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CcpA Is Important for Growth and Virulence of *Enterococcus faecium*

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The collagen adhesin Acm was the first virulence determinant reported to be important for the pathogenesis of *Enterococcus faecium* in a rat infective endocarditis model. We had previously reported that there was a slight growth delay associated with *acm* allelic replacement (*cat*) mutant strain TX6051 used in that study. Recently, we generated a nonpolar markerless *acm* deletion mutant and did not observe a delay in growth. We therefore performed comparative genome sequence analysis of wild-type strain TX82 and TX6051 and found a single mutation, a nonsense mutation in the *ccpA* gene of TX6051. After correcting this mutation, the growth defect of TX6051 was abolished, implicating a role for CcpA in the growth of *E. faecium*. To confirm this, we created a *ccpA* deletion mutant of TX82, which also exhibited a slight delay in growth. Furthermore, the *ccpA* deletion mutant was attenuated ($P = 0.0024$) in a mixed-inoculum (TX82 plus TX82 Δ*ccpA*) rat endocarditis model and also in an *in vitro* competitive growth assay; a *ccpA*-complemented strain showed neither reduced growth nor reduced virulence. We also found attenuation in the endocarditis model with the new *acm* deletion mutant although not as great as that previously observed with TX6051 carrying the *ccpA* mutation. Taken together, our data confirm the role of Acm in the pathogenesis of endocarditis. We also show that CcpA affects the growth of *E. faecium*, that an intact *ccpA* gene is important for full virulence, and that a *ccpA* mutation was partly responsible for the highly attenuated phenotype of TX6051.

Enterococcus faecium has become one of the most problematic causes of nosocomial infections in the United States over the last 2 decades and is found as a cause of various infections, including endocarditis, bacteremia in neutropenic patients, urinary tract infections, and transplant- and device-associated infections (1). A recent survey indicated that slightly more than one-third of the hospital-associated infections caused by enterococci can be attributed to *E. faecium* when the organisms were identified to the species level (2). Infections with multidrug-resistant *E. faecium* strains are a major clinical threat due to limited treatment options. The emergence of resistance to ampicillin and the subsequent acquisition of resistance to vancomycin have been associated with the progression of this harmless commensal organism into an important nosocomial pathogen in the United State (3–5). In addition, *E. faecium* isolates recovered from hospital-associated infections (HA clade or subclade A1) (4, 6) are also characterized by the presence of many putative or confirmed virulence-associated traits, including the expression of collagen adherence mediated by Acm (adhesin of collagen from *E. faecium*) (7), Esp (enterococcal surface protein) (8), a plasmid encoding a hyaluronidase-like protein (Hyl_{fm}, now annotated family 84 glycosyltransferase) (9), MSCRAMMs (microbial surface component recognizing adhesive matrix molecules) (10, 11), EmpABC (forming *E. faecium* pili [previously known as $EbpABC_{fm}$]) (11), and a putative phosphotransferase system important for gut colonization during antibiotic treatment (12). Moreover, the presence of insertion sequence (IS) elements and a high level of recombination events have also contributed to the evolution and rise of this organism in the nosocomial environment (13, 14).

The ability of *E. faecium* to adhere to collagen is mediated primarily by Acm (15). Acm is expressed only by HA clade strains, and an inactive gene was found in many commensal isolates via insertion sequences or stop codons; however, *acm* was found intact in all infective endocarditis isolates (16). Previously, we reported that in a rat endocarditis model, the *acm* allelic replacement mutant (TX6051 [TX82 Δ*acm*::*cat*]) was highly attenuated compared to wild-type (WT) strain TX82 (17). We also observed that TX6051 produced small colonies and that the doubling time was long compared to the wild type (7). However, colony counts were equal beyond about 9 h. Since the genetic tools to restore the deleted gene had not yet been developed and the plasmid vectors that we tested were not stable *in vivo*, we were not able to determine if the growth defect and attenuation were attributed solely to the deletion of the *acm* gene. With the development of efficient genetic manipulation techniques (18, 19), we recently generated a markerless, nonpolar deletion of *acm*, which exhibited growth characteristics similar to those of the wild type. This prompted us to look for the actual cause of the growth defect associated with TX6051.

