Molecular analysis of artisanal Italian cheeses reveals *Enterococcus italicus* sp. nov.

M. Grazia Fortina, G. Ricci, D. Mora and P. L. Manachini

Correspondence

M. Grazia Fortina
grazia.fortina@unimi.it

Department of Food Science and Microbiology – Industrial Microbiology Section, University of Milan, Via Celoria 2, 20133 Milan, Italy

The taxonomic positions of seven atypical *Enterococcus* strains, isolated from artisanal Italian cheeses, were investigated in a polyphasic study. By using 16S rRNA gene sequencing, DNA–DNA hybridization and intergenic transcribed spacer analysis, as well as by examining the phenotypic properties, the novel isolates were shown to constitute a novel enterococcal species. Their closest relatives are *Enterococcus sulfureus* and *Enterococcus saccharolyticus*, having a 16S rRNA gene sequence similarity of 96.7%. This group of strains can be easily differentiated from the other *Enterococcus* species by DNA–DNA hybridization and by their phenotypic characteristics: the strains do not grow in 6.5% NaCl, and they do not produce acid from L-arabinose, melezitose, melibiose, raffinose or ribose. The name *Enterococcus italicus* sp. nov. is proposed for this species, with strain DSM 15952^T (=LMG 22039^T) as the type strain.

Enterococci constitute a large proportion of the autochthonous bacteria associated with the mammalian gastrointestinal tract and can cause infection from this endogenous source. The incidence of infections caused by enterococci, together with the increasing difficulty of treating such infections because of multiple antibiotic resistance, put these organisms, especially *Enterococcus faecium* and *Enterococcus faecalis*, among the emerging human pathogens (Morrison *et al.*, 1997; Robredo *et al.*, 2000). However, enterococci are ubiquitous and can be found as free-living micro-organisms in soil, on plants and in large numbers in dairy products, where, in some cases, they predominate with respect to lactobacilli and lactococci (Franz *et al.*, 1999; Giraffa, 2002). The presence of enterococci in dairy products has long been considered as an indicator of inadequate sanitary conditions during the production and processing of milk. In contrast, many authors suggest that enterococci may have a potentially desirable role in some cheeses, because of their proteolytic and lipolytic activities, in the development of typical flavours and for the production of enterocins with anti-*Listeria* activity (Ennahar & Deschamps, 2000; Giraffa *et al.*, 1997). The important question is whether enterococci originating from food and from community sources possess an equally pathogenic potential, or whether differences in pathogenicity exists, perhaps in relation to the different species. In this context,

correct species identification is of great importance to both medical and food microbiologists.

Since the revival of the genus *Enterococcus* by Schleifer & Kilpper-Bälz (1984), phylogenetic studies have established, on the basis of 16S rRNA gene sequences, the presence of at least four ‘species groups’ (Devriese *et al.*, 1993; Devriese & Pot, 1995; Stiles & Holzapfel, 1997), as follows: the ‘faecium group’, comprising *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus hirae*, *Enterococcus mundtii* and the newly described *Enterococcus villorum* (Vancanneyt *et al.*, 2001); the ‘avium group’, comprising *Enterococcus avium*, *Enterococcus raffinosus*, *Enterococcus malodoratus* and *Enterococcus pseudoavium*; the ‘gallinarum group’, comprising *Enterococcus gallinarum* and *Enterococcus casseliflavus*; and a fourth group, comprising *Enterococcus columbae* and *Enterococcus cecorum*. Other described enterococci form individual lines of descent, e.g. *Enterococcus sulfureus*, *Enterococcus saccharolyticus*, *Enterococcus asini* and *Enterococcus faecalis*. Recently, two novel species, *Enterococcus haemoperoxidus* and *Enterococcus moraviensis* (Švec *et al.*, 2001), have been shown to be phylogenetically closely related to *Enterococcus faecalis*. However, 16S rRNA gene sequence similarities between enterococcal species are mostly high and exceed the generally accepted value of 97% for species differentiation. For this reason, at the genetic level, only the percentage of DNA–DNA hybridization can clearly indicate their species identity.

