

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Furoxan nitric oxide donors disperse *Pseudomonas aeruginosa* biofilms, accelerate growth, and repress pyoverdine production

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1647035> since 2017-09-18T07:58:06Z

Published version:

DOI:10.1021/acscchembio.7b00256

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This is the author's final version of the contribution published as:

Poh, Wee Han; Barraud, Nicolas; Guglielmo, Stefano; Lazzarato, Loretta; Fruttero, Roberta; Rolando, Barbara; Rice, Scott A.. Furoxan nitric oxide donors disperse *Pseudomonas aeruginosa* biofilms, accelerate growth, and repress pyoverdine production. ACS chemical biology. 12 pp: 2097-2106. DOI: 10.1021/acscchembio.7b00256

The publisher's version is available at:

<http://pubs.acs.org/doi/pdf/10.1021/acscchembio.7b00256>

When citing, please refer to the published version.

Link to this full text:

<http://hdl.handle.net/2318/1647035>

1 **Furoxan nitric oxide donors disperse *Pseudomonas aeruginosa* biofilms, accelerate**
2 **growth and repress pyoverdine production**

3 Wee Han Poh¹, Nicolas Barraud², Stefano Guglielmo³, Loretta Lazzarato³, Barbara Rolando³,
4 Roberta Fruttero³, Scott A. Rice^{1,4*}

5

6 1 Singapore Centre for Environmental Life Sciences Engineering, Nanyang
7 Technological University, Singapore

8 2 Genetics of Biofilms Unit, Department of Microbiology, Institut Pasteur, Paris, France.

9 3 Dipartimento di Scienza e Tecnologia del Farmaco, The University of Torino

10 4 School of Biological Sciences, Nanyang Technological University, Singapore

11

12 Running title: The effect of furoxans on *P. aeruginosa* biofilms

13

14 *Corresponding author

15 RSCOTT@ntu.edu.sg

16 Tel: +65 6592 7944

17 Fax: +65 6316 7349

18

19 **Key words:**

20 Furoxans, 1, 2, 5 Oxadiazole N-Oxides, nitric oxide donors, *Pseudomonas aeruginosa*,
21 Biofilms, Pyoverdine, Biofilm Dispersal

22

23 **Abstract**

24 The use of nitric oxide (NO) as a signal for biofilm dispersal has been shown to increase the
25 susceptibility of many biofilms to antibiotics, promoting their eradication. The delivery of NO
26 to biofilms can be achieved by using NO-donors with different kinetics and properties of NO
27 release that can influence their efficacy as biofilm control agents. In this study, the kinetics of
28 three furoxan-derivatives were evaluated. The effects of these NO-donors, which have an
29 advantageous pharmacological profile of slower onset with an extended duration of action, on
30 *Pseudomonas aeruginosa* growth, biofilm development and dispersal were also characterized.
31 Compound LL4254, which showed a fast rate of NO release, induced biofilm dispersal at
32 approximately 200 μ M. While LL4212 and LL4216 have a slower rate of NO release, both
33 compounds could induce biofilm dispersal, under the same treatment conditions, when used at
34 higher concentrations. Further, LL4212 and LL4216 were found to promote *P. aeruginosa*
35 growth in iron-limited minimal medium, leading to a faster rate of biofilm formation and
36 glucose utilization, and ultimately resulted in early dispersal of biofilm cells through carbon
37 starvation. High concentrations of LL4216 also repressed production of the siderophore
38 pyoverdine by more than 50-fold, via both NO_x-dependent and NO_x-independent mechanisms.
39 The effects on growth and pyoverdine levels exerted by the furoxans appeared to be mediated
40 by NO-independent mechanisms, suggesting functional activities of furoxans in addition to
41 their release of NO and nitrite. Overall, this study reveals that secondary effects of furoxans
42 are important considerations for their use as NO-releasing dispersal agents, and that these
43 compounds could be potentially re-designed as pyoverdine inhibitors.

44 **Introduction**

45 Bacterial cells growing within biofilms can be up to a thousand times more resistant to anti-
46 microbial agents than their planktonic counterparts, making their eradication very difficult ^{1, 2}.
47 This enhanced tolerance to stress, immune defenses and antibiotics may partly explain the
48 observation that biofilms are associated with up to 80% of all microbial infections and the
49 majority of chronic and recurrent infections including pneumonia in cystic fibrosis patients,
50 wound infections and medical implant-associated infections ^{1, 3, 4}.

