Title: Auranofin inhibits virulence in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is widely attributed as the leading cause of hospital-acquired infections. Due to intrinsic antibiotic resistance mechanisms, as well as the ability to form biofilms, *P. aeruginosa* infections are notoriously difficult to treat. *P. aeruginosa* employs multiple virulence mechanisms to establish infections, and the global virulence regulator, Vfr, plays an important role in modulating these mechanisms. An attractive strategy to combat *P. aeruginosa* infections is the use of anti-virulence compounds. Here, we report the identification of auranofin, an FDA-approved drug, that attenuates virulence in *P. aeruginosa*. We present evidence that auranofin exerts its action by targeting Vfr, which in turn leads to inhibition of quorum sensing (QS), Type III secretion (T3SS) and Type IV pili (TFP). Consistent with potent inhibition of QS and TFP expression, we show that auranofin attenuates biofilm maturation, and when used in combination with colistin, displays strong synergy in eradicating *P. aeruginosa* biofilms both in flow chambers and in a murine implant model. Auranofin may have immediate applications as an anti-virulence drug against *P. aeruginosa* infections.

37 **Introduction**

38 *Pseudomonas aeruginosa* is a prevalent opportunistic pathogen known for its potent
39 virulence, environmental versatility and multi-drug resistance. Its role as the leading cause of
40 hospital-acquired infections (HAIs) among immuno-compromised patients is well
41 documented[1]. Notably, it is one of the main causes of pneumonia in cystic fibrosis (CF)
42 patients[2], and wound infections in surgical patients or burn victims[3]. Furthermore, *P.*
43 *aeruginosa* has been known to thrive in a variety of ecological niches[4], allowing colonization
44 on abiotic surfaces like catheters and other implanted medical devices[5,6]. Increasing incidences
45 of multi-drug resistant (MDR) *P. aeruginosa* infections[7,8] in the clinic pose a serious threat to
46 global health, underscoring the need to develop new strategies to combat these pathogens.

47

48 *P. aeruginosa* carries several intrinsic antibiotic resistance mechanisms involving the
49 collaboration of restricted uptake through the outer membrane[9], the use of energy-dependent
50 efflux pumps[10,11], and expression of β-lactamases'[12,13]. In addition, *P. aeruginosa*, like many
51 other bacteria, exhibits a form of social behavior known as biofilm formation[14], which further
52 increases its tolerance to antibiotics[15]. A biofilm is a community of bacteria encased in a
53 polymeric mesh consisting of extracellular DNA (eDNA), proteins and polysaccharides. In the
54 clinic, significantly higher patient mortality has been associated with the formation of biofilms
55 in the lungs of CF patients[16]. Specifically, the presence of biofilms increases the complexity of
56 treatment due to the function of the extracellular matrix as a physical barrier against, and
57 sponge for, antimicrobial drugs[17]. The small pore size of the biofilm mesh effectively reduces
58 penetration of lysozymes and macromolecular drugs, presumably decreasing the immediate
59 concentrations of these agents surrounding the cells. Additionally, the biofilm matrix is also
60 highly negatively charged due to the presence of eDNA and polysaccharides, which protect the
61 colony from positively-charged aminoglycosides[18]. Consequently, the inhibition of biofilm

62 formation, maturation and/or function remains a principal consideration in the search for new
63 treatment options against *P. aeruginosa*.
64
65 An attractive alternative strategy to control bacterial infections has been the use of small
66 molecules that block virulence. Such anti-virulence compounds may reduce the ability of
67 bacteria to infect the host[19,20], and/or attenuate bacterial defenses against host immune cells[21],
68 thereby facilitating clearance of the infection site[22]. The virulence of *P. aeruginosa* is multi-
69 faceted but largely controlled by the global virulence factor regulator, Vfr[23]. Vfr is a cyclic
70 adenosine monophosphate (cAMP)-dependent transcription factor that coordinates expression
71 of roughly 200 genes[24,25]. It regulates virulence directly by activating expression of secretion
72 systems[26] (e.g. Type III) and the pilus formation machinery[27] (Type IV). The Type III secretion
73 system (T3SS) in *P. aeruginosa* mediates cytotoxicity against host cells through the delivery
74 of molecular effectors that can disrupt host processes[28]. The Type IV Pilus (TFP) is important
75 for twitching motility[29], and often associated with the establishment and maturation of *P.*
76 *aeruginosa* biofilms. Vfr also controls quorum sensing (QS)[30], a cell-cell communication
77 network involving the *las*-, *rhl*- and *pqs*-encoded systems, which plays a role in the production
78 of several virulence factors including extracellular proteases, biosurfactants and toxins[21], and
79 is important for biofilm maturation[31] and immune evasion. While many small molecules that
80 inhibit QS and T3SS have been reported[32–34], no inhibitors of Vfr and TFP have been
81 discovered to date.
82
83 In this work, we identified auranofin, an FDA-approved, gold(I)-based anti-
84 inflammatory drug, as a potent inhibitor of virulence in *P. aeruginosa*. We show that auranofin
85 inhibits QS, T3SS and TFP formation at low micromolar concentrations. It does this in part by
86 targeting cysteines in Vfr and abolishing the ability of Vfr to bind DNA. Using flow cell biofilm

