**Enterococcus crotali** sp. nov., isolated from faecal material of a timber rattlesnake

Richard W. McLaughlin,1,2,* Patricia Lynn Shewmaker,3 Anne M. Whitney,3 Ben W. Humrighouse,3 Ana C. Lauer,3 Vladimir N. Loparev,3 Christopher A. Gulvik,3 P. A. Cochran2 and Scot E. Dowd4

**Abstract**

A facultatively anaerobic, Gram-stain-positive bacterium, designated ETRF1T, was found in faecal material of a timber rattlesnake (*Crotalus horridus*). Based on a comparative 16S rRNA gene sequence analysis, the isolate was assigned to the genus *Enterococcus*. The 16S rRNA gene sequence of strain ETRF1T showed >97% similarity to that of the type strains of *Enterococcus rotai*, *E. caccae*, *E. silesiacus*, *E. haemoperoxidus*, *E. ureasiticus*, *E. moraviensis*, *E. plantarum*, *E. quebecensis*, *E. ureilyticus*, *E. termitis*, *E. rivorum* and *E. faecalis*. The organism could be distinguished from these 12 phylogenetically related enterococci using conventional biochemical testing, the Rapid ID32 Strep system, comparative pheS and rpoA gene sequence analysis, and comparative whole genome sequence analysis. The estimated in silico DNA–DNA hybridization values were <70%, and average nucleotide identity values were <96%, when compared to these 12 species, further validating that ETRF1T represents a unique species within the genus *Enterococcus*. On the basis of these analyses, strain ETRF1T (=CUG 65857=LMG 28312) is proposed as the type strain of a novel species, *Enterococcus crotali* sp. nov.

At the time of writing, there are 50 validly named species in the genus *Enterococcus* on the List of Prokaryotic Names with Standing in Nomenclature (LSPN; www.bacterio.net [11]). Several species are common inhabitants of the intestinal tracts of both humans and animals [2]. Members of the genus have been isolated from fermented foods, such as pickles [3] and cheese [4], or used to make foods, such as soy yogurt [5]. In several countries, *Enterococcus faecium* is used as a probiotic [6, 7]. However, several species are associated with human infections [8]. In the past, a combination of phenotypic and molecular methods has been used to identify enterococcal species [9–11]. Using a polyphasic approach, we describe a novel *Enterococcus* species (represented by strain ETRF1T) isolated from faecal material of a timber rattlesnake.

To study the gut microbiome of the timber rattlesnake, bacteria were isolated from faecal material inoculated onto a sheep blood agar plate containing 10 µg rifampicin ml−1 and incubated aerobically at 37 °C for 48 h. Small, convex, circular and off-white colonies were visualized. A pure culture was obtained and assessed for phenotypic characteristics by using conventional biochemical testing [11–13], and the API Rapid ID32 Strep system (bioMerieux) according to the manufacturer’s instructions. For the ID32 testing, all strains were grown on Columbia agar with colistin/naladixic acid at 37 °C for 24 h and tests were repeated two to three times. The nearly full-length 16S rRNA gene of the rattlesnake strain ETRF1T was amplified and sequenced as previously described [14], but using a subset of eight sequencing primers (fD1-5p, F785, R357, F357, BSF1099, BSR1114, BSR1407 and rP2-5p). Pairwise sequence comparisons were made between the target species consensus sequence (accession no. KF803651, 1483 bp) and sequences deposited in GenBank and CDC MicrobeNet (http://microbenet.cdc.gov) nucleotide databases using the BLAST N algorithm. Comparative 16S rRNA gene sequence analysis has limited use in interspecies differentiation of the genus *Enterococcus*. Previous studies [10, 15] have shown that several distinct *Enterococcus* species have high or sometimes identical sequence similarity, and the proposed cut-off value of 98.7% for species differentiation [16] is not valid with this genus. For this study, we followed the recommendations suggested by Tindall et al. [17] and used a cut-off of 97%. Comparative 16S rRNA gene analysis revealed >97% sequence similarity with 12 previously described *Enterococcus* species: *E. rotai* (100%), *E. caccae* (99.9%), *E. silesiacus* (99.9%), *E. haemoperoxidus* (99.8%), *E.**
ureasiticus (99.8%), E. moraviensis (99.7%), E. plantarum (99.7%), E. quebecensis (99.7%), E. ureilyticus (99.7%), E. termitis (99.3%), E. rivorum (98.1%) and E. faecalis (97.4%). These 12 species have been previously shown to group within the E. faecalis clade [18–23]. The 16S rRNA gene sequences from strain ETRF1\textsuperscript{T} and these 12 phylogenetically related Enterococcus species were then aligned using the CLUSTAL W algorithm [24] and trimmed to a 1483 bp consensus. The program MEGA version 6 [25] was used to create a phylogenetic tree by the neighbour-joining method. To quantify the stability of the groups, a bootstrap resampling analysis of 1000 trees from the data set was performed (Fig. 1). The 16S rRNA gene sequence of strain ETRF1\textsuperscript{T} was positioned in the E. faecalis group on a branch with E. rotai, E. silesiacus, E. caccae, E. ureasiticus and E. ureilyticus.

