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Deletion of σ^{54} (*rpoN*) Alters the Rate of Autolysis and Biofilm Formation in *Enterococcus faecalis*

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Transcription initiation is a critical step in bacterial gene regulation and is often controlled by transcription regulators. The alternate sigma factor (σ^{54}) is one such regulator that facilitates activator-dependent transcription initiation and thus modulates the expression of a variety of genes involved in metabolism and pathogenesis in bacteria. This study describes the role of σ^{54} in the nosocomial pathogen *Enterococcus faecalis*. Biofilm formation is one of the important pathogenic mechanisms of *E. faecalis*, as it elevates the organism's potential to cause surgical site and urinary tract infections. Lysis of bacterial cells within the population contributes to biofilm formation by providing extracellular DNA (eDNA) as a key component of the biofilm matrix. Deletion of *rpoN* rendered *E. faecalis* resistant to autolysis, which in turn impaired eDNA release. Despite the significant reduction in eDNA levels compared to the parental strain, the *rpoN* mutant formed more robust biofilms as observed using laser scanning confocal microscopy and Comstat analysis, indicating and emphasizing the presence of other matrix components. Initial adherence to a polystyrene surface was also enhanced in the mutant. Proteinase K treatment at early stages of biofilm development significantly reduced the accumulation of biofilm by the *rpoN* mutant. In conclusion, our data indicate that other factors in addition to eDNA might contribute to the overall composition of the enterococcal biofilm and that the regulatory role of σ^{54} governs the nature and composition of the biofilm matrix.

As opportunistic pathogens, enterococci are the third leading cause of hospital-acquired or associated infections, as they are responsible for 11.2% of surgical site infections (SSI), 14.9% of urinary tract infections (UTI), and 16% of reported bloodstream infections (25). The ability to form a biofilm is an important aspect of the lifestyle of the organism, as biofilm formation is thought to be a property associated with the establishment of SSI and UTI (34), both of which can serve as foci to establish bloodstream infections. Biofilms are aggregates of bacteria that are covered in exoplomer matrix and are more resistant to antibiotics than their planktonic counterparts (15, 26). In several bacterial species, nucleic acids, polysaccharides, proteins, and lipids constitute the exopolymer matrix (19). The components of the biofilm matrix form a physical barrier that enhances the inaccessibility of the biofilm cells to antibiotics and the immune system, thereby making the infection difficult to eradicate (33). Extracellular DNA (eDNA) serves as an important biofilm matrix component in several microbial model systems, including but not limited to *Neisseria meningitidis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* (2, 23, 29, 32, 36, 47, 48, 54). The expression of the two secreted *E. faecalis* proteases, gelatinase and serine protease, is regulated in a quorum-dependent manner by the Fsr regulatory system (22, 45, 46), and these proteases direct biofilm development by modulating the eDNA matrix via regulation of the extent of autolysis (54) in a fratricidal manner (51). In an attempt to identify other factors that govern eDNA release in *E. faecalis*, we identified *rpoN*, which encodes σ^{54}, in a preliminary transposon mutagenesis screen.

Transcription initiation is one of the important stages of gene regulation, and sigma factors play a crucial role in determining the controlled response of a subset of genes tied to a given environmental stimulus. Sigma factors reversibly bind to RNA polymerases and drive promoter-specific transcription initiation. In prokaryotes, two distinct families of sigma factors have been studied, sigma 70 (σ^{70}) and σ^{54}. The σ^{70} family also includes several related alternate sigma factors. Sigma 54 shares no structural homology with sigma 70, possesses a distinct consensus binding sequence (−24/−12; TTGGCACNNNNNTTGCT) and, unlike sigma 70, facilitates activator-dependent transcription initiation (24, 38).

Sigma 54 plays an important role in the virulence of several bacteria but does not share the same function in all pathogens (30). In *Vibrio fischeri*, σ^{54} influences biofilm formation, motility, and symbiotic colonization of squid and negatively regulates bioluminescence (58). Quorum-sensing regulation in *Vibrio cholerae* O1 strains is dependent on *rpoN* (28). Sigma 54 is required for biofilm formation by *Burkholderia cenocepacia* as well as its survival within macrophages (51). In the major food-borne pathogen *Listeria monocytogenes*, σ^{54} is essential for its osmotolerance potential (41) and is responsible for mesentericin sensitivity (14, 41), whereas in *Pseudomonas aeruginosa*, σ^{54} influences the activity of isocitrate lyase (21), alginate biosynthesis (6), and pilin and flagellin production, in addition to several other virulence determinants (44). Sigma 54 also regulates biofilm formation, enterocyte effacement, acid tolerance, flagellar biosynthesis, and several other processes in *Escherichia coli* (3, 49, 60).

In *E. faecalis*, σ^{54} is responsible for sensitivity to class IIa bacteriocins, such as mesentericin and divercin (9, 13). The basis for the class IIa bacteriocin sensitivity is due to the role of σ^{54} in regulating four distinct sugar phosphotransferase systems (PTSs)

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

Strain	Genotype or description	Reference
V583	Parental strain	Clinical isolate (50)
VI01	V583 Δ*rpoN*	This study
VI40	VI01 full-length *rpoN* markerless complement	This study
VT09	V583(pMV158GFP), GFP$^+$ Tet^r	54
VI29	VI01(pMV158GFP) GFP$^+$ Tet^r	This study
VI41	VI40(pMV158GFP) GFP$^+$ Tet^r	This study

that are dependent on four known σ^{54} enhancer binding proteins (LpoR, MphR, MpoR, and MptR) (13). MptD, a component of the mannose PTS, is thought to serve as the cellular receptor for the class IIa bacteriocins (24). However, additional roles for σ^{54} in enterococcal biology remain to be elucidated.

In this study, we investigated the role of σ^{54} in eDNA release, autolysis, and biofilm formation, and we demonstrate a functional role for σ^{54} in regulating initial adherence of cells to substrate as well as the overall composition of the biofilm matrix.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. *E. coli* Electro10 Blue (E10-Blue; Stratagene) was used for construction of plasmids and was cultured in Luria-Bertani (LB) broth supplemented with appropriate antibiotics. *E. faecalis* strains were cultured in either Trypticase soy broth containing 0.25% glucose (TSB) or Todd-Hewitt broth (THB; BD Biosciences) containing appropriate antibiotics whenever required. Chloramphenicol (Cm) and spectinomycin (Spec) were used for selection of *E. coli* at concentrations of 10 μg/ml and 150 μg/ml, respectively. For *E. faecalis*, Cm, Spec, and tetracycline (Tet) were used at 15 μg/ml, 500 μg/ml, and 15 μg/ml, respectively. When required, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Amresco) was used at a concentration of 80 μg/ml for both *E. coli* and *E. faecalis*.

