UNIVERSITY^{OF} BIRMINGHAM University of Birmingham Research at Birmingham

Biofilm inhibitor taurolithocholic acid alters colony morphology, specialized metabolism, and virulence of pseudomonas aeruginosa

Condren, Alanna R; Kahl, Lisa Juliane; Boelter, Gabriela; Kritikos, George; Banzhaf, Manuel; Dietrich, Lars E P; Sanchez, Laura M

DOI: 10.1021/acsinfecdis.9b00424

License: None: All rights reserved

Document Version Peer reviewed version

Citation for published version (Harvard):

Condren, AR, Kahl, LJ, Boelter, G, Kritikos, G, Banzhaf, M, Dietrich, LEP & Sanchez, LM 2020, 'Biofilm inhibitor taurolithocholic acid alters colony morphology, specialized metabolism, and virulence of pseudomonas aeruginosa', ACS Infectious Diseases, vol. 6, no. 4, pp. 603-612. https://doi.org/10.1021/acsinfecdis.9b00424

Link to publication on Research at Birmingham portal

Publisher Rights Statement:

This document is the Accepted Manuscript version of a Published Work that appeared in final form in ACS Infectious Diseases, copyright © American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see: https://doi.org/10.1021/acsinfecdis.9b00424

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

•Users may freely distribute the URL that is used to identify this publication.

•Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.

•User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?) •Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

1 Title 2 Biofilm inhibitor taurolithocholic 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 Alanna R. Condren¹, Lisa Juliane Kahl², Gabriela Boelter³, George Kritikos³, Manuel Banzhaf³, 10 Lars E. P. Dietrich², and Laura M. Sanchez^{1*} 11 12 13 Affiliations 1. Department of Pharmaceutical Sciences, University of Illinois at Chicago, Chicago, IL 14 60612 15 16 2. Department of Biological Sciences, Columbia University, New York, NY 10027 17 3. Institute of Microbiology & Infection and School of Biosciences, University of Birmingham, 18 Edgbaston, Birmingham, UK. 19 **Author Contributions** 20 Wrote the paper: ARC, LK, GB, GK, MB, LD, LMS 21 Conceptualized the research: ARC, LMS 22 Conducted the experiments: ARC, LK, MB, GK, GB 23 Provided strains: LK, LD

- 24 Supervised the research: MB, LD, LMS
- 25 **Correspondence**
- 26 sanchelm@uic.edu
- 27

28 Abstract

29 Biofilm inhibition by exogenous molecules has been an attractive strategy for the development 30 of novel therapeutics. We investigated the biofilm inhibitor taurolithocholic acid (TLCA) and its 31 effects on the specialized metabolism, virulence and biofilm formation of the clinically relevant 32 bacterium Pseudomonas aeruginosa strain PA14. Our study shows that TLCA alters specialized 33 metabolism, thereby affecting *P. aeruginosa* colony biofilm physiology. We observed an 34 upregulation of metabolites correlated to virulence such as the siderophore pyochelin. A wax 35 moth virulence assay confirmed that treatment with TLCA increases virulence of *P. aeruginosa*. 36 Based on our results, we believe that future endeavors to identify biofilm inhibitors must 37 consider how a putative lead is altering the specialized metabolism of a bacterial community to 38 prevent pathogens from entering a highly virulent state.

Keywords: biofilms, taurolithocholic acid, bile acid, *Pseudomonas aeruginosa*, virulence
 40

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

48 P. aeruginosa, one of the ESKAPE microorganisms, is often referred to as a "ubiquitous" bacterium because of its ability to adapt to a wide variety of environments and hosts.⁴ P. 49 50 aeruginosa can be found in the lung of cystic fibrosis patients, colonizing large open wounds of burn victims in hospitals, or invading the cornea of the human eye leading to permanent vision 51 52 loss.⁵⁻⁷ P. aeruginosa infections are often complicated by the fact that it readily forms a multicellular aggregate known as a biofilm - a state which contributes towards its resistance to 53 antibiotics.⁸ The minimum bactericidal concentration for cells in a biofilm state is estimated to be 54 55 10-1000 times higher than their planktonic counterparts complicating treatment of biofilm infections.⁹ Yet, there are currently no biofilm inhibitors on the market in the US.¹⁰ 56

57 There are, however, several biofilm inhibitors used for in vitro analysis of biofilm dispersal.¹¹ One example is taurolithocholic acid (TLCA), a bile acid which efficiently inhibits 58 biofilm formation and induces dispersion of mature P. aeruginosa biofilms.^{12,13} TLCA 59 60 demonstrated a low micromolar biofilm inhibitory concentration (BIC₅₀) against P. aeruginosa at 38.4 µM compared to other lithocholic and bile acid derivatives.¹² Bile acids are a class of acidic 61 62 steroids that play a physiological role in digestion by solubilizing dietary fats.¹⁴ Though bile acids 63 are classified as detergents, the steroid control cholesterol 3-sulfate, does not inhibit biofilm 64 formation nor do other bile acids tested at concentrations up to 1 mM. However, all lithocholic bile acids have specific bioactivity against P. aeruginosa which varies based on the conjugation to 65 glycine or taurine.¹² Additionally, the reported BIC₅₀ of TLCA was in the low micromolar range 66

while the maximum critical micelle concentration for these bile acids ranges from 8 to 12 mM.¹⁴ *P. aeruginosa* tightly regulates biofilm formation using molecular signaling networks and a wellcharacterized arsenal of specialized metabolites.^{15–19} Therefore, we hypothesized that TLCA
treatment induces *P. aeruginosa* to alter the production of specific specialized metabolites leading
to the reported biofilm inhibition/dispersion.

72 We observed altered morphology of colony biofilms and changes in specialized 73 metabolism when P. aeruginosa PA14 is exposed to TLCA. An increase in pyochelin production 74 when TLCA is present lead us to perform a wax moth virulence model which confirmed that TLCA treated cells are significantly more virulent than non-treated cells.²⁰ The observed increase in 75 76 virulence led to an investigation into how TLCA may be inducing an increase in virulence factors 77 such as pyochelin. We performed an iron starvation tolerance assay as well as mutant studies via 78 imaging mass spectrometry (IMS) which concluded that TLCA treatment does not make P. 79 *aeruginosa* cells sensitive to iron starvation and a knockout of the pgsH ($\Delta pgsH$) and phzA1-G1

80 *phzA2-G2* (*\(\Deltaphz\)*) gene clusters also leads to an increase in pyochelin production as observed for

the wild-type strain.²¹ Taken together, while TLCA has shown promising bioactivity towards biofilm inhibition, it appears that biofilm inhibition (or dispersion) ultimately leads to the bacterium becoming more virulent in a host model, which is supported by the observed alteration in specialized metabolism.

