Bacillus niabensis sp. nov., isolated from cotton-waste composts for mushroom cultivation

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A group of five bacilli, designated strains 4T12, 4T19^T, 5M45, 5M53 and 5T52, isolated from cotton-waste composts for mushroom cultivation, were examined. These strains were Gram-positive, aerobic, motile, spore-forming rods. 16S rRNA gene sequence analyses revealed that the isolates belonged to the genus *Bacillus*, showing the highest levels of similarity (approx. 96.6–96.9 %) with respect to *Bacillus herbersteinensis* DSM 16534^T. The values for DNA–DNA hybridization (approx. 85–96 %) among these five strains revealed that they belong to the same species. The major menaquinone present was MK-7 and the predominant cellular fatty acids were anteiso-C_{15:0} (approx. 24.5–33.9 %) and C_{16:0} (approx. 15.1–34.1 %). The DNA G + C contents were 37.7–40.9 mol%. On the basis of physiological, biochemical, chemotaxonomic and comparative genomic analyses, the five isolates represent a novel species of the genus *Bacillus*, for which the name *Bacillus niabensis* sp. nov. is proposed. The type strain is 4T19^T (=KACC 11279^T =DSM 17723^T).

Cotton waste is frequently used in Korea as a medium for the cultivation of oyster mushrooms. In the course of a study of the changes in microbial communities involved in the composting of cotton wastes, the temperature of the material was raised gradually from room temperature to 65 °C, and five bacterial strains were isolated.

Samples were spread on tryptic soy agar (TSA; Difco) at pH 7.0 and incubated at 30 °C for 3 days. Light-yellow colonies were produced on TSA and nutrient agar (Difco). Gram staining was performed with a Gram-stain kit (Difco) according to the manufacturer's protocol. A KOH test and an L-alanine aminopeptidase assay were also performed (Gegersen, 1978). The cell morphology was observed by using transmission electron microscopy (model 912AB; LEO) and phase-contrast microscopy (AXIO; Zeiss) after cultivation on TSA for 2 days.

Various physiological tests were carried out according to Gordon *et al.* (1973) and Claus & Berkeley (1986). The

Voges–Proskauer test was performed along with tests for catalase activity, temperature ranges for growth (from 5 to 65 °C, using 5 °C increments), resistance to lysozyme (0.001 %), growth in the presence of NaCl (at 0, 3, 5, 7, 10 and 15 %), pH range for growth (pH 4–9 in increments of 1.0 pH unit), acid production from carbohydrates (D-glucose, L-arabinose, D-xylose and D-mannitol), gas formation from glucose, utilization of citrate and propionate, production of indole and dihydroxyacetone, deamination of phenylalanine, decomposition of casein and tyrosine and liquefaction of gelatin. The oxidase test and tests for the hydrolysis of aesculin, gelatin and starch were conducted according to Smibert & Krieg (1994). Motility tests were performed on motility medium, which was composed of 0.1 % yeast extract, 0.01 % K₂HPO₄ and 0.2 % agar. Anaerobic growth was tested in serum bottles by adding thioglycolate (1 g l⁻¹) to tryptic soy broth medium and replacing the upper airspace with nitrogen gas. To test for the reduction of nitrate and nitrite, each isolate was inoculated into three serum bottles (25 ml) containing tryptic soy broth medium, while nitrate and nitrite were added as KNO₃ and NaNO₂ at concentrations of 10 mM. The reduction of nitrate and nitrite was monitored using an ion chromatograph (model 790 personal IC; Metrohm) equipped with a conductivity detector and an anion-exchange column (Metrosep Anion Supp 4; Metrohm). The urease test was performed according to Kim *et al.* (2006).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 4T12, 4T19^T, 5M45, 5M53 and 5T52 are DQ176420, AY998119, DQ176421, DQ176422 and DQ176423, respectively.

Phase-contrast micrographs of the novel strains and tables showing their phenotypic characteristics and fatty acid compositions and levels of DNA–DNA hybridization between the novel isolates are available as supplementary material with the online version of this paper.