In-frame markerless deletion of *rpoN*. An *E. coli-Enterococcus* temperature-sensitive cloning vector, pLT06 (56), was used to generate an isogenic in-frame deletion of *rpoN* in *E. faecalis* V583. Upstream and downstream regions flanking *rpoN* (*ef0782*) were amplified by PCR from a V583 genomic template by using the primer pair RpoNP1/RpoNP2 and RpoNP3/RpoNP4, respectively (Table 3). The primers RpoNP1/RpoNP2 and RpoNP3/RpoNP4 were designed with EcoRI/BamHI and BamHI/PstI restriction sites, respectively. The resultant PCR products were digested with BamHI, ligated, and reamplified with primers RpoNP1 and RpoNP4. For the construction of the deletion vector, the amplified product was digested with EcoRI and PstI followed by ligation to similarly digested pLT06. The ligation was electroporated into competent E10-Blue cells for propagation, and blue colonies were selected on LB agar containing chloramphenicol and X-Gal at 30°C. Clones were screened for the appropriate insert by using the primers OriF and SeqR. A positive plasmid designated pKS70 was confirmed by restriction digestion and electroporated into *E. faecalis* V583 cells (12), and VI01 was subsequently generated following the protocol previously described (56) and confirmed by PCR using the primers RpoNUp and RpoNDown. Using pKS70, ~98% of the *rpoN* gene was deleted, leaving seven codons at the 5′ end and two codons at the 3′ end. The next adjacent gene is *ef0783*, which encodes an O-acetyltransferase. This gene is located approximately 200 bp downstream of *rpoN*, and the strategy used to delete *rpoN* does not alter the expression of *ef0783* (data not shown).

TABLE 2 Plasmid constructs used in this study

Plasmid	Description	Reference
pLT06	Deletion vector; chloramphenicol resistance	56
pKS70	pLT06 containing engineered *rpoN* deletion (2-kb EcoRI/PstI fragment)	This study
pVI12	pLT06 containing full-length *rpoN*	This study
pMV158GFP	Gram-positive replicative vector: GFP$^+$ Tet^r	1

TABLE 3 Oligonucleotides used in this study

Primer	Sequence (5′–3′)
RpoNP1	GAGAGAATTCACAACGGTACAGTAAAATGG
RpoNP2	CTCTGGAATCCCATTCGTTGCTCAAATTTCAT
RpoNP3	GAGAGGATCCGAGTAAACAACCAAAGATTAT
RpoNP4	CTCTCTGCAGGAACTAAGGCACTTAAACCA
RpoN UP	AGTCCAAGGAAGAGTCGTG
RpoN DOWN	AAGACAGTGGCTGCCAAAC
OriF	CAATAATCGCATCCGATTGCA
SeqR	CCTATTATACCATATTTTGGAC

Markerless complementation of VI01 (Δ*rpoN*). The temperature-sensitive cloning vector pLT06 (56) was used to generate markerless gene complementation of *rpoN* in VI01. The *rpoN* gene (*ef0782*) along with flanking regions was amplified by PCR from a V583 genomic template using primers RpoNP1 and RpoNP4 (Table 3). For the construction of *rpoN* markerless complementation vector pVI12, the amplified product was digested with EcoRI and PstI followed by ligation with similarly digested plasmid vector pLT06. The ligation was electroporated into competent E10-Blue cells for propagation, and blue colonies were selected on LB agar containing chloramphenicol and X-Gal at room temperature. Clones were screened for the appropriate insert by using the primers OriF and SeqR. A positive plasmid designated pVI12 was confirmed by restriction digestion and electroporated into *E. faecalis* VI01 cells (12), and VI40 (markerless complement) was generated following the protocol previously described (56) and was confirmed by PCR using primers RpoNUp and RpoNDown.

2DG resistance. *E. faecalis* V583, VI01, and VI40 were grown on LB agar containing 0.2% fructose and 10 mM 2-deoxy-D-glucose (2DG) (24). 2DG is a toxic homologue of glucose and enters cells via the mannose PTS permease (5). In *E. faecalis*, mannose PTS expression is controlled by σ^{54}. Strains resistant to 2DG do not express a functional mannose PTS permease (24). Hence, growth on medium containing 2DG was used as a marker to confirm deletion of *rpoN*.

Detection and precipitation of extracellular DNA. Overnight cultures were centrifuged for 10 min at 13,000 rpm, and the resulting supernatant was filtered (0.2-μm pore size; Nalgene) to obtain cell-free supernatants. The supernatants were tested for the presence of eDNA by using 1 μM SYTOX Green (Invitrogen, Molecular Probes).

The eDNA was also precipitated from the culture filtrate with an equal volume of isopropanol. The precipitated eDNA was washed in 75% ethanol, air dried, and dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 8.0) and visualized on 1% agarose gels after staining with ethidium bromide.

Autolysis assay. The autolysis assay was performed as previously described (15).

Quantitative detection of eDNA in biofilms. eDNA in biofilms was quantified using a previously described protocol with some modifications (36). Briefly, biofilms were grown in a 96-well polystyrene plate in TSB for 24 h at 37°C. After 24 h, the supernatant was discarded and the biofilm was suspended in resuspension buffer (50 mM Tris-Cl [pH 8], 10 mM EDTA, 500 mM NaCl). The resuspended biofilm was centrifuged, and eDNA was quantified in the supernatant with 1 μM SYTOX Green (Invitrogen, Molecular Probes).

CSLM. Confocal laser scanning microscopy (CLSM) was performed on 1-day-old biofilms as described previously (54). *E. faecalis* strains VI01 and VI40 were transformed with pMV158GFP (39) to generate VI29 and VI41, respectively, both of which expressed green fluorescent protein

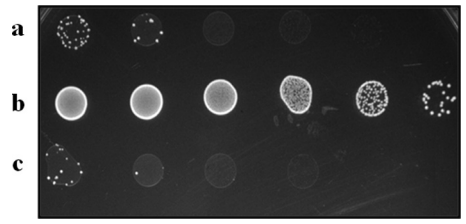

FIG 1 2DG resistance analysis of VI01. Wild-type strain, V583 (a) and the complement strain VI40 (c) are sensitive to 2DG because of a functional *mpt* operon under the control of intact *rpoN*. *rpoN* mutant VI01 (b) is resistant to 2DG. This confirms the deletion and complementation of *rpoN* in *E. faecalis*.

