Bacillus galactosidilyticus sp. nov., an alkali-tolerant β -galactosidase producer

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A novel *Bacillus* isolate from raw milk and four strains from diverse origins that were identified previously as *Bacillus lentus*, *Bacillus firmus* and *Bacillus circulans* showed a high degree of similarity in amplified rDNA restriction analysis, SDS-PAGE and routine phenotypic tests, whilst 16S rDNA sequence comparisons and DNA relatedness data showed that this taxon was different from related *Bacillus* species. On the basis of these data, *Bacillus galactosidilyticus* sp. nov. is proposed, with the type strain LMG 17892^T (=DSM 15595^T=Logan B2188^T=MB 800^T).

Bacillus lentus and *Bacillus firmus*, which were both described in the 1930s (Werner, 1933; Gibson, 1935) and included in the Approved List of Bacterial Names (Skerman *et al.*, 1980), are two species that have long been recognized as being genetically and phenotypically heterogeneous. Typical strains of both species can readily be differentiated from each other and from other *Bacillus* species with traditional and API tests, as *B. lentus* produces acid from a wider range of carbohydrates. DNA homology data suggest that the two species are distinct, but intermediate strains can be allocated to either species (Logan & Berkeley, 1981; Claus & Berkeley, 1986). In the first comprehensive phenotypic study of the genus *Bacillus* that incorporated these species (Gordon *et al.*, 1973), although only three strains of *B. lentus* (cautiously classified by the authors as unassigned strains) were included, it was already recognized that these few strains of *B. firmus* and *B. lentus* may represent a spectrum-like species. Some years later, Gordon *et al.* (1977) asked the taxonomic question 'Do *B. firmus* and *B. lentus* represent a series or a species?'. Numerous

alkaliphilic *Bacillus* strains have been classified as *B. firmus*, *B. lentus* (divided into three groups) or '*B. firmus*–*B. lentus* intermediates', the latter group accounting for nearly half of strains investigated (Gordon & Hyde, 1982). This phenotypic heterogeneity was further demonstrated in a numerical study by Priest *et al.* (1988), which classified environmental isolates that were related to *B. firmus* and had been described previously as '*B. firmus*–*B. lentus* intermediates' in three phenetic clusters. Genetic heterogeneity of the species was demonstrated in several studies. Several DNA homology groups have been described in *B. firmus* (Fahmy *et al.*, 1985) and, in another study, five *B. lentus* strains showed low DNA homologies with each other (and with *B. firmus*) (Seki *et al.*, 1983). The 'complex' of alkaliphilic *B. firmus*–*B. lentus* intermediate strains, many of which were investigated previously by Gordon & Hyde (1982), was divided into separate clusters on the basis of genomic DNA G + C content and physiological properties (Fritze *et al.*, 1990). This study suggested the presence of numerous species rather than a 'continuum' of strains, but no further taxonomic studies have been undertaken.

B. lentus is isolated frequently from raw and pasteurized milk. In an elaborate taxonomic study of the aerobic spore-forming flora of raw milk, a novel *Bacillus* strain (LMG 17892^T) was isolated, which was closely related phylogenetically to *B. lentus* (Heyndrickx & Scheldeman, 2002). On the basis of a polyphasic taxonomic study, a close relationship was found between this strain and four other culture collection strains that were labelled originally as *B. lentus*, *B. firmus* or *Bacillus circulans*. As a result, a novel

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Abbreviation: ARDRA, amplified rDNA restriction analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Bacillus galactosidilyticus* LMG 17892^T and *Bacillus* sp. R-6760, both determined in this work, are AJ535638 and AJ535639, respectively.

Supplementary figures showing a full phylogenetic tree and normalized computer profiles from SDS-PAGE analysis are available as supplementary material in IJSEM Online.

species, *Bacillus galactosidilyticus* sp. nov., is proposed. The proposal of this species is a further step in resolving the taxonomic enigma of the *Bacillus lentus*–*Bacillus firmus* complex.