Here, we show that the previously reported, highly attenuated phenotype exhibited by an *acm* allelic replacement mutant is partly mediated by the presence of a nonsense mutation in the *ccpA* gene and that CcpA, a global transcriptional regulator of carbon catabolite repression (CR) in Gram-positive bacteria, affects the growth of *E. faecium*. We also demonstrate the importance of CcpA in infective endocarditis in a rat model, likely due to its effect on growth, and confirm the contribution of Acm to virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference(s) or source
Strains		
E. faecium		
TX82	Endocarditis isolate; Van^r Amp^r	7
TX6051	TX82 Δ*acm*::*cat*; *acm* allelic replacement mutant with an incidental *ccpA* premature stop codon	7; this study
TX6086	TX82 Δ*acm*; nonpolar markerless *acm* deletion mutant	This study
TX6130	TX6051::*ccpA* nucleotide mutation corrected	This study
TX6127	TX82 Δ*ccpA*; *ccpA* deletion mutant	This study
TX6140	TX6127 complemented with *ccpA* (*in situ* in the chromosome)	This study
TX6145	TX6127 complemented with 300 bp of the *ccpA* gene	This study
E. coli		
DH5α	*E. coli* cloning host	Invitrogen
EC1000	*E. coli* host strain, provides RepA	45
E. faecalis CK111	Conjugative donor for genetic manipulations	18
Plasmids		
pHOU1	Plasmid for mutagenesis; Gen^r	19
TX6143	Plasmid for *ccpA* gene deletion; 820 bp upstream and 912 bp downstream of the *ccpA* gene cloned into pHOU1	This study
TX6144	Plasmid for initial 300-bp complementation of the *ccpA* gene; 452 bp upstream of the start codon of *ccpA* along with the initial 300 bp of *ccpA* and 507 bp downstream of the stop codon of the *ccpA* gene cloned into pHOU1	This study
TX6146	Plasmid for correcting the *ccpA* gene mutation of TX6051; fragment containing 863 bp upstream and 572 bp downstream of the *ccpA* stop codon cloned into pHOU1	This study
TX6147	Plasmid for *acm* gene deletion; flanking regions of the *acm* gene cloned into pHOU1	This study
TX6148	Plasmid for second-step complementation (restoration) of the *ccpA* gene; fragment containing bp 21–1020 of the *ccpA* gene along with 507 bp of downstream sequence cloned into pHOU1	This study

[a] Amp^r, ampicillin resistance; Gen^r, gentamicin resistance; Van^r, vancomycin resistance.

grown at 37°C in Luria-Bertani (LB) broth or agar, and enterococci were grown in brain heart infusion (BHI) or M17 broth or agar (Difco Laboratories). For enterococci, the following antibiotic concentrations were used: 10 μg/ml chloramphenicol, 10 μg/ml erythromycin, and 200 μg/ml gentamicin. For *E. coli*, the concentrations used were 30 μg/ml kanamycin and 25 μg/ml gentamicin.

Genomic sequencing. Sequence reads of the *E. faecium* strains (TX82 and TX6051) were generated with the Illumina IIx genome analyzer and assembled at the Washington University Genome Institute. Genome-wide variant detection (read mapping) and comparisons of contigs were performed by using standard methods as described previously (20).

Construction of a markerless nonpolar deletion mutant of *acm*. The *acm* gene along with the upstream region (1,036 bp) and the downstream region (473 bp) were PCR amplified by using specific primers (data not shown), cloned into pBluescript, and subcloned into pHOU1 between BamHI and EcoRI sites; pHOU1 contains a *pheS** allele, encoding a phenylalanine tRNA synthetase with altered substrate specificity, which confers susceptibility to *p*-chloro-phenylalanine (*p*-Cl-Phe) (17). The *acm* coding region, except for the 239 bp at the 5′ end, was deleted by digestion with MscI, followed by self-ligation and transformation into competent *E. coli* EC1000 cells, as described previously (19). This recombinant plasmid, our deletion construct (TX6147), was then electroporated into CK111 and transferred to TX82 by filter mating. Single-crossover integrants were selected on BHI plates containing gentamicin and erythromycin and then replated onto MM9YEG medium (M9-based medium supplemented with yeast extract, salts, and glucose) containing *p*-Cl-Phe (7 mM) to select for excision of pHOU1. Excision of pHOU1 was confirmed by an absence of growth on BHI-gentamicin agar; colonies lacking *acm* were detected by PCR, and one of these was designated TX6086. The correct deletion was confirmed by sequencing of the PCR product obtained by using outside primers specific for the flanking regions of the cloned gene and by pulsed-field gel electrophoresis (PFGE).

Correction of the mutation in the *ccpA* gene of TX6051. To correct the mutation in the *ccpA* gene of TX6051, part of the *ccpA* coding region (863 bp at the C-terminal end) together with the downstream sequence (572 bp) was PCR amplified and cloned into plasmid pHOU1 between PstI and SphI sites, and this construct (TX6146) was introduced into TX6051 and processed as described above. Replacement of the mutated *ccpA* gene with the wild-type *ccpA* gene (generating TX6130) was confirmed by the absence of growth on BHI-gentamicin agar, by sequencing of the PCR product obtained using primers specific for the flanking regions of the cloned gene, and by PFGE.

