Phylogenetic position of the North American isolate of *Pasteuria* that parasitizes the soybean cyst nematode, *Heterodera glycines*, as inferred from 16S rDNA sequence analysis

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A 1341 bp sequence of the 16S rDNA of an undescribed species of *Pasteuria* that parasitizes the soybean cyst nematode, *Heterodera glycines*, was determined and then compared with a homologous sequence of *Pasteuria ramosa*, a parasite of cladoceran water fleas of the family *Daphnidae*. The two *Pasteuria* sequences, which diverged from each other by a dissimilarity index of 7%, also were compared with the 16S rDNA sequences of 30 other bacterial species to determine the phylogenetic position of the genus *Pasteuria* among the Gram-positive eubacteria. Phylogenetic analyses using maximum-likelihood, maximum-parsimony and neighbour-joining methods showed that the *Heterodera glycines*-infecting *Pasteuria* and its sister species, *P. ramosa*, form a distinct line of descent within the *Alicyclobacillus* group of the *Bacillaceae*. These results are consistent with the view that the genus *Pasteuria* is a deeply rooted member of the *Clostridium*–*Bacillus*–*Streptococcus* branch of the Gram-positive eubacteria, neither related to the actinomycetes nor closely related to true endospore-forming bacteria.

Keywords: *Heterodera glycines*, *Pasteuria* sp., phylogeny, 16S rDNA sequence, soybean cyst nematode

INTRODUCTION

Pasteuria spp. are Gram-positive, mycelial and endospore-forming bacteria traditionally classified in the *Actinomycetales* (Sayre & Starr, 1989). Four species of *Pasteuria* have been described, the first of which, *Pasteuria ramosa* (the type species), parasitizes cladoceran water fleas of the family *Daphnidae* (Ebert *et al.*, 1996; Metchnikoff, 1888; Sayre *et al.*, 1979, 1983). The other three species of *Pasteuria* are parasites of plant-parasitic nematodes, against which they have shown great potential as biological control agents (Atibalentja *et al.*, 1998; Brown *et al.*, 1985; Chen *et al.*, 1996, 1997; Duponnois & Ba, 1998; Giblin-Davis, 1990; Gowen *et al.*, 1998; Nishizawa, 1987; Weibelzahl-Fulton *et al.*, 1996). The three species of nematode-infecting *Pasteuria* are as follows: *Pasteuria penetrans*,

which occurs on root-knot nematodes, i.e. *Meloidogyne* spp. (Sayre & Starr, 1985; Starr & Sayre, 1988); *Pasteuria thornei*, which affects root-lesion nematodes, i.e. *Pratylenchus* spp. (Starr & Sayre, 1988); and *Pasteuria nishizawae*, which is parasitic on cyst nematodes of the genera *Heterodera* and *Globodera* (Sayre *et al.*, 1991a, b). Members of the genus *Pasteuria* are widespread and have been reported from 323 nematode species belonging to 116 genera including plant-parasitic, entomopathogenic, predatory and free-living nematodes (Chen & Dickson, 1998; Ciancio *et al.*, 1994; Sayre & Starr, 1988; Sturhan, 1988).

Attempts to culture *Pasteuria* spp. *in vitro* have not been successful (Bishop & Ellar, 1991; Williams *et al.*, 1989). As a result, the taxonomy of the genus *Pasteuria* has been marked by a series of errors and confusion (Sayre & Starr, 1989), as it relies mainly on morphological, developmental and pathological characteristics including the size and shape of the sporangium and endospore, ultrastructure, life cycle and host range (Davies *et al.*, 1990; Giblin-Davis *et al.*, 1990; Metchnikoff, 1888; Noel & Stanger, 1994; Sayre & Starr,

Abbreviations: ML, maximum likelihood; MP, maximum parsimony; NJ, neighbour-joining; RDP, Ribosomal Database Project.

The GenBank accession number for the 16S rDNA sequence of the North American isolate of *Pasteuria* reported in this paper is AF134868.

