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Enterococcus asini sp. nov. isolated from the caecum of donkeys (*Equus asinus*)

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Several Gram-positive, non-spore-forming and non-motile bacteria consisting of pairs or chains of cocci were isolated during an investigation of the bacterial flora of the caecum of donkeys. Physiological and metabolic data indicated that the strains belong to the genus *Enterococcus*; phenotypic traits of these organisms were not consistent with any of the currently known *Enterococcus* species. 16S rRNA gene sequence analysis placed these strains in the genus *Enterococcus*. Their closest relatives are *Enterococcus avium*, *Enterococcus faecium* and *Enterococcus pseudoavium* with a sequence similarity of 97·4%. This group of strains can be differentiated from the other *Enterococcus* spp. by their phenotypic characteristics: strains do not grow in 6·5% NaCl; they do not produce acid from mannitol, sorbitol, sorbose, sucrose, raffinose, ribose and tagatose; they produce acid from D-xylose; they are able to utilize pyruvate; and they present a negative reaction on arginine. The name *Enterococcus asini* sp. nov. is proposed for these strains; the type strain is AS2^T (= DSM 11492^T).

Keywords: *Enterococcus asini* sp. nov., *Enterococcus*, *Streptococcus*, 16S rRNA analysis, phenotypic analysis

INTRODUCTION

During an investigation of the bacterial flora of the caecal contents of donkeys, colonies isolated on M17 glucose agar plates showed very uniform traits and formed a homogeneous group. Their physiological and phenotypic characteristics and growth behaviour were similar to strains classically described in both streptococci and enterococci. Streptococci and enterococci have been routinely isolated from intestinal or faecal sources of animal and human origin (5, 7–9). However, the strains isolated from donkey caecum could not be assigned to any known species.

In previous years, streptococci belonging to the faecal group or serological group D were divided in two physiologically different sub-groups: the enterococcal division, which comprised *Streptococcus faecium* and *Streptococcus faecalis*, and the viridans division, which comprised *Streptococcus bovis* and *Streptococcus equinus*. Results based on nucleic acid hybridization with 23S rDNA and 23S rRNA probes and DNA–DNA homology indicated that the enterococcal and non-enterococcal members of group D streptococci belonged to different genera. *S. faecium* and *S. faecalis* were transferred to the genus *Enterococcus* as *Enterococcus faecium* and *Enterococcus faecalis* (23). Physiologically, enterococci are related to streptococci by their ability to grow at 10 and 45 °C, in 6·5% NaCl, at pH 9·6 or in the presence of 40% bile. However, some of the more recently described species do not have some of these characteristics. For example, *Enterococcus columbae* failed to grow in 6·5% NaCl (7) and *Enterococcus cecorum* failed to grow at 10 °C and in 6·5% NaCl and did not react with group D antiserum (8). It has been proposed to amend the definition of the genus *Enterococcus* as given by Schleifer & Kilpper-Bälz (23): strains may or may not grow at 10 °C and in 6·5% NaCl and some strains do not react with group D antiserum (28).

The small subunit of rRNA is universally recognized as a powerful molecular chronometer and is currently the most widely used molecule for elucidating phylogenetic relationships between organisms. 16S rRNA reverse sequencing studies have not only confirmed the separateness of the genus *Enterococcus* from the genus *Streptococcus* and revealed the existence of different clusters within the genera (29), but have also been invaluable in classifying new species (7, 17) or in reclassifying existing species (28).

The GenBank accession number for the sequence reported in this paper is Y11621.

Therefore, in addition to analysis of the phenotypic features of the strains isolated from donkey caecum, we determined the complete 16S rDNA sequence of one strain representative of this group in an attempt to clarify its phylogenetic position and taxonomic status. As a result of this study, we propose that these strains should be placed in a new species of the genus *Enterococcus*, *Enterococcus asini*.

METHODS

Bacterial strains, growth, temperature and biochemical tests. Sixteen strains were isolated on M17 (26) glucose (0·5% w/v) agar plates from the caecal contents of a fistulated donkey fed a maintenance diet of alfalfa hay. Strains were maintained on the same medium. Cultures were routinely incubated at 37 °C.

