The Enterococcus faecalis EbpA Pilus Protein: Attenuation of Expression, Biofilm Formation, and Adherence to Fibrinogen Start with the Rare Initiation Codon ATT

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ABSTRACT The endocarditis and biofilm-associated pili (Ebp) are important in Enterococcus faecalis pathogenesis, and the pilus tip, EbpA, has been shown to play a major role in pilus biogenesis, biofilm formation, and experimental infections. Based on in silico analyses, we previously predicted that ATT is the EbpA translational start codon, not the ATG codon, 120 bp downstream of ATT, which is annotated as the translational start. ATT is rarely used to initiate protein synthesis, leading to our hypothesis that this codon participates in translational regulation of Ebp production. To investigate this possibility, site-directed mutagenesis was used to introduce consecutive stop codons in place of two lysines at positions 5 and 6 from the ATT, to replace the ATT codon in situ with ATG, and then to revert this ATG to ATT; translational fusions of ebpA to lacZ were also constructed to investigate the effect of these start codons on translation. Our results showed that the annotated ATG does not start translation of EbpA, implicating ATT as the start codon; moreover, the presence of ATT, compared to the engineered ATG, resulted in significantly decreased EbpA surface display, attenuated biofilm, and reduced adherence to fibrinogen. Corroborating these findings, the translational fusion with the native ATT as the initiation codon showed significantly decreased expression of β-galactosidase compared to the construct with ATG in place of ATT. Thus, these results demonstrate that the rare initiation codon of EbpA negatively regulates EbpA surface display and negatively affects Ebp-associated functions, including biofilm and adherence to fibrinogen.

IMPORTANCE Enterococcus faecalis is among the leading causes of serious infections in the hospital setting, and the endocarditis and biofilm-associated pili (Ebp) have been shown to play significant roles in E. faecalis pathogenesis. Understanding the regulation of virulence is important for the development of new approaches to counteract multidrug-resistant pathogens. We previously predicted that ATT, which has been reported to start protein synthesis only in rare instances, is the most likely translational start codon of EbpA in E. faecalis. Here, we demonstrate that ATT is the initiation codon of EbpA and, relative to a constructed ATG start codon, results in smaller amounts of EbpA on the surface of the cells, attenuating biofilm formation and fibrinogen adherence, phenotypes associated with the ability of E. faecalis to cause infections. This provides the first example of pilus regulation through the use of an ATT initiation codon.
The fsr system was shown to be a weak repressor (13). Furthermore, environmental conditions, including bicarbonate (13) and the presence of serum (7), positively affect Ebp production.

Several reports have shown that EbpC forms the backbone polymer, while EbpA and EbpB are present at the tip and at the base of the pilus fiber, respectively (5–7, 14). In addition, our electron microscopy studies indicated that the majority of the EbpA protein is found on the surface of E. faecalis OG1RF cells (6), and analyses of the contribution of each structural subunit of the Ebp revealed the importance of EbpA to pilus biogenesis (5, 6, 14). We previously reported that deletion of ebpA resulted in the formation of fewer but extremely long pili compared to wild-type (WT) OG1RF, which suggested a role for EbpA in the initiation as well as termination of pilus polymerization (6). We corroborated the role of EbpA as a factor that influences the length of pili by controlled overexpression of EbpA from a nisin-inducible promoter in an \( \Delta \text{ebp}A \) mutant, which led to a gradual decrease in pilus length as the concentration of nisin was increased (6). Deletion of ebpA also affected the overall levels of the other Ebp pilin subunits, without altering the transcription levels of the downstream genes (6). EbpA has been demonstrated to play a crucial role in biofilm formation and in a model of urinary tract infection, with the ebpA deletion mutant attenuated approximately to the same level as that observed with the ebpABC operon deletion (6). In addition, Nielsen et al. showed the contribution of EbpA in the colonization of bladders and intrablisters implants in CAUTIs and revealed that the metal ion-dependent adhesion site (MIDAS) motif present in EbpA’s von Willebrand factor A (VWA) domain is important for pilus function (5). More recently, this group demonstrated that immunization with EbpA inhibits binding of E. faecalis to fibrinogen and provides protection in catheter-related infections, with the ebpA deletion mutant attenuated approximately to the same level as that observed with the ebpABC operon deletion (6).