Phenotypically, there are no definite characteristics that distinguish the enterococci unequivocally from the other Gram-positive, catalase-negative, coccus-shaped bacteria or within the genus, at the species level. The ability to grow at 10 and 45 °C, in 6.5% NaCl, at pH 9.6 and on selective

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Enterococcus italicus* TP1.5^T is AJ582753.

DNA–DNA relatedness values and the long-fatty-acid composition of *Enterococcus italicus* sp. nov. and other enterococcal species are available as supplementary material in IJSEM Online.

media can also be possessed by some non-enterococcal species, while these criteria can exclude several novel enterococcal species, such as *Enterococcus columbae*, which fails to grow in 6.5% NaCl (Devriese *et al.*, 1990), or *Enterococcus haemoperoxidus* (Švec *et al.*, 2001), which shows a clear positive catalase reaction.

For these reasons, a polyphasic approach is desirable for correctly identifying 'unusual' enterococcal strains. Today, some aspects of the taxonomic and genotypic diversity within the genus *Enterococcus* can also be further investigated through the use of recently developed molecular techniques, such as intergenic ribosomal PCR, randomly amplified polymorphic DNA and PFGE (Angeletti *et al.*, 2001; Naïmi *et al.*, 1997; Poyart *et al.*, 2000; Quednau *et al.*, 1998).

Recently, during a study on the natural bacterial population obtained from raw cows' milk used in the production of artisanal Italian cheeses (Fortina *et al.*, 2003), we isolated some atypical *Enterococcus* strains that seemed to represent a novel species of *Enterococcus*. In the present paper, we describe a polyphasic study of a collection of seven isolates: five originated from Toma piemontese cheese and two were from Robiola piemontese cheese. The results demonstrated that the strains represent a novel species, for which the name *Enterococcus italicus* sp. nov. is proposed.

Strains TP1.5^T, TP1.3, TP2.3, TP1.D and TP3.D were isolated from Toma piemontese cheese, while strains RP1 and RP4 were isolated from Robiola piemontese cheese. They were routinely maintained at 4 °C after growth at 37 °C for 18 h in M17 glucose broth or agar (Difco). On this agar, colonies appeared circular and smooth, with entire margins; after 3 days growth, they were 1 mm in diameter.

Cell morphology was studied using phase-contrast microscopy. Gram staining was done as described by Salle (1961). Growth was checked on kanamycin/aesculin/azide agar (Merck), blood agar and skimmed-milk medium. Sugar-fermentation patterns were determined by using a microtitre plate assay and a basal M17 medium containing chlorophenol red as indicator and the desired filter-sterilized carbohydrate at a final concentration of 0.2% (w/v). The reproducibility was verified by repeated analyses. A pH-dependent change in the indicator was documented for up to 5 days. Only definitive turnovers were rated as positive. Biochemical tests were also performed using API galleries 50 CH and 20 STREP (bioMérieux) according to the manufacturer's instructions. Susceptibility to antibiotics was evaluated after overnight growth at 37 °C in M17 glucose broth containing different concentrations of antibiotic and using 10⁵ cells ml⁻¹ as the initial inoculum.

DNA was isolated and purified as described previously (Fortina *et al.*, 2001a). The DNA base composition was determined in 0.1 × SSC buffer (1 × SSC is 0.15 mol NaCl l⁻¹ plus 0.015 mol sodium citrate l⁻¹) by using the thermal denaturation method described by Marmur & Doty

