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Two novel species Enterococcus lemanii sp. nov. and Enterococcus eurekensis sp. nov., isolated from a swine-manure storage pit.

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Abstract:	A polyphasic taxonomic study using morphological, biochemical, chemotaxonomic and molecular genetic methods was performed on six strains of an unknown Gram-positive, nonspore-forming, facultative anaerobic coccus-shaped bacterium isolated from a swine-manure storage pit. On the basis of 16S rRNA, RNA polymerase -subunit (rpoA), and the 60-kilodalton chaperonin (cpn60) gene sequence analyses, it was shown that all the isolates were enterococci but formed two separate lines of descent. Pairwise 16S rRNA sequence comparisons demonstrated that the two novel organisms were most closely related to each other (97.9 %) and to Enterococcus aquimarinus (97.8%). Both organisms contained major amounts of C16:0, C16:1 7c, C16:1 7c, and C18:1 7c /12t/9t as the major cellular fatty acids. Based on biochemical, chemotaxonomic, and phylogenetic evidence, the names Enterococcus lemanii sp. nov. (type strain PC32T = CCUG 61260T = NRRL B-59661T) and Enterococcus eurekensis sp. nov. (type strain PC4BT = CCUG 61259 T = NRRL B-59662 T) are proposed for the hitherto undescribed species.

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Two novel species *Enterococcus lemanii* sp. nov. and *Enterococcus eurekensis sp. nov.*, isolated from a swine-manure storage pit.

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Abstract

A polyphasic taxonomic study using morphological, biochemical, chemotaxonomic and molecular genetic methods was performed on six strains of unknown Gram-positive, nonspore-forming, facultative anaerobic coccus-shaped bacteria isolated from a swine-manure storage pit. On the basis of the 16S rRNA, RNA polymerase α-subunit (*rpo*A) and 60-kilodalton chaperonin (cpn60) gene sequence analyses, it was shown that all the isolates were enterococci but formed two separate lines of descent. Pairwise 16S rRNA gene sequence comparisons demonstrated that the two novel organisms were most closely related to each other (97.9 %) and to *Enterococcus aquimarinus* (97.8%). Both organisms contained major amounts of $C_{16:0}$, $C_{16:1}$ ω7c, $C_{16:1}$ ω7c, and $C_{18:1}$ ω7c /12t/9t as the major cellular fatty acids. Based on biochemical, chemotaxonomic and phylogenetic evidence, the names *Enterococcus lemanii* sp. nov. (type strain PC32^T = CCUG 61260^T = NRRL B-59661^T) and *Enterococcus eurekensis* sp. nov. (type strain PC4B^T = CCUG 61259 ^{T} = NRRL B-59662 ^{T}) are proposed for these hitherto undescribed species.

Key words: *Enterococci*-16S rRNA -phylogeny-taxonomy-manure-swine

Introduction

Enterococci are Gram-positive cocci that are found in a wide range of environments that include foodstuffs, plants and water; in addition they are often considered to be commensal inhabitants of warm-blooded animals but can also be found associated with reptiles and insects (Svec and Devriese 2009). Thus enterococci are commonly found in the faeces of humans and other animals and their presence in water can be considered an indication of faecal pollution (Marks 2001; Miescier and Cabelli 1982). Associated with intensive modern livestock production systems are the lagoon treatment or deep pit storage methods to manage liquid swine manure. The production of odorous chemicals that include ammonia, organic acids and alcohols, and sulphides by microorganisms, and an understanding of the underlying processes of the production of these compounds is the focus of ongoing research on swine manure management (Miller 2001; Le et al. 2005; Feilberg et al. 2010). Furthermore, storage facilities such as lagoons are subject to leakages that may contaminate surrounding water sources and a better understanding of the microorganisms present, including enterococcal species, may provide useful public health information (Harwood et al. 2000; Marks 2001). Previous research from our laboratories on the bacterial populations present in swine faeces and manure stored in underground pits has demonstrated that the vast majority of the microorganisms are low mol % G + C, Gram-positive anaerobic bacteria (Whitehead and Cotta 2001; Cotta et al. 2003). These ecosystems have also been found to be a rich source of new and novel bacterial genera and species (for example, see Whitehead et al 2003; Whitehead et al. 2005; Cotta et al 2009). The present study deals with our continuing investigation into the microflora of underground manure storage pits and the description of six strains of Gram-positively staining, facultatively anaerobic, coccus-shaped organisms. Comparative 16S rRNA gene sequence analyses confirmed that all the isolates were

enterococci and formed two separate branches. Pairwise analysis demonstrated that the two novel organisms were most closely related to *Enterococcus aquimarinus.* In addition to phenotypic traits the distinctiveness of the two species was confirmed using the sequences of the housekeeping genes *rpo*A and *cpn60*, pairwise analysis again indicating that these formed a group with *Enterococcus aquimarinus*.

Based on phenotypic, chemotaxonomic and phylogenetic considerations, it is proposed that the two unknown species originating from underground swine manure pits be classified as novel species of *Enterococcus,* for which we propose the names *Enterococcus lemanii* sp. nov. (type strain PC4B^T = CCUG 61259 ^{T} = NRRL B-59662 ^{T}) and *Enterococcus eurekensis* sp. nov. (type strain PC32^T = CCUG 61260^T = NRRL B-59661^T).

