

Library Screen Identifies *Enterococcus faecalis* CcpA, the Catabolite Control Protein A, as an Effector of Ace, a Collagen Adhesion Protein Linked to Virulence

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The *Enterococcus faecalis* cell wall-anchored protein Ace is an important virulence factor involved in cell adhesion and infection. Expression of Ace on the cell surface is affected by many factors, including stage of growth, culture temperature, and environmental components, such as serum, urine, and collagen. However, the mechanisms that regulate or modulate Ace display are not well understood. With interest in identifying genes associated with Ace expression, we utilized a whole-cell enzyme-linked immunosorbent assay (ELISA)-based screening method to identify mutants from a transposon insertion mutant library which exhibited distinct Ace surface expression profiles. We identified a *ccpA* insertion mutant which showed significantly decreased levels of Ace surface expression at early growth phase versus those of wild-type OG1RF. Confirmation of the observation was achieved through flow cytometry and complementation analysis. Compared to the wild type, the *E. faecalis ccpA* mutant had an impaired ability to adhere to collagen when grown to early exponential phase, consistent with the lack of Ace expression in the early growth phase. As a key component of carbon catabolite regulation, CcpA has been previously reported to play a critical role in regulating expression of proteins involved in *E. faecalis* carbohydrate uptake and utilization. Our discovery is the first to associate CcpA with the production of a major *E. faecalis* virulence factor, providing new insights into the regulation of *E. faecalis* pathogenesis.

Enterococcus faecalis, a commensal organism of the gastrointestinal tract, is also known to be an opportunistic pathogen, a major cause of hospital-acquired infections, and a growing public health concern due to its increasing resistance to multiple antibiotics. A group of surface proteins, the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), play an important role in the virulence of *E. faecalis* by encouraging adherence and colonization to host tissue. Among them, Ace (adhesin of collagen from *E. faecalis*) plays an important role in adherence, presumably by mediating binding to extracellular matrix proteins, including both collagen and laminin (1). Ace has significant sequence homology with and a structural organization similar to that of Cna, a known collagen binding virulence factor of *Staphylococcus aureus* (2), and homology to Acm, a collagen adhesion protein in *Enterococcus faecium* (3). The association of Ace expression with virulence has been reported in many publications, including recent reports that an *ace* deletion mutant was significantly attenuated in both a rat endocarditis model and a murine urinary tract infection model, suggesting that Ace is involved in the pathogenesis of *E. faecalis* in both infectious endocarditis and urinary tract infections (4–6).

The surface expression of Ace has been shown to be regulated by many factors, including growth phase, temperature, and medium components, such as serum and collagen. As previously reported, Ace is maximally displayed on the surface of *E. faecalis* OG1RF in exponential phase and decreases to undetectable levels by stationary phase (7, 8). Growth at 46°C also significantly increases Ace expression compared to growth at 37°C; furthermore, reverse transcription-quantitative PCR (qRT-PCR) revealed an

18-fold increase in *ace* mRNA levels in cells grown in the presence of collagen IV compared to levels in the control (9). However, many of the genetic determinants that regulate Ace expression have yet to be evaluated.

Recently, we developed a whole-cell enzyme-linked immunosorbent assay (ELISA)-based library screening method to identify genetic elements that are involved in the display of *E. faecalis* cell surface proteins (8, 10). From a 536-membered *E. faecalis* OG1RF transposon (Tn) insertion mutant library, one mutant, an *fsrB* insertion mutant, was identified with significantly higher Ace expression levels at late growth phase. Further analysis demonstrated that *E. faecalis* mutants with disruption or deletion of *fsrB* or *gelE*, known to be regulated by *fsrB*, demonstrated higher Ace surface expression levels than the wild type and that gelatinase encoded by *gelE* is responsible for cleavage of Ace from the cell wall. These findings revealed one mechanism which *E. faecalis* utilizes to regulate Ace surface display. At high cell density, activation of the *fsr* quorum-sensing system leads to elevated production of gelatinase, which in turn cleaves Ace from the cell surface.

In this study, we utilized the same screening strategy to identify genes which affect Ace expression in early growth phase. An *E.*

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TABLE 1 Strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) | Reference or source |
|------------------------------|---|---------------------|
| Strains | | |
| <i>E. faecalis</i> | | |
| OG1RF | Wild-type strain, Fus ^r Rif ^r p-Cl-Phe ^r | 32 |
| OG1RF Tn library | OG1RF Tn insertion mutants | 13 |
| CK111 | Conjugative donor | 12 |
| Δ ccpA mutant | Deletion of ccpA from OG1RF | This study |
| pMSP/ccpA::Tn917 strain | ccpA::Tn917-containing plasmid pMSP3535-tet | This study |
| pMSP-ccpA/ccpA::Tn917 strain | ccpA::Tn917-containing plasmid pMSP-ccpA | This study |
| TX5467 | OG1RF ace (Δ ace) deletion mutant | 6 |
| JH2-2 | | |
| CL14 | JH2-2 ccpA insertion mutant | 33 |
| <i>E. coli</i> | | |
| XL1-Blue | Cloning host | Stratagene |
| EC1000 | Host for pCJK47 | 12 |
| Plasmids | | |
| pGEM-T Easy | Cloning plasmid | Promega |
| pMSP3535 | Nisin-inducible expression vector, Em ^r | 34 |
| pMSP3535-tet | Nisin-inducible expression vector | This study |
| pMSP-ccpA | pMSP3535-tet carrying a ccpA fragment encoding residues 1–333 | This study |
| pCJK47 | Plasmid for markerless exchange | 12 |
| pMC1 | Deletion of ccpA allele in pCJK47 | This study |

faecalis transposon insertion mutant with disruption of its *ccpA* gene was identified from the library screen with significantly lower levels of Ace surface expression than those in other mutant strains. The *ccpA* gene encodes the catabolite control protein A, which is a key regulator for carbon catabolite repression (CCR) (11). The involvement of *ccpA* in the regulation of Ace expression demonstrates a role for this gene in *E. faecalis* virulence factor surface presentation and, subsequently, the infection process.

