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Regulation of Autolysis-Dependent Extracellular DNA Release by *Enterococcus faecalis* Extracellular Proteases Influences Biofilm Development[▽]

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Enterococci are major contributors of hospital-acquired infections and have emerged as important reservoirs for the dissemination of antibiotic resistance traits. The ability to form biofilms on medical devices is an important aspect of pathogenesis in the hospital environment. The *Enterococcus faecalis* Fsr quorum system has been shown to regulate biofilm formation through the production of gelatinase, but the mechanism has been hitherto unknown. Here we show that both gelatinase (GelE) and serine protease (SprE) contribute to biofilm formation by *E. faecalis* and provide clues to how the activity of these proteases governs this developmental process. Confocal imaging of biofilms suggested that GelE$^-$ mutants were significantly reduced in biofilm biomass compared to the parental strain, whereas the absence of SprE appeared to accelerate the progression of biofilm development. The phenotype observed in a SprE$^-$ mutant was linked to an observed increase in autolytic rate compared to the parental strain. Culture supernatant analysis and confocal microscopy confirmed the inability of mutants deficient in GelE to release extracellular DNA (eDNA) in planktonic and biofilm cultures, whereas cells deficient in SprE produced significantly more eDNA as a component of the biofilm matrix. DNase I treatment of *E. faecalis* biofilms reduced the accumulation of biofilm, implying a critical role for eDNA in biofilm development. In conclusion, our data suggest that the interplay of two secreted and coregulated proteases—GelE and SprE—is responsible for regulating autolysis and the release of high-molecular-weight eDNA, a critical component for the development of *E. faecalis* biofilms.

Bacteria are often found in nature as communities of sessile surface-adherent populations covered in a slimy matrix composed of exopolysaccharides, protein, and DNA (9, 19, 23). Bacteria present within these communities (also referred to as biofilms) exhibit social behavior analogous to that found in higher organisms in that they can communicate and rapidly adapt to changing growth environments (5, 23, 55).

The gram-positive opportunistic pathogen, *Enterococcus faecalis* develops persistent biofilm-like vegetations on implant devices, including orthopedic implants, urethral stents, catheters, and heart valves, making it a leading cause of nosocomial infection (29). Enterococci are becoming increasingly resistant to many conventional antibiotics (22). Compounding the drug resistance phenotypes displayed by clinical isolates is the observation that enterococci growing as biofilms are more resistant to vancomycin, ampicillin, and linezolid than their planktonic counterparts (44). Epidemiological data also suggest enterococci to be important reservoirs for the transmission of antibiotic resistance genes among different species of bacteria (7, 56).

Of the factors reported to be important for *E. faecalis* biofilm formation (29), the enterococcal surface protein (Esp) and the secreted metalloprotease, gelatinase (GelE), are known to be expressed as variable traits (33, 47). More recently, Tendolkar et al. (51a) identified a locus from a clinical *E. faecalis* urinary tract isolate that they termed biofilm enhancer in *Enterococcus* (*bee* locus). The genes from this locus resemble the pilin biosynthetic genes identified by Nallapareddy et al. (33a) and have been shown to contribute to biofilm formation, but were found to be present in less than 5% of clinical isolates. It is noteworthy that Arciola et al. (3) recently correlated the presence of the *esp* gene and high phenotypic expression of gelatinase with the ability of *E. faecalis* epidemic clones from orthopedic implant infections to form biofilms. The *esp* gene that encodes the surface-associated Esp is located on a 153-kb pathogenicity island, and its expression significantly increases the bacterial cell surface hydrophobicity and attachment on a substratum (51, 52). The expression of GelE is dependent on the *fsr* regulatory system (38, 39) and is known to vary among strains of *E. faecalis* due to a defined 23.9-kb deletion in the genome that encompasses the *fsr* genes (33). The *fsr* locus consists of four genes, designated *fsrA*, *fsrB*, *fsrC*, and *fsrD* (32). The *fsrC* and *fsrA* genes encode a two-component sensor kinase-response regulator pair (39). The *fsrD* codes for a peptide lactone that functions in a cell-density-dependent manner (31). FsrB is thought to be responsible for the proteolytic cleavage and cyclization of FsrD (32). It is likely that FsrC sensor histidine kinase senses the accumulation of the FsrD peptide in the extracellular space, leading to activation of the response regulator FsrA. The gene encoding GelE is located immediately adjacent to the 3′ end of *fsrC* and is cotranscribed with *sprE*, which encodes a secreted serine protease (38, 39). Mutations in the *fsr* locus and its downstream target *gelE* resulted in poor biofilm-forming capabilities, indicating that biofilm formation in *Enterococcus* is dependent on quorum sensing (20, 30, 36). Mutants defective in *fsr* quorum signaling were restored to wild-type biofilm levels by the addition of purified

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TABLE 1. *E. faecalis* strains used in this study

Strain	Relevant genotype	Complementation in pAT28	Relevant phenotype[a]	Origin
V583	Parental		GelE$^+$ SprE$^+$	Clinical isolate
VT01	Δ*gelE*		GelE$^-$ SprE$^+$	V583
VT02	Δ*sprE*		GelE$^+$ SprE$^-$	V583
VT03	Δ*gelE-sprE*		GelE$^-$ SprE$^-$	V583
VT05	*gelE*	*gelE* promoter *gelE*	GelE$^+$ SprE$^+$ Spec^r	pVT05→VT01
VT07	*gelE sprE*	*gelE* promoter *gelE sprE*	GelE$^+$ SprE$^+$ Spec^r	pVT07→VT03
VT08	*sprE*	*gelE* promoter *sprE*	GelE$^+$ SprE$^+$ Spec^r	pVT08→VT02
VT09	Parental		GelE$^+$ SprE$^+$ Gfp Tet^r	pMV158gfp→V583
VT10	Δ*gelE*		GelE$^-$ SprE$^+$ Gfp Tet^r	pMV158gfp→VT01
VT11	Δ*sprE*		GelE$^+$ SprE$^-$ Gfp Tet^r	pMV158gfp→VT02
VT12	Δ*gelE-sprE*		GelE$^-$ SprE$^-$ Gfp Tet^r	pMV158gfp→VT03

[a] Spec^r, spectinomycin resistance; Tet^r, tetracycline resistance.

GelE, indicating that GelE alone is a major contributor to biofilm development (20).

