***Corynebacterium mooreparkense* sp. nov. and *Corynebacterium casei* sp. nov., isolated from the surface of a smear-ripened cheese**

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Ten isolates each of two different bacterial species isolated from the surface of a smear-ripened cheese were found to exhibit many characteristics of the genus *Corynebacterium*. The isolates were Gram-positive, catalase-positive, non-spore-forming rods that did not undergo a rod/coccus transformation when grown on complex media. Chemotaxonomic investigation revealed that the strains belonged unambiguously to the genus *Corynebacterium*. Their cell walls contained arabinose, galactose and short-chain mycolic acids (C₂₂ to C₃₆) and their peptidoglycan contained meso-diaminopimelic acid. The G+C content of the DNA was 51–60 mol%. MK-9 (H₂) was the principal menaquinone. The 16S rDNA sequences of four isolates of each bacterium were determined and aligned with those of other members of the coryneform group. Phylogenetic analysis showed that the strains represented two new sublines within the genus *Corynebacterium*; *Corynebacterium variabile* and *Corynebacterium ammoniagenes* were their nearest known phylogenetic neighbours. *Corynebacterium variabile* and *Corynebacterium ammoniagenes* showed the highest levels of sequence homology with the isolates; however, DNA–DNA hybridization studies indicated that the *Corynebacterium* strains isolated from the cheese smear did not belong to either *Corynebacterium variabile* or *Corynebacterium ammoniagenes* (26 and 46% chromosomal similarity, respectively). On the basis of the phylogenetic and phenotypic distinctiveness of the unknown isolates, it is proposed that the bacteria be classified as two new *Corynebacterium* species, for which the names *Corynebacterium mooreparkense* sp. nov. and *Corynebacterium casei* sp. nov. are proposed. Type strains have been deposited in culture collections as *Corynebacterium mooreparkense* LMG S-19265^T (= NCIMB 30131^T) and *Corynebacterium casei* LMG S-19264^T (= NCIMB 30130^T).

Keywords: *Corynebacterium mooreparkense* sp. nov., *Corynebacterium casei* sp. nov., cheese microflora

INTRODUCTION

Bacterially surface-ripened cheeses (e.g. Tilsit and Limburger) are characterized by the development of a viscous, red/orange smear, comprising yeasts, bacteria

and moulds, on the surface of the cheese during ripening, which makes a major contribution to the flavour of the cheese (Reps, 1993). Yeasts, which dominate at the beginning of the ripening, metabolize the lactic acid produced by the lactic starters to CO₂ and H₂O, increase the pH from 5.0 to > 6.0 and produce growth factors (e.g. pantothenic acid) that permit the growth and development of a Gram-positive, catalase-positive, salt-tolerant bacterial

The GenBank accession numbers for the 16S rRNA gene sequences of strains LMG S-19264^T and LMG S-19265^T are AF267148 and AF267152, respectively.

Table 1. Isolates studied in this work

Abbreviations: DPC, Dairy Products Research Centre, Teagasc, Moorepark, Fermoy, County Cork, Ireland; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; LMG, Laboratorium voor Microbiologie, Rijksuniversiteit, Gent, Belgium; PHLS, Public Health Laboratory Services, Newcastle upon Tyne, UK; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK; NCFB, National Collection of Food Bacteria, Aberdeen, UK.

Taxon	Strain(s)
<i>Corynebacterium mooreparkense</i> sp. nov.	DPC 5305, 5306, 5307, 5308, 5309, 5312, 5313, 5314, 5315, LMG S-19265 ^T (= DPC 5310 ^T)
<i>Corynebacterium casei</i> sp. nov.	DPC 5293, 5294, 5297, 5299, 5300, 5301, 5302, 5303, LMG S-19264 ^T (= DPC 5298 ^T)
<i>Corynebacterium variabile</i>	NCDO 2102, NCFB 2097 ^T , NCFB 2099
<i>Corynebacterium ammoniagenes</i>	DSM 20306 ^T
<i>Brevibacterium linens</i>	DPC 4892 ^T /NCIMB 9909 ^T
<i>Listeria innocua</i>	LMG 11387 ^T /DPC 5072 ^T , LMG 13568/DPC 5073
<i>Staphylococcus aureus</i>	PHLS

microflora composed primarily of members of the *Micrococcaceae* and coryneform bacteria (El-Erian, 1969; Lenoir, 1984). Early studies reported that *Brevibacterium linens* represents 30 % of the bacterial microflora of smear-ripened cheeses (Brandl, 1980; Busse, 1989). Recently, the importance of other bacteria to the ripening process has been emphasized, e.g. *Corynebacterium ammoniagenes*, *Corynebacterium insidiosum*, *Corynebacterium variabile*, *Curtobacterium betae* and *Curtobacterium poinsettiae* have been identified by Valdes-Stauber *et al.* (1997) and Eliskases-Lechner & Ginzing (1995) in different smear cheeses.

