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Capsular Polysaccharide Production in *Enterococcus faecalis* and Contribution of CpsF to Capsule Serospecificity▽†

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Many bacterial species produce capsular polysaccharides that contribute to pathogenesis through evasion of the host innate immune system. The gram-positive pathogen *Enterococcus faecalis* was previously reported to produce one of four capsule serotypes (A, B, C, or D). Previous studies describing the four capsule serotypes of *E. faecalis* were based on immunodetection methods; however, the underlying genetics of capsule production did not fully support these findings. Previously, it was shown that capsule production for serotype C (Maekawa type 2) was dependent on the presence of nine open reading frames (*cpsC* to *cpsK*). Using a novel genetic system, we demonstrated that seven of the nine genes in the *cps* operon are essential for capsule production, indicating that serotypes A and B do not make a capsular polysaccharide. In support of this observation, we showed that serotype C and D capsule polysaccharides mask lipoteichoic acid from detection by agglutinating antibodies. Furthermore, we determined that the genetic basis for the difference in antigenicity between serotypes C and D is the presence of *cpsF* in serotype C strains. High-pH anion-exchange chromatography with pulsed amperometric detection analysis of serotype C and D capsules indicated that *cpsF* is responsible for glucosylation of serotype C capsular polysaccharide in *E. faecalis*.

Enterococcus faecalis is a gram-positive bacterium commonly found as a commensal organism in the gastrointestinal tracts of most mammals. *E. faecalis* is one of the leading causes of hospital-acquired urinary tract infections, bacteremia, and surgical-site infections (29). The development of multiple antibiotic resistances, including resistance to vancomycin, makes treatment of enterococcal infections difficult (11). The 2004 National Nosocomial Infections Surveillance report indicated that nearly 30% of enterococci isolated from clinical settings were resistant to vancomycin, constituting a 12% rise from the previous 5 years (26). The development of alternative therapies to treat enterococcal infections has frequently been suggested due to rising percentages of antibiotic-resistant enterococcal strains (13–15, 19).

Capsular polysaccharides are major contributors to the virulence of many microorganisms. The presence of capsule allows these microbes to escape detection and clearance by the host immune system (9, 27, 30, 41). There have been several publications regarding the role of cell wall polysaccharides in the pathogenesis of enterococcal infections (10, 13, 17, 37, 43). Several attempts have been made to establish a serotyping system for *E. faecalis* capsular polysaccharides (16, 23, 35, 36). These serotyping schemes include differences in capsular polysaccharide antigens but are also based on differences in surface antigens, including lipoteichoic acid (16, 38). To date, only one study has linked genetic evidence with capsule production (12). Two loci that have been reported to contain putative genes for capsule production are the *epa* and *cps* operons (10, 42). The polysaccharide produced by the *epa* locus is thought to be the cell wall rhamnopolymer (10), but it cannot be detected on the surface of the bacterium (43). Although rhamnopolymer production is reported to be abrogated by mutation (43), the full nature of rhamnopolymer production is yet to be determined for many *E. faecalis* strains. Probing the genomes of serotype A and B strains with a probe specific to the *cps* locus, including the genes *cpsA* and *cpsB*, identified a single ClaI restriction fragment for serotypes A and B (16). However, multiple ClaI restriction fragments were identified in serotypes C and D (16), suggesting that the genes responsible for capsule production in serotypes C and D were absent in serotypes A and B. Furthermore, the hybridization pattern between serotype C and D strains indicated a single restriction fragment polymorphism, but the basis on which genes were different between the two serotypes was not fully characterized (16). Studies based on the serotyping scheme proposed by Hufnagel et al. (17) have shown that serotype C and D strains are much more resistant to opsonophagoctyosis by neutrophils in the presence of normal human serum. More recently, a study by McBride et al. indicated that serotype C clinical isolates harbored a greater repertoire of antibiotic resistance cassettes and were more likely to possess multiple virulence factors than the other serotypes, suggesting that the presence of the capsule is associated with pathogenic lineages of *E. faecalis* (17, 24).

It is essential to understand the underlying mechanisms of capsule production in *E. faecalis* because of ongoing efforts to develop alternative therapies targeting capsule. Here, we used a novel vector system for creating isogenic, in-frame deletion mutants to analyze the genetic basis for capsule production and serotype specificity. Our results show that only serotype C and D strains of *E. faecalis* produce capsular polysaccharides, based on the observation that deletions of *cpsC*, *cpsD*, *cpsE cpsG*, and *cpsI* abolish the production of capsule. In conjunction with these observations, we also demonstrated that the presence of capsule prevents detection of lipoteichoic acid on the surface of serotype

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TABLE 1. *E. faecalis* strains used in this study

Strain	Description[a]	Reference
V583	Serotype C	31
FA2-2	Serotype C	7
MMH594	Serotype C	18
OG1RF	Serotype B	25
12030	Serotype A	14
12107	Serotype B	14
E-1	Serotype NT	4
Maekawa type 1	Serotype B	23
Maekawa type 2	Serotype C	23
Maekawa type 5	Serotype D	23
Maekawa type 7	Serotype A	23
Maekawa type 8	Serotype NT	23
Maekawa type 11	Serotype NT	23
Maekawa type 18	Serotype D	23
LT01	FA2-2 Δ*cpsF*	This study
LT02	V583 Δ*cpsF*	This study
LT03	LT01 + pLT10	This study
LT04	LT02 + pLT10	This study
LT05	FA2-2 Δ*cpsC*	This study
LT06	V583 Δ*cpsC*	This study
LT07	LT05 + pLT34	This study
LT08	LT06 + pLT34	This study
LT09	T-5 + pLT10	This study
LT10	T-18 + pLT10	This study
LT11	OG1RF + pLT10	This study
LT15	V583 Δ*cpsD*	This study
LT17	V583 Δ*cpsE*	This study
LT19	V583 Δ*cpsG*	This study
LT21	V583 Δ*cpsH*	This study
LT23	V583 Δ*cpsI*	This study
LT25	LT15 + pLT25	This study
LT27	LT17 + pLT32	This study
LT29	LT19 + pLT33	This study
LT31	LT21 + pLT14	This study
LT33	LT23 + pLT35	This study

[a] Strains marked "NT" were nontypeable by conventional serotyping methods (16).