1989; Sayre *et al.*, 1991a, b; Starr & Sayre, 1988; Sturhan *et al.*, 1994). For the nematode-infecting *Pasteuria*, the taxonomic value of endospore morphometrics and host range was questioned when these characteristics were reported to reflect an evolutionary adaptation that occurred during host–nematode speciation (Ciancio, 1995). In addition, many of the *Pasteuria* isolates reported display a high degree of morphological similarity and others exhibit cross-generic parasitism of nematodes (Chen & Dickson, 1998). Clearly, morphological, developmental and pathological characteristics are not sufficient to accommodate the increasing number of *Pasteuria* isolates collected from soil and plant nematodes.

During the last few years, the analysis of 16S rDNA and rRNA sequences has been used for the identification and classification of bacteria, including those that cannot be cultured (Relman *et al.*, 1990, 1992; Woese, 1987). Recently, Ebert *et al.* (1996) questioned the position of the genus *Pasteuria* in the *Actinomycetales* based on the analysis of the 16S rDNA sequence of *P. ramosa*. Like other investigators (Sayre & Starr, 1985; Sayre & Wergin, 1977), those authors observed some morphological similarities between *P. ramosa* and *Thermoactinomyces* spp. Unfortunately, they could not resolve the phylogenetic relationships between these organisms and *Pasteuria* spp., since there was no *Thermoactinomyces* sequence for comparison. Apart from that of *P. ramosa*, no 16S rDNA sequence has been reported for *Pasteuria* spp., especially those infecting nematodes. Our objectives, therefore, were as follows: to determine the 16S rDNA sequence of a nematode-infecting *Pasteuria*, namely the North American isolate of *Pasteuria* that parasitizes the soybean cyst nematode, *Heterodera glycines* Ichinohe (Noel & Stanger, 1994); to compare this sequence with that of *P. ramosa* (Ebert *et al.*, 1996); and to resolve the phylogenetic position of the genus *Pasteuria*.

METHODS

Source of endospores. *Pasteuria*-infected *H. glycines* females and cysts were selected, on the basis of their opaque appearance, from the nematodes extracted from the rhizosphere of 3-month-old soybean plants grown in naturally infested soil in a greenhouse. The selected nematodes were transferred individually into 100 µl tap water in 1.6 ml microfuge tubes in which they were crushed with a tissue grinder; 10 µl of the resulting suspension was examined microscopically for the presence of *Pasteuria* endospores. Positive fractions were pooled in a 1.6 ml microfuge tube to form the stock suspension, which was then forced through a 5-µm-pore polycarbonate membrane filter (Poretics) to remove nematode debris. The endospore concentration of the suspension was determined with a Levy–Hausser counting chamber (Arthur H. Thomas) and the material was stored at 4 °C until used.

DNA extraction from *Pasteuria* endospores. DNA extraction was based on a modification of a procedure described by Ebert *et al.* (1996). Specifically, 500 µl of a stock suspension

containing 2×10^6 endospores ml⁻¹ was transferred into a clean 1.6 ml microfuge tube, which was then incubated for 10 min in a boiling water-bath. After cooling to room temperature (24 °C), the suspension was centrifuged for 15 min in an Eppendorf microcentrifuge at maximum speed (approx. 16000 g) and the pellet was resuspended with 200 µl 25 mg lysozyme ml⁻¹ solution in 10 mM Tris/HCl buffer, pH 8.0. The mixture was incubated at 37 °C for 30 min, at which point a 10% solution of SDS was added to a final concentration of 2% (v/v); this was followed by an additional 30 min incubation at 37 °C. The endospores, which are resistant to heat, lysozyme and detergent treatments, were pelleted as before and washed twice by resuspending the pellet in 500 µl 10 mM Tris/HCl buffer, pH 7.0; this was followed by 15 min centrifugation at high speed. The pellet from the last wash was resuspended in 500 µl buffered solution (40 mM Tris/HCl, pH 8.0, 10 mM NaCl, 6 mM MgCl₂ and 10 mM CaCl₂) containing 10 µg (50 U ml⁻¹) RQ1 RNase-free DNase ml⁻¹ (Promega) to destroy any DNA released from contaminating microorganisms. The DNase reaction was conducted at 37 °C for 30 min, at which point the endospores were pelleted, washed twice and resuspended with 500 µl of a solution containing 50 U RNase One ml⁻¹ (Promega) in RNase One buffer (10 mM Tris/HCl, pH 7.5, 5 mM EDTA and 200 mM sodium acetate). After 30 min incubation at 37 °C, proteinase K (50 µg ml⁻¹) and SDS (0.5%, v/v) were added and the mixture was incubated further at 55 °C for 2 h. The endospores were pelleted again, washed twice and resuspended in 500 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) to produce the pre-lysis suspension.

