Transfer of *Bacillus ehimensis* and *Bacillus chitinolyticus* to the genus *Paenibacillus* with emended descriptions of *Paenibacillus ehimensis* comb. nov. and *Paenibacillus chitinolyticus* comb. nov.

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The taxonomic status of *Bacillus ehimensis* and *Bacillus chitinolyticus* was examined, based on their 16S rDNA sequences, DNA–DNA hybridization and other taxonomic characteristics. A phylogenetic analysis using 16S rDNA sequences revealed that the two species belong to the genus *Paenibacillus*. In particular, *B. ehimensis* KCTC 3748^T and *B. chitinolyticus* KCTC 3791^T were found to be phylogenetically closely related to *Paenibacillus koreensis* YC300^T (98.3 % sequence similarity) and *Paenibacillus chinjuensis* WN9^T (95.2 % sequence similarity), respectively. DNA–DNA hybridization values between *B. ehimensis* KCTC 3748^T and *P. koreensis* YC300^T were less than 26 %. An experiment using *Paenibacillus*-specific PCR primers, PAEN515F and 1377R, revealed that *B. ehimensis* and *B. chitinolyticus* had the same amplified 16S rDNA fragment as members of the genus *Paenibacillus*. Accordingly, it is proposed that *B. ehimensis* and *B. chitinolyticus* be transferred to the genus *Paenibacillus* as *Paenibacillus ehimensis* comb. nov. and *Paenibacillus chitinolyticus* comb. nov., respectively.

The genus *Paenibacillus* was first proposed by Ash *et al.* (1993), based on phylogenetic data from 16S rDNA sequences, and then emended by Shida *et al.* (1997). Members of the genus *Paenibacillus* produce ellipsoidal spores in swollen sporangia, are facultatively anaerobic or strictly aerobic, rod-shaped, and have G + C contents ranging from 45 to 54 mol% (Ash *et al.*, 1993).

Bacillus ehimensis and *Bacillus chitinolyticus*, both of which exhibit chitinolytic activity, were first described by Kuroshima *et al.* (1996). At that time, these authors studied the morphological and biochemical characteristics of *B. ehimensis* and *B. chitinolyticus*, but did not describe their phylogenetic relationship with related taxa. However, the current authors believe that *B. ehimensis* and *B. chitinolyticus* are closer to the genus *Paenibacillus* than the genus *Bacillus*, based on a phylogenetic analysis of their 16S rDNA sequences. Therefore, to clarify the taxonomic position of *B. ehimensis* and *B. chitinolyticus*, the current study examined their phenotypic and chemotaxonomic characteristics, 16S rDNA sequences, and phylogenetic relationship.

The following strains were used in this study: *B. chitinolyticus* KCTC 3791^T; *B. ehimensis* KCTC 3748^T, KCTC 3747 and KCTC 3757; *Bacillus sphaericus* KCTC 3346^T; *Bacillus subtilis* KCTC 3135^T; *Brevibacillus brevis* KCTC 3743^T; *Halobacillus halophilus* KCTC 3685^T; *Jeotgalibacillus alimentarius* KCTC 13914^T; *Paenibacillus chinjuensis* WN9^T; *Paenibacillus koreensis* YC300^T; *Paenibacillus polymyxa* KCTC 3627^T; *Paenibacillus validus* KCTC 3401^T; and *Virgibacillus pantothenicus* KCTC 3539^T. All strains, except for *H. halophilus* and *J. alimentarius*, were cultured on trypticase soy agar (Difco). *H. halophilus* KCTC 3685^T and *J. alimentarius* KCTC 13914^T were cultivated on marine agar 2216 (Difco). All strains were incubated for 48 h at 30 °C.

For cellular fatty acid analysis, *B. ehimensis* KCTC 3748^T, KCTC 3747 and KCTC 3757; *B. chitinolyticus* KCTC 3791^T; *P. koreensis* YC300^T and *P. chinjuensis* WN9^T were cultured on trypticase soy agar for 48 h at 30 °C. The fatty acids were then analysed as previously described (Lee *et al.*, 1996; Yang *et al.*, 1993).

The whole-cell fatty acid compositions of the strains are shown in Table 1. The dominant fatty acids for the studied strains were C_{15:0} anteiso, C_{16:0} iso, C_{17:0} anteiso, C_{16:0}, and C_{15:0} iso. Detailed fatty acid compositions of the strains are available as Supplementary Table A in IJSEM Online.

