**Bacillus caseinilyticus** sp. nov., an alkali- and thermotolerant bacterium isolated from a soda lake

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A novel Gram-stain-positive, rod-shaped, motile, endospore-forming and proteolytic bacterial strain, SP\(^T\), was isolated from Lonar soda lake, in India. On the basis of 16S rRNA gene sequence analysis it was identified as belonging to the class *Firmibacteria* and was most closely related to *Bacillus cellulosilyticus* DSM 2522\(^T\) (96.7 \%) and other members of the genus *Bacillus* (<95.9 \%). Strain SP\(^T\) was catalase- and oxidase-positive. The cell-wall peptidoglycan of strain SP\(^T\) contained *meso*-diaminopimelic acid. Polar lipids included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three phospholipids, two aminolipids and two unknown lipids. The predominant isoprenoid quinone was MK-7. Anteiso-C\(_{15}:0\) (26.8 \%) was the predominant fatty acid and significant proportions (>5 \%) of iso-C\(_{15}:0\) (20.9 \%), C\(_{16}:0\)(\(\omega7\)c alcohol (6.3 \%), iso-C\(_{16}:0\) (6.3 \%) and anteiso-C\(_{17}:0\) (5.3 \%) were also detected in strain SP\(^T\). The DNA G+C content of strain SP\(^T\) was 38.9 mol\%. The results of phylogenetic, chemotaxonomic and biochemical tests allowed a clear differentiation of strain SP\(^T\) from all other members of the genus *Bacillus*. Strain SP\(^T\) represents a novel member of the genus *Bacillus*, for which the name *Bacillus caseinilyticus* sp. nov. is proposed. The type strain is SP\(^T\) (=MCC 2612\(^T\)=JCM 30246\(^T\)).

The genus *Bacillus* comprises rod-shaped, endospore-forming, aerobic or facultatively anaerobic members which are low G+C Gram-positive bacteria (Claus & Berkeley, 1986). Members of the genus *Bacillus* are ubiquitous and are found in a variety of environments ranging from desert sands (Zhang et al., 2013; Baik et al., 2013; Sarethy et al., 2014) to marine sediments (You et al., 2014). The alkaliphilic species of the genus *Bacillus* have important industrial applications because of their ability to produce extracellular enzymes (Fujinami & Fujisawa, 2010; Sarethy et al., 2011). During a study on the microbial population in the alkaliphilic Lonar soda lake, India, an aerobic, Gram-stain-positive, proteolytic bacterium designated strain SP\(^T\) was isolated. In this report, we describe the results of a polyphasic study aimed at the characterization of strain SP\(^T\).

Strain SP\(^T\) was isolated from the alkaline Lonar Lake, located at Buldhana, Maharashtra, India (latitude 19° 58’ 22.63” N, longitude 76° 30’ 30.76” E), which is a unique basaltic rock meteorite impact crater, situated in the formerly volcanic Deccan trap geological region. The strain was initially isolated on a skimmed-milk agar medium (Atlas, 1993) consisting of (g l\(^{-1}\)) casein enzyme hydrolysate (5), yeast extract (2.5), glucose (1) and agar (15) with pH 7.0 prepared initially in 900 ml distilled water. Skimmed-milk solution (100 ml) was prepared separately by adding 28 g skimmed-milk powder to 100 ml distilled water and autoclaving, cooling and adding to the above media. Pure culture was obtained by repeated streaking of the isolate on nutrient agar plates consisting of (g l\(^{-1}\)) peptone (5), NaCl (5), beef extract (1.5), yeast extract (1.5) and agar (15) with a final pH of 7.5 and was then preserved at 4 °C for further use.

