

1 OprF impacts *Pseudomonas aeruginosa* biofilm matrix eDNA levels in a nutrient-dependent
2 manner

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9 Running title: OPRF IN *P. AERUGINOSA* BIOFILM MAINTENANCE

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14 **ABSTRACT**

15 The biofilm matrix is composed of exopolysaccharides, eDNA, membrane vesicles, and
16 proteins. While proteomic analyses have identified numerous matrix proteins, their functions in
17 the biofilm remain understudied compared to the other biofilm components. In the
18 *Pseudomonas aeruginosa* biofilm, several studies have identified OprF as an abundant matrix
19 protein and, more specifically, as a component of biofilm membrane vesicles. OprF is a major
20 outer membrane porin of *P. aeruginosa* cells. However, current data describing the effects of
21 OprF in the *P. aeruginosa* biofilm is limited. Here we identify a nutrient-dependent effect of OprF
22 in static biofilms, whereby $\Delta oprF$ cells form significantly less biofilm than wild type when grown
23 in media containing glucose or low sodium chloride concentrations. Interestingly, this biofilm
24 defect occurs during late static biofilm formation and is not dependent on the production of PQS,
25 which is responsible for outer membrane vesicle production. Furthermore, while biofilms lacking
26 OprF contain approximately 60% less total biomass than those of wild type, the number of cells
27 in these two biofilms is equivalent. We demonstrate that *P. aeruginosa* $\Delta oprF$ biofilms with
28 reduced biofilm biomass contain less eDNA than wild-type biofilms. These results suggest that
29 the nutrient-dependent effect of OprF is involved in the maintenance of mature *P. aeruginosa*
30 biofilms by retaining eDNA in the matrix.

31 **IMPORTANCE**

32 Many pathogens form biofilms, which are bacterial communities encased in an extracellular
33 matrix that protects them against antibacterial treatments. The roles of several matrix
34 components of the opportunistic pathogen *Pseudomonas aeruginosa* have been characterized.
35 However, the effects of *P. aeruginosa* matrix proteins remain understudied and are untapped
36 potential targets for antibiofilm treatments. Here we describe a conditional effect of the abundant
37 matrix protein OprF on late-stage *P. aeruginosa* biofilms. A $\Delta oprF$ strain formed significantly
38 less biofilm in low sodium chloride or with glucose. Interestingly, the defective $\Delta oprF$ biofilms did
39 not exhibit fewer resident cells but contained significantly less extracellular DNA (eDNA) than
40 wild type. These results suggest that OprF is involved in matrix eDNA retention in mature
41 biofilms.

42 INTRODUCTION

43 Biofilms are aggregates of bacterial cells encased in a self-produced extracellular matrix. The
44 matrix protects resident cells from external assaults and is composed of exopolysaccharides,
45 extracellular DNA (eDNA), membrane vesicles, and proteins (1). Many studies have reported
46 the effects of exopolysaccharides, eDNA, and membrane vesicles on biofilm function. However,
47 relatively few have investigated the roles of biofilm matrix proteins, even though matrix proteins
48 have been suggested to play many vital functions in the biofilm (2, 3). Since the late 2000s,
49 researchers have used proteomic approaches to identify biofilm matrix proteins and gain insight
50 into their roles, including several studies in the model biofilm organism *Pseudomonas*
51 *aeruginosa*. Four different studies have identified OprF as an abundant matrix protein (4-7).
52 Additionally, homologs of *P. aeruginosa* OprF have been identified in biofilm matrices of other
53 organisms (8-10).

54
55 Within the *P. aeruginosa* biofilm, two populations of OprF protein exist: cell-associated and
56 matrix-associated. In its more established cell-associated role, OprF is an OmpA family member
57 and the major non-specific porin in *P. aeruginosa*, where it facilitates diffusion across the outer
58 membrane (11). Multiple studies have examined biofilm formation after deletion of *oprF* or
59 *ompA*, which eliminates both the cell- and matrix-associated protein pools (12-14). However, the
60 impact of OprF and its OmpA homologs on biofilm formation is somewhat conflicting and may
61 depend on conditions, such as oxygen or nutrient availability (15). One study shows that under
62 aerobic conditions, a *P. aeruginosa oprF* interruption mutant produces twice as much biofilm as
63 the parental strain (13). This result conflicts with a separate study in which an *oprF* mutant
64 produced less biofilm when grown under anaerobic conditions (12). Furthermore, the OprF
65 homolog OmpA, which is abundant in *Escherichia coli* biofilms (16), increases biofilm formation
66 on hydrophobic surfaces (17). Mirroring this effect, in the pathogen *Acinetobacter baumannii*,
67 *ompA* mutants are deficient in biofilm formation on abiotic surfaces and have decreased

68 attachment to host cells (18). Together, these data suggest that OprF may play an important
69 role in biofilm function.

70

71 Within the biofilm matrix, OprF is highly abundant in membrane vesicles, which are a major
72 matrix component involved in biofilm structure and cell-to-cell signaling (5, 19). Two membrane
73 vesicle synthesis pathways have been established: the bilayer couple model, which produces
74 outer membrane vesicles (OMVs), and the explosive cell lysis model, which results in
75 membrane vesicles (20, 21). Interestingly, OprF has been suggested to play a role in OMV
76 production via the bilayer couple model. An OprF mutant overproduces OMVs relative to wild-
77 type cells due to its overproduction of the quorum-sensing signal PQS (22). Since increased
78 production of PQS and OMVs is correlated with biofilm dispersal (23), OprF may be important
79 for this stage of the biofilm lifecycle. However, the role of vesicle-associated OprF in the biofilm
80 is currently unknown (11).

