The Majority of a Collection of U.S. Endocarditis Enterococcus faecalis Isolates Obtained from 1974 to 2004 Lack Capsular Genes and Belong to Diverse, Non-Hospital-Associated Lineages

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Eighty-one endocarditis-derived Enterococcus faecalis isolates that were collected from individual patients in the United States between 1974 and 2004 were sequence typed and analyzed for the presence of various genes, including some previously associated with virulence. Overall, using our previously described trilocus sequence typing (TLST), 44 different sequence types (STs) were found within this collection; 26 isolates were singletons (a unique TLST sequence type [ST]), some STs contained multiple isolates (up to 6 isolates), and 16% of the isolates (13 isolates) could be grouped by additional sequence typing into clonal cluster 21 (CC21). Of note, only four isolates (7%) of the 56 whose multilocus sequence types were determined were found to belong to one of the previously described hospital-associated clonal clusters CC2 and CC9, and only 15% and 37% of all isolates had high-level resistance to gentamicin and streptomycin, respectively, including 10% that were resistant to both. We also found that 64% of the isolates lacked the genes for production of capsule polysaccharide, which has been proposed to enhance the pathogenic potential of the hospital-associated clonal clusters. In summary, while our collection is not a random sample of cases of E. faecalis endocarditis, these results indicate that nonencapsulated strains belonging to non-hospital-associated lineages were predominant among endocarditis E. faecalis isolates recovered during this time period.

Enterococci are Gram-positive commensal organisms of the gastrointestinal tract that have emerged in recent decades as the second most common organisms isolated from health care-associated infections, after staphylococci (1, 2). More longstanding is its role as a cause of endocarditis, which was documented as early as 1899 (3–5). Endocarditis is a bacterial infection of the valves and/or inner lining of the heart and is uniformly fatal without antibiotic treatment (6). Enterococci cause 5 to 15% of cases of infective endocarditis (IE) (2, 7) and are third behind staphylococci and streptococci; however, in the hospital setting, enterococci are second behind staphylococci as a cause of endocarditis (6, 8, 9). Historically, one species of enterococci, Enterococcus faecalis, causes most cases of enterococcal endocarditis (10–13).

Treatment of enterococcal infections often presents a notable therapeutic challenge to physicians due to the ease of acquiring and transferring antimicrobial drug resistance, including high-level aminoglycoside resistance and glycopeptide resistance (14), along with their intrinsic resistance to various antibiotics, such as cephalosporins, and their relative resistance to penicillins (14).

The identification and genotypic characterization of particular lineages that may be more fit, more virulent, and/or more antibiotic resistant (and thus capable of causing more-problematic infections) are important objectives when trying to understand the epidemiology of infectious diseases, especially for emerging or evolving pathogens, such as enterococci. In addition, typing studies of other organisms have often found that certain clones can be associated with specific disease states (15–18). Previously, a multilocus sequence typing (MLST) scheme based on 7 housekeeping genes of E. faecalis identified two high-risk clonal clusters (CCs), CC2 and CC9, which seem to be particularly adapted to the hospital environment, containing predominately hospital-derived isolates (19). Of note, the majority of β-lactamase-producing (Bla+) isolates from outbreaks in the 1980s (Bla+ vancomycin-resistant endocarditis [BVE] lineage) and the first documented vancomycin-resistant enterococci (VRE) in the United States (E. faecalis strain V583) belong to CC2, and high-level gentamicin resistance was most frequently found within CC9 (19–22).

