Available online at www.sciencedirect.com
SCIENCE DIRECT®
Systematic and Applied Microbiology 28 (2005) 323–327
SYSTEMATIC AND APPLIED MICROBIOLOGY
www.elsevier.de/syapm

Bacillus nematocida sp. nov., a novel bacterial strain with nematotoxic activity isolated from soil in Yunnan, China

Xiao-Wei Huang, Qiu-Hong Niu, Wei Zhou, Ke-Qin Zhang*

Laboratory for Conservation and Utilization of Bio-Resources, Yunnan University, Kunming, Yunnan 650091, P.R. China

Received 25 November 2004

Abstract

An endospore-forming bacterium, designated strain B-16^T, was isolated from a forest soil sample in Yunnan, China. The isolate presented remarkable nematotoxic activity against nematode *Panagrellus redivivus*. The organism was strictly aerobic, motile, spore forming and rod shaped, catalase- and oxidase-positive. The predominant isoprenoid quinone was menaquinone 7 (MK-7). The major cellular fatty acid profiles were anteiso-$C_{15:0}$ (48.67%), iso-$C_{15:0}$ (13.45%), $C_{16:0}$ (9.06%) and anteiso-$C_{17:0}$ (8.29%). The DNA G + C content was 46%. Phylogenetic analyses based on 16S rDNA sequence revealed that isolate belongs to the genus *Bacillus*. Strain B-16^T exhibited high 16S rDNA similarity with its closest neighbors *Bacillus vallismortis* (99.79%), *B. subtilis* (99.43%), *B. atrophaeus* (99.43%), *B. amyloliquefaciens* (99.36%), *B. licheniformis* (98.0%) and less than 97.0% with all the other relative type strains in the genus *Bacillus*. The phenotypic and genotypic characteristics and DNA–DNA relatedness data indicate that strain B-16^T should be distinguished from all the relative species of genus *Bacillus*. Therefore, on the basis of the polyphasic taxonomic data presented, a new species of the genus *Bacillus*, *B. nematocida*, with the type strain B-16^T (= CGMCC 1128^T) is proposed.

The GenBank accession number for the sequence reported in this paper is AY820954.

Keywords: *Bacillus nematocida* sp. nov.; Polyphasic taxonomy; 16S rDNA

Introduction

Plant-parasitic nematodes cause serious losses to a variety of agricultural crops worldwide. In 2000, nematodes cost cotton growers across the belt 4.3% of their crop, which translated into an economic loss valued over $300 million. Since the traditional methods based on the use of nematicides and antihelminthic drugs are associated with major environmental and health concerns, the development of biocontrol agents to control nematodes is of major importance [4].

Because of the fast breeding, ease cultivation and production compared with fungi, nematophagous bacteria have been used extensively as bioinsecticides against nematodes in soil, and levels of control equivalent to those of chemical pesticides developed [22]. In this work, we present the polyphasic taxonomic characterization of strain B-16^T with remarkable nematotoxic activity against nematode *Panagrellus redivivus*. Genotypic and phenotypic data showed that the strain B-16^T should be recognized as a new species of the genus *Bacillus*, for which the name *Bacillus nematocida* sp. nov. is proposed.

*Corresponding author. Tel.: +86 871 5034878; fax: +86 871 5034838.
E-mail address: kqzhang1@yahoo.com.cn (K.-Q. Zhang).

0723-2020/$ - see front matter © 2005 Elsevier GmbH. All rights reserved.
doi:10.1016/j.syapm.2005.01.008

Methods

Isolation, screening and culture condition

Strain B-16^T was isolated by using the dilution plating method from a soil sample in Yunnan Province, the south-west of China. The medium used for selective isolation was LB agar medium and incubated at 37 °C for about 2–3 days. The bacteria with significant nematotoxic activities were screened according to the method described by Åhman et al. [1] with modification. The strain B-16^T was maintained on LB agar slants at 4 °C and as glycerol suspensions (20%, v/v) at –20 °C. Biomass for chemical and molecular systematic studies was obtained by growing in shake flasks (about 150 rpm) of LB medium broth at 37 °C for 2–3 days.