(GFP) constitutively. VT09 [V583(pMV158GFP)] (54) along with VI29 and VI41 were used for confocal imaging. Briefly, biofilms were grown on sterile glass coverslips placed in six-well tissue culture plates. The coverslip was submerged in 5 ml of TSB containing tetracycline for plasmid maintenance. After 24 h of growth, the biofilm was gently washed with sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 [pH 7.4]) and stained with 1 μM SYTOX orange (Invitrogen) for 6 to 7 min. The coverslip were inverted on a clean glass slide and sealed using clear nail polish. The biofilm was visualized using a Zeiss LSM 5 Pa laser scanning confocal microscope.

Macroscopic biofilm. To visualize the biofilms formed by VT09, VI29, and VI41 macroscopically, biofilms were grown as described for confocal analysis, with the exception that after 24 h of growth, the biofilms were gently washed with sterile phosphate buffer, then fresh TSB was added, and the biofilms were grown for an additional 24 h, at which time the biofilm was washed and imaged with an AlphaImager system (Alpha Innotech, San Leandro, CA).

Adherence assay. Adherence of *E. faecalis* strains to flat-bottom polystyrene plates (BRAND, Germany) was tested using a previously described protocol (27) with some modifications. Cultures grown overnight were diluted 1:10 in fresh TSB, and 200 μl was transferred to a flat-bottom 96-well polystyrene microtiter plate. After 2 h of incubation at 37°C, the supernatant was discarded and the wells were gently washed with sterile PBS. The adherent cells were resuspended in 200 μl PBS by vigorous pipetting, diluted, and plated on THB agar for colony counting. Also, the initial load was calculated by plating the diluted culture on THB agar for colony counting. The adherence potential of the strains was calculated as the percentage of initial load that adhered.

Proteinase K treatment of biofilm. Biofilms were grown on 96-well round-bottom tissue culture-treated polystyrene plates (Techno Plastic Products, Switzerland) as previously described (22). At 6, 12, and 24 h, the biofilms were treated with 1 μg/ml proteinase K (Amresco), and this treatment remained for the remainder of the experiment. The 24-h treatment was allowed to stand for 1 h prior to processing the biofilm. An untreated control was included to determine the effect of treatment. After 24 h of growth, the biofilm was quantified by the crystal violet staining method (22). Each assay was performed in triplicate and repeated four times.

Statistical analysis. Statistical analysis of quantitative detection of eDNA, adherence assay results, and Comstat analysis of biofilms were performed using GraphPad Prism 4 software (San Diego, CA). One-way analysis of variance followed by Dunn's multiple comparison tests was performed to determine statistical significance.

RESULTS

Construction of the *E. faecalis* V583 isogenic *rpoN* mutant and its complement. The *rpoN* deletion mutant VI01 (Δ*rpoN*) was constructed using the markerless deletion vector pKS70. Initial growth curves of the wild-type V583 strain, the *rpoN* deletion mutant (VI01), and its complement (VI40) were assessed in TSB. No alterations in the growth of the 3 strains were observed (see Fig. S1 in the supplemental material). The 2DG-resistant phenotype was confirmed by growth on medium containing 2DG. VI01 grew to the final dilution of 10^{-8}, while the parental strain V583 and the *rpoN* complement VI40 were significantly inhibited and grew only at dilutions of 10^{-3} and 10^{-4}. Complementation confirmed that there were no polar effects of the gene deletion and attributed the 2DG-resistant phenotype to the targeted deletion of *rpoN* (Fig. 1).

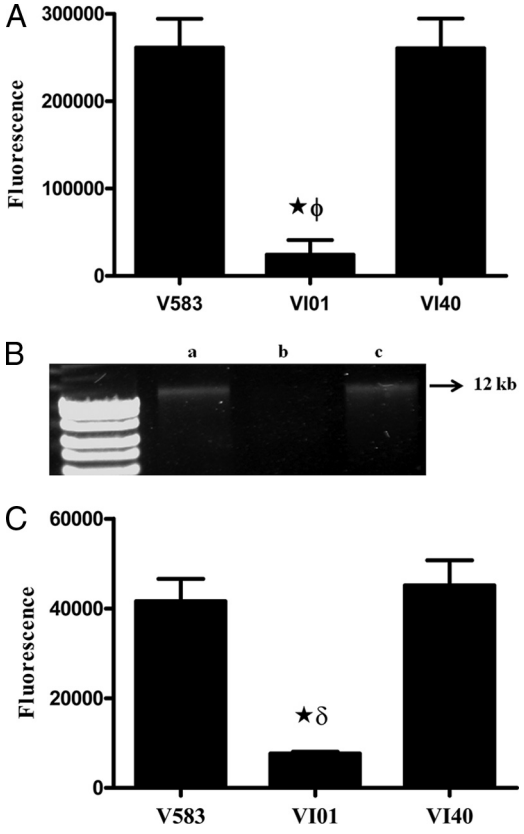

FIG 2 (A) Quantitative detection of eDNA in culture supernatants with SYTOX Green. eDNA was quantified in the culture supernatants of cultures grown overnight by using 1 μM SYTOX Green. Assays were performed in quadruplicate, and error bars indicate the standard errors of the means. ★, significant decrease ($P < 0.001$) relative to wild-type V583; ϕ, significant decrease ($P < 0.001$) relative to *rpoN* complement (VI40). (B) Qualitative detection of eDNA in culture supernatant by isopropanol precipitation. Lane a, wild type (V583); lane b, *rpoN* mutant (VI01); lane c, *rpoN* complement (VI40). (C) Detection of eDNA in biofilm by using SYTOX Green. Assays were performed in sextuplets, and error bars indicate standard errors of means. ★, significant decrease ($P < 0.001$) relative to wild-type V583; δ, significant decrease ($P < 0.001$) relative to the *rpoN* complement (VI40).