The hospital-associated lineages (CC2 and CC9) of E. faecalis have also been found to produce capsular polysaccharide (CPS) (23), and capsule production has been associated with the evasion of host defenses (24). Capsule-producing E. faecalis strains were found to be much more resistant than nonencapsulated strains to complement-mediated opsonophagocytosis, by masking C3 bound on the surface and by preventing bacterial surface antigens, such as lipoteichoic acid (LTA), from being detected by agglutinating antibodies (24, 25). Capsular polysaccharide is encoded by a locus containing up to 9 open reading frames (cpsC to cpsK), at least 6 of which (cpsC, cpsD, cpsE, cpsG, cpsI, and cpsK) are essential for capsule biosynthesis (25–27). The upstream genes cpsA and cpsB (present in all E. faecalis strains and initially thought to be involved in capsule production) are transcribed from a different promoter and are not part of the operon (28). Furthermore, pre-
vious studies classified *E. faecalis* isolates into four serotypes, serotypes A to D, which include the Maekawa serotyping strains T1, T2, and T5, representing the three prototypical capsule locus polymorphisms (29). Serotypes A and B (containing Maekawa type 1) are nonencapsulated and contain only *cpsA* and *cpsB*. Serotype C (including Maekawa type 2) contains *cpsC* to *cpsK*, while serotype D (containing Maekawa type 5) is like serotype C but also lacks *cpsF* (23, 25, 29, 30). Also of interest, isolates of Maekawa type 2 have been noted for harboring more virulence and antibiotic resistance traits than the other types (27).

With recent clinical reports suggesting 15% to 39% of enterococcal endocarditis to be nosocomial (31–33), we chose to examine and to identify whether particular lineages or clones of *E. faecalis* might be associated with infective endocarditis by trilocus sequence typing (TLST), MLST, *cps* genotyping, and pulsed-field gel electrophoresis (PFGE) analysis, using a >30-year collection of 81 endocarditis isolates from across the United States (19, 21, 34–39). In addition, these isolates were tested for resistance to high levels of aminoglycosides and were characterized for the presence or absence of genes within the pathogenicity island (PAI), virulence-associated genes, and genes encoding microbial surface components recognizing adhesion matrix molecules (MSCRAMMs).

**MATERIALS AND METHODS**

**Bacterial isolates.** Eighty-one *E. faecalis* isolates, each from individual patients with infective endocarditis, were studied. These isolates were recovered over 31 years (with the earliest isolate collected in 1974 and the latest in 2004) from patients from a variety of states (including Connecticut, Iowa, Illinois, Massachusetts, Minnesota, Missouri, Ohio, Texas, and Wisconsin); many have been described previously (19, 21, 34–39). In addition, these isolates were tested for resistance to high levels of aminoglycosides and were characterized for the presence or absence of genes within the pathogenicity island (PAI), virulence-associated genes, and genes encoding microbial surface components recognizing adhesion matrix molecules (MSCRAMMs).

**Sequence-based typing.** Genomic DNA was extracted using the Qia-gen DNeasy tissue kit (Valencia, CA), from single-colony-derived cultures grown in brain heart infusion (BHI) broth (BectonDickson, Sparks, MD). All isolates were typed by TLST using a previously described procedure that assesses intragenic fragments of *ace*, *sala*, and *lsa* (34). The TLST sequence type (STT) of isolate TX0052 was previously published (34). Select strains were also typed by MLST, as described by Ruiz-Garbajosa et al. (19). MLST sequence types (STMs) of 9 isolates were published previously (34, 38) (see Table S1 in the supplemental material). Sequence alignments for each of the gene fragments were performed by the Jotun Hein method (40, 41), using the MegAlign program of DNASTAR software (Madison, WI). Distinct allelic types for *ace*, *sala*, or *lsa* not described previously (34) were assigned the next consecutive number (see Tables S2 to S4 in the supplemental material). STT’s previously assigned were assigned as published, with the exception of STT-T512 (allele profile *ace*-12, *sala*-33, *lsa*-34) (34), which was reclassified as STT-T317 to match the corresponding MLST type. STT-T512 was then arbitrarily reassigned to TLST allelic profile *ace*-17, *sala*-20, *lsa*-45. STT’s not described previously for isolates typed by both MLST and TLST (34) were assigned sequential numbers beginning with T500 when the STT either was not determined or corresponded to another previously published STT allelic profile (34); when possible, the STT number assigned was matched to the STT. STTs were assigned in accordance with the database available at http://efaecalis.mlst.net. When two or more isolates had the same STT and related PFGE pattern, MLST was performed on at least one isolate of the group, and then that MLST was assigned to the other members of the group and designated the “inferred” STT (see Table S1 in the supplemental material).

**PFGE.** PFGE was performed as previously described (42). The PFGE patterns were interpreted using the criteria suggested by Tenover et al. (43).