Methods

A number of isolates designated PC32^T (= CCUG 61260 ^{T} = NRRL B-59661^T), PPC27A (= CCUG 61269) and PPC38 (= CCUG 61261), PC4B^T (= CCUG 61259^T = NRRL B-59662^T), PPC15 (= CCUG 61268) and PPC107 (= CCUG 61272) were recovered from a swine manure storage pit located near Peoria, IL, USA. Isolations and enumerations were performed by plating samples that were serially diluted in anaerobic buffer onto habitat simulating media containing either 40% (v/v) substrate depleted rumen fluid (Dehority and Grubb 1976; Leedle and Hespell 1980) or 80% (v/v) clarified swine manure slurry (Slurry medium; 8000 x g, 20 min, 4^{o}C) (Cotta et al. 2003). The media used in these experiments were prepared anaerobically using the method of Hungate as modified by Bryant (1972). The basic media contained macrominerals, microminerals, buffers, reducing agents and other components as in the RGM medium as described by Hespell et al. (1987) or anaerobic BHI medium as described by Whitehead and Flint

(1995). No additional volatile fatty acids were added to slurry containing media. Glucose, xylose, cellobiose, maltose, starch (0.05% w/v each) and peptone (0.3% w/v) were provided as complex carbon, nitrogen and energy sources. Plates were initially incubated anaerobically in a 96% carbon dioxide, 4% hydrogen atmosphere at room temperature for manure slurry samples to simulate the pit environment (Cotta et al. 2003). The new isolates were subsequently determined to grow equally well at 37°C and cultures were routinely grown at this temperature. Single colonies were picked and repeatedly streaked out until pure cultures were obtained.

Gram–staining was performed with the BD Gram Stain Kit (Sigma, St. Louis, IL), according to manufacturer's instructions. Cell morphology and motility was examined via phase contrast microscopy using an Olympus DB12 microscope. The strains were biochemically characterised by using a combination of conventional tests, the API Rapid ID 32S API Rapid ID 32A, API 50CH and API ZYM systems, according to the manufacturer's instructions (API bioMériux, Marcy L'Etoile , France). All biochemical tests were performed in duplicate. Coagulase activity was determined using the Fluka Coagulase Test kit (Sigma, St. Louis, IL), according to the manufacturer's instructions. *Staphylococcus aureus* was used as a positive control. Bile Esculin Agar was obtained from Difco. The presence of Lancefield A,B,C,D,F and G group antigens was assayed using the SLIDEX Strepto Plus kit (Biomerieux, Durham, NC), according to the manufacturer's instructions. Other classical phenotypic tests were performed as described by Tindall et al. (2007). Hydrogen production was determined by analyses of headspace gases after 24 hrs of growth on BHI using a Hewlett Packard HP 5890 Gas Chromatograph equipped with a packed column of Carbosieve G (100/120 mesh, 3 mm x 2.0 mm id, Supelco) heated to 170°C and detected by thermoconductivity with argon as the carrier (10 ml/min) and the reference gas (15 ml/min).

For the determination mol% of G+C content and PCR of genes used for phylogenetic analyses, DNA was isolated by the method of Saito and Miura (1963). To provide a rapid means of identification and determine the phylogenetic position of the swine manure isolates, 16S rRNA gene sequence analysis was performed as described by Whitehead and Cotta (2004). Sequences for *rpo*A genes were amplified as described by Naser et al. (2005). Sequences for *cpn60* were obtained as described previously by Vermette et al. (2010), and closely related sequences were obtained from the cpnDB chaperonin sequence database (http://www.cpndb.ca/cpnDB/home.php). For the 16S rRNA and *rpo*A genes the closest known relatives of the new isolates were determined by performing database searches of EMBL / GenBank using the program FASTA (Pearson and Lipman 1988). These sequences and those of other related strains were aligned with the newly determined sequences using the program Clustal-W (Thompson et al., 1994) contained within the program SEQtools (Rasmussen 2002). The resulting multiple sequence alignment was corrected manually to remove the first 100 nucleotide bases to reduce alignment ambiguities using the program GeneDoc (Nicholas et al. 1997). Phylogenetic analysis was performed on 1320 shared nucleotides and the phylogenetic tree was constructed according to the neighbor-joining method (Saitou and Nei, 1987) with the programs SEQtools and TREEVIEW (Page 1996). The stabilities of the groupings were estimated by bootstrap analysis (1000 replications) using the same programs. Where necessary, pairwise sequence comparisons were determined using the same programs. For the *rpoA* gene (612 nucleotide bases) and *cpn60* gene (551 nucleotide bases) the same method of phylogenetic analyses were used. All major branching nodes were confirmed by maximum parsimony (data not shown). RAPD-PCR analyses were carried out with primers m13 and D11344 as previously

described (Descheemaeker et al. 1997; Andrighetto et al. 1998). Determination of mol % G+C was carried out by thermal denaturation of chromosomal DNA using a Beckman model DU 640 spectrophotometer equipped with a high performance temperature controller and Tm analysis software (Johnson, 1994).

Fatty acid methyl ester (FAME) analysis was performed using gas chromatography, performed in a standardised protocol using a system similar to that of the MIDI Sherlock MIS system (www.ccug.se/pages/CFA_method_2008.pdf) as described previously (Miller 1982; Sasser 1990). Cells were grown for 3 days on Chocolate agar (Brain Heart Infusion, Difco 241830) under anaerobic conditions at 37°C. Analysis was carried out with a Hewlett Packard HP 5890 Gas Chromatograph equipped with a phenyl methyl silicone fused silica capillary column (HP-5 25m x 0.2 mm x 0.33 mm film thickness) and a flame ionization detector. Hydrogen was used as the carrier gas. The temperature program was initiated at 170 °C and increased at 5 °C min^{-1} to a final temperature of 270 °C. Integration of peaks and further calculations was performed by a HP 3396A integrator. Fatty acid methyl esters (FAMEs) were identified and quantified, and the relative amount of each fatty acid was expressed in terms of the percentage of total fatty acids in the profile of the strain.

Results and Discussion

16S rRNA gene sequence analysis demonstrated that the six isolates belonged to two distinct but closely related groups. Group 1 consisted of three isolates i.e. $PC32^T$ (= CCUG 61260^T = NRRL 61260^T), PPC27A (= CCUG 61269) and PPC38 (= CCUG 61261); Group 2 consisted of three

isolates i.e. PC4B^T (= CCUG 61259^T), PPC15 (= CCUG 61268) and PPC107 (= CCUG 61272). The strains in each group had a number of similar features that included being Gram-positive, non-motile coccal or ovoid-shaped cells in single cells, pairs, or short chains. All strains were catalase- and oxidase-negative.