Sigma 54 alters eDNA in the supernatant of planktonic and biofilm cultures. On the basis of phenotype characterization from a preliminary transposon mutagenesis screen, we tested for eDNA in the supernatants of planktonic cultures by using SYTOX Green. A smaller amount of eDNA was detected in VI01 culture supernatants than with the wild-type V583 strain, whereas the markerless complementation of the *rpoN* mutant restored the phenotype to wild type (Fig. 2A). eDNA in the supernatants of the various strains was also confirmed by visualization on an ethidium bromide-stained 1% agarose gel after precipitation of eDNA with isopropanol (Fig. 2B).

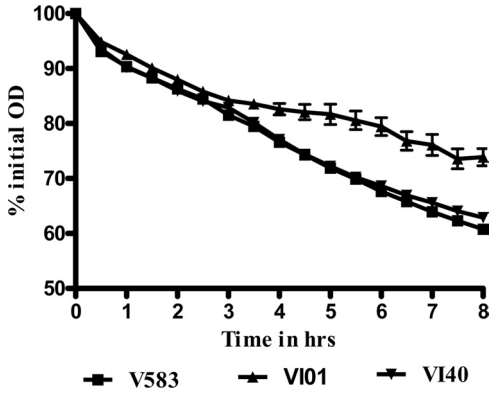

FIG 3 RpoN alters the rate of autolysis in *E. faecalis*. Differences in autolysis rates of the wild type (V583), *rpoN* mutant (VI01), and complemented strain (VI40) are plotted as the percentage of the initial optical density (OD) at 600 nm. Assays were performed in triplicate and repeated four times; error bars represent standard errors of means.

Given the fact that planktonic growth and biofilms are two different lifestyles of the bacterium, we tested to see the effect of *rpoN* deletion on eDNA during biofilm development. As observed in planktonic cultures, a lesser amount of eDNA was detected in the VI01 biofilm than in the wild type, and this was attributed to the deletion of *rpoN*, as the complementation restored the eDNA detected in the biofilm to wild-type levels (Fig. 2C).

Sigma 54 alters the rate of autolysis in *E. faecalis* V583. Because eDNA release in *E. faecalis* is dependent upon cell death by autolysis (54) and the *rpoN* mutant is defective in eDNA release, we hypothesized that σ^{54} may differentially modify the rate of autolysis in *E. faecalis*. In the autolysis assay, we observed that VI01 showed a significant decrease in the rate of autolysis, a phenotype readily complemented by introducing the gene in single copy to its native locus (Fig. 3).

Sigma 54 alters biofilm development by *E. faecalis*. eDNA has been shown to be an important matrix component in *E. faecalis* biofilms (54). The decreased levels of eDNA in VI01 led us to the hypothesis that VI01 may form less-dense biofilms than the wild-type V583. However, CLSM analysis of 24-h-old biofilms grown on glass coverslips showed that VI29 (Δ*rpoN*, GFP$^+$) formed a thicker biofilm (as measured based on *z*-stack thickness, using an LSM image examiner) than those formed by the wild-type strain VT09 or the complemented strain VI41 (Fig. 4b). The appearance of the VI29 biofilm suggested early initiation of microcolony development, which was confirmed by macroscopic examination of the biofilms after 2 days of growth on coverslips (Fig. 4a). Despite the increased thickness and overall biofilm biomass of the *rpoN* mutant strain (VI29) compared to the parental and complemented strains (Table 4), very few random dead cells and DNA

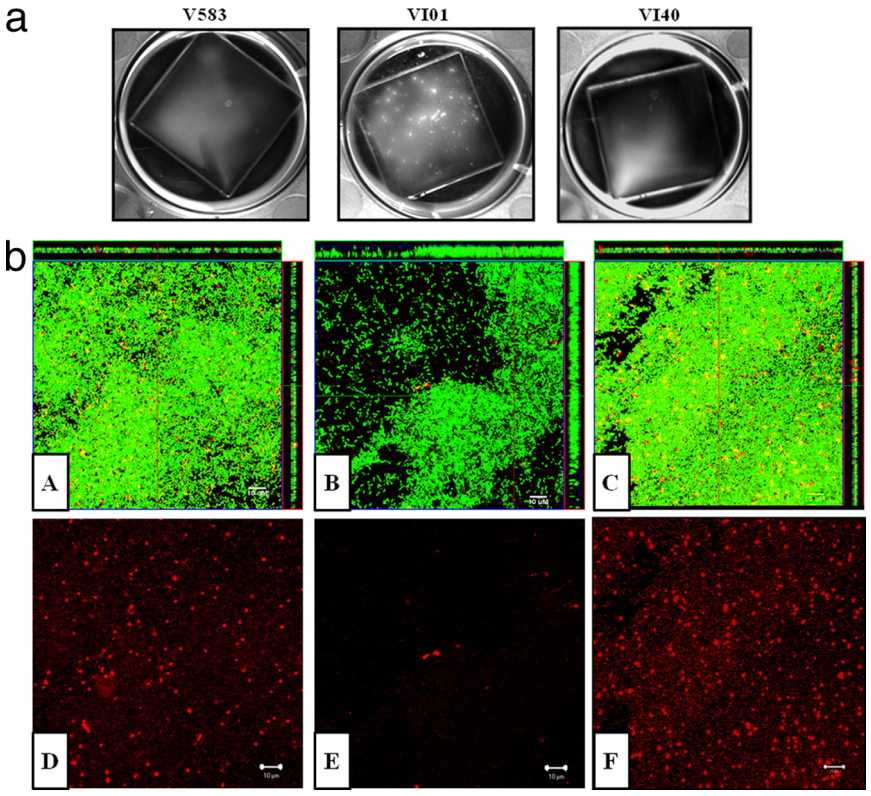

FIG 4 (a) Macroscopic views of biofilms (produced by V583, VI01, and VI40) grown on glass coverslips in TSB medium. (b) Confocal analysis of 1-day-old biofilms grown on glass coverslips. The wild type, mutant, and complement constitutively expressed GFP from pMV158gfp as mentioned in Materials and Methods. Biofilms were grown on glass coverslips in TSB medium. Dead cells and eDNA were stained with SYTOX orange (1 μM). Live bacteria appear green, while dead cells and eDNA are red. Biofilm orthogonal projections are shown for VT09 (A), VI29 (B), and VI41 (C) and show merged green and red staining. Panels D, E, and F correspond to dead cell and eDNA staining of VT09, VI29, and VI41 biofilms, respectively (matched pairs to biofilms in panels A, B, and C). Bars, 10 μm.

