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Deletion of Sigma54 (*rpoN*) alters the rate of autolysis and biofilm formation in *Enterococcus faecalis*

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Abstract

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* that elevates its 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 indicates 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.

Introduction

As opportunistic pathogens, enterococci, are the third leading cause of hospital acquired or associated infections responsible for 11.2 % surgical site infection (SSI), 14.9% of urinary tract infection (UTI) and 16% of the reported blood stream infections (25). The ability to form 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 serve as foci to establish blood stream infections. Biofilms are aggregates of bacteria that are covered in exoploymer 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 enhance 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, Pseudumonas aeruginosa, E. 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 by regulating 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 sigma 54 (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 squids and negatively regulates bioluminescence (58). Quorum sensing regulation in *V. cholerae* O1 strains is dependent on *rpoN* (28). Sigma 54 is required for biofilm formation by *Burkholderia cenocepacia* as well as survival within macrophages (51). In the major food-borne pathogen *Listeria monocytogenes* , σ^{54} is essential for its osmotolerance potential (42) and is responsible for mesentericin sensitivity (14, 42) whereas in *Pseudomonas aeruginosa*, σ^{54} influences the activity of isocitrate lyase (21) , alginate biosynthesis (6) and in 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 *E. 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 (PTS) systems that are dependent on four known σ^{54} enhancer binding proteins (LpoR, MphR, MpoR, and MptR) (13) . MptD, a component of the mannose PTS system 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 investigate the role of σ^{54} in eDNA release, autolysis and biofilm formation and 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 Method

Bacterial strains and growth conditions:

The bacterial strains and plasmids used in this study are listed in Tables 1 and 2 respectively. *E. coli* Electroten Blue (Strategene) 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 a concentration of 10µg/ml and 150µg/ml respectively. For *E. faecalis*, Cm, Spec and tetracycline (Tet) were used at 15µg/ml, 500µg/ml and 15µg/ml respectively. When required, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; AMRESCO) was used at a concentration of 80µg/ml for both *E. coli* and *E. faecalis*.

In-frame Markerless deletion of *rpoN*

An *E.coli*-enterococcal temperature sensitive cloning vector,pLT06 (56), was used to generate 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 using the primer pair RpoNP1/RpoNP2 and RpoNP3/RpoNP4 respectively (refer 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 re-amplified 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 using the primers OriF and SeqR. A positive plasmid designated pKS70 was confirmed by restriction digest and electroporated into *E. faecalis* V583 cells (12) and VI01 was subsequently generated following the protocol previously described (56) and confirmed by PCR using 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-acetyl transferase. This gene is located approximately 200 bps downstream of *rpoN* and the strategy used to delete *rpoN* does not alter the expression of *ef0783* (data not shown).

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 (refer 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 using the primers OriF and SeqR. A positive plasmid designated pVI12 was confirmed by restriction digest and electroporated into *E. faecalis* VI01 cells (12) and VI40 (markerless complement) was generated following the protocol previously described (56) and confirmed by PCR using primers RpoNUp and RpoNDown.

2-Deoxy-D-Glucose (2DG) resistance

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

Detection and precipitation of extracellular DNA

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

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

Autolysis assay

Autolysis assay was performed as previously described (15).

Quantitative detection of eDNA in biofilm

eDNA in biofilm was quantified using a previously described protocol (36). Briefly, biofilms were grown in 96-well polystyrene plate in TSB for 24 hrs at 37°C. After 24 hrs the supernatant was discarded and the biofilm was suspended in resuspension buffer (50mM Tris-Cl pH 8,

10mM EDTA, 500mM NaCl). The resuspended biofilm was centrifuged and eDNA was quantified in the supernatant using 1μM SYTOX® Green (Invitrogen, Molecular Probes).

Confocal Laser Scanning Microscopy (CLSM)

CLSM was performed on one-day old biofilms as described previously (54). *E. faecalis* strains VI01 and VI40 were transformed with pMV158GFP (39) to generate VI29 and VI41, respectively, and both express 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 5ml of TSB broth containing tetracycline for plasmid maintenance. After 24 hrs of growth, the biofilm was gently washed with sterile phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4 pH 7.4) and stained with 1μM SYTOX Orange® (Invitrogen) for 6 to 7 minutes. The coverslip were inverted on a clean glass slide and sealed using clear nail polish. The biofilm was visualized using Zeiss LSM 5 Pascal laser scanning confocal microscope.

