Enterococcus faecium Biofilm Formation: Identification of Major Autolysin AtlA_{Efm}, Associated Acm Surface Localization, and AtlA_{Efm}-Independent Extracellular DNA Release

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ABSTRACT Enterococcus faecium is an important multidrug-resistant nosocomial pathogen causing biofilm-mediated infections in patients with medical devices. Insight into E. faecium biofilm pathogenesis is pivotal for the development of new strategies to prevent and treat these infections. In several bacteria, a major autolysin is essential for extracellular DNA (eDNA) release in the biofilm matrix, contributing to biofilm attachment and stability. In this study, we identified and functionally characterized the major autolysin of E. faecium E1162 by a bioinformatic genome screen followed by insertional gene disruption of six putative autolysin genes. Insertional inactivation of locus tag EfmEI162_2692 resulted in resistance to lysis, reduced eDNA release, deficient cell attachment, decreased biofilm, decreased cell wall hydrolysis, and significant chaining compared to that of the wild type. Therefore, locus tag EfmEI1162_2692 was considered the major autolysin in E. faecium and renamed atlA_{Efm}. In addition, AtlA_{Efm} was implicated in cell surface exposure of Acm, a virulence factor in E. faecium, and thereby facilitates binding to collagen types I and IV. This is a novel feature of enterococcal autolysins not described previously. Furthermore, we identified (and localized) autolysin-independent DNA release in E. faecium that contributes to cell-cell interactions in the atlA_{Efm} mutant and is important for cell separation. In conclusion, AtlA_{Efm} is the major autolysin in E. faecium and contributes to biofilm stability and Acm localization, making AtlA_{Efm} a promising target for treatment of E. faecium biofilm-mediated infections.

IMPORTANCE Nosocomial infections caused by Enterococcus faecium have rapidly increased, and treatment options have become more limited. This is due not only to increasing resistance to antibiotics but also to biofilm-associated infections. DNA is released in biofilm matrix via cell lysis, caused by autolysin, and acts as a matrix stabilizer. In this study, we identified and characterized the major autolysin in E. faecium, which we designated AtlA_{Efm}, atlA_{Efm} disruption resulted in resistance to lysis, reduced extracellular DNA (eDNA), deficient cell attachment, decreased biofilm, decreased cell wall hydrolysis, and chaining. Furthermore, AtlA_{Efm} is associated with Acm cell surface localization, resulting in less binding to collagen types I and IV in the atlA_{Efm} mutant. We also identified AtlA_{Efm}-independent eDNA release that contributes to cell-cell interactions in the atlA_{Efm} mutant. These findings indicate that AtlA_{Efm} is important in biofilm and collagen binding in E. faecium, making AtlA_{Efm} a promising target for treatment of E. faecium infections.
creased by 60%. A decrease of only 20% in the OD600 was detected in the AtlAEfm-deficient mutant of \textit{E. faecium}, indicating a significant role for AtlAEfm in cell separation, a finding which suggests that eDNA contributes to the chaining phenotype in the AtlAEfm-deficient mutant.

RESULTS

Identification of putative \textit{E. faecium} autolysins. Six putative autolysin-encoding genes were selected by bioinformatic analysis, and single-crossover mutants were constructed. Locus tag EfmE1162_2692 has the highest similarity and sequence coverage compared to AtlA in \textit{E. faecalis} (51% amino acid similarity and 80% sequence coverage) (see Table S1 in the supplemental material). Growth curves of these six mutants in brain heart infusion (BHI) broth showed no difference compared to the wild type; however, in tryptic soy broth (TSBg) medium with 1% glucose (TSBg), all strains grew to a lower optical density at 600 nm (OD\text{600}) than with growth in BHI medium (Fig. S1A and B). Mutant 0587::pWS5 showed earlier log phase in TSBg than did other strains.

The putative autolysin locus tag EfmE1162_2692 is resistant to Triton X-100, exerts peptidoglycan hydrolysis activity, and is involved in cell separation and cell adhesion. Several functional assays were performed to characterize the autolysis activity of the six predicted autolysins. In early stationary phase, bacterial cells are known to be more vulnerable to cell lysis when exposed to Triton X-100 while major autolysin mutants show less lysis (18). Triton X-100 lysis assays of E1162 wild type and the six putative autolysin mutants revealed that with the exception of mutant 2692::pWS9, the OD\text{600} of the wild type and the mutants was decreased by 60%. A decrease of only 20% in the OD\text{600} was detected in mutant 2692::pWS9, confirming a resistance to lysis (Fig. 1). Therefore, we renamed locus tag EfmE1162_2692 to \textit{atlAEfm}.

Although the observed phenotype of 2692::pWS9 is similar to that of other major autolysins, we attempted to construct a markerless mutant of \textit{atlAEfm} (see Materials and Methods), but unfortunately, this was unsuccessful. As an alternative, we selected from a mariner transposon library in E1162 a mutant (\textit{atlAEfm}\textasciitilde{genta}) that contained an insertion of a gentamicin resistance gene at position 278 relative to the start of the gene. This insertion of a single resistance gene is considerably smaller than pWS9 and even less likely to result in polar effects on adjacent genes that encode a type 2 phosphatidic acid phosphatase family protein (upstream) and a leucyl-tRNA synthetase (downstream). However, to confirm that all observed phenotypes were attributed only to insertional inactivation of \textit{atlAEfm}, we complemented \textit{atlAEfm}\textasciitilde{genta} in \textit{trans}, resulting in \textit{atlAEfm}\textasciitilde{genta}+\textit{atlAEfm}.

