Mycetocola reblochoni sp. nov., isolated from the surface microbial flora of Reblochon cheese

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Four Gram-positive, aerobic, non-sporulating, rod-shaped bacteria isolated from the surface microflora of Reblochon cheese at the late stage of ripening had chemotaxonomic properties characteristic of members of the family Microbacteriaceae. The isolates had virtually identical SDS-PAGE whole-organism protein patterns, shared many chemical and phenotypic characteristics and formed an independent branch in the Microbacteriaceae 16S rRNA gene tree that was most closely related to the type strains of Mycetocola species. The new isolates had chemotaxonomic properties consistent with their classification in the genus Mycetocola but were readily distinguished from recognized members of this taxon based on DNA–DNA relatedness, whole-organism protein and phenotypic data. The combined genotypic and phenotypic data indicate that the isolates should be classified in the genus Mycetocola as members of a novel species, for which the name Mycetocola reblochoni sp. nov. is proposed. The type strain is LMG 22367T (=R-20377T =BRB-1L41T =DSM 18580T).

The largely undefined microbial communities present on the surfaces of smear-ripened cheeses (Eliskases-Lechner & Ginzinger, 1995; Valdes-Stauber et al., 1997) include members of novel bacterial taxa. Corynebacterium casei and Microbacterium gubbeenense, for instance, were isolated from the surface of the Irish farmhouse surface-ripened cheese Gubbeen (Brennan et al., 2001a, b, 2002), and Arthrobacter bergerei and Arthrobacter arilaitensis were isolated from different smear-ripened cheeses (Irlinger et al., 2005). During the course of a study into the biodiversity of the surface flora of Reblochon cheese, a traditional smear-ripened cheese manufactured from cow’s milk in a restricted area of the northern French Alps, a homogeneous group of coryneform strains were isolated and provisionally assigned to the family Microbacteriaceae Park et al. 1995 emend. Rainey et al.1997. Representative members of this group plus the type strains of recognized Mycetocola species were the subject of a polyphasic taxonomic study, which showed that the Reblochon strains represent a novel species of the genus Mycetocola.

At the time of writing, the family Microbacteriaceae comprises 21 genera with validly published names, including the recently described genera Frondicola and Labedella (Lee, 2007). The family includes coccoid, rod-shaped and mycelium-forming strains that have a β-type cell-wall peptidoglycan and unsaturated menaquinones. The constituent genera can be distinguished readily based on chemotaxonomic characteristics, such as the diamino acid of the cell-wall peptidoglycan (γ-aminobutyric acid, 2,4-diaminobutyric acid, lysine and ornithine) and fatty acid, menaquinone and polar lipid components (Evtushenko & Takeuchi, 2006; Sheridan et al., 2003; Manaia et al., 2004; Lee, 2007).

Bacteria were isolated from Reblochon cheese following suspension and homogenization (stomacher; Interscience) of samples of the red smear surface in sodium citrate (2 %,
After tenfold dilutions and plating onto plate count agar (PCA; Biokar Diagnostic) supplemented with 3% (w/v) sodium chloride, cultures were isolated, purified and screened by using rep-PCR. They were then grown for 1–2 days at 30 °C on tryptoncase soy broth (TSB; Difco) containing 1.5% agar (Oxoid). DNA isolation and electrophoresis were performed according to Gevers et al. (2001) and PCR was performed with the primer BOXA1R following Versalovic et al. (1994). Band patterns were analysed by using Pearson’s product–moment correlation coefficient and UPGMA cluster analysis with BioNumerics software (Applied Maths), and were then compared with a database consisting of type and representative strains of the most common species present on red smear cheeses (data not shown). A homogeneous cluster consisting of eight isolates (LMG 22367T, LMG 23020, LMG 23021, LMG 23022, R-20391, R-20398, R-20413 and R-20423) remained unidentified; the taxonomic position of these isolates is the subject of the present study.