The genus *Corynebacterium* was created by Lehmann & Neumann (1896) for the 'Diphtherie Bacillus'. The genus is restricted to Gram-positive, catalase-positive, non-spore-forming, non-acid-fast, aerobic or facultatively anaerobic, slightly curved club-shaped rods containing meso-diaminopimelic acid in the cell-wall peptidoglycan as well as an arabinogalactan polymer and short-chain mycolic acids (C₂₂ to C₃₆). The cellular fatty acids present are predominantly straight-chain saturated or monosaturated. The G+C content is 51–65 mol % (Collins & Cummins, 1986).

Only in recent years has the heterogeneity of the genus *Corynebacterium* been recognized, mainly because of the application of sophisticated molecular and chemotaxonomic techniques. The genus *Corynebacterium* can be divided into medical and non-medical species. The non-medical *Corynebacterium* species have been isolated from a variety of habitats, including soil (*Corynebacterium glutamicum*), faeces (*Corynebacterium ammoniagenes*, *Corynebacterium glutamicum*), plant material (*Corynebacterium variabile*, *Corynebacterium glutamicum* and *Corynebacterium callunae*) and dairy products (*Corynebacterium flavesens*, *Corynebacterium ammoniagenes* and *Corynebacterium variabile*). *Corynebacterium variabile* now accommodates *Arthrobacter variabilis* and

Caseobacter polymorphus (Collins, 1987a; Collins *et al.*, 1989; Liebl, 1992). In a numerical taxonomy study of coryneform bacteria isolated from a smear-ripened cheese, Seiler (1986) found that 20 % of white- and yellow-coloured coryneform-like bacteria were *Brevibacterium ammoniagenes*, which was subsequently reclassified as *Corynebacterium ammoniagenes* (Collins, 1987b).

This paper reports two novel *Corynebacterium* species isolated from the surface of an Irish farmhouse smear-ripened cheese. By using a polyphasic approach to taxonomy, including pulsed-field gel electrophoresis (PFGE), cell wall and phenotypic analyses, we have been able to describe 10 isolates of each previously unknown species of the genus *Corynebacterium*; on the basis of the results of this study, two new species, *Corynebacterium mooreparkense* sp. nov. and *Corynebacterium casei* sp. nov., are proposed.

METHODS

Strains. The isolates included in this study were taken from the surface of an Irish farmhouse smear-ripened cheese; those that were examined are listed in Table 1. Strains were cultivated for 5 d at 30 °C on Funke medium containing 1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) malt extract, 0.5 % (w/v) Casamino acids, 1.5 % (w/v) bacteriological agar No. 1 and 1 % (v/v) Tween 80 (Funke *et al.*, 1993). *Listeria innocua* LMG 11387^T and LMG 13568 and a strongly haemolytic *Staphylococcus aureus* strain received from the Public Health Laboratory Services, Newcastle upon Tyne, UK, were used for analysis of the Christie–Atkins–Munch–Petersen reaction. *B. linens* (NCIMB 9909^T) was used as the positive control for detecting methanethiol production. Biomass for chemotaxonomic analysis was obtained by growing the strains aerobically at 30 °C for 3 d in Funke broth on an orbital shaker at approximately 150 r.p.m. and was harvested by centrifugation. Biomass for molecular systematics studies was scraped from the surface of Funke agar plates, resuspended in 200 µl extraction solution [2 % (w/v) Triton X-100, 1 % SDS, 100 mM NaCl,

10 mM EDTA, 10 mM Tris/HCl, pH 8.0; autoclaved at 121 °C for 15 min] and stored at -20 °C until required.