Growth curves of \textit{atlAEfm}\textasciitilde{genta} in BHI medium showed no difference compared to the wild type, while in TSBg, \textit{atlAEfm}\textasciitilde{genta} reached a higher OD in stationary phase (see Fig. S1A and B in the supplemental material). As shown in \textit{atlAEfm}\textasciitilde{genta}::pWS9, \textit{atlAEfm}\textasciitilde{genta} is also resistant to lysis when analyzed in the Triton X-100 assay (Fig. 1).

\textit{AtlAEfm} contains six LysM domains which are conserved in other major autolysins and have been shown to be able to bind and hydrolyze peptidoglycan from the cell wall, causing cell death (19). To confirm hydrolysis of peptidoglycan by \textit{AtlAEfm} supernatants of the wild type, all predicted autolysin mutants, and the complemented strain \textit{atlAEfm}\textasciitilde{genta}+\textit{atlAEfm} were subjected to zymography. In Fig. 2, three autolytic bands of 75, 50, and 37 kDa were observed. Mutants 2692::pWS9 and \textit{atlAEfm}\textasciitilde{genta} lacked the 75-kDa band compared to the wild type and the complemented strain, a band which is the expected size of the major autolysin \textit{AtlAEfm}. The second band of approximately 50 kDa was missing from the mutant 1800::pWS6. Fifty kilodaltons is the expected size of locus tag EfmE1162_1800 (46.8 kDa), indicating that this gene possibly encodes a secondary autolysin. None of the screened mutants lacked the third autolytic band (37 kDa).

By hydrolyzing peptidoglycan, autolysins can play a role in cell separation and growth. Chaining of bacterial cells, a result of impaired cell partitioning, has been observed in other species when the major autolysin gene is inactivated. In overnight cultures, long chains of \textit{atlAEfm}\textasciitilde{genta} were observed, implying the cell separation defect (see Fig. S2A to H in the supplemental material). No chaining was observed in the other predicted autolysin mutants or in the complemented \textit{atlAEfm} mutant.

Since in other bacterial species, autolysins are also implicated in early attachment of cells, we subsequently studied early adhesion on polystyrene of the wild type and the six putative autolysin mutants. Only \textit{atlAEfm}\textasciitilde{genta} had substantially fewer cells attached.

FIG 1  Autolysis induction by Triton X-100 (0.02%). \textit{E. faecium} wild type, predicted autolysin mutants, and the complemented \textit{AtlAEfm}-deficient strain were incubated in the Bioscreen C system at 37°C with continuous shaking, and absorbance at 600 nm (OD\text{600}) was recorded every 15 min for 21 h. On the x axis, time is indicated in hours (h); on the y axis is the percentage of surviving cells in suspension.

FIG 2  Zymography assay. Hydrolysis of peptidoglycan was analyzed in an SDS-PAGE gel (12%) with crude wild-type cell wall. Lane 1, Precision Plus Protein standards dual color (Bio-Rad); lane 2, wild type; lane 3, 2692::pWS9; lane 4, 1800::pWS6; lane 5, 0244::pWS4; lane 6, 0587::pWS5; lane 7, 0008::pWS7; lane 8, 1543::pWS8; lane 9, \textit{atlAEfm}\textasciitilde{genta}; lane 10, \textit{atlAEfm}\textasciitilde{genta}+\textit{atlAEfm}.
than the wild type (Fig. 3), while attachment was restored in the complemented strain atlAEfm::genta.

In conclusion, atlAEfm::genta not only was less vulnerable to Triton X-100 and less capable of initial adherence but also reduced peptidoglycan hydrolysis activity and exhibited long chaining, supporting that this gene serves as the actual major autolysin in E. faecium.

**AtlAEfm is a prerequisite for normal biofilm development in E. faecium.** We studied the effect of autolysin mutants on biofilm formation in three different models: polystyrene, semistatic, and flow cell models. In the polystyrene model, all predicted autolysin mutants were tested, and only atlAEfm::genta formed 80% less biofilm than the wild type while biofilm formation was restored in the complemented mutant (see Fig. S3 in the supplemental material).

Due to this result, only atlAEfm::genta was tested in the other assays. In the semistatic model, biofilm formation of atlAEfm::genta was considerably reduced compared to that of the wild type. Average thickness was reduced by 80% while biomass was reduced by 90% relative to that of the wild type. This phenotype was complemented in atlAEfm::genta + atlAEfm (Fig. 4A to C).

In a flow cell model, biofilm formation of the AtlAEfm mutant was also attenuated. In this model, wild-type cells firmly attached to the slide, formed small clumps, and developed into thick biofilm covering the whole slide within a few hours. In contrast, atlAEfm::genta was impaired in initial attachment and subsequently formed only small and thin clumps, which were not stable enough to develop to mature biofilm at 17 h of growth (see Movie S1A in the supplemental material). Again, the wild-type

**FIG 3** Adhesion assay. The wild type, predicted autolysin mutants, and the complemented AtlAEfm-deficient strain at an OD660 of 0.3 were incubated for 2 h at 37°C without shaking in a 96-well plate. Cell attachment was measured by absorbance of crystal violet at 595 nm.

