The Fsr Quorum-Sensing System of *Enterococcus faecalis* Modulates Surface Display of the Collagen-Binding MSCRAMM Ace through Regulation of *gelE*[▽]

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Ace, a known virulence factor and the first identified microbial surface component recognizing adhesive matrix molecule (MSCRAMM) of *Enterococcus faecalis* is associated with host cell adherence and endocarditis. The Fsr quorum-sensing system of *E. faecalis*, a two-component signal transduction system, has also been repeatedly linked to virulence in *E. faecalis*, due in part to the transcriptional induction of an extracellular metalloprotease, gelatinase (GelE). In this study, we discovered that disruption of the Fsr pathway significantly increased the levels of Ace on the cell surface in the latter phases of growth. Furthermore, we observed that, in addition to *fsrB* mutants, other strains identified as deficient in GelE activity also demonstrated a similar phenotype. Additional experiments demonstrated the GelE-dependent cleavage of Ace from the surface of *E. faecalis*, confirming that GelE specifically reduces Ace cell surface display. In addition, disruption of the Fsr system or GelE expression significantly improved the ability of *E. faecalis* to adhere to collagen, which is consistent with higher levels of Ace on the *E. faecalis* surface. These results demonstrate that the display of Ace is mediated by quorum sensing through the action of GelE, providing insight into the complicated world of Gram-positive pathogen adhesion and colonization.

Enterococcus faecalis, a normal commensal organism of the gastrointestinal tract, is also known to be an opportunistic pathogen and a cause of hospital-acquired infections. Enterococci are one of the three major contributors to nosocomial infections in U.S. hospitals and account for as many as 20% of infectious endocarditis cases (17, 27). Pathogenesis in enterococci is associated with the ability to express surface proteins which enable colonization and biofilm formation in its human host. With antibiotic resistance on the rise, immunotherapy to target these surface antigens is strongly being considered as an alternative approach. Understanding surface antigen expression and regulation is a critical first step in the development of the next generation of strategies toward effective therapy and prevention.

A major family of bacterial surface proteins being considered as immunotherapy targets are the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) (30). These extracellular matrix adhesion proteins play an important role in Gram-positive bacterial virulence by mediating adherence and colonization to host tissues which is an early step toward clinical infection (33). Ace (adhesin to collagen of

E. faecalis) was the first MSCRAMM discovered in enterococci (39). Ace consists of a ligand binding A domain important for interaction with collagen and laminin, a section of repeats that defines the B domain, and a C-terminal cell-wall anchoring region, containing an LPXTG-like motif for covalent attachment to the peptidoglycan. Ace shares structural organization and significant sequence similarity with other Gram-positive MSCRAMMs, including Cna, a known collagen binding virulence factor of *Staphylococcus aureus* (34), and Acm, a collagen adhesion protein in *Enterococcus faecium* (26). As with Cna and Acm, the presence of Ace on the cell surface correlates with the ability of the bacteria to adhere to collagen (24, 25, 49). It has also been demonstrated that Ace is commonly expressed in human infection (25) and has been closely associated with *E. faecalis* virulence. Isogenic strains with *ace* deletions were recently shown to be significantly attenuated compared to the wild type in both a murine urinary tract infection model (13) and an experimental endocarditis infection model (45).

Regulation of Ace surface display is of broad interest due to a defined role of Ace in virulence and the potential role for Ace as a target for vaccination or passive protection. Early investigations demonstrated that growth at elevated temperatures (46°C) improved the ability of *E. faecalis* to adhere to collagens (51). This was later shown to be specifically associated with increased levels of Ace on the bacterial cell surface (8, 24, 25). In addition, studies have demonstrated that *ace* is transcriptionally upregulated upon exposure of cells to serum, urine, and collagen (8, 23, 24, 41), showing that various envi-

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ronmental factors affect *ace* expression. Finally, and of particular interest to our investigation, it was shown that Ace demonstrated a growth-phase-dependent profile in which surface display is prominent during early exponential and exponential phase and yet inexplicably diminished as cells reached the stationary phase (8). Despite these efforts, however, the mechanisms that regulate or modulate Ace display are not well understood.

