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Full Paper

Enterococcus faecium 129 BIO 3B is classified as *Enterococcus lactis* 129 BIO 3B

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***Enterococcus faecium* 129 BIO 3B is a lactic acid bacterium that has been safely used as a probiotic product for over 100 years. Recently, concerns about its safety have arisen because some species of *E. faecium* belong to the vancomycin-resistant enterococci. The groups of *E. faecium* with less pathogenic potential have been split into a separate species (*Enterococcus lactis*). In this study, I investigated the phylogenetic classification and safety of *E. faecium* 129 BIO 3B as well as *E. faecium* 129 BIO 3B-R, which is naturally resistant to ampicillin. Mass spectrometry and basic local alignment search tool analysis using specific gene regions failed to differentiate 3B and 3B-R into *E. faecium* or *E. lactis*. However, multilocus sequence typing successfully identified 3B and 3B-R as the same sequence types as *E. lactis*. Overall genome relatedness indices showed that 3B and 3B-R have high degrees of homology with *E. lactis*. Gene amplification was confirmed for 3B and 3B-R with *E. lactis* species-specific primers. The minimum inhibitory concentration of ampicillin was confirmed to be 2 µg/mL for 3B, which is within the safety standard for *E. faecium* set by the European Food Safety Authority. Based on the above results, *E. faecium* 129 BIO 3B and *E. faecium* 129 BIO 3B-R were classified as *E. lactis*. The absence of pathogenic genes except for *fms21* in this study demonstrates that these bacteria are safe for use as probiotics.**

Key words: *Enterococcus faecium*, *Enterococcus lactis*, probiotics, lactic acid bacteria, multilocus sequence typing (MLST), average nucleotide identity (ANI), digital DNA–DNA hybridization (dDDH)

INTRODUCTION

Enterococci are Gram-positive, catalase-negative facultative anaerobic bacteria that belong to the lactic acid bacteria group. *Enterococcus faecium* is ubiquitous in nature and is found in many foods, mainly in fermented products of animal origin such as cheese and dry sausages [1]. Some *Enterococcus* strains are also used as medicines exhibiting beneficial effects on the host [2]. The safety of *E. faecium* use has been questioned, as vancomycin-resistant enterococci bacteria, which typically cause nosocomial infections in clinical practice, also belong to *E. faecium*.

Streptococcus faecalis 129 BIO 3B (3B) has been used as a probiotic product since 1917. This bacterium was classified as *S. faecalis* by species identification based on its properties. In 1984, Schleifer *et al.* showed using DNA–DNA and DNA–rRNA hybridization that *S. faecalis* and *Streptococcus faecium* belong to the genus *Enterococcus* [3]. Furthermore, in 1990, Woese *et al.* proposed a phylogenetic system for all organisms based on small subunit rRNA gene sequences [4], which further confirmed that the two *Streptococcus* species mentioned above belong to the genus *Enterococcus*. This taxonomic change is documented in *Bergey's Manual of Systematic Bacteriology Volume 3: The Firmicutes* (2009), in which *S. faecalis* was revised to *Enterococcus faecalis*, *S. faecium* was revised to *E. faecium*, and *S. faecalis* 129 BIO 3B was classified as *E. faecium* 129 BIO 3B [5]. Additionally, in 2021, Belloso Daza *et al.* reported that there was a genetic and evolutionary difference between hospital-derived *E. faecium* (clade A), environmental *E. faecium* (clade B), and mixed groups of *Enterococcus lactis*. They reported that since the mixed group of clade B and *E. lactis* did not have the pathogenic gene markers *IS16*, hyl_{Efm}, and *esp*, and did not have an allelic profile of penicillin-binding protein 5 (PBP5) associated with ampicillin resistance, the *E. faecium* clade B strain should be classified as *E. lactis* [6]. *E. lactis* was first isolated from a milk sample in 2006 [7], and it was later confirmed to be a possible variant of *E. faecium* [8]. In 2012, *E. lactis* was proposed as an independent species of the genus *Enterococcus*, with *E. lactis* BT 159^T=DSM 23655^T=LMG 25958^T being the type strain [8]. The taxonomic name has gone through some historical changes, and *E. faecium* 129 BIO 3B has been used for over 100 years. As there have been no reports of infections caused by *E. faecium* 129 BIO 3B thus far and the strain has a high degree of safety, it is believed to possibly be *E. lactis*.