After 48hrs of anaerobic growth at 37^{0}C on blood agar plates, colonies varied in diameter from 0.5-1-2 mm in diameter and were whitish-grey in color and smooth and flat. No hemolysis was observed. Growth was observed at 10^{o}C, 30 ^{o}C, 37 ^{o}C and 45^{o}C. Optimal growth was observed with Brain Heart Infusion (BHI) at 37 ^{o}C. Growth was also observed in the presence of 6.5% NaCl. Growth on BHI and Bile Esculin Agar plates under aerobic conditions at 37^o mirrored that on blood agar. All strains produced whitish colonies capable of hydrolysis of esculin. Such variation in growth on Bile Esculin Agar by different enterococcal species and strains has been previously demonstrated (Weiss et al. 2005). Lancefield A, B,C,D,F and G group antigens were not detected. According to the manufacturer's information such a result is not unusual with hemolysis-negative species. The most useful biochemical and enzymatic tests used to distinguish *E. lemanii*, *E. eurekensis* and *E.aquimarinus* are given in Table 1, with the presence or absence of leucyl glycine arylamidase, glycine arylamidase, esterase (C_4), and N-acetyl-β-glucosaminidase being the most discriminative. Cellular fatty acid analysis demonstrated that both novel organisms contain major amounts of $C_{16:0}$, $C_{16:1}$ ω7c, $C_{16:1}$ ω7c, and $C_{18:1}$ ω7c /12t/9t (Table 2). Using identical growth conditions *E. eurekensis* PC4B^T contained almost twice as much $C_{14:0.}$as *E. lemanii* PC32^T. The fatty acid profiles demonstrate that $C_{14:0}$, $C_{18:2}$ ω6,9c / ante$C_{18:0}$, and $C_{18:1}$ ω7c /12t/9t are particular useful is the differentiation between the two novel strains and *E. aquimarinus*.

RAPD-PCR analyses of the strains using primers m13 and D11344 indicated that the strains are of separate clonal lineage and are distinct from *E. aquimarinus* (Supplementary Fig. 1 and 2).

To investigate the phylogenetic affinity of the unknown isolates the 16S rRNA gene was amplified by PCR and sequenced. Sequence searches of EMBL/GenBank revealed that the unknown organisms were members of the phylum *Firmicutes* and most closely related to members of the genus *Enterococcus*. A phylogenetic tree, constructed by the neighbor-joining method, depicting the phylogenetic affinity of the novel bacteria as exemplified by strain PC4B^T and PC32^T, is shown in Fig. 1. Pairwise comparisons between the two novel species (1415 nb) demonstrated that the sequences represented two distinct but closely related lines of decent within the enterococci. Each new enterococcal group contained sequences of very high homogeneity (99.5-100%) and were 97.8% related to each other. For a continuous stretch of 1500 nucleotide bases, PC32^T, PPC27A and PCC38 differed by a total of only 6 bases; likewise PC4B^T, PPC15 and PPC107 differed by only 5 bases confirming the genetic homogeneity of the two groups. The two organisms formed a cluster with *Enterococcus aquimarinus* (Svec et al. 2005) with 16S rRNA gene sequence similarity values of 97.8%. There is no precise correlation between 16S rRNA sequence divergence and species delineation when considering different genera, but it is generally recognised that divergence values of 3% or more are significant (Stackebrandt and Goebel 1994). However, more recent information demonstrates that this value can be decreased to 1.3% without loss of resolution (Stackebrandt and Ebers 2006) in that corresponding DNA-DNA hybridization values remain below 70%, the generally accepted limit for species delineation (Wayne et. al 1987).

Many enterococci share high levels of sequence similarity based on 16S rRNA gene sequence analysis and Nasser et al. (2005) demonstrated the usefulness of housekeeping genes as

alternative phylogenetic and identification tools. Therefore, we further investigated the distinctiveness of the unidentified strains using *rpo*A gene sequence analysis. Phylogenetic analysis employing *rpo*A as shown in Fig 2, again showed that the novel organisms clustered with *Enterococcus aquimarinus*, strains PC32^T and PC4B^T possessing sequence similarity values of 90.9% and 92.7% respectively. Although Naser et al. (2005) demonstrated that interspecies variations of the *rpo*A could be based upon similarity values of <97%, Sistek et al. (2011) have demonstrated this value can be increased to <99%. Similarly, sequence analysis using the universal 60 kDa chaperonin gene (*cpn60*) has been shown to offer superior discrimination between closely related organisms at the species level and has been applied to enterococci (Vermette et al. 2009). A phylogenetic tree demonstrating the phylogenetic relationships of the *cpn60* sequences of the two novel organisms within the enterococci is shown in Fig. 3. Pairwise sequence analyses demonstrated that the novel organisms formed a cluster with *E. aquimarinus* although each displayed low sequence similarities of approximately 83%, demonstrating the separateness of these species. In all three phylogenetic analyses undertaken, the two novel species form a robust cluster with *E. aquimarinus* supported by significant bootstrap values. However, the branching node shared by *E. eurekensis* and *E. aquimarinus* is not supported by high bootstrap values demonstrating that *E. eurekensis* and *E. lemanii* can be interchangeable reflecting their identical rRNA gene similarity values (97.8%) to *E. aquimarinus*.

The GenBank accession numbers for the 16S rRNA, *rpoA*, and *cpn60* gene sequences of *E. lemanii* strains are PC4B^T: AF445305, JQ003583, JQ038128; PPC15: AF445307, JX087947, JQ999956; PPC107: AF445284, JX087946, JQ999959. The GenBank accession numbers for the 16S rRNA, *rpoA*, and *cpn60* gene sequences of *E. eurekensis* strains are: PC32^T: AF445301, JQ003584,

JQ38129; PPC27A: AF445278, JQ087948, JQ999956; PPC38: AF445300, JX087949, JQ999958.

