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Construction of Improved Temperature-Sensitive and Mobilizable Vectors and Their Use for Constructing Mutations in the Adhesin-Encoding *acm* Gene of Poorly Transformable Clinical *Enterococcus faecium* Strains

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Inactivation by allelic exchange in clinical isolates of the emerging nosocomial pathogen *Enterococcus faecium* has been hindered by lack of efficient tools, and, in this study, transformation of clinical isolates was found to be particularly problematic. For this reason, a vector for allelic replacement (pTEX5500ts) was constructed that includes (i) the pWV01-based gram-positive *repAts* replication region, which is known to confer a high degree of temperature intolerance, (ii) *Escherichia coli oriR* from pUC18, (iii) two extended multiple-cloning sites located upstream and downstream of one of the marker genes for efficient cloning of flanking regions for double-crossover mutagenesis, (iv) transcriptional terminator sites to terminate undesired readthrough, and (v) a synthetic extended promoter region containing the *cat* gene for allelic exchange and a high-level gentamicin resistance gene, *aph(2″)-Id*, to distinguish double-crossover recombination, both of which are functional in gram-positive and gram-negative backgrounds. To demonstrate the functionality of this vector, the vector was used to construct an *acm* (encoding an adhesin to collagen from *E. faecium*) deletion mutant of a poorly transformable multidrug-resistant *E. faecium* endocarditis isolate, TX0082. The *acm*-deleted strain, TX6051 (TX0082Δ*acm*), was shown to lack Acm on its surface, which resulted in the abolishment of the collagen adherence phenotype observed in TX0082. A mobilizable derivative (pTEX5501ts) that contains *oriT* of Tn*916* to facilitate conjugative transfer from the transformable *E. faecalis* strain JH2Sm::Tn*916* to *E. faecium* was also constructed. Using this vector, the *acm* gene of a nonelectroporable *E. faecium* wound isolate was successfully interrupted. Thus, pTEX5500ts and its mobilizable derivative demonstrated their roles as important tools by helping to create the first reported allelic replacement in *E. faecium*; the constructed this *acm* deletion mutant will be useful for assessing the role of *acm* in *E. faecium* pathogenesis using animal models.

In today's world, enterococci, especially some strains of *Enterococcus faecium*, are best known in the clinical setting as multidrug-resistant opportunists causing difficult-to-treat hospital-acquired infections, including infective endocarditis (32, 33). While in the past clinical isolates of *Enterococcus faecalis* outnumbered those of *E. faecium* by approximately 9:1, this ratio has changed in some U.S. hospitals to ~6:4, and this increase parallels the increase in vancomycin resistance of *E. faecium* (20, 33, 58). An important theme relating to strains causing *E. faecium* infections in hospitals today is that, in addition to acquiring antibiotic resistances, they seem to have lost the harmless, commensal nature of the strains colonizing healthy individuals in the community and often contain either new genes (e.g., esp_{fm}, encoding a potential enterococcal surface protein, or *hyl*, encoding a potential hyaluronidase [16, 25, 44, 62]) or a functional form of genes (e.g., *acm*, encoding a collagen adhesin [37]), thus presumably enhancing their ability to survive and/or cause infection in the clinical setting. While esp_{fm} and *hyl* genes were found to be rare in isolates from healthy volunteers and in isolates from animal origin (44), the *acm* gene was present in these isolates as an inactive gene (37 and S. R. Nallapareddy and B. E. Murray, unpublished results). To study virulence traits of *E. faecium*, it is necessary to have appropriate genetic methods. However, tools for constructing targeted mutations in *E. faecium* are rudimentary or contain resistant genes frequently present in clinical isolates.

Several methods have been previously developed for targeted mutagenesis of enterococci and found to be useful for genetic manipulation of *E. faecalis*. One of these makes use of plasmids that are suicidal in gram-positive hosts (13, 15, 43, 51). A second method of allelic replacement, involving electroporation of *E. faecalis* with DNA from a pool of mini-γδ-mutagenized cosmid clones, is applicable for genes with detectable phenotypes (28, 51). The disadvantage of these two techniques is the requirement for efficient transformation, since selection depends on both successful bacterial transformation and successful integration in the same step. However, the problem with enterococci is that, although some strains are readily transformable using electroporation, others are not. A third method of delivery integration is based on pORI19/pG$^+$host3 and requires individual transformation with two different plasmids (45). Other methods based on conjugation systems (30, 56) and temperature-sensitive (ts) plasmids (1, 12, 47) have also been used intermittently for *E. faecalis* mutagenesis to circumvent the difficulty of electroporation. However, these vectors were constructed for specialized uses and lack

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
E. faecium		
TX1330	Fecal isolate from community volunteer; Amp^s, Chl^s, Ery^s, Gen^s, Kan^r, Str^s, Tet^s; Cn-Adh^-	8, 37
TX2466	Blood isolate; Ery^i, Kan^r, Gen^s, Van^r	This study
TX0082	Endocarditis isolate; Amp^r, Chl^s, Ery^r, Kan^r, Gen^s, Van^r; Cn-Adh^+	This study
TX2555	Perineal wound isolate; Chl^s, Ery^r, Kan^r, Gen^s, Van^r; Cn-Adh^+	37
TX6051	TX0082Δ*acm, acm* deletion mutant of TX0082, Chl^r; Cn-Adh^-	This study
TX6054	TX2555*acm*::pTEX6052, *acm* insertion disruption mutant of TX2555, Gen^r, Cn-Adh^-	This study
E. faecalis		
JH2Sm::Tn*916*	JH2 harboring Tn*916*; Gen^s, Str^r, Tet^r	6
TX6053	JH2Sm::Tn*916*(pTEX6052); Str^r, Tet^r, Gen^r	This study
OG1RF	Laboratory strain; Rif^r, Fus^r, Chl^s, Gen^s	35
E. casseliflavus		
UC73	*aph*(2″)-*Id* containing strain; Gen^r	59
E. coli		
DH5α	*E. coli* host strain for routine cloning	Stratagene
TX5500	DH5α(pTEX5500ts); Chl^r, Gen^r	This study
TX5501	DH5α(pTEX5501ts); Chl^r, Gen^r	This study
TX6050	DH5α(pTEX6050); Chl^r, Gen^r	This study
TX6052	DH5α(pTEX6052); Gen^r	This study
Plasmids		
pAM401	Shuttle plasmid; Chl^r and, in *E. coli*, Tet^r	64
pAT18	Shuttle plasmid; Ery^r	60
pTV1-OK	pWV01 replicon-based ts vector; Kan^r, Ery^r	14
pTV1-ts	pE194 replicon-based ts vector; Chl^r, Ery^r	66
pAM401ts	pIP501 replicon-based ts vector; Chl^r	60
pFW14	Streptococcal integration vector; Chl^r	42
pHS1	*E. faecalis* mobilizable ts plasmid containing $oriT_{Tn916}$; Kan^r, Gen^r	1
pTEX5500ts	Shuttle plasmid, ts in gram-positive hosts; Chl^r, Gen^r	This study
pTEX5501ts	Mobilizable ts vector containing $oriT_{Tn916}$; Chl^r, Gen^r	This study
pTEX6050	Plasmid for *acm* deletion with flanking regions of the *acm* binding domain cloned into pTEX5500ts; Chl^r, Gen^r	This study
pTEX6052	*acm* insertion construct, intragenic fragment of *acm* cloned into pTEX5501ts with deletion of *cat*; Gen^r, Chl^s	This study

[a] Chl, chloramphenicol; Ery, erythromycin; Fus, fusidic acid; Gen, gentamicin; Kan, kanamycin; Rif, rifampin; Tet, tetracycline; Str, streptomycin; Van, vancomycin; and ts, temperature sensitive. Superscript "s" designates sensitivity, "r" designates resistance, and "i" designates intermediate susceptibility (MIC of 2 to 4 μg/ml for Ery); for aminoglycosides "r" is defined for enterococci as MIC > 500 for Gen and > 2,000 for Kan. Cn-Adh^+, adherence to collagen types I and IV; Cn-Adh^-, no adherence to collagen types I and IV.

many characteristics associated with cloning vectors, such as availability of unique recognition sites and appropriate markers that would be useful in mutagenesis of clinical isolates of *E. faecium*. Additionally, the ts-based plasmids used in mutagenesis of other gram-positive bacteria usually contain remnants of enterococcal transposons Tn*916* and Tn*917*, thus posing potential problems with recombination into these elements which are common in clinical isolates.