Two novel strains, LMG 17892^T (=DSM 15595^T=Logan B2188^T=MB 800^T) and R-6760, were freshly isolated from raw milk after heat-treatment (10 min at 80 °C and 30 min at 100 °C, respectively) for pasteurization of the sample and activation of spores, plating on brain heart infusion (BHI; Oxoid) supplemented with Bacteriological agar no. 1 (15 g l⁻¹; Oxoid) and filter-sterilized vitamin B₁₂ (1 mg l⁻¹) and incubation at 37 °C for 48 h. The other culture collection strains included in this study, with their respective isolation sources (if known), were as follows: strain LMG 12353 [=Logan B0353=NRRL NRS-769; listed as a *B. firmus* strain by Gordon *et al.* (1973)], isolated from partially decomposed wheat grain; *B. lentus* R-15577 (=Logan B0437; Wellcome collection); *B. lentus* R-16004 (=Logan B0439; Wellcome collection); and *B. circulans* LMG 12396 (=Logan B0419), isolated from infant bile. The type strains of *B. lentus* (LMG 16798^T=NCIMB 8773^T=Logan B0779^T) and *B. circulans* (LMG 13261^T=DSM 11^T) and another authentic strain of *B. lentus* (R-16003=Logan B0436) were also included for some analyses. All strains were maintained on trypticase soy agar (TSA) that contained 5 mg MnSO₄ l⁻¹ to enhance sporulation, as described by Logan *et al.* (2000). Amplified rDNA restriction analysis (ARDRA) was performed as described previously (Heyndrickx *et al.*, 1996). Almost-complete 16S rDNA sequences were obtained for strains LMG 17892^T and R-6760, as described by Scheldeman *et al.* (2002) with the modification that an Applied Biosystems ABI 310 sequencer (Perkin Elmer Applied Biosystems) was used. Sequences were used for phylogenetic analysis with BioNumerics software (Applied Maths) as described by Logan *et al.* (2002). For determination of DNA G+C content of and DNA–DNA hybridization between strains LMG 17892^T, LMG 12353, LMG 12396, R-6760 and some *Bacillus* reference strains, approximately 1 g biomass was harvested from agar plates and DNA was purified as described by Logan *et al.* (2000) with the following modifications: lysozyme was dissolved in TE buffer with an increased EDTA concentration (1.21 g Tris/HCl, 6 ml 0.5 M EDTA; pH 8.0 in 1 l MilliQ water) and cell suspensions were incubated with lysozyme until viscosity increased (usually after 10–15 min). The DNA G+C content was determined by HPLC, using further specifications given by Logan *et al.* (2000). DNA–DNA hybridization was performed by using a modification of the microplate method described by Ezaki *et al.* (1989), as described by Willems *et al.* (2001). A hybridization temperature of 37 °C was used. Cells of strains LMG 17892^T, LMG 12353, LMG 12396, R-15577, R-16004 and R-6760 were obtained as described by Heyndrickx *et al.* (1998) and subjected to SDS-PAGE analysis of whole-cell proteins, according to Pot *et al.* (1994). SDS-PAGE data were collected and interpreted as described by Vauterin & Vauterin (1992). For GC analysis

of methylated cellular fatty acids, cells of the same strains were grown and analysed as described by Heyndrickx *et al.* (1998) and Vauterin *et al.* (1991). All six strains, as well as the above-mentioned type strains and authentic strains of *B. lentus*, *B. firmus* and *B. circulans*, were characterized phenotypically by the methods of Logan & Berkeley (1984); other characters were determined and data were analysed numerically as described by Logan *et al.* (2000).

Comparison of the ARDRA pattern of strains LMG 17892^T and R-6760 with those in a database of over 1000 authentic strains of aerobic, endospore-forming bacteria did not yield any identification. On the other hand, >90 % similarity was found between strain LMG 17892^T and four strains in the database that did not cluster with the type strain of the respective species to which they were assigned originally, i.e. *B. firmus* LMG 12353, *B. lentus* R-15577, *B. lentus* R-16004 and *B. circulans* LMG 12396 (data not shown). The ARDRA pattern of strain R-6760 showed 87.5 % similarity to that of strain LMG 17892^T. In 16S rDNA sequence comparisons with entries in GenBank/EMBL, the closest ungapped FASTA matches with a species with a validly published name that were achieved for strain LMG 17892^T (1486 bp; accession no. AJ535638) and strain R-6760 (1423 bp; accession no. AJ535639) were 96.3 and 96.6 %, respectively, with *B. lentus* (accession no. AB021189). On the other hand, 16S rDNA similarities of 97.4 and 97.1 %, respectively, were found with an unidentified strain in GenBank/EMBL, TGS137, which was isolated from a composter (accession no. AB020192). Strains LMG 17892^T and R-6760 showed 98.3 % 16S rDNA sequence similarity to each other. In phylogenetic cluster analysis by using neighbour-joining (Fig. 1), both strains clustered closely together in the radius of the genus *Bacillus*, with *B. lentus* and related species as

