Bacillus ruris sp. nov., from dairy farms

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Four novel ellipsoidal spore-forming *Bacillus* isolates with swollen sporangia, isolated from raw milk and feed concentrate, showed a high level of similarity in SDS-PAGE, fatty acid methyl esters and routine phenotypic tests. However, 16S rRNA gene sequence comparisons showed that this taxon was different from other related *Bacillus* species, and only a low level of DNA relatedness was found with the closest phylogenetic and phenotypic relative, *Bacillus galactosidilyticus*. This taxon could be differentiated from *B. galactosidilyticus* on the basis of morphological differences, stronger acid reactions with a wide range of substrates after 48 h incubation, and qualitative and quantitative differences in fatty acid content. On the basis of these data, a novel species, *Bacillus ruris* sp. nov., is proposed, with LMG 22866^T (=DSM 17057^T) as the type strain.

In the study of some milk and dairy-farm isolates that led to the proposal of the novel species *Bacillus galactosidilyticus*, a strain (R-6760^T) was encountered which showed a high degree of phylogenetic relatedness (98·3 % 16S rRNA gene sequence similarity) with respect to the type strain of this species (Heyndrickx *et al.*, 2004). However, on the basis of a low DNA relatedness value with respect to *B. galactosidilyticus*, it was concluded that this strain represented the core of another novel *Bacillus* species. In the course of a polyphasic taxonomic characterization of aerobic, endospore-forming isolates from dairy farms, three more isolates were found that showed a close relationship to each other and to this strain. As a result, a novel species, *Bacillus ruris* sp. nov., is proposed.

The previously reported (Heyndrickx *et al.*, 2004) raw-milk strain R-6760^T (=Logan B3037^T=MB 1669^T) and the novel feed-concentrate strain LMG 22867 (=R-7400=Logan B3038) were deposited in the BCCM/LMG (Belgian Co-ordinated Collections of Micro-organisms/ Laboratory of Microbiology Ghent) public collection (Ghent University, Belgium) as LMG 22866^T and LMG 22867, respectively. Two other novel strains, R-7794 (=Logan B3039) and R-8025 (=Logan B3040), were each isolated from feed concentrate. All four strains were isolated from a different dairy farm, after heat treatment (30 min at 100 °C) for selection of potentially highly heat-resistant spores such as those of *Bacillus sporothermodurans*, by means of plating on brain-heart infusion (Oxoid) supplemented with bacteriological agar no. 1 (15 g l^{-1}) (Oxoid) and filter-sterilized vitamin B_{12} (1 mg l^{-1}) and incubation at 37 °C for 48 h. Genomic DNA of strain LMG 22866^T was purified as described by Logan *et al.* (2000), with the modifications described by Heyndrickx *et al.* (2004). The G+C content of the DNA was determined by using HPLC as described by Logan *et al.* (2000). Cells of strains LMG 22866^T, LMG 22867, R-7794 and R-8025 were obtained as described by Heyndrickx *et al.* (1998) and subjected to SDS-PAGE analysis of whole-cell proteins according to the method of Pot *et al.* (1994). The SDS-PAGE data were collected and interpreted as described by Vauterin & Vauterin (1992). For GC analysis of the fatty acid methyl esters, cells of the strains were grown and analysed as described by Heyndrickx *et al.* (1998) and Vauterin *et al.* (1991). The strains were characterized phenotypically by the methods of Logan & Berkeley (1984); further characteristics were determined and the data were numerically analysed as described by Logan *et al.* (2000).

In comparisons of 16S rRNA gene sequences with entries in the EMBL database, the closest ungapped FASTA matches

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LMG 22866^T is AJ535639.

A UPGMA clustering of normalized SDS-PAGE patterns of whole-cell proteins of isolates belonging to *Bacillus ruris* sp. nov. and the *Bacillus galactosidilyticus* type strain and a table showing the cellular fatty acid methyl ester profiles of strains of *B. ruris* sp. nov. and of *B. galactosidilyticus* are available as supplementary material in IJSEM Online.

with a species with a validly published name achieved for strain LMG 22866^T (1423 bp; EMBL accession no. AJ535639) was 98·3 % with *B. galactosidilyticus* (EMBL accession no. AJ535638) and 96·6 % with *Bacillus lentus* (EMBL accession no. AB021189). On the other hand, a 16S rRNA gene sequence similarity of 99·9 % was found with entries of unidentified low-G+C Gram-positive isolates from compost deposited in GenBank/EMBL (accession nos AB116129, AB116128 and AB116144). In a phylogenetic cluster analysis using neighbour-joining (Fig. 1), it was shown that strain LMG 22866^T clustered close to the type strain of *B. galactosidilyticus* in the radius of the genus *Bacillus* and with *B. lentus* and related species as the closest phylogenetic relatives.