Published online ahead of print on 23 January 2004 as DOI 10.1099/ijse.0.02765-0.

Additional data relating to whole-cell fatty acid compositions are available as supplementary material in IJSEM Online.

Table 1. Whole-cell fatty acid compositions of *B. ehimensis*, *B. chitinolyticus*, *P. koreensis* and *P. chinjuensis*.

A *cis* isomer is indicated by the suffix *c*. For unsaturated fatty acids, the position of the double bond can be located by counting from the methyl (ω) end of the carbon chain. Trace, <0.5 %.

Fatty acids	<i>B. ehimensis</i> KCTC 3748 ^T	<i>B. ehimensis</i> KCTC 3747	<i>B. ehimensis</i> KCTC 3757	<i>P. koreensis</i> YC300 ^T	<i>B. chitinolyticus</i> KCTC 3791 ^T	<i>P. chinjuensis</i> WN9 ^T
Saturated fatty acids						
C _{16:0}	7.1	6.3	7.7	8.5	6.9	8.9
Unsaturated fatty acids						
C _{16:1} ω 11 <i>c</i>	5.2	4.9	5.8	5.4	Trace	1.8
Branched fatty acids						
C _{15:0} iso	8.1	7.1	6.6	6.0	3.6	5.9
C _{15:0} anteiso	52.9	55.8	50.8	43.4	58.7	55.0
C _{16:0} iso	8.6	7.7	11.2	12.2	8.4	6.4
C _{17:0} iso	3.3	2.9	2.5	5.5	2.0	6.3
C _{17:0} anteiso	8.0	7.2	7.1	11.9	7.3	11.9

The 16S rDNA sequences for *B. chitinolyticus* KCTC 3791^T and *B. ehimensis* strains KCTC 3748^T, KCTC 3747 and KCTC 3757 were determined as follows. Two universal primers described by Stackebrandt & Liesack (1993), 27F and 1492R, were used for the PCR amplification of the 16S rDNA. The amplified PCR product was then purified using a QIAquick PCR purification kit (Qiagen). Finally, the purified 16S rDNA was sequenced using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) and automatic DNA sequencer (Model 377; Applied Biosystems).

The 16S rDNA sequences were aligned with representative sequences from the genera *Bacillus* and *Paenibacillus* and related taxa using CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Evolutionary distance matrices were calculated using the algorithm of Jukes & Cantor (1969) along with the DNADIST program included in the PHYLIP software package (Felsenstein, 1993). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987), as implemented in the NEIGHBOR program from the same software package. The stability of the groupings was assessed by a bootstrap analysis of 1000 datasets using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE from the PHYLIP software package. The GenBank accession numbers for the 16S rDNA sequences used for the phylogenetic analysis are shown in Fig. 1.

Nearly complete 16S rDNA sequences (nucleotide positions 28 to 1491, *Escherichia coli* numbering) were determined for *B. chitinolyticus* KCTC 3791^T and *B. ehimensis* strains KCTC 3748^T, KCTC 3747 and KCTC 3757. The primary structures of the 16S rDNA sequences were compared between the strains and between closely related taxa. A phylogenetic tree based on K_{nuc} values (Fig. 1) indicated that *B. chitinolyticus* KCTC 3791^T and *B. ehimensis* strains KCTC 3748^T, KCTC 3747 and KCTC 3757 were all clustered with the genus *Paenibacillus*.

B. ehimensis strains KCTC 3748^T, 3747 and 3757 showed identical 16S rDNA sequences, suggesting that they are members of the same species, and were most closely related to *P. koreensis* YC300^T (98.3 % sequence similarity). At the

Fig. 1. Phylogenetic tree, based on 16S rDNA sequence data, showing the position of strains of *B. ehimensis* and *B. chitinolyticus* in relation to other species from the genus *Paenibacillus* and related taxa. Numbers indicate bootstrap values greater than 600. Bar, 0.1 nucleotide substitutions per site.

same time, *B. chitinolyticus* KCTC 3791^T was found to be phylogenetically closely related to *P. chinjuensis* WN9^T (95.2 % sequence similarity).

