SUPPLEMENTAL FIGURES

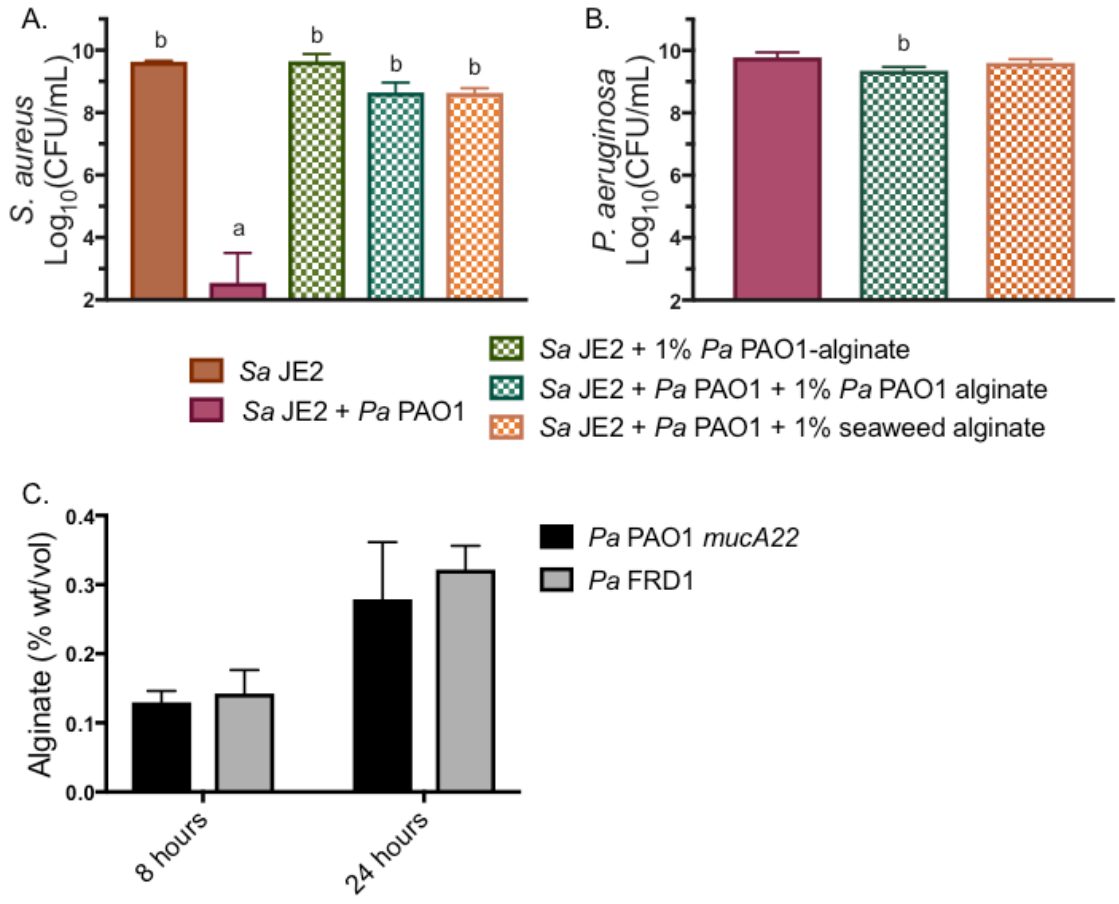


Figure S1. Exogenous alginate protects *S. aureus* JE2 from *P. aeruginosa* PAO1 in co-culture. **A)** *S. aureus* JE2 and **B)** *P. aeruginosa* PAO1 growth after 8 hours of liquid co-culture in TSB +/- 1% seaweed-derived or *P. aeruginosa* PAO1-derived alginate. CFU/ml were enumerated and $\log_{10}$ transformed. Dashed boxes indicate the presence of alginate in the culture. Significance was determined by one-way ANOVA with Dunnett's post-test. a, $p<0.0001$ with *S. aureus* JE2 as the reference. b, $p<0.0001$ for panel **A** and $p<0.05$ for panel **B** with *P. aeruginosa* PAO1 + *S. aureus* JE2 as the reference. **C)** Alginate production by mucoid strain *P. aeruginosa* PAO1 *mucA22* and mucoid clinical isolate *P. aeruginosa* FRD1 after 8 and 24 hours liquid culture in flasks.