Construction of a *ccpA* deletion mutant and its complementation. To construct a *ccpA* deletion mutant of TX82, genomic DNA regions flanking the *ccpA* gene (820 bp upstream and 912 bp downstream) were amplified by overlap extension PCR using primers with BamHI and PstI restriction sites and cloned into plasmid pHOU1 (TX6143), and the deletion mutant (TX82 Δ*ccpA*, designated TX6127) was prepared as described above.

To construct a *ccpA* chromosomally complemented strain (i.e., restoration of the wild-type gene back into its native location), the *ccpA* gene along with the upstream and downstream sequences were amplified by using overlapping primers, which ensured the removal of the BamHI site in the *ccpA* gene (silent mutation), in order to make a distinction between the wild type and strains with *ccpA* complemented in the native chromosomal site. Because of the difficulty in the cloning of this *ccpA* complementation construct (the entire *ccpA* gene along with the upstream and downstream sequences) in *E. coli*, a two-step complementation strategy was employed. Initially, a construct (TX6144) was made, including the upstream genomic sequence (452 bp) of the *ccpA* gene along with the initial 300 bp of *ccpA* and 507 bp from the stop codon of the *ccpA* gene, and was used to insert the first 300 bp of the *ccpA* gene back into the *ccpA* deletion mutant (TX6127), resulting in TX6145 (TX82 Δ*ccpA*::300 bp *ccpA* [*ccpA* deletion mutant complemented with the initial 300 bp of the *ccpA* gene]). This was followed by the introduction of a construct (TX6148) containing the *ccpA* gene (21 bp from the start codon to bp 1020) and the downstream sequence (507 bp) into strain TX6145 (TX82 Δ*ccpA*::300 bp *ccpA*) to create TX6140 (TX82 Δ*ccpA*::*ccpA*).

Growth curves. Cultures grown overnight were inoculated into BHI broth at a dilution of 1:100. The cultures were then grown at 37°C, and

aliquots were removed at 0, 2, 4, 6, 9, and 24 h for determination of their CFU on BHI agar.

Experimental endocarditis. Aortic valve endocarditis was induced in white Sprague-Dawley rats according to our previously reported method (21). The wild type and mutants were tested in a mixed-infection competition assay by inoculating a mixture of bacteria (approximately 1:1 by the optical density [OD]) intravenously via the tail vein, 24 h after catheterization; the cultures were then serially diluted and plated to determine the actual CFU and percentage of each strain that went into the inoculum. Animals were sacrificed 48 h after infection; hearts were aseptically removed; the aortic valves were excised, weighed, and homogenized in 1 ml of saline; and dilutions were plated onto BHI medium. After 24 h, all colonies that grew from an aortic valve (up to 47/rat, thus accommodating two rats' colonies and controls in a 96-well microtiter plate) were picked into wells of microtiter plates containing BHI medium, grown overnight, and then replica plated onto a filter overlaid onto BHI agar. DNA lysates from colonies were hybridized under high-stringency conditions (22), using intragenic DNA probes of *acm*, *ccpA*, and *ddl* (23) to generate the percentages of wild-type and mutant colonies of the bacteria recovered from aortic valves. Data were expressed as percentages of WT and mutant colonies per aortic valve and analyzed by the paired *t* test using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). The rat experimental procedures were carried out in accordance with the institutional policies and the guidelines stipulated by the Animal Welfare Committee, University of Texas Health Science Center at Houston.

Growth competition assay. For the growth competition assay, 0.1 ml each of cultures (~2×10^9 bacteria/ml) of TX82 and TX6127 grown overnight were added to 4.8 ml of BHI broth and grown at 37°C with gentle shaking, aliquots were removed at 4 and 24 h, and dilutions were plated onto BHI medium. Even though the strains were distinguishable by colony size differences, for specificity, we employed DNA hybridization using intragenic DNA probes of *acm* and *ccpA* to determine the percentages of wild-type and mutant colonies. The experiment was performed three times, and statistical analyses were performed by two-way analysis of variance (ANOVA).

Biofilm assay. Cultures of TX82 or TX6127 grown overnight were diluted 1:100 in tryptic soy broth (TSB) containing 0.25% glucose, and 200 μl of this mixture was inoculated into the wells of 96-well polystyrene microtiter plates. After 24 h of incubation at 37°C, biofilm formation was quantified by measuring the OD at 570 nm (OD_{570}) of crystal violet-stained wells, as described previously (24, 25). The assay was repeated twice with a minimum of 8 wells each time. Statistical analyses were performed by the Mann-Whitney test.