Phenotypic characteristics

Morphological properties were examined by light microscopy (Olympus microscope BH-2) and Hitachi H-800 transmission electron microscopy. Gram staining was carried out using the KOH lysis test method [2] combined with standard Gram reaction. Metabolic properties were determined using API ID 32 E test kits (bioMerieux) according to the manufacturer's instructions. Other physiological and biochemical tests were performed as described previously [12,13]. Color determination was done with color chips from the ISCC-NBS COLOR CHARTS Standard Samples No. 2106 [7].

Menaquinone and cellular fatty acid compositions

Menaquinones were isolated using the methods of Minnikin et al. [16] and separated by HPLC [10]. The whole-cell fatty acid compositions were determined by using the standard Microbial Identification System (MIDI Inc.) of Sasser [18] and organisms that had been grown for 24 h at 37 °C on Trypticase soy agar.

DNA extraction and amplification of 16S rDNA

Extraction of genomic DNA and amplification of 16S rDNA were done as described by Xu et al. [21]. 16S rDNA was amplified by PCR using TaKaRa Ex *Taq* (TaKaRa Biotechnology) and primers A 8-27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and B 1523-1504r (5′-TAAGGAGGTGATCCAGCCGC-3′). The PCR reaction parameters included: an initial 5 min for pre-denaturation at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 56 °C, 3 min at 72 °C and a final extension of 5 min at 72 °C and then cooled to 4 °C. The 1.5 kb amplified 16S rDNA fragment was separated by agarose gel electrophoresis. The purified fragment was directly sequenced using a *Taq* DyeDeoxy terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed with an ABI PRISMTM 377 DNA sequencer (Applied Biosystems Inc.). Sequencing primers used included primer A, primer B and primer C (5′-AGGGTTGC GCTCGTTG-3′).

Analysis of sequence data

The 16S rDNA sequence of the test strain was aligned manually against corresponding sequences of most related *Bacillus* strains obtained from the GenBank database. Phylogenetic analysis was performed using the software packages PHYLIP [6] and molecular evolutionary genetics analysis (MEGA) version 2.1 [11] after multiple alignment of data by CLUSTALX [19]. Distances (distance options according to the Kimura [8,9] two-parameter mode) and clustering with the neighbor-joining method [17]. Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1000 resamplings [5].

Nucleotide sequence accession numbers

The 16S rDNA sequence of strain B-16^T determined in this study has been deposited in GenBank under the accession number AY820954. The accession numbers of the reference strains, which are closely related to strain B-16^T, are indicated in Fig. 1.

DNA–DNA reassociation and G + C content determination

The DNA for the base composition and renaturation studies was prepared following the method of Marmur [14]. In brief, 2 × SSC of the salt concentration and 70.5 °C of hybridization temperature were employed. The renaturation rates of genomic fragments from pairs of strains were determined spectrophotometrically with a model 1601 UV spectrophotometer equipped with a Thermoelectric Cell Temperature Controller (SHIMADZU). The equation of De Ley et al. [3] was used to calculate the extent of DNA–DNA reassociation.

The G + C content was estimated and calculated by the thermal melting procedure described by Marmur and Doty [15].