DNA–DNA hybridization was carried out by a membrane-filter technique, using a DIG High Prime DNA labelling and detection starter kit II (Roche Molecular Biochemicals). Genomic DNA (200 ng) was denatured by the alkaline method and immobilized on a nylon membrane (Hybond-N+; Amersham) by applying a low vacuum. DNA preparations (1 µg) were labelled using the DIG High Prime DNA labelling and detection starter kit II, according to the manufacturer's protocol. Membranes were prehybridized in hybridization solution at 52 °C for 30 min. Hybridization was carried out in a hybridization solution containing labelled DNA (25 ng ml⁻¹) at 52 °C for 16 h. After hybridization, membranes were washed twice in primary washing solution (2 × SSC and 0.1 % SDS), and then washed twice in secondary washing solution (0.5 × SSC and 0.1 % SDS) at 68 °C. Detection reagents were added to the membranes for 5 min at room temperature, and then excess liquid was squeezed out. Membranes were exposed to autoradiography film (Hyperfilm-ECL; Amersham) for 10 min, and signal intensities were determined using the TINA 2.0 program. The signal produced by self-hybridization was taken as 100 %, and percentage hybridization values were calculated from duplicate samples.

The DNA–DNA hybridization results confirmed that *B. ehimensis* strains KCTC 3748^T, 3747 and 3757 were members of the same species (Kuroshima *et al.*, 1996). They exhibited high levels of relatedness (93–105 %) with each other. The DNA–DNA hybridization values between *B. ehimensis* KCTC 3748^T and *P. koreensis* YC300^T were less than 26 %. The phylogenetic definition of a species generally includes 'strains with approximately 70 % or greater DNA–DNA relatedness' (Wayne *et al.*, 1987). According to the available compiled data, organisms that have less than 97.0 % sequence similarity will not reassociate to more than 60 %, regardless of the hybridization method employed (Stackebrandt & Goebel, 1994). We did not determine the DNA–DNA hybridization value between *B. chitinolyticus* KCTC 3791^T and *P. chinjuensis* WN9^T because the sequence similarity between the two strains was 95.2 %. The phylogenetic and DNA–DNA hybridization results demonstrate that *B. ehimensis* and *B. chitinolyticus* are phylogenetically closer to the genus *Paenibacillus* than to the genus *Bacillus* and are not related to any of the previously described *Paenibacillus* taxa at the species level.

The specific forward primer PAEN515F (5'-GCTCGGAGAGTGACGGTACCTGAGA-3') and universal reverse primer 1377R were used in PCR amplifications for presumptive identification of strains belonging to the genus *Paenibacillus*. The PCR conditions followed the method of Shida *et al.* (1997). The universal primers 27F and 1492R were used for the PCR amplification of the 16S rDNA.

The results of the 16S rDNA fragment amplification for the

Fig. 2. Agarose gel electrophoresis of PCR products generated with control primers (a) and *Paenibacillus*-specific primers (b). Lanes: M, size markers (1 kb ladder); 1, *B. ehimensis* KCTC 3748^T; 2, *B. ehimensis* KCTC 3747; 3, *B. ehimensis* KCTC 3757; 4, *P. koreensis* YC300^T; 5, *B. chitinolyticus* KCTC 3791^T; 6, *P. chinjuensis* WN9^T; 7, *P. polymyxa* KCTC 3627^T; 8, *P. validus* KCTC 3401^T; 9, *B. subtilis* KCTC 3135^T; 10, *B. sphaericus* KCTC 3346^T; 11, *Brevibacillus brevis* KCTC 3743^T; 12, *H. halophilus* KCTC 3685^T; 13, *V. pantothenicus* KCTC 3539^T; 14, *J. alimentarius* KCTC 13914^T.

three *B. ehimensis* strains, one *B. chitinolyticus* strain, four *Paenibacillus* strains, two *Bacillus* strains, one *Brevibacillus* strain, one *Halobacillus* strain, one *Virgibacillus* strain and one *Jeotgalibacillus* strain are shown in Fig. 2. The control reaction resulted in the predicted band of 1.5 kb from all tested strains (Fig. 2a). However, the PCR amplifications with primers PAEN515F and 1377R produced a 0.8 kb PCR fragment from the *Paenibacillus* strains, the three *B. ehimensis* strains and the *B. chitinolyticus* strain (Fig. 2b), suggesting that *B. ehimensis* and *B. chitinolyticus* are indeed members of the genus *Paenibacillus*.