RESULTS

The growth defect of TX6051 is due to a nonsense mutation in its *ccpA* gene. In order to determine whether the growth delay observed with TX6051 is indeed due to the effect of the deletion of *acm*, we constructed a markerless, nonpolar *acm* gene deletion mutant. Surprisingly, this mutant did not exhibit any growth defect, unlike TX6051 (Fig. 1), while both strains showed a loss of collagen binding (data not shown). This suggested that there may be one or more extraneous mutations in the genome of TX6051 which caused the growth defect. This prompted us to perform genomic sequencing of TX6051, which revealed the presence of a nonsense mutation in the *ccpA* gene. We did not find any mutations other than the *ccpA* single nucleotide nonsense mutation. The domain organization of the CcpA protein is shown in Fig. 2A. The *ccpA* gene encodes a protein of 339 amino acids (aa) that contains a helix-turn-helix (HTH) DNA-binding domain of the LacI family of transcriptional regulators at the N terminus (aa 15 to 66); a C-terminal ligand-binding domain (aa 71 to 337), which exhibits the type 1 periplasmic protein-binding fold; and a linker (aa 55 to 64) connecting these two domains (26).

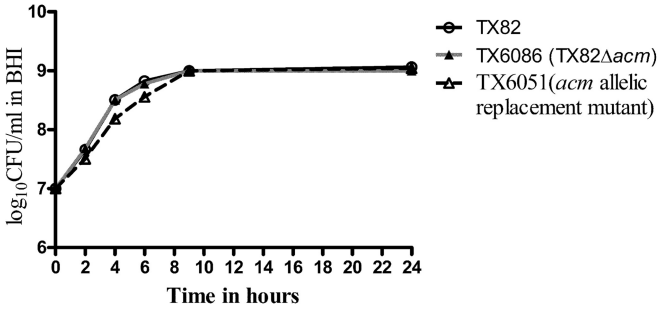

FIG 1 Effect of *acm* deletion on growth of TX82. Shown are growth curves of wild-type *E. faecium* strain TX82, its nonpolar *acm* deletion mutant (TX6086), and the previously described *acm* allelic replacement mutant (TX6051). Cells were grown in BHI broth at 37°C, and aliquots were removed at 0, 2, 4, 6, 9, and 24 h for determination of their CFU counts on BHI agar. Data are representative of two independent experiments.

The nonsense mutation in the *ccpA* gene of TX6051 occurred at the 299th amino acid, converting glutamine to a stop codon and thereby causing premature termination (Fig. 2B). To confirm that the observed growth defect is due solely to the mutation in the *ccpA* gene, we corrected the *ccpA* mutation in TX6051 and examined the growth curve; correction of the *ccpA* nonsense mutation completely abrogated the growth defect associated with TX6051 (Fig. 2C), suggesting that the C-terminal (aa 71 to 337) ligand-binding domain is responsible for the regulation of growth.

Deletion of *ccpA* affects growth of TX82. Since our data showed an effect of CcpA on growth, we next sought to determine the consequence of the deletion of the complete *ccpA* gene on growth and virulence. For this, we constructed a markerless nonpolar *ccpA* gene mutant (TX6127) in *E. faecium* TX82. As with TX6051, *ccpA* deletion mutant colonies were small compared to those of TX82 and TX6140, the *ccpA* chromosomally complemented strain (Fig. 3A). In addition, it showed a growth defect in broth almost identical to that observed for TX6051 carrying the *ccpA* single nucleotide mutation, in the lag phase and early log phase, compared to TX82 (Fig. 3B).

CcpA is important for virulence of *E. faecium* in experimental endocarditis. Next, we wanted to determine whether CcpA is important for the infection process during endocarditis. For this, 9 catheterized rats were inoculated intravenously through the tail vein with a mixture of wild-type strain TX82 and the *ccpA* deletion mutant (TX6127). Although we had aimed for approximately equal CFU/ml estimated by equal ODs, the actual bacterial mean CFU determined for TX82 and TX6127 from the inocula were 2.1×10^8 CFU/rat (representing 13% of the inoculum) and 1.4×10^9 CFU/rat (representing 87% of the inoculum), respectively. Even though the inoculum mixture contained a higher percentage of the *ccpA* deletion mutant than of the wild type, plating of the bacteria recovered from aortic valves showed a (geometric) mean of 6.3×10^4 CFU/g of aortic valve, ranging from 1.3×10^1 to 7.9×10^6 CFU/g. This was followed by Southern hybridization of individual colonies, which revealed that 68% of the total bacteria were wild-type strain TX82, compared to only 32% for the *ccpA* deletion mutant (TX6127) ($P = 0.0024$) (Fig. 4A). This demonstrates a clear dominance of TX82 over the *ccpA* deletion mutant and indicates that CcpA is important in infective endocarditis.