51 The insensitivity of biofilms to conventional antimicrobial treatments appears to be
52 multifactorial and involves: (i) a protective barrier of self-produced extracellular polymeric
53 substances (EPS) that can inactivate or reduce diffusion of bactericidal compounds ⁵, (ii)
54 enhanced lateral transfer of genes including those from drug resistant and extremely drug
55 resistant (XDR) strains ⁶ and (iii) the presence of dormant-like persister cells ⁷. Given the
56 central role played by biofilms in promoting antibiotic resistance and causing the failure of
57 therapeutic treatments, there is an urgent need to develop alternative strategies specifically
58 aimed at biofilm development processes. Towards this end, the dispersal phase of the biofilm
59 life cycle has been targeted as a key stage that can be manipulated to control biofilms ⁸. During
60 dispersal, bacterial cells transit from a biofilm to a planktonic mode of growth, rendering them
61 more susceptible to various antibiotics ^{9, 10}. Biofilms disperse in response to several
62 environmental cues, such as changes in carbon levels and iron levels ¹¹⁻¹³. In addition, low
63 nanomolar concentrations of the gaseous free radical nitric oxide (NO) were found to induce
64 biofilm dispersal in *Pseudomonas aeruginosa*, an opportunistic pathogen and the model
65 organism for biofilm studies ⁹, as well as several other bacterial species ⁸. Furthermore,
66 exposure of pre-established biofilms to the NO-donor compound sodium nitroprusside (SNP)
67 increased the susceptibility of biofilm cells towards several antimicrobial agents, including the
68 antibiotics tobramycin and ceftazidime in *P. aeruginosa* biofilms grown both on abiotic
69 surfaces and in ex vivo sputum samples of CF patients ^{9, 10}. Recently, the use of NO gas
70 delivered at low dose to CF patients, together with intravenous tobramycin and ceftazidime,
71 reduced *P. aeruginosa* biofilms and improved lung function when compared to placebo-treated
72 control patients in proof of concept clinical studies ¹⁴. These observations suggest that the use
73 of NO, together with conventional antibiotics, represents a promising alternative for the
74 treatment of chronic biofilm infections.

75

76 The delivery of NO for medical purposes, in addition to the gaseous form, is typically achieved
77 by using soluble donors. These compounds spontaneously release NO when dissolved in
78 aqueous solutions and the kinetics of release are a function of their donor chemistry. In this
79 way, donors can be tuned to optimize the release of NO. Furoxans are heterocyclic compounds
80 containing a 1,2,5-oxadiazole 2-oxide ring and two substituent groups at positions 3 and 4 of
81 the furoxan ring (Figure 1) ¹⁵. These substituents influence several properties of the furoxan
82 compound, such as its solubility, the rate of NO production, and whether NO-release is thiol-
83 activated or could occur spontaneously ¹⁵⁻¹⁷. This flexibility allows for the design of furoxan
84 compounds that generate varying fluxes of NO when administered ¹⁷. In general, furoxans have
85 an advantageous pharmacological profile of a slow onset with an extended duration of action,
86 as compared to other NO donors ^{15, 18}. In turn, furoxans as such, or used in designing NO-donor
87 hybrid drugs, have been observed to be active against a variety of targets and have been
88 assessed for use in cardiovascular diseases, neurological and inflammatory disorders ¹⁹⁻²¹. More
89 recently, a few furoxan derivatives were found to inhibit ABC transporters in MDR tumor cells
90 ^{22, 23}

91 Despite the many applications of furoxans, they have not been tested for their activity against
92 biofilms. In this study, we evaluated the release of nitrogen oxide species (NO_x), namely NO
93 and nitrite (NO₂⁻), from three furoxans, LL4212, LL4216 and LL4254, and studied their effect
94 on *P. aeruginosa* biofilm development, where they induced biofilm dispersal. LL4212 and
95 LL4216 were, additionally found to affect bacterial growth under iron-limited conditions in an
96 NO_x-independent manner, increasing the rates of glucose utilization and in turn leading to the
97 earlier onset of glucose-starvation-induced dispersal of *P. aeruginosa* biofilms. Further,
98 LL4216 was found to reduce expression of the siderophore pyoverdine in an NO_x-independent
99 manner. Taken together, the study indicated that, depending on their backbone, furoxans may
100 have secondary effects on bacterial growth and is an important consideration for their design
101 and use as NO-releasing agents.

102

103 **Results and Discussions**

104 **Kinetics of NO_x release from LL4254, LL4212 and LL4216**

105 The study of the kinetics of NO_x release from the furoxan compounds indicated that both NO
106 and nitrite (NO₂⁻) were spontaneously released from the furoxans under physiologically

107 relevant conditions (pH, temperature). The results, expressed as percent mol/mol of NO_x
108 released with respect to the concentration of furoxan in solution (Figure 2a – c), showed two
109 types of NO_x-production behavior. For LL4254, NO was the predominant species released,
110 accounting for 82% of the total NO_x detected (~ 95%) by 24 h (Figure 2a); the liberation
111 occurred very fast, with 80% of the total NO_x produced within the first 30 min. In contrast,
112 NO₂⁻ was the dominant species generated from LL4212 and LL4216 and liberation occurred in
113 an almost linear fashion over a few hours. By 24 h, 83% and 32% of the NO_x species were
114 detected as NO₂⁻ from LL4212 and LL4216, respectively, while only 2.6% and 1.2% were
115 measured as NO (Figure 2b, c). An alternative chemiluminescence-based NO detection method,
116 also confirmed the results obtained with the DAN assay (data not shown).

