Title

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Journal

Applied and Environmental Microbiology, 80(6)

ISSN

0099-2240

Authors

Kopit, Lauren M

Kim, Eun Bae

Siezen, Roland J

et al.

Publication Date

2014-03-15

DOI

10.1128/aem.03859-13

AEM
Journals.ASM.org

Safety of the Surrogate Microorganism *Enterococcus faecium* NRRL B-2354 for Use in Thermal Process Validation

Lauren M. Kopit,[a] Eun Bae Kim,[a,b] Roland J. Siezen,[c] Linda J. Harris,[a,d] Maria L. Marco[a]

Department of Food Science & Technology, University of California, Davis, Davis, California, USA[a]; Department of Animal Products and Food Science, College of Animal Life Sciences, Kangwon National University, Chuncheon, Kangwon-do, Republic of Korea[b]; Microbial Bioinformatics, Ede, Netherlands[c]; Western Center for Food Safety, University of California, Davis, Davis, California, USA[d]

Enterococcus faecium **NRRL B-2354 is a surrogate microorganism used in place of pathogens for validation of thermal processing technologies and systems. We evaluated the safety of strain NRRL B-2354 based on its genomic and functional characteristics. The genome of *E. faecium* NRRL B-2354 was sequenced and found to comprise a 2,635,572-bp chromosome and a 214,319-bp megaplasmid. A total of 2,639 coding sequences were identified, including 45 genes unique to this strain. Hierarchical clustering of the NRRL B-2354 genome with 126 other *E. faecium* genomes as well as *pbp5* locus comparisons and multilocus sequence typing (MLST) showed that the genotype of this strain is most similar to commensal, or community-associated, strains of this species. *E. faecium* NRRL B-2354 lacks antibiotic resistance genes, and both NRRL B-2354 and its clonal relative ATCC 8459 are sensitive to clinically relevant antibiotics. This organism also lacks, or contains nonfunctional copies of, enterococcal virulence genes including *acm*, *cyl*, the *ebp* operon, *esp*, *gelE*, *hyl*, IS*16*, and associated phenotypes. It does contain *scm*, *sagA*, *efaA*, and *pilA*, although either these genes were not expressed or their roles in enterococcal virulence are not well understood. Compared with the clinical strains TX0082 and 1,231,502, *E. faecium* NRRL B-2354 was more resistant to acidic conditions (pH 2.4) and high temperatures (60°C) and was able to grow in 8% ethanol. These findings support the continued use of *E. faecium* NRRL B-2354 in thermal process validation of food products.**

Enterococcus faecium is a commensal organism of mammalian digestive tracts and is important for the production of fermented food products, including cheese and sausage (1). Certain strains of *E. faecium* were shown to have beneficial, or probiotic, effects on animal (2–4) and human (5–7) health. However, strains of *E. faecium* have also been associated with nosocomial infections (8). Over the past 30 years, the number of enterococcal infections has increased, with a growing number of illnesses specifically attributed to *E. faecium* (9). *E. faecium* infections are of particular concern because of the high incidence of antibiotic resistance among many hospital-associated strains. For this reason, *E. faecium* was identified as an important problem organism requiring new treatment methods (10).

Recent studies have shown that there is a significant evolutionary distance between hospital- and community-associated strains of *E. faecium*. Differences between these strains include phenotypic (11), gene-specific (12, 13), and whole-genome and proteome level (14–19) distinctions. There are currently over 200 publicly available *E. faecium* draft genomes. The best-characterized genomes are for strain TX16 (also referred to as DO), isolated from an individual with endocarditis (20), and strain Aus0004, isolated from the bloodstream of a hospitalized patient (21). Several draft genomes of community-associated strains are also currently available in public databases, but none have been characterized in depth.

The taxonomic classification of *E. faecium* strain NRRL B-2354 has gone through numerous revisions. It was originally isolated from dairy utensils in 1927 by G. J. Hucker (22) and in 1960 was deposited in the U.S. Department of Agriculture (USDA) Agricultural Research Service NRRL culture collection as NRRL B-2354. In 1979, the strain was placed within the American Type Culture Collection (ATCC) as *Micrococcus freudenreichii* ATCC 8459. However, it was later found to lack many of the characteristics typical of *M. freudenreichii* (23) and was reclassified to an undetermined species of *Pediococcus*. Recently, 16S rRNA gene sequencing and biochemical assays led to the conclusion that strain NRRL B-2354 is most similar to members of the *E. faecium* species (24), a finding that led to reclassification of the strain assignment at NRRL and ATCC.

The thermal tolerance of *E. faecium* NRRL B-2354 on almonds is similar to that of *Salmonella enterica* serovar Enteritidis phage type 30. This strain is recommended and widely used as a surrogate for *Salmonella* in the validation of commercial thermal processes that are used for almonds (25–28). *E. faecium* NRRL B-2354 is also considered to be a suitable surrogate for food-borne pathogens in thermal processes used for dairy products (29), juice (30), and meat (24). Surrogate organisms are inoculated into or onto food products that are subsequently sent through food processing equipment located in commercial food processing facilities. Because of the risks associated with introducing a pathogen into a food processing facility, it is preferred to use a nonpathogenic surrogate organism that has been adequately characterized. Despite the long history of use of *E. faecium* NRRL B-2354 as a

Received 25 November 2013 **Accepted** 6 January 2014
Published ahead of print 10 January 2014
Editor: M. W. Griffiths
Address correspondence to Maria L. Marco, mmarco@udavis.edu.
L.M.K. and E.B.K. contributed equally to this work.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03859-13.

doi:10.1128/AEM.03859-13
The authors have paid a fee to allow immediate free access to this article.

surrogate, concern over *E. faecium* in clinical settings supports the need to further evaluate the characteristics of this particular strain. Therefore, we examined the genome of *E. faecium* NRRL B-2354 for the presence of virulence factors, evaluated the expression of those genes and environmentally relevant phenotypes, and quantified resistance to several clinically important antibiotics.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. faecium* NRRL B-2354 was obtained from the USDA Agricultural Research Service Culture Collection (Peoria, IL; receiving date, 22 July 2011; http://nrrl.ncaur.usda.gov). *E. faecium* ATCC 8459 (receiving date, 26 July 2011), *Enterococcus faecalis* ATCC 29212 (receiving date, 26 July 2011), and *Bacillus cereus* ATCC 14579 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). *E. faecium* TX0082 was provided by Barbara Murray (University of Texas Medical School, Houston). *E. faecium* 1,231,502 was provided Michael Gilmore (Harvard University, Boston, MA). *Enterococcus* strains were routinely cultured in brain heart infusion (BHI) agar or broth (dehydrated medium; Difco, Becton, Dickinson [BD], Franklin Lakes, NJ), incubated overnight at 37°C.