The 16S rRNA gene of each isolate was amplified by means of a PCR with two universal primers, as described previously (Kwon *et al.*, 2003). The sequences of amplified 16S rRNA genes were analysed using a DNA sequencer (ABI3100; Applied Biosystems). The 16S rRNA gene sequences were aligned using CLUSTAL W software (Thompson *et al.*, 1994). Nucleotide similarity values were calculated from the alignment. An evolutionary distance matrix was constructed using a two-parameter model (Kimura, 1980), and phylogenetic trees for the datasets were inferred with the neighbour-joining method (Saitou & Nei, 1987) using MEGA, version 3.0 (Kumar *et al.*, 2004). The stability of relationships was assessed by performing bootstrap analyses of the neighbour-joining data (based on 1000 resamplings).

DNA–DNA hybridization was carried out using the filter hybridization method described by Seldin & Dubnau (1985). Probe labelling was conducted by using the non-radioactive DIG-High prime system (Roche), and hybridized DNA was visualized using a DIG luminescent detection kit (Roche). DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad). Chemotaxonomic characteristics of these strains in this study were determined using cells grown at 30 °C on TSA for 24 h. Isoprenoid quinones were analysed by HPLC (Shimadzu) as described previously (Groth *et al.*, 1996). DNA G+C contents (mol%) were determined by performing HPLC analysis of deoxyribonucleosides, as described by Mesbah *et al.* (1989). Analysis of the fatty acid methyl esters was performed by GLC according to the instructions for the Microbial Identification System (MIDI).

The five strains, 4T19^T, 4T12, 5M45, 5M53 and 5T52, showed similar morphologies. The cells were rod-shaped, occurring singly or in filamentous chains. Single cells were 0.5–0.7 µm in diameter and 2.0–3.0 µm in length. Spores were ellipsoidal or oval, occurring subterminally or terminally in swollen sporangia (see Supplementary Fig. S1 in IJSEM Online).

The isolates were motile on the medium used in this study. The growth temperature range for the isolates was 15–40 °C and the pH range for growth was pH 6.0–8.0 (optimum, pH 7.0), these ranges being different from those for *Bacillus litoralis* and *Bacillus herbersteinensis*. The isolates differed from each other with regard to growth ranges for NaCl (%): strain 4T19^T grew in the presence of NaCl (w/v) at concentrations up to 5%, 4T12 could grow with NaCl concentrations up to only 3% and the other isolates did not grow in the presence of 3% NaCl. All isolates were positive for catalase, urease, nitrate reduction, acid production from D-glucose, L-arabinose, D-xylose and D-mannitol and hydrolysis of aesculin, gelatin and starch. All isolates were negative for oxidase, phenylalanine deamination, the formation of indole and dihydroxyacetone, in the Voges–Proskauer test and for the utilization of citrate and propionate. Detailed results of the physiological characterizations are given in the species description, and

features that serve to distinguish between the isolates and related *Bacillus* species are listed in Table 1 and in Supplementary Table S1 (available in IJSEM Online).

For the phylogenetic analysis of the 16S rRNA gene sequences, representative sequences retrieved from GenBank were aligned from position 37 (5') to position 1330 (3') (*Escherichia coli* numbering; Brosius *et al.*, 1978). All of the isolates, which fell within the radiation of the genus *Bacillus* including *Bacillus subtilis*, formed a compact cluster with 100% bootstrap support (Fig. 1). The 16S rRNA gene sequence similarity between strain 4T19^T and the other isolates was approximately 99.5–99.7%. However, strain 4T19^T exhibited relatively low levels of 16S rRNA gene sequence similarity with respect to *Bacillus* species. The closest relatives of strain 4T19^T were the type strains of *B. herbersteinensis* (96.7%), *B. litoralis* (96.2%) and *Bacillus cohnii* (96.0%).

DNA–DNA hybridization was performed to determine the genetic relatedness between all five isolates. The DNA–DNA relatedness value between strain 4T19^T and the other isolates was 91–96%, meaning that they represent the same species (Wayne *et al.*, 1987) (Supplementary Table S2). Meanwhile, the low levels of 16S rRNA gene sequence similarity (<97%) between 4T19^T and the type strains of *Bacillus* species with validly published names confirm that this strain represents a species that is distinct from recognized *Bacillus* species (Stackebrandt & Goebel, 1994).

Fatty acid analysis revealed some differences between the isolates and related *Bacillus* species (Table 1). The major fatty acid components of the isolates were anteiso-C_{15:0}, C_{16:0}, anteiso-C_{17:0}, iso-C_{16:0} and iso-C_{14:0} (Supplementary Table S3). The isolates contained an unsaturated menaquinone with seven isoprene units (MK-7) as the predominant isoprenoid quinone. The G+C contents of the isolates were in the range 37.7–40.9 mol%, which is higher than the values for *B. litoralis*, *B. herbersteinensis*, *B. cohnii* and *Bacillus fastidiosus*.