MATERIALS AND METHODS

Chemicals. Unless otherwise indicated, all culture media were purchased from Difco and all chemicals were purchased from Sigma (St. Louis, MO). Bacto brain heart infusion (BHI), Bacto tryptic soy broth without glucose (TSB), and Luria broth (LB) were prepared as described by the manufacturer (Becton, Dickinson). Bacto agar was used as a solidifying agent for all semisolid media. Oligonucleotides were purchased from Invitrogen (La Jolla, CA).

Bacteria strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cell cultures were grown at 37°C in LB medium, while *E. faecalis* strains were grown at 37°C in BHI, TSB, or tryptic soy agar. When required, the growth medium was supplemented with antibiotics at the following concentrations: 100 μ g/ml ampicillin, 200 μ g/ml erythromycin, and 10 μ g/ml tetracycline. For culture conditions specific for in-frame deletion mutant construction, refer to the work of Kristich et al. (12).

Whole-cell ELISA library screen. The whole-cell ELISA screen was performed as previously described (10), with some modifications. The Tn library of cells was cultured for 24 h before being diluted 1:10 into new wells containing BHI medium and subsequently cultured at 37°C for 1.5 h. Anti-Ace monoclonal antibody (MAb) 70 at 2 μ g/ml was used to label cells, followed by the addition of 100 μ l of a 1:3,000 dilution of goat anti-mouse IgG-horseradish peroxidase (HRP) (Jackson

TABLE 2 Primers designed and used in this study

| Primer function and name | Sequence (5'–3') |
|--------------------------|----------------------------------|
| <i>ccpA</i> cloning | |
| Forward primer | GCGCGGATCCATAGGAGAAGAAACATGG |
| Reverse primer | CCGGCTCGAGTTATTTTGTGTAACC |
| <i>ccpA</i> deletion | |
| ccpAFlank1F | CGGAATTCGCCATTATCACCATTGATGGC |
| ccpAFlank1R | GCCGTGTCAATGCGCTTAT |
| ccpAFlank2F | CTTTGTGTCATTAGTTTTGTAATAAGCGCATT |
| | GACACGGCGACAACACGAGAAACAGTAGCC |
| ccpAFlank2R | GCTCTAGAGCCGGTTGACTGATGGCCTC |
| ccpAUpF | ATAAGCGCATTGACACGGC |
| ccpADownR | CTGTTTCCTGTTGTCAATGGTA |
| ccpAOuterF | CAAGATGTTCTTTCGGCGTCA |
| ccpAOuterR | GTGTGCCTTGTGTTCTGT |
| <i>ace</i> qRT-PCR set 1 | |
| Forward primer | AACAGCAACGGCGACTCAAC |
| Reverse primer | AACGGATAGTCTCGCTCAATTTG |
| <i>ace</i> qRT-PCR set 2 | |
| Forward primer | GGCAACGAGAACTCCCAAA |
| Reverse primer | ACACCTAAAACGGCAAATGTACTTG |
| <i>gelE</i> qRT-PCR set | |
| Forward primer | CTAATCCAGAAATTGGTGCGGA |
| Reverse primer | AGCCATGGTTTCTGGTTGTCC |
| <i>gyrB</i> qRT-PCR | |
| Forward primer | ACCAACACCGTGCAAGCC |
| Reverse primer | CAAGCCAAAACAGGTCGCC |
| <i>srtA</i> qRT-PCR set | |
| Forward primer | ATTGATGACGTTCTGGTCA |
| Reverse primer | TTTAATAGGCGTCGTTGCTG |

ImmunoResearch). After 1 h of incubation at 37°C, the wells were washed three times with phosphate-buffered saline (PBS)–0.2% Tween 20 (PBST). Tetra-methylbenzidine (TMB) substrate was added, and the mixture was incubated at room temperature for 10 min before the reaction was stopped with the addition of 1 M H₂SO₄. The absorbance of each well was measured at an optical density at 450 nm (OD₄₅₀).

Flow cytometry analysis. Briefly, cells grown in BHI broth were collected in the early log phase of growth, centrifuged at 10,000 \times g, and resuspended to an OD₆₀₀ of 0.5 in PBS-bovine serum albumin (BSA) at 100 μ l in a 1.5-ml Eppendorf tube. Diluted (PBS-BSA), purified anti-Ace MAb 70 at 10 μ g/ml (100 μ l) was added, and the mixture was allowed to stand for 30 min at room temperature. Following centrifugation and washing with 1 ml PBS-BSA, 100 μ l of R-phycoerythrin (PE)-labeled goat F(ab')₂ anti-mouse IgG (Fc) (Jackson ImmunoResearch) was added, and the mixture was incubated for 30 min at room temperature. The bacteria were washed twice with 1 ml PBS-BSA/tube and resuspended in 0.5 ml PBS-BSA, followed by 0.5 ml 2% paraformaldehyde/tube in PBS. Fluorescence analysis was carried out with a Becton, Dickinson FACSCalibur flow cytometer.

Complementation of *E. faecalis* *ccpA* mutant. To create the pMSP3535-tet vector, the *tetM* gene from HH22 was amplified by PCR using the primer pair AB262/AB263, shown in Table 2. The resulting 2,594-bp fragment was subcloned into pGEM-T Easy (Promega), digested using BglII, and ligated with pMSP3535 digested with BglII to yield the plasmid pMSP3535-tet. A 1,017-bp fragment containing the *E. faecalis* *ccpA* gene and its ribosome binding site was PCR amplified from *E. faecalis* OG1RF (primers are listed in Table 2) and cloned under the control of the nisin promoter of the shuttle vector pMSP3535-tet. The resulting plasmid, pMSP-ccpA, was transformed into the *ccpA* insertion mutant by

electroporation. Nisin was added to the culture medium to induce exogenous expression of the *ccpA* gene. The cells were cultured in BHI with 25 ng/ml nisin overnight before being diluted to an OD of 0.1 in the same medium and subsequently cultured at 37°C for 1 h and harvested for flow cytometry analysis using anti-Ace MAb 70.