To ascertain the absence of vegetative cells in the pre-lysis suspension, 15 µl was examined microscopically and 100 µl of a 1:10 dilution in sterile distilled water was spread on each of two Luria agar plates, which were incubated at 30 °C for a week. As positive controls, two Luria agar plates were spread with equivalent amounts of the stock suspension that had not been subjected to either heat or enzyme treatments and incubated at 30 °C. Aliquots (10 µl) of the pre-lysis suspension and the suspensions derived from representative colonies of the bacteria that grew on control plates were used in separate PCR reactions (see below), the products of which were sequenced to determine the identity of the DNAs in the suspensions. The remaining 455 µl of the pre-lysis suspension was transferred into a fresh screw-capped 1.6 ml microfuge tube containing 860 mg acid-washed glass beads (0.150–0.212 mm in diameter) and 500 µl Tris buffer (pH 8.0)-equilibrated phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). Lysis of endospores was achieved with four 30 s pulses of bead-beating in a Mini-BeadBeater (BiosPec Products) operated at high speed in a cold room (4 °C). The mixture was incubated on ice for 5 min between pulses of bead-beating. The lysate was centrifuged at maximum speed for 10 min and the aqueous phase was transferred into a fresh 1.6 ml microfuge tube. The handling of the supernatant, thereafter, was according to standard procedures (Sambrook *et al.*, 1989), which included chloroform:isoamyl alcohol (24:1, v/v) extraction followed by DNA precipitation with 3 M sodium acetate (pH 5.2) and 2 vols ice-cold ethanol (100%), then washing with 70% ethanol. The final pellet was resuspended with 50 µl TE buffer to make the post-lysis suspension, which was stored at –20 °C until used.

DNA amplification and sequencing. The PCR primers (PrDNA-1 and PrDNA-2) were designed (Table 1) to amplify an approximately 1400 bp region of the 16S rDNA,

Table 1. Primers used for PCR amplification and sequencing of the 16S rDNA of the *Heterodera glycines*-infecting *Pasteuria*

Name*	Direction	5' → 3' sequence	Position of 5' nucleotide†
1	Forward	GCGGCGTGCCTAATACA	39
2	Reverse	ACGGGCGGTGTGTACAAG	1388‡
3	Forward	TACGGGAGGCAGCAGTA	342
4	Reverse	TACTGCTGCCTCCCGTA	342‡
5	Forward	CCCTGGTAGTCCACGCG	794
6	Reverse	CGCGTGGACTACCAGGG	794‡
7	Forward	TCGAGAGAGTGCTGTGCC	1011
8	Reverse	GGGCACAGCACTCTCTCGA	1011‡

* Primers were designated by the generic name 'PrDNA-*X*', where *X* is a number from 1 to 8.

† Numbers refer to equivalent positions in the *E. coli* sequence (Brosius *et al.*, 1978).