DNA sequencing, assembly, and annotation. One colony of *E. faecium* NRRL B-2354 strain was inoculated into 15 ml of BHI broth and incubated at 37°C under static conditions for 8 h. Cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS; 80 g NaCl, 2 g KCl, 26.8 g $Na_2HPO_4 \cdot 7H_2O$, and 2.4 g KH_2PO_4 in 800 ml H_2O, pH 7.3). Genomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

A 500-bp insert library was prepared for 100-bp, paired-end sequencing in the Illumina HiSeq 2000 as previously reported (31). A total of 4,044 Mbp of 100-bp paired-end reads were obtained and were quality filtered at a quality score of ≥20 at each nucleotide position. After quality filtering, 656 Mbp of 80-bp or longer Illumina reads was obtained. The filtered reads (≥80 bp) were assembled into contigs by using the Ray 1.7 sequence assembler (32) with a 31-bp k-mer. The filtered Illumina reads were assembled into 49 contigs (>100 bp; total length, 2,841,503 bp; average length, 57,989 bp; maximum length, 198,831 bp; N50 length, 138,902 bp; GC content, 37.84%). The genomic DNA was also sequenced using the PacBio RS sequencer with C2 chemistry (1 × 90 min and 2 × 45 min) (21). PacBio RS sequencing produced a total of 292 Mbp with an average length of 2,328 bp (maximum length, 14,914 bp; N50 length, 3,385 bp) after removing adaptor sequences. PacBio reads 6 kbp or longer were used to close gaps between the contigs. Errors found in PacBio reads were corrected by using the Illumina reads (33). All DNA sequencing was performed at the UC Davis Genome Center (http://www.genomecenter.ucdavis.edu).

The gap-closed and error-corrected genome sequences were annotated for protein coding sequences (CDS), rRNA genes, and tRNA genes by manual annotation and by Rapid Annotation Using Subsystem Technology (RAST) (34), RNAmmer 1.2 Server (35), and tRNAscan-SE 1.21 (36) with default options for bacteria. The genome was also screened for the presence of antibiotic resistance (AR) genes found in the Antibiotic Resistance Database (ARDB) (37).

Hierarchical clustering and phylogenetic analysis. A total of 126 genome sequences and annotations for 125 *E. faecium* strains, including two versions of the *E. faecium* DO (TX16) genome were obtained from public databases in April 2013 (see Table S1 in the supplemental material). The genomes were screened for 7,017 orthologs of protein coding sequences identified in a previous report (19). For hierarchical clustering of *E. faecium* strains, gene ortholog distances between two strains were calculated according to the Euclidean distance method. Bootstrapping was performed using Pvclust with a 10,000 resampling option (38).

Phylogenetic comparisons of PBP5 protein sequences (39) and multilocus sequence typing (MLST) for seven housekeeping genes (*adk*, *atpA*, *ddl*, *gdh*, *gyd*, *pstS*, and *purK*) (40) were performed for all *E. faecium* genomes using MEGA 5 (41).

Circular genome alignment. *E. faecium* genome sequences were fragmented into 500-bp sequences and then aligned to the *E. faecium* NRRL B-2354 genome as a reference using GASSST (42). The alignment was visualized in concentric circles using perl scripts. Protein coding sequences, tRNA, rRNA, AR genes (37), virulence factor (VF) genes (8), and mobile elements (ME) were designated as previously described (19). ME elements included phage genes or transposon, transposase, integrase, and insertion sequences (IS) based on the genome annotations. Genes with GC contents that were high (GC% ≥ mean + 1.5 × standard deviation [SD]) or low (GC% ≤ mean − 1.5 × SD) were also identified.

Detection of virulence-associated genes. Certain *Enterococcus* VF genes were examined for their presence in the NRRL B-2354 genome by using NCBI BLAST+ (43). The presence of *esp*, *gelE*, and *hyl* was also examined by PCR according to previously described protocols (44, 45) (see Table S2 in the supplemental material). Positive controls used for PCR were *E. faecalis* ATCC 29212 (*gelE*) and *E. faecium* 1,231,502 (*esp* and *hyl*).

Electron microscopy. Negative staining was accomplished with standard techniques utilizing 1% ammonium molybdate (46). Transmission electron microscopy was performed with a Philips CM120 Biotwin lens (FEI Company, Hillsboro, OR), and the camera used was a Gatan MegaScan model 794/20 digital camera (2K × 2K; Pleasanton, CA). Microscopy and staining were performed at The University of California, Davis, School of Medicine, Department of Pathology and Laboratory Medicine, Electron Microscopy Lab.

Production of gelatinase and hemolysin. The ability to hydrolyze gelatin was determined by examining for zones of turbidity around colonies after growth overnight at 37°C on Todd-Hewitt agar supplemented with 3% (wt/vol) gelatin (47). Hemolysin was measured by examining for zones of clearing around colonies after growth overnight at 37°C on tryptic soy agar (TSA) supplemented with 5% (vol/vol) defibrinated horse blood (47).

Adherence to collagen type I. Adhesion to collagen was evaluated using a previously described method (48), with several modifications. Rat tail collagen (type I) in 0.02 M acetic acid (BD) at a concentration of 15 μg per well was used. *E. faecium* cells from overnight cultures grown in BHI broth were collected by centrifugation at 805 × *g*, suspended in PBS to an optical density at 600 nm (OD_{600}) of 1.0, and added to the wells. Adhesion was calculated according to relative absorbance in a microplate spectrophotometer (Biotek, Winooski, VT) as follows: $OD_{595(\text{collagen+BSA+bacteria})} - OD_{595(\text{BSA+bacteria})}$.

Adherence to fibrinogen and fibronectin. The ability to adhere to fibrinogen and fibronectin was examined using previously described methods (49), except that 200 μl human fibrinogen (Calbiochem, Merck KGaA, Darmstadt, Germany) in 50 mM sodium carbonate buffer (pH 9.6), 15 μg per well, or 200 μl fibronectin (Calbiochem) in 50 mM sodium carbonate buffer (pH 9.6), 15 μg per well, was used.

Biofilm formation on polystyrene. The ability of cells to adhere to polystyrene plates was evaluated using a previously described method (50) with several modifications. Cells were grown overnight in BHI broth at 37°C, and 200 μl of a 1:20 dilution of the cultures in BHI broth was added to a sterile 96-well polystyrene microtiter plate (BD). After incubating for 24 h at 37°C, wells were washed three times with PBS, dried in an inverted position for 15 min, and stained with 1% (wt/vol) crystal violet for 15 min. The wells were rinsed again with PBS, and the crystal violet was solubilized in 200 μl of an ethanol and acetone solution (80:20, vol/vol). The OD_{595} was determined using a microplate spectrophotometer (Biotek).

Antibiotic susceptibility testing. Antibiotic resistance was determined at the University of California, Davis Medical Center Clinical Laboratory, Sacramento, CA (http://www.ucdmc.ucdavis.edu/pathology/services/), using the BD Phoenix 100 Automated Microbiology system (BD).