We previously predicted that the most likely translational start codon of EbpA in E. faecalis OG1RF is the very rarely used triplet ATT (AUU) as the initiation factor 3 (IF3) discriminates against noncanonical start codons (18). As observed in other prokaryotic species, in E. faecalis OG1RF, the use of ATT as the initiation of protein synthesis is very rare, and there is no evidence indicating that other E. faecalis proteins, besides EbpA, start translation with this codon. In addition, when our search of several other sequenced E. faecalis strains found conservation of this codon, we postulated that this codon participates in regulation of protein synthesis. Then, we constructed a derivative of OG1RF in which the ATT codon of ebpA was replaced with ATG and investigated the effect of this change on translation, EbpA and EbpC surface display, biofilm formation, and adherence to fibrinogen.

**RESULTS**

Introduction of stop codons indicates that ATT (AUU) is the start codon of EbpA protein synthesis. The ebpA gene is the first gene of the ebpABC operon that encodes the E. faecalis Ebp (Fig. 1). The current genome annotation of the E. faecalis strain V583 indicates that EbpA is an 1,103-residue protein with a VWA domain; however, closer examination of the operon sequence revealed that no recognizable RBS is present upstream of the ebpA annotated start codon, ATG (7). In addition, no recognizable signal peptide or cleavage site was found downstream of the ATG annotated as the start codon of the predicted 1,103-residue EbpA protein (EbpA-1103) (see Fig. S1A in the supplemental material). We predicted that the most likely, but very unusual, start codon of ebpA is the triplet ATT, located 9 bp downstream of a recognizable RBS and 120 bp upstream of the currently annotated ATG start codon (Fig. 1) (7). In the majority of mRNAs, translation initiation from the codon AUG that in prokaryotes codes for formylmethionine; nevertheless, other NUG codons are occasionally found as translational starts (17, 18). In only very few instances has the AUU codon been reported as the translational start for protein synthesis in bacteria (19, 20). The rarity of AUU as a start codon is attributed to the fact that initiation factor 3 (IF3) discriminates against noncanonical start codons (18). As observed in other prokaryotic species, in E. faecalis OG1RF, the use of ATT as the initiation of protein synthesis is very rare, and there is no evidence indicating that other E. faecalis proteins, besides EbpA, start translation with this codon. In addition, when our search of several other sequenced E. faecalis strains found conservation of this codon, we postulated that this codon participates in regulation of EbpA expression. To test this hypothesis and our prediction of ATT as the start codon of EbpA protein synthesis, we first used site-directed mutagenesis to introduce, in E. faecalis OG1RF, two successive stop codons between the ATT predicted initiation codon and the currently annotated ATG start codon, to experimentally show that the ATT indeed determines the start of EbpA protein synthesis. Then, we constructed a derivative of OG1RF in which the ATT codon of ebpA was replaced with ATG and investigated the effect of this change on translation, EbpA and EbpC surface display, biofilm formation, and adherence to fibrinogen.

**FIG 1** Schematic representation of the ebpABC-bps locus of E. faecalis OG1RF. The locus consists of three genes, ebpA, ebpB, and ebpC, encoding the pilin subunits, and bps, encoding a class C sortase. The putative promoter region (with the −35 and −10 promoter boxes), the predicted transcriptional start (+1), the predicted ribosomal binding site (RBS), the ATT postulated start codon, and the ATG annotated start codon are shown. The positions of the two successive stop codons introduced to generate strain TXS731 are also shown.
TABLE 1  Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) | Reference or source |
|-------------------|-----------------------------|---------------------|
| **E. faecalis** | | |
| OG1RF | Laboratory strain; Rif\(^r\); Fus\(^r\) | 35 |
| TX5731 | Oligotyping with two point mutations at nucleotides 13 and 16 after the putative ebpA ATT initiation codon that changed two lysine residues (AAG and AAA) to two stop codons (TAG and TAA, respectively) (ebpA\(_{STOP}\)) | This study |
| TX5732 | Restored Oligotyping ebpA\(_{ATT}\); the mutated ebpA\(_{ATT}\) codon was restored to wild-type ebpA\(_{ATT}\) | This study |
| TX5608 | Oligotyping for ebpABC; ebpABC operon deletion mutant | 12 |
| TX5620 | Oligotyping for ebpA; ebpA deletion mutant | 6 |
| **E. coli** | | |
| TG1 | E. coli host strain used for routine cloning | 36 |
| EC1000 | E. coli host strain for cloning of RepA-dependent plasmids | |
| **Plasmids** | | |
| pGEM-T Easy | Plasmid used for initial cloning of PCR fragments; Amp\(^r\) | Promega |
| pHOU1 | Conjugative donor plasmid used for the introduction of point mutations into *E. faecalis*; confers Gen\(^r\) and carries the counterselectable pheS\(^r\) gene | 31 |
| pSD2 | Plasmid used to construct the translational *lacZ* fusions; the *lacZ* gene lacks a promoter, an RBS, and a start codon; confers Amp\(^r\) and Erm\(^r\) | 32 |
| pTEX5749 | pSD2 plasmid containing a fragment from −261 bp upstream to 31 bp downstream of the ATT start codon of the *ebpA* gene of *E. faecalis* OG1RF(pSD2-ebpA\(_{ATT}\);*lacZ*) | This study |
| pTEX5750 | pSD2 plasmid containing a fragment from −261 bp upstream to 31 bp downstream of the mutated ATG start codon of the *ebpA* gene of *E. faecalis* TX5731(pSD2-ebpA\(_{ATT}\);*lacZ*) | This study |