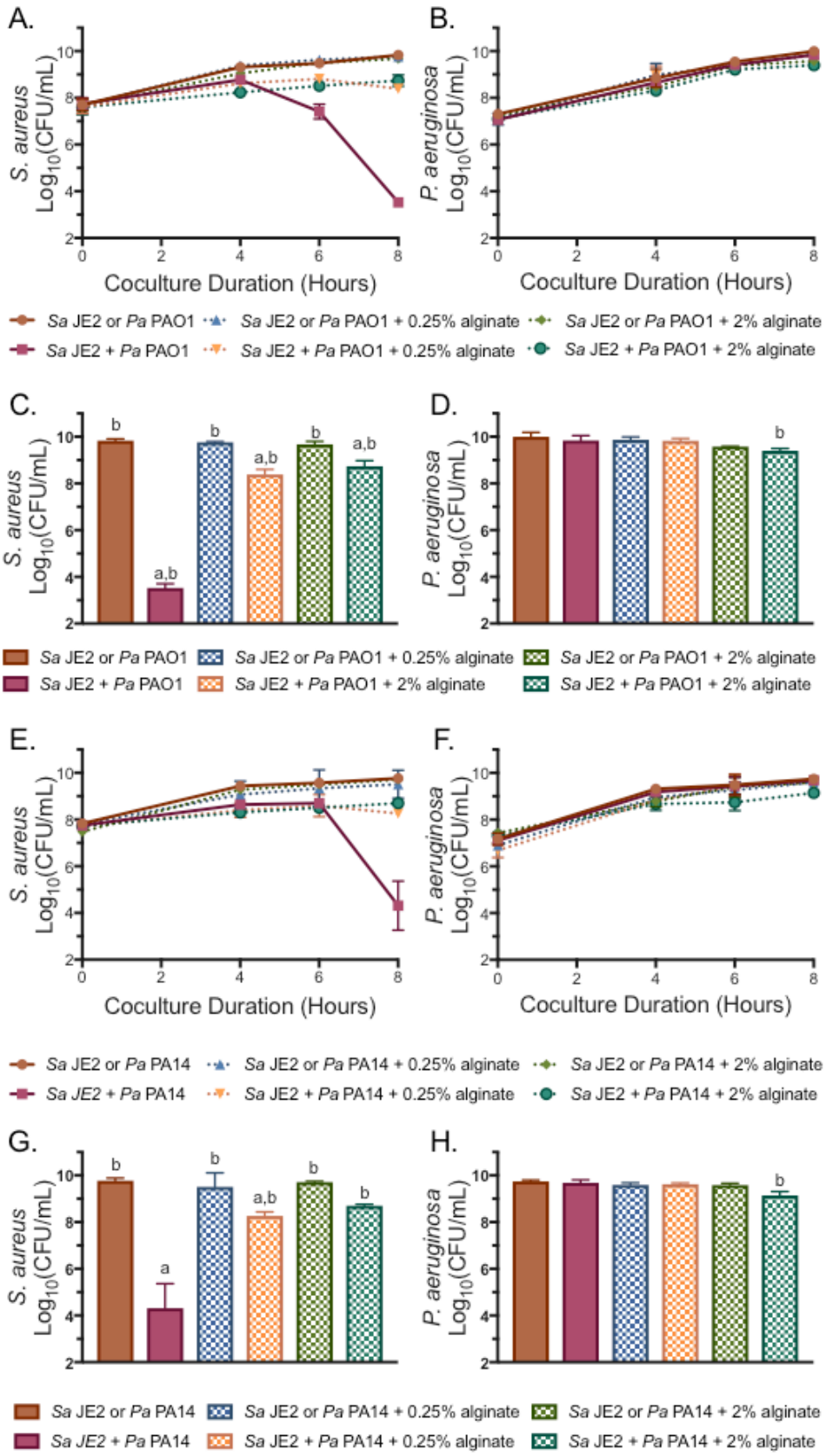

A.
B.
S. aureus Log10(CFU/mL)
P. aeruginosa Log10(CFU/mL)
Coculture Duration (Hours)
Sa JE2 or Pa PAO1
Sa JE2 or Pa PAO1 + 0.25% alginate
Sa JE2 or Pa PAO1 + 2% alginate
Sa JE2 + Pa PAO1
Sa JE2 + Pa PAO1 + 0.25% alginate
Sa JE2 + Pa PAO1 + 2% alginate
C.
D.
b
a,b
E.
F.
Sa JE2 or Pa PA14
Sa JE2 or Pa PA14 + 0.25% alginate
Sa JE2 or Pa PA14 + 2% alginate
Sa JE2 + Pa PA14
Sa JE2 + Pa PA14 + 0.25% alginate
Sa JE2 + Pa PA14 + 2% alginate
G.
H.
a

Figure S2. Exogenous alginate protects *S. aureus* JE2 from *P. aeruginosa* in co-culture.

Liquid co-cultures of *S. aureus* JE2 and *P. aeruginosa* in TSB +/- seaweed-derived alginate added at 0.25% or 2%. CFU/ml were $\log_{10}$ transformed. **A)** *S. aureus* JE2 and **B)** *P. aeruginosa* PAO1 growth curves over 8 hours in monoculture or the indicated co-cultures. CFU/ml were enumerated at indicated time points. **C)** *S. aureus* JE2 and **D)** *P. aeruginosa* PAO1 growth after 8 hours. **E)** *S. aureus* JE2 and **F)** *P. aeruginosa* PA14 growth curves over 8 hours. CFU/ml were enumerated at indicated time points. **G)** *S. aureus* JE2 and **H)** *P. aeruginosa* PA14 growth after 8 hours. Dotted lines/dashed boxes indicate the presence of alginate in the culture. Significance was determined by one-way ANOVA with Dunnett's post-test. a, $p<0.05$ with *S. aureus* JE2 as the reference. b, $p<0.001$ with *P. aeruginosa* + *S. aureus* JE2 as the reference.

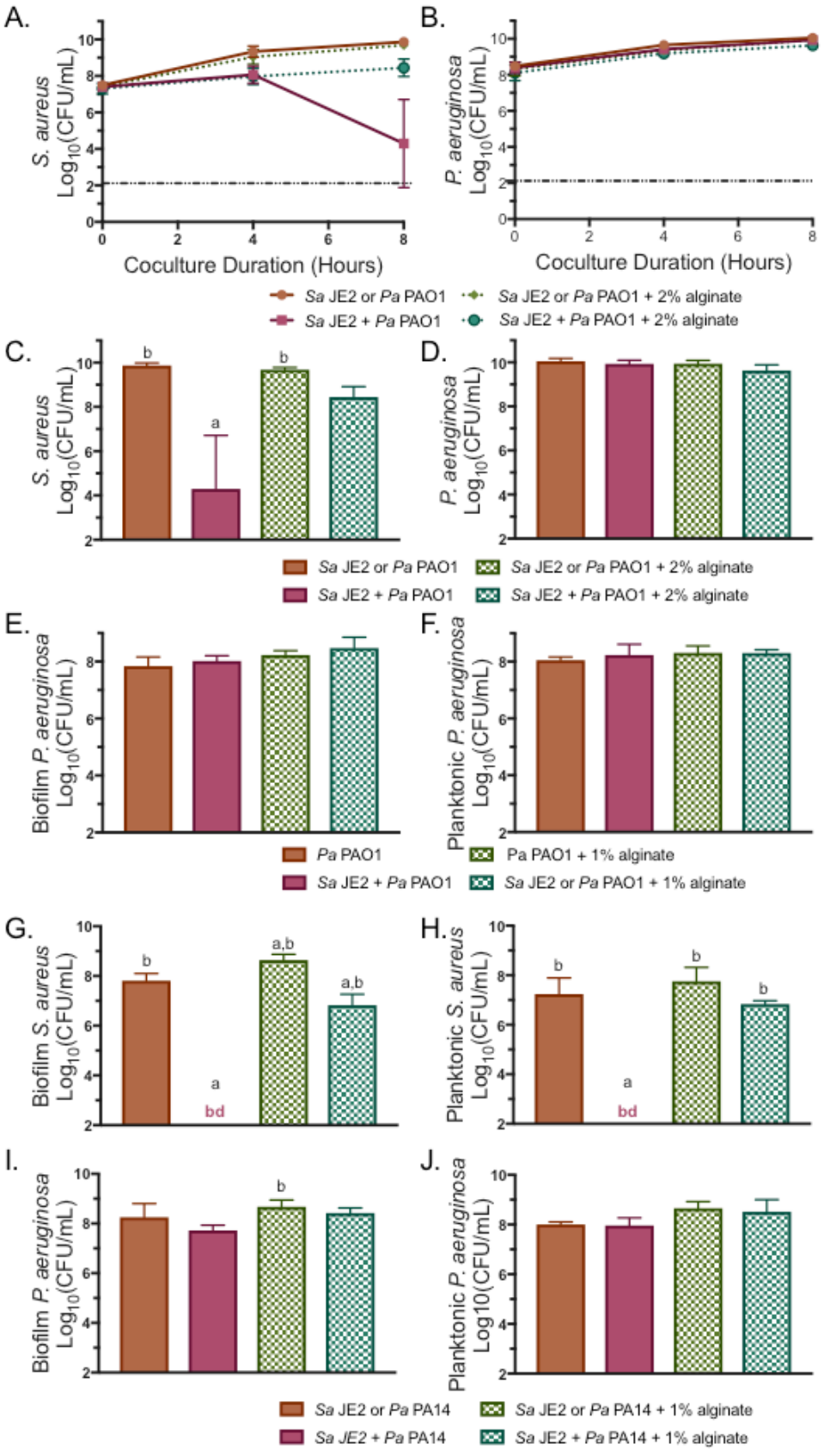
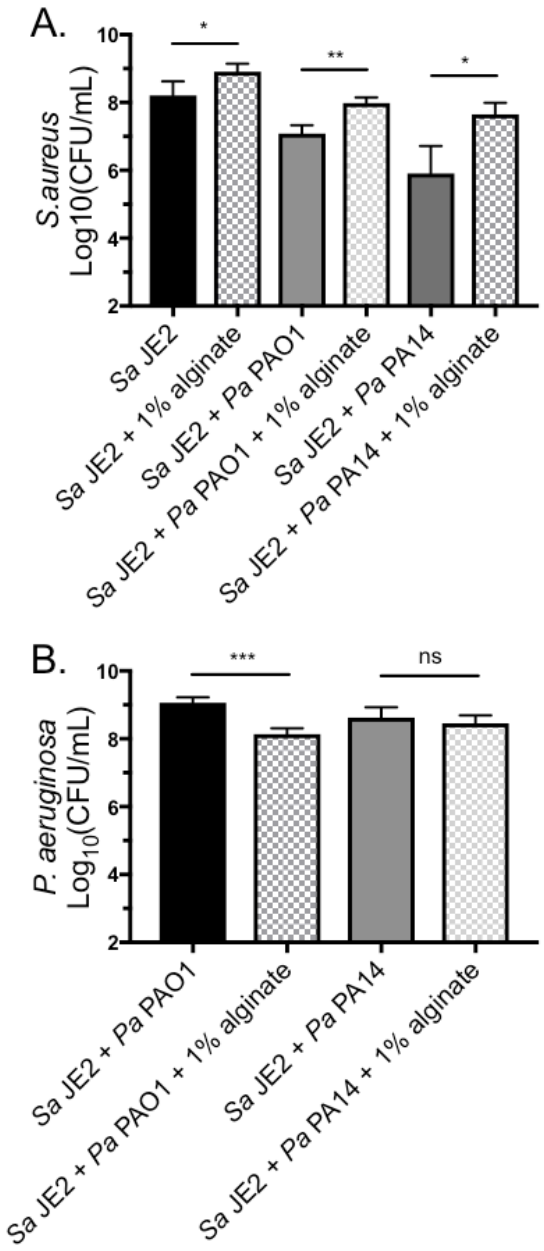