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Therefore, in this study, I verified the latest classification and safety of *E. faecium* 129 BIO 3B (3B) and its naturally ampicillin-resistant strain, *E. faecium* 129 BIO 3B-R (3B-R).

MATERIALS AND METHODS

Strains

E. faecium 129 BIO 3B and *E. faecium* 129 BIO 3B-R stains were obtained from Biofermin Pharmaceutical Co., Ltd. (Kobe, Japan). *E. lactis* DSM 23655^T was purchased from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany). *E. faecium* TMU 2126, 2127, 2128, 2129, 2130, 2134, 2154, 2155, 2156, and 2157 were isolated from clinical specimens.

Bacterial species identification using MALDI-TOF-MS

The bacterial strains were cultivated on 5% sheep blood agar plates at 35°C. Proteins from the strains were extracted via an existing ethanol/formic acid method according to the Bruker Daltonics protocol. Bacterial species identification via matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) was performed using a MALDI Biotyper (Bruker Japan K.K., Kanagawa, Japan) with a stainless-steel target plate. Spectra were analyzed in the Bruker Biotyper 3.1 BDAL (RUO) version 11.0 (DB-10833 MSP) library with the flexAnalysis 3.4 software. Following the manufacturer's recommendations, a score of 2.0 or above indicated correct identification at the species level, scores between 1.700 and 1.999 indicated identification at the genus level, and a score below 1.7 was considered unreliable.

16S rRNA gene and 16S-23S ITS region sequence analysis

The 16S rRNA gene sequence was amplified with universal primers 8UA (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1485B (5′-ACGGGCGGTGTGTRC-3′), as previously described [9]. A homology search for 3B and 3B-R was conducted using the EzBioCloud (https://www.ezbiocloud.net) database. For gene sequence analysis of the internal transcribed spacer (ITS) region according to the method of Jensen, Webster, and Straus [10], a homology comparison with the nucleotide sequence of the type strain was conducted using the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) BLAST website (https://blast.ncbi.nlm.nih.gov/) database.

Whole genome analysis of E. faecium *129 BIO 3B and 3B-R*

E. faecium 129 BIO 3B was cultured in GAM Broth "*Nissui*" (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 0.7% glucose (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 0.1% polysorbate 80 (Junsei Chemical Co., Ltd., Tokyo, Japan) for 18 hr at 37°C. DNA extraction was performed using a DNeasy Blood & Tissue Kit (QIAGEN K.K., Tokyo, Japan).

Draft genome analysis of 3B and 3B-R was conducted using the pyrosequencing method. Libraries were prepared using TruSeq DNA Sample Preparation Kit v2 (Illumina, Inc., San Diego, CA, USA) according to the TruSeq DNA Sample Preparation v2 Guide Rev. A (Illumina, Inc.) manual. The samples to be analyzed were physically fragmented into several hundreds of base pairs using a Covaris S-series focused-ultrasonicator (Covaris, Woburn, MA, USA) and ligated with indexed adapters. The size-prepared DNAs were used as templates and amplified using PCR to prepare a sequence library. Cluster formation as a template for sequencing using a sequence library was conducted following the cBot User Guide Rev. F and TruSeq PE Cluster Kit v3 Reagent Prep Guide Rev. B manuals to obtain the base sequence of the template DNA. TruSeq PE Cluster Kit v3-cBot-HS was used as the reagent, and a cBot was used as the instrument. Sequence analysis was conducted on a HiSeq 2000 using TruSeq PE Cluster Kit v3-HS according to the CASAVA (Consensus Assessment of Sequence and Variation) v1.8 User Guide Rev. B manual, HiSeq Control Software (HCS) v1.4.8, Real Time Analysis (RTA) v1.12.4, and CASAVA v1.8.1. Sequence analysis yielded 71 contigs (>1 kb) for 3B and 117 for 3B-R.