Macroscopic Biofilm

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

Adherence Assay

Adherence of *E. faecalis* strains to flat-bottom polystyrene plates (Brand plates, Germany) was tested using a previously described protocol (27) with some modification. Overnight grown

cultures were diluted 1:10 in fresh TSB and 200 ul was transferred to a flat bottom 96 well polystyrene microtiter plates. After 2 hrs 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

Biofilm 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-hr time points, the biofilm were treated with 1µg/ml proteinase K (Amresco) and this treatment remained for the remainder of the experiment. The 24 hour treatment was allowed to stand for 1 hour prior to processing the biofilm. An untreated control was included to determine the effect of treatment. After 24 hrs of growth, the biofilm was quantified with 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 and comstat analysis of biofilm was 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 (Figure S1). The 2DG resistant phenotype was confirmed by growth on media containing 2DG. VI01 grew to the final dilution of 10^{-8}, whilst the parental strain V583 and the *rpoN* complement VI40 were significantly inhibited and grew only at a dilution 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).

Sigma 54 alters eDNA in the supernatant of planktonic and biofilm cultures.

On the basis of phenotype characterization of a preliminary transposon mutagenesis screen, we tested for eDNA in the supernatants of planktonic cultures using SYTOX green. A lower amount of eDNA was detected in VI01 culture supernatant in comparison to the wild type V583 strain whereas the markerless complementation of *rpoN* mutant restored the phenotype to wild type levels (Fig 2a). eDNA in the supernatants of the various strains was also confirmed by visualization on an EtBr-stained 1% agarose gel after precipitation of eDNA with isopropanol (Fig 2b).

Given the fact that planktonic growth and biofilm are two different lifestyles of the bacteria, 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 VI01 biofilm than the wild type

which 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 dependant 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 the biofilm development of *E. faecalis*

eDNA has been shown to be an important matrix component in *E. faecalis* biofilm (54). The decreased levels of eDNA in VI01 led us to the hypothesis that VI01 may form less dense biofilm in comparison to the wild type V583. However, CLSM analysis of 24 hr old biofilm grown on glass coverslip showed that VI29 [Δ*rpoN*, Gfp^{+}] formed thicker biofilm (as measured by the Z stack thickness using LSM image examiner) than those formed by the wild type strain VT09 or the complement strain VI41 (Fig. 4). The appearance of the VI29 biofilm suggested early initiation of microcolony development, which was confirmed by macroscopic examination of the biofilms after 2 days growth on coverslips (Fig. 4). Despite the increased thickness and overall biofilm biomass of the *rpoN* mutant [VI29] compared to the parental and complemented strains (Table 4) , very few random dead cells and DNA (as detected by SYTOX Orange staining) were observed within the biofilm. Consistent with earlier observations on the role of cell death and 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 strain VI41 (Fig 4).

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 initial inoculum that adhered to 96 well microtiter plates after two hours. 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 biofilm.

On the basis of the macroscopic observation and CLSM of the VI29 biofilm and the relative lack of eDNA detection in 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 affect of Proteinase K treatment on biofilm development. The wild type and complemented strain exhibited decreased biofilm when treated with proteinase K only after 24 hr of biofilm growth .In contrast, reduction in VI01 biofilm was significant when treated with proteinase K after 6 hr biofilm growth and continued to respond to treatment after 12 hr and 24 hr of biofilm growth (Fig 6).

Discussion

The role of σ^{54} in regulating numerous biological properties, including those that relate to virulence has been well documented in a variety of bacterial species (3, 10, 21, 41, 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 PTS systems (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 affect 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.