In order to conclusively show the importance of CcpA in endocarditis, we infected 10 rats with an inoculum mixture of *ccpA*

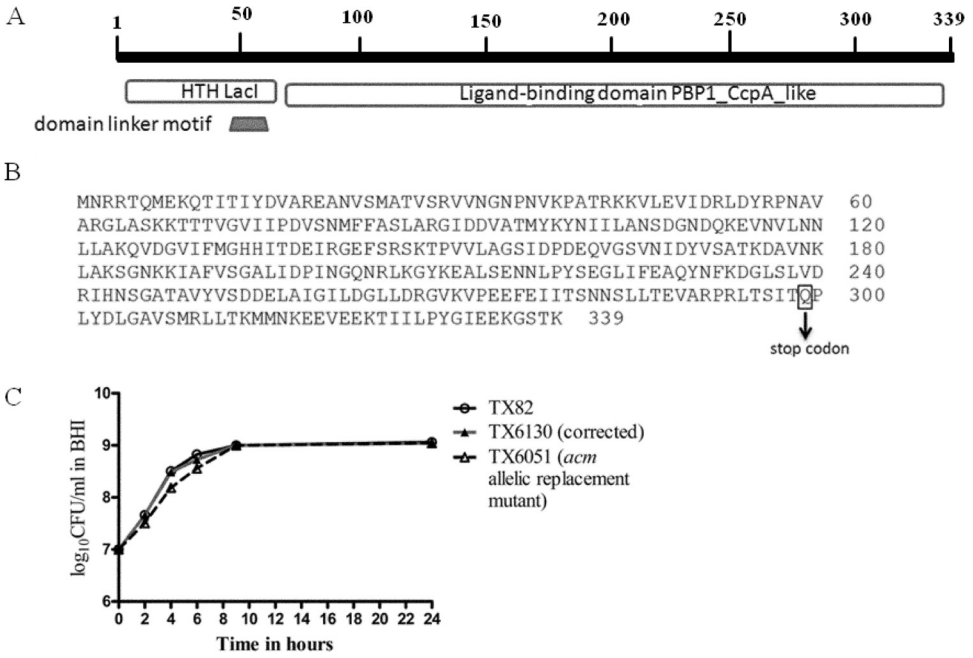

FIG 2 Diagrammatic representation of the CcpA protein and effect of the *ccpA* nonsense mutation on growth of TX82. (A) Schematic representation of the CcpA protein indicating conserved domains. (B) Amino acid sequence of CcpA of TX82 indicating the position of the nonsense mutation. (C) Growth curve of wild-type *E. faecium* strain TX82, the previously described *acm* allelic replacement mutant (TX6051), and TX6051 with a corrected *ccpA* gene (TX6130). Cells were grown in BHI broth at 37°C, and aliquots were removed at 0, 2, 4, 6, 9, and 24 h for determination of their CFU counts on BHI agar. Data are representative of two independent experiments.

deletion mutant strain TX6127 (1.4×10^9 bacteria/rat, representing 73% of the inoculum) and *ccpA* chromosomally complemented strain TX6140 (5.2×10^8 bacteria/rat, representing 27% of the inoculum). The geometric mean CFU recovered from aortic valves was 4.5×10^4 CFU/g of aortic valve, ranging from 1.3×10^1 to 2.8×10^7 CFU/g. The *ccpA* chromosomally complemented strain was recovered at a significantly higher percentage (81.5%) than the *ccpA* deletion mutant (18.5%) ($P = 0.0018$). Taken together, these findings demonstrate the importance of CcpA for virulence in experimental endocarditis (Fig. 4B).

Deletion of *ccpA* also causes reduced competitiveness in mixed *in vitro* growth and reduced biofilm formation capacity. Since the *ccpA* deletion mutant (TX6127) showed attenuation in the endocarditis model, we next sought to determine whether this effect was correlated with an *in vitro* mixed-growth defect or reduced biofilm formation. For this, we performed a growth competition assay. The percentage of colonies recovered was higher for wild-type strain TX82 than for *ccpA* deletion mutant strain TX6127 (77.3% versus 23.7% and 75.3% versus 24.7% for the wild type and the *ccpA* deletion mutant at 4 h and 24 h, respectively; $P < 0.001$ by two-way ANOVA), suggesting that the growth defect plays a major role in the attenuated phenotype exhibited by the *ccpA* deletion mutant in the endocarditis model (Fig. 5). Next, we measured biofilm formation of these strains, grown independently in microtiter plates, and observed that the *ccpA* deletion mutant exhibited reduced biofilm formation compared to that of the wild type (median OD_{570} values of 1.486 and 1.933 for TX6127 and TX82, respectively; $P < 0.0001$) (Fig. 6). This raises the possibility that the capacity to