In spite of a number of available vector systems, due to low transformability of the host as well as some limitation in each of the vector system(s) described above, we and others have had limited success in generating targeted mutations in *E. faecium* genes. The two *E. faecium* genes that were interrupted, albeit with low efficiency, in our laboratory were generated in the moderately transformable *E. faecium* strain TX1330 (also known as SE-34), a fecal isolate, isolated from a healthy community volunteer (50, 55). It is important to note that this *E. faecium* fecal strain does not contain esp_{fm} or *hyl*, nor does it express *acm* (the *acm* gene of this strain is a pseudogene), the three potential virulence-related genes described to date for this species (37, 44).

In order to circumvent the limitations described above, in the present study we engineered *Escherichia coli*-enterococcal cloning vectors that are conditionally suicidal in enterococci at nonpermissive growth temperatures and which harbor antibiotic genes that are selectable in both *Escherichia coli* and enterococci. Appropriate *acm* gene fragments were cloned into these new vectors and then used to introduce allelic replacement or disruption mutations in the *acm* gene of clinical strains of *E. faecium*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli*, *E. faecalis*, and *E. faecium* strains used in this study are listed in Table 1. Enterococcal isolates initially identified to the species level by biochemical tests were confirmed by colony hybridization (49) using an intragenic *ace* probe for *E. faecalis* (9, 36) and an *aac*(6′)-*Ii* probe for *E. faecium* (9). *E. coli* strains were grown in Luria-Bertani media (Difco Laboratories, Detroit, Mich.). Enterococci were grown in either

brain heart infusion (BHI) or Todd-Hewitt (TH) broth or agar (Difco Laboratories) at 37°C, unless a different growth temperature is specified. The following antibiotic concentrations were used with enterococci: chloramphenicol, 10 μg/ml; erythromycin, 15 μg/ml (for selection of transformants); erythromycin, 200 μg/ml [for selection of naturally resistant *E. faecium* strains due to *msrC* (50) plus *erm*(B)]; gentamicin, 125 μg/ml; and tetracycline, 10 μg/ml. With *E. coli*, the concentrations used were chloramphenicol, 10 μg/ml; erythromycin, 200 μg/ml; and gentamicin, 25 μg/ml. All antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). All plasmids used in this study are listed in Table 1. All constructs were given TX numbers as shown in Table 1. Plasmids from these constructs were assigned respective pTEX numbers.

Standard molecular techniques. Specific primers were purchased either from Invitrogen (Carlsbad, Calif.) or from Sigma-Genosys (The Woodlands, Tex.). Restriction enzymes and DNA modification enzymes were purchased mostly from Invitrogen, and reactions were carried out under the recommended conditions. Bovine collagen type I was purchased from Cohesion Technologies, Inc. (Palo Alto, Calif.), collagen type IV was purchased from Sigma Chemical Co., and fibrinogen was purchased from Enzyme Research Laboratories (South Bend, Ind.). Tran^{35}S label and bovine serum albumin (BSA) were purchased from MP Biomedicals Inc. (Irvine, Calif.). All other chemicals used in the investigation were of molecular biology grade. Chromosomal DNA from *E. faecium* isolates was prepared following the hexadecyltrimethyl ammonium bromide method described earlier (63). Plasmid DNA isolation from *E. coli* used the Wizard Plus SV minipreps DNA purification system (Promega Corporation, Madison, Wis.) and, from enterococci, by a previously described method (65). General recombinant DNA techniques such as ligation and agarose gel electrophoresis were performed using standard methods (46). When necessary, DNA fragments were purified with low-melting-point agarose gels followed by purification using QIAquick-gel extraction kits (QIAGEN, Inc., Valencia, Calif.). PCRs were performed with a Perkin-Elmer GeneAmp PCR system 9700 using the optimized buffer B (1× buffer: 60 mM Tris-HCl [pH 8.5], 15 mM ammonium sulfate, and 2 mM $MgCl_2$) obtained from Invitrogen. PCR-generated fragments were purified using the Wizard PCR DNA Cleanup System (Promega Corporation). Recombinant plasmids were generated in *E. coli* DH5α. Agarose plugs containing genomic DNA were digested with SmaI, and pulsed-field gel electrophoresis (PFGE) was performed using previously described methods (34) but with different ramped pulse times, 2 s and 28 s. Southern blotting was performed using Hybond-N$^+$ nylon membranes and a 0.4 N NaOH solution. The RadPrime DNA Labeling System (Invitrogen) was used to label the probes with [α-^{32}P]dCTP (GE Healthcare, Piscataway, NJ), and hybridizations were carried out using high-stringency conditions (35). DNA sequencing reactions were performed by the *Taq* dye-deoxy terminator method and an automated ABI Prism sequencer (Applied Biosystems, Foster City, Calif.). Sequences were assembled using SeqMan program of DNASTAR software (Lasergene; Madison, Wis.).

Bacterial electrotransformation and conjugation. Electroporation of *E. coli*, *E. faecalis*, and *E. faecium* was carried out using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as described previously (28, 50). Broth-mating experiments were performed as previously described (57), except for a donor: recipient ratio of 2:1. In brief, overnight cultures of 0.5 ml of donor and 0.25 ml of recipient were added to 4.25 ml of fresh BHI broth, and the mixture was incubated without shaking at 28°C overnight. After vortexing, cells were harvested by centrifugation at 4,000 rpm and 4°C and resuspended in 200 μl of saline followed by plating on BHI agar plates with 200 μg/ml erythromycin and 125 μg/ml gentamicin. Plates were checked for colonies for up to 96 h (at 24-h intervals) of incubation at 28°C. For some strains, in an effort to increase the conjugation frequency, filter-mating experiments were also performed as previously described (57), except for a donor/recipient ratio of 20:1. In brief, 1 ml of an overnight culture of donor (grown at 28°C in BHI broth containing 125 μg/ml gentamicin and 10 μg/ml tetracycline to increase the transfer frequency [6]) and 50 μl of an erythromycin-resistant recipient were added to 4.5 ml of fresh BHI broth, and the mixture was then passed through a 0.45-μm membrane filter (Millipore, Bedford, Mass.). The cells trapped on the filters were incubated at 28°C overnight and were then suspended in 1 ml of saline. Appropriate dilutions of the mixture were transferred to BHI agar plates containing 200 μg/ml erythromycin and 125 μg/ml gentamicin. Plates were assessed for colonies after 24 and 48 h of incubation at 28°C.

Determination of plasmid segregation rates. TX0082 transformed with pTEX5500ts (construction of this plasmid is described in Results and Discussion) was grown at 28°C with 10 μg of chloramphenicol and 125 μg of gentamicin per ml until the optical density at 600 nm (OD_{600}) reached approximately 1. The culture was then diluted 1:1,000 with fresh BHI broth and grown overnight (16 h) without antibiotic selection at 28, 37, and 42°C. Initial and final numbers of TX0082 cells with and without pTEX5500ts were determined by quantitative plate counting on agar plates with and without antibiotics after growth at 28°C. Plasmid segregation rates were derived from these CFU using the following formula described by Framson et al. (11): percent loss per generation = $[1 - (F_f/F_i)^{1/g}] \times 100$, where F is the frequency of the plasmid-mediated resistance phenotype in the population, f is the final CFU, i is the initial CFU, and g is the number of generations intervening between the final and initial CFU (determined on nonselective media) according to the formula $g = [\log_e (\text{final CFU/initial CFU})]/\log_e (2)$.

