Importance of Two *Enterococcus faecium* Loci Encoding Gls-like Proteins for In Vitro Bile Salts Stress Response and Virulence

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General stress proteins, Gls24 and GlsB, were previously shown to be involved in bile salts resistance of *Enterococcus faecalis* and in virulence. Here, we identified 2 gene clusters in *Enterococcus faecium* each encoding a homolog of Gls24 (Gls33 and Gls20; designated on the basis of their predicted sizes) and of GlsB (GlsB and GlsB1). The sequences of the *gls33* and *gls20* gene clusters from available genomes indicate distinct lineages, with those of hospital-associated CC17 isolates differing from non-CC17 by ~7% and ~3.5%, respectively. Deletion of an individual locus did not have a significant effect on virulence in a mouse peritonitis model, whereas a double-deletion mutant was highly attenuated ($P < .004$) versus wild-type. However, mutants lacking either *gls33-glsB*, *gls20-glsB1,* or both all exhibited increased sensitivity to bile salts. These results suggest that *gls*-encoded loci may be important for adaptation to the intestinal environment, in addition to being important for virulence functions.

Enterococci have emerged as important opportunistic pathogens over the past 3 decades and now rank second among pathogens isolated from health care-associated infections in humans [1, 2]. Recent surveillance data have revealed a significant change in the ratio of *E. faecium* to *E. faecalis* isolated from nosocomial infections; as recently as the 1990s, *E. faecium* represented <10% of total enterococcal infections, but now it accounts for more than one-third of enterococcal clinical isolates identified to the species level in the United States and in some European hospitals [2–4]. Concurrently, clinical *E. faecium* isolates are much more resistant to multiple antibacterial agents, particularly ampicillin and vancomycin [5–7], than are *E. faecalis* isolates, making treatment of infections caused by this organism problematic. Furthermore, *E. faecium* isolates from nosocomial sources more often carry virulence-associated genes, such as an intact *acm* gene (encoding a collagen-binding adhesin) [8, 9], *hyl* (a putative hyaluronidase) [10–12], and *esp* (enterococcal surface protein) [13, 14], and most of the 14 *fms* genes encoding *E. faecium* surface proteins of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family [15–17], including pili [15,18–20].

Bacteria are known to encounter various stress conditions in their natural environments and in the host when causing infections. Although these processes and their determinants remain uncharacterized for *E. faecium,* several factors involved in stress response in other gram-positive bacteria are thought to be involved in bacterial adaptation to hostile environments in the host and have been associated with virulence [21–25]. Previous studies with *E. faecalis* have shown that growth in the absence of glucose elicits the production of 42 glucose starvation proteins [26]. Because the expression of one of these proteins,

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Gls24, was shown to be induced under other stress conditions also, such as the presence of bile salts and cadmium chloride, it was designated as a general stress (Gls) protein. Insertional inactivation of the *gls24* gene [27, 28] led to a reduced survival rate in the presence of bile salts. Subsequently, our mutation and complementation data identified *glsB*, located immediately downstream of *gls24*, as also being involved in bile salts tolerance of *E. faecalis* strain OG1RF. Disruption of *gls24* in *E. faecalis* OG1RF resulted in significant attenuation in a mouse peritonitis model [27] and a rat endocarditis model [29], whereas no effect was observed with *glsB* in either model, indicating that *gls24*, but not *glsB*, is important for *E. faecalis* virulence. Our data also demonstrated protection by anti-Gls24 immune serum against *E. faecalis* infection in the mouse peritonitis model, suggesting that Gls24 may be useful as a target for immunotherapy [27].

In this study, we used in silico analysis to search for *E. faecalis gls24* homologs in *E. faecium* and found 2 closely located gene clusters, each containing a homolog of *gls24* and of *glsB*. The 2 *gls24-glsB*-like gene pairs were deleted independently and in combination to evaluate their role in bile salts response and in a mouse peritonitis model.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Escherichia coli, *E. faecalis,* and *E. faecium* strains and plasmids used are listed in Table 1. Plasmid constructs were given pTEX numbers, and their host strains were assigned respective TX numbers. *E. coli* strains were grown routinely in Luria-Bertani media (Difco Laboratories) and enterococci in either brain heart infusion (BHI) or Todd-Hewitt (TH) broth or agar (Difco Laboratories) at 37°C. The following antibiotic concentrations were used with enterococci: chloramphenicol, 10 μg/mL; erythromycin, 15 μg/mL, gentamicin, 125 μg/mL. With *E. coli*, the concentrations used were chloramphenicol, 10 μg/mL; erythromycin, 200 μg/mL; and gentamicin, 25 μg/mL.

