

1 **Title**

2 Biofilm inhibitor tauroolithocholic acid alters colony morphology, specialized metabolism, and
3 virulence of *Pseudomonas aeruginosa*

4

5 **Short Title**

6 Biofilm inhibition alters colony morphology, specialized metabolism, and virulence of *P.*
7 *aeruginosa*

8

9 **Authors**

10 Alanna R. Condren¹, Lisa Juliane Kahl², George Kritikos³, Manuel Banzhaf³, Lars E. P. Dietrich²,
11 and Laura M. Sanchez^{1*}

12

13 **Affiliations**

14 1. Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at
15 Chicago, Chicago, IL 60612

16 2. Department of Biological Sciences, Columbia University, New York, NY 10027

17 3. Institute of Microbiology & Infection and School of Biosciences, University of
18 Birmingham, Edgbaston, Birmingham, UK.

19 **Author Contributions**

20 Wrote the paper: ARC, LK, GK, MB, LD, LMS

21 Conceptualized the research: ARC, LMS

22 Conducted the experiments: ARC, LK, MB, GK

23 Provided strains: LK, LD

24 Supervised the research: MB, LD, LMS

25 **Correspondence**

26 sanchelm@uic.edu

27 **Abstract**

28 Biofilm inhibition by exogenous molecules has been an attractive strategy for the development
29 of novel therapeutics. We investigated the biofilm inhibitor tauroolithocholic acid (TLCA) and its
30 effects on the specialized metabolism, virulence and biofilm formation of the clinically relevant
31 bacterium *Pseudomonas aeruginosa* strain PA14. Our study shows that TLCA alters specialized

32 metabolism, thereby affecting *P. aeruginosa* colony biofilm physiology. We observed an
33 upregulation of metabolites correlated to virulence such as the siderophore pyochelin. A wax
34 moth virulence assay confirmed that treatment with TLCA increases virulence of *P. aeruginosa*.
35 Based on our results, we believe that future endeavors to identify biofilm inhibitors must
36 consider how a putative lead is altering the specialized metabolism of a bacterial community to
37 prevent pathogens from entering a highly virulent state.

38 **Introduction**

39
40 The ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella*
41 *pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species)
42 have been deemed a severe threat, as the major cause of nosocomial infections, by evolving
43 mechanisms to “escape” the biocidal action of antibiotics [1,2]. The Center for Disease Control
44 and Prevention estimates that costs related to nosocomial infections, which have increased in
45 frequency in all countries regardless of income or industrial development, are between \$680 to
46 \$5,683 USD on average per patient [3].

47 *P. aeruginosa*, one of the ESKAPE microorganisms, is often referred as a “ubiquitous”
48 bacterium because of its ability to adapt to a wide variety of environments and hosts [4]. *P.*
49 *aeruginosa* can be found in the lung cavity of cystic fibrosis patients, colonizing large open
50 wounds of burn victims in hospitals, or invading the cornea of the human eye leading to
51 permanent vision loss [5–7]. *P. aeruginosa* infections are often complicated by the fact that it
52 readily forms a multicellular aggregate known as a biofilm - a state which contributes towards its
53 resistance to antibiotics [8]. The minimum bactericidal concentration for cells in a biofilm state is
54 estimated to be 10-1000 times higher than their planktonic counterparts complicating treatment
55 of biofilm infections [9]. *P. aeruginosa* tightly regulates biofilm formation using molecular
56 signaling networks and a well-characterized arsenal of specialized metabolites [10–14].
57 Specialized metabolites encompass the bioactive small molecules produced by a given

58 organism outside of those needed for primary metabolism [15]. Yet, there are currently no
59 biofilm inhibitors on the market in the US [16].

60 There are, however, several biofilm inhibitors used for *in vitro* analysis of biofilm
61 dispersal [17]. One example is tauro lithocholic acid (TLCA), a bile acid which efficiently inhibits
62 biofilm formation and induces dispersion of mature *P. aeruginosa* biofilms [18,19]. TLCA
63 demonstrated a low micromolar biofilm inhibitory concentration (BIC₅₀) against *P. aeruginosa* at
64 38.4 μ M compared to other lithocholic and bile acid derivatives [18]. Bile acids are a class of
65 acidic steroids that play a physiological role in digestion by solubilizing dietary fats [20]. Though
66 bile acids are classified as detergents, the steroid control cholesterol 3-sulfate, does not inhibit
67 biofilm formation nor do other bile acids tested at concentrations up to 1 mM. However, all
68 lithocholic bile acids have specific bioactivity against *P. aeruginosa* which varies based on the
69 conjugation to glycine or taurine [18]. Additionally, the reported BIC₅₀ of TLCA was in the low
70 micromolar range while the maximum critical micelle concentration for these bile acids ranges
71 from 8 to 12 mM [20]. Therefore, we hypothesized that when TLCA enters the microorganism's
72 chemical space, it induces *P. aeruginosa* to alter the production of specific specialized
73 metabolites leading to the reported biofilm inhibition/dispersion.

74 We observed altered morphology of colony biofilms and changes in specialized
75 metabolism when *P. aeruginosa* PA14 is exposed to TLCA. An increase in pyochelin production
76 when TLCA is present lead us to perform a wax moth virulence model which confirmed that
77 TLCA treated cells are significantly more virulent than non-treated cells [21]. The observed
78 increase in virulence lead to an investigation into how TLCA may be inducing an increase in
79 pyochelin production to increase virulence. We performed an iron starvation tolerance assay as
80 well as mutant studies via imaging mass spectrometry (IMS) which concluded that TLCA
81 treatment does not make *P. aeruginosa* cells sensitive to iron starvation and a knockout of the
82 *pqsH* (Δ *pqsH*) and *phzA1-G1 phzA2-G2* (Δ *phz*) gene clusters also leads to an increase in

83 pyochelin production as observed for the wild-type strain [22]. Taken together, while TLCA has
84 shown promising bioactivity towards biofilm inhibition, it appears that biofilm inhibition (or
85 dispersion) ultimately leads to the bacterium becoming more virulent in a host model, which is
86 supported by the observed alteration in specialized metabolism.

87 **Methods & Materials**

88 *TLCA stock solution preparation.* Taurolithocholic acid was purchased from Sigma Aldrich
89 ($\geq 98\%$). A 0.5 M stock solution was made by dissolving TLCA in methanol. This solution was
90 then sterile filtered with a 0.22 μm sterile filter and stored at -80°C .

