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Psychrobacillus gen. nov. and proposal for reclassification of *Bacillus insolitus* Larkin & Stokes, 1967, *B. psychrotolerans* Abd-El Rahman et al., 2002 and *B. psychrodurans* Abd-El Rahman et al., 2002 as *Psychrobacillus insolitus* comb. nov., *Psychrobacillus psychrotolerans* comb. nov. and *Psychrobacillus psychrodurans* comb. nov.☆

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ABSTRACT

The taxonomic status of three *Bacillus* species, *Bacillus insolitus*, *B. psychrodurans* and *B. psychrotolerans* was reexamined using a polyphasic approach. In our analysis, these three *Bacillus* species formed a cluster separate from other members of *Bacillus* rRNA group 2 [5] and from *Bacillus* sensu stricto. These three species shared high 16S rRNA gene sequence similarities between them (97.8–99.7%) and showed closest sequence similarity (95.3–96.3%) to *Paenisporosarcina quisquiliarum* gen. nov., sp. nov. [18]. Sequence similarities with other related genera ranged between 90.9% and 94.5%. Phylogenetic coherence of the three species was supported by phenotypic characteristics, such as growth at low temperatures, negative oxidation and assimilation of many carbohydrates, MK8 as the major isoprenoid quinine and broadly similar polar lipid profiles. All three species had a similar peptidoglycan type of the variation A4β and similar genomic G + C contents (35.7–36.6 mol% [1]). Genomic relatedness among them was shown to be less than 70% and justified their separate species status [1]. These three species could be differentiated from each other and from related taxa on the basis of phenotypic, including chemotaxonomic, characteristics and ribotype patterns. On the basis of our analysis, we propose a new genus *Psychrobacillus* gen. nov. and to transfer *B. insolitus*, *B. psychrodurans* and *B. psychrotolerans* to the new genus as *Psychrobacillus insolitus* comb. nov. (type species of the genus; type strain W16B^T = DSM 5^T), *P. psychrodurans* comb. nov. (type strain 68E3^T = DSM 11713^T) and *P. psychrotolerans* comb. nov. (type strain 3H1^T = DSM 11706^T).

On the basis of the 16S rRNA gene sequence analysis, heterogeneity in the genus *Bacillus* was first pointed out by Ash et al. [5] and they recognized five distinct groups. Members belonging to rRNA group 3 were placed in a new genus *Paenibacillus* [6]. In their analysis, *Bacillus* rRNA group 2 was comprised of six *Bacillus* species and the genus *Sporosarcina*. The genus *Bacillus*, at present, is heterogeneous and the need for reclassification of many of its members has been indicated by many authors [2,3,9,14,15,31] Subsequently, three species from the *Bacillus* rRNA group 2 of Ash et al. [5] were transferred to the genus *Sporosarcina* [31] and two to *Lysinibacillus* [2]. Taxonomic status of the sole remaining member of the original six species, *Bacillus insolitus*, has not been reexamined so far. In spite of the uncertain taxonomic position, several new species were still being added to *Bacillus* rRNA group 2 (*Bacillus silvestris* [25]; *Bacillus pycnus* and *B. neidei* [22]; *Bacillus psychrodurans* and *Bacillus psychrotolerans* [1]; *Bacillus odysseyi* [19]; *B. arvi* and *B. arenosi* [12]; *Bacillus massiliensis* [11]; *Bacillus decisifrondis* [32]). Of these, *B. arvi*, *B. arenosi* and *B. neidei* were recently transferred to the genus *Viridibacillus* [3] and *B. silvestris* to a new genus *Solibacillus* [17]. In an attempt to bring homogeneity in the genus *Bacillus*, Kämpfer et al. [15] suggested a set of chemotaxonomic properties present in the type species of the genus, *Bacillus subtilis*, "should in future constitute the 'core characteristics' of the genus". Unfortunately, many of these properties were not reported for most of the recently described species of *Bacillus*.

In our study of prokaryotic diversity of a landfill, we isolated a few strains showing affiliation to *Bacillus* rRNA group 2. Polyphasic taxonomic characterization of one of these strains designated

☆ The GenBank accession number for the 16S rRNA sequence of *Bacillus insolitus* DSM 5^T is AM980508.