**FIG 4** Confocal microscopy images of the semistatic biofilm model and the flow cell biofilm model. Biofilm of the wild type (A), atlAEfm::genta (B), and atlAEfm::genta + atlAEfm (C) was grown for 24 h on polystyrene-coated glass in TSBg at 120 rpm at 37°C. Cells were stained with propidium iodide (red). The average thickness and biomass of biofilms were measured at five random positions and analyzed with Comstat/Matlab software. The average thickness was 1.86 μm for the wild type, 0.14 μm for atlAEfm::genta, and 0.84 μm for atlAEfm::genta + atlAEfm. The mean biomass was 0.73 μm³/μm² for the wild type, 0.03 μm³/μm² for atlAEfm::genta, and 0.45 μm³/μm² for atlAEfm::genta + atlAEfm. The difference between the wild type and the mutant was significant ($P < 0.005$). Pictures were taken at ×63 magnification with a 2.5 optical zoom. In the flow cell model, biofilm was grown for 17 h in a Stovall flow cell system in 1:10 TSB with 1% glucose (0.13 ml/min) at 37°C. Pictures were taken at ×40 magnification with a 2.5 optical zoom for the wild type (D), a 3.5 optical zoom for atlAEfm::genta (E), and a 2.5 optical zoom for atlAEfm::genta + atlAEfm (F). Cells were stained with Syto 9 (green) and propidium iodide (red).
phenotype was restored when the mutant was complemented (Fig. 4D to F).

**AtlAEfm** is cell surface localized at the septum and is secreted in biofilm. In other bacterial species, the major autolysin has been identified both in the supernatant of planktonic cells and localized to the bacterial surface (11, 20). We used AtlAEfm-specific antibodies to detect cell surface expression of AtlAEfm in planktonic cells. Flow cytometry analysis demonstrated that AtlAEfm was detected at the wild-type cell surface, with higher expression in log phase than stationary phase (data not shown). AtlAEfm antibodies were specific, since in all of the following experiments, incubations with secondary antibodies alone yielded no signal.

In addition, confocal microscopy showed that AtlAEfm was also localized at the septum of cells in log phase, supporting a role in cell separation, as already concluded from the chaining phenotype in the mutant (Fig. 5A). As expected, no AtlAEfm signal was observed from the mutant (Fig. 5B). In the complemented strain (Fig. 5C), the same septum localization as that identified in the wild type was observed. In 24-h biofilms, AtlAEfm was not specifically localized at the septum of the cells but was identified between cells in the matrix and randomly around a subset of cells. In the mutant, again, no AtlAEfm was detected, while the complemented strain exhibited the same localization of AtlAEfm as the wild type (Fig. 5D to F).

**AtlAEfm contributes to eDNA release and eDNA-dependent biofilm formation.** By exerting lysis activity, autolysins can contribute to eDNA release in planktonic cells and also in biofilm, thereby contributing to biofilm formation and stability. The presence of eDNA in *E. faecium* wild type, atlAEfm::genta, and atlAEfm::genta + atlAEfm was analyzed by isolation of DNA present in the supernatant of overnight cultures. As shown in Fig. S4 in the supplemental material, supernatant of atlAEfm::genta contained less DNA than the wild type and the complemented strain. To further investigate the role of eDNA in biofilm, we studied the effect of DNase in a semistatic biofilm model. Incubation with DNase at a t of 0 reduced biofilm biomass and average biofilm thickness by 94% and 91%, respectively, in the wild type and atlAEfm::genta + atlAEfm after 24 h of biofilm growth (Fig. 6A, B, E, and F). These results show that eDNA is important in biofilm formation. Unexpectedly, incubation of the mutant with DNase leads to separation of bacterial chains into single cells and increased cell attachment but not clump and biofilm formation (Fig. 6C and D). Similar to the biofilm semistatic model, chains were separated within 5 min during planktonic growth in an eight-well chamber slide when DNase was added to atlAEfm::genta (Movie S1B).

To further investigate how DNase treatment results in chain separation, we incubated 4-h and 24-h biofilms and overnight-cultured planktonic cells with double-stranded DNA (dsDNA) antibodies. Using confocal microscopy, we were able to detect DNA at the cell septum in wild-type 4-h early biofilm (Fig. 7A and B) and between the chains of overnight planktonic growth of atlAEfm::genta (Fig. 7C and D), results which concur with our observations of the effect of DNase on cell separation in the chained atlAEfm::genta and suggest that eDNA contributes to the chaining
phenotype in the AtlAEfm-deficient mutant. Immunoelectron microscopy clearly showed the chaining phenotype in the AtlAEfm-deficient mutant, confirmed by \( /H9251\)-dsDNA gold incubation labeling of eDNA at the cell septum of \( \text{atlAEfm::genta} \) chains from overnight cultures (Fig. 7E and F). In both methods, incubation with only secondary antibodies of the same isotype did not give specific signals (see Fig. S5E and F in the supplemental material). In 4-h and 24-h \( \text{atlAEfm::genta} \) biofilms, DNA was also observed between the cells (Fig. S5B and C).

