NOTE

***Bacillus pycnus* sp. nov. and *Bacillus neidei* sp. nov., round-spored bacteria from soil**L. K. Nakamura,¹ O. Shida,² H. Takagi² and K. Komagata³¹ Microbial Properties Research Unit, National Center for Agricultural Utilization Research, 1815 N. University Street, Peoria, IL 61604, USA² Research Laboratory, Higeta Shoyu Co. Ltd, Choshi, Chiba 288, Japan³ Department of Agricultural Chemistry, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156, Japan

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***Bacillus sphaericus sensu lato* currently consists of seven or more groups of unrelated taxa, one of which is *B. sphaericus sensu stricto* and another of which is *Bacillus fusiformis*. Members of two groups (groups 6 and 7), in common with all other *B. sphaericus*-like organisms, are unable to grow anaerobically or to use common hexoses, pentoses and hexitols as sources of carbon, have G+C contents of 34–36 mol% and form round spores. Groups 6 and 7 can be differentiated from other *B. sphaericus*-like organisms by low DNA relatedness and by variations in whole-cell fatty acid composition. Unique characteristics of group 6 include the ability to oxidize β -hydroxybutyrate, the non-requirement for biotin and thiamin and failure to grow in 5% NaCl. Distinctive traits of group 7 include the inability to oxidize pyruvate and a requirement for biotin, thiamin and cystine for growth. These data show that groups 6 and 7 represent two novel species, for which the names *Bacillus pycnus* sp. nov. and *Bacillus neidei* sp. nov., respectively, are proposed; the corresponding type strains are NRRL NRS-1691^T (= JCM 11075^T) and NRRL BD-87^T (= JCM 11077^T).**

Keywords: *Bacillus pycnus*, *Bacillus neidei*, novel species, 16S rDNA

The round-spored, mesophilic species *Bacillus sphaericus* was first described by Neide (1904). Because early differentiation tests were ineffective, all round-spored mesophiles were classified as *B. sphaericus*; the lack of adequate differentiating tools deterred taxonomic study of mesophilic, round-spored bacteria. However, the discovery of mosquitocidal activity among some of these organisms (Kellen *et al.*, 1965) and the development of molecular-biological techniques have rekindled taxonomic and phylogenetic examination of the round-spored organisms. Taxonomic heterogeneity of *B. sphaericus* had been suggested by variability of insecticidal activity among strains and was confirmed by comparative studies using DNA similarity assessment (Krych *et al.*, 1980), numerical taxonomy (Alexander & Priest, 1990; Priest *et al.*, 1988), randomly amplified polymorphic DNA fingerprinting (Woodburn *et al.*, 1995) and rRNA gene restriction (Aquino de Muro *et al.*, 1992). These developments led to the discovery of two more mesophilic species, *Bacillus fusiformis* (Priest *et al.*, 1988) and *Bacillus silvestris* (Rheims *et al.*, 1999). In a phylogenetic study based on 16S rDNA sequences (Nakamura, 2000), *B. sphaericus*-like species segregated into seven groups wherein group 3 represented *B. sphaericus* and group 2

comprised *B. fusiformis*; group 1 appeared to be a subgroup of 2. Groups 4–7 were unknown taxa.

In the present study, DNA relatedness evaluation and phenotypic characterization were used to differentiate groups 6 and 7 from each other, from representative strains of groups 4 and 5 and from type strains of recognized round-spore-forming relatives.

DNA was extracted from 24-h cultures from groups 1 (NRRL B-23269), 4 (NRRL NRS-593), 5 (NRRL B-1876), 6 (NRRL NRS-1691^T, NRRL NRS-1693, NRRL NRS-1694, NRRL NRS-1695) and 7 (NRRL BD-87^T, NRRL BD-101, NRRL BD-103) and the following type cultures: '*Bacillus aminovorans*' DSM 4337, *B. fusiformis* DSM 2898^T, *Sporosarcina globispora* NRRL NRS-1533^T, *Bacillus insolitus* NRRL NRS-1531^T, *Marinibacillus marinus* DSM 1297^T, *Sporosarcina psychrophila* IFO 15381^T, *B. silvestris* DSM 12223^T, *B. sphaericus* JCM 2502^T and *Ureibacillus thermosphaericus* DSM 10633^T. DNA reassociation was carried out using the method of Ezaki *et al.* (1989). Probes were prepared with DNA from *B. sphaericus* JCM 2502^T, *B. fusiformis* DSM 2898^T, NRRL NRS-1691^T, NRRL NRS-1694, NRRL BD-87^T and NRRL BD-103.