81

82 Here we identified a nutrient-dependent biofilm defect in $\Delta oprF$ strains of *P. aeruginosa*. Upon
83 dissection of the medium components, we found that $\Delta oprF$ biofilm formation was significantly
84 reduced in the presence of glucose or low sodium chloride concentrations without affecting
85 overall bacterial growth. The biofilm defect in the absence of OprF occurs during late-stage
86 biofilm development and is not dependent on PQS production. Interestingly, we observed
87 equivalent numbers of cells in mature wild-type biofilms and $\Delta oprF$ biofilms (that have reduced
88 biofilm biomass). However, there was a significant reduction in eDNA in $\Delta oprF$ biofilms.
89 Together, our data suggest that OprF is involved in the retention of eDNA during mature biofilm
90 maintenance under certain growth conditions.

91

92 RESULTS

93 $\Delta oprF$ cells exhibit a nutrient-dependent biofilm defect

94 Since OprF is an abundant *P. aeruginosa* matrix protein (5, 6), we tested the effect of deleting
95 *oprF* on biofilm formation. We deleted *oprF* from *P. aeruginosa* PAO1 and confirmed via whole
96 genome sequencing that our engineered deletion allele was the only difference between this
97 strain and the parental strain. We also inserted an arabinose-inducible *oprF* at a neutral site in
98 the chromosome in the $\Delta oprF$ background. This strain expressed OprF at levels similar to wild
99 type upon addition of 0.5% arabinose, but not in the absence of inducer (Fig. S1). Using
100 standard microtiter biofilm assays (24), we compared the $\Delta oprF$ biofilm formed in two common
101 growth media: tryptic soy broth (TSB) and lysogeny broth (LB). While forming more biofilm than
102 the exopolysaccharide-deficient $\Delta pslD$ negative-control strain in both media, $\Delta oprF$ formed 57.3
103 $\pm 3.8\%$ S.D. (N=3; $p < 0.01$, ANOVA with post hoc Bonferroni) less biofilm than wild type in
104 TSB, but an equivalent amount of biofilm to wild type in LB (Fig. 1A). This difference in biofilm
105 formation was not due to growth rate differences in these media (see Fig. S2), and the biofilm
106 defect was rescued in the inducible *oprF* strain when 0.5% arabinose was added. To determine
107 if the $\Delta oprF$ biofilm defect in TSB exists in other *P. aeruginosa* strains, we constructed $\Delta oprF$
108 mutants in three other backgrounds: the tomato plant isolate E2, the water isolate MSH10, and
109 the UTI isolate X24509 (25). Similar to PAO1, biofilm defects were observed in all three $\Delta oprF$
110 mutants when grown in TSB (Fig. S3A). Furthermore, the established *oprF* interruption mutant
111 strain H636, which is made from a H103-based PAO1 background (26), exhibited a significant
112 biofilm defect when grown in TSB (Fig. S3B). However, in agreement with a previously
113 published study (13), the H636 strain produced approximately double the biofilm biomass as the
114 H103 parental strain in LB (Fig. S3C). This H636 result conflicts with our $\Delta oprF$ strain biofilm
115 phenotype in LB (Fig 1A), suggesting that the interruption mutation of *oprF* in H636 may be
116 polar or that the H636 strain may have acquired secondary mutations. Nonetheless, since all

117 $\Delta oprF$ strains that we tested had a biofilm defect in TSB, we continued our studies using our *P.*
118 *aeruginosa* PAO1 $\Delta oprF$ strain.

119

120 **Glucose and low sodium chloride reduce $\Delta oprF$ biofilm formation**

121 While LB and TSB are both rich media with peptic digests as primary carbon sources, three
122 notable ingredients differ between the two: sodium chloride (NaCl), glucose, and dipotassium
123 phosphate (K_2HPO_4)(Table S1). To determine if these media components affect $\Delta oprF$ biofilm
124 formation, we measured the static biofilm formed when strains were grown in media in which the
125 concentrations of these components were individually altered to match that of the other medium.
126 First, biofilms were grown in TSB or LB, each containing 5 or 10 g/L NaCl. Since reducing the
127 NaCl concentration below 5 g/L decreases cell viability in *oprF* mutants (27), we did not test
128 sodium chloride concentrations below this threshold. While $\Delta oprF$ formed less biofilm than wild
129 type in TSB (with 5 g/L NaCl; original formula), $\Delta oprF$ formed biofilms similar to those of wild
130 type when the NaCl concentration was increased to 10 g/L (with no other change in TSB) (Fig.
131 1B, S4A). The reciprocal effect was observed with LB, where $\Delta oprF$ formed biofilms similar to
132 wild type in the original medium (with 10 g/L NaCl), but less biofilm than wild type when NaCl
133 was reduced to 5 g/L (Fig. 1C, S4A). This reduced biofilm formation mirrors $\Delta oprF$ biofilms
134 formed in TSB, which also contain 5 g/L NaCl. Changing the glucose concentration had a
135 similar effect. Removing glucose from TSB resulted in $\Delta oprF$ biofilm biomass similar to that of
136 wild type (Fig. 1B, S4B), mirroring the phenotype of $\Delta oprF$ biofilms formed in LB, which does not
137 contain glucose (Fig. 1C, S4B). When glucose was added to LB, $\Delta oprF$ formed less biofilm than
138 wild type, similar to biofilm formed by the mutant in TSB (which contains glucose). Changing the
139 amount of K_2HPO_4 did not change the $\Delta oprF$ biofilm phenotype in either medium (Fig. 1B, 1C,
140 S4C). These biofilm phenotypes were not the result of growth defects, as the planktonic growth
141 rates of wild type and $\Delta oprF$ strains in these altered media were statistically equivalent (Fig. S2).