A.
S. aureus Log10(CFU/mL)
Coculture Duration (Hours)
B.
P. aeruginosa Log10(CFU/mL)
Coculture Duration (Hours)
Sa JE2 or Pa PAO1
Sa JE2 + Pa PAO1
Sa JE2 or Pa PAO1 + 2% alginate
Sa JE2 + Pa PAO1 + 2% alginate
C.
S. aureus Log10(CFU/mL)
D.
P. aeruginosa Log10(CFU/mL)
E.
Biofilm P. aeruginosa Log10(CFU/mL)
F.
Planktonic P. aeruginosa Log10(CFU/mL)
Pa PAO1
Pa PAO1 + 1% alginate
Sa JE2 + Pa PAO1
Sa JE2 or Pa PAO1 + 1% alginate
G.
Biofilm S. aureus Log10(CFU/mL)
H.
Planktonic S. aureus Log10(CFU/mL)
bd
I.
Biofilm P. aeruginosa Log10(CFU/mL)
J.
Planktonic P. aeruginosa Log10(CFU/mL)
Sa JE2 or Pa PA14
Sa JE2 + Pa PA14
Sa JE2 or Pa PA14 + 1% alginate
Sa JE2 + Pa PA14 + 1% alginate

Figure S3. Exogenous alginate protects *S. aureus* JE2 from *P. aeruginosa* in co-culture.

A-D) Liquid co-cultures of *S. aureus* JE2 and *P. aeruginosa* PAO1 in TSB +/- seaweed-derived alginate added at 0.25% or 2%. *P. aeruginosa* initial density was 10X higher than *S. aureus* density based on OD_{600}. CFU/ml were log_{10} transformed. **A)** *S. aureus* JE2 and **B)** *P. aeruginosa* PAO1 growth curves over 8 hours. CFU/ml were enumerated at indicated time points. The dashed line at 2 indicates the limit of detection. **C)** *S. aureus* JE2 and **D)** *P. aeruginosa* PAO1 growth after 8 hours. **E-F)** Biofilm co-cultures of *P. aeruginosa* PAO1 and *S. aureus* JE2 for 16 hrs in MEM L-gln L-arg +/- 1% seaweed-derived alginate. CFU/ml were enumerated and log_{10} transformed. *P. aeruginosa* PAO1 **E)** biofilm and **F)** planktonic growth. **G-J)** Biofilm co-cultures of *P. aeruginosa* PA14 and *S. aureus* JE2 for 16 hours in MEM L-gln L-arg +/- 1% seaweed-derived alginate. CFU/ml were enumerated and log_{10} transformed. *S. aureus* JE2 **G)** biofilm and **H)** planktonic and *P. aeruginosa* PA14 **I)** biofilm and **J)** planktonic growth. Dotted lines/dashed boxes indicate the presence of alginate in the culture. Significance was determined by one-way ANOVA with Dunnett's post-test. a, $p<0.05$ with *S. aureus* JE2 as the reference. b, $p<0.05$ with *P. aeruginosa* + *S. aureus* JE2 as the reference.



43

44 **Figure S4. Exogenous alginate protects *S. aureus* JE2 from *P. aeruginosa* in minimal medium**

45 **liquid co-culture. A)** *S. aureus* JE2 and **B)** *P. aeruginosa* PAO1 and *P. aeruginosa* PA14 growth

46 after 20 hours co-culture in 1/2X MEM L-gln L-arg +/- 1% alginate. CFU/ml were enumerated

47 and $\log_{10}$ transformed. Significance determined by unpaired t-test. *p<.05, **p<.01, ***p<.001.