Genomic DNA was extracted and purified according to the method of Marmur (1961). The 16S rRNA gene sequence of strain SP\(^T\) was obtained by PCR as described previously (Vishnuvardhan Reddy et al., 2013). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SP\(^T\) is LK026324.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.
et al., 2012). The CLUSTAL W algorithm of MEGA 5 (Tamura et al., 2011) was used for sequence alignments and the phylogenetic analysis of the 16S rRNA gene was performed using MEGA 5. Distances were calculated by using the Kimura correction in a pairwise deletion manner (Kimura, 1980). Neighbour-joining, minimum-evolution, maximum-likelihood and maximum-parsimony methods in the MEGA 5 software (Tamura et al., 2011) were used to reconstruct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure. The G+C content (mol%) of the DNA of strain SP^T was determined by HPLC (Mesbah et al., 1989).

The almost complete 16S rRNA gene sequence (1433 bp) of strain SP^T was obtained. The results of phylogenetic analysis of the 16S rRNA gene sequence suggested that strain SP^T formed a subclade within the genus Bacillus (neighbour-joining tree is shown as Fig. 1), and sequence similarities with the nearest phylogenetic members of the genus were in agreement with the EzTaxon-e server result. EzTaxon-e server search analysis revealed that strain SP^T was most closely related to Bacillus cellulolyticus DSM 2522^T (96.7 %), B. vedderi JaH^T (95.9 %), B. polygoni YN-1^T (95.8 %), B. clarkii DSM 8720^T (95.8 %), B. agaradhaerens DSM 8721^T (94.2 %) and other members of the genus Bacillus (<94.2 %). DNA–DNA hybridizations play an important role in species delineation in micro-organisms and this is applicable mostly in cases where 16S rRNA gene sequence similarities are 97 % or higher. The nearest neighbours of strain SP^T as noted above share less than 97 % 16S rRNA gene sequence similarity and so DNA–DNA hybridization was not carried out in the present study. The G+C content (mol%) of the

![Fig. 1. Phylogenetic analysis of strain SP^T with other closely related members of the genus Bacillus based on 16S rRNA gene sequences available from the EMBL database (accession numbers are given in parentheses). Multiple alignments, distance calculations (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining, maximum-likelihood, minimum-evolution and maximum-parsimony methods were performed by using the software package MEGA version 5 (Tamura et al., 2011). Bootstrap values based on 1000 replications are shown as percentages at branching points. Bar, 0.01 substitutions per nucleotide position. Filled circles indicate branches that clade differently using different algorithms tested.](image-url)
DNA of strain SP\textsuperscript{T} was 38.9 mol\%, which was similar to that of the nearest phylogenetic neighbours (Table 1).

The phenotypic features of strain SP\textsuperscript{T} were determined following the minimum standards for describing new taxa of aerobic, endospore-forming bacteria recommended by Logan et al. (2009). Morphological properties such as cell shape, cell size and motility were observed by phase-contrast light microscopy (MLX; Magnus). The pH (range pH 6.0–12.0, with intervals of 0.5 pH units, tested after sterilization. Utilization of various substrates as sole carbon and energy sources, or carbon, nitrogen and energy sources, was determined using a basal medium with the following composition (g l\textsuperscript{−1}): yeast extract (0.01), KH\textsubscript{2}PO\textsubscript{4} (0.5), MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O (0.2), (NH\textsubscript{4})\textsubscript{2}HPO\textsubscript{4} (1.0) and NaCl (60). To this liquid medium, 0.1 % (w/v) filter-sterilized substrate was added. Carbohydrates were used at a final concentration of 0.2 % (w/v) and the tests for their utilization were performed as described by Ventosa et al. (1982). Antibiotic sensitivity tests of the strain were performed using the standard disc assay method (Ventosa et al., 1982).