The effect of exogenous AtlAEfm on phenotypes of wild-type and mutant strains was studied using confocal microscopy. For this, the wild type, \( \text{atlAEfm::genta} \), and \( \text{atlAEfm::genta/H11001} \) were incubated with or without AtlAEfm in BHI in an eight-well chamber. When AtlAEfm was added, as with DNase, chains started to be released into separate cells in the AtlAEfm-deficient mutant, but unlike DNase, clumping of cells occurred in all strains. It is important to mention that this phenomenon was observed in BHI, a medium in which \( E. faecium \) forms less-established biofilms (see Movie S1C in the supplemental material). In 4-h and 24-h \( \text{atlAEfm::genta} \) biofilms, DNA was also observed between the cells (Fig. S5B and C).

FIG 6 Effect of DNase in biofilm formation. Confocal images of wild-type biofilm, cultured without (A) and with (B) DNase, \( \text{atlAEfm::genta} \) biofilm cultured without (C) and with (D) DNase, and \( \text{atlAEfm::genta/atlAEfm} \) biofilm cultured without (E) and with (F) DNase. Twenty-four-hour biofilm was grown in a semistatic model in TSBgl at 120 rpm at 37°C. Cells were stained with propidium iodide. When DNase was added, the average thickness was 0.068 \( \mu \text{m} \) for the wild type, 0.099 \( \mu \text{m} \) for \( \text{atlAEfm::genta} \), and 0.10 \( \mu \text{m} \) for \( \text{atlAEfm::genta/atlAEfm} \). The mean biomass was 0.019 \( \mu \text{m}^3/\mu \text{m}^2 \) for the wild type, 0.037 \( \mu \text{m}^3/\mu \text{m}^2 \) for \( \text{atlAEfm::genta} \), and 0.035 \( \mu \text{m}^3/\mu \text{m}^2 \) for \( \text{atlAEfm::genta/atlAEfm} \). The difference between biofilms with and without DNase was significant for the wild type and the complemented AtlAEfm-deficient strain (P < 0.05). Pictures were taken at \( \times63 \) magnification with a 2.5 optical zoom.

FIG 7 Confocal microscopy and transmission electron microscopy close-up images of eDNA. Pictures were taken at \( \times63 \) magnification with 6.6 and 15.5 optical zooms for wild-type 4-h biofilm (A and B) and 27.0 optical zooms for overnight-cultured \( \text{atlAEfm::genta} \) chains from overnight cultures (Fig. 7E and F). In both methods, incubation with only secondary antibodies of the same isotype did not give specific signals (see Fig. S5E and F in the supplemental material). In 4-h and 24-h \( \text{atlAEfm::genta} \) biofilms, DNA was also observed between the cells (Fig. S5B and C).

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These results indicate that AtlAEfm plays different roles in biofilm, where it contributes to biofilm stability via eDNA release, and during planktonic growth, where it may induce cell clumping.

**AtlAEfm influences surface protein localization and binding to collagen types I and IV.** Some autolysins are known to act as adhesins and may bind extracellular matrix (ECM) proteins (microbial surface components recognizing adhesive matrix molecules [MSCRAMMs]). Therefore, we verified the role of AtlAEfm in binding to ECM proteins using a whole-cell enzyme-linked immunosorbent assay (ELISA). When the wild type, \( \text{atlAEfm::genta} \), and \( \text{atlAEfm::genta/atlAEfm} \) were incubated with \( \alpha\)-dsDNA and goat \( \alpha\)-mouse Alexa 488 (green). The bacterial membrane was stained with FM 95-5 (red). Transmission electron microscopy of \( \text{atlAEfm::genta} \) overnight cultures (E and F). Cells were incubated with \( \alpha\)-dsDNA and rabbit \( \alpha\)-mouse of the same isotype. Immunogold particles are seen linked to wire-like structures between the cells as well as to polymeric structures outside cells, which likely represents released DNA.

Since AtlAEfm seemed not directly involved in collagen binding, we hypothesized that AtlAEfm contributed to cell surface expression of another collagen-binding surface protein. Nallapareddy et al. (21) described Acm, a collagen-binding adhesin,
Like the major autolysin of N-terminal domain, the function of which is still unknown (23), sins to the division septum and poles (24), and a T/E-rich rules that afford peptidoglycan affinity and possibly target autolys- dase activity (23), a C-terminal domain, composed of LysM mod- domains, a central catalytic domain, responsible for glucosamini- 

which, so far, is the only E. faecium surface protein that specifically binds to collagen types I and IV.

Confocal microscopy using α-Acm antibodies revealed that Acm was localized at the septum and at the poles in the wild type and the complemented strain, while in atlAEfm::genta, Acm was localized only at the septum (Fig. 9A to C). Control antibodies from the same isotype gave no labeling (see Fig. S6B in the supplemental material). Absence of exposed Acm at the poles of the AtlAEfm-deficient mutant most probably resulted from the defect in cell separation in this strain and may explain the reduced binding to collagen types I and IV. Specificity of α-Acm antibodies was confirmed, since flow cytometry revealed, unlike with the E1162 wild type, no Acm expression in strain E980, a commensal isolated with a transposon insertion in atlAEfm (Fig. S6A).

To confirm the later hypothesis, the AtlAEfm-deficient mutant was tested for collagen type I and IV binding in a whole-cell ELISA in the presence of DNase, which, as demonstrated above, resolves the long chains in this mutant. As expected, incubation of the atlAEfm::genta mutant with DNase restored binding to collagen types I and IV (Fig. 8).

