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Deletion of *sigma54* (*rpoN*) alters the rate of autolysis and biofilm formation in *Enterococcus faecalis*

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How to cite this manuscript

If you make reference to this version of the manuscript, use the following information:

Iyer, V. S., & Hancock, L. E. (2012). Deletion of *sigma54* (*rpoN*) alters the rate of autolysis and biofilm formation in *Enterococcus faecalis*. Retrieved from <http://krex.ksu.edu>

Published Version Information

Citation: Iyer, V. S., & Hancock, L. E. (2012). Deletion of *sigma54* (*rpoN*) alters the rate of autolysis and biofilm formation in *Enterococcus faecalis*. *Journal of Bacteriology*, 194(2), 368-375.

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Digital Object Identifier (DOI): doi:10.1128/JB.06046-11

Publisher's Link: <http://jb.asm.org/content/194/2/368.full.pdf+html>

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

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17 Running Title : Role of σ^{54} in *Enterococcus faecalis* V583

18 Key Words : *Enterococcus faecalis*, σ^{54} (*rpoN*), Biofilm, , Extracellular DNA, Autolysis.

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20

21 **Abstract**

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

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43 **Introduction**

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

64 Transcription initiation is one of the important stages of gene regulation and sigma factors play a
65 crucial role in determining the controlled response of a subset of genes tied to a given

66 environmental stimulus. Sigma factors reversibly bind to RNA polymerases and drive promoter
67 specific transcription initiation. In prokaryotes, two distinct families of sigma factors have been
68 studied, sigma 70 (σ^{70}) and sigma 54 (σ^{54}). The σ^{70} family also includes several related alternate
69 sigma factors. Sigma 54 shares no structural homology with sigma 70, possesses a distinct
70 consensus binding sequence (-24/-12;TTGGCACNNNNNTTGCT) and unlike sigma 70,
71 facilitates activator dependent transcription initiation (24, 38).

72 Sigma 54 plays an important role in the virulence of several bacteria but does not share the same
73 function in all pathogens (30). In *Vibrio fischeri*, σ^{54} influences biofilm formation, motility and
74 symbiotic colonization of squids and negatively regulates bioluminescence (58). Quorum sensing
75 regulation in *V. cholerae* O1 strains is dependent on *rpoN* (28). Sigma 54 is required for biofilm
76 formation by *Burkholderia cenocepacia* as well as survival within macrophages (51). In the
77 major food-borne pathogen *Listeria monocytogenes*, σ^{54} is essential for its osmotolerance
78 potential (42) and is responsible for mesentericin sensitivity (14, 42) whereas in *Pseudomonas*
79 *aeruginosa*, σ^{54} influences the activity of isocitrate lyase (21), alginate biosynthesis (6) and in
80 pilin and flagellin production in addition to several other virulence determinants (44). Sigma 54
81 also regulates biofilm formation, enterocyte effacement, acid tolerance, flagellar biosynthesis
82 and several other processes in *E. coli* (3, 49, 60).

83 In *E. faecalis*, σ^{54} is responsible for sensitivity to class IIa bacteriocins such as mesentericin and
84 divercin (9, 13). The basis for the class IIa bacteriocin sensitivity is due to the role of σ^{54} in
85 regulating four distinct sugar phosphotransferase (PTS) systems that are dependent on four
86 known σ^{54} enhancer binding proteins (LpoR, MphR, MpoR, and MptR) (13). MptD, a
87 component of the mannose PTS system is thought to serve as the cellular receptor for the class

88 Ila bacteriocins (24). However, additional roles for σ^{54} in enterococcal biology remain to be
89 elucidated.

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

93

94 **Materials and Method**

95 **Bacterial strains and growth conditions:**

96 The bacterial strains and plasmids used in this study are listed in Tables 1 and 2 respectively. *E.*
97 *coli* Electroten Blue (Stratagene) was used for construction of plasmids and was cultured in
98 Luria-Bertani (LB) broth supplemented with appropriate antibiotics. *E. faecalis* strains were
99 cultured in either Trypticase soy broth containing 0.25% glucose (TSB) or Todd-Hewitt broth
100 (THB; BD Biosciences) containing appropriate antibiotics whenever required. Chloramphenicol
101 (Cm) and spectinomycin (Spec) were used for selection of *E. coli* at a concentration of 10µg/ml
102 and 150µg/ml respectively. For *E. faecalis*, Cm, Spec and tetracycline (Tet) were used at
103 15µg/ml, 500µg/ml and 15µg/ml respectively. When required, X-Gal (5-bromo-4-chloro-3-
104 indolyl-β-D-galactopyranoside; AMRESCO) was used at a concentration of 80µg/ml for both *E.*
105 *coli* and *E. faecalis*.