The mechanism by which GelE positively regulates biofilm formation has hitherto been unknown. It was hypothesized that GelE, like Esp, may be able to modify the bacterial cell surface hydrophobicity by virtue of its ability to cleave substrates at hydrophobic residues (6, 27, 28). An alternate hypothesis involves the ability of GelE to activate cell wall autolysins (48, 54). SprE has also been shown to be an important virulence factor since an *sprE* gene disruption resulted in decreased virulence in a mouse peritonitis model (39, 50), a *Caenorhabditis elegans* model (15, 49), and a rabbit endophthalmitis model (14).

In the present study, we investigated the role of both extracellular secreted proteases in biofilm formation by comparing isogenic single Δ*gelE* and Δ*sprE* and double protease Δ*gelE-sprE* deletion mutants of *E. faecalis* V583. Further, the ability to regulate autolysis with the concomitant release of extracellular DNA (eDNA) was shown to be a key contributor to the overall development of *E. faecalis* biofilms.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The different strains and plasmids used in the present study are listed in Tables 1 and 2, respectively. Strains were cultured in Todd-Hewitt broth (THB) or M17 medium (Difco Laboratories) and grown at 37°C unless otherwise indicated. *Escherichia coli* EC1000 was used for plasmid constructions. The antibiotics used for selection in *E. coli* were chloramphenicol, kanamycin, and spectinomycin at concentrations of 10, 50, and 150 μg/ml, respectively, and those used for *E. faecalis* included chloramphenicol, tetracycline, and spectinomycin at concentrations of 15, 15, and 750 μg/ml, respectively.

Construction of *E. faecalis* V583 in-frame protease deletion mutants. In-frame deletions of *gelE*, *sprE*, and *gelE-sprE* were constructed by using pLT06, an *E. coli* enterococcal temperature-sensitive cloning vector that had selectable and counterselectable markers that aided in the selection of mutants containing the targeted deletions (L. Thurlow and L. E. Hancock, unpublished results). The vector pLT06 is a derivative of pCJK47 (25), retaining the counterselection properties on DL-*p*-chlorophenylalanine containing agar due to the presence of the *pheS* dominant-negative allele (25). In addition, pLT06 contains a chloramphenicol resistance marker and origin of replication from pWV01 (26).

Flanking regions (~1 kb) from both the 5′ and the 3′ ends of the targeted proteases were PCR amplified with the primers listed in Table 3. For the construction of pVT01 (*gelE* deletion), the primers GelEP1 and GelEP2 were used to amplify the region 5′ to *gelE* on the V583 genome. The primers GelEP3 and GelEP4 were used to amplify the region 3′ to *gelE*. GelEP1 and GelEP2 contained EcoRI and XhoI sites, respectively, and GelEP3 and GelEP4 contained SalI and BamHI restriction sites to facilitate cloning. Each PCR product was digested with the corresponding restriction enzymes, and both products were ligated into pLT06 cut with EcoRI and BamHI, prior to electroporation into *E. coli* EC1000. The correct constructs were identified by selection on LB agar plates containing chloramphenicol at 10 μg/ml, screened by restriction digest analysis, and further sequenced for verification. A similar approach was used in the construction of pVT02 (*sprE* deletion) using the primer pairs SprEP1 and SprEP2, as well as SprEP3 and SprEP4; and for pVT03 (*gelE-sprE* deletion) the primer pairs GelEP1 and GelEP2 were used, along with SprEP3 and SprEP4. The isolated plasmids were electroporated into electrocompetent *E. faecalis* V583 (10). *E. faecalis* V583 transformants were selected by growth at 28°C on THB agar containing chloramphenicol at 15 μg/ml and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 120 μg/ml. Blue colonies were inoc-

TABLE 2. Plasmids used in this study

Plasmid	Description	Source or reference
pLT06	Integration vector, Cm^r derivative of pCJK47	L. Thurlow, unpublished data
pVT01	pLT06 containing a 2.0-kb EcoRI/BamHI fragment containing engineered *gelE* deletion	This study
pVT02	pLT06 containing a 2.0-kb EcoRI/BamHI fragment containing engineered *sprE* deletion	This study
pVT03	pLT06 containing a 2.0-kb EcoRI/BamHI fragment containing engineered *gelE sprE* deletion	This study
pAT28	Broad-host-range shuttle vector, spectinomycin resistance	53
pVT05	pAT28 containing 1748-bp EcoRI/XhoI fragment containing the native *gelE* promoter along with full-length *gelE*	This study
pVT07	pAT28 containing 2687-bp EcoRI/BamHI fragment containing the native *gelE* promoter and full-length *gelE sprE*	This study
pVT08	pAT28 containing 2123-bp EcoRI/BamHI fragment containing the native *gelE* promoter, a truncated *gelE*, and full-length *sprE*	This study
pMV158gfp	Gram-positive replicative vector expressing a Gfp reporter	1

TABLE 3. Oligonucleotides used in this study

Primer	Sequence (5′–3′)
GelEP1	GAGAGAATTCGATTGGCTTAGTCATTGAAGC T
GelEP2	CTCTCTCGAGAAAGATGCCTGTACCTAAAATG
GelEP3	GAGAGTCGACCAGGTAAACCAACCAAGTGAAT
GelEP4	CTCTGGATCCCCGTGATTCTGGAAATTCCGAG
SprEP1	GAGAGAATTCGTGAACGCTACAGATGGA ACAA
SprEP2	CTCTCTCGAGTTCATTCATTGACCAGAACAGA
SprEP3	GAGAGTCGACCTCGGAATTTCCAGAATCACGG
SprEP4	CTCTGGATCCAGGTTCAGCGTTATCTACTAAG
GelEUp	CGCCAGAGATTTCACCTGACT
GelEDown	GGTACTTTTATCGTAACTTACAC
SprEUp	CAATCGGTTGATGCAATCGGTG
SprEDown	GTACGAGCATTCGCAGTAAATTC
GelEprom	GAGAGAATTCGCTATGGTATTGAGTTATGAGG
Ef1091-5′	GCTATGTTGGCTACTCAAGTG
Ef1091-3′	TGTCCTGCAGGTGCTTTTAC
Ef0887-5′	GAGAGAATTCGAAGGAATTGTCATTGTGCG
Ef0887-3′	CAAAACGGATCCGACTCGC
Ef2490-5′	GAGAGTCGACAGCAAAAGGGTTGTTGAAATA
Ef2490-3′	CTCTGCATGCCCTACTTTCTCTGTTACTTAAT
Ef2488-5′	GAGAGGATCCTAAACAGGGGAGTGTGTGA CATG
Ef2488-3′	GAGAGCATGCCAAACACCAAATGCATTATTTA
Ef2194-5′	GGAACAACGCTAAACTTTTAC
Ef2194-3′	CGCTCCTATTCTGCTGCTAA
OriF	CAATAATCGCATCCGATTGCA
SeqR	CCTATTATACCATATTTTGGAC

