Bacillus infantis sp. nov. and *Bacillus idriensis* sp. nov., isolated from a patient with neonatal sepsis

Kwan Soo Ko,[1,2] Won Sup Oh,[2] Mi Young Lee,[1] Jang Ho Lee,[3] Hyuck Lee,[4] Kyong Ran Peck,[2] Nam Yong Lee[3] and Jae-Hoon Song[1,2]

[1]Asian-Pacific Research Foundation for Infectious Diseases (ARFID), Seoul 135-710, Korea

[2,3]Division of Infectious Diseases[2] and Department of Laboratory Medicine[3], Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea

[4]Dong-A University Hospital, Busan 602-715, Korea

Correspondence
Won Sup Oh
wsoh@smc.samsung.co.kr

Two Gram-positive bacilli, designated as strains SMC 4352-1^T and SMC 4352-2^T, were isolated sequentially from the blood of a newborn child with sepsis. They could not be identified by using conventional clinical microbiological methods. 16S rRNA gene sequencing and phylogenetic analysis revealed that both strains belonged to the genus *Bacillus* but clearly diverged from known *Bacillus* species. Strain SMC 4352-1^T and strain SMC 4352-2^T were found to be closely related to *Bacillus firmus* NCIMB 9366^T (98·2 % sequence similarity) and *Bacillus cibi* JG-30^T (97·1 % sequence similarity), respectively. They also displayed low DNA–DNA reassociation values (less than 40 %) with respect to the most closely related *Bacillus* species. On the basis of their polyphasic characteristics, strain SMC 4352-1^T and strain SMC 4352-2^T represent two novel species of the genus *Bacillus*, for which the names *Bacillus infantis* sp. nov. (type strain SMC 4352-1^T = KCCM 90025^T = JCM 13438^T) and *Bacillus idriensis* sp. nov. (type strain SMC 4352-2^T = KCCM 90024^T = JCM 13437^T) are proposed.

The genus *Bacillus* comprises aerobic or anaerobic, endospore-forming, Gram-positive bacteria. More than 100 bacterial species are included in the genus *Bacillus*, although many species have been transferred into other genera, e.g. *Paenibacillus*, *Brevibacillus* and *Alicyclobacillus* (http://www.bacterio.cict.fr/b/bacillus.html; Logan & Turnbull, 2003). Most are widely distributed saprophytes, but some are pathogenic, including *Bacillus alvei*, *B. anthracis*, *B. brevis*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. licheniformis*, *B. macerans*, *B. pumilus*, *B. sphaericus*, *B. subtilis* and *B. thuringiensis* (Logan & Turnbull, 2003). In this paper, we report on the isolation of two bacterial strains, SMC 4352-1^T and SMC 4352-2^T, from a newborn child. 16S rRNA gene sequencing, DNA–DNA hybridization and biochemical testing showed that these two bacterial isolates constitute two novel species of the genus *Bacillus*.

A 5-day-old female newborn child was admitted to our neonatal intensive-care unit because of cyanosis after birth. Transthoracic echocardiography showed the presence of a large (35 mm diameter) patent ductus arteriosus. On day 5 of hospitalization, fever and hypotension developed, and two sets of blood cultures were found to harbour Gram-positive bacilli. However, these isolates could not be identified by using conventional methods, such as VITEK (bioMérieux) and Microscan (Dade-Microscan), in the clinical microbiology laboratory.

When the bacterial isolates were tested repeatedly with API 50 CH kits (bioMérieux) to characterize their biochemical traits, they were both found to be positive for D-xylose, galactose, glucose, fructose, mannitol, sorbitol, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, maltose, melibiose, sucrose, trehalose, raffinose, starch, glycogen and gluconate. Cellobiose, lactose and inulin were positive only in the case of strain SMC 4352-1^T, whereas glycerol, ribose, mannose, inositol, xylitol, gentiobiose and 5-ketogluconate were positive only for strain SMC 4352-2^T. Because conventional biochemical tests failed to identify these isolates to given species in the clinical microbiology laboratory, we subjected them to 16S rRNA gene sequence analysis in order to identify them.

