

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

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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 (BIC<sub>50</sub>) against *P. aeruginosa* at  
64 38.4  $\mu$ 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 BIC<sub>50</sub> 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* ( $\Delta$ *pqsH*) and *phzA1-G1 phzA2-G2* ( $\Delta$ *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  $\mu\text{m}$  sterile filter and stored at  $-80^\circ\text{C}$ .

91 *Colony Morphology Studies.* Liquid agar was prepared by autoclaving 1% tryptone (Teknova),  
92 1% agar (Teknova) mixture. The liquid agar was cooled to  $60^\circ\text{C}$  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  $\mu\text{M}$ , 250  $\mu\text{M}$ , 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  $37^\circ\text{C}$  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  $37^\circ\text{C}$ , 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  $\mu\text{l}$  spots of subculture were  
102 spotted onto a morphology plate, with not more than four colonies per plate. Colony biofilms  
103 were grown at  $25^\circ\text{C}$  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  $55^\circ\text{C}$  before adding TLCA. A

107 calculated aliquot of sterilized TLCA stock solution was added to achieve the desired  
108 concentration of 250  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ 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:  $\pm 0.2$  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  $\mu$ L of a  $2.5 \times 10^3$  CFU/mL  
152 solution and incubated at 37°C for 2 hours. After this incubation time, larvae were injected with  
153 20  $\mu$ L of compound or PBS. The final concentration of TLCA and sodium nitroprusside (SNP)  
154 was 250  $\mu$ 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  $\mu$ 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,  $\Delta pqsA-C$ ,  $\Delta pqsH$ ,  $\Delta phz$ ,  $\Delta pqsA-C::pqsA-C$ , 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  $\mu$ 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 H<sub>2</sub>O (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 ( $\Delta phz$ ) over produces extracellular matrix which  
201 causes  $\Delta phz$  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  $\mu\text{M}$ ) 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  $\Delta phz$   
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  $\Delta phz$   
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  $\Delta phz$ -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  $\mu\text{M}$  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,  $\Delta phz$ , 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 ( $\Delta phz$ ) 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  $\Delta phz$  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.

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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  $\Delta phz$  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  $\Delta phz$  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.