Fig. 1. Neighbour-joining clustering of 16S rDNA sequences (rooted with *Bacillus sphaericus* as reference), based on a selection of 16S rDNA sequences of the nearest neighbours to *Bacillus galactosidilyticus* LMG 17892^T and *Bacillus* sp. R-6760 from GenBank/EMBL (accession numbers are given in parentheses). Only the nearest neighbours are shown here; a more complete tree is given as supplementary material in IJSEM Online.

the closest phylogenetic relatives (a more complete tree that includes *Bacillus subtilis*, the type species of the genus *Bacillus*, is given as supplementary material in IJSEM Online).

From SDS-PAGE analysis, it follows that five strains (LMG 17892^T, LMG 12396, LMG 12353, R-16004 and R-15577) formed a homogeneous group at a similarity level of 83 %, with strain R-6760 linked to this group at 80 % similarity (a figure showing UPGMA clustering of normalized SDS-PAGE patterns is given as supplementary material in IJSEM Online).

In API 50 CHB tests (data not shown), the five strains LMG 17892^T, LMG 12396, LMG 12353, R-15577 and R-16004 gave weak and varied reactions and did not present a consistent profile; they showed consistent results in API 20 E tests and in their morphological characters. In numerical analysis of API tests and other phenotypic characters, they clustered together at 88 % S_G and with isolates of *Bacillus shackletonii* from Candlemas Island (Logan *et al.*, 2004) at 86 % S_G. These two groups merged with the group of unreactive strains (containing *B. firmus*) at 86 % S_G. Phenotypic characters that distinguish the five above-mentioned strains from the phenotypically related species *B. shackletonii* and from the phylogenetically related species *B. lentus* are shown in Table 1. Strain R-6760 showed some phenotypic similarity to strains LMG 17892^T, LMG 12396, LMG 12353, R-15577 and R-16004, but gave much stronger reactions in API 50 CHB tests, did not show any casein hydrolysis and produced very swollen sporangia.

DNA homology values (data not shown) revealed high DNA interrelatedness (93.5–99.0 %) within the above-mentioned SDS-PAGE group of strains (LMG 17892^T, LMG 12396, LMG 12353, R-15577 and R-16004), whereas a low DNA homology value (32.5 %) was found between strains LMG 17892^T and R-6760. DNA homology values with the type strains of *B. lentus* and *B. circulans* were low (<30 %).

The genetic and phenotypic data presented above show that strains LMG 17892^T, LMG 12353, LMG 12396, R-15577 and R-16004 belong to a novel *Bacillus* species, for which we propose the name *Bacillus galactosidilyticus* sp. nov. Strain R-6760 represents the core of another novel *Bacillus* species, of which more strains must be identified before a formal species proposal can be made.

Description of *Bacillus galactosidilyticus* sp. nov.

Bacillus galactosidilyticus (ga.lac.to.si.di.ly'ti.cus. N.L. neut. n. *galactosidum* galactoside; N.L. masc. adj. *lyticus* lysing, dissolving; N.L. masc. adj. *galactosidilyticus* referring to a positive ONPG test, revealing β -galactosidase activity).

Cells are 0.7–0.9 μ m in diameter and 2–5 μ m in length, motile in a tumbling motion, Gram-positive or -variable, small, plump, round-ended rods that occur singly or in

Table 1. Some characters for distinguishing between *B. galactosidilyticus*, *B. shackletonii* and *B. lentus*

Taxa: 1, *B. galactosidilyticus*; 2, *B. shackletonii*; 3, *B. lentus*. Characters are scored as: +, >85 % positive; +/w, positive or weakly positive; v, variable (26–74 % positive); –, 0–15 % positive; w, weakly positive reaction.