87 assays, we demonstrate that auranofin is able to attenuate biofilm maturation. Furthermore,
88 auranofin displays synergistic effects in eradicating *P. aeruginosa* biofilms *in vitro*, when used
89 in combination with colistin. We propose that auranofin can potentially be re-purposed as an
90 anti-virulent drug to treat *P. aeruginosa* infections.

91 **Results**

92 **Auranofin inhibits QS in *Pseudomonas aeruginosa***

93 We initially discovered auranofin in a screen to identify small molecules that inhibit
94 QS in *P. aeruginosa*. We screened ~6,000 compounds from various libraries using a *lasB-*
95 *gfp*(ASV) strain[35], where expression of a fusion construct between LasB N-terminus and an
96 unstable GFP variant (GFP(ASV)) is controlled by constitutively expressed LasR, the cognate
97 transcriptional regulator of the *las* pathway. Inhibitors of this pathway should reduce GFP
98 fluorescence in this assay. In addition, since the *las* pathway controls both the *rhl* and *pqs*
99 pathways, compounds identified in this screen could potentially shut down the entire QS
100 network. We found that auranofin exhibits sub-micromolar (IC_{50}) inhibition of LasB-GFP
101 expression with minimal effects on cell growth (Fig. 1a, S1). Auranofin also strongly inhibits
102 fluorescence in two other reporter strains, *rhlA-gfp*(ASV) and *pqsA-gfp*(ASV)[36], both under
103 the control of their respective transcriptional activators, RhlR and PqsR (Fig. 1b&c). To
104 validate our findings, we examined the effects of auranofin on the levels of RNA transcripts
105 for key QS-regulated genes, as well as on the production of various QS-controlled virulence
106 factors. Auranofin results in the down-regulation of *lasA* and *rhlA* transcripts (Fig. S2), and
107 significantly reduces the levels of elastase (LasB), rhamnolipids and pyocyanin (Fig. 2). Taken
108 together, these results establish that auranofin is a potent QS inhibitor.

109

110 **Auranofin inhibits expression of Type III secretion system (T3SS) and Type IV pili (TFP)**
111 **in *P. aeruginosa***

112 To gain insights into the mechanism(s)-of-action of auranofin, we compared the protein
113 profiles of *P. aeruginosa* in the presence and absence of auranofin. Consistent with it being a
114 QS inhibitor, treatment with auranofin caused reduction in the levels of proteins involved in or
115 regulated by QS (Table S1). Interestingly, we also observed down-regulation of proteins

116 involved in T3SS and TFP formation. We confirmed that T3SS was down-regulated using
117 immunoblot analyses (Fig. S4). We also showed that auranofin is able to inhibit TFP-dependent
118 motility, including swarming and twitching[37,38] (Fig. 3). Our observations here are, in part, due
119 to transcriptional down-regulation of these systems (Fig. S2). These results imply that
120 auranofin either has multiple targets, and/or it targets a transcriptional regulator upstream of
121 QS, T3SS and TFP.
122

123 **Auranofin inhibits Vfr *in vitro* but has other targets in cells**

124 QS, T3SS and TFP formation are positively regulated by Vfr[25]. We asked if auranofin
125 directly targets Vfr function. To test this, we examined the ability of auranofin to inhibit the
126 function of Vfr in binding to promoter sequences *in vitro* using electrophoretic mobility shift
127 assays (EMSA). We first showed that purified Vfr is able to bind DNA fragments containing
128 the promoter regions of representative QS (*lasR*) and virulence (*toxA*, *regA*) genes (Fig. S3),
129 as previously reported[39]. We further demonstrated that auranofin effectively prevents Vfr
130 binding to these promoters in a dose-dependent manner, indicating that it inhibits Vfr function
131 *in vitro* (Fig. 4a, c & d). Gold(I)-based compounds are thiophilic, and auranofin has previously
132 been shown to react covalently with cysteine residues within target proteins[40,41]. To ascertain
133 if its inhibition of Vfr proceeded via covalent modification of cysteines, we generated a
134 cysteine-less Vfr variant (VfrΔ5) in which all five cysteines were replaced by serine residues.
135 This variant is functional as judged by its ability to bind the *lasR, toxA and regA* promoters in
136 vitro (Fig. S3). Remarkably, auranofin is no longer able to prevent binding of VfrΔ5 to these
137 same promoters (Fig. 4b, c & d). These results establish that auranofin inhibits Vfr *in vitro* via
138 its cysteine residues.