The eight strains were isolated from Reblochon cheeses manufactured in September 2002 in three different farmhouses. The cheeses were made from raw milk without the addition of starter surface microflora and were ripened on wooden shelves. All of the isolates were recovered at the late stage of ripening, i.e. after about 15 days. Four representative strains, LMG 22367T, LMG 23020, LMG 23021 and LMG 23022, were selected for further taxonomic study. The reference type strains used in the present study, Mycetocola lacteus LMG 22187T, Mycetocola saprophilus LMG 22188T and Mycetocola tolaasinivorans LMG 22189T, were obtained from the BCCM/LMG Bacteria Collection (http://www.belspo.be/bccm/lgm.htm).

The phylogenetic position of the representative isolates was determined by 16S rRNA gene sequence analysis. Cells of the organisms were grown in brain heart infusion broth (Difco) for 5 days at 30 °C, and the biomass was checked for purity, harvested by centrifugation, washed in NaCl/EDTA buffer (0.1 M EDTA, 0.1 M NaCl, pH 8.0) and stored at −20 °C until required. Genomic DNA, extracted as described by Sambrook & Russell (2001), was used as template for PCR amplification and sequencing following the procedure of Kim et al. (1998). The resultant almost-complete 16S rRNA gene sequences (1541 nt) were aligned manually with corresponding sequences of representatives of the genera classified in the family Microbacteriaceae, retrieved from the RDP and GenBank databases, by using the pairwise alignment option and 16S rRNA secondary structure information held in the PHYDIT program (available at http://plaza.snu.ac.kr/~jchun/phydit/).

Phylogenetic trees were inferred by using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms from the PHYDIT program. Evolutionary distance matrices for the least-squares and neighbour-joining algorithms were generated by using the distance model of Jukes & Cantor (1969). The resultant unrooted tree topologies were evaluated in a bootstrap analysis (Felsenstein, 1985) of the neighbour-joining dataset by using the SEQBOOT and CONSENSE options from the PHYLIP package (Felsenstein, 1993). The resultant phylogenetic analyses showed that the new isolates formed an independent phyletic line among the actinomycete genera classified in the family Microbacteriaceae (Stackebrandt et al., 1997; Lee, 2007) (Fig. 1). The new isolates shared 16S rRNA gene sequence similarities of 99.9–100% and were most closely related to the type strains of Mycetocola species, namely Mycetocola lacteus LMG 22187T, Mycetocola saprophilus LMG 22188T and Mycetocola tolaasinivorans LMG 22189T. The relationship between the new isolates and the Mycetocola strains was supported by all of the tree-making algorithms and by a high level (80%) of bootstrap support in the neighbour-joining analysis. Mycetocola strains were not included in the initial rep-PCR screening as members of this taxon have not been found previously amongst the surface flora of red smear cheeses.

SDS-PAGE of whole-organism proteins, a method routinely used for species delineation, was carried out as a screening method to reveal relationships between the representative isolates and the Mycetocola reference strains. Mycetocola lacteus LMG 22187T, Mycetocola saprophilus LMG 22188T and Mycetocola tolaasinivorans LMG 22189T and the Reblochon isolates were cultivated as indicated for the rep-PCR analyses. Whole-organism protein extracts were prepared and SDS-PAGE was performed as described by Pot et al. (1994). Densitometric analysis, normalization and interpolation of protein profiles, and numerical analyses were carried out by using Gelcompar software package, versions 3.1 and 4.0 (Applied Maths). The Reblochon isolates formed a homogeneous cluster that was sharply separated from the Mycetocola reference strains (Fig. 2).

The DNA G + C content of strain LMG 22367T was determined. Cells were cultivated in TSB for 24 h at 28 °C. DNA was extracted from 0.75–1.25 g wet weight of cells by using the protocol described by Pitcher et al. (1989) with the following modifications: the washed cell pellet was resuspended and lysed in a buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) containing RNase (200 µg ml−1; Sigma), mutanolysin (100 U ml−1; Sigma) and lysozyme (25 mg ml−1; SERVA) for 1 h at 37 °C. Before the addition of GES reagent, proteinase K (200 µg ml−1; Merck) was added to the mixture for 15 min and the DNA was degraded enzymically into nucleosides, as described by Mesbah et al. (1989). The nucleoside mixture was separated by HPLC by using a Waters SymmetryShield C8 column maintained at 37 °C with 0.02 M NH4H2PO4 (pH 4.0) and 1.5% acetonitrile as the solvent. Non-methylated λ-phage DNA (Sigma) was used as the calibration reference. The DNA G + C content of strain LMG 22367T was 70 mol%, a value higher than the 64–65 mol% reported for recognized members of the genus Mycetocola (Tsukamoto et al., 2001).