Fig. 1. Neighbour-joining tree showing the phylogenetic position of groups 6 and 7 among the mesophilic and psychrophilic round-spored *Bacillus* species and selected oval-spored species. The tree is based on 1321-nt sequences. Confidence limits estimated from bootstrap analyses (500 replicates) appear at the nodes. This is a modification of the tree generated by Nakamura (2000). A maximum-parsimony tree generated from the sequence data showed a similar topology. *Alicyclobacillus cycloheptanicus* was designated the outgroup species for the analysis. *B. thermosphaericus* has been reclassified as *Ureibacillus thermosphaericus* (Fortina et al., 2001); *B. globisporus*, *B. psychrophilus* and *B. pasteurii* have been reclassified as *Sporosarcina globispora*, *Sporosarcina psychrophila* and *Sporosarcina pasteurii*, respectively (Yoon et al., 2001a); *B. marinus* has been reclassified as *Marinibacillus marinus* (Yoon et al., 2001b); and *B. stearothermophilus* has been reclassified as *Geobacillus stearothermophilus* (Nazina et al., 2001). Bar, 1 nt substitution per 10 nt.

Substrate oxidation profiles were obtained using the BIOLOG GP system under conditions prescribed by the manufacturer. Other physiological traits were examined using the methods of Gordon *et al.* (1973). Antibiotic sensitivity was assessed by placing com-

mercially prepared antibiotic discs on TGY agar plates spread-inoculated with the test organisms. The test plates were incubated at 28 °C and the extent of inhibition was determined at 48 h. Decomposition of Tweens 40 and 80 was determined by the method

of Breuil & Gounot (1972). Vitamin and amino acid requirements were determined using the method of Proom *et al.* (1955).

The extent of similarity among strains based on fatty acid composition data (Nakamura, 2000) was estimated using the simple matching coefficient and clustering was based on the unweighted pair group method with arithmetic averages (Sneath & Sokal, 1973). All cultures were grown on trypticase soy agar for 24 h at 30 °C. Cell-wall peptidoglycan composition was analysed using the method described by Suzuki *et al.* (1993).

In a phylogenetic tree based on 16S rDNA sequences, strains of groups 4–7 were positioned within a clade that consisted generally of mesophilic, round-spored species, namely *B. sphaericus*, *B. fusiformis* and *B. silvestris*; the thermophile, *U. thermosphaericus*, is situated outside the clade [Fig. 1; this tree is a modification of one generated by Nakamura (2000)]. High bootstrap values validate the uniqueness of groups 4–7 within the clade of round-spored organisms. Groups 6 and 7 were more closely related to each other than to the other mesophilic groups.

High levels of DNA relatedness (97–100 %) were observed between NRRL NRS-1691^T and other group 6 strains, namely NRRL NRS-1693, NRRL NRS-1694 and NRRL NRS-1695; 100 % relatedness was also observed between NRRL NRS-1694 and two other group 6 strains (NRRL NRS-1693 and NRRL NRS-1695). Low DNA relatedness values, of 27 and 19 %, respectively, were determined between NRRL NRS-1694 and two group 7 strains, NRRL BD-87^T and NRRL BD-103. Similarly, low levels of DNA relatedness were observed between NRRL NRS-1694 and representative strains from groups 1 (NRRL B-23269), 4 (NRRL NRS-593) and 5 (NRRL B-1876); values were respectively 23, 27 and 15 %. Finally, the levels of DNA relatedness between NRRL NRS-1694 and the type strains of existing round-spored species (*B. aminovorans* DSM 4337, *B. fusiformis* DSM 2898^T, *S. globispora* NRRL NRS-1533^T, *B. insolitus* NRRL NRS-1531^T, *M. marinus* DSM 1297^T, *Sporosarcina pasteurii* DSM 33^T, *S. psychrophila* IFO 15381^T, *B. silvestris* DSM 12223^T, *B. sphaericus* JCM 2502^T and *U. thermosphaericus* DSM 10633^T) ranged from 1 to 18 %.