\(^a\) Amp, ampicillin; Erm, erythromycin; Gen, gentamicin; Fus, fusidic acid; Rif, rifampin.

cleavage sites (see Fig. S1C and D). Furthermore, protein alignments of EbpA-1103 and EbpA-1143 with EmpA (EbpA homolog in *Enterococcus faecium*) support the ATT start for EbpA protein synthesis.

To experimentally confirm that the designated ATG (located 120 bp downstream of our predicted ATT start) is not the start codon for EbpA protein synthesis, we used site-directed oligonucleotide mutagenesis to generate the strain TX5731, in which two lysine residues, AAG and AAA at positions 5 and 6 after the ATT, respectively, were changed to two stop codons, TAG (amber) and TAA (ocher) (Fig. 1; Table 1). Our rationale was that, if translation starts from ATT, the introduction of a stop codon would signal termination of translation and no EbpA protein would be produced; for this purpose, we introduced two stop codons in order to avoid readthrough, as was reported when only one stop codon was inserted at the 5′ end of a chromosomal copy of a gfp reporter gene in *Bacillus subtilis* (21). In contrast, if the currently annotated ATG is the start of EbpA synthesis, the introduction of the stop codons before this ATG would not affect EbpA translation. As we predicted, flow cytometry showed that *E. faecium* OG1RF displayed a strong EbpA signal (Fig. 2A), while strain TX5751 was negative for EbpA surface display (Fig. 2B). Next, we investigated the conservation of this unusual codon as the start of EbpA protein synthesis and found 100% conservation of the ATT codon in the 347 bp of E. faecalis strains with available genome sequences in NCBI (data not shown). In addition, we interrogated the genome annotation for EbpA homologs in other enterococcal species, including *E. faecium*, *Enterococcus hirae*, *Enterococcus casseliflavus*, *Enterococcus munditii*, and *Enterococcus gallinarum* and found that, in all but *E. gallinarum*, ATG was annotated as the start codon of these EbpA homologs (see Fig. S2 in the supplemental material). Furthermore, a recognizable RBS was present appropriately upstream of these ebpA-like genes’ ATG annotated start codons. Interestingly, no recognizable RBS was observed upstream of the annotated ebpA ATC start codon in *E. gallinarum* (see Fig. S2), raising the possibility of an alternative start for EbpA protein synthesis in this enterococcal species.

The start codon ATT affects EbpA surface display. The use of a rare start codon as the translational start of EbpA, along with its conservation in all published *E. faecalis* genomes, led us to the hypothesis that this codon may play a role in the regulation of Ebp expression. To investigate this, we constructed a single-nucleotide variant, named TX5731, in which the ebpA ATG triplet of *E. faecalis* OG1RF was replaced with ATG (ebpA\(_{ATT}\)), and then explored the effect of this initiation codon change on the levels of EbpA surface display. Whole-cell enzyme-linked immunosorbant assay (WC-ELISA) using anti-recombinant EbpA (anti-rEbpA), performed after growing the cells to exponential phase in TSBG medium (trypic soy broth supplemented with 0.25% [vol/vol] glucose), revealed that strain TX5731 carrying ATG as the initiation codon of ebpA had significantly increased amounts of EbpA on the surface compared to OG1RF (P < 0.001) (Fig. 3). To confirm that the differences observed between WT OG1RF and its ebpA\(_{ATT}\) mutant (TX5731) were due to the mutation in the ebpA start codon, we generated a revertant strain (TX5732), by replacing the ATG of TX5731 with the original ebpA start codon ATT (ebpA\(_{ATT}\)), in the native location. TX5732 displayed EbpA levels on the surface similar to those observed on WT OG1RF, demonstrating the role of the initiation codon in the regulation of EbpA protein levels (Fig. 3). In addition, WC-ELISA did not show any EbpA on the surface of the strain carrying the two consecutive stop codons after the ATT initiation codon, TX5731, nor on the ebpA deletion mutant, TX5620 (Fig. 3). EbpC surface display was also investigated under the same growth conditions, and the results
revealed a small but nonsignificant increase in EbpC on the surface of TX5731 compared to OG1RF and TX5732 (see Fig. S3 in the supplemental material). As expected, no surface display of either EbpA or EbpC was observed on the surface of TX5608, the ebpABC operon deletion mutant (Fig. 3; see also Fig. S2) (6, 7). Similarly, when the cells were grown in BHI-S (brain heart infusion broth supplemented with 40% [vol/vol] horse serum), the ebpA ATG mutant, TX5731, also showed a significant increase in surface display of EbpA compared to the ebpA ATT counterparts, OG1RF and TX5732 ($P < 0.001$) (data not shown). Quantitation of surface-localized EbpA protein was also performed by flow cytometry. The mutant strain TX5731 with ATG showed increased levels of EbpA on the cell surface, compared to OG1RF and TX5732, corroborating the results obtained with WC-ELISA (data not shown).

