‘Candidatus Pasteuria aldrichii’, an obligate endoparasite of the bacterivorous nematode Bursilla

R. M. Giblin-Davis, G. Nong, J. F. Preston, D. S. Williams, B. J. Center, J. A. Brito and D. W. Dickson

Correspondence
R. M. Giblin-Davis
giblin@ufl.edu

1University of Florida-IFAS, Fort Lauderdale Research and Education Center, 3205 College Avenue, Fort Lauderdale, FL 33314-7719, USA
2Microbiology and Cell Science, University of Florida, PO Box 110700, Gainesville, FL 32611-0700, USA
3Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Bureau of Nematology, 1911 SW 34th Street, Gainesville, FL 32608, USA
4Department of Entomology and Nematology, University of Florida-IFAS, PO Box 110620, Gainesville, FL 32611-0620, USA

A novel bacterium of the genus Pasteuria was discovered parasitizing bacterivorous nematodes of the genus Bursilla, in selected bermudagrass (Cynodon) field plots in Davie, FL, USA. Soil containing this bacterium was sampled and supplied with bi-weekly inoculations of cultured species of the genus Bursilla in order to build and maintain a source of endospores for continuous in vivo conservation of the bacteria for further study and characterization. 16S rRNA gene sequence similarities supported its congeneric ranking with other members of the genus Pasteuria that have been identified from nematodes and cladocerans. There were, however, no clear sister candidates for this organism, which supported the evidence of endospore ultrastructure and host-range studies, suggesting it belonged to a novel taxon. Because members of the genus Pasteuria cannot yet be isolated, definitive type strains could not be maintained; therefore, the name ‘Candidatus Pasteuria aldrichii’ is proposed for this organism.

The genus Pasteuria appears to be a well-defined group of Gram-positive, ‘mycelial’, endospore-forming, endoparasitic prokaryotes with worldwide distribution, which are found in many different groups of soil-inhabiting nematodes and freshwater cladocerans (Preston et al., 2003). At the time of writing, the genus comprised four recognized species, Pasteuria ramosa (Metchnikoff, 1888), Pasteuria penetrans (Sayre & Starr, 1985), Pasteuria thornei (Starr & Sayre, 1988) and Pasteuria nishizawae (Sayre et al., 1991; emended by Noel et al., 2005), and one ‘Candidatus’ taxon, ‘Candidatus Pasteuria usgae’ (Giblin-Davis et al., 2003).

Although more than 300 members of the genus Pasteuria have been reported from many different trophic groups of nematodes, including predatory, free-living, fungivorous and bacterivorous forms, most of the recognized species of the genus Pasteuria have been described as being associated with plant-parasitic nematodes (Chen & Dickson, 1998; Preston et al., 2003; Sturhan et al., 2005). This bias is probably due to a vested interest in obtaining a greater understanding of the potential of these bacteria as biological control agents for this subset of economically important nematodes and is not an indication that any particular affinity of these bacteria for a specific trophic group of nematodes exists (Sturhan et al., 2005).

Because of the obligately parasitic nature of members of the genus Pasteuria, culturing has not been possible until recently (Hewlett et al., 2004), and even then is only possible via proprietary means without any suitable contingency for the establishment and sustained maintenance of a standard type culture collection. Previous work, therefore, was done with a typological approach that relied mostly upon the ultrastructure of mature endospores and on the results of host-range studies to delineate recognized taxa (Metchnikoff, 1888; Sayre & Starr, 1985; Sayre et al., 1988, 1991; Starr & Sayre, 1988; Ebert et al., 1996; Giblin-Davis et al., 2001; Atibalentja et al., 2004; Noel et al., 2005). However, advances in sequencing have given rise to a molecular phylogenetic approach for the delineation of

Abbreviations: MCL, maximum composite likelihood; MP, maximum-parsimony; NJ, neighbour-joining; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of ‘Candidatus Pasteuria aldrichii’ is GU186440.
taxa or phylotypes of members of the genus *Pasteuria* (Ebert et al., 1996; Anderson et al., 1999; Atibalanjta et al., 2000; Bekal et al., 2001; Preston et al., 2003; Sturhan et al., 2005; Bishop et al., 2007).