DNA relatedness values of 100 % were measured between NRRL BD-87^T and other group 7 strains (NRRL BD-101 and NRRL BD-103). The levels of DNA relatedness between NRRL BD-101 and NRRL BD-103 were also 100 %. Low DNA relatedness values were noted between NRRL BD-87^T and representative strains from groups 1 (NRRL B-23269), 4 (NRRL NRS-593) and 5 (NRRL B-1876); the respective values were 20, 17 and 19 %. Low levels of DNA relatedness, ranging from 3 to 19 %, were measured between NRRL BD-87^T and the type strains of the existing round-spored species listed above. DNA relatedness

Table 1. Traits useful for differentiating the *B. sphaericus*-like groups

The BIOLOG system was used to evaluate substrate oxidation. Substrates listed are those giving 0 or 100 % reactions. L-Glutamate was used by all strains; 64 substrates were not used at all and variable reactions were obtained with 26 substrates. All organisms tested were sensitive to chloramphenicol, erythromycin, tetracycline and tobramycin. Groups: 1 and 2 (*n* = 20), *B. fusiformis*; 3 (*n* = 7), *B. sphaericus*; 4 (*n* = 10), unknown; 5 (*n* = 7), unknown; 6 (*n* = 3), *B. pycnus* sp. nov.; and 7 (*n* = 4), *B. neidei* sp. nov.

Character	Group					
	1 and 2	3	4	5	6	7
Substrate oxidized:						
Pyruvate	+	+	+	+	+	–
β-Hydroxybutyrate	–	–	–	–	+	–
L-Alanine	+	+	+	+	–	–
Glycyl L-glutamate	+	+	+	+	–	+
2'-Deoxyadenosine	+	+	+	+	–	+
Inosine	+	+	–	+	–	+
AMP	+	+	–	–	–	+
UMP	+	+	–	–	–	+
Growth in 5 % NaCl	+	+	+	+	–	+
Resistance to streptomycin	+	+	–	–	–	–
Decomposition of:						
Tween 40	+	+	+	+	–	–
Tween 80	+	+	+	–	–	–
Vitamin requirement:						
Biotin	+	+	+	+	–	+
Thiamin	+	+	+	+	–	+
Cystine requirement	–	–	–	–	–	+

data show that groups 6 and 7 are genetically distinct taxa.

Phenotypic data collated from this and previous studies (Table 1) demonstrated the phenotypic distinctiveness of groups 6 and 7. Traits that differentiate group 6 from all the other taxa were β-hydroxybutyrate oxidation, growth inhibition by 5 % NaCl and non-requirement for vitamins. Unique traits of group 7 were failure to oxidize pyruvate and a growth requirement for cystine.

The dendrogram based on fatty acid composition shown in Fig. 2 shows clear separation of groups 6 and 7 from each other, from groups 4 and 5 and from the existing named mesophilic species. Numerical analysis of substrate-oxidation patterns segregated the organisms similarly (Nakamura, 2000). The predominant fatty acids found in group 6 were 15:0iso (70.3 %), 15:0anteiso (8.1 %) and 16:1ω7cis alcohol (6.0 %). Smaller amounts of 16:0 (3.1 %) and 16:1ω11cis (1.4 %) fatty acids were also found.

In group 7, the most abundant fatty acids were 15:0iso (23.6 %), 15:0anteiso (17.8 %) and 16:1ω11cis

Fig. 2. Dendrogram showing the relationship of groups 6 and 7 to the other *B. sphaericus*-like species. A matrix consisting of 47 strains and 12 fatty acids was used. The fatty acid compositional data were obtained in a previous study (Nakamura, 2000). Similarity was estimated by means of the simple matching coefficient (S_{sm}) and clustering was based on the unweighted pair group method with arithmetic averages (Sneath & Sokal, 1973).

(14.5%). Other fatty acids observed were 14:0iso (5.0%), 15:0 (1.7%), 16:1 ω 7*cis* alcohol (4.7%) and 16:0iso (8.1%).

L-Lysine and D-glutamic acid were the key amino acids found in the cell wall peptidoglycans of NRRL NRS-1691^T and NRRL BD-87^T. Like many of the round-spored species (Stackebrandt *et al.*, 1987), the peptidoglycan type for groups 6 and 7 was L-Lys-D-Glu.

Based on these results, groups 6 and 7 merit recognition as two novel species, for which the respective names *Bacillus pycnus* sp. nov. and *Bacillus neidei* sp. nov. are proposed. The respective type strains are NRRL NRS-1691^T (= JCM 11075^T) and NRRL BD-87^T (= JCM 11077^T).

Description of *Bacillus pycnus* sp. nov.

Bacillus pycnus (pyc'nus. Gr. adj. *pyknos* thick; N.L. adj. *pycnus* thick, referring to thick cells).