**Generation of probes and hybridization.** Colony lysate hybridizations were performed under high-stringency conditions (44) with probes labeled using the RadPrime DNA labeling kit (Invitrogen, Carlsbad, CA). PCR products (primers and amplification conditions as reported in the respective references) used for probes included amplified internal fragments of *cps* (capsular polysaccharide) genes (*cpsA* to *cpsK*) (26); two genes within the putative PAI, i.e., *cbh* (putative bile acid hydrolase) and *ef571* (putative DNA-binding response regulator) (45, 46); a few other genes suggested to be virulence-associated genes, including *ge264* (general stress response protein) (21, 47), *hydA* (hyaluronidase, with MSCRAMM-type features) (21, 48), *hylB* (putative hyaluronidase) (21), *hec3* (biofilm enhancement) (49, 50), *ef3056* (srtA; housekeeping sortase) (51), and *ef2524* (a class A sortase that is part of an integrated plasmid remnant region of strain V583 [efacC]) (51-54); and several Ig-like fold-containing putative surface adhesion/MSCRAMM-type genes, including *ef0089*, *ef1269*, *ef2224*, *ef1896*, *ef1824*, *ef2505*, and *ef2347* (21, 48).

**Capulse locus genotypes.** The *cps* genotypes are based on the hybridization profiles for the presence or absence of 11 genes, i.e., *cpsA* to *cpsK*, and are correlated with the three Maekawa prototypical capsule locus polymorphisms; Maekawa type 1 (*cps* serotypes A and B) possesses *cpsA* and *cpsB* only and corresponds to *cps* genotype 1, Maekawa type 2 (*cps* serotype C) possesses *cpsA* to *cpsK* and corresponds to *cps* genotype 2, and Maekawa type 5 (*cps* serotype D) possesses *cpsA* to *cpsK* except for *cpsF* and corresponds to *cps* genotype 5 (23, 26, 27, 30). Serotypes were inferred based on *cps* genotypes in accordance with a previous study by Hufnagel et al. (27).

**Resistance profiles.** Resistance to high levels of streptomycin and gentamicin (Sigma-Aldrich) were determined according the protocol presented by the Clinical and Laboratory Standards Institute (35).

**RESULTS AND DISCUSSION**

**Sequence types and major lineages.** Using TLST (34), we assessed the genetic relatedness of the 81 endocarditis-derived *E. faecalis* isolates, to determine whether there might be a predominant lineage associated with this infection. All strains and their origin, isolation year, STT, STM, and *cps* genotype are listed in Table S1 in the supplemental material. For the individual genes of the TLST scheme, a total of 23, 26, and 32 allele types were found for *ace*, *sala*, and *lsa*, respectively, in this collection (see Tables S2 to S4 in the supplemental material), including 13 *ace* (14 *sala*), and 14 (lsa) new alleles. Forty-four different STT’s were found among the 81 isolates, of which 58 isolates belonged to 36 previously unpublished types. Approximately 36% of the IE isolates (29/81 isolates) represented a single isolate per STT, of which 26 of the 29 were new STT’s not yet described and thus were singletons, that is, distinct types that did not match the STT of any other strain in our collection (34). The remaining 52 isolates formed sequence type (ST) groups containing 2 to 6 isolates. The largest TLST types within this collection were STT-T40 (n = 6), STT-T511 (n = 6), STT-T55 (n = 5), STT-157 (n = 5), STT-20 (n = 4), STT-44 (n = 4), STT-6 (n = 3), STT-287 (n = 3), STT-317 (n = 3), and STT-T523 (n = 3). The STT’s with two isolates included STT-T21, STT-T62, STT-T107, STT-T283, and STT-T525. Isolates in the largest ST groups, STT-T40 and STT-T511, were predominately collected at the Mayo Clinic (a regional referral center for IE cases) in Rochester, Minnesota, between 1974 and 1994. However, the five isolates of the next largest clonal lineage, STT-T55, were acquired from hospitals in Iowa, Connecticut, and Massachusetts between 1975 and 1993; the five
isolates of STT-157 were also acquired from diverse locations (Illinois, Ohio, Minnesota, Texas, and Massachusetts) and over longer time periods (between 1975 and 2002). The more recent isolates (collected since 2000; n/H110059) were represented by 8 distinct types (Table 1; also see Table S1 in the supplemental material).