ulated into fresh THB containing chloramphenicol at 15 μg/ml. Cultures were grown overnight at 28°C, diluted 1:100 into fresh medium, and grown for an additional 2.5 h at 28°C and then shifted to 42°C for an additional 2.5 h to favor single-site integration of plasmids into the *E. faecalis* V583 genome. Serial dilutions of the integrants were plated onto THB agar plates supplemented with 15 μg of chloramphenicol/ml. Colony PCR was used to confirm single-site integration for each construct using vector-specific primers OriF or SeqR, along with primers targeted to regions 5′ or 3′ to the site of insertion (GelEUp, GelEDown, SprEUp, or SprEDown). A positive colony was then cultured in the absence of selection until the culture reached stationary phase (~2×10^9 CFU/ml). Serial dilutions were prepared, and fresh medium (THB) was inoculated such that it contained 100 CFU/ml. Serial dilutions (1:500 and 1:1,000) were plated on MM9YEG agar supplemented with 10 mM DL-*p*-Cl-Phe and X-Gal at 120 μg/ml. Counterselection using DL-*p*-Cl-Phe has been shown to favor the selection of colonies that have lost the plasmid (25). Colony PCR using the primers GelEUp and GelEDown for VT01, SprEUp and SprEDown for VT02, and GelEUp and SprEDown for VT03 were used to confirm the gene deletion in the genome. Phenotypic confirmation of the protease deletions were also visualized on THB agar containing 1.5% skim milk.

Complementation of *E. faecalis* V583 in-frame protease deletion mutants. In-frame protease deletions of *E. faecalis* V583 were complemented with full-length *gelE*, *sprE*, and *gelE-sprE*, each with the native *gelE* promoter region in a pAT28 vector (53) and were denoted pVT05, pVT08, and pVT07, respectively. The *gelE* complement insert was cloned by PCR amplification of V583 genome with primers GelEprom and SprEP2 and subsequently inserted as an EcoRI/XhoI fragment into pAT28 cut with EcoRI/SalI. The *gelE-sprE* double protease complement construct was cloned by PCR amplification of V583 genome using primers GelEprom and GelEP4 and inserted as an EcoRI/BamHI fragment into EcoRI/BamHI-cut pAT28 vector. Plasmid pVT08 was constructed by digesting pVT07 with AflII and NcoI, followed by Klenow treatment to make it blunt ended, and then the molecule was circularized by self-ligation to obtain an in-frame deletion of *gelE*. These constructs were transformed into the corresponding protease deletion mutants, and phenotypic complementation was confirmed by zymography using skim milk at a final concentration of 0.02% as a substrate (24).

Biofilm assay on polystyrene microtiter plates. Biofilm formation on polystyrene was quantified with crystal violet staining method as previously described (20). Each assay was performed in octuplicate and repeated five times. Statistical significance was calculated by using Dunnett's test (GraphPad Software, San Diego, CA).

Cell surface hydrophobicity assay. The cell surface hydrophobicities of *E. faecalis* V583 and isogenic protease mutant strains were carried out as previously described (43). The percentage of bacterial adhesion to hydrocarbon was calculated as follows: $[1 - (OD_F/OD_I)] \times 100$, where OD_I and OD_F are the optical densities of cells resuspended in PUM buffer (100 mM potassium phosphate [pH 7.1], 30 mM urea, 800 μM $MgSO_4 \cdot H_2O$) determined at the beginning and the end of the experiment, respectively. Statistical significance was computed by using the Dunnett's test (GraphPad Software, San Diego, CA).

Autolysis assay. Autolysis assay was carried out as previously reported (11).

Isolation of eDNA from *E. faecalis* planktonic culture supernatants. Supernatants from 24-h-old grown cultures were passed through a sterile syringe filter (0.2-μm pore size; Nalgene) and concentrated ~20-fold using a 10-kDa cutoff membrane (YM-10 Centricon centrifugal filter devices; Millipore) according to the manufacturer's instructions. The concentrated samples were loaded on a 1% agarose gel and stained with ethidium bromide to visualize high-molecular-weight DNA. Densitometric spot comparisons were performed by using Alphaimager software (Alpha-Innotec, San Leandro, CA).

eDNA from culture supernatants was isolated by using the Wizard genomic DNA purification kit according to the manufacturer's instructions, and chromosomal DNA was isolated as previously described (37). For comparative PCR, primers listed in Table 3 were designed to amplify genes from regions of the *E. faecalis* V583 genome, including Ef0887, Ef1091, Ef2194, Ef2488, Ef2490, and Ef1818 (*gelE*).

Laser scanning confocal microscopy. *E. faecalis* strains V583, VT01, VT02, and VT03 were transformed with pMV158GFP (34) to constitutively express Gfp for confocal imaging. The resulting strains were designated VT09, VT10, VT11, and VT12, respectively. Confocal microscopy was performed on *E. faecalis* biofilms grown on glass coverslips. Sterile glass coverslips were placed on the bottom of six-well tissue culture plates and submerged with 5 ml of M17 broth, seeded with a 1:100 dilution from an overnight culture (approximately 5 to 10×10^6 CFU), and grown for 24 h at 37°C. For 2-, 3-, and 4-day-old biofilms, the culture supernatants were replaced with fresh medium daily. Just prior to imaging, biofilms were gently rinsed three times with sterile phosphate-buffered saline, followed by 10 min of staining with 5 ml of propidium iodide (PI; 1 μM). The coverslips were mounted on a microscope slide and sealed with clear nail polish to prevent dehydration. Slides were visualized by using a Zeiss LSM 5 Pascal laser scanning confocal microscope. The LSM 5 system was equipped with a Zeiss Axioplan 2 MOT research microscope, a fully motorized stage, a Plan Apohromat objective (×63/1.4 oil) and differential contrast interference. Dual fluorescence emission imaging of green fluorescent protein (GFP) and PI was accomplished using a 488-nm line of 458/488/514 argon gas ion laser to excite GFP and a 543-nm line of HeNe laser to excite PI. A secondary HFT 545 dichroic was used to split the emission signals into two signals, the shorter wavelengths passed through a band-pass 505- to 530-nm filter to image GFP fluorescence, and the longer wavelength passed through a long-pass 560-nm filter to image PI fluorescence. For z-series, the Airy units of the longer and shorter wavelengths were adjusted to give an optical slice thickness of 0.7 μm, and this thickness was used as the slice interval. Biofilm quantification was carried out using the COMSTAT analysis package (21). Volumetric analysis (μm^3) of representative confocal images portraying regions within the biofilm stained by PI were carried out using the 3D Object counter plug-in in the NIH Image J software. For determination of statistical significance, the data were natural log transformed, and an unpaired *t* test was performed using GraphPad (GraphPad Software, San Diego, CA).