‡ The position of the 5' nucleotide in the complementary sequence.

based on a comparison of published sequences from related Gram-positive bacteria including *Bacillus subtilis*, *Bacillus caldolyticus*, *Bacillus validus*, *Thermoactinomyces vulgaris* and *Thermoactinomyces candidus* (accession numbers of published sequences used in this study are listed at the end of this section). Additional primers were designed (Table 1) on the basis of preliminary sequencing results. The 50 µl PCR reactions [10 µl sample, 1× buffer (20 mM Tris/HCl, pH 8.4, 50 mM KCl), 1.5 mM MgCl₂, 0.2 µM each primer, 0.2 mM each dNTP and 2.5 U *Taq* DNA Polymerase (Life Technologies)] were carried out in a GeneAmp PCR System 9700 (Perkin-Elmer, Applied Biosystems) using the following regimen: 94 °C for 10 min; 30 cycles each of 1 min denaturation at 94 °C, 1 min primer annealing at 52 °C and 2 min extension at 72 °C; final extension at 72 °C for 5 min; and incubation at 4 °C. The sizes of the amplicons were determined by loading 10 µl on to a 1% agarose gel alongside a 1 kbp DNA ladder (Life Technologies). Unincorporated primers, dNTPs and salts were removed from the amplicons using the QIAquick PCR purification kit (QIAGEN). The concentration of the eluted DNA (50 µl DNA in 10 mM Tris/HCl buffer, pH 8.5) was estimated by comparing band intensities on agarose gel and by UV-spectrophotometry.

The purified amplicons were sequenced using the ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA Polymerase, FS (Perkin-Elmer, Applied Biosystems), according to a protocol suggested by the manufacturers (except that the annealing temperatures were adjusted for each primer). The 20 µl sequencing reactions (8 µl terminator ready reaction mix, 75 ng DNA template and 3.2 µM primer) were conducted in the same GeneAmp PCR System 9700 used for PCR amplification. Sequencing products were purified prior to electrophoresis using Centri-Sep spin columns (Princeton Separations) according to the manufacturer's instructions. The sequencing products were dried in a vacuum microcentrifuge and forwarded to the Genetic Engineering Laboratory of the University of Illinois at Urbana-Champaign, where the pellets were resuspended and loaded into an automated ABI PRISM 377 sequencer according to the manufacturers' instructions.

Phylogenetic analysis. The various fragments of DNA sequence obtained from both strands were assembled with the multiple sequence editor SEQUENCHER (Gene Codes). The

consensus sequence, hereafter referred to as Hg *Pasteuria* (*Heterodera glycines*-infecting *Pasteuria*), was submitted for sequence-similarity searches using the BLAST program (Altschul *et al.*, 1990) and the SIMILARITY_RANK feature of the Ribosomal Database Project (RDP; Maidak *et al.*, 1999). The SUGGEST_TREE option provided an approximate placement of Hg *Pasteuria* in the RDP maximum-likelihood tree. In addition to the sequences suggested by the above search methods, a set of sequences from a previous alignment involving *P. ramosa* (Ebert *et al.*, 1996) and sequences from *Anacystis nidulans* and *Escherichia coli* (the latter two sequences were used as the outgroup) were retrieved from public databases and aligned with Hg *Pasteuria*, using the graphical multiple alignment software CLUSTALX 1.64b (Thompson *et al.*, 1997). The multiple alignment was edited manually to remove regions with long stretches of leading or trailing gaps. A measure of signal content was obtained using Relative Apparent Synapomorphy Analysis (RASA) with the program RASA 2.2 (Lyons-Weiler *et al.*, 1996) before the data were subjected to phylogenetic analyses using maximum-likelihood (ML), maximum-parsimony (MP) and distance-based methods. The robustness of trees was assessed through bootstrap analysis (Felsenstein, 1985). The fastDNAmL-loop script of fastDNAmL 1.1.1a (Felsenstein, 1981; Olsen *et al.*, 1994) was used for ML analysis with the following option settings: empirical base frequencies, a transition:transversion ratio of 2.0, global rearrangement, N_BEST 3 and MAXJUMBLE 10. The fastDNAmL-boot script of the same software was also used to generate 100 bootstrap trees, which were fed into the CONSENSE program in the PHYLIP package (Felsenstein, 1993) to compute bootstrap proportions. MP analyses were based on informative sites only and were conducted with PAUP 3.1 (Swofford, 1993), using a heuristic search method with 100 random sequence-addition replicates, steepest descent, MULTIPARS and TBR branch-swapping option settings. The same search method and options were used for bootstrap analysis of the MP trees based on 100 replicates with 10 random sequence addition replicates each. The PHYLO_WIN program (Galtier *et al.*, 1996) was used to compute pair-wise evolutionary distances either from all sites or from parsimony sites only and to construct neighbour-joining (NJ) trees (Saitou & Nei, 1987) based on LogDet-corrected distances (Lake, 1994; Lockhart *et al.*, 1994). The NJ trees were evaluated through bootstrap resampling, with 1000 replicates each.