142 These results indicate that $\Delta oprF$ biofilm formation is dependent on the NaCl and glucose
143 concentrations.

144

145 **$\Delta oprF$ biofilm phenotype is not due to changes in osmolarity or metal concentrations**

146 Since altering concentrations of major media solutes may impact medium osmolarity, we tested
147 if the medium osmolarity is related to the $\Delta oprF$ biofilm defect by measuring the osmolarity of
148 the various TSB and LB media with a vapor pressure osmometer and then correlating these
149 measurements to the amounts of $\Delta oprF$ static biofilm biomass formed in the media. While there
150 was a weak positive correlation between media osmolarity and $\Delta oprF$ biofilm formation, the
151 relationship was not statistically significant (Fig. S5). We noted that the effect of osmolarity
152 appeared to be driven by the changes in sodium chloride concentration within each medium
153 (Fig. S5, squares). In comparison, glucose, which impacted $\Delta oprF$ biofilm formation, did not
154 alter media osmolarity (Fig. S5, triangles). Assuming that the medium components impact
155 biofilm formation through the same mechanism, we conclude that changes in osmolarity are not
156 the major driving force behind the effect on $\Delta oprF$ biofilm formation.

157

158 Changes to media formulations can also affect the concentrations of biologically relevant
159 metals. To determine the concentrations of iron, manganese, nickel, cobalt, copper,
160 molybdenum, sodium, potassium, magnesium, calcium, and zinc, we performed inductively
161 coupled plasma mass spectrometry (ICP-MS) for each base medium and variant. While
162 concentrations of sodium and potassium were altered when changes were made to sodium
163 chloride or dipotassium phosphate levels, metal concentrations primarily tracked with TSB or LB
164 base media (Fig. S6). Furthermore, there was no significant correlation between individual metal
165 concentrations and $\Delta oprF$ biofilm formation ($p > 0.05$, Pearson's). These results suggest that
166 the nutrient-dependent effect of OprF in biofilm formation is not due to differential metal
167 concentrations.

168

169 **OprF affects late-stage biofilm maturation in TSB**

170 Biofilm formation occurs in distinct stages (28). The nutrient-dependent effects of OprF detailed
171 above reflect mature static biofilm phenotypes and do not address when the $\Delta oprF$ biofilm
172 defect in TSB begins. To pinpoint these potential time-dependent effects of OprF in biofilm
173 formation, we performed static microtiter biofilm assays in TSB for 1, 4, 8, 16, and 24 hours
174 (29). There was no defect in the attached biomass of $\Delta oprF$ relative to that of wild type at any
175 time point between 1-16 hours (Fig. 2). Unexpectedly, at 8 hours, $\Delta oprF$ formed more biofilm
176 than wild type in TSB (Fig. 2C). However, by the 16-hour time point, $\Delta oprF$ biofilm levels once
177 again matched those of wild type (Fig. 2D). These results suggest that the $\Delta oprF$ defect does
178 not begin in the early stages of static biofilm formation. Instead, between the 16h and 24h time
179 points, $\Delta oprF$ static biofilm biomass decreased by $36.8 \pm 9.0\%$ S.D. (N=3), while wild type
180 increased $27.1 \pm 16.1\%$ S.D. (N=3). This suggests that without OprF, the static biofilm cannot
181 maintain its biomass in TSB. Investigation of mature biofilm maintenance is ideally performed
182 under continuous media flow, as it allows the biofilm to form for several days (30). However, the
183 $\Delta oprF$ strain does not form biofilms on glass slides under media flow (unpublished data, E.K.
184 Cassin and B.S. Tseng), limiting our methods to static assays. Combined with our earlier data
185 on the nutrient-dependent effects of OprF, these data suggest that OprF is involved in the
186 maintenance of mature *P. aeruginosa* biofilms in the presence of glucose or low sodium.

187

188 **The $\Delta oprF$ TSB biofilm defect is not dependent on PQS biosynthesis**

189 The role of OprF in late-stage biofilm formation is interesting because planktonic *oprF* mutants
190 make more OMVs than wild type cells and OMV production increases just before dispersal (22,
191 23). We hypothesized that the $\Delta oprF$ biofilm defect in TSB may be due to an increased OMV
192 production, resulting in early dispersion and less biofilm biomass relative to wild type. Since the
193 increased OMV production of *oprF* mutants is due to PQS overproduction and deletion of PQS

194 biosynthesis genes in an *oprF* mutant significantly decreases OMV production (22), we tested if
195 deleting *pqsA* or *pqsH* in the $\Delta oprF$ strain would rescue the $\Delta oprF$ biofilm defect in TSB. Since
196 PqsA is involved in the first steps of PQS biosynthesis and PqsH in the final step, a $\Delta oprF\Delta pqsA$
197 strain does not produce PQS or the PQS precursor HHQ, while a $\Delta oprF\Delta pqsH$ strain produces
198 HHQ, but not PQS (31). While the $\Delta pqsA$ and $\Delta pqsH$ single deletion strains formed biofilms
199 equal to wild type, both $\Delta oprF\Delta pqsA$ and $\Delta oprF\Delta pqsH$ formed biofilms equivalent to those of
200 $\Delta oprF$ in TSB (Fig. 3), suggesting that increased PQS, and thereby OMV production, from the
201 $\Delta oprF$ mutant strain is not responsible for the mature biofilm defect in TSB.