Growth curves. Overnight cultures were inoculated in BHI broth at a dilution of 1:100. The cultures were then grown at 37°C with shaking in an orbital shaker, and the aliquots were removed hourly from 0 to 12 h and at 24 h for determining CFU on BHI agar and also to measure the absorbance at 600 nm (OD_{600}) with a spectrophotometer.

Cell wall-associated protein extraction and Western blotting. Protein extracts from *E. faecium* strains were prepared using mutanolysin (Sigma Chemical Co.) as described previously (37). Protein concentrations were estimated by bicinchoninic acid assay (Pierce, Rockford, Ill.). Equal concentrations of mutanolysin extracts were electrophoresed in 4 to 12% NuPAGE Bis-Tris gels (Invitrogen) under reducing conditions in morpholinepropanesulfonic acid buffer and were transferred to a polyvinylidene difluoride membrane according to the protocol supplied by Invitrogen. Membranes were then probed with anti-AcmA polyclonal antiserum or preimmune serum (antibody I) (37) followed by Protein A horseradish peroxidase conjugate (antibody II) and developed with 4-chloronaphthol in the presence of H_2O_2.

Adherence assay. Adherence of *E. faecium* to collagen, fibrinogen, and BSA was determined in four independent experiments using Tran^{35}S-labeled bacteria by a previously described assay (37).

Nucleotide sequence accession numbers. Nucleotide sequences of pTEX5500ts and pTEX5501ts were submitted to GenBank under accession numbers DQ208936 and DQ208937.

RESULTS AND DISCUSSION

The recent increase in multidrug-resistant *E. faecium* infections and its emergence as a nosocomial pathogen highlight the need for genetic studies addressing the mechanism of bacterial pathogenesis of this species. Although we and others have reported the presence of homologues of known virulence factors among *E. faecium* strains (e.g., $efaA_{fm}$, esp_{fm}, *hyl*, and *acm*) (25, 37, 44, 49, 62), the relative contributions of these genes to *E. faecium* pathogenicity have not been assessed, largely due to the lack of tools for replacement of a wild-type gene with a mutated gene. Our repeated attempts to generate targeted mutations in some *E. faecium* clinical strains with suicide, temperature-sensitive, and/or mobilizable vector systems (1, 51, 56) developed for *E. faecalis* mutagenesis were unsuccessful. Hence, the aims of this study were to develop a delivery and screening system of *E. faecium* and to validate the applicability of this system for generating targeted mutations in the *acm* gene of *E. faecium* clinical isolates. As a first step, some clinical strains were screened for their ability to acquire plasmid pAM401.

Clinical strains of *E. faecium* are poorly transformable. We and others previously found that *E. coli-E. faecalis* shuttle plasmids (e.g., pAM401, pWM401, and pAT18) can be transformed into and will replicate in selected strains of *E. faecium*, although they do so with some instability in the absence of selection pressure. However, the three *E. faecium* strains that we previously reported as transformable (37) either lack the virulence factors of interest (44) or contain a pseudogene (37). In this study, diverse *E. faecium* strains containing potential virulence genes were screened for their transformation capability using electroporation conditions optimized for *E. faecium* (50) and a pIP501-derived high-copy-number shuttle plasmid, pAM401 (64). Results from this preliminary experi-

TABLE 2. Transformation efficiencies of various strains of *E. faecium* with high-copy-number shuttle vector pAM401

Strain (reference)	Source	No. of transformants/μg[a]
TX1330 (8, 37)	Fecal isolate from community volunteer	2,113 ± 1,313
D344-S (37, 48)	Laboratory recipient	955 ± 503
GE-1 (10)	Recipient (ATCC 51558)	222 ± 86
TX0054 (37)	Endocarditis	0
TX0082 (this study)	Endocarditis	33 ± 8
TX2535 (37)	Endocarditis	0
TX2699 (this study)	Endocarditis	0[b]
TX2095c (this study)	Catheter tip	0[b]
TX2092 (this study)	Rectum	9 ± 3
TX2084 (this study)	Urine	0
TX2555 (37)	Wound	0

[a] All experiments were carried out with 500 ng of pAM401 DNA isolated from *E. coli* DH5α. Electroporation was performed with a Bio-Rad Gene Pulser apparatus with the following conditions: 12.5 kV (per 1-cm cuvette), 25 mF capacitance, and 400 Ω resistance. These values represent the means and standard deviations of three independent competent cell preparations.

[b] Sporadically, some breakthrough colonies were found on chloramphenicol plates. These colonies did not contain pAM401 and, upon restreaking, were unable to grow on chloramphenicol plates.

TABLE 3. Transformation efficiencies of *E. faecium* TX1330 with various plasmids

Plasmid (replicon, type)[a]	Size (kb)	Antibiotic marker[b]	No. of transformants[c]/μg
pAM401 (pIP501, θ)	10.4	Chl	2,113 ± 1,313
pTV1-OK (pWV01, RC)	11	Ery	64 ± 6
pTV1-ts (pE194, RC)	12.4	Chl	13 ± 3
pAM401ts (pIP501, θ)[d]	10.4	Chl	19 ± 2

[a] The replicon is derived from the replication apparatus of the original plasmid. The replication mechanism used by the plasmid is RC, rolling circle, or θ, theta.

[b] Antibiotic markers used to select for the plasmid transformants. Ery, erythromycin; Chl, chloramphenicol.

[c] These values represent the means and standard deviations of three independent competent cell preparations.

[d] This plasmid is highly unstable in *E. faecium*.

ment using a total of 11 human-derived *E. faecium* strains, including 8 clinical isolates, revealed that strains varied markedly in their ability to acquire this plasmid, with most failing to do so (Table 2). As is evident from Table 2, among the tested strains, the community-derived fecal *E. faecium* isolate TX1330 exhibited high transformation efficiency, in the range of 1,200 to 4,000 CFU/μg of plasmid. Similar transformation efficiencies were noted for pAT18 (with the pAMβ1 replicon) with this strain. Two other strains that were found to be moderately transformable were D344-S (a laboratory recipient isolate and spontaneous mutant of D344 [48]) and an American Type Culture Collection (ATCC 51558) recipient isolate, GE-1 (10). Their efficiencies of transformation were 100-fold less than that of pAM401 transformation into *E. faecalis* OG1X (64). While some clinical strains exhibited low-level transformation with pAM401 (e.g., an endocarditis isolate TX0082 yielded 33 ± 8 CFU/μg of plasmid), others appeared to be nontransformable (e.g., wound isolate TX2555) (Table 2). Additionally, in the absence of selective pressure, ca. 6 to 16% of overnight cultures were found to have lost pAM401 in three independent experiments. Poor transformability increases the difficulty of using suicidal vectors and motivated us to explore conditional replicons that may be applicable for *E. faecium*.

Transformation and stability of conditional replication based ts plasmids in *E. faecium*. At least two thermosensitive broad-host-range vector families (derived from pWVO1 and pE194) have been shown to be useful for high-efficiency gene inactivation and replacement in gram-positive bacteria (2, 3, 5, 17, 41, 54). In order to test the applicability of these systems in *E. faecium*, the ability of three established ts plasmids, pTV1-OK (14), pTV1-ts (66), and pAM401ts (60), derived from pWV01, pE194, and pIP501 replicons, respectively, were assessed to transform and replicate in the transformable *E. faecium* strain, TX1330, at permissive temperature (established as 30°C for other gram-positive bacteria). All three plasmids were found to transform, although poorly, and replicate in TX1330 at 28°C (Table 3). At 30°C, recovery of transformants was found to be reduced to approximately half of what we recovered at 28°C. Among these three ts plasmids, pTV1-OK showed maximum recovery, followed by pAM401ts, and pTV1-ts being the least (Table 3). Restriction digestions of plasmids analyzed from transformants grown at 28°C identified that pTV1-OK and pTV1-ts were relatively stable in *E. faecium* at permissive temperature and maintained their original molecular sizes. In contrast, pAM401ts in *E. faecium* was unstable with multiple deletions and different colonies showed different digestion patterns. Since pTV1-OK is not suitable for *E. faecium* clinical strains (due to lack of appropriate markers and the presence of Tn*917*), we selected pHS1 (a pWV01ts-based *E. faecalis* suicidal vector [1]) and introduced it in two clinical strains (TX0082 and TX2466) at 28°C. We next cloned an *acm* intragenic fragment into pHS1 and introduced this plasmid into *E. faecium* TX0082 by electroporation, selected at 28°C, with subsequent growth of an individual colony at 42°C. In addition to the expected single-crossover recombination into *acm* at the nonpermissive temperature (42°C), persistence of the plasmid was noticed in TX0082 even after >10 serial passages at 42°C. Upon sequencing, we found that RepA of this construct was not that of the more ts version found in pVE6004 (29) but rather is like the parental plasmid pWV01 (27), which is able to replicate at higher temperatures. This observation warranted the need for a vector that would be more reliably nonreplicative at elevated temperatures.