139 To determine if Vfr is the main target of auranofin in cells, we complemented the Δ*vfr*
140 strain with wild-type Vfr and the VfrΔ5 variant, and examined the levels of elastase, T3SS

141 expression and twitching motility in the presence and absence of auranofin. Both wild-type Vfr
142 and the VfrΔ5 variant were able to complement the Δ*vfr* strain in these assays (Fig. S4). We
143 showed that auranofin inhibits all three phenotypes in the wild-type Vfr complemented strain,
144 as expected. Surprisingly, auranofin was still able to inhibit these phenotypes in the VfrΔ5
145 complemented strain, unlike what we have observed *in vitro*. While we have demonstrated that
146 auranofin does inhibit Vfr via its cysteine residues *in vitro*, our data suggests auranofin may
147 have other targets, in addition to Vfr.
148
149 **Auranofin impedes the development of *P. aeruginosa* biofilms**
150 Both TFP and QS are required during biofilm development[42,43]. TFP are important for
151 initial surface attachment of cells, as well as for the organization of cells into the typical
152 "mushroom" architecture of mature *P. aeruginosa* biofilms. QS is also critical for biofilm
153 maturation, although the mechanisms here are less understood. Since auranofin inhibits both
154 TFP formation and QS networks, we expect it to also have an impact on the development of *P.*
155 *aeruginosa* biofilms. To test this, we monitored biofilm formation and maturation in the
156 presence and absence of auranofin over a period of 72 h in continuous flow chambers. The
157 formation of fully matured, canonical mushroom-shaped biofilm structures for wild-type *P.*
158 *aeruginosa* was readily observed within 72 h (Fig. 5). Interestingly, while auranofin did not
159 have obvious effects on the initial surface attachment of cells, the formation of mushroom-
160 shaped structures was less pronounced; biofilms formed in the presence of auranofin appeared
161 more flat, similar to biofilms of QS or TFP-null strains[44]. Our data clearly indicate that despite
162 permitting biofilm attachment and growth, auranofin attenuates the progression of biofilm
163 maturation, possibly by inhibiting QS and TFP.
164
165

166 **Auranofin exhibits synergistic effects with colistin in killing *P. aeruginosa* biofilms**

167 Since auranofin affects the maturation of the biofilm, we asked whether it may display
168 synergy with colistin, a last resort drug commonly used to treat *P. aeruginosa* infections. We
169 evaluated the combined effects of auranofin and colistin by monitoring the ratio of live versus
170 dead cells within *P. aeruginosa* flow cell biofilms after treatment. As previously reported,
171 colistin treatment alone results in a sharp decline in cell viability across a 2-day old biofilm,
172 with the exception of several surviving colistin-tolerant subpopulations[44] (Fig. 6a).
173 Remarkably, adding auranofin prior to biofilm formation allows subsequent colistin additions
174 to completely eradicate the *P. aeruginosa* biofilm. In fact, co-treatment of a 2-day biofilm with
175 auranofin and colistin also results in complete killing. These results demonstrate synergy
176 between auranofin and colistin in a flow cell biofilm.

177 To test whether auranofin and colistin can work together *in vivo*, we surgically inserted silicone
178 implants coated with 1-day old *P. aeruginosa* biofilms into the peritoneal cavity of mice, and
179 subsequently treated them locally with colistin, auranofin, or a combination of both drugs.
180 Remarkably, combination therapy of auranofin and colistin resulted in significantly lower
181 bacterial titer counts from the implant than either auranofin or colistin alone (Fig. 6b). This
182 confirms the synergy between auranofin and colistin in a murine model.