A *Pasteuria*-like bacterium was found parasitizing a bacteriovorous nematode of the genus *Bursilla* in soil from bermudagrass (*Cynodon*) field plots at the Fort Lauderdale Research and Education Center, University of Florida, Davie, FL, USA (Giblin-Davis et al., 2004). In an effort to further study and characterize this bacterium, the host was isolated and cultured on TSB (Tryptic Soy Broth) agar. The identity of the host was determined morphologically and molecularly using 18S rDNA, D2D3 LSU rDNA and mtCOI gene sequences following the methods of Ye et al. (2007). The nematode host was confirmed to have an identical near full-length 18S rDNA gene sequence to *Bursilla* sp. PS1179 (GenBank accession no. U73452) (referred to as *Mesorhabditis* by Baldwin et al., 1997 but as *Bursilla* by Kiontke & Fitch, 2005), which belongs to the Pleiorhabditis group of nematode worms Kiontke & Fitch (2005). The host nematode, designated *Bursilla* sp. RGD244, is available from the Fort Lauderdale Research and Education Center and *Bursilla* sp. PS1179 is available from the laboratories of Dr David Fitch and Dr Lynn Carta (http://www.nyu.edu/projects/fitch/WSRN/strains/ps1179.html).

**In vivo culturing of nematodes/bacteria**

*Bursilla* sp. RGD244 nematodes were mass-produced on mixed bacterial cultures to produce sentry nematodes for identifying soil containing the novel *Pasteuria*-like organism. Once soil from transect sampling was identified as yielding heavily parasitized hosts, it was heated to 50 °C for 2 hours to decontaminate it of other nematodes and supplied with bi-weekly inoculations of cultured populations (50 000–50 000) of un-infested *Bursilla* sp. RGD244 nematodes in order to build a source of endospores. For initial establishment of *in vivo* culture, un-infested *Bursilla* sp. were inoculated into soil that had been harvested from a single transect location (the original culture was established on 16 July 2003) and their numbers were increased by bi-weekly inoculations of *Bursilla* sp. RGD244 for several months. To reduce potential contamination from other strains of the genus *Pasteuria*, the infested hosts were extracted from the soil using sugar-flotation-centrifugation (Jenkins, 1964) at 48 h post-inoculation and 500 were hand-picked and inoculated into 100 ml autoclaved Margate fine sand with 4 % organic matter (OM). Bi-weekly additions of ~30 000–50 000 un-infested *Bursilla* sp. RGD244 nematodes were made into this soil for 5 months prior to mixing it, as a starter, into 1 l of autoclaved soil in a screened plastic box (13 × 11 × 18 cm). Boxed soil received bi-weekly inoculations of ~30 000–50 000 healthy uninfected *Bursilla* sp. RGD244 nematodes in about 20 ml deionized water for about 1 year before starting over again (Giblin-Davis et al., 2004). This method of building up and maintaining high densities of *Bursilla–Pasteuria* in soil has proven to be reliable over the past 6 years.

Bioassay of these soils after about 2–3 months revealed that un-infested *Bursilla* sp. RGD244 sentinels consistently yielded >80 % of the nematodes of all stages (except the L1 and egg) with 1 to >100 attached bacterial endospores within 24 h of exposure. Replicate experiments assessing the storability of the *Bursilla–Pasteuria* soil culture suggested that dry storage should not be performed for more than about 9 months without renewal of bi-weekly inoculations of cultured populations (30 000–50 000) of un-infested nematodes in order to rejuvenate/maintain the culture. In addition, when the *Bursilla–Pasteuria* soil culture was kept frozen at −20°C for 5 days and then bioassayed with sentinel nematodes, spores that had attached to the nematodes were observed detaching when heat was applied to immobilize the nematodes for observation. This suggests that freezing is not an acceptable method for storage of *Bursilla–Pasteuria* soil cultures.