C and D strains but not on unencapsulated strains. Our data also show that CpsF is responsible for the difference in serospecificity between serotype C and D strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All relevant bacterial strains are listed in Table 1. *Escherichia coli* EC-1000 (20) and Electro-10 Blue (Stratagene) were used for plasmid construction. *E. coli* clones were grown in Luria-Bertani (LB) broth supplemented with the appropriate antibiotics when required (32). *E. faecalis* strains were cultivated in Todd-Hewitt broth supplemented with the appropriate antibiotics when needed (THB; Becton, Dickinson and Company, Sparks, MD). When required for selective growth of *E. coli*, chloramphenicol (Cm) was used at 10 μg/ml and spectinomycin was used at 150 μg/ml. When required for the selective growth of *E. faecalis*, Cm was used at 15 μg/ml and spectinomycin was used at 750 μg/ml. For detection of β-galactosidase activity, 5-bromo-4-chloro-3-indolyl-â-d-galactopyranoside (X-Gal) was used at 80 μg/ml for *E. coli* and 120 μg/ml for *E. faecalis*.

Dot blot analysis. We performed dot blots with DNA from representative *E. faecalis* strains, including FA2-2, V583, MMH594, Maekawa types 1, 2, 4, 5, 7, 8, 11, and 18, and strains OG1RF, 12030, 12107, and E-1 to determine the presence of *cps* operon genes. Purified DNA from each strain was denatured in 0.4 M NaOH to a concentration of 1 μg/ml and spotted onto nylon membranes. The membranes were rinsed several times with Tris-EDTA buffer, pH 8.0. DNA was cross-linked to the membrane using UV irradiation. Gene-specific radiolabeled probes were generated by PCR using primers (see Table S1 in the supplemental material) for each of the *cpsA* through *cpsK* genes and the downstream gene, *hcp*. Membrane strips were placed in 12 hybridization tubes to be probed independently by each gene-specific probe. Following hybridization, membrane strips were aligned adjacent to one another beginning with the strip probed by the *cps*A-specific probe and continuing through to *hcp*. These membranes were then exposed to X-ray film for autoradiography.

Construction of pLT06. For descriptions of all primers and plasmids, see Table S1 in the supplemental material. pLT06 is a combination of pCJK47 (20), pGB354 (3), and pCASPER (6) (see Fig. 2). The *ermC* cassette in pCJK47 was replaced with the Cm acetyltransferase (*cat*) gene from the *Streptococcus agalactiae* plasmid pGB354. The vector pCJK47 was digested with the restriction enzymes BglII and NsiI, resulting in 5.8-kb and 0.9-kb fragments. The *cat* gene from pGB354 was amplified by PCR with the primers Cat5′ and Cat3′. The resulting PCR product was cloned as a blunt-end fragment into a 5.8-kb fragment of pCJK47 (T4 DNA polymerase treated). The resulting construct was called pKS05. pKS05 was subsequently digested with SmaI and EcoNI, and the 5.7-kb fragment containing P-*pheS*, *cat*, and *lacZ* was gel extracted (QIAquick gel extraction kit; Qiagen), followed by Klenow treatment (Bioline). pCASPER was digested with EcoRV and PshAI, and the 2.15-kb product containing *orfB*, *orfC*, *repA*(Ts), and *orfD* was gel extracted. The 5.75-kb pKS05 product and the 2.15-kb product of pCASPER were blunt end ligated, resulting in pLT06.

Construction of markerless exchange vectors. Vector pLT06 was used to create in-frame deletions of *cpsC*, *cpsD*, *cpsE*, *cpsF*, *cpsG*, *cpsH*, and *cpsI* in *E. faecalis* strains V583 and FA2-2. Relevant primers are listed in Table S1 in the supplemental material. Fragments (1.0 kb each) were PCR amplified upstream and downstream of the gene targeted for mutation. The PCR products were ligated and reamplified, resulting in a 2.0-kb product. The 2.0-kb PCR product was digested by restriction enzymes as described in Table 2 and ligated with pLT06. The ligated products were electroporated into *E. coli* Electro 10 Blue (E10B) for propagation and grown on LB plates containing Cm and X-Gal at 30°C. Blue colonies were screened for the presence of the ~2.0-kb inserts using primers OriF and KS05SeqR. Positive clones were grown overnight in liquid LB medium containing Cm at 30°C. The plasmid was purified using the QIAprep spin miniprep kit (Qiagen). The ~2.0-kb inserts from each construct were sequenced using the primers OriF and KS05SeqR to ensure that no mutations arose during cloning. The resulting deletion constructs, pLT08, pLT13, pLT16, pLT18, pLT22, pLT23, and pLT24, were used to generate the *cpsF*, *cpsH*, *cpsD*, *cpsE*, *cpsC*, *cpsG*, and *cpsI* deletions, respectively.