Standard Molecular Techniques

DNA from *E. faecium* isolates was prepared following the hexadecyltrimethyl ammonium bromide method [35]. Agarose plugs containing genomic DNA were digested with *Sma*I, and pulsed-field gel electrophoresis (PFGE) was performed as described previously [36], but with ramped pulse times beginning with 2 s and ending with 28 s, at 200 V for 23 h.

Construction of pTEX5504ts Vector

To construct plasmid pTEX5504ts, a polymerase chain reaction (PCR)-amplified *erm*(B)-containing fragment (using primers erm F and erm R; Supplementary Table 1) from the *E. faecalis* shuttle vector pMSP3535 [32] was digested with *Apa*I and *Hin*dIII and then ligated into similarly digested pTEX5501ts [33].

Construction of Deletion Mutants

E. faecium deletion mutants were constructed by allelic replacement, as described previously [33]. Briefly, upstream and downstream fragments (both ~1 kb) flanking *gls33* and *glsB* were amplified from *E. faecium* TX1330 genomic DNA using primers designed from the sequenced strain TX16 (for primers, see Supplementary Table 1) and ligated into pTEX5504ts; the construct was then transformed into *E. coli* DH5α to obtain TX6059 (Table 1) and confirmed by sequencing. After electroporation into *E. faecium* TX1330 (wild-type [WT]), single-crossover integrants (TX1330::pTEX6059; see Table 1) were selected on BHI plates that contained gentamicin and erythromycin. One single cross-over integrant grown for 12 serial passages at 42°C was serially diluted, plated, and grown at 37°C on nonselective media. These plates were then replica plated onto erythromycin and gentamicin plates to identify colonies that had lost the integrated plasmid by double crossover recombination. This deletion mutant, designated as TX6060 [TX1330Δ*gls33-glsB*::*erm*(B)], was confirmed by sequencing, hybridization, and PFGE.

The same protocol was used to construct a clean *gls20-glsB1* deletion, except that another temperature-sensitive vector derivative, pTEX5501ts [33], was used. The upstream and downstream fragments flanking *gls20-glsB1* were cloned in this vector, resulting in pTEX6065.The resulting deletion mutant strain was designated as TX6066 (TX1330Δ*gls20-glsB1*).

Attempts to create a *gls20-glsB1* deletion in the *gls33-glsB* deletion mutant (TX6060), using the above construct (pTEX6065, above and in Table 1), were unsuccessful, so another reverse primer (gls20-B1del1b R, Supplementary Table 1) was designed for amplification of the upstream flanking region of *gls20-glsB1* and to construct pTEX6065a (Table 1). As a result, the double deletion mutant, designated as TX6067 (TX1330Δ*gls33-glsB*::*erm* Δ*gls20-glsB1*), includes a 68-bp 5′ segment of the *gls20* coding sequence.

Complementation Constructs

To complement the TX1330Δ*gls33-glsB*::*erm* and TX1330Δ*gls20-glsB1* mutants *in trans*, a 1250-bp fragment containing the *gls33-glsB* open reading frames plus the *gls33* ribosomal binding site (see Supplementary Table 1 for primers) and a 853-bp fragment containing the *gls20-glsB1* open reading frames plus the *gls20* ribosomal binding site (Supplementary Table 1) were cloned under control of the P2 promoter of the shuttle vector, pAT392 [34]. The resulting constructs were designated as TX6064 (TX6060 with pAT392::*gls33-glsB*) and TX6071 (TX6066 with pAT392::*gls20-glsB1*).