The four strains LMG 22866^T, LMG 22867, R-7794 and R-8025 showed very similar patterns in SDS-PAGE analyses, and clustered at a similarity level of 91 %. Nevertheless, some variation in the SDS-PAGE patterns was visible. These data together with data for the strains from different farms indicate that the organisms were not duplicate isolates. The *B. galactosidilyticus* type strain, LMG 17892^T, showed a similarity of 83 % to this group of strains (a UPGMA clustering of normalized SDS-PAGE patterns is available as a supplementary figure in IJSEM Online).

The fatty acid methyl ester data revealed a dominance of anteiso-$C_{15:0}$, $C_{15:0}$ and $C_{16:0}$, representing about 28, 14 and 33 %, respectively, of the total fatty acid content in this group of strains (see the supplementary table available in IJSEM Online). Some qualitative and quantitative differences in fatty acid content could be observed between the group of four strains described above and the phylogenetically closest relative, *B. galactosidilyticus*: the group of four strains contained small amounts of $C_{17:0}$, but no $C_{13:0}$ or $C_{16:1}\omega11c$ (*B. galactosidilyticus* contained trace amounts of the last two fatty acids but lacked the former fatty acid) and they contained more $C_{15:0}$ and less iso-$C_{15:0}$ in comparison with *B. galactosidilyticus*.

In the API 50 CHB tests (bioMérieux; data not shown) the four strains LMG 22866^T, LMG 22867, R-7794 and R-8025 gave strong acid reactions with a wide range of substrates after 48 h incubation. In general, the four strains presented consistent phenotypic profiles, with similar results in API 20E tests (bioMérieux) and in morphological observations. In a numerical analysis of the API tests, the four strains formed a distinct cluster at 87·5 % S_G, which then joined with a larger cluster containing strains of *B. galactosidilyticus*, *Bacillus firmus* and members of the *Bacillus cereus* group at only 72·5 % S_G. Phenotypic characteristics that distinguish the four above-mentioned strains from some phenotypically similar and phylogenetically related *Bacillus* species are shown in Table 1.

It had been reported previously that strain LMG 22866^T (=R-6760^T) showed only a low level of DNA relatedness (32·5 %) with the type strain of *B. galactosidilyticus*, its closest relative in phylogenetic terms (Heyndrickx *et al.*, 2004). The genetic and phenotypic data presented above show that strains LMG 22866^T, LMG 22867, R-7794 and R-8025 belong to a novel *Bacillus* species, for which we propose the name *Bacillus ruris* sp. nov.

Description of *Bacillus ruris* sp. nov.

Bacillus ruris (ru′ris. L. neut. n. *rus* the country, the farm; L. gen. n. *ruris* from the country, the farm).

Cells are rods and coccoid rods 0·5–0·8 μm in diameter and 1·0–2·0 μm in length, motile, Gram-positive, occurring singly and in pairs as well as in chains of three to four cells. They bear ellipsoidal endospores that lie in subterminal, paracentral and central positions within swollen sporangia (Fig. 2). After 2 days on trypticase soy agar, colonies are smooth, flat and butyrous, approximately 1 mm in diameter, their edges are usually irregular, and they are creamy or off-white in colour, with opaque centres. The type strain, LMG 22866^T, is facultatively anaerobic and catalase-positive. Growth of the type strain occurs at 30 and 40 °C but not at 50 °C, and between pH 6 and 11. Casein is not hydrolysed. In API 20E tests, the ONPG reaction is positive, and nitrate is reduced to nitrite; arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase, citrate utilization,

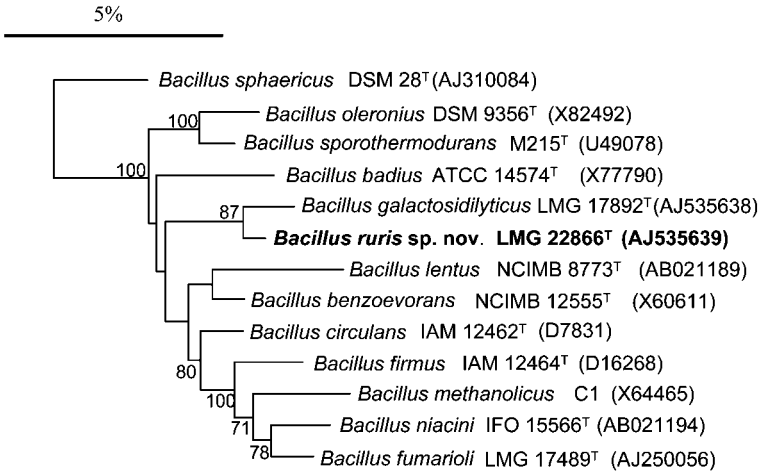

Fig. 1. Neighbour-joining clustering (showing bootstrap values above 70 %) of 16S rRNA gene sequences (rooted with *Bacillus sphaericus* as the reference), based on a selection of 16S rRNA gene sequences of the nearest neighbours of *B. ruris* LMG 22866^T, taken from GenBank/EMBL (accession nos are given in parentheses). Bar, percentage of nucleotide substitutions per nucleotide position.