Construction of the *ccpA* in-frame deletion mutant. The primer pairs *ccpA*Flank1F/*ccpA*Flank1FR and *ccpA*Flank2F/*ccpA*Flank2FR were used to amplify the sequences upstream and downstream of the *ccpA* gene. The forward primer of flank 1 was designed to incorporate an EcoRI site, and the reverse primer of flank 2 was designed to incorporate an XbaI site. These two amplicons were joined by crossover PCR, resulting in a 5' EcoRI site and 3' XbaI site, which were used for insertion into the recipient pCJ47 vector to generate pMC1. The plasmid pMC1 was then introduced into *E. faecalis* CKIII by electroporation and then conjugated into *E. faecalis* OG1RF. Allelic exchange was performed as described by Kristich et al. (12).

The mutant resulted in an in-frame deletion of nucleotides 57 to 916 out of a total open reading frame of 1,059 nucleotides. The mutant was verified using two sets of primers (Table 2) outside the deleted region.

Quantitative real-time PCR. Cells grown to an OD₆₀₀ equivalent to ~1 in BHI were collected at early log phase and mid-log phase for RNA extraction. Total RNA was prepared using a NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA) and reverse transcribed to cDNA using SuperScript II reverse transcriptase and random primers (Invitrogen, CA) according to the method provided by the manufacturer. Quantitative PCR of cDNA was performed using a SYBR green PCR master mix kit and an ABI 7900 real-time PCR system (Applied Biosystems). The expression of *ace* and the reference gene *gyrB* were analyzed using primer pairs listed in Table 2. For each primer set, a reference curve was established using the genomic DNA purified from wild-type OG1RF cells. The amount obtained for *ace* transcripts was normalized with the amount of *gyrB* transcripts.

Gelatinase plate assay. Briefly, gelatinase activity analysis was performed by inoculating overnight-cultured *E. faecalis* strains on Todd-Hewitt agar plates containing 3% gelatin, and the strains were grown overnight at 37°C. The gelatinase activities of different strains were determined by their halo formation.

Cell fractionation and Western blotting. Cell wall-associated proteins were prepared and cell lysates and culture media were generated by following previously published protocols, with some modification. Briefly, OG1RF and Δ *ccpA* cells grown in BHI broth were collected in exponential phase (OD, 0.4) and stationary phase (OD, 1.8) by centrifugation at 10,000 × g for 5 min. Culture medium supernatants were collected, concentrated to a 1/10 volume using a YM-10 spin column, and subjected to SDS-PAGE analysis. Two OD₆₀₀ units of cells were washed twice with 20 mM Tris-HCl, 10 mM MgCl₂, 0.5 M sucrose (pH 7.0) (TMS) buffer and incubated in 100 μl of the same buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF)-EDTA-free Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and 10 U mutanolysin at 37°C for 1 h. After centrifugation at 12,000 × g for 10 min, the supernatant cell wall extracts were collected for SDS-PAGE analysis. The protoplasts were resuspended in 100 μl of lysis buffer (20 mM Tris-HCl [pH 8.0], 200 mM NaCl, 1 mM EDTA, 1× protease inhibitor cocktail [Roche]) and mixed with 50 μl 0.1-mm-diameter zirconia beads (BioSpec Products, Bartlesville, OK), and the mixture was disrupted using a mini-beadbeater for 1 min. The cell lysates were centrifuged at 12,000 × g for 30 min to remove cell debris. The supernatants (cell lysate) were collected for SDS-PAGE analysis. The samples were loaded on 4% to 12% NuPAGE Novex Bis-Tris gels (Invitrogen) under reducing conditions in MOPS (morpholinepropanesulfonic acid) buffer and transferred to an Immobilon-P PVDF membrane (Millipore, Billerica, MA) according to the manufacturer's protocol. Membranes were then probed with anti-Ace MAb 70 (8) followed by IR800-conjugated goat anti-mouse IgG antibody (LI-COR Biosciences, Lincoln, NE), and DNA was detected using the LI-COR Odyssey scanner (LI-COR Biosciences).

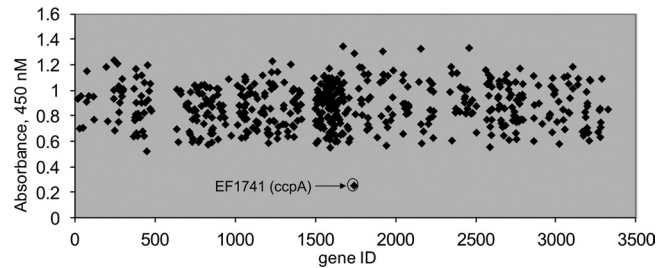


FIG 1 Identification of genes involved in the surface expression of Ace at early growth phase. The 540 Tn917 insertion mutants of OG1RF in the Tn library are represented by the gene identification (gene ID) number (EF number) on the x axis. Relative whole-cell ELISA signals are represented on the y axis. An early-growth-phase culture of each mutant was labeled with anti-Ace MAb and then with a goat anti-mouse IgG-HRP conjugate and developed with the TMB substrate. Absorbance values were determined by using a Thermo Multiskan EX plate reader.