48

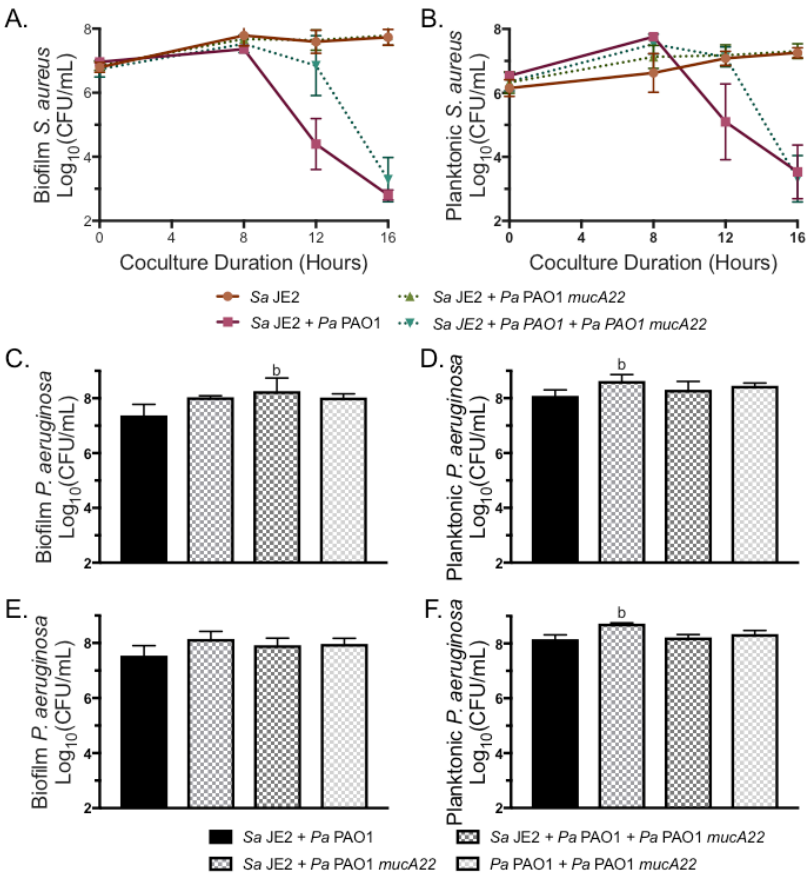


Figure S5. Culture with mucoid *P. aeruginosa* PAO1 *mucA22* delays *S. aureus* JE2 killing by wild-type *P. aeruginosa* PAO1. Biofilm tri-culture on plastic with *S. aureus* JE2, *P. aeruginosa* PAO1, and *P. aeruginosa* PAO1 *mucA22* in MEM L-gln L-arg. CFU/ml were enumerated on MSA and $\log_{10}$ transformed. Growth curve of *S. aureus* JE2 viability in the **A)** biofilm and **B)** planktonic fraction. Dashed lines indicate the presence of a mucoid strain in the culture. *P. aeruginosa* PAO1 growth in the **C)** biofilm and **D)** planktonic fraction after 12 hours and *P. aeruginosa* PAO1 growth in the **E)** biofilm and **F)** planktonic fraction after 16 hours co-culture. Dashed boxes indicate the presence of a mucoid strain in the culture. Significance was determined by one-way ANOVA with Dunnett's post-test. a, $p<0.05$ with *S. aureus* JE2 as the reference. b, $p<0.05$ with *P. aeruginosa* PAO1 + *S. aureus* JE2 as the reference.

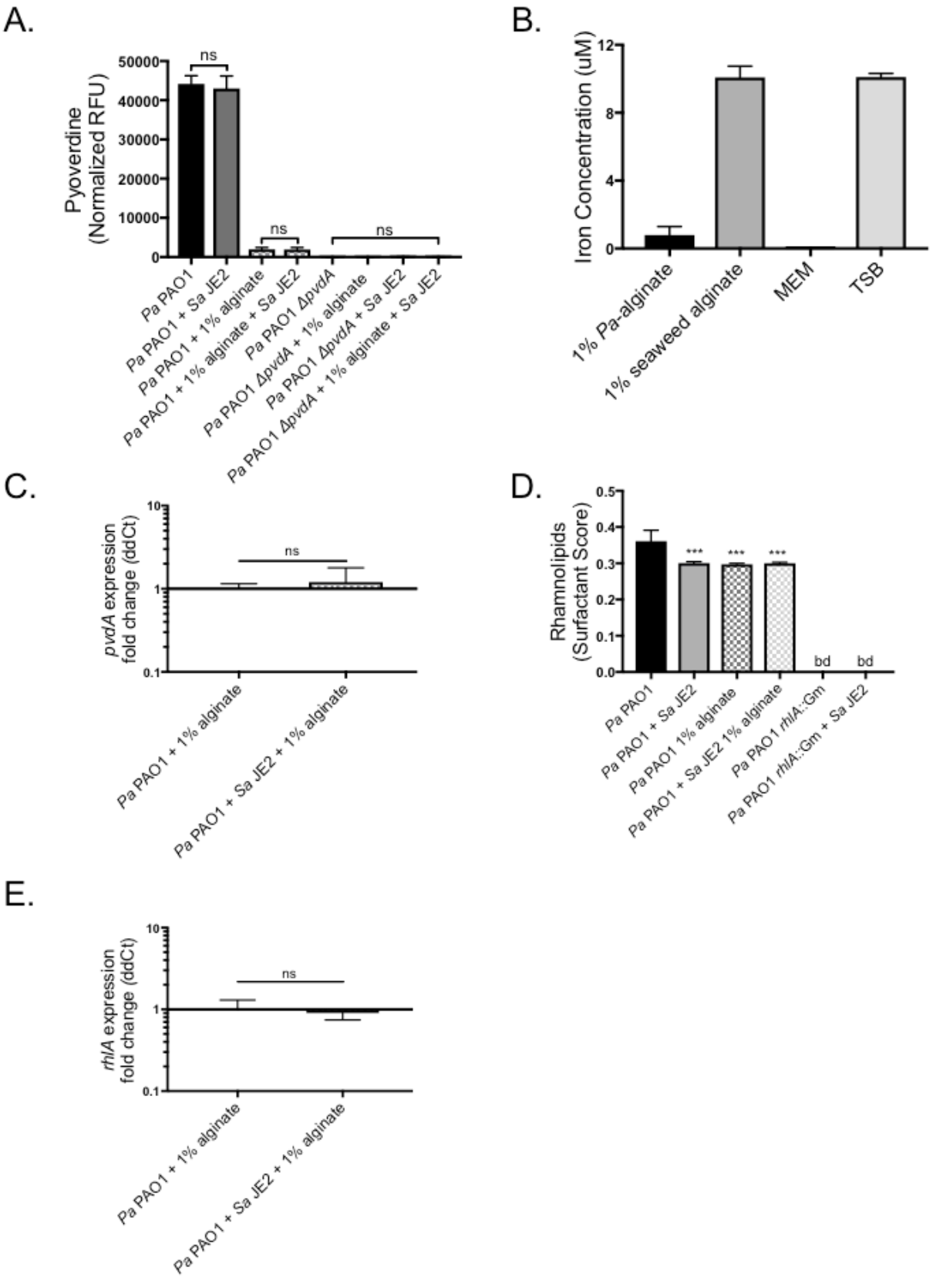

A.
Pyoverdine (Normalized RFU)
50000
40000
30000
20000
10000
0
ns
ns
ns
Pa PAO1
Pa PAO1 + Sa JE2
Pa PAO1 + 1% alginate
Pa PAO1 + 1% alginate + Sa JE2
Pa PAO1 ΔpvdA
Pa PAO1 ΔpvdA + 1% alginate
Pa PAO1 ΔpvdA + Sa JE2
Pa PAO1 ΔpvdA + 1% alginate + Sa JE2
B.
Iron Concentration (uM)
12
8
4
0
1% Pa-alginate
1% seaweed alginate
MEM
TSB
C.
pvdA expression fold change (ddCt)
10
1
0.1
ns
Pa PAO1 + 1% alginate
Pa PAO1 + Sa JE2 + 1% alginate
D.
Rhamnolipids (Surfactant Score)
0.5
0.4
0.3
0.2
0.1
0.0
***
***
***
bd
bd
Pa PAO1
Pa PAO1 + Sa JE2
Pa PAO1 1% alginate
Pa PAO1 + Sa JE2 1% alginate
Pa PAO1 rhlA::Gm
Pa PAO1 rhlA::Gm + Sa JE2
E.
rhlA expression fold change (ddCt)
10
1
0.1
ns
Pa PAO1 + 1% alginate
Pa PAO1 + Sa JE2 + 1% alginate

