Allobacillus halotolerans gen. nov., sp. nov. isolated from shrimp paste

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A novel bacterial strain designated B3AT, isolated from shrimp paste, was investigated by a polyphasic taxonomic approach. Cells stained Gram-positive and were aerobic, non-pigmented, sporulating and rod-shaped with a polar flagellum. 16S rRNA gene sequence analysis indicated that strain B3AT belonged to the class Bacilli and was a member of the family Bacillaceae. Strain B3AT shared low levels of 16S rRNA gene sequence similarity (94.0%) with members of other genera in the family Bacillaceae and was most closely related to Halalkalibacillus halophilus BH2T (93.8% sequence similarity). The isolate was able to grow at 20–45 °C, with 0.5–15% NaCl and at pH 6–9. Menaquinone with seven isoprene units (MK-7) was the major respiratory quinone and 16 : 0 iso, 15 : 0 anteiso and 14 : 0 iso were the major fatty acids. The major polar lipids were diphosphatidylglycerol and phosphatidylglycerol. The characteristic diamino acid of the peptidoglycan was meso-diaminopimelic acid. The DNA G+C content was 45.3 mol%. On the basis of 16S rRNA gene sequence analysis in combination with chemotaxonomic and physiological data, strain B3AT represents a novel genus and species in the family Bacillaceae for which the name Allobacillus halotolerans is proposed. The type strain of Allobacillus halotolerans is B3AT (=BCRC 17939T =LMG 24826T).

During the characterization of micro-organisms present in shrimp paste, a white-transparent bacterial strain, designated B3AT, was isolated and incubated on marine 2216 agar (MA; BD Difco) or in marine 2216 broth (MB; BD Difco) at 37 °C for 2 days. Subcultivation was done on MA at 37 °C for 24 h to 48 h. On this medium, strain B3AT was able to grow at 20–45 °C but not at 15 or 50 °C. Halalkalibacillus halophilus BH2T was obtained from the Japan Collection of Microorganisms (JCM) and used as a reference strain for phenotypic and genotypic tests.

The 16S rRNA gene sequence of strain B3AT was determined and analysed as described previously (Chen et al., 2001). Analysis of the sequence data was performed with the software packages BioEdit (Hall, 1999) and MEGA version 3.1 (Kumar et al., 2004), after multiple alignments of the data by CLUSTAL X (Thompson et al., 1997).

The 16S rRNA gene sequence of strain B3AT was a continuous stretch of 1431 bp. Phylogenetic analyses (distance options according to the Kimura-2 model; Kimura, 1983) were carried out using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) methods. The neighbour-joining tree was reconstructed with a distance matrix calculated using the method described by Jukes & Cantor (1969). The stability of the tree topology was evaluated by bootstrap analysis (based on 1000 replications). 16S rRNA gene sequence analysis indicated that strain B3AT belonged to the class Bacilli and was a member of the family Bacillaceae. Sequence similarity calculations using BioEdit software (over 1400 bp) indicated that strain B3AT is distinct from any other genera in the family Bacillaceae and showed low levels of 16S rRNA gene sequence similarity (<94.0%) with the members of the genera Alkalibacillus, Aquisalibacillus, and Halalkalibacillus.
Bacillus, Filobacillus, Halalkalibacillus, Piscibacillus and Tenuibacillus (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). The novel strain was closely related to *H. halophilus* BH2<sup>T</sup> (Echigo et al., 2007) (93.8% 16S rRNA gene sequence similarity). The overall topologies of the phylogenetic trees obtained either with neighbour-joining, maximum-likelihood or maximum-parsimony methods were similar. Since strain B3AT and *H. halophilus* BH2<sup>T</sup> formed a stable clade, the latter was used as a reference strain for subsequent phenotypic and genotypic analyses.