Generation of deletion mutants. *E. faecalis* V583 and FA2-2 were used for the generation of isogenic, in-frame *cps* deletion mutants. Both V583 and FA2-2 are classified as serotype C strains and contain *cpsF* (16). Deletion constructs were transformed by electroporation into V583 and FA2-2 as described previously (8). Transformed bacteria were grown on Todd-Hewitt broth (THB) plates containing Cm and X-Gal at 30°C. Blue colonies were screened for the presence of the engineered deletion constructs by colony PCR using the primers OriF and KS05SeqR. Colonies that were positive for the deletion constructs were inoculated into 5.0 ml of THB containing Cm and grown overnight at 30°C. The cultures were back diluted 1:1,000 in fresh THB with 15 μg/ml Cm and grown for 2.5 h at 30°C, followed by shifting to 42°C for 2.5 h to force single-site integration by homologous recombination. Following incubation at 42°C, the cells were serially diluted and plated on THB containing Cm and X-Gal. Blue colonies growing at 42°C were screened for the targeted integration using PCR with primers flanking the site of integration. Positive integration clones were serially passaged from overnight cultures for two successive days in THB with no selection at 30°C to force the second site recombination event. Following serial passage at 30°C, the cultures were plated by serial dilution on MM9YEG plates (20) containing 10 mM *p*-chloro-phenylalanine and X-Gal at 37°C. The resulting white colonies were screened for the deletion of the target genes by PCR. Genomic DNA from colonies containing the deletions were purified and sequenced to confirm gene deletions. The resulting deletion mutants are listed in Table 1.

Complementation of deletion mutants. The markerless gene deletions were complemented in *trans* by cloning target genes in a pAT28 plasmid background (39). The promoter region for the *cps* operon (*cpsC* promoter) was PCR amplified from the plasmid pCPSC2 using the primers Vlac1 and Vlac2 (12, 28). The amplified product was cloned as an EcoRI/BamHI fragment into pAT28, generating pLT09 (Table 2). PCR-amplified gene products were generated for *cpsC*, *cpsD*, *cpsE*, *cpsF*, *cpsG*, *cpsH*, *cpsI* from purified V583 genomic DNA using primers listed in Table S1 in the supplemental material. The amplified products were cloned into pLT09, generating the complementation plasmids pLT10 (*cpsF*), pLT14 (*cpsH*), pLT25 (*cpsD*), pLT32 (*cpsE*), pLT33 (*cpsG*), pLT34 (*cpsC*), and pLT35 (*cpsI*) (Table 2). The complementation vectors were transformed by electroporation into the corresponding deletion mutants (Table 1), resulting in strains LT03, LT04, LT07, LT08, LT25, LT27, LT29, LT31, and LT33. The serotype D strains T-5 and T-18 and the serotype B strain OG1RF were complemented with pLT10, generating strains LT09, LT10, and LT11, respectively (Table 1).

TABLE 2. Plasmid constructs used in this study

Plasmid	Description	Reference
pCJK47	Conjugative donor plasmid, carries $OriT_{pCF10}$, *lacZ*, and *pheS*, used in pLT06 construction	20
pGB354	Contains Cat^r, used in the construction of pLT06	3
pCASPER	Contains *orfB*, *orfC*, *RepA*(Ts), and *orfD*, used in pLT06	6
pAT28	Broad-range shuttle vector, spectinomycin resistant	39
pCPSC2	Source of the CpsC promoter used in pLT09	12
pKS05	pCJK47 derivative containing Cat^r	This study
pLT06	Deletion construct used for making mutants	This study
pLT08	pLT06 containing a 2.0-kb EcoRI/PstI fragment containing engineered *cpsF* deletion	This study
pLT09	pAT28 containing a 398-bp EcoRI/BamHI fragment containing the native CpsC promoter	This study
pLT10	pLT09 containing 851-bp SalI/SphI fragment containing *cpsF*	This study
pLT13	pLT06 containing a 2.0-kb EcoRI/PstI fragment containing engineered *cpsH* deletion	This study
pLT14	pLT09 containing a 447-bp BamHI/SphI fragment containing *cpsH*	This study
pLT16	pLT06 containing a 2.0-kb BamHI/SmaI fragment containing engineered *cpsD* deletion	This study
pLT18	pLT06 containing a 2.0-kb EcoRI/PstI fragment containing engineered *cpsE* deletion	This study
pLT22	pLT06 containing a 2.0-kb EcoRI/PstI fragment containing engineered *cpsC* deletion	This study
pLT23	pLT06 containing a 2.0-kb SmaI/SphI fragment containing engineered *cpsG* deletion	This study
pLT24	pLT06 containing a 2.0-kb EcoRI/PstI fragment containing engineered *cpsI* deletion	This study
pLT25	pLT09 containing a 1,418-bp BamHI/SphI fragment containing *cpsD*	This study
pLT32	pLT09 containing a 2,562-bp BamHI/SalI fragment containing *cpsE*	This study
pLT33	pLT09 containing a 2,555-bp SalI/SphI fragment containing *cpsG*	This study
pLT34	pLT09 containing a 1,319-bp BamHI/SphI fragment containing *cpsC*	This study
pLT35	pLT09 containing a 1,192-bp BamHI/SphI fragment containing *cpsI*	This study

Determination of serospecificity by ELISA and slide agglutination. Serotype C strains, including FA2-2 and V583, can be detected by enzyme linked immunosorbent assay (ELISA) or agglutination using the Maekawa type 2 (MT2) antibody (12, 23). However, serotype D strains, such as Maekawa serotypes T-5, T-6, and T-18, cannot be detected by ELISA or agglutinated by MT2 antibodies (16, 23). We used the MT2 antibodies to compare the serospecificities of V583, FA2-2, LT01, LT03, T-5, LT09, T-18, LT10, OG1RF, and LT11.