DNase I treatment of biofilms. To assess the significance of eDNA for *E. faecalis* biofilms, 6-, 12-, and 24-h-old biofilms were treated with 100 Kunitz units per ml of DNase I. The control contained denatured DNase I that was heated at 100°C for 15 min. The biofilms were imaged by using confocal laser scanning microscopy (CLSM).

RESULTS

Construction and complementation of *E. faecalis* V583 isogenic protease mutants. Kristich et al. recently developed a *pheS* counterselectable vector system, pCJK47 to generate markerless in-frame isogenic deletion mutations in *E. faecalis* OG1RF (25). However, this vector system was unsuitable for studies with strain V583 due to the unavailability of selectable resistance markers, as well as difficulty associated with conjugal mating of strains possessing multiple plasmids. In the present

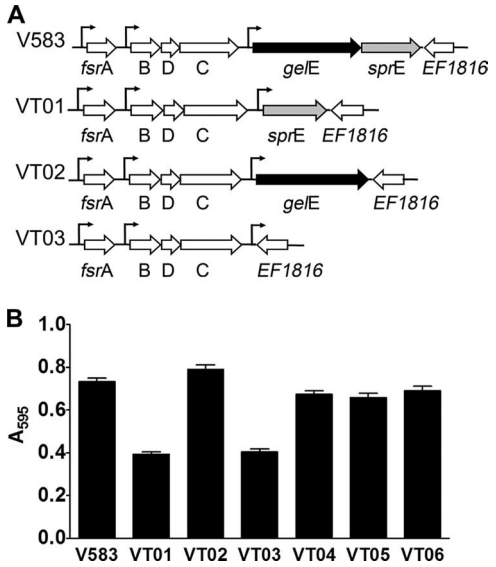

FIG. 1. Extracellular protease deletion mutations affect *E. faecalis* V583 biofilm development. (A) Diagrammatic depiction of extracellular protease deletions. VT01, VT02, and VT03 correspond to *E. faecalis* V583 strains harboring Δ*gelE*, Δ*sprE*, and Δ*gelE-sprE* protease deletions, respectively. Solid lines indicate chromosome, boxed arrows indicate genes, and curved arrows indicate promoter regions. The schematic is not drawn to scale. (B) Biofilm formation of extracellular protease mutants on polystyrene microtiter plates. The biofilm density within microtiter plate wells was assayed as a function of crystal violet stain retained by the biofilm biomass. Mutant strains complemented with *gelE*, *sprE*, and *gelE-sprE* are designated VT04, VT05, and VT06, respectively. Assays were performed in triplicate, and error bars indicate the standard error of the mean.

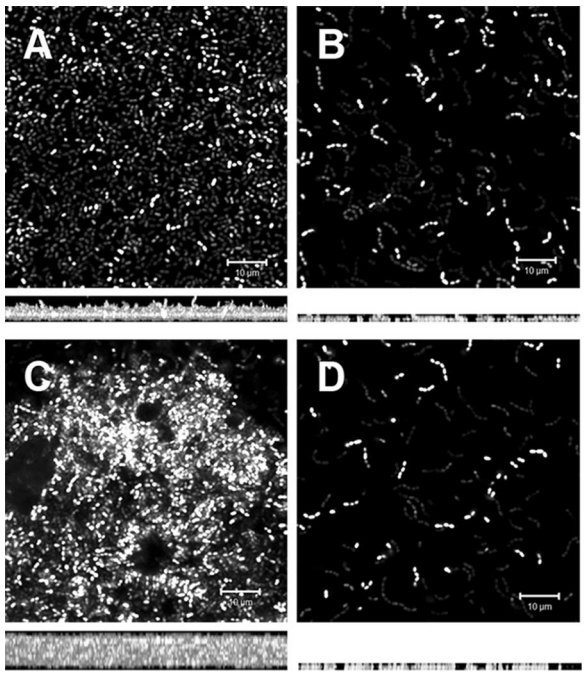

FIG. 2. Confocal analysis of 1-day-old biofilms of *E. faecalis* wild type and isogenic protease deletion mutants. All strains constitutively expressed Gfp from pMV158GFP (see Materials and Methods) and were grown on glass coverslips in M17 medium. Panels A, B, C, and D are representative biofilm projections of VT09, VT10, VT11, and VT12, respectively. Below each panel is the z-projection for the corresponding image, and the depth of the biofilm is indicated by the height of the z-stack (see Table 4). The inset scale bar represents 10 μm.

study, we used the plasmid pLT06 (a derivative of pCJK47), which encodes resistance to chloramphenicol and contains a temperature-sensitive replication origin from pWV01 (26).

Extracellular protease deletion mutants VT01 (Δ*gelE*), VT02 (Δ*sprE*), and VT03 (Δ*gelE-sprE*) (Fig. 1A) were constructed by using the markerless exchange vectors pVT01, pVT02, and pVT03, respectively. The respective plasmids were integrated into the V583 genome by homologous recombination. Subsequent plasmid excision was counterselected by plating on medium containing DL-*p*-chlorophenylalanine as described previously (25). Roughly 50% of the isolates growing in the presence of DL-*p*-chlorophenylalanine yielded the expected gene deletion for each of the plasmid constructs. The proteolytic phenotypes of the mutants were compared to V583 and were consistent with previous reports (24). Strains VT01 and VT03 lacked a zone of proteolysis on skim milk agar, whereas strain VT02 showed a smaller zone compared to V583 (data not shown).