117 Previous studies showed that thiol groups such as those present in L-cysteine (L-cys) can
118 promote NO_x liberation from furoxan compounds and increase their activity ¹⁷. Since thiol
119 groups are commonly produced by bacteria, in the form of metabolites or proteins, they are
120 highly relevant in the context of bacterial infection treatments and their effects on NO_x release
121 from LL4212, LL4216 and LL4254 were investigated. ~~Exogenous addition of L-cys reduced~~
122 ~~the extent of NO_x liberated from the fast releasing LL4254 but promoted their production from~~
123 ~~the slower releasing LL4212 and LL4216 (Figure 2d – f). At t = 1 h, the presence of L-cys~~
124 ~~reduced the amount of NO and NO₂⁻ generated from LL4254 to 4% and 36%, respectively,~~
125 ~~from an initial 70% and 90% in the absence of L-cys (Figure 2d). At t = 4 h, NO_x production~~
126 ~~increased from 26% to 28% in the presence of L-cys for LL4212, and from 8% to 20% for~~
127 ~~LL4216. NO production from these compounds also increased in the presence of L-cys, from~~
128 ~~1.8% to 2.2% for LL4212 and from 0.8% to 1.5% for LL4216 (Figure 2 e, f).~~

129 Exogenous addition of L-cys reduced the extent of NO_x liberated from the fast releasing compound
130 LL4254, promoted their production from the slower releasing LL4216 while did not affected the NO_x
131 production from LL4212 (Figure 2d – f). At t = 1 h, the presence of L-cys reduced the amount of NO
132 and NO₂⁻ generated from LL4254 to 4% and 36%, respectively, from the initial 70% and 90% in the
133 absence of L-cys (Figure 2d). At t = 4 h, NO₂⁻ production increased from 8% to 20% in the presence
134 of L-cys for LL4216. NO production from LL4216 and LL4212 was not influenced by L-cys (Figure
135 2 e, f).

136

137

138 **Furoxan NO_x-donors can induce *P. aeruginosa* biofilm dispersal**

139 To determine if the NO-releasing furoxans could induce biofilm dispersal, *P. aeruginosa*
140 biofilms were treated with 100 μ M, 200 μ M or 500 μ M of the furoxans for 1 h before
141 quantification of biofilm biomass by CV staining. At 200 μ M or above, LL4254 reduced the
142 biofilm biomass by >70% compared to untreated control samples ($p < 0.0001$) (Figure 3a, c).
143 This biomass reduction, which was associated with an increase in OD₆₀₀ reading of the
144 supernatant (data not shown), occurred after 1 h treatment, and therefore cannot be linked to
145 growth effects but instead is a clear indication of biofilm dispersal events. These results were
146 in agreement with previous studies, where NO was found to induce *P. aeruginosa* biofilm
147 dispersal^{9, 24}. Dispersal by LL4254 occurred in a concentration-dependent manner. In contrast,
148 LL4212 and LL4216 were unable to induce significant biofilm dispersal at concentrations
149 between 100 μ M and 500 μ M.

150 To assess if dispersal was caused by NO or other non-specific interactions between *P.*
151 *aeruginosa* and the LL4254 backbone, the assay was repeated in the presence of the NO
152 scavenger cPTIO. Under the conditions used, cPTIO alone did not have a significant effect on
153 both planktonic and biofilm growth (data not shown). Addition of cPTIO reduced dispersal by
154 LL4254 from an average of 73% to 18%, suggesting that the effect of LL4254 on dispersal was
155 NO-dependent (Figure 3b, d).

156 NO release from LL4254 was established as an important factor for inducing biofilm dispersal.
157 As the rate of both NO and NO₂⁻ generated from LL4212 and LL4216 was much lower than
158 that of LL4254 under cell-free physiological conditions (Figure 2), it is likely that at the same
159 compound concentration, both LL4212 and LL4216 produce a lower amount of NO, This may
160 in turn influence the effective NO concentration perceived by the bacteria at a given time, as
161 NO generated may be consumed by cellular processes, reaction with oxygen, and reactions
162 with other chemical compounds present in the medium^{25, 26}, hence limiting the extent of
163 biofilm dispersal. Therefore, the assay was repeated using 3.6 mM LL4212 and LL4216, which
164 correspond to the amount of NO_x released from approximately 400 μ M and 200 μ M LL4254
165 within 1 h. The furoxan stocks were solubilized in DMSO, and while at low concentrations (\leq
166 0.5% v/v) DMSO did not significantly alter *P. aeruginosa* growth or biofilm formation in our
167 assays (data not shown), at a higher level of 3.6% v/v (the amount present when adding 3.6
168 mM furoxan), DMSO reduced the amount of biofilm formed by approximately 14% (Figure
169 3f). Therefore, samples to which an equivalent volume of DMSO was added, were used as a
170 control for comparison of the extent of biofilm dispersal induced with 3.6 mM furoxan
171 treatments. At 3.6 mM, LL4212 and LL4216 dispersed 48% and 70% of the biofilm biomass

172 respectively (Figure 3e, f). Decrease in biofilm biomass observed was concomitant with an
173 increase in planktonic biomass, thus confirming that the observed effects correlated with
174 dispersal of bacteria from the biofilm. Although LL4216 alone, under physiological, cell-free
175 conditions has a slower rate of NO_x release than LL4212, in this experiment, LL4216 was
176 found to induce a larger extent of dispersal than LL4212. This is likely due to the presence of
177 nucleophiles, e.g. thiol groups, that are produced or secreted by the bacteria¹⁵⁻¹⁷ that promoted
178 NO_x release from LL4212, LL4216 and LL4254 to different extents, as supported in cell-free
179 NO kinetics experiments (Figure 2).

