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Examination into the taxonomic position of *Bacillus thermotolerans* Yang et al., 2013, proposal for its reclassification into a new genus and species *Quasibacillus thermotolerans* gen. nov., comb. nov. and reclassification of *B. encimensis* Dastager et al., 2015 as a later heterotypic synonym of *B. badius*☆

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ABSTRACT

Two novel Gram-staining positive, rod-shaped, moderately halotolerant, endospore forming bacterial strains 5.5LF 38TD and 5.5LF 48TD were isolated and taxonomically characterized from a landfill in Chandigarh, India. The analysis of 16S rRNA gene sequences of the strains confirmed their closest identity to *Bacillus thermotolerans* SgZ-8T with 99.9% sequence similarity. A comparative phylogenetic analysis of strains 5.5LF 38TD, 5.5LF 48TD and *B. thermotolerans* SgZ-8^T confirmed their separation into a novel genus with *B. badius* and genus *Domibacillus* as the closest phylogenetic relatives. The major fatty acids of the strains are iso-$C_{15:0}$ and iso-$C_{16:0}$ and MK-7 is the only quinone. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The digital DNA-DNA hybridization (DDH) and ortho average nucleotide identity (ANI) values calculated through whole genome sequences indicated that the three strains showed low relatedness with their phylogenetic neighbours. Based on evidences from phylogenomic analyses and polyphasic taxonomic characterization we propose reclassification of the species *B. thermotolerans* into a novel genus named *Quasibacillus thermotolerans* gen. nov., comb. nov with the type strain SgZ-8^T (= CCTCC AB2012108^T = KACC 16706^T). Further our analyses also revealed that *B. encimensis* SGD-V-25^T is a later heterotypic synonym of *Bacillus badius* DSM 23^T.

The family *Bacillaceae* currently comprises 57 genera and 618 validly published species names (http://www.bacterio.net/classifphyla.html#bacillaceae). The members of the family *Bacillaceae* are ubiquitous in distribution and have been isolated from a wide range of habitats such as soil [19], soda ponds [11], high altitude [54], spacecraft assembly facility [39], deep sea sediment [6], humus [62] etc. Ash et al. [3] for the first time addressed the issue of inconsistencies in the systematics of the genus and split 51 *Bacillus* species into five different rRNA groups based on evolutionary relationships of their 16S rRNA genes. Later, Nielsen et al. [40] proposed a sixth rRNA group to accommodate the alkaliphilic and alkalitolerant species. In spite of earlier classification of the *Bacillus* species into different taxonomic groups, it is generally agreed that the genus requires further revision with some organisms clearly in need of being reclassified to novel or existing genera [26–28]. The current situation results from the genus being used as a general repository for Gram staining-positive, endospore-

☆ **New Taxa-Firmicutes**: The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the strains 5.5LF 48TD, 5.5LF 38TD and SgZ8^T are EF112395, FM958153 and MF359551 respectively. The NCBI accession numbers for the draft genome sequences of the strains 5.5LF 48TD, 5.5LF 38TD, *B. badius* DSM 23^T and *B. thermotolerans* SgZ-8^T are JWIR00000000, JWIQ00000000, JXLP00000000 and JWJE00000000, respectively.

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forming bacteria without the comprehensive examination of their physiological, chemotaxonomic and phylogenetic relationships. Consequently, the taxonomy of the genus *Bacillus* has undergone extensive revision to resolve the issue of phenotypic and phylogenetic heterogeneity during the last two decades by proposing reclassification of existing strains into new genera such as creation of a new genus *Paenibacillus* and transfer of species belonging to rRNA group 3 into it [4], *Bacillus brevis* and *Bacillus aneurinolyticus* to the genera *Brevibacillus* and *Aneurinibacillus* respectively [52], *Bacillus fusiformis* and *Bacillus sphaericus* to *Lysinibacillus* [1], *Bacillus arvi*, *Bacillus arenosi* and *Bacillus neidei* to *Viridibacillus* [2] and *Bacillus globisporus*, *Bacillus psychrophilus* and *Bacillus pasteurii* to the genus *Sporosarcina* [63]. Similarly there have been other proposals to resolve the taxonomic ambiguity that remains [14,15,20,27,28,53,57,65].

During the investigation of prokaryotic diversity from a landfill soil sample [29], two strains designated as 5.5LF 38TD and 5.5LF 48TD were isolated after dilution plating of the samples on tryptic soy broth (TSB) diluted 100 times and solidified with agarose (1.5% w/v). The strains were maintained in 10% glycerol suspensions at −70 °C. The strain *Bacillus thermotolerans* SgZ-8^T isolated from a compost demonstration plant [62] was obtained as a reference culture from the original authors of the paper. In the present paper, strains 5.5LF 38TD, 5.5LF 48TD and *Bacillus thermotolerans* SgZ-8^T were taxonomically characterized using a phylogenomics approach as well as following the minimal standards of description of aerobic endospore forming bacteria [33]. The strains *B. badius* DSM 23^T, *Domibacillus robiginosus* DSM 25058^T, *D. iocasae* DSM 29979^T and *Bacillus encimensis* SGD-V-25^T were included for comparative phenotypic and genomic analyses as reference strains. All the biochemical tests and maintenance of strains were done at 30 °C.