TABLE 4 Comstat analysis of 1-day-old biofilm[a]

Strain	Biomass ($\mu m^3/\mu m^2$)	Avg thickness (μm)
VT09	2.09 ± 0.522§	2.40 ± 0.589§
VI29	4.723 ± 1.28	5.97 ± 2.01
VI41	2.19 ± 0.638§	2.14 ± 0.624§

[a] Values are means ± standard deviations. §, significantly different ($P < 0.05$) from VI29 result.

(as detected by SYTOX orange staining) were observed within the biofilm. Consistent with earlier observations on the role of cell death and of eDNA as a matrix component (54), regions within the wild-type VT09 contained concentrated foci of DNA and dead cells, which was phenocopied by the complement VI41 strain (Fig. 4b).

Deletion of *rpoN* increases adherence to polystyrene plates. In order to determine whether increased biofilm formation by the *rpoN* mutant was due to its initial adherence ability, we calculated the percentage of the initial inoculum that adhered to 96-well microtiter plates after 2 h. Adherence of VI01 to a polystyrene plate was significantly enhanced in comparison to the wild type. In addition, markerless complementation of VI01 (VI40) reduced the adherence potential to wild-type levels (Fig. 5).

Sigma 54 modulates the composition of *E. faecalis* V583 biofilms. On the basis of the macroscopic observations and CLSM of the VI29 biofilm and the relative lack of eDNA detection for this mutant, we hypothesized a role for a different polymer matrix that promotes biofilm formation in the *rpoN* mutant. To test the role of proteins in VI01 biofilm, we examined the effect of proteinase K treatment on biofilm development. The wild-type and complemented strains exhibited decreased biofilm when treated with proteinase K only after 24 h of biofilm growth. In contrast, reduction in VI01 biofilm was significant when treated with proteinase K after 6 h of biofilm growth and continued to respond to treatment after 12 h and 24 h of biofilm growth (Fig. 6).

DISCUSSION

The role of σ^{54} in regulating numerous biological properties, including those related to virulence, has been well documented for a variety of bacterial species (3, 10, 21, 42, 49, 51, 52, 55, 58, 59). However, its role in *E. faecalis* has been limited to observations made regarding its contribution to sensitivity to class IIa bacteriocins through the regulation of sugar PTSs (9, 13, 22). Identification of σ^{54} as a potential regulatory protein in the cascade of biofilm development was an interesting breakthrough, and we focused our efforts on elucidating its effect on *E. faecalis* V583 biofilm. The role of autolysis (54) and fratricide (53) has been well documented in enterococcal biofilm formation and has been shown to be important in providing eDNA as a key biofilm matrix component. However, the observation that biofilm formation was enhanced in the *rpoN* mutant despite the increased resistance to autolysis and the absence of eDNA was an unexpected finding.

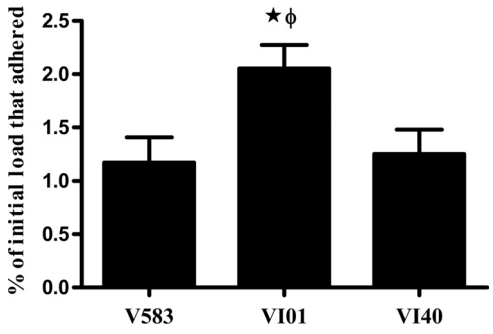

FIG 5 Polystyrene plate adherence assay results. Deletion of *rpoN* increased adherence of *E. faecalis* to 96-well polystyrene plates. ★, significant increase ($P < 0.05$) relative to wild-type V583; ϕ, significant increase ($P < 0.05$) relative to the *rpoN* complement (VI40).

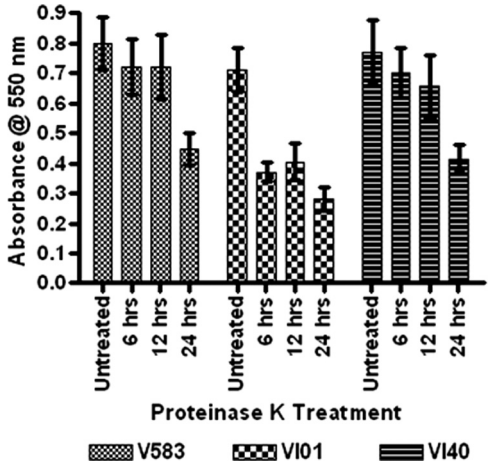

FIG 6 Proteinase K inhibits biofilm development of the *rpoN* mutant on polystyrene plates. Biofilms were seeded at time zero for V583, VI01, and VI40, and the untreated biofilms were stained 24 h later. At the indicated times after seeding the biofilm, proteinase K (1 μg/ml) was added, and the treatment was allowed to continue for the remainder of the assay. Each assay was performed in triplicate and repeated four times. Error bars indicate standard errors of the means.

One possible explanation for the increased resistance to autolysis observed in the *rpoN* mutant could be novel modifications of the cell wall or alteration of the modifications, such as O-acetylation (43) or D-alanylation (17) on the cell wall that protect against lysis. Deletion of *rpoN* did not alter the autolysin profile of *E. faecalis* when the micrococcal cell wall was used as a zymogram substrate (data not shown), ruling out the possibility of inactive autolysins. Also, the deletion of *rpoN* did not have a measurable effect on the secretion of the extracellular proteases GelE and SprE (data not shown), which have been previously shown to contribute to autolysis in *E. faecalis* (54, 57). A significant reduction in cell death due to impaired cell lysis occurred in *E. faecalis* V583 (Δ*rpoN*) planktonic and biofilm cultures, suggesting the requirement of a functional σ^{54} for regulation of susceptibility to cell lysis. In *P. aeruginosa*, deletion of *rpoN* abolishes cell death in the microcolonies during biofilm maturation and has been related to the expression of surface structures (type 4 pili and flagella) whose expression is regulated by σ^{54} (56). Additionally, σ^{54}-dependent gene regulation promotes phage-induced lysis in *P. aeruginosa* (10). There are seven phages associated with *E. faecalis* V583, with one of them being a part of the core genome (37). It will be interesting to test the role of σ^{54}-dependent transcription of phage particle proteins and host cell lysis and the contribution of σ^{54} to biofilm development.