Morphological, physiological and biochemical characterization. The rod/coccus transformation was studied by the procedure of Cure & Keddie (1973). Cell morphology was determined by phase-contrast microscopy following growth on Funke agar and motility was determined by the hanging drop method. Catalase activity was determined by the presence of bubbles upon application of a 3% (v/v) H₂O₂ solution to a colony. The ability to grow anaerobically was examined. Whether the strains respired or fermented glucose was determined by using a modification of the medium of Hugh & Leifson (1953) containing bacteriological agar No. 1 (0.2%, w/v), tryptone (1.0%, w/v), yeast extract (0.1%, w/v), glucose (1.0%, w/v) and bromthymol blue (0.004%, w/v) at pH 7.2, dispensed into 10 ml aliquots and then autoclaved at 121 °C for 15 min. Duplicate test tubes were inoculated by stabbing; one test tube was sealed with paraffin and both were then incubated aerobically. The methods used to determine the biochemical profiles were as described by Funke *et al.* (1993). Commercially available API CORYNE and API ZYM kits were used to determine phenotypic and enzymic activities (bioMérieux); the results were read after 24 h incubation, except in the case of acid production, which was determined after 72 h. Fermentation profiles were determined using the API 50CH system with 50CHE medium (bioMérieux) and strips were read after 24, 48, 72 and 240 h incubation. The API 20E system (bioMérieux) was used to determine biochemical profiles and the final readings were performed after 48 h. Ability to hydrolyse tyrosine was determined on the medium described by Nash & Krenz (1991). Unless otherwise stated, all biochemical tests were performed at 30 °C.

Detection of methanethiol production. The method used to detect methanethiol production was that described by Pitcher & Malnick (1984), using milk/5,5'-dithiobis-2-nitrobenzoic acid (DTNB) medium. Skimmed milk (30 g) was diluted in 500 ml distilled water. A separate solution (500 ml) contained 2% (w/v) bacteriological agar No. 1, 0.2% (w/v) L-methionine, 0.2% (w/v) K₂HPO₄ and 0.24% (w/v) DTNB (Sigma). Both solutions were autoclaved at 121 °C for 10 min, after which both solutions were tempered to 50 °C. The solutions were mixed and 2 ml volumes were used to form slopes in Bixoux bottles. Duplicate slopes were inoculated with loopfuls of culture from a Funke agar plate. The bottles were capped tightly and incubated at 30 °C for 10 d. The presence of a bright yellow colour due to the formation of an aryl mercaptan was considered a positive result.

Chemotaxonomic methods. The isomer of diaminopimelic acid in the cell wall of all of the strains was determined by TLC of whole-organism hydrolysates on cellulose acetate sheets (Staneck & Roberts, 1974). Menaquinones were extracted with chloroform/methanol (2:1) from freeze-dried biomass and were purified by preparative TLC (Collins, 1994). The purified menaquinones were resuspended in 50 µl *n*-hexane and 10 µl of each sample was then injected onto a Spherisorb ODS column (250 × 4.6 mm i.d., 5 µm particle size; Jones Chromatography) fitted to a Pharmacia LKB HPLC instrument. Acetonitrile/2-propanol (75:25) was used as the mobile phase, and the absorbance of the eluate was monitored at 254 nm. Retention times and peak areas were determined using an integrator (HP3396A; Hewlett Packard). Polar lipids, extracted from freeze-dried biomass of all the isolates, were examined by two-dimensional TLC and were identified using the procedures of Minnikin *et al.*

(1984). Samples for sugar determinations were prepared from freeze-dried biomass and were separated by TLC (Staneck & Roberts, 1974). An alkali procedure was used to prepare mycolic acid methyl esters for two-dimensional TLC (Minnikin, 1993). Mycolic acid methyl esters were purified from preparative TLC plates, visualized by spraying with 0.01% (w/v) ethanolic Rhodamine 6G and examined under UV light (366 nm). Silica gel, containing mycolic acids, scraped from the TLC plates was extracted twice with diethyl ether (3 ml for each extraction) and the extracts were evaporated to dryness in a fume hood. Chain length was determined by electron-impact mass spectrometry with an ionization energy of 70 eV.

Genomic DNA extraction. All isolates were cultivated on Funke agar for 5 d at 30 °C. Biomass was scraped from the plates and lysed by resuspension in 200 µl extraction solution containing 20 µl proteinase K (10 mg ml⁻¹), 20 µl RNase A (10 mg ml⁻¹) and 20 µl lysozyme (50 mg ml⁻¹) and incubation for 1 h at 37 °C (Sambrook *et al.*, 1989). The cleared lysates were extracted by grinding with acid-washed glass beads (425–600 µm; Sigma) and phenol/chloroform/isoamyl alcohol (25:24:1), vortexed for 4 min and then centrifuged at 14000 r.p.m. for 5 min in a microcentrifuge. The clear, upper phase, which contained the DNA, was transferred to a sterile microcentrifuge tube and washed twice with phenol/chloroform/isoamyl alcohol and then with chloroform/isoamyl alcohol (24:1) to remove traces of phenol that might inhibit subsequent molecular procedures. The DNA was then precipitated with absolute ethanol at -20 °C, washed twice with 70% ethanol, dried at 37 °C for 45 min and dissolved in sterile water and the final concentration was estimated by agarose gel electrophoresis and ethidium bromide staining (Sambrook *et al.*, 1989).