202

203 **Mature $\Delta oprF$ biofilms in TSB contain cell numbers equal to that of wild type**

204 Static microtiter biofilm assays use crystal violet to stain surface-attached biomass as a proxy
205 for total biofilm formation (29). Since crystal violet stains many biofilm components, including
206 biofilm cells and the extracellular matrix, it is an indiscriminate indicator of surface-attached
207 biomass. Therefore, we performed biofilm cell viability assays (29) in tandem with microtiter
208 biofilm assays to tease apart which major components of the biofilm are affected by OprF.
209 Surprisingly, despite the 60% decrease in total biofilm biomass in a side-by-side crystal violet
210 staining (Fig. 4A), $\Delta oprF$ static microtiter biofilms in TSB contain approximately the same
211 number of cells as that of wild type (Fig. 4B). Furthermore, to verify that the 60% decrease in
212 $\Delta oprF$ static biofilms was not due to differential crystal violet staining between strains, we
213 stained planktonic wild type and $\Delta oprF$ cells. These strains stain equivalently with crystal violet
214 at the cell densities observed in the biofilm cell viability assays (Fig. S7). These results suggest
215 that mature $\Delta oprF$ static biofilms in TSB contain less matrix, while biofilm cells remain attached
216 to the surface and that OprF is involved in maintaining or retaining the mature biofilm matrix.

217

218 **$\Delta oprF$ biofilms in TSB contain less eDNA than that of wild type**

219 Since crystal violet stains negatively charged molecules, we reasoned that less eDNA in the
220 biofilm could result in less biofilm biomass in the static biofilm assays. To quantify the eDNA in
221 $\Delta oprF$ biofilms, we grew static $\Delta oprF$ or wild type biofilms in TSB and stained them with the
222 eDNA-specific fluorophore DiTO-1. Static $\Delta oprF$ biofilms grown in TSB exhibit more eDNA-
223 associated signal than the $\Delta psfD$ biofilm-negative control strain, but $58.6 \pm 4.5\%$ S.D. (N=3) less
224 eDNA signal than wild-type biofilms (Fig. 5). This significant defect suggests that in the absence
225 of OprF, eDNA is lost from the mature biofilm matrix. Furthermore, when combined with our
226 earlier results, these results suggest that under certain conditions, OprF is involved in retaining
227 eDNA in the mature *P. aeruginosa* biofilm matrix.

228

229 **DISCUSSION**

230 Our results highlight that growth conditions, specifically glucose and sodium concentrations,
231 impact *P. aeruginosa oprF* mutant biofilm phenotypes. *P. aeruginosa* $\Delta oprF$ strains formed
232 significantly less biofilm in TSB than LB. The decrease in $\Delta oprF$ biofilm in TSB occurred
233 between 16-24 hours and did not result in fewer *P. aeruginosa* cells. Instead, $\Delta oprF$ biofilms in
234 TSB contained significantly less eDNA than wild-type biofilms. The mechanisms underlying how
235 glucose and low sodium led to decreased biofilms in cells lacking OprF is an exciting topic for
236 future studies, as is determining how matrix-associated OprF affects eDNA levels.

237

238 Bouffartigues and colleagues previously found that an *oprF* interruption mutant forms
239 approximately twice as much biofilm as the parental strain in LB, suggesting that a lack of OprF
240 results in the overproduction of biofilm (13). Our results in LB using the same *oprF* interruption
241 mutant strain agree with this conclusion. While these results follow the overall trend we saw in
242 our $\Delta oprF$ strain in TSB and LB (Fig. 1), we did not observe hyperbiofilm formation in our $\Delta oprF$
243 strain in LB. Since both strains are of the PAO1 lineage and whole genome sequencing of our

244 $\Delta oprF$ strain confirmed that no other differences exist between this strain and the parental, the
245 difference in biofilm phenotypes suggests that there may be additional genetic factors at play. It
246 is possible that the insertion in *oprF* in H636 affects biofilm formation or that the strain has
247 accumulated secondary mutations within or outside the *oprF* interruption that affect biofilm
248 formation in LB. These possibilities could be sorted out via future whole genome sequencing of
249 H636 and comparing it to its parental strain.

250

251 Matrix-associated OprF, a membrane protein containing many hydrophobic residues, is
252 abundant in biofilm membrane vesicles (4, 5). OMV production in biofilms is dependent on PQS
253 production (32), but in our experiments, abolishing PQS production did not impact the $\Delta oprF$
254 biofilm phenotype (Fig. 3). In a wild-type biofilm, cells produce OMVs via the bilayer couple
255 model with PQS, and MVs via explosive cell lysis (32). In $\Delta pqsA$ biofilms, MVs are still produced
256 (32), and we saw no defect in biofilm formation (Fig. 3). Similarly, MVs are likely still produced
257 by cell lysis in the defective biofilms of both the $\Delta oprF$ and $\Delta oprF\Delta pqs$ strains. Notably, these
258 mutant strains would produce vesicles with no OprF. Given that these strains exhibit 60% less
259 biofilm than wild type, we conclude that this decline is due to the lack of OprF, independent of
260 OMV production. Overall, the results of the current study indicate that in a $\Delta oprF$ background,
261 PQS-mediated OMV synthesis is not related to the decrease in biofilm observed in TSB, which
262 raises several questions outside the scope of this study: 1) do $\Delta oprF$ mutants in a biofilm
263 produce more OMVs, as has been reported for planktonic *oprF* mutants (22)? 2) is matrix-
264 associated OprF found only in vesicles? 3) how do glucose and low sodium affect the typical
265 functions of OprF in biofilms? Further research probing these questions would expand our
266 understanding of the roles of OprF and OmpA homologs in biofilm matrices.