Representative isolates of each STT containing two or more isolates were also typed by MLST; the MLST types for 9 isolates were published previously (34, 38). Overall, 26 isolates representing 18 distinct STTs were differentiated into 17 different types by MLST. As in our previous study (34), the concordance of discrimination between MLST and TLST was extremely high, with the discordant isolates differing only by 1/3,297 total nucleotides (nt).

### TABLE 1

<table>
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<th>STT (ace, salA, lsa) types</th>
<th>No. of isolates</th>
<th>STM</th>
<th>CPS type</th>
<th>Year(s) of collection</th>
<th>Isolation location(s)</th>
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</table>

a The data are ordered according to ST’s based on intragenic sequences of ace, salA, and lsa. See Table S1 in the supplemental material for additional isolate information. ST’s not described previously (34) that had MLST data were assigned, when possible, the same type number as the corresponding STM. Type numbers assigned previously by TLST were assigned as published; therefore, identical corresponding STM numbers were not always possible (specifically STT-20 and STT-33). ST’s for which MLST data were not available, the STM was not in the database, or the STM corresponded to another published STT (such as for STM-4 and STM-34) were assigned arbitrary numbers beginning with T500.

b STMs are according to the seven-housekeeping-gene scheme and were assigned in accordance with the database at http://efaecalis.mlst.net.

c STMs include direct and inferred types. The inferred types were based on groups of isolates with identical STs for which MLST had been performed for at least one isolate of the group and which were also related by PFGE.

d NT, new type (i.e., a new STM not yet assigned in the MLST database) (the allele types were gdh-9, ggd-5, pslS-4, gki-16, aroE-1, xpt-11, and yqil-8).

e The cps genotypes were assigned in accordance with the three prototypical capsule polymorphisms; the cps type 1 possesses cpsA and cpsB only, as in Maekawa strain T1 (corresponding to serotypes A and B), the cps type 2 possesses cpsA to cpsK, as in Maekawa strain T2 (corresponding to serotype C), and the cps type 5 possesses cpsA to cpsK except for cpsF, as in Maekawa strain T5 (corresponding to serotype D) (23, 26).
(thus, only one allele difference) by MLST in all cases in which two MLST types were identified within one TLST type and therefore are considered very closely related (see Table S1 in the supplemental material).

Our data also indicate that some isolates in different ST’s belong to the same lineages based on MLST allelic variation. For example, ST T -21 (n = 2) and ST T -157 (n = 5) are single-locus variants (SLVs) of each other that differ by only 4 nt in salA and are 99.8% identical over the 2,559 total concatenated nt of the three trilocus genes. The corresponding MLST data corroborated the very close relationships between ST M -21 and ST M -157, which differ by 1 nt in gki (99.9% identical over the concatenated 7 gene sequences, consisting of 3,297 nt). Therefore, all 7 isolates would be considered part of CC21, a clonal cluster previously defined in the MLST scheme (19, 23). In addition, the 6 isolates in ST T -T511 differ from ST T -21 by only 1 nt in lsa and are 99.9% identical to each other over concatenated ace, lsa, and salA in trilocus typing; by MLST, these isolates were all classified as ST M -21. Thus, 13 isolates in total can be placed in CC21. Another close relationship was identified for ST -283 (n = 2) and ST -62 (n = 2), which are double-locus variants in both multilocus and trilocus typing (99.7% and 99.4% identical in concatenated gene sequences by MLST and TLST, respectively). ST T -40 (n = 6) and ST T -55 (n = 5) also clustered closely together in Fig. 1, on the basis of the concatenated sequences of the TLST alleles. They are 99.8% identical over the three loci (SLVs differing by 4 nt in lsa); although they differ by 3/7 genes by MLST, they are 99.6% identical (12 nt difference) in the 7-allele concatenated sequences and thus are closely related. Additional possible relationships are described in the supplemental material. However, the largest lineages remained CC21 (n = 13), ST T -40 (n = 6), and ST T -55 (n = 5).