Results

Phenotypic characteristics

The strain B-16^T was aerobic, Gram-positive. Cells are bacilli that are 0.8–1.2 μm wide by 2.0–3.5 μm long and occurring singly or in short chains, motile and spore forming. Colonies of B-16^T were opaque, smooth,

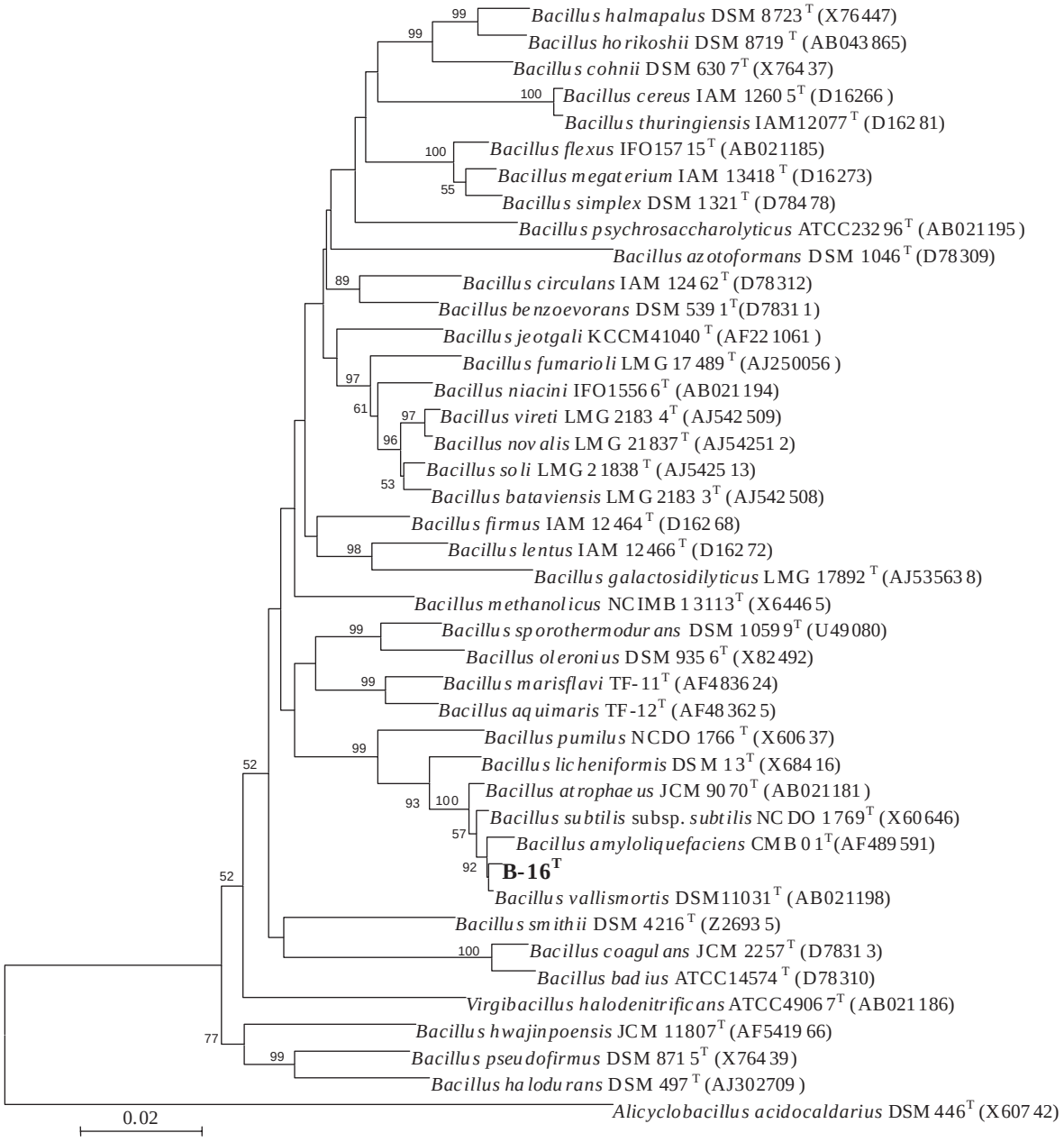

Fig. 1. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rDNA sequences, showing the position of strain B-16^T among phylogenetic neighbors. Numbers on branch nodes are bootstrap values (1000 resamplings). The sequence of *Alicyclobacillus acidocaldarius* DSM 446^T (X60742) was used as root. Bar, 1% sequence divergence.

