Agrococcus casei sp. nov., isolated from the surfaces of smear-ripened cheeses

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Seven Gram-positive, coryneform bacteria with virtually identical whole-organism protein patterns were isolated from the surface of smear-ripened cheeses. Representatives of these strains were the subject of a polyphasic study designed to establish their taxonomic status. The organisms formed a distinct branch in the Microbacteriaceae 16S rRNA gene tree and were most closely related to members of the genus Agrococcus, sharing sequence similarities of 95.4–98.7 %. The chemotaxonomic profiles of the strains were consistent with their classification in the genus Agrococcus. The combined genotypic and phenotypic data show that the isolates should be classified in the genus Agrococcus as representatives of a novel species. The name Agrococcus casei sp. nov. is proposed for this taxon. Isolate R-17892T (= DSM 18061T = LMG 22410T) is the type strain of Agrococcus casei sp. nov.

The complex consortium of micro-organisms found on the surfaces of smear-ripened cheeses includes major populations of coryneform bacteria (catalase-positive irregular rods and cocci), staphylococci (catalase-positive cocci) and yeasts (Valdés-Stauber et al., 1997; Carnio et al., 1999; Bockelmann & Hoppe-Seyler, 2001). Until recently, cheese coryneform bacteria were assigned to groups based on a few subjectively weighted morphological and staining properties (Piton-Malleret & Gorrieri, 1992; Eliskases-Lechner & Ginzinger, 1995). Such studies have been replaced by polyphasic taxonomic investigations, which show that the coryneform component of smear-ripened cheeses contains members of novel taxa, as exemplified by the isolation and description of Corynebacterium casei and Microbacterium gubbeenense from the surface of Gubbeen cheese (Brennan et al., 2001a, b, 2002) and Arthrobacter arilaitensis and Arthrobacter bergerei from diverse French smear-ripened cheeses (Irlinger et al., 2005). The present study was designed to determine the taxonomic status of a homogeneous group of coryneform bacteria isolated from smear-ripened cheeses and presumptively assigned to the genus Agrococcus.

The genus Agrococcus was proposed by Groth et al. (1996) for two Gram-positive, coryneform bacteria that could be distinguished from members of other genera classified in the family Microbacteriaceae using genotypic and phenotypic criteria. The genus currently contains four species with validly described names: Agrococcus baldri Zlamala et al. 2002; Agrococcus citreus Wieser et al. 1999; Agrococcus jenensis Groth et al. 1996; and Agrococcus lahaulensis Mayilraj et al. 2006. These taxa contain organisms isolated from compost soil and sandstone, a medieval wall painting, air and soil, respectively. Members of these species can be

Abbreviation: DAB, diaminobutyric acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of Agrococcus casei LMG 224107, LMG 22411, LMG 22330 and LMG 22447 are DQ168427, DQ168426, DQ168425 and DQ168424, respectively.
distinguished using a combination of phenotypic markers and together form a distinct branch in the Microbacteriaceae 16S rRNA gene tree (Wieser et al., 1999; Lin et al., 2004).

Bacteria were isolated from Gubbeen, Livarot and Tilsit cheeses following suspension and homogenization (stomacher; Interscience) of samples of the surfaces of the cheeses in sodium citrate (2%, w/v). Tenfold dilutions were plated onto plate count agar (Biokar Diagnostic) supplemented with 3% (w/v) sodium chloride. The isolates were grown for up to 2 days at 30 °C on tryptic soy broth (TSB; Difco) containing 1.5% agar (Oxoid). DNA isolation and electrophoresis were done according to Gevers et al. (2001) and rep-PCR was carried out with the primer set BOXAIR according to Versalovic et al. (1994). Band patterns analysed using the Pearson product moment coefficient and the UPGMA clustering algorithm with Bionumerics software (Applied Maths) were compared with a database composed of type and reference strains representing species that are common on the surfaces of smear-ripened cheeses (data not shown). A homogeneous group comprising seven isolates (LMG 22410^T, LMG 22411, LMG 22330, LMG 22447, R-21294, R-17921 and R-17924) remained unidentified. The taxonomic position of these isolates was the subject of the present study.

Five strains, LMG 22410^T (= DSM 18061^T = R-17982)\(^2\) = BG1: 37: 24), LMG 22411 (= R-17926 = BG1: 37: 9), R-21294 ( = BG4: L10), R-17921 ( = BG1: 23: 67) and R-17924 (BG1: 37: 21), were isolated from Gubbeen cheese, one strain, LMG 22447 ( = R-19148 = BIV-1M43), was from Livarot cheese, and one strain, LMG 22330 ( = R-18889 = B-Y413), was from Tilsit cheese. Four representative strains, namely isolates LMG 22410^T, LMG 22411, LMG 22330 and LMG 22447, were selected for further study. The reference type strains used in the present study were A. balidri LMG 23057^T ( = DSM 14215^T), A. citreus LMG 23056^T ( = DSM 12453^T) and A. jenensis LMG 23058^T ( = DSM 9580^T).

