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Reevaluation of the Taxonomic Status of Recently Described Species of *Enterococcus*: Evidence that *E. thailandicus* Is a Senior Subjective Synonym of "*E. sanguinicola*" and Confirmation of *E. caccae* as a Species Distinct from *E. silesiacus*[▽]

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Several of the more recently proposed new species of *Enterococcus* are nearly identical based on 16S rRNA gene sequence analysis and phenotypic traits. In the present study, DNA-DNA reassociation experiments, in conjunction with sequencing of the 16S rRNA and *rpoB* genes, provided evidence that "*Enterococcus sanguinicola*" and *Enterococcus thailandicus* actually represent the same species. In contrast, *Enterococcus caccae* and *Enterococcus silesiacus*, two other species with nearly identical 16S rRNA gene sequences, were confirmed to be separate species.

The number of species included in the genus *Enterococcus* has expanded considerably in the last two decades, and over 35 valid species are currently recognized (6) (http://www.bacterio.cict.fr). Several of them are associated with infections in humans, while other species have been isolated only from nonhuman sources to date. However, the differentiation of some of the enterococcal species based on comparative 16S rRNA gene sequencing and phenotypic testing alone is sometimes inconclusive for accurate identification. Strains of these *Enterococcus* species require a polyphasic approach using a combination of techniques, such as 16S rRNA gene sequencing, additional gene target sequencing, analysis of whole-cell protein profiles, phenotypic tests, and often DNA-DNA reassociation to conclusively determine their taxonomic status. In 2004, a single isolate, designated CDC PNS-E2, was determined to be a new *Enterococcus* species, and its phenotypic characteristics were described by our group (3). Formal species designation was not proposed at that time in deference to the recommendation in minute 10 of the July 2002 meeting of the International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of Staphylococci and Streptococci (15), which refers to the description of new species based on a single isolate. Subsequently an additional strain was identified, and the epithet "*Enterococcus sanguinicola*" was proposed to accommodate these two strains recovered from clinically relevant human sources (2). This denomination was effectively but not validly published. Around this same time frame, a new species, designated *Enterococcus thailandicus*, comprising a single isolate from fermented sausage, was described (10). Although the source was not clinical, this isolate had phenotypic traits that were very similar and a 16S rRNA gene sequence that was identical to those of the *E. sanguinicola* isolates, indicating the need to further evaluate their taxonomic position. Two additional species of *Enterococcus*, *Enterococcus caccae* (4) and *Enterococcus silesiacus* (10), were also described within a short period of time and were indistinguishable by 16S rRNA gene sequence comparisons. However, there are several phenotypic differences between strains of these two taxa. The purpose of the present study was to reevaluate the taxonomic status of these proposed four species of *Enterococcus*.

Two *E. sanguinicola* strains, ATCC BAA-781^T and CCUG 47884, isolated from blood cultures from two patients (2, 3), and *E. caccae* ATCC BAA-1240^T, recovered from a stool sample obtained from a healthy individual (4), were obtained from the CDC *Streptococcus* Laboratory culture collection (Atlanta, GA). *E. thailandicus* (NRBC 101867^T) isolated from fermented sausage (10) was obtained from the NITE Biological Research Center (NBRC) culture collection (Japan), and *E. silesiacus* (LMG 23085^T), isolated from drinking water (9), was obtained from the BCCM/LMG bacterial collection (Belgium).