Survival at low pH, at high temperature, or in the presence of ethanol. For acid and thermal stress tolerance tests, *E. faecium* strains were first grown in BHI overnight at 37°C and washed twice in physiological saline (0.85% [wt/vol] NaCl, pH 7). To measure survival at low pH, washed cells

were suspended in physiological saline with an adjusted pH of 2.4 (acidified with 5 M HCl). Suspensions were sampled at 10-min intervals for 60 min, and serial dilutions were prepared in physiological saline for plating onto BHI agar. Plates were incubated at 37°C overnight for CFU enumeration. Thermal tolerance was determined by dispensing 50 μl of the *E. faecium* cells in physiological saline (pH 7) into 200 μl microcentrifuge tubes and incubating in a C1000 Thermal Cycler (Bio-Rad Laboratories, Foster City, CA) at either 50°C or 60°C. Cell survival was determined every 10 min for 60 min by CFU enumeration using serial dilutions of separate 50-μl aliquots cooled to 21 to 23°C. Ethanol tolerance was determined by incubation of approximately 10^7 *E. faecium* cells at 37°C in BHI broth adjusted to contain either 12% (vol/vol) additional water or 8% or 12% (vol/vol) ethanol. Growth was measured at an OD_{600} every 15 min over 24 h in a microplate spectrophotometer (Biotek).

Nucleotide sequence accession numbers. The genome sequence and gene annotation information have been deposited at GenBank under the accession numbers CP004063 (chromosome) and CP004064 (plasmid pNB2354_1).

RESULTS

Genome sequencing, assembly, and annotation of *E. faecium* NRRL B-2354. The genome of *E. faecium* NRRL B-2354 was sequenced, assembled, and annotated to yield one chromosome (2,635,572 bp) and one plasmid (214,319 bp, designated pNB2354_1) (see Fig. S1 and Table S3 in the supplemental material). The GC content of the NRRL B-2354 chromosome (38.03%) is similar to that of other *E. faecium* strains, including TX16 (also known as DO) (38.15%) (20) and Aus0004 (38.36%) (21). The GC content of the plasmid is lower (35.98%) than that of the chromosome but similar to that of the megaplasmids of other *E. faecium* strains (see Table S3 in the supplemental material). A total of 2,639 CDS, 18 rRNA (5S, 16S, and 23S), and 49 tRNA genes were predicted in the assembled annotated genome (see Table S3). The genome of the clonal deposit of this strain at the ATCC, strain ATCC 8459, was also sequenced and found to share over 99% sequence identity with the strain NRRL B-2354 (data not shown).

Hierarchical clustering and phylogenetic analysis. Hierarchical clustering was performed using strain NRRL B-2354 and 126 other *E. faecium* genome sequences according to the presence/absence of orthologous genes as previously described (19). The genome of strain NRRL B-2354 clustered with other nonclinical (NC), or community, *E. faecium* isolates in a clade containing few clinical (CL) strains (Fig. 1). Specifically, the NRRL B-2354 genome clustered with other community *E. faecium* strains in the NC2 clade. This newly described clade is significantly enriched in nonclinical strains ($P = 0.036$, Fisher's exact test) among which 26 of the 32 strains available for comparison had nonclinical, or community, origins. The genome of *E. faecium* NRRL B-2354 was most similar to that of strain E1050, a fecal isolate from a healthy volunteer.

Gene-targeted comparisons using the *pbp5* gene and MLST were also performed to further evaluate the relationships between *E. faecium* NRRL B-2354 and other *E. faecium* strains. The *pbp5-R* genotype is associated with ampicillin resistance, and PBP5 amino acid sequences separated *E. faecium* strains into two different clades (39, 51). A phylogenetic tree of PBP5 protein sequences from *E. faecium* strains showed that the PBP5 amino acid sequence of strain NRRL B-2354 clustered together with many community-associated (nonclinical) strains of strains belonging to the NC2 clade (see Fig. S2 in the supplemental material). Notably, these strains along with NRRL B-2354 exhibit a *pbp5-R* genotype (51).

In contrast, MLST analysis did not show clear NC and CL strain separation (see Fig. S3 in the supplemental material). Examination of the *E. faecium* NRRL B-2354 genome revealed that this strain belongs in sequence type ST32 according to six of the seven housekeeping genes used for sequence typing (*atpA*, *ddl*, *gdh*, *gyd*, *pstS*, and *purK*). This MLST pattern is highly associated with NC *E. faecium* strains, unlike other sequence types (ST) (see Table S4 in the supplemental material). Sequence comparisons of *adk*, the remaining gene commonly used for enterococcal MLST, identified a single-nucleotide change in this gene in NRRL B-2354 compared to the other members of ST32. Because this difference might represent a novel MLST group, strain NRRL B-2354 was assigned a novel sequence type (ST860) in the *E. faecium* MLST database (http://efaecium.mlst.net/) (40) (see Table S1 in the supplemental material).

Unique genes in the NRRL B-2354 genome. Genome comparisons identified 45 unique genes in NRRL B-2354 not present in the other 125 *E. faecium* strains examined. These genes are colocalized in the genome in five locations (designated SP1 to SP5) (Fig. 2). Many of the genes specific to strain NRRL B-2354 are ME or colocalized to ME genes (Fig. 2; see Table S5 in the supplemental material). A total of 25 of the 45 genes have functional assignments and include a putative glycosyltransferase, an amidohydrolase domain protein, a transcriptional antiterminator, a DEAD/DEAH box helicase-like protein, and DNA repair protein RadC (Fig. 2; see Table S5 in the supplemental material).

Antibiotic resistance and related genes in NRRL B-2354. Antibiotic resistance genes were not found in the NRRL B-2354 genome (Fig. 2 and Table 1). Based on MICs, *E. faecium* NRRL B-2354 is sensitive to vancomycin, streptomycin, gentamicin, and ampicillin (Table 1), antibiotics commonly used separately or in tandem to treat enterococcal infections (52). The strain exhibited intermediate sensitivity to erythromycin and was sensitive to the cephalosporins cefoxitin and cefazolin, despite cephalosporin resistance being intrinsic to most enterococci (53) (Table 1).

Presence of *cas* and the number of AR, VF, and ME genes. The genome of *E. faecium* NRRL B-2354 lacks *cas* genes encoding clustered regularly interspaced short palindromic repeat (CRISPR)-associated proteins involved in bacterial immunity against foreign DNA (54). For at least certain *E. faecium* strains, the number of *cas* genes is negatively correlated with the number of AR genes (55). To examine whether this trait was common to *E. faecium* from both NC and CL origins, the numbers of AR genes were compared among strains lacking CRISPR-*cas* systems. Notably, *cas*-negative NC strains contained significantly lower numbers of AR genes than did strains with a CL background (Fig. 3). The numbers of VF and ME genes are also significantly lower in NC than in CL isolates (Fig. 3). Such observations were not statistically examined for *cas*-positive *E. faecium* strains due to the low number of those strains for which genome sequences were available.