The phylogenetic positions of the four representative cheese isolates were determined by 16S rRNA gene sequence analysis. Biomass from growth in brain-heart infusion broth (BHI; Difco) for 5 days at 30 °C 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 was extracted as described by Sambrook & Russell (2001) and used as a template for PCR amplification and sequencing following the procedure of Kim et al. (1998). The resultant almost complete 16S rRNA gene sequences (1466–1470 nt) were manually aligned with corresponding sequences of representatives of the genera classified in the family Microbacteriaceae, retrieved from the GenBank and RDP databases, using the pairwise alignment option and 16S rRNA secondary structure information held in the program PHYDIT (available at http://plaza.snu.ac.kr/~jchun/phydit/).

Phylogenetic trees were inferred using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms from the PHYLIP suite of programs (Felsenstein, 1993). The distance model of Jukes & Cantor (1969) was used to generate evolutionary distance matrices for the least-squares and neighbour-joining algorithms. The resultant unrooted tree topologies were evaluated in a bootstrap analysis (Felsenstein, 1985) of the neighbour-joining dataset using the CONSENSE and SEQBOOT options from the PHYLIP package. Phylogenetic analyses showed that the four isolates formed a distinct lineage within the evolutionary radiation occupied by genera classified in the family Microbacteriaceae (Fig. 1). This taxon was most closely related to four representatives of the genus Agrococcus, namely A. baldri, A. citreus, A. lahaulensis and A. jenensis, with 16S rRNA gene sequence similarities of 95.4–98.7%.

The four isolates were the subject of chemotaxonomic studies designed to determine whether they had chemical features that were consistent with their classification in the genus Agrococcus. The strains were grown in BHI broth for 5 days at 30 °C and the resultant biomass was washed twice in distilled water and freeze-dried. Established methods were used for the extraction and analysis of fatty acids (Komagata & Suzuki, 1987), muramic acid type (Uchida et al., 1999), polar lipids (Minnikin et al., 1984) and whole-organism sugars (Schaal, 1985). Qualitative analysis of the amino acids was performed using a standard procedure (Schleifer & Kandler, 1972; Schleifer, 1985). The peptidoglycan structures of isolates LMG 22411 and LMG 22330 were determined using the DSMZ Identification Service. To this end, quantitative analyses of amino acids of the isolates in total hydrolysates were performed by GC and GC-MS, as described by Mackenzie (1987).

The isoprenoid quinone composition of strain LMG 22410^T was determined using modifications of established procedures (Collins & Kroppenstedt, 1987; Kroppenstedt, 1985). Isoprenoid quinones were extracted from lyophilized cells (500 mg) with chloroform:methanol (2:1, v/v) for 2 h on a magnetic stirrer. The extract was filtered and the cell biomass was re-extracted for another 2 h. Extracts were combined and evaporated (Zymark evaporator) to dryness at 40 °C; the lipids were dissolved in 200 μl acetone. Isoprenoid quinones were purified by TLC on Kieselgel 60F254 plastic-backed TLC sheets (Merck) using the solvent system hexane:diethyl ether (85:15, v/v). Isoprenoid quinones were visualized under UV light (254 nm) and eluted from the TLC plate with diethyl ether. The eluate was dried, dissolved in 200 μl 2-propanol and filtered through a 2 μm hollow fibre syringe filter (DynaGard). The purified extract (5 μl) was analysed by HPLC on a reversed-phase column (250 × 4.6 mm Lichrospher 100 RP-18 endcapped; Merck) at 40 °C, eluted with acetonitrile:2-propanol (65:35, v/v) at 1 ml min^{−1} using an HP-1090 HPLC pump (Hewlett Packard) and an HP-1090 diode array.
detector with HP-chemstation software. Isoprenoid quinones were identified by their retention times using bacterial quinone extracts of known composition for comparison. A spectrum of 200–400 nm at the apex of the peak was determined using a diode array detector.