The strains were initially compared on the basis of their phenotypic characteristics by using conventional biochemical tests, as previously described (7, 12), and the API Rapid ID32 Strep system (bioMérieux, Inc., Durham, NC) according to the manufacturer's instructions. In addition, the AccuProbe *Enterococcus* identification test was performed as described by the manufacturer (Gen-Probe, Inc., San Diego, CA). Considering the *Enterococcus* identification scheme previously described (12), *E. sanguinicola* and *E. thailandicus* were placed in phenotypic group II (Table 1) based upon acid formation from mannitol and sorbose and hydrolysis of arginine. Group II currently includes 6 other valid species: *E. casseliflavus*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. haemoperoxidus*, and *E.*

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TABLE 1. Phenotypic characteristics of *Enterococcus sanguinicola*, *Enterococcus thailandicus*, *Enterococcus caccae*, *Enterococcus silesiacus*, and physiologically related species of *Enterococcus*

Species	Phenotypic characteristic[a]													
	MAN	SOR	ARG	ARA	SBL	RAF	TEL	MOT	PIG	SUC	PYU	MGP	TRE	XYL
Group II														
E. faecium	+[d]	−	+	+	V	V	−	−	−	+[d]	−	−	+	−[d]
E. casseliflavus	+	−	+[d]	+	V	+	−[d]	+[d]	+[d]	+	V	+	+	+
E. gallinarum	+	−	+[d]	+	−	+	−	+[d]	−	+	−	+	+	+
E. mundtii	+	−	+	+	V	+	−	−	+	+	−	−	+	+
E. faecalis	+[d]	−	+[d]	−	+	−	+	−	−	+[d]	+	−	+	−[d]
E. haemoperoxidus[b]	+[c]	−	+[c]	−	−	−	−	−	−	+	−	+	+	−
E. sanguinicola	+	−	+	−	−	−	V[e]	−	−	+	−	V	+	−
E. thailandicus[b]	**+**	−	**+**	−	−	−	−	−	−	**+**	−	−	**+**	−
Group IV														
E. aquimarinus[b]	−	−	−	+	−	+	−	−	−	+	−	+	+	+
E. phoeniculicola[b]	−	−	−	+	−	+	−	−	−	+	−	+	+	+
E. cecorum[b]	−	−	−	−	−	+	−	−	−	+	+	−	+	−
E. sulfureus	−	−	−	−	−	+	−	−	+	+	−	+	+	−
E. asini[b]	−	−	−	−	−	−	−	−	−	+	−	−	+	+
E. caccae	−	−	−	−	−	−	−	−	−	**+**	**+**	**+**[c]	**+**	−
E. silesiacus[b]	−	−	−	−	−	−	−	−	−	**+**	−	**+**[c]	**+**	**+**
E. termitis[b]	−	−	−	−	−	−	−	−	−	+	+	+	+	+

[a] Based on the conventional identification scheme proposed by Teixeira et al. (12). Abbreviations and symbols: MAN, mannitol; SOR, sorbose; ARG, arginine; ARA, arabinose; SBL, sorbitol; RAF, raffinose; TEL, 0.04% tellurite; MOT, motility; PIG, pigment; SUC, sucrose; PYU, pyruvate; MGP, methyl-α-D-glucopyranoside; TRE, trehalose; XYL, xylose; +, 90% or more of the strains are positive; −, 90% or more of the strains are negative; V, variable (11 to 89% of the strains are positive). Data for species other than *E. sanguinicola*, *E. thailandicus*, *E. caccae*, and *E. silesiacus* were taken from the work of Teixeira et al. (12).

[b] Phenotypic characteristics based on data from type strains.

[c] Late positive (3 days of incubation or longer).

[d] Occasional exceptions occur (<3% of strains show atypical reactions).

[e] Weak reaction.

mundtii. No biochemical reactions were observed to distinguish between *E. sanguinicola* and *E. thailandicus*. The two *E. sanguinicola* isolates were recovered from human blood indicating the potential to cause invasive infections in humans while the formal type strain *E. thailandicus* was isolated from a nonhuman source (fermented sausage). Interestingly, one of the *E. sanguinicola* isolates was vancomycin resistant, due to presence of the *vanA* gene. *Van*A is an important antimicrobial resistance marker with propensity to rapidly spread to other bacteria and it is often associated with *E. faecalis* and *E. faecium*, which belong to the same phenotypic group of enterococcal species. No additional conventional biochemical trait was found that could differentiate the *E. sanguinicola* strain from the *E. thailandicus* type strain, so the API Rapid ID32 Strep system was used to look for additional biochemical markers. The phenotypic profile obtained by this system for the 3 strains was classified as "doubtful profile" with the closest similarity to *E. hirae* with 67.7% of confidence.