jagged and 1.0–2.0 mm in diameter after incubation for 2 days at 37 °C. The pH and temperature range for growth were 6.0–8.5 and 4–50 °C, and the optimum growth pH and temperature were 7.0–8.0 and 20–37 °C, respectively. Catalase- and oxidase-positive. The activities for ornithine decarboxylase, β-glucosidase, lysine decarboxylase, urease, α-glucosidase, α-galactosidase, α-maltosidase, L-aspartique arylamidase and lipase were positive, *N*-acetyl-β-glucosaminidase, arginine dihydrolase, β-galactosidase and β-glucuronidase were negative. Strain B-16^T could utilize glucose, maltose, ribose, mannose, mannitol, sucrose, xylose, fructose, salicin, dextrin, galactose, starch, arabinose, cellobiose, laetrile, sorbitol and rhamnose. Acid was only produced from *N*-acetyl-β-glucosamine, lactose and aesculin. It was positive for milk coagulation and peptonization, gelation liquefaction, Tween 80 decomposition and starch hydrolysis, but negative for growth in cellulose, H_2S and melanin production (shown in Table 1).

Table 1. Phenotypic characteristics that distinguish strain B-16^T from the other most related five type strains of genus *Bacillus*

Character	B-16^T	*B. vallismortis* DSM 11031^T	*B. subtilis* NCDO 1769^T	*B. atrophaeus* JCM 9070^T	*B. amyloliquefaciens* CMB 01^T	*B. licheniformis* DSM 13^T
Oxidase activity	+	+	+	−	+	+
Maximum growth temperature (°C)	50	50	50	50	50	55
Minimum growth temperature (°C)	4	10	10	10	10	15
Acid from						
Glucose	−	+	+	+	+	+
Arabinose	−	+	+	+	+	+
Xylose	−	+	+	+	+	+
Mannitol	−	+	+	+	+	+
Lactose	+	−	−	−	+	−
Tween 80 decomposition	+	+	W	+	ND	ND
G+C mol%	46	43	43	42	43	46

Note: The following phenotypic characteristics are same in all the related type strains: Gram-positive, catalase-positive, motile, spore-forming rod cells. Casein and starch are decomposed. Nitrate is reduced to nitrite. +, positive; −, negative; w, utilized weakly; d, doubtful; ND, not determined.

Chemotaxonomic characteristics

The predominant menaquinone was MK-7. The major cellular fatty acid profiles were anteiso-$C_{15:0}$ (48.67%), iso-$C_{15:0}$ (13.45%), $C_{16:0}$ (9.06%) and anteiso-$C_{17:0}$ (8.29%).

Phylogenetic analysis

The almost complete 16S rDNA sequence of strain B-16^T consisting of 1511 bp was determined. Preliminary comparison of the sequence against the GenBank database indicated that members of the genus *Bacillus* were the closest phylogenetic neighbors. Binary similarity values of this strain and other species of the genus *Bacillus* ranged between 92.3% (*B. azotoformans* DSM 1046^T) and 99.79% (*B. vallismortis* DSM 11031^T). The phylogenetic tree of *Bacillus* species is shown in Fig. 1. The closest phylogenetic neighbors of strain B-16^T are *B. vallismortis* DSM 11031^T (99.79%), *B. subtilis* NCDO 1769^T (99.43%), *B. atrophaeus* JCM 9070^T (99.43%), *B. amyloliquefaciens* CMB 01^T (99.36%) and *B. licheniformis* DSM 13^T (98.0%), which have higher than 97% 16S rDNA sequence similarity.