Next, a Fosmid clone was prepared. The sequence between each contig was determined by assembling the results obtained through contig sequencing, and the full-length sequence was analyzed.

Whole genome analysis of E. lactis *DSM 23655^T*

E. lactis DSM 23655^T was cultured in GAM broth with 0.7% glucose and 0.1% polysorbate 80 for 18 hr at 37°C. After culturing, the broth was centrifuged at 14,000 × *g* for 5 min. The supernatant was discarded, and the sediment (bacteria) was suspended in phosphate-buffered saline (Nacalai Tesque, Inc., Kyoto, Japan) and centrifuged at 14,000 × *g* for 5 min. After discarding the supernatant, DNA was extracted from the bacterial cells.

The DNA was purified from the cells, an MGIEasy FS DNA Library Prep Set (MGI Tech Co., Ltd., Tokyo, Japan) was used according to the user manual to prepare the library, and adapters were added using an MGIEasy DNA Adapters-96 (Plate) Kit (MGI Tech Co., Ltd.).

By following the user manual instructions, circularized DNA was prepared using the prepared PCR product and an MGIEasy Circularization Kit (MGI Tech Co., Ltd.), and DNA nanoballs (DNB) were prepared using a DNBSEQ G-400 RS High-throughput Sequencing Set (MGI Tech Co., Ltd.). A DNBSEQ-G400 sequencer (MGI Tech Co., Ltd.) was used with 2 × 200 bp paired-end reads to obtain a scaffold-level genome sequence from the prepared DNB via sequence analysis.

Multilocus sequence typing (MLST) analysis

First, 143 and 23 *E. faecium* and *E. lactis* genomes, respectively, were obtained from NCBI. Complete genomes were used for 3B and 3B-R, and scaffold-level gene sequences were used for *E. lactis* DSM 23655^T. MLST analysis of seven *E. faecium* housekeeping genes (*atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, and *adk*) was conducted, and allelic profiles and sequence types (STs) were obtained from the PubMLST website (https://pubmlst.org/). A minimum spanning tree (MST) was created using PHYLOViZ (https://www.phyloviz.net/) based on sequence data types.

Overall genome relatedness indices (OGRIs): Average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH)

dDDH was performed using the Genome-to-Genome Distance Calculator (https://ggdc.dsmz.de) [11]. For this analysis, the distance values were computed using the total number of identities within high scoring segment pairs (HSPs) per total HSP length. Formula 2 (highly recommended if the genomes submitted for

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analysis are incomplete) was used. DDH values ≥70% indicated that the tested strain represented the same species as the type strain [12].

Average nucleotide identity (ANI) analysis was performed using the ANI calculator (https://ezbiocloud.net/). The resulting values were classified using 95% as the cut-off value for same-species determination [13].

Confirmation of amplification using 3B and 3B-R E. lactis*-specific primers*

DNA was extracted from bacterial strains using a MORA Extraction kit (AMR Inc., Gifu, Japan). *E. lactis*-specific PCR primers were designed for the phenylalanyl-tRNA synthase alpha subunit (*pheS*) gene. The base sequence was GAAACAATCGTCAAAATCACG, forward, and TGGTCAGACTCTACTTCGTAACC, reverse, and the amplified product was 383 bp.

The DNA amplification reaction was conducted by denaturing the DNA at 95°C for 1 min, followed by 38 cycles of 95°C for 15 sec, 63°C for 15 sec, and 72°C for 20 sec. DNA amplification products were confirmed by agarose gel electrophoresis and ethidium bromide staining. The *E. lactis* ECTC 21015 strain DNA was used as a positive control.