Bacterial DNA for the amplification of the 16S rRNA gene was extracted using a boiling lysis method (Ko *et al.*, 2005a, b). Colonies on blood agar plates were suspended in lysis buffer (100 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA and 1 % Triton X-100) and then incubated at 90 °C for 10 min. The mixture was then briefly centrifuged and the aqueous

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SMC 4352-1^T and SMC 4352-2^T are AY904032 and AY904033, respectively.

Fatty acid compositions of strains SMC 4352-1^T and SMC 4352-2^T are detailed in a supplementary table in IJSEM Online.

phase was used as template for a PCR. The 16S rRNA gene was amplified using the universal primers 16S-F0 (5′-GATCCTGGCTCAGGACGAAC-3′) and 16S-R0 (5′-CTTGTTACGACTTCACCCCA-3′) (Hong *et al.*, 2003; Zhu *et al.*, 2002). Template DNA and 20 pmol each primer were added to the PCR mixture tubes (AccuPower PCR PreMix; Bioneer), and the reaction mixtures were then subjected to 35 amplification cycles. Each cycle consisted of 30 s at 95 °C for denaturation, 30 s at 60 °C, and 1 min at 72 °C for extension, followed by a final extension at 72 °C for 5 min. Amplified PCR products were purified for sequencing using a PCR purification kit (CoreOne). Purified PCR products were sequenced directly using the PCR amplification primers and primer 16S-F5 (5′-TATTGGGCGTAAAGCGAGCGC-3′) (Ko *et al.*, 2005a, b). The 16S rRNA gene sequences of the two novel bacterial strains and those of other *Bacillus* species retrieved from GenBank were aligned using the CLUSTAL X program (Thompson *et al.*, 1997). The phylogenetic relationships among the two novel strains and other *Bacillus* species were determined by using the neighbour-joining, maximum-parsimony and maximum-likelihood methods within PAUP, version 4.0 (Swofford, 1999).

The 16S rRNA gene sequences of strains SMC 4352-1^T (1367 bp) and SMC 4352-1^T (1437 bp) showed a pairwise similarity of 95·2 %, indicating that they belong to the same genus but to different species. Comparisons with the GenBank database revealed that their sequences did not match those of any known bacterium. The bacterium with the greatest pairwise similarity to strain SMC 4352-1^T was *B. firmus* NCIMB 9366^T (98·2 %), whereas *Bacillus cibi* JG-30^T most closely matched strain SMC 4352-2^T, showing a pairwise similarity of 97·1 %. Initially, we retrieved and included the 16S rRNA gene sequences of nearly all *Bacillus* species with validly published names. Of these, 19 *Bacillus* species found to have a close relationship to the two novel bacterial strains in the initial analysis were selected and analysed (Fig. 1). A phylogenetic tree constructed using the neighbour-joining method suggested that strains SMC 4352-1^T and SMC 4352-2^T are members of the genus *Bacillus* but they represent distinct species. Strain SMC 4352-1^T clustered with *B. firmus* and *Bacillus siralis* with a low bootstrap value ($<$50 %), whereas strain SMC 4352-2^T clustered with *B. cibi* – a relationship supported by bootstrap value of 100 %. Other methods of phylogenetic reconstruction, such as maximum parsimony and maximum likelihood, showed relationships similar to those presented in Fig. 1.