Collagen adhesion assay. A collagen adhesion assay was carried out as previously described (8), with some modification. Briefly, Microton 600 ELISA plates were coated with human collagen IV (Sigma) at 10 μg/ml in PBS overnight at 4°C. The ELISA plates were blocked with 200 μl PBS-BSA/well. The overnight cultures of OG1RF, Δ *ccpA*, *ccpA*::Tn917, and Δ *ace* cells were diluted into 125-ml centrifuge tubes containing 20 ml BHI broth and incubated for 1 or 4 h at 37°C. Each cell strain at a density equivalent to an OD₆₀₀ of 1 was resuspended in 1 ml of PBS-BSA, and 100 μl/well was added to the collagen-coated plates for 1 h at room temperature under static conditions. The wells were washed, and the remaining attached bacterial cells were fixed with Bouin's solution. After additional washes, a 1% solution of crystal violet was applied and removed by using water flushes. The absorption of the solubilized (80:20, ethanol-acetone) crystal violet-stained cells was determined with a Multiskan EX plate reader with a 595-nm filter.

RESULTS

A library screen identified a gene which regulates Ace surface display in early growth phase. Based on the characteristic surface expression profile of Ace in wild-type OG1RF cells, namely, high levels of Ace at a low cell density (early growth phase), with the cell density significantly decreasing in later phases of growth, screening for mutants affecting Ace surface expression can be used to detect increases as well as decreases of Ace surface expression. We have previously reported that screening stationary-phase-grown *E. faecalis* cells identified *fsrB* as a gene negatively associated with Ace surface display (8). In this study, we applied a similar strategy to an *E. faecalis* OG1RF insertion library with 536 unique gene disruption mutants (13) to identify factors that influence Ace surface display during exponential phase (1.5 h). As shown in Fig. 1, the ELISA readouts for the vast majority of the mutants were between OD_{450s} of 0.6 and 1.2, indicating a high level of Ace surface expression on cells cultured to exponential phase. The only mutant in this library that exhibited a significantly lower ELISA reading was a *ccpA* (EF_1741) insertion mutant with an OD₄₅₀ of 0.26. The cell densities of all mutants grown under these conditions were determined, with no significant growth variability observed. These results indicated that disruption of the *ccpA* gene in this insertion (*ccpA*::Tn917) mutant altered the Ace surface expression profile.

***ccpA* is required for Ace surface expression.** To confirm the whole-cell ELISA results, we analyzed the growth and Ace expression profile of the *ccpA* insertion mutant. Compared to the wild

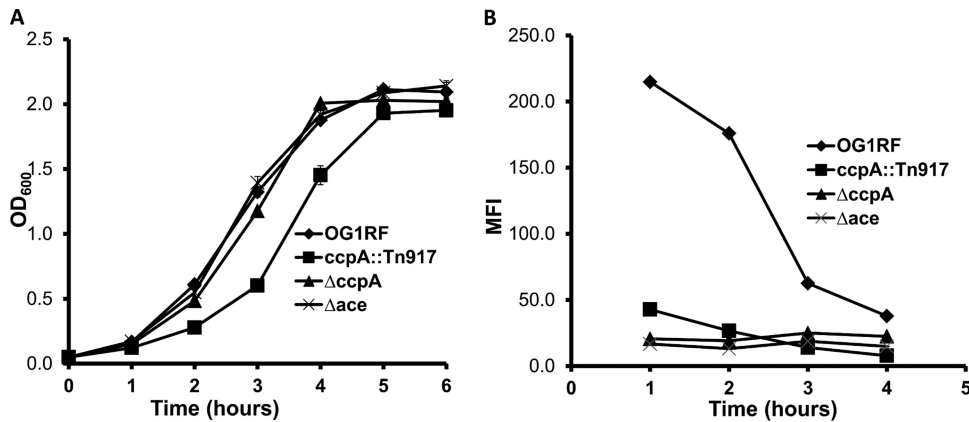


FIG 2 Growth and Ace surface expression of wild-type OG1RF and *ccpA* mutants. (A) Growth curves of *E. faecalis* OG1RF, the *ccpA::Tn917* strain, the $\Delta ccpA$ mutant, and the Δace mutant were determined by measuring OD₆₀₀ at various time points. Cells were grown in BHI broth medium at a starting OD₆₀₀ of 0.05. (B) Levels of the bacterial-surface Ace expression of OG1RF, the *ccpA::Tn917* strain, the $\Delta ccpA$ mutant, and the Δace mutant were determined via flow cytometry at various time points using anti-Ace MAb 70 as the primary antibody and monitoring the mean fluorescence intensity of the population using R-phycoerythrin (PE)-labeled goat F(ab')₂ anti-mouse IgG (Fc). Plotted data are representative of 3 independent experiments.

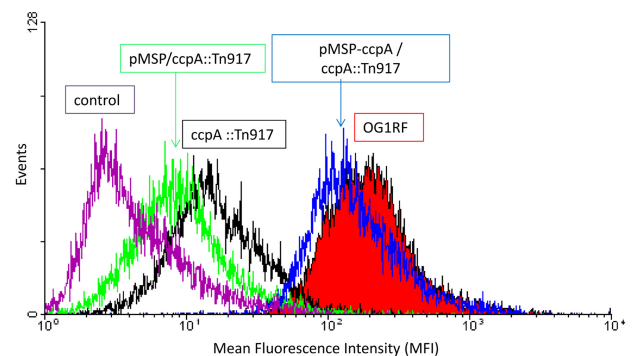
type or an *ace* deletion mutant, the *ccpA::Tn917* strain has a slightly longer lag time to exponential phase but has ODs equal to those of the wild type and *ace* deletion mutant by 4 h of growth (Fig. 2A). Since Ace surface display is higher for cells at low cell density than at high cell density due to the absence of gelatinase cleavage early on, this lower growth rate of the *ccpA::Tn917* mutant than that of wild-type cells cultured for same period in theory might be predicted to positively affect surface Ace. However, the *ccpA::Tn917* strain exhibited an expression profile similar to that of the *ace* deletion mutant throughout all growth phases by flow cytometry analysis (Fig. 2B). In comparison, wild-type strain OG1RF demonstrated much higher levels of Ace expression at early time points (mean fluorescent intensities [MFIs] of 215 and 176 at 1 and 2 h of culture, compared to MFIs of 43 and 27 for the *ccpA::Tn917* strain collected at the same time points). These results clearly demonstrate that insertion of the transposon in the *ccpA* gene altered Ace surface display.