Accession numbers. The accession numbers of published 16S rDNA sequences used in this study are as follows: *Actinomyces bovis*, X81061; *Alicyclobacillus acidocaldarius*, X60742; *Alicyclobacillus acidoterrestris*, X60743; *Alicyclobacillus cycloheptanicus*, X51928; strain ALV, M79375, M79376, M80290; *Anacystis nidulans*, X03538, K01982, X01296; *Aureobacterium liquefaciens*, X77444; *Bacillus caldolyticus*, Z26924; *Bacillus methanolicus*, X64465, S42879; *Bacillus schlegelii*, Z26934; *Bacillus subtilis*, D64126; *Bacillus thermocatenulatus*, Z26926; *Bacillus thermocloacae*, Z26939; *Bacillus thermoruber*, Z26921; *Bacillus thuringiensis* (strain IAM12077), D16281; *Bacillus tusciae*, Z26933; *Bacillus validus*, M77489; *Clostridium difficile*, X73450; *Clostridium polysaccharolyticum*, X71858; *Escherichia coli*, J01859, K02555, M24828, M24833–M24837, M24911, M24996; *Flavobacterium heparinum*, M11657, M61766, M81326; *Heliobacterium chlorum*, M11212; strain HTA1417, AB002649; *Lactobacillus brevis* (isolate L63), D37785; *Megasphaera elsdenii*, M26493; *Oxalophagus oxalicus*, Y14581; *Paenibacillus apiarius*, U49247; *Paenibacillus validus* (strain DSM3037), D78320; *Pasteuria ramosa* (*Daphnia* endosymbiotic bacterium), U34688; *Planococcus citreus*, X62172, S49897; strain Rt8B.4, L35151; *Saccharococcus thermophilus*, X70430; *Thermoactinomyces candidus*, M77490; *Thermoactinomyces vulgaris*, M77491; type 0803 unidentified filamentous bacterium, X86071.

RESULTS

PCR amplification and sequencing of *Pasteuria* DNA

Microscopic examinations revealed that the stock suspension of *Pasteuria* endospores contained many contaminating bacteria. In contrast, no contaminants were observed in the pre-lysis suspension. Likewise, no bacterial growth was observed on the Luria agar plates that were spread with the pre-lysis suspension and incubated for a week at 30 °C. Conversely, the control plates spread with the stock suspension contained several bacterial colonies after 48 h incubation under similar conditions. PCR reactions using the suspensions of representative bacterial colonies that grew on control plates as the template produced a single band, each of which was of the expected size (1400 bp). DNA sequences of those products matched the 16S rDNA of *F. heparinum*, *A. liquefaciens* or type 0803 of an unidentified filamentous bacterium following sequence-similarity searches with the BLAST program (Altschul *et al.*, 1990). PCR reactions with either the pre- or the post-lysis suspension as the template also yielded a single product, each of which was of the expected size (1400 bp). DNA sequences of the products originating from the pre- or the post-lysis suspensions were identical. At least two fragments of DNA sequence were generated with each of the eight primers used to amplify and sequence DNA from either the pre- or the post-lysis suspension, for a total of 18 fragments derived from both DNA strands. When each of the 18 fragments was submitted for sequence-similarity searches using the BLAST program (Altschul *et al.*, 1990), the 16S rDNA sequence of *P. ramosa* was always retrieved as the highest scoring segment with the query sequence. The 18 fragments