**Life-cycle description**

Timed developmental studies of the *Bursilla–Pasteuria* interaction using soil cultures at room temperature (24–25°C) (four repeat experiments; n=10 for each experiment) showed that a mean of five spores were attached and ~30 % had penetration tubes within 24 h (Fig. 1a). Within the 24 h period after the initial 24 h of exposure (up to 48 h total) to a soil culture, a mean of five spores were attached, ~50 % had penetration tubes and 21 % of the total attached spores were associated with small pseudocoelic microcolonies that were the diameter of the spore or less (Fig. 1b). Within the 48 h period after the initial 24 h exposure time (72 h total), a mean of five spores were attached and 57 % of the total attached spores were associated with small to medium pseudocoelic microcolonies (ranging in length from the diameter of the spore to about 0.5 times the diameter of the nematode). Within the 72 h period after the initial 24 h exposure time (96 h total), a mean of five spores were attached and 52 % of the total attached spores were associated with medium to large pseudocoelic microcolonies (ranging in length from <0.5 to 2 times the diameter of the nematode) (Fig. 1c); there were also many colony fragments visible in the host nematode pseudocoel. Within the 96 h period after the initial 24 h exposure time (120 h total), a mean of six spores were attached, very few of the attached spores were associated with distinct colonies and the nematodes were filled with vegetative stage and maturing endospores, often forming tetrad of spores (each within their mother cell); penetration tubes under spores often appeared more distinct than in previous time periods. Within the 120 h period after the initial 24 h exposure time (144 h total), a mean of six spores were attached and nematodes varied from being completely filled with spores that were showing engulfment and maturation with parallel fibre formation to being filled with scattered clumps of spores and vegetative cells. Within the 144 h period after the initial 24 h exposure time (168 h total), a mean of four spores were attached and the majority of sampled nematodes were filled with mature endospores (Fig. 1d).
Endospore attachment and host-range studies

Host-range attachment studies of the Bursilla–Pasteuria interaction were conducted using a soil culture and cultured isolates of phylogenetically diverse nematodes, namely: Bursilla sp. RGD244 [belonging to the Pleiorhabditis group of Kiontke & Fitch (2005) and clade V of Blaxter et al. (1998)]; Caenorhabditis elegans (Eurhabditis, clade V); Acrostichus rhynchophori RGD193 (Diplogastridae, clade V); Koeneria sp. RGD228 (Diplogastridae, clade V); Myolaimus sp. RGD233 (Myolaimidae, clade IV); Aphelenchus avenae RGD103 (Aphelenchidae, clade IV); Ditylenchus sp. RGD199 (Tylenchidae, clade IV) and Bursaphelenchus xylophilus (Aphelenchoideidae, clade IV). The Bursilla–Pasteuria soil culture was assayed with Bursilla sp. RGD244 sentinel nematodes first and shown to produce high encumbrance levels (100% of hermaphrodites with a mean cuticular load of 83 spores per nematode) after a 24 h exposure. One litre of this soil was removed and dried for 6 days at room temperature before dividing into 30 g aliquots, each placed into a 35 ml plastic cup. Cultures of each of the above-mentioned species were harvested by the Baermann funnel method (Southey, 1970) and ~1000 nematodes of each species were concentrated into 6 ml water for inoculation. There were four replicate experiments per species. After 24 h of exposure to the culture, 15 g soil per cup was removed, nematodes were extracted separately using the sugar-flotation-centrifugation method (Jenkins, 1964) and >25 nematodes were examined under a compound microscope for attached spores of the Pasteuria-like bacterium. Of the Bursilla sp. RGD244 nematodes examined, 99% were encumbered with between 5 and 72 spores. None of the other test species were encumbered with attached spores, nor were Bursilla sp. RGD244 nematodes that had been inoculated into autoclaved soil from a soil culture as a control. A month-long exposure experiment was conducted with the 15 g soil culture remaining in each of the cups by inoculating them with 500 test nematodes in 3 ml deionized water each week for 4 weeks before harvesting again, 24 h after the final inoculation to see if the host’s attachment status had changed. Nematodes were harvested as described above. About 90% of the examined Bursilla sp. RGD244 nematodes were encumbered with between 1 and 76 spores. Again, none of the other test species were encumbered with attached spores. Thus, from these broad tests of host specificity within nematode species of clades IV and V, it appears that the Bursilla–Pasteuria interaction is relatively host-specific, or at least to the level of the Pleiorhabditis group of nematode worms. However, the specificity of the Bursilla–Pasteuria interaction needs further verification with more members of this group, such as the closely related gonochoristic species of the Pleiorhabditis group, available from the Caenorhabditis Genetics Center (CGC), e.g. Mesorhabditis longisepiculosa DF5017 or Teratorhabditis palmurum DF5019.