180

181 **Furoxans accelerate *P. aeruginosa* biofilm development and enhance glucose utilization**

182 Because furoxans have a slower rate of release and can produce NO_x over longer periods of
183 time, we then assessed whether these compounds can constantly prevent the switch for
184 attachment of free-floating bacteria, maintain cells in a planktonic mode of growth and thus
185 inhibit biofilm formation over time. Furoxans were added to M9GC medium together with the
186 *P. aeruginosa* at the time of inoculation. After 6 h, wells that had been treated with LL4212
187 and LL4216 showed a significant concentration-dependent reduction in biofilm biomass, with
188 increases in planktonic growth, compared to untreated wells, with 100 μM of LL4216 reducing
189 biofilm biomass by > 70% ($p < 0.0001$) (Figure 4a, c).

190 To further characterize the impact of furoxans on biofilm formation, biofilm biomass of
191 untreated control groups and groups treated with 500 μM LL4212 or 200 μM LL4216, both of
192 which reduced the biofilm biomass at $t = 6$ h by > 80%, was quantified over time (Figure 4b).
193 Surprisingly, addition of LL4212 and LL4216 promoted both biofilm formation and planktonic
194 growth over the first 4 h and 3 h, respectively (Figure 4b). Subsequently, the biofilm dispersed
195 while planktonic growth continued to increase. A similar sharp decrease in biofilm biomass
196 during growth had already been observed in *P. aeruginosa* biofilms and was linked to biofilm
197 dispersal due to the sudden depletion of the carbon source and the onset of starvation²⁷. The
198 levels of glucose in the biofilm cultures were then determined over time and the extent of
199 biofilm reduction at $t = 6$ h was consistent with a reduction in glucose concentration (Figure
200 4d, e). The effect was the most pronounced with 500 μM LL4216, which led to complete
201 glucose depletion by $t = 6$ h.

202 While NO is known to affect biofilm dispersal, a potential role in regulating glucose utilization
203 and growth has not been observed before. To determine if the effects of LL4212 or LL4216 on
204 glucose consumption were dependent on NO, biofilm prevention experiments were carried out
205 with furoxans added together with the NO scavenger cPTIO, or using NO-depleted furoxans
206 which had been incubated in culture medium for 24 h before inoculating with *P. aeruginosa*,
207 thus resulting in exhaustion of NO released from the donor compounds. The data show that
208 after 6 h, *P. aeruginosa* biofilms had dispersed under both conditions (Figure 5a – c),
209 suggesting that the effects of LL4212 and LL4216 on biofilm formation were not due to NO.

210 These observations of the impact of LL4212 and LL4216 on biofilms did not correlate with a
211 typical dispersal response to NO resulting in biofilm prevention. To further elucidate the effect
212 of the furoxans on biofilms, we then examined their impact in our assay on a known marker of
213 biofilm dispersal in *P. aeruginosa*, including when induced by NO, which is a decrease in the
214 production of the iron chelating siderophore pyoverdine²⁸. A similar decrease in pyoverdine
215 levels was also previously observed upon dispersal with 200 μ M of LL4254 and 3.6 mM of
216 LL4212 or 4216 (Figure 3e). The results revealed that in the presence of 500 μ M NO-
217 exhausted LL4216, pyoverdine levels were reduced by at least 50-fold compared to untreated
218 biofilms, even though there was no free NO present. In contrast, the use of 500 μ M of NO-
219 depleted LL4212 had no effect on the pyoverdine levels with respect to the untreated controls
220 (Figure 5c). To control for a potential direct influence of LL4216 on the pyoverdine fluorescent
221 signal, 500 μ M of LL4216 were added to filtered, cell-free M9GC medium collected from
222 microtiter plate cultures grown under the same conditions, which contained pyoverdine.
223 Pyoverdine levels were found to be relatively stable in filtered medium without any compound
224 and LL4216 at 500 μ M only slightly decreased pyoverdine relative fluorescent units (RFU) by
225 6.3% and 6.9%, after 6 h and 24 h respectively, compared to untreated cell-free solutions. In
226 contrast, when 500 μ M LL4216 was added to a non-filtered culture inoculated with *P.*
227 *aeruginosa* at $t = 0$ h, pyoverdine expression was > 99% lower than that of the control after
228 both 6 h and 24 h, suggesting that LL4216 actively repressed pyoverdine expression, rather
229 than simply affecting the fluorescence signal from pyoverdine (Figure 5d). Thus, these results
230 suggest that the furoxans can reduce pyoverdine synthesis in *P. aeruginosa*, via both NO-
231 independent (LL4216) and NO-dependent (LL4212) mechanisms.