Virulence factors. The *E. faecium* NRRL B-2354 genome lacks several genes encoding VF that are associated with this species (Table 2). Specifically, IS*16*, a common marker of hospital-associated strains (56), is absent from strain NRRL B-2354, as are genes coding for gelatinase (*gelE*) (57), hyaluronidase (*hyl*) (58), cytolysin (*cyl*) (59), and a virulence and biofilm formation protein (*esp*) (60). These VF are commonly found in hospital-associated strains of *E. faecium* (47). The *in silico* findings for several of these

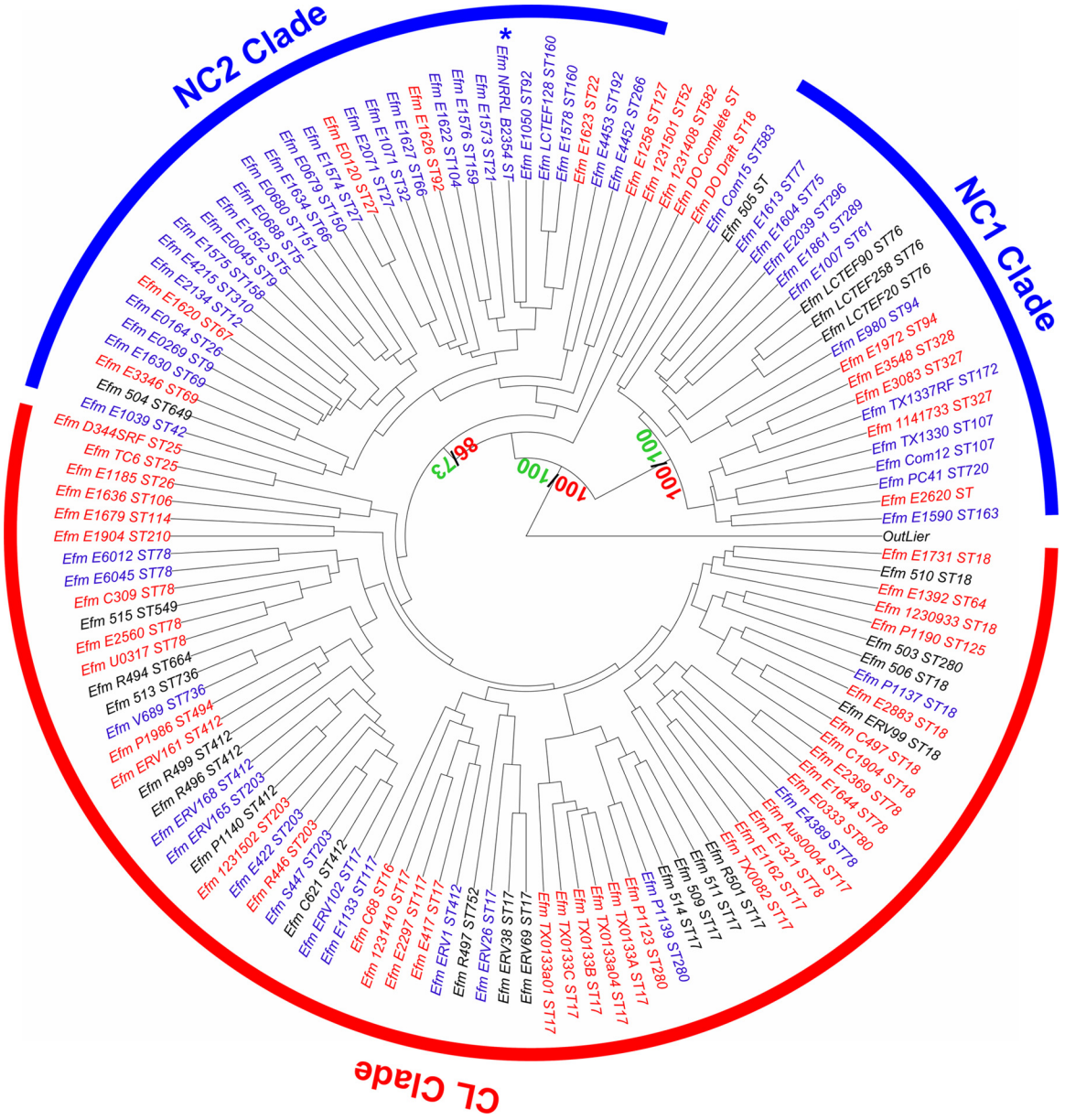

FIG 1 Hierarchical clustering of 127 *E. faecium* genomes. Genome comparisons were based on the presence/absence of 7,017 orthologs found in the *E. faecium* pangenome. Nonclinical strains are labeled in blue, clinical strains in red, and strains with unidentified origins in black. An artificial outlier was included. Strain NRRL B-2354 is indicated by an asterisk. Red and green values in certain nodes indicate approximately unbiased and biased probabilities for the bootstrapping, respectively.

genes were confirmed by PCR (data not shown). The absence of *gelE* was also confirmed by the inability of *E. faecium* NRRL B-2354 to hydrolyze gelatin during growth on laboratory culture medium (see Fig. S4 in the supplemental material). Although another putative hemolysin (AGE29035) was annotated in the genome of *E. faecium* NRRL B-2354, hemolytic activity was not detected for *E. faecium* NRRL B-2354 (see Fig. S4 in the supplemental material).

The *scm* gene encoding a collagen I and IV adhesion (61) and the *acm* gene encoding a collagen I adhesion associated with endocarditis (62) were found in strain NRRL B-2354 (Table 2). Although *scm* appeared to be intact, *acm* contained a 1,059-bp insertion 52 bp into the coding region of the gene (Fig. 4A; see Fig. S5 in the supplemental material). This insertion is an integrase that shares 100% nucleotide identity with an integrase from *E. faecium* Aus0004 (21). The integrase contains many stop codons in the reading frame of *acm* (Fig. 4A), indicating that *acm* is not expressed. Strain NRRL B-2354 and its clonal relative ATCC 8459 also adhered to collagen in significantly smaller amounts than *E. faecium* TX0082 (ST17) (Fig. 4B). *E. faecium* TX0082 (ST17) contains an intact *acm* gene and was previously shown to bind collagen (63).

Pili are associated with enterococcal virulence and biofilm formation (64, 65). Strain NRRL B-2354 contains genes in the *ebp* and *pil* operons coding for pilus production (65, 66) (Table 2).