Morphological tests such as Gram staining and endospore staining were performed as per Smibert and Krieg [55]. For examining motility, cells (grown for 1–2 days on TSBA) were observed by the hanging drop method and checked under a phase contrast microscope at 1000× magnification (Olympus model BX 51TRF). Oxidase and catalase tests were done using oxidase discs (Hi-Media laboratories) and 3% (v/v) H_2O_2 in order to confirm the presence of cytochrome c oxidase and superoxide dismutase, respectively [55]. Hydrolysis of starch, gelatin, urea and casein were determined as per standard procedures [55]. Nitrate reduction, indole production, methyl red and Voges–Proskauer were performed according to the recommendations of Lányi [31]. Range and optimum parameters of temperature, salt concentrations and pH (using biological buffers as explained by Breznak and Costilow [12]) were investigated using agar plates and plotting growth curves by growing the strains in TSB under shaking conditions (150 r.p.m.) at 30 °C and at different temperatures. Anaerobic growth was checked by using Merck anaerobic jar with Anaerocult A sachet for a week to create an oxygen free CO_2 atmosphere. Other biochemical tests such as utilization/fermentation of carbon sources, antibiotic resistance and enzymatic activities were completed using API 20NE, API 50CH, API 20E, API ZYM strips and the OMNILOG GEN III system according to manufacturer's instruction.

For determining cellular fatty acids, the fatty acid methyl esters were obtained from late log phase grown cells by saponification, methylation, extraction and washing according to the instructions of the MIDI system (MIS operating manual version 6.1, [48]). The samples were injected into an Agilent model 7890A gas chromatograph equipped with a 7693 autosampler and a flame-ionization detector fitted with a 5% phenylmethyl silicone column (0.2 mm × 25 m). The injection-port temperature was 300 °C and the oven temperature was set between 170 and 300 °C, increasing at a rate of 1 °C min^{-1}. Fatty acid profiles were compared with those in the MIS library of MIDI (TSBA 6.0). Menaquinones were extracted from 200 mg dry cell mass with a 10% aqueous solution of 0.3% (w/v) NaCl in methanol and petroleum ether (60–80 °C boiling point) at a ratio of 1:1. The upper phase was collected and dried in a Turbo Vap LV evaporator (Zymark). The residue was dissolved in 100 μl acetone. The extract was developed on a TLC plate (20 × 20 cm Silica gel 60 F_{254}; Merck) using petroleum ether (boiling point 60–80 °C) and diethyl ether (85:15, v/v). Purified menaquinones were dissolved in 2-propanol, analyzed by reverse-phase TLC according to Collins and Jones [13]. These were then separated by reverse-phase HPLC (Shimadzu Uflc xr 20A model) using acetonitrile/*iso*-propanol at a flow rate of 1 ml min^{-1} with detection at a wavelength of 254 nm. Extraction of polar lipids was done based on the protocol of Bligh and Dyer [9]. Two-dimensional TLC was run for identification of polar lipids according to procedures described by Komagata and Suzuki [25]. The diagnostic diamino acid of the peptidoglycan was determined in the whole-cell hydrolysate according to Protocol 1 mentioned elsewhere [49]. For the analyses of the ribosomal protein profiles, matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) was performed [59]. Furthermore, riboprinting was undertaken using an automated Riboprinter (Dupont) with *Eco*RI as the restriction enzyme in order to analyze the diversity of the ribotype pattern within strains of the different species [50].

Extraction of genomic DNA, amplification and sequencing of 16S rRNA gene were performed as reported earlier by Verma et al. [59]. The 16S rRNA gene sequence was then used as a query to check for the related phylogenetic neighbours against the database of the type strains of the prokaryotes with validly published names in EzTaxon server (http://www.ezcloud.net/eztaxon) [23]. For the analyses of the *gyrB* gene, the amino acid sequences of the protein were used since the BLASTn search gave very low identity values. The sequences of the closest species were retrieved using translated BLAST (BLAST X). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6.0 [58].

For the preparation of genomic DNA from samples (5.5LF 38TD, 5.5LF 48TD, *B. thermotolerans* SgZ-8^T, *B. badius* DSM 23^T, DSM 30822, DSM 5610 and *Domibacillus iocasae* DSM 29979^T) extraction was performed using Genomic Tip kit100/G (Qiagen). Library preparation was carried out according to the TruSeq DNA sample preparation protocol (Illumina, Inc., San Diego, CA) at C-CAMP, Bengaluru and Bioserve Technologies, Hyderabad, India. In brief, 1 μg of bacterial DNA was sheared to an average length of 300–400 bp. End repair, A-tailing and adapter ligation (∼120 base adapters) was performed according to paired-end DNA sample preparation kit (Illumina, Inc.). Size selection of adapter ligated DNA was done in range of 400–550 bases for DNA library. The insert size was taken in range of 280–430 bases for DNA library. PCR enrichment was performed for 8 cycles and the samples were validated on a bioanalyzer. Libraries were sequenced in a Paired End 100 base run, using TruSeq PE Cluster Kit v3-cBot-HS for cluster generation on C-bot and TruSeq SBS Kit v3-HS (Catalog No.: PE-401-3001) for sequencing on the Illumina HiSeq 1000 platform according to recommended protocols. The *de novo* assembly of paired end reads for all the organisms were completed using SPAdes v3.1 [7] and CLC Genomics Workbench software version 7.0.3 using default settings. The contigs thus obtained for all the organisms were scaffolded using independently SSPACE v2.0 [10] and the gaps were filled by GapFiller v1.10 [38]. The gap-filled scaffolds thus obtained, were broken into contigs where gaps were not filled. Functional annotation was carried out by RAST (Rapid Annotation using Subsystem Technology) [5,41], tRNA was predicted by tRNAscan-SE 1.23 [34] and rRNA genes by RNAmmer 1.2 [30]. The genome sequences of *B. encimensis* SGD-V-25^T, *Domibacillus robiginosus* WS 4628^T, *D. indicus* SD111^T, *D. enclensis* NIO-1016^T and *D. tundrae* PAMC 80007^T were downloaded from the NCBI website (http://www.ncbi.nlm.nih.gov/genome). For calculating the overall genomic relatedness, digital DDH values were determined by uploading the fasta files