Analysis of carbohydrate metabolism

First, 3B and 3B-R were cultured in GAM broth with 0.7% glucose and 0.1% polysorbate 80 for 18 hr at 37°C. Carbohydrate metabolism was then examined using API 50 CH strips (bioMérieux SA, Marcy-l'Étoile, France) according to the included protocol.

Confirmation of pathogenic genes

Identification of the primary pathogenic genes involved probing the genome of interest using the NCBI Basic Local Alignment Search Tool (BLAST) to confirm the presence of the putative pathogenic genes identified by Freitas *et al.* [14] and European Food Safety Authority (EFSA) [15]. The targets were determined by confirming amplification of the genes that code for the following using polymerase chain reaction (PCR): insertion sequence *IS16* (AF507977.1), hyaluronidase-like protein hyl_{Efm} (HMPREF0351_12988), genomic island *orf1481*-encoded sugar-binding protein (EAN09962.1), putative phosphotransferase *ptsD* (MBG7632288.1), pili gene cluster PGC-1 including pili protein *fms21* (ACI49671.1), nidogen-binding LPXTG surface adhesin *sgrA* (AFK59147.1), and enterococcal surface protein *esp* (EFAU004_02750). The DNA amplification reaction was conducted by denaturing the DNA at 95°C for 1 min, followed by 38 cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 20 sec. DNA amplification products were confirmed via agarose gel electrophoresis and ethidium bromide staining.

Identification of bacterial species and antimicrobial susceptibility testing using automated equipment

A MicroScan WalkAway 96 Plus system (Beckman Coulter K.K., Tokyo, Japan) and the Pos Combo 3.1 J panel (PC 3.1 J) were used for bacterial strain identification and antimicrobial susceptibility testing. The criteria of the Clinical and Laboratory Standards Institute (CLSI) were used for minimum inhibitory concentration (MIC) susceptibility and resistance breakpoints [16].

RESULTS

Bacterial species identification: MALDI-TOF-MS, 16S rRNA gene, and ITS region gene sequence homology

Bacterial species identification using MALDI-TOF-MS is a method that involves ionization of the proteins in the test bacterium and comparison of its mass spectrum pattern with those in a database to identify the bacterial species. The *E. faecium* scores were high for both 3B (score: 2.21) and 3B-R (score: 2.13). However, *E. lactis* was not registered in database of MALDI biotyper. Regarding the 16S rRNA gene base sequence homology, 3B showed 99.1% (1133/1134) homology to *E. faecium*^{T} LMG 11423 (AJ301830) and 99.7% (1133/1136) homology to *E. lactis*^{T} BT 159 (GU983697). Similarly, 3B-R was 99.6% (1472/1476) homologous to *E. faecium*^{T} LMG 11423 (AJ301830) and 99.2% (1426/1437) homologous to *E. lactis*^{T} BT 159 (GU983697). The ITS region gene sequence showed that 3B and 3B-R were both 98.6% (138/140) homologous to *E. faecium*^{T} ATCC 19434.

MLST analysis

The allelic profile for 3B and 3B-R was as follows: *atpA*, 6; *ddl*, 6; *gdh*, 4; *purK*, 4; *gyd*, 3; *pstS*, 3; and *adk*, 27. The ST was ST812. Additionally, when the MST was created together with 143 *E. faecium* and 24 *E. lactis* STs, *E. lactis* formed two clusters. ST812 (two strains) was associated with another *E. lactis* ST, namely ST39 (two strains; Fig. 1).