(1962) and the equation of Owen & Hill (1979). DNA from *Escherichia coli* strain B (Sigma) was used as the internal standard. The extent of DNA reassociation was determined as previously described (Fortina *et al.*, 2001b) by using the optical renaturation rates method with a model Response spectrophotometer equipped with an Advance Kinetics Graphic version 1.3 thermoprogrammer (CIBA–Corning Diagnostics). For all samples tested, the reaction was carried out under optimal conditions (25 °C below the melting temperature) in 5 × SSC, containing 20% (v/v) DMSO. The equation of Seidler & Mandel (1971) was used to calculate the extent of DNA–DNA reassociation.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were performed as described previously (Fortina *et al.*, 2003). Pairwise alignment similarities were calculated using the Ribosomal Database Project service (Maidak *et al.*, 2001). Phylogenetic reconstructions were done by using the TREECON software (version 1.3b) (Van de Peer & De Wachter, 1994) and the model of Jukes & Cantor (1969). Amplification of the 16S–23S rRNA gene intergenic transcribed spacer region was performed as previously described (Fortina *et al.*, 2003), employing the primers G1 (5'-GAAGTCGTAACAAGG-3') and L1 (5'-CAAGGCAT-CCACCGT-3') (Jensen *et al.*, 1993). Following amplification, the reaction mixtures were electrophoresed in 3% agarose gel in TAE buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8) at 100 V for 2 h, before staining with ethidium bromide. A DNA ladder (50 bp; Amersham Pharmacia Biotech) was used as the molecular size marker.

Analysis of fatty acid methyl esters was performed by GC using the Microbial Identification System (MIDI) at the DSMZ (Braunschweig, Germany).

The strains were Gram-positive with spherical or ovoid cells that occurred in pairs and short chains, catalase-negative, non-motile and non-endospore-forming. The strains did not grow on kanamycin/aesculin/azide agar, showed α-haemolysis on blood agar and were able to grow in skimmed-milk medium, in which they caused clotting within 48 h incubation. All of the strains were susceptible to vancomycin. Growth was enhanced slightly by cultivation in a 5% CO₂ atmosphere and was more abundant at 37 °C in comparison with 30 or 25 °C. Growth occurred at pH 9.6 and at 42 °C, but was reduced at 10 °C. The ability to grow at 45 °C was variable among the strains tested. The novel isolates failed to grow in M17 broth containing 6.5% NaCl, as for strains of *Enterococcus cecorum* (Devriese *et al.*, 1983) and *Enterococcus columbae* (Devriese *et al.*, 1990). The ability to grow in 6% NaCl was possessed only by the type strain, TP1.5^T, while all isolates grew in 5% NaCl.

The almost complete 16S rRNA gene sequence (1396 bp) of TP1.5^T had the highest similarity to 16S rRNA gene sequences of enterococci: pairwise sequence alignments indicated that the closest relatives of strain TP1.5^T are *Enterococcus saccharolyticus* and *Enterococcus sulfureus*

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences available from the GenBank/EMBL/DBJ database (accession numbers in parentheses), showing the relationship between *Enterococcus italicus* sp. nov. and other enterococci. The sequence of the 16S rRNA gene of *Tetragenococcus halophilus* was used as an outgroup. Sequences were aligned using the Ribosomal Database Project service. A neighbour-joining tree was constructed using TREECON software (version 1.3b) and includes bootstrap probability values (%) at the branch-points (500 tree replications).

(96.7%) and *Enterococcus gallinarum* (95.4%). Lower sequence similarities (<95%) were found with other described species of the genus *Enterococcus*. The 16S rRNA genes of the species of the genera *Melissococcus* and *Tetragenococcus*, for which a close relationship with the enterococcal strains has been previously demonstrated, exhibited 90.4 and 88.8% sequence identity, respectively, with the 16S rRNA gene sequence of strain TP1.5^T. Strain TP1.5^T was more distantly related to the streptococcal and lactococcal species tested (<84% sequence identity). A tree depicting the phylogenetic affinity of strain TP1.5^T with other enterococci is shown in Fig. 1. It should be noted that the 16S rRNA gene sequence of strain TP1.5^T showed 99% similarity to those of two unidentified strains (accession nos AF349934 and AY321375) and of a strain deposited as *Enterococcus sulfureus* (accession no. AY184239). These strains, isolated from an artisanal Sicilian cheese, from human clinical specimens and from a fermented maize beverage, respectively, could belong to *Enterococcus italicus* sp. nov., indicating the possibility that it occurs in other sources. Whether or not these enterococci belong to *Enterococcus italicus* remains to be determined.