One possible explanation for increased resistance to autolysis observed in the *rpoN* mutant could be novel modifications of the cell wall or altering 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 using micrococcal cell wall as a zymogram substrate (data not shown) ruling out the possibility of inactive autolysins. Also, the deletion of *rpoN* does not have a measurable affect on the secretion of the extracellular proteases, GelE and SprE, which have been previously shown to contribute to autolysis in *E. faecalis* (54, 57) (data not shown). 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} to regulate 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 lysis and its contribution 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 to unravel factors associated with *E. faecalis* biofilm development. Cellular processes regulated by σ^{54} will be 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 strain could grow on 2DG at a much lower frequency (10^{-4} and 10^{-5}) relative to the *rpoN* mutant parallels a recent report by Flanagan et al. (18) in which 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 an 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* K12, *rpoN* deletion enhances biofilm formation (3). Such different effects of *rpoN* on biofilm forming potential of bacteria provides a clear example of how 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 the 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 *Streptococci* (40) it has been shown that defect in initial adherence of the bacteria affect biofilm formation and subsequently influences the virulence of these pathogens. Our data indicates a similar influence of 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 in VI01 despite the significant reduction in eDNA led us to test for the presence of other matrix components 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). These 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 promotes cellular adhesion. In *S. aureus*, a biofilm defect in mutants that over produce extracellular protease was rectified by the addition of α2 macroglobulin – a general protease inhibitor, indicating a vital role for proteins in either cellular adhesion or biofilm matrix (4). Similarly in *B. 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 hours growth in the biofilm DNase has a minimal affect at 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 following only at 24 hours and not at earlier timepoints. This suggests the time dependent involvement of different polymers in the overall development of the biofilm.

Complementation studies of the *rpoN* mutant using a low copy 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 selectioin, 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 in *L. monocytogenes* (42). 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 towards gram- negative species with *P. aeruginosa, Vibrio* spps 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. Amongst the organisms queried, only *L. monocytogenes, B. subtilis, C. difficle* and *C. perfringens* appeared to have homologues, whereas in *S. aureus, S. pneumoniae*, and *S. pyogenes* homologs to σ^{54} were absent. The basis for this distribution among enteric adapted organisms as well as the potential genes regulated by σ^{54} awaits 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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Reference:

1. **Acebo, P., C. Nieto, M. A. Corrales, M. Espinosa, and P. Lopez.** 2000. Quantitative detection of *Streptococcus pneumoniae* cells harbouring single or multiple copies of the gene encoding the green fluorescent protein. Microbiol. **146:**1267-1273.
2. **Bass, J. I. F., D. M. Russo, M. L. Gabelloni, J. R. Geffner, M. Giordano, M. Catalano, A. Zorreguieta, and A. S. Trevani.** 2010. Extracellular DNA: A Major Proinflammatory Component of *Pseudomonas aeruginosa* Biofilms. J. Immunol **184:**6386-6395.
3. **Belik, A. S., N. N. Tarasova, and I. A. Khmel.** 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, B. R., M. Thoendel, A. J. Roth, and A. R. Horswill.** 2010. Identification of Genes Involved in Polysaccharide-Independent *Staphylococcus aureus* Biofilm Formation. Plos One **5**.
5. **Bond, D. R., B. M. Tsai, and J. B. Russell.** 1999. Physiological characterization of *Streptococcus bovis* mutants that can resist 2-deoxyglucose-induced lysis. Microbiology-Sgm **145:**2977-2985.
6. **Boucher, J. C., M. J. Schurr, and V. Deretic.** 2000. Dual regulation of mucoidy in *Pseudomonas aeruginosa* and sigma factor antagonism. Mol. Microbiol. **36:**341-351.
7. **Branda, S. S., F. Chu, D. B. Kearns, R. Losick, and R. Kolter.** 2006. A major protein component of the *Bacillus subtilis* biofilm matrix. Mol. Microbiol. **59:**1229-1238.
8. **Byrd, M. S., B. Pang, M. Mishra, W. E. Swords, and D. J. Wozniak.** 2010. The *Pseudomonas aeruginosa* Exopolysaccharide Psl Facilitates Surface Adherence and NF-kappa B Activation in A549 Cells. Mbio **1:**4.
9. **Calvez, S., A. Rince, Y. Auffray, H. Prevost, and D. Drider.** 2007. Identification of new genes associated with intermediate resistance of *Enterococcus faecalis* to divercin V41, a pediocin-like bacteriocin. Microbiol. **153:**1609-1618.
10. **Ceyssens, P. J., V. Mesyanzhinov, N. Sykilinda, Y. Briers, B. Roucourt, R. Lavigne, J. Robben, A. Domashin, K. Miroshnikov, G. Volckaert, and K. Hertveldt.** 2008. The genome and structural Proteome of YuA, a new *Pseudomonas aeruginosa* phage resembling M6. J. Bacteriol. **190:**1429-1435.
11. **Clarke, S. R., and S. J. Foster.** 2006. Surface adhesins of *Staphylococcus aureus*, p. 187-225, Adv. Microb. Physiol., Vol 51, vol. 51. Academic Press Ltd, London.
12. **Cruzrodz, A. L., and M. S. Gilmore.** 1990. High-efficiency introduction of plasmid DNA into glycine treated *Enterococcus faecalis* by electroporation. Mol. Gen. Genet. **224:**152-154.
13. **Dalet, K., C. Briand, Y. Cenatiempo, and Y. Hechard.** 2000. The *rpoN* gene of *Enterococcus faecalis* directs sensitivity to subclass IIa bacteriocins. Curr. Microbiol. **41:**441-443.
14. **Dalet, K., Y. Cenatiempo, P. Cossart, Y. Hechard, and C. European Listeria Genome.** 2001. A sigma(54)-dependent PTS permease of the mannose family is responsible for sensitivity of *Listeria monocytogenes* to mesentericin Y105. Microbiol. **147:**3263-3269.
15. **Del Papa, M. F., L. E. Hancock, V. C. Thomas, and M. Perego.** 2007. Full activation of *Enterococcus faecalis* gelatinase by a C-terminal proteolytic cleavage. J. Bacteriol. **189:**8835-8843.
16. **Dunne, W. M.** 2002. Bacterial adhesion: Seen any good biofilms lately? Clin. Microbiol. Reviews **15:**155-66.
17. **Fabretti, F., C. Theilacker, L. Baldassarri, Z. Kaczynski, A. Kropec, O. Holst, and J. Huebner.** 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., D. Clewell, and C. Sedgley.** 2010. Presented at the 3rd International ASM Conference on Enterococci, Portland, OR.
19. **Flemming, H. C., and J. Wingender.** 2010. The biofilm matrix. Nature Rev. Microbiol **8:**623-633.
20. **Guiton, P. S., C. S. Hung, L. E. Hancock, M. G. Caparon, and S. J. Hultgren.** Enterococcal biofilm formation and virulence in an optimized murine model of foreign body-associated urinary tract infections. Infect. Immun. **78:**4166-75.
21. **Hagins, J. M., J. A. Scoffield, S. J. Suh, and L. Silo-Suh.** 2010. Influence of RpoN on isocitrate lyase activity in *Pseudomonas aeruginosa*. Microbiol. **156:**1201-1210.
22. **Hancock, L. E., and M. Perego.** 2004. The *Enterococcus faecalis* fsr two-component system controls biofilm development through production of gelatinase. J. Bacteriol. **186:**5629-5639.
23. **Harmsen, M., M. Lappann, S. Knochel, and S. Molin.** 2010. Role of extracellular DNA during biofilm Formation by *Listeria monocytogenes*. Appl. Environ. Microbiol. **76:**2271-2279.
24. **Hechard, Y., C. Pelletier, Y. Cenatiempo, and J. Frere.** 2001. Analysis of sigma(54)-dependent genes in *Enterococcus faecalis*: a mannose PTS permease (EIIMan) is involved in sensitivity to a bacteriocin, mesentericin Y105. Microbiol. **147:**1575-1580.
25. **Hidron, A. I., J. R. Edwards, and J. Patel.** 2009. 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 (vol 29, pg 996, 2008). Infect. Control Hosp. Epidemiol. **30:**1.
26. **Hoiby, N., T. Bjarnsholt, M. Givskov, S. Molin, and O. Ciofu.** 2010. Antibiotic resistance of bacterial biofilms. International J. Antimicrob. Agts. **35:**322-332.
27. **Huelves, L., G. del Prado, V. Rodriguez-Cerrato, M. Gracia, C. Cenjor, J. J. Granizo, C. Ponte, and F. Soriano.** 2007. Adherence of *Streptococcus pneumoniae* to polystyrene plates, effect of serum on adhesion, and virulence in the gerbil otitis media model. Microb. Pathogen. **43:**114-119.
28. **Ishikawa, T., P. K. Rompikuntal, B. Lindmark, D. L. Milton, and S. N. Wai.** 2009. Quorum sensing regulation of the two *hcp* alleles in *Vibrio cholerae* O1 Strains. Plos One **4:**9.
29. **Jefferson, K. K.** 2004. What drives bacteria to produce a biofilm? FEMS Microbiol. Lett. **236:**163-173.
30. **Kazmierczak, M. J., M. Wiedmann, and K. J. Boor.** 2005. Alternative sigma factors and their roles in bacterial virulence. Microbiol. Mol. Biol. Rev. **69:**527-43.
31. **Kim, H. S., M. A. Lee, S. J. Chun, S. J. Park, and K. H. Lee.** 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., H. Claus, T. van Alen, M. Harmsen, J. Elias, S. Molin, and U. Vogel.** 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., and A. von Holy.** 2006. Bacterial biofilms within the clinical setting: what healthcare professionals should know. J. Hosp. Infect. **64:**313-325.
35. **Ma, L., K. D. Jackson, R. M. Landry, M. R. Parsek, and D. J. Wozniak.** 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, E. E., K. C. Rice, B. R. Boles, J. L. Endres, D. Ranjit, L. Chandramohan, L. H. Tsang, M. S. Smeltzer, A. R. Horswill, and K. W. Bayles.** 2009. Modulation of eDNA Release and Degradation affects *Staphylococcus aureus* Biofilm Maturation. Plos One **4:**12.
37. **McBride, S. M., V. A. Fischetti, D. J. LeBlanc, R. C. Moellering, and M. S. Gilmore.** 2007. Genetic diversity among *Enterococcus faecalis*. Plos One **2**.