The enriched lineages among our collection of U.S. IE isolates were also among common lineages, particularly CC21 and CC40, found in a subset of 51 diverse U.S. nonendocarditis clinical E. faecalis isolates in a 2007 study by McBride et al. (23). Furthermore, five clonal lineages, i.e., CC2 (n = 14), CC9 (n = 9), CC40 (n = 8), CC8 (n = 5), and CC21 (n = 4), represented 78% (n = 40) of the U.S. nonendocarditis subset examined by McBride et al. (23) with MLST. Of those, the hospital-associated clonal lineages CC2 and CC9 were the largest clonal clusters, representing 27% (14/51 isolates) and 18% (9/51 isolates) of the isolates, respectively (23). In contrast, considerably more diversity was found among our collection of U.S. IE isolates (44 different ST’s among the 81 isolates, of which 36% were singletons), and only 7% (4 of 56 isolates with direct or inferred MLST types) belonged to a high-risk hospital-associated MLST lineage (CC21) (versus 27% above). Our results are consistent with those of a 2011 study by Larsen et al. (56), which also found a diversity of ST’s among 20 IE E. faecalis isolates from Danish hospitals typed by MLST, with at most three isolates per ST (ST M -72, n = 3; ST M -97, n = 3; ST M -55, n = 2; ST M -306, n = 2; the remaining 10 STs were singletons); one isolate belonging to CC40 and one belonging to CC21, which were common in our collection, were also found, while no IE isolates belonged to CC2 or CC9. It is interesting to note that of 6 of our IE isolates belong to ST -40, which is also associated with commensal organisms in Danish pigs (46, 56).

Investigation of the large clonal lineages by PFGE. The aforementioned larger TLST clonal lineages and MLST CC21 (containing ST M -21 and ST M -157) were also examined by PFGE. Although at times PFGE can be too discriminatory to detect the relatedness of organisms in a ST, all isolates within the respective TLST lineages were considered by PFGE to be possibly to probably related (see Fig. S1a and b in the supplemental material). Also, although most of the isolates of ST T -40 were collected in one hospital, the lineage contained several distinct PFGE subtypes. On the other hand, although the isolates of ST T -55 were from different geographical locations, subtypes from different states were very closely related (≤2 band differences) by PFGE, i.e., TX0002 from Connecticut (1993) and TX0030 from Iowa (1983), as well as TX0027 from Iowa (1975) and TX0048 from Massachusetts (before 1980) (see Fig. S1a in the supplemental material). Among the 13 isolates within the large MLST CC21 (ST T -21, ST T -157, and ST T -T511), each had a related PFGE pattern within the respective TLST-defined lineage, but there were multiple PFGE types overall. In conclusion, the clonal lineages most often found were represented by several subtypes, but overall these lineages were enriched within the endocarditis collection (see Fig. S1b in the supplemental material).

cps gene locus types. The endocarditis isolates were further characterized for their cps gene locus type. Approximately 64% harbored cpsA and cpsB only (indicating Maekawa type 1, with inferred serotypes A and B), 14% harbored cpsA to cpsK (indicating Maekawa type 2, with inferred serotype C), and 22% harbored cpsA to cpsK but lacked cpsF (indicating Maekawa type 5, with inferred serotype D) (23, 26, 27, 30). The cps locus type was invariant between strains of the same ST.

CPS and endocarditis isolates. Although a previous study found nearly a quarter of enterococcal endocarditis to be nosocomial (endocarditis developing >72 h after admission in association with a hospital-based procedure during that hospitalization or within the preceding 8 weeks) (32), our study found only four isolates in MLST types (direct or inferred) that are part of CC2 (ST T -2, n = 1; ST T -6, n = 3) and none belonged to CC9, the two CCs that are hospital associated (19). Also, in contrast to CC2 and CC9, which were found to be cps genotype 2 and genotype 5, respectively (23), the predominant CC and/or clonal lineages found to be enriched among the endocarditis isolates (MLST CC21 and ST T -40, ST T -55, ST T -20, and ST T -44) were nonencapsulated (cpsA and cpsB only). Interestingly, although capsular polysaccharide has been found to enhance the pathogenicity of E. faecalis by masking detection of C3 and LTA antibodies, therefore evading complement-mediated opsonization (24) and thus possibly contributing to the persistence of encapsulated strains in hospital-associated infections (23), the lack of capsule among the lineages enriched among the IE isolates suggests that other virulence factors play more key roles in establishing the infection of endocardial tissue; it is also possible that capsule may interfere with the function of these factors.

