***Enterococcus porcinus* sp. nov. and *Enterococcus ratti* sp. nov., associated with enteric disorders in animals**

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Recent insights have been brought to the taxonomy of the genus *Enterococcus* by studies applying whole-cell protein analysis and DNA–DNA reassociation experiments, in addition to conventional physiological tests. Using these techniques, a group of 10 strains resembling the physiological group III of enterococcal species was characterized. Five strains were recovered from pigs and five from rats with enteric disorders. On the basis of the results of conventional physiological tests, the most likely identity of these strains was *Enterococcus durans* or *Enterococcus hirae*. Analysis of the electrophoretic whole-cell protein profiles showed two distinct clusters of virtually indistinguishable profiles: one composed of strains isolated from pigs, and one composed of strains isolated from rats. These protein profiles were not similar to the profiles of any previously described *Enterococcus* species. The results of DNA–DNA relatedness experiments were consistent with the results of the protein-profile analysis. The high levels of DNA relatedness found for pig isolates demonstrated that they belong to a new enterococcal species, for which the designation *Enterococcus porcinus* sp. nov. is proposed (type strain = DS 1390-83^T = ATCC 700913^T = CCUG 43229^T = NCIMB 13634^T). Strains isolated from rats were found to comprise another new species, for which the designation *Enterococcus ratti* sp. nov. is proposed (type strain = DS 2705-87^T = ATCC 700914^T = CCUG 43228^T = NCIMB 13635^T). This report provides data on the phenotypic and genotypic characterization of these two new enterococcal species, which may represent diarrhoeagenic pathogens for animals.

Keywords: *Enterococcus*, taxonomy, DNA–DNA reassociation, whole-cell protein profiles

INTRODUCTION

The enterococci comprise an important group of intestinal bacteria whose classification has undergone considerable changes in recent years. Since the recognition of *Enterococcus* as a separate genus (Schleifer & Kilpper-Balz, 1984), several new species have been described as a result of improvements in the methods for their identification, combined with a growing interest in their role as opportunistic pathogens.

The GenBank accession numbers for the 16S rDNA sequence of *Enterococcus porcinus* and the 16S rRNA sequence of *Enterococcus ratti* are AF335596 and AF326472, respectively.

However, the differentiation of some of the species belonging to the genus *Enterococcus* remains problematic because of the overlap of phenotypic characteristics. Furthermore, some strains do not possess all of the typical characteristics, and questions may arise as to their precise identification. This is particularly applicable to the species included in physiological group III. This group is composed of arginine-positive enterococcal species (*Enterococcus dispar*, *Enterococcus durans* and *Enterococcus hirae*) that are characterized by their inability to produce acids from mannitol, sorbitol and sorbose, and are, therefore, difficult to distinguish from unusual mannitol-negative variants of *Enterococcus faecium* and *Enterococcus*

Table 1. Phenotypic characteristics of *Enterococcus porcinus*, and *Enterococcus ratti* strains and type or reference strains of physiologically related enterococcal species

Strains: 1, *E. durans* ATCC 19432^T; 2, *E. hirae* ATCC 8043^T; 3, *E. dispar* ATCC 61266^T; 4, *E. faecium* DS 954-82 (mannitol-negative variant); 5, *E. porcinus*; 6, four *E. ratti* strains; 7, *E. ratti* SS 1506. All strains grew in the presence of 6.5% NaCl, as well as at 10 and 45 °C; were positive for pyrrolidonyl arylamidase activity, leucine aminopeptidase activity, hydrolysis of aesculin in the presence of bile and hydrolysis of arginine; were susceptible to vancomycin, non-pigmented and non-motile; produced acids from maltose and ribose; reacted with AccuProbe *Enterococcus* genetic probe and with group D antiserum; did not produce gas in *Lactobacillus* deMan–Rogosa–Sharpe broth; did not tolerate tellurite; and did not produce acid from inulin, sorbitol or sorbose. v, Reaction varied according to strain; w, weak and/or delayed reaction; MGP, methyl α -D-glucopyranoside.