38. **Merrick, M. J.** 1993. In a class of its own - The RNA Polymerase sigma factor 54 (sigma N). Mol. Microbiol. **10:**903-909.
39. **Nieto, C., and M. Espinosa.** 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, A. H., R. J. Lamont, and H. F. Jenkinson.** 2009. *Streptococcus* adherence and colonization. Microbiol. Mol. Biol. Rev. **73:**407-450.
41. **O'Toole, R., D. L. Milton, P. Horstedt, and H. Wolf-Watz.** 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. Microbiol. **143:**3849-3859.
42. **Okada, Y., N. Okada, S. Makino, H. Asakura, S. Yamamoto, and S. Igimi.** 2006. The sigma factor RpoN (sigma 54) is involved in osmotolerance in *Listeria monocytogenes*. FEMS Microbiol. Lett. **263:**54-60.
43. **Pfeffer, J. M., H. Strating, J. T. Weadge, and A. J. Clarke.** 2006. Peptidoglycan O acetylation and autolysin profile of *Enterococcus faecalis* in the viable but nonculturable state. J. Bacteriol. **188:**902-908.
44. **Potvin, E., F. Sanschagrin, and R. C. Levesque.** 2008. Sigma factors in *Pseudomonas aeruginosa*. FEMS Microbiol. Rev. **32:**38-55.
45. **Qin, X., K. V. Singh, G. M. Weinstock, and B. E. Murray.** 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., K. V. Singh, G. M. Weinstock, and B. E. Murray.** 2000. Effects of *Enterococcus faecalis fsr* genes on production of gelatinase and a serine protease and virulence. Infect. Immun. **68:**2579-2586.
47. **Qin, Z. Q., Y. Z. Ou, L. A. Yang, Y. L. Zhu, T. Tolker-Nielsen, S. Molin, and D. Qu.** 2007. Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis. Microbiology-Sgm **153:**2083-2092.
48. **Rice, K. C., E. E. Mann, J. L. Endres, E. C. Weiss, J. E. Cassat, M. S. Smeltzer, and K. W. Bayles.** 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, J. T., J. A. Tietjen, C. W. Walsh, J. E. Gustafson, and T. S. Whittam.** 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. Microbiol. **156:**719-730.
50. **Sahm, D. F., J. Kissinger, M. S. Gilmore, P. R. Murray, R. Mulder, J. Solliday, and B. Clarke.** 1989. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. **33:**1588-1591.
51. **Saldias, M. S., J. Lamothe, R. Wu, and M. A. Valvano.** 2008. *Burkholderia cenocepacia* requires the RpoN sigma factor for biofilm formation and intracellular trafficking within macrophages. Infect. Immun. **76:**1059-1067.
52. **Stevens, M. J. A., D. Molenaar, A. de Jong, W. M. De Vos, and M. Kleerebezem.** 2010. Sigma(54)-mediated control of the mannose phosphotransferase sytem in *Lactobacillus plantarum* impacts on carbohydrate metabolism. Microbiol. **156:**695-707.
53. **Thomas, V. C., Y. Hiromasa, N. Harms, L. Thurlow, J. Tomich, and L. E. Hancock.** 2009. A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis*. Mol. Microbiol. **72:**1022-1036.
54. **Thomas, V. C., L. R. Thurlow, D. Boyle, and L. E. Hancock.** 2008. Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development. J. Bacteriol. **190:**5690-5698.