267

268 OprF significantly affects the *P. aeruginosa* biofilm when grown under certain conditions. It is
269 tempting to assume that the 60% decline in $\Delta oprF$ biofilms grown in TSB (Fig. 4A) is a

270 proportional loss of all biofilm components. However, the static microtiter biofilm assay
271 quantifies total biomass with crystal violet that stains the negatively charged components of the
272 biofilm, namely cell surfaces, matrix membrane vesicles, and eDNA. Our biofilm cell viability
273 assays demonstrate that $\Delta oprF$ biofilms do not lose 60% of their cells (Fig. 4B). Instead, the
274 $\Delta oprF$ biofilms contain approximately 60% less eDNA than wild-type biofilms (Fig. 5). eDNA is
275 an essential matrix component primarily produced by biofilm cell lysis (21, 33). It has been
276 proposed that membrane vesicles stabilize the matrix of wild-type biofilms through their
277 interactions with eDNA (34). Therefore, OprF, which is abundant in membrane vesicles, may be
278 involved – directly or indirectly – in these eDNA interactions and thereby in biofilm structural
279 maintenance.

280

281 The maintenance of mature biofilms as an active, discrete stage in the biofilm lifecycle has been
282 a recent topic of discussion (30). In this model, established biofilms respond to environmental
283 changes to persist as a community. In a static microtiter biofilm, these changes include
284 depletion of nutrients and waste accumulation over time. Our data indicate that OprF affects
285 mature static biofilms in TSB, with the established $\Delta oprF$ biofilm decreasing between 16-24
286 hours of incubation. This phenotype suggests that in the absence of OprF, biofilm formation
287 progresses to maturity and subsequently degrades. When combined with our biofilm cell viability
288 results (Fig. 4), mature $\Delta oprF$ biofilm degradation does not appear to be due to dispersion since
289 cell numbers are maintained. Therefore, we hypothesize that OprF may be involved in matrix
290 retention in mature static biofilm maintenance via 1) matrix-bound OprF interactions with eDNA
291 or 2) intracellular regulatory effects of deleting *oprF*. Future research into these lines of
292 questioning is necessary and will contribute to an expanded understanding of the role of OprF in
293 mature biofilm maintenance.

294

295 **METHODS AND MATERIALS**

296 **Bacterial strains and growth conditions**

297 Bacterial strains, oligonucleotides, and plasmids used in this study are in Tables S4-S6. Strains
298 produced for this study were constructed using allelic exchange, as in (35), and described in
299 Supplemental Methods. Liquid lysogeny broth (LB) and tryptic soy broth (TSB) were prepared
300 according to the recipe in Table S1. The PAO1 $\Delta oprF+oprF$ strain containing *oprF* under an
301 arabinose-inducible promoter was grown in media containing 0.5% L-arabinose (Sigma Aldrich).
302 Unless otherwise noted, strains were grown at 37°C in specified media with 250 RPM shaking
303 or on semi-solid LB containing 1.5% Bacto agar.

304

305 **Static microtiter biofilm assays**

306 Static biofilms were grown as described in (24). Overnight cultures of bacteria grown in
307 appropriate media were diluted 1:100, and 100 μ L was seeded into sterile 96-well polystyrene
308 plates (Greiner Bio-One, #650101). Plates were incubated at 37°C without shaking for 24 h
309 unless otherwise noted. Planktonic cells were removed by triplicate washes in deionized water.
310 Attached biofilm biomass was stained with 0.1% crystal violet for 15 min and washed as above.
311 Stained biomass was solubilized using 30% acetic acid, transferred to a flat-bottom 96-well
312 plate (Greiner Bio-One, #655090), and the absorbance at OD₅₅₀ was read in a Synergy Hybrid
313 HTX Microplate Reader (BioTek Instruments). Absorbance from blank media wells was
314 subtracted from raw OD₅₅₀ readings. Absorbance value of each strain was normalized to the
315 average absorbance of the wild-type or parental strain. Four to ten technical replicates within
316 each biological replicate were averaged, and the average measurement of three biological
317 replicates were used to statistically compare biofilm formation by 1-way ANOVA with post hoc
318 Tukey HSD for assays with 1 independent variable or 2-way ANOVA with post hoc Bonferroni
319 for assays with 2 independent variables. All statistical analyses were performed in IBM SPSS.

320

321 **Biofilm cell viability assays**

322 Biofilms were grown as above in static microtiter biofilm assays. Following 24-hr incubation,
323 planktonic cells were removed by washing with sterile deionized water poured over plates three
324 times. Half of the wells in each plate were scraped with sterile flat toothpicks in 125 μ L sterile
325 PBS to remove attached biofilm biomass. Solubilized biomass was serially diluted, spread on
326 LB agar, and incubated at 37°C. CFU/well (100 μ L/well) was enumerated after 24 h. The other
327 half of the wells in each plate were stained with crystal violet, as detailed in static microtiter
328 biofilm assays above. Four technical replicates within each biological replicate were averaged,
329 and the average CFU/well of the three biological replicates was used to statistically compare
330 cell counts by 1-way ANOVA with post hoc Tukey HSD.