To confirm that the *ccpA* gene is associated with *E. faecalis* expression of Ace, we genetically complemented the *ccpA::Tn917* strain using the plasmid pMSP-*accA*, a nisin-controlled expression vector carrying the *E. faecalis* *ccpA* gene. As shown in Fig. 3, the complemented pMSP-*ccpA/ccpA::Tn917* strain exhibited an Ace expression phenotype that resembled that of wild-type OG1RF (MFI, 225 versus 264 by flow cytometry), which was significantly higher than that of the *ccpA::Tn917* mutant strain alone or the *ccpA::Tn917* mutant with the empty vector (MFIs, 23 and 16, respectively). This result demonstrated that *ccpA* is required for *E. faecalis* Ace surface expression in early growth phase.

To ensure that the effects of the transposon insertion mutant were indeed due to loss of *ccpA* and not due to polar effects on surrounding genes and to further confirm the effect of *ccpA* on Ace expression, an in-frame deletion of *ccpA* was generated in the wild-type OG1RF strain ($\Delta ccpA$ mutant). As demonstrated in Fig. 2A, the $\Delta ccpA$ strain shows a growth profile similar to that of wild-type OG1RF. In addition, its Ace surface expression resembles that of the insertion mutant, which is consistently low in all growth phases. As shown in Fig. 2B, the Ace expression level of the $\Delta ccpA$ strain in early growth (1 h of culture) is about 13-fold lower than that of the wild-type strain (MFI, 215 versus 16).

Ace surface expression is glucose independent. Since *ccpA* plays an important role in carbon catabolite repression (CCR), we evaluated whether Ace expression is regulated through a CCR-mediated mechanism. To test this possibility, we studied whether different carbon sources affected Ace expression. As shown in Table 3, similar levels of Ace surface expression were detected by flow cytometry on wild-type OG1RF cells grown in TSB without sugar (MFI, 314.97), 0.5% glucose (phosphoenolpyruvate-sugar-phosphotransferase system [PTS] sugar) (MFI, 291.62), and 0.5% galactose (non-PTS) (14) (MFI, 258.4). In comparison, Ace surface expression levels on both *ccpA* mutants are consistently lower in all three media. All together, these results suggest that the regulatory effect of CcpA on Ace production may be through a CCR-independent mechanism.

The regulation on Ace expression by *ccpA* is not at the transcriptional level. CcpA has a well-defined function as a transcriptional regulator via binding to target DNA at catabolite-responsive element (CRE) sites to alter gene transcription (15). To test



| | OG1RF | <i>ccpA::Tn917</i> | pMSP/ <i>ccpA::Tn917</i> | pMSP- <i>ccpA</i> / <i>ccpA::Tn917</i> | (-) control |
|-----|--------|--------------------|--------------------------|---|-------------|
| MFI | 264.24 | 23.35 | 16.39 | 224.78 | 8.14 |

FIG 3 Complementation of *ccpA* restores Ace surface display. The nisin-inducible vector pMSP3535-tet alone and with a *ccpA* gene insertion were introduced into the *ccpA::Tn917* mutant strain, and cells were analyzed for Ace expression using flow cytometry. Plotted data are representative of 3 independent experiments.

TABLE 3 Ace surface expression with different carbon sources^a

| Strain | Medium | MFI |
|-----------------------------|-----------|--------|
| OG1RF | TSB | 314.97 |
| | TSB + Glu | 291.62 |
| | TSB + Gal | 258.4 |
| <i>ccpA</i> ::Tn917 strain | TSB | 39.88 |
| | TSB + Glu | 53.55 |
| | TSB + Gal | 49.21 |
| Δ <i>ccpA</i> mutant | TSB | 44.28 |
| | TSB + Glu | 43.4 |
| | TSB + Gal | 44.69 |

^a Bacterial surface Ace expression was determined via flow cytometry using 1-h-cultured cells grown in various media at a starting OD₆₀₀ of 0.05.

whether loss of *ccpA* results in regulation of Ace expression at the transcriptional level, we utilized qRT-PCR to quantitatively determine *ace* mRNA levels in wild-type and Δ *ccpA* strains. Two sets of primers targeting each end of *ace* mRNA were chosen to ensure detection of the full-length mRNA product. As shown in Fig. 4, no significant reduction of *ace* mRNA levels in the *ccpA* deletion mutant can be observed using either primer pair at analyzed time points. In fact, we observed the *ace* mRNA levels in the Δ *ccpA* mutant to be about 1-fold higher than those of the wild-type strain in early-stage culture (1 h). Nonetheless, these results indicate that the phenotype of decreased Ace expression in the Δ *ccpA* mutant is not due to regulation at the transcriptional level.

As an LPXTG motif cell wall anchor protein, Ace requires the function of sortases for surface display, specifically, for attachment to peptidoglycan (16). It has been shown that OG1RF contains only one class A sortase, which is responsible for Ace anchoring to the cell wall (17). To study whether loss of *ccpA* affects Ace display through the function of sortase A, we compared the *srtA* mRNA levels of the wild-type and Δ *ccpA* strains using qRT-PCR analysis. As shown in Fig. 4, no significant change was observed in the mRNA levels, suggesting that CcpA does not affect *srtA* transcription.