$\beta$-Galactosidase production is reduced by the presence of ATT as the start codon of an ebpA-lacZ fusion. To explore the direct contribution of the EbpA initiation codon to translational efficiency, two translational reporter fusions, pTEX5749 and pTEX5750, were generated by amplifying a 292-bp fragment from -261, including the promoter region of ebpA, to 31 bp downstream of the translational start codon from E. faecalis OG1RF and TX5731, respectively (Fig. 4A). These fragments were fused to the reporter gene lacZ (Fig. 4A), and after electroporation of the fusion constructs into E. faecalis OG1RF, TX5731, and TX5732, $\beta$-galactosidase activity was assayed following growth in TSBG (Fig. 4B) and BHI-S (see Fig. S4 in the supplemental material). Under both growth conditions and in each of the strain backgrounds, $\beta$-galactosidase activity from cells carrying the reporter fusion pTEX5750 with ATG as the start codon was significantly greater than that from cells carrying the reporter fusion pTEX5749 with the triplet ATT as the initiation codon of translation ($P < 0.001$) (Fig. 4B; see also Fig. S4). It is interesting that strain TX5731 carrying either pTEX5749 or pTEX5750 expressed significantly higher levels of the reporter protein than did OG1RF and TX5732 carrying the corresponding reporter fusions ($P < 0.001$), which could suggest a positive-feedback loop controlling EbpA expression. In contrast, no differences in $\beta$-galactosidase activity were observed between OG1RF and TX5732 (Fig. 4B; see also Fig. S4). In addition, we observed that in BHI-S, $\beta$-galactosidase activity was increased approximately 3-fold over that in TSBG-grown cells in both the ATT (pTEX5749) and ATG (pTEX5750) constructs, but the relationship of $\beta$-galactosidase from the ATT and the ATG start codon was still maintained (see Fig. S4 versus Fig. 4B). Importantly, the differences in $\beta$-galactosidase activity are not a consequence of differences in growth rate, as OG1RF and its deriva-
tives carrying the two plasmids, pTEX5749 and pTEX5750, exhibited equivalent growth kinetics (data not shown).

**ATT as the initiation codon of ebpA translation correlates with less biofilm formation.** Previous studies showed that deletion of ebpA had a marked effect on the ability of *E. faecalis* OG1RF to form biofilm (6). We therefore investigated the impact of the ebpA initiation codon on early biofilm development (3 h) and on mature biofilm (24 h). When we scored biofilm after 3 h of static incubation, the strain carrying ATG as the initiation codon of EbpA, TX5731, showed a significant increase in biofilm density compared to the strains carrying ATT, WT OG1RF, and the revertant TX5732 (median for TX5731, 0.68, versus 0.58 and 0.57 for WT and TX5732, respectively; \( P < 0.001 \)) (Fig. 5A). A smaller but still significant increase was observed in biofilm density after 24 h of static incubation of the ebpA\(_{ATT}\) mutant, TX5731, compared to OG1RF and TX5732 (\( P < 0.001 \)). Consistent with previous findings (6, 7, 11, 12), a marked reduction in biofilm formation was observed when the ebpABC operon (TX5608) or ebpA (TX5620) had been deleted (\( P < 0.001 \)) (Fig. 5A and B). In addition, TX5751, the strain in which two successive stop codons were introduced after the ATT EbpA start codon, showed reduction in biofilm density comparable to these two deletion mutants, corroborating the role played by EbpA in biofilm formation. The greater difference in biofilm density observed at the earlier time