Previously, we developed a whole-cell enzyme-linked immunosorbent assay (ELISA)-based library screening strategy to identify genes that are involved in *E. faecalis* surface protein expression (5). In the present study we use this powerful tool to explore the mechanism behind the growth-phase-dependent profile of Ace on the cell surface. Interestingly, we identified a mutant of the Fsr quorum-sensing system of *E. faecalis* which retains Ace on its surface in late growth phase. We further demonstrated that Fsr mediates Ace surface levels through its regulation of the metalloprotease gelatinase (GelE), which we showed directly cleaves Ace, subsequently impacting the ability of cells to adhere to collagen. Both Ace and the Fsr quorum-sensing pathway of *E. faecalis* have been extensively studied for their roles in enterococcal virulence and for their occurrence in clinical isolates (4, 11, 13, 35, 45, 52). Our discovery demonstrates a link between these two independently important factors and builds a more complete understanding of the complexities of the mechanisms regulating adherence and colonization.

MATERIALS AND METHODS

Chemicals. Unless otherwise indicated, all culture media were purchased from Difco Laboratories (Detroit, MI), and all chemicals were purchased from Sigma (St. Louis, MO). Brain heart infusion broth (BHI) was prepared as described by the manufacturer. Oligonucleotides were purchased from Invitrogen (La Jolla, CA).

Bacterial strains. The *E. faecalis* strains utilized in the present study included OG1RF (21), TX5467 (OG1RF Δace) (45), TX5266 (OG1RF $\Delta fsrB$) (37), TX5264 (OG1RF $\Delta gelE$) (43), TX5471 (OG1RF $\Delta gelE/\Delta sprE$) (the present study), TX5243 (OG1RF *sprE* insertion mutant [$\Delta sprE$]) (38), V583 (40), JH2-2 (10), HH22 (19), TX0104 (sequenced strain derived from enterococcal endocarditis patient), 11700 (American Type Culture Collection [ATCC]), 51188 (ATCC), and OG1RF transposon (Tn) library (6).

Development of antibodies. Recombinant Ace domain A (rAce; amino acids 32 to 380) was amplified by PCR from OG1RF by using the primers AceDI1BamHIFor (5'-GCAGGATCCGAGTTGAGCAAAAGTTCATCG-3') and AceDI1KpnRev (5'-CTCGGTACCTTATCAGTCTGTCTTTTCACTTGTTCTG-3') and ligated into pQE30 (Qiagen, Inc.) using BamHI and KpnI recognition sites. Upon transformation into M15 *Escherichia coli* harboring pREP4 (Qiagen, Inc.), rAce was expressed and purified as previously described (39). To generate monoclonal antibodies (MAbs) against Ace, BALB/c mice were immunized with the rAce protein according to standard techniques (21). The splenocytes were collected and fused with SP2/O mouse myeloma cells as previously described (12). MAbs from single-cell clones were evaluated for binding specificity to rAce and native surface displayed antigen via ELISAs and whole-cell ELISAs, respectively (8, 36), followed by kinetic screening for the highest affinity clones using surface plasmon resonance as previously described (3). Polyclonal anti-GelE antibody goat serum was generated at Bethyl Laboratories (Montgomery, TX) using recombinant antigen generated in pML53 encoding an inactive GelE E137A mutant that was previously described (9).

Flow cytometry analysis. Ace surface expression on various *E. faecalis* strains was detected by anti-Ace MAb 70 labeling. Flow cytometry analysis was performed as previously described (5). Cells grown in BHI were harvested at specific time points as indicated, washed twice in phosphate-buffered saline (PBS), and labeled for 1 h at 25°C with 5 μ g of anti-Ace MAb 70/ml in 2% bovine serum albumin (BSA) in PBS (PBS-BSA). After a second wash step, the cells were labeled with R-phycoerythrin (PE)-labeled goat F(ab')₂ anti-mouse IgG (Fc) (Jackson ImmunoResearch) and incubated an additional 30 min at 25°C. *E. faeca-*

lis cells were then fixed in 1% paraformaldehyde in PBS and analyzed with a Becton Dickinson FACSCalibur flow cytometer.