CcpA affects Ace protein production. Since Ace RNA levels are not reduced in the *ccpA* deletion mutant, the regulation of Ace surface expression may occur at the translational level or during posttranslational events, including secretion, cell wall anchoring, or protease digestion. In our previous study of Ace surface expression (8), we demonstrated that Ace surface display is associated with the FSR quorum-sensing system through gelatinase, which functions as an extracellular protease to cleave the cell wall-anchored Ace from the enterococcal cell surface. Since deletion of *ccpA* does not affect Ace expression at the transcriptional level, it is possible that it alters Ace surface display through regulation of *gelE* expression. To test this hypothesis, we first compared the mRNA levels of *gelE* in the wild-type and Δ *ccpA* strains. Although the Δ *ccpA* strain showed higher levels of *gelE* mRNA than OG1RF at the late growth phase (4 h of culture), this still could not explain the Ace-deficient phenotype observed in the Δ *ccpA* mutant at the early growth phase since no significant difference was observed in *gelE* mRNA levels in the cells cultured to early growth phase (Fig. 4). At the protein level, both the wild-type and Δ *ccpA* strains are gelatinase positive, as demonstrated by a gelatinase plate assay whose results are shown in Fig. 5A, suggesting that *gelE* expression is not affected by a mutation in *ccpA* in OG1RF.

To further confirm whether gelatinase plays a role in *ccpA*-dependent Ace expression, we analyzed the Ace expression of a *ccpA* mutant, CL14 (generously provided by Axel Hartke), derived from an *fsr*-deficient strain, JH2-2 (18, 19); this deficiency results in attenuated gelatinase production. As demonstrated by the gelatinase plate assay whose results are shown in Fig. 5A, both JH2-2 and CL14 are gelatinase negative. The Ace surface expression levels during the early growth phase of JH2-2 and CL14 were compared, as shown in Fig. 5B. Like OG1RF and the *ccpA*::Tn917 mutant, the *ccpA* mutant strain of JH2-2 (CL14) showed significantly decreased Ace surface expression compared to that of JH2-2 (MFI, 19.49 versus 143.93), suggesting that gelatinase is indeed not involved in the *ccpA*-dependent Ace regulation process.

To test if CcpA affects Ace secretion or cell wall anchoring, we further explored the presence of Ace in protoplast lysates, mutanolysin-treated cell wall extracts, and conditioned culture media collected at the exponential and stationary phases of growth of OG1RF and the Δ *ccpA* mutant using SDS-PAGE and anti-Ace MA b 70 probes (8). High-molecular-weight Ace bands were detected in the cytoplasmic and cell wall extracts of exponential-phase OG1RF cells (Fig. 6A, lanes 2 and 4), consistent with our previous flow cytometry results showing that Ace is readily detected on OG1RF cells grown to exponential phase. In comparison, no high-molecular-weight Ace band was detected in the cytoplasmic or cell wall extracts of exponential-phase Δ *ccpA* cells (Fig. 6A, lanes 3 and 5), suggesting less Ace production. Ace degradation products (around 37 kDa) were also detected in the cell wall fractions, which may result from protease cleavage of Ace during mutanolysin processing, and is consistent with previously published results (16). Although Ace degradation products were seen in both Δ *ccpA* and OG1RF cell wall extracts, it was more abundant in the OG1RF extracts, representing OG1RF's higher level of Ace surface expression. In the stationary phase, intact Ace is still detected in the cytoplasm of OG1RF but not in the Δ *ccpA* mutant (Fig. 6B, lanes 2 and 3). Minimal levels of Ace can be detected in the cell wall extracts of either OG1RF or the Δ *ccpA*

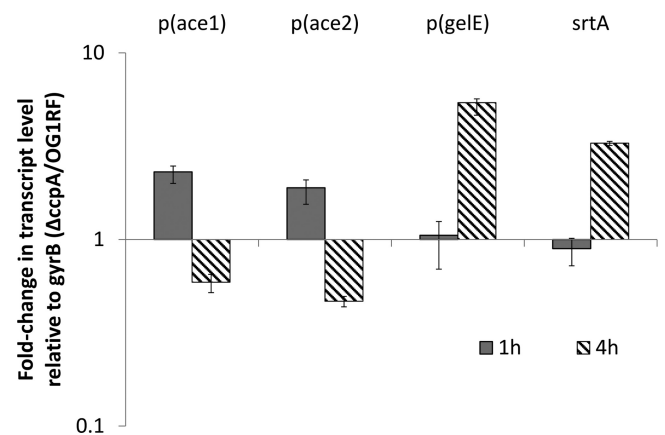


FIG 4 Comparison of Ace and GelE mRNA levels in wild-type OG1RF and the Δ *ccpA* mutant by qRT-PCR analysis. RNA was isolated from the early-log and early-stationary-phase-cultured wild-type and Δ *ccpA* cells. Two sets of *ace* primers were used to target both the 5' and 3' ends of *ace*. The fold change in gene expression corresponds to the ratio of the transcript level of the *ace* gene in the Δ *ccpA* strain to that of the wild type. The level of the *ace* transcript was determined in triplicate and normalized using *gyrB* transcript levels. Data shown here are averaged from three independent experiments.