OGRIs: ANI and dDDH of enterococci

ANI was used to compare the homology of *E. faecium* with its relatives 3B and 3B-R. In the case of ANI, ≥95% homology indicates that the compared strains are of the same species; 3B was 94.75% homologous to *E. faecium*^{T} and 97.99% homologous to *E. lactis*^{T}. Additionally, 3B-R was 94.81% homologous to *E. faecium*^{T} and 97.92% homologous to *E. lactis*^{T}. Therefore, 3B and 3B-R were at least 95% homologous only with *E. lactis*^{T} (Table 1). ANI and dDDH are said to be correlated [17], and dDDH was used in this study in a manner similar to ANI to compare the homology between *E. faecium* and its relatives as well as 3B and 3B-R. dDDH regards strains to be homogeneous subspecies when the in silico DDH values calculated from the pairwise distances between genomes are 79–80% [18], and it regards them to be homogeneous when they are 80% or more. The DDH values for 3B were 60.3% with *E. faecium*^{T} and 83.3% with *E. lactis*^{T}. Additionally, those for 3B-R were 60.1% with *E. faecium*^{T} and 83.0% with *E. lactis*^{T}. Therefore, 3B and 3B-R were at least 80% homologous to only *E. lactis*^{T} (Table 1). Similar to ANI, dDDH showed higher homology to *E. lactis*^{T} than *E. faecium*^{T}.

Confirmation of amplification for 3B and 3B-R using E. lactis*-specific primers*

Gene amplification was confirmed when DNA extracted from 3B and 3B-R was used to conduct PCR using *E. lactis*-specific primers. All 10 strains of *E. faecium* isolated from clinical specimens were negative.

Carbohydrate metabolism

E. lactis^T, 3B, and 3B-R did not metabolize glycerol. Carbohydrate metabolism similar to that of *E. lactis*^{T} was observed in both 3B and 3B-R (Table 2).

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Confirmation of 3B and 3B-R pathogenic genes

Amplification of the seven target pathogenic genes was confirmed using PCR, and none of the pathogenic genes were amplified, except for *fms21*, in 3B and 3B-R (Fig. 2).

Antimicrobial susceptibility test

Using automated equipment, 3B was identified as *E. faecium* with profile code 616757544. The MIC of ampicillin (ABPC) was 2 µg/mL, which was judged as "susceptible (S)" according to the CLSI criteria. On the other hand, 3B-R could not be judged due to poor growth, but the MIC was 400 µg/mL based on the agar plate dilution method according to the antimicrobial susceptibility measurement method of the Japanese Society of Chemotherapy (Biofermin Pharmaceutical Co., Ltd., 2021. BIOFERMIN-R® Powder and BIOFERMIN-R® Tablets drug interview forms).

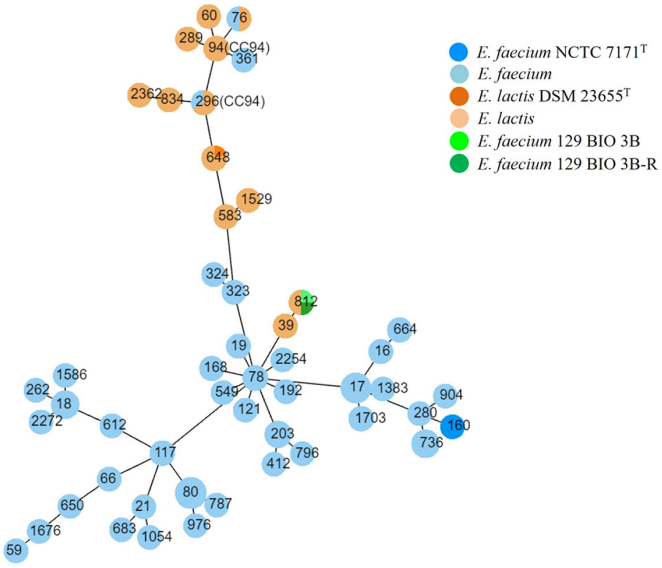

Fig. 1. Minimum spanning tree (MST) of *E. faecium* 129 BIO 3B, *E. faecium* 129 BIO 3B-R, *E. faecium* (n=143), and *E. lactis* (n=24).

At each node, the dark blue, light blue, dark orange, light orange, dark green, and light green colors indicate *E. faecium* NCTC7171^{T}, *E. faecium*, *E. lactis* DSM23655^{T}, *E. lactis*, *E. faecium* 129 BIO 3B, and *E. faecium* 129 BIO 3B-R, respectively. Nodes are labeled with the corresponding STs. Links are labeled with absolute distances.