Construction of a $\Delta gelE \Delta sprE$ deletion mutant of *E. faecalis* OG1RF. A 2,526-bp region encoding *gelE* and *sprE* genes was deleted by using the suicide vector pTEX4577 (46). The 5' and 3' flanking regions of the *gelE-sprE* region were amplified by PCR using two sets of primers—(i) gelsprDelF1 (5'-ATTGT TGGATCCGTTGTTACTTTTGTGTTTTG-3') and gelsprDelR1 (5'-TTTT GAATTCCTTCTTTTATTTCTTTTCCCAAC-3') and (ii) gelsprDelF2 (5'-T TTTGAATTCGTAAGTTACGATAAAAAGTACCTTG-3') and gelsprDelR2 (5'-TTTGGTACCTACTTAAAAAATTCATTTCATTC-3')—and then purified and ligated together by linkers (EcoRI) incorporated in gelsprDelR1 and gelsprDelF2. The 5' and 3' flanking regions were cloned into pTEX4577 by using two linkers (with BamHI and KpnI recognition sites) incorporated in the two outside primers, gelsprDelF1 and gelsprDelR2. The resulting construct, pTEX5319, was then transformed into OG1RF by electroporation, and single-crossover mutants were selected on Todd-Hewitt (TH) agar supplemented with 2,000 μ g of kanamycin/ml. One of the single-crossover recombinants was grown for six daily serial passages at 37°C. The culture from the sixth passage was serially diluted and plated on TH agar containing 3% gelatin to score for the loss of gelatinase activity. Colonies that were gelatinase production negative were then scored for the loss of kanamycin resistance and were further confirmed by sequencing. Pulsed-field gel electrophoresis (20) was used to verify that the deletion mutants were not contaminants. One of these colonies with the correct deletion was designated TX5471 (OG1RF $\Delta gelE \Delta sprE$).

Whole-cell ELISA library screen. The whole-cell ELISA screen was performed as previously described (5) with some modifications. The Tn library of cells was cultured for 24 h before being diluted 1:10 into new wells containing BHI and subsequently cultured at 37°C for 4 h. Anti-Ace 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-HRP (Jackson ImmunoResearch). After 1 h of incubation at 37°C, the wells were washed three times with PBS-0.2% Tween 20. Tetramethylbenzidine (TMB) substrate was added, followed by incubation at room temperature for 10 min, before being stopped by the addition of 1 M H₂SO₄. The absorbance of each well was measured at an optical density at 450 nm (OD₄₅₀).

Gelatinase plate assay. Briefly, gelatinase activity analysis was performed by inoculating overnight cultured *E. faecalis* strains on TH agar plates containing 3% gelatin and grown overnight at 37°C. The gelatinase activities of different strains were determined by a "halo" formation (44).

Purification of gelatinase. Natively expressed GelE was produced and purified as previously described (9, 14) with modification. Briefly, *E. faecalis* OG1RF $\Delta sprE$ was used for GelE production to ensure no SprE contamination. Overnight culture was inoculated into 1-liter cultures with an OD₆₀₀ of 0.05 and grown for an additional 6 h. The culture medium was centrifuged and filtered with 0.45- and then 0.22- μ m-pore-size filters. Ammonium sulfate crystal was added to achieve a saturation of 60% at 4°C and allowed to stand overnight. The slurry was centrifuged and decanted, and the resulting pellet was resuspended into 50 mM Tris-HCl-1 mM CaCl₂ (pH 7.8) and dialyzed overnight at 4°C. The dialyzed sample was further concentrated with a Millipore stirred-cell concentrator containing a 10,000-molecular-weight cutoff membrane. Concentrates were applied at 1 ml/min to a preconditioned (dialysis buffer) HiPrep 16/60 Sephacryl S-200 high-resolution column (GE Healthcare). Eluted protein fractions were analyzed for gelatinase activity on gelatin agar plates as previously described (44). Active fractions were pooled, concentrated, and characterized using SDS-PAGE to determine protein purity. Aliquots of the purified GelE were stored at -20°C in 50 mM Tris-HCl-1 mM CaCl₂ (pH 7.8).