106 **In-frame Markerless deletion of *rpoN***

107 An *E.coli*-enterococcal temperature sensitive cloning vector, pLT06 (56), was used to generate
108 isogenic in-frame deletion of *rpoN* in *E. faecalis* V583. Upstream and downstream regions
109 flanking *rpoN* (EF0782) were amplified by PCR from a V583 genomic template using the primer
110 pair RpoNP1/RpoNP2 and RpoNP3/RpoNP4 respectively (refer Table 3). The primers
111 RpoNP1/RpoNP2 and RpoNP3/RpoNP4 were designed with EcoRI/BamHI and BamHI/PstI
112 restriction sites respectively. The resultant PCR products were digested with BamHI, ligated and
113 re-amplified with primers RpoNP1 and RpoNP4. For the construction of the deletion vector, the
114 amplified product was digested with EcoRI and PstI followed by ligation to similarly digested
115 pLT06. The ligation was electroporated into competent E10-Blue cells for propagation and blue

116 colonies were selected on LB agar containing chloramphenicol and X-Gal at 30°C. Clones were
117 screened for the appropriate insert using the primers OriF and SeqR. A positive plasmid
118 designated pKS70 was confirmed by restriction digest and electroporated into *E. faecalis* V583
119 cells (12) and VI01 was subsequently generated following the protocol previously described (56)
120 and confirmed by PCR using primers RpoNUp and RpoNDown. Using pKS70, ~ 98% of the
121 *rpoN* gene was deleted leaving seven codons at the 5' end and two codons at the 3' end. The
122 next adjacent gene is *ef0783*, which encodes an O-acetyl transferase. This gene is located
123 approximately 200 bps downstream of *rpoN* and the strategy used to delete *rpoN* does not alter
124 the expression of *ef0783* (data not shown).

125 **Markerless Complementation of VI01 [Δ *rpoN*]**

126 The temperature sensitive cloning vector pLT06 (56), was used to generate markerless gene
127 complementation of *rpoN* in VI01. The *rpoN* gene (EF0782) along with flanking regions was
128 amplified by PCR from a V583 genomic template using primers RpoNP1 and RpoNP4 (refer
129 Table 3). For the construction of *rpoN* markerless complementation vector pVI12, the amplified
130 product was digested with EcoRI and PstI followed by ligation with similarly digested plasmid
131 vector pLT06. The ligation was electroporated into competent E10-Blue cells for propagation
132 and blue colonies were selected on LB agar containing chloramphenicol and X-Gal at room
133 temperature. Clones were screened for the appropriate insert using the primers OriF and SeqR.
134 A positive plasmid designated pVI12 was confirmed by restriction digest and electroporated into
135 *E. faecalis* VI01 cells (12) and VI40 (markerless complement) was generated following the
136 protocol previously described (56) and confirmed by PCR using primers RpoNUp and
137 RpoNDown.

138 **2-Deoxy-D-Glucose (2DG) resistance**

139 *E. faecalis* V583, VI01 and VI40 were grown on LB agar containing 0.2% fructose and 10 mM
140 2-Deoxy-glucose (2DG) (24). 2DG is a toxic homologue of glucose and enters the cells via the
141 mannose PTS permease (5). In *E. faecalis*, the mannose PTS expression is controlled by σ^{54} .
142 Strains resistant to 2DG do not express a functional mannose PTS permease (24). Hence growth
143 on media containing 2DG was used as a marker to confirm deletion of *rpoN*.

144 **Detection and precipitation of extracellular DNA**

145 Overnight cultures were centrifuged for 10 minutes at 13,000 rpm and the resulting supernatant
146 filtered (0.2 μ m pore size; Nalgene) to obtain cell free supernatants. The supernatants were
147 tested for the presence of e-DNA using 1 μ M SYTOX[®] Green (Invitrogen, Molecular Probes).
148 The eDNA was also precipitated from the culture filtrate using an equal volume of isopropanol.
149 The precipitated eDNA was washed in 75% ethanol, air-dried and dissolved in TE buffer (10mM
150 Tris-Cl, 1mM EDTA, pH 8.0) and visualized on 1% agarose gels after staining with ethidium
151 bromide.