The four representative isolates contained diaminobutyric acid (DAB) as the wall diamino acid. In addition, alanine, glycine and glutamic acid were detected in the two isolates that were the subject of additional studies. Cell wall Ala:Gly:DAB:Glu ratios were 2.9:1.3:0.9:1.0 and 1.8:1.0:0.7:1.0 for strains LMG 22411 and LMG 22330, respectively. The partial hydrolysates of these strains included the peptides Gly–Glu and Ala–Ala, but not L-Ala–D-Glu. The qualitative amino acid composition of strains LMG 22411 and LMG 22330 corresponded to peptidoglycan type B2 (Schleifer & Kandler, 1972). The isolates contained N-acetylated muramic acid. They contained diphosphatidylglycerol, phosphatidylglycerol, an unknown glycolipid and unknown polar lipids as major polar lipids and four unknown phospholipids as minor components. Whole-organism sugars galactose, glucose and mannose were present and menaquinones MK-10 (4 % of total), MK-11 (71 % of total) and MK-12 (20 % of total) were the predominant isoprenologues. The fatty acid profiles of the strains were rich in 12-methyltetradecanoic (antei-C15:0; 44.9–56.1 % of total), 14-methylhexadecanoic (anteoi-C17:0; 26.9–30.0 % of total) and 13-methyltetradecanoic (iso-C15:0; 8.2–11.6 % of total) acids, with smaller proportions of 14-methylpentadecanoic acid (iso-C16:0; 2.9–8.0 % of total) and traces of hexadecanoic acid (C16:0).

These chemotaxonomic characteristics are consistent with the classification of the isolates in the genus Agrococcus (Groth et al., 1996; Wieser et al., 1999; Zlamala et al., 2002), but they also suggest that the isolates belong to a separate species.

The DNA G+C content was determined for strain LMG 22410T. DNA was extracted from biomass using the protocol described by Pitcher et al. (1989) modified by lysing the washed cell pellet in 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, then adding protease K (200 μg ml⁻¹; Merck) to the mixture for 15 min before addition of guanidine isothiocyanate. The DNA was digested enzymically to nucleotides (Mesbah et al., 1989). The nucleotides were analysed by HPLC on a Waters SymmetryShield C8 column maintained at 37°C with 0.02 M NH₄H₂PO₄ (pH 4.0) and 1.5 % acetonitrile as eluent; non-methylated φ-phil DNA (Sigma) was used as the calibration reference. The DNA G+C content of strain
Agrococcus casei sp. nov. was isolated and a screening procedure to establish relationships between the commonly used for species delineation, was performed as SDS-PAGE of whole-organism proteins, a procedure package GELCOMPAR versions 3.1 and 4.0 (Applied Maths). and numerical analyses were achieved using the software analysis, normalization and interpolation of protein profiles carried out according to Pot et al. (1994). Densitometric analysis, normalization and interpolation of protein profiles and numerical analyses were achieved using the software package GELCOMPAR versions 3.1 and 4.0 (Applied Maths). The isolates formed a homogeneous group that was distinct from that of the Agrococcus type strains studied (Fig. 2).

The representative isolates and type strains of the three Agrococcus species studied were examined for a range of phenotypic properties using standard procedures: acid production from sugars (Hugh & Leifson, 1953); catalase activity, degradation tests and growth on organic acids as sole carbon sources (Gordon & Mihm, 1957, 1962); aesculin hydrolysis (Williams et al., 1983); allantoin and urea hydrolysis (Cowan, 1974); nitrate reduction and hydrogen sulphide production (Gordon, 1968); degradation of Tweens (Sierra, 1957); and the breakdown of glycerol tributyrate using tributyrin agar (Sigma). All tests were incubated at 30°C for 14 days. API ZYM tests (API bioMérieux) were performed following the manufacturer’s instructions. The isolates shared a range of phenotypic properties that enabled them to be distinguished from the Agrococcus type strains studied (Table 1).

It can be concluded from the genotypic and phenotypic data that the newly isolated strains should be classified as representatives of a novel species in the genus Agrococcus. The name proposed for this taxon is Agrococcus casei sp. nov.

**Description of Agrococcus casei sp. nov.**

Agrococcus casei (ca.se’i. L. gen. n. casei of cheese, named because the organism was isolated from smear-ripened cheeses).

**Table 1.** Characteristics that enable Agrococcus casei sp. nov. to be distinguished from the type strains of Agrococcus species

<table>
<thead>
<tr>
<th>Characteristic</th>
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<td>+†</td>
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</table>

*Data taken from Mayilraj et al. (2006). ND, Not determined. †Data not in line with Wieser et al. (1999).
strains were isolated from the surfaces of smear-ripened cheeses.

The type strain is R-17892t² (DSM 18061T = LMG 22410T), isolated from the surface of Gubbeen cheese. The DNA G+C content of the type strain is 65 mol%.

Acknowledgements

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References