**DISCUSSION**

In this study, we identified and characterized the major autolysin in E. faecium, AtlAEfm, and demonstrated that AtlAEfm, as in other species, is directly involved in biofilm formation, cell separation, cell wall hydrolysis, and eDNA release (22). Furthermore, the AtlAEfm-deficient mutant displayed attenuated cell localization of Acm.

Based on sequence similarity, AtlAEfm is composed of three domains, a central catalytic domain, responsible for glucosaminidase activity (23), a C-terminal domain, composed of LysM modules that afford peptidoglycan affinity and possibly target autoly- sins to the division septum and poles (24), and a T/E-rich N-terminal domain, the function of which is still unknown (23). Like the major autolysin of E. faecalis (14), AtlAEfm contains a lysozyme-like superfamily domain and six LysM domains which are not present in any of the other putative autolysins analyzed. Lysozyme-like superfamily domains are known to be involved in the hydrolysis of beta-1,4-linked polysaccharides (25). Moreover, atlAEfm is present in all sequenced E. faecium strains, suggesting a highly conserved and essential function.

Five types of experiments indicated that AtlAEfm is the major autolysin of E. faecium. First, the AtlAEfm-deficient mutant dem- onstrated considerably less cell lysis in the presence of Triton X-100 than did the wild type. Second, in the zymography, a lytic band of 75 kDa, which is identical to the predicted molecular mass of AtlAEfm, was identified as AtlAEfm. Third, the AtlAEfm-deficient mutant showed decreased early adhesion compared to that of the wild type. Fourth, devisel cell separation by chain formation was observed only in the AtlAEfm-deficient mutant, and finally, the AtlAEfm-deficient mutant had 80% less biofilm production than the wild type in a polystyrene assay. All phenotypes were able to be restored by complementation in trans. These five lines of evidence demonstrate that AtlAEfm plays a major role in E. faecium E1162 autolysis and should be considered the major autolysin. In addition to AtlAEfm, we identified a second autolysin (locus tag EfmE1162_1800) by zymography. In E. faecalis, a second autolysin, designated AtlB, has lytic properties and can act as a surrogate of AtlA but is less efficient in cell separation, probably due to the presence of fewer LysM domains (26). Also in E. faecium, locus tag EfmE1162_1800 has only two LysM domains, compared to the six domains in AtlAEfm. The exact function of this second autolysin remains to be investigated.

Confocal microscopic observations revealed that the AtlAEfm-deficient mutant formed long chains of cells compared to the wild type in planktonic growth and biofilm. The formation of long chains of cells upon inactivation of the major autolysin has also been observed previously (27, 28) and implies that autolysin plays a role in cell separation. Our findings that AtlAEfm was localized at the septum of cells in log phase and that addition of exogenous AtlAEfm resolved chained cells into single cells support a role of AtlAEfm in cell separation. Schlag et al. (19) elucidated the mechanism underpinning localized peptidoglycan hydrolysis in staphylococci. Although wall teichoic acid prevents S. aureus Atl from binding to the cell wall, binding of Atl through its amidase domain at the cell septum is increased, presumably due to a lower wall teichoic acid concentration (19). This mechanism explains why autolysin-derived peptidoglycan hydrolases are active primarily at the site of cell separation (29, 30). In E. faecalis, cell chaining in an Atl mutant disappeared after 24 h of culture, indicating that eventually AtlB can cleave the cell septum in the absence of AtlA (31). However, this does not apply to E. faecium, since chaining was also observed in overnight-cultured atlAEfm::genta and in all other conditions tested.

An unexpected finding was that addition of DNase to the AtlAEfm-deficient mutant also resulted in chains resolving into
single cells. This means that for the first time we have demonstrated that DNA plays a role in cell-cell interactions in *E. faecium*. In *E. faecalis*, DNA can be secreted by metabolically active cells in the absence of lysis, and this actively secreted eDNA is present at the cell septum and important in early biofilm formation (32). As in *E. faecalis*, we were able to demonstrate that in wild-type *E. faecium* and in the AtlAEfm-deficient mutant, eDNA is present at the cell septum. Specifically, the fact that this was also seen in the AtlAEfm-deficient mutant supports the hypothesis that also in *E. faecium*, eDNA was able to be released in the absence of a major autolysin and is involved in cell separation. Together, these results indicate that both peptidoglycan and eDNA are involved in cell-cell interactions upon cell division that allow both AtlAEfm and DNase to separate the bacterial chains. The exact organization of peptidoglycan and eDNA at the cell septum and the possible interaction between these two components are currently not known and remain to be elucidated.

We also demonstrated the presence of AtlAEfm in the biofilm matrix. As in *E. faecalis*, AtlAEfm might be involved in autolysis of a subpopulation in the biofilm by a fratricidal mechanism (14). In *E. faecalis*, this is mediated by the protease GelE and coordinated by a two-component system. Such a regulatory mechanism of AtlAEfm expression is currently unknown in *E. faecium*.

Incubation of a single layer of AtlAEfm-deficient mutant cells with purified AtlAEfm resulted in clump formation, suggesting that secretion of AtlAEfm contributes to this process. This indicates that AtlAEfm may have different functions during planktonic and biofilm lifestyles of *E. faecium*.

Biofilm formation is a multistep process that requires bacterial adhesion to a substrate followed by cell-cell interactions, resulting in multiple layers of biofilm. Apart from impaired initial adherence of the AtlAEfm-deficient mutant, which may be attributed to the chaining phenotype (33–35), the biofilm deficiency also seemed to result from reduced stability of the matrix in biofilm clumps. This is most probably due to reduced amounts of eDNA but might also be due to a lack of cohesive activity of AtlAEfm when secreted in biofilm.