Rods are 1.0–1.5 $\times$ 3.0–5.0 μ m (determined from photomicrograph). Gram-positive. Motile. Round spores form in swollen sporangia. Agar colonies are non-pigmented, translucent, thin, smooth, circular, entire and the mean diameter is about 1 mm after 24 h of incubation on TGY agar at 28 °C. Catalase is produced. Strictly aerobic. Nitrate is not reduced to nitrite.

Acetylmethylcarbinol, dihydroxyacetone, indole and H₂S are not produced. The pH in Voges–Proskauer broth ranges from 7.2 to 7.6. Starch, casein, tyrosine, urea, Tweens 40 and 80 and egg-yolk lecithin are not decomposed. Common hexoses, pentoses, hexitols, disaccharides and trisaccharides are not fermented. Grows at pH 5.7, but not in the presence of 0.001% lysozyme and 5% NaCl. Sensitive to chloramphenicol, tobramycin, streptomycin, erythromycin and tetracycline. Biotin, thiamin and cystine are not required for growth. Based on the BIOLOG GP method, pyruvate and β -hydroxybutyrate are oxidized. Citrate, propionate, L-alanine, glycyl L-glutamate, 2'-deoxyadenosine, inosine, AMP and UMP are not oxidized. Optimum growth temperature is 28–30 °C (minimum, 5–10 °C; maximum, 40–45 °C). Whole-cell fatty acids detected are 15:0iso (70.3%), 15:0anteiso (8.1%), 16:1 ω 7*cis* alcohol (6.0%), 16:0iso (3.1%) and 16:1 ω 11*cis* (1.4%). Cell wall peptidoglycan type is L-Lys-D-Glu. Isolated from soil. G+C content is 35 mol%. Type strain is NRRL NRS-1691^T (= JCM 11075^T).

Description of *Bacillus neidei* sp. nov.

Bacillus neidei (nei'de.i. N.L. gen. n. *neidei* of Neide, in recognition of the early microbiologist E. Neide).

Rods are about 1.0 $\times$ 3.0–5.0 μ m (determined by photomicrograph). Gram-positive. Motile. Round spores form in swollen sporangia. Agar colonies are non-pigmented, translucent, thin, smooth, circular, entire and the mean diameter is about 1 mm after 24 h of incubation on TGY at 28 °C. Catalase is produced. Strictly aerobic. Nitrate is not reduced to nitrite. Acetylmethylcarbinol, dihydroxyacetone, indole and H₂S are not produced. The pH in Voges–Proskauer broth ranges from 7.2 to 7.6. Starch, casein, tyrosine, urea, Tweens 40 and 80 and egg-yolk lecithin are not decomposed. Common hexoses, pentoses, hexitols, disaccharides and trisaccharides are not fermented. Grows at pH 5.7 and in 5% NaCl, but not in the presence of 0.001% lysozyme. Sensitive to chloramphenicol, tobramycin, streptomycin, erythromycin and tetracycline. Biotin, thiamin and cystine are required for growth. Based on the BIOLOG GP method, pyruvate and β -hydroxybutyrate are oxidized. Citrate, propionate, L-alanine, glycyl L-glutamate, 2'-deoxyadenosine, inosine, AMP and UMP are not oxidized. Optimum growth temperature is 28–30 °C (minimum, 5–10 °C; maximum, 40–45 °C). Whole-cell fatty acids detected are 14:0iso (5.0%), 15:0 (1.7%), 15:0iso (23.6%), 15:0anteiso (17.8%), 16:0 (5.4%), 16:1 ω 7*cis* alcohol (4.7%), 16:0iso (8.1%) and 16:1 ω 11*cis* (14.5%). Cell wall peptidoglycan type is L-Lys-D-Glu. Isolated from soil. G+C content is 35 mol%. Type strain is NRRL BD-87^T (= JCM 11077^T).

References

- Alexander, B. & Priest, F. G. (1990). Numerical classification and identification of *Bacillus sphaericus* including some strains pathogenic for mosquito larvae. *J Gen Microbiol* **136**, 367–376.