The unidentified organisms from swine manure were found to be morphologically and biochemically consistent with their assignment to the genus *Enterococcus*. In addition to their 16S rRNA, *rpo*B, and *cpn60* sequences, the two novel organisms may also be differentiated from each other and *E. aquimarinus* by characteristics shown in Table 1 and Supplementary Figures 1 and 2. Based on phenotypic, chemotaxonomic, and phylogenetic evidence that demonstrates the separateness of the novel organisms from other members of the genus *Enterococcus*, we consider the unidentified organisms merit classification in two new species of the genus *Enterococcus*, as *Enterococcus lemanii* sp. nov. (type strain PC4B^T = CCUG 61259^T = NRRL B-59662^T) and *Enterococcus eurekensis* sp. nov. (type strain PC32^T = CCUG 61260^T = NRRL B-59661^T).

Description of *Enterococcus lemanii* sp. nov.

lemanii: le.ma'ni.i N.L. gen. mas. n. of Leman, named after the late American veterinarian Allen D. Leman for his contributions toward swine disease and swine production.

Cells consist of Gram-positive, catalase- and oxidase-negative, non-motile coccal or ovoid-shaped cells in single cells, pairs, or short chains. After 48hrs of anaerobic growth at 37^{0}C on blood agar plates, colonies are 1-2 mm in diameter, grey, smooth and flat. No hemolysis is observed on blood agar. Facultatively anaerobic. Grows at 45^o C but slow growth is observed at 10^o C. Grows in BHI with 6.5% NaCl. Catalase, urease and nitrate reduction activity is negative. Hydrolyses esculin and starch but not indole or hippurate. Voges-Proskauer negative. Coagulase activity is negative. Lancefield A, B,C,D,F and G group antigens are not detected. Classical

methods show that cellobiose, fructose, glucose, lactose, maltose and sucrose are utilized but arabinose and xylose are not. In the API 50CH tests, strain PC4B^T provides a positive reaction for eusculin hydrolyisis with weakly positive reactions for N-acetyl glucosamine, Cellibiose, Glucose, Fructose, Maltose, Mannose, Melibiose, Lactose, Salicin, Sorbose, D and L-xylose. Using the API Rapid ID32An test kit positive reactions are obtained for *N*-acetyl-β-glucosaminidase, alanine arylamidase (weak), arginine arylamidase (weak), β-galactosidase, α-glucosidase (weak), β-glucosidase, mannose, and raffinose. Negative reactions are obtained with alkaline phosphatase, arginine dihydrolase, α- fucosidase, β-glucuronidase, glutamic acid decarboxylase, glutamyl glutamic acid arylamidase, glycine arylamidase, indole hydrolysis, leucyl glycine arylamidase, nitrate reduction, proline arylamidase and urease. Reactions for α-galactosidase, histidine arylamidase, leucine arylamidase, phenyl alanine arylamidase, pyroglutamic acid arylamidase, serine arylamidase and tyrosine arylamidase are weak or strain dependent. α-arabinosidase and β-galactosidase 6-phosphatase and are strain dependent. Employing the API Rapid ID32S system positive reactions are produced from α-galactosidase, glycyl-tryptophan arylamidase, lactose, maltose, pyroglutamic acid arylamidase, D-raffinose, sucrose and trehalose. Negative reactions are obtained with acetoin, β-glucosidase, β-glucuronidase, alkaline phosphatase, alanyl phenylalanine proline arylamidase, arginine dihydrolase, D-arabitol, cyclodextrin, glycogen, hippurate, β-mannosidase, melezitose, pullulan, sorbitol, tagatose and urease. L-arabinose, *N*-acetyl-β-glucosaminidase, β-galactosidase, mannitol, melibiose, methyl β-D-glucopyranoside and D-ribose are strain dependent. Using the API ZYM system reactions for α-chymotrypsin, β-galactosidase and leucine arylamidase are positive or weakly positive depending on the strain. No activity is detected for *N*-acteyl-β-glucosaminidase, alkaline phosphatase, *N*-AS-BI-phosphohydrolase, cystine arylamidase,

esterase (C4) and esterase lipase (C8), α-glucosidase, β-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase, α-fucosidase, trypsin or valine arylamidase. Acid phosphatase and α-galactosidase are strain dependent. Major fatty acids are $C_{16:0}$, $C_{16:1}$ ω7c, $C_{16:1}$ ω7c, and $C_{18:1}$ ω7c /12t/9t. No hydrogen production is observed after 24 hr growth on BHI. The G+C content of the DNA of the type strain is 36.0 mol%. Isolated from a swine manure storage pit. Habitat range not known. The type strain is PC4B^T (= CCUG 61259^T = NRRL B-59662^T).

Description of Enterococcus eurekensis sp. nov.

eurekensis: eu. re.ken'sis N.L. masc. adj. pertaining to Eureka, a city in Illinois, USA, from where the type strain was isolated.

Cells consist of Gram-positive, catalase- and oxidase-negative, non-motile coccal or ovoid-shaped cells in single cells, pairs, or short chains. Growth is observed at 10^{o}C, 30^{o}C, 37^{o}C, and 45^{o}C. Optimal growth is observed with Brain Heart Infusion (BHI) at 37^{o}C. Growth is also observed in the presence of 6.5% NaCl. Growth on BHI and Bile Esculin Agar plates under aerobic conditions at 37^o is comparable to that on blood agar. All strains produced whitish colonies (0.5-2mm) capable of hydrolysis of esculin. No hemolysis is observed on blood agar. Facultatively anaerobic. Grows at 45^{o}C but slow growth is observed at 10^{o}C. Growth in BHI with 6.5% NaCl. Indole production is negative. Catalase, urease and nitrate reduction activity is negative. Hydrolyses esculin and starch but not indole or hippurate. Voges-Proskauer negative. Coagulase activity is negative. Lancefield A, B,C,D,F and G group antigens are not detected. Classical methods show that cellobiose, fructose, glucose, lactose, maltose and sucrose are utilized but arabinose and xylose are not. Using the API 50CH test system PC32^T gives positive reactions for N-acetyl glucosamine, cellibiose, eusculin hydrolyisis, glucose, Fructose, Mannose,