DNA base composition. The base composition of the genomic DNA of all of the strains was determined by reverse-phase HPLC according to the method of Tamaoka (1994). Analysis was performed on a Supelcosil LC-185 column (4.6 × 15 cm, 5 µm particle size; Supelco) with 0.5 M KH₂PO₄ and methanol (95:5) as the mobile phase; detection was performed at 245 nm. Molar G+C ratios were calculated by the method of Mesbah *et al.* (1989).

Sequencing of genes encoding 16S rRNA. A large fragment (~1500 bp) of the 16S rRNA gene was amplified from DNA by a PCR with universal primers for use in phylogenetic studies. The first oligonucleotide, CO1, for the 5'-end of the 16S rRNA gene, had the sequence 5'-AGTTTGA-TCTGGCTCAG-3', and the second oligonucleotide, CO2, for the 3'-end, had the sequence 5'-TACCTTGTTACGAC-CTT-3' (Beresford & Condon, 1991). Each reaction mixture (50 µl) contained 5 µl 10 × NH₄ buffer, 1.5 µl 50 mM MgCl₂, 1 µl dNTP master mix containing 12.5 µmol of each dNTP, 2.5 µl 20 µM stock of each primer, 0.5 µl chromosomal DNA, 0.25 µl (5 U µl⁻¹) *Taq* polymerase (Bioline) and sterile water to 50 µl. A rapid cyclor PCR instrument (Bio/Gene) was used for thermal amplification and was programmed as follows: 35 cycles of 95 °C for 45 s, 45 °C for 30 s and 74 °C for 40 s. Amplified 16S rRNA was purified from 1.5% agarose gel by using a Nucleo-spin gene clean kit (Bio/Gene) and sequenced using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). Nucleotide sequences were obtained automatically using a 373A DNA sequencer (Applied Biosystems) and the protocol and software recommended by the manufacturer. The following forward and reverse primers (*Escherichia coli* numbering system; Brosius *et al.*, 1978) were used at a concentration of 16 pM: universal

Table 2. Percentage 16S rRNA sequence similarities between *Corynebacterium mooreparkense* sp. nov. (LMG S-19265^T) and *Corynebacterium casei* sp. nov. (LMG S-19264^T) and some closely related speciesC., *Corynebacterium*. Accession numbers are given in parentheses.

