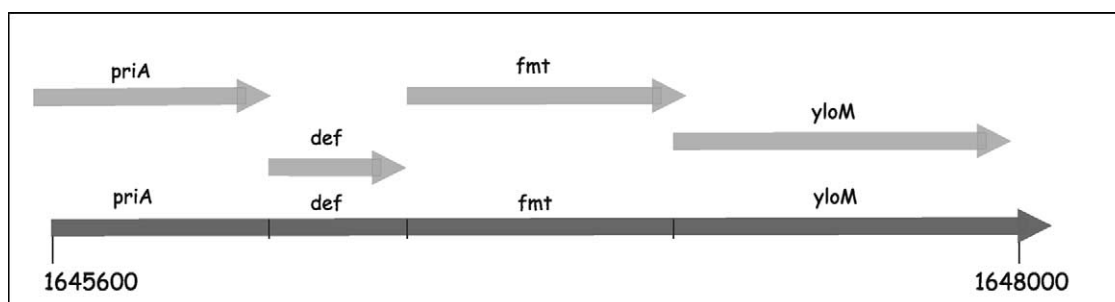


Fig. 4. Genome synteny between *Pasteuria penetrans* and *Bacillus subtilis*. In this *Pasteuria* contig, cho#3019 (light grey), the four predicted genes exhibit conservation of sequence (BlastP, e-value <math>< 1.0e^{-10}</math>), size, gene order and spacing with their presumed orthologues in *B. subtilis* (dark grey).

4.3. *Pasteuria* genomic sequencing

Based on the size of the *Bacillus* genome (~4.2 Mb) and our own preliminary genome sequencing, we estimate that the *Pasteuria* genome is no larger than 4.2 Mb. We have constructed four genomic libraries from the broad host-range *P. penetrans* strain RES147, and using a whole shotgun sequencing approach, we have (to date) analysed 9,074 sequencing reads, resulting in 2,840,162 bp of primary, quality-trimmed genomic sequence. This initial sequence assembled into approximately 1,500 contigs, spanning more than 1.5 Mb of the *P. penetrans* genome.

Approximately 50% of the sequences have yielded significant (e-value $\leq 1.0e^{-10}$) similarities to known genes in the NCBI database. Fig. 3 summarises the deduced function of the approximately 1,400 genes thus far identified. We have observed significant genome colinearity between *P. penetrans* and *Bacillus subtilis* in our larger contiguous sequences. Fig. 4 depicts such a region in which gene order, spacing, and orientation are completely conserved between the two bacterial species. This finding is extremely important, as it permits us to model the *Pasteuria* sequence using the wealth of biology (including complete genome sequences) of *B. subtilis*, *Bacillus anthracis* and *Bacillus halodurans*, and we anticipate this close relationship will aid significantly in annotation of the *Pasteuria* genome. Further, it permits us to pose questions, and to test hypotheses that go much further than simply understanding the unique biology of this bacterial, hyper-parasite of nematodes. What, for example, are the differences in gene content and/or regulation that dictate one species to be a facultative mammalian pathogen (*B. anthracis*), one to be

a fastidious invertebrate parasite (*P. penetrans*), and two to be free-living (*B. subtilis* and *B. halodurans*)? How do these different life styles influence the way in which the different species interact with their environment to regulate key life-stage transitions (e.g., vegetative growth vs. sporulation)? What were the structural and regulatory changes required to permit these four species to occupy these diverse ecological niches, and what were the evolutionary processes that lead to such adaptation? A complete genome sequence will underpin our ability to address these and many more questions about the biology, ecology and evolution of *P. penetrans*. And, of course, the sequence may yield additional HGT candidates, and perhaps even suggest a mechanism for genetic exchange.

Acknowledgements

We thank Mark Burke for bioinformatic support and advice, and Reenah Schafer for help with figures. This research was supported in part by NSF plant genome award DBI-0077503 to D.M.B. and C.H.O., and a Syngenta Biotechnology, Inc. CFAP Award to D.M.B. and C.H.O. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

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