Table 1. Bacterial Strains and Plasmids Used in This Study

Strains/plasmids	Relevant characteristics	Reference or source
Strains		
Enterococcus faecium		
TX16 (DO)	Sequenced strain; endocarditis isolate, Ery^r, Gm^s	[30]
TX1330	Sequenced strain; faecal isolate from a healthy volunteer, Ery^s, Gm^s	[31]
TX6060	TX1330Δ*gls33-glsB*::*erm*(B), single deletion mutant; Ery^r	This study
TX6066	TX1330Δ*gls20-glsB1*, single deletion mutant	This study
TX6067	TX1330Δ*gls33-glsB*::*erm* Δ*gls20-glsB1*, double deletion mutant, Ery^r	This study
TX6064	TX6060 with pAT392:: *gls33-glsB,* complementation construct, Ery^r, Gm^r	This study
TX6071	TX6066 with pAT392::*gls20-glsB1,* complementation construct, Gm^r	This study
TX6063	TX6060 with pAT392, Ery^r, Gm^r	This study
TX6070	TX6066 with pAT392, Gm^r	This study
Escherichia coli		
DH5α	*E. coli* cloning host	Stratagene
TX6059	DH5α (pTEX6059)	This study
TX6065	DH5α (pTEX6065)	This study
TX6065a	DH5α (pTEX6065a)	This study
TX6072	DH5α with pAT392::*gls33-glsB*	This study
TX6073	DH5α with pAT392::*gls20-glsB1*	This study
Plasmids		
pMSP3535	Shuttle plasmid used as a source of *erm*(B) to create pTEX5504ts	[32]
pTEX5501ts	Temperature-sensitive suicide plasmid, used for deletion mutagenesis of *E. faecium,* Chl^r, Gm^r	[33]
pTEX5504ts	Derived from pTEX5501ts, used for deletion mutagenesis of *E. faecium,* Ery^r, Gm^r	This study
pAT392	Vector used for complementation, Gm^r	[34]
pTEX6059	Plasmid for *gls33-glsB* deletion; flanking regions of the *gls33-glsB* on either side of *erm*(B) cloned into pTEX5504ts; Ery^r, Gm^r	This study
pTEX6065	Plasmid for *gls20-glsB1* deletion; flanking regions of *gls20-glsB1* amplified using primers gls20-B1del1F, gls20-B1del1a R and gls20-B1del2F, gls20-B1del2R and cloned into pTEX5501ts; Chl^r, Gm^r,	This study
pTEX6065a	Plasmid for *gls20-glsB1* deletion; flanking regions of the *gls20-glsB1* amplified using primers gls20-B1del1F, gls20-B1del1b R and gls20-B1del2F, gls20-B1del2R and cloned into pTEX5501ts; Chl^r, Gm^r	This study
pTEX6072	Plasmid for complementation; fragment containing *gls33-glsB* (1250 bp) cloned to pAT392	This study
pTEX6073	Plasmid for complementation; fragment containing *gls20-glsB1* (853 bp) cloned to pAT392	This study

NOTE. Superscript ''s'' designates sensitivity, ''r'' designates resistance. Chl, chloramphenicol; Ery, erythromycin; Gm, gentamicin.

Growth Curves

Growth curves in BHI were determined as described previously [33].

Reverse Transcriptase (RT)-PCR

Isolation of total RNA from *E. faecium* TX1330, TX6060, TX6066, and TX6067, grown to mid-exponential phase in BHI, and RT-PCR (see Supplementary Table 1 for primers) were performed as described previously [15]. A 585-bp fragment of the gyrase gene (*gyrA*) of TX1330 was amplified as an internal control, and reactions without reverse transcriptase were used as additional controls to confirm the absence of DNA contamination in the total RNA preparation.

Antibodies

Anti-Gls24 antibodies raised in rabbits against *E. faecalis* OG1RF recGls24 [27] were affinity purified using recGls24 as the binding ligand, as described previously [9].

Protein Extraction and Western Blot

Surface protein extracts from *E. faecium* were prepared using 0.2% Zwittergent 3–12 (Calbiochem) as described previously [27]. Protein extracts were electrophoresed by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were incubated with affinity-purified anti-Gls24-specific antibodies followed by horseradish peroxidase-conjugated goat anti-rabbit antibodies. The blots were then developed with Supersignal West Pico chemiluminescent substrate (Pierce).

Bile Salts Resistance Assay

Resistance to bile salts was determined as described previously [27]. In brief, after 24 h growth in BHI at 37°C, *E. faecium* WT and mutants were centrifuged and resuspended in the same volume of fresh BHI. From these, 20 μL was then inoculated into 1 mL of fresh BHI containing 0.3% bile salts. After a 30 min

incubation at 37°C, *E. faecium* cells were serially diluted and plated. The percentage of survival was calculated using a previously described equation [27].

Mouse Peritonitis Model

Four inocula (ranging from 2.6×10^8 to 3.8×10^9) of *E. faecium* TX1330 (WT), *gls* mutants, and complemented strains, grown in BHI broth premixed with 50% sterile rat fecal extract, were individually injected intraperitoneally into outbred ICR mice (Harlan Sprague Dawley) using 10 mice per inoculum, and mice were observed for 5 days, following our previously published method [37]. Preapproved guidelines of the Animal Welfare Committee of the University of Texas Health Science Center at Houston were followed throughout the animal experiments.