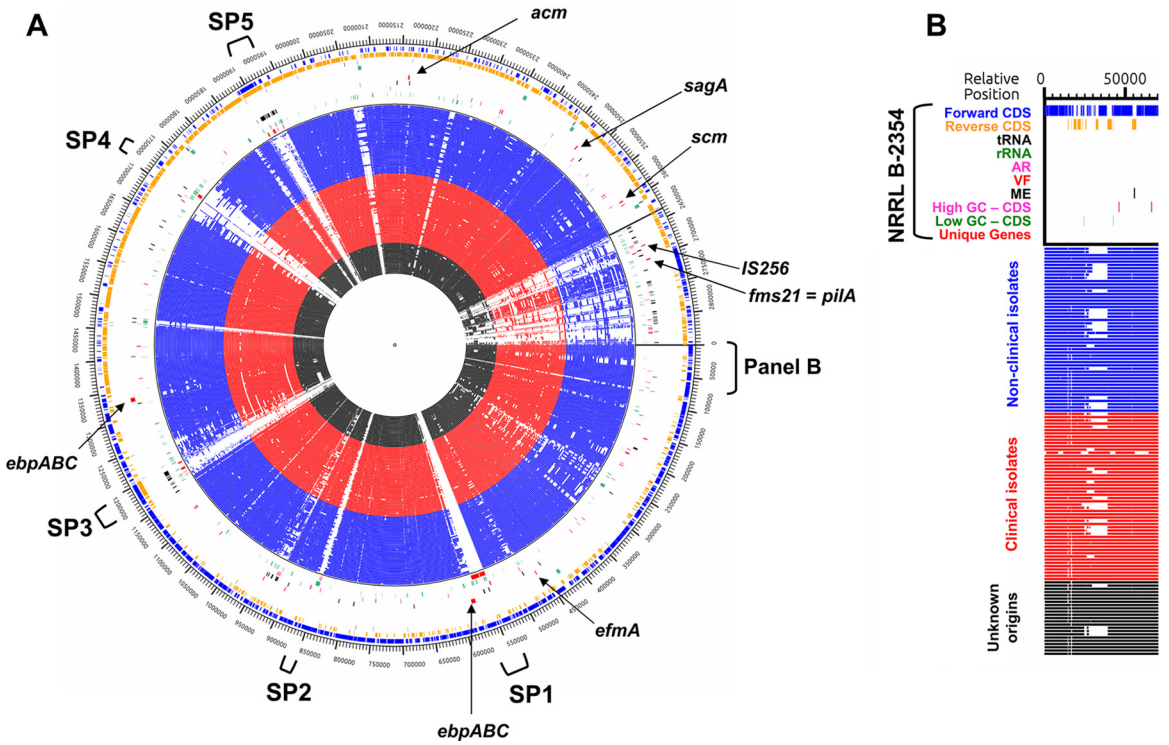

FIG 2 Alignment of 127 *E. faecium* genomes. (A) Circular alignment of *E. faecium* genomes. The outside of the circle is the NRRL B-2354 reference genome to which the other genomes are aligned. Genes identified as potential VF in NRRL B-2354 are indicated. Loci unique to NRRL B-2354 are also indicated (SP1 to SP5). (B) Key to circular alignment map. Gene types and strain origins are depicted in the same order (outwards to inwards) in the circular map. AR, antibiotic resistance genes; VF, virulence factors; ME, mobile genetic elements.

TABLE 1 Antibiotic resistance of *E. faecium* NRRL B-2354[a]

Class and antibiotic	MIC (mg/liter)	Sensitivity	No. of screened AR gene types from ARDB[c]
Aminoglycosides			
Gentamicin	Sensitive[b]	Sensitive	737
Streptomycin	Sensitive[b]	Sensitive	869
Cephalosporins			
Cefazolin	<2	Sensitive	1,393
Cefoxitin	8	Sensitive	844
Glycopeptides			
Vancomycin	<0.5	Sensitive	300
Macrolides			
Erythromycin	2	Intermediate	1,092
Penicillins			
Ampicillin[d]	<0.125	Sensitive	1,896
Penicillin	<1	Sensitive	1,896
Quinolones			
Levofloxacin	2	Sensitive	273
Tetracyclines			
Minocycline	<1	Sensitive	597
Tetracycline	<0.5	Sensitive	597

[a] The same results were found for *E. faecium* ATCC 8459.
[b] Numerical MICs were not determined.
[c] No AR genes were detected in our study; ARDB data are from reference 37.
[d] Certain *pbp5* genotypes are associated with ampicillin resistance in *E. faecium*, but they were not considered here because of the variation in ampicillin resistance and sensitivity phenotypes among strains containing this gene.

However, the transcriptional regulator *ebpR*, the promoter for the cotranscribed *ebpA*, *ebpB*, and *ebpC* genes, and the first 688 bp of *ebpA* are absent from strain NRRL B-2354 (Fig. 5A). In total, the deletion encompasses approximately 2 kb in comparison to strain TX0082, a strain confirmed to produce pili encoded by the *ebp* operon (65). Similarly, pili were detected on the surface of TX0082 but not *E. faecium* NRRL B-2354 (see Fig. S6 in the supplemental material).

Because *ebp*-encoding pili are associated with biofilm formation (65), the capacity of the strains to form biofilms on polystyrene was also measured. Biofilm formation according to cell staining intensities was 5-fold lower for strains NRRL B-2354 and ATCC 8459 than for TX0082 (Fig. 4B). Notably, *E. faecium* B-2354 has a reduced capacity to form biofilms even though it contains *efaA*, a manganese-dependent gene encoding an endocarditis-specific antigen involved in biofilm formation (67, 68) (Table 2).

E. faecium NRRL B-2354 contains *sagA*, a virulence gene associated with endocarditis (Table 2) (69). SagA contributes to binding to fibrinogen, fibronectin, and collagen type I and IV laminin (70) and is also present in TX0082 (67). Accordingly, both *E. faecium* strains as well as ATCC 8459 bound to fibrinogen and fibronectin at similar levels (see Fig. S7 in the supplemental material).

Tolerance to low pH, heat, and ethanol. After 20 min of incubation at pH 2.4, *E. faecium* NRRL B-2354 survived in 10- and 100-fold-larger amounts than the clinical strains TX0082 and 1,231,502 (ST203) (Fig. 6A). Within 50 min of incubation at the acidic pH, NRRL B-2354 exhibited only a 3-log decline, whereas viable cell numbers of the two clinical isolates were reduced by 7 log (Fig. 6A).