183 **Discussion**

184 Auranofin is approved by the FDA for the treatment of rheumatoid arthritis. It has been
185 shown to reduce inflammation of joints in rheumatic patients[45]; however, the precise
186 mechanism of how this occurs is not clear. Auranofin also exhibits anti-cancer and anti-
187 parasitic properties[40,46,47]. Furthermore, auranofin is able to kill a broad spectrum of Gram-
188 positive bacteria, and also mycobacteria[41]. All of these activities may be attributed to
189 auranofin's ability to disrupt the thiol-redox balance in cells, in part by targeting the
190 thioredoxin reductases in each of these systems[48]. Interestingly, auranofin is largely ineffective
191 against most Gram-negative bacteria, presumably because of lack of penetration of the
192 bacterial outer membrane[49]. Here, we have demonstrated that auranofin is additionally able to
193 inhibit virulence in *P. aeruginosa*. Despite not being able to accumulate at high concentrations
194 in cells, auranofin causes downregulation of QS, T3SS and TFP formation. One possible target
195 is Vfr, which controls all of these phenotypes. Consistently, we have shown that auranofin
196 inhibits the ability of Vfr to bind to target promoter sequences in vitro. The fact that a cysteine-
197 less Vfr variant is not inhibited in vitro indicates that auranofin also targets cysteines within
198 this protein. Interestingly, *P. aeruginosa* does not gain resistance against auranofin even when
199 expressing cysteine-less Vfr, suggesting that it has multiple targets in cells. This is probable,
200 given that auranofin is a general thiol-targeting reagent[40]. Understanding the mechanism of
201 action of auranofin would require identifying these unknown targets.

202 Current clinical treatment options of *P. aeruginosa* infections involve the usage of
203 carbapenems, aminoglycosides and ciprofloxacin, to name a few[16]. These drugs are often used
204 in combination or singularly in treatment. Unfortunately, many strains of *P. aeruginosa* have
205 developed resistance to such compounds and drastically reduce the pool of available treatment
206 options for clinicians[7]. Colistin remains one of the few drugs effective against most strains of
207 *P. aeruginosa*. Its usage, however, remains limited and is confined to last resort situations due

208 to its toxicity[50]. We have shown that auranofin and colistin combine synergistically to
209 completely eradicate a *P. aeruginosa* biofilm, something not observed with colistin treatment
210 alone. This synergy is also observed in a murine implant model. We believe that the synergy
211 between auranofin and colistin works in both directions. It has been shown that disrupting outer
212 membrane permeability (here with the use of colistin) allows auranofin to penetrate the cell
213 envelope and kill Gram-negative bacteria, through its potential action on thioredoxin reductase.
214 At the same time, the inhibition of TFP and QS (by auranofin) during biofilm development
215 may prevent the formation of colistin-tolerant subpopulations within the biofilm, thereby
216 allowing complete eradication. The unique synergistic effects of auranofin and colistin may be
217 exploited as a new treatment regimen for *P. aeruginosa* infections. Given that auranofin is
218 already an FDA-approved drug, it may not be in the very far future that this approach can be
219 tested.

220 **Materials and Methods**

221 Detailed methods can be found in SI Appendix, Materials and Methods. All bacterial strains
222 and plasmids are summarized in Table S2 and S3. Oligonucleotides for PCR are listed in Table
223 S4.

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233 **Contributions**

234 L.Y., M.G., and S.-S.C. conceived the project and directed the study. Z.H. conducted the QS
235 inhibitor screening assay. L.Z.W.T. conducted all biochemical experiments. J.K.H.Y. and
236 M.M.S.S. performed the mouse model and the flow cell biofilm experiments. B.Y.W.
237 conducted the rhamnolipid analysis with supervision from S.F.Y.L.. L.Z.W.T. and S.-S.C.
238 wrote the manuscript with input from all authors.

239

240 **Competing financial interests**

241 M.G., S.-S.C., L.Y., L.Z.W.T., and Z.H. have filed an international patent application
242 (PCT/SG2016/050436, filed 6th September 2016) on the work described in this manuscript.

243

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366

367 **Figure Legends**

368

369 **Figure 1.** Auranofin inhibits all levels of QS with minimal effects on cell growth.
370 Representative fluorescence induction profiles (relative fluorescence units normalized to cell
371 density, over time) for (a) LasR-mediated expression of LasB-GFP, (b) RhlR-mediated
372 expression of P_{rhlA}-*gfp*, and (c) PqsR-mediated expression of P_{pqsA}-*gfp*, in the presence of
373 indicated concentrations of auranofin, whose chemical structure is shown on the top. Dose-
374 dependent inhibition curves for each assay are plotted on the right. Error bars represent
375 standard deviation from technical triplicates.

376

377 **Figure 2.** The production of key QS-controlled virulence factors is reduced in the presence of
378 auranofin. (a) Elastase activity in the supernatants of PAO1 and Δ*lasIrhlI* cultures treated with
379 indicated concentrations of auranofin. (b) Relative abundance of rhamnolipid fragments
380 isolated from PAO1 cells treated with 20 μM auranofin, as quantified using multiple reaction
381 monitoring mass spectrometry. The chemical structure of rhamnolipid and its measured
382 fragments are shown on the right. (c) Relative levels of pyocyanin in supernatants of PAO1
383 and Δ*lasIrhlI* cultures treated with indicated concentrations of auranofin, as measured at
384 absorbance at 691 nm. Error bars represent standard deviation from biological triplicates.