The cellular fatty acid compositions of the two novel strains were examined using GC (6890A; Hewlett Packard) and a MIDI aerobe method (Chem Station, version 4.02) at MicroID (Seoul, Korea). For fatty acid analysis, the strains were grown at 35 °C on blood agar for about 24 h. The cellular fatty acid profiles determined were compared with published profiles. Analysis of the cellular fatty acid composition was performed for strains SMC 4352-1^T and

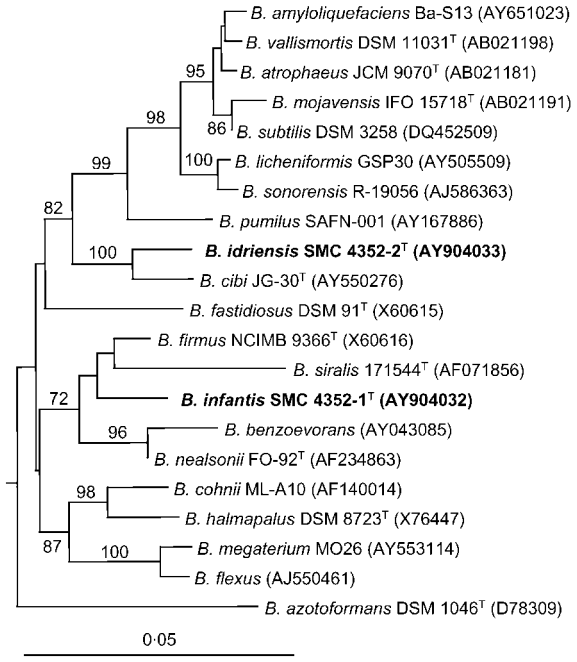

Fig. 1. Neighbour-joining phylogenetic tree, based on almost-complete sequences of 16S rRNA genes, showing the relationships among the two novel isolates and species of the genus *Bacillus*. Bootstrap values are shown as percentages from 1000 replications at branch points. Species names and accession numbers are given as cited in the GenBank database. *Bacillus niacini* S147 (GenBank accession no. AY509227) was used as an outgroup (not shown). Bar, 5 substitutions per 100 nucleotides.

SMC 4352-2^T, the results of which are shown in Supplementary Table S1 available in IJSEM Online. The profiles of the two strains differed: in strain SMC 4352-1^T, the predominant fatty acid was iso-$C_{15:0}$ (44·0 %), followed by anteiso-$C_{15:0}$ (30·9 %) and anteiso-$C_{17:0}$ (7·4 %); in strain SMC 4352-1^T, however, the predominant fatty acid was anteiso-$C_{15:0}$ (26·0 %), followed by iso-$C_{15:0}$ (18·0 %) and anteiso-$C_{17:0}$ (6·9 %).

DNA–DNA reassociation was measured fluorometrically by using the microplate hybridization method described by Ezaki *et al.* (1989). Strain SMC 4352-1^T showed 36 % DNA–DNA reassociation with *B. firmus* ATCC 8247^T, while strain SMC 4352-2^T showed 23 % DNA–DNA reassociation with *B. cibi* KCTC 3880^T. The DNA G+C contents of strains SMC 4352-1^T and SMC 4352-2^T were determined spectrophotometrically using the thermal denaturation method (Marmur & Doty, 1962) and found to be 40·8 and 41·2 mol%, respectively.

Although these two strains were isolated from a neonate, their association with neonatal sepsis could not be determined. In addition to these two isolates, several other bacterial organisms, such as vancomycin-susceptible enterococci, *Acinetobacter lwoffii*, *Alcaligenes xylosoxidans*

and methicillin-resistant, coagulase-negative staphylococci, were also isolated from the patient. Since these strains were isolated from blood obtained via a central venous catheter, which is prone to contamination, they may not be pathogens but rather contaminants or colonizers. *B. firmus* and *B. cibi*, the closest relatives of these two strains, also do not cause human disease (Logan & Turnbull, 2003; Yoon *et al.*, 2005), although their pathogenicity remains to be investigated.