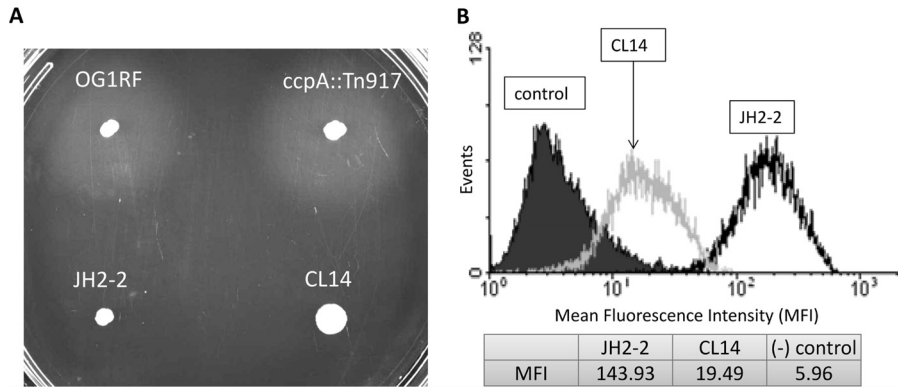


FIG 5 Gelatinase production and Ace expression of JH2-2 and the corresponding *ccpA* mutant. (A) The GelE activities of *E. faecalis* OG1RF, OG1RF *ccpA*::Tn917, JH2-2, and CL14 were determined by a gelatinase plate assay. The gelatinase activities of different strains were determined by assessing halo formation. (B) Surface Ace expression of *E. faecalis* JH2-2 and its *ccpA* insertion mutant CL14 was determined by flow cytometry after labeling Ace with anti-Ace MAb 70 primary antibody and R-phycoerythrin (PE)-labeled goat F(ab')₂ anti-mouse IgG (Fc) secondary antibody using 1-h-cultured cells grown in BHI at a starting OD₆₀₀ of 0.05. The control population represents JH2-2 culture labeled with secondary antibody alone. Plotted data are representative of 3 independent experiments.

mutant, consistent with the result of our flow cytometry, in which very little Ace was detected on stationary-phase cells. Cleaved products of Ace were detected in the culture media of both OG1RF and the Δ *ccpA* mutant, and again, they were more abundant in OG1RF. Altogether, less Ace is detected in all fractions of the Δ *ccpA* mutant than in OG1RF, suggesting that Ace protein production is lower in the Δ *ccpA* mutant than in OG1RF.

Loss of *ccpA* impacts the ability of *E. faecalis* to adhere to collagen. Since loss of Ace has been shown to decrease cellular adhesion to collagen, we evaluated the direct effect of the *ccpA* mutants on collagen adhesion. Compared to the wild type, the *ccpA* deletion mutant, the insertion mutant, and an *ace* deletion mutant all demonstrated significant reductions of collagen adhesion when cells were evaluated in early exponential phase (1 h) (Fig. 7). This is as expected, since only at early growth phase do wild-type OG1RF cells have high levels of Ace on their surfaces. In the late growth phase, all the tested strains showed reduced adhesion due to the absence of Ace on the surface (Fig. 7).

DISCUSSION

Carbon catabolite repression (CCR) is a common negative regulatory mechanism that bacteria utilize to inhibit catabolite-repres-

sive genes in the presence of a rapidly metabolized carbon source (20). In Gram-positive bacteria, such as *Bacillus subtilis*, the repression of gene transcription is mediated through the interaction of three components: CcpA of the LacI/GalR family of transcriptional regulators, the phosphocarrier HPr protein of the phosphoenolpyruvate-sugar-phosphotransferase system (PTS), and the CRE site of the target genes (21). In the presence of glucose, HPr kinase is activated, resulting in the phosphorylation of HPr at Ser46 [HPr(Ser46-P)]. The complex of CcpA and HPr(Ser46-P) is then formed and binds to CREs of catabolite-sensitive operons to block gene transcription. The majority of the catabolite-sensitive operons are systems involved in the uptake and utilization of alternative carbohydrates. As an example, the expression of the *B. subtilis* gluconate operon (*gnt*) is repressed upon association of the CcpA-HPr complex with the CRE site located far downstream of the *gnt* transcription initiation site (22).

Despite the fact that CcpA is a key component of the CCR machinery, its role is not limited to repression of catabolite-repressive genes. In fact, CcpA, as a global transcriptional regulator, acts as both a repressor and an activator. Genes are activated by CcpA, including *ackA* and *pta* for the formation of acetate (23)

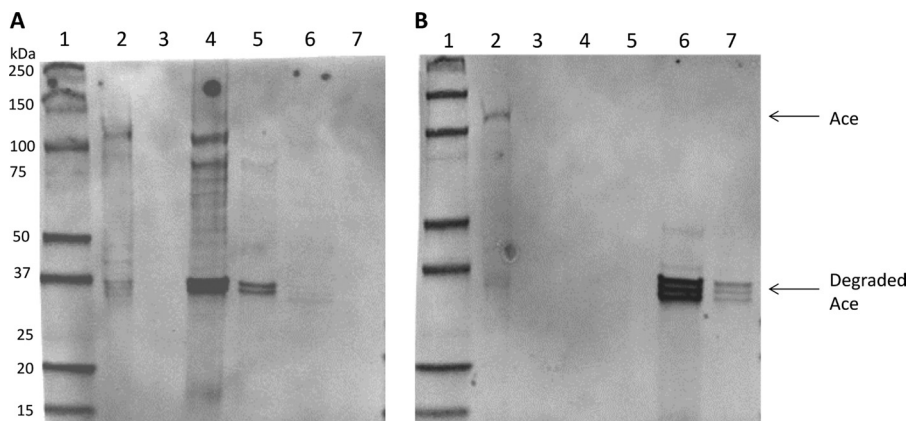


FIG 6 Comparison of Ace protein production levels by *E. faecalis* OG1RF and Δ *ccpA*. Fractions of wild-type OG1RF and Δ *ccpA* mutant cells were prepared at exponential phase (A) or stationary phase (B) and analyzed by Western blotting using anti-Ace MAb 70. Lanes: 1, prestained protein markers; 2, OG1RF cytosolic lysates; 3, Δ *ccpA* cytosolic lysates; 4, OG1RF cell wall extracts; 5, Δ *ccpA* cell wall extracts; 6, OG1RF culture medium; 7, Δ *ccpA* cell culture medium.