91 *Colony Morphology Studies.* Liquid agar was prepared by autoclaving 1% tryptone (Teknova),
92 1% agar (Teknova) mixture. The liquid agar was cooled to 60°C and Congo Red (EMD; final
93 concentration 40 $\mu\text{g}/\text{mL}$) and Coomassie Blue (EMD; final concentration: 20 $\mu\text{g}/\text{mL}$) were
94 added. TLCA stock solution, dissolved in MeOH, was vortexed for 2-3 minutes until clear. TLCA
95 was added to liquid agar, different amounts were added to reach the final concentrations of 100
96 μM , 250 μM , and 1 mM TLCA. Sixty mL of liquid agar mixture was poured into square plates
97 (LDP, 10 cm x 10 cm x 1.5 cm) and left to solidify for ~18-24 hours. Precultures of *P.*
98 *aeruginosa* PA14 were grown in LB for 12-16 h at 37°C while shaking at 250 rpm. Subcultures
99 were prepared as 1:100 dilutions of precultures into fresh LB media and shaking for 2.5 hours at
100 37°C , at which point all subcultures had reached mid-exponential phase (optical density of ~0.4-
101 0.6 at 500 nm). Morphology plates were dried for 20-30 min and 10 μl spots of subculture were
102 spotted onto a morphology plate, with not more than four colonies per plate. Colony biofilms
103 were grown at 25°C and high humidity ($+90\%$) for up to 5 days. Images were taken every 24
104 hours with a Keyence VHX-1000 microscope.

105 *Imaging mass spectrometry experiments.* TLCA and the respective vehicle were imbedded into
106 liquid agar prior to plating. LB agar was autoclaved and cooled to 55°C before adding TLCA. A

107 calculated aliquot of sterilized TLCA stock solution was added to achieve the desired
108 concentration of 250 μ M. Plates were stored in 4 \square refrigerator until needed. *P. aeruginosa*
109 PA14 was plated on Bacto LB agar and grown overnight at 30 \square . A colony from the plate was
110 then used to inoculate a 5 mL LB liquid culture of *P. aeruginosa* and grown overnight at 30 \square
111 shaking at 225 rpm. Overnight liquid cultures (5 μ L) was spotted on thin agar plates (10 mL of
112 agar in 90 mm plate) embedded with either TLCA or the vehicle (MeOH) and incubated for 48
113 hours at 30 \square . Humidity/moisture was removed from environment during growth by placing a
114 small amount of DrieRite in the incubator. Following 48 hours of growth, colonies were excised
115 from agar plates using a razor blade and transferred to an MSP 96 target ground steel target
116 plate (Bruker Daltonics). An optical image of the colonies on the target plate was taken prior to
117 matrix application. A 53 μ m stainless steel sieve (Hogentogler Inc) was used to coat the steel
118 target plate and colonies with MALDI matrix. The MALDI matrix used for the analysis was a 1:1
119 mixture of recrystallized \square -cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic
120 acid (DHB) (Sigma). The plate was then placed in an oven at 37 \square for approximately four hours
121 or until the agar was fully desiccated. After four hours, excess matrix was removed from target
122 plate and sample with a stream of air. Another optical image was taken of the desiccated
123 colonies on the target plate. The target plate and desiccated colony were then introduced into
124 the MALDI-TOF mass spectrometer (Bruker Autoflex Speed) and analyzed with FlexControl
125 v.3.4 and FlexImaging v.4.1 software. The detector gain and laser power must be optimized for
126 each imaging run to achieve acceptable signal to noise, as an example, in figure 2, the detector
127 gain and laser power were set at 3.0x and 41% respectively. Range of detection was from 100
128 Da to 3,500 Da with ion suppression set at 50 Da in positive reflectron mode. The raster size
129 was set to 500 μ m and the laser was set to 200 shots per raster point at medium (3) laser size.

130 *Statistical analysis of Imaging Data.* SCiLS software (Bruker, version 2015b) was used to run
131 statistical analysis of raw imaging data. Settings for analysis was as follows: Normalization: root

132 mean square (RMS), error: ± 0.2 Da, and weak denoising for segmentation. Using “Find
133 Discriminative Values (ROC)” for unbiased analysis, PA14 control colony was selected as class
134 1 and TLCA treated colony as class 2 and SCiLS identified signals that were significantly
135 upregulated in class 1 (MeOH control colony) with a threshold of 0.75 corresponding to a
136 Pearson correlation of $p < 0.05$. In our report, these signals are referred to as “downregulated”
137 since they have a higher intensity in the control. The same analysis was performed with the
138 classes flipped to identify signals that were upregulated in TLCA condition. Statistical analysis
139 was completed after calculating mass error (**Table S2**) of three biological replicates (N=3)
140 (**Figures S3-S5**) and signals were only considered significant if altered regulation was observed
141 in two or more replicates.

142 *Iron Stress Tolerance Assay:* The protocol for the iron stress tolerance assay used was exactly
143 as described by Chua *et al.* [22], with the exceptions that strain PA14 rather than PAO1 and a 1
144 mM of TLCA and 2’2-bipyridine (DIPY) were used. The OD at 600 nm of each liquid culture was
145 measured every 15 minutes for 16 hours.

146 *Galleria mellonella treatment assays.* *Galleria mellonella* larvae (greater wax moth) were
147 purchased from TrueLarv UK Ltd. (Exeter, UK) and stored at 15°C prior to use. The assay was
148 performed as described previously [23], except for the following differences: PA14 wild-type
149 (WT) cells were grown exponentially for 2 hours, washed with PBS buffer and adjusted to an
150 $OD_{(600)}$ of 0.1. The PA14 culture was further diluted with PBS and plated to determine the CFU
151 of the inoculation suspension. Larvae were inoculated with 20 μ L of a 2.5×10^3 CFU/mL
152 solution and incubated at 37°C for 2 hours. After this incubation time, larvae were injected with
153 20 μ L of compound or PBS. The final concentration of TLCA and sodium nitroprusside (SNP)
154 was 250 μ M. For controls, uninfected larvae were administered the same compound dose to
155 monitor for toxicity. In parallel one group of larvae received two sterile 20 μ L PBS injections to
156 control for unintentional killing by the injections. In total 25 larvae were used per condition, split

157 in two independent experiments. In total 10 larvae were used for the PBS only condition.
158 Survival of a larvae was determined by the ability to respond to external stimuli (poking). Larvae
159 survival was estimated using the Kaplan-Meier estimator.[24] Survival estimates were
160 subsequently compared using the log-rank test [25]. Resulting p-values were corrected using
161 the Benjamini-Hochberg method [26] (**Figure S3**).

162 *pqs mutants IMS analysis.* Mutants proceeded through the same protocol as the WT (PA14)
163 bacterial colonies (**Table S10**). IMS sample prep and experimentation was completed at the
164 same settings as described in “Imaging mass spectrometry experiments” section. Statistical
165 analysis was completed after calculating mass error (**Table S4-S8**) of three biological replicates
166 (N=3) and signals were only considered significant if altered regulation was observed in two or
167 more replicates.