E. caccae and *E. silesiacus* were placed in phenotypic Group IV using the same *Enterococcus* identification scheme (Table 1). Group IV also includes 6 additional species: *E. aquimarinus*, *E. asini*, *E. cecorum*, *E. phoeniculicola*, *E. sulfureus*, and *E. termitis*. The type strains of *E. caccae* and *E. silesiacus* were distinguished from one another based on the following conventional biochemical tests: pyruvate utilization and production of acids from melibiose and xylose. In addition, results for the Accuprobe *Enterococcus* test were also useful in distinguishing between these two strains. *E. caccae* tests pyruvate positive, melibiose negative, xylose negative, and probe positive, while *E. silesiacus* tests pyruvate negative, melibiose positive, xylose positive, and probe negative. *E. caccae* is lactose negative in the API Rapid ID32 Strep system; however, lactose is positive in the longer incubated conventional test and will probably not be a reliable criterion when additional strains are isolated and tested. Moreover, *E. caccae* tests saccharose/sucrose positive in the API Rapid ID32 Strep system, while *E. silesiacus* tests negative. However, both are positive by the conventional biochemical method, so these two differences would probably not be reliable for differentiation. Acid production from tagatose may also be useful in differentiating these two species. The API Rapid ID32 Strep system misidentified *E. caccae* isolates as *E. durans* with a confidence level of 87% and misidentified *E. silesiacus* as *E. durans* with a confidence level of 99.9%.

The phylogenetic positions of the isolates were initially determined by comparative analysis of the 16S rRNA gene sequences, as previously described (3). Sequencing reaction products were purified using Centri-Sep plates (Princeton Separations, Princeton, NJ). Reaction products were electrophoresed on an ABI 3130 or 3730 genetic analyzer using the POP-7 polymer (Applied Biosystems). Chromatograms were assembled and analyzed in the Seqmerge software program (Wisconsin Package, version 10.3; Accelrys, Inc., San Diego, CA). The sequences were aligned using the CLUSTAL-W algorithm (13) and trimmed to a 1,493-bp consensus, and a neighbor-joining tree was created (Fig. 1A) using MEGA 4.0 (8). The 16S rRNA gene sequence analysis (1,483 bp) comparing *E. thailandicus* to *E. sanguinicola* showed identical sequences. The 16S RNA gene sequence analysis (1,483 bp) comparing *E. silesiacus* to *E. caccae* also showed complete sequence identity. These findings, along with the limited number of strains available for compilation of biochemical data,