Statistical Analyses

For the bile salts assay, survival rates of WT versus *gls* mutants were compared using 1-way ANOVA (analysis of variance) with Bonferroni correction for multiple comparisons. The unpaired *t* test was used for complementation constructs versus the respective vector controls. For the peritonitis model, comparison of survival curves at similar inocula was performed using a log-rank test.

RESULTS AND DISCUSSION

Paralogous *gls* Loci in *E. faecium*

Because factors involved in the adaptation of *E. faecium* to stress environments have not been described, we searched the genomes of *E. faecium* strains TX16 (DO; endocarditis-derived) and TX1330 (healthy human volunteer feces-derived) for homologs of *gls* genes of *E. faecalis* that assist in resistance to bile salts [27, 28]. In both genomes, we identified 2 genes encoding close homologs of *E. faecalis gls24* (88%–90% similarity with Gls24 of *E. faecalis* V583 and 87% similarity between the 2 *E. faecium* paralogs). One is encoded as part of a 9-gene cluster and the second as part of a 4-gene cluster, separated by ~3.2 (TX16) or 12.1 (TX1330) kb regions (Figure 1A; see below). Each of these Gls24 homologs was followed by an 80 aa ORF exhibiting >90% similarity with GlsB of *E. faecalis* V583 (ca. 98% similarity between the 2 paralogs). The *gls24* homolog found in the 9-gene cluster has an additional 130 aa at the N-terminus, compared with the *E. faecalis* homolog and the other *E. faecium* paralog, and was designated as Gls33 based on its predicted molecular mass of 33 kDa. The Gls24 homolog of the 4-gene cluster was named as *gls20* (predicted protein 20 kDa) and the 2 GlsB homologs as GlsB and GlsB1, respectively. Of note, although *E. faecalis* OG1RF has a single *gls24* locus, *E. faecalis* V583 has an additional *gls*-like locus in its pathogenicity island [38]. The percent similarities among different ORFs of each *gls* cluster of *E. faecium* and also versus *E. faecalis* are shown in Supplementary Table 2.

Transcriptional Analysis of the *gls33* and *gls20* Loci Confirmed That These Genes Are Co-Transcribed as 9- and 4-Gene Operons, Respectively

As shown in Figure 1A, in both TX16 and TX1330, *gls33* and *glsB* are preceded by 7 ORFs oriented in the same direction and separated from each other by -8 (overlapping) to 79 bp (*orf1* to *orf7*), whereas *gls20* and *glsB1* are preceded by 2 similarly oriented ORFs (*orfA* and *orfB*, separated by 12 bp). Annotations for the genes encoding ORF1 to ORF7 and ORFA to ORFB are listed in Supplementary Table 2. Because of short or overlapping intergenic regions and the absence of identifiable rho-independent transcriptional terminator-like sequences, we predicted that *orf1* to *glsB* and *orfA* to *glsB1* may be co-transcribed.

RT-PCR of messenger RNA (mRNA) isolated from TX1330, using primers spanning the intergenic regions of the 2 gene clusters, indicated that the genes from *orf1* to *glsB* and from *orfA* to *glsB1* are expressed by TX1330 cells during mid-exponential growth and that the genes in each cluster are expressed as a single mRNA transcript (Figure 1B). No PCR products were detected with primer pairs designed to amplify upstream regions of *orf1* and *orfA*, which begin before their predicted promoter sequences and extend into their 5′ intragenic regions, indicating that the genes further upstream are not part of these operons. A strong hair-pin loop 133 bp downstream of *glsB* (free energy -22.9 kcal/mol) and another 43 bp downstream of *glsB1* (-19.9 kcal/mol) were identified as transcriptional terminators for these operons. Consistent with these predictions, no PCR product was detected with primer pairs that include the 3′ end of *glsB* or *glsB1* and extend beyond the predicted transcriptional terminators. Taken together, our analyses indicate that both gene clusters, from *orf1* to *glsB* and *orfA* to *glsB1*, are transcribed as single polycistronic mRNA and, thus, are organized as operons. However, the gene compositions of both of these operons are only partially similar to the 6-gene *gls* operon present in *E. faecalis* [27].