Accordingly, comparative taxonomic studies were performed among strain B-16^T, *B. vallismortis* DSM 11031^T, *B. subtilis* NCDO 1769^T, *B. atrophaeus* JCM 9070^T, *B. amyloliquefaciens* CMB 01^T and *B. licheniformis* DSM 13^T to determine whether strain B-16^T could be considered as a new species of the genus *Bacillus* or would be assigned to one of these species.

The strain B-16^T showed some differences from the other five most closest neighbors in some phenotypic data (Table 1). DNA–DNA relatedness tests were performed among strain B-16^T and the other five related species under optimal conditions (2 × SSC at 70.5 °C), and DNA–DNA reassociation similarity between strain B-16^T and *B. vallismortis* DSM 11031^T, *B. subtilis* NCDO 1769^T, *B. atrophaeus* JCM 9070^T, *B. amyloliquefaciens* CMB 01^T and *B. licheniformis* DSM 13^T were with 45.5%, 38.6%, 15.2%, 21.3% and 13.2%, respectively. DNA–DNA relatedness provided decisive evidence that the new isolate B-16^T and the other related type strains are members of different genomic species [20]. The G+C content for strain B-16^T was 46 mol%, which lies within the range for the genus *Bacillus*.

Therefore, based on the above phenotypic and genotypic results, we consider strain B-16^T to represent a novel new species of genus *Bacillus*, for which we propose the name *B. nematocida* sp. nov.

Description of *B. nematocida* sp. nov.

B. nematocida (ne.ma.to' ci.da. N.L. plur. n. Nematoda sytematic zoological name of a phylum (common name roundworms), L.masc. suffix n. -cida killer, L. masc. n. nematocida a killer of nematods). The organism is strictly aerobic, motile, spore forming and rod shaped, catalase- and oxidase-positive. The activity for ornithine decarboxylase, β-glucosidase, lysine decarboxylase, urease, α-glucosidase, α-galactosidase, α-maltosidase, L-aspartique arylamidase and lipase are positive, *N*-acetyl-β-glucosaminidase, arginine dihydrolase, β-galactosidase and β-glucuronidase are negative. It could utilize glucose, maltose, ribose, mannose, mannitol, sucrose, xylose, fructose, salicin, dextrin,

galactose, starch, arabinose, cellobiose, laetrile, sorbitol and rhamnose. Acid is only produce from *N*-acetyl-β-glucosamine, lactose and aesculin. It is positive for milk coagulation and peptonization, gelation liquefaction, Tween 80 decomposition and starch hydrolysis, but negative for growth in cellulose, H_2S and melanin production. The predominant menaquinone is MK-7. The cellular fatty acid profiles (>1%) are anteiso-$C_{15:0}$ (48.67%), iso-$C_{15:0}$ (13.45%), $C_{16:0}$ (9.06%), anteiso-$C_{17:0}$ (8.29%), iso-$C_{17:0}$ (4.53%), $C_{15:0}$ (4.01%), iso-$C_{16:0}$ (3.61%) and iso-$C_{14:0}$ (2.98%). The DNA G + C content is 46 mol%. Its optimum growth temperature and pH are at 20–37 °C and 7.0–8.0, respectively. It was isolated from soil in Yunnan Province, the west of China. The type strain is strain B-16^T (= CGMCC 1128^T).

Acknowledgements

The authors are grateful to Prof. Hans G. Trüper for the Latin construction of the species name. We thank Dr. Wen-Jun Li's kind help in technical support and in preparing the manuscript. This research was supported by National Natural Science Foundation of China (Project No. 30230020) and Department of Science and Technology of Yunnan Province (Project Nos. 2004C0004Q and 2004C0001Z).

References

[1] J. Åhman, T. Johansson, M. Olsson, P.J. Punt, C.A. van den Hondel, A.S. Tunlid, Improving the pathogenicity of a nematode-trapping fungus by genetic engineering of a subtilisin with nematotoxic activity, Appl. Environ. Microbiol. 689 (2002) 3408–3415.