E. faecalis V583 isogenic protease mutants (VT01, VT02, and VT03) were complemented with full-length genes of *gelE*, *sprE*, and *gelE-sprE in trans* under the control of the native *gelE* promoter. Complementation confirmed that the protease-negative phenotypes were a result of targeted protease deletions and not due to polar effects of gene mutations elsewhere on the chromosome (data not shown).

Biofilm formation of *E. faecalis* V583 isogenic protease mutants. Quantitative analysis of biofilms formed by the protease deletion mutants on polystyrene confirmed previous findings (20). VT01 (Δ*gelE*) and the double protease deletion strain VT03 (Δ*gelE-sprE*) were significantly reduced in biofilm biomass compared to strain V583 (Dunnett's test, $P < 0.05$) (Fig. 1B). Interestingly, deletion of *sprE* (VT02) marginally increased the biofilm biomass, although this did not appear to be statistically significant (Dunnett's test, $P = 0.30$). Complementation of the protease-negative strains restored biofilm formation to near wild-type levels, suggesting no polar effects for the deletion mutations (Fig. 1B).

Given the differences in biofilm biomass on polystyrene, we sought to determine whether mutant cells exhibited any differences in primary biofilm mat formation on a glass substrate. CLSM analysis of the structural and spatial organization of 24-h-old biofilms (Fig. 2) showed a dense and compact parental V583 biofilm (VT09). Consistent with our earlier observations, VT10 (Δ*gelE*) and VT12 (Δ*gelE-sprE*) displayed poor biofilms (decreased by ca. 60 and 50%, respectively, compared to VT09; Table 4) and was composed mainly of isolated and sparse distributions of cells on the glass surface. In contrast, biofilms of VT11 (Δ*sprE*) were more dense than those formed by the parental strain (increased by ca. 55%; see Table 4) and appeared to have a rugged, mountainous surface terrain consistent with an early initiation of microcolony development.

Extracellular proteases do not affect the cell surface hydrophobicity of *E. faecalis* V583. To determine whether the ability of GelE to enhance biofilm formation resulted from an in-

TABLE 4. COMSTAT analysis of wild-type and isogenic protease mutant biofilm images

Day	Biofilm	Mean ± SD		
		Biomass ($\mu m^3/\mu m^2$)	Mean thickness (μm)	Maximum thickness (μm)
1	VT09	6.7 ± 0.93	6.3 ± 0.98	6.3 ± 0.98
	VT10	2.6 ± 0.37	2.4 ± 0.41	2.45 ± 0.49
	VT11	10.5 ± 0.33	10.1 ± 0.48	10.15 ± 0.49
	VT12	3.0 ± 0.48	2.4 ± 0.49	2.45 ± 0.5
4	VT09	5.6 ± 0.38	7.9 ± 1.04	11.2 ± 0.0
	VT10	0.035 ± 0.04	0.022 ± 0.027	8 ± 2.26
	VT11	11.61 ± 0.46	19.9 ± 0.01	20.8 ± 0.0
	VT12	0.194 ± 0.04	0.28 ± 0.03	7.2 ± 2.26

crease in overall cell surface hydrophobicity, we tested whether a Δ*gelE* mutation would decrease the overall hydrophobicity of cells. The assay was carried out by quantifying the population of bacteria that were able to separate into an organic phase (*n*-hexadecane) depending on the degree of cell surface hydrophobicity displayed. The presence or absence of either protease in both single- and double-deletion protease mutants did not result in significant differences in partitioning into the *n*-hexadecane phase relative to the wild-type V583 strain (Fig. 3, Dunnett's test, $P < 0.05$).

Extracellular proteases modify the rate of *E. faecalis* V583 autolysis. Given the ability of enterococcal proteases to modify autolysins (48) and based on the observations seen using confocal imaging of biofilms, we hypothesized that GelE and SprE may differentially regulate the autolysis rates of *E. faecalis*. We observed that VT01 (Δ*gelE*) and VT03 (Δ*gelE-sprE*) exhibited a decrease in the rate of autolysis compared to V583 (Fig. 4A), a finding consistent with observations reported by Waters et al. (54). In contrast, VT02 (Δ*sprE*) displayed a significant increase in the rate of autolysis compared to V583 (Fig. 4A, Student *t* test, $P < 0.05$).

eDNA in *E. faecalis* V583 culture supernatants. Based on the altered rates of autolysis, we hypothesized that eDNA resulting from cell lysis would be more abundant in culture supernatants of *E. faecalis* V583 than mutants deficient in GelE production. Concentrated (20-fold) supernatant fractions were assessed for the presence of eDNA by agarose gel electrophoresis. High-molecular-weight DNA was detected in V583 and VT02 (Δ*sprE*) fractions (Fig. 4B, lanes 1 and 3), but not in mutants VT01 and VT03 (Fig. 4B, lanes 2 and 4), a finding consistent

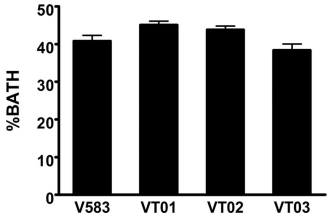

FIG. 3. Cell surface hydrophobicity of *E. faecalis* V583 and extracellular protease mutants. The overall measure of hydrophobicity of wild-type and mutant populations were calculated as the percent bacteria that adhered to hydrocarbon (BATH). Assays were performed in triplicate, and error bars represent the standard error of the mean.

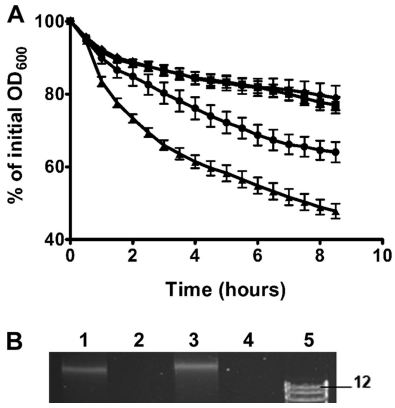

FIG. 4. Extracellular proteases influence autolysis rates and eDNA release. (A) Differences in autolysis rates of V583 (●) and extracellular protease mutants VT01 (■), VT02 (▲), and VT03 (◆) are exhibited as percent values of the initial optical density at 600 nm (OD_{600}). Assays were performed in quadruplicate, and error bars denote the standard error of the mean calculated from three independent assays. (B) High-molecular-weight bacterial chromosomal DNA was detected by ethidium bromide staining, after 20-fold concentration of 24-h-old culture supernatants. Lanes: 1, V583; 2, VT01; 3, VT02; 4, VT03; and 4, 1-kb DNA ladder showing the 12-, 10-, and 8-kb bands (the 12-kb band is labeled in lane 5).