were assembled with the multiple sequence editor SEQUENCHER (Gene Codes) to produce a 1341 bp consensus sequence referred to as the Hg *Pasteuria* sequence. This sequence not only included the *Pasteuria*-specific oligonucleotide, 5'-CATTTCTTCTCCCGATG-3' (Ebert *et al.*, 1996), but also contained two oligonucleotides (5'-TACACCCCAGAGGATGCA-3' and 5'-GGGCACAGCACTCTCTCGA-3') that searches of the RDP database of 16S rDNA probes revealed to be unique to the North American isolate of *Pasteuria* [the above sequences are from the minus strand and the positions of the 5' residues in the complementary strand are 440, 69 and 1011, respectively, according to the *E. coli* numbering system (Brosius *et al.*, 1978)].

Phylogenetic relationships between Hg *Pasteuria* and other bacterial species

The edited DNA sequence alignment comprised 1336 sites, including 531 informative sites under parsimony, which were unambiguously aligned for 32 taxa. On the basis of observed (uncorrected) distances, Hg *Pasteuria* shared 93.2% sequence identity with *P. ramosa*, as opposed to the 87.3, 84.7, 83.3, 80.4 and 75.1% sequence identity found between Hg *Pasteuria* and *T. vulgaris*, *A. cycloheptanicus*, *B. subtilis*, *A. bovis* and *E. coli*, respectively. For comparison, sequence similarities between *A. acidocaldarius* and *A. acidoterrestris* and between *T. vulgaris* and *T. candidus* were 99.4 and 98.5%, respectively. The analysis of signal content, after removal of invariant and autapomorphic sites, indicated a significant (tRASA = 12.992, $P < 0.001$) cladistic structure in the data. Phylogenetic inference using ML placed Hg *Pasteuria* and *P. ramosa* at the base of a clade that contained *Alicyclobacillus* spp., *B. tusciae* and a strain (ALV) of a facultatively thermophilic iron-oxidizer (Fig. 1). Six equally parsimonious trees, 2972 steps long with a rescaled consistency index of 0.2 each, were recovered by MP analysis. Four of the six trees (and, hence, the consensus tree) concurred with the ML tree in placing Hg *Pasteuria* and *P. ramosa* in the same branch as *Alicyclobacillus* spp., *B. tusciae* and ALV, there being similar bootstrap values for these relationships (result not shown). The other two most parsimonious trees recovered the *Pasteuria* (Hg *Pasteuria* and *P. ramosa*) as a separate cluster between the branch leading to *Alicyclobacillus* spp. and *B. tusciae* and the branch leading to *Thermoactinomyces* spp. and HTA1417, a strain of an unidentified low-G+C-DNA Gram-positive bacterium (result not shown). Similar topology was obtained when the NJ tree was constructed from LogDet-transformed distances based on all sites (result not shown). However, when LogDet-transformed distances were calculated from parsimony sites only, the NJ tree was consistent with both the ML and MP consensus trees regarding the position of *Pasteuria* spp. (Fig. 2). Unlike the ML tree, the NJ and MP consensus trees placed *B. schlegelii* at the base of the branch leading to the *Alicyclobacillus* group, although

Fig. 1. Phylogenetic reconstruction based on maximum-likelihood analysis of 16S rDNA sequences from 32 bacterial species including Hg *Pasteuria* (*Heterodera glycines*-infecting *Pasteuria*). *A. nidulans* (cyanobacteria) and *E. coli* (*Proteobacteria*) were used as outgroup taxa. Bootstrap proportions (100 replicates) are given for relevant branches only; the scale represents the mean number of nucleotide substitutions per site.

the bootstrap support for this position was poor (18 and 11%, respectively, for the NJ and the MP consensus trees). Strain ALV was not recovered with *Pasteuria* spp. in any of the NJ trees.