Apart from biofilm formation, cell attachment through binding to ECM proteins is an important step in colonization. We observed reduced binding of the AtlAEfm-deficient mutant to collagen types I and IV, most likely due to altered localization of Acm. Peptidoglycan hydrolases, in general, have been described to be involved in surface protein assembly, specifically proteins related to secretion systems in Gram-negative bacteria (36). However, this is the first documentation of the role of a major autolysin in protein surface localization in Gram-positive bacteria. This observation implies an essential contribution of AtlAEfm to *E. faecium* colonization, since Acm seems to be the major collagen-binding surface protein. Collagen type I is the most common collagen in the human body and is found in all tissues, while collagen type IV is present exclusively in the basal lamina (37), for instance in the gut, which is an important niche of *E. faecium*.

**FIG 9** Confocal microscopy images of surface-localized Acm in planktonic cells. Pictures were taken at ×63 magnification with 5.9 and 29.5 optical zooms for the wild type (A), 4.6 and 12.0 optical zooms for atlAEfm::genta (B), and 6.5 and 39.1 optical zooms for atlAEfm::genta + atlAEfm (C). Planktonic cells were cultured in BHI broth until stationary phase at 37°C. Cells were incubated with α-Acm and goat α-rabbit Alexa 488 (green). The bacterial membrane was stained with FM95-5 (red).
The results presented here provide insights into how AtlA_Efm, a newly identified major autolysin in *E. faecium*, plays an important role in cell lysis and separation, biofilm formation, cell adhesion, and Acm localization and thus may contribute to the pathogenesis of *E. faecium* infections. Expression, distribution, and activation of the autolysin are probably tightly controlled in *E. faecium,* and these mechanisms need to be elucidated.

Nosocomial *E. faecium* infections are caused by multiresistant strains, which tend to even become panresistant. In addition, current drugs fail to eradicate biofilms and new drugs are barely needed. Although animal models did not show a reduction in virulence of AtlA-deficient mutants in *E. faecalis* (38), we expect AtlA_Efm to behave differently. First, genetic inactivation of the major autolysin AtlA_Efm affects not only biofilm formation but also binding to the ECM collagen types I and IV. Second, after prolonged growth, no apparent replacement of AtlA_Efm function by the secondary autolysin, as in *E. faecalis*, occurs. Therefore, AtlA_Efm may serve as a promising candidate for development of new targeted strategies in the treatment of *E. faecium* biofilm-related infections (39).

### MATERIALS AND METHODS

**Identification of putative *E. faecium* autolysins.** A bioinformatic screen was performed to identify putative autolysins in the *E. faecium* E1162 genome (40). Using BLASTp (http://www.ncbi.nlm.nih.gov/), six genes were selected for further characterization based on amino acid similarity to atlA from *E. faecalis* V583 (see Table S1 in the supplemental material).

**Bacterial strains, plasmds, growth conditions, and determination of growth curves.** *E. faecium* and *Escherichia coli* strains used in this study are listed in Table S2 in the supplemental material. The ampicillin-resistant *E. faecium* strain E1162, recently sequenced in our lab, was used throughout this study (40). Unless otherwise mentioned, *E. faecium* was grown in BH at 37°C. For biofilm assays, TSBg was used. *E. coli* DH5α, BL21 (Invitrogen), and ECI000 (41) were grown in Luria-Bertani (LB) medium. Where necessary, antibiotics (Sigma-Aldrich, Saint Louis, MO) were used at the following concentrations: gentamicin, 300 μg/ml (*E. faecium*) and 25 μg/ml (*E. coli*); spectinomycin, 300 μg/ml (*E. faecium*) and 100 μg/ml (*E. coli*); erthyromycin, 50 μg/ml with added lincomycin at 50 μg/ml (*E. faecium*) and 150 μg/ml (*E. coli*); and ampicillin, 150 μg/ml (*E. coli*). Determination of growth curves in triplicate was described by Zhang et al. (42) using a Bioscreen C instrument (Oy Growth Curves AB).

**Gene disruption and mutation complementation.** Predicted autolysin genes were disrupted and complemented as described before (43). Gene-specific primers and modified pWS3 vectors are mentioned in Table S3 in the supplemental material, resulting in single-crossover mutant strains in Table S2. To confirm the phenotype of 2692::pWS9, we tried to construct a markerless mutant using the approach previously described by us (42); Table S2 in the supplemental material, resulting in single-crossover mutant strains in Table S3. To confirm the phenotype of 2692::pWS9, we tried to construct a markerless mutant using the approach previously described by us (42); Table S2 in the supplemental material, resulting in single-crossover mutant strains in Table S3. To confirm the phenotype of 2692::pWS9, we tried to construct a markerless mutant using the approach previously described by us (42); Table S2 in the supplemental material, resulting in single-crossover mutant strains in Table S3. To confirm the phenotype of 2692::pWS9, we tried to construct a markerless mutant using the approach previously described by us (42); Table S2 in the supplemental material, resulting in single-crossover mutant strains in Table S3. To confirm the phenotype of 2692::pWS9, we tried to construct a markerless mutant using the approach previously described by us (42); Table S2 in the supplemental material, resulting in single-crossover mutant strains in Table S3. To confirm the phenotype of 2692::pWS9, we tried to construct a markerless mutant using the approach previously described by us (42); Table S2 in the supplemental material, resulting in single-crossover mutant strains in Table S3. To confirm the phenotype of 2692::pWS9, we tried to construct a markerless mutant using the approach previously described by us (42); Table S2 in the supplemental material, resulting in single-crossover mutant strains in Table S3. To confirm the phenotype of 2692::pWS9, we tried to construct a markerless mutant using the approach previously described by us (42); Table S2 in the supplemental material, resulting in single-crossover mutant strains in Table S3. To confirm the phenotype of 2692::pWS9, we tried to construct a markerless mutant using the approach previously described by us (42); Table S2 in the supplemental material, resulting in single-crossover mutant strains in Table S3. To confirm the phenotype of 2692::pWS9, we tried to construct a markerless mutant using the approach previously described by us (42); Table S2 in the supplemental material, resulting in single-crossover mutant strains in Table S3. To confirm the phenotype of 2692::pWS9, we tried to construct a markerless mutant using the approach previously described by us (42); Table S2 in the supplemental material, resulting in single-crossover mutant strains in Table S3.