Table 1. Characteristics used to distinguish strain SP\textsuperscript{T} from the type strains of phylogenetically related species of the genus Bacillus

<table>
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<th>Characteristic</th>
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<td>C</td>
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<td>NaCl range (% w/v)</td>
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<td>0–9</td>
<td>3–14</td>
<td>0–16</td>
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<td>5</td>
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<td>10–45</td>
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<td>Temp. optimal (°C)</td>
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<td>29–31</td>
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<td>ND</td>
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<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>ND</td>
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<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
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<td>Casein</td>
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<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Gelatin</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Starch</td>
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<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Tween 20</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>38.9</td>
<td>39.6</td>
<td>38.3</td>
<td>42.9</td>
<td>42.4–43.0</td>
<td>39.3–39.5</td>
</tr>
<tr>
<td>Menaquinone content (%)</td>
<td>MK6 (4 %) and</td>
<td>MK7</td>
<td>MK7</td>
<td>MK7</td>
<td>MK7</td>
<td>MK6 (1 %) and</td>
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<td></td>
<td>MK7 (96%) (100 %)</td>
<td>(100 %)</td>
<td>(100 %)</td>
<td>(100 %)</td>
<td>(100 %)</td>
<td>(99 %)</td>
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</tbody>
</table>

H\textsubscript{2}S production, indole production, oxidase and catalase activities, were carried out as described by Smibert & Krieg (1981, 1994), in the modified Horikoshi II medium described above or the specified medium. In both cases, 10 % NaHCO\textsubscript{3} was autoclaved separately and only added after sterilization. Utilization of various substrates as sole carbon and energy sources, or carbon, nitrogen and energy sources, was determined using a basal medium with the following composition (g l\textsuperscript{−1}): yeast extract (0.01), KH\textsubscript{2}PO\textsubscript{4} (0.5), MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O (0.2), (NH\textsubscript{4})\textsubscript{2}HPO\textsubscript{4} (1.0) and NaCl (60). To this liquid medium, 0.1 % (w/v) filter-sterilized substrate was added. Carbohydrates were used at a final concentration of 0.2 % (w/v) and the tests for their utilization were performed as described by Ventosa et al. (1982). Antibiotic sensitivity tests of the strain were performed using the standard disc assay method (Ventosa et al., 1982).

Colonies of strain SP\textsuperscript{T} grown on nutrient agar were colourless, circular (1.0–2.0 mm in diameter), convex and opaque with entire margin. Cells were Gram-stain-positive and motile rods with 0.1–0.2 μm diameter and 3.5–4.2 μm length. The strain formed a terminal endospore (Fig. S1, available in the online Supplementary Material). Growth occurred at a pH range of pH 7.0–10.5 with an optimum at pH 9. NaCl was not essential for growth and could be tolerated at up to 28 % (w/v) with optimum growth at 6 % (w/v). Optimum growth occurred at 37 °C within a range of 15–60 °C. Casein, aesculin, tyrosine and DNA were hydrolysed whereas starch, cellulose, hippingate, xanthine, hypoxanthine and Tween 20 were not hydrolysed by strain SP\textsuperscript{T}. Gelatin was liquefied. Oxidase, catalase,
lipase and urease activities were positive. Indole production from tryptophan was negative. Strain SP\textsuperscript{T} did not produce \( \text{H}_2\text{S} \), but showed positive results for nitrate reduction, citrate utilization and the Voges–Proskauer test. The strain showed negative results for the methyl red test. Strain SP\textsuperscript{T} was a facultative anaerobe and the substrates which supported growth are given in the species description. The strain was sensitive to gentamicin (120 \( \mu \text{g} \)), vancomycin (30 \( \mu \text{g} \)), tetracycline (30 \( \mu \text{g} \)), streptomycin (10 \( \mu \text{g} \)), penicillin (10 \( \mu \text{g} \)), ampicillin (10 \( \mu \text{g} \)), amikacin (30 \( \mu \text{g} \)), erythromycin (15 \( \mu \text{g} \)) and ciprofloxacin (5 \( \mu \text{g} \)), and resistant to kanamycin (30 \( \mu \text{g} \)) and nalidixic acid (30 \( \mu \text{g} \)). The differentiation characteristics of strain SP\textsuperscript{T} from the related species of the genus \textit{Bacillus} are summarized in Table 1.