Organization of Both *gls* Loci Is Highly Conserved among Sequenced *E. faecium* Isolates of Clinical and Nonclinical Origin

Our most recent searches with other currently available *E. faecium* draft genomes (by the Broad Institute, MA, and available at NCBI [39]) also identified *orf1,2,3,4,5,6,7-gls33-glsB* and *orfA,B-gls20-glsB1* operons in 8 of the 9 genomes; the missing *orf1-glsB* locus in one of the genomes may be attributable to the incomplete status of this genome assembly (currently 220 contigs). The sequences of these clusters from the 11 strains (including TX16 and TX1330) were found to represent 2 distinct groups. Group A consists of 6 strains (all from clinical origin and

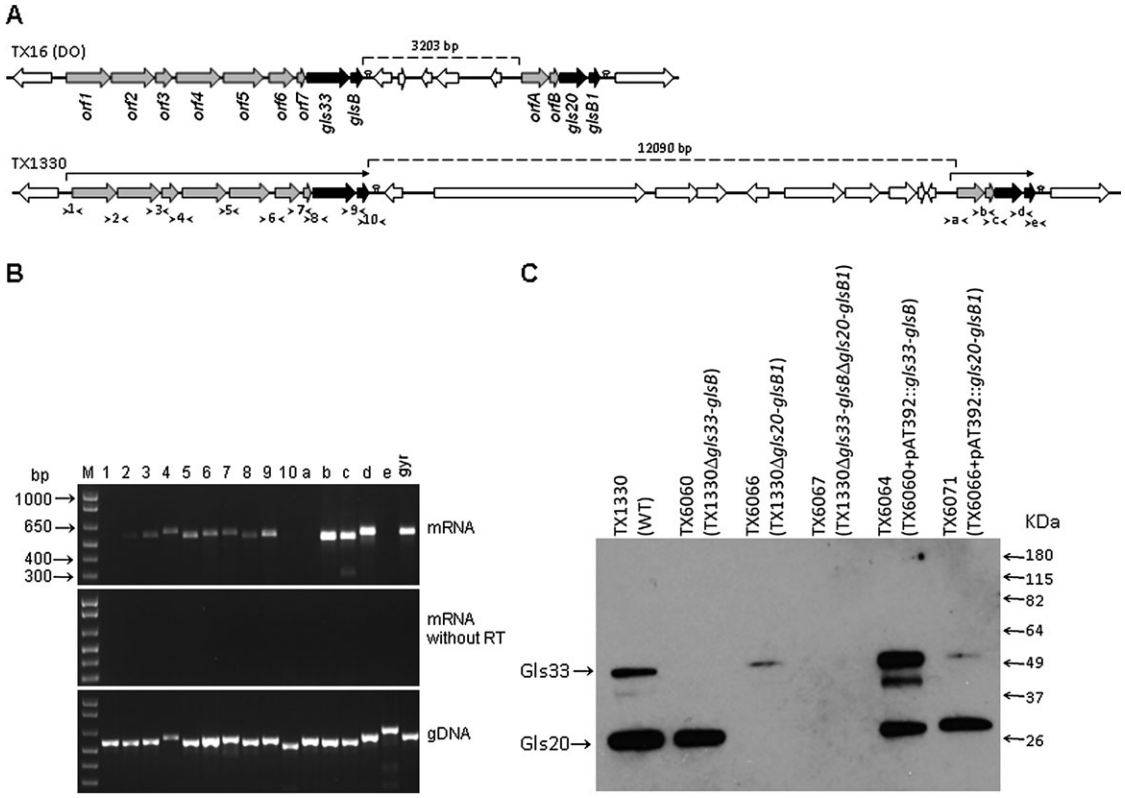

Figure 1. Organization of the 2 *gls* loci of *Enterococcus faecium* and their transcriptional and protein expression analysis. *A,* Schematic representation of these loci in *E. faecium* TX16 and TX1330. The annotations were predicted from bioinformatics analysis for *orf1* to *orf7* and *orfA* to *orfB,* which are shown in Supplementary Table 2. Predicted transcriptional terminators downstream of *glsB* and *glsB1* are indicated with lollypops. Arrows above each of the 2 *gls* clusters of TX1330 indicate messenger RNA (mRNA) co-transcripts. Location of each intergenic primer pair for reverse transcriptase polymerase chain reaction (RT-PCR) is shown with arrow heads (see panel *B* below). *B,* RT-PCR analysis of the 2 *gls* clusters of TX1330. Top gel, RT-PCR with DNase-treated total RNA (30 ng) isolated from mid-exponential cells; middle gel, control reaction of the same RNA preparation amplified without reverse transcriptase; bottom gel, control reaction amplified with genomic TX1330 DNA. An intragenic region of the gyrase gene was used as an additional control. Numbering and lettering of the lanes corresponds to the intergenic primer pairs shown in panel A. M, molecular weight marker. *C,* Western blot analysis of Zwittergent whole-cell extracts from *E. faecium* TX1330, its 3 *gls* deletion mutants, and complementation derivatives. The membrane was probed with affinity-purified anti-Gls24 (*E. faecalis*) antibodies [26], which cross-react with both Gls33 and Gls20. Locations of bands corresponding to Gls33 and Gls20 are indicated with arrows.