Characteristic	1	2	3	4	Clinical isolates		
					5	6	7
Efrotomycin susceptibility	+	+	—	+	+	+	+
Hippurate hydrolysis	—	—	+	+	—	v	—
Pyruvate utilization	—	—	+	—	—	—	—
Voges–Proskauer reaction	+	—	—	+	—	v	—
Acid production from:							
Arabinose	—	—	—	+	—	—	—
Glycerol	—	—	+	—	—	—	—
Lactose	+	+	+	+	+	v	+
Mannitol	—	—	—	—	—	—	+
Melibiose	—	+	w	+	+	—	—
MGP	—	—	+	—	—	—	—
Raffinose	—	+	w	—	—	—	—
Sucrose	—	+	+	—	—	—	—
Trehalose	—	+	w	+	w	—	+
Xylose	—	—	—	—	+	—	—

faecalis, the two predominant enterococcal species (Facklam *et al.*, 1999; Teixeira *et al.*, 1995).

The *Streptococcus* Laboratory at the Centers for Disease Control and Prevention serves as a reference laboratory for the State and City Health Departments of the United States. The majority of the cultures examined for identification or for typing in this laboratory are isolated from human patients. However, on occasion, the laboratory is asked to confirm the identity of isolates recovered from non-human sources. In 1983, we received a few enterococcal strains isolated from the intestines of piglets with severe diarrhoeal disease. These isolates were identified as *Streptococcus durans* on the basis of the information available at that time, although it was noted that they did not have the exact characteristics of most of the strains belonging to this species. A few years later, a strain showing similar characteristics, recovered from a pig with diarrhoea in Canada, was referred to us for identification. In 1987, the Reference Laboratory was asked to examine a few cultures of *Enterococcus*-like bacteria isolated from diarrhoea-like illness in a rat colony. Micro-organisms with similar characteristics were received for identification in subsequent years. These cultures were reported as probable enterococci, but they were not identified to the species level. Additional phenotypic studies indicated that the isolates from diarrhoeic rats and piglets were closely

related to *E. durans* and *E. hirae*. Before the completion of our studies, Uhl *et al.* (1998) reported the isolation of a new enterococcal species from the small intestines of diarrhoeic neonatal rats, and provisionally named the isolates '*Enterococcus rattus*'. As the characteristics of the isolates resembled those of the rat isolates we were examining, a representative strain was kindly provided for inclusion in our studies.

To resolve the true taxonomic position of the pig and rat isolates, we performed an analysis of whole-cell protein profiles and DNA–DNA reassociation experiments, and also conducted physiological tests, comparing these isolates with the type strains of all known enterococcal species. The results revealed that the isolates represented two new enterococcal species. In the present report, the phenotypic and genotypic characteristics of strains belonging to these two additional physiological group III enterococcal species isolated from animals with enteric disorders are described. The name *Enterococcus porcinus* sp. nov. is proposed for the strains isolated from pigs, and the name *Enterococcus ratti* sp. nov. is proposed for the strains isolated from rats.

METHODS

Bacterial strains. The study included 10 strains that resembled physiologic group III enterococcal species isolated from the intestines or faeces of animals with diarrhoea.

Fig. 1. (a) SDS-PAGE profiles of whole-cell protein extracts of *Enterococcus porcinus* strains and of strains belonging to physiologically related enterococcal species. Lanes: 1 and 13, molecular mass markers (kDa); 2, *Enterococcus faecalis* ATCC 19433; 3, *Enterococcus faecium* ATCC 19434; 4, *Enterococcus dispar* ATCC 51266; 5, *Enterococcus durans* ATCC 19432; 6, *Enterococcus hirae* ATCC 8043; 7, *Enterococcus ratti* DS 2705-87^T; 8, *Enterococcus porcinus* DS 1390-83^T; 9, DS 1391-83; 10, DS 1393-83; 11, DS 432-84; 12, SS 1646. (b) Dendrogram resulting from the computer-assisted analysis of the protein profiles shown in (a). The scale represents mean percentages of similarity.

They comprised five strains recovered from pigs and five strains from rats. Four of the pig strains (DS 1390-83, DS 1391-83, DS 1393-83 and DS 432-84) were from cases reported to occur in the USA, and one (SS 1646) was from Canada. All five rat strains (DS 2705-87^T, DS 2254-91, DS 3567-95, DS 3568-95 and SS 1506) were isolated from cases reported to occur in the USA; strain SS 1506 was kindly provided by Dr F. R. Cockerill III of the Mayo Clinic, Rochester, MN, USA. The type strains of all enterococcal species described to date, as well as strain DS 954-82 (previously confirmed as a mannitol-negative variant of *E. faecium*; Teixeira *et al.*, 1995), were included for comparative purposes.