Enhancement of biofilm formation in the absence of a well-characterized matrix component in the *rpoN* mutant indicates that a substantial knowledge gap still exists in unraveling factors

associated with *E. faecalis* biofilm development. Cellular processes regulated by σ^{54} are attractive in this regard, to begin revealing the interplay between metabolism and biofilm development, as one of the few characterized roles for σ^{54} is the regulation of four sugar PTS pathways. It is noteworthy that deletion of the genes encoding the four known enhancer binding proteins (LpoR, MphR, MpoR, and MptR) did not reduce eDNA release, impair autolysis, or alter biofilm development (data not shown), suggesting that σ^{54} might act as a repressor of genes independent of enhancer protein function. The idea that σ^{54} levels in the cell or within the population might be regulated raises an interesting experimental question. Our observation that the wild-type and *rpoN* complement strains could grow on 2DG at a much lower frequency (10^{-4} and 10^{-5}, respectively) relative to the *rpoN* mutant parallels a recent report by Flanagan et al. (18) that resistance to the *E. faecalis* plasmid-encoded bacteriocin MC4-1 (a class IIa bacteriocin) was dependent on point mutations within the *rpoN* gene that occurred at high frequency (10^{-3} to 10^{-4}). This resistance was shown to be reversible to a susceptible phenotype by point mutations that also occurred within *rpoN* as second-site suppressors. These combined observations suggest that there are hot spots for mutation within *rpoN* and could be a mechanism for phase variation within the *E. faecalis* population.

In *Vibrio vulnificus*, σ^{54} positively regulates the gene encoding ADP-glycero-manno-heptose-6-epimerase (*gmhD*), which is responsible for production of lipopolysaccharide and exopolysaccharide, both of which are required for biofilm formation (31), while in *B. cenocepacia* σ^{54} controls motility, which in turn plays a role in biofilm formation (51). However, in *E. coli* K-12, *rpoN* deletion enhances biofilm formation (3). Such different effects of *rpoN* on the biofilm-forming potential of bacteria provide a clear example of how the knowledge regarding a gene whose function was first reported to be restricted to nitrogen assimilation has evolved to govern virulence-related functions in addition to bacterial metabolism. Our data provide additional support for the expanding role of σ^{54} in the world of low-GC Gram-positive bacteria.

Biofilm formation is a multistep process that begins with the attachment of bacteria to a substrate, followed by colonization via further recruitment of more bacteria or by cell division. Initial attachment of a bacterial cell to a surface is an important stage in biofilm development and determines the fate of this process. In *P. aeruginosa* (8, 35), *Staphylococcus aureus* (11), and *Streptococcus* spp. (40) it has been shown that a defect in initial adherence of a bacterium affects biofilm formation and subsequently influences the virulence of these pathogens. Our data indicate a similar influence of the attachment process in enterococcal biofilm development, wherein deletion of *rpoN* increases the adherence potential of the pathogen, which subsequently results in a more dense biofilm.

Other than DNA, other molecules, such as proteins and polysaccharides, have been suggested to be important constituents in the polymer matrix of several bacteria (16, 19). Robust biofilm formation by VI01 despite the significant reduction in eDNA led us to test for the presence of other matrix components by using compounds capable of dissolving the aforementioned components. The reduced ability of VI01 (Δ*rpoN*) to form a biofilm when treated with proteinase K suggests a role for protein in either adhesion or matrix composition to promote *E. faecalis* biofilm and is consistent with recent observations by Guiton et al. (20). Those authors observed that colonization of an implanted piece of urinary catheter as well as the bladder epithelium was dependent on a functioning sortase enzyme for the proper anchoring of proteins to the cell wall, which in turn promoted cellular adhesion. In *S. aureus*, a biofilm defect in mutants that overproduce extracellular protease was rectified by the addition of α_2-macroglobulin, a general protease inhibitor, indicating a vital role for proteins in either cellular adhesion or the biofilm matrix (4). Similarly, in *Bacillus subtilis*, TasA is required for the structural integrity and development of biofilms (7). In *E. faecalis* biofilms (54), eDNA is known to be a crucial matrix component in the early stages of biofilm development, but by 24 h of growth in the biofilm DNase has a minimal effect on disrupting the biofilm. Here we show that in *E. faecalis* V583, proteins are likely to serve as important matrix components during the later stages of biofilm development, as a reduction in biomass was observed only at 24 h and not at earlier time points. This suggests the time-dependent involvement of different polymers in the overall development of the biofilm.

Complementation studies of the *rpoN* mutant by using a low-copy-number plasmid did not result in complete reversal of the phenotype to wild-type levels in experiments that involved stressing of cells (osmotic shock and 2DG toxicity) (data not shown). This was primarily due to plasmid loss in the absence of selection and suggested a survival advantage for *E. faecalis* in the absence of σ^{54} under certain stress conditions. The inability to fully complement an *rpoN* mutant has also been reported for *L. monocytogenes* (41). Similarly, in a *V. fischeri* squid colonization model (58), the level of colonization varied with the complemented strain, and only some animals exhibited wild-type levels of colonization. For this reason, we utilized a complementation strategy that restored the function of the gene by placing it at its native locus in single copy.

A literature survey for σ^{54} and its biological roles revealed a bias toward Gram-negative species, with *P. aeruginosa*, *Vibrio* spp., and *E. coli* being the most studied. In an attempt to identify the distribution of *rpoN* in low-GC Gram-positive organisms, we performed a BLAST search using σ^{54} of *E. faecalis* V583 as the query. Among the organisms queried, only *L. monocytogenes*, *B. subtilis*, *C. difficile*, and *C. perfringens* appeared to have homologues, whereas in *S. aureus*, *S. pneumoniae*, and *S. pyogenes* homologues to σ^{54} were absent. The basis for this distribution among enterically adapted organisms as well as the potential genes regulated by σ^{54} await further study.

In conclusion, the results from this study show that σ^{54} in *E. faecalis* V583 contributes to cell death and eDNA release and that in its absence, *E. faecalis* adapts an alternate matrix to establish biofilms. Understanding the mechanism underlying the phenotypes observed in this study is the main focus of ongoing studies in our laboratory.