Figure S6. Contributions of *S. aureus* and iron in alginate co-cultures. A) *P. aeruginosa* PAO1 was grown on plastic in 1/2X MEM L-gln L-arg +/- 1% alginate for 16 hours at 37^{o}C with 5% CO_2. Supernatants were collected from the planktonic fraction. Pyoverdine was quantified by measuring RFU of the supernatants at 400 nm excitation and 460 nm emission and normalizing to CFU/mL of the planktonic fraction. **B)** Iron was quantified in MEM L-gln, TSB, and seaweed alginate. For alginate, μg/g quantifications were converted to μM that 1% alginate contributes to the solution. **C-E)** *P. aeruginosa* PAO1 was grown in TSB in liquid culture for 8 hours +/- 1% alginate. Supernatants were collected by centrifuging to remove cell debris, and sterile filtering. Cell pellets were snap frozen for expression analyses. **C)** *pvdA* expression was quantified by q-RT-PCR and ddCt was calculated relative to *P. aeruginosa* PAO1 + 1% alginate *rpoD* expression. Significance for four biological replicates was measured by paired t-test. ns, not significant. **D)** Rhamnolipid production by *P. aeruginosa* PAO1 quantified by drop collapse and normalized to CFU/mL to determine surfactant score. Significance determined by one-way ANOVA with Dunnett's post-test comparison to *P. aeruginosa* PAO1. *p>0.05, **p<0.01, ***p<0.001, ****p<0.0001. **E)** *rhlA* expression was quantified by q-RT-PCR and ddCt was calculated relative to *P. aeruginosa* PAO1 + 1% alginate *rpoD* expression. Significance for three biological replicates was measured by paired t-test. ns, not significant.

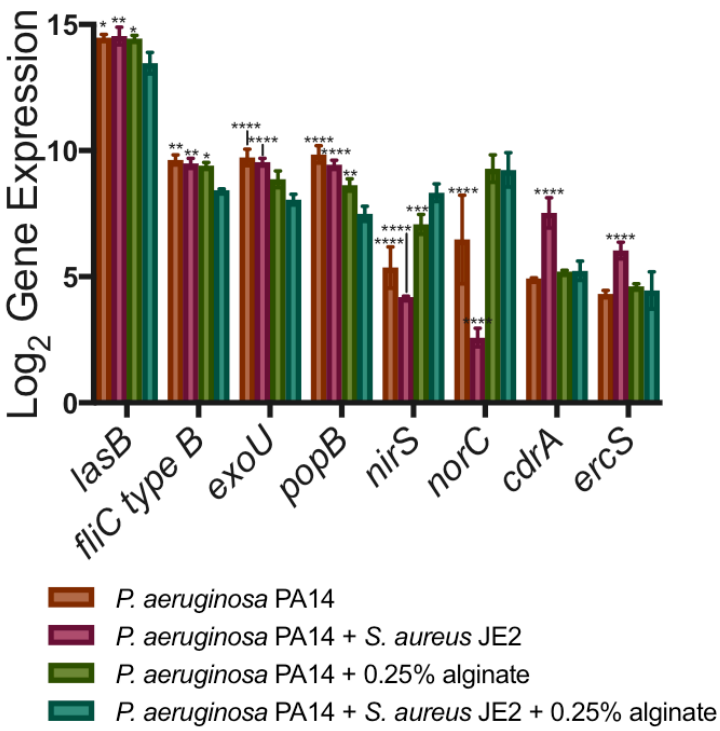


Figure S7. Gene expression patterns of significantly differentially expressed *P. aeruginosa* transcripts. Expression of genes identified in Figure 7 C-D to be differentially expressed in the presence of alginate. Gene expression was analyzed by two-way ANOVA followed by Tukey's multiple comparisons. All displayed statistical comparisons are in reference to *P. aeruginosa* PAO1 + *S. aureus* JE2 + 0.25% alginate. All statistical data is available in supplemental table 4. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

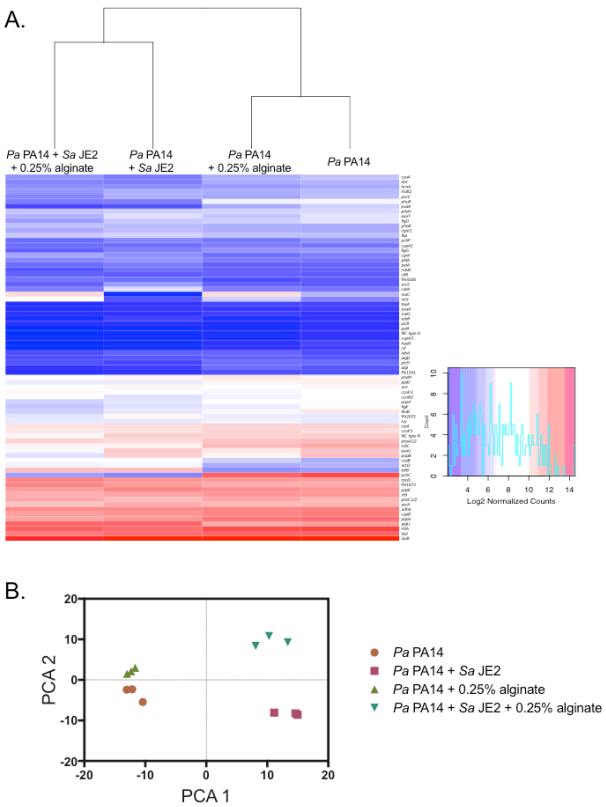


Figure S8. *P. aeruginosa* PA14 gene expression changes in response to *S. aureus* JE2 and alginate. Transcriptional profiling of *P. aeruginosa* by Nanostring revealed that the presence of *S. aureus* has a stronger impact on overall *P. aeruginosa* gene expression than exogenous alginate. *P. aeruginosa* PA14 was cultured for 8 hours in TSB +/- 0.25% alginate and +/- *S. aureus* JE2. Nanostring counts were normalized to positive controls and three housekeeping genes (*rpoD, ppiD, fbp*) and $\log_2$ transformed. **A)** Clustering by Euclidean distance of the average of three biological replicates. Image was generated in R with the Heatmap.2 function. **B)** Principal component analysis of all biological replicates. Samples separate more strongly on the presence of *S. aureus* (PCA 1) than on the presence of exogenous alginate (PCA 2). However, this clustering disappears when iron-acquisition genes are removed from the analysis (data not shown).