Bursilla sp. RGD244 nematodes at all stages of development were inoculated into the Bursilla–Pasteuria soil culture for 96 h and then extracted, measured and examined for encumbrance levels. There were five, relatively discrete, size classes (8–11 nematodes were measured for each size class) of Bursilla sp. RGD244: L1 (240–290 μm); L2 (300–385 μm); L3 (525–625 μm); L4 (675–750 μm) and hermaphroditic females (675–925 μm). The percentage encumbrance was 0% for the L1 size class, 38% for L2, 60% for L3 and 100% for L4 and adult hermaphrodites with 1–11 spores attached.

Morphometrics and ultrastructure

Using light microscopy, the mean endospore diameter in the Bursilla–Pasteuria culture was $4.3 \pm 0.2 \mu m$ (range,
3.7–4.8 μm), the mean central body diameter was 1.5 ± 0.1 μm (range, 1.3–1.8 μm) and the mean core diameter was 0.8 ± 0.1 μm (range, 0.7–0.9 μm) (n=44 externally attached spores). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to determine the external and internal ultrastructure of the Bursilla–Pasteuria complex, relative to other recognized species of the genus Pasteuria. For SEM, Bursilla sp. RGD244 nematodes were inoculated into the Bursilla–Pasteuria soil culture for 24 h and then extracted. Twenty hermaphrodites with spores attached were hand-picked and transferred into 3 % (v/v) glutaraldehyde, post-fixed in 2 % osmium tetroxide for 12 h at 22 °C, rinsed in deionized water, dehydrated in a graded ethanol series, critical-point dried with liquid CO2, mounted on a stub with double sticky tape, sputter-coated with 20 nm of gold-palladium and viewed with a Hitachi S-4000 Field Emission scanning electron microscope at 7 kV (Giblin-Davis et al., 2001). For TEM, Bursilla sp. RGD244 nematodes were inoculated into the Bursilla–Pasteuria soil culture for 24 h and then extracted. Forty hermaphrodites with spores attached were hand-picked and transferred into sterile deionized water and kept at room temperature for 48, 72 and 120 h before being transferred, live, into 2 % formaldehyde (prepared from paraformaldehyde) and 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and fixed overnight at 4 °C. After repeated rinsing in buffer, specimens were post-fixed in 2 % osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2 for 3.5 h at 22 °C. The nematodes were rinsed in water, fixed with 1 % aqueous uranyl acetate, dehydrated using 100 % ethanol, transferred into 100 % acetone and infiltrated with Spurr’s epoxy resin. Blocks were sectioned on an RMC MT-6000-XL ultramicrotome and nearly-serial sections with silver refraction (75 nm) were picked up on copper grids with a 0.35 % Formvar coating reinforced with a light carbon film. Sections were post-stained with 5 % aqueous uranyl acetate and lead citrate before viewing in a Zeiss EM-10CA transmission electron microscope at 80 kV. The external and internal ultrastructure of the Bursilla–Pasteuria complex was typologically different from all previously described interactions between members of the genus Pasteuria and nematode worms (see taxon description) (Fig. 2).