Construction of pTEX5500ts and its mobilizable derivative. Since currently available vectors often contain regions of DNA from enterococci as markers, which may lead to undesired recombination, the minimal functional regions were amplified using specific primers containing rare restriction sites. The streptococcal integration vector pFW14, containing two multiple-cloning sites (MCS), was used as the backbone for pTEX5500ts construction (Fig. 1). The vector pFW14 is a derivative of pFW8 (42) in which the *aad9* promoter driving *cat* gene expression has been replaced by a synthetic promoter region from pFW11 (42) that contains two extended −10 and −35 boxes. To clone a minimal gram-positive ts replicon region by restriction digestion and ligation (and also to add commonly used restriction sites to MCS-II of pFW14), we first introduced ClaI, PvuI, EcoRI, KpnI, EcoRV, and SstII restriction sites into the MCS-II. This was done by treating the appropriately designed synthetic primer duplexes with T4

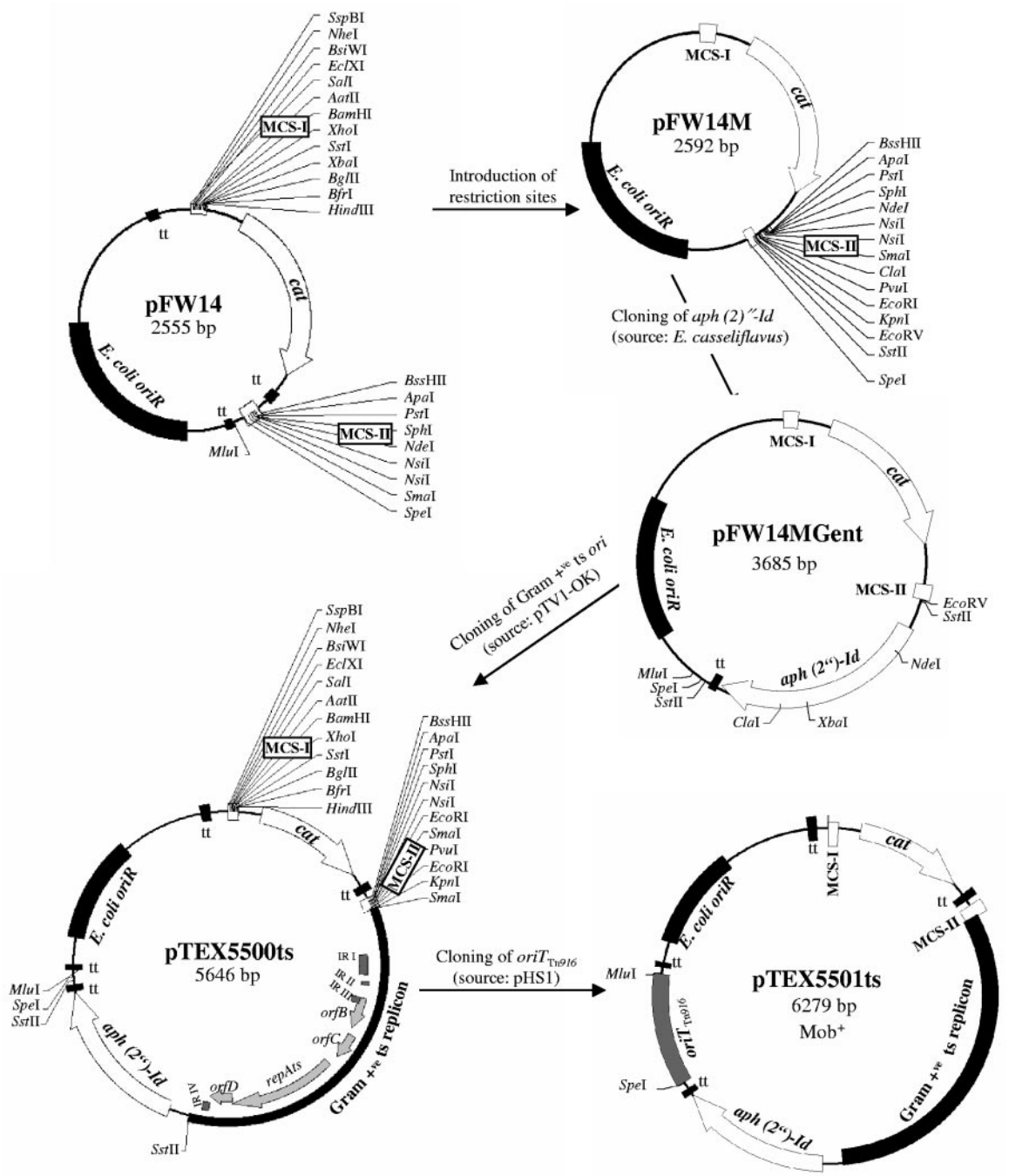

FIG. 1. Construction of pTEX5500ts, a temperature-sensitive (ts) delivery vector, and its mobilizable derivative, pTEX5501ts. Both vectors contain a ts version of the broad-host-range pWV01 replicon for replication in gram-positive hosts at permissive temperatures and *oriR* derived from pUC18 to replicate in *E. coli*. Arrow directions in each plasmid indicate the direction of transcription. Only relevant restriction sites are shown. Maps of pTV1-OK and pHS1, not shown here, have been previously published (1, 14). MCS, multiple cloning sites; *cat*, the chloramphenicol acetyltransferase gene from pC194; tt, transcriptional terminator sites (tts from pFW14 are not shown in pFW14M and pFW14Mgent constructs); *E. coli oriR*, origin of replication derived from pUC18; *aph*(*2''*)-*Id*, gentamicin resistance gene from *E. casseliflavus*; gram-positive ts replicon, temperature-sensitive origin of replication from pTV1-OK; IR, inverted repeats; $oriT_{Tn916}$, origin of transfer region of Tn*916*; and Mob$^+$, mobilizable by *trans*-activation from strains harboring Tn*916*.

DNA polymerase and T4 polynucleotide kinase (52) and subsequent cloning into the SmaI site of pFW14. This intermediate construct was designated pFW14M (Fig. 1). The gentamicin resistance gene *aph*(*2''*)-*Id* was then amplified from *Enterococcus casseliflavus* strain UC73 (59) using primers GentF and GentR (Table 4). This PCR-amplified fragment containing the *aph*(*2''*)-*Id* gene and its promoter and terminator regions was digested with SstII and then ligated to similarly