Incubation at 50°C for 60 min was not detrimental to the via-

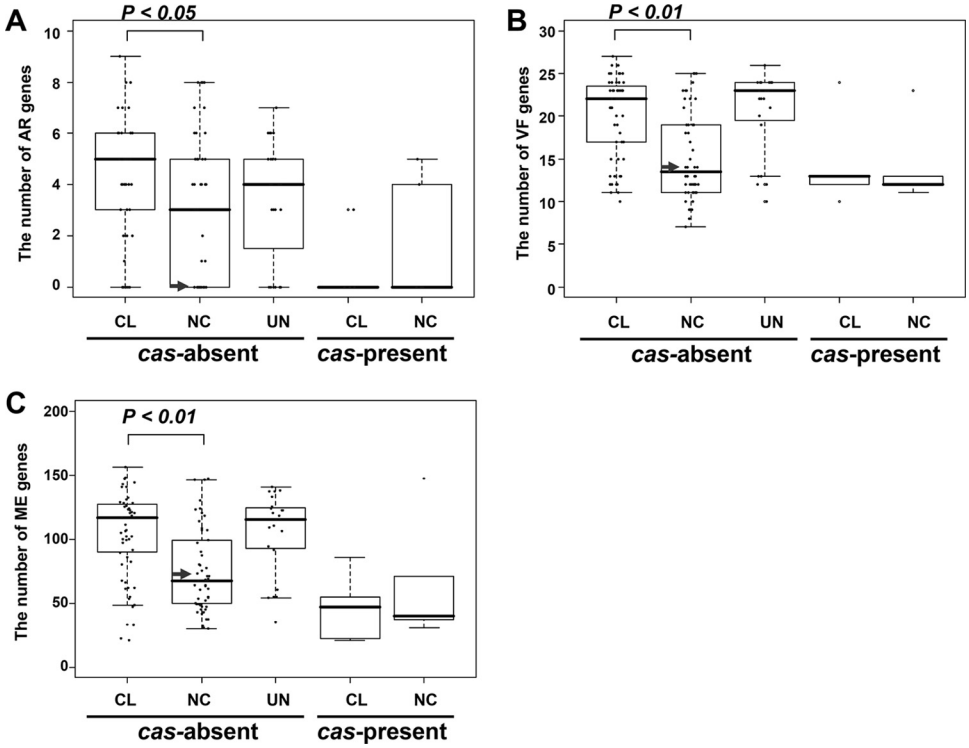

FIG 3 Numbers of AR, VF, and ME genes in strains with or without *cas* genes. Three types of genes, AR (A), VF (B), and ME (C), that have important roles in virulence of *E. faecium* were counted and compared statistically between CL and NC *E. faecium* strains (Student's *t* test). Strains with unidentified origins (UN) were also shown for reference. The arrow indicates the value of NRRL B-2354 in each panel.

bility of *E. faecium* NRRL B-2354, TX0082, and 1,231,502 (Fig. 6B). At an incubation temperature of 60°C for 10 min, there was a decline of over 7 log in viability of strains TX0082 and 1,231,502 (Fig. 6B). In contrast, a 2-log decline was observed for NRRL B-2354 after 60 min at 60°C (Fig. 6B).

E. faecium NRRL B-2354, TX0082, and 1,231,502 were unable to grow in BHI in the presence of 12% ethanol (data not shown). When the culture medium contained 8% ethanol, only NRRL B-2354 was able to grow (Fig. 6C). *E. faecium* NRRL B-2354 exhibited a longer lag phase in this medium, and the cells reached a 1.3-fold-lower final optical density than did cells grown in BHI lacking ethanol (data not shown).

DISCUSSION

E. faecium NRRL B-2354, a commonly used strain with a long history in food products and thermal process validation, lacks the

TABLE 2 Presence of virulence factors in *E. faecium* NRRL B-2354

Gene[a]	Function	Length of reference gene (bp)	% Coverage	Nucleotide identity (%)	Reference
acm	Adhesion to collagen and other extracellular proteins	2,166	100	99	64
cyl	Cytolysin, hemolysis	7,500	ND[b]	ND	60
ebpR	Regulatory gene for enterococcus biofilm and pilus (*ebp*) operon	1,392	ND	ND	65
ebpA	Pilin subunit	3,390	80	99	65
ebpB	Pilin subunit	1,422	100	99	65
ebpC	Pilin subunit	1,878	100	99	65
$efaA_{fm}$	Adhesion protein, plays role in endocarditis	951	100	100	68
esp	Enterococcal surface protein	2,315	ND	ND	61
gelE	Gelatinase	6,088	ND	ND	58
hyl	Hyaluronidase	1,662	ND	ND	59
IS*16*	Mobile insertion sequence	1,188	ND	ND	57
pilA	Major pilin subunit	1,977	100	99	67
pilE	Secreted surface protein	756	100	100	67
pilF	Minor pilin subunit	2,091	100	99	67
sagA	Adhesion protein	1,575	100	100	70
scm	Surface protein, adhesion to extracellular proteins	1,983	100	100	62

[a] The *E. faecium* NRRL B-2354 genome was compared to the *E. faecium* TX16 (DO) genome, except for the following: *E. faecium* Aus0004 (*esp*), *E. faecalis* V583 (*cyl*, *gelE*), *E. faecium* U37 (IS*16*), and *E. faecium* E1165 (*pilA*, *pilE*, *pilF*).
[b] ND, not detected.

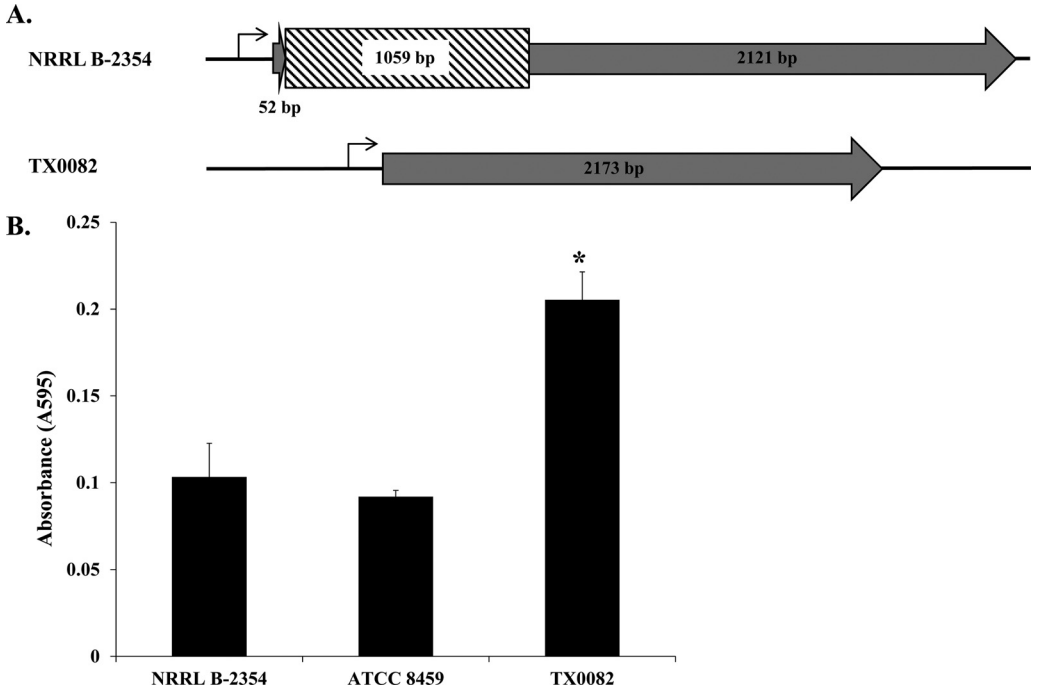

FIG 4 NRRL B-2354 *acm* gene insertion and impaired collagen adherence. (A) Schematic diagram of the *acm* gene in *E. faecium* NRRL B-2354 and clinical strain *E. faecium* TX0082. The genes are 99% identical in the coding regions (gray); however, a 1,059-bp insertion sequence (hatched lines) is located 52 bp from the start codon in NRRL B-2354. (B) *E. faecium* binding to type I collagen. Absorbance was significantly higher for TX0082 than for the other two strains (Tukey's honestly significant difference [HSD], $P < 0.05$). The averages ± SD of three replicates per strain are shown.

majority of virulence factors known for this species and is sensitive to medically relevant antibiotics. These features are consistent with its genomic relationship to nonclinical (NC), or community, strains of *E. faecium*. Overall, the findings of this study support the continued use of *E. faecium* NRRL B-2354 and its clonal relative ATCC 8459 in the validation of processing equipment used for thermal treatment of food products.