Fatty acids, quinones and polar lipids of strains SP\textsuperscript{T} and \textit{B. cellulolysiticus} DSM 2522\textsuperscript{T} (Nogi \textit{et al.}, 2005) were analysed from cells grown in Horikoshi-I medium (Nogi \textit{et al.}, 2005) at 37 °C with \( \text{pH} \) 9 and 6 \% (w/v) \( \text{NaCl} \). Cells were harvested by centrifugation (10,000 \( g \) for 15 min at 4 °C) on reaching a cell density of 70 \% of the maximum optical density (100 \%, 0.8 OD\textsubscript{540}) and the lyophilized pellet was used for analysis. Cellular fatty acids of strains SP\textsuperscript{T} and \textit{B. cellulolysiticus} DSM 2522\textsuperscript{T} were methylated, separated and identified according to the instructions for the Microbial Identification System (version 6.0; MIDI; peak identification based on RTSBA6 database) (Sasser, 1990; www.midi-inc.com). Fatty acid methyl ester analysis was outsourced to Royal Research Laboratories, Secunderabad, India. Polar lipids were extracted from 1 g freeze-dried cells with methanol/chloroform/saline (2 : 1 : 0.8, by vol.) as described by Kates (1986) and were separated using silica gel TLC (Kieselgel F254; Merck) by two-dimensional chromatography using chloroform/methanol/water (75 : 32 : 4, by vol.) in the first dimension and chloroform/methanol/acetic acid/water (86 : 15 : 5 : 4, by vol.) in the first dimension and chloroform/methanol/acetate acid/water (86 : 15 : 5 : 4, by vol.) in the second dimension (modified after Tindall 1990a, b; Oren \textit{et al.}, 1996). Total polar lipids profiles were detected by spraying with 5 \% ethanolic molybdophosphoric acid and further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates), Dragendorff’s reagent (quaternary nitrogen) or \( \alpha \)-naphthol (specific for sugars) (Kates, 1972; Oren \textit{et al.}, 1996). Quinones of strains SP\textsuperscript{T} and \textit{B. cellulolysiticus} DSM 2522\textsuperscript{T} were determined by extraction with chloroform/methanol (2 : 1, v/v) mixture, purified by TLC and analysed by HPLC (Tamaoka \textit{et al.}, 1983). The peptidoglycan of strains SP\textsuperscript{T} and \textit{B. cellulolysiticus} DSM 2522\textsuperscript{T} was isolated after disruption of the cells by shaking with glass beads and subsequent trypsin digestion, according to the method of Schleifer (1985). The cell wall was hydrolysed for amino acid analysis and \( \alpha \)-amidase as described by Schleifer & Kandler (1972) and Hasegawa \textit{et al.} (1983).

Whole-cell fatty acid analysis of strain SP\textsuperscript{T} revealed that anteiso-C\textsubscript{15:0} (26.8 \%) and iso-C\textsubscript{15:0} (20.9 \%) were the predominant fatty acids present, whereas in \textit{B. cellulolysiticus} DSM 2522\textsuperscript{T} C\textsubscript{16:0} (37.9 \%), anteiso-C\textsubscript{15:0} (23.7 \%) and iso-C\textsubscript{15:0} (14.5 \%) were detected as the major fatty acids. The other significant proportions (>5 \%) of fatty acids detected in strain SP\textsuperscript{T} included C\textsubscript{16:1} \%7c alcohol (6.3 \%), iso-C\textsubscript{16:0} (6.3 \%) and anteiso-C\textsubscript{17:0} (5.3 \%). Similarly, notable proportions (>5 \%) of C\textsubscript{18:0} (6.2 \%) and anteiso-C\textsubscript{17:0} (5.7 \%) were detected in \textit{B. cellulolysiticus} DSM 2522\textsuperscript{T} (Table S1). Polar lipids of strain SP\textsuperscript{T} included diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), three phospholipids, two amino lipids and two unknown lipids (Fig. S2). The strain differed from \textit{B. cellulolysiticus} DSM 2522\textsuperscript{T} by the absence of a phospholipid (PL2) and the presence of three phospholipids (PL1, PL3 and PL4). Further, an additional aminolipid (AL1) was present in only strain SP\textsuperscript{T}. These profiles are somewhat similar to the polar lipid profile of \textit{Bacillus subtilis} subsp. \textit{subtilis} DSM 10\textsuperscript{T} (Kämpfer \textit{et al.}, 2006), with DPG, PG and PE as the major polar lipids. The major quinone of strain SP\textsuperscript{T} was MK-7 (96 \%) with traces of MK-6 (4 \%).