331

332 **Biofilm eDNA fluorescence assays**

333 Biofilms were grown as above in static microtiter biofilm assays. Following 24-hr incubation,
334 planktonic cells were removed by washing with sterile deionized water poured over plates three
335 times. Half of the wells in the plate were stained with eDNA-specific DiTO-1 (1 μ M, AAT
336 Bioquest, #17575) for 15 min. Stain was removed by pipetting and rinsed with 100 μ L
337 phosphate buffered saline in triplicate. Attached, stained biomass was removed by scraping with
338 sterile toothpicks, as in biofilm cell viability assays above, in each well containing 125 μ L sterile
339 PBS. Scraped, stained biomass was transferred to a flat-bottomed, black-walled 96-well plate
340 (Greiner Cellstar, #655090) and the fluorescence (Ex: 485/20, Em: 528/20) and absorbance
341 (OD_{600}) were measured in a Synergy Hybrid HTX Microplate Reader (BioTek Instruments). One
342 quarter of the unstained wells were processed for cell viability and one quarter were processed
343 for crystal violet staining to assess total biofilm formation, as above. The background fluorescent
344 signal from wells incubated with media only was subtracted from total fluorescence, and the
345 average total fluorescence from four technical replicates per biological replicate were averaged.
346 The average fluorescence per biological replicate was normalized to the average OD_{600} value

347 per strain. Average fluorescence/OD₆₀₀ of the three biological replicates was used to statistically
348 compare strain fluorescence by 1-way ANOVA with post hoc Tukey HSD.

349

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358 **AUTHOR CONTRIBUTIONS**

359 Conceptualization: E.K. Cassin, B.S. Tseng; Formal analysis, investigation, methodology, or
360 visualization of data: E.K. Cassin, S.A. Araujo-Hernandez, D.S. Baughn, M.C. Londono, D.Q.
361 Rodriguez, B.S. Tseng; Writing – original draft: E.K. Cassin, B.S. Tseng; and Writing – review
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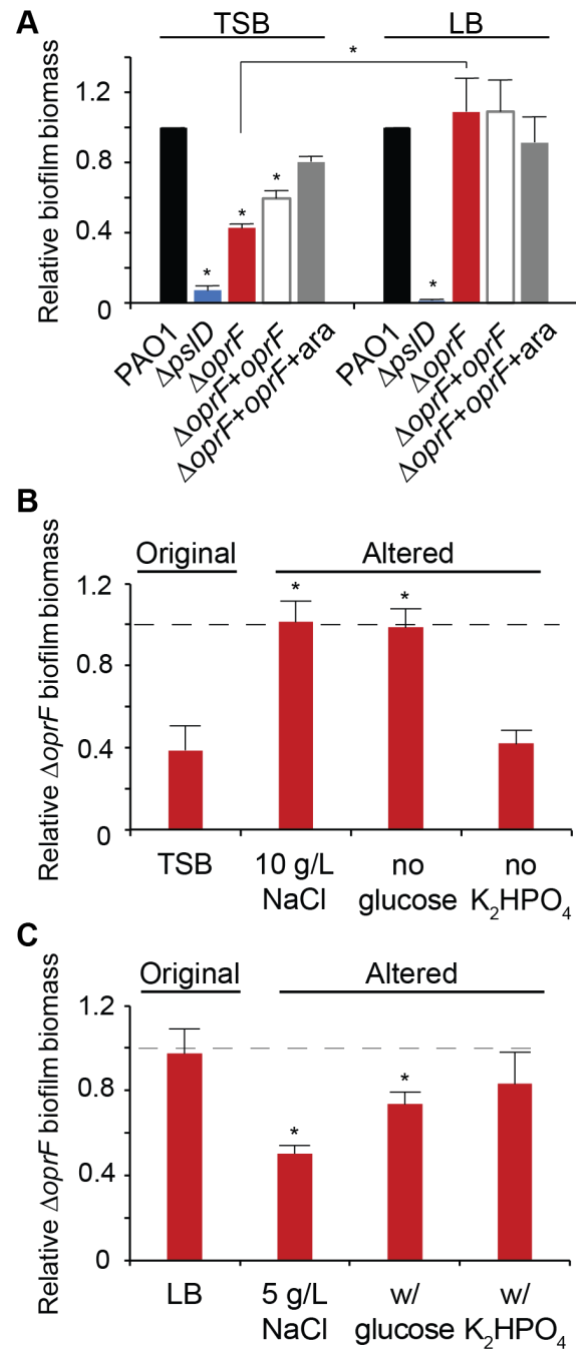
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- 461