152 **Autolysis assay**

153 Autolysis assay was performed as previously described (15).

154 **Quantitative detection of eDNA in biofilm**

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

158 10mM EDTA, 500mM NaCl). The resuspended biofilm was centrifuged and eDNA was
159 quantified in the supernatant using 1 μ M SYTOX[®] Green (Invitrogen, Molecular Probes).

160 **Confocal Laser Scanning Microscopy (CLSM)**

161 CLSM was performed on one-day old biofilms as described previously (54). *E. faecalis* strains
162 VI01 and VI40 were transformed with pMV158GFP (39) to generate VI29 and VI41,
163 respectively, and both express Gfp constitutively. VT09 [V583 (pMV158GFP)] (54) along with
164 VI29 and VI41 were used for confocal imaging. Briefly, biofilms were grown on sterile glass
165 coverslips placed in six-well tissue culture plates. The coverslip was submerged in 5ml of TSB
166 broth containing tetracycline for plasmid maintenance. After 24 hrs of growth, the biofilm was
167 gently washed with sterile phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 10mM
168 Na₂HPO₄, 2mM KH₂PO₄ pH 7.4) and stained with 1 μ M SYTOX Orange[®] (Invitrogen) for 6 to
169 7 minutes. The coverslip were inverted on a clean glass slide and sealed using clear nail polish.
170 The biofilm was visualized using Zeiss LSM 5 Pascal laser scanning confocal microscope.

171 **Macroscopic Biofilm**

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

177 **Adherence Assay**

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

180 cultures were diluted 1:10 in fresh TSB and 200 μ l was transferred to a flat bottom 96 well
181 polystyrene microtiter plates. After 2 hrs of incubation at 37°C, the supernatant was discarded
182 and the wells were gently washed with sterile PBS. The adherent cells were resuspended in 200
183 μ l PBS by vigorous pipetting, diluted and plated on THB agar for colony counting. Also, the
184 initial load was calculated by plating the diluted culture on THB agar for colony counting. The
185 adherence potential of the strains was calculated as the percentage of initial load that adhered.

186 **Proteinase K treatment of Biofilm**

187 Biofilm were grown on 96 well round-bottom tissue-culture treated polystyrene plates (Techno
188 Plastic Products ,Switzerland) as previously described (22). At 6-,12-, and 24-hr time points, the
189 biofilm were treated with 1 μ g/ml proteinase K (Amresco) and this treatment remained for the
190 remainder of the experiment. The 24 hour treatment was allowed to stand for 1 hour prior to
191 processing the biofilm. An untreated control was included to determine the effect of treatment.
192 After 24 hrs of growth, the biofilm was quantified with the crystal violet staining method (22).
193 Each assay was performed in triplicate and repeated four times.

194 **Statistical Analysis**

195 Statistical analysis of quantitative detection of eDNA, adherence assay and comstat analysis of
196 biofilm was performed using GraphPad Prism 4 software (San Diego, CA). One way analysis of
197 variance followed by Dunn's multiple comparison tests was performed to determine statistical
198 significance.

199 **Results**

200 **Construction of the *E. faecalis* V583 isogenic *rpoN* mutant and its complement**

201 The *rpoN* deletion mutant VI01 [$\Delta rpoN$] was constructed using the markerless deletion vector
202 pKS70. Initial growth curves of the wild-type V583 strain, the *rpoN* deletion mutant (VI01) and
203 its complement (VI40) were assessed in TSB. No alterations in the growth of the 3 strains were
204 observed (Figure S1). The 2DG resistant phenotype was confirmed by growth on media
205 containing 2DG. VI01 grew to the final dilution of 10^{-8} , whilst the parental strain V583 and the
206 *rpoN* complement VI40 were significantly inhibited and grew only at a dilution of 10^{-3} and 10^{-4} .
207 Complementation confirmed that there were no polar effects of the gene deletion and attributed
208 the 2DG resistant phenotype to the targeted deletion of *rpoN* (Fig 1).