Table 1. Results (%) for average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH)

Strain	OGRI	*E. faecium*^{T}	*E. lactis*^{T}
E. faecium 129 BIO 3B	ANI	94.75	97.99
	dDDH	60.3	83.3
E. faecium 129 BIO 3B-R	ANI	94.81	97.92
	dDDH	60.1	83.0

E. faecium^{T}, *E. faecium* NCTC 7171^{T} (GCA_900447735.1); *E. lactis*^{T}, *E. lactis* DSM 23655^{T}.
OGRI: overall genome relatedness indice.

Table 2. Differential acid production of enterococci

Acid production from:	*E. lactis* [8]	*E. faecium* [8]	*E. faecium* 129 BIO 3B	*E. faecium* 129 BIO 3B−R
L-Arabinose	+	+	+	+
Glycerol	−	+	−	−
Mannitol	+	+	+	−
Melezitiose	−	−	−	−
Melibiose	+	v	+	+
Raffinose	−	−	−	−
Ribose	+	+	+	+
Salicin	+	+	+	+
Sorbitol	−	v	−	−
Sucrose	−	v	+	−
D-Xylose	−	−	−	−

+: positive; −: negative; v: variable.

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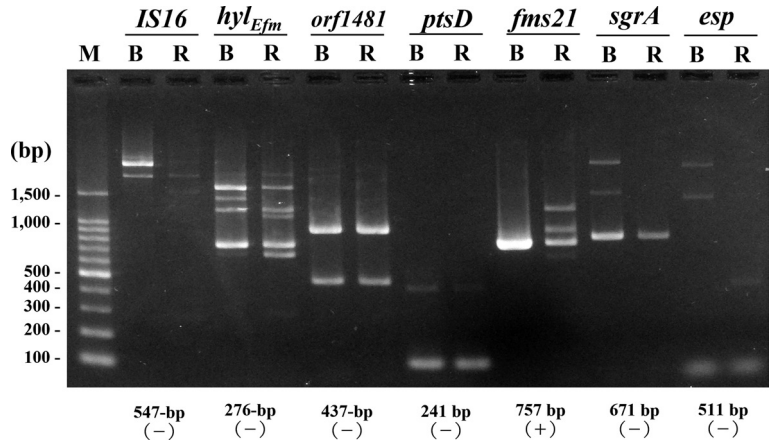

Fig. 2. Confirmation of *E. faecium* 129 BIO 3B and *E. faecium* 129 BIO 3B-R pathogenic genes by polymerase chain reaction (PCR). Lanes: M, marker; B, *E. faecium* 129 BIO 3B; R, *E. faecium* 129 BIO 3B-R.

DISCUSSION

In this study, I examined the phylogenetic classifications of 3B and its naturally ampicillin-resistant strain 3B-R. The results showed that these strains were classified as *E. lactis* and did not possess pathogenic genes strongly associated with nosocomial infections.

First, I identified bacterial species by protein analysis using MALDI-TOF-MS and then identified bacterial species using 16S rRNA gene and ITS region gene sequences. The results showed that 3B and 3B-R were highly homologous to both *E. lactis* and *E. faecium*; hence, the strains could not be distinguished. However, Kim *et al.* reported that they were able to distinguish *E. faecium* and *E. lactis* using MALDI-TOF-MS [19]. The accuracy of MALDI-TOF MS depends on the reference microbial database, and currently, the MALDI BioTyper database does not have a reference spectrum for *E. lactis* [19]. Kim *et al.* constructed their own database by adding reference spectra of *E. lactis* to the existing database and succeeded in distinguishing *E. faecium* and *E. lactis* [19]. This difference in the database used is possibly the reason why *E. faecium* and *E. lactis* could not be distinguished in the present study. As the MALDI BioTyper database is further developed, it may become possible in the future to distinguish *E. faecium* and *E. lactis* more conveniently than PCR.