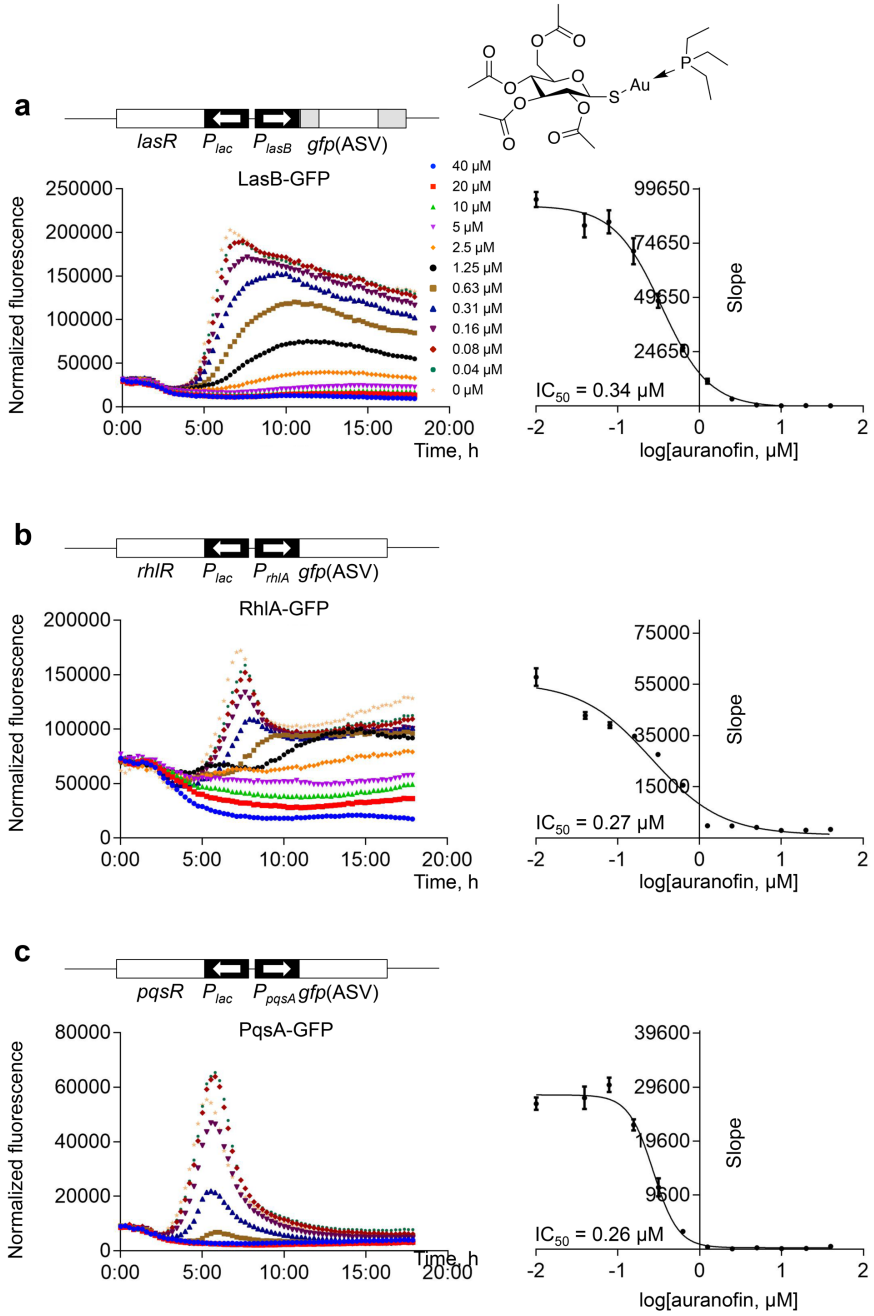
385

386 **Figure 3.** Motility phenotypes that depend on TFP (i.e. swarming and twitching) are reduced
387 in the presence of auranofin. (A) Swarming (0.5% LB agar), (b) swimming (0.3% LB agar),
388 and twitching (1% LB agar) motilities of PAO1 and PAO1 Δ*vfr* strains in the presence or
389 absence of 20 μM auranofin. For twitching, cells at the base of the plates were stained with
390 Coomassie blue.