Salicin, Sorbose, Maltose, Ribose, L-xylose; weakly positive reactions for Arbuin, Dulcitol, Lactose, Melibiose, Raffinose, Sucrose and Trehalose. Using the API Rapid ID32An, positive reactions are obtained for *N*-acetyl-β-glucosaminidase, alanine arylamidase, α-arabinosidase, arginine arylamidase, β-galactosidase 6-phosphatase, α-glucosidase, β-glucosidase, glycine arylamidase, histidine arylamidase, leucine arylamidase, leucyl glycine arylamidase, mannose, phenyl alanine arylamidase, proline arylamidase, pyroglutamic acid arylamidase, raffinose, serine arylamidase and tyrosine arylamidase. Negative reactions are obtained with alkaline phosphatase, arginine dihydrolase, α- fucosidase, β-glucuronidase, glutamic acid decarboxylase, glutamyl glutamic acid arylamidase, indole hydrolysis, nitrate reduction and urease. β-galactosidase is strain dependent. Employing the API Rapid ID32S system positive reactions are produced from L-arabinose, α-galactosidase, glycyl-tryptophan arylamidase, lactose, maltose, mannitol, melezitose, methyl β-D-glucopyranoside, pyroglutamic acid arylamidase, D-raffinose, sucrose and trehalose. Negative reactions are obtained with acetoin, β-glucosidase, alkaline phosphatase, alanyl phenylalanine proline arylamidase, arginine dihydrolase, D-arabitol, cyclodextrin, β-glucuronidase, glycogen, hippurate, melibiose, pullulan, D-ribose, sorbitol, tagatose and urease. *N*-acetyl-β-glucosaminidase, β-galactosidase, and β-mannosidase were found to be strain dependent. Using the API ZYM system, positive reactions are obtained for *N*-acteyl-β-glucosaminidase, α-chymotrypsin, β-galactosidase, and leucine arylamidase. No activity is detected for alkaline phosphatase, *N*-AS-BI-phosphohydrolase, esterase (C4), esterase lipase (C8), α-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase, α-fucosidase, or trypsin. Acid phosphatase, cystine arylamidase, α-galactosidase, and β-glucosidase are weakly positive or strain dependant. Valine arylamidase is strain dependent. The G+C content of the DNA of the type strain is 37.9 mol%. Major fatty acids are $C_{14:0}$, $C_{16:0}$, $C_{16:1}$ ω7c, $C_{16:1}$ ω7c and $C_{18:1}$ ω7c

/12t/9t. No hydrogen production is observed after 24 hr growth on BHI. Isolated from a swine manure storage pit. Habitat range not known. The type strain is PC32^T (= CCUG 61260^T = NRRL B-61260^T)

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Table 1. Characteristics useful in differentiating Enterococcus *eurekensis* sp. nov., *Enterococcus lemanii* sp. nov., and *E. aquimarinus*.

[a]**Characteristic**	*Enterococcus eurekensis* (3 strains)	*Enterococcus lemanii* (3 strains)	*Enterococcus aquimarinus* CCUG 51308^T
API Rapid ID32An			
Arginine arylamidase	+	w	-
β-galactosidase	d	+	+
Leucyl glycine arylamidase	**+**	**-**	**-**
Leucine arylamidase	+	w	-
Pyroglutamic acid arylamidase	+	w	+
Alanine arylamidase	+	w	-
Glycine arylamidase	**+**	**-**	**-**
Histidine arylamidase	+	w	+
Serine arylamidase	+	w	-
API ZYM			
Esterase (C_4)	**-**	**-**	**+**
Esterase lipase (C8)	-	-	w
N-acetyl-β-glucosominidase	**+**	**-**	**+**
Hydrogen from BHI Growth	-	-	+
DNA G +C content (mol%)	36.0	37.9	38.7
Source	Swine manure	Swine manure	Sea water

+, positive; -, negative; d, strain dependant; w, weakly positive. Biochemical tests given in bold typeface are the most useful.

Full profiles for API Rapid ID32S, API Rapid ID32An and API ZYM for all strains are available at http://www.ccug.se. Additional API test systems are also available for the Type strains at the same address.

Table 2. Cellular fatty acid compositions (%) of *Enterococcus eurekensis* sp. nov., *Enterococcus lemanii* sp. nov., and *E. aquimarinus*.

Fatty acid[a]	***E. eurekensis* sp. nov. CCUG 61259^T**	***E. lemanii* sp. nov. CCUG 61260^T**	***E. aquimarinus* CCUG 51308^T**
$C_{13:0}$	1.8	2.7	0.6
iso-2OH-$C_{13:0}$	0.9	0.6	1.5
$C_{14:0}$	**11.2**	6.6	**11.6**
$C_{14:1}\omega7c$	0.3	-	-
$C_{16:0}$	**22.2**	**18.0**	**18.1**
$C_{16:1}\ \omega7c$	**17.8**	**16.1**	**24.0**
$C_{16:1}\ \omega7c$ / iso-2OH-$C_{15:1}$ $\omega7c$ / $C_{16:1}\ \omega6c$	2.2	3.3	-
$C_{16:1}\ \omega7c$	**18.9**	**16.1**	**24.0**
$C_{18:0}$	0.4	0.8	4.3
$C_{18:1}\ \omega9c$	0.4	2.0	6.3
$C_{18:2}\ \omega6,9c$ / ante$C_{18:0}$	0.4	2.0	**11.8**
$C_{18:1}\ \omega7c$ /12t/9t	**42.8**	**50.6**	**22.5**