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as SK 55^T led to the proposal of a novel genus *Paenisporosarcina* and reclassification of *Sporosarcina macmurdoensis* as *P. macmurdoensis* [18]. Detailed study of these strains also prompted us to reexamine the taxonomic status of three psychrotolerant *Bacillus* species, *Bacillus insolitus* DSM 5^T, *Bacillus psychrotolerans* DSM 11706^T and *Bacillus psychrodurans* DSM 11713^T, because of their phylogenetic closeness to the genus *Paenisporosarcina* [18]. In a previous study, Abd El Rahman et al. [1] investigated many strains of these three *Bacillus* species and, based on data from biochemical tests and DNA–DNA hybridization experiments, concluded separate species status for each group of strains. We examined several phenotypic and chemotaxonomic (fatty acids, menaquinones, polar lipids) properties of the type strains of these three species. In addition, we also investigated many biochemical properties (using the Biolog system and by API 50CH) and fatty acids of additional strains of the three species [strains 84E1 (DSM 22887) and 4H2 (DSM 11718) belonging to *B. psychrotolerans*, strains 67E1 (DSM 11712) and 61E1 (DSM 22889) belonging to *B. psychrodurans*, and strain T16B (DSM 2272) belonging to *B. insolitus* (Supplementary Tables S2, S3 and S4)]. This was carried out in order to take care of the intraspecies variations in the phenotypic properties of the bacterial species. Furthermore, riboprinting was also undertaken using an automated Riboprinter (Dupont) with EcoRI as the restriction enzyme [4] in order to analyze the diversity of the ribotype pattern within strains of the different species. On the basis of a polyphasic taxonomic analysis of the type strains, along with phenotypic characteristics of other strains (mentioned above), we show in this study that these three species are distinct from other members of *Bacillus* rRNA group 2 and they merit reclassification into a novel genus. We, therefore, propose creation of a new genus *Psychrobacillus* and to transfer these three *Bacillus* species to this genus. It is appropriate to mention here that a novel generic status of *B. insolitus* was speculated earlier in the extensive phylogenetic study of this group by Stackebrandt and Swiderski [28].

Eight strains of these three species were tested for acid production from, and oxidation of, various substrates using API 50CH strips (bioMèrieux, France) and Biolog GP2 plates (Biolog Inc., USA), respectively, according to the manufacturers' instructions, except that incubation was carried out at 20–25 °C depending on the growth behavior of strains. In addition, *B. psychrotolerans* DSM 11706^T, *B. psychrodurans* DSM 11713^T and *B. insolitus* DSM 5^T were subjected to further biochemical characterization using API ZYM and API NE test kits (bioMèrieux, France), according to the manufacturer's instructions except that incubation was carried out at 22 °C. Motility was determined using the hanging drop method and by observing cells under a phase contrast microscope (Zeiss, Germany). For analysis of cellular fatty acids, all eight strains were grown on TSBA at 20–25 °C according to their growth behavior. Fatty acid methyl esters were extracted and analyzed using the Microbial Identification System (MIDI), as described previously [23]. For analyses of isoprenoid quinones and polar lipids, *B. psychrotolerans* DSM 11706^T and *B. psychrodurans* DSM 11713^T were cultivated on TSB, and *B. insolitus* DSM 5^T was grown on marine broth for 2 days in a rotary shaker (200 rpm) and harvested by centrifugation at 3000 × *g*. Isoprenoid quinones were extracted and purified as described previously [26]. The purified quinones were separated by reversed phase HPLC (SCL-10AVP, Shimadzu) using the solvent system of acetonitrile and isopropanol in a ratio of 65:35 with a flow rate of 1 mL min^{-1} monitored at a wavelength of 269 nm. Extraction of polar lipids was carried out based on the modified protocol of Bligh and Dyer [7]. Two-dimensional TLC was run for identification of polar lipids according to procedures described by Komagata and Suzuki [16]. Lipid spots were detected using the following spray reagents: molybdatophosphoric acid (5%; w/v) in absolute ethanol, molybdenum blue spray reagent (1.3%, Sigma), ninhydrin (0.2%; w/v) in acetone and anisaldehyde reagent (Sigma) for detection of total lipids, phospholipids, aminolipids and glycolipids, respectively.