The type species of the genus \textit{Bacillus} (\textit{Bacillus subtilis} subsp. \textit{subtilis}) also contains a quinone system predominant in MK-7 (Collins & Jones, 1980). The cell-wall peptidoglycan of strain SP\textsuperscript{T} contained \textit{meso-diaminopimelic acid} (m-DAP) as the diagnostic diaminoc acid. Almost all species of the genus \textit{Bacillus} tested have vegetative cell walls made up of peptidoglycan containing m-DAP. The exceptions (\textit{Bacillus sphaericus} and the related species \textit{Bacillus pasteurii} and \textit{Bacillus globisporus}) contain lysine instead. But even those species, as all others, contain m-DAP in the peptidoglycan of their spore cortex (Slepecky & Hemphill, 2006).

The phenotypic and genotypic distinctiveness of strain SP\textsuperscript{T} support the proposal of the isolate as a representative of a novel member of the genus \textit{Bacillus}, for which the name \textit{Bacillus caseinilyticus} sp. nov. is proposed.

**Description of \textit{Bacillus caseinilyticus} sp. nov.**

\textit{Bacillus caseinilyticus} [\textit{ca.se.in.i.ly tic.us}. N.L. neut. n. casein-\textit{inum} casein; N.L. masc. adj. lyticus (from Gr. masc. adj. \textit{lutikos}) able to loosen, able to dissolve; N.L. masc. adj. casein-\textit{inum} casein-dissolving].

Cells are motile, rod-shaped, Gram-stain-positive and form terminal endospores, which are elliptoidal in shape, in non-swollen sporangia. Facultative anaerobe. Positive for nitrate reduction, and oxidase and catalase activities. Optimal growth occurs after 24–48 h of incubation on nutrient agar at 37 °C (range 15–60 °C). Growth occurs between pH 7.0 and 10.5 (optimum pH 9.0). NaCl is not essential for growth; optimum growth occurs with 6 \% NaCl and up to 28 \% is tolerated. Casein, tyrosine, aesculin and DNA are hydrolysed whereas cellulose, starch, hippurate, xanthine, hypoxanthine and Tween 20 are not hydrolysed. Nitrate but not nitrite is reduced and gelatin is liquefied. Lipase and urease activities are positive. Indole production from tryptophan is negative; does not produce \( \text{H}_2\text{S} \), but shows positive results for citrate utilization and Voges–Proskauer test. Negative results for the methyl red test. Acids but not gas are produced from raffinose, sucrose, D-glucose, maltose, lactose, D-xyllose, D-sorbitol,
D-arabinose, cellobiose, trehalose, mannose, D-mannitol and melibiose. Although growth is supported by inulin, salicin, D-galactose, lactose, maltose, raffinose, melibiose and D-mannitol; mannose, D-xylose, trehalose, raffinose, rhamnose, inositol or fructose as the sole carbon source do not yield either gas or acid. Ammonium chloride, nitrate and urea are the most suitable nitrogen sources, but growth is also observed with glutamate and aspartate. Major (>5%) fatty acids are anteiso-C₁₅:₀, iso-C₁₅:₀, C₁₆:₀ 10MeC alcohol, iso-C₁₆:₀ and anteiso-C₁₇:₀. Polar lipids include diphasphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three phospholipids, two amino lipids and two unknown lipids. The predominant isoprenoid quinone is MK-7. The diamino acid in the cell-wall peptidoglycan is meso-diaminopimelic acid.

The type strain is SPᵀ (= MCC 2612ᵀ = JCM 30246ᵀ). The type strain was isolated from a sediment sample of Lonar Lake, India. The DNA G+C content of the type strain is 38.9 mol%.

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References