Overnight cultures were diluted 1:100 in fresh THB supplemented with the appropriate antibiotics and were allowed to grow to mid-log phase (optical density at 600 nm of 0.6). Log-phase cells were washed three times with equal volumes of phosphate-buffered saline (PBS), aliquoted (50 μl) into wells of a high-binding 96-well Costar plate (Corning), and allowed to adhere overnight at 4°C. Simultaneously, MT2 antibodies were diluted 1:1,000 in PBS and were absorbed against T-5 cells in PBS overnight at 4°C to remove any cross-reactivity. Following overnight incubation, the ELISA plates were washed three times in PBS-Tween 20 (PBS-T) (0.05%) and blocked with 5.0% skim milk in PBS for 2 h. Plates were subsequently washed three times with PBS-T, and the primary MT2 antibodies were added at a dilution of 1:1,000 and allowed to bind overnight at 4°C. The plates were washed again with PBS-T, and goat antirabbit secondary antibodies conjugated to horse-radish peroxidase (Jackson ImmunoResearch, West Grove, PA) were added to the wells. The plates were incubated at room temperature for 2 h, followed by washing with PBS-T three times, followed by washing with PBS three times to remove residual detergent. The ELISA was developed in the presence of the *o*-phenylenediamine dihydrochloride (Sigma) substrate for 30 min in the dark. The ELISA plates were analyzed using a PowerWave XS 96-well plate reader (Bio-Tek instruments) at an optical density of 490 nm.

Slide agglutination assays were performed as described previously (23). Serotype A antiserum contains antibodies directed toward *E. faecalis* lipoteichoic acid (LTA) (16). Briefly, 5.0 μl of serum was added to 15.0 μl of test cells on a glass slide and gently rotated for 1 min. Agglutination was determined by visual clumping of the cells. Sterile PBS was used in place of antiserum as a negative control.

Preparation and purification of cell wall carbohydrates. Cell wall carbohydrates and capsular polysaccharides were isolated and purified as described previously with slight modifications (10, 14). Briefly, bacteria were grown in two or four liters of THB supplemented with 1% glucose at 37°C to mid-log phase. Cells were washed in 300 ml of Tris-sucrose solution (10 mM Tris-Cl [pH 8.0], 25% sucrose), and the resulting cell pellets were resuspended in Tris-sucrose solution with lysozyme (1 mg/ml), mutanolysin (10 U/ml), and 0.05% sodium azide and incubated with gentle rocking at 37° for 16 h. Following incubation, the samples were centrifuged and the supernatants were treated with RNase A (100 μg/ml) and DNase (10 U/ml) and incubated for 4 h at 37°C with gentle agitation. Pronase (50 μg/ml) was added to the samples and additionally incubated at 37°C for 16 h. The supernatants were collected and passed through a 0.2-μm filter, followed by extensive dialysis against distilled water. The samples were then lyophilized and resuspended in a minimal volume of gel filtration buffer (50 mM Tris base–150 mM NaCl–0.05% sodium azide, pH 7.0) and were run over an S-400 size exclusion column (GE Healthcare Bio-Sciences, Uppsala, Sweden). Collected fractions were analyzed for capsular polysaccharide content using acrylamide gel electrophoresis and the cationic dye Stains-All for detection as described previously (10). Fractions containing capsular polysaccharide were pooled, extensively dialyzed against distilled water, lyophilized, and resuspended in a minimal volume of 50 mM Tris buffer (pH 8.0). The sample was applied to an anion exchange Q-Sepharose column for further purification (GE Healthcare Bio-Sciences, Uppsala, Sweden). Bound capsular polysaccharide was eluted using a stepwise gradient starting with 50 mM Tris (pH 8.0) and ending with 50 mM Tris–1 M NaCl (pH 8.0). Determination of fractions containing capsular polysaccharide was carried out as described above. Capsular polysaccharide containing fractions were pooled, extensively dialyzed against distilled water, lyophilized, and used for downstream applications.

Small-scale cell wall carbohydrate preparations for determining production of capsular polysaccharide were performed as stated above with slight modifications. Cells were grown in 25 ml of THB supplemented with 1% glucose until they reached an optical density at 600 nm of 0.6 to 0.8. The cells were harvested, washed with 2.0 ml of Tris-sucrose solution, and treated with lysozyme and mutanolysin at the same concentrations listed above for 16 h at 37°C. The cell suspensions were centrifuged, and the pellets were discarded. The remaining supernatants were treated with RNase (100 μg/ml) and DNase (10 U/ml) for 4 h before final treatment with pronase as described above. Remaining impurities were extracted with 500 μl of chloroform, and the remaining carbohydrates were precipitated with ethanol at a final concentration of 75% at −80°C for 30 min. The resulting pellets were air dried and resuspended in 100 μl of sterile distilled water, and 25 μl was loaded onto an acrylamide gel as described previously. The gels were stained in Stains-All following electrophoresis. Stained gels showed the presence of three distinct staining regions, with the highest-molecular-weight band corresponding to capsular polysaccharide (10).

Carbohydrate compositional analysis. Analysis of purified capsular polysaccharide was performed at the Glycotechnology Core Resource at the University of California, San Diego, using high-pH anion-exchange chromatography using a Dionex DX 500 high-performance liquid chromatography system (Dionex, Sunnyvale, CA) with pulsed amperometric detection (ED40; Dionex) (HPAEC-PAD). Samples were hydrolyzed with 2 M trifluoroacetic acid at 100°C for 5 h, dried, and resuspended in 25 μl distilled water. Sugars were eluted with 120 mM sodium hydroxide at a flow rate of 0.4 ml/min. The carbohydrate composition of each polysaccharide was determined by comparison to known carbohydrate standards that were prepared under identical conditions.