On the basis of the polyphasic data, the five isolates represent a novel species of the genus *Bacillus*, for which the name *Bacillus niabensis* sp. nov. is proposed.

Description of *Bacillus niabensis* sp. nov.

Bacillus niabensis (niab.en'sis. N.L. masc. adj. *niabensis* arbitrary name formed from NIAB, the acronym for the National Institute of Agricultural Biotechnology, Korea, where taxonomic studies on this species were performed).

Cells are Gram-positive, motile, spore-forming rods, 0.5–0.7 × 2.0–3.0 µm, motile by means of single polar flagella. Cells form ellipsoidal or oval spores that occur subterminally or terminally in swollen sporangia. Colonies are yellowish white, 2–3 mm in diameter and circular with clear margins after incubation on TSA for 2 days. Growth occurs at temperatures ranging from 15 to 50 °C, the optimum being 30 °C. Growth occurs at pH 6.0–8.0, the optimum being pH 7.0. Capable of growing anaerobically.

Table 1. Characteristics that serve to differentiate the novel strains from recognized *Bacillus* species

Taxa: 1, strains 4T12, 4T19^T, 5M45, 5M53 and 5T52 (data from this study); 2, *B. litoralis* (Yoon & Oh, 2005); 3, *B. herbersteinensis* (Wieser *et al.*, 2005); 4, *B. cibi* (Yoon *et al.*, 2005); 5, *B. indicus* (Suresh *et al.*, 2004); 6, *B. cohnii* (Priest *et al.*, 1988; Täubel *et al.*, 2003; Suresh *et al.*, 2004); 7, *B. megaterium* (Priest *et al.*, 1988; Täubel *et al.*, 2003; Suresh *et al.*, 2004); 8, *B. fastidiosus* (Claus & Berkeley, 1986); 9, *B. flexus* (Priest *et al.*, 1988; Täubel *et al.*, 2003; Suresh *et al.*, 2004). +, Positive; –, negative; w, weakly positive; v, variable; NG, no growth; ND, no data available. Data in parentheses are for the type strain. The cells of all taxa listed are rod-shaped and catalase-positive.

Characteristic	1	2	3	4	5	6	7	8	9
Spore position*	S, T	C, S or T	T	C or S	S	T	C	ND	ND
Spore shape†	E	E	ND	E	ND	E	E	E	ND
Sporangium shape	Swollen	Swollen	Not swollen	Swollen	Slightly swollen	Swollen	Swollen	Not swollen	ND
Oxidase activity	–	+	+	+	ND	+	–	ND	ND
Anaerobic growth	+	–	ND	–	–	ND	–	–	ND
pH range	6–8	5.5–7.5	7–12	5.5–7.5	6–7	5	4.5–9.5	ND	4.5–9.5
Optimum pH	7	7.5	8–9	6.5–7.5	7	9	7	ND	7
NaCl (%) range	(0–5)	2–10	0–5	0–12	0–2	0–10 (w)	0–5	0–5	0–10 (w)
Temperature (°C) range	15–50	4–45	4–28	10–40	15–37	10–47	10–40	10–40	17–40
Acid from:									
L-Arabinose	+	+	–	–	ND	ND	V	NG	ND
D-Glucose	+	+	–	+	–	(–)	+	NG	+
D-Mannitol	+	–	–	–	–	(–)	V	NG	+
D-Xylose	+	+	–	–	–	(–)	V	NG	–
Hydrolysis of:									
Aesculin	+	+	ND	+	+	(–)	+	ND	–
Casein	–	+	ND	+	ND	v (+)	+	–	ND
Gelatin	+	+	ND	–	+	+	+	–	–
Starch	+	+	+	+	ND	+	+	–	+
Nitrate reduction	+	–	–	–	–	+	–	–	–
Urease activity	+	+	ND	–	–	–	+	ND	–
DNA G + C content (mol%)	37.7–40.8	35.2	36.2–36.9	45	41.2	33.9–35.0	37.3–37.6	34.3–35.1	38.4
Major fatty acid(s)	ai-C _{15:0} , C _{16:0}	ai-C _{15:0} , i-C _{15:0}	ai-C _{15:0} , i-C _{15:0}	i-C _{15:0} , i-C _{14:0}	i-C _{15:0} , ai-C _{15:0}	ai-C _{17:0} , i-C _{15:0} , ai-C _{15:0} or i-C _{15:0} , ai-C _{15:0}	ai-C _{15:0}	ND	ai-C _{15:0} , i-C _{15:0}

*C, Central or paracentral; S, subterminal; T, terminal.