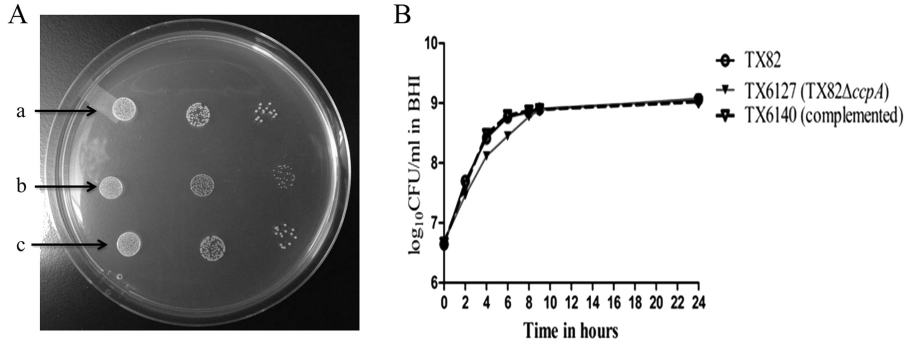

FIG 3 Effect of *ccpA* deletion on growth of TX82. (A) Colony characteristics of wild-type *E. faecium* strain TX82 (a), the *ccpA* deletion mutant (TX6127) (b), and TX6127 complemented with *ccpA* (TX6140) (c). A serially diluted suspension of bacteria grown overnight was spotted onto BHI agar plates. (B) Growth curve of wild-type *E. faecium* strain TX82, its *ccpA* deletion mutant (TX6127), and TX6127 complemented with *ccpA* (TX6140). Data are representative of two independent experiments.

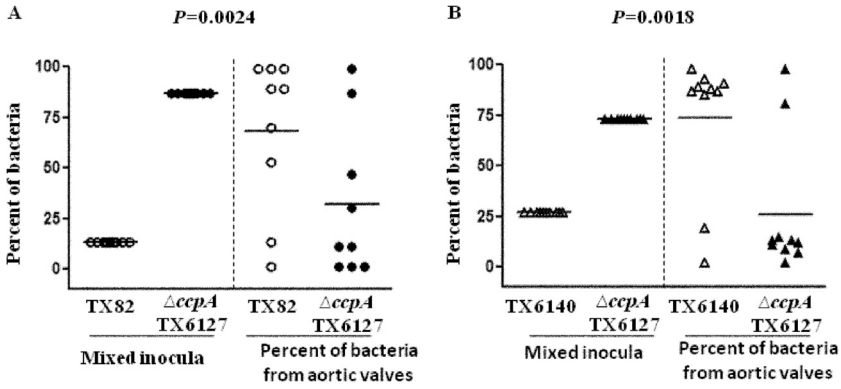

FIG 4 Attenuation of *ccpA* deletion mutant strain TX6127 in a rat endocarditis model. (A) Percentages of wild-type (TX82) and *ccpA* deletion mutant (TX6127) bacteria recovered from the initial inoculum (left) and from aortic valves of 9 rats 48 h after infection (right). Horizontal lines indicate means ($P = 0.0024$ by paired *t* test) for the percentage of total bacteria in the aortic valve versus that in the inoculum. (B) Percentages of *ccpA* chromosomally complemented (TX6140) and *ccpA* deletion mutant (TX6127) bacteria recovered from the initial inoculum (left) and from aortic valves 48 h after infection of 10 rats (right). Horizontal lines indicate means ($P = 0.0018$ by paired *t* test) for the percentage of total bacteria in the aortic valve versus that in the inoculum for each rat.

form biofilm may also contribute to the attenuated phenotype of the *ccpA* deletion mutant in the infective endocarditis model.

Acm also contributes to the infectivity of TX82 in experimental endocarditis. Next, we wanted to determine whether Acm actually has a role in infective endocarditis in the absence of a *ccpA* mutation. Toward this goal, we tested wild-type strain TX82 and its markerless nonpolar *acm* deletion mutant (TX6086) in the endocarditis model. For this, 16 rats were used, as described above, with a mixture of the wild type and the *acm* deletion mutant. The CFU of the wild type and mutant strain TX6086 in the inoculum were 2.5×10^9 CFU (38%) and 4×10^9 CFU (62%) for TX82 and TX6086, respectively. The mean percentages of CFU recovered from aortic valves (geometric mean of 3.9×10^5 CFU/g of aortic valve, ranging from 4.4×10^1 to 5.1×10^7 CFU/g) were 48% and 52% for TX82 and TX6086, respectively ($P = 0.0213$), indicating an advantage of TX82 over the *acm* mutant (TX6086), even in the absence of the *ccpA* mutation (Fig. 7).