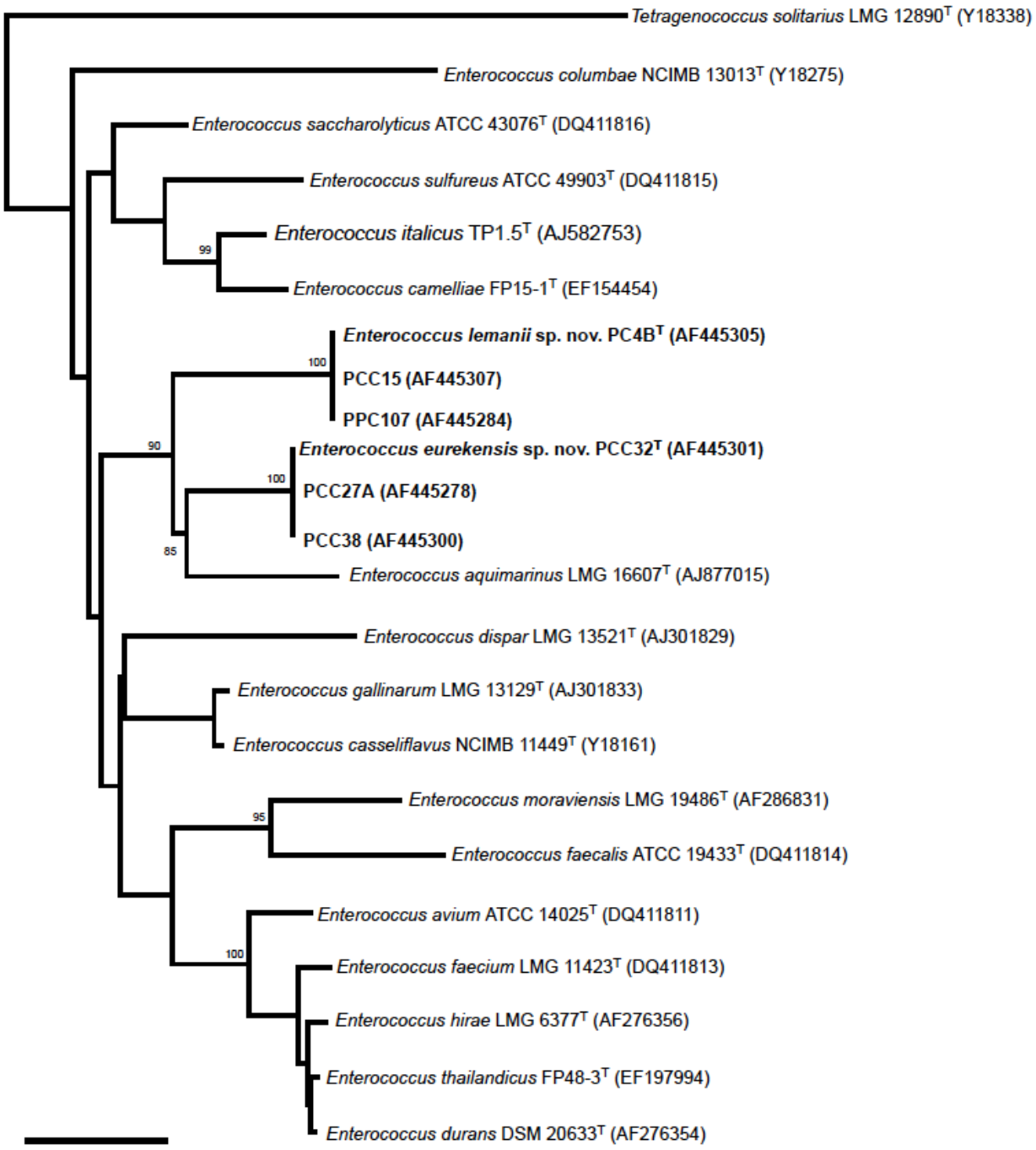
[a] Data obtained from this study, all growth conditions were identical as given in the text. Bold values represent major products