Conventional biochemical phenotypic testing (with an incubation period of up to 7 days) and Rapid ID32 STREP testing were performed on strain ETRF1\textsuperscript{T} and the type strains of the 12 phylogenetically related species: E. rotai LMG 26676\textsuperscript{T} [23], E. caccae BAA-1240\textsuperscript{T} [21], E. silesiacus LMG 23085\textsuperscript{T} [19], E. haemoperoxidus LMG 19487\textsuperscript{T} [18], E. ureasiticus DSM 23328\textsuperscript{T} [22], E. moraviensis LMG 19486\textsuperscript{T} [18], E. plantarum LMG 26214\textsuperscript{T} [26], E. quebecensis LMG 26306\textsuperscript{T} [22], E. ureilyticus LMG 26676\textsuperscript{T} [23], E. termitis LMG 8895\textsuperscript{T} [19], E. rivorum LMG 25899\textsuperscript{T} [27] and E. faecalis ATCC 19433\textsuperscript{T} [28]. The key biochemical reactions distinguishing strain ETRF1\textsuperscript{T} from the 12 phylogenetically related species are shown in Table 1. Some of the reactions for the rapid ID32 STREP testing did not match those published by Sistek et al. [22], and we were unable to determine the reason for the discrepancy in the results between the two labs.

We have observed that the media, growth conditions and inoculum density can cause results to vary in some of the Rapid ID32 STREP system tests. In addition, in our conventional testing, acid was produced from sorbitol, raffinose and melibiose; however, these tests were negative using the rapid ID32 STREP system. Additional phenotypic characteristics for strain ETRF1\textsuperscript{T} are provided in the species description. These findings (16S rRNA gene and phenotypic comparison data) indicated the need for additional genetic sequence analysis.

A sub-culture of strain ETRF1\textsuperscript{T} was sent to Molecular Research LP (MR DNA; www.mrdnalab.com) for whole genome sequencing. DNA was isolated using a Qiaegen mini DNA isolation kit. Using an Ion Xpress library kit and the Ion Torrent PGM sequencer (Thermo Fisher Scientific), genomic DNA libraries were then sequenced following the manufacturer’s guidelines. Using NGEN (DNastar) the genome was assembled, and annotation of the genome was performed using RAST [29, 30]. Twenty contigs were constructed which totalled 3.9 Mbp having greater than 50× average coverage. Annotation in RAST revealed 67 RNAs, 3909 coding sequences and 352 subsystems. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under accession AYPQ00000000. The version described in this paper is version AYPQ01000000.