On the basis of their biochemical characteristics and cellular fatty acid profiles, the two isolates were demonstrated to represent two different species (Table 1 and Supplementary Table S1); this was confirmed by the results of 16S rRNA gene sequence comparisons and DNA–DNA hybridizations. The 16S rRNA gene sequence of strain SMC 4352-1^T showed the highest level of pairwise similarity to that of the type strain of *B. firmus* (98·2 %), while that of strain SMC 4352-2^T showed the highest level of pairwise similarity to that of the type strain of *B. cibi* (97·1 %). These pairwise similarities are sufficient to consider strains SMC 4352-1^T and SMC 4352-2^T as belonging to novel species, as many *Bacillus* species having sequence pairwise similarities >98·5 % are considered as different species. For example, the 16S rRNA gene sequences of the type strains of *Bacillus bataviensis*, *B. soli*, *B. dretensis*, *B. novalis* and *B. vireti*, which are distinct species, show 98·7–99·6 % pairwise similarity, whereas the type strains of *B. mojavensis*, *B. subtilis*, *B. amyloliquefaciens*, *B. vallismortis* and *B. atrophaeus* show 98·5–99·6 % pairwise similarity and yet they are regarded as distinct species. In addition, DNA–DNA hybridization results supported species differentiation of the two strains, because their values for DNA–DNA reassociation with the closest species were below 40 %. Phenotypically, strain SMC 4352-1^T could be differentiated from *B. firmus* in that it was positive for utilization of glycogen, inulin and salicin (Table 1). Strain SMC 4352-2^T was positive for utilization of mannitol and salicin, while *B. cibi* was negative for both (Table 1). Thus, our data suggest that the two isolates represent novel *Bacillus* species.

Table 1. Comparison of the biochemical characteristics of strains SMC 4352-1^T and SMC 4352-2^T and related species

Taxa: 1, strain SMC 4352-1^T; 2, strain SMC 4352-2^T; 3, *B. cibi* KCTC 3880^T; 4, *B. firmus*; 5, *B. siralis*; 6, *B. nealsonii*; 7, *B. megaterium*. Biochemical profiles of strains SMC 4352-1^T, SMC 4352-2^T and *B. cibi* KCTC 3880^T were determined using API 50 CH, and those of related species were retrieved from Logan & Turnbull (2003), Pettersson *et al.* (2000) and Venkateswaran *et al.* (2003). Symbols: +, positive; −, negative; V, variable; ND, no data available. All strains are negative for utilization of D-arabinose.

Biochemical reaction	1	2	3	4	5	6	7
Utilization of:							
Glycerol	−	+	V	−	−	+	+
Glycogen	+	+	+	−	−	−	+
Inulin	+	−	−	−	ND	−	+
Mannitol	+	+	−	V	−	−	+
Salicin	+	+	−	−	−	+	+
Trehalose	+	+	+	V	−	+	+
Starch	+	+	+	+	−	ND	+

On the basis of the above biochemical data, cellular fatty acid compositions and molecular phylogenetic results, strains SMC 4352-1^T and SMC 4352-2^T represent two novel species of *Bacillus*, for which we propose the names *Bacillus infantis* sp. nov. and *Bacillus idriensis* sp. nov.

Description of *Bacillus infantis* sp. nov.

Bacillus infantis (in.fan′tis. L. gen. n. *infantis* of an infant, baby, the putative source of the type strain).

Aerobic, Gram-positive, catalase-positive, oxidase-negative bacillus. Grows well on blood agar at 37 °C. When assayed with the API 50 CH system, it is positive for utilization of D-xylose, galactose, glucose, fructose, mannitol, sorbitol, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, maltose, melibiose, sucrose, trehalose, raffinose, starch, glycogen, gluconate, cellobiose, lactose and inulin and is negative for utilization of glycerol, ribose, mannose, inositol, xylitol, gentiobiose and 5-ketogluconate. The major fatty acid is iso-$C_{15:0}$ (44·0 %), followed by anteiso-$C_{15:0}$ (30·9 %) and anteiso-$C_{17:0}$ (7·4 %), and its 16S rRNA gene sequence shows most similarity (98·2 %) to that of the type strain of *B. firmus*. DNA–DNA reassociation with *B. firmus* ATCC 8247^T is 36 %. The DNA G+C content is 40·8 mol%.