belonging to the hospital-associated genogroup CC17 [40]) with >99% identity to each other over the entire *orf1-gls33-glsB* cluster but only ~93% identity with the 5 group B isolates (3 derived from community human feces and 2 from human blood, all non-CC17 and belonging to unrelated STs that differ by more than 5 alleles). Similarly, the *orfA-gls20-glsB1* cluster showed 100% identify within Group A isolates but ~96.5% identity with the B isolates. Interestingly, of the 8 *E. faecium* draft genomes where both *gls* loci are located in the same contig, the distance between the 2 loci (*glsB* and *orfA*) is ~3.2 kb in all 4 group A isolates (including TX16) and ~12.1 kb in the 4 group B isolates (including TX1330). More than 90% of the ~3.2 kb spacer region of TX16 is also present in the longer spacer of TX1330, which is predicted to carry 5 additional ORFs. Thus, our analysis suggests that there are 2 common variants of this region in *E. faecium,* in terms of sequence length and gene composition.

Characterization of *gls33-glsB* and *gls20-glsB1* Deletion Mutants

We initially constructed an insertion mutant of *gls33* by single cross-over mutagenesis in TX1330 and tested it in a mouse peritonitis model. Although our results showed delayed mortality of the *gls33* insertion mutant, compared with WT TX1330, this difference did not reach statistical significance (data not shown). To circumvent disadvantages associated with single cross-over mutants (eg, instability of the mutant or possible expression of residual *gls33*), we next generated single and double deletion mutants of the *gls* loci in TX1330 (Supplementary Figure 1A, panel A). Comparison of growth curves of TX1330 (WT), TX6060 (TX1330Δ*gls33-glsB::erm*(B), TX6066 (TX1330Δ*gls20-glsB1*), and TX6067 [TX1330Δ*gls33-glsB::erm*(B) Δ*gls20-glsB1*] showed almost identical growth rates (OD and CFU) in BHI (data not shown), indicating that these genes are not essential for in vitro growth of *E. faecium*.

i. mRNA Studies. Analysis of the genes located immediately upstream of *gls33* (*orf6-orf7*) and *gls20* (*orfA-orfB*) in these 3 mutants by *RT-PCR* showed that the gene deletions had no effect on expression of the upstream genes (versus WT), indicating that *gls33-glsB* and *gls20-glsB1* are not involved in transcriptional autoregulation of these operons (Supplementary Figure 1B). Furthermore, the transcription of the remaining *gls* gene pair in each of the 2 single deletion mutants showed no difference from the WT (Supplementary Figure 1B), suggesting that the 2 *gls* loci do not cross-regulate each other's expression, at least not in BHI.

ii. Protein Studies. Because Gls24 of *E. faecalis* is similar to Gls33 and Gls20 (see above) and, thus, antibodies against these proteins may cross-react, we used an affinity-purified polyclonal anti-Gls24 antibody in a Western blot analysis of surface protein extracts from WT TX1330 and the 3 deletion mutants. We detected 2 bands in the TX1330 extract (Figure 1C) corresponding to Gls33 and Gls20, although they migrated at ~50 kDa and ~28 kDa in SDS-PAGE, respectively (our previous study with Gls24 of *E. faecalis* also showed migration at ~28 kDa; this may be due to the pI values of these proteins). In addition, the larger band was absent in the *gls33-glsB* deletion (TX6060) and the lower band in the *gls20-glsB1* deletion (TX6066), whereas both were absent in the double deletion mutant (TX6067). Hence, this confirms the lack of protein expression of Gls33 and Gls20 in surface extracts of their respective deletion mutants. Furthermore, complementation of the deleted genes in trans restored expression of Gls33 and Gls20 to WT levels in TX6064 (TX6060 with pAT392::*gls33-glsB*) and TX6071 (TX6066 with pAT392::*gls20-glsB1)*, respectively (Figure 1C). The extra band in TX6064 is likely to be a degradation product of the over-expressed protein. Because TX6064 is a complementation construct of a single deletion mutant lacking only Gls33, it is expected to have the other band, which is Gls20. A similar band pattern was observed in >3 experiments.