168 *PQS complementation.* The complementation strains *P. aeruginosa* PA14 $\Delta pqsA-C::pqsA-C$,
169 $\Delta pqsH::pqsH$, and $\Delta pqsL::pqsL$ were constructed as described in Jo *et al.* [57]. Primers LD1 &
170 LD4, LD168 & LD171, and LD9 & LD12 were used to amplify the *pqsA-C*, *pqsH* and *pqsL*
171 genes, respectively (**Table S10**). Correct constructs were confirmed by PCR and sequencing
172 and complemented into the original deletion site, following the same procedure as for deletion.

173 *Bacterial Extraction for PCA and PCH Fold-change.* Bacterial growth for quantification
174 proceeded exactly as described in “Imaging mass spectrometry experiments”. Each strains
175 (PA14 WT, $\Delta pqsA-C$, $\Delta pqsH$, Δphz , $\Delta pqsA-C::pqsA-C$, and $\Delta pqsH::pqsH$) was plated in
176 duplicate. After 48 hours, a spatula was used to separate the entire colony and surrounding
177 agar from petri dish. Samples were transferred to a glass vial. Each vial was filled with 2-3 mL of
178 DI water and homogenized using a spatula. Vials were either flash frozen or stored at -80°C prior
179 to lyophilization. Upon dryness, 3mL of MeOH was added to each vial and samples were

180 sonicated for 30 minutes to extract organics. Total weights of each sample dry biomass were
181 used to achieve a 10mg/mL solution of each extract. 10mg/mL solutions were centrifuged at
182 10,000 rpm for 2 minutes before transferring to HPLC vials for analysis. (Repeated for a total of
183 two biological replicates).

184 *PCA and PCH Fold-change analysis.* PCA standard was purchased from ChemScience (>97%).
185 An HPLC method previously described by *Adler et al.* was used on an Agilent 1260 Infinity to
186 isolate and identify PCA through retention time matching with standard (32.5 min at 250 nm;
187 **Figure S7**) with a Phenomenex C18 analytical column (150 x 4.6 mm; 5 μ m) and a flow rate of
188 0.5 mL/min [27]. Area under the curve (AUC) was used to quantify fold-change between PA14
189 WT and other strains/conditions. For PCH fold-change quantification, a gradient of 10%-85%
190 ACN (0.1% TFA) and H₂O (0.1% TFA) over 25 minutes with the two PCH stereoisomers
191 (pyochelin I and II) eluting at 16.0 & 16.2 minutes respectively at 210 nm. Area reported for fold
192 change analysis was achieved by combining the AUC of the peaks for pyochelin I and II. Fold-
193 change analysis was determined averaging the areas of EICs for two independent biological
194 cultures (N=2), with three technical replicates (n=3), and compared to PA14 WT with no TLCA
195 (control).

196 **Results & Discussion**

197 Phenazines constitute one of the most notable families of specialized metabolites
198 produced by *P. aeruginosa*. Phenazines are redox-active compounds that have been implicated
199 in balancing redox homeostasis in the hypoxic regions of biofilms, thereby regulating biofilm
200 morphology [28–32]. A phenazine-null mutant (Δphz) over produces extracellular matrix which
201 causes Δphz biofilms to have a characteristic hyper wrinkled morphology that allows for
202 increased access to oxygen due to a higher surface area-to-volume ratio [32–35]. To
203 characterize the effect of TLCA on *P. aeruginosa* biofilms, we grew PA14 colonies in a colony
204 morphology assay on solid agar supplemented with Congo Red, Coomassie Blue, and TLCA (0,

205 100, 250, and 1000 μM) for five days (**Figure S1**). Wild type PA14 initially forms a smooth
206 colony but initiates wrinkle structure development after 70 hours (**Figure 1**). In contrast, a Δphz
207 mutant showed enhanced production of biofilm matrix, leading to an earlier onset of wrinkling
208 between 24 and 48 hours. Addition of TLCA to the medium promoted a hyper-wrinkled
209 morphology after 94 hours. The colony continued to wrinkle over time, resembling the Δphz
210 mutant (118 hours; **Figure 1**). This morphology phenotype was also observed at lower
211 concentrations, but it was most dramatic at 1 mM TLCA, which is a physiologically relevant
212 concentration [36] (**Figure S1**). Based on this assay, it appears that TLCA induces matrix
213 production, possibly by downregulating phenazines.

214 The putative decrease in phenazine production as indicated by the Δphz -like colony
215 morphology in the presence of TLCA was queried alongside other changes in specialized
216 metabolism using imaging mass spectrometry (IMS). We employed matrix-assisted laser
217 desorption/ionization time-of-flight IMS (MALDI-TOF IMS), because it provides a robust,
218 untargeted analysis of the specialized metabolites produced by *P. aeruginosa in situ* [37,38]. *P.*
219 *aeruginosa* colonies were grown on thin agar (2-3 mm) embedded with the vehicle or 250 μM of
220 TLCA for 48 hours. The colonies and their respective agar controls were then prepared for IMS
221 analysis. Twelve known specialized metabolites were identified and visualized from *P.*
222 *aeruginosa* colonies (**Figure 2**). Orthogonal analytical techniques were used to confirm
223 identities of all twelve metabolites (**Figure S2**). Following a combination of manual and
224 statistical analyses using SCiLS lab, eight of the twelve specialized metabolites were observed
225 to have significant altered regulation in the presence of TLCA ($p < 0.05$; **Table S1**). The identified
226 specialized metabolites represent four broad classes of molecular families including the
227 phenazines, quinolones, rhamnolipids, and siderophores. We found that the phenazines
228 pyocyanin (PYO) and phenazine-1-carboxamide (PCN) are significantly downregulated when
229 TLCA is present, supporting our hypothesis that TLCA exposure causes hyper-wrinkled colonies

230 by downregulating phenazine production. We did not observe a statistically significant change in
231 phenazine-1-carboxylic acid (PCA) production in the presence of TLCA. Since methylated
232 phenazines like PYO, but not PCN and PCA were shown to inhibit colony wrinkling [39] our data
233 is consistent with TLCA affecting colony morphology by modulating phenazine production.