391

392 **Figure 4.** Auranofin inhibits Vfr binding to target promoter sequences in vitro by reacting with
393 cysteine residues. Polyacrylamide gels showing specific binding of (a) Vfr, (b) VfrΔ5 to P_{lasR},
394 or both proteins to (c) P_{toxA} and (d) P_{regA}, in the presence of indicated concentrations of
395 auranofin. The coding region of the gene *rsaL* is used as a probe to control for non-specific
396 binding.
397
398 **Figure 5.** Auranofin attenuates the progression of *P. aeruginosa* biofilms. Confocal
399 fluorescence microscopy images illustrating the formation and maturation of PAO1-*gfp*
400 biofilms over three days in the presence or absence of 1 μM under continuous flow conditions.
401 Scale bar = 20 μm
402
403 **Figure 6.** Auranofin displays strong synergy in eradicating *P. aeruginosa* biofilms when used
404 in combination with colistin. (a) Confocal fluorescence microscopy images of two-day old
405 PAO1-*gfp* biofilms before and after treatment with 10 μg/ml colistin. Auranofin was not added
406 (top panels), added prior to biofilm formation (middle panels), or added together with colistin
407 (bottom panels). Dead cells were revealed by staining with propidium iodide. Each panel
408 represents a square surface area of 212 x 212 μm. (b) Mean c.f.u per ml of *in vivo* PAO1
409 biofilms obtained from silicone implants in the peritoneal space of mice, treated with auranofin,
410 colistin or a combination of both drugs. *P<0.01, Student's *t*-test.
411

412 **Figure 1**



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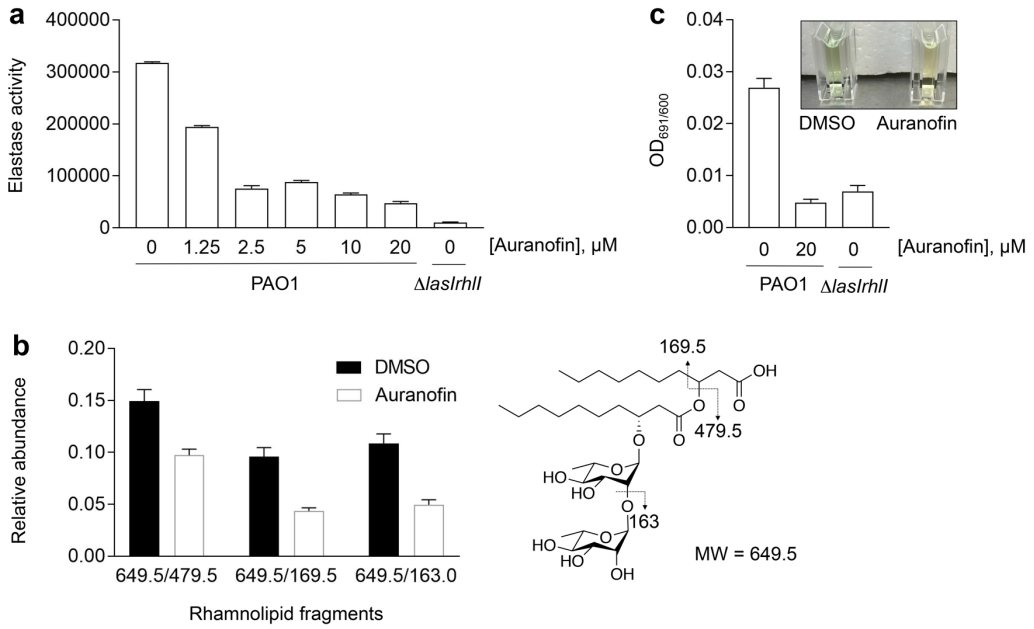
414 **Figure 2**



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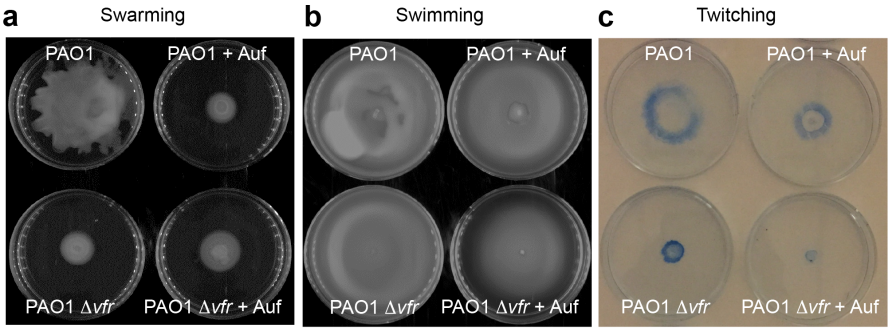