Contribution of the Two *gls* Loci to Survival in the Presence of Bile Salts

To study the role of the *gls33-glsB* and *gls20-glsB1* gene pairs in resistance to bile salts, the different mutants and complementation derivatives were examined for viability upon exposure to 0.3 % bile salts, a concentration that is physiologically relevant [41]. All 3 *gls* deletion mutants were more sensitive to bile salts than TX1330 ($P < .001$; ANOVA). The mean percentages of survival ± SD of TX6060, TX6066, and TX6067 were 64% ± 2%, 70% ± 4%, and 17% ± 8%, respectively, compared with WT (Figure 2). The reduction by the double deletion mutant versus each of the single deletion mutant was also significant ($P < .001$). As shown in Figure 2, complementation of the 2 single deletions, TX6060 and TX6066, with *gls33-glsB* (TX6064) and *gls20-glsB1* (TX6071), respectively, resulted in restoration of tolerance to bile salts to levels ⩾WT (119 ± 12 by TX6064, $P < .001$ versus the vector alone control TX6063 and versus TX6060; 108 ± 15 by TX6071, $P < .001$ versus the vector alone control TX6070 and versus TX6066). These results are in agreement with the restored protein expression observed by Western blots (see above). Thus, our data indicate that both *gls* loci are required for full tolerance of bile salts at concentrations likely to be encountered by enterococci in the upper human intestinal tract, under the conditions tested.

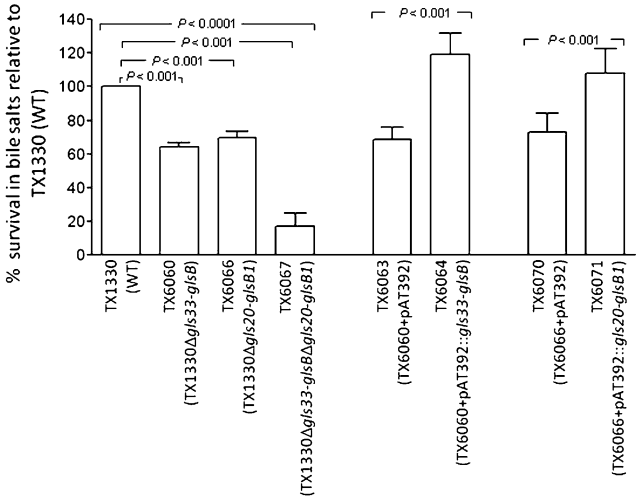

Figure 2. Comparison of three *gls* deletion mutants and complementation constructs versus wild-type (WT) and vector alone controls for survival after an exposure to bile salts. The mean and SD for combined results are shown from ⩾5 independent experiments. Survival rates of WT versus the 3 *gls* deletion mutants were compared by analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons, and complementation constructs versus the vector only controls by the unpaired *t* test.

Importance of the *gls*33-*glsB* and *gls20-glsB1* Loci for *E. faecium* Virulence

To test the effects of different *gls* deletions in a mouse peritonitis model, mice were infected with a range of CFUs. As shown in Figure 3A and B, neither the *gls33-glsB* (TX6060) nor the *gls20-glsB1* deletion (TX6066) mutants showed attenuation versus the WT in either total death or time to death over a period of 5 days. Similarly, no difference was observed with complementation constructs TX6064 and TX6071 versus vector alone controls TX6063 and TX6070, respectively (data not shown). Of note, we also found that ~95% of TX6064 and TX6071 colonies isolated from peritoneal fluid of mice had lost the complementing plasmid in vivo. Using a range of inocula (4.0×10^8 to 3.8×10^9 CFU), the double deletion mutant, TX6067, was significantly ($P < .004$) attenuated versus WT in this model (Figure 3C), indicating that, although both *gls33-glsB* and *gls20-glsB1* are important for virulence in this model, the individual genes are able to compensate for each other's absence. Because of the observed instability of the complementing plasmid noted above,