85 Results & Discussion

86 Phenazines constitute one of the most notable families of specialized metabolites 87 produced by *P. aeruginosa*. Phenazines are redox-active compounds that have been implicated 88 in balancing redox homeostasis in the hypoxic regions of biofilms, thereby regulating biofilm 89 morphology.^{22–26} A phenazine-null mutant (Δphz) over produces extracellular matrix which causes

 Δphz mutant biofilms to have a characteristic hyper wrinkled morphology that allows for increased



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

104 days of growth, colonies that were exposed to TLCA showed a similar hyper-wrinkled biofilm

105 structure to that of the untreated $\triangle phz$ mutant. (N =3)

106 The putative decrease in phenazine production as indicated by the Δphz -like colony 107 morphology in the presence of TLCA was gueried alongside other changes in specialized 108 metabolism using imaging mass spectrometry (IMS). We employed matrix-assisted laser 109 desorption/ionization time-of-flight IMS (MALDI-TOF IMS), because it provides a robust, 110 untargeted analysis of the specialized metabolites produced by *P. aeruginosa in situ*.^{31,32} *P.* 111 aeruginosa colonies were grown on thin agar (2-3 mm) with the vehicle or 250 µM of TLCA for 48 112 h. The colonies and their respective agar controls were then prepared for IMS analysis. Twelve 113 known specialized metabolites were identified and visualized from *P. aeruginosa* colonies (Figure 114 2). Orthogonal analytical techniques were used to confirm identities of all twelve metabolites 115 (Figure S2). Following a combination of manual and statistical analyses using SCiLS lab, eight 116 of the twelve specialized metabolites were observed to have significant altered regulation in the 117 presence of TLCA (p<0.05; Table S1). The specialized metabolites represent four broad classes 118 of molecular families including the phenazines, guinolones, rhamnolipids, and siderophores. We 119 found that the phenazines pyocyanin (PYO) and phenazine-1-carboxamide (PCN) are 120 significantly downregulated when TLCA is present, supporting our hypothesis that TLCA exposure 121 causes hyper-wrinkled colonies by downregulating phenazine production. We did not observe a 122 statistically significant change in phenazine-1-carboxylic acid (PCA) production in the presence 123 of TLCA. Since N-methylated phenazines like PYO were shown to inhibit colony wrinkling our 124 data is consistent with TLCA affecting colony morphology by modulating phenazine production.³³



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

125

Twelve specialized metabolites produced by *P. aeruginosa* were identified and visualized. Signal intensity is displayed as a heat map and shows that exposure to TLCA altered regulation of highlighted specialized metabolites compared to control. * denotes the signal is significantly upor down-regulated in two biological replicates within the colony and ** denotes the signal was significant over all three biological replicates (p<0.05).

132 P. aeruginosa is reported to produce up to 50 quinolones which are specialized 133 metabolites that play specific roles in signaling and/or virulence. For example, both 2-heptyl-4-134 quinolone (HHQ) and Pseudomonas quinolone signal (PQS) are specifically known for their 135 signaling properties but have also demonstrated antifungal bioactivity.^{34–40} The N-oxide guinolone. 136 4-hydroxy-2-heptylguinoline-N-oxide (HQNO), has recently been shown to have antimicrobial 137 activity towards Gram-positive bacteria and contributes to *P. aeruginosa*'s virulence.^{34–39} When 138 P. aeruginosa was treated with TLCA, we observed a significant upregulation of HHQ, PQS, and 139 4-hydroxy-2-nonylquinoline (HNQ) in the P. aeruginosa colony. PQS is a well-characterized signaling molecule in *P. aeruginosa* guorum sensing cascade.⁴¹ Though IMS is a valuable tool for 140 141 identifying and visualizing the chemical composition of a sample, it cannot differentiate between 142 constitutional isomers like HQNO and PQS (m/z 260; Figure 2). In order to differentiate these two 143 metabolites, we used a combination of tandem mass spectrometry and knockout mutants to 144 demonstrate that PQS was represented by the signal that is retained in the colony (center, 145 upregulated) and HQNO corresponds to the signal that was excreted into the agar (outer signal, 146 downregulated). HHQ and PQS are well established signaling molecules that are required for phenazine production in *Pseudomonas aeruginosa*, However, since we observed decreased
 phenazine levels despite an increase in quinolone production⁴² our results suggest that TLCA is
 attenuating phenazine production in a quinolone-independent manner.

150 Based on the IMS analyses, treatment of TLCA seems to induce the production of 151 biosurfactants. Surfactants, such as rhamnolipids, are amphipathic small molecules that P. 152 aeruginosa produces to increase surface adhesion and motility.43 Rha-Rha-C10-C10 and Rha-153 Rha-C12-C10/C10-C12 production were produced at elevated levels after treatment with TLCA 154 (Figure 2). However, while the upregulation was not determined to be statistically significant 155 overall biological replicates, the trend observed is worth noting. In colony biofilms, TLCA markedly 156 increases matrix production and leads to increased spreading and wrinkling. This wrinkly spreader 157 phenotype is reminiscent of the phenazine-null mutant as seen in Figure 1.

158 In addition to guinolones and phenazines, we detected changes in the levels of the 159 siderophore pyochelin. Siderophores are iron chelators that allow bacteria to acquire iron from the surrounding environment.⁴⁴ Siderophores have been known to sequester iron from host 160 161 proteins and simultaneously act as signals for biofilm development.¹⁷ The IMS results show a 162 significant upregulation of pyochelin in the presence of TLCA (Figure 2). Pyochelin is produced by the biosynthetic pathway pchA-I which is activated by the presence of both iron and the ferric 163 164 uptake regulator (Fur).¹⁷ Additionally, previous work has shown that an increase in iron-bound 165 PQS can indirectly increase siderophore production by activating the siderophore gene clusters 166 pvd and pch; our IMS experiments with TLCA treatment of wild-type PA14 are in line with these results (Figure 2).⁴⁵ Siderophores have antimicrobial activity and contribute to virulence.^{46,47} 167 168 Hence, the TLCA-dependent upregulation of pyochelin raised the question of whether TLCA-169 exposed P. aeruginosa become hypervirulent.

Many bacteria, like the ESKAPE pathogens, exhibit distinct lifestyle states depending on
surrounding environmental factors. Chua *et al.* recently described characteristics of the dispersed

172 cell state using sodium nitroprusside (SNP) as a biofilm-dispersing agent.²¹ They found that 173 dispersed cells are characterized by altered physiology, increased virulence against 174 macrophages and *C. elegans*, and extreme sensitivity to iron starvation.²¹ Having observed an 175 increase in pyochelin production when exposing *P. aeruginosa* to TLCA (**Figure 2**), we sought to 176 determine if TLCA treated cells were hypervirulent using a *Galleria mellonella* (greater wax moth) 177 larvae virulence assay.