232 Finally, because another major NO_x released from LL4212 and LL4216 is NO₂⁻, its effect on
233 biofilm formation in our assay was investigated. As opposed to NO, NO₂⁻ is stable in solution

234 and can be added directly to the culture medium When 100 – 500 μM of exogenous nitrite were
235 added at $t = 0$ h in place of LL4212 and LL4216, there was no significant effect on planktonic
236 growth, biofilm formation or pyoverdine production in *P. aeruginosa* (Supplementary figure
237 3).

238 Collectively, these results suggest that LL4212 and LL4216 likely increase the growth rates of
239 *P. aeruginosa* through increased rates of glucose metabolism and cause biofilm dispersal via a
240 carbon or glucose starvation-induced response ^{11, 12}. The use of NO depleted LL4212 or
241 LL4216 in M9GC medium induced the same effects. Further, the addition of exogenous
242 sources of nitrite, the main NO_x species released from LL4212 and LL4216, did not affect
243 planktonic growth, biofilm formation or pyoverdine production. This indicates that the
244 backbone or by-product of NO_x released from these two compounds, but not NO or NO_2^- , were
245 responsible for the increased growth.

246

247 **LL4212 and LL4216 are not utilized as direct carbon or nitrogen sources by *P. aeruginosa***

248 One possibility to explain the accelerated biofilm formation and increased growth of *P.*
249 *aeruginosa* in the presence of the furoxans, is that these compounds may be used as carbon or
250 nitrogen sources for metabolism. To determine the impact of the furoxans on metabolism, *P.*
251 *aeruginosa* was inoculated in various modified M9 media with or without available carbon or
252 nitrogen sources. In M9 medium supplemented with casamino acids and glucose as sources of
253 carbon (M9GC), or M9 medium supplemented with glucose only (M9G), the addition of
254 500 μM LL4212 or LL4216 increased both the growth rates and growth yields of *P.*
255 *aeruginosa* (Figure 6a, Supplementary figure 4), which agrees with our previous observations.
256 In contrast, the addition of LL4212 and LL4216 to M9 medium with glucose but without any
257 nitrogen source (M9-N), did not significantly alter growth rates, although there was a slight
258 increase in total growth (Figure S2c). In M9 medium without any carbon source (M9S), *P.*
259 *aeruginosa* did not grow at all whether in the absence or presence of LL4212 and LL4216
260 (Figure Supplementary figure 2d).

261 This hence suggests that the compounds are most likely not utilized as a direct source of energy
262 for *P. aeruginosa*, and may influence growth through interfering with processes related to
263 glucose metabolism and increases in ATP production (Supplementary figure 6). This latter

264 point is also supported by the observation that glucose was more rapidly depleted in the
265 presence of these compounds.

266

267 **LL4216 inhibits pyoverdine production under both low and high iron conditions in *P.***
268 ***aeruginosa*, but under high iron conditions reduces the growth rate**

269 Another nutrient essential for growth in *P. aeruginosa* is iron, which is typically limiting in
270 M9. However, bacteria can acquire essential trace amounts from contaminants in the water or
271 components used to prepare the medium, typically by producing siderophores like pyoverdine,
272 which has high iron affinity. Since the furoxans appeared to influence both growth and
273 pyoverdine production in *P. aeruginosa*, we assessed if these effects may be associated with
274 iron uptake. As expected, the addition of 500 μM LL4212 and LL4216 to M9GC resulted in
275 increased growth and reduced pyoverdine levels (Figure 6b, c). In contrast, under the same
276 conditions, LL4254 did not affect growth or pyoverdine production. In M9GC medium, the
277 increase in growth rate showed a clear concentration dependence for LL4212, while there was
278 only a slight increase in growth rate for LL4216 at 500 μM . With respect to pyoverdine
279 production, LL4212 repressed pyoverdine only when added at 500 μM , while LL4216 showed
280 a clear concentration-dependent reduction.

281 In M9GCFe, *P. aeruginosa* displayed an increased growth rate of about 8-fold compared to
282 M9GC. In M9GCFe, LL4212 addition resulted in a slight reduction in growth rate relative to
283 the control while LL4216 reduced the growth rate in a concentration dependent fashion, with
284 a 38% reduction when added at 500 μM (Figure 6b). The presence of added iron also generally
285 reduced pyoverdine production. While LL4212 only slightly further reduced this production,
286 by 12% when added at the highest concentration tested, the effects were more pronounced with
287 LL4216, which at 500 μM induced a drastic further reduction in pyoverdine to background
288 levels. The addition of LL4254 had no influence on growth rate and pyoverdine production in
289 either medium (Figure Supplementary figure 5c, f). Taken together these data suggest that
290 LL4212 and LL4216 may have opposite impacts on *P. aeruginosa* growth depending on the
291 availability of iron, resulting in increased growth under iron limited conditions, while reducing
292 growth when iron is replete.