234 *P. aeruginosa* is reported to produce up to 50 quinolones which are specialized
235 metabolites that play specific roles in signaling and/or virulence. For example, both 2-heptyl-4-
236 quinolone (HHQ) and *Pseudomonas* quinolone signal (PQS) are specifically known for their
237 signaling properties but have also demonstrated antifungal bioactivity [40–46]. The N-oxide
238 quinolone, 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), has recently been shown to have
239 antimicrobial activity towards Gram-positive bacteria and contributes to *P. aeruginosa*'s
240 virulence [40–45]. When exposed to TLCA, we observed a significant upregulation of HHQ,
241 PQS, and 4-hydroxy-2-nonylquinoline (HNQ) within the colony. PQS is a well-characterized
242 signaling molecule in *P. aeruginosa* quorum sensing cascade [47]. Though IMS is a valuable
243 tool for identifying and visualizing the chemical composition of a sample, it cannot differentiate
244 between constitutional isomers like HQNO and PQS (m/z 260; **Figure 2**). In order to
245 differentiate these two metabolites, we used a combination of tandem mass spectrometry and
246 knockout mutants to demonstrate that PQS was represented by the signal that is retained in the
247 colony (center, upregulated) and HQNO corresponds to the signal that was excreted into the
248 agar (outer signal, downregulated) (**Figure S3**). HHQ and PQS are well established signaling
249 molecules that are required for phenazine production in *Pseudomonas aeruginosa*, However,
250 since we observed decreased phenazine levels despite an increase in quinolone production [48]
251 our results suggest that TLCA is attenuating phenazine production in a quinolone-independent
252 manner.

253 Based on the IMS analyses, treatment of TLCA seems to induce the production of
254 biosurfactants. These compounds, such as rhamnolipids, are amphipathic small molecules that

255 *P. aeruginosa* produces to increase surface adhesion and motility [49]. Rha-Rha-C10-C10 and
256 Rha-Rha-C12-C10/C10-C12 production were produced at elevated levels after treatment with
257 TLCA (**Figure 2**). However, while these results were not statistically significant, the trend is
258 worth noting because increases in rhamnolipid production would agree with the previously
259 reported bioactivity of TLCA. In colony biofilms, TLCA markedly increases matrix production and
260 leads to increased spreading and wrinkling. This wrinkly spreader phenotype is reminiscent of
261 the phenazine-null mutant as seen in **Figure 1**.

262 In addition to quinolones and phenazines, we detected changes in the levels of the
263 siderophore pyochelin. Siderophores are iron chelators that allow bacteria to acquire iron from
264 the surrounding environment [50]. Siderophores have been known to sequester iron from host
265 proteins and simultaneously act as signals for biofilm development [12]. The IMS results show a
266 significant upregulation of pyochelin in the presence of TLCA (**Figure 2**). Pyochelin is produced
267 by the biosynthetic pathway *pchA-I* which is activated by the presence of both iron and the ferric
268 uptake regulator (Fur) [12]. Additionally, previous work has shown that an increase in iron-
269 bound PQS can indirectly increase siderophore production by activating the siderophore gene
270 clusters *pvd* and *pch*. Our IMS experiments on wild-type PA14 (**Figure 2**) confirm this
271 relationship due to the increase of both PQS and pyochelin in the presence of TLCA.
272 Siderophores have antimicrobial activity and contribute to virulence [51,52]. Hence, the TLCA-
273 dependent upregulation of pyochelin raised the question of whether TLCA-exposed *P.*
274 *aeruginosa* become hypervirulent.

275 Many bacteria, like the ESKAPE pathogens, exhibit distinct lifestyle states depending on
276 surrounding environmental factors. Chua *et al.* recently described characteristics of the
277 dispersed cell state using sodium nitroprusside (SNP) as a biofilm-dispersing agent [22]. They
278 found that dispersed cells are characterized by altered physiology, increased virulence against
279 macrophages and *C. elegans*, and extreme sensitivity to iron starvation [22]. Having observed
280 an increase in pyochelin production when exposing *P. aeruginosa* to TLCA (**Figure 2**), we

281 sought to determine if TLCA treated cells were hypervirulent using a *Galleria mellonella* (greater
282 wax moth) larvae virulence assay.

283 *G. mellonella* larvae have been shown to be an ideal model for studying microbial
284 pathogenesis of several ESKAPE pathogens since they are easily infected, inexpensive, and
285 produce a similar immune response as vertebrates and mammals [53–57]. Larvae were injected
286 with a *P. aeruginosa* or vehicle load, and exposed to SNP, TLCA, or no dispersing agent two
287 hours post infection. In the infected TLCA-exposed larvae, a 50% decrease in survival rate was
288 observed 17 hours post infection compared to the infected larvae that did not receive a
289 dispersing agent (**Figure 3**). In the *G. mellonella* model, the potency of treatment is gauged by
290 the survival curve, therefore a shift in this curve will indicate increased or decreased virulence of
291 *P. aeruginosa* as compared to the 50% survival rate of non-treated infected larvae [58].

292 Addition of SNP or TLCA lead to a significant decrease in survival with 50% of the larvae
293 succumbing to infection 1-2 hours earlier than the PA14 control. Exposure to either of the biofilm
294 inhibitors, SNP or TLCA, did not affect the survival rate of uninfected larvae. TLCA-dispersed
295 cells are also hypervirulent and have an analogous trend to SNP-dispersed cells. This confirms
296 that TLCA-dispersed cells are significantly more virulent than planktonic or biofilm cells as
297 previously observed with SNP-dispersed cells (**Table S3**; $p < 0.05$).

298 Having confirmed that TLCA increases virulence in *P. aeruginosa*, we were interested in
299 testing whether *P. aeruginosa* TLCA-treated cells would also be sensitive to iron stress and
300 whether this may be related to the increase in pyochelin production. In SNP-dispersed cells,
301 pyoverdine the other main siderophore produced by *P. aeruginosa*, was markedly decreased
302 and these dispersed cells showed a sensitivity to iron starvation [22]. However, we were unable
303 to detect pyoverdine in either the wild type or TLCA-treated *P. aeruginosa* via mass
304 spectrometry, but were able to detect pyochelin which Chua *et al.* did not measure (Figure 2).
305 Therefore, we recapitulated the iron starvation assay performed by Chua *et al.* to determine if
306 TLCA exposure induces sensitivity to iron starvation as shown for SNP-dispersed cells.

307 Iron starvation was induced by exposing PA14 cells to the iron chelator 2'2-bipyridine
308 (DIPY; **Figure 4**). This assay was performed with TLCA-dispersed biofilm cells, which were
309 generated from pellicles (biofilms grown at the air-liquid interface) (**Figure 4A & B**), and
310 planktonic cells, where exposure to TLCA acted to inhibit biofilm formation (**Figure 4C & D**).
311 Proliferation of the cells was monitored by measuring optical density at 600 nm of each
312 condition over 16 hours. TLCA exposure alone does not alter proliferation compared to the
313 untreated control (**Figure 4B**). While exposure to DIPY, which induces iron starvation,
314 significantly decreases proliferation ($p < 0.05$) of biofilm-derived cells during the log phase of
315 growth (120-540 mins) (**Figure 4B**). However, there is no significant difference between biofilm
316 cells and TLCA-dispersed biofilm cells when exposed to iron starvation. We observe the same
317 trends when inhibiting biofilm formation of planktonic cells with TLCA (**Figure 4D**). Therefore,
318 unlike for SNP, TLCA-dispersed cells are not sensitive to iron starvation.