Characterization of the strains. The strains were tested for their phenotypic characteristics by conventional physiological tests and serological grouping, as described pre-

Fig. 2. (a) SDS-PAGE profiles of whole-cell protein extracts of *E. ratti* strains and of strains belonging to physiologically related enterococcal species. Lanes: 1 and 13, molecular mass markers (kDa); 2, *E. faecalis* ATCC 19433; 3, *E. faecium* ATCC 19434; 4, *E. dispar* ATCC 51266; 5, *E. durans* ATCC 19432; 6, *E. hirae* ATCC 8043; 7, *E. porcinus* DS 1390-83^T; 8, *E. ratti* DS 2705-87^T; 9, DS 2254-91; 10, DS 3567-95; 11, DS 3568-95; 12, SS 1506. (b) Dendrogram resulting from the computer-assisted analysis of the protein profiles shown in (a). The scale represents mean percentages of similarity.

viously (Facklam & Collins, 1989; Facklam *et al.*, 1999). Reactivity with the AccuProbe *Enterococcus* culture confirmation test (Gen-Probe) was assayed as directed by the manufacturer.

Analysis of whole-cell protein profiles by SDS-PAGE. Strains were grown on trypticase soy/sheep-blood agar plates for 24 h at 37 °C. Preparation of extracts and analysis of whole-cell protein profiles by one-dimensional SDS-PAGE were performed as outlined by Merquior *et al.* (1994).

DNA-DNA reassociation experiments. Harvesting and lysis of the bacterial cells were performed according to the recommendations of Teixeira *et al.* (1995). Extraction and purification of DNA and the determination of DNA relatedness by using the hydroxyapatite hybridization method were done as described by Brenner *et al.* (1982). DNA-hybridization experiments were performed at 55 °C for optimal DNA reassociation, and at the stringent reassociation temperature of 70 °C.

Table 2. Levels of DNA relatedness of *Enterococcus porcinus*, *Enterococcus ratti* and other enterococcal species

ATCC; American Type Culture Collection; T, type strain; DS, diagnostic strain from the culture collection of the Centers for Disease Control and Prevention; SS, standard strain from the culture collection of the Centers for Disease Control and Prevention; RBR, relative binding ratio; %D, percentage of divergence, calculated to the nearest 0.5%; RBR at 55 °C of DS 1390-83^T or DS 2705-87^T with strains of all other enterococcal species ranged from 6 to 16.

Source of unlabelled DNA	Labelled DNA from:											
	<i>E. durans</i> ATCC 19432 ^T			<i>E. hirae</i> ATCC 8043 ^T			<i>E. porcinus</i> DS 1390-83 ^T			<i>E. ratti</i> DS 2705-87 ^T		
	RBR at 55 °C	%D	RBR at 70 °C	RBR at 55 °C	%D	RBR at 70 °C	RBR at 55 °C	%D	RBR at 70 °C	RBR at 55 °C	%D	RBR at 70 °C
Reference strains												
<i>E. durans</i> ATCC 19432 ^T	100	0.0	100	31			25			22		
<i>E. hirae</i> ATCC 8043 ^T	22			100	0.0	100	33			29		
<i>E. dispar</i> ATCC 51266 ^T							11			7		
<i>E. faecium</i> ATCC 19434 ^T	21			28			23			21		
<i>E. faecalis</i> ATCC 19433 ^T	13			9			9			9		
Clinical strains												
<i>E. porcinus</i> DS 1390-83 ^T	24			31			100	0.0	100	30		
<i>E. porcinus</i> DS 1391-83	20			36			95	0.5	93			
<i>E. porcinus</i> DS 1393-83	21			41			97	0.5	98			
<i>E. porcinus</i> DS 432-84	22			39			93	1.1	98			
<i>E. porcinus</i> SS 1646	22			37			95	0.5	96			
<i>E. ratti</i> DS 2705-87 ^T	17			31						100	0.0	100
<i>E. ratti</i> DS 2254-91	16			29						91	1.0	83
<i>E. ratti</i> DS 3567-95	21			30						99	0.5	99
<i>E. ratti</i> DS 3568-95	17			30						91	0.5	95
<i>E. ratti</i> SS 1506	25			34			35			82	2.3	86

Analysis of chromosomal DNA restriction profiles by PFGE.