**Molecular phylogenetic analysis**

For molecular phylogenetic analysis Bursilla sp. RGD244 nematodes were inoculated into the Bursilla–Pasteuria soil culture for 24 h and then extracted. Twenty hermaphrodites with spores attached were hand-picked, transferred into sterile deionized water and kept at room temperature for 48 h before being transferred into a 0.5 ml tube to be gently smashed with a pipette tip to release the vegetative bacterial cells. The suspension was centrifuged at 5 000 g for 5 min and all but 1 μl of the supernatant was removed. The cell pellet was resuspended in 9 μl sample buffer from a GenomiPhi kit (GE healthcare) and heated for 3 min in a boiling water bath. After cooling on ice for 3 min, 9 μl reaction buffer and 1 μl enzyme mix were added for PCR amplification according to the protocols of the GenomiPhi kit. The 16S rRNA gene fragments were amplified with a general primer pair used for most bacteria (27F: 5’-AGAGTTTGATCMTGGCTCAG-3’; 1100R: 5’-GGTTGCGCTGTTGTTG-3’) in a Bio-Rad iCycler using enzyme blends (5 U Taq DNA polymerase per reaction and 0.2 U ProofStart DNA polymerase per reaction; Qiagen) and a touchdown PCR protocol with the following conditions: 1 cycle of 95 °C for 5 min, 21 cycles of 95 °C for 30 s followed by an initial annealing temperature of 60 °C for 30 s decreasing by 0.5 °C per cycle and an extension step of 72 °C for 1 min, followed by an additional 20 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min and a final extension step of 72 °C for 10 min. PCR products were obtained at the predicted size of ~1 kb. PCR products were re-treated with Taq DNA polymerase (Qiagen) at 72 °C for 10 min and then cloned into pCR4-TOPO following the protocol of the supplier (Invitrogen). The products of ligation were transformed into chemically competent Escherichia coli TOP10 cells. Colonies containing recombinant plasmids were chosen for sequencing. Endonuclease restriction digestion demonstrated that the inserts were without an EcoRI site. Two sequences of 982 and 1 003 bp were obtained from the clones, which showed 99.6 % similarity with only four base pairs not matching (three substitutions; one T/C, one T/G and one A/G; and one gap resulting from insertion or deletion).

In order to extend the above-mentioned ~1 kb 16S rRNA gene sequence of the novel Pasteuria-like bacteria, bacterial genomic DNA was released from 20 infected nematodes and randomly amplified with a GenomiPhi kit as described above. PCR fragments were amplified with a specific primer for the 5’ end of the previously sequenced ~1 kb fragment and a reverse primer containing the consensus sequence (1392r: 5’-ACGGGCGGTGTGTRC-3’) at the 3’-end of the gene sequence, using HotStar High Fidelity kit (Qiagen). PCR products were cloned into the pCR2.1-TOPO vector and transformed into E. coli TOP10 recipients. Transformants were detected by real-time PCR using an iCycler (Bio-Rad) and determining a product melting point of 89 °C. Seven clones were selected on the basis of RT-PCR parameters (melting point) and the same size of insert being released upon digestion with EcoRI. The DNA inserts in these clones were then sequenced. Three of these contained identical sequences for the 540 bp sequence extending from position 853 from the start of the full-length 16S rRNA gene, providing a nearly complete (1393 bp) sequence for the gene encoding 16S rRNA in the novel Pasteuria-like bacterium. The four other clones were not related to members of the genus Pasteuria, but were most closely related to other members of the phylum Firmicutes, for example, members of the families Paenibacillaceae, Enterobacteraceae and Xanthomonadaceae such as species of the genera Cohnella, Photobacterium and Stenotrophomonas. The complete 16S rRNA sequence
obtained in this study has been made available in GenBank under accession no. GU186440.