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

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

217 Given the fact that planktonic growth and biofilm are two different lifestyles of the bacteria, we
218 tested to see the effect of *rpoN* deletion on eDNA during biofilm development. As observed in
219 planktonic cultures, a lesser amount of eDNA was detected in VI01 biofilm than the wild type

220 which was attributed to the deletion of *rpoN* as the complementation restored the eDNA detected
221 in the biofilm to wild type levels (Fig 2c).

222 **Sigma 54 alters the rate of autolysis in *E. faecalis* V583.**

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

228 **Sigma 54 alters the biofilm development of *E. faecalis***

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

241 concentrated foci of DNA and dead cells, which was phenocopied by the complement strain
242 VI41 (Fig 4).

243

244

245 **Deletion of *rpoN* increases adherence to polystyrene plates**

246 In order to determine whether increased biofilm formation by the *rpoN* mutant was due to its
247 initial adherence ability, we calculated the percentage of initial inoculum that adhered to 96 well
248 microtiter plates after two hours. Adherence of VI01 to a polystyrene plate was significantly
249 enhanced in comparison to the wild type. In addition, markerless complementation of VI01
250 [VI40] reduced the adherence potential to wild type levels (Fig 5).

251 **Sigma 54 modulates the composition of *E. faecalis* V583 biofilm.**

252 On the basis of the macroscopic observation and CLSM of the VI29 biofilm and the relative lack
253 of eDNA detection in this mutant, we hypothesized a role for a different polymer matrix that
254 promotes biofilm formation in the *rpoN* mutant. To test the role of proteins in VI01 biofilm, we
255 examined the affect of Proteinase K treatment on biofilm development. The wild type and
256 complemented strain exhibited decreased biofilm when treated with proteinase K only after 24 hr
257 of biofilm growth .In contrast, reduction in VI01 biofilm was significant when treated with
258 proteinase K after 6 hr biofilm growth and continued to respond to treatment after 12 hr and 24
259 hr of biofilm growth (Fig 6).

260

261 Discussion

262 The role of σ^{54} in regulating numerous biological properties, including those that relate to
263 virulence has been well documented in a variety of bacterial species (3, 10, 21, 41, 49, 51, 52,
264 55, 58, 59). However its role in *E. faecalis* has been limited to observations made regarding its
265 contribution to sensitivity to class IIa bacteriocins through the regulation of sugar PTS systems
266 (9, 13, 22). Identification of σ^{54} as a potential regulatory protein in the cascade of biofilm
267 development was an interesting breakthrough and we focused our efforts on elucidating its affect
268 on *E. faecalis* V583 biofilm. The role of autolysis (54) and fratricide (53) has been well
269 documented in enterococcal biofilm formation and has been shown to be important in providing
270 eDNA as a key biofilm matrix component. However, the observation that biofilm formation was
271 enhanced in the *rpoN* mutant despite the increased resistance to autolysis and the absence of
272 eDNA was an unexpected finding.

273 One possible explanation for increased resistance to autolysis observed in the *rpoN* mutant could
274 be novel modifications of the cell wall or altering the modifications such as o-acetylation (43) or
275 d-alanylation (17) on the cell wall that protect against lysis. Deletion of *rpoN* did not alter the
276 autolysin profile of *E. faecalis* when using micrococcal cell wall as a zymogram substrate (data
277 not shown) ruling out the possibility of inactive autolysins. Also, the deletion of *rpoN* does not
278 have a measurable affect on the secretion of the extracellular proteases, GelE and SprE, which
279 have been previously shown to contribute to autolysis in *E. faecalis* (54, 57) (data not shown). A
280 significant reduction in cell death due to impaired cell lysis occurred in *E. faecalis* V583 $\Delta rpoN$
281 planktonic and biofilm cultures suggesting the requirement of a functional σ^{54} to regulate
282 susceptibility to cell lysis. In *P. aeruginosa*, deletion of *rpoN* abolishes cell death in the
283 microcolonies during biofilm maturation and has been related to the expression of surface

284 structures (Type 4 pili and flagella) whose expression is regulated by σ^{54} (56). Additionally, σ^{54}
285 dependent gene regulation promotes phage induced lysis in *P. aeruginosa* (10). There are seven
286 phages associated with *E. faecalis* V583 with one of them being a part of the core genome (37).
287 It will be interesting to test the role of σ^{54} dependent transcription of phage particle proteins and
288 host lysis and its contribution to biofilm development.