For comparative genomic analyses, genome sequences for four of the 12 phylogenetically related Enterococcus species were retrieved from GenBank (E. caccae AJAU00000000, E. haemoperoxidus ASVY00000000, E. moraviensis AJAS00000000 and E. faecalis ASDA01000000). The genome
Table 1. Phenotypic characteristics that distinguish strain ETRF1<sup>T</sup> from 12 phylogenetically related Enterococcus species

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sequences for E. rotai, E. silesiacus, E. ureasiticus, E. plantarum, E. quebecensis, E. ureilyticus, E. termitis and E. rivorum were determined in this study (GenBank accession numbers CP013655, CP013614, MIJZ00000000, MIKA00000000, MIKB00000000, MIKC00000000, MIJY00000000 and MIEK00000000, respectively). The genomic DNA for these eight Enterococcus species type strains was prepared according to the DOE Joint Genome Institute CTAB protocol (http://jgi.doe.gov/wp-content/uploads/2014/02/JGI-Bacterial-DNA-isolation-CTAB-Protocol-2012.pdf). One microgram of DNA per sample was diluted in 50 µl of PCR-grade water and sheared on a Covaris M220 Focused-ultrasonicator for 32 s at a duty factor of 20 % and 200 cycles per burst to generate 500 bp fragments according to the manufacturer’s instructions. Genomic DNA Libraries were constructed on a Biomek FX<sup>P</sup> Laboratory Automation Workstation (Beckman Coulter) using SPRIworks HT Fragment Library Kits (Beckman Coulter) following the manufacturer’s instructions. Libraries were labelled using Illumina indices from a TrueSeq DNA Sample Prep Kit (Illumina) and sequenced on an Illumina MiSeq v2 500 cycle reagent kits. E. rotai and E. silesiacus sequencing data were assembled using the CLC Genomics Workbench v8.0.2 de novo assembly utility [31]. To close the genome sequences of E. rotai and E. silesiacus, contigs were ordered and joined through the use of data generated via optical mapping. Optical maps were generated using Opgen’s ARGUS whole genome optical mapping system. Genomic DNA was treated with AffIII and assembled into maps of single chromosomes of lengths 3 921 923 bp (E. silesiacus) and 3 714 469 bp (E. rotai). Contigs assembled via the CLC
Genomics Workbench were then converted to *in silico* maps using AflII enzyme cut sites, mapped to the isolate-specific OpGen optical map and ordered in MapSolver v3.2.0. For six of the eight reference genomes determined in this study (*E. ureasiticus, E. plantarum, E. quebecensis, E. wilyoticus, E. termitis* and *E. rivorum*) where only short-read (Illumina) data were generated, we removed PhiX sequences (NC_001422.1) with BBduk v36.19 using a 31-nmer query and allowing for single nucleotide polymorphism. Next, adapters were trimmed using Trimmomatic v0.36 [32]. A 30 bp sliding window requiring at least a Phred score of 20 was used in Trimmomatic to discard low-quality read fragments. The sister reads and broken single reads that passed these filters and that were at least 50 bp in length were used to create a *de novo* assembly in SPAdes v3.9.0 [33] using k-mers of size 21, 33, 55, 77 and 97. Contigs with at least 5× horizontal coverage and 750 bp in length were used in subsequent analyses and deposited into NCBI PRJNA330680. Prodigal v2.6.2 [34] was used to extract protein sequences from each assembly, and genome completeness was evaluated using model-specific cut-off values with HHMER v3.1b2 [35] on 672 taxonomic protein markers (259 marker sets) for the genus *Enterococcus* in CheckM v1.0.6 [36]. For the six genomes, completeness averaged 98.2 % (SD, 0.70 %), which classifies all as near complete status. For reference, we computed completeness for *E. silesiacus* LMG 23085T which was 99.47 % complete.

Comparative sequence analysis of the phenylalanine-tRNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*) genes have shown them to be better discriminating targets for the genus *Enterococcus* than 16S rRNA [20]. These authors state that *Enterococcus* strains within the same species have at least 99 % *rpoA* and at least 97 % *pheS* gene sequence similarity and that different species at most have 97 % *rpoA* gene sequence similarity and 86 % *pheS* gene sequence similarity. A more recent study proposed threshold values for interspecies similarity of <99 % for *rpoA* gene sequences and of ≦90 % for *pheS* gene sequences [22]. Both studies used partial *rpoA* and *pheS* gene sequences. Using these criteria on full-length genes extracted from whole genome sequence assemblies, the *rpoA* gene sequence could be used for species distinction of strain ETRF1T from the 12 other species, as shown in Table S1 (available in the online Supplementary Material). *E. cappaceae* showed the greatest *rpoA* gene sequence similarity of 98.9 %; however, the greatest *pheS* gene sequence similarity (90.4 %) was with *E. ureasiticus*. This is slightly above the newly proposed ≦90 % threshold for interspecies discrimination, so perhaps the threshold should be raised to ≦91 % when full-length *pheS* gene sequences are used. The other 11 species were distinguished from ETRF1T using these new criteria for minimum thresholds. The full-length *rpoA* and *pheS* gene sequences suggest a genetically distinct and novel *Enterococcus* species (Table S1, Figs S1 and S2).