As well as microscopic and morphological investigations, fermentation, enzymic activities and growth were studied. Fermentation tests and enzymic assays were carried out in API galleries (20A, 20Strep and 50CH) according to the manufacturer's instructions (bioMérieux). Tests for growth at pH 9·6, in 4·0 and 6·5% NaCl, with 40% bile and at 10 and 45 °C were performed in duplicate in M17 glucose broth medium. Results were recorded after 24, 48 and 72 h. Survival at 60 °C for 30 min was tested on pre-warmed culture medium. The main fermentation products (succinate, lactate, formate, acetate, ethanol) were determined using kits according to the manufacturer's protocol (Boehringer Mannheim).

Physiological and metabolic behaviour data were collected from previously published information on strains *Enterococcus avium* (2, 8–10, 30), *Enterococcus casseliflavus* (9, 10), *E. cecorum* (8, 28), *E. columbae* (7), *Enterococcus durans* (9, 10), *Enterococcus gallinarum* (2, 8, 9, 30), *E. faecalis* (2, 8–10, 23, 30), *E. faecium* (2, 8–10, 23, 30), *Enterococcus malodoratus* (9, 10), *Enterococcus sulfureus* (17) and *S. bovis* (2, 8, 12). Similarities between the caecal isolates and the enterococcal and streptococcal strains were calculated from the proportion of shared features to the total number of features studied using a simple matching coefficient (25). A dendrogram was constructed from the similarity matrix by the unweighted pair group method with arithmetic mean (UPGMA) (24). To test the goodness-of-fit of cluster analysis, a cophenetic value matrix was calculated and compared with the original UPGMA-clustered similarity matrix using the NTSYS-PC analysis package (version 1.8; Exeter Software, Setauket, NY, USA). Matrix correlation was estimated by the correlation coefficient r, which is the normalized Mantel test statistic Z.

Determination of G + C content. Cells were lysed with SDS as previously described (16). DNA was isolated and purified as described by Meyer & Schleifer (18). The G + C content was estimated from the A_{245}/A_{270} ratio of the total DNA content of the strain (27). DNAs from *Clostridium perfringens* (G + C content, 31·0 mol%), calf thymus (43·2 mol%) and *Escherichia coli* B (51·3 mol%), all purchased from Sigma, were used as standards.

16S rDNA sequencing. The strain AS2^T was grown overnight at 37 °C on M17 glucose broth. Cells were harvested by centrifugation (12000 ***g***, 10 min), washed twice with sterile distilled water, then resuspended in 50 mM Tris (pH 8·2) to give an OD_{600} of 1·0. A crude bacterial extract was obtained by cell wall digestion at 55 °C for 2 h in the presence of 60 μg proteinase K ml^{-1} and the enzyme was inactivated by boiling for 10 min. PCR amplification of 16S rDNA was done as previously described (15). Amplification was confirmed by analysing 5 μl PCR reaction mixture on a 0·9% agarose minigel (21).

Plasmid pT7Blue(R) (Novagen) was used for cloning and sequencing PCR-amplification products as recommended by the manufacturer. *E. coli* DH5α (Stratagene) served as the host strain. Bacteria were grown overnight at 37 °C in agitated cultures in Luria–Bertani broth (1). Ampicillin (50 μg ml^{-1}) was added in selective medium. Double-stranded DNA from the recombinant plasmid was purified with a QIAGEN Tip 100 Plasmid kit. 16S rDNA was sequenced according to the dideoxynucleotide chain-termination method (22) using the T7 Sequencing kit (Pharmacia). Both strands were sequenced using synthetic oligonucleotide primers (Eurogentec) as recommended by the manufacturer.

Sequence and phylogenetic analysis. Analysis was based on 16S rDNA sequences published previously or available from the GenBank database. Nucleotide sequences were aligned by the CLUSTAL program (13) from the BISANCE software (6). A matrix of pairwise distances corrected for multiple base substitutions by the method of Kimura (14) was computed with the DNADIST program of the Phylogenetic Inference Package (PHYLIP) (11) and a phylogenetic tree was constructed with the NEIGHBOR program (20). The 16S rDNA sequence of strain AS2^T has GenBank accession number Y11621.