**FIG 4** \( \beta \)-Galactosidase expression from ebpA::lacZ translational fusions is dependent on the identity of the start codon. (A) Schematic representation of the ebpA::lacZ fusions pTEX5749 (pSD2-ebpA\(_{ATT}\)::lacZ) and pTEX5750 (pSD2-ebpA\(_{ATG}\)::lacZ) carrying ATT and ATG as the start of EbpA::LacZ fusion protein synthesis, respectively. (B) \( \beta \)-Galactosidase activity in *E. faecalis* OG1RF and its ebpA start codon derivatives, TX5731 and TX5732, containing either pTEX5749 (gray bars) or pTEX5750 (green bars) after growth to mid-log phase in TSBG. Bars represent the means ± standard deviations of results from four independent assays, each with two duplicates. The mean values were compared using ANOVA with Bonferroni’s posttest (***, \( P < 0.001 \); ns, \( P > 0.05 \)).
point than at 24 h between the strain carrying ATG as the start codon of EbpA (TX5731) and the strains carrying ATT (OG1RF and TX5732) may be related to the data of Bourgogne et al., who demonstrated that ebpA expression peaked at log phase, followed by a decline during stationary phase (13). Hence, our results corroborate the importance of EbpA in biofilm production and demonstrate that the levels of EbpA protein on the surface of the cells are important for E. faecalis biofilm formation, in particular during its initial stages.

**ATT as the initiation codon of ebpA translation correlates with less binding to fibrinogen.** Nallapareddy et al. demonstrated that Ebp-deficient mutants of E. faecalis OG1RF showed reduced binding to fibrinogen and to collagen type I (8); in addition, Flores-Mireles et al. confirmed that EbpA mediates attachment of E. faecalis to host fibrinogen (15). Therefore, we investigated the abilities of OG1RF, its ebpA<sub>ATG</sub> initiation codon mutant (TX5731), and the revertant strain ebpA<sub>ATT</sub> (TX5732) to bind to fibrinogen. A slight but significant increase in binding to fibrinogen was observed when the initiation codon of ebpA was changed to ATG (TX5731) compared to OG1RF and TX5732 with the ATT initiation codon (P < 0.05 and P = 0.01, respectively) (Fig. 6). This result therefore indicates that the ATT initiation codon of EbpA also affects E. faecalis adherence to fibrinogen.

**DISCUSSION**

In bacteria, the most frequently used translational start codon is AUG (90% of the Escherichia coli mRNAs); however, alternative initiation codons, including GUG (8%) and UUG (1%), are occasionally found (17, 18). In contrast, the triplet AUU has been reported in only two instances in E. coli, namely, the pcnB gene encoding poly(A) polymerase (PAPI) (19) and the infC gene encoding translation initiation factor IF3 (20). In both instances, expression of the corresponding proteins was limited by the presence of AUU as the translational initiation codon (19, 20, 22). We analyzed the genome of E. faecalis OG1RF and determined that, in 81.5% of the open reading frames (ORFs), AUG is annotated to be the initiation codon while, in 10% and 8.5% of the instances, the codons GUG and UUG are predicted to initiate protein synthesis, respectively. However, we observed that the most likely translational start codon of EbpA in E. faecalis OG1RF is the rare triplet AUU (corresponding to ATT in the DNA) (Fig. 1), while the E. faecalis infC gene, encoding IF3, is annotated to start with the canonical ATG. The presence of a rare start codon as the most likely start of EbpA protein synthesis and its conservation in all sequenced E. faecalis strains led us to the hypothesis that this codon plays a role in the translational regulation of EbpA expression. First, we experimentally confirmed that ATG is not the start of EbpA translation by introducing two successive stop codons in between the ATT that we predicted is the initiation codon and the

![FIG 5](image-url) Effect of the ebpA initiation codon on biofilm formation. Bacterial cells grown for 3 h (A) or 24 h (B) in TSBG were analyzed for biofilm formation using a crystal violet-based assay. Bars represent the means of absorbance at 570 nm ± standard deviations from four independent assays (40 wells per strain). ANOVA with Bonferroni’s posttest was used to compare biofilm density values (***, P < 0.001; ns, P > 0.05).