**Gene expression and purification of recombinant AtlA_Efm.** The E1162 *atlA_Efm* gene was amplified with primers *atlA_Efm*-comp-BamHI-R and *atlA_Efm*-comp-XhoI-F (Table S3) and cloned in pMSP3535, resulting in pMSP3535. pMSP3536 was introduced in *E. coli* by electroporation, resulting in *atlA_Efm*-genta + *atlA_Efm*-genta. The purified *atlA_Efm* was obtained by the method described by Bardoel et al. (49).

**Zymography.** A zymographic analysis of cell wall-associated hydro- lases was conducted as described by Liu et al. (45) and Ju et al. (46) with some modifications. Briefly, extracellular murein hydrolyses of the wild type, all mutants, and the complemented strain *atlA_Efm*-genta + *atlA_Efm*-genta were collected from 50 ml BHI medium at an OD660 of 0.3. To prepare substrates for the zymogram, 300 ml of wild-type overnight BHI culture was used to extract the cell wall from the bacteria. Lytic activity as a result of cell wall hydrolysis appeared as a white band on a blue background.

**Initial polystyrene adherence assay.** The initial adherence assay was performed in triplicate as described previously with some modifications (47). *E. faecium* strains cultured overnight in BHI were diluted up to an OD660 of 0.05 and grown until an OD660 of 0.3. Two-hundred-microliter bacterial suspensions were added in triplicate to the polystyrene plate. Bacteria were stained with crystal violet and eluted with 96% ethanol.

**Biofilm polystyrene assay.** The biofilm polystyrene assay was performed in triplicate as described previously with some modifications (47). In brief, plate-grown bacteria were resuspended in TSBg and the OD660 was measured. Cell suspensions were diluted to an OD660 of 0.01 and incubated for 24 h. The plates were washed and stained as described above.

**Biofilm semistatic model and CLSM.** For the biofilm semistatic model, overnight bacterial cultures were diluted to an OD660 of 0.01 in 6 ml TSBg and added to a coverslip coated with poly-γ-L-lysine (0.45 μm; diameter, 12 mm; Becton Dickinson) inside a well from a six-well polystyrene plate (Corning Inc.). When applied, 1.5 μg/μl of DNase (Roche) was titrated at the start of the biofilm (*t* = 0). Biofilms were grown at 37°C for 24 h at 120 rpm. After 24 h, the coverslips were washed once with 0.85% NaCl, and the biofilms were chemically fixed in 8% glutaraldehyde (Merck) for 20 min and washed again with 0.85% NaCl. The biofilms were stained with 15 μg/ml propidium iodide (PI) in 0.85% NaCl for 15 min. After incubation, the stain was removed and coverslips were transferred to glass microscope slides and analyzed by a confocal laser scanning microscope (CLSM) (Leica SP5), equipped with an oil plan-Neofluar ×63/1.4 objective. PI was excited at 633 nm. Z stacks were taken with an interval of 0.42 μm. Pictures were analyzed with LAS AF software (Leica), and biofilm was quantified using Comstat (48)/Matlab R2010b software (the MathWorks). The average thickness and biomass of the biofilms were measured at five randomly chosen positions.

**Flow cell biofilm model.** Dynamic biofilms were studied in a Stovall flow cell system (Life Science, Inc., Greensboro, NC). Twenty-four-hour TSB cultures were diluted until an OD660 of 0.01 and inoculated in the flow chambers. Biofilms were grown in TSB diluted in PBS (1:10 [vol/vol]) with 1% glucose under a flow of 0.13 ml/min during 17 h. Biofilm development was scanned at regular intervals of 7 min (×40 objective) with a DFC360 FX digital camera kit SP5 (Leica). After 17 h, the flow was increased to 0.5 ml/min to wash away loose cells. Final biofilms were stained with live/dead stain (BAC light kit; Invitrogen), and images were acquired using a confocal microscope (Leica SP5).

**cDNA isolation.** Total cDNA from the E1162 wild type, *atlA_Efm*-genta, and *atlA_Efm*-genta + *atlA_Efm*-genta was isolated from BHI overnight cultures with isopropanol and ethanol precipitation as described previously (14).