TABLE 4. Primers used in this study

Primer name	Type	Sequence (5′ → 3′)[a]
GentF	Forward	TTCCCCGCGGAGATAGGTTATGCAAGATTTTTATATG
GentR	Reverse	TTCCCCGCGGATTCCGGATTCTAAAAAAGGATTGCTA
5500-MCS-IF[b]	Forward	GACAATACTGATAAGATAATATATC
5500-MCS-IR[b]	Reverse	TTACGTTACGTTATTAGTTATAG
5500-MCS-IIF[b]	Forward	TTTGTTTTATTATTTAATATTTGG
5500-MCS-IIR[b]	Reverse	GCCGATAACTAAACGAAATAAACG
oriTF	Forward	GGACTAGTTTTTATACTCCCCTTGTACCAAAAG
oriTR	Reverse	GGACGCGTCAAAGGACGAATATGTCGCCTAAAG
AcmDelF1	Forward	CGCGGATCCTATCTCTTGAAACGTTCTTAGAATTGC
AcmDelR1	Reverse	CCCAAGCTTACTATATCATCTCTCAAAAATCCTTTC
AcmDelF4	Forward	AAAACTGCAGAATGAGAACCAAGACGGCAAACGAC
AcmDelR6	Reverse	TCCCCCGGGCCAGCAGGTACAGTGACTCCATTAGC
AcmUp11	Forward	TTTGTAAATAATTGGATGGCAGATT
CmR	Reverse	AAAGCATTTTCAGGTATAGGTG
CmF	Forward	GAATGACTTCAAAGAGTTTTATG
AcmDownR11	Reverse	AACCGAACAAGAAGCTTATGAACTA
AcmF2	Forward	CAGGCAGAGATATCAGCAG
AcmR1	Reverse	ATTCTCATTTGTAACGACTAGC
AcmInsF	Forward	CGCGGATCCACGAATATTACAGATGGAGGAAACA
Acm19R	Reverse	CGGGGTACCTTAATTTTTAACTGTATGATTGAAACTTTC

[a] Introduced restriction sites are underlined.
[b] These primers flanking each of the MCS will facilitate DNA sequencing across the vector-insert junctions.

digested pFW14M, and this construct was designated pFW14MGent (Fig. 1). The gentamicin resistance gene of this vector functions as a spacer as well. We next ligated the ts replicon (obtained by filling in the eluted ~2.5-kb XbaI fragment of pTV1-OK with T4 DNA polymerase, followed by treating with T4 polynucleotide kinase and finally digesting with KpnI) and the previously KpnI- and EcoRV-digested pFW14MGent. This construct was designated pTEX5500ts (Fig. 1). To construct plasmid pTEX5501ts, the PCR-amplified *oriT* fragment (using primers oriTF and oriTR) from the *E. faecalis* mobilizable vector pHS1 (1) was digested with SpeI and MluI and then ligated into similarly digested pTEX5500ts (Fig. 1).

With the aim of improving the selection efficiency of these vectors, we sought to utilize a counter selection based on sucrose sensitivity, proven to be functional in some other gram-positive bacteria (4, 21, 39, 40). Cloning of *sacB* derived from *Bacillus subtilis* (encoding levansucrase) under the extended synthetic promoter of pFW14 followed by electroporation in *E. faecium* strain TX1330 resulted in normal growth of the transformant even in medium containing sucrose. At this stage, it is unknown whether the *sacB* product is nonfunctional in *E. faecium* or whether the toxicity due to the *sacB* product was complemented by an alternate mechanism of sucrose resistance. Similarly, with the aim of facilitating detection of allelic exchange events, blue-white selection as a reporter system was also investigated. However, growth of *E. faecium* on plates with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside rendered a light-blue colonial phenotype indistinguishable from colonies containing the *lacZ* construct we made from pORI280 (26). Additionally, use of a new marker encoded by *emtA* (31) was also found to be ineffective due to the observed background growth of avilamycin-susceptible *E. faecium* when electrocompetent cells (~1×10^{10} CFU) were plated.

Properties of the newly derived ts replicon vector, pTEX5500ts. pTEX5500ts contains (i) *E. coli oriR* of pUC18 derived from the streptococcal integration vector pFW14 (42); (ii) a gram-positive *repAts* replicon from pTV1-OK (14) which is functional in *E. faecium* at a permissive temperature; (iii) two distinct multiple cloning sites (MCSs) on either side of the *cat* gene to provide several alternative sites for cloning of flanking regions for double-crossover mutagenesis; (iv) a synthetic functional promoter region (with two extended −35 and −10 boxes) to overcome relatively low expression levels of the pC194-based *cat* gene (19, 42, 61); (v) a gentamicin resistance gene *aph*(2′′)-*Id* from *E. casseliflavus* (59); (vi) transcriptional terminator sites (tts) to terminate undesired readthrough and to prevent destabilizing effects of cloned inserts; and (vii) primer sites flanking each of the MCSs (5500-MCS-IF, 5500-MCS-IR, 5500-MCS-IIF, and 5500-MCS-IIR [Table 4]) that facilitate screening the vector by PCR or for DNA sequencing across the vector-insert junctions. The *cat* gene encoding chloramphenicol resistance can be used as a marker for selecting chromosomal integration. The gentamicin resistance marker located on the vector can be used to distinguish double-crossover recombinants after plasmid excision. Both resistance markers are functional in *E. coli* and enterococcal backgrounds. Restriction sites adjacent to each of these markers will be helpful for replacing them with alternative markers, as needed. Although the tt positioned downstream of the *cat* gene may have some disadvantages due to a possible polar effect, in this study, it was found to be necessary for cloning of the AcmDn fragment (see below) in MCS-II of pTEX5500ts. A similar observation of the necessity of introduction of tts for efficient cloning of streptococcal DNA fragments was reported in previous studies (7). However, it is possible to use this vector to generate a nonpolar mutation of polycistronic genes by cloning the flanking regions contiguously in-frame followed by deletion of the *cat* cassette including its terminator.

Upon complete sequencing of pTEX5500ts, an additional SmaI site in the 5′ region of the ts replicon and creation of an EcoRI site due to a single mutation in MCS-II were noticed.

Since these restriction sites are also part of MCS-II, these sites still can be used in cloning procedures. However, XbaI of MCS-I and NdeI and ClaI of MCS-II are unusable because of their secondary occurrence in *aph(2'')-Id*. These plasmids should be high copy number in *E. coli* due to the pUC18-based replicon, are stable after growth at 37°C, and should be low copy number (the pGK12 parent of the pWV01 replicon is known to occur in three copies per cell in lactococci [24]) in gram-positive hosts at permissive temperatures.

Replication, temperature sensitivity, and segregation stability of pTEX5500ts. To ensure replication, both postelectroporation incubation and growth on selective media (48 to 96 h) were performed at 28°C. When the primary selection plates contained more than one antibiotic (i.e., both chloramphenicol and gentamicin), either no transformants were found or they were very rare. Using gentamicin for selection, transformation with successful replication was tested with TX1330 and four clinical isolates. Among these, TX1330 yielded more colonies, as anticipated from the above results, in the range of transformation efficiencies noted with pTV1-OK (ca. 80 CFU/μg of plasmid). Two clinical strains that we were able to transform with pTEX5500ts are TX0082 and TX2466, albeit with poor efficiency, i.e., 13 and 9 CFU/μg of plasmid, respectively. The trend of these results is consistent with those obtained with pAM401 (Table 2), suggesting that the transformation variability is a property of the specific strain and not plasmid instability. Colonies that appeared on selective plates were grown in BHI broth with gentamicin, and plasmid DNA isolated from overnight cultures was detected by agarose gel electrophoresis (data not shown).

In *E. faecium*, pTEX5500ts was found to be relatively stable, showing 48% retention (stability seen in three independent experiments was 60, 35, and 49%) of the chloramphenicol resistance phenotype following 16 h of growth at 28°C in the absence of antibiotics. In order to confirm the permissive and nonpermissive temperatures for the replication of pTEX5500ts in *E. faecium*, the stability of this plasmid was analyzed at different temperatures. The ratio of the number of CFU on selective plates to the number of CFU on nonselective plates dropped by ca. 3,100 and ca. 4,700-fold at 37 and 42°C, respectively. The segregation rates of the chloramphenicol resistance phenotype from TX0082 in broth culture in the absence of antibiotic selection at different temperatures were as follows: at 37°C, 59% per generation; at 42°C, 64% per generation; and at 28°C, 5% per generation. These results confirm that pTEX5500ts exhibits a dramatic loss in plasmid replication between 28 and 37 or 42°C, as observed with pWV01ts-based plasmids in other gram-positive pathogens. In subsequent experiments, a culture temperature of 42°C completely cured the plasmid from TX0082 after five serial passages. The nonpermissive temperature of 42°C found for pTEX5500ts in *E. faecium* does not limit the applicability of this system, since these organisms are characterized by high heat tolerance and grow relatively well up to ≥45°C.