![FIG 6](image-url) Effect of the ebpA initiation codon on the adherence of E. faecalis to immobilized fibrinogen. Bars represent means ± standard deviations of absorbance measured at 570 nm (for 4 wells per strain). The mean values between E. faecalis OG1RF and its derivatives were analyzed using ANOVA with Bonferroni’s posttest (***, P < 0.001; **, P = 0.01; *, P = 0.05; ns, P > 0.05).
currently annotated ATG start codon of ebpA; as we expected, these stop codons abolished EbpA surface display (Fig. 2 and 3). Then, we constructed a derivative of OG1RF in which we replaced, in its native location, the ATT start codon of ebpA with ATG (TX5731) and then reverted this ATG to ATT (TX5732) and demonstrated that E. faecalis OG1RF and TX5732, carrying ATT as the start of EbpA protein synthesis, had reduced levels of EbpA on their surfaces compared to the strain TX5731, carrying ATG as the translational start (Fig. 3). We previously demonstrated that EbpA levels influence the length and number of pilus fibers, which led us to propose that EbpA is important for initiation as well as termination of pilus polymerization (6). It could be suggested that increased levels of EbpA protein observed on TX5731 with the ATT change (Fig. 3) may result in a small increase in the number of pilus fibers, albeit with decreased pilus length, consistent with a small (but nonsignificant) increase in EbpC surface display (see Fig. S3 in the supplemental material); however, we postulate that a major consequence of the ATT start codon is on EbpA levels exposed on E. faecalis cells, as we previously described that there are considerable amounts of EbpA monomers on the cell surface (6).

We inferred that the reduced levels of EbpA on the surface of E. faecalis OG1RF and TX5732 are a consequence of reduced rates of translation when ATT is present as the start of EbpA protein synthesis. Our results using translational reporter fusions to lacZ also indicate that, in E. faecalis, ATG is a more efficient start codon for the initiation of EbpA translation than ATT (Fig. 4; also see Fig. S4 in the supplemental material), which is in accordance with the hierarchy of start codon efficiencies proposed in E. coli (23). Although other signals and factors play a role in the rate of translation initiation (23), including the Shine–Dalgarno sequence and the initiation factors IF1, IF2, and IF3 (24), evidence in E. coli indicates that, in the presence of an ATT start codon, IF3 increases the dissociation of the initiation complex, which includes the 30S ribosomal subunit, the specific initiator tRNA, the mRNA, and the three initiation factors (18). It is interesting that β-galactosidase activity, which is a reflection of the levels of the EbpA-LacZ fusion protein inside the cell, was increased approximately 3.5-fold when the reporter fusion start codon was ATG versus ATT (Fig. 4), but a more modest increase (approximately 1.5-fold) was observed in EbpA surface display on TX5731 carrying ATG as the ebpA start codon than on the strains carrying ATT (OG1RF and TX5732) (Fig. 3). One possibility that could explain this difference would be the existence of additional regulatory mechanisms participating in the regulation of EbpA levels on the surface of the cells. In light of the current model of Ebp assembly in E. faecalis (6, 14), one could infer that modulating the ratios of the major backbone subunit, EbpC, to the minor subunits, EbpA and EbpB, could be important for pilus biogenesis, since an individual pilus fiber is composed of multiple EbpC subunits while in theory only one EbpA and one EbpB subunit are required for the tip and base of one pilus fiber, respectively (6, 14). Therefore, it seems plausible that the ATT start codon of ebpA is a way to regulate the ratio of EbpA to EbpC in E. faecalis.