For phylogenetic analysis, alignments were performed with sequences of related taxa obtained from GenBank, using CLUSTAL_X version 1.81 using Mycobacterium tuberculosis as an outgroup. The aligned results were converted to MEGA version 4.0 (Kumar et al., 2008) format and phylogenetic analyses were performed using MEGA version 4.0 software with the neighbour-joining (NJ), maximum-parsimony (MP) and maximum composite likelihood (MCL) methods (with bootstrap analysis based on 1000 replicates) as described in the manual for the program (http://www.megasoftware.net/manual.pdf).

NJ and MP analyses of near full-length 16S rRNA gene sequences (Figs 3 and 4) suggested that the Pasteuria-like bacterium occupied a unique taxon, but grouped with other species of the genus Pasteuria. Using phylogenetic analyses of multiple loci, Charles et al. (2005) inferred that members of the genus Pasteuria occur within the Gram-positive, spore-forming Bacillus–Clostridium supergroup. Therefore, the results of 16S rRNA gene sequence analysis of the Pasteuria-like bacterium strongly support the assertion that it is a previously undocumented organism of the genus Pasteuria.

Based on the development, life cycle, morphology, ultrastructure and phylogenetic position of the novel organism, previously referred to as the Pasteuria sp. R-1 isolate (Giblin-Davis et al., 2004), it represents a novel taxon in the genus Pasteuria, for which the name ‘Candidatus Pasteuria aldrichii’ is proposed. The bacterium was identified from a bacterivorous nematode of the genus Bursilla collected from bermudagrass (Cynodon) field-plot soil at the Fort Lauderdale Research and Education Center, University of Florida, FL, USA (26°05′12″ N 80°14′26″ W). The following description is based upon data presented herein.

**Description of ‘Candidatus Pasteuria aldrichii’**

‘Candidatus Pasteuria aldrichii’ (al.dri’chi.i. N.L. masc. gen. n. aldrichii of Aldrich, named in honour of Dr Henry Aldrich for his many contributions to research and teaching in microbiology at the University of Florida).

‘Candidatus Pasteuria aldrichii’ (Firmicutes) NC; G++; M; NAS (GenBank no. GU186440); morphology (see following...
Fig. 3. Molecular analysis of the near full-length 16S rRNA gene sequences of ‘Candidatus Pasteuria aldrichii’ and related taxa. The tree was reconstructed with MEGA version 4 software by using the neighbour-joining (NJ) method. Bootstrap values >50 %, based on 1000 replicates, are given at branch points. GenBank accession numbers are given in parentheses. Strain numbers and type strains have been provided where appropriate. Bar, 0.02 substitutions per nucleotide position.

Fig. 4. Molecular analysis of sequences of the near full-length 16s rRNA gene of ‘Candidatus Pasteuria aldrichii’. The tree was reconstructed with MEGA version 4 software by using the maximum-parsimony (MP) method. Bootstrap values >50 %, based on 1000 replicates, are given at branch points. GenBank accession numbers are given in parentheses. Strain numbers and type strains have been provided where appropriate.
described species of the genus Pasteuria relative to the peripheral fibre layers is about 2:1 in 'Pasteuria penetrans' as inferred from 16S rDNA sequence analysis. Int J Syst Evol Microbiol 50, 605–613.


Acknowledgements

Special thanks to Dr Henry Aldrich for early discussions concerning this project and Drs Karin Kiontke and Weimin Ye for help with the molecular identification of the nematode species.

References


that is easily cultured on a bacteriovorous nematode, *Bursilla* sp. in soil. *J Nematol* 36, 319–320.