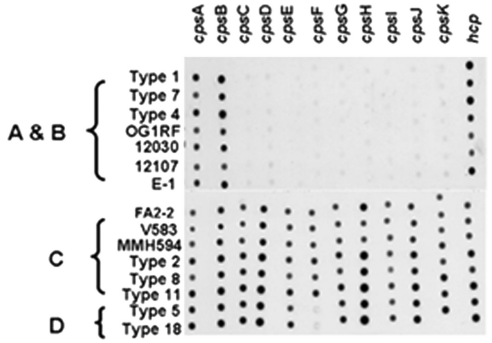

FIG. 1. Dot blot analysis of the four putative serotypes of *E. faecalis*. Serotypes A and B (top) hybridize only to *cpsA*, *cpsB*, and the control gene *hcp*, which sits outside of the capsule locus. The serotype C strains (middle) hybridize to all the genes in the *cps* locus (*cpsC* to *cpsK*), as well as the *cpsA*, *cpsB*, and *hcp* genes. Serotype D strains (bottom) hybridize to all genes of the *cps* locus except *cpsF*.

RESULTS

Dot blot analysis of the capsule locus from serotype A, B, C, and D strains. Dot blot analysis was performed for representatives of the four *E. faecalis* serotypes. *E. faecalis* serotype A or B strains E-1, OG1RF, type 1, type 4, type 7, 12030, and 12107, serotype C strains FA2-2, V583, MMH594, type 2, type 8, and type 11, and serotype D strains type 5 and type 18 were used. Blotting was performed to determine the presence or absence of specific capsule operon genes (*cpsC* to *cpsK*), as well as the conserved flanking genes *cpsA*, *cpsB*, and *hcp*, which are known to reside adjacent to the capsule operon. All serotypes contained the genes *cpsA*, *cpsB*, and *hcp* (Fig. 1). Only serotypes C and D contained the genes *cpsC* to *cpsK*, with the only identifiable difference between the two serotypes being that serotype D strains lacked *cpsF* (Fig. 1).

Construction of pLT06 and generation of *cps* operon deletion mutants. The development of pCJK47 by Kristich et al. was one of the first vector systems for generating gene deletion mutations in *E. faecalis*. (20). Limitations of this system involved the necessity of conjugally mating the plasmid construct from a donor strain (20). This delivery method is inefficient for delivery of cloned DNA into target strains that harbor endogenous plasmids, such as the vancomycin-resistant strain V583. Another noted obstacle associated with this system is the mobilization and unwanted transfer of genomic DNA from the donor strain into the recipient strain. The *erm* resistance cassette used in pCJK47 for selection was also unsuitable for work with V583 due to inherent resistant to erythromycin.

To counter these limitations, we constructed an improved vector system, pLT06, to generate markerless in-frame deletions of *cps* operon genes (Fig. 2). Insertional inactivation techniques would not have been suitable to assess the contributions of the individual *cps* operon genes to capsule production or serospecificity. The pLT06 vector contains components of pCJK47, including *lacZ* and the counterselectable marker P-*pheS* (Fig. 2). pLT06 also contains the chloramphenicol acetyltransferase (*cat*) marker from pGB354 for selection purposes and *orfB*, *orfC*, *repA*(Ts), and *orfD* from pCASPER. The combination of genes comprising pLT06 allowed for direct transformation by electroporation of cloned DNA into target *E. faecalis* strains. The plasmid can replicate in *E. faecalis* at permissive temperatures of 30°C but cannot replicate at the nonpermissive temperature of 42°C due to the temperature-sensitive nature of the *repA* gene. Flanking regions of the gene targeted for deletion were cloned into pLT06 to serve as templates for targeted recombination. Derivatives of pLT06 designed to delete the targeted genes are forced to integrate into the host genome through single-site homologous recombination when grown at nonpermissive temperatures in the presence of Cm. If recombination does not occur, then the subsequent clones of the host cell harboring pLT06 will not survive since they will not carry the *cat* cassette for resistance to chloramphenicol. Clones containing properly integrated pLT06 constructs were serially passaged at the permissive temperature in THB without selection to induce the second site recombination event and subsequent loss of pLT06. Bacteria harboring integrated or circularized pLT06 constructs should not grow on MM9YEG agar due to the presence of the *p*-chloro-phenylalanine substrate and the P-*pheS* cassette (20). White colonies from the MM9YEG plates were screened by PCR to confirm deletion of the target gene. Approximately 50% of the screened colonies harbored the desired mutation. PCR amplification from the *cpsE-cpsG* junction in serotype C strains (FA2-2 and V583), the corresponding *cpsF* mutants (LT01 and LT02), and serotype D strains (T-5 and T-18) shows a 2.8-kb amplicon in strains containing *cpsF* and a 2.0-kb amplicon in strains lacking *cpsF*. DNA sequence analysis of LT01 and LT02 and complementation with *cpsF* with pLT10 showed that a nonpolar deletion was generated using pLT08.

Determination of capsular polysaccharide production in serotypes A, B, C, and D. Cell wall polysaccharides were purified from parental and mutant strains to assess capsule production. This method of detection allows for the most direct and solid evidence of the presence of a capsule as opposed to an antibody-based method, which could falsely detect other cell wall antigens (38). Small-scale cell wall polysaccharide preparations were loaded on polyacrylamide gels, electrophoresed, and stained with the cationic dye Stains-All. The high-molecular-weight dark blue band corresponds to capsular polysaccharide and correlates with previously described high-molecular-weight *E. faecalis* capsule (10). The light blue band immediately below the capsule corresponds to the rhamnopolymer, and the low-molecular-weight dark blue smear corresponds to teichoic acid as described previously (10). From results shown in Fig. 3, it is clear that serotype A and B strains (lanes B and C, showing 12030 and OG1RF, respectively) do not produce the high-molecular-weight capsular polysaccharide. Interestingly, the serotype A strain 12030 did not appear to produce detectable rhamnopolymer; however, the basis for this observation is not known at the present time. Consistent with genetic data (Fig. 1), all serotype C and D strains produced the high-molecular-weight band corresponding to capsular polysaccharide (Fig. 3).