Species	Similarity (%) to:	
	<i>C. mooreparkense</i>	<i>C. casei</i>
<i>C. accolens</i> CCUG 28779 (X80500)	93	93
' <i>C. acetoacidophilum</i> ' CIP 103773 (X84240)	92	94
<i>C. afermentans</i> CIP 103499 ^T (X82054)	93	92
<i>C. ammoniagenes</i> CIP 101283 ^T (X82056)	93	98
<i>C. amycolatum</i> CIP 103452 ^T (X82057)	94	92
<i>C. argentoratense</i> CIP 104296 ^T (X83955)	93	92
' <i>C. asperum</i> ' CIP 52:13 (X82050)	93	93
<i>C. auris</i> DMMZ 328 ^T (X81873)	92	92
<i>C. bovis</i> NCTC 3224 (X84444)	95	93
<i>C. callunae</i> NCIMB 10338 (X84251)	92	94
<i>C. casei</i> LMG S-19264 ^T (AF267152)	93	100
<i>C. confusum</i> DMMZ 3259 (Y15887)	92	93
<i>C. coyleae</i> DMMZ 214 ^T (X96497)	92	93
<i>C. cystitidis</i> ATCC 29593 ^T (D37914)	90	91
<i>C. diphtheriae</i> NCTC 11397 (X84248)	95	93
<i>C. durum</i> IBS G15036 ^T (Z97069)	90	91
' <i>C. fastidiosum</i> ' CIP 103808 (X84245)	92	92
<i>C. flavescens</i> NCDO 1320 (X84441)	93	95
' <i>C. genitalium</i> ' NCTC 11859 (X84253)	92	91
<i>C. glucuronolyticum</i> DSM 44120 ^T (X86688)	88	92
<i>C. glutamicum</i> CIP 10025 (X84257)	92	94
<i>C. jeikeium</i> NCTC 11913 ^T (X84250)	94	92
<i>C. kutscheri</i> CIP 103423 (X81871)	93	92
<i>C. macginleyi</i> ATCC 104099 ^T (X80499)	94	92
<i>C. matruchotii</i> CIP 8182 ^T (X82065)	91	91
<i>C. minutissimum</i> NCTC 10288 ^T (X84678)	93	92
<i>C. mooreparkense</i> LMG S-19265 ^T (AF267148)	100	92
<i>C. mucifaciens</i> DMMZ 2278 ^T (Y11200)	92	92
<i>C. mycetoides</i> NCTC 9864 ^T (X84241)	91	91
<i>C. pilosum</i> NCTC 11862 (X84246)	93	95
<i>C. propinquum</i> CIP 103792 ^T (X81917)	91	91
<i>C. pseudodiphtheriticum</i> CIP 103420 ^T (X81918)	91	91
<i>C. pseudotuberculosis</i> NCTC 3450 (X84255)	94	92
<i>C. renale</i> CIP 103421 (X84249)	96	92
' <i>C. segmentosum</i> ' NCTC 934 (X84437)	93	92
<i>C. seminale</i> CIP 104297 ^T (X84375)	90	91
<i>C. striatum</i> NCTC 764 (X84442)	94	93
<i>C. terpenotabidum</i> IFO 14764 ^T (AB004730)	97	92
<i>C. thomssenii</i> DSM 44276 ^T (AF010474)	93	95
' <i>C. tuberculoearicum</i> ' ATCC 35692 (X84247)	92	92
<i>C. ulcerans</i> NCTC 7910 ^T (X84256)	95	91
<i>C. urealyticum</i> DSM 7109 (X84439)	93	92
<i>C. variabile</i> NCDO 2097 ^T (X53185)	98	92
<i>C. vitaeruminis</i> NCTC 20294 (X84680)	93	92
<i>C. xerosis</i> DSM 20743 (X84446)	94	93
<i>Turicella otitidis</i> DSM 8821 ^T (X73976)	90	90

M13 forward–reverse primers, p127f (5'-AGAGTTTGAT-CMTGGTGAG-3', positions 8–27), p357f (5'-CTACGG-GRSGCAG-3', 342–357), p693f (5'-AATTCCTGGTGTA-

GCGGT-3', 675–692), p926f (5'-AAACTCAAAGGAAT-TGACGG-3', 907–926) and p1208f (5'-GACGTCAAGT-CATCATGCC-3', 1190–1208) (Lane, 1991).

Phylogenetic analysis. The 16S rRNA sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) with *Corynebacterium* nucleotide sequences derived from the Ribosomal Database Project (Maidak *et al.*, 1996) or from the EMBL/GenBank databases (Table 2). Evolutionary distance matrices were generated by using the neighbour-joining method of Saitou & Nei (1987). A phylogenetic tree was constructed according to the neighbour-joining method, using the program NEIGHBOR (Felsenstein, 1993). The stability of the grouping was estimated by bootstrap analysis (1000 replications) using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE. The PHYLIP package (Felsenstein, 1993) was used to generate a tree and was viewed using TREEVIEW software (Page, 1996). The root of the unrooted tree based on the neighbour-joining method was estimated by using the following five outgroups: *Dietzia maris* (X81920), *Gordonia amarae* (X80635), *Nocardia asteroides* (X53205), *Rhodococcus erythropolis* (X76691) and *Tsukamurella paurometabola* (X53200).

DNA–DNA hybridization. DNA–DNA hybridization studies were carried out at Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, by the thermal denaturation method. DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980), using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program of Jahnke (1992).