Gelatinase-Ace cleavage assay(s). The Ace cleavage was carried out using either purified GelE described above or conditioned medium (CM) obtained from sterile-filtered overnight cultures of various *E. faecalis* strains. For rAce cleavage, reaction mixtures containing 3 μ g of rAce and various amounts of purified GelE (range, from 0 to 1.5 μ g) in 0.1 ml of PBS were incubated for 1 h at 37°C. The enzyme activity was stopped by the addition of SDS-PAGE sample buffer containing 2-mercaptoethanol and boiling. Purified GelE alone (1.5 μ g) and rAce (3.0 μ g) were diluted in 0.1 ml of PBS and incubated similarly to serve as controls. Samples were applied onto SDS-PAGE gradient gels (4 to 20%) (NuSep, Inc.), followed with Coomassie blue protein staining (Sigma).

For cleavage of cell surface-displayed Ace, $\Delta gelE \Delta sprE$ cells were cultured at 37°C to achieve an OD₆₀₀ of 0.3. An equivalent of 10 OD₆₀₀ units per tube was centrifuged, and pellets were resuspended into 100- μ l volumes of selected CM from either *E. faecalis* OG1RF wild-type, $\Delta gelE \Delta sprE$, or $\Delta gelE \Delta sprE$ strains. After 30 min of incubation at 37°C, the cell suspensions were centrifuged, and the resulting supernatants were analyzed by Western blotting for the presence of Ace or GelE. Cell pellets of $\Delta gelE \Delta sprE$ strains from each reaction in CM were resuspended and analyzed by flow cytometry as described above.

FIG. 1. Screening of a Tn insertion library identified an *fsrB* mutant (Δ *fsrB*) that had significant levels of surface-expressed Ace in the stationary phase. Each member of the Tn library is represented by gene identification (ID) number (ef number) on the *x* axis. Relative whole-cell ELISA signals are represented on the *y* axis. Each mutant was labeled with anti-Ace MAb, followed by a goat anti-mouse IgG-HRP conjugate and developed with TMB substrate. Absorbance values were determined by using a Thermo Multiskan EX plate reader.

Collagen adhesion assay. A collagen adhesion assay was used as previously published (39), with some modification. Briefly, Microton 600 ELISA plates (Greiner) were coated with human collagen IV (Sigma) at 10 μ g/ml in PBS overnight at 4°C. The ELISA plates were blocked with 200 μ l of PBS-BSA/well. OG1RF wild-type, Δ *fsrB*, Δ *gelE*, and Δ *ace* strains were cultured in BHI for 18 h at 37°C. The overnight bacterial cultures were diluted in BHI to an OD₆₀₀ of 0.05 and grown for 1 or 4 h at 37°C. One OD₆₀₀ equivalent of each cell strain was resuspended in 100 μ l of PBS-BSA and 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 Boulin's solution. After additional washes, 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 (18).

RESULTS

Mutation in *fsrB* results in maintenance of Ace on the cell surface. In an effort to explore the regulation mechanism of Ace surface display in *E. faecalis*, we utilized a whole-cell ELISA-based screening method to identify genes that affect the Ace surface display profile. First, a high-affinity mouse anti-Ace MAb generated against *E. faecalis* rAce was selected for use in subsequent assays for its affinity and ability to recognize native antigen. It has been reported that Ace in *E.*

faecalis OG1RF has a unique display profile, in which Ace surface expression is robust in early exponential phase and drastically decreases in the latter growth phases (8). To identify factors that alter Ace surface display, we screened for mutants which maintained Ace on the surface in late growth phase. An *E. faecalis* OG1RF Tn917 insertion library with 540 unique gene disruption mutants (6) was screened for Ace surface display after culture for 4 h. As shown in Fig. 1, an insertion mutation in *fsrB* (*ef1821*) exhibited a significantly higher level of Ace surface display compared to all other insertion mutants.