PAI, virulence-associated, and MSCRAMM genes. Three factors, i.e., Ace (adhesin to collagen of E. faecalis), Ebp pili (endocarditis- and biofilm-associated pili), and Gls24 (glucose starvation protein), which are ubiquitous among E. faecalis isolates (except for a single fecal isolate, of 472 tested, that lacked the ebp locus) (21, 38, 57, 58), have previously been implicated as playing roles in infection, since disruption of the genes encoding Ace, Ebp, Gls24 resulted in attenuation in animal models of IE (58–60). Thus, we searched for the presence of additional factors that may play roles in pathogenicity by colony hybridization of selected genes within the PAI, virulence-associated genes, and genes
FIG 1 Phylogenetic tree of genetic relationships determined by TLST among 81 E. faecalis strains. The phenogram is based on the matrix of pairwise sequence divergences in the concatenated composite sequences of three gene fragments (ace, salA, and bla) and was generated by the Jotun Hein method of the DNASTAR software package. The length of each pair of branches represents the distance between sequence pairs.
en encoding MSCRAMMs. In addition to the genes ace (48, 51), ebp (38), and gbs24 (24) and as found with previous surveys of E. faecalis, the MSCRAMM-encoding genes, ef0089, ef1269, and ef2222, as well as ef3056 (srtA), were found in all isolates in this collection (data not shown). The other sortase gene present in the mobile genetic element efaCl of strain V583 (52, 53) (but not OG1RF), ef2524, was found in only 31% of the isolates (25/81 isolates). The next most frequently found genes were hylA and hylB, which were present in 70/81 isolates (86%) and 67/81 isolates (83%), respectively. Only one isolate, TX0079 (a singleton), was found to have neither of the putative hyaluronidase genes. The bee3 gene was found in only two strains, both singletons, i.e., TX0025 (STT-T509) and TX0006 (STT-T506) and TX0006 (STT-T509). The remaining MSCRAMM-encoding genes were found as follows: ef1824 was present in 51% of the isolates (41/81), ef1896 was present in 35% of the isolates (28/81), and ef2347 was present in 6% of the isolates (5/81). Of the genes within the putative PAI, cbh was present in 59% of the isolates (48/81) and ef571 was present in 14% of the isolates (11/81 isolates) (Fig. 2). Also, unlike the cps gene locus types, some isolates within a specific clonal cluster/lineage had genetic variation in the presence of the other genes tested. Variation within isolates of the same ST suggests recent acquisition or loss of these genes by an isolate.

**Resistance to high levels of aminoglycosides.** Approximately 15% of the isolates (12/81 isolates) and 37% of the isolates (30/81 isolates) exhibited resistance to gentamicin 500 μg/ml and streptomycin 2,000 μg/ml, respectively, including 8 isolates (10%) that were resistant to both gentamicin and streptomycin, which was seen only in isolates collected after 1990. In general, the trend for aminoglycoside resistance increased over the years, except that 60% of the isolates collected between 1974 and 1980 (n = 15) were resistant to streptomycin 2,000 μg/ml, whereas 32% collected after 1980 (n = 66) were resistant (Fig. 3).

**Concluding remarks.** Although we found considerable diversity among IE isolates, we also identified several lineages that occurred more often than others, including CC21 (containing STM-21 and STM-157) with nearly 16% (n = 13) of the isolates, as well as STM-40 (n = 6) and STM-55 (n = 5). Of particular interest, only 7% of 56 IE isolates for which MLST was performed or results were inferred were part of the hospital-associated CCs (four isolates belonged to ST-2 and ST-6, which are part of CC2). We also found that only 36% of our isolates had cpsA to cpsF (with or without cpsF), indicating that the majority of isolates are noncapsulated, unlike the common hospital-associated lineages. Whether the clonal lineages and cps locus types associated with hospitals will increase as hospital-acquired enterococcal endocarditis increases remains a question for the future.

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