PFGE analysis. Biomass was scraped from Funke agar plates, washed twice in 1 M NaCl, 10 mM Tris/HCl (pH 7.6), and resuspended in 200 µl of the same solution. The resulting suspensions were then heated to 37 °C for 15 min, mixed with an equal volume of 2% low-melting-point agarose (Bio-Rad) in 0.125 M EDTA (pH 7.6) and left to solidify in moulds (Bio-Rad). Cells were lysed *in situ* in agarose blocks by gentle shaking for 18 h at 37 °C in EC buffer (1 M NaCl; 6 mM Tris/HCl; 100 mM EDTA; 1%, w/v, Sarkosyl; pH 7.6) containing 10 mg ml⁻¹ lysozyme. Blocks were shaken in proteinase K solution (0.5 mg ml⁻¹ in 0.5 M EDTA, 1% Sarkosyl, pH 8.0) for 24 h at 55 °C and twice in 1 mM PMSF in TE 10/1 (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and then stored in TE 10/100 at 4 °C until required. Slices (1–2 mm) were cut from the agarose blocks with sterile coverslips and washed three times at room temperature with gentle shaking in TE 10/0.1. Slices were washed once at 4 °C for 30 min with restriction buffer (New England Biolabs) and then incubated for 4 h with 20 U *SpeI* (New England Biolabs) in a total volume of 100 µl. Following digestion, slices were loaded into wells of a 1% PFGE-grade agarose gel (Bio-Rad) and the gel was run in 0.5 × Tris/borate buffer, using a CHEF-DR III PFGE apparatus and a cooling module (Bio-Rad) at 1 V (6 V cm⁻¹) for 16 h at 14 °C, with the pulse ramped from 1 to 20 s. Low-range PFGE markers (New England Biolabs) were used to determine the sizes of fragments. Gels were stained with ethidium bromide (0.5 µg ml⁻¹) in water, destained in water and then photographed using Polaroid type 667 film.

RESULTS AND DISCUSSION

The 10 isolates of *Corynebacterium mooreparkense* sp. nov. and of *Corynebacterium casei* sp. nov. were Gram-

positive, catalase-positive, non-spore-forming, non-motile, irregularly shaped rods, 1–3 µm in length; they were not acid-fast. They did not undergo a rod/coccus transformation. The cell walls of all 10 *Corynebacterium mooreparkense* and *Corynebacterium casei* isolates were found to be type IV (Schleifer & Kandler, 1972), containing *meso*-diaminopimelic acid, arabinose, galactose and short-chain mycolic acids (C₂₂ to C₃₆). The menaquinones in the cell wall were MK-9 (H₂). Analysis of the polar lipids from the cell wall showed the presence of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylglycerol mannosides. The G+C content of DNA of the strains was found to be 60 mol% for *Corynebacterium mooreparkense* sp. nov. and 51 mol% for *Corynebacterium casei* sp. nov. These are features typical of the genus *Corynebacterium* (Collins & Cummins, 1986).

Corynebacterium mooreparkense sp. nov. isolates grown on Funke agar plates at 30 °C for 5 d formed cream-coloured, circular, matt, entire, low-concave colonies, approximately 1 mm in diameter. All of the isolates were aerobic and oxidase- and ONPG-negative and produced acid aerobically. Eight of the 10 isolates (DPC 5305, 5306, 5307, 5308, 5309, 5314 and 5415 and LMG S-19265^T) were positive for pyrazinamidase activity, but only one isolate (DPC 5312) exhibited pyrrolidonyl arylamidase activity. One of the 10 isolates (DPC 5313) produced acid from glycogen. All of the isolates produced methanethiol from methionine. Biochemical patterns obtained with the API CORYNE strips gave the profile 2003314, which could not be identified but allowed the strains to be placed in the genus *Corynebacterium*.

Strains of *Corynebacterium casei* sp. nov. grown on Funke agar at 30 °C for 5 d formed cream, circular, matt, entire, low-concave colonies, approximately 1 mm in diameter. All strains were facultative anaerobes and produced acid anaerobically from glucose. They were oxidase- and ONPG-negative. One strain (DPC 5303) did not exhibit valine arylamidase activity. Six of the 10 isolates (DPC 5293, 5294, 5297, 5302 and 5303 and LMG S-19264^T) were capable of producing naphthol-AS-β1-phosphohydrolase. One strain (DPC 5301) had α-fucosidase activity and three strains (LMG S-19264^T, DPC 5297 and DPC 5300) were positive for pyrrolidonyl arylamidase activity. Only three strains (DPC 5293, 5301 and 5302) were capable of producing methanethiol from methionine. Aesculin and tyrosine were not hydrolysed. Individual strains were capable of producing acid from trehalose (DPC 5293) or from 5-ketogluconate (DPC 5294). Biochemical patterns for the cheese isolates obtained with the API CORYNE strips gave a profile corresponding to 3100304, confirming that these isolates belonged to the genus *Corynebacterium*.