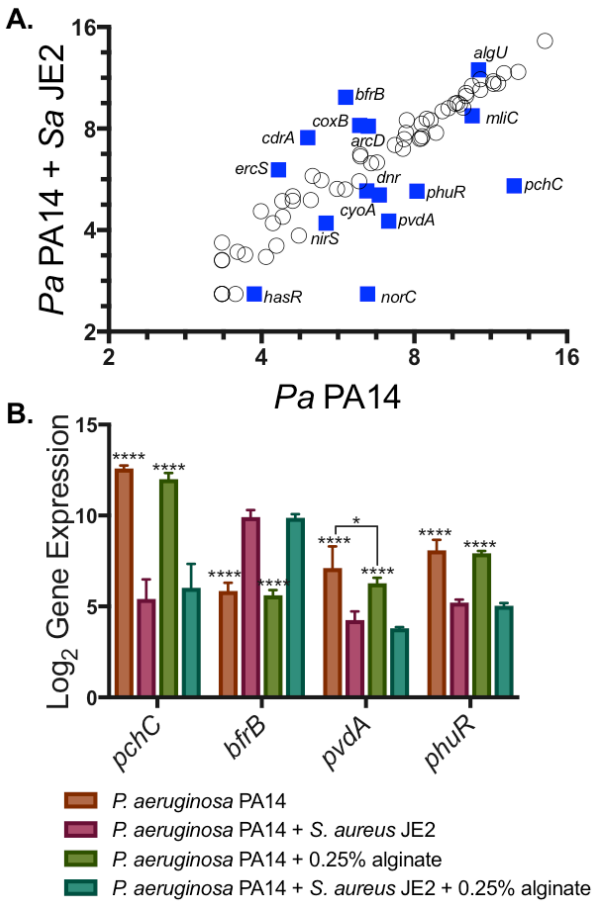


Figure S9. *S. aureus* alters the expression of *P. aeruginosa* iron acquisition genes. Raw Nanostring counts were normalized to positive controls and three housekeeping genes (*rpoD, ppiD, fbp*) and $\log_2$ transformed. Three biological replicates per condition. **A)** Significantly differentially expressed genes were determined by unpaired *t*-test followed by the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (with *q* 1% for false discovery), and are marked by blue squares and labeled with the gene name. **B)** Iron acquisition genes with the largest expression changes between *P. aeruginosa* PA14 and *P. aeruginosa* PA14 + *S. aureus* JE2. * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. All displayed statistical comparisons are in reference to the *P. aeruginosa* PAO1 + *S. aureus* JE2 condition unless otherwise indicated. All statistical data is available in supplemental table 4.

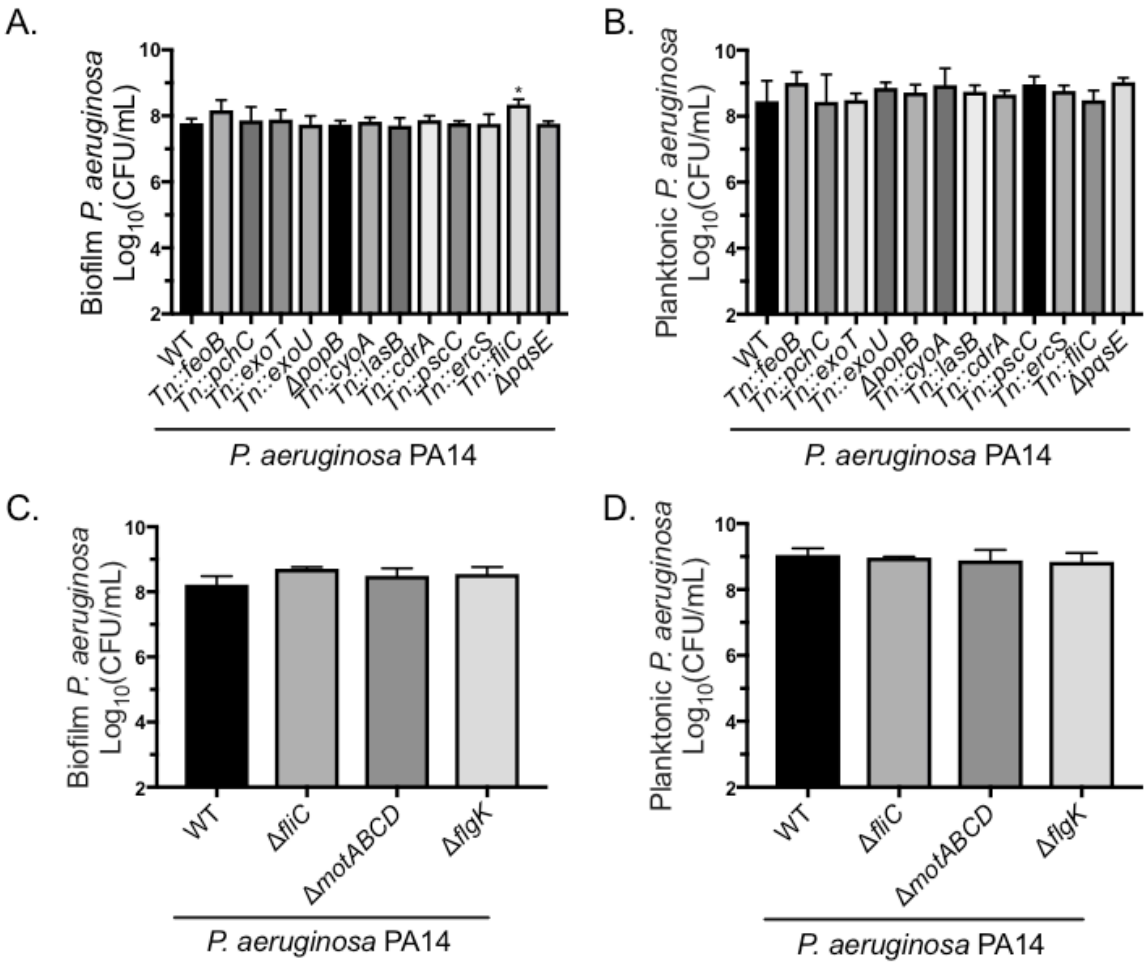
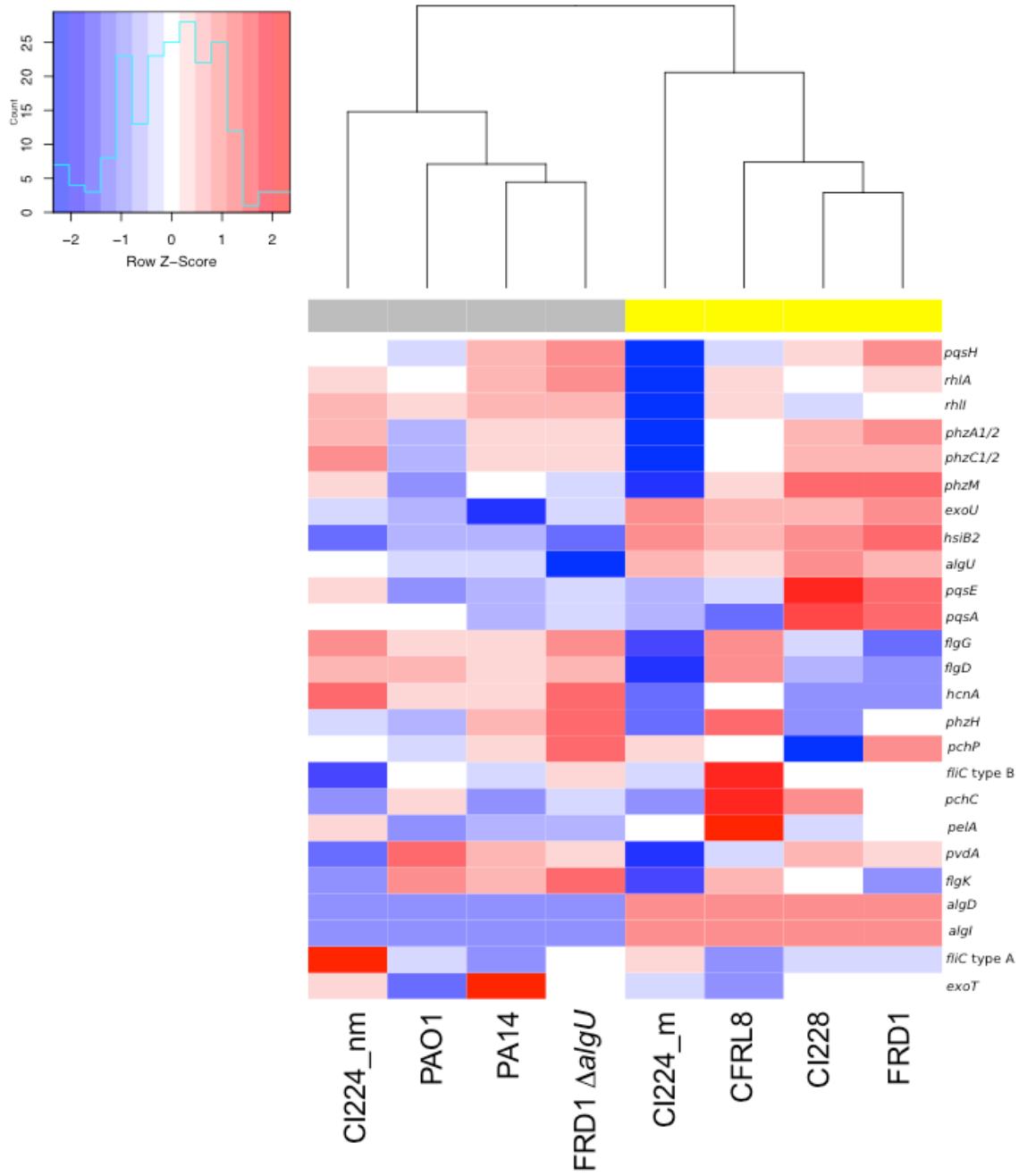