- Aquino de Muro, M., Mitchell, W. J. & Priest, F. G. (1992).** Differentiation of mosquito-pathogenic strains of *Bacillus sphaericus* from non-toxic varieties by ribosomal RNA gene restriction patterns. *J Gen Microbiol* **138**, 1159–1166.
- Breuil, C. & Gounot, A. M. (1972).** Recherches preliminaries sur les bacteries lipolytiques psychrophiles des sols et des eaux. *Can J Microbiol* **18**, 1445–1451.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989).** Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Fortina, M. G., Pukall, R., Schumann, P., Mora, D., Parini, C., Manachini, P. L. & Stackebrandt, E. (2001).** *Ureibacillus* gen. nov., a new genus to accommodate *Bacillus thermosphaericus* (Andersson *et al.* 1995), emendation of *Ureibacillus thermosphaericus* and description of *Ureibacillus terrenus* sp. nov. *Int J Syst Evol Microbiol* **51**, 447–455.
- Gordon, R. E., Haynes, W. C. & Pang, C. H.-N. (1973).** Genus *Bacillus*. *Agriculture Handbook* no. 427. Washington, DC: United States Department of Agriculture.
- Kellen, W. R., Clark, T. B., Lindegren, J. E., Ho, B. C., Rogoff, M. H. & Singer, S. (1965).** *Bacillus sphaericus* Neide as a pathogen of mosquitoes. *J Invertebr Pathol* **7**, 442–448.
- Krych, V. K., Johnson, J. L. & Yousten, A. A. (1980).** Deoxyribonucleic acid homologies among strains of *Bacillus sphaericus*. *Int J Syst Bacteriol* **30**, 476–484.
- Nakamura, L. K. (2000).** Phylogeny of *Bacillus sphaericus*-like organisms. *Int J Syst Evol Microbiol* **50**, 1715–1722.
- Nazina, T. N., Tourova, T. P., Poltarau, A. B. & 8 other authors (2001).** Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. *Int J Syst Evol Microbiol* **51**, 433–446.
- Neide, E. (1904).** Botanische Beschreibung einiger sporenbildenden Bakterien. *Zentbl Bakteriol Parasitenkd Infektionskr Hyg Abt II* **12**, 337–352.
- Priest, F. G., Goodfellow, M. & Todd, C. (1988).** A numerical classification of the genus *Bacillus*. *J Gen Microbiol* **134**, 1847–1882.
- Proom, H. & Knight, B. C. J. G. (1955).** The minimal nutritional requirements of some species in the genus *Bacillus*. *J Gen Microbiol* **13**, 474–480.
- Rheims, H., Frühling, A., Schumann, P., Rohde, M. & Stackebrandt, E. (1999).** *Bacillus silvestris* sp. nov., a new member of the genus *Bacillus* that contains lysine in its cell wall. *Int J Syst Bacteriol* **49**, 795–802.
- Sneath, P. H. A. & Sokal, R. R. (1973).** *Numerical Taxonomy*. San Francisco: W. H. Freeman.
- Stackebrandt, E., Ludwig, W., Weizenegger, M., Dorn, S., McGill, T. J., Fox, G. E., Woese, C. R., Schubert, W. & Schleifer, K.-H. (1987).** Comparative 16S rRNA oligonucleotide analyses and murein types of round-spore-forming bacilli and non-spore-forming relatives. *J Gen Microbiol* **133**, 2523–2529.
- Suzuki, K., Goodfellow, M. & O'Donnell, A. G. (1993).** Cell envelopes and classification. In *Handbook of New Bacterial Systematics*, pp. 195–250. Edited by M. Goodfellow & A. G. O'Donnell. London: Academic Press.
- Woodburn, M. A., Yousten, A. A. & Hilu, K. H. (1995).** Random amplified polymorphic DNA fingerprinting of mosquito-pathogenic and nonpathogenic strains of *Bacillus sphaericus*. *Int J Syst Bacteriol* **45**, 212–217.
- Yoon, J.-H., Lee, K.-C., Weiss, N., Kho, Y. H., Kang, K. H. & Park, Y.-H. (2001a).** *Sporosarcina aquimarina* sp. nov., a bacterium isolated from seawater in Korea, and transfer of *Bacillus globisporus* (Larkin and Stokes 1967), *Bacillus psychrophilus* (Nakamura 1984) and *Bacillus pasteurii* (Chester 1898) to the genus *Sporosarcina* as *Sporosarcina globispora* comb. nov., *Sporosarcina psychrophila* comb. nov. and *Sporosarcina pasteurii* comb. nov., and emended description of the genus *Sporosarcina*. *Int J Syst Evol Microbiol* **51**, 1079–1086.
- Yoon, J.-H., Weiss, N., Lee, K.-C., Lee, I.-S., Kang, K. H. & Park, Y.-H. (2001b).** *Jeotgalibacillus alimentarius* gen. nov., sp. nov., a novel bacterium isolated from jeotgal with L-lysine in the cell wall, and reclassification of *Bacillus marinus* Rüger 1983 as *Marinibacillus marinus* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **51**, 2087–2093.