319 The discrepancy in iron starvation sensitivity might be attributed to their different effects
320 on pyoverdine production. Though we were unable to detect pyoverdine in our IMS analysis,
321 iron starvation might be prevented by the increased presence of pyochelin in colony biofilms
322 that were exposed to TLCA (Figure 2). Despite their differing effects on iron starvation
323 sensitivity, both SNP and TLCA have previously been shown to readily disperse biofilms, likely
324 through different mechanisms of action [18,22]. SNP, a nitric oxide donor, has been shown to
325 disperse mature biofilms by producing nitrosative stress inside of the biofilm structure [21].
326 Since TLCA cannot act as a nitric oxide donor and it does not cause cell lysis, TLCA must act
327 via an alternative mechanism to disperse biofilms [18]. This, along with results, supports our
328 hypothesis that TLCA exposure induces alterations to *P. aeruginosa*'s specialized metabolism.

329 PQS is a major quorum sensing signal and iron-bound PQS upregulates siderophore
330 production [59]. The increase in pyochelin production *in situ* and the enhanced virulence *in vivo*
331 lead us to investigate the contribution of the *pqs* gene cluster to the TLCA-mediated effect. We
332 tested four mutants with deletions in quinolone and phenazine biosynthetic genes: $\Delta pqsA-C$,

333 *ΔpqsH*, *ΔpqsL*, and *Δphz*. *ΔpqsA-C* does not produce any quinolones, while *ΔpqsH* cannot
334 produce PQS and *ΔpqsL* cannot produce N-oxide quinolones such as HQNO [48,60,61]. The
335 phenazine-null mutant, *Δphz*, is a double-deletion of the two redundant core phenazine
336 biosynthetic gene clusters *phzA1-G1* and *phzA2-G2* [48,60,61]. Using IMS, we investigated if
337 any of the four mutants would recapitulate the TLCA-dependent increase in pyochelin
338 production that we observed for the WT. No variation in pyochelin production for *ΔpqsA-C* and
339 *ΔpqsL* mutants were found, while *ΔpqsH* mutant produced significantly more pyochelin in
340 response to TLCA, mimicking the trend observed in the WT ($p < 0.05$; **Figure S6**). This result
341 puts into question our earlier assumption that PQS and pyochelin production are positively
342 correlated. We also observed a significant increase in pyochelin production in the *Δphz* mutant
343 (**Figure S6**). Previous work has shown that increasing PCA concentrations allows PA14
344 siderophore-null mutants to still develop biofilms and sequester iron [62]. This may be due to
345 phenazine's ability to mediate the reduction of Fe(III) to the bioavailable Fe(II) [62]. *pqsH* and
346 *phz* gene clusters are necessary for phenazine production, hence a decrease or lack of
347 phenazine production due to TLCA exposure might lead to the observed increase in pyochelin
348 production (**Figures 2 & S6**).

349 Since our IMS results were inconclusive regarding the effect of TLCA on PCA production
350 in colony biofilms (**Figure 2**), we subjected bacterial colony extracts from wild-type PA14,
351 *ΔpqsH*, and *Δphz* to an HPLC analysis to measure fold change across biological (N=3) and
352 technical replicates (n=3) (**Figure 5A**). When considering fold changes greater than +/- 1, PCA
353 production was not altered by TLCA exposure in the wild type and *ΔpqsH* strains (not produced
354 by *Δphz* mutant). However, an increase in pyochelin production was observed in the TLCA-
355 exposed *Δphz* mutant (+2.20) (**Figure 5B**). When the *ΔpqsH* mutant is complemented
356 (*ΔpqsH::pqsH*) there is increased production of pyochelin compared to wild-type whether the
357 colony is TLCA-treated or not (+2.43 and +1.81, respectively) (**Table S9**). There was no notable

358 difference in production of pyochelin in the complementation strain whether it was TLCA-treated
359 or not, which recapitulates the wild-type data. Though the fold change of PCA and pyochelin
360 was comparable in both wild-type PA14 and $\Delta pqsH$, the change in the production of these two
361 metabolites in the phenazine-null mutant, Δphz , suggests that a lack of phenazine production is
362 correlated to the observed increase in pyochelin production from cells exposed to TLCA.

363 **Conclusion**

364 In this study we demonstrate that the endogenous enteric biofilm inhibitor, TLCA, can alter
365 colony morphology, specialized metabolism, and virulence of *P. aeruginosa*. Our biological and
366 chemical studies confirm what is already known about TLCA's bioactivity and offers insight into
367 the chemical communication occurring between the cells upon treatment with a known biofilm
368 inhibitor. TLCA-dispersed biofilm cells are not sensitive to iron starvation, as previously reported
369 for the biofilm-dispersing agent SNP, however dispersion via either agent induces a significant
370 increase in virulence *in vivo*, implying that the mechanism of action of the two biofilm inhibitors
371 is different. Our IMS analysis of mutant strains revealed that when exposed to TLCA, a lack of
372 PQS ($\Delta pqsH$) or phenazine production (Δphz) lead to an increase in pyochelin production,
373 matching the results observed in our colony morphology and IMS WT experiments. Therefore,
374 PQS or iron-bound PQS is not responsible for activating the *pchA-I* gene cluster (**Figure 6A**).
375 However, IMS analysis highlighted that a significant increase in pyochelin production was
376 observed in the Δphz mutant confirming that the *phz* gene cluster plays a role in increased
377 pyochelin production when TLCA is present (**Figure 6B**). Though these results are not
378 conclusive regarding the mechanism of TLCA dispersal since biofilm inhibition and dispersal
379 cannot be tested using colony biofilms as a model system, it does support the hypothesis that
380 TLCA is acting as an environmental cue to induce *P. aeruginosa* to alter its metabolic signaling
381 throughout the bacterial community, thus disrupting the biofilm life cycle.

382 More work is needed to determine the mechanism of action of TLCA, but our work
383 shows that through TLCA exposure *P. aeruginosa* bacterial communities increase the

384 production of virulence factors such as pyochelin and consequently develop hypervirulence *in*
385 *vivo*. Others have documented similar phenomena by reporting an increase in virulence *in vivo*
386 as a consequence of exposure to a biofilm inhibitor in organisms such as, Group A
387 *Streptococcus*, *Vibrio cholerae*, and *Candida albicans* [63–67]. Taken into consideration
388 previous literature, our work promotes that future investigations must consider how biofilm
389 inhibitors alter the chemical environment within a bacterial biofilm and that biofilm inhibition as a
390 treatment strategy should be closely monitored for variations in specialized metabolism leading
391 to undesired side effects such as increased virulence.