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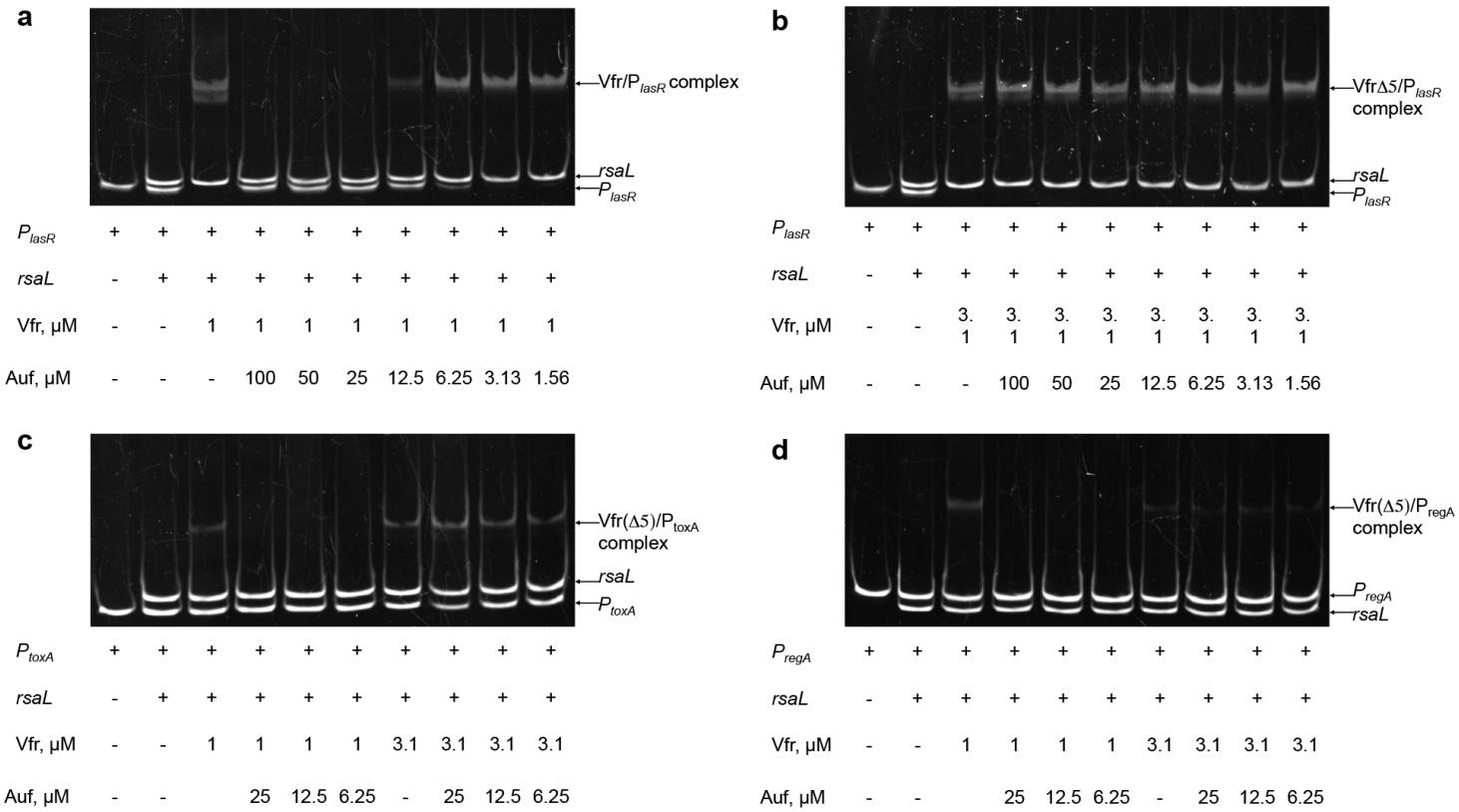
418 **Figure 4**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Vfr/P lasR complex
rsaL
P lasR
P lasR + + + + + + + + + +
rsaL - + + + + + + + + +
Vfr, μM - - 1 1 1 1 1 1 1 1
Auf, μM - - - 100 50 25 12.5 6.25 3.13 1.56
b
VfrΔ5/P lasR complex
rsaL
P lasR
P lasR + + + + + + + + + +
rsaL - + + + + + + + + +
Vfr, μM - - 3.1 3.1 3.1 3.1 3.1 3.1 3.1 3.1
Auf, μM - - - 100 50 25 12.5 6.25 3.13 1.56
c