DISCUSSION

We have previously shown that the expression of a functional *acm* gene confers a collagen adherence phenotype to clinical *E. faecium* strains and found evidence that Acm expression occurred *in vivo* in all patients with infective endocarditis, even when not expressed *in vitro* (16). In addition, *acm* allelic replacement mutant strain TX6051 was found to be highly attenuated in a rat endocarditis model and exhibited a slight growth delay compared to wild-type TX82 (7, 17). To follow up on this finding, we recently created a nonpolar markerless *acm* deletion mutant and did not observe any growth delay or defect, as was seen with *acm* allelic replacement mutant strain TX6051, which suggested that the presence of one or more extraneous mutations in the genome of TX6051 might have caused the defect in growth. By comparative genomic sequence analysis of the wild type and the *acm* allelic replacement mutant, we then found that there is a nonsense mutation in the *ccpA* gene of TX6051, leading to premature termination of CcpA.

CcpA is a global transcriptional regulator belonging to the LacI-GalR family of transcriptional regulators and is the major regulator of carbon catabolite repression (CR) in low-G+C Gram-positive bacteria. CR refers to the repression of metabolism of nonpreferred carbon sources when preferred sugar sources are available. When glucose or other easily metabolized sugars are present, Hpr kinase is activated and causes the phosphorylation of

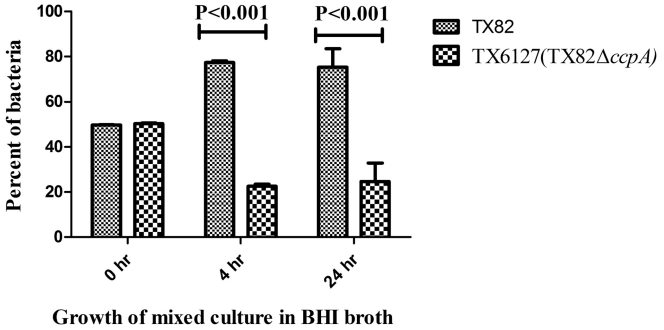

FIG 5 Effect of *ccpA* deletion on competitive growth in a mixed culture. Equal quantities of cultures (2×10^9 bacteria/ml) of the wild type (TX82) and the *ccpA* deletion mutant (TX6127) grown overnight were added to BHI broth and grown at 37°C with gentle shaking. Aliquots were removed at 4 and 24 h, and dilutions were plated onto BHI agar. The bars represent the means ± standard errors of percentages of each strain in the mixed culture in BHI broth at 4 and 24 h after inoculation ($P < 0.001$by two-way ANOVA).

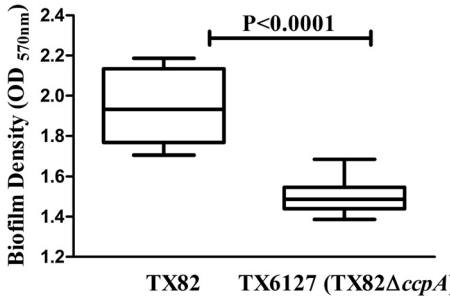

FIG 6 Effect of *ccpA* deletion on biofilm formation by TX82. Biofilm formation by the wild type (TX82) and the *ccpA* deletion mutant (TX6127) was quantitated by measuring the OD_{570} of crystal violet-stained wells. Horizontal lines indicate median OD_{570} values, and interquartile ranges, with the minimum and maximum values marked by whiskers, represent combined data from two independent assays with 8 wells per experiment. Statistical analyses were performed by a Mann-Whitney test.

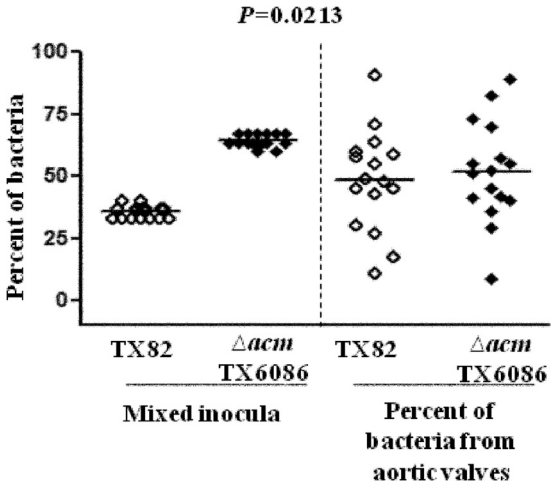

FIG 7 Attenuation of a nonpolar *acm* deletion mutant (TX6086) in a rat endocarditis model. Percentages of wild-type and *acm* deletion mutant (TX6086) bacteria recovered from the initial inoculum (left) and from aortic valves 48 h after infection of 16 rats (right) are shown. Horizontal lines indicate means ($P = 0.0213$ by paired *t* test) for the percentage of total bacteria in the aortic valve versus that in the inoculum for each rat.