462 **FIGURES AND FIGURE LEGENDS**

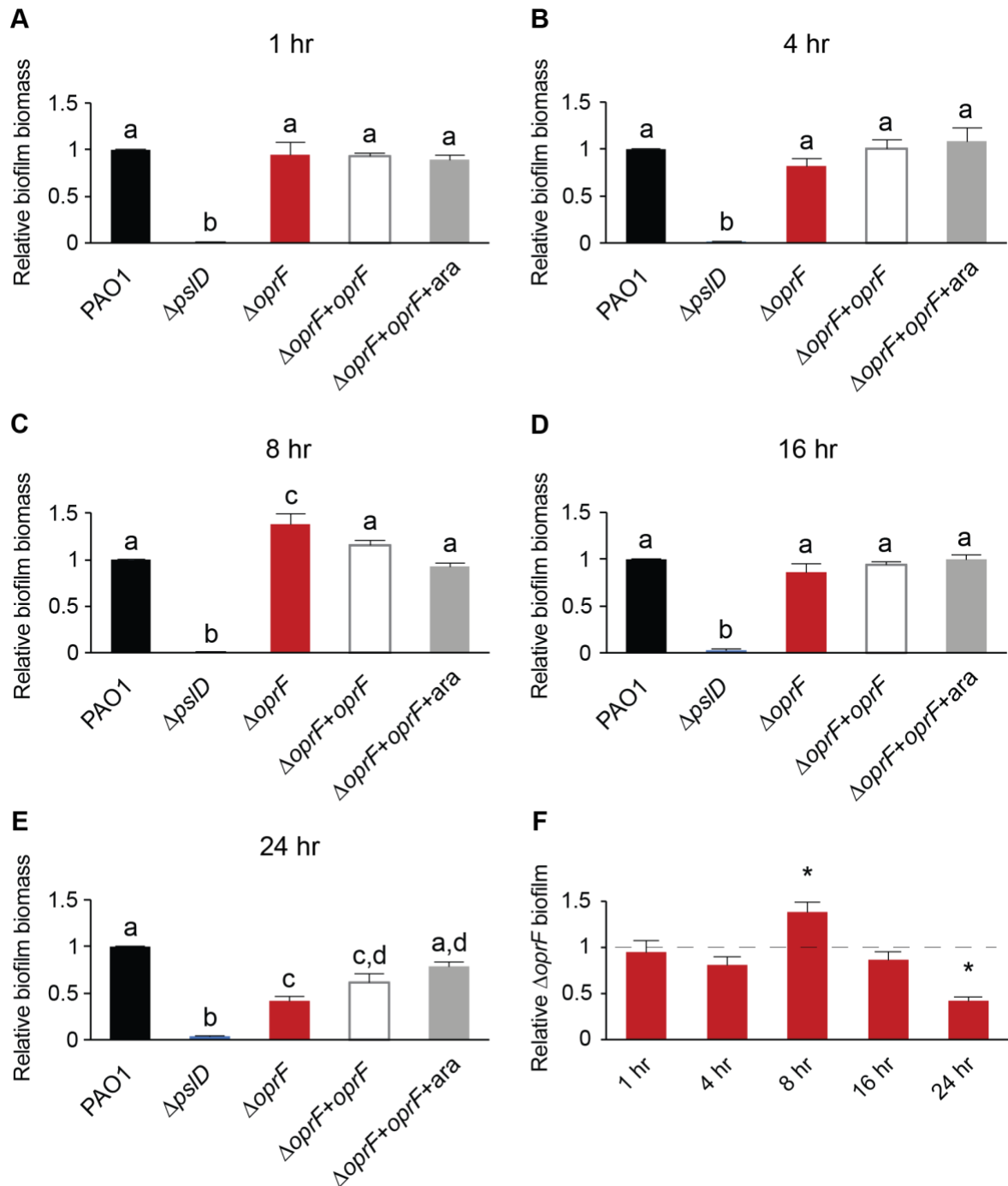
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464

465 **Figure 1. $\Delta oprF$ forms less biofilm in TSB than in LB, due to lower sodium chloride**
466 **concentration and presence of glucose**

467 (A) 24-hour static microtiter biofilm assays of *P. aeruginosa* PAO1 (WT, black), $\Delta pslD$ (blue),
468 $\Delta oprF$ (red), and a $\Delta oprF attTn7::P_{BAD^-} oprF$ restoration strain ($\Delta oprF + oprF$) without (white) and
469 with (gray) 0.5% arabinose (ara) in the indicated media. Error bars, SEM (N = 3); asterisk over
470 error bar, statistically different from WT in the same medium ($p < 0.05$; two-way ANOVA with
471 post hoc Bonferroni). Statistical difference between $\Delta oprF$ strains in different media are
472 indicated by a bar and asterisk. (B and C) Biofilm formation of $\Delta oprF$ strain in variations of TSB
473 and LB: unaltered, altered NaCl concentrations, altered glucose concentrations, and altered
474 K_2HPO_4 concentrations (left to right). Biofilm formation is normalized to WT in each respective
475 medium. Dashed line, normalized amount of WT biofilm formation in each medium; error bars,
476 SEM (N = 3); asterisk over error bar, statistically different from $\Delta oprF$ in the original medium ($p <$
477 0.05 ; two-way ANOVA with post hoc Bonferroni). See Figure S4 and Tables S2-S3 for full
478 comparisons.