Average nucleotide identity (ANI) represents a good measure of the evolutionary and genetic distance between bacterial strains [37]. ANI values correlate well with DNA–DNA hybridization (DDH) values and the conserved DNA percentage [38]. Goris et al. [38] has demonstrated that DDH values can be replaced by ANI if the genome sequences are available. The cut-off for the species boundary has been proposed at 95–96 % [39]. *In silico* DDH using the Genome-to-Genome Distance calculator (GGDC) has also been demonstrated to produce reliable estimates of wet-lab DDH values [40].

The ETRF1T genome was compared to the 12 phylogenetically related *Enterococcus* species by ANI using default values (http://enve-omics.ce.gatech.edu/ani/), and it had <95 % ANI to all 12 species. The *in silico* DDH values generated by the GGDC were below the threshold of 70 %, also indicating that strain ETRF1T represents a unique species (Table S2).

Using the phenotypic tests listed in Table 1, strain ETRF1T can be differentiated from its phylogenetically closest known relatives. Additional phenotypic results are given in the species description.

The phenotypic data shown in Table 1, the 16S rRNA, *pheS* and *rpoA* comparative gene sequence data shown in Table S1 and Figs 1, S1 and S2, and whole genome analyses data shown in Table S2 establish that strain ETRF1T represents a novel *Enterococcus* species, for which the name *Enterococcus crotali* sp. nov. is proposed.

### DESCRIPTION OF ENTEROCoccus Crotali SP. NOV.

*Enterococcus crotali* (cro.ta’li. N.L. gen. n. crotali of the timber rattlesnake *Crotalus horridus*, the host organism from which the type strain was isolated).

This organism is a facultative anaerobe. Cells are Gram-stain-positive. Colonies on Columbia agar or tripticase soy agar (TSA) with 5 % sheep blood are not pigmented, circular with an entire margin, shiny and 1 mm in diameter after 24 h of growth at 35 °C. No growth occurs on TSA without sheep blood. Cells are motile. Catalase negative, but positive for pyrogallatic acid arylamidase and leucine amidopeptidase. Positive for growth with bile aesculin, aesculin, soluble starch, dextrin, melibiose, raffinose, ribose, sucrose, trehalose, methyl β-D-glucopyranoside, maltose, starch, saliva, malt, colloidal starch, α-D-glucopyranoside, methyl β-D-glucopyranoside, tagatose and xylose. (Using the Rapid ID32 system, acid is not produced from growth in de Man, Rogosa and Sharpe (MRS) *Lactobacillus* broth. Acid is produced from glycerol, inulin, lactose, maltose, mannotol, melibiose, raffinose, ribose, sorbitol, sorbose, sucrose, trehalose, methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, tagatose and xylose. (Using the Rapid ID32 system, acid is not produced from sorbitol, raffinose or melibiose.) Acid is not produced from arabinose, pullulan, d-arabitol, cyclodextrin, glycogen or melezitose. Produces α-galactosidase, β-galactosidase, N-acetyl-β-glucosaminidase and glycl-tryptophan arylamidase. Does not produce β-galactosidase, β-

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glucuronidase, alkaline phosphatase or alanine-phenylalanine-proline arylamidase.

The type strain, ETRF\textsuperscript{T}(=CCUG 65857\textsuperscript{T}=LMG 28312\textsuperscript{T}), originated from faecal material of a dead timber rattlesnake in Minnesota. The CDC Streptococcus Laboratory designation for this strain is SS1930\textsuperscript{T}.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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