**Cloning, expression, and purification of recombinant AtlA_Efm.** The E1162 *atlA_Efm* gene was amplified with primers *atlA_Efm*-exp-BamHI-R and *atlA_Efm*-exp-NotI-F (primers (see Table S3 in the supplemental material) and cloned in frame in the expression plasmid (Table S2), expressed and purified as previously described by Bardol et al. (49).

**α-Acm polyclonal rabbit antisera.** Highly specific polyclonal rabbit antisera were prepared by Eurogentec (Seraing, Belgium) according to their classic 28-day anti-protein polyclonal antibody protocol (https://secure.eurogentec.com/products/custom-polyclonal-antibodies.html). Two specific-pathogen-free rabbits were immunized with 200 μg purified AtlA_Efm. For eliciting Acm polyclonal antisera,
rabbots were immunized with a synthetic peptide (KLH- WEPVPDAAETKRLDNGC) based on the amino acid sequence (locus tag EfmE1162_1704) according to the 28-day-antipeptide protocol of Eurogentec.

Flow cytometry. AtlAEfm and Acm were detected at the surface of E. faecium E1162, atlAEfm::genta, atlAEfm::gente + atlAEfm, and E. faecium E980 grown in BHI until an OD600 of 0.3 or stationary phase by flow cytometry as described previously (47). α-AtlAEfm and α-Acm rabbit immune serum (1:100) were used to detect AtlAEfm and Acm. AtlAEfm, Acm, and eDNA localization. AtlAEfm, Acm, and eDNA localization in E. faecium was performed on single cells and in the semistatic biofilm model. For single cells, 5 μl PBS, fixed with 3% paraformaldehyde for 15 min, and washed again with PBS. To identify AtlAEfm and Acm, cells were incubated with 3 ml PBS, fixed with 3% paraformaldehyde for 15 min, and washed again with PBS. To identify atlAEfm or α-Acm IgG antibody (1:500 in PBS with 1% bovine serum albumin [BSA]) or monoclonal α-dsDNA (Abcam, Cambridge, MA) (1: 1,000 in PBS with 1% BSA) for 1 h on ice and subsequently washed with PBS. A secondary IgG Alexa Fluor 488 goat anti-rabbit or α-mouse antibody (Life Technology) (1:500 in PBS with 1% BSA) was added and incubated for an additional 1 h on ice to detect binding to first antibodies. Cells were washed once more and incubated with 5 μg/ml FM-5-95 dye (Invitrogen) for 2 min on ice. Fluorescence was analyzed in the confocal microscope (Leica S5P). Alexa 488 and FM-5-95 were excited at 488 nm. As a control, bacteria were treated as described above but omitting the first antibodies (α-AtlAEfm, α-Acm, and α-dsDNA).

eDNA localization in the AtlAEfm-deficient mutant by transmission immunoelectron microscopy. Transmission immunoelectron microscopy was performed as described previously with some modifications (50). eDNA in atlAEfm::gente overnight culture was labeled with 1:50-diluted specific monoclonal α-dsDNA mouse immune serum (Abcam, Cambridge, MA) in PBS containing 1% bovine serum albumin. For the negative control, this primary antibody step was omitted. Grids were examined using a FEI Tecnai T20 transmission electron microscope at a magnification of ×30,000 to ×65,000, and images were recorded using a digital 4-kb–by–4-kb charge-coupled device (CCD) camera.

Effect of AtlAEfm and DNase on E. faecium strains. The effect of AtlAEfm and DNase on the phenotype of the E1162 wild type, atlAEfm::gente, and atlAEfm::gente + atlAEfm was analyzed during bacterial growth under a confocal microscope. The wild type, atlAEfm::gente, and atlAEfm::gente + atlAEfm were grown in BHI at 37°C for 24 h. After overnight growth, cells were diluted to an OD600 of 0.05 in BHI and inoculated with and without purified AtlAEfm (5 μg) or DNase (1.5 μg/μl) in a Lab-Tek chambered eight-well coverglass (Thermo, Fisher Scientific). Cell growth was recorded at regular intervals of 3 min (×40 objective) with the DFC360 FX digital camera kit SP5 (Leica).

ELISA. Binding of the E1162 wild type, atlAEfm::gente, and atlAEfm::gente + atlAEfm to immobilized fibrinogen, fibronectin, collagen type I, collagen type III, and collagen type IV was assayed by a whole-cell ELISA as described previously (51).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl/doi:10.1128/mBio.00154-13/-/DCSupplemental.

Movie S1, AVI file, 1.16 MB.
Figure S1, TIF file, 0.8 MB.
Figure S2, TIF file, 0.1 MB.
Figure S3, TIF file, 0.8 MB.
Figure S4, TIF file, 0.1 MB.
Figure S5, TIF file, 0.8 MB.
Figure S6, TIF file, 0.1 MB.
Table S1, DOC file, 0.1 MB.
Table S2, DOC file, 0.1 MB.
Table S3, DOC file, 0.1 MB.

ACKNOWLEDGMENTS
Part of this work was supported by ZonMW VENI grant 91610058 to H.L.L. from the Netherlands Organization for Health Research and Development; by a personal Research Grant from the European Society of Clinical Microbiology and Infectious Diseases–ESCMID (2011) to H.L.L.; and by the European Union Seventh Framework Programme (FP7-HEALTH-2011-single-stage) under grant agreement no. 282004, EvoTAR, to R.J.L.W.

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