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

307 In *Vibrio vulnificus*, σ^{54} positively regulates the gene encoding an ADP-glycero-manno-heptose-
308 6-epimerase (*gmhD*) which is responsible for production of lipopolysaccharide and
309 exopolysaccharide, both of which are required for biofilm formation (31) while in *B.*
310 *cenocepacia*, σ^{54} controls motility which in turn plays a role in biofilm formation (51).
311 However, in *E. coli* K12, *rpoN* deletion enhances biofilm formation (3). Such different effects of
312 *rpoN* on biofilm forming potential of bacteria provides a clear example of how a gene whose
313 function was first reported to be restricted to nitrogen assimilation has evolved to govern
314 virulence related functions in addition to bacterial metabolism. Our data provide additional
315 support for the expanding role of σ^{54} in the world of low-GC gram-positive bacteria.

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

325 Other than DNA, other molecules such as proteins and polysaccharides have been suggested to
326 be important constituents in the polymer matrix of several bacteria (16, 19). Robust biofilm in
327 VI01 despite the significant reduction in eDNA led us to test for the presence of other matrix
328 components using compounds capable of dissolving the aforementioned components. The
329 reduced ability of VI01 ($\Delta rpoN$) to form a biofilm when treated with proteinase K suggests a

330 role for protein in either adhesion or matrix composition to promote *E. faecalis* biofilm and is
331 consistent with recent observations by Guiton et al (20). These authors observed that
332 colonization of an implanted piece of urinary catheter as well as the bladder epithelium was
333 dependent on a functioning sortase enzyme for the proper anchoring of proteins to the cell wall,
334 which in turn promotes cellular adhesion. In *S. aureus*, a biofilm defect in mutants that over
335 produce extracellular protease was rectified by the addition of $\alpha 2$ macroglobulin – a general
336 protease inhibitor, indicating a vital role for proteins in either cellular adhesion or biofilm matrix
337 (4). Similarly in *B. subtilis*, TasA is required for the structural integrity and development of
338 biofilms (7). In *E. faecalis* biofilms (54), eDNA is known to be a crucial matrix component in the
339 early stages of biofilm development, but by 24 hours growth in the biofilm DNase has a minimal
340 affect at disrupting the biofilm. Here we show that in *E. faecalis* V583, proteins are likely to
341 serve as important matrix components during the later stages of biofilm development as a
342 reduction in biomass was observed following only at 24 hours and not at earlier timepoints. This
343 suggests the time dependent involvement of different polymers in the overall development of the
344 biofilm.

345 Complementation studies of the *rpoN* mutant using a low copy plasmid did not result in
346 complete reversal of the phenotype to wild type levels in experiments that involved stressing of
347 cells (osmotic shock and 2DG toxicity)(data not shown). This was primarily due to plasmid loss
348 in the absence of selection, and suggested a survival advantage for *E. faecalis* in the absence of
349 σ^{54} under certain stress conditions. The inability to fully complement an *rpoN* mutant has also
350 been reported in *L. monocytogenes* (42). Similarly, in a *V. fischeri* squid colonization model
351 (58), the level of colonization varied with the complemented strain and only some animals

352 exhibited wild type levels of colonization. For this reason, we utilized a complementation
353 strategy that restored the function of the gene by placing it at its native locus in single copy.

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

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

365

366 **Acknowledgments**

367 We are grateful to Dr. Vinai Thomas for the preliminary screen of the *E. faecalis* transposon
368 library, and to Dr. Dan Boyle for assistance with confocal imaging. This work was supported by
369 Public Health Service grant AI77782 from the National Institutes of Health.

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532 **Figure Legends**

533 **Figure 1**

534 2-Deoxy-D-Glucose (2DG) resistance analysis of VI01. Wild type strain, V583, (a) and the
535 complement strain VI40 (c) are sensitive to 2DG because of functional *mpt* operon under the
536 control of intact *rpoN*. RpoN mutant VI01 (b) is resistant to 2DG. This confirms the deletion and
537 complementation of *rpoN* in *E.faecalis*.

538 **Figure 2**

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

549 **Figure 3**

550 RpoN alters rate of autolysis in *E.faecalis*. Difference in autolysis rates of wild type (V583),
551 *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 (1 μ M). 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 μ m.

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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571

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 remainder of the assay. Each
577 assay was performed in triplicate and repeated four times. Error bars indicate standard error of
578 the mean.