392 **Acknowledgements**

393 We thank Drs. Atul Jain and Terry Moore for assisting with the recrystallization of the MALDI
394 matrices. Funding was provided by Grant K12HD055892 from the National Institute of Child Health
395 and Human Development (NICHD) and the National Institutes of Health Office of Research on
396 Women's Health (ORWH) (L.M.S.); University of Illinois at Chicago Startup Funds (L.M.S.);
397 American Society for Pharmacognosy research startup grant (L.M.S.); NIH/NIAID grant
398 R01AI103369 and an NSF CAREER award (L.E.P.D). ARC was supported in part by the National
399 Science Foundation Illinois Louis Stokes Alliance for Minority Participation Bridge to the
400 Doctorate Fellowship (grant number 1500368) and a UIC Abraham Lincoln retention fellowship.

401 **References**

- 402 1. Rice LB. Federal funding for the study of antimicrobial resistance in nosocomial pathogens:
403 no ESKAPE. *J Infect Dis.* 2008;197: 1079–1081.
- 404 2. Rice LB. Progress and challenges in implementing the research on ESKAPE pathogens.
405 *Infect Control Hosp Epidemiol.* 2010;31 Suppl 1: S7–10.
- 406 3. Kouchak F, Askarian M. Nosocomial infections: the definition criteria. *Iran J Med Sci.*
407 2012;37: 72–73.
- 408 4. Kramer A, Schwebke I, Kampf G. How long do nosocomial pathogens persist on inanimate
409 surfaces? A systematic review. *BMC Infect Dis.* 2006;6: 130.
- 410 5. Spencer WH. *Pseudomonas aeruginosa* infections of the eye. *Calif Med.* 1953;79: 438–
411 443.
- 412 6. Norbury W, Herndon DN, Tanksley J, Jeschke MG, Finnerty CC. Infection in Burns. *Surg*

- 413 Infect . 2016;17: 250–255.
- 414 7. Finch S, McDonnell MJ, Abo-Leyah H, Aliberti S, Chalmers JD. A Comprehensive Analysis
415 of the Impact of *Pseudomonas aeruginosa* Colonization on Prognosis in Adult
416 Bronchiectasis. *Ann Am Thorac Soc*. 2015;12: 1602–1611.
- 417 8. Hall CW, Hinz AJ, Gagnon LB-P, Zhang L, Nadeau J-P, Copeland S, et al. *Pseudomonas*
418 *aeruginosa* Biofilm Antibiotic Resistance Gene *ndvB* Expression Requires the RpoS
419 Stationary-Phase Sigma Factor. *Appl Environ Microbiol*. 2018;84. doi:10.1128/AEM.02762-
420 17
- 421 9. Wu H, Moser C, Wang H-Z, Høiby N, Song Z-J. Strategies for combating bacterial biofilm
422 infections. *Int J Oral Sci*. 2015;7: 1–7.
- 423 10. Mukherjee S, Moustafa DA, Stergioula V, Smith CD, Goldberg JB, Bassler BL. The PqsE
424 and RhlR proteins are an autoinducer synthase-receptor pair that control virulence and
425 biofilm development in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*. 2018;115:
426 E9411–E9418.
- 427 11. Heeb S, Fletcher MP, Chhabra SR, Diggle SP, Williams P, Cámara M. Quinolones: from
428 antibiotics to autoinducers. *FEMS Microbiol Rev*. 2011;35: 247–274.
- 429 12. Banin E, Vasil ML, Greenberg EP. Iron and *Pseudomonas aeruginosa* biofilm formation.
430 *Proc Natl Acad Sci U S A*. 2005;102: 11076–11081.
- 431 13. Cezairliyan B, Vinayavekhin N, Grenfell-Lee D, Yuen GJ, Saghatelian A, Ausubel FM.
432 Identification of *Pseudomonas aeruginosa* phenazines that kill *Caenorhabditis elegans*.
433 *PLoS Pathog*. 2013;9: e1003101.
- 434 14. Das T, Kutty SK, Tavallaie R, Ibugo AI, Panchompoo J, Sehar S, et al. Phenazine virulence
435 factor binding to extracellular DNA is important for *Pseudomonas aeruginosa* biofilm
436 formation. *Sci Rep*. 2015;5: 8398.
- 437 15. Davies J. Specialized microbial metabolites: functions and origins. *J Antibiot* . 2013;66:
438 361–364.
- 439 16. Stewart PS. Prospects for Anti-Biofilm Pharmaceuticals. *Pharmaceuticals* . 2015;8: 504–
440 511.
- 441 17. Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim HO. Agents that inhibit
442 bacterial biofilm formation. *Future Med Chem*. 2015;7: 647–671.
- 443 18. Sanchez LM, Cheng AT, Warner CJA, Townsley L, Peach KC, Navarro G, et al. Biofilm
444 Formation and Detachment in Gram-Negative Pathogens Is Modulated by Select Bile
445 Acids. *PLoS One*. 2016;11: e0149603.
- 446 19. Sanchez LM, Wong WR, Riener RM, Schulze CJ, Lington RG. Examining the fish
447 microbiome: vertebrate-derived bacteria as an environmental niche for the discovery of
448 unique marine natural products. *PLoS One*. 2012;7: e35398.
- 449 20. Monte MJ, Marin JJG, Antelo A, Vazquez-Tato J. Bile acids: chemistry, physiology, and
450 pathophysiology. *World J Gastroenterol*. 2009;15: 804–816.