with a decreased rate of autolysis in strains lacking GelE. Densitometric determination of band intensity between DNA present in V583 and VT02 culture supernatants indicated a ~2-fold increase in the amount of eDNA from an $SprE^-$ mutant, a finding consistent with a role for SprE as a negative regulator of autolysis. Initiation of DNA release in V583 culture supernatants followed expression of GelE in the transition to stationary phase (data not shown), a finding consistent with the earlier observation that GelE initiates autolysis. Finally, comparative PCR using eDNA and chromosomal DNA as templates confirmed that eDNA was indeed chromosomal in nature since amplification with primer pairs targeted to randomly distributed regions of the V583 genome could be amplified from both templates (data not shown).

Tracking cell death in enterococcal biofilms. Because autolysis and biofilm formation of *E. faecalis* was directly dependent on the presence of gelatinase, we questioned whether biofilms formed by the parental strain would contain foci of lysed cells compared to VT01 (Δ*gelE*). To test this, biofilms of V583 and VT01 expressing Gfp (VT09 and VT10, respectively) were grown over a period of 3 days and were stained for the presence of DNA and dead cells with PI. Regions within the biofilm of VT09 contained concentrated foci of DNA (as detected by PI staining) in contrast to the few random dead cells in VT10 biofilms (Fig. 5). ImageJ analysis software was used to quantify the amount of PI-stained volumes within the biofilm as a measure of eDNA present in the biofilm. From this analysis, it is apparent that a common feature shared by both $GelE^-$ mutant and wild-type cell populations is the presence of damaged cells capable of taking up PI, and this cell population is accounted for in our analysis. A property unique to the wild-type cells compared to the $GelE^-$ mutant is the presence of larger volumes of PI staining associated with lysed cells. The mean values for PI-stained volumes is ~4.4-fold higher in the

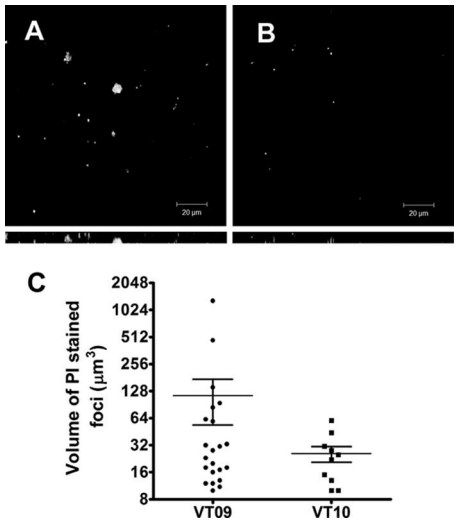

FIG. 5. Bacterial cell death and eDNA release in 3-day-old biofilms. *E. faecalis* biofilms grown in M17 medium over a period of 3 days were stained with PI (1 μM) before being visualized by CLSM. (A) Top-down view of VT09 biofilm displaying discrete foci of lysed bacteria, along with dead bacterial cells. (B) View of isolated dead bacterial cells within the VT10 biofilm. Below each panel is the z-projection for the corresponding image, and the depth of the biofilm is indicated by the height of the z-stack. The inset scale bar represents 20 μm. (C) Volumetric analysis of PI-stained foci for VT09 and VT10 biofilms. Vertical scatter plots with each of the values of stained foci (cubic microns) are shown along with the mean and standard error of the mean.

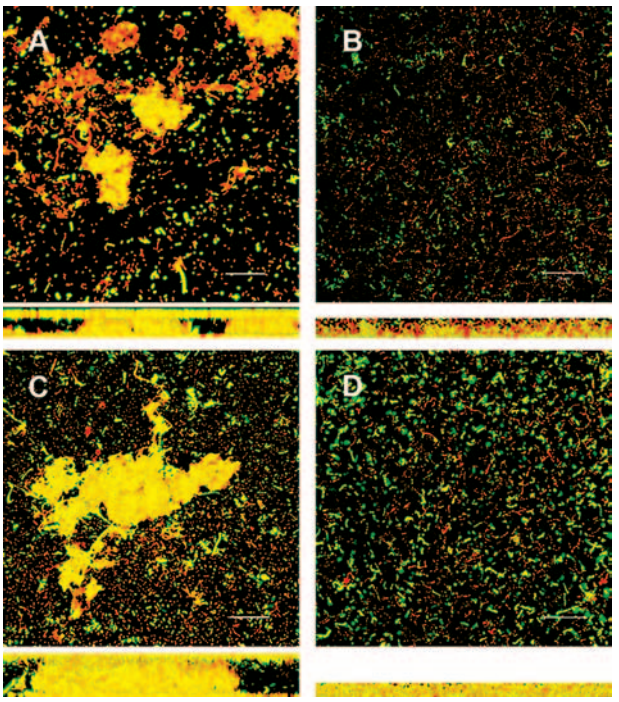

FIG. 6. Comparison of biofilm architectures and relative eDNA localization. Four-day-old Gfp-expressing strains of *E. faecalis* V583 and isogenic protease mutants were grown in M17 and stained for the presence of eDNA with PI (1 μM) as indicated in Materials and Methods. Live bacteria are green, and eDNA and dead cells are visualized in red. High concentrations of eDNA laced among live bacteria present on each raised microcolony and surroundings appear in shades of yellow. Panels A, B, C, and D are representative biofilm projections of VT09, VT10, VT11, and VT12, respectively. Below each panel is the z-projection for the corresponding image, and the depth of the biofilm is indicated by the height of the z-stack (see Table 4). The inset scale bar represents 20 μm.

wild-type strain (113.5 ± 59.88) than in the GelE$^-$ mutant (25.80 ± 5.09), and this was shown to be statistically significant ($P = 0.0004$) by using an unpaired t test, after data transformation, to account for the fact that stained volumes present in the V583 biofilms were not normally distributed compared to VT01 biofilms. A graph of this analysis is shown in Fig. 5C, and the *z*-stack image comparing V583 and VT01 biofilms stained with PI is also shown in Fig. 5. Collectively, these results suggest that GelE enhances biofilm formation by inducing lysis in discrete pockets of cells that appear to initiate biofilm development.