Construction of a deletion mutation in the *acm* gene of the collagen-binding *E. faecium* endocarditis isolate, TX0082. To demonstrate the applicability of our new vector for targeted gene disruption, the *acm* gene was chosen. The choice of *acm* is because of our hypothesis that Acm plays a role in *E. faecium* pathogenesis, including endocarditis (37). This is based on our earlier observation of the predominance of a functional *acm* gene in clinical isolates but a pseudogene in fecal and animal isolates (37 and S. R. Nallapareddy and B. E. Murray, unpublished results) and also data for the importance of the *Staphylococcus aureus* collagen adhesin Cna (an Acm homologue) in animal models, including endocarditis (18, 23, 38). Figure 2 outlines the protocol that was used for *acm* gene replacement in *E. faecium*. A 1,106-bp DNA fragment designated AcmUp (Fig. 2), encompassing the region upstream of *acm*, was amplified from TX0082 genomic DNA template using primers AcmDelF1 and AcmDelR1 (Table 4), digested with BamHI and HindIII and ligated with similarly digested pTEX5500ts. Similarly, a 1,031-bp DNA fragment designated AcmDn (Fig. 2), encompassing some of the coding region of the 3′ end of *acm* and the downstream region, was amplified from the same genomic DNA template using primers AcmDelF4 and AcmDelR4 (Table 4). The AcmDn PCR product digested with PstI and SmaI was ligated to similarly digested pTEX5500ts:: AcmUp. This in vitro-ligated construct for generating an *acm* deletion (designated pTEX6050 [Fig. 2]) was transformed into *E. coli* to obtain TX6050 and was then introduced into electrocompetent cells of TX0082 which were allowed to recover at room temperature for 2 h; the latter step was found to be essential for transformation of plasmids into poorly transformable strains. The cells were then plated on gentamicin plates at the permissive temperature (28°C) to select for transformants. After overnight growth at elevated temperature (42°C), the cells were plated on chloramphenicol plates and incubated at 37°C. The single-crossover integration (TX0082::pTEX6050) was tested by PCR (primers sets AcmUp11 and CmR as well as AcmDownR11 and CmF were used for verifying integration into AcmUp and AcmDn regions, respectively [Table 4]), which confirmed that integration was specific and occurred either in AcmUp or in AcmDn regions in different colonies. Also, 40 of 40 gentamicin-resistant colonies tested showed integration. However, PFGE followed by Southern blot probing showed persistence of free plasmid, in addition to single-crossover integration, after overnight growth at 42°C. One of the integrants was picked and was grown for eight serial passages at a culture temperature of 42°C to completely cure the plasmid. The cultures from the fifth passage onward were serially diluted and plated at 37°C on nonselective media to select for plasmid excision by double-crossover recombination. These master plates were then replica plated to chloramphenicol plates and gentamicin plates to identify colonies that retained the *cat* gene and not the vector. Double crossovers were expected to be chloramphenicol resistant and gentamicin sensitive, as shown in Fig. 2. We found 4 of ~5,000 colonies in which the *acm* gene replacement had occurred from the plates from the eighth passage. One of these colonies was designated TX6051 (TX0082Δ*acm*). The deletion was indicated by PCR with primers AcmUpF11 and AcmDownR11, and the strain's identity was confirmed by PFGE (Fig. 3A). Sequencing of this PCR product confirmed the correct deletion of *acm* from −38 to +1677 (including the ribosome-binding site, signal sequence, complete A domain [binding domain], and 13 amino acids of the B domain) and is replaced by *cat* (Fig. 3B). Southern hybridizations using the deleted fragment of *acm* (amplified from TX0082 using primers AcmF2 and AcmR1 [Table 4]), the *cat* gene (amplified from pTEX5500ts using primers

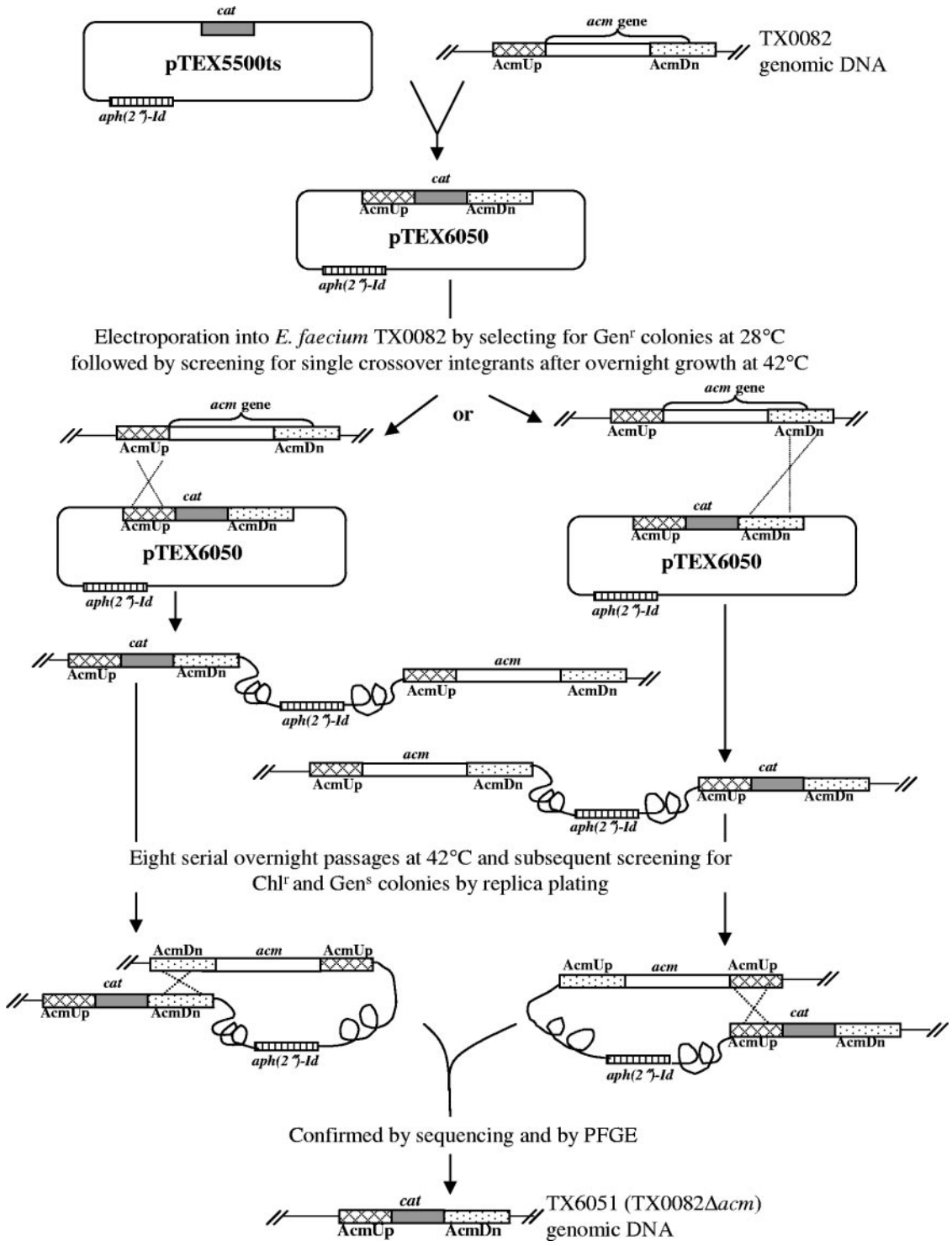

FIG. 2. Protocol used for replacing the *acm* wild-type sequence on the TX0082 chromosome with the *cat* gene. The gene replacement construct (pTEX6050) carrying in vitro-altered sequences (AcmUp, the region upstream of *acm* [shown by the cross-hatched box], and AcmDn, part of the *acm* 3′ region as well as the downstream region [shown by the dotted box]) was transformed into *E. coli*. At permissive temperature (28°C), pTEX6050 was introduced into TX0082 by electroporation. Colonies were screened for an integration event when the temperature was shifted to 42°C. One of the integrants was grown for eight serial overnight passages at 42°C to completely cure the plasmid. The culture from the eighth passage was serially diluted and plated at 37°C on nonselective media to select for double crossover recombination. Upon replica plating to chloramphenicol plates and gentamicin plates, the colonies that retained the *cat* gene only were identified. One of these colonies was designated TX6051 (TX0082Δ*acm*). The gray box represents the *cat* gene coding for chloramphenicol resistance, and the striped box represents the *aph*(*2″*)-*Id* gene coding for gentamicin resistance.