The morphology of bacterial cells was observed by phase-contrast microscopy (Leica DM 2000) and scanning electron microscopy (S-3500N; Hitachi) (Supplementary Fig. S2) using cells grown in MB at 37 °C for 6 h (lag growth phase), 18 h (exponential phase) and 36 h (stationary phase). Motility was tested by the hanging drop method. Flagellar staining was performed using the Spot Test flagella stain (BD Difco). The Gram stain set (BD Difco), the Ryu non-staining KOH method (Powers, 1995) and Bactident Aminopeptidase test strips (Merck) were used to ascertain Gram reaction. Presence of endospores was checked by the method of Wirtz-Conklin (Murray et al., 1999). Accumulation of poly-β-hydroxybutyrate granules was observed by light microscopy after staining cells with Sudan black. Colony morphology was examined using a stereoscopic microscope (SMZ 800; Nikon). Details of cell morphology are given in the species description.

The optimum pH range for growth was determined in MB using appropriate biological buffers such as glycine/HCl, citrate/Na<sub>2</sub>HPO<sub>4</sub>, phosphate buffer and glycine/NaOH for adjusting the pH to 3.0–4.0, 4.0–8.0, 6.0–8.0 and 9.0–11.0 (at 1.0 pH unit intervals), respectively. pH was adjusted prior to sterilization and post-sterilization controls revealed only minor changes. NaCl requirement was determined using nutrient broth (BD Difco) containing 0, 0.5 and 1.0–20.0 % (w/v) NaCl (at 1.0 % intervals). The temperature range for growth was examined at 4, 10, 15, 20, 25, 30, 35, 37, 40, 42, 45 and 50 °C in MB. Growth was determined by measuring the turbidity (OD<sub>600</sub>) of cultures grown at various pH, NaCl concentrations and temperatures.

Anaerobic growth was assessed using an Oxoid AnaeroGen system (Miller et al., 1995). Catalase, oxidase and DNase activities and hydrolysis of starch, casein, gelatin and Tweens 20, 40, 60 and 80 were determined using standard methods (Gerhardt et al., 1994). The commercially available API 20NE, API ZYM (bioMérieux) and MicroPlate GP2 (Biolog) microtest systems were used, according to the manufacturers’ instructions, to determine biochemical properties, enzyme activities and carbohydrate oxidation pattern. The API ZYM strip was read after 4 h of incubation at 37 °C, whereas API 20NE and MicroPlate GP2 were read after 72 h at 37 °C. The three commercial systems were inoculated with a cell suspension in artificial seawater.

Polar lipids were analysed by two-dimensional TLC as described by Ventosa et al. (1993). The cellular polar lipids of strain B3AT were diphosphatidylglycerol, phosphatidylglycerol, minor to trace amounts of two unknown phospholipids and one aminolipid (Supplementary Fig. S3).

Fatty acid methyl esters were prepared, separated and identified according to the instructions of the Microbial Identification System (Microbial ID; Sasser, 1990). The fatty acid constituents of strain B3AT were 16:0 iso (28.8%), 15:0 anteiso (28.0%), 14:0 iso (14.6%), 17:1 anteiso ω9c (6.8%), 17:0 anteiso (5.6%), 15:0 iso (5.3%), 16:1 ω7c alcohol (2.5%), 16:0 (2.0%), 18:0 (1.9%) and 18:1 iso H (1.7%) (Supplementary Table S1).

Isoprenoid quinones of strain B3AT were extracted and separated as described by Minnikin et al. (1984) and analysed by HPLC as described by Collins (1985). Strain B3AT contained menaquinone MK-7 as the major respiratory quinone (at a peak area ratio of approximately 93%) and MK-6 as the minor respiratory quinone (at approximately 7%). The diagnostic cell-wall amino acid of strain B3AT was determined by TLC (Staneck & Roberts, 1974). The isolate contained meso-diaminopimelic acid.

For guanine-plus-cytosine (G+C) content determination, DNA was prepared and degraded enzymically into nucleosides.
as described by Mesbah et al. (1989). The nucleoside mixture obtained was then separated by high-performance liquid chromatography. The DNA G+C content of strain B3A\textsuperscript{T} was 45.3 ± 1.0 mol%.