[2] G. Cerny, Studies on aminopeptidase for the distinction of Gram-negative from Gram-positive bacteria, Eur. J. Appl. Microbiol. Biotechnol. 5 (1978) 113–122.

[3] J. De Ley, H. Cattoir, A. Reynaerts, The quantitative measurement of DNA hybridization from renaturation rates, Eur. J. Biochem. 12 (1970) 133–142.

[4] L.W. Duncon, Current options for nematode management, Annu. Rev. Phytopathol. 29 (1991) 469–490.

[5] J. Felsenstein, Confidence limits on phylogenies: an approach using the bootstrap, Evolution 39 (1985) 783–791.

[6] J. Felsenstein, PHYLIP (Phylogeny Inference Package), version 3.5c, Department of Genetics, University of Washington, Seattle, USA, 1993.

[7] K.L. Kelly, Inter-Society Color Council-National Bureau of Standards Color Name Charts Illustrated with Centroid Colors, US Government Printing Office, Washington, DC, 1964.

[8] M. Kimura, A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequence, J. Mol. Evol. 16 (1980) 111–120.

[9] M. Kimura, The Neutral Theory of Molecular Evolution, Cambridge University Press, Cambridge, 1983.

[10] R.M. Kroppenstedt, Separation of bacterial menaquinones by HPLC using reverse phase (RP 18) and a silver loaded ion exchanger as stationary phases, J. Liq. Chromatogr. 5 (1982) 2359–2387.

[11] S. Kumar, K. Tamura, I.B. Jakobsen, M. Nei, MEGA2: molecular evolutionary genetics analysis software, Bioinformatics 17 (2001) 1244–1245.

[12] W.-J. Li, H.-H. Chen, P. Xu, Y.-Q. Zhang, P. Schumann, S.-K. Tang, L.-H. Xu, C.-L. Jiang, *Yania halotolerans* gen. nov., sp. nov., a novel member of the suborder *Micrococcineae* from saline soil in China, Int. J. Syst. Evol. Microbiol. 54 (2004) 525–531.

[13] W.-J. Li, H.-H. Chen, Y.-Q. Zhang, P. Schumann, E. Stackebrandt, L.-H. Xu, C.-L. Jiang, *Nesterenkonia halotolerans* sp. nov. and *Nesterenkonia xinjiangensis* sp. nov., actinobacteria from saline soils in the west of China, Int. J. Syst. Evol. Microbiol. 54 (2004) 837–841.

[14] J. Marmur, A procedure for the isolation of deoxyribonucleic acid from microorganisms, J. Mol. Biol. 3 (1961) 208–218.

[15] J. Marmur, P. Doty, Determination of base composition of deoxyribonucleic acid from its denaturation temperature, J. Mol. Biol. 5 (1962) 109–118.

[16] D.E. Minnikin, A.G. O'Donnell, M. Goodfellow, G. Alderson, M. Athalye, A. Schaal, J.H. Parlett, An integrated procedure for the extraction of isoprenoid quinines and polar lipids, J. Microbiol. Meth. 2 (1984) 233–241.

[17] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.

[18] M. Sasser, Identification of bacteria by gas chromatography of cellular fatty acids, USFCC Newslett. 20 (1990) 16.

[19] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D. Higgins, The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Res. 24 (1997) 4876–4882.

[20] L.G. Wayne, D.J. Brenner, R.R. Colwell, et al., International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics, Int. J. Syst. Bacteriol. 37 (1987) 463–464.

[21] P. Xu, W.J. Li, L.H. Xu, C.L. Jiang, A microwave-based method for genomic DNA extraction from Actinomycetes, Microbiology (Chinese) 30 (4) (2003) 73–75.

[22] X.S. Zhou, H.K. Kaya, K. Heungens, G.B. Heidi, Response of ants to a deterrent factor(s) produced by the symbiotic bacteria of entomopathogenic nematodes, Appl. Environ. Microbiol. 68 (2002) 6202–6209.