- 451 21. Barraud N, Hassett DJ, Hwang S-H, Rice SA, Kjelleberg S, Webb JS. Involvement of nitric
452 oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J Bacteriol.* 2006;188: 7344–7353.
- 453 22. Chua SL, Liu Y, Yam JKH, Chen Y, Vejborg RM, Tan BGC, et al. Dispersed cells represent
454 a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. *Nat Commun.*
455 2014;5: 4462.
- 456 23. Desbois AP, Coote PJ. Wax moth larva (*Galleria mellonella*): an in vivo model for assessing
457 the efficacy of antistaphylococcal agents. *J Antimicrob Chemother.* 2011;66: 1785–1790.
- 458 24. Kaplan EL, Meier P. Nonparametric Estimation from Incomplete Observations. *J Am Stat*
459 *Assoc.* Taylor & Francis; 1958;53: 457–481.
- 460 25. Bland JM, Altman DG. The logrank test. *BMJ.* 2004;328: 1073.
- 461 26. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful
462 Approach to Multiple Testing. *J R Stat Soc Series B Stat Methodol.* 1995;57: 289–300.
- 463 27. Adler C, Corbalán NS, Seyedsayamdost MR, Pomares MF, de Cristóbal RE, Clardy J, et al.
464 Catechol siderophores protect bacteria from pyochelin toxicity. *PLoS One.* 2012;7:
465 e46754.
- 466 28. Recinos DA, Sekedat MD, Hernandez A, Cohen TS, Sakhtah H, Prince AS, et al.
467 Redundant phenazine operons in *Pseudomonas aeruginosa* exhibit environment-
468 dependent expression and differential roles in pathogenicity. *Proc Natl Acad Sci U S A.*
469 2012;109: 19420–19425.
- 470 29. Price-Whelan A, Dietrich LEP, Newman DK. Pyocyanin alters redox homeostasis and
471 carbon flux through central metabolic pathways in *Pseudomonas aeruginosa* PA14. *J*
472 *Bacteriol.* 2007;189: 6372–6381.
- 473 30. Price-Whelan A, Dietrich LEP, Newman DK. Rethinking “secondary” metabolism:
474 physiological roles for phenazine antibiotics. *Nat Chem Biol.* 2006;2: 71–78.
- 475 31. Mentel M, Ahuja EG, Mavrodi DV, Breinbauer R, Thomashow LS, Blankenfeldt W. Of two
476 make one: the biosynthesis of phenazines. *Chembiochem.* 2009;10: 2295–2304.
- 477 32. Okegbe C, Fields BL, Cole SJ, Beierschmitt C, Morgan CJ, Price-Whelan A, et al. Electron-
478 shuttling antibiotics structure bacterial communities by modulating cellular levels of c-di-
479 GMP. *Proc Natl Acad Sci U S A.* 2017;114: E5236–E5245.
- 480 33. Briard B, Bomme P, Lechner BE, Mislin GLA, Lair V, Prévost M-C, et al. *Pseudomonas*
481 *aeruginosa* manipulates redox and iron homeostasis of its microbiota partner *Aspergillus*
482 *fumigatus* via phenazines. *Sci Rep.* 2015;5: 8220.
- 483 34. Wang Y, Newman DK. Redox reactions of phenazine antibiotics with ferric (hydr)oxides and
484 molecular oxygen. *Environ Sci Technol.* 2008;42: 2380–2386.
- 485 35. Ramos I, Dietrich LEP, Price-Whelan A, Newman DK. Phenazines affect biofilm formation
486 by *Pseudomonas aeruginosa* in similar ways at various scales. *Res Microbiol.* 2010;161:
487 187–191.
- 488 36. Hofmann AF, Eckmann L. How bile acids confer gut mucosal protection against bacteria.

- 489 Proc Natl Acad Sci U S A. 2006;103: 4333–4334.
- 490 37. Cleary JL, Condren AR, Zink KE, Sanchez LM. Calling all hosts: Bacterial communication in
491 situ. *Chem*. 2017;2: 334–358.
- 492 38. Galey MM, Sanchez LM. Spatial Analyses of Specialized Metabolites: The Key to Studying
493 Function in Hosts. *mSystems*. 2018;3. doi:10.1128/mSystems.00148-17
- 494 39. Sakhtah H, Koyama L, Zhang Y, Morales DK, Fields BL, Price-Whelan A, et al. The
495 *Pseudomonas aeruginosa* efflux pump MexGHI-OpmD transports a natural phenazine that
496 controls gene expression and biofilm development. *Proc Natl Acad Sci U S A*. 2016;113:
497 E3538–47.
- 498 40. Filkins LM, Graber JA, Olson DG, Dolben EL, Lynd LR, Bhujra S, et al. Coculture of
499 *Staphylococcus aureus* with *Pseudomonas aeruginosa* Drives *S. aureus* towards
500 Fermentative Metabolism and Reduced Viability in a Cystic Fibrosis Model. *J Bacteriol*.
501 2015;197: 2252–2264.
- 502 41. Phelan VV, Moree WJ, Aguilar J, Cornett DS, Koumoutsi A, Noble SM, et al. Impact of a
503 transposon insertion in *phzF2* on the specialized metabolite production and interkingdom
504 interactions of *Pseudomonas aeruginosa*. *J Bacteriol*. 2014;196: 1683–1693.
- 505 42. Machan ZA, Taylor GW, Pitt TL, Cole PJ, Wilson R. 2-Heptyl-4-hydroxyquinoline N-oxide,
506 an antistaphylococcal agent produced by *Pseudomonas aeruginosa*. *J Antimicrob*
507 *Chemother*. 1992;30: 615–623.
- 508 43. Hoffman LR, Déziel E, D’Argenio DA, Lépine F, Emerson J, McNamara S, et al. Selection
509 for *Staphylococcus aureus* small-colony variants due to growth in the presence of
510 *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*. 2006;103: 19890–19895.
- 511 44. Biswas L, Biswas R, Schlag M, Bertram R, Götz F. Small-colony variant selection as a
512 survival strategy for *Staphylococcus aureus* in the presence of *Pseudomonas aeruginosa*.
513 *Appl Environ Microbiol*. 2009;75: 6910–6912.
- 514 45. Voggu L, Schlag S, Biswas R, Rosenstein R, Rausch C, Götz F. Microevolution of
515 cytochrome *bd* oxidase in *Staphylococci* and its implication in resistance to respiratory
516 toxins released by *Pseudomonas*. *J Bacteriol*. 2006;188: 8079–8086.
- 517 46. Kim K, Kim YU, Koh BH, Hwang SS, Kim S-H, Lépine F, et al. HHQ and PQS, two
518 *Pseudomonas aeruginosa* quorum-sensing molecules, down-regulate the innate immune
519 responses through the nuclear factor- κ B pathway. *Immunology*. 2010;129: 578–588.
- 520 47. Häussler S, Becker T. The *Pseudomonas* quinolone signal (PQS) balances life and death in
521 *Pseudomonas aeruginosa* populations. *PLoS Pathog*. 2008;4: e1000166.
- 522 48. Gallagher LA, McKnight SL, Kuznetsova MS, Pesci EC, Manoil C. Functions required for
523 extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J Bacteriol*. 2002;184:
524 6472–6480.
- 525 49. Donlan RM. Biofilms: microbial life on surfaces. *Emerg Infect Dis*. 2002;8: 881–890.
- 526 50. Cornelis P, Dingemans J. *Pseudomonas aeruginosa* adapts its iron uptake strategies in
527 function of the type of infections. *Front Cell Infect Microbiol*. 2013;3: 75.