RESULTS AND DISCUSSION

The sixteen strains isolated on M17 agar from donkey caecal contents appeared as pairs or short chains of Gram-positive cocci that were non-motile, non-spore-forming and grew in the absence of oxygen at temperatures prevailing in the caecum (38–40 °C). They were able to grow on 40% bile and 4% NaCl, were catalase-negative and reacted with group D antigen. These features are common to both streptococci and enterococci. Some of the characteristics that distinguish the caecal isolates from known species of the genera *Enterococcus* and *Streptococcus* which have similar phenotypic or genotypic properties are summarized in Table 1. The main fermentation end-products from glucose metabolism were L-lactate (74·5% of the carbon recovery), ethanol (4·0% of the carbon recovery) and acetate (1·8% of the carbon recovery). The growth yield was 0·24 g dry biomass (g sugar used)$^{-1}$.

The strains reacted with group D antigen, grew in the presence of 40% bile and were pyrrolidonylaryl-aminidase-positive, which suggested that they could be members of the genus *Enterococcus* (9). However, their failure to grow in 6·5% NaCl, like strains of *E. columbae* (7) and *E. cecorum* (8), indicated that they conform to the definition of the genus *Enterococcus* as amended by Devriese *et al.* (7). These strains did not produce acid from mannitol, sorbitol and sorbose, like the Division III *Enterococcus* species (9), which comprises *Enterococcus dispar* (5), *E. durans*, *Enterococcus hirae* and an asaccharolytic variant of *E. faecalis*. However, in contrast to the members of Division III,

Table 1. Physiological and metabolic characteristics that differentiate donkey caecum strains from other enterococci and from streptococci

+, > 90% positive; −, < 10% positive; ±, 10–90% positive.

Characteristic	*E. asini**	*E. cecorum*	*S. bovis*	*E. avium*	*E. faecalis*	*E. faecium*	*E. gallinarum*	*E. sulfureus*	*E. columbae*	*S. equinus*‡
Growth at:										
10 °C	±	−	−	±/−†	+	+	+	+		−
40 °C	+	+	+	+	+	+	+	+		+
45 °C	±	+	+	+	+	+	+	−		+
Survival at 60 °C, 30 min	+	−	−	+	+	+	±/+†			+
Growth in:										
4·0% NaCl	±/−[a]			+	+	+	+	+		+
6·5% NaCl	−	−	−	±/+†	+	+	+	+	−	−
40% bile	+	+	+	+	+	+	+		+	+
Acetoin production	+	+		+	+	+	+		+	
Hippurate hydrolysis	+	−	−	−	+	±	+	−	−	−
Pyrrolidonylarylaminidase	+	−	−	+	+	+	+	+	−	
α-Galactosidase	−	±	+	−	−	−	+	+	+	
β-Glucuronidase	−	+	−	−	−	−	+	−	−	
β-Galactosidase	−	±	−	−	−	+	+	+	±	
Alkaline phosphatase	−	+	−	−	−	−	−	−	+	
Arginine dihydrolase	−	−	−	±/−†	+	+	−	−	−	−
Group D antigen	+	−	+	+	+	+	+		−	+
Acid from:										
Glycerol	−	−	−	±	+	±/−†	±	−	−	−
D-Arabinose	−	−	−	+	±	−	±	−	−	−
L-Arabinose	−	−	−	±	−	+	+	−	+	−
Ribose	−	+	−	+	+	+	+	+	+	+
D-Xylose	+	−	−	−	+	+	+	−	+	−
L-Xylose	−	−	−	+	±	−	±	−		−
Adonitol	−	−	−	+	−	−	−	−	−	−
L-Sorbose	−	−	−	+	−	−	−		−	−
Rhamnose	+[a]	−	−	±	±	±	−	−	±	±
Mannitol	−	−	±	+	+	±/+†	+	−	+	±
Sorbitol	−	−	−	+	+	−	−	−	+	−
Methyl α-D-glucoside	±[b]	−	−	+	−	±	+	+	+	−
Lactose	+[c]	+	+	+	+	+	+	+	±	−
Melibiose	−	+	+	±/+†	−	±/+†	+	+	+	−
Sucrose	−	+	+	±	±	+	+	+	+	±
Inulin	−	+	±	−	−	±	±	−	+	±
Melezitose	−	+	−	+	+	−	−	+	±	±
D-Raffinose	−	+	+	−	−	±	+	+	+	±
Starch	±[d]	±	+	−	−	−	−		+	−
D-Tagatose	−	±/−†	−	+	+	±	+	−	±	
Gluconate	−	−	−	±	+	±	+	+	−	±
2-Ketogluconate	−	±/−†	−	+	−	−	−	+	±	

* a, Reaction is still weak after 72 h; b, 3/16 positive strains (strains 6, 7 and 9); c, 15/16 strains positive (strain 4 was negative); d, 13/16 strains positive (strains 3, 8 and 10 were negative).