Vfr(Δ5)/P toxA complex
rsaL
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Vfr, μM - - 1 1 1 1 3.1 3.1 3.1 3.1
Auf, μM - - 25 12.5 6.25 - 25 12.5 6.25
d
Vfr(Δ5)/P regA complex
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rsaL
P regA + + + + + + + + + +
rsaL - + + + + + + + + +
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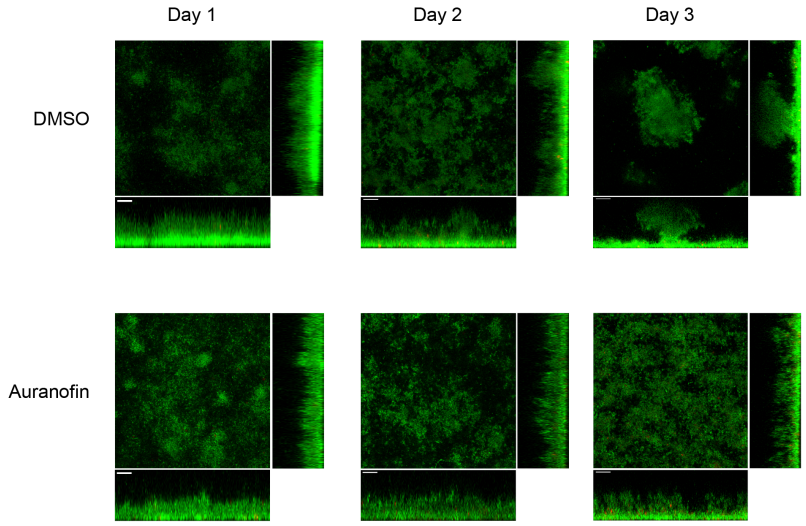
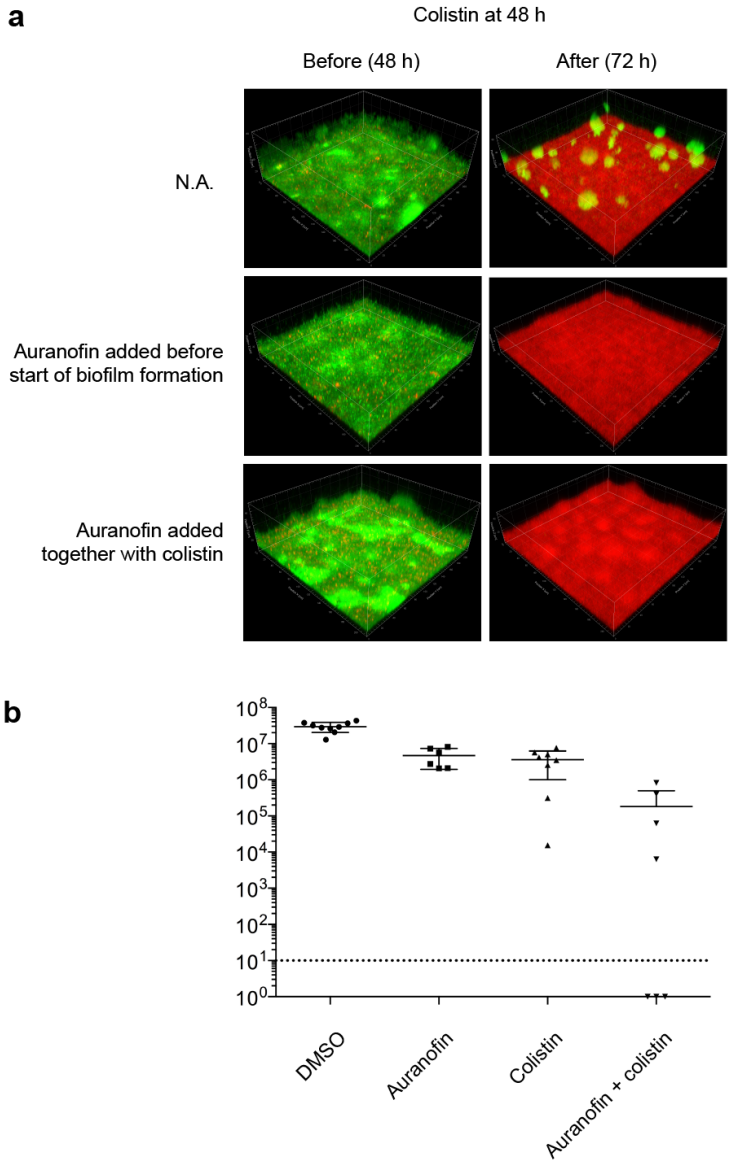
Figure 5



421 **Figure 6**



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