Figure S10. *P. aeruginosa* PA14 mutants do not have growth defects. Growth of *P. aeruginosa* PA14 mutants after 16 hours co-culture on plastic with *S. aureus* JE2 in MEM L-gln L-arg. **A)** Biofilm and **B)** planktonic fractions for initial mutant screen. **C)** Biofilm and **D)** planktonic fractions for flagellar mutant screen. Significance determined by one-way ANOVA with Dunnett's post-test comparison to wild-type *P. aeruginosa* PA14. *p<0.05.



118

119 **Figure S11. Gene expression of *P. aeruginosa* clinical isolates and nonmucoid revertants.**

120 Heatmap and dendrogram of a subset of genes in the Nanostring codeset from *P. aeruginosa*

121 clinical isolates cultured in lysogeny broth (LB) for 8-11 hours (Gifford AH, Willger SD, Dolben

122 EL, Moulton LA, Dorman DB, Bean H, Hill JE, Hampton TH, Ashare A, Hogan DA. 2016. Infect

123 Immun 84:2995–3006.). Log_2 transformation of Nanostring counts were normalized to positive

controls from a single biological replicate. Expression values are displayed as within-row Z-scores. Yellow indicates mucoid strains, and gray indicates non-mucoid strains. Image generated in R with Heatmap.2 function. This dataset contains expression data for the isogenic nonmucoid revertants of *P. aeruginosa* FRD1 and *P. aeruginosa* CI2224. Here we display a direct comparison of gene expression for these six clinical isolates and two laboratory *P. aeruginosa* strains of a subset of genes in the Nanostring codeset. Interestingly, isolates cluster primarily on mucoidy, and the mucoid isolates which were better able to kill *S. aureus* (*P. aeruginosa* FRD1 and *P. aeruginosa* CI228) clustered together within those groups. Mucoid *P. aeruginosa* FRD1 decreases some genes as expected given its mucoid phenotype, including *flgG, flgD, flgK*, and *fliC.* However, *rhlA, phz,* and *pvdA* expression remain high; all of these genes are known to contribute to the ability of *P. aeruginosa* to kill *S. aureus* in co-culture (24, 32, 33, 57–59). *P. aeruginosa* CI228 has a similar gene expression pattern to *P. aeruginosa* FRD1, but *pvdA* expression is even higher in this strain. *P. aeruginosa* CI2224, which is similar to *P. aeruginosa* CFBRPA32 in the ability to kill *S. aureus* (that is, both strains show limited ability to kill *S. aureus*) downregulates a large number of genes coding for known virulence factors, including those genes required for the production of rhamnolipids (*rhlA, rhlI*), phenazines (*phzA, phzC*), quorum sensing factors (*pqsH, pqsA*), and flagella (*flgD, flgG, fliC*). It is not immediately clear based on these data why this isolate is able to kill *S. aureus* at the late time points. *P. aeruginosa* CFRL8, which is the poorest of these four strains at killing *S. aureus*, highly expresses *pchC* and *fliC*, but has lower *pqsA* expression relative to the other strains. To determine whether we could broadly correlate the expression of any particular genes with changes in virulence towards *S. aureus*, we plotted the expression of each gene against *S. aureus* survival

146 in co-culture at both 16 and 24 hours for each *P. aeruginosa* strain where we have Nanostring
147 data and performed a linear regression (data not shown). We observed by this method that no
148 individual gene's expression is significantly correlated with *S. aureus* survival. Taken together,
149 these data suggest that across clinical mucoid strains, the relative production of a variety of
150 transcripts such as those encoding pyoverdine, rhamnolipids, flagella, and phenazines
151 determines whether isolates of *P. aeruginosa* effectively outcompete *S. aureus,* but the specific
152 mechanisms vary on a strain-by-strain basis.