Captions for Figure

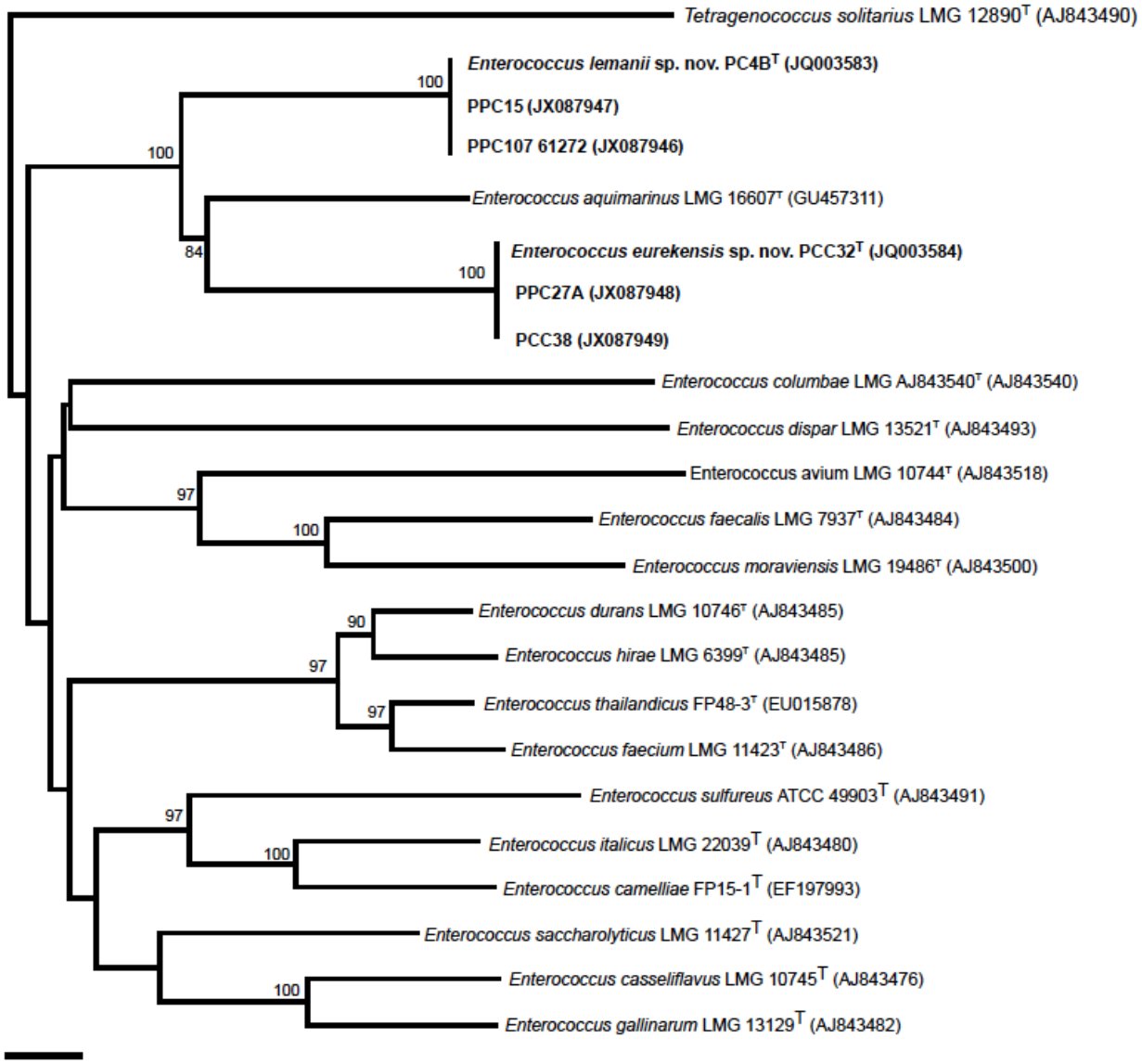
Fig. 1 16S rRNA tree showing the phylogenetic relationships of *Enterococcus lemanii* sp. nov. and *Enterococcus eurekensis* sp. nov., and some other *Enterococcus* species. The tree constructed using the neighbour-joining method was based on a comparison of approx. 1320 nucleotides. *Tetragenococcus solitarius* LMG 12890^T was used as the outgroup and bootstrap values, expressed as a percentage of 1000 replications, are given at branching points; only significant valuves are shown. The bar represents a sequence divergence range of 1 %

Fig. 2 *rpo*A tree showing the phylogenetic relationships of *Enterococcus lemanii* sp. nov. and *Enterococcus eurekensis* sp. nov., and some other *Enterococcus* species. The tree constructed using the neighbour-joining method was based on a comparison of approximately 615 nucleotides. *T. solitarius* LMG 12890^T was used as the outgroup and bootstrap values, expressed as a percentage of 1000 replications, are given at branching points; only significant values are shown. The bar represents a sequence divergence range of 1 %

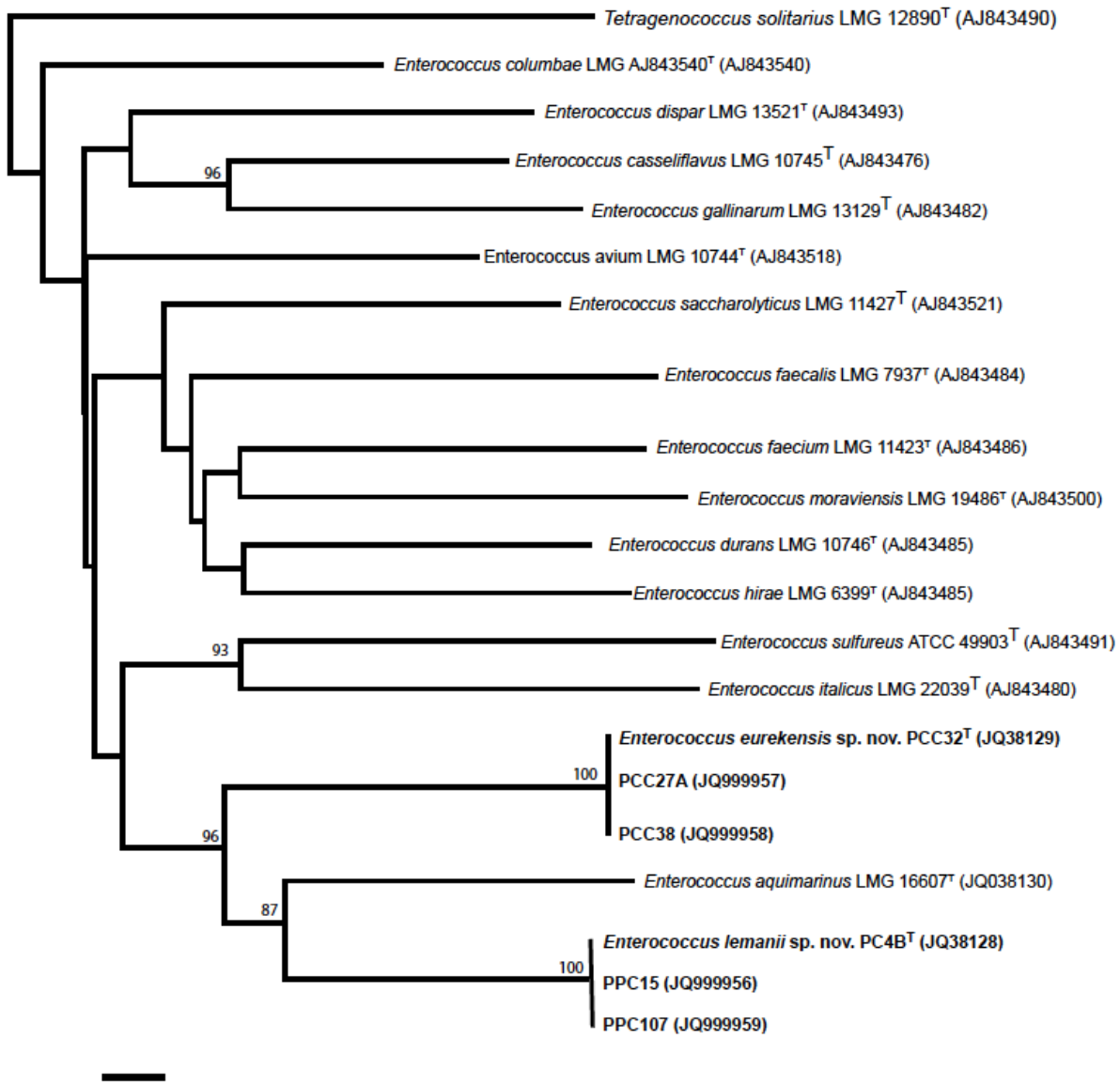
Fig. 3 Phylogenetic relationships of *Enterococcus lemanii* sp. nov. and *Enterococcus eurekensis* sp. nov., and some other *Enterococcus* species based on the *cpn60* gene. The tree constructed using the neighbour-joining method was based on a comparison of approximately 605 nucleotides. *T. solitarius* LMG 12890^{T} was used as the outgroup and bootstrap values, expressed as a percentage of 1000 replications, are given at branching points; only significant values are shown. The bar represents a sequence divergence range of 1 %