PFGE of *SpeI* restriction digests of four strains of *Corynebacterium mooreparkense* sp. nov. (DPC 5305,

Fig. 1. PFGE of *SpeI* restriction digests of *Corynebacterium mooreparkense* sp. nov. strains LMG S-19265^T, DPC 5307, DPC 5315 and DPC 5305 (lanes 2–5) and *Corynebacterium casei* sp. nov. strains LMG S-19264^T, DPC 5300, DPC 5294 and DPC 5302 (lanes 6–9). Lane 1, low-range PFGE molecular size markers.

5307 and 5315 and LMG S-19265^T) showed that they shared the same profile (Fig. 1), implying that these isolates are identical clones. The 16S rRNA sequences of *Corynebacterium casei* sp. nov. DPC 5294, 5300, 5302 and LMG S-19264^T showed only 99% similarity; analysis of *SpeI* restriction digests of these four strains (Fig. 1) showed that they also had identical profiles, implying that they are, in fact, identical clones.

Gene sequences were examined to establish the phylogenetic relationships of the isolates. Almost-complete 16S rRNA gene sequences (> 1400 nucleotides) of four strains of each of the two new species were determined. Comparative sequence analysis of isolates revealed 100% similarity between the four *Corynebacterium mooreparkense* sp. nov. isolates (DPC 5305, 5307 and 5315 and LMG S-19265^T) and 99% similarity between the four *Corynebacterium casei* sp. nov. isolates (DPC 5294, 5300 and 5302 and LMG S-19264^T), demonstrating their homogeneity. Sequence searches using the FASTA program with the EMBL/GenBank and Ribosomal Database Project databases (Maidak *et al.*, 1996) revealed that the sequences of the new species are closely related to species of the genus *Corynebacterium*; 16S rRNA sequence similarities of > 90% were found (Table 2). A tree showing the phylogenetic relationships between the unidentified bacteria and members of the genus *Corynebacterium* is shown in Fig. 2. It was evident from the 16S rRNA data that the closest phylogenetic relatives of *Corynebacterium mooreparkense* sp. nov. and *Corynebacterium casei* sp. nov. were *Corynebacterium variabile* and *Corynebacterium ammoniagenes*, respectively. A sequence divergence of approximately 2% was found between *Corynebacterium mooreparkense* and *Corynebacterium variabile*, and between *Cory-*

nebacterium casei and *Corynebacterium ammoniagenes*, corresponding to 30 nucleotide differences in each case, indicating that the strains isolated from the cheese surface were phylogenetically distinct from their nearest neighbour. In view of the high levels of 16S rRNA sequence relatedness between the two unknown isolates and some *Corynebacterium* species, chromosomal DNA–DNA hybridization experiments were performed to establish whether the cheese isolates represented distinct species. The isolates designated as *Corynebacterium mooreparkense* sp. nov. and *Corynebacterium casei* sp. nov. displayed low levels of reassociation with *Corynebacterium variabile* (26%) and *Corynebacterium ammoniagenes* (46%) (their nearest phylogenetic neighbours), respectively.

On the basis of the results of the physiological, chemical and molecular genetic analyses, it was concluded that the *Corynebacterium* isolates described above should be classified as two new species of the genus *Corynebacterium*, for which we propose the names *Corynebacterium mooreparkense* sp. nov. and *Corynebacterium casei* sp. nov. Features that distinguish *Corynebacterium mooreparkense* sp. nov. from *Corynebacterium variabile* include the ability to hydrolyse urea and aesculin and their distinctive carbon fermentation patterns (Table 3). Those characteristics that allow discrimination between *Corynebacterium casei* sp. nov. and *Corynebacterium ammoniagenes* are the absence of urease and gelatinase activities and the ability to produce acid from glucose but not from maltose or sucrose (Table 3). *Corynebacterium mooreparkense* sp. nov. and *Corynebacterium variabile* can be distinguished from *Corynebacterium casei* sp. nov. and *Corynebacterium ammoniagenes* by their inability to grow anaerobically.

Description of *Corynebacterium mooreparkense* sp. nov.

Corynebacterium mooreparkense (moore.par.ken'se. N. L. gen. n. *mooreparkense* of Moorepark, named because the organism was isolated at the Dairy Products Research Centre, Moorepark, Fermoy, County Cork, Ireland).