Chromosomal DNA for PFGE was obtained as described by Teixeira *et al.* (1997) and digested with the restriction enzyme *Sma*I according to the manufacturer's instructions (Boehringer Mannheim). Restriction fragments were separated by PFGE in 1.2% agarose gels (SeaKem GTG Agarose; FMC BioProducts) by using a CHEF-DRIII system (Bio-Rad). The following parameters were used: pulse time increasing from 5 to 35 s; running timing, 22 h; voltage gradient, 6 V cm⁻¹; temperature, 13 °C; included angle, 120°. Analysis of chromosomal DNA-fragmentation profiles was performed by visual inspection of the photographic register of the ethidium-bromide-stained gels, in view of the criteria suggested by Tenover *et al.* (1995).

Computer-assisted analysis of the protein profiles and PFGE profiles. The protein profiles and the PFGE profiles were also analysed and compared by using Molecular Analyst Fingerprinting Plus software package, version 1.12 (Bio-Rad). The percentage similarities of the banding patterns were estimated using the Dice coefficient and were clustered by using the unweighted pair group method with arithmetic averages.

RESULTS AND DISCUSSION

The enterococci are generally recognized as being present in the gastrointestinal tracts of most animals as commensals rather than as aetiological agents of enteric disorders. However, infections with certain enterococcal strains have been associated with diarrhoea in neonates of different animal species (Collins *et al.*, 1988; Drolet *et al.*, 1990; Etheridge *et al.*, 1988; Etheridge & Vonderfecht, 1992; Tzipori *et al.*, 1984).

The 10 isolates included in the present study were recovered from pigs (five strains) and rats (five strains) with diarrhoea. The pathogenic properties of some of the strains isolated from rats (Etheridge *et al.*, 1988; Etheridge & Vonderfecht, 1992) and pigs (Drolet *et al.*, 1990) have been investigated previously, but their precise identification was not achieved. The first proposal that bacterial cultures isolated from neonatal rats with diarrhoea represented a new enterococcal species was made only in 1998 (Uhl *et al.*, 1998). Except for a few reactions, including the production of acids from D-mannitol, the phenotypic traits of their isolates were very similar to those of the strains previously isolated from rats with diarrhoea and referred to us for identification. We then compared those isolates to determine if they were different unique species or phenotypic variants of a single species.

Growth of pig strains on blood-agar plates and in Todd-Hewitt broth was evident after 18–24 h incubation at 37 °C, whereas rat isolates grew more slowly under the same conditions (Table 1). All of the clinical isolates were positive for pyrrolidonyl arylamidase activity, leucine aminopeptidase activity, hydrolysis of aesculin in the presence of bile, and hydrolysis of arginine. Growth occurred at 10 and at 45 °C and in broth containing 6.5% NaCl. Weak and/or delayed reactions were noticed with the rat isolates. All strains were susceptible to vancomycin and efrotomycin, non-motile and non-pigmented; all were negative for the production of gas in *Lactobacillus* deMan–Rogosa–Sharpe broth, and for pyruvate utilization. They all produced acids from maltose and D-ribose, and none produced acids from L-arabinose, glycerol, inulin,

Fig. 3. (a) PFGE profiles of *Sma*I-digested genomic DNA of *E. porcinus* (lanes: 2, DS 1390-83^T; 3, DS 1391-83; 4, DS 1393-83; 5, DS 432-84; 6, SS 1646) and *E. ratti* isolates (lanes: 7, DS 2075-87; 8, DS 2254-91; 9, DS 3567-95; 10, DS 3568-95; 11, SS 1506). Lanes 1 and 12, molecular mass markers, (lambda DNA concatemers ranging from 48.5 to 1018.5 kb). (b) Dendrogram resulting from the computer-assisted analysis of the PFGE profiles shown in (a). The scale represents mean percentages of similarity.

methyl α -D-glucopyranoside, D-raffinose, D-sorbitol, L-sorbose and sucrose. Additionally, they all reacted with the AccuProbe *Enterococcus* genetic probe and with anti-group D serum, although weaker reactions were observed for the rat isolates.