293 **LL4212 and LL4216 may affect biofilm and planktonic growth via influences on glucose**
294 **utilization and pyoverdine production in *P. aeruginosa***

295 The decrease in pyoverdine levels upon LL4212 and LL4216 addition may be in part due to
296 NO release, which is known to repress the expression of pyoverdine related genes ²⁴. Indeed,
297 when NO-depleted LL4212 was used, no repression in pyoverdine production was observed.
298 In contrast, the effect of LL4216 on pyoverdine appeared to be independent of NO, as the use
299 of the NO-depleted compound still induced a decrease in pyoverdine levels (Figure 5c). Overall,
300 the results show that decreases in pyoverdine production with LL4212 are predominantly
301 mediated by NO while similar decreases in LL4216-treated samples were either due to the
302 presence of NO or mediated directly by the LL4216 backbone. Despite these differences, both
303 compounds could promote bacterial growth in *P. aeruginosa* and no correlation between
304 growth rate and pyoverdine levels was observed (Figure 6b, c). Thus, the furoxans likely exert
305 independent effects on growth and pyoverdine levels, especially in the absence of added iron.

306 We initially hypothesized that LL4216 may function as a siderophore in place of pyoverdine.
307 Repression of pyoverdine production by LL4216, while still being able to take up iron, could
308 then direct metabolites towards energy generation and growth away from the costly production
309 of pyoverdine. For example, pyoverdine production which has been previously estimated to
310 require approximately 10% of additional carbon consumption per growth unit in a closely
311 related species, *Pseudomonas putida*, grown under iron limitation ^{29, 30}. However, when
312 supplemented with ferric chloride, no further increase in growth was observed with addition of
313 500 μM of LL4216, suggesting that LL4216 does not function as an alternative iron scavenger
314 and further supporting that LL4216 exerts growth-related effects independent of pyoverdine
315 production and iron acquisition.

316

317 **Conclusions**

318 The results suggest that furoxans with fast and slow NO release profiles could be used at low
319 and high concentrations, respectively, to promote biofilm dispersal. Slow NO-releasing
320 furoxans were also found to be potent at preventing biofilm formation over longer periods of
321 time, in a similar manner as slow NO-releasing polymers had been previously found to inhibit
322 biofilm formation in *P. aeruginosa* ³¹, although a potential role of NO in mediating the effects
323 of furoxans on biofilm prevention could not be clearly determined here. Interestingly, slow
324 NO-releasing furoxans may be useful at higher concentrations to, in one single treatment,
325 induce dispersal of pre-established biofilms and prevent re-formation of a biofilm over
326 extended times.

327 In addition to inducing biofilm dispersal via NO, the furoxans LL4212 and LL4216 also
328 influenced growth and pyoverdine production of *P. aeruginosa*. The data suggest that the two
329 furoxans exert effects on growth and pyoverdine levels independently of each other and further
330 work is needed to elucidate these mechanisms. While the increased growth rate would not
331 normally be advantageous from the perspective of pathogen control, if the compounds strongly
332 repress siderophore production, they may facilitate control in the host, where iron is severely
333 limited. In this respect, there may be interest in developing these compounds as pyoverdine
334 inhibitors³². This is relevant as *P. aeruginosa* is predominantly found in the lungs of cystic
335 fibrosis patients, with pyoverdine expression being a major factor accounting for *P. aeruginosa*
336 virulence, antibiotic resistance and biofilm maturation³³⁻³⁵. Further studies would be required
337 for the successful development of furoxans as dispersal agents or for their use in other
338 applications.

339 **Materials and methods**

340 **Nitric oxide donors and scavengers**

341 The furoxans LL4212 (3-((2-(dimethylamino)ethyl)oxy)-4-phenylfuroxan), LL4216 (3-((2-
342 aminoethyl)thio)-4-phenylfuroxan) and LL4254 (4-(phenylsulfonyl)-3-((2-
343 dimethylamino)ethyl)thio)furoxan) were synthesized as previously reported by Sorba *et al.*¹⁷.
344 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (carboxy-
345 PTIO, cPTIO) was purchased from Sigma Aldrich (# C221).

346 **Nitrite measurements by Griess reaction (total NO_x evaluation)**

347 The total release of NO_x was evaluated as nitrite (NO₂⁻) by Griess reaction. Furoxan compounds
348 were incubated at 37°C in 50 mM phosphate buffer, pH 7.4 at 0.1 mM concentration in the
349 absence or in the presence of 0.5 mM L-cysteine (L-cys) (5 times mol/mol excess). At regular
350 intervals, the presence of nitrite in the reaction mixture was determined by the Griess assay: 1
351 mL of the reaction mixture was treated with 250 µL of the Griess reagent (4% w/v
352 sulphanilamide, 0.2 % w/v *N*-naphthylethylenediamine dihydrochloride, 1.47 M phosphoric
353 acid). After 10 min at room temperature, the absorbance was measured at 540 nm. A calibration
354 curve was obtained using standard solutions of sodium nitrite at 10 µM to 80 µM. The yield in
355 nitrite was expressed as percent NO₂⁻ (mol/mol, relative to the initial compound concentration)
356 ± SEM.

357 **NO measurements by DAN (2,3-diaminonaphthalene) method**

358 NO release was quantified using a 2,3-diaminonaphthalene (DAN)-based chemical assay,
359 which is based on the immediate reaction of NO with oxygen (O₂) to form dinitrogen trioxide
360 (N₂O₃), which then reacts with non-fluorescent DAN to form the highly fluorescent 2,3-
361 naphthotriazole (NAT) that can be quantified by RP-HPLC. Compounds were incubated at
362 37°C in 50 mM phosphate buffer, pH 7.4 at 0.1 mM concentration with 0.2 mM DAN in the
363 absence or presence of L-cys at 0.5 mM. At fixed time points, NAT in the reaction mixture was
364 evaluated by HPLC analysis according to previously published protocol ³⁶.