Comparative genomics approaches have previously concluded that there is a significant evolutionary distance between clinical and community isolates of *E. faecium* (11–19). In the present study, whole-genome, PBP5, and MLST comparisons revealed that *E. faecium* NRRL B-2354 is most similar to nonclinical strains. This result is in agreement with the original isolation of *E. faecium* NRRL B-2354 from dairy utensils. Among nonclinical strains, NRRL B-2354 belongs to the newly identified NC2 clade (34). Although the origins of NC2 are currently unclear (16, 17, 19), it is notable that NC2 strains share similar PBP5 amino acid sequences associated with ampicillin resistance. Divergence of NC2 clade strains, including NRRL B-2354, might therefore be in accordance with *pbp5* evolution.

E. faecium NRRL B-2354 contains 45 unique genes not present in the 125 other *E. faecium* genomes examined here. The majority of these genes encode phage-associated proteins or have unknown function. These genes were distributed among five loci (SP1 to SP5) throughout the genome, suggestive of separate gene integration events. Further investigation is needed to elucidate whether these genes confer unique functionality to NRRL B-2354, particularly with regard to its association with dairy products and high levels of environmental stress tolerance.

Like other NC strains, *E. faecium* NRRL B-2354 has fewer ME, AR, and VF genes than strains isolated from clinical settings. A lower abundance of those genes is also related to the smaller genome sizes of NC strains (16, 19). In contrast to the low number of ME, NRRL B-2354 lacks CRISPR-*cas* systems associated with protection against ME-associated foreign DNA, including AR and VF genes (55). The significantly lower number of ME in *cas*-negative NC strains supports the possibility that other factors are also important to ME susceptibility in *E. faecium*.

The lack of AR genes in *E. faecium* NRRL B-2354 is in agreement with the sensitivity of this strain to medically relevant antibiotics, including but not limited to vancomycin. Although this strain contains a *pbp5-R* allele, it is also sensitive to ampicillin. Resistance of *E. faecium* to either vancomycin or ampicillin severely limits treatment options for enterococcal infections. While strain NRRL B-2354 exhibited an intermediate level of resistance to erythromycin, this trait is common among other food-associated *E. faecium* (71).

E. faecium NRRL B-2354 also lacks or contains nonfunctional copies of the majority of known and established enterococcal virulence factors. This includes *esp*, *hyl*, and IS*16* commonly found in clinical isolates (56, 72). Those loci were specified by the European Food Safety Authority (EFSA) as targets for the safety evaluation of *E. faecium* strains intended as additives for animal feed (73). EFSA recommends examining for the presence of *esp*, *hyl*, and IS*16* as well as sensitivity to ampicillin as exclusion criteria (73). *E. faecium* NRRL B-2354 lacks these genes and, as discussed above, is sensitive to ampicillin. Therefore, this strain meets the requirements for safety by the EFSA guidelines. These results were shared with American Type Culture Collection and were deemed sufficient for ATCC to classify the clonal strain in the biosafety level 1 (BSL-1) category (Brian Beck, ATCC, personal communication). Furthermore, based on this information, USDA agreed to remove

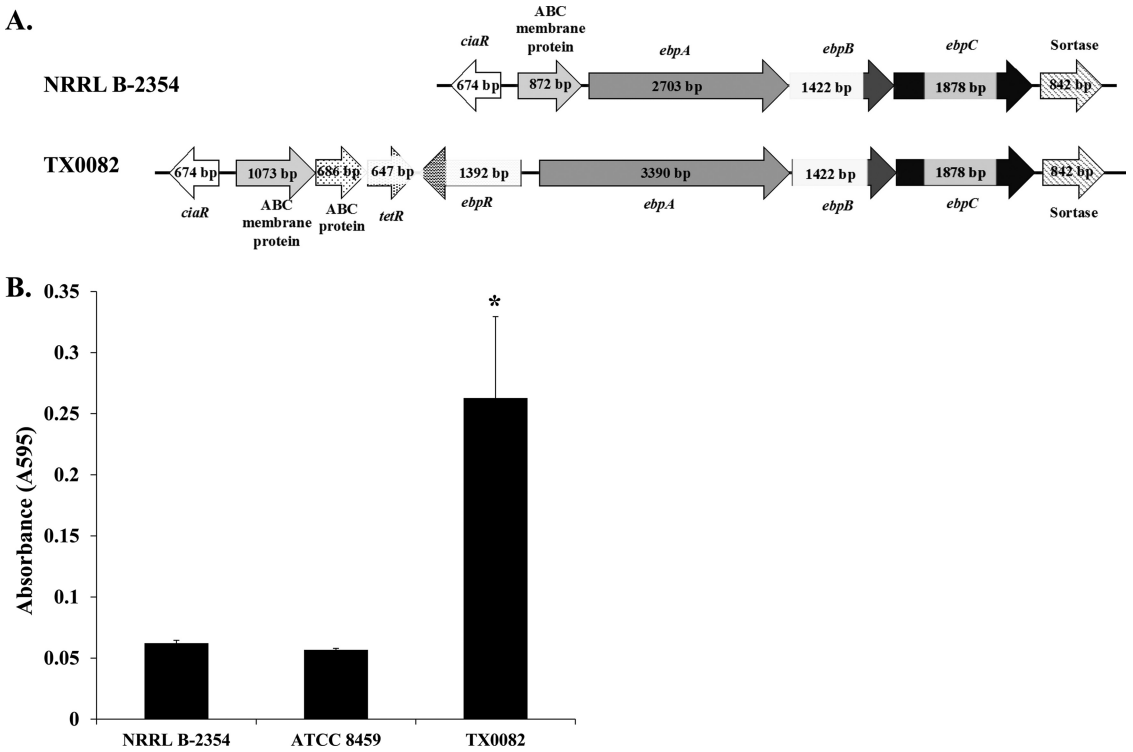

FIG 5 Ebp locus and biofilm formation in *E. faecium* NRRL B-2354. (A) Schematic diagram of the *ebp* operon in *E. faecium* NRRL B-2354 and TX0082. (B) *E. faecium* biofilm formation on polystyrene. Absorbance was significantly higher for TX0082 than for the other two strains (Tukey's HSD, $P < 0.05$). The averages ± SD of three replicates per strain are shown.

reference to BSL-2 for NRRL B-2354 (Todd Ward, personal communication).

Additionally, we examined the NRRL B-2354 genome for other *E. faecium* VF, including secreted enzymes and cell surface proteins. Genotype and phenotype assessments confirmed that *E. faecium* NRRL B-2354 lacks the capacity to produce gelatinase and cytolysin. These enzymes are most often found in CL-associated strains of *E. faecium* and have been directly linked to enterococcal virulence in animal models of infection (74, 75). Cell surface proteins associated with enterococcal virulence include functions in biofilm formation and adhesion to extracellular matrix proteins such as collagen, fibrinogen, and fibronectin (65, 76). *E. faecium* NRRL B-2354 contains partial or nonfunctional copies of *acm* encoding a collagen I adhesin and the *ebp* operon for pilus production.