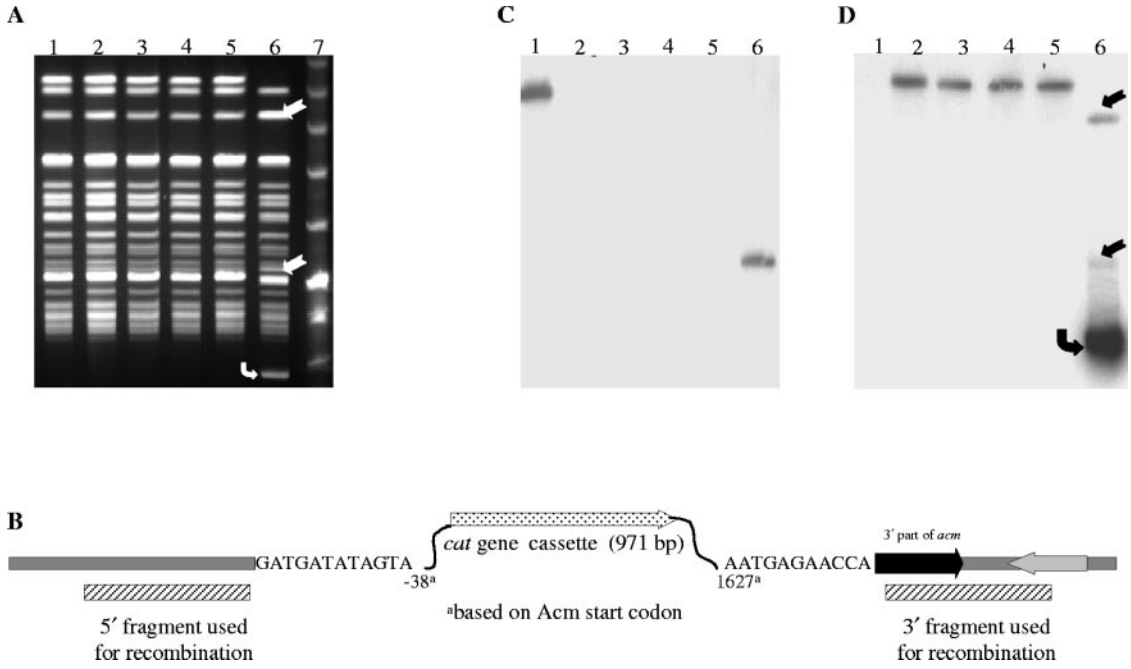

FIG. 3. Confirmation of allelic replacement of the *acm* gene of TX6051. (A) PFGE analysis of SmaI-digested genomic DNA of TX0082 (lane 1), four double-crossover colonies that retained the chloramphenicol resistance (lanes 2 to 5), a colony resulting from a single-crossover integration event (TX0082AcmUp::pTEX6050) (lane 6), and a molecular weight marker (lane 7). Two fragments marked with straight arrows in lane 6 are due to a SmaI site in the inserted plasmid. The extrachromosomal (nonintegrated) plasmid band is marked with a bent arrow. (B) Illustration of a 4,474-bp Δ*acm* region of *E. faecium* TX6051 (TX0082Δ*acm*). Sequencing confirmed that 1,663 bp of the *acm* locus is replaced with a 971-bp *cat* cassette. The 5′ and 3′ regions that were used for recombination events are shown by cross-striped boxes. (C and D) Southern blot analysis of the digests shown in lanes 1 to 6 of panel A. Panel C was probed with the deleted fragment of *acm*, and panel D was probed with the *cat* gene. Hybridization results obtained with pTEX5500ts are identical to those obtained with its *cat* gene, as shown in panel D.

5500-MCS-IF and 5500-MCS-IIR [Table 4]), and pTEX5500ts as probes confirmed the absence of unintegrated plasmid (Fig. 3C and D). Since in silico analysis as well as mRNA analysis of the *acm* locus (data not shown) confirmed the monocistronic nature of *acm* (and thus predicts absence of a polar effect with this system), we used a replacement strategy in this study to introduce the *cat* marker into the mutant to take advantage of the discriminatory capability; this will facilitate distinguishing between wild-type and mutant bacteria after in vivo animal experiments with mixed cultures.

Characterization of the *acm* mutant. (i) Growth characteristics. TX6051 (TX0082Δ*acm*) colonies appeared smaller in size compared to the wild type (TX0082) when grown overnight either on BHI or TH agar. However, no abnormality in cell shape was noticed when the *acm* mutant was observed by light microscopy at various time points of growth. To further characterize the behavior of the *acm* mutant, growth was monitored by CFU and OD_{600}. As shown in Fig. 4A, the doubling time of the *acm* mutant (TX6051) was longer than that of the wild type (TX0082) in the lag and early log phases. Also, the final culture density of the *acm* mutant in BHI was reproducibly two times lower than that of the wild type. The growth of a single-crossover construct (TX0082AcmUp::pTEX6050), which does not cause an interruption of *acm*, was similar to the wild type (Fig. 4A), suggesting that the presence of *cat* had no effect on the observed slow growth of *acm* mutant. Similar observations were made when growth was monitored by OD_{600}. Southern hybridization results showed localization of a single copy of the *cat* gene to the *acm* locus without evidence of a second integration elsewhere in the genome of TX6051 (Fig. 3D).

(ii) Lack of Acm in surface preparations of TX6051 and lack of adherence of this *acm* mutant to collagen(s). Western blots of cell wall-associated proteins released by mutanolysin were probed with anti-Acm polyclonal antibodies, which we previously raised against the A domain of Acm (37). A single immunoreactive protein of ~86 kDa was detected in an endocarditis isolate TX2535 (37) and in TX0082 but not in TX6051 (Fig. 4B). Probing of mutanolysin extracts of these isolates with preimmune rabbit serum showed no reactive bands in all three isolates (data not shown).

E. faecium TX0082 and its isogenic Δ*acm* strain TX6051 were tested for their ability to adhere to immobilized collagen types I and IV, fibrinogen, and BSA. Strain TX0082 adhered to collagen(s) and fibrinogen, whereas the *acm* mutant (TX6051) was completely defective in adhering to collagen types I and IV but not to fibrinogen (Fig. 4C). This genetic evidence corroborates our earlier paper indicating that Acm mediated collagen adherence of *E. faecium* based on results with recombinant Acm and complementation of *acm*-pseudogene containing *E. faecium* isolates (37). Using this *acm* deletion mutant, the role of *acm* in *E. faecium* pathogenesis will be tested in animal models including endocarditis.