The distinctive phenotypic characteristics of *B. ehimensis*, *B. chitinolyticus*, and phylogenetically related species and subspecies in the genus *Paenibacillus* are taken from previously described data (Chung *et al.*, 2000; Danne *et al.*, 2002; Heyndrickx *et al.*, 1995, 1996; Kuroshima *et al.*, 1996; Nakamura, 1984, 1987; Yoon *et al.*, 2001) and are given in Table 2.

Accordingly, based on the above results, it is proposed that *Bacillus ehimensis* and *Bacillus chitinolyticus* be transferred to the genus *Paenibacillus* as *Paenibacillus ehimensis* comb. nov. and *Paenibacillus chitinolyticus* comb. nov., respectively.

Description of *Paenibacillus ehimensis* (Kuroshima *et al.* 1996) comb. nov.

Paenibacillus ehimensis (e.hi.men'sis. N. L. masc. adj. ehimensis referring to Ehime Prefecture, Japan, the source of the soil samples from which the organisms were isolated).

Table 2. Distinctive phenotypic characteristics of *Bacillus ehimensis*, *Bacillus chitinolyticus* and closely related *Paenibacillus* strains

Species: 1, *B. ehimensis* (data from Kuroshima *et al.*, 1996); 2, *P. koreensis* (data from Chung *et al.*, 2000); 3, *P. validus* (data from Heyndrickx *et al.*, 1995; Nakamura, 1984); 4, *P. naphthalenovorans* (data from Danne *et al.*, 2002); 5, *B. chitinolyticus* (Kuroshima *et al.*, 1996); 6, *P. chinjuensis* (data from Yoon *et al.*, 2002); 7, *P. chondroitinus* (data from Nakamura, 1987); 8, *P. alginolyticus* (Nakamura, 1987); 9, *P. larvae* subsp. *larvae* (data from Heyndrickx *et al.*, 1996); 10, *P. larvae* subsp. *pulvifaciens* (Heyndrickx *et al.*, 1996). +, 90 % or more of strains positive; –, 90 % or more of strains negative; V, 11–89 % of strains positive; NT, not tested.

Characteristic	1	2	3	4	5	6	7	8	9	10
Anaerobic growth	–	–	–	–	–	+	–	–	+	+
Oxidase	+	+	–	NT	V	+	–	–	–	–
Nitrate reduction	+	+	–	V	+	–	–	–	V	+
Acid from:										
Arabinose	+	NT	–	–	–	–	+	+	–	–
Glucose	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	–	–	+	+	–	–
Xylose	+	+	+	–	–	–	+	+	–	–
Production of:										
Acetylmethylcarbinol	–	NT	V	NT	+	NT	–	–	V	V
Indole	NT	–	+	–	NT	NT	–	–	+	+
Hydrolysis of:										
Casein	V	+	–	–	V	+	–	–	+	+
Starch	+	+	V	V	–	+	+	+	–	–
Use of citrate	–	–	–	V	–	–	–	+	–	–
Growth at 50 °C	+	+	+	NT	–	–	–	–	–	–
G + C content (mol%)	52–55	54	50–52	49	51–53	53	47–48	47–49	42–43	42–43

Basonym: *Bacillus ehimensis* Kuroshima *et al.* 1996.

The description is the same as that given by Kuroshima *et al.* (1996). The type strain is KCTC 3748^T (= IFO 15659^T).

Emended description of *Paenibacillus chitinolyticus* (Kuroshima *et al.* 1996) comb. nov.

Paenibacillus chitinolyticus (chi.ti.no.ly'ti.cus. N.L. n. *chitinum* chitin; M.L. adj. *lyticus* dissolving; N.L. adj. *chitinolyticus* decomposing chitin).

Basonym: *Bacillus chitinolyticus* Kuroshima *et al.* 1996.

The description is the same as that given by Kuroshima *et al.* (1996). The type strain is KCTC 3791^T (= IFO 15660^T).

Acknowledgements

The authors would like to thank Professor Hans G. Trüper for his advice in naming the organisms and Dr Y. R. Chung and Dr J.-H. Yoon for donating *Paenibacillus koreensis* YC300^T and *Paenibacillus chinjuensis* WN9^T, respectively. This research was supported by a grant from KRIIBB Research Initiative Program.

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