Character	1	2*	3
Gram staining	+ /v	+	+
Anaerobic growth	+	+	–
Growth at pH 10.5	+	–	–
Casein hydrolysis	w	+	–
Citrate utilization	–	–	v
Urease production	v	–	+
Nitrate reduction	+	–	+
Acid from:			
L-Arabinose	v	–	–
Ribose	v	–	+ /w
D-Xylose	v	–	+ /w
L-Xylose	–	–	+ /w
Galactose	v	–	+ /w
Rhamnose	v	–	+ /w
Mannitol	–	w	+ /w
Lactose	v	–	+ /w
Melibiose	v	–	+ /w
Sucrose	v	–	+ /w
D-Melezitose	v	–	+ /w
D-Raffinose	v	–	+ /w
Starch	v	–	+ /w
Gentiobiose	v	–	+ /w

*Data from Logan *et al.* (2004).

pairs, and occasionally in short chains. They bear ellipsoidal endospores that lie in central, paracentral and subterminal positions within slightly swollen sporangia (Fig. 2). After 2 days on TSA, colonies are approximately 1 mm in diameter, smooth, flat and butyrous; their edges are usually irregular with pointed projections that may spread and become rhizoid in old cultures; they are creamy or off-white in colour, with opaque centres. Organisms are facultatively anaerobic and catalase-positive. Growth occurs at 30 and 40 °C, but not at 50 °C. Growth occurs between pH 6 and 10.5, but not at pH 5 or below. Casein hydrolysis is very weak. In API 20E tests, *o*-nitrophenyl β -D-galactoside reaction is positive; arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are negative; citrate is not utilized; hydrogen sulphide is not produced; urease production is variable; Voges–Proskauer reaction is negative; indole is not produced; and gelatin is not hydrolysed. Nitrate is reduced to nitrite. Hydrolysis of aesculin is positive. In the API 50 CHB gallery, acid is produced without gas, often weakly, from *N*-acetylglucosamine, D-fructose and D-glucose. Acid production from the following carbohydrates is variable and, when positive, is usually very weak: amygdalin, L-arabinose, arbutin,

Fig. 2. Photomicrograph of sporangia and vegetative cells of *B. galactosidilyticus* LMG 17892^T, viewed by phase-contrast microscopy; ellipsoidal spores lie centrally, paracentrally and subterminally in slightly swollen sporangia. Bar, 2 µm.

D-cellobiose, galactose, gentiobiose, inulin, lactose, maltose, mannitol, D-mannose, D-melezitose, D-melibiose, methyl D-glucoside, D-raffinose, rhamnose, ribose, salicin, starch, sucrose, D-trehalose, D-turanose and D-xylose. Acid is not produced from the following carbohydrates: adonitol, D-arabinose, D- or L-arabitol, dulcitol, erythritol, D- and L-fucose, glycerol, gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate, glycogen, D-lyxose, methyl D-mannoside, *meso*-inositol, sorbitol, L-sorbose, D-tagatose, L-xylose, methyl xyloside and xylitol. Major cellular fatty acids (mean percentage $\pm$ SD of total fatty acids) are: C_{14:0} (8.32 $\pm$ 2.51 %), iso-C_{15:0} (12.71 $\pm$ 1.72 %), anteiso-C_{15:0} (32.60 $\pm$ 1.94 %) and C_{16:0} (27.42 $\pm$ 3.04 %). The following fatty acids are present in smaller amounts (mean percentage $\pm$ SD of total fatty acids): iso-C_{14:0} (3.96 $\pm$ 0.73 %), C_{15:0} (5.31 $\pm$ 1.08 %), iso-C_{16:0} (2.45 $\pm$ 0.44 %) and anteiso-C_{17:0} (4.49 $\pm$ 0.72 %). Trace amounts (<1 % of total fatty acids) of the fatty acids iso-C_{13:0} and iso-C_{17:0} are also present. DNA G + C content is 37.7 mol% for the type strain and 35.7–38.2 mol% for other strains.

The type strain is LMG 17892^T (=DSM 15595^T=Logan B2188^T=MB 800^T). The 16S rRNA gene sequence of the type strain is deposited at GenBank/EMBL under accession no. AJ535638. In the variable characters listed above, the type strain is negative for urease hydrolysis and acid is produced without gas from arbutin, D-cellobiose, D-melibiose, D-melezitose, D-raffinose, starch, sucrose and D-trehalose.

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