Use of a mobilizable derivative of pTEX5500ts to interrupt the *acm* gene of a nonelectroporable *E. faecium* strain. A mobilizable derivative of pTEX5500ts, designated pTEX5501ts

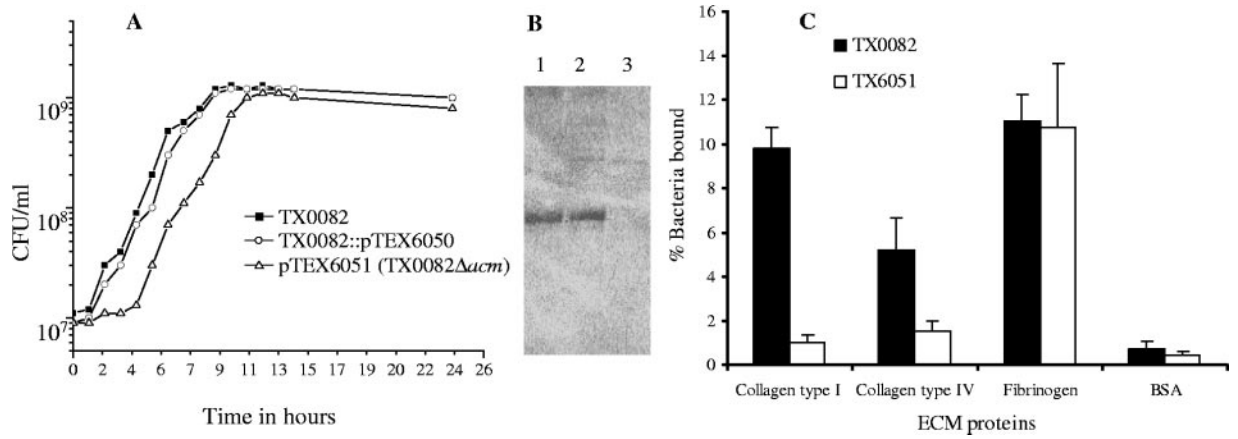

FIG. 4. Characterization of TX6051. (A) Growth curve of wild-type *E. faecium* strain TX0082, its isogenic *acm* deletion mutant (TX6051), and a colony resulting from a single-crossover integration event (TX0082AcmUp::pTEX6050). Aliquots of culture were withdrawn every hour through the growth cycle for the measurement of CFU. (B) Western blots of mutanolysis extracts of *E. faecium* isolates (lane 1, TX2535; lane 2, TX0082; and lane 3, TX6051). Samples were probed with polyclonal antiserum raised against recombinant AcmA (37). (C) Adherence of wild-type *E. faecium* TX0082 and its isogenic *acm* deletion mutant (TX6051) to immobilized collagen types I and IV, fibrinogen, and BSA. Adherence was tested in wells coated with 1 μg of extracellular matrix (ECM) proteins. Bars represent the mean percentages of cells bound ± standard deviations for eight wells. Results are from four independent experiments. BSA was used as a negative control.

was created by adding *oriT* from Tn*916* in order to transfer it by conjugation from *E. faecalis* JH2Sm::Tn*916* (6). To generate the plasmid for insertional inactivation, an 846-bp DNA fragment designated AcmIns, which is an intragenic region of *acm*, was amplified from TX2555 genomic DNA template using primers AcmInsF and Acm19R. The AcmIns PCR product was digested with BamHI and KpnI and ligated to a similarly digested 5.24-kb BamHI and KpnI gel-eluted fragment from pTEX5501ts, yielding pTEX6052 (pTEX5501ts::*acm*). This plasmid was electroporated into *E. coli* DH5α to obtain TX6052, and from there it was introduced into JH2Sm::Tn*916* by electroporation, selecting for gentamicin resistance after growth at 28°C; a resulting colony was chosen and designated TX6053. Using the principle of *trans*-activation (Tn*916* presence in the donor strain activates conjugal transfer of coresident plasmids carrying $oriT_{Tn916}$ [1, 22]), pTEX6052 from *E. faecalis* JH2Sm::Tn*916* was mobilized to the nonelectroporable (in our hands) *E. faecium* strain TX2555 by broth mating. Transconjugants were selected for resistance to 125 μg/ml gentamicin and 200 μg/ml erythromycin (present in the recipient). PFGE analysis confirmed the strain identity of the transconjugant (data not shown). Selected gentamicin-resistant colonies were shown to stably maintain pTEX6052 after growth at 28°C. Subsequently, a culture of TX2555(pTEX6052) was shifted to 42°C (to cause plasmid loss), grown for 10 serial passages, serially diluted, and plated on gentamicin at 37°C. PFGE analysis followed by probing of Southern blots showed complete loss of detectable plasmid in some colonies (data not shown). This insertional mutant strain of *acm* was designated TX6054.

Growth patterns and adherence characteristics of wild-type TX2555 versus TX6054 (data not shown) were essentially the same as we observed with wild-type TX0082 versus TX6051. We also showed the stability of the *acm* insertion of TX6054, without evidence of excised plasmid DNA by Southern blots using pTEX6052 as a probe, after two overnight in vitro passages at 37°C and without antibiotic (data not shown). Our ability to disrupt the *acm* gene in a nontransformable *E. faecium* strain using mobilization and recombination events indicate that the same technique could be used for allelic replacement, as described above with pTEX5500ts and TX0082.

Conjugal transfer of a pTEX5501ts-derived plasmid from *E. faecalis* JH2Sm::Tn*916* to diverse *E. faecium* clinical strains. By broth mating, the transfer of pTEX6052 (pTEX5501ts::*acm* intragenic fragment) was successful only with the recipient strain TX2555 (1×10^{-8}), and no transconjugants were seen

TABLE 5. Transferability of plasmid pTEX6052 (a pTEX5501ts derivative) from *E. faecalis* JH2Sm::Tn*916* to diverse *E. faecium* clinical strains by *trans*-activation

Recipient (source of isolation, place of origin)	Antibiotic selection (μg/ml)	Transfer frequency[e]
TX2442 (blood, Belgium)[a]	Gen, 125; Ery, 200	3×10^{-9}
TX2535 (endocarditis, Houston, Tex.)[b,c,d]	Gen, 125; Ery, 200	3.8×10^{-7}
TX2067 (clinical isolate, Boston, Mass.)[c]	Gen, 125; Ery, 200	2.0×10^{-7}
TX2492 (blood, Houston, Tex.)[b,c,d]	Gen, 125; Ery, 200	3.6×10^{-8}

[a] Vancomycin resistant, VanA type.
[b] Vancomycin resistant, VanB type.
[c] Ampicillin resistant.
[d] Isolates TX2535 and TX2492 were from different hospitals.
[e] Transfer frequencies are expressed as number of transconjugants per donor CFU.

with TX2535 and TX2466. Subsequent experiments with filter matings demonstrated the transferability of pTEX6052 into diverse *E. faecium* clinical strains of different PFGE types, and the transfer frequencies varied with different strains (Table 5). From every mating, randomly selected transconjugants were analyzed for plasmid content and the strain identity was confirmed by PFGE (data not shown). These results suggest that mobilization from an *E. faecalis* donor is a convenient method for introducing pTEX5501ts derivatives into diverse clinical strains of *E. faecium* and even into the strains that have been recalcitrant to standard electroporation.

Replication of pTEX5500ts in *E. faecalis*. To assess the functionality of this plasmid in *E. faecalis* mutagenesis, transformation and stability of pTEX5500ts was also tested with *E. faecalis* OG1RF. This plasmid was transformed with better efficiency (420 CFU/μg of plasmid) into *E. faecalis* OG1RF than *E. faecium*. The permissive temperature as well as plasmid loss rates were similar to what we noted for *E. faecium*. Since pWV01-based *repATs* has been used for allelic replacement of several gram-positive bacteria, including lactococci (3), streptococci (41), staphylococci (17, 54), and *Desulfitobacterium* (53), these new vectors should be applicable for mutagenesis of a broad range of hosts.

In summary, to overcome the limitation of poor transformability of clinical *E. faecium* isolates, a ts replicon derived from the low-copy-number plasmid pWV01 was used to construct a thermosensitive vector which is functional in *E. faecium* at permissive temperature and suicidal at nonpermissive temperature. Using this vector, the *acm* gene of an endocarditis-derived *E. faecium* strain was successfully deleted, and this is the first example to our knowledge of allelic replacement in this species. More importantly, the transferability of mobilizable derivatives of this vector was demonstrated with nonelectroporable *E. faecium* clinical strains. These vectors will be particularly useful for genes that do not confer easily screenable phenotypes. The improved cloning vector properties of this system should also be helpful for mutagenesis of other gram-positive bacteria as well.

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