Determination of serospecificity between serotype C and D strains. Given that the only genetic difference between serotype C and D strains is the presence of *cpsF* in serotype C strains, we hypothesized that CpsF was the sole contributor to differences in antigenicity between serotype C and D strains. We performed an ELISA with MT2 antiserum to detect the serotype C antigenic determinant (23). MT2 antiserum has

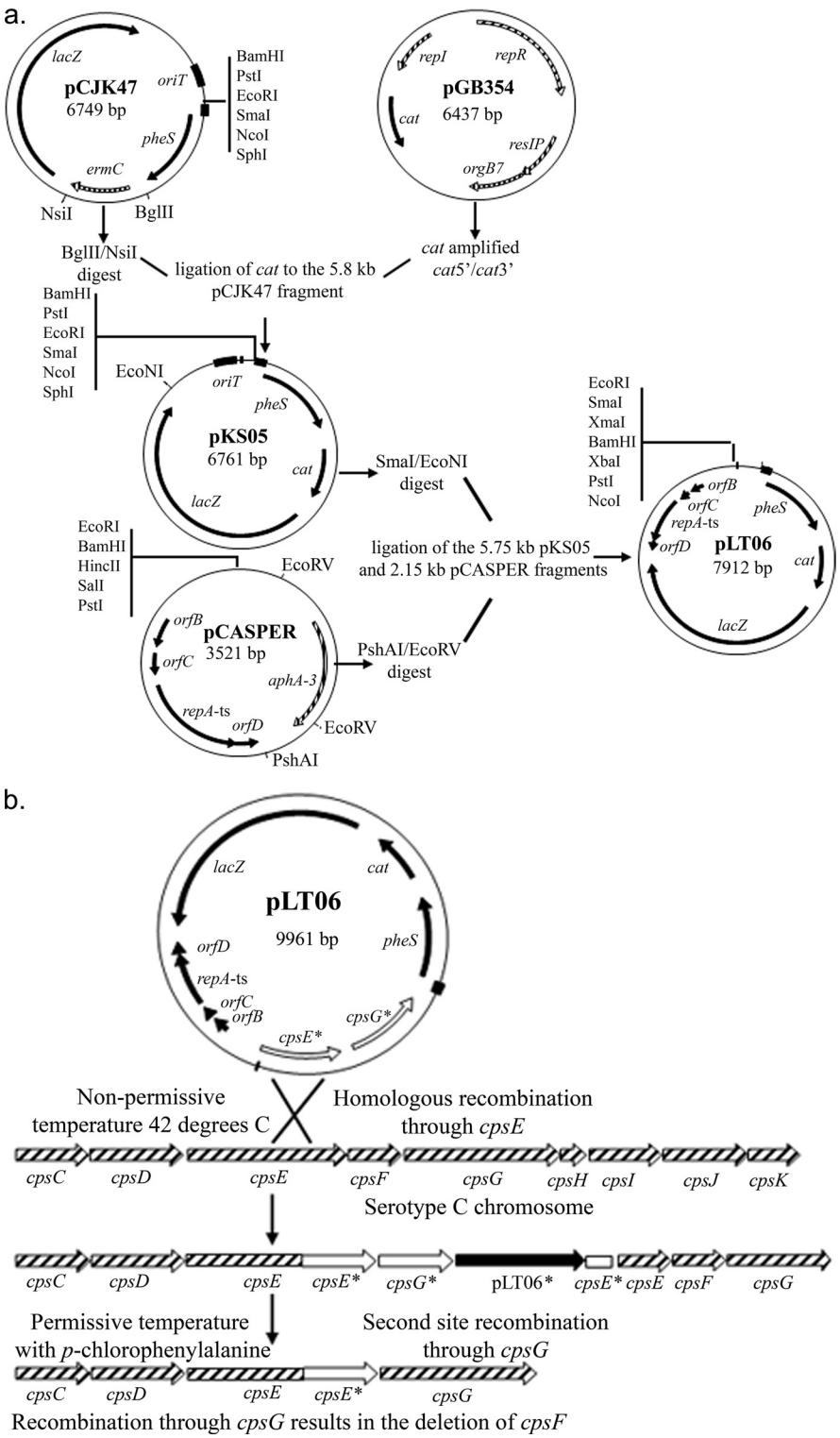

FIG. 2. (a) Strategy for the construction of plasmid pLT06, used in this study for construction of isogenic, in-frame deletion mutants of *E. faecalis*. See Materials and Methods for details. The *erm* marker from pCJK47 was replaced with the *cat* marker from pGB354, resulting in pKS05. *oriT* from pKS05 was replaced with an enterococcal origin of replication and the temperature-sensitive *repA* gene, resulting in pLT06. pLT06 was subsequently used to engineer all of the isogenic, in-frame deletion mutants used in this study. (b) Diagram of the generation of the in-frame, isogenic *cpsF* mutation using pLT08. Integration through homologous recombination of pLT08 into the *E. faecalis* genome took place at the nonpermissive temperature of 42°C. Strains harboring the integrated plasmid were serially passaged at the permissive temperature of 30°C in the absence of the selecting antibiotic Cm. Serial passaging induced the second site homologous recombination event and the excision of the plasmid. Bacteria were plated on medium containing ρ-chlorophenylalanine and X-Gal to screen for isolates that lost the plasmid. White colonies were screened by PCR for the deletion event, and isolated DNA was sequenced to confirm that an in-frame deletion had occurred.