Table 1. Characteristics useful for distinguishing between *B. ruris* sp. nov. and some phenotypically similar and phylogenetically related *Bacillus* species

With the exception of microscopic observations, anaerobic growth and casein hydrolysis, all characteristics were determined using tests in the API 20E and 50 CHB systems. Symbols: +, more than 85 % of the strains positive; (+), 75–84 % of the strains positive; V, variable (26–74 % of the strains positive); (−), 16–25 % of the strains positive; −, 0–15 % of the strains positive; +/W, positive or weakly positive; W, weak positive reaction; V/W, variable, and weak when positive.

Characteristic	*B. ruris*	*B. galactosidilyticus*	*B. firmus*	*B. cereus*	*B. licheniformis*	*B. subtilis*
Swollen sporangia*	+	+	−	−	−	−
Anaerobic growth	+	+	+	+	+	−
Gelatin hydrolysis	−	−	+	+	+	+
Casein hydrolysis	−	W	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+
Voges–Proskauer	−	−	+	+	+	+
Acid from:						
N-Acetylglucosamine	+	+/W	V/W	+	+	(−)
L-Arabinose	+	V	−	−	+	+
Amygdalin	−	V/W	−	−	+	+
β-Gentiobiose	−	V/W	−	−	+	+
Glycerol	−	−	+	+	+	+
Lactose	+	V/W	−	−	+	V
D-Mannose	+	V/W	(+)	−	+	+
D-Melezitose	+	V/W	−	−	−	−
Melibiose	+	V/W	−	−	V	V
Starch	+	V/W	+	+	+	+
Sucrose	+	V/W	+	V	+	+
D-Tagatose	−	−	−	−	+	−
D-Xylose	+	V/W	−	−	+	+

*The sporangial swelling shown by *B. galactosidilyticus* is only slight.

hydrogen sulphide production, urease production, the Voges–Proskauer reaction, indole production and gelatin hydrolysis are all negative. Hydrolysis of aesculin is positive. In the API 50 CHB gallery, acid is produced without gas from *N*-acetylglucosamine, L-arabinose, D-glucose, D-fructose, lactose, D-mannose, D-melezitose, D-melibiose, ribose, starch, sucrose, D-trehalose and D-xylose. Acid production from the following carbohydrates is variable: D-cellobiose, galactose, glycogen, inulin, maltose, mannitol, methyl D-glucoside, D-raffinose and salicin. Acid is not produced from adonitol, amygdalin, D-arabinose, D-arabitol, L-arabitol, arbutin, dulcitol, erythritol, D-fucose, L-fucose, *β*-gentiobiose, gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate, glycerol, *myo*-inositol, D-lyxose, methyl D-mannoside, methyl xyloside, rhamnose, sorbitol, L-sorbose, D-tagatose, D-turanose, xylitol or L-xylose. The major cellular fatty acids (mean percentage ± standard deviation of total fatty acids) are: 14:0 (6·24±3·67 %), iso-15:0 (7·43±1·15 %), anteiso-15:0 (27·59±6·80 %), 15:0 (14·14±0·47 %) and 16:0 (32·79±7·72 %). The following fatty acids are present in smaller amounts (mean percentage ± standard deviation of total fatty acids): iso-14:0 (2·24±0·68 %), iso-16:0 (2·82±0·44 %), anteiso-17:0 (3·49±2·22 %) and 17:0 (1·79±0·50 %). Small amounts of iso-17:0 and 18:0 fatty acids may also be present in some strains. The DNA G+C content of the type strain is 39·2 mol%.

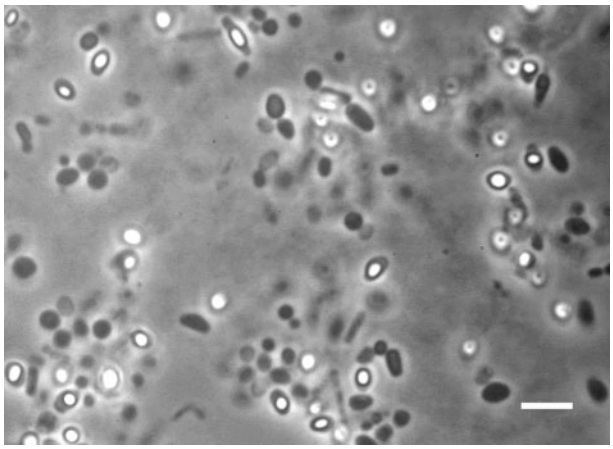

Fig. 2. Photomicrograph of sporangia and vegetative cells of *B. ruris* LMG 22866^T viewed by phase-contrast microscopy. Vegetative cells are rods and coccoid rods, and ellipsoidal spores lie centrally, paracentrally and subterminally within swollen sporangia. Bar, 2 μm.

The type strain is LMG 22866^T (=DSM 17057^T=Logan B3037^T=MB 1669^T). With regard to the variable

characteristics listed above, the type strain is positive for the production of acid without gas from D-cellobiose, galactose, glycogen, inulin (weak reaction), maltose, mannitol (weak reaction), methyl D-glucoside, D-raffinose and salicin (weak reaction).

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