To demonstrate whether the phenotype observed in the *fsrB* insertion mutant was due to the disruption of *fsrB* directly, we analyzed the Ace surface display levels of an isogenic deletion mutant of *fsrB* (Δ *fsrB*) previously reported (37). Comparison of Ace surface display by OG1RF and Δ *fsrB* at various growth phases revealed distinctly different Ace display profiles between the two strains as monitored by flow cytometry (Fig. 2A). Ace on the surface of the *fsrB* deletion strain was high at early exponential phase and modestly decreased as it entered the exponential and stationary phases compared to a more rapid decrease of Ace on the surface observed in the wild-type OG1RF strain. We also showed that the loss of *fsrB* did

FIG. 2. The surface expression of Ace is affected by *fsrB* and EDTA. (A) Ace expression on the cell surface of OG1RF (with or without EDTA) and Δ *fsrB* cells at various growth phases were determined by flow cytometry using anti-Ace MAb as the primary antibody. Mean fluorescence intensity (MFI) was determined by flow cytometry. (B) The growth of *E. faecalis* OG1RF in BHI alone or BHI + 0.1 mM EDTA and of Δ *fsrB* cells was determined by measuring the OD₆₀₀ at the indicated time points. The data are presented as the averages of three independent experiments.

FIG. 3. Gelatinase activity correlates with maintenance of Ace surface expression. The GelE activity (A) and Ace surface expression (B) in selected *E. faecalis* strains (spots 1 to 7) and mutants (spots 8 to 10) of *E. faecalis* OG1RF were determined. In panel A, gelatinase activity was detected by inoculating overnight-cultured *E. faecalis* strains on a TH agar plate containing 3% gelatin, followed by growth overnight at 37°C. The gelatinase activities of different strains were determined by assessing the "halo" formation. In panel B, selected strains were cultured overnight, diluted to an OD₆₀₀ of 0.05, and cultured for 4 h. Surface Ace expression was confirmed via anti-Ace MAb labeling. Histograms representing strains that demonstrated gelatinase activity are represented in white, while those without gelatinase activity are shaded.

not significantly change the bacterial growth rate (Fig. 2B), excluding the possibility that the difference in Ace expression was growth rate related. Together, both whole-cell ELISA and flow cytometry analysis indicated that the phenotype of prolonged Ace display on the *E. faecalis* cell surface is a consequence of the loss of *fsrB* expression.

The loss of FsrB function affects Ace surface display through the activity of an extracellular protease. It has long been recognized that the Fsr system regulates the expression of the extracellular protease GelE and the serine protease (SprE), both of which could potentially mediate the cleavage of surface proteins. In addition, maximal GelE secretion had been shown to occur as cells reach stationary phase (22, 37), a finding consistent with the observed timing of growth-phase-dependent loss of Ace surface display. To evaluate whether extracellular proteases can influence Ace surface display, we compared the growth-phase-dependent surface display of Ace on OG1RF cells grown in the presence or absence of EDTA. EDTA is a metalloprotease inhibitor and inhibits GelE activity by chelating the metal ions required for metalloprotease enzymatic activity. Using levels that did not significantly affect the growth rate of the *E. faecalis* culture (0.1 mM) (Fig. 2B), we demonstrated that cells grown with EDTA exhibited

prolonged Ace surface display in the late growth phases (Fig. 2A) similar to Ace display on the *fsrB* mutant. These results suggest that protease activity decreased Ace surface display during late growth phase and that inhibition of such activity can at least partially prevent the decrease of Ace on the cell surface.

To expand on this observation, we tested a number of *E. faecalis* strains for their ability to secrete active GelE using a gelatinase plate assay and compared these results to each strain's ability to maintain Ace display after 4 h of culture to late growth phase. The panel of strains evaluated included (i) those observed to have similar Ace expression profiles as wild-type OG1RF (TX0104, HH-22, V583, and 51188), (ii) those that maintained significant levels of Ace surface expression in stationary phase (JH2-2 and 11700), and (iii) in-frame deletion mutants of OG1RF that lacked *fsrB* (37), *gelE* (43), or *gelE* and *sprE* (generated in the present study). The results demonstrated a clear association between maintaining Ace surface display in the stationary phase and the lack of gelatinase activity of each strain (Fig. 3). This suggested that the secretion of GelE plays an important role in Ace surface display, possibly through enzymatic cleavage of Ace from the cell wall. Since Δ*gelE* strains retain the ability to produce active SprE (43), this

FIG. 4. (A) Cleavage of Ace from surface of cells with CM containing GelE. OG1RF $\Delta gelE$ $\Delta sprE$ cells were collected and centrifuged. The pellets were resuspended into selected CM from the indicated strains (OG1RF wild type or *E. faecalis* $\Delta gelE$, $\Delta sprE$, or $\Delta gelE$ $\Delta sprE$ strains). These CM-treated cells were centrifuged, and the resulting supernatants were used in Western blots for the detection of Ace or GelE. (B) Flow cytometry analysis of $\Delta gelE$ $\Delta sprE$ cells treated with CM from the strains indicated on the histogram demonstrated that the loss of surface Ace expression correlates with the presence of GelE in CM.

suggested that SprE alone is not sufficient to remove Ace from the cell surface.