178 G. mellonella larvae have been shown to be an ideal model for studying microbial 179 pathogenesis of several ESKAPE pathogens since they are easily infected, inexpensive, and produce a similar immune response as vertebrates and mammals.^{48–52} In the *G. mellonella* model, 180 181 the potency of treatment is measured via a Kaplan-Meier curve, therefore a shift at the 50% 182 survival rate compared to the appropriate control will indicate increased or decreased virulence of *P. aeruginosa*.⁵³ To observe how TLCA and SNP treatment altered virulence of *P. aeruginosa* 183 184 infected larvae, TLCA or SNP were injected with *P. aeruginosa* and larvae survival was monitored 185 for 25 h. Treatment of uninfected larvae with either SNP or TLCA did not affect larvae survival 186 (Figure 3; Table S2). Within 15 h, 50% of larvae infected with P. aeruginosa succumbed to 187 infection and treatment with SNP or TLCA led to a significant decrease in survival with 50% of the 188 larvae succumbing to infection 2 h earlier than the infected control group (Figure 3; p<0.05). 189 These results indicate that TLCA treatment increases virulence, mimicking what was previously reported from SNP treatment.²² Given the high dose of TLCA administered we also sought to 190 191 control for an immunological response. Larvae were treated with taurocholic acid (TCA), a 192 derivative of TLCA which has been previously shown to have no biofilm inhibition against P. 193 aeruginosa.¹⁸ Treatment with TCA altered survival slightly however only TLCA treatment caused 194 a significant increase in virulence compared to control group (Figure S3A). This suggests that 195 treatment with bile acids induces an innate immunological response but the observed increase in 196 virulence from TLCA treatment is significant compared to TCA treatment or control group.

197 In continuing to explore how specialized metabolites impact virulence, we used the Δphz 198 mutant in the wax moth assay. We observed the Δphz mutant takes longer than WT to reduce 199 the population to 50%, therefore, the Δphz mutant is intrinsically less virulent (Figure S3B). 200 TLCA treatment of the Δphz mutant infected larvae induce a significant increase in virulence 201 compared to the control (Figure S3B; p<0.05), however, TCA treatment induced the same 202 increase in virulence. This similarity in response to both bile acids indicates that the observed 203 shift in the survival curve of the Δphz mutant is due to an immunological response from the bile 204 acids. Based on our in vivo assays, WT PA14 cells treated with TLCA are significantly more 205 virulent, and TLCA treatment is linked to specialized metabolism since the increase in virulence 206 was lost when the Δphz mutant was tested.



207

Figure 3: Virulence assay with Galleria mellonella (greater wax moth). Using *G. mellonella* as an infection model revealed that regardless of agent (250 μM), WT PA14 treated cells show a
 significant increase in virulence compared to controls (N=3).

211 In a previous report, SNP-dispersed *P. aeruginosa* PAO1 cells resulted in decreased

212 production of the siderophore pyoverdine and also showed a sensitivity to iron starvation when

competing with exogenous iron chelator DIPY.²² We were unable to detect pyoverdine in either 213 214 the wild type or TLCA-treated *P. aeruginosa* PA14 via mass spectrometry, but we were able to 215 detect pyochelin in our IMS experiments which Chua et al. did not measure (Figure 2). Having 216 confirmed that TLCA treatment increases virulence in *P. aeruginosa*, we next tested whether *P.* 217 aeruginosa TLCA-treated cells would also be sensitive to iron stress due to increased pyochelin 218 production. Therefore, we recapitulated the iron starvation assay performed by Chua et al. to 219 determine if TLCA exposure induced sensitivity to iron starvation as shown previously for SNP-220 dispersed cells.

221 Iron starvation was induced by exposing PA14 cells to the iron chelator 2'2-bipyridine 222 (DIPY; Figure 4). This assay was performed with TLCA or SNP treated cells, which were 223 generated using two different methods: 1) from pellicles (biofilms grown at the air-liquid interface 224 in standing liquid cultures; Figure 4A-C), and 2) planktonic cells (shaken liquid cultures; Figure 225 **4D-F**). Treatment of *P. aeruginosa* PA14 with either TLCA or SNP does not alter proliferation 226 compared to the DIPY control (Figure 4B). Regardless of agent used for treatment, P. aeruginosa 227 PA14 cells do not show an increase in sensitivity to iron starvation (Figure S4). Therefore, TLCA 228 and SNP treatment does not increase P. aeruginosa PA14's sensitivity to iron starvation. The 229 same trend occurred with *P. aeruginosa* PA14 Δphz (Figure 4C).

230 Using WT PA14 planktonic cells, we observe no change in growth regardless of condition 231 (Figure 4E). Interestingly, TLCA treatment of the Δphz mutant planktonic cells showed a 232 significant increase in sensitivity compared to SNP treated cells (Figure 4F). Based on our data, 233 dispersed biofilm cells and WT planktonic cells show no sensitivity to iron starvation. However, 234 planktonic cells of the *Aphz* mutant show significant sensitivity when biofilm formation is inhibited 235 via TLCA treatment. This result agrees with our hypothesis that TLCA is altering the specialized 236 metabolism of PA14 and phenazines may help alleviate sensitivity to iron starvation during TLCA 237 treatment since phenazines are capable of carrying oxidation and reduction of iron species to increase bioavailability.²⁷ With our previous morphology assay and IMS experiments, the iron
starvation assay confirms that TLCA treatment likely effects the phenazine production which leads
to altered morphology and metabolism in response to biofilm inhibition from an exogenous small
molecule treatment.

242 Of note, we do not observe the same sensitivity of SNP treatment with iron starvation as 243 reported by Chua et al. which is likely due to the difference in strains used for this assay (PA14 244 vs PAO1). The discrepancy in iron starvation sensitivity between SNP and TLCA might be 245 attributed to different pyoverdine production capabilities of these strains. Though we were unable 246 to detect pyoverdine in our IMS analysis, we would speculate that iron starvation might be 247 prevented by the increased production of pyochelin in colony biofilms that were treated with TLCA 248 (Figure 2). Despite their differing effects on iron starvation sensitivity, both SNP and TLCA have 249 previously been shown to readily disperse biofilms, likely through different mechanisms of action.^{18,22} SNP, a nitric oxide donor, has been shown to disperse mature biofilms by producing 250 251 nitrosative stress inside of the biofilm structure.²¹ Since TLCA cannot act as a nitric oxide donor 252 and it does not cause cell lysis, TLCA must act via an alternative mechanism to disperse biofilms.¹⁸ This supports our hypothesis that TLCA's bioactivity is achieved through inducing 253 254 changes in *P. aeruginosa*'s specialized metabolism.





Planktonic PA14 cells also show no sensitivity to iron starvation regardless of agent used. (F) Interestingly, treatment of the Δphz mutant with TLCA lead to a significant increase in sensitivity to iron starvation and the population was unable to fully recover over the 16 hour experiment. * denotes p<0.05.