Since the 16S rRNA gene sequence of *B. insolitus* DSM 5^T available in the GenBank database (X60642) was found to be of poor quality, it was sequenced again and the sequence was deposited (AM980508). Partial sequences of the 16S rRNA gene of *B. psychrodurans* DSM 11713^T and *B. psychrotolerans* DSM 11706^T and other strains were also determined to check the authenticity of the strains. The genomic DNA of the strains was isolated according to the method described by Pitcher et al. [24]. The 16S rRNA gene was amplified by PCR using the universal primers 8-27F (5′-AGAGTTTGATC- CTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The amplification reaction and purification of the product was carried out as mentioned earlier [23]. The amplified 16S rDNA was sequenced by the dideoxy chain terminator method using the BigDye Terminator kit followed by capillary electrophoresis on an ABI 310 genetic analyzer (Applied Biosystems, USA). The primers used for sequencing were 357F (5′-CTCCTACGGGAGGCAGC-AG-3′), 685R (5′-TCTACGCATTTCACCGCTAC-3′), 926F (5′-AAACT-CAAAGGAATTGACGG-3′), and 907R (5′-CCGTCAATTCMTTTRAG-TTT-3′). The 16S rRNA gene sequences of *B. psychrodurans* DSM 11713^T, *B. psychrotolerans* DSM 11706^T and *B. insolitus* DSM 5^T, along with sequences of other closely related taxa, were retrieved from the GenBank database and aligned using the Clustal X software [29], and the alignments were edited manually. The 16S rRNA gene sequence similarities were calculated from the alignment. Gaps at the 5′ and 3′ ends of the alignment were omitted from further analysis. Evolutionary distance matrices were calculated by using the algorithm of Jukes and Cantor [13] with the DNADIST program within the TREECON software package [30]. A phylogenetic tree was constructed using the neighbour-joining method [27] and bootstrap analysis was performed to assess the confidence limits of the branching. Trees based on the maximum parsimony and maximum likelihood methods were also constructed using the Phylip software package version 3.5c [10].

Results of biochemical analysis revealed that strains of the three species were non-reactive towards most of the substrates in oxidation and acid production tests (Supplementary Table S3 and S4). Out of the 95 substrates in the Biolog microplate most of the strains were unable to oxidize more than five substrates (except strain DSM 11712 which oxidized six substrates). Similarly, out of the 50 carbon sources tested in API 50 CH strips none of the strains could produce acid from more than three substrates (Supplementary Table S4). These observations were in agreement with previous and recent findings that species belonging to the so-called *Bacillus* rRNA group 2 are not reactive towards most of the substrates and these tests alone offer little diagnostic importance [2,3,12,21,22]. Analysis of cellular fatty acids of eight strains revealed that anteiso-$C_{15:0}$ was the most predominant fatty acid (30.2–50.5%; Supplementary Table S2). The other major fatty acids present were $C_{16:1}\omega7c$ alcohol (7.5–16.5%; 4.71% in *Bacillus psychrodurans* strain 67E1), iso-$C_{14:0}$ (6.2–21.1%) and iso-$C_{15:0}$ (10.5–21.9%; 4.7% in *B. insolitus* DSM 5^T). As compared to its closest relative *Paenisporosarcina quisquiliarum*, the three species contained relatively higher amounts of anteiso-$C_{15:0}$ and iso-$C_{14:0}$, and lower amount of iso-$C_{15:0}$. Moreover, as compared to *B. subtilis*, the three species showed both quantitative and qualitative variations in the amount of a few fatty acids, such as $C_{16:1}\omega7c$ alcohol and iso-$C_{14:0}$ (Supplementary Table S2). These three species were also characterized by the presence of A4β type peptidoglycan with ornithine as the diaminoacid at position 3 of the peptide subunit [1], whereas all other genera of *Bacillus* group 2 contained A4α type peptidoglycan with lysine as the diaminoacid (Table 1). This is one of the most important chemotaxonomic parameters which distin-