The type strain, SMC 4352-1^T (=KCCM 90025^T=JCM 13438^T), was isolated from a newborn child with sepsis.

Description of *Bacillus idriensis* sp. nov.

Bacillus idriensis (id.ri.en′sis. N.L. masc. adj. *idriensis* arbitrary specific epithet pertaining to IDRI, the Infectious Disease Research Institute, where this study was performed).

Aerobic, Gram-positive, catalase-positive, oxidase-negative bacillus. Grows well on blood agar at 37 °C. When assayed with the API 50 CH system, it is positive for utilization of D-xylose, galactose, glucose, fructose, mannitol, sorbitol, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, maltose, melibiose, sucrose, trehalose, raffinose, starch, glycogen, gluconate, glycerol, ribose, mannose, inositol, xylitol, gentiobiose and 5-ketogluconate, but negative for utilization of cellobiose, lactose and inulin. The predominant fatty acid is anteiso-$C_{15:0}$ (26·0 %), followed by iso-$C_{15:0}$ (18·0 %) and anteiso-$C_{17:0}$ (6·9 %), and its 16S rRNA gene sequence shows most similarity (97·1 %) to that of the type strain of *B. cibi*. DNA–DNA reassociation with *B. cibi* KCTC 3880^T is 23 %. The DNA G+C content is 41·2 mol%.

The type strain, SMC 4352-2^T (=KCCM 90024^T=JCM 13437^T), was isolated from a newborn child with sepsis.

Acknowledgements

This work was partly supported by the Asian-Pacific Research Foundation for Infectious Diseases (ARFID) and the Samsung Biomedical Research Institute (SBRI, C-A5-319-1).

References

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.

Hong, T., Heibler, N. & Tang, Y.-W. (2003). "*Bacillus hackensackii*" sp. nov., a novel carbon dioxide sensitive bacterium isolated from blood culture. *Diagn Microbiol Infect Dis* **45**, 143–147.

Ko, K. S., Lee, N. Y., Oh, W. S., Lee, J. H., Ki, H. K., Peck, K. R. & Song, J. H. (2005a). *Tepidimonas arfidensis* sp. nov., a novel Gram-negative and thermophilic bacterium isolated from the bone marrow of a patient with leukemia in Korea. *Microbiol Immunol* **49**, 785–788.

Ko, K. S., Peck, K. R., Oh, W. S., Lee, N. Y., Lee, J. H. & Song, J. H. (2005b). New species of *Bordetella*, *Bordetella ansorpii* sp. nov., isolated from the purulent exudates of an epidermal cyst. *J Clin Microbiol* **43**, 2516–2519.

Logan, N. A. & Turnbull, P. C. B. (2003). *Bacillus* and other aerobic endospore-forming bacteria. In *Manual of Clinical Microbiology*, 8th edn, pp. 445–460. Edited by P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller & R. H. Yolken. Washington, DC: American Society for Microbiology.

Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.

Pettersson, B., de Silva, S. K., Uhlén, M. & Priest, F. G. (2000). *Bacillus siralis* sp. nov., a novel species from silage with a higher order structural attribute in the 16S rRNA genes. *Int J Syst Evol Microbiol* **50**, 2181–2187.

Swofford, D. L. (1999). PAUP – phylogenetic analysis using parsimony, version 4.0. Sunderland, MA: Sinauer Associates.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Venkateswaran, K., Kempf, M., Chen, F., Satomi, M., Nicholson, W. & Kern, R. (2003). *Bacillus nealsonii* sp. nov., isolated from a spacecraft-assembly facility, whose spores are γ-radiation resistant. *Int J Syst Evol Microbiol* **53**, 165–172.

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**, 733–756.

Zhu, X. Y., Zhong, T., Pandva, Y. & Joerger, R. D. (2002). 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. *Appl Environ Microbiol* **68**, 124–137.