SUPPLEMENTAL METHODS

Alginate preparation and quantification. *P. aeruginosa* PAO1 *mucA22* was cultured in 25mL TSB overnight shaking at 225 RPM and 37^{o}C. Samples were mixed with equal volume of 0.85% saline, and debris were removed by centrifugation (12,000 *g* for 30 min). The culture supernatant was mixed with an equal volume of 2% cetyl pyridinium chloride, and the precipitated alginate was collected by centrifugation (12,000 *g* for 10 min at room temperature). The pellet was dissolved in 1 M NaCl, precipitated with isopropanol, and dissolved in a reduced volume of DI water to concentrate the samples. The pellet was precipitated again with cold isopropanol and dissolved in DI water. The concentration of alginate in solution was determined by the carbazole method described by Knutson and Jeanes (1), in which a solution of alginate (50 µl, diluted with DI water to dynamic range of the assay) was mixed with 200 µl of borate-sulfuric acid reagent (10 mM H_3BO_3 in concentrated H_2SO_4) and 50 µl of carbazole reagent (0.1% in ethanol). The mixture was then incubated at 100°C for 10 min, and absorbance at 550 nm was determined by spectrophotometer. The alginate concentration was determined by extrapolation from a standard curve (0 to 1 g/ml) of alginic acid from seaweed.

Biofilm cultures. Strains were centrifuged and resuspended in 1X MEM L-gln and each strain was inoculated at a final concentration of .05 OD/mL in 100uL total volume in a plastic 96-well plate. After 1 hour at 37^{o}C with 5% CO_2, 90uL of media was removed to select for surface-attached cells and replaced with fresh 1X MEM L-gln L-arg (for co-cultures with *P. aeruginosa* mutants) or ½X MEM +/- 1% alginate (for alginate-containing experiments). At the indicated time points, the planktonic fraction was removed plated to quantify CFU/ml. The biofilm

fraction was collected by adding 50ul of PBS + 0.1% Triton to each well and shaking for 30 minutes prior to plating for CFU/ml.

Supernatant preparation. Liquid cultures or the planktonic fraction of biofilm cultures were collected and centrifuged (2 min, 13,000 rpm) to remove cell debris. Supernatant was removed and sterilized by .22 μm filtration.

Siderophore Quantification. 100uL of *P. aeruginosa* supernatants from each sample was aliquoted in triplicate in a 96-well flat-bottom plate. Relative fluorescence units were quantified at 400nm excitation and 460nm emission. Measurements were normalized to CFU/mL.

Drop Collapse Assay. *P. aeruginosa* supernatants were serially diluted ½X in PBS and were scored as the reciprocal of the highest dilution at which the drop collapsed. Scores were normalized to CFU/mL.

q-RT-PCR. Media was removed from cultures (centrifuge 2 min, 14,000 rpm at 4^{o}C) after 8 hours of culture. Cell pellets were immediately frozen in ethanol cooled with dry ice. RNA was isolated with TRIzol (Zymo Research) as described by the manufacturer. DNA was removed by three sequential treatments with Turbo-DNAse treatment (Invitrogen), and cDNA was prepared (RevertAid First Strand cDNA synthesis kit). Briefly, 1uL 100uM random hexamer primer was added to 600 ng RNA, and volume was adjusted to 12uL with nuclease-free water. RNA was incubated at 65^{o}C for 5min and placed on ice for 1min. 4uL 5X reaction buffer and 2uL 10mM dNTPs were then added to each sample. 2uL water was added to NRT controls, and 1uL RiboLock RNase Inhibitor and 1uL RevertAid H Minus Reverse Transcriptase were added to non-NRT samples. cDNA was synthesized by the following protocol: 25^{o}C 5 min, 50^{o}C 60 min, 70^{o}C 5 min.

Supernatant Extraction Procedure for LC-MS/MS. For detection of *P. aeruginosa* secondary metabolites, 100 μL of supernatant was extracted with 500 μL of 1:1 ethyl acetate:methanol (Fisher Scientific). After a 1 hr incubation at room temperature, the extraction was dried, resuspended in 100% methanol (Fisher Scientific) containing benzethonium, and centrifuged prior to analysis.

LC-MS/MS Analysis. Mass spectrometry was performed using a Bruker Daltonics Maxis II HD qTOF mass spectrometer equipped with a standard electrospray ionization source. The mass spectrometer was tuned by infusion of Tuning Mix ESI-TOF (Agilent Technologies) at a 3 μL/min flow rate. For accurate mass measurements, a wick saturated with Hexakis (1H,1H,2H-difluoroethoxy) phosphazene ions (Synquest Laboratories, m/z 622.1978) located within the source was used as a lock mass internal calibrant. Samples were introduced by an Agilent 1290 Ultra Performance Liquid Chromatography (UPLC) system using a 5 μL injection volume. Extracts were separated using a Phenomenex Kinetex 2.6μm C18 column (2.1 mm x 50 mm). A 9 minute linear water-acetonitrile gradient from 98:2 to 2:98 water:acetonitrile containing 0.1% formic acid was utilized. The flow rate was 0.5 mL/min. The mass spectrometer was operated in data dependent positive ion mode, automatically switching between full scan MS and MS/MS acquisitions. Full scan MS spectra (m/z 50 – 1500) were acquired in the TOF and the top five most intense ions in a particular scan were fragmented using collision induced dissociation using the stepping function in the collision cell.

LC-MS/MS Feature Finding and Molecular Annotation. LC-MS/MS data was converted from .d format to .mzXML format using Bruker Daltonics CompassXport after applying lock-mass calibration. Feature finding was performed on the .mzXML files using mzMine (version 2.39)

using the parameters listed in Supplemental Table 8 (2). As methanolic resuspension of the sample extracts was influenced by presence of alginate, sample features were normalized to the peak area of benzethonium (m/z 412.3215), a standard present in the methanol. Features were annotated by comparing the exact mass and MS/MS spectra with reported values. Annotation of the features was validated by comparing the experimental data (exact mass, MS/MS, and retention time) to data acquired under identical conditions on a mixture of commercial standards for PYO (Sigma), PCA (Ark Pharm), HHQ (Ark Pharm), HQNO (Cayman Chemical), PQS (Chemodex), and rhamnolipids (AGAE Technologies, 90% pure). Annotation of these features is level 1 (3). Annotation of pyochelin is level 2, as a commercial standard is not available. All data including the raw files, mzXML files, metadata table, and feature analysis are available via MassIVE (accession number: MSV000084224). Statistical analyses of relative quantitation between samples were conducted in GraphPad Prism (v7.04).

Nanostring. Total RNA was prepared as described for q-RT-PCR from *P. aeruginosa* PA14 cultured for 8 hours in flasks in TSB +/- 0.25% alginate. Total RNA was also prepared from both *P. aeruginosa* PA14 and *P. aeruginosa* PAO1 prepared as follows: Cultures were grown to mid-log phase in flasks in 25mL TSB and then 10mL from each flask moved to a new flask containing 10mL TSB or 10mL TSB + 2% alginate, for a final concentration of 1% alginate. The previously published PAV2 codeset(4) was incubated with total RNA as described by the manufacturer's protocol. Briefly, two probes complementary to each transcript of interest are incubated with the RNA sample to allow for hybridization.

Strains. All strains are listed in Supplemental Table 9.

Literature Cited.

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