DNA–DNA hybridizations were performed between strain LMG 22367T and Mycetocola lacteus LMG 22187T,
Mycetocola saprophilus DSM 15178T and Mycetocola tolaisinivorans DSM 15179T by using genomic DNA prepared according to the protocol described above. The microplate method was employed as described by Ezaki et al. (1989) and Goris et al. (1998), by using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with unlabelled single-stranded DNA which was bound non-covalently to microplate wells. Hybridizations were performed at 50°C in a hybridization mixture (2× SSC, 5× Denhardt’s solution, 2.5% dextran sulfate, 50% formamide, 100 μg denatured salmon sperm DNA ml−1, 1250 ng biotinylated probe DNA ml−1). Levels of DNA–DNA relatedness were calculated as means based on at least two independent hybridization experiments; reciprocal reactions were performed and considered as independent hybridization experiments. Levels of DNA–DNA relatedness of 6–8% were found between strain LMG 22367T and the type strains of recognized Mycetocola species, suggesting that the Reblochon isolates represent a novel species.

Peptidoglycan analyses were carried out to establish whether strains LMG 22367T, LMG 23020, LMG 23021 and LMG 23022 represented a novel Mycetocola species or a new genus in the family Microbacteriaceae. Towards this end, the three isolates and the type strains of the three recognized Mycetocola species were grown in brain heart infusion broth (Difco) for 5 days at 28°C, and the resultant biomass was washed twice in distilled water and freeze-dried. Purified peptidoglycan preparations were obtained after disruption of cells by shaking with glass beads and subsequent trypsin digestion, according to the method of Schleifer (1985). The amino acids and peptides in the cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates by using the solvent systems described by Schleifer (1985). The N-terminal amino acids of the interpeptide bridges were detected by dinitrophenylation, as described by Schleifer (1985). The molar ratios of the amino acids were determined by GC and GC-MS of N-heptafluorobutyryl amino acid isobutyl esters (MacKenzie, 1987; Groth et al., 1996). A standard procedure was also used to determine the muramic acid type (Uchida et al., 1999).

Cell-wall hydrolysates of the novel strains contained major amounts of lysine as the diagnostic cell-wall diamino acid; the molar ratios of lysine, alanine, glycine and glutamic acid were estimated to be 1.0:0.7:1.1:1.8 for strain LMG 23020, 1.0:0.6:1.1:1.6 for strain LMG 22367T, 1.0:6.8:1.6:2.0 for strain LMG 23021, 1.0:0.6:1.0:1.6 for Mycetocola lacteus DSM 15177T, 1.0:0.5:0.8:1.7 for Mycetocola saprophilus DSM 15178T and 1.0:0.7:1.0:1.7 for Mycetocola tolaisinivorans DSM 15179T; the unusually high alanine content found for strain LMG 23021 might be due to contaminating protein. The peptidoglycan preparations of the Mycetocola type strains, like those of the new isolates (except strain LMG 23021), had a low alanine content, and more than one glutamic acid residue per lysine residue. Alanine occurred as the N-terminal amino acid of the interpeptide bridge in Mycetocola saprophilus DSM 15178T, Mycetocola tolaisinivorans DSM 15179T and strains LMG 22367T, LMG 23020 and LMG 23021. The peptide patterns of the partially hydrolysed peptidoglycan of all of the novel strains were very similar, consisting of the dipeptides L-Ala–D-Glu, Gly–D-Glu and L-Lys–D-Ala; these data justify the conclusion that these strains have a β-type peptidoglycan.
Strain LMG 22367T and the type strains of the *Mycetocola* species were examined for additional chemotaxonomic markers. Polar lipids and isoprenoid quinones were extracted from freeze-dried biomass following the procedure described by Minnikin et al. (1984). The polar lipids were separated by using a standard TLC technique (Minnikin et al., 1984) and the isoprenoid quinone extracts were filtered and reduced to dryness in a stream of nitrogen. The resultant dried preparations were dissolved in 200 μl propanol and filtered by using a Dyna Gard 0.2-μl syringe filter and 5 μl of the filtered extract was separated by HPLC following Kroppenstedt (1985). Fatty acid methyl esters were prepared and analysed following Klatte et al. (1994) by using the standard Microbial Identification System (MIDI system; http://www.midi-inc.com/).