Meanwhile, the MLST results in the present study showed that both 3B and 3B-R had the same STs as *E. lactis* and that their STs were different from those of *E. faecium*. Therefore, the entire genomes of 3B and 3B-R were decoded, and the homology of their entire base sequences with *E. lactis* and *E. faecium* was compared using ANI and dDDH. The results showed that both 3B and 3B-R had the highest homology with *E. lactis*. Furthermore, a known characteristic difference in carbohydrate metabolism between *E. faecium* and *E. lactis* is that *E. faecium* metabolizes glycerol, whereas *E. lactis* does not [8]. Therefore, carbohydrate metabolism was examined, and it was found that, like *E. lactis,* 3B and 3B-R did not metabolize glycerol. These results suggested that 3B and 3B-R may be classified as *E. lactis*. Based on the above results, 3B and its naturally ABPC-resistant strain 3B-R should be classified as *E. lactis*.

Although 16S rRNA gene sequence analysis has been the standard method used for phylogenetic classification, the presence of high variability within multiple copies of the 16S rRNA gene sequence is thought to prevent the accurate classification of bacteria [20]. Previous research has also shown that *E. faecium* generally has 6 copies of the rrn operon, and WGS analysis has confirmed intraspecies variation in rrn sequences [6]. Additionally, variability in 16S rRNA gene sequences within the genome is a common phenomenon in bacteria [21] and can lead to incorrect phylogenetic tree assignments [22]. Therefore, the use of 16S rRNA gene sequences alone for taxonomic identification of *E. faecium* and its relatives (such as *E. lactis*) is thought to have limitations. To confirm whether 3B and 3B-R are distinguished from *E. lactis* rather than *E. faecium*, *E. lactis*-specific primers were generated for the *pheS* gene in the present study. However, Belloso Daza *et al.* distinguished *E. faecium* from *E. lactis* using mutations in the *gluP* gene, a rhomboid protease [23]. Although mutations in the *gluP* gene were not examined in the present study, this method could have been used to prove that 3B and 3B-R are *E. lactis*.

E. faecium is known to possess various pathogenic genes, whereas *E. lactis* has been reported to have no pathogenic genes [24]. Therefore, PCR amplification of pathogenic genes strongly associated with clinical *E. faecium* (i.e., *IS16*, hyl_{Efm}, *orf1481*, *ptsD, fms21*, *sgrA*, and *esp*) was performed to verify this for 3B and 3B-R [14]. *IS16*, hyl_{Efm}, and *esp* are the three pathogenic genes that the criteria of the EFSA states should not be in strains that can be used in food [15]. The results revealed none of the abovementioned pathogenic genes except for *fms21* in 3B and 3B-R, which means that they meet the EFSA criteria; hence, they have low pathogenicity. To date, there have been no reports of infections caused by 3B-R, as in the case of 3B, but this bacterium is resistant to ampicillin, cephalosporins, aminoglycosides, macrolides, tetracyclines, nalidixic acid, and other types of penicillin. Naturally, this is outside the EFSA ampicillin MIC criteria (MIC ≤2 mg/L) [15]. However, it is not necessarily resistant to all antibiotics. For example, since 3B-R does not have resistance to new quinolone antibiotics [25], it is thought that control is possible should 3B-R cause an infection.

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Additionally, 3B-R is a naturally resistant strain of 3B that does not have resistance genes such as the β-lactamase gene and does not have a plasmid (data not shown). Hence, there is no risk of transmitting resistance to other bacteria, and 3B-R is thought to be as safe as 3B. As a topic for future study, there is a need to verify the safety of using living organisms to corroborate the safety of 3B, 3B-R, and *E. lactis* in foods and pharmaceutical products.

In conclusion, 3B and 3B-R, which are used as probiotics, were classified as *E. lactis*. Based on this result, the *E. faecium* in other marketed probiotics may also be classified as *E. lactis*. Additionally, these bacteria were found to be genetically safe because they did not possess pathogenic genes specified by the EFSA as unsafe or other pathogenic genes except for one that is strongly associated with clinical *E. faecium*.

CONFLICT OF INTEREST

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