268 PQS is a major guorum sensing signal and iron-bound PQS upregulates siderophore production.⁴⁵ The increase in pyochelin production *in situ* and the enhanced virulence *in vivo* lead 269 270 us to investigate the contribution of the pgs gene cluster to the TLCA-mediated effect. We tested 271 four mutants with deletions in quinolone and phenazine biosynthetic genes: ΔpqsA-C, ΔpqsH, 272 $\Delta pgsL$, and Δphz . $\Delta pgsA$ -C does not produce any quinolones, while $\Delta pgsH$ cannot produce PQS and $\Delta pqsL$ cannot produce N-oxide quinolones such as HQNO.^{42,48,49} The phenazine-null mutant, 273 274 Δphz , is a double-deletion of the two redundant core phenazine biosynthetic gene clusters phzA1-G1 and phzA2-G2.^{42,48,49} Using IMS, we investigated if any of the four mutants would recapitulate 275 276 the TLCA-dependent increase in pyochelin production that we observed for the WT. No variation 277 in pyochelin production for $\Delta pqsA$ -C and $\Delta pqsL$ mutants was found, while $\Delta pqsH$ mutant 278 produced significantly more pyochelin in response to TLCA, mimicking the trend observed in the 279 WT (p<0.05; Figure S5). This result puts into question our earlier assumption that PQS and 280 pyochelin production are positively correlated. We also observed a significant increase in 281 pyochelin production in the Δphz mutant (**Figure S5**). Previous work has shown that increasing 282 PCA concentrations allows PA14 siderophore-null mutants to still develop biofilms and sequester 283 iron.⁵⁰ This may be due to phenazine's ability to mediate the reduction of Fe(III) to the bioavailable 284 Fe(II).⁵⁰ pgsH and phz gene clusters are necessary for phenazine production, hence a decrease 285 or lack of phenazine production caused by TLCA treatment might be responsible for the observed 286 increase in pyochelin production (Figures 2 & S5).

Since our IMS results were inconclusive regarding the effect of TLCA on PCA production in colony biofilms (**Figure 2**), we subjected bacterial colony extracts from wild-type PA14, $\Delta pqsH$, and the Δphz mutant to an HPLC analysis to measure fold change across biological (N=3) and technical replicates (n=3) (**Figure 5A**). When considering fold changes greater than +/- 1, PCA 291 production was not altered by TLCA exposure in the wild type and $\Delta pqsH$ strains (not produced 292 by the Δphz mutant). When monitoring pyochelin production, only TLCA treatment of the Δphz 293 mutant showed a fold change increase in pyochelin production compared to control (+2.20) 294 (Figure 5B). When the $\Delta pqsH$ mutant is complemented ($\Delta pqsH::pqsH$) there is increased 295 production of pyochelin compared to the wild-type regardless of whether the colony is TLCA-296 treated or not (Table S10). There was no notable difference in production of pyochelin in the 297 complementation strain whether it was TLCA-treated or not, which recapitulates the wild-type 298 data. Though the fold change of PCA and pyochelin was comparable in both wild-type PA14 and 299 $\Delta pqsH$, the change in the production of these two metabolites in the phenazine-null mutant, Δphz , 300 suggests that a lack of phenazine production is correlated to the observed increase in pyochelin 301 production from cells exposed to TLCA.



303

Figure 5: IMS and fold change analysis of wild type PA14, $\Delta pqsH$, and Δphz mutants. (A) IMS and HPLC fold-change analysis revealed a 250 µM treatment of TLCA induced no change in PCA production however (B) there was an increase in pyochelin production in the Δphz mutant, resembling the trend observed in WT. \dagger [†] denotes the observed regulation was statistically

308 309 significant over three biological replicates in IMS experiments (p<0.05).

310 **Conclusion**

311 In this study we demonstrate that the mammalian endogenous enteric metabolite, TLCA, can alter 312 colony morphology, specialized metabolism, and virulence of P. aeruginosa. Our biological and 313 chemical studies confirm what is already known about TLCA's bioactivity and offers insight into 314 the chemical communication occurring between the cells upon treatment with a known biofilm 315 inhibitor. TLCA-treated cells are not sensitive to iron starvation, as previously reported for the 316 biofilm-dispersing agent SNP, however treatment with either agent induces a significant increase 317 in virulence in vivo, implying that the mechanism of action of the two biofilm inhibitors is different. 318 Our IMS analysis of mutant strains revealed that when exposed to TLCA, a lack of PQS ($\Delta pqsH$) 319 or phenazine production (Δphz) lead to an increase in pyochelin production, matching the results 320 observed in our colony morphology and IMS WT experiments. Therefore, PQS or iron-bound PQS 321 is not responsible for activating the pchA-I gene cluster (Figure 6A). However, IMS analysis 322 highlighted that a significant increase in pyochelin production was observed in the Δphz mutant 323 confirming that TLCA treatment not only affects phenazine production but could also play a role 324 in the observed increased pyochelin production when TLCA is present (Figure 6B). Though these 325 results are not conclusive regarding the mechanism of TLCA since biofilm inhibition and dispersal 326 cannot be tested using colony biofilms as a model system, it does support the hypothesis that 327 TLCA is acting as an environmental cue to induce *P. aeruginosa* to alter its metabolic signaling 328 throughout the bacterial community.



329

Figure 6: *pqs* **metabolic pathway under TLCA exposure.** (A) Represents the canonical pathway for pyochelin production with the role iron-bound PQS plays in indirectly (dashed-line) activating pyochelin production. (B) However, TLCA treatment of the WT or $\Delta pqsH$, and Δphz mutants leads to an increase in pyochelin production and subsequently virulence supporting that these genes play a role in the observed increase in pyochelin production from TLCA exposure.

336 More work is needed to determine the mechanism of action of TLCA, but our work shows 337 that through TLCA-treated P. aeruginosa bacterial communities increase the production of 338 virulence factors, such as pyochelin, in vitro and induced hypervirulence in vivo. Others have 339 documented similar phenomena by reporting an increase in virulence in vivo as a consequence 340 of treatment with a biofilm inhibitor in organisms such as, Group A Streptococcus, Vibrio cholerae, and Candida albicans.⁵¹⁻⁵⁵ Based on previous literature, our work supports that future 341 342 investigations should measure the chemical environment within a biofilm, and that biofilm 343 inhibition as a treatment strategy should be closely monitored for undesired side effects, such as 344 increased virulence.

345 **Experimental Section**

346 *TLCA stock solution preparation.* Taurolithocholic acid was purchased from Sigma Aldrich
 347 (≥98%). A 0.5 M stock solution was made by dissolving TLCA in methanol. This solution was then

348 sterile filtered with a 0.22 μ m sterile filter and stored at -80°C.

349 Colony Morphology Studies. Liquid agar was prepared by autoclaving 1% tryptone (Teknova), 1% 350 agar (Teknova) mixture. The liquid agar was cooled to 60°C and Congo Red (EMD; final 351 concentration 40 µg/mL) and Coomassie Blue (EMD; final concentration: 20 µg/mL) were added. 352 TLCA stock solution, dissolved in MeOH, was vortexed for 2-3 min until clear. TLCA was added 353 to liquid agar, different amounts were added to reach the final concentrations of 100 μ M, 250 μ M, 354 and 1 mM TLCA. 60 mL of liquid agar mixture was poured into square plates (LDP, 10 cm x 10 355 cm x 1.5 cm) and left to solidify for ~18-24 h. Precultures of P. aeruginosa PA14 were grown in 356 LB for 12-16 h at 37°C while shaking at 250 rpm. Subcultures were prepared as 1:100 dilutions 357 of precultures into fresh LB media and shaking for 2.5 h at 37°C, at which point all subcultures 358 had reached mid-exponential phase (optical density of ~0.4-0.6 at 500 nm). Morphology plates 359 were dried for 20-30 min and 10 µL spots of subculture were spotted onto a morphology plate. 360 with not more than four colonies per plate. Colony biofilms were grown at 25°C and high humidity 361 (+90%) for up to 5 d. Images were taken every 24 h with a Keyence VHX-1000 microscope.