Sensitivity of strain B3A\textsuperscript{T} to different antibiotics was analysed by the diffusion method on MA. The following antibiotic discs (Oxoid) were used: ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), rifampicin (5 μg), penicillin G (10 U), streptomycin (10 μg) and tetracycline (30 μg). The effect of antibiotics on cell growth was assessed after 2 days of incubation at 37 °C and susceptibility was scored based on the distance from the edge of the clear zone to the disc.

Detailed results of the physiological characterization of strain B3A\textsuperscript{T} are provided in the genus and species descriptions and in Table 1. Analysis of the 16S rRNA gene sequence showed that the isolate represented a novel lineage within the family Bacillaceae. Phenotypic properties, such as reduced NaCl range for growth, non-alkaliphilic nature, presence of C4 esterase, trypsin and α-chymotrypsin activities, and its ability to oxidize more carbohydrates in the Biolog GP2 test system (Table 1), distinguish strain B3A\textsuperscript{T} from \textit{H. halophilus} BH2\textsuperscript{T}.

The quantities of fatty acids 15:0 iso and 17:0 anteiso in strain B3A\textsuperscript{T} are much lower than those of \textit{H. halophilus} BH2\textsuperscript{T}, while the quantity of fatty acid 14:0 iso is much higher (Table 1 and Supplementary Table S1). As well as these differences, strain B3A\textsuperscript{T} shows a very high DNA G+C content (45.3 mol%) compared to \textit{H. halophilus} BH2\textsuperscript{T} (35.1 mol%).

In addition, several chemotaxonomic and physiological properties, such as Gram-staining, cell motility, alkaliphilic nature, optimal growth pH, ability to grow at pH 10, with 0.5 % NaCl or at 45 °C, presence of oxidase activity, ability to reduce nitrate to nitrite, cell-wall peptidoglycan type/variant and the distinctive major fatty acid, distinguish strain B3A\textsuperscript{T} from its closely related phylogenetic neighbours, \textit{Alkalibacillus}, \textit{Aquisalibacillus}, \textit{Filobacillus}, \textit{Piscibacillus} and \textit{Tenuibacillus} (Supplementary Table S2). On the basis of these results, we suggest that strain B3A\textsuperscript{T} represents a novel genus and species in the family \textit{Bacillaceae}, for which the name \textit{Allobacillus halotolerans} gen. nov., sp. nov. is proposed.

**Description of \textit{Allobacillus} gen. nov.**

\textit{Allobacillus} (Al.lo.ba.cillus Gr. pref. -allos another; L. masc. n. bacillus a small staff or rod; N.L. masc. n. \textit{Allobacillus} another bacillus or rod).

\begin{table}[h]
\centering
\caption{Differential characteristics of strain B3A\textsuperscript{T} and \textit{Halalkalibacillus halophilus} BH2\textsuperscript{T}}
\begin{tabular}{|l|l|l|}
\hline
Characteristic & Strain B3A\textsuperscript{T} & \textit{H. halophilus} BH2\textsuperscript{T} \\
\hline
Salinity range for growth (optimum) (%) & 0.5–15 (6) & 5–25 (12) \\
\hline
pH range for growth (optimum) & 6–9 (7) & 5–10 (9) \\
\hline
Temperature range for growth (optimum) (°C) & 20–45 (37) & 20–40 (37) \\
\hline
API ZYM tests & & \\
\hline
C4 esterase & + & – \\
Trypsin & + & – \\
α-Chymotrypsin & + & – \\
\hline
Oxidation of (Biolog GP2): & & \\
\hline
\textit{d}-Fructose & + & – \\
\textit{α}-D-Glucose & + & – \\
Maltose & + & – \\
Maltotriose & + & – \\
\textit{d}-Mannitol & – & + \\
\textit{d}-Mannose & + & – \\
\textit{d}-Psicose & + & – \\
Raffinose & – & + \\
\textit{d}-Ribose & + & – \\
Sucrose & + & – \\
Trehalose & + & – \\
Turanose & + & – \\
\textit{d}-Xylose & + & – \\
\hline
Predominant fatty acids & 16:0 iso (28.8 %), 15:0 anteiso (47.5 %), 17:1 & 15:0 anteiso (47.5 %), 17:1 \\
15:0 anteiso (28.0 %), 14:0 iso (14.6 %) & & 15:0 anteiso (47.5 %), 17:1 \\
14:0 iso (14.6 %) & & \\
\hline
DNA G+C content (mol%) & 45.3 & 35.1 \\
\hline
\end{tabular}
\end{table}
Cells are Gram-positive, sporulating and aerobic. Spores are spherical, located terminally, with swollen sporangia. KOH test and L-alanine aminopeptidase are negative. meso-diaminopimelic acid is present in the peptidoglycan. The major fatty acids are 16:0 iso, 15:0 anteiso and 14:0 iso. The major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. The major respiratory quinone is MK-7. The DNA G+C content of the type strain of the type species is 45.3 mol%. The type species is *Allobacillus halotolerans*.