DISCUSSION

Provision of clean *Pasteuria* DNA for PCR amplification

The absence of vegetative cells in the pre-lysis suspension of endospores, as evidenced by both microscopic and cultural observations, shows that the heat and lysozyme treatments were effective in destroying contaminating bacteria present in the stock suspension. The identity of the DNA amplified from the pre- and post-lysis suspensions of endospores indicates that, following destruction (by DNase and RNase) of the nucleic acids released (mostly from contaminating bacteria) under the heat and lysozyme treatments, proteinase K and SDS were able to destabilize *Pasteuria* endospores sufficiently to enable release of the DNA amplified during the PCR reaction. The assumption that the amplified DNA originated from

Pasteuria rather than from contaminating bacteria is supported by the fact that the resulting sequence was not only very similar to that of *P. ramosa*, the only *Pasteuria* sequence currently available in the public domain, but also contained the *Pasteuria*-specific oligonucleotide (Ebert *et al.*, 1996). The identity between the amplicons from the pre- and post-lysis suspensions also suggests that the task of extracting and sequencing DNA from *Pasteuria* endospores can now be simplified by eliminating unnecessary steps such as glass-bead beating and subsequent DNA purification.

Hg *Pasteuria* and *P. ramosa* are closely related but distinct species

According to current criteria for delineating bacterial species (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987) Hg *Pasteuria* and *P. ramosa* are closely related but different species. Both *Pasteuria* belong to a cluster of thermophilic endospore-forming bacilli that differ enough from the other members of the genus *Bacillus* to warrant their assignment into separate genera within the *Bacillaceae* (Wisotzkey *et al.*, 1992). The genus

Fig. 2. Neighbour-joining tree derived from the analysis of pair-wise evolutionary distances between the 16S rDNA sequences of 32 bacterial species including *Hg Pasteuria* (*Heterodera glycines*-infecting *Pasteuria*). *A. nidulans* (cyanobacteria) and *E. coli* (Proteobacteria) were used as outgroup taxa. Distances were calculated from parsimony sites only, and the LogDet transformation, which corrects for asymmetric base compositions, was applied. In spite of its usefulness in identifying the correct tree, the LogDet transformation generally does not give the amount (or rate) of change along individual branches of the tree, so no scale is provided. Bootstrap proportions (1000 replicates) are indicated for relevant branches only.

Alicyclobacillus, formerly *Bacillus*, was the first of these 'new genera' to be proposed. It includes thermoacidophilic bacilli that share the rare phenotype of possessing ω -alicyclic fatty acid as the major natural lipid component of the membrane (Wisotzkey *et al.*, 1992). In contrast, the thermophilic chemolithoautotrophs *B. tusciae* and *B. schlegelii*, which group peripherally with *Alicyclobacillus* spp., do not contain ω -alicyclic fatty acid in their membranes (Rainey *et al.*, 1994; Wisotzkey *et al.*, 1992). It would be interesting to know if *Pasteuria* spp., which clearly form a distinct line of descent within the '*Alicyclobacillus* group', exhibit the ω -alicyclic fatty acid phenotype. The position of strain ALV, an asporogenous and Gram-negative bacterium that clusters with the Gram-positive eubacteria (Lane *et al.*, 1992), was not consistent in this study and a previous one (Ebert *et al.*, 1996). Its membership of the '*Alicyclobacillus* group' as suggested by the ML and MP methods [which are

sensitive to DNA base compositions (Galtier & Gouy, 1995; Lake, 1994; Lockhart *et al.*, 1994)] did not stand up when evolutionary distances were corrected for bias using the LogDet transformation (Lake, 1994; Lockhart *et al.*, 1994).