365 HPLC analyses were performed with a HP 1200 chromatograph system (Agilent Technologies)
366 equipped with a quaternary pump (G1311A), a membrane degasser (G1322A), a multiple
367 wavelength UV detector (G1365D) and a fluorescence detector (G1321A) integrated in the
368 HP1200 system. Data analysis was performed using a HP ChemStation system (Agilent
369 Technologies). The sample was eluted on a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm,
370 5 μm; Agilent) with an injection volume of 20 μL. The mobile phase consisted of 65% of 15
371 mM potassium phosphate buffer (pH 8.0) and 35% acetonitrile at a flow rate of 1.0 mL min⁻¹.
372 The fluorescence signals were obtained using an excitation and emission wavelength of 355
373 and 460 nm, respectively (gain factor = 10). Data analysis was performed by with Agilent
374 ChemStation. The values obtained from integration of the peak of NAT were interpolated in a
375 calibration line, prepared using NaNO₂ (in acidic conditions) as a standard. Briefly sodium
376 nitrite standard solutions were acidified with HCl (pH 2) in the presence of excess DAN (0.2
377 mM). After 10 min, the reaction mixture was diluted in phosphate buffer at pH 7.4 (NO final
378 concentration 1 to 80 μM) and analyzed by HPLC.

379

380 **Bacterial strains and growth conditions**

381 *P. aeruginosa* PAO1 (ATCC BAA-47) was maintained on agar plates of Luria-Bertani medium
382 with 10 g/L NaCl (LB10) (644520, Difco). Cultures were grown overnight in LB10 medium at
383 37°C with 200 rpm shaking (Infors HT, orbit diameter 25 mm). Overnight PAO1 cultures were
384 subsequently diluted 200 times to an OD₆₀₀ = 0.005 in various culture media depending on the
385 assay, made of M9 salts (M9S) (48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM
386 NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, pH 7.0) supplemented with different carbon and
387 nitrogen sources: M9GC (M9S, 0.04 % w/v glucose; 0.2 % w/v casamino acid), M9G (M9S,
388 0.4 % w/v glucose), M9-N (M9G made without any NH₄Cl), M9GCFe (M9GC, 3 μM FeCl₃)
389 or M9GCNO₂ (M9GC, 100 μM to 500 μM KNO₂) medium.

390 **Growth studies**

391 Overnight cultures of *P. aeruginosa* were washed three times in M9S made without NH₄Cl and
392 diluted to an OD₆₀₀ of 0.005 in 200 µl of M9GC, M9G, M9-N, M9S, M9GCFe, or M9GCNO₂
393 medium, which were added to each well of a 96 well plate and incubated statically at 37°C for
394 24 h. Furoxans were added at the time of inoculation (t = 0 h). The growth of *P. aeruginosa*
395 was monitored spectroscopically at 600 nm, while pyoverdine production was quantified by
396 measurement of fluorescence intensity (excitation at 398 nm and emission at 460 nm)³⁷. All
397 measurements were carried out using a microtiter plate reader (Infinite 200 pro, Tecan). Growth
398 rates were determined by calculating changes in OD₆₀₀ over time during exponential growth
399 phase while growth yields refer to OD₆₀₀ values recorded at late stationary phase

400 **Biofilm assays**

401 Overnight cultures of *P. aeruginosa* were diluted 1:200 in fresh M9 medium. One mL of the
402 diluted culture was added into a well of a 24-well polystyrene plate (142475, Nunclon), which
403 was subsequently incubated at 37°C with 200 rpm shaking (Infors HT, orbit diameter 25 mm)
404 for no more than 6 h. To assess the effect of furoxan on biofilm dispersal, compounds were
405 added into each well after 5 h incubation (t = 5 h) to a final concentration of 100 µM, 200 µM,
406 500 µM or 3.6 mM, and the plates were incubated for a further 1 h. To assess the effect of each
407 compound on biofilm formation, the furoxans were added into each well at the time of
408 inoculation (t = 0 h) and biofilms were allowed to form over the next 6 h. For experiments
409 involving NO scavengers, c-PTIO was added into each well to a final concentration of 0.5 mM
410 at the same time as furoxans. For experiments involving NO-depleted M9GC medium
411 containing LL4212 or LL4216, 500 µM of each furoxan were first added to M9GC medium
412 and incubated at 37°C for 24 h prior to inoculation with *P. aeruginosa*. Biofilm biomass was
413 quantified by crystal violet (CV) staining as described by Barraud *et al.* (2014)³⁸. Planktonic
414 growth and pyoverdine production were quantified as described above.