Since the expression of GelE and SprE may only be optimally activated after the establishment of a quorum of bacteria on a surface, we hypothesized that we would see more prominent defects in the differentiation of the biofilm at later stages of development rather than the initial stages of attachment and proliferation. Consistent with this hypothesis, we observed that VT10 (Δ*gelE*) and VT12 (Δ*gelE-sprE*) were able to form a primary biofilm matt on a glass surface within 48 to 72 h of growth (data not shown). However, unlike the parental VT09 or VT11 (Δ*sprE*), even after 96 h of growth these two strains were not able to differentiate into microcolonies (Fig. 6). PI staining of the dead bacteria and eDNA in 4-day-old biofilms revealed clusters of dead bacteria around the base and stalk of a microcolony, whereas live bacteria interspersed with DNA frequently occupied the top of microcolonies within biofilms (Fig. 6). This suggested that pockets or clusters of dead cells that are dependent on the expression of GelE visualized at an earlier phase of biofilm development (Fig. 5) may actually be sites of initial microcolony development. Consistent with a role for SprE in negatively regulating GelE activity, we observed significantly more biofilm biomass (107% increase compared to the wild type) in an SprE$^-$ mutant after 96 h of growth than in the parental strain (Fig. 6 and Table 4).

Functional role of eDNA in enterococcal biofilms. To determine whether eDNA of *E. faecalis* played a structural role in biofilm development, we analyzed the affect of DNase I on biofilm formation. Static biofilms grown on glass substrates were treated with DNase I after 6, 12, and 24 h of growth. Biofilm defects were most pronounced after early treatments of DNase I at 6 and 12 h of biofilm growth (Fig. 7). The affect of DNase I treatment at later stages of development was less significant, as exhibited by the 24-h treatment.

DISCUSSION

The importance of extracellular proteases of *E. faecalis* in pathogenesis has been well demonstrated in a number of biological models (14, 15, 49, 50). Components of the host innate immune response are known to be cleaved by the proteolytic activity of gelatinase and include LL37 (45), α-defensin (46), and the complement components C3a and C3b (35), providing a mechanism for host immune evasion. GelE has also been shown to cleave fibrin, possibly enhancing efficient dissemina-

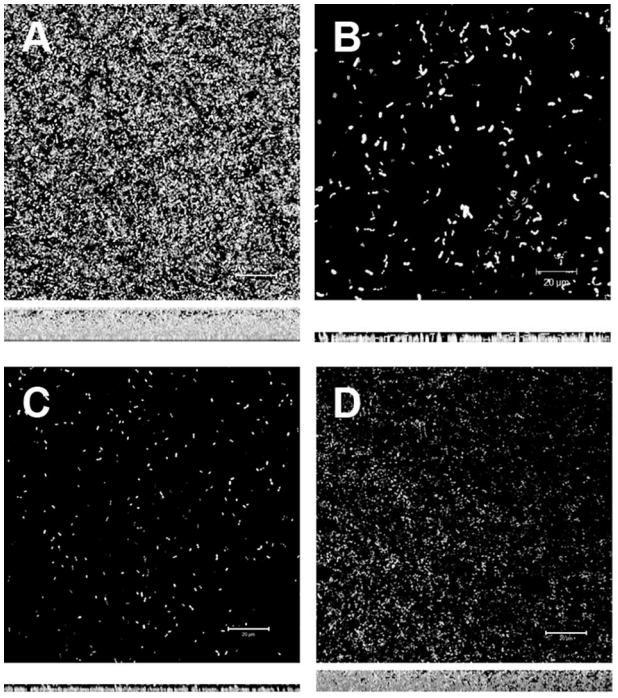

FIG. 7. DNase I inhibits biofilm formation at early stages of development. V583 biofilms grown on glass coverslips were treated with DNase I after 6, 12, and 24 h of growth (represented in panels B, C, and D, respectively) and analyzed after 26 h by CLSM. The biofilm micrograph on the far left (panel A) shows a control experiment with heat-inactivated DNase I introduced after 6 h of biofilm development. Below each panel is the z-projection for the corresponding image, indicating the depth of the biofilm. The inset scale bar represents 20 μm.

tion of the organism in vivo (54). Aside from its proteolytic affects on host factors, gelatinase has also been shown to have a positive role in *E. faecalis* biofilm development (20, 25). Hence, our present focus was to elucidate the mechanism behind GelE-dependent biofilm development and to further examine the role of SprE in that process.

A speculative role for GelE in biofilm development included its potential ability to increase cell surface hydrophobicity by cleaving surface polypeptides at hydrophobic residues (6, 27). Although cell surface hydrophobicity has previously been proposed to be a key factor in the initial attachment of bacteria to a substratum (12), our analysis of the different protease mutants does not support a role for GelE or SprE in altering cell surface hydrophobicity since the deletion of either protease singly or in tandem resulted in minimal changes. A second hypothesis centered on the ability of GelE to alter rates of autolysis, based on observations by Shockman and Cheney (48) and Waters et al. (54). Our data appear to confirm the importance of autolysis in driving the development of *E. faecalis* biofilms, since we observed altered rates of autolysis, changes in eDNA release, and differences in biofilm development in mutants defective in extracellular protease production. The contributions of both proteases to the process of biofilm development was readily observed only after confocal analysis. We did not initially observe a contribution for SprE in the microtiter plate biofilm assay. The apparent discrepancy between the two assays is consistent with observations reported by Tendolkar et al. (51) in which the plate assay significantly underestimated biofilm biomass compared to confocal imaging and COMSTAT analysis.

The major autolysin, AtlE of *Staphylococcus epidermidis* was recently shown to contribute to biofilm development through the generation of eDNA upon autolytic activation (40). A role for muramidase 2, a major autolysin of *E. faecalis*, in biofilm formation was reported by Mohamed et al. (30), and these authors concluded that it played a major role in the initial adherence phase of biofilm development. The findings reported by Qin et al. (40) that eDNA is an integral component of the biofilm matrix in *S. epidermidis* biofilms may warrant a reevaluation of the role of autolytic processes in biofilm development in *E. faecalis*. The observed alterations in eDNA release that are dependent on protease activity and appear to mediate the ability of *E. faecalis* to develop microcolonies within biofilms suggest that autolytic processes may govern not only initial attachment but also the subsequent development of the biofilm. Our findings have not only confirmed the role for GelE in activating autolysis since its deletion resulted in autolysis and biofilm defects but also provide direct evidence that SprE is involved in negatively regulating autolysis, eDNA release, and biofilm maturation.