† Differences appeared in the literature.

‡ Data were obtained from reference 2.

the strains isolated from donkey caecum were arginine-negative. They shared metabolic features with the asaccharolytic variant of *E. faecalis*, i.e. they were able to grow on pyruvate and did not produce acid from sucrose and raffinose, but differed from this strain by their inability to grow in 6·5% NaCl, to utilize ribose and tagatose and in having a negative reaction on arginine.

A dendrogram was constructed from phenotypic features of the caecal isolates and from data available for reference species of the genera *Enterococcus* and *Streptococcus* (Fig. 1). The sixteen isolates clustered together with a similarity coefficient of 98·3%. This group was clearly distinct from the known species of *Enterococcus* and *Streptococcus* included in the analysis.

G+C content

The DNA G+C content of the isolate was 39·4 mol%, a value similar to the published figures for both streptococci and enterococci (2, 7, 8, 10, 12, 17, 19, 23).

16S rDNA sequence analysis

Amplification of the 16S RNA gene yielded the expected PCR product of approximately 1·5 kb. The

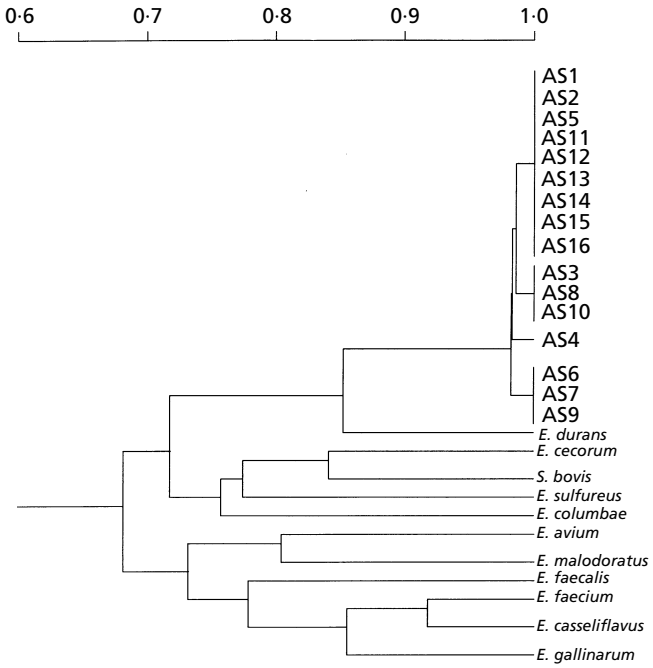

Fig. 1. Dendrogram (UPGMA) showing phenotypic relationships between the donkey caecal isolates (AS1–AS16) and reference strains of *Enterococcus* spp. and *Streptococcus* spp. Bar indicates the level of similarity; $r = 0{\cdot}95$.