All of the organisms gave a simple polar lipid pattern that consisted of two phospholipids, a major diphosphatidylglycerol spot, a faint phosphatidylglycerol spot and a prominent glycolipid that was visualized by spraying with anisaldehyde. All of the strains contained unsaturated menaquinones with ten isoprene units as the predominant isoprenologue, although strain LMG 22367T produced smaller proportions of MK-9 and MK-11 compared with the other strains and lacked MK-8 (Table 1). It can be seen from this table that the stains also shared similar fatty acid profiles, with 12-methyl tetradecanoic acid as the major component, although all four strains could be separated based on quantitative differences in major fatty acid components.

The presence of lysine in the cell-wall peptidoglycan of the new isolates readily distinguished them from genera of the family *Microbacteriaceae* that contain other cell-wall diamino acids (Zhang et al., 2007). Additional chemical markers could be used to distinguish the new isolates from genera in the family that contain lysine in the peptidoglycan. Thus, strain LMG 22367T could be distinguished from *Frigoribacterium* and *Microcella* strains based on its major fatty acid and menaquinone components (Kämpfer et al., 2000; Tiago et al., 2005), and strains LMG 22367T, LMG 23020 and LMG 23022 could be distinguished from members of the genera *Microbacterium* and *Okibacterium* as the former contain N-acetylated muramic acid and the latter N-glycolylated muramic acid (Takeuchi & Hatano, 1998; Evtushenko et al., 2002). By contrast, strain LMG 22367T and the type strains of *Mycetocola lacteus*, *Mycetocola saprophilus* and *Mycetocola tolaasinivorans* showed very similar chemotaxonomic profiles based on the present and previous data (Tsukamoto et al., 2001).

**Table 1. Fatty acid and menaquinone profiles (%) of strain LMG 22367T and the type strains of recognized *Mycetocola* species**

<table>
<thead>
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<th>Component</th>
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<td>Fatty acids</td>
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<tr>
<td>anteiso-13 : 0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.09</td>
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<tr>
<td>iso-14 : 0</td>
<td>0.42</td>
<td>0.56</td>
<td>0.59</td>
<td>0.83</td>
</tr>
<tr>
<td>14 : 0</td>
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<td>0.85</td>
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<td>anteiso-15 : 1</td>
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<td>anteiso-15 : 0</td>
<td>44.48</td>
<td>47.88</td>
<td>48.71</td>
<td>50.31</td>
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<td>15 : 0</td>
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<td>1.02</td>
<td>1.09</td>
<td>2.07</td>
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<td>iso-16 : 0</td>
<td>10.23</td>
<td>11.2</td>
<td>11.18</td>
<td>9.73</td>
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<td>16 : 0</td>
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<td>iso-17 : 0</td>
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<td>0.95</td>
<td>0.90</td>
<td>0.22</td>
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<td>17.61</td>
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<td>17 : 0</td>
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<td>0.48</td>
<td>0.46</td>
<td>0.52</td>
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<tr>
<td>18 : 0</td>
<td>–</td>
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<tr>
<td>Menaquinones</td>
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<td>9.8</td>
</tr>
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<td>MK-11</td>
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<td>8.7</td>
<td>9.1</td>
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LMG 22367T could be distinguished from *Frigoribacterium* and *Microcella* strains based on its major fatty acid and menaquinone components (Kämpfer et al., 2000; Tiago et al., 2005), and strains LMG 22367T, LMG 23020 and LMG 23022 could be distinguished from members of the genera *Microbacterium* and *Okibacterium* as the former contain N-acetylated muramic acid and the latter N-glycolylated muramic acid (Takeuchi & Hatano, 1998; Evtushenko et al., 2002). By contrast, strain LMG 22367T and the type strains of *Mycetocola lacteus*, *Mycetocola saprophilus* and *Mycetocola tolaasinivorans* showed very similar chemotaxonomic profiles based on the present and previous data (Tsukamoto et al., 2001).
Table 2. Differential characteristics between strain LMG 22367\textsuperscript{T} and the type strains of recognized Mycetocola species

<table>
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<td>Dextrin</td>
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<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Galactose</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
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<tr>
<td>Mannose</td>
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<td>Raffinose</td>
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<td>Salicin</td>
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<td>Sucrose</td>
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<tr>
<td>α-Galactosidase</td>
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<td>β-Galactosidase</td>
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<td>α-Glucosidase</td>
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<td>Leucine arylamidase</td>
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<td>Trypsin</td>
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<td>Valine arylamidase</td>
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Table 2. cont.