†E, Ellipsoidal or oval.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of the novel strains in relation to selected *Bacillus* species. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

NaCl growth range is variable, being in the range 0–5 % (w/v). Positive for catalase, urease, acid production from D-glucose, L-arabinose, D-xylose and D-mannitol and hydrolysis of aesculin, gelatin and starch. Negative for oxidase, formation of indole and dihydroxyacetone, phenylalanine deamination, utilization of citrate and propionate, degradation of tyrosine, growth with lysozyme and in the Voges–Proskauer test. Nitrate is reduced under anaerobic conditions. The major cellular fatty acids are anteiso- $C_{15:0}$, $C_{16:0}$, anteiso- $C_{17:0}$ and iso- $C_{16:0}$. The major quinone is MK-7. The DNA G+C contents are 37.7–40.9 mol%, as determined by HPLC.

The type strain, 4T19^T (=KACC 11279^T =DSM 17723^T), was isolated from cotton-waste composts in Suwon, Korea.

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References

- Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci U S A* **75**, 4801–4805.
- Claus, D. & Berkeley, R. C. W. (1986). Genus *Bacillus* Cohn 1872. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1105–1140. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Gordon, R. E., Haynes, W. C. & Pang, C. H.-N. (1973). *The Genus Bacillus*. US Department of Agriculture Handbook no. 427. Washington, DC: US Department of Agriculture.
- Gregersen, T. (1978). Rapid method for distinction of Gram-negative from Gram-positive bacteria. *Eur J Appl Microbiol Biotechnol* **5**, 123–127.
- Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.
- Kim, B. Y., Lee, S. Y., Weon, H. Y., Kwon, S. W., Go, S. J., Park, Y. K., Schumann, P. & Fritze, D. (2006). *Ureibacillus suwonensis* sp. nov., isolated from cotton waste composts. *Int J Syst Evol Microbiol* **56**, 663–666.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Kwon, S. W., Kim, J. S., Park, I. C., Yoon, S. H., Park, D. H., Lim, C. K. & Go, S. J. (2003). *Pseudomonas koreensis* sp. nov., *Pseudomonas umsongensis* sp. nov. and *Pseudomonas jinjuensis* sp. nov., novel species from farm soils in Korea. *Int J Syst Evol Microbiol* **53**, 21–27.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Priest, F. G., Goodfellow, M. & Todd, C. (1988). A numerical classification of the genus *Bacillus*. *J Gen Microbiol* **134**, 1847–1882.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Seldin, L. & Dubnau, D. (1985). DNA homology among *Bacillus polymyxa*, *Bacillus macerans*, *Bacillus azotofixans*, and other nitrogen-fixing *Bacillus* strains. *Int J Syst Bacteriol* **35**, 151–154.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Spanka, R. & Fritze, D. (1993). *Bacillus cohnii* sp. nov., a new, obligately alkaliphilic, oval-spore-forming *Bacillus* species with ornithine and aspartic acid instead of diaminopimelic acid in the cell wall. *Int J Syst Bacteriol* **43**, 150–156.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Suresh, K., Prabakaran, S. R., Sengupta, S. & Shivaji, S. (2004). *Bacillus indicus* sp. nov., an arsenic-resistant bacterium isolated from an aquifer in West Bengal, India. *Int J Syst Evol Microbiol* **54**, 1369–1375.

- Täubel, M., Kämpfer, P., Buczolits, S., Lubitz, W. & Busse, H.-J. (2003).** *Bacillus barbaricus* sp. nov., isolated from an experimental wall painting. *Int J Syst Evol Microbiol* **53**, 725–730.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Wieser, M., Worliczek, H., Kämpfer, P. & Busse, H.-J. (2005).** *Bacillus herbersteinensis* sp. nov. *Int J Syst Evol Microbiol* **55**, 2119–2123.
- Yoon, J.-H. & Oh, T.-K. (2005).** *Bacillus litoralis* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **55**, 1945–1948.
- Yoon, J.-H., Lee, C.-H. & Oh, T.-K. (2005).** *Bacillus cibi* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. *Int J Syst Evol Microbiol* **55**, 733–736.