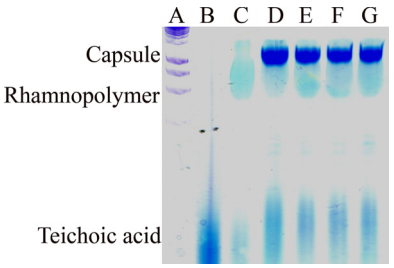

FIG. 3. Acrylamide gel stained with Stains-All, showing the presence/absence of capsule production in serotype A to D strains. The high-molecular-weight bands correspond to capsular polysaccharide as described previously (10). The serotype A strain 12030 (B) and the serotype B strain OG1RF (C) do not produce the capsule band. The serotype C strains V583 and FA2-2 (D and E) and the serotype D strains T-5 and T-18 (F and G) produce the high-molecular-weight capsule band.

been shown to be specific for the serotype C antigen (12). While the serotype C strain FA2-2 was detected by the MT2 antiserum, LT01 (FA2-2 Δ*cpsF*) and the serotype D strains T-5 and T-18 were not detected by the MT2 antiserum (Fig. 4). Strain LT03 (FA2-2 Δ*cpsF* pLT10), along with the serotype D strains LT09 (T-5) and LT10 (T-18), containing the complementation vector pLT10, were detected by the MT2 antiserum. As expected, the serotype B strain OG1RF was not detected by the MT2 antiserum after transformation with pLT10 (LT11) (Fig. 4).

Capsule production alters detection of lipoteichoic acid by slide agglutination. Recently it was discovered that agglutinating antibodies generated to the serotype A strain 12030 were directed toward LTA and not toward capsule as described previously (16, 38). This suggests that sera developed for serotyping and detecting serotype A strains should recognize other strains with exposed LTA. We used serotype A antisera in agglutination assays to determine if our mutant strains could be agglutinated. No agglutination was observed for FA2-2 and LT01 (FA2-2 Δ*cpsF*), but 12030 and LT05 (FA2-2 Δ*cpsC*) agglutinated in the presence of these antibodies (Table 3). This suggests that the presence of capsule in serotype C and D strains protects LTA from detection by agglutinating antibodies.

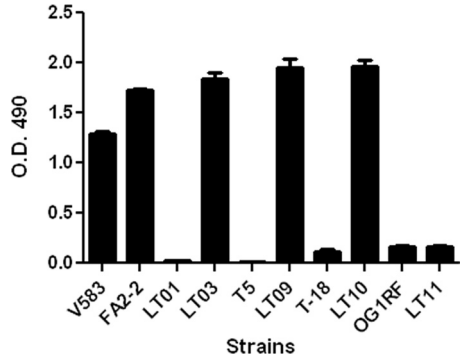

FIG. 4. CPS ELISA using MT2 antibodies to detect serotype C capsule. Serotype C strains V583 and FA2-2 show reactivity with the MT2 antibody. The *cpsF* deletion mutant LT01 is not detected by the antibody, but complementation of LT01 (FA2-2 Δ*cpsF*) with pLT10 (LT03) restores reactivity to the antibody. The serotype D strains T-5 and T-18 are not detected by the serotype C antibody. However, LT09 (T-5 plus pLT10) and LT10 (T-18 plus pLT10) are seroconverted to serotype C strains when complemented with *cpsF*. The serotype B strain OG1RF is not detected by the MT-2 antibody before or after (LT11) complementation with pLT10, indicating that serotype conversion cannot occur in a strain that does not produce capsular polysaccharide. O.D. 490, optical density at 490 nm.

TABLE 3. Slide agglutination using serotype A antiserum

Strain	Agglutination result
FA2-2	Negative
FA2-2 Δ*cpsF*	Negative
FA2-2 Δ*cpsC*	Positive
12030	Positive

Comparison of serotype C and D capsule polysaccharides by HPAEC-PAD. We used purified capsular polysaccharide from FA2-2 (serotype C) and LT01 (FA2-2 Δ*cpsF*/serotype D) to determine the contribution of *cpsF* to the difference in antigenicity between serotype C and D strains. Capsular polysaccharides were purified as described in Materials and Methods. Analysis comparing the FA2-2 capsule to the LT01 capsule indicated a difference in the ratio of glucose to galactose between the two capsule serotypes (for strains FA2-2 and FA2-2 Δ*cpsF*, galactose values were both 1.0 while glucose values were 4.4 and 3.0, respectively), indicating that CpsF could be a glucosyltransferase.

Contributions of *cps* operon genes to capsule production. We generated in-frame deletions of *cpsCDEFGHI* in the serotype C strain V583 to determine their contribution to capsule production. Figure 5 clearly shows that genes *cpsC*, *cpsD*, *cpsE*, *cpsG*, and *cpsI* are essential for production of the high-molecular-weight capsular polysaccharide. Further, these phenotypes were not due to polar effects on downstream genes, since complementation of each gene in *trans* restores capsule production. The genes *cpsF* and *cpsH* are the only genes in the *cps* operon that are not essential for capsule production (Fig. 5).

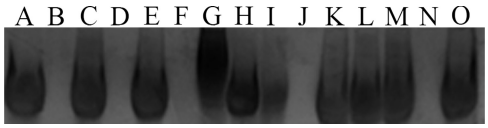

FIG. 5. Polyacrylamide gel stained with Stainz-all, showing the high-molecular-weight capsule bands of capsule mutants and complemented mutants. Lanes: A, V583; B, LT06 (V583 Δ*cpsC*); C, LT08 (V583 Δ*cpsC* plus pLT10); D, LT15 (V583 Δ*cpsD*); E, LT25 (V583 Δ*cpsD* plus pLT25); F, LT17 (V583 Δ*cpsE*); G, LT27 (V583 Δ*cpsE* plus pLT32); H, LT02 (V583 Δ*cpsF*); I, LT04 (V583 Δ*cpsF* plus pLT10); J, LT19 (V583 Δ*cpsG*); K, LT29 (V583 Δ*cpsG* plus pLT33); L, LT21 (V583 Δ*cpsH*); M, LT31 (V583 Δ*cpsH* plus pLT14); N, LT23 (V583 Δ*cpsI*); O, LT33 (V583 Δ*cpsI* plus pLT35). Only the genes *cpsF* and *cpsH* are not essential for capsule production. Deletion of the genes *cpsC*, *cpsD*, *cpsE*, *cpsG*, and *cpsI* completely abrogates capsule production. This observation supports the evidence that serotypes A and B do not produce capsule based on the absence of essential genes for capsule production in these strains. Complementation of these deletions restores capsule production.