Similarly, DNA–DNA hybridization results (Supplementary Table A in IJSEM Online) for the seven novel strains and the type strain of the seven reference *Enterococcus* species tested confirmed the species identity of the isolates. These DNA–DNA relatedness values clearly differentiated the strains from the phylogenetically closest species, *Enterococcus saccharolyticus*, *Enterococcus sulfureus* and *Enterococcus gallinarum*, and revealed high levels of similarity between strains.

PCR amplification of the intergenic transcribed spacer between the 16S and 23S rRNA genes produced an amplification profile characteristic of the novel enterococcal species. This fingerprint pattern showed genetic homogeneity in the cheese isolates and separated them from all

enterococcal species included in this study by the presence of three major bands with approximate sizes of 400, 380 and 300 bp (Fig. 2).

The fatty acid profile of strain TP1.5^T was compared with those of its closest relatives, *Enterococcus saccharolyticus* and *Enterococcus sulfureus*. Qualitatively, all three strains showed roughly the same fatty acid pattern, but there were significant quantitative differences. The major non-hydroxylated long-chain fatty acids of strain TP1.5^T were C_{16:0} (27.9%) and C_{18:1ω7c} (60.6%); C_{19:0cycloω8c} was present in small amounts (2.5%). *Enterococcus sulfureus* produced significantly smaller amounts of C_{18:1ω7c} and

Fig. 2. Intergenic transcribed spacer-PCR profiles of *Enterococcus italicus* sp. nov. and other enterococcal species. Lanes: 1–7, *Enterococcus italicus* strains; 8, *Enterococcus faecalis* NCDO 588; 9, *Enterococcus durans* NCDO 595; 10, *Enterococcus faecium* ATCC 19434^T; 11, *Enterococcus sulfureus* DSM 6905^T; 12, *Enterococcus gallinarum* DSM 20628^T; 13, *Enterococcus avium* DSM 20679^T; 14, *Enterococcus saccharolyticus* DSM 20726^T; 15, negative control; M, 50-base-pair ladder (Amersham Pharmacia Biotech).

very large amounts of C_{19:0}cycloω8c. *Enterococcus saccharolyticus* showed more balanced amounts of C_{16:0} and C_{18:1ω7c}; C_{19:0}cycloω8c was not detectable (Supplementary Table B in IJSEM Online).

In complete agreement with the phylogenetic and genotypic examinations described above, the phenotypic studies clearly separated all the novel strains from the other reference species. Indeed, the novel species can be readily distinguished from the other enterococci by using a combination of physiological and biochemical tests (Table 1). In particular, utilization of ribose confirms the separateness of the cheese isolates from all tested enterococcal species. *Enterococcus italicus* can also be differentiated from its closest relatives, *Enterococcus saccharolyticus* and *Enterococcus sulfureus*, by its inability to acidify L-arabinose, melezitose, melibiose and raffinose.

The results of the present study allowed us to assign the strains tested to a novel species, for which we propose the name *Enterococcus italicus* sp. nov. The 16S rRNA gene sequence and DNA–DNA hybridization analyses indicate that all of the cheese isolates form a homogeneous group

that can be differentiated from other *Enterococcus* species. Further support for the distinctiveness of the novel species comes from phenotypic evidence.

Description of *Enterococcus italicus* sp. nov.

Enterococcus italicus (i.ta'li.cus. L. masc. adj. *italicus* from Italy, where the bacterium was first isolated).