The description of the biochemical and morphological characteristics was based on results for 10 isolates. Cells are aerobic, Gram-positive, catalase-positive and oxidase-negative, non-motile, non-spore-forming, partially acid-fast, irregularly shaped rods, 1–3 µm in length. Colonies on Funke media are cream, circular, matt, entire and low-concave, 1 mm in diameter. Acid is produced from glucose, ribose, mannose and fructose but not from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl β-xyloside, galactose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, salicin,

Fig. 2. Unrooted tree showing the phylogenetic relationships of *Corynebacterium mooreparkense* sp. nov., *Corynebacterium casei* sp. nov. and other members of the genus *Corynebacterium*. The tree, constructed by the neighbour-joining method, was based on a comparison of approximately 1400 nucleotides. Bootstrap values, expressed as percentages of 1000 replications, are given at the branch points.

cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Urea and gelatin are

hydrolysed. Nitrate is not reduced. Hydrolysis of aesculin, tyrosine and ONPG, and the Christie–Atkins–Munch–Petersen reaction, are negative. Esterase, esterase/lipase, lipase, leucine arylamidase and tryptophan deaminase activities are present in the

Table 3. Characteristics used to differentiate *Corynebacterium mooreparkense* sp. nov. and *Corynebacterium casei* sp. nov. from their nearest phylogenetic neighbours

The number of strains analysed of each species is shown. Test results are scored as: +, positive; –, negative. *C.*, *Corynebacterium*. All taxa were negative for production of acid from maltose and sucrose and for the Christie–Atkins–Munch–Petersen reaction.

Characteristic or test	<i>C. mooreparkense</i> sp. nov. (n = 10)	<i>C. variabile</i> (n = 3)	<i>C. casei</i> sp. nov. (n = 10)	<i>C. ammoniagenes</i> (n = 1)
Anaerobic growth	–	–	+	+
Nitrate reductase	–	+	+	+
Urease	+	+	–	+
Gelatinase	+	–	–	+
Aesculin hydrolysis	+	+	–	–
Pyrazinamidase	+	+	–	–
Alkaline phosphatase	–	–	+	+
Production of acid from glucose*	+	–	+	–

* Acid production data were obtained by using the API 50CH and API 20E systems.

organism. The isolates do not possess valine arylamidase, chymotrypsin, α -galactosidase, α -mannosidase, β -galactosidase, pyrazinamidase, alkaline phosphatase, cystine arylamidase, acid phosphatase, trypsin, naphthol-AS- β 1-phosphohydrolase, *N*-acetylglucosaminidase or α -fucosidase activity. All strains produce acetoin from pyruvate and methanethiol from methionine. The cell wall of strains of *Corynebacterium mooreparkense* sp. nov. was found to be type IV, containing *meso*-diaminopimelic acid, arabinose, galactose and short-chain mycolic acids (C_{22} to C_{36}). The menaquinones in the cell wall are of the MK-9 (H_2) type. Analysis of polar lipid of the cell wall revealed the presence of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylglycerol mannosides. The G + C content of the isolates is 60 mol%. The type strain is LMG S-19265^T (= DPC 5310^T).

Description of *Corynebacterium casei* sp. nov.

Corynebacterium casei (ca'se.i. L. gen. n. *casei* of cheese, named because the organism was isolated from cheese).

The description of the biochemical and morphological characteristics was based on results from 10 isolates. Cells are Gram-positive, facultatively anaerobic, irregularly shaped rods, 1–3 μ m in length; they are catalase-positive, oxidase-negative, non-motile, non-spore-forming and partially acid-fast. Colonies on Funke media are cream, circular, matt, entire, low-concave and 1 mm in diameter. Acid is produced from glucose, ribose, mannose and fructose but not from glycogen, glycerol, erythritol, D-arabinose, L-arabi-

nose, D-xylose, L-xylose, adonitol, methyl β -xyloside, galactose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, inulin, melezitose, raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate or 2-ketogluconate. Nitrate is reduced to nitrite. Aesculin, urea, gelatin, tyrosine and ONPG are not hydrolysed. The Christie–Atkins–Munch–Petersen reaction is negative. Alkaline phosphatase, esterase, esterase/lipase, leucine arylamidase, cystine arylamidase, acid phosphatase and pyrazinamidase activities are present. The isolates do not produce lipase, trypsin, chymotrypsin, α -galactosidase, α -mannosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetylglucosaminidase or α -mannosidase. The cell wall of strains of *Corynebacterium casei* sp. nov. was found to be type IV, containing *meso*-diaminopimelic acid, arabinose, galactose and short-chain mycolic acids (C_{22} to C_{36}). The menaquinones in the cell wall were of the MK-9 (H_2) type. Analysis of the polar lipid of the cell wall revealed the presence of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylglycerol mannosides. The G + C content of the strains was 51 mol%. The type strain is LMG S-19264^T (= DPC 5298^T).

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