362 Imaging mass spectrometry experiments. TLCA and the respective vehicle (MeOH) were added 363 into liquid agar prior to plating. LB agar was autoclaved and cooled to 55 °C before adding TLCA. 364 The final concentration of TLCA in each agar plate was 250 µM. Plates were stored in 4°C 365 refrigerator until needed. P. aeruginosa PA14 was plated on Bacto LB agar and grown overnight 366 at 30°C. A colony from the plate was then used to inoculate a 5 mL LB liquid culture of P. 367 aeruginosa and grown overnight at 30°C shaking at 225 rpm. Overnight liquid cultures (5 µL) was 368 spotted on thin agar plates (10 mL of agar in 90 mm plate) embedded with either TLCA or the 369 vehicle (MeOH) and incubated for 48 h at 30°C. Humidity/moisture was removed from environment 370 during growth by placing a small amount of DrieRite in the incubator. Following 48 h of growth, 371 colonies were excised from agar plates using a razor blade and transferred to an MSP 96 target 372 ground steel target plate (Bruker Daltonics). An optical image of the colonies on the target plate 373 was taken prior to matrix application. A 53 µm stainless steel sieve (Hogentogler Inc) was used 374 to coat the steel target plate and colonies with MALDI matrix. The MALDI matrix used for the 375 analysis was a 1:1 mixture of recrystallized α -cyano-4-hydroxycinnamic acid (CHCA) and 2,5-376 dihydroxybenzoic acid (DHB) (Sigma). The plate was then placed in an oven at 37°C for 377 approximately 4 h or until the agar was fully desiccated. After 4 h, excess matrix was removed 378 from target plate and sample with a stream of air. Another optical image was taken of the 379 desiccated colonies on the target plate. The target plate and desiccated colony were then 380 introduced into the MALDI-TOF mass spectrometer (Bruker Autoflex Speed) and analyzed with 381 FlexControl v.3.4 and FlexImaging v.4.1 software. The detector gain and laser power were set at 382 3.0× and 41% respectively. Range of detection was from 100 Da to 3,500 Da with ion suppression 383 set at 50 Da in positive reflectron mode. The raster size was set to 500 µm and the laser was set 384 to 200 shots per raster point at medium (3) laser size.

385 Statistical analysis of Imaging Data. SCiLS software (Bruker, version 2015b) was used to run

statistical analysis of raw imaging data. Settings for analysis was as follows: Normalization: root mean square (RMS), error: ∓0.2 Da, and weak denoising for segmentation. Using "Find Discriminative Values (ROC)" for unbiased analysis, PA14 control colony was selected as class 1 and TLCA treated colony as class 2 and SCiLS identified signals that were significantly upregulated in class 1 (MeOH control colony) with a threshold of 0.75 corresponding to a Pearson correlation of p<0.05. In our report, these signals are referred to as "downregulated" since they</p> have a higher intensity in the control. The same analysis was performed with the classes flipped

to identify signals that were upregulated in TLCA condition. Statistical analysis was completed after calculating mass error (**Table S3**) of three biological replicates (N=3) (**Figures S6S-8**) and signals were only considered significant if altered regulation was observed in two or more replicates.

397 Iron Stress Tolerance Assay: The protocol for the iron stress tolerance assay used was exactly 398 as described by Chua *et al.*,²¹ with the exceptions that strain PA14 rather than PAO1 and a 1 mM 399 of TLCA and 2'2-bipyridine (DIPY) were used. The OD at 600 nm of each liquid culture was 400 measured every 15 min for 16 h.

401 Galleria mellonella treatment assays. Galleria mellonella larvae (greater wax moth) were 402 purchased from TrueLarv UK Ltd. or Livefoods, UK and stored at 15°C prior to use. The assay was performed as described previously,⁵⁶ except for the following differences: PA14 wild-type 403 404 (WT) cells were grown exponentially for 2 h, washed with PBS buffer and adjusted to an OD₍₆₀₀₎ 405 of 0.1. The PA14 culture was further diluted with PBS and plated to determine the CFU of the inoculation suspension. Larvae were inoculated with 20 µL of a 2.5 × 10³ CFU/mL solution and 406 407 20 µL of compound or PBS. The final concentration of TLCA and sodium nitroprusside (SNP) was 408 250 µM. For controls, uninfected larvae were administered the same compound dose to monitor 409 for toxicity. In parallel one group of larvae received sterile PBS injections to control for 410 unintentional killing by the injections. Survival of a larvae was determined by the ability to respond 411 to external stimuli (poking). To increase robustness of the G. mellonella treatment assay several, 412 independent experiments were pooled to draw conclusions. All raw data can be found in supplementary data set 1. Larvae survival was estimated using the Kaplan-Meier estimator.⁵⁷ 413 Survival estimates were subsequently compared using the log-rank test.⁵⁸ Resulting p-values 414 were corrected using the Benjamini-Hochberg method (Tables S2 & S4).59 415

416 pqs mutants IMS analysis. Mutants proceeded through the same protocol as the WT (PA14) 417 bacterial colonies . IMS sample prep and experimentation was completed at the same settings as 418 described in "Imaging mass spectrometry experiments" section. Statistical analysis was 419 completed after calculating mass error (**Table S5-S9**) of three biological replicates (N=3) and 420 signals were only considered significant if altered regulation was observed in two or more 421 replicates.

422 PQS complementation. The complementation strains P. aeruginosa PA14 △pqsA-C::pqsA-C,

423 $\triangle pqsH::pqsH$, and $\triangle pqsL::pqsL$ were constructed as described in Jo *et al.* [57]. Primers LD1 &

LD4, LD168 & LD171, and LD9 & LD12 were used to amplify the *pqsA-C*, *pqsH* and *pqsL* genes, respectively (**Table S10**). Correct constructs were confirmed by PCR and sequencing and complemented into the original deletion site, following the same procedure as for deletion.

427 Bacterial Extraction for PCA and PCH Fold-change. Bacterial growth for quantification proceeded

428 exactly as described in "Imaging mass spectrometry experiments". Each strain (PA14 WT, $\Delta pqsA$ -

429 *C*, $\triangle pqsH$, $\triangle phz$, $\triangle pqsA$ -*C*::pqsA-*C*, and $\triangle pqsH$::pqsH) was plated in duplicate. After 48 h,

430 colonies and surrounding agar were excised, transferred, and extracted with MeOH.. Total
431 weights of each sample dry extract was used to achieve a 10 mg/mL solution of each extract for
432 HPLC analysis (N=3, n=3).