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REFERENCES

1. **Acebo P, Nieto C, Corrales MA, Espinosa M, Lopez P.** 2000. Quantitative detection of *Streptococcus pneumoniae* cells harbouring single or

multiple copies of the gene encoding the green fluorescent protein. Microbiology **146**:1267–1273.
2. **Bass JIF, et al.** 2010. Extracellular DNA: a major proinflammatory component of *Pseudomonas aeruginosa* biofilms. J. Immunol. **184**:6386–6395.
3. **Belik AS, Tarasova NN, Khmel IA.** 2008. Regulation of biofilm formation in *Escherichia coli* K12: effect of mutations in the genes *hns*, *strA*, *lon*, and *rpoN*. Mol. Gen. Microbiol. Virol. **23**:159–162.
4. **Boles BR, Thoendel M, Roth AJ, Horswill AR.** 2010. Identification of genes involved in polysaccharide-independent *Staphylococcus aureus* biofilm formation. Plos One **5**:e10146.
5. **Bond DR, Tsai BM, Russell JB.** 1999. Physiological characterization of *Streptococcus bovis* mutants that can resist 2-deoxyglucose-induced lysis. Microbiology **145**:2977–2985.
6. **Boucher JC, Schurr MJ, Deretic V.** 2000. Dual regulation of mucoidy in *Pseudomonas aeruginosa* and sigma factor antagonism. Mol. Microbiol. **36**:341–351.
7. **Branda SS, Chu F, Kearns DB, Losick R, Kolter R.** 2006. A major protein component of the *Bacillus subtilis* biofilm matrix. Mol. Microbiol. **59**: 1229–1238.
8. **Byrd MS, Pang B, Mishra M, Swords WE, Wozniak DJ.** 2010. The *Pseudomonas aeruginosa* exopolysaccharide Psl facilitates surface adherence and NF-kappa B activation in A549 cells. Mbio **1**(3):e00140–10.
9. **Calvez S, Rince A, Auffray Y, Prevost H, Drider D.** 2007. Identification of new genes associated with intermediate resistance of *Enterococcus faecalis* to divercin V41, a pediocin-like bacteriocin. Microbiology **153**: 1609–1618.
10. **Ceyssens PJ, et al.** 2008. The genome and structural proteome of YuA, a new *Pseudomonas aeruginosa* phage resembling M6. J. Bacteriol. **190**: 1429–1435.
11. **Clarke SR, Foster SJ.** 2006. Surface adhesins of *Staphylococcus aureus*. Adv. Microb. Physiol. **51**:187–224.
12. **Cruz-Rodz AL, Gilmore MS.** 1990. High-efficiency introduction of plasmid DNA into glycine treated *Enterococcus faecalis* by electroporation. Mol. Gen. Genet. **224**:152–154.
13. **Dalet K, Briand C, Cenatiempo Y, Hechard Y.** 2000. The *rpoN* gene of *Enterococcus faecalis* directs sensitivity to subclass IIa bacteriocins. Curr. Microbiol. **41**:441–443.
14. **Dalet K, Cenatiempo Y, Cossart P, Hechard Y, European Listeria Genome Consortium.** 2001. A σ^{54}-dependent PTS permease of the mannose family is responsible for sensitivity of *Listeria monocytogenes* to mesentericin Y105. Microbiology **147**:3263–3269.
15. **Del Papa MF, Hancock LE, Thomas VC, Perego M.** 2007. Full activation of *Enterococcus faecalis* gelatinase by a C-terminal proteolytic cleavage. J. Bacteriol. **189**:8835–8843.
16. **Dunne WM.** 2002. Bacterial adhesion: seen any good biofilms lately? Clin. Microbiol. Rev. **15**:155–166.
17. **Fabretti F, et al.** 2006. Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. Infect. Immun. **74**:4164–4171.
18. **Flanagan S, Clewell D, Sedgley C.** 2010. *Enterococcus faecalis* alternates between resistance and sensitivity to the class IIa enterococcal bacteriocin MC4-1, abstr. 35A.3rd Int. ASM Conf. Enterococci. ASM Press, Washington, DC.
19. **Flemming HC, Wingender J.** 2010. The biofilm matrix. Nat. Rev. Microbiol. **8**:623–633.
20. **Guiton PS, Hung CS, Hancock LE, Caparon MG, Hultgren SJ.** 2010. Enterococcal biofilm formation and virulence in an optimized murine model of foreign body-associated urinary tract infections. Infect. Immun. **78**:4166–4175.
21. **Hagins JM, Scoffield JA, Suh SJ, Silo-Suh L.** 2010. Influence of RpoN on isocitrate lyase activity in *Pseudomonas aeruginosa.* Microbiology **156**: 1201–1210.
22. **Hancock LE, Perego M.** 2004. The *Enterococcus faecalis* fsr two-component system controls biofilm development through production of gelatinase. J. Bacteriol. **186**:5629–5639.
23. **Harmsen M, Lappann M, Knochel S, Molin S.** 2010. Role of extracellular DNA during biofilm Formation by *Listeria monocytogenes.* Appl. Environ. Microbiol. **76**:2271–2279.
24. **Hechard Y, Pelletier C, Cenatiempo Y, Frere J.** 2001. Analysis of σ^{54}-dependent genes in *Enterococcus faecalis*: a mannose PTS permease (EIIMan) is involved in sensitivity to a bacteriocin, mesentericin Y105. Microbiology **147**:1575–1580.
25. **Hidron AI, et al.** 2008. Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infect. Control Hosp. Epidemiol. **29**: 996–1011.
26. **Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O.** 2010. Antibiotic resistance of bacterial biofilms. Int. J. Antimicrob. Agents **35**:322–332.
27. **Huelves L, et al.** 2007. Adherence of *Streptococcus pneumoniae* to polystyrene plates, effect of serum on adhesion, and virulence in the gerbil otitis media model. Microb. Pathog. **43**:114–119.
28. **Ishikawa T, Rompikuntal PK, Lindmark B, Milton DL, Wai SN.** 2009. Quorum sensing regulation of the two *hcp* alleles in *Vibrio cholerae* O1 strains. Plos One **4**:9.
29. **Jefferson KK.** 2004. What drives bacteria to produce a biofilm? FEMS Microbiol. Lett. **236**:163–173.
30. **Kazmierczak MJ, Wiedmann M, Boor KJ.** 2005. Alternative sigma factors and their roles in bacterial virulence. Microbiol. Mol. Biol. Rev. **69**: 527–543.
31. **Kim HS, Lee MA, Chun SJ, Park SJ, Lee KH.** 2007. Role of NtrC in biofilm formation via controlling expression of the gene encoding an ADP-glycero-manno-heptose-6-epimerase in the pathogenic bacterium, *Vibrio vulnificus.* Mol. Microbiol. **63**:559–574.
32. **Lappann M, et al.** 2010. A dual role of extracellular DNA during biofilm formation of *Neisseria meningitidis.* Mol. Microbiol. **75**:1355–1371.
33. **Lewis K.** 2001. Riddle of biofilm resistance. Antimicrob. Agents Chemother. **45**:999–1007.
34. **Lindsay D, von Holy A.** 2006. Bacterial biofilms within the clinical setting: what healthcare professionals should know. J. Hosp. Infect. **64**: 313–325.
35. **Ma L, Jackson KD, Landry RM, Parsek MR, Wozniak DJ.** 2006. Analysis of *Pseudomonas aeruginosa* conditional Psl variants reveals roles for the Psl polysaccharide in adhesion and maintaining biofilm structure postattachment. J. Bacteriol. **188**:8213–8221.
36. **Mann EE, et al.** 2009. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. Plos One **4**:12.
37. **McBride SM, Fischetti VA, LeBlanc DJ, Moellering RC, Gilmore MS.** 2007. Genetic diversity among *Enterococcus faecalis.* Plos One **2**:e582.
38. **Merrick MJ.** 1993. In a class of its own: the RNA polymerase sigma factor 54 (sigma N). Mol. Microbiol. **10**:903–909.
39. **Nieto C, Espinosa M.** 2003. Construction of the mobilizable plasmid pMV158GFP, a derivative of pMV158 that carries the gene encoding the green fluorescent protein. Plasmid **49**:281–285.
40. **Nobbs AH, Lamont RJ, Jenkinson HF.** 2009. *Streptococcus* adherence and colonization. Microbiol. Mol. Biol. Rev. **73**:407–450.
41. **Okada Y, et al.** 2006. The sigma factor RpoN (sigma 54) is involved in osmotolerance in *Listeria monocytogenes.* FEMS Microbiol. Lett. **263**: 54–60.
42. **O'Toole R, Milton DL, Horstedt P, Wolf-Watz H.** 1997. RpoN of the fish pathogen *Vibrio* (*Listonella*) *anguillarum* is essential for flagellum production and virulence by the water-borne but not intraperitoneal route of inoculation. Microbiology **143**:3849–3859.
43. **Pfeffer JM, Strating H, Weadge JT, Clarke AJ.** 2006. Peptidoglycan O acetylation and autolysin profile of *Enterococcus faecalis* in the viable but nonculturable state. J. Bacteriol. **188**:902–908.
44. **Potvin E, Sanschagrin F, Levesque RC.** 2008. Sigma factors in *Pseudomonas aeruginosa.* FEMS Microbiol. Rev. **32**:38–55.
45. **Qin X, Singh KV, Weinstock GM, Murray BE.** 2001. Characterization of *fsr*, a regulator controlling expression of gelatinase and serine protease in *Enterococcus faecalis* OG1RF. J. Bacteriol. **183**:3372–3382.
46. **Qin X, Singh KV, Weinstock GM, Murray BE.** 2000. Effects of *Enterococcus faecalis fsr* genes on production of gelatinase and a serine protease and virulence. Infect. Immun. **68**:2579–2586.
47. **Qin ZQ, et al.** 2007. Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis. Microbiology **153**:2083–2092.
48. **Rice KC, et al.** 2007. The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus.* Proc. Natl. Acad. Sci. U. S. A. **104**:8113–8118.
49. **Riordan JT, Tietjen JA, Walsh CW, Gustafson JE, Whittam TS.** 2010. Inactivation of alternative sigma factor 54 (RpoN) leads to increased acid resistance, and alters locus of enterocyte effacement (LEE) expression in Escherichia coli O157:H7. Microbiology **156**:719–730.
50. **Sahm DF, et al.** 1989. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis.* Antimicrob. Agents Chemother. **33**: 1588–1591.