the phosphocarrier protein HPr, which is a component of the phosphoenol pyruvate-carbohydrate phosphotransferase system (PTS) (27). This initiates complex formation between CcpA and Hpr (Ser46-P), which binds to CREs (catabolite-responsive elements) of catabolite-responsive genes and either represses or activates gene transcription. The role of CcpA in growth and sugar utilization was previously demonstrated for many low-G+C Gram-positive bacteria, including *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus mutans*, and *Lysinibacillus sphaericus* (28–31). However, its role in *E. faecium* is not yet known. Thus, we sought to determine whether the *ccpA* mutation was responsible for the transient growth delay observed for TX6051 by correcting the mutation in the *ccpA* gene of TX6051 as well as by creating a *ccpA* gene deletion mutant. After the nonsense mutation in *ccpA* was corrected, the transient growth defect observed for TX6051 reverted to normal, and after deletion of the *ccpA* gene, a reduced initial growth rate and smaller colony size were observed. Furthermore, the reduced growth and the smaller colonies caused by the deletion of the *ccpA* gene were completely reversed by chromosomal complementation of the *ccpA* deletion mutant with an intact *ccpA* gene. Thus, the present study demonstrates that CcpA is important for the regulation of growth and colony size in *E. faecium*.

Despite the fact that most of the transcriptional regulatory functions of CcpA are associated with the uptake and utilization of carbohydrates, CcpA is also involved in the regulation of amino acid biosynthesis, sporulation, and toxin production (32–35). CcpA has been linked to biofilm formation in many bacteria, including *S. aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Clostridium perfringens* (36–39). Interestingly, CcpA-dependent regulation has also been demonstrated for the expression of many virulence factors in Gram-positive bacterial pathogens, including *S. aureus*, *Bacillus anthracis*, group A streptococci, and *E. faecalis* (30, 40–43). Moreover, deletion of *ccpA* led to decreased virulence of *S. pneumoniae* in a pneumonia model and reduced colonization of the nasopharynx (41). Similarly, a *ccpA* deletion mutant strain of *B. anthracis* was highly attenuated in a murine model of infection (42). Attenuation of virulence was also observed when the *ccpA* gene of *Streptococcus suis* was deleted (44). Nevertheless, until now, it had not been shown that CcpA played any part in the pathogenesis of enterococcal infections or endocarditis. In the present study, when rats were inoculated intravenously with a mixture of TX82 and the *ccpA* deletion mutant, TX82 was found to have a statistically significant advantage over the *ccpA* deletion mutant with respect to the capacity to infect aortic valves, implicating CcpA as being important for full infectivity in the pathogenesis of endocarditis. This was further confirmed by the increased recovery of a *ccpA*-complemented strain from aortic valves compared to the recovery of *ccpA* deletion mutant strain when a mixture of both strains was used to infect rats. The increase in the percentage of recovered colonies observed in the presence of *ccpA* may be due to the upregulation of expression of certain virulence-associated genes, which in turn might provide enhanced adherence, colonization, and persistence properties to *E. faecium*, as observed for other pathogens (41, 42, 44). Alternatively, CcpA might also favor the increased uptake and utilization of carbohydrates from the surroundings, thereby increasing the metabolic fitness and success of *E. faecium* during the infection process. Thus, in order to determine the effect of the growth defect on the attenuated virulence phenotype exhibited by the *ccpA* deletion mutant, we performed a growth competition assay, and the results suggested that the wild-type strain outcompetes the *ccpA* mutant strain when they are mixed and grown competitively, attributing a main role for the growth defect in the reduced-virulence property. However, biofilm formation, a key factor in the pathogenicity and persistence properties of many microbes, was also found to be reduced in the *ccpA* deletion mutant strain compared to the wild type when these strains were independently grown into a biofilm in microtiter plates. Thus, our results suggest that the attenuation observed with the *ccpA* deletion mutant may be attributable to the pleiotropic regulatory effects of this transcriptional regulator, possibly affecting genes involved in carbon utilization which may affect fitness as well as possibly having an effect on virulence genes, a subject which we are currently pursuing (S. R. Somarajan and B. E. Murray, unpublished data).

As it became evident that CcpA is important in the pathogenesis of infective endocarditis and also contributed to the attenuation of TX6051, we sought to clarify whether Acm actually has a role in endocarditis, in the absence of a *ccpA* mutation. After infecting rats with a mixture of the wild type and the nonpolar markerless *acm* deletion mutant, we found that the *acm* deletion mutant infected valves at a significantly lower level than did wild-type strain TX82, although the level of colonization was not as drastically reduced as the level that we observed in our previous study with the *acm* allelic replacement mutant harboring the *ccpA* mutation (17); nonetheless, the results showed that Acm is important in the pathogenesis of infective endocarditis, which may also account for the presence of anti-Acm antibodies in all patients with *E. faecium* endocarditis (16).

In conclusion, our study indicates that Acm and CcpA contribute independently to the pathogenesis of infective endocarditis. It is not yet clear how CcpA affects the pathogenesis of *E. faecium* infections, and this subject warrants future study.

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