Previous reports have identified and characterized SprE as a virulence factor whose activity is altered in the presence of GelE (24). This activity is similar in nature to that reported for the corresponding homologous extracellular proteases of *S. aureus*, where the metalloprotease aureolysin processes the cotranscribed SspA (V8 protease) (41). In *S. aureus*, SspA is known to alter the autolytic profile (41), which is consistent with our observations for the role of GelE and SprE in regulating autolysis. Because our data suggest that SprE prevents early maturation of biofilms by negatively regulating GelE activity, we postulated whether there would be a fitness cost associated with the bacterial cell in the absence of SprE. Our observations suggest that the quick biofilm maturation phenotype of VT02 is associated with cell surface perturbations that may be disadvantageous at a planktonic level of existence. For instance, the SprE$^-$ mutant is at least fourfold more sensitive to vancomycin compared to wild-type V583 (data not shown). Hence, it would seem that the trade-off for rapid biofilm development is costly and, in an evolutionary sense, unstable.

It has been observed in several model systems that eDNA serves as an important matrix component of microbial biofilms (2, 40, 42, 57). Consistent with a role for eDNA as a matrix component, we observed that treating a developing biofilm with DNase I at 6 and 12 h postinoculation resulted in diminished biofilm accumulation compared to a heat-inactivated DNase I control. In contrast, the addition of DNase I at 24 h showed only a marginal reduction in biofilm accumulation, suggesting that changes in the matrix composition may take place at later stages of development. Consistent with our findings, the observation that disrupting biofilms with DNase I treatment works better at earlier stages of development has been reported for *Pseudomonas aeruginosa* (57) and *S. aureus* (42) biofilms.

Although the factors regulating the spatial death of a subpopulation of bacterial cells in a biofilm are not clear, the extracellular nature of the proteases and their opposing phe-

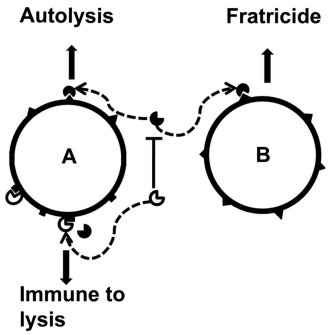

FIG. 8. Model of GelE-mediated lysis in *E. faecalis* biofilm development. The model presents two mechanisms by which GelE could mediate lytic activity. The first mechanism is referred to as autolysis (A), and gelatinase (◕) from the producer cell could activate a putative autolysin (▲) on the cell surface, resulting in autolysis. The presence of SprE (◔) is predicted to regulate the GelE-mediated autolysin activation. The second mechanism, referred to as fratricide (allolysis) (B), allows for the diffusion of GelE (◕) from the producer cell (A) to a susceptible sibling (B), wherein the sibling cell undergoes lysis following autolysin (▲) activation by GelE. The extent of bystander or sibling lysis would potentially be regulated by the presence of SprE (◔) in the environment. The mechanism of SprE-mediated regulation is unknown but may involve alteration of the putative autolysin, rendering it to an inactive form (■).

notypes may play a role in this process. Our current model (Fig. 8) proposes two possible means by which these proteases may exert their regulatory affects on biofilm development. The first mechanism involves an autolytic pathway, wherein GelE localizes to the cell wall of the producing cell to activate autolysis. If insufficient levels of SprE are present to control the autolytic activation induced by GelE, then that cell will likely undergo autolysis. The second mechanism would involve an allolytic or fratricidal event, wherein GelE freely diffuses from the producer cell to a target sibling cell to activate autolysins present on the sibling cell wall. A delay in responding to the quorum signal by siblings would render them susceptible to the action of autolysins activated by GelE secreted from another cell. SprE would also likely be present in this extracellular environment, but differences in diffusion and affinity for the cell wall may likely give rise to regions in the biofilm where GelE could act independently of SprE activity. In the rare instances in which GelE would function independently of SprE, a sibling cell would lyse providing the necessary eDNA scaffold on which a developing biofilm could form. Consistent with the above model is the fact that only a few pockets within the observed biofilms give rise to cell lysis, which is indicative of the fact that the process is highly regulated.

In recent times, bacterial death in biofilms has been compared to programmed cell death in eukaryotes (4). Often such comparisons propagate the idea that defective cells within a biofilm population are eliminated in response to environmental challenges due to their altruistic suicidal acts (4). Our model adds to this complexity by proposing that GelE-mediated lysis appears to be an important aspect of biofilm development by *E. faecalis*. The cytotoxic activity of GelE toward the producer cell (autolysis) or sibling cells (allolysis) by the activation of autolysins may result in the release of eDNA crucial for the early development of biofilms. Although in this case a subpopulation of cells may not be defective per se, their inability to produce the immunity factor (SprE) would result in their death. Allolysis has also recently been referred to as microbial fratricide ("sibling killing sibling"), and this term has been applied to the competence developmental program in *Streptococcus pneumoniae* (18), and a model was proposed on how this process might contribute to the development of biofilms (16). Allolysis (18) and cannibalism (17) regulate the differentiation of competent cells in *S. pneumoniae* and sporulation in *Bacillus subtilis*, respectively. Consistent with these fratricidal systems, a model for how fratricide in *E. faecalis* regulates the development of biofilms is also proposed (Fig. 8). Interestingly, all three processes of differentiation may be considered attributes of multicellularity resulting from cell-cell communication and involve quorum sensing, killing factors, and immunity proteins (8, 13). In *E. faecalis* biofilm development, quorum sensing is mediated through a peptide lactone (FsrD) originally characterized as the gelatinase biosynthesis-activating pheromone (31). The extracellular accumulation of the peptide triggers expression of both GelE (the effector) and SprE (the regulator). Both proteases are cotranscribed, suggesting an equal number of both molecules in the extracellular milieu. For this reason, we anticipate that most cells would be protected from autolysis or allolysis. However, within discrete foci, the balance of these two proteases may not be the same, giving rise to effector-mediated processes in the absence of regulatory control. Ongoing studies will better clarify which of the two mechanisms (autolysis versus fratricide) plays the dominant role in *E. faecalis* biofilm development.

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