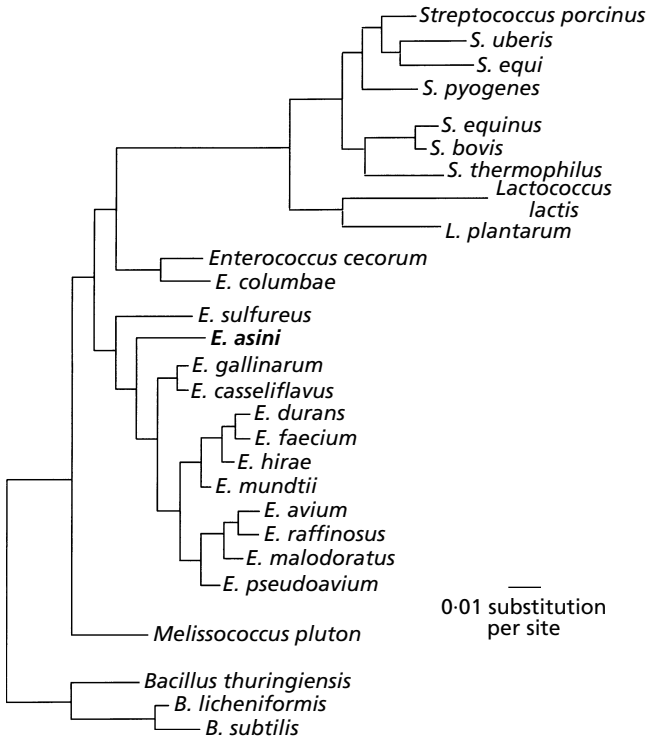

Fig. 2. Dendrogram showing the phylogenetic interrelationship of *E. asini* with other enterococcal species and species belonging to other genera. The tree is based on sequence homology of the total stretches available and clustering by the neighbour-joining method.

almost complete nucleotide sequence of the 16S rRNA gene of the caecal isolate AS2^T was determined. This sequence was compared to those of physiologically related species of *Enterococcus* and Gram-positive organisms with low G+C content. Similarity values and the number of nucleotide differences were calculated for a continuous stretch of 1551 bases [corresponding to positions 8 and 1541 in the *E. coli* 16S rRNA gene (3)] (data not shown). The sequence of strain AS2^T differed from all the sequences included in this analysis. The most similar 16S rDNAs (97·4–94·6%) were those of the *Enterococcus* species. The 16S ribosomal sequence of AS2^T displayed lower relatedness with sequences of Division III enterococci than with members of the other groups, as defined on the basis of their metabolic characteristics. It presented the highest levels of sequence similarity with *E. avium* (Division I), *E. faecium* (Division II) and *Enterococcus pseudoavium* (Division I) (97·4% identity each). It also exhibited high levels of similarity with the sequences of *E. durans* (Division III) and *Enterococcus mundtii* (Division II) (97·3% each). AS2^T was more distantly related to the streptococcal species tested (91·0–88·5% sequence identity). The 16S rDNA of the species *Melissococcus pluton*, for which a close relationship with the enterococcal strains has been previously demonstrated (4), exhibited 94·4% sequence identity with the 16S rDNA sequence of strain AS2^T.

An unrooted phylogenetic tree constructed by the neighbour-joining method is shown in Fig. 2. The tree topology indicated that the donkey strain was phylogenetically classified as a member of the genus *Enterococcus*. However, like *E. sulfureus* and the group formed by *E. cecorum* and *E. columbae*, this strain formed a distinct line of descent within the genus, which confirmed the results of phenotypic analysis. It is suggested that the strains isolated from donkey caecum be designated a new species of the genus *Enterococcus* for which the name *Enterococcus asini* is proposed.

Description of *Enterococcus asini* sp. nov.

Enterococcus asini (a.si′ni. M.L. gen. sing. *asini* of donkeys, *Equus asinus*).

Cells are Gram-positive cocci, ovoid and occur mostly in pairs or short chains. Non-pigmented and non-motile. Facultatively anaerobic. Catalase-negative. Lancefield group D-antigen-positive. Grow at 40 ° C but growth at 10 and 45 °C is weak. No growth in 6·5% NaCl. Grow in a 40% bile M17 broth. Acid is produced from D-xylose, galactose, D-glucose, D-fructose, D-mannose, *N*-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, trehalose, starch and β-gentibiose. Weak reactions can occur on rhamnose. Acid is not produced from glycerol, erythritol, D- and L-arabinose, ribose, L-xylose, adonitol, methyl α-D-glucoside, β-methyl-xyloside, L-sorbose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, melibiose, sucrose, inulin,

melezitose, D-raffinose, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, gluconate and 2- and 5-ketogluconate. Aesculin and hippurate hydrolysis are positive. Strains are positive for leucine arylaminidase, pyrrolidonyl-arylaminidase and β-glucosidase and negative for α-galactosidase, β-galactosidase, β-glucuronidase, alkaline phosphatase and arginine dihydrolase. The reactions of the type strain are the same as the majority of the reactions for *E. asini* given in Table 1 with the exceptions indicated by the footnotes. The G+C content of DNA is about 39·4 mol% as determined by the UV absorbancy ratio. At the molecular level, this species can be differentiated from other *Enterococcus* species by the sequence of 16S rRNA gene. Strain AS2^T is the type strain of *E. asini* and has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as strain DSM 11492^T.

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