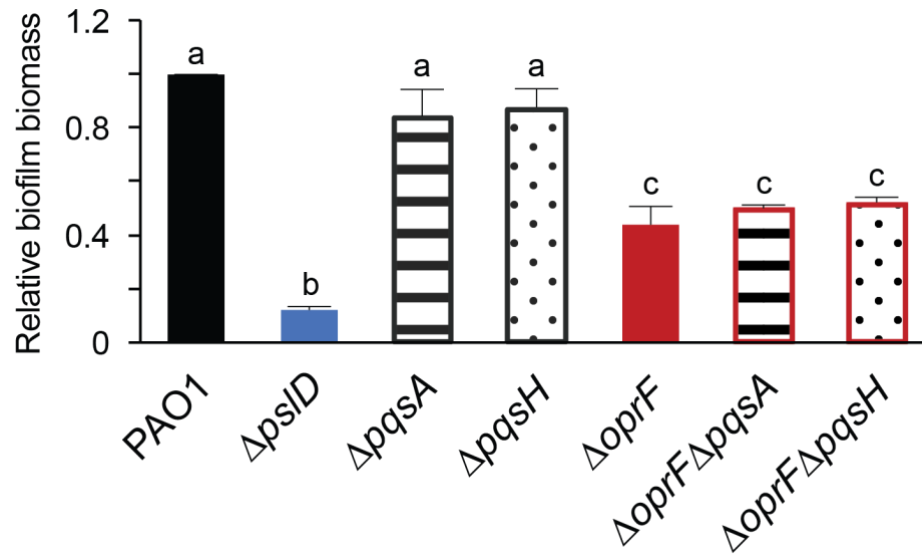


479

480 Figure 2. OprF affects late biofilm maturation in TSB

481 (A) 1-hour static microtiter biofilm assays were performed in TSB with PAO1 (WT, black), $\Delta pslD$
482 (blue), $\Delta oprF$ (red), and a $\Delta oprF attTn7::P_{BAD}-oprF$ restoration strain ($\Delta oprF + oprF$) with (white)
483 and without (gray) 0.5% arabinose (ara). (B) 4-hour, (C) 8-hour, (D) 16-hour, and (E) 24-hour

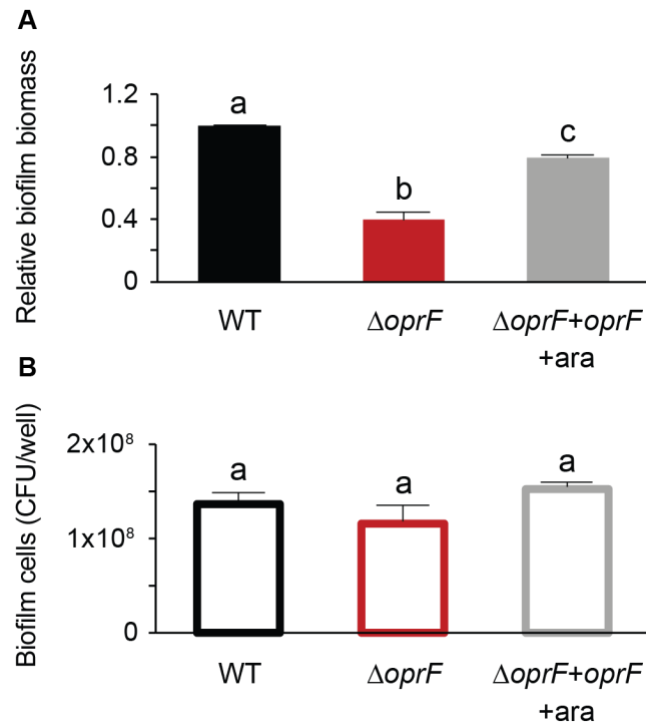
484 assays were performed with the same strains and media. (F) Static $\Delta oprF$ biofilm formation
485 relative to WT (dashed line) at respective time points is represented. Biofilm formation is
486 normalized to WT in each respective medium. Error bars, SEM (N = 3); letters, statistical
487 groupings ($p < 0.01$; one-way ANOVA with post hoc Tukey HSD); asterisk, statistically different
488 from WT at the same time point.



489

490 **Figure 3. $\Delta oprF$ biofilm defect in TSB is independent of PQS**

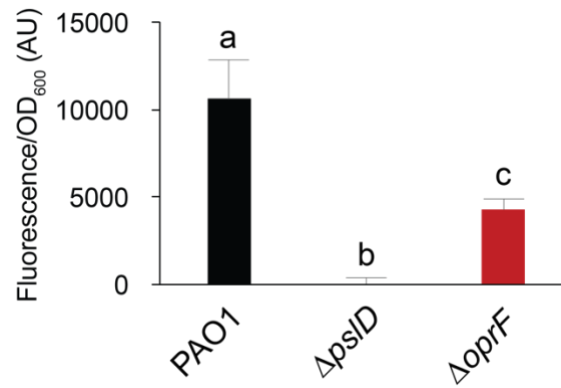
491 24-hour static microtiter biofilm assays were performed in TSB with PAO1 (black), $\Delta psID$ (blue),
492 $\Delta pqsA$ (stripes), $\Delta pqsH$ (dots), $\Delta oprF$ (red), $\Delta oprF\Delta pqsA$ (stripes with red outline), and
493 $\Delta oprF\Delta pqsH$ (dots with red outline). Biofilm formation is normalized to WT. Error bars, SEM (N =
494 3); letters, statistical groupings ($p < 0.01$; one-way ANOVA with post hoc Tukey HSD).



495

496 **Figure 4. $\Delta oprF$ exhibits no biofilm cell viability defect in TSB**

497 Side-by-side 24-hour (A) static microtiter biofilm and (B) biofilm cell viability assays were
498 performed in TSB in the same 96-well plate with *P. aeruginosa* PAO1 (WT, black), $\Delta oprF$ (red),
499 and the $\Delta oprF$ *attTn7::P_{BAD}-oprF* restoration strain with 0.5% arabinose ($\Delta oprF + oprF + ara$;
500 gray). Biofilm formation is normalized to WT. Error bars, SEM (N = 3); letters, statistical
501 groupings (p < 0.05; one-way ANOVA with post hoc Tukey HSD).



502

503 **Figure 5. OprF affects biofilm eDNA levels in TSB**

504 24-hour static microtiter biofilms grown in TSB with PAO1 (black), ΔpsID (blue), and ΔoprF (red)
505 were stained with the eDNA-specific dye DiTO-1. Fluorescence intensity from each strain was
506 normalized to respective biofilm cell numbers (via absorbance at OD₆₀₀). Error bars, SEM (N =
507 3); letters, statistical groupings (p < 0.05; one-way ANOVA with post hoc Tukey HSD).