- 528 51. Kang D, Kirienko DR, Webster P, Fisher AL, Kirienko NV. Pyoverdine, a siderophore from
529 *Pseudomonas aeruginosa*, translocates into *C. elegans*, removes iron, and activates a
530 distinct host response. *Virulence*. 2018;9: 804–817.
- 531 52. Cox CD. Effect of pyochelin on the virulence of *Pseudomonas aeruginosa*. *Infect Immun*.
532 1982;36: 17–23.
- 533 53. Browne N, Heelan M, Kavanagh K. An analysis of the structural and functional similarities
534 of insect hemocytes and mammalian phagocytes. *Virulence*. 2013;4: 597–603.
- 535 54. Jander G, Rahme LG, Ausubel FM. Positive correlation between virulence of *Pseudomonas*
536 *aeruginosa* mutants in mice and insects. *J Bacteriol*. 2000;182: 3843–3845.
- 537 55. Insua JL, Llobet E, Moranta D, Pérez-Gutiérrez C, Tomás A, Garmendia J, et al. Modeling
538 *Klebsiella pneumoniae* pathogenesis by infection of the wax moth *Galleria mellonella*. *Infect*
539 *Immun*. 2013;81: 3552–3565.
- 540 56. Jønsson R, Struve C, Jenssen H, Krogfelt KA. The wax moth *Galleria mellonella* as a novel
541 model system to study Enteroaggregative *Escherichia coli* pathogenesis. *Virulence*. 2017;8:
542 1894–1899.
- 543 57. Peleg AY, Monga D, Pillai S, Mylonakis E, Moellering RC Jr, Eliopoulos GM. Reduced
544 susceptibility to vancomycin influences pathogenicity in *Staphylococcus aureus* infection. *J*
545 *Infect Dis*. 2009;199: 532–536.
- 546 58. Koch G, Nadal-Jimenez P, Cool RH, Quax WJ. Assessing *Pseudomonas* virulence with
547 nonmammalian host: *Galleria mellonella*. *Methods Mol Biol*. 2014;1149: 681–688.
- 548 59. Diggle SP, Matthijs S, Wright VJ, Fletcher MP, Chhabra SR, Lamont IL, et al. The
549 *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional
550 roles in quorum sensing and iron entrapment. *Chem Biol*. 2007;14: 87–96.
- 551 60. Lépine F, Milot S, Déziel E, He J, Rahme LG. Electrospray/mass spectrometric
552 identification and analysis of 4-hydroxy-2-alkylquinolines (HAQs) produced by
553 *Pseudomonas aeruginosa*. *J Am Soc Mass Spectrom*. 2004;15: 862–869.
- 554 61. Bredenbruch F, Nimtze M, Wray V, Morr M, Müller R, Häussler S. Biosynthetic pathway of
555 *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines. *J Bacteriol*. 2005;187: 3630–3635.
- 556 62. Wang Y, Wilks JC, Danhorn T, Ramos I, Croal L, Newman DK. Phenazine-1-carboxylic
557 acid promotes bacterial biofilm development via ferrous iron acquisition. *J Bacteriol*.
558 2011;193: 3606–3617.
- 559 63. Dow JM, Crossman L, Findlay K, He Y-Q, Feng J-X, Tang J-L. Biofilm dispersal in
560 *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence
561 to plants. *Proc Natl Acad Sci U S A*. 2003;100: 10995–11000.
- 562 64. Rossmann FS, Racek T, Wobser D, Puchalka J, Rabener EM, Reiger M, et al. Phage-
563 mediated dispersal of biofilm and distribution of bacterial virulence genes is induced by
564 quorum sensing. *PLoS Pathog*. 2015;11: e1004653.
- 565 65. Uppuluri P, Chaturvedi AK, Srinivasan A, Banerjee M, Ramasubramaniam AK, Köhler JR,
566 et al. Dispersion as an important step in the *Candida albicans* biofilm developmental cycle.

567 PLoS Pathog. 2010;6: e1000828.

568 66. Hay AJ, Zhu J. Host intestinal signal-promoted biofilm dispersal induces *Vibrio cholerae*
569 colonization. *Infect Immun*. 2015;83: 317–323.

570 67. Connolly KL, Roberts AL, Holder RC, Reid SD. Dispersal of Group A streptococcal biofilms
571 by the cysteine protease SpeB leads to increased disease severity in a murine model.
572 *PLoS One*. 2011;6: e18984.

573 66. Connolly KL, Roberts AL, Holder RC, Reid SD. Dispersal of Group A streptococcal biofilms
574 by the cysteine protease SpeB leads to increased disease severity in a murine model.
575 *PLoS One*. 2011;6: e18984.

576

577

578 **Figure Legends**

579 **Figure 1: The effect of TLCA on colony biofilm formation in *P. aeruginosa* PA14.**

580 After five days of growth, colonies that were exposed to TLCA showed a similar hyper-wrinkled
581 biofilm structure to that of the untreated Δphz mutant.

582 **Figure 2: MALDI-TOF IMS analysis of *P. aeruginosa* after exposure to TLCA.**

583 Twelve characterized specialized metabolites produced by *P. aeruginosa* were identified and
584 visualized. Signal intensity is displayed as a heat map and shows that exposure to TLCA altered
585 regulation of highlighted specialized metabolites compared to control. * denotes the signal is
586 significantly up- or down-regulated in two biological replicates within the colony and □ denotes
587 significance in the surrounding agar ($p < 0.05$). Two asterisks (**) denote the signal was
588 significant over all three biological replicates.

589 **Figure 3: Virulence assay with *Galleria mellonella* (greater wax moth).**

590
591
592 Using *G. mellonella* as an infection model revealed that regardless of biofilm dispersing agent,
593 dispersed biofilm cells show a significant increase in virulence compared to controls ($p < 0.05$).
594

595 **Figure 4: PA14 Iron starvation assay.**

596
597 (A & B) shows that when biofilm dispersed *P. aeruginosa* cells are exposed to TLCA there are
598 no changes in proliferation. When exposed to iron starvation, we observe no significant
599 difference between biofilm and dispersed biofilm cells. Confirming TLCA dispersed cells are not
600 more sensitive to iron starvation than biofilm cells. (C & D) We observe the same relationship
601 with planktonic cells and planktonic cells inhibited from entering the biofilm state. However,
602 planktonic cells do show a sensitivity to iron starvation. * denotes $p < 0.05$.
603

604 **Figure 5: IMS and fold change analysis of wild type PA14, *pqsH*, and *phz* mutants.**

605
606 (A) IMS analysis revealed TLCA exposure induced no change in PCA production however (B)
607 there was an increase in pyochelin production in the Δphz mutant, resembling the trend
608 observed in wild-type experiments. □□ denotes the observed regulation was statistically
609 significant over three biological replicates in IMS experiments ($p < 0.05$).
610

611 **Figure 6: *pqs* metabolic pathway under TLCA exposure.**

612
613 (A) Represents the canonical pathway for pyochelin production with the role iron-bound PQS
614 plays in indirectly (dashed-line) activating pyochelin production. (B) However, TLCA treatment of
615 the WT, *pqsH*, and *phz* mutants leads to an increase in pyochelin production and subsequently
616 virulence supporting that these genes play a role in the observed increase in pyochelin
617 production from TLCA exposure.