Ebp are considered one of the major virulence factors of E. faecalis, playing a role in biofilm formation, adherence to fibrinogen, and the ability of E. faecalis to cause endocarditis and infection in mouse models of ascending UTI and CAUTI (4–8, 14, 15). EbpA has been demonstrated to be the most important pilin in biofilm formation (6, 15), while deletion of ebpC, encoding the major pilin, had a minor effect (6) despite abrogating pilus formation; this suggests that EbpA, in monomeric or dimeric form, on the surface of E. faecalis cells is capable of sustaining biofilm formation even when it is not part of a pilus polymer (6, 15). In addition, it has been shown that the MIDAS motif present in EbpA’s VWA domain is crucial for EbpA-mediated biofilm formation and fibrinogen binding (16). Proteins containing VWA domains, which are widely distributed among the three domains of life, Eukarya, Archaea, and Bacteria, often participate in cell adhesion and protein–protein interactions (25). Furthermore, other VWA-containing tip pilin proteins, including PilA of Streptococcus agalactiae and Rga of Streptococcus pneumoniae, have also been implicated in binding to ECM proteins (26, 27). Considering the demonstrated role of EbpA in biofilm formation (6, 15) and fibrinogen adherence (16), we believed that identity of the EbpA start codon would impact these processes, which have been associated with the ability of E. faecalis to cause infection. Indeed, we found that the presence of ATT, compared to ATG, as the start codon of EbpA protein synthesis resulted in less biofilm formation (Fig. 5) and decreased adherence to fibrinogen (Fig. 6). Although the reasons behind the advantages or disadvantages of negatively regulating the levels of pilation are unknown, it has been suggested that high expression of pilin surface proteins could involve a fitness cost to the bacteria due to the selective pressure exerted by the immune system (28). Indeed, Danne et al. demonstrated in Streptococcus galilolyticus that a mutant overexpressing pili showed reduced survival in human blood compared to a nonpiliated mutant (28). In addition, they demonstrated that THP-1 human macrophages showed better opsonophagocytosis of highly piliated bacterial cells than did their nonpiliated counterparts. Although we cannot discard the possibility that ATT as the start codon of EbpA protein synthesis has a role in E. faecalis human infections, the conservation of this rare codon in all sequenced E. faecalis strains implies that this change appeared long before enterococci became common human colonizers and pathogens, and we speculate that its presence aided E. faecalis in some way in the environment or an early host, perhaps due to the decreased adherence observed in weakly piliated cells, thus favoring dispersal. Regardless of why this change occurred, our results, taken together, provide the first example of pilus regulation through the use of a very rare initiation codon and support our hypothesis that “ATTenuation starts with ATT.”

MATERIALS AND METHODS

Bacterial strains, plasmids, and routine growth conditions. Bacterial strains and plasmids used in this study and their relevant characteristics are listed in Table 1. E. faecalis strains were routinely grown at 37°C using brain heart infusion (BHI) agar (Becton, Dickinson [BD], France). Tryptic soy broth (BD) supplemented with 0.25% (vol/vol) glucose (TSBG) and BHI broth supplemented with 40% (vol/vol) horse serum (Sigma-Aldrich, Saint Louis, MO) (BHI-S) were used for some experiments. Escherichia coli strains used for cloning experiments were grown at 37°C in/on Luria-Bertani (LB) medium (BD). Growth characteristics of OG1RF and its derivatives were assessed in BHI broth based on the optical density at 600 nm (OD600). In addition, samples were taken at 0, 3, 6, 8, and 24 h for CFU determination on BHI agar, as previously described (29).

Construction of mutants. Specific point mutations were generated by modifying a previously described methodology (30), based on the pHO1 vector (31) that carries the pheS allele that confers susceptibility to p-chloro-phenylalanine. Strains TX5751 and TX5731 were constructed using E. faecalis OG1RF as the parental strain, while a revertant strain, named TX5732, was generated by placing back the ebpAstart codon into the TX5731 strain background (Table 1). In brief, two external primers...
ers, ebpA-Ext-F-BamHI and ebpA-Ext-R-PstI, containing the BamHI and PstI restriction sites, respectively, and two internal complementary primers containing the desired change were designed (see Table S1 in the supplemental material). First, two independent PCRs using the respective external and internal primers were carried out. Then, the two PCR amplimers were joined together by a crossover PCR and the generated fragments, now containing the desired mutations (confirmed by sequencing), were cloned into the pGEM vector and then subcloned into pHOU1 (31) using the BamHI and PstI sites. The recombinant pHOU1 plasmids were propagated in E. coli EC1000 and then electroporated into E. faecalis CK11 (30). Subsequently, the recombinant pHOU1 plasmids were transferred into E. faecalis OG1RF (or TX5731) by filter mating with CK111, followed by culturing the gentamicin-resistant colonies that integrated the plasmid on MM9–yeast extract-glucose (MM9YE) medium supplemented with 10 mM p-chloro-phenylalanine as described in references 30 and 31, to select for the colonies from which the plasmid had excised. Sequencing and pulsed-field gel electrophoresis (PFGE) were performed to detect the mutations in the correct background.

Construction of translational lacZ fusion vectors and β-galactosidase assay. The region extending from 261 bp upstream to 31 bp downstream of the predicted ebpA ATT translational start codon was PCR amplified from E. faecalis OG1RF and its ebpA2* allele, mutant TX5731, digested with SalI and BamHI, and cloned into the pSD2 vector (32) (Table 1). The reporter gene of pSD2, lacZα, lacks a promoter, an RBS, and a coding sequence. Constructs, harboring either the native (pTEX5749) or the mutated (pTEX5750) form of the ebpA start codon, were propagated in E. coli TG1 before electroporation into E. faecalis OG1RF, TX5731, and TX5732. β-Galactosidase activity was assayed in TSBG or BHI-S, as previously described (33).