The strains recovered from pigs also produced acids from lactose, melibiose, D-trehalose and D-xylose, but failed to form acids from D-mannitol and sucrose. The strains obtained from rats were all negative for the production of acids from melibiose and D-xylose, and showed variable reactions for the production of acids from lactose. Only one strain isolated from a rat (SS 1506) was able to produce acids from D-mannitol and D-trehalose. On the basis of the results of conventional

tests, the strains isolated from pigs and rats were assigned to enterococcal group III. The most likely identities of these strains were phenotypic variants of *E. durans* or *E. hirae*.

To clarify the identification, analysis of the electrophoretic whole-cell protein profiles was performed and the protein profiles were compared with those obtained for the type strains of all of the enterococcal species. Two distinct clusters of virtually indistinguishable profiles were found: one was composed of strains isolated from pigs (Fig. 1), and the other was composed of strains isolated from rats (Fig. 2). These protein profiles were not identical to the profiles of any previously described *Enterococcus* species.

The results of DNA–DNA relatedness experiments were consistent with the results of the protein-profile analysis (Table 2). In view of the three major criteria for species-level DNA–DNA relatedness (Wayne *et al.*, 1987), all of the strains isolated from pigs were shown to have low levels of relatedness to the type strains of all known enterococcal species, as well to a strain (DS 2705-87^T) representative of those isolated from rats. On the other hand, they all had species-level relatedness to the pig isolate (DS 1390-83^T) that was selected as the reference strain. They had levels of relatedness ranging from 93 to 97% at optimal conditions (55 °C), percentages of divergence ranging from 0.5 to 1.0%, and levels of relatedness ranging from 93 to 98%, under stringent conditions (70 °C). The high levels of DNA relatedness found for pig isolates demonstrated that they belong to a new enterococcal species, which we propose to designate as *Enterococcus porcinus* sp. nov.

The strains isolated from rats were found to represent another new species. In addition to showing low levels of relatedness to the type strains of all known enterococcal species, as well as to strain DS 1390-83^T isolated from pigs, they all conformed to the criteria for species-level relatedness in experiments performed with the reference rat isolate (DS 2705-87^T). Their levels of relatedness ranged from 82 to 99%, at optimal conditions; the percentage divergence ranged from 0.5 to 2.5%, and their levels of relatedness under stringent conditions ranged from 83 to 99%. These isolates were related at the species level to a strain (SS 1506) representative of the recently reported new enterococcal species provisionally named '*E. ratti*' (Uhl *et al.*, 1998). As a detailed description of strain SS 1506 was not previously available and as it may represent an unusual variant of physiological group III (because of its positive reaction in the mannitol test), we propose strain DS 2705-87^T as the type strain for this new species, for which we propose the name *Enterococcus ratti* sp. nov.

The PFGE profiles generated after digestion of chromosomal DNA with *Sma*I indicated low levels of genetic diversity among both the *E. porcinus* isolates and those of *E. ratti* (Fig. 3), despite their occasional differences in terms of geographic area and period of

Table 3. Phenotypic characteristics useful for the differentiation of *Enterococcus* species included in physiological group III

Species: 1, *E. durans*; 2, *E. porcinus*; 3, *E. ratti*; 4, *E. hirae*; 5, *E. dispar*; 6, *E. faecalis* (mannitol-negative variant); 7, *E. faecium* (mannitol-negative variant). v, Variable reaction.

Characteristic	1	2	3	4	5	6	7
Litmus milk reaction*	+ / +	+ / -	- / -	+ / +	+ / -	v/v	+ / v
Pyruvate utilization	-	-	-	-	+	+	-
Hippurate hydrolysis	+	-	v	-	+	v	v
Tellurite tolerance	-	-	-	-	-	+	-
Acid production from:							
Arabinose	-	-	-	-	-	-	+
Glycerol	-	-	-	-	+	v	-
Lactose	+	+	v	+	+	v	+
Melibiose	+	+	v	+	+	-	+
Methyl α -D-glucopyranoside	-	-	-	-	+	-	-
Raffinose	-	-	-	+	+	-	v
Sucrose	-	-	-	+	+	-	-
Trehalose	+†	+	v	+	+	-	+†
Xylose	-	+	-	-	-	-	-

* + / +, Production of acids and clot; + / -, production of acids; - / -, no reactions; v/v, variable reactions.