Ace is cleaved by gelatinase. To pursue the hypothesis that Ace is cleaved directly by GelE, we evaluated the effect of CM containing or lacking GelE on cells producing surface displayed Ace. To maximize Ace protein release over a limited time period, we incubated OG1RF $\Delta gelE$ $\Delta sprE$ cells (which maintain Ace display) with culture media of different strains harvested at the late exponential phase, thus ensuring maximal Fsr-induced GelE levels in the CM. As shown by Western blot analysis in Fig. 4A, GelE is present in CM from $\Delta sprE$ and OG1RF wild-type culture media but absent in CM generated in $\Delta gelE$ $\Delta sprE$ and $\Delta gelE$ cells, as shown by detection with polyclonal anti-GelE antibody serum. Incubation of exponentially grown $\Delta gelE$ $\Delta sprE$ cells with CM yielded a soluble product identified as digested Ace fragment (Fig. 4A) only when the CM contained GelE, as determined by Western blotting. Flow cytometry analysis of the treated cells confirmed the loss of Ace on the surface only on cells treated with GelE containing CM (Fig. 4B).

The cleavage of Ace by GelE was further confirmed using recombinant Ace domain A and purified GelE. As shown in Fig. 5, upon the addition of purified GelE (0.015 to 15 μ g/ml), the rAce is digested to yield smaller fragments. This suggests the existence of at least one GelE cleavage site in Ace domain A. At lower GelE concentrations (0.015 to 0.15 μ g/ml), one major digested band was observed. At higher GelE concentrations (0.45 to 15 μ g/ml), multiple digestion fragments appeared, while the amount of the initial digested fragment decreased, suggesting secondary digestion sites. Altogether, these results indicated that cleavage by GelE is responsible for the disappearance of Ace from *E. faecalis* cell surface.

Cleavage of Ace by GelE affects the ability of *E. faecalis* to adhere to collagen. Since loss of Ace from the surface has previously been shown to inhibit cellular adhesion to collagen (24), we evaluated four *E. faecalis* strains—the OG1RF, $\Delta fsrB$, $\Delta gelE$, and Δace strains—for their ability to adhere to collagen. For cells analyzed in the early growth phase (1 h culture), only the Δace strain exhibited a significantly lower binding capacity to collagen IV compared to the other strains. For late-phase cultures (4 h of culture), however, both $\Delta fsrB$ and $\Delta gelE$ strains demonstrated a more robust ability to adhere to collagen IV coated plates compared to wild-type OG1RF, likely due to the higher levels of Ace surface expression compared to the wild type. (Fig. 6). This is consistent with findings by Tomika et al. demonstrating that GelE⁺ *E. faecalis* isolates adhered less efficiently to collagen-coated surfaces (49). It is also notable that collagen adhesion at the early growth phase is significantly higher than at the late growth phase in all strains except the Δace strain (Fig. 6). As demonstrated (Fig. 2A), Ace levels on the cell surface do decrease from early to late phase even when the GelE activity is inhibited (+EDTA or $\Delta fsrB$). The loss of surface Ace expression due to factors independent of GelE may account for differences in early- versus late-phase collagen adhesion in the $gelE$ and $fsrB$ mutants.

DISCUSSION

Due to surface localization, antigenicity, and their role in virulence, MSCRAMMs are of considerable interest as targets in vaccination and passive protection strategies (30). As a consequence, it is crucial to improve our knowledge of how levels of MSCRAMM proteins are regulated. The results presented here provide strong evidence that surface display of Ace, the

FIG. 5. GeIE cleavage of rAce analyzed by SDS-PAGE. Sample lanes from left to right represent reaction mixtures containing 30 µg of rAce/ml with various amounts of purified GeIE (0.015, 0.045, 0.15, 0.45, 1.5, 4.5, and 15 µg/ml), rAce (30 µg/ml) alone, or GeIE (15 µg/ml) alone. Molecular mass standards are indicated to the right in kilodaltons.

first MSCRAMM described in *E. faecalis*, is regulated by the quorum-sensing mediated secretion of GeIE.