PCA and PCH Fold-change analysis. PCA standard was purchased from ChemScence (>97%).
An HPLC method previously described by Adler *et al.* was used on an Agilent 1260 Infinity to
isolate and identify PCA through retention time matching with standard (32.5 min at 250 nm; **Figure S9**) with a Phenomenex C18 analytical column (150 x 4.6 mm; 5 μm) and a flow rate of
0.5 mL/min.⁶⁰ Area under the curve (AUC) was used to quantify fold-change between PA14 WT
and other strains/conditions. For PCH fold-change quantification, a gradient of 10%-85% ACN

(0.1% TFA) and H₂0 (0.1% TFA) over 25 min with the two PCH stereoisomers (pyochelin I and
II) eluting at 16.0 & 16.2 min respectively at 210 nm (Figure S10). Area reported for fold change
analysis was achieved by combining the AUC of the peaks for pyochelin I and II. Fold-change
analysis was determined by averaging the AUC's from three independent biological cultures

443 (N=3), with three technical replicates (n=3), and compared to PA14 WT with no TLCA (control).

444 Supporting information

445 Materials and methods expanded information, strain information, molecular networking used to 446 verify the structures identified with imaging mass spectrometry, IMS replicates, fold change HPLC

447 analysis, mass error reports and statistical analysis reports, data availability.

448 Acknowledgements

- 449 We thank Drs. Atul Jain and Terry Moore for assisting with the recrystallization of the MALDI matrices,
- 450 Dr. Michael Federle for access to Biotek plate reader, and Dr M. Lisandra Zepeda Mendoza for
- 451 assistance with statistics. Funding was provided by Grant K12HD055892 from the National Institute
- 452 of Child Health and Human Development (NICHD) and the National Institutes of Health Office of
- 453 Research on Women's Health (ORWH) (L.M.S.); University of Illinois at Chicago Startup Funds
- 454 (L.M.S.); American Society for Pharmacognosy research startup grant (L.M.S.); NIH/NIAID grant
- 455 R01AI103369 and an NSF CAREER award (L.E.P.D). ARC was supported in part by the National
- 456 Science Foundation Illinois Louis Stokes Alliance for Minority Participation Bridge to the Doctorate
- 457 Fellowship (grant number 1500368) and a UIC Abraham Lincoln retention fellowship.

458 **References**

- 459 (1) Rice, L. B. Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE. *J. Infect. Dis.* 2008, *197* (8), 1079–1081.
 461 (2) Rice, L. B. Progress and Challenges in Implementing the Research on ESKAPE Pathogens. *Infect. Control Hosp. Epidemiol.* 2010, *31 Suppl 1*, S7–S10.
- 463 (3) Kouchak, F.; Askarian, M. Nosocomial Infections: The Definition Criteria. *Iran. J. Med.*464 Sci. 2012, 37 (2), 72–73.
- 465 (4) Kramer, A.; Schwebke, I.; Kampf, G. How Long Do Nosocomial Pathogens Persist on 466 Inanimate Surfaces? A Systematic Review. *BMC Infect. Dis.* **2006**, *6*, 130.
- 467 (5) Spencer, W. H. Pseudomonas Aeruginosa Infections of the Eye. *Calif. Med.* 1953, 79
 468 (6), 438–443.
- 469 (6) Norbury, W.; Herndon, D. N.; Tanksley, J.; Jeschke, M. G.; Finnerty, C. C. Infection in

- 470 Burns. Surg. Infect. 2016, 17 (2), 250-255.
- 471 (7) Finch, S.; McDonnell, M. J.; Abo-Leyah, H.; Aliberti, S.; Chalmers, J. D. A 472 Comprehensive Analysis of the Impact of Pseudomonas Aeruginosa Colonization on 473 Prognosis in Adult Bronchiectasis. Ann. Am. Thorac. Soc. 2015, 12 (11), 1602–1611.
- Hall, C. W.; Hinz, A. J.; Gagnon, L. B.-P.; Zhang, L.; Nadeau, J.-P.; Copeland, S.; Saha, 474 (8)
- 475 B.; Mah, T.-F. Pseudomonas Aeruginosa Biofilm Antibiotic Resistance Gene ndvB 476 Expression Requires the RpoS Stationary-Phase Sigma Factor. Appl. Environ. Microbiol. 477 2018, 84 (7). https://doi.org/10.1128/AEM.02762-17.
- 478 (9) Wu, H.; Moser, C.; Wang, H.-Z.; Høiby, N.; Song, Z.-J. Strategies for Combating 479
 - Bacterial Biofilm Infections. Int. J. Oral Sci. 2015, 7 (1), 1-7.
- 480 Stewart, P. S. Prospects for Anti-Biofilm Pharmaceuticals. Pharmaceuticals 2015, 8 (3), (10)481 504-511.
- 482 (11)Rabin, N.; Zheng, Y.; Opoku-Temeng, C.; Du, Y.; Bonsu, E.; Sintim, H. O. Agents That 483 Inhibit Bacterial Biofilm Formation. Future Med. Chem. 2015, 7 (5), 647-671.
- 484 Sanchez, L. M.; Cheng, A. T.; Warner, C. J. A.; Townsley, L.; Peach, K. C.; Navarro, G.; (12)485 Shikuma, N. J.; Bray, W. M.; Riener, R. M.; Yildiz, F. H.; et al. Biofilm Formation and 486 Detachment in Gram-Negative Pathogens Is Modulated by Select Bile Acids. PLoS One 487 **2016**, *11* (3), e0149603.
- 488 (13) Sanchez, L. M.; Wong, W. R.; Riener, R. M.; Schulze, C. J.; Linington, R. G. Examining 489 the Fish Microbiome: Vertebrate-Derived Bacteria as an Environmental Niche for the 490 Discovery of Unique Marine Natural Products. PLoS One 2012, 7 (5), e35398.
- 491 (14) Monte, M. J.; Marin, J. J. G.; Antelo, A.; Vazquez-Tato, J. Bile Acids: Chemistry, 492 Physiology, and Pathophysiology. World J. Gastroenterol. 2009, 15 (7), 804–816.
- 493 Mukherjee, S.; Moustafa, D. A.; Stergioula, V.; Smith, C. D.; Goldberg, J. B.; Bassler, B. (15) 494 L. The PasE and RhIR Proteins Are an Autoinducer Synthase-Receptor Pair That 495 Control Virulence and Biofilm Development in Pseudomonas Aeruginosa. Proc. Natl. 496 Acad. Sci. U. S. A. 2018, 115 (40), E9411–E9418.
- 497 (16) Heeb, S.; Fletcher, M. P.; Chhabra, S. R.; Diggle, S. P.; Williams, P.; Cámara, M. 498 Quinolones: From Antibiotics to Autoinducers. FEMS Microbiol. Rev. 2011, 35 (2), 247-499 274.
- 500 (17)Banin, E.; Vasil, M. L.; Greenberg, E. P. Iron and Pseudomonas Aeruginosa Biofilm 501 Formation. Proc. Natl. Acad. Sci. U. S. A. 2005, 102 (31), 11076–11081.
- 502 (18) Cezairliyan, B.; Vinayavekhin, N.; Grenfell-Lee, D.; Yuen, G. J.; Saghatelian, A.; 503 Ausubel, F. M. Identification of Pseudomonas Aeruginosa Phenazines That Kill 504 Caenorhabditis Elegans. PLoS Pathog. 2013, 9 (1), e1003101.
- 505 (19) Das, T.; Kutty, S. K.; Tavallaie, R.; Ibugo, A. I.; Panchompoo, J.; Sehar, S.; Aldous, L.; 506 Yeung, A. W. S.; Thomas, S. R.; Kumar, N.; et al. Phenazine Virulence Factor Binding to Extracellular DNA Is Important for Pseudomonas Aeruginosa Biofilm Formation. Sci. 507 508 Rep. 2015, 5, 8398.
- 509 (20) Barraud, N.; Hassett, D. J.; Hwang, S.-H.; Rice, S. A.; Kjelleberg, S.; Webb, J. S. 510 Involvement of Nitric Oxide in Biofilm Dispersal of Pseudomonas Aeruginosa. J. 511 Bacteriol. 2006, 188 (21), 7344-7353.
- 512 (21) Chua, S. L.; Liu, Y.; Yam, J. K. H.; Chen, Y.; Vejborg, R. M.; Tan, B. G. C.; Kjelleberg, S.; Tolker-Nielsen, T.; Givskov, M.; Yang, L. Dispersed Cells Represent a Distinct Stage 513 514 in the Transition from Bacterial Biofilm to Planktonic Lifestyles. Nat. Commun. 2014, 5, 515 4462.
- 516 (22) Recinos, D. A.; Sekedat, M. D.; Hernandez, A.; Cohen, T. S.; Sakhtah, H.; Prince, A. S.; 517 Price-Whelan, A.; Dietrich, L. E. P. Redundant Phenazine Operons in Pseudomonas 518 Aeruginosa Exhibit Environment-Dependent Expression and Differential Roles in 519 Pathogenicity. Proc. Natl. Acad. Sci. U. S. A. 2012, 109 (47), 19420–19425.
- 520 Price-Whelan, A.; Dietrich, L. E. P.; Newman, D. K. Pyocyanin Alters Redox (23)