51. **Saldias MS, Lamothe J, Wu R, Valvano MA.** 2008. *Burkholderia cenocepacia* requires the RpoN sigma factor for biofilm formation and intracellular trafficking within macrophages. Infect. Immun. **76**:1059–1067.
52. **Stevens MJA, Molenaar D, de Jong A, De Vos WM, Kleerebezem M.** 2010. σ^{54}-mediated control of the mannose phosphotransferase sytem in *Lactobacillus plantarum* impacts on carbohydrate metabolism. Microbiology **156**:695–707.
53. **Thomas VC, et al.** 2009. A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis.* Mol. Microbiol. **72**:1022–1036.
54. **Thomas VC, Thurlow LR, Boyle D, Hancock LE.** 2008. Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development. J. Bacteriol. **190**: 5690–5698.
55. **Thompson LS, Webb JS, Rice SA, Kjelleberg S.** 2003. The alternative sigma factor RpoN regulates the quorum sensing gene *rhlI* in *Pseudomonas aeruginosa.* FEMS Microbiol. Lett. **220**:187–195.
56. **Thurlow LR, Thomas VC, Hancock LE.** 2009. Capsular polysaccharide production in *Enterococcus faecalis* and contribution of CpsF to capsule serospecificity. J. Bacteriol. **191**:6203–6210.
57. **Waters CM, Antiporta MH, Murray BE, Dunny GM.** 2003. Role of the *Enterococcus faecalis* GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. J. Bacteriol. **185**:3613–3623.
58. **Wolfe AJ, Millikan DS, Campbell JM, Visick KL.** 2004. *Vibrio fischeri* σ^{54} controls motility, biofilm formation, luminescence, and colonization. Appl. Environ. Microbiol. **70**:2520–2524.
59. **Yang TC, Leu YW, Chang-Chien HC, Hu RM.** 2009. Flagellar biogenesis of *Xanthomonas campestris* requires the alternative sigma Factors RpoN2 and FliA and is temporally regulated by FlhA, FlhB, and FlgM. J. Bacteriol. **191**:2266–2275.
60. **Zhao K, Liu MZ, Burgess RR.** 2010. Promoter and regulon analysis of nitrogen assimilation factor, σ^{54}, reveal alternative strategy for *E. coli* MG1655 flagellar biosynthesis. Nucleic Acids Res. **38**:1273–1283.