***Pasteuria* spp. and *Thermoactinomyces* spp. are not as closely related as their morphology would suggest**

At the suprageneric level, *Pasteuria* spp. were considered morphologically closer to the *Actinomycetales*, especially *T. vulgaris* (Sayre & Starr, 1985; Sayre & Wergin, 1977), than to the *Bacillaceae* (Mankau, 1975). As mentioned earlier, Ebert *et al.* (1996) questioned the position of the genus *Pasteuria* in the *Actinomycetales* on the basis of the 16S rDNA sequence of *P. ramosa*, but they were unable to provide a definitive answer to the question. To clarify the issue of whether *Pasteuria* spp. are members of the *Actino*-

mycetales, we have considered both the traditional and modern concepts of the actinomycetes. Traditionally, the actinomycetes were defined as bacteria that have the ability to form branching hyphae at some stage of their development (Gottlieb, 1973). This viewpoint was represented in this study by the sequences of *T. candidus* and *T. vulgaris*. According to modern taxonomists, the Gram-positive division of eubacteria consists of four subdivisions, two of which can be readily distinguished on the basis of DNA composition (Fox *et al.*, 1980; Stackebrandt & Woese, 1981a; Woese, 1987). The actinomycete subdivision, which was represented by *A. bovis*, includes bacteria with high G+C content (≥ 55 mol%) and the *Clostridium*–*Bacillus*–*Streptococcus* subdivision contains bacteria with low G+C content (≤ 50 mol%). The two minor subdivisions of Gram-positive bacteria, the photosynthetic subdivision and the subdivision of species with Gram-negative walls, were represented by *H. chlorum* and *M. elsdenii*, respectively.

Several taxa traditionally associated with the actinomycetes have been shown to belong to the low-G+C-content subdivision of the Gram-positive bacteria (Fox *et al.*, 1980; Goodfellow & Cross, 1983; Stackebrandt *et al.*, 1983; Stackebrandt & Woese, 1981a, b; Woese, 1987). Specifically, *T. vulgaris*, which has long been regarded as a ‘good actinomycete’ (Goodfellow & Cross, 1983), has been reclassified in the *Bacillaceae*, thereby indicating that (from a phylogenetic standpoint) possession of hyphae is not as informative as endospore formation (Stackebrandt *et al.*, 1983; Stackebrandt & Woese, 1981b). *Pasteuria* spp. and *Thermoactinomyces* spp. are not closely related either, in spite of their common ability to form branching mycelia. In only three instances in this study (two of six equally parsimonious trees and the NJ tree derived from all sites) were members of the two genera close to each other. As discussed earlier for strain ALV, the G+C content could have shifted the position of *Pasteuria* spp. in the two MP trees, since MP is more sensitive to DNA base composition bias than the ML method (Galtier & Gouy, 1995; Lake, 1994; Lockhart *et al.*, 1994). Although the LogDet transformation effectively corrects for unequal base compositions, it is still not clear which sites should be used for the correction. There are some indications that inclusion of all sites could result in serious underestimation of the amount of change, particularly for anciently diverged sequences (Lockhart *et al.*, 1994).

The results presented herein are consistent with the view that the genus *Pasteuria* is a deeply rooted member of the *Clostridium*–*Bacillus*–*Streptococcus* branch of the Gram-positive eubacteria, neither related to the actinomycetes nor closely related to true endospore-forming bacteria (Berkeley & Ali, 1994). We are currently designing *Pasteuria*-specific primers that will allow the amplification and sequencing of *Pasteuria* DNA from limited amounts of endospores. These procedural improvements are likely to stimulate the sequencing of 16S rDNA from many isolates of

Pasteuria (including *P. nishizawae*, which was not available during the present study). As more of these sequences become available, it should be possible to validate species and to determine the phylogenetic relationships among the various isolates of *Pasteuria*.

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NOTE ADDED IN PROOF

While this paper was in press, the 16S rDNA sequence for *Pasteuria penetrans* was published by Anderson *et al.* (1999). Thus, our statement in the Introduction that no nematode-infecting *Pasteuria* species had been sequenced no longer holds true. *P. penetrans* is a different species to that studied in the present paper.

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