521 Homeostasis and Carbon Flux through Central Metabolic Pathways in Pseudomonas 522 Aeruginosa PA14. J. Bacteriol. 2007, 189 (17), 6372-6381. Price-Whelan, A.; Dietrich, L. E. P.; Newman, D. K. Rethinking "Secondary" Metabolism: 523 (24) 524 Physiological Roles for Phenazine Antibiotics. Nat. Chem. Biol. 2006, 2 (2), 71-78. 525 (25) Mentel, M.; Ahuja, E. G.; Mavrodi, D. V.; Breinbauer, R.; Thomashow, L. S.; 526 Blankenfeldt, W. Of Two Make One: The Biosynthesis of Phenazines. Chembiochem 527 2009, 10 (14), 2295-2304. 528 Okegbe, C.; Fields, B. L.; Cole, S. J.; Beierschmitt, C.; Morgan, C. J.; Price-Whelan, A.; (26)Stewart, R. C.; Lee, V. T.; Dietrich, L. E. P. Electron-Shuttling Antibiotics Structure 529 Bacterial Communities by Modulating Cellular Levels of c-Di-GMP. Proc. Natl. Acad. Sci. 530 531 U. S. A. 2017, 114 (26), E5236-E5245. 532 (27) Briard, B.; Bomme, P.; Lechner, B. E.; Mislin, G. L. A.; Lair, V.; Prévost, M.-C.; Latgé, J.-533 P.; Haas, H.; Beauvais, A. Pseudomonas Aeruginosa Manipulates Redox and Iron 534 Homeostasis of Its Microbiota Partner Aspergillus Fumigatus via Phenazines. Sci. Rep. 535 2015. 5. 8220. 536 Wang, Y.; Newman, D. K. Redox Reactions of Phenazine Antibiotics with Ferric (28) 537 (hydr)oxides and Molecular Oxygen. Environ. Sci. Technol. 2008, 42 (7), 2380-2386. 538 (29) Ramos, I.; Dietrich, L. E. P.; Price-Whelan, A.; Newman, D. K. Phenazines Affect Biofilm 539 Formation by Pseudomonas Aeruginosa in Similar Ways at Various Scales. Res. 540 Microbiol. 2010, 161 (3), 187–191. Hofmann, A. F.; Eckmann, L. How Bile Acids Confer Gut Mucosal Protection against 541 (30)542 Bacteria. Proc. Natl. Acad. Sci. U. S. A. 2006, 103 (12), 4333-4334. 543 (31) Cleary, J. L.; Condren, A. R.; Zink, K. E.; Sanchez, L. M. Calling All Hosts: Bacterial 544 Communication in Situ. Chem 2017, 2 (3), 334-358. 545 Galey, M. M.; Sanchez, L. M. Spatial Analyses of Specialized Metabolites: The Key to (32) 546 Studying Function in Hosts. mSystems 2018, 3 (2). 547 https://doi.org/10.1128/mSystems.00148-17. 548 (33) Sakhtah, H.; Koyama, L.; Zhang, Y.; Morales, D. K.; Fields, B. L.; Price-Whelan, A.; Hogan, D. A.; Shepard, K.; Dietrich, L. E. P. The Pseudomonas Aeruginosa Efflux Pump 549 550 MexGHI-OpmD Transports a Natural Phenazine That Controls Gene Expression and 551 Biofilm Development. Proc. Natl. Acad. Sci. U. S. A. 2016, 113 (25), E3538-E3547. 552 (34) Filkins, L. M.; Graber, J. A.; Olson, D. G.; Dolben, E. L.; Lynd, L. R.; Bhuju, S.; O'Toole, 553 G. A. Coculture of Staphylococcus Aureus with Pseudomonas Aeruginosa Drives S. 554 Aureus towards Fermentative Metabolism and Reduced Viability in a Cystic Fibrosis 555 Model. J. Bacteriol. 2015, 197 (14), 2252-2264. 556 (35) Phelan, V. V.; Moree, W. J.; Aquilar, J.; Cornett, D. S.; Koumoutsi, A.; Noble, S. M.; Pogliano, K.; Guerrero, C. A.; Dorrestein, P. C. Impact of a Transposon Insertion in 557 558 phzF2 on the Specialized Metabolite Production and Interkingdom Interactions of 559 Pseudomonas Aeruginosa. J. Bacteriol. 2014, 196 (9), 1683–1693. 560 (36) Machan, Z. A.; Taylor, G. W.; Pitt, T. L.; Cole, P. J.; Wilson, R. 2-Heptyl-4-561 Hydroxyguinoline N-Oxide, an Antistaphylococcal Agent Produced by Pseudomonas 562 Aeruginosa. J. Antimicrob. Chemother. 1992, 30 (5), 615–623. 563 (37) Hoffman, L. R.; Déziel, E.; D'Argenio, D. A.; Lépine, F.; Emerson, J.; McNamara, S.; Gibson, R. L.; Ramsey, B. W.; Miller, S. I. Selection for Staphylococcus Aureus Small-564 565 Colony Variants due to Growth in the Presence of Pseudomonas Aeruginosa. Proc. Natl. 566 Acad. Sci. U. S. A. 2006, 103 (52), 19890-19895. Biswas, L.; Biswas, R.; Schlag, M.; Bertram, R.; Götz, F. Small-Colony Variant Selection 567 (38) 568 as a Survival Strategy for Staphylococcus Aureus in the Presence of Pseudomonas 569 Aeruginosa. Appl. Environ. Microbiol. 2009, 75 (21), 6910-6912. 570 (39) Voggu, L.; Schlag, S.; Biswas, R.; Rosenstein, R.; Rausch, C.; Götz, F. Microevolution of 571 Cytochrome Bd Oxidase in Staphylococci and Its Implication in Resistance to