DISCUSSION

Previous reports of capsule production in *E. faecalis* have focused on differences in antigenicity between cell surface polymers (16, 22). One study divided *E. faecalis* into 21 different serogroups based on differences in agglutination to polyclonal antibodies generated to heat-killed cells (23). The antiserum used in this study was possibly detecting capsule as well as other surface antigens (16). A more recent study grouped strains of *E. faecalis* into four capsular serotypes (A to D) based on serospecificity (16). This study inferred that serotypes A and B shared a locus similar to that of serotypes C and D that was responsible for capsule production in all four serotypes. Accordingly, the capsular antigen of serotype A was purified and compositionally analyzed and the structure deduced by nuclear magnetic resonance analysis (14, 40). However, it was recently reported that the serotyping antibody used to classify serotype A isolates actually recognized LTA and that the determined structure of the serotype A capsule corresponded to LTA (38). To date, only one genetic locus had been determined to be responsible for capsule production in *E. faecalis* (10). The capsule locus described by Hancock et al. is comprised of nine genes (*cpsC-cpsK*) that directly contribute to the expression of a capsular polysaccharide in *E. faecalis* (10). We have shown that serotype C and D strains contained all genes of the *cps* locus described by Hancock et al. (preceded by *cpsA* and *cpsB*), with the variation between serotypes C and D being attributed to the presence (serotype C) or absence (serotype D) of *cpsF*.

Previous studies have shown that the genes *cpsA* and *cpsB* are not part of the capsule operon since they are transcribed from a different promoter (12). Attempts to mutate these genes never resulted in the recovery of viable isolates (12). However, reactive capsule antigen could be produced in a heterologous host (*E. coli*) by complementation with the *cpsC-cpsK* operon (12). The absence of capsule production by serotypes A and B (Fig. 3) highlights the fact the CpsA and CpsB play no role in capsule production. Therefore, based on sequence homology, we propose renaming *cpsA* to *uppS*, consistent with its function as an undecylprenyl pyrophosphate synthetase. We also propose to rename *cpsB* to *cdsA* since it shares strong sequence similarity with known cytidyl transferase proteins. Both UppS and CdsA are known to be essential proteins in other bacterial systems (1), which explains the inability to recover such mutants for *E. faecalis* (12).

We have demonstrated that the production of capsule prevents detection of LTA by agglutinating antibodies (Table 3). This observation is consistent with the argument that LTA is shielded from agglutinating antibodies by capsule. Our observations support a role for CpsF in determining serospecificity between serotype C and D strains. Compositional analysis suggests that CpsF is responsible for the altered ratios of glucose to galactose present in the capsules of serotypes C and D (Table 4). Additionally, we propose that serotypes C and D are the only *E. faecalis* serotypes that produce a capsular polysaccharide, which is supported with the data in Fig. 3 and the underlying genetics known to contribute to capsule production (Fig. 1 and 5).

CpsF has no known sequence similarity to any characterized protein, thus making it difficult to predict a possible contribution to serotype differences. Purified capsular polysaccharide extracts from FA2-2 (serotype C) and LT01 (serotype D) were analyzed by HPAEC-PAD to determine the possible contribution of CpsF. HPAEC-PAD analysis revealed a difference in glucosylation of the polysaccharides, with FA2-2 containing an extra glucose relative to galactose compared to data for LT01 (FA2-2 Δ*cpsF*) (Table 4). The ratio of glucose to galactose for the serotype C strain FA2-2 is identical to results of previous compositional analysis (10). This result indicates that CpsF is a putative glucosyltransferase, but ongoing studies to reveal the structure of the repeating unit will provide solidifying evidence for the functional role of CpsF.

Opsonophagocytic killing of both serotype C and D strains by healthy human sera is drastically reduced compared to that of the unencapsulated serotype A and B strains (17). Additional studies with the serotype B strain, OG1RF, demonstrated the presence of protective antibodies in normal serum, leading to clearance of *E. faecalis* (2). This could be due by the presence of opsonizing anti-LTA antibodies present in normal human serum (17). Presumably, the presence of capsule in serotype C and D strains masks LTA from detection by the circulating anti-LTA antibodies. Serotype A antibodies that recognize LTA (38) cannot recognize or agglutinate the encapsulated serotype C and D strains (Table 3). However, these same antibodies readily recognize and agglutinate the unencapsulated strain LT05 (V583 Δ*cpsC*) and the serotype A strain 12030. These observations are consistent with the increased virulence associated with serotype C and D strains (24). Furthermore, LTA is a pathogen-associated molecular pattern that is recognized by the pattern recognition receptor Toll-like receptor 2 (33, 34). Recognition of LTA results in increased cytokine production and neutrophil recruitment to the site of infection (5, 21). Presumably, the presence of capsule would attenuate the host innate immune response. Currently, we are conducting studies to determine the effects of capsule on innate immune system evasion.

In summary, the results presented in this study argue that only *E. faecalis* serotypes C and D produce a true capsular polysaccharide while serotypes A and B do not. We provide empirical proof that CpsF is the basis for the difference in antigenicity between serotype C and D strains. Finally, the inability to detect LTA on the surface of encapsulated strains indicates that the capsule of *E. faecalis* may play a role in evasion of the host innate immune response. Future studies will aim to address such questions in order to develop targeted therapies to treat infections caused by multidrug-resistant *E. faecalis*.

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