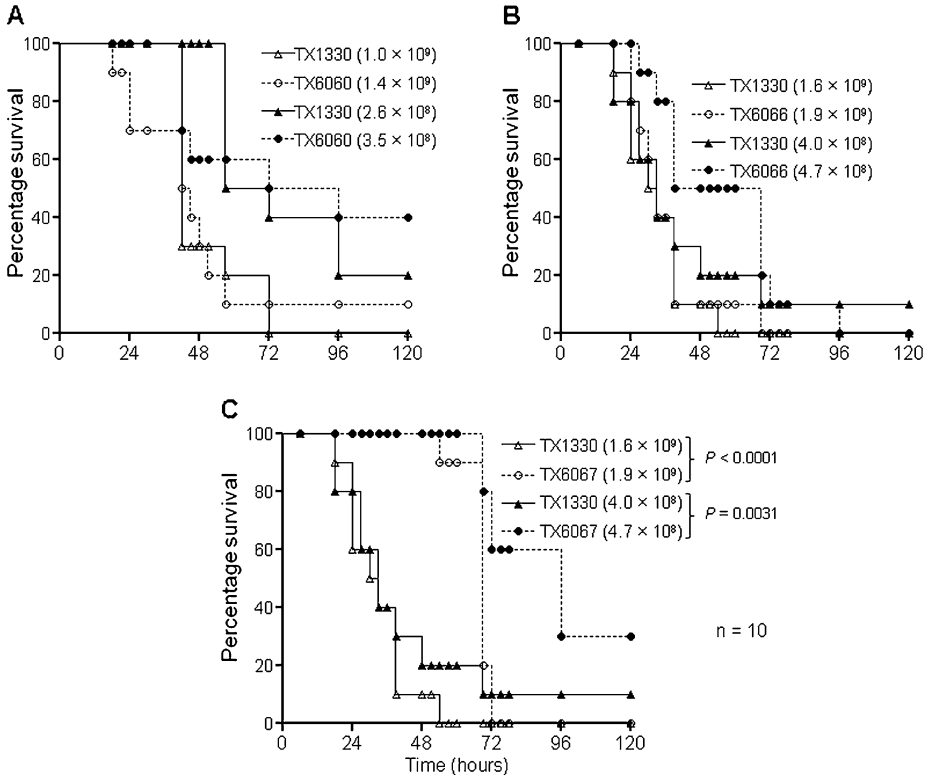

Figure 3. Effect of the *gls* deletions on *Enterococcus faecium* virulence in a mouse peritonitis model. Kaplan-Meier survival curves after injecting 2 different inocula of *(A)* wild-type (WT) and TX6060 (TX1330Δ*gls33-glsB*), *(B)* WT and TX6066 (TX1330Δ*gls20-glsB1*), and *(C)* WT and TX6067 (TX1330Δ*gls33-glsB*Δ*gls20-glsB1*). A log-rank test was used for comparisons of survival rates. Survival rates of each single deletion mutant at 2 inocula shown above were nonsignificant versus WT, with *P* values .1 to .7.

we did not attempt further to complement the double deletion mutant, because other vectors have also shown instability.

In summary, we have identified 2 closely located conserved paralogs, *gls33-glsB* and g*ls20-glsB1*, encoding Gls-like proteins in *E. faecium*. RT-PCR analysis demonstrated that both loci are expressed as larger operons, with the *gls33-glsB* operon encompassing 7 additional upstream genes and the g*ls20-glsB1* operon encompassing 2 additional upstream genes. Analysis of regions containing *gls* genes from available sequenced genomes identified that (1) the 2 *gls* clusters are close to each other in all strains, (2) the *gls* region occurs as 2 common variants with the 2 operons separated by either a 3.2 or a 12.1 kb region in different strains, and (3) the 2 variants show ~7% sequence divergence between their *gls33-glsB* operons and ~3.5% between their *gls20-glsB1*operons. Importantly, 1 of the variants was exclusively found in the hospital-associated CC17 genogroup, suggesting the possibility that evolution of this locus preceded CC17 emergence. Deletion of each *gls*-like locus resulted in decreased tolerance to bile salts and, in our assay, a double mutant lacking both loci showed additional decreased tolerance. Furthermore, in the peritonitis model, single gene pair deletions were not significantly attenuated, whereas deletion of both *gls33-glsB* and g*ls20-glsB* resulted in significantly prolonged survival, compared with WT. Thus, *gls33-glsB* and *gls20-glsB1* both play a role in bile salts tolerance and in experimental peritonitis, although one appears to be able to substitute, at least partially, the role of the other in the latter.

Supplementary Data

Supplementary data are available at http://jid.oxfordjournals.org/ online.

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