Flow cytometry. Flow cytometry analysis of E. faecalis strains was performed as previously described (12) with minor modifications. Cells grown in TSBG to mid-logarithmic phase were collected by centrifugation and washed twice with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS; pH 7.4). The bacterial cells were resuspended in 100 μl of the previously described affinity-purified polyclonal antibodies against EbpA (7) (1 μg/ml) and incubated for 30 min at room temperature (RT). After a washing step, secondary labeling was performed with a 1:100 dilution of phycoerythrin-conjugated goat anti-rabbit IgG for 30 min, and cells were washed and fixed with 1% paraformaldehyde for analysis using a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA).

WC-ELISA. EbpA and EbpC surface display by OG1RF and the panel of mutants was evaluated as described above, with minor modifications (6). Briefly, bacteria grown overnight in TSBG were diluted in the same medium to an OD500 of 0.1 and incubated until they reached mid-logarithmic phase. Cells were collected by centrifugation and washed twice with PBS before they were resuspended in 50 mM carbonate-bicarbonate buffer, pH 9.6, to an OD500 of 1.0. Immulon 1B plate wells (Thermo Scientific, Woburn, MA) were coated for 1 h with E. faecalis cells, followed by two washes with PBS containing 0.05% Tween 20 (PBS-T). The wells were then blocked for 1 h with 2% bovine serum albumin (BSA), followed by a 1 h incubation with affinity-purified polyclonal antibodies against EbpA and EbpC, respectively (1:5,000 dilution of 1 mg/ml) (7). After three washes with PBS-T to remove the unbound antibodies, goat anti-rabbit F(ab′)2 fragment conjugated to alkaline phosphatase (AP) (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:5,000 dilution) was added and incubated for 1 h. Next, the wells were washed twice with PBS-T and once with PBS, followed by the addition of AP substrate solution. The absorbance at 405 nm was measured with a microplate reader (Thermo Scientific, Waltham, MA).

Biofilm formation assay. Biofilm density was measured as previously described (34), with some modifications. In brief, E. faecalis strains from overnight cultures in TSBG broth were diluted in the same medium to an OD500 of 0.1 and grown statically for 3 or 24 h at 37°C in 96-well polystyrene plates (BD, Franklin Lakes, NJ). The plates were gently washed with PBS, and then the cells were fixed with Bouin’s solution (Sigma-Aldrich Co., St. Louis, MO) for 30 min. After two washes with PBS, bacterial cells were stained with a 1% crystal violet solution (Sigma-Aldrich Co., St. Louis, MO) for 30 min. Excess crystal violet was removed by rinsing thoroughly with distilled water followed by the addition of ethanol-acetone (80:20) to solubilize the dye and dissolve the biofilms. The absorbance at 570 nm was measured with a microplate reader (Thermo Scientific, Waltham, MA). Two independent experiments were performed in duplicate (8 wells per strain each in duplicate).

Fibrinogen binding assay. E. faecalis adherence to immobilized fibrinogen was assayed using the CytoSelect cell adhesion assay kit (Cell Biolabs, San Diego, CA). First, E. faecalis OG1RF and its derivatives from overnight cultures grown at 37°C in BHI-S were normalized to an OD600 of 0.05 and cultured to mid-log phase. Bacterial cells were collected by centrifugation, washed three times with PBS, and resuspended in 0.5% BSA to an OD600 of 1.0. A volume of 150 μl of the cell suspension was added to the fibrinogen-precoted wells and incubated for 1 h at 37°C. The unbound bacteria were removed by gently washing each well two times with PBS. Next, 200 μl of the cell stain solution was added to each well and incubated for 10 min at RT, followed by two washes with deionized water. After air-drying the wells, 200 μl of extraction solution was added per well. The plate was incubated for 10 min on a shaker, and then 150 μl from each extracted sample was transferred to a 96-well microtiter plate (Becton, Dickinson, Franklin Lakes, NJ). The absorbance at 570 nm was measured with a microplate reader (Thermo Scientific, Waltham, MA).

Statistical analyses. Analysis of variance (ANOVA) with Bonferroni’s multiple comparison posttest was used to compare the results. GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00467-15/-/DCSupplemental.

Figure S1, TIF file, 1.9 MB.
Figure S2, TIF file, 1.0 MB.
Figure S3, TIF file, 2.1 MB.
Figure S4, TIF file, 1.1 MB.
Table S1, DOXX file, 0.1 MB.

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