55. **Thompson, L. S., J. S. Webb, S. A. Rice, and S. Kjelleberg.** 2003. The alternative sigma factor RpoN regulates the quorum sensing gene *rhlI* in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **220:**187-195.
56. **Thurlow, L. R., V. C. Thomas, and L. E. Hancock.** 2009. Capsular polysaccharide production in *Enterococcus faecalis* and contribution of CpsF to capsule serospecificity. J. Bacteriol. **191:**6203-6210.
57. **Waters, C. M., M. H. Antiporta, B. E. Murray, and G. M. Dunny.** 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, A. J., D. S. Millikan, J. M. Campbell, and K. L. Visick.** 2004. *Vibrio fischeri* sigma(54) controls motility, biofilm formation, luminescence, and colonization. Appl. Environ. Microbiol. **70:**2520-2524.
59. **Yang, T. C., Y. W. Leu, H. C. Chang-Chien, and R. M. Hu.** 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., M. Z. Liu, and R. R. Burgess.** 2010. Promoter and regulon analysis of nitrogen assimilation factor, Sigma(54), reveal alternative strategy for *E. coli* MG1655 flagellar biosynthesis. Nucleic Acids Res. **38:**1273-1283.

Figure Legends

Figure 1

2-Deoxy-D-Glucose (2DG) resistance analysis of VI01. Wild type strain, V583, (a) and the complement strain VI40 (c) are sensitive to 2DG because of 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*.

Figure 2

a) Quantitative detection of eDNA in culture supernatants using SYTOX green. eDNA was quantified in the culture supernatants of overnight grown cultures using 1µM SYTOX green. Assays were performed in quadruplets and error bars indicate the standard error of mean. ★, significant *P* values of less than 0.001 relative to wild type V583; ϕ, significant P values of less than 0.001 relative to *rpoN* complement (VI40). **b)** Qualitative detection of eDNA in culture supernatant by isoporpanol precipitation. a, Wildtype (V583); b. *rpoN* mutant (VI01); c. *rpoN* complement (VI40). **c)** Detection of eDNA in biofilm using SYTOX green. Assays were performed in sextuplets and error bars indicate standard error or mean. ★, significant *P* values of less than 0.001 relative to wild type V583; δ, significant *P* values of less than 0.001 relative to *rpoN* complement (VI40).

Figure 3

RpoN alters rate of autolysis in *E.faecalis*. Difference in autolysis rates of wild type (V583), *rpoN* mutant (VI01) and complemented strain (VI40) are plotted as percent of initial optical

552 density at 600 nm. Assays were performed in triplicates and repeated four times; error bars
553 represent standard error of mean.

554 **Figure 4**

555 a) Macroscopic view of biofilm grown on glass coverslip. Biofilms were grown on glass
556 coverslip in TSB media. A : VT09 ; B: VI29 ; C: VI41

557 b) Confocal analysis of 1-day old biofilms grown on glass coverslip. The wild type, mutant and
558 complement constitutively express Gfp from pMV158gfp as mentioned in materials and
559 methods. Biofilms were grown on glass coverslips in TSB media. Dead cells and eDNA were
560 stained with SYTOX orange (1µM). Live bacteria appear green while dead cells and eDNA are
561 red. Panel A, B and C represent biofilm orthogonal projections for VT09, VI29 and VI41
562 respectively, exhibiting merged green and red staining. Panel D, E and F correspond to dead cell
563 and eDNA staining in VT09, VI29 and VI41 biofilm respectively (matched pair to biofilms in
564 Panel A, B and C). The scale bar represents 10 µm.

565 **Figure 5**

566 Polystyrene plate adherence assay. Deletion of *rpoN* increases adherence of *E.faecalis* to 96-well
567 polystyrene plates. ★, significant *P* values of less than 0.05 relative to wild type V583; ϕ,
568 significant *P* values of less than 0.05 relative to *rpoN* complement (VI40).

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572 **Figure 6**

573 Proteinase K inhibits biofilm development of *rpoN* mutant on polystyrene plates. Biofilms were
574 seeded at time zero for V583, VI01, and VI40 and the untreated biofilms were stained 24 hours
575 later. At the indicated times, proteinase K (1µg/ml) was added at either 6, 12, or 24 hours after
576 seeding the biofilm, and the treatment was allowed to stand for the reminder of the assay. Each
577 assay was performed in triplicate and repeated four times. Error bars indicate standard error of
578 the mean.