572 Respiratory Toxins Released by Pseudomonas, J. Bacteriol. 2006, 188 (23), 8079-8086. 573 (40) Kim, K.; Kim, Y. U.; Koh, B. H.; Hwang, S. S.; Kim, S.-H.; Lépine, F.; Cho, Y.-H.; Lee, G. 574 R. HHQ and PQS, Two Pseudomonas Aeruginosa Quorum-Sensing Molecules, down-Regulate the Innate Immune Responses through the Nuclear Factor-kappaB Pathway. 575 576 Immunology 2010, 129 (4), 578–588. 577 (41) Häussler, S.; Becker, T. The Pseudomonas Quinolone Signal (PQS) Balances Life and 578 Death in Pseudomonas Aeruginosa Populations. PLoS Pathog. 2008, 4 (9), e1000166. 579 Gallagher, L. A.; McKnight, S. L.; Kuznetsova, M. S.; Pesci, E. C.; Manoil, C. Functions (42) 580 Required for Extracellular Quinolone Signaling by Pseudomonas Aeruginosa. J. 581 Bacteriol. 2002, 184 (23), 6472–6480. 582 (43) Donlan, R. M. Biofilms: Microbial Life on Surfaces. Emerg. Infect. Dis. 2002, 8 (9), 881-583 890. 584 (44) Cornelis, P.; Dingemans, J. Pseudomonas Aeruginosa Adapts Its Iron Uptake Strategies 585 in Function of the Type of Infections. Front. Cell. Infect. Microbiol. 2013. 3, 75. 586 Diggle, S. P.: Matthiis, S.: Wright, V. J.: Fletcher, M. P.: Chhabra, S. R.: Lamont, I. L.: (45) 587 Kong, X.; Hider, R. C.; Cornelis, P.; Cámara, M.; et al. The Pseudomonas Aeruginosa 4-588 Quinolone Signal Molecules HHQ and PQS Play Multifunctional Roles in Quorum 589 Sensing and Iron Entrapment. Chem. Biol. 2007, 14 (1), 87-96. 590 Kang, D.; Kirienko, D. R.; Webster, P.; Fisher, A. L.; Kirienko, N. V. Pyoverdine, a (46) 591 Siderophore from Pseudomonas Aeruginosa, Translocates into C. Elegans, Removes 592 Iron, and Activates a Distinct Host Response. Virulence 2018, 9 (1), 804-817. 593 (47) Cox, C. D. Effect of Pyochelin on the Virulence of Pseudomonas Aeruginosa. Infect. 594 Immun. 1982, 36 (1), 17–23. 595 Lépine, F.; Milot, S.; Déziel, E.; He, J.; Rahme, L. G. Electrospray/mass Spectrometric (48) Identification and Analysis of 4-Hydroxy-2-Alkylquinolines (HAQs) Produced by 596 597 Pseudomonas Aeruginosa. J. Am. Soc. Mass Spectrom. 2004, 15 (6), 862–869. 598 (49) Bredenbruch, F.; Nimtz, M.; Wray, V.; Morr, M.; Müller, R.; Häussler, S. Biosynthetic 599 Pathway of Pseudomonas Aeruginosa 4-Hydroxy-2-Alkylguinolines. J. Bacteriol. 2005, 187 (11), 3630-3635, 600 601 (50) Wang, Y.; Wilks, J. C.; Danhorn, T.; Ramos, I.; Croal, L.; Newman, D. K. Phenazine-1-602 Carboxylic Acid Promotes Bacterial Biofilm Development via Ferrous Iron Acquisition. J. 603 Bacteriol. 2011, 193 (14), 3606–3617. 604 (51) Dow, J. M.; Crossman, L.; Findlay, K.; He, Y.-Q.; Feng, J.-X.; Tang, J.-L. Biofilm 605 Dispersal in Xanthomonas Campestris Is Controlled by Cell-Cell Signaling and Is 606 Required for Full Virulence to Plants. Proc. Natl. Acad. Sci. U. S. A. 2003, 100 (19), 607 10995-11000. 608 (52) Rossmann, F. S.; Racek, T.; Wobser, D.; Puchalka, J.; Rabener, E. M.; Reiger, M.; 609 Hendrickx, A. P. A.; Diederich, A.-K.; Jung, K.; Klein, C.; et al. Phage-Mediated 610 Dispersal of Biofilm and Distribution of Bacterial Virulence Genes Is Induced by Quorum 611 Sensing. PLoS Pathog. 2015, 11 (2), e1004653. 612 Uppuluri, P.; Chaturvedi, A. K.; Srinivasan, A.; Banerjee, M.; Ramasubramaniam, A. K.; (53)Köhler, J. R.; Kadosh, D.; Lopez-Ribot, J. L. Dispersion as an Important Step in the 613 614 Candida Albicans Biofilm Developmental Cycle. PLoS Pathog. 2010, 6 (3), e1000828. 615 Hay, A. J.; Zhu, J. Host Intestinal Signal-Promoted Biofilm Dispersal Induces Vibrio (54) 616 Cholerae Colonization. Infect. Immun. 2015, 83 (1), 317-323. 617 Connolly, K. L.; Roberts, A. L.; Holder, R. C.; Reid, S. D. Dispersal of Group A (55)618 Streptococcal Biofilms by the Cysteine Protease SpeB Leads to Increased Disease 619 Severity in a Murine Model. PLoS One 2011, 6 (4), e18984. 620 Desbois, A. P.; Coote, P. J. Wax Moth Larva (Galleria Mellonella): An in Vivo Model for (56) 621 Assessing the Efficacy of Antistaphylococcal Agents. J. Antimicrob. Chemother. 2011, 622 66 (8), 1785–1790.

- 623 (57) Kaplan, E. L.; Meier, P. Nonparametric Estimation from Incomplete Observations. *J. Am.*624 Stat. Assoc. 1958, 53 (282), 457–481.
- 625 (58) Bland, J. M.; Altman, D. G. The Logrank Test. *BMJ* **2004**, 328 (7447), 1073.
- 626 (59) Benjamini, Y.; Hochberg, Y. Controlling the False Discovery Rate: A Practical and
 627 Powerful Approach to Multiple Testing. J. R. Stat. Soc. Series B Stat. Methodol. 1995,
 628 57 (1), 289–300.
- 629 (60) Adler, C.; Corbalán, N. S.; Seyedsayamdost, M. R.; Pomares, M. F.; de Cristóbal, R. E.;
 630 Clardy, J.; Kolter, R.; Vincent, P. A. Catecholate Siderophores Protect Bacteria from
 631 Pyochelin Toxicity. *PLoS One* **2012**, 7 (10), e46754.