† Exceptions occur occasionally.

isolation, suggesting that variability among strains may be restricted within each of these new species. The DNA-fragmentation profiles of *E. porcinus* strains were not identical but were all highly related, with differences related to a few bands. Four of the five *E. ratti* strains had identical PFGE profiles, which were also related to that obtained for the fifth strain (SS 1506).

Although the number of strains is still low, this report provides data on the phenotypic and genotypic characterization of two new enterococcal species that may represent aetiological agents associated with diarrhoeal diseases in animals.

On the basis of our findings, we suggest that the two new species (*E. porcinus* and *E. ratti*) be included in the conventional test scheme used to identify the species of *Enterococcus* and some related genera. The use of a combination of the characteristics shown in Table 3 is recommended for their differentiation from the other enterococcal species included in physiological group III. In addition, electrophoretic whole-cell protein profile analysis can be recommended as a reliable and relatively simple method for their differentiation from other closely related enterococcal species, since protein profiles correlate with results of DNA relatedness experiments.

Description of *Enterococcus porcinus* sp. nov.

Enterococcus porcinus sp. nov. (por.ci'nus. L. adj. *porcinus* of or pertaining to pigs).

Gram-positive cocci that occur in pairs or short chains. Colonies are non-pigmented and α -haemolytic on

sheep-blood agar. Facultatively anaerobic and catalase-negative. Growth occurs at 10 °C, at 45 °C and in broth containing 6.5 % NaCl. All of the strains are non-motile and are susceptible to vancomycin and efrotomycin. Positive for pyrrolidonyl arylamidase activity, leucine aminopeptidase activity, hydrolysis of aesculin in the presence of bile, and hydrolysis of arginine. Negative for the production of gas and for pyruvate utilization. Acids are produced from lactose, maltose, melibiose, D-ribose, D-trehalose and D-xylose, but not from L-arabinose, glycerol, inulin, D-mannitol, methyl α -D-glucopyranoside, D-raffinose, D-sorbitol, L-sorbose or sucrose. Strains react with the AccuProbe *Enterococcus* genetic probe and with Lancefield group D antiserum. Isolated from the intestines and faeces of pigs with diarrhoea. The type strain is DS 1390-83^T (=ATCC 700913^T =CCUG 43229^T =NCIMB 13634^T). The description of the type strain is similar to the description of the species. The GenBank accession number for the 16S rDNA sequence of DS 1390-83^T is AF335596.

Description of *Enterococcus ratti* sp. nov.

Enterococcus ratti sp. nov. (rat'ti. L. gen. masc. n. *ratti* of the rat).

Gram-positive cocci that occur in pairs or short chains. Colonies are non-pigmented and α -haemolytic or non-haemolytic on sheep-blood agar. Facultatively anaerobic and catalase-negative. Growth occurs at 10 °C, at 45 °C and in broth containing 6.5 % NaCl. All of the strains non-motile and are susceptible to vancomycin and efrotomycin. Strains are positive for pyrrolidonyl arylamidase activity, leucine aminopeptidase activity,

hydrolysis of aesculin in the presence of bile, and hydrolysis of arginine. Negative for the production of gas and for pyruvate utilization. Acids are produced from maltose and D-ribose, but not from L-arabinose, glycerol, inulin, melibiose, methyl α -D-glucopyranoside, D-raffinose, D-sorbitol, L-sorbose, sucrose or D-xylose. Most strains are also negative for the production of acids from D-mannitol and D-trehalose. Variable results may be obtained for lactose. Reactions are usually weak or delayed. Strains react with the AccuProbe *Enterococcus* genetic probe, and weak reactivity with Lancefield group D antiserum is observed. Isolated from the intestines and faeces of rats with diarrhoea. The type strain is DS 2705-87^T (=ATCC 700914^T =CCUG 43228^T =NCIMB 13635^T). In most respects the description of the type strain resembles the description of the species. The type strain is non-haemolytic, and negative for the production of acids from lactose. The GenBank accession number for the 16S rDNA sequence of *E. ratti* is AF326472.

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