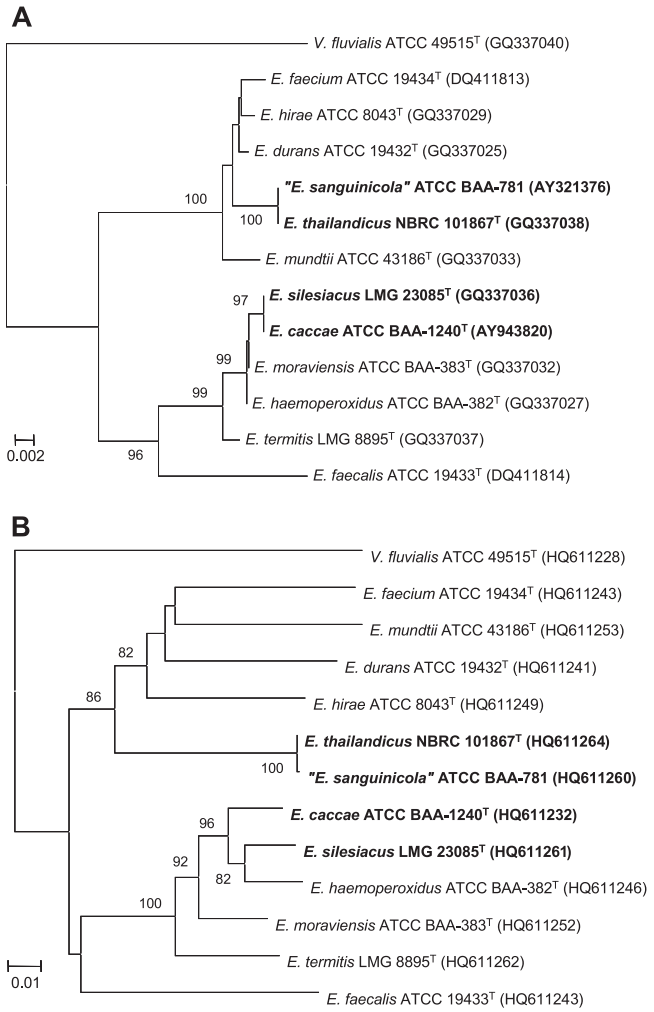

FIG. 1. Phylogenetic trees based on comparative analysis of the 16S rRNA (A) or *rpoB* (B) gene sequences, showing the relationships among the type strains of *Enterococcus sanguinicola*, *Enterococcus thailandicus*, *Enterococcus caccae*, *Enterococcus silesiacus*, and other selected species of *Enterococcus*. GenBank accession numbers are given in parentheses. The neighbor-joining method was used to create the dendrograms, and 1,000 resamplings at the nodes are displayed as percentages. Scale bars indicate numbers of substitutions per nucleotide position. *V. fluvialis*, *Vagococcus fluvialis*.

indicated the need for analysis of additional genetic targets and DNA-DNA hybridization experiments.

A ~1,300-bp portion of the RNA polymerase beta subunit (*rpoB*) gene from each isolate was amplified with HotStarTaq polymerase (Qiagen, Valencia, CA) using the primers UnivrpoB3F (5′-ATGGGNDCGNAAYATGCA) and UnivrpoB23R (5′-GAYATGGAYGTNTGYGC) in a 50-μl PCR. The thermal cycling conditions were as follows: 95°C for 5 min; 15 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a decrease in the annealing temperature by 1°C at each cycle; 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; 72°C for 10 min; and a final hold at 4°C. The PCR amplicons were purified through a Nucleospin column (Clontech) and eluted in 120 μl PCR-grade water. Cycle sequencing was performed as described for the 16S rRNA gene above using the primers UnivrpoB3F, UnivrpoB23R, UnivrpoBseq1 (5′-GGNGAYAARNTNKSNRR), and Univrpo Bseq2 (5′-YYNSMNANYTTRTCNCC). Comparative analysis of an 1,177-bp section of the *rpoB* gene of the *E. sanguinicola* and *E. thailandicus* strains showed a similarity of 99.9%, while *rpoB* sequences of *E. caccae* and *E. silesiacus* revealed 96.7% sequence identity. Considering the criterion of similarity greater than 97% as indicative of species-level relatedness (5), such findings suggested that *E. sanguinicola* and *E. thailandicus* may constitute a single taxon, while *E. caccae* and *E. silesiacus* should be considered separate species.