Cells are facultatively anaerobic, non-motile, non-spore-forming, catalase-negative, non-pigmented, Gram-positive cocci that occur in pairs or short chains. Colonies are whitish-grey, circular and smooth, with entire margins; 1 mm in diameter after 3 days growth on M17 glucose agar. Growth is enhanced slightly by cultivation in a 5 % CO₂ atmosphere and is optimal at 37 °C. Growth occurs at 42 °C, but is weak at 10 °C. The ability to grow at 45 °C is variable among the strains. Strains grow at pH 9.6 and in 5 % NaCl. A few strains are positive for growth in 6 % NaCl; no growth is seen in 6.5 % NaCl. The strains do not grow on kanamycin/aesculin/azide agar, show α-haemolysis on blood agar and are able to grow in skimmed-milk medium, where they cause clotting within 48 h incubation. All strains are susceptible to vancomycin. Homofermentative, producing lactic acid and no gas. Acid is produced from galactose, D-glucose, D-fructose, D-mannose, methyl α-D-glucoside (often delayed), N-acetylglucosamine, aesculin, salicin, maltose, lactose, sucrose, trehalose and starch. Weak reactions can occur on cellobiose. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-xyloside, L-sorbose, rhamnose, dulcitol, inositol, methyl α-D-mannoside, amygdalin, melibiose, inulin, melezitose, D-raffinose, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Strain-dependent reactions are observed with mannitol (4/7 strains), β-gentiobiose (3/7 strains), D-tagatose (3/7 strains), arbutin (3/7 strains) and sorbitol (5/7 strains). Positive results are obtained in API tests for acetoin production, aesculin hydrolysis, pyrrolidonyl arylamidase and leucine arylamidase. Strains are negative for hippurate hydrolysis, α-galactosidase, β-glucuronidase, β-galactosidase, alkaline phosphatase and arginine dihydrolase. The DNA G + C content ranges from 39.9 to 41.1 mol%, as determined by melting temperature. The strains form a distinct relatedness group, as determined by DNA–DNA hybridization. The strains can also be differentiated from the other species of *Enterococcus* by their unique intergenic transcribed spacer profile and the sequence of their 16S rRNA genes.

Strains were isolated from dairy products in Piedmont, Italy. The type strain is DSM 15952^T (= LMG 22039^T).

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Table 1. Biochemical tests useful for the differentiation of *Enterococcus italicus* sp. nov. from other enterococci

Taxa: 1, *Enterococcus italicus*; 2, *Enterococcus saccharolyticus*; 3, *Enterococcus sulfureus*; 4, *Enterococcus faecalis*; 5, the *Enterococcus faecium* group; 6, the *Enterococcus avium* group; 7, the *Enterococcus gallinarum* group; 8, the *Enterococcus cecorum* group. Data for taxa other than *Enterococcus italicus* were obtained from Collins *et al.* (1984), Farrow *et al.* (1984), Martinez-Murcia & Collins (1991), Devriese *et al.* (1993), Vancanneyt *et al.* (2001) and De Graef *et al.* (2003). +, Positive; D, different or variable; D+, usually positive; D−, usually negative; −, negative; W, weak reaction; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8
Pigment production	−	−	+	−	−	(+) ^a *	−	D ^b −
Growth in 6.5 % NaCl	−	+	+	+	+	+	+	−
Growth at 10 °C	W	+	+	+	+	D+	+	D−
Acid production from:								
L-Arabinose	−	−	−	−	D ^c	D ^d	+	D+ ^e
Melezitose	−	+	+	D+	−	D ^d	−	D
Melibiose	−	+	+	−	D+ ^f	D	+	+
Raffinose	−	+	+	−	D−	D ^g	D	+
Ribose	−	+	+	+	+	+	+	+
Sorbitol	D	+	−	D+	−	(+) ^f	+	D−

*Discrepant results are indicated by: a, positive in *Enterococcus mundtii*; b, positive in *Enterococcus casseliflavus*; c, positive in *Enterococcus faecium*, *Enterococcus mundtii* and *Enterococcus canis*; d, positive in *Enterococcus avium* and *Enterococcus raffinosus*; e, negative in *Enterococcus cecorum*; f, positive in *Enterococcus canis*; g, negative in *Enterococcus avium* and *Enterococcus pseudoavium*.

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