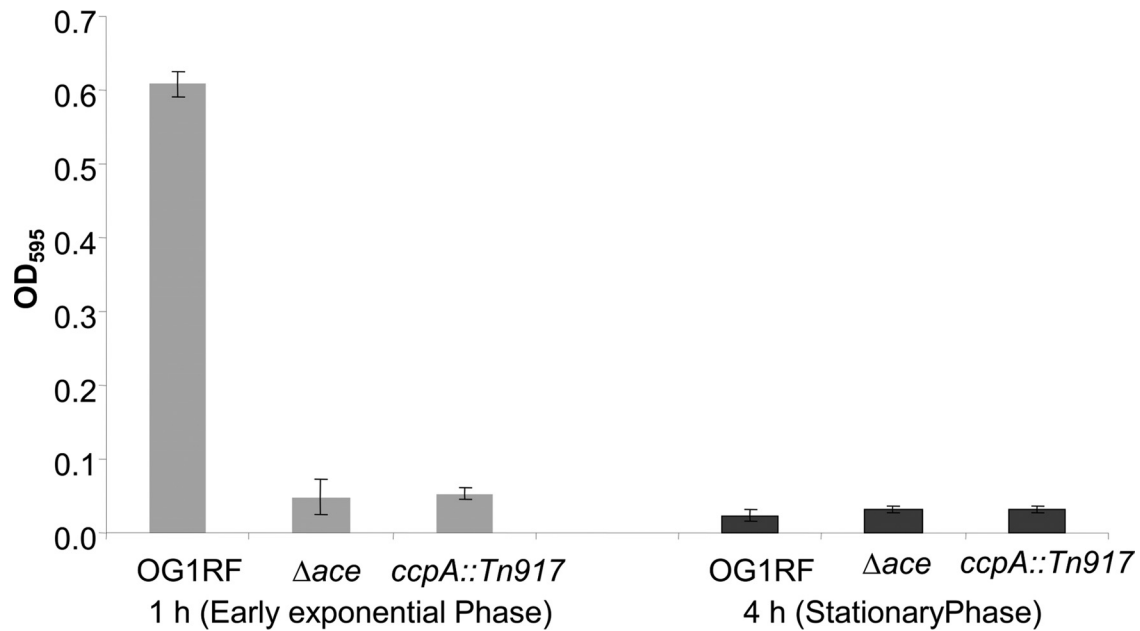


FIG 7 Collagen adhesion of *E. faecalis* OG1RF and mutant strains at different growth phases. Net adherence values were determined ($A_{\text{Net}} = A_{\text{collagen}} - A_{\text{PBS}}$). Adhesion and standard deviation calculations were averaged from triplicate samples. The data provided are from one of three representative experiments.

and the *ilv-leu* operon for amino acid biosynthesis (24), suggesting the involvement of CcpA in anabolism as well. Moreover, in *S. aureus*, a large amount of CcpA-dependent gene regulation occurs in the absence of glucose, suggesting a glucose-independent CcpA regulatory mechanism (25).

Recently, the involvement of CcpA in bacterial virulence has been unveiled, as several reports directly associate CcpA with expression of virulence factors in *S. aureus*, group A streptococci (GAS), and other human pathogens (26–30). In *S. aureus*, it has been demonstrated that *ccpA* regulates the expression of a number of virulence factors, including the *agr* quorum-sensing system, protein A, and alpha-hemolysin (26). In GAS, CcpA is reported to influence the transcription of several well-characterized virulence factors, including cytolysin, streptolysin S, and a number of other virulence-related genes (30). An *in vitro* binding assay demonstrated binding between recombinant GAS CcpA and the streptolysin S-encoding gene *pel* (*sagA*) promoter region (27), suggesting the direct involvement of CcpA in streptolysin S transcriptional regulation. All these new discoveries suggest another important functional role of CcpA as a modulator of the expression of virulence-related genes as well, although mechanisms involved in these regulation processes are still largely unknown.

In *E. faecalis*, the role of CcpA in regulating the expression of catabolite-repressive and starvation-inducible proteins has been documented (18, 31). Our discovery in this report demonstrates for the first time a direct link between *E. faecalis* *ccpA* and virulence factor production. Although the mechanism by which *ccpA* regulates Ace surface expression is still not clear, we have demonstrated that the Ace mRNA level is not decreased in the *ccpA* mutant compared to that of the wild type, indicating that the regulation of Ace expression does not occur at the transcriptional level. Western blot analysis demonstrated that Ace protein is less in the *ccpA* mutant than in the wild type in all fractions, including cytosolic lysate, cell wall extracts, and culture media, suggesting

that the loss of *ccpA* affects Ace protein production rather than secretion or cell wall anchoring. In addition, our results using the gelatinase-negative strain CL14 demonstrated that the decrease of Ace expression in the *ccpA* mutants is not associated with the function of gelatinase. Furthermore, the presence of various carbon sources in the culture media did not significantly change Ace surface display, suggesting that the regulatory effect of CcpA on Ace production is not through a CCR-mediated mechanism.

Our library screen method once again (8, 10) proved to be of great value in studying genetic determinants involved in bacterial surface protein expression, since it can detect changes that occur at any level from transcription to surface anchoring. Since Ace mRNA levels are not affected by deletion of *ccpA*, the link between these two factors would not have been discovered through traditional genetic or transcriptome analysis. Further application of this screening strategy will yield enhanced understanding of the regulation of protein production in the postgenomic era.

In conclusion, our study demonstrated that *ccpA* is required for Ace surface display in *E. faecalis*. Disruption of the *ccpA* gene leads to significant decreases of Ace expression at early growth phase, which in turn diminished the ability of the mutant strains to adhere to collagen. Our discovery that *ccpA* is required for the surface expression of a major *E. faecalis* virulence factor demonstrates a role for *ccpA* in the regulation of enterococcal pathogenesis.

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