E. faecium NRRL B-2354 does contain complete and hence likely functional copies of *sagA*, *scm*, *efaA*, and the *pilA* operon. The majority of these genes apparently did not contribute to the phenotypes tested here (i.e., collagen adherence, pilus production, and biofilm formation). Overall, these genes are not as well characterized as other known *E. faecium* virulence determinants (8). Although NRRL B-2354 adhered to fibrinogen and fibronectin, possibly through SagA, this phenotype has also been described for probiotic *Lactobacillus* strains (77, 78) and has been detected for dairy-associated strains of *E. faecium* (79). Such functions might be important for intestinal colonization and regarded as "niche factors," as has been suggested for probiotic lactobacilli (80).

Unlike the clinical strains TX0082 and 1,231,502, *E. faecium* NRRL B-2354 exhibited a heightened capacity to survive environmental stresses. These are useful characteristics for a surrogate microorganism which should exhibit levels of stress tolerance similar to those of the human pathogens that they are intended to mimic, such as strains of *Salmonella*. Stress tolerance in lactic acid bacterium relatives of *E. faecium* is due to a variety of metabolic and stress response pathways (81, 82). It is notable that *E. faecium* NRRL B-2354 exhibited superior acid, heat, and ethanol stress tolerance levels, possibly indicating that similar mechanisms are involved in conferring to this strain the capacity to survive/grow under those conditions. *E. faecium* NRRL B-2354 contains numerous genes coding for stress-responsive proteins, including an F_oF_1 ATPase, certain transcriptional regulators (*ctsR*), and chaperones and proteases (*dnaK*, *groEL*, *grpE*, *ftsH*, *htrA*, *clpB*, *clpC*, *clpE*, *clpP*, and *clpX*) (data not shown). However, the genomes of the hospital-associated strains TX0082 and 1,231,502 also contain the majority of these genes. Hence, future studies should investigate the specific mechanisms by which *E. faecium* NRRL B-2354 and not TX0082 and 1,231,502 can survive environmental stresses. This information would also be useful for predicting which organisms would be suitable surrogates.

Despite its common occurrence in foods, *E. faecium* has not been causally linked to food-borne infection (1). Instead, experimental and clinical infections caused by this species appear to be the result of contact by certain strains of this species to extraintestinal sites on the body through catheters, surgeries, or poor sanitation (8). Hence, *E. faecium* NRRL B-2354 presents a clear example of the need for strain- and application-specific evaluations rather than species level designations on safety. Future studies should further clarify the exact mechanisms of *E. faecium* pathogenesis and distinguish between strains that have acquired distinct

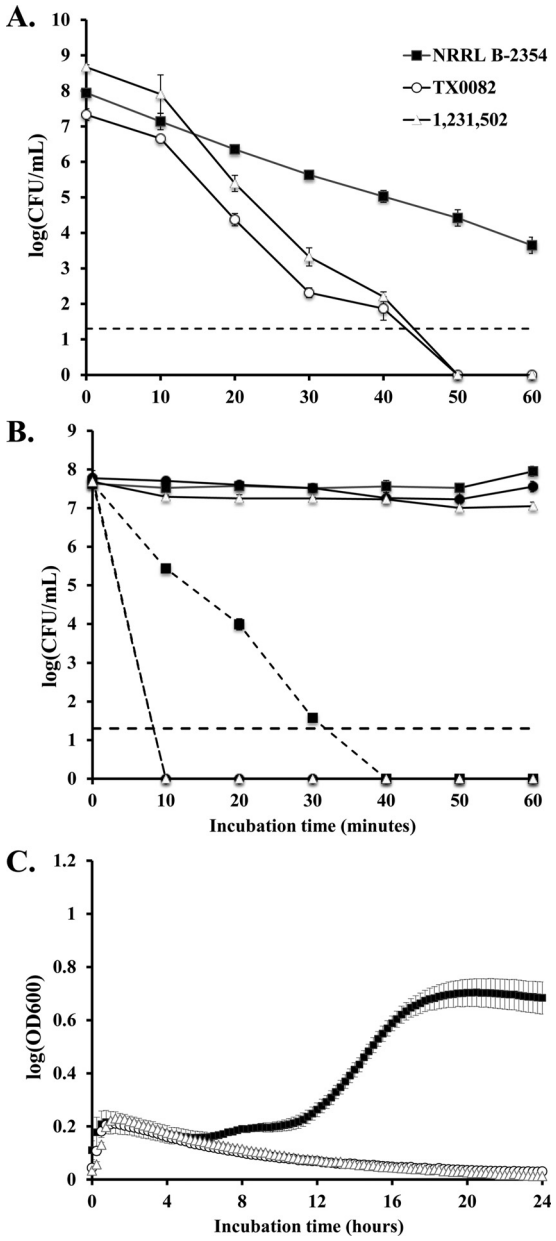

FIG 6 Stress responses to low pH, heat, and ethanol. (A) Survival at pH 2.4. Numbers of viable cells of *E. faecium* NRRL B-2354, TX 0082, and 1,231,502 were determined at intervals of 10 min during incubation in physiological saline adjusted to pH 2.4. Detection limit was 20 CFU/ml. The averages ± SD of three replicates per strain are shown. (B) Thermal survival of *E. faecium* in physiological saline. Numbers of viable cells of *E. faecium* NRRL B-2354, TX 0082, and 1,231,502 were determined at 10-min intervals during incubation at 50°C (solid lines) and 60°C (dashed lines). Detection limit (black dashed line) was 20 CFU/ml. The averages ± SD of three replicates per strain are shown. (C) Ethanol tolerance of *E. faecium*. Growth of *E. faecium* strains NRRL B-2354, TX 0082, and 1,231,502 was monitored during incubation in 8% ethanol for 24 h with absorbance at OD_{600} measured every 15 min. The averages ± SD of three replicates per strain are shown.

traits for colonization of extraintestinal sites on the human body and those that benefit food safety and human health.

Presently, there are few bacterial surrogate strains available to validate processes used to control food-borne pathogens in food processing (83, 84). As for any bacterial strain, *E. faecium* NRRL B-2354 should be handled with appropriate care; however, it lacks the genomic and phenotypic characteristics that define strains of this species responsible for nosocomial infections. The data presented here along with the long history of safe use of *E. faecium* NRRL B-2354 and its clonal representative ATCC 8459 support its continued role in the safe production of foods.

ACKNOWLEDGMENTS

We thank Grete Adamson and Patricia Kysar from the University of California, Davis, School of Medicine, Department of Pathology and Laboratory Medicine, Electron Microscopy Lab, Davis, CA, for their assistance with the electron microscopy. We thank Lex Overmars for assistance with genome assembly. We also thank the UC Davis Medical Center for their assistance with the antibiotic resistance testing. We also kindly thank Barbara Murray for providing strain *E. faecium* TX0082, Michael Gilmore for providing strain 1,231,502, and Catherine Strong for *Bacillus cereus* strain ATCC 14579.

This project was supported by the Almond Board of California.

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