Since the Fsr quorum-sensing system is known to transcriptionally regulate a number of genes (2, 22, 37), we first considered the potential effect of a mutation of *fsr* on *ace* transcript levels. However, preliminary analysis of *ace* transcript abundance at different growth phases comparing the $\Delta fsrB$ strain to the wild-type strain showed no obvious differences between the two (data not shown). This is consistent with previous microarray analysis, which failed to identify *ace* as one of the genes transcriptionally regulated by Fsr (2), and suggested that a posttranscriptional mechanism might be responsible for the Ace surface display phenotype seen in the *fsr* mutants.

The Fsr system is widely known for transcriptionally regulating the expression of the extracellular proteases GeIE, a metalloprotease, and SprE, a serine protease. Monitoring GeIE secretion over time previously demonstrated that maximum GeIE activity occurred as cells reached the stationary phase in culture (22, 37). Since this event correlated with the growth-phase-dependent loss of surface-displayed Ace (8) (Fig. 2A), we were compelled to consider these proteases as suspects in the link between quorum sensing and Ace display. Demonstration that EDTA (a chelator of divalent cations) prevented the rapid loss of Ace suggested that a metalloprotease was responsible for cleavage (Fig. 2A). Subsequent experiments showed that (i) the $\Delta geIE$ mutant, which retains SprE expression, maintained Ace surface expression, (ii) CM

FIG. 6. Mutants that retain Ace expression in the stationary phase maintain the ability to adhere to collagen. OG1RF and selected mutants were cultured overnight in BHI. The overnight cultures were subsequently diluted to an OD₆₀₀ of 0.05 and incubated for an additional 1 h (early phase) or 4 h (late phase). Adhesion and standard deviation calculations were averaged from four wells per experiment, and the data are representative of three independent experiments.

from wild-type and $\Delta sprE$ strains but not *gelE* mutants were able to cleave Ace from the cell surface, and (iii) purified GelE cleaved recombinant Ace domain A. These experiments provided strong evidence that GelE activity was responsible for the loss of Ace surface display.

In work by Hall et al. (8), the loss of Ace from the surface into the medium was also considered as a possible explanation for the rapid disappearance of Ace from the cell surface in the latter stages of growth. However, attempts to demonstrate Ace accumulation in the medium were unsuccessful in their studies. In our present work, observation of an Ace cleavage product (Fig. 4A) in the medium required the knowledge that GelE was a major contributing factor in Ace surface depletion. By using CM harvested at the late exponential phase, thus ensuring maximal Fsr-induced GelE levels and exposing $\Delta gelE \Delta sprE$ cells (which maintain high levels of Ace display into stationary phase) to CM, we maximized Ace protein cleavage and thus release over a limited time frame. Whether this soluble cleavage product has the ability to bind collagen or provides a benefit to *E. faecalis* survival will be addressed in future studies. Our inability to visualize Ace simply by Western blot analysis of the CM of cells grown to stationary phase (Fig. 4A) is consistent with Hall's observations and suggests that there may be downstream cleavage events that degrade Ace further in culture over time.

Commonly considered a virulence factor of *E. faecalis*, GelE is linked to a number of virulence-related phenotypes. Multiple accounts have associated the Fsr system and GelE in the establishment of biofilm, functioning to encourage lysis and subsequent eDNA release of a subpopulation of cells which could aid in colonization establishment through biofilm formation (9, 18, 47). This is consistent with work by Singh et al. (44) demonstrating a decreased *E. faecalis* bacterial burden at the primary site of infection upon disruption of GelE in a rat endocarditis model.