For DNA-DNA reassociation studies, bacterial cells were grown in flasks containing 2 liters of Todd-Hewitt broth for 18 to 24 h and harvested by centrifugation. The cells were lysed, and DNA was extracted as previously described (11). The techniques and reassociation of DNA using the hydroxyapatite method were as previously described (1). The temperatures used for DNA reassociation were 55°C (optimal conditions) and 70°C (stringent conditions). The percent divergence (*% D*) was calculated, considering that a decrease of 1°C in thermal stability of a heterologous DNA duplex compared with that of the homologous duplex correlates to 1% unpaired DNA bases. DNA-DNA reassociation is considered the "gold standard" for species relatedness. The three criteria for defining a species include relatedness of greater than 70% under optimal conditions, relatedness of greater than 60% under stringent conditions, and divergence of less than 5% (14). The relative binding ratio at the optimal and stringent temperatures for the two *E. sanguinicola* blood isolates and the *E. thailandicus* sausage isolate was greater than 70%, and the divergence was less than

TABLE 2. Levels of DNA relatedness between *Enterococcus sanguinicola* and *Enterococcus thailandicus* and between *Enterococcus caccae* and *Enterococcus silesiacus*

Source of unlabeled DNA	Result with labeled DNA from[a]:					
	E. sanguinicola ATCC BAA781T			*E. caccae* ATCC BAA-1240T		
	RBR at 55°C	*% D*	RBR at 70°C	RBR at 55°C	*% D*	RBR at 70°C
E. sanguinicola ATCC BAA-781T	100	0.0	100	NP	NP	NP
E. sanguinicola CCUG 47884	75	0.0	84	NP	NP	NP
E. thailandicus NRBC 101867T	73	0.5	77	NP	NP	NP
E. caccae ATCC BAA-1240T	NP[b]	NP	NP	100	0.0	100
E. silesiacus LMG 23085T	NP	NP	NP	40	11.0	NP

[a] RBR, relative binding ratio; *% D*, percentage of divergence, calculated to the nearest 0.5%.
[b] NP, not performed.

1% (Table 2), which meets the criteria for the definition of species-level relatedness (14). Therefore, results of DNA-DNA reassociation experiments between the two *E. sanguinicola* strains and the *E. thailandicus* strain confirm that these three strains actually belong to the same taxon. According to the rules of nomenclature, since *E. thailandicus* is the earliest formally published and valid epithet (10), it should be considered the senior subjective synonym of *E. sanguinicola* and must be used to name this taxon. Therefore, *E. thailandicus* should include strains previously described as CDC PNS-E2 (3) and later designated *E. sanguinicola* (2). Clinical laboratories should be aware on the occurrence of this additional enterococcal species potentially associated with *vanA* gene acquisition and transmission.

In contrast, results of DNA-DNA reassociation experiments with strains of *E. caccae* and *E. silesiacus* confirmed that they are two valid species even though 16S rRNA gene sequencing could not resolve them. The relative binding ratio at the optimal temperatures for the *E. caccae* and *E. silesiacus* type strains was 40%, and the divergence was 11%, indicating that these strains represent distinct species (Table 2). Our observations indicate that potentially conventional biochemical tests, such as pyruvate utilization and production of acids from melibiose and xylose, may be used to reliably distinguish these species. In addition, *E. caccae* tests positive using the Accuprobe *Enterococcus* genetic probe, while *E. silesiacus* tests negative.

Results of DNA-DNA reassociation experiments corroborate the *rpoB* sequencing data and indicate that analysis of the *rpoB* gene sequence is a reliable component for use in distinguishing isolates of the enterococcal species included in the present study.

On the basis of our findings, isolates previously proposed as *E. sanguinicola* should be assigned to the species *E. thailandicus*, while *E. caccae* and *E. silesiacus* should remain as distinct species. In addition to clarifying the taxonomic status of these proposed new species of *Enterococcus*, the results of the present study highlight the need to use more than a single gene sequence for resolution of enterococcal species. For that, *rpoB* gene sequencing may represent a useful additional tool. As more closely related enterococcal species are being recognized, it is also important to test enterococcal isolates from different sources for a wide variety of biochemical characteristics in order to prepare more-comprehensive identification schemes. The potential clinical significance of these infrequently isolated enterococcal species, especially when associated with relevant antimicrobial resistance markers, should draw attention to the need for the application of identification procedures of greater precision, allowing their proper recognition and characterization and improving our knowledge of their routes of transmission.

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