Cotta et al, Fig 1.



Cotta et al, Fig 2.



Cotta et al, Fig 3.

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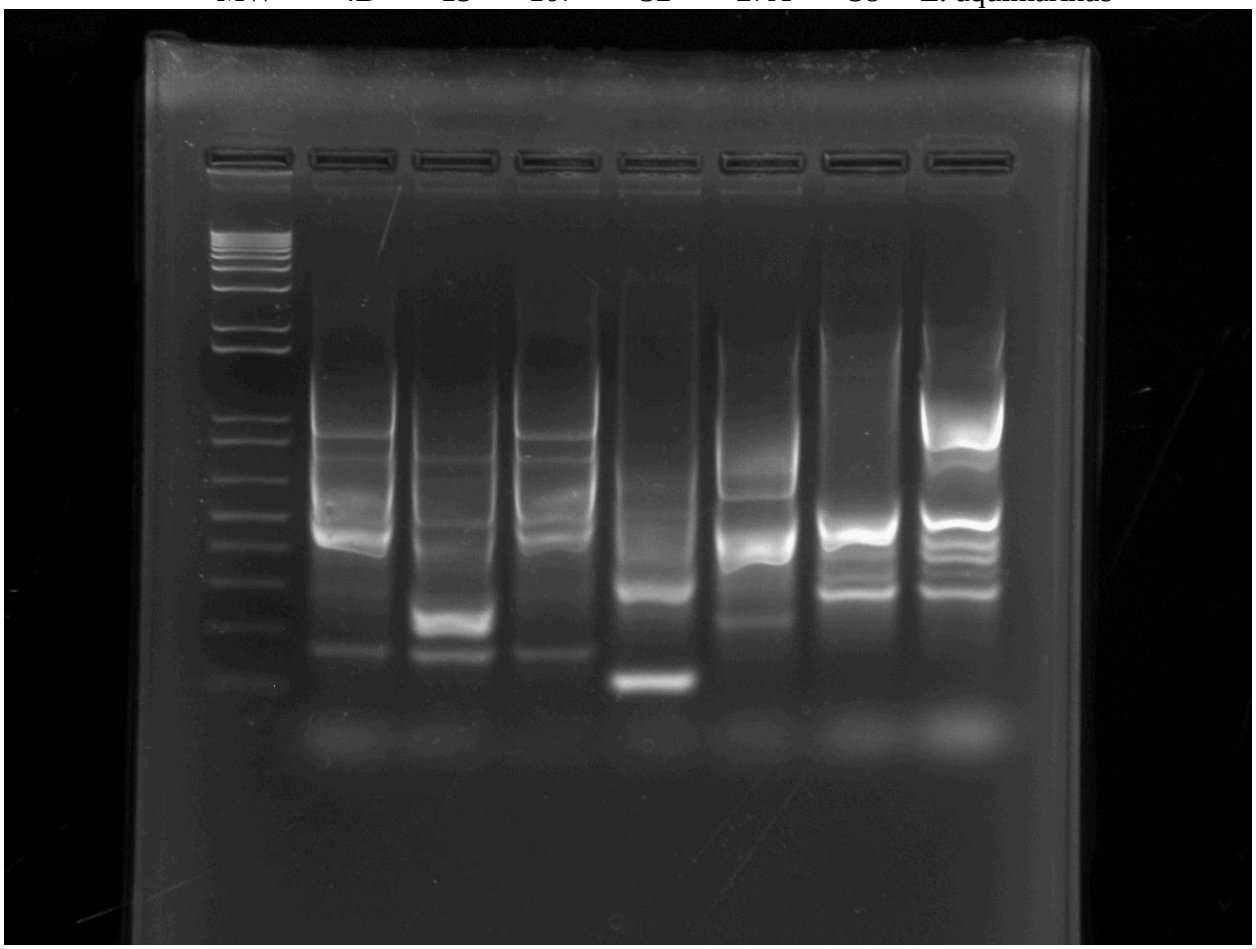
Michael A. Cotta, Terence R. Whitehead, Enevold Falsen, Edward Moore, and Paul A. Lawson. Supplemental Fig. 1. RAPD-PCR of Enterococcus Strains Using m13 Primer

MW 4B 15 107 32 27A 38 E. aquimarinus



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Michael A. Cotta, Terence R. Whitehead, Enevold Falsen, Edward Moore, and Paul A. Lawson. Supplemental Fig. 2. RAPD-PCR of Enterococcus Strains Using D11344 Primer