In addition to assisting in the establishment of biofilm, it has been suggested that GelE may also play a role in dissemination. In a rabbit model of endocarditis, GelE did not significantly affect the bacterial burden at the primary site of infection but did mediate increased levels of bacterial burden at disseminated sites of infection (7, 48). Consistent with a role of GelE in dissemination, Waters et al. observed several functions of GelE that could encourage dissemination of *E. faecalis* in high-population-density environments. These include GelE's role in degrading misfolded surface protein Asc10, its ability to activate muramidase-1 autolysin (42) which decreases the chaining of *E. faecalis*, and its ability to cleave fibrin, the major component of the matrix layer surrounding established vegetation (50). Citing GelE's ability to hydrolyze fibrin, Thurlow et al. recently provided histology findings that the matrix layer surrounding *E. faecalis* is significantly smaller in GelE⁺ strains (48), suggesting that this may improve the ability of *E. faecalis* to disseminate from a vegetation.

Our observation that GelE mediates cleavage of Ace from the cell surface could also suggest a role for GelE in dissemination where, in established vegetations, and directed by quorum sensing, cleavage of Ace could aid in the release of cells from the collagen matrix to encourage the dissemination of bacteria from the primary site of infection. Since GelE is also known to also cleave collagen (14), it is interesting to spec-

ulate that GelE could potentially encourage disassociation of *E. faecalis* from collagen through either loss of the displayed Ace collagen-binding domain or by cleaving the collagen to which it is bound. A role for Fsr in dissemination is also consistent with a recent report demonstrating that the Agr quorum-sensing system of *S. aureus*, which is the equivalent of the Fsr system of *E. faecalis*, mediates the dispersal of cells in established biofilms through the regulation of extracellular proteases (1). In their report, Boles et al. specifically propose MSCRAMM cleavage as a possible participating factor in bacterial dissemination, a process which is likely multifaceted.

Another possible role for GelE-mediated cleavage of Ace from the surface of *E. faecalis* is that it could provide a method to evade the immune system. Upon establishment of an infection, when initial adherence is no longer required, it may be beneficial for the bacteria to remove surface adhesins, such as Ace, which are known to trigger an immune response in the host (25). Known for its role in immune system evasion, it has been demonstrated that GelE disrupts the polymorphonuclear neutrophil-mediated killing of *E. faecalis* through the cleavage of C3 and iC3b (31, 32). In addition, it has also been demonstrated that GelE is involved in the degradation of C5a (48), providing further evidence of GelE's role in evading clearance by the immune system.

Mediation of MSCRAMM surface display via quorum-sensing regulation of a metalloprotease is probably not limited to this example in *E. faecalis* and may represent a more global regulation mechanism. In *S. aureus*, the fibrinogen binding MSCRAMM ClfB was reported to have a similar growth-phase-dependent expression profile (15). Similar to our findings with Ace, cleavage of ClfB was identified to be within the matrix protein binding domain and inhibited the cell's ability to adhere to its matrix protein (fibrinogen), a finding consistent with our observations with Ace (and bacterial adhesion to collagen). Cleavage of ClfB was partially attributed to aureolysin, a metalloprotease of *S. aureus* regulated in part by the Agr quorum-sensing system of *S. aureus* (29), thus supporting the role of quorum-sensing in impacting MSCRAMM display in this organism. This finding in *S. aureus*, coupled with our discovery in *E. faecalis*, supports the theory of a common mechanism in Gram-positives to regulate the presence of MSCRAMM proteins on the cell surface. In addition to metalloprotease-mediated cleavage, a number of other extracellular proteases have been identified in Gram-positives that are capable of MSCRAMM cleavage, including SpeB, a cysteine protease (28), and V8 protease, a serine protease (16) from *Streptococcus pyogenes* and *S. aureus*, respectively. Identifying additional MSCRAMMs cleaved via quorum-sensing-regulated proteases by monitoring growth-phase-dependent changes in MSCRAMM surface expression patterns is ongoing.

Future work will focus on the role of protease-mediated MSCRAMM cleavage and how this mechanism may benefit *E. faecalis* through different stages of infection, including adhesion, biofilm formation, and dissemination. Also, with considerable research efforts focusing on MSCRAMMs for passive protection and vaccination strategies, our discovery could have significant impact when considering the utilization of MSCRAMMs as targets, influencing decisions as to which MSCRAMM to target, what domain to target, or whether the metalloprotease itself might be a target as a

means of preventing bacterial infection, evasion of the immune system, and dissemination.

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