**Description of Allobacillus halotolerans** *sp. nov.*

*Allobacillus halotolerans* (ha.lo.to’le.rans. Gr. n. hals halos salt; L. part. adj. tolerans tolerating; N.L. part. adj. halotolerans salt-tolerating).

Positive for oxidase and catalase. Negative for DNase, lipase (corn oil) and hydrolysis of starch, casein, gelatin and Tween 20, 40, 60 and 80. No accumulation of poly-beta-hydroxybutyrate granules. After 48 h of growth on MA agar at 25 °C, cells have a mean size of about 0.6 μm (width) by 2.0–3.0 μm (length) and are motile. Colonies are round, entire, convex and white-transparent. Colonies are approximately 0.6–0.8 mm in diameter on MA agar after 48 h of incubation at 37 °C. Optimal growth occurs at 37 °C, with 6% NaCl and at pH 7. In API 20NE tests, negative for nitrate and nitrite reduction, indole production, hydroxybutyrate granules. After 48 h of growth on MA agar, D-lactic acid methyl ester, pyruvic acid methyl ester, D-psicose, D-ribose, sucrose, trehalose, turanose, D-xylose, D-mannose, 3-methyl-D-glucose, methyl D-glucosamine, β-galactosidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

The following compounds are oxidized in the Biolog GP2 test system: dextrin, Tween 40, Tween 80, mannann, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, D-galacturonic acid, D-fructose, α-D-glucose, maltose, maltotriose, D-mannose, 3-methyl-D-glucose, methyl α-D-glucoside, D-psicose, D-rhamnose, trehalose, turanose, D-xylitol, lactose, methyl β-D-glucoside, α-ketoglutaric acid, D-lactic acid methyl ester, pyruvic acid methyl ester, γ-hydroxybutyric acid, α-ketovaleric acid, L-lactic acid, pyruvic acid, L-alanine, D-alanine, L-alanyl glycin, L-serine, 2,3-butanediol, glycerol, adenosine, 2′-deoxy adenosine, thymidine and uridine. The type strain is sensitive to gentamicin, chloramphenicol, kanamycin, streptomycin, penicillin G, ampicillin, novobiocin, tetracycline and rifampicin but is resistant to nalidixic acid. The fatty acid constituents are 16:0 iso, 15:0 anteiso, 14:0 iso, 17:1 anteiso ω9c, 17:0 anteiso, 15:0 iso, 16:1 ω7c alcohol, 16:0, 18:0 and 18:1 iso H. Additional phenotypic properties are listed in Table 1.

The type strain, B3A² (=BCRC 17939T =LMG 24826T), was isolated from shrimp paste.

**Acknowledgements**

W. M. C. was supported by the National Science Council, Taiwan, ROC (grants NSC 96-2320-B-022-001-MY2 and 96-2313-B-022-001-MY3).

**References**