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<td>Degradation of:</td>
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<td>Casein</td>
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<td>DNA</td>
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<td>–</td>
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<td>Tween 60</td>
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<td>Growth at pH 10.0</td>
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<td>–</td>
<td>+</td>
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<tr>
<td>Hydrolysis of urea</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Reduction of nitrate</td>
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<td>+</td>
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<td>Utilization of sole carbon and nitrogen sources</td>
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<td>l-Asparagine</td>
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<td>l-Histidine</td>
<td>–</td>
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The colonial and cellular morphology of the four representative isolates was examined, as was their ability to grow on PY agar (Tsukamoto et al., 2001) over ranges of temperature and pH. Cellular morphology was examined by electron microscopy following growth on PY agar for 3 days at 30 °C and negative staining with phosphotungstic acid, as described by (Tsukamoto et al., 2001). Cells were Gram-positive, non-sporulating, non-motile rods that grew well under aerobic conditions. They formed smooth, circular, convex colonies on brain heart infusion agar (Difco). Growth was observed between 20 and 30 °C, but not at 37 °C, and from pH 7.0 to 10, but not at pH 12. All of these properties are consistent with the assignment of the strains to the genus Mycetocola (Tsukamoto et al., 2001).

The four representative isolates and the type strains of the three recognized Mycetocola species were examined for a range of biochemical and physiological properties as outlined by Kim et al. (1998). In addition, API ZYM tests (bioMérieux) were performed following the manufacturer’s instructions. It is clear from the data in Table 2 that the new isolates share many phenotypic properties, some of which allow them to be distinguished from the Mycetocola type strains.

It can be concluded from the genotypic and phenotypic data presented that the Reblochon cheese isolates represent a novel species of the genus Mycetocola, for which the name Mycetocola reblochoni sp. nov. is proposed.

Description of Mycetocola reblochoni sp. nov.

Mycetocola reblochoni (re.blo.cho’ni. N.L. neut. n. reblochonum Reblochon cheese; N.L. gen. n. reblochoni of a Reblochon cheese, to denote that the first strains were isolated from the surface of Reblochon cheese).

Aerobic, Gram-positive, asporogenous, non-motile, catalase-positive, rod-shaped actinomycetes (0.3 × 1.1–1.5 μm) that form circular, convex, smooth, shiny colonies on PY agar. Growth occurs between 20 and 30 °C, but not at 37 °C, and between pH 7.0 and pH 10, but not at pH 12.0. Does not hydrolyse allantoin. Additional phenotypic properties are given in Table 2. The organism has
chemotaxonomic properties consistent with its classification in the genus *Mycetocola* and forms a distinct phylectic line in the *Mycetocola* 16S rRNA gene tree. The G+C content of the DNA of the type strain is 70.0 mol%.

The type strain, LMG 22367T (=R-20377T = BRB-1L41T = DSM 18580T), was isolated from the surface of Reblochon cheese at the late stage of ripening. Reference strains LMG 23020 (=BRB-4LJ1 = R-20402), LMG 23021 (=BRB-4LB3 = R-203813) and LMG 23022 (=BRB-3LJ5 = R-203891) were isolated from the same source.

**Acknowledgements**

The study was carried out with financial support from the European Union [project QLK-CT-2001-02228: Biodiversity and anti-listerial activity of surface microbial consortia from Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese. Acronym SCM (Smear cheese microflora)].

We are indebted to Bruno Mathieu (Syndicat Interprofessionnel du Reblochon) for helpful discussions, to Jennifer Gregor (Corynebacteriaceae) for excellent technical assistance and to Jean Euzéby (Ecole Nationale Vétérinaire, France) for help with naming the new species.

**References**