415 ***P. aeruginosa* glucose utilization assay**

416 The utilization of glucose by *P. aeruginosa* in M9 medium was measured using the GO assay
417 kit (GAGO20, Sigma). Experiments were carried out as described in the biofilm assays.
418 Subsequently, the medium from each well was filtered and diluted in ultrapure water to obtain
419 a glucose concentration of approximately between 20 to 80 µg mL⁻¹. Glucose standards were
420 prepared per the manufacturer's instructions. Each volume of the standard or sample was mixed

421 with two volumes of the assay reagent and incubated at 37°C statically for 30 min. The reaction
422 was stopped by adding two volumes of 12 N H₂SO₄. Glucose concentrations were determined
423 with a microtiter plate reader (absorbance at 540 nm) and interpolating to the standard curve.

424 **Statistics**

425 All statistical tests were carried out using Graphpad Prism 7.0. Results from biofilm and growth
426 assays were analyzed with one-way ANOVA followed by Dunnett's test for multiple
427 comparison ($\alpha= 0.05$) against a relevant control group. Geometric means were used in
428 statistical tests involving pyoverdine measurements and analyzed using ANOVA as described
429 above.

430

431 **Acknowledgements**

432 We acknowledge financial support from the Singapore Centre for Environment Life Sciences
433 Engineering, whose research is supported by the National Research Foundation Singapore,
434 Ministry of Education, Nanyang Technological University and National University of
435 Singapore, under its Research Centre of Excellence Programme. N. Barraud is supported by
436 the French Government's Investissement d'Avenir program, Laboratoire d'Excellence
437 "Integrative Biology of Emerging Infectious Diseases" (grant n°ANR-10-LABX-62-IBEID).
438 The authors also thank A. Gasco for helpful discussions.

439

440 **Supporting information**

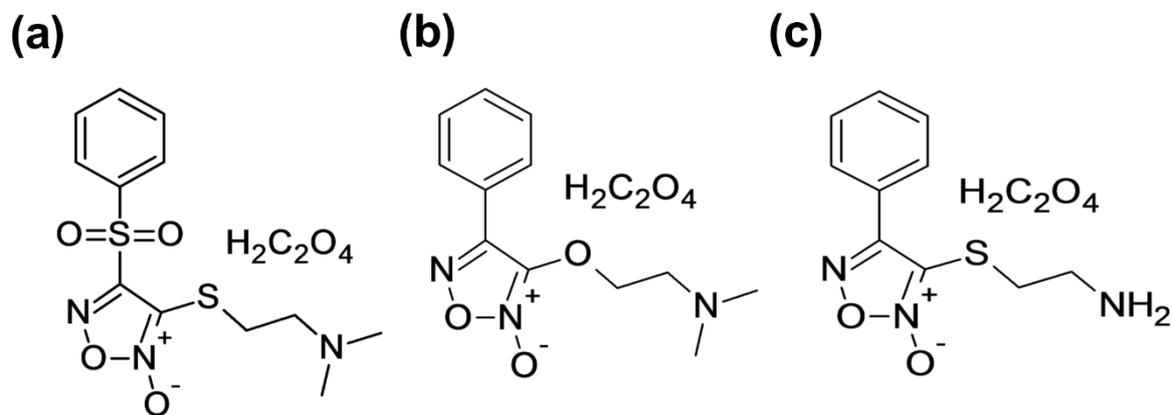
441 *Supporting information available:* This material is available free of charge *via* the internet.

442 References

- 443 1. Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010) Antibiotic resistance of
444 bacterial biofilms, *Int. J. Antimicrob. Agents* 35, 322-332.
- 445 2. Stewart, P. S., and William Costerton, J. (2001) Antibiotic resistance of bacteria in biofilms, *The*
446 *Lancet* 358, 135-138.
- 447 3. Attinger, C., and Wolcott, R. (2012) Clinically addressing biofilm in chronic wounds, *Advan.*
448 *Wound Care* 1, 127-132.
- 449 4. Høiby, N., Ciofu, O., and Bjarnsholt, T. (2010) *Pseudomonas aeruginosa* biofilms in cystic
450 fibrosis, *Fut. Microbiol.* 5, 1663-1674.
- 451 5. Mulcahy, H., Charron-Mazenod, L., and Lewenza, S. (2008) Extracellular DNA chelates cations
452 and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms, *PLoS Path.* 4,
453 e1000213.
- 454 6. Roberts, A. P., and Mullany, P. (2010) Oral biofilms: a reservoir of transferable, bacterial,
455 antimicrobial resistance, *Expert review of anti-infective therapy* 8, 1441-1450.
- 456 7. Lewis, K. (2007) Persister cells, dormancy and infectious disease, *Nat Rev Micro* 5, 48-56.
- 457 8. Barraud, N., Kelso, M. J., Rice, S. A., and Kjelleberg, S. (2015) Nitric oxide: a key mediator of
458 biofilm dispersal with applications in infectious diseases, *Curr. Pharm. Des.* 21, 31-42.
- 459 9. Barraud, N., Hassett, D. J., Hwang, S.-H., Rice, S. A., Kjelleberg, S., and Webb, J. S. (2006)
460 Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*, *J. Bacteriol.*
461 188, 7344-7353.
- 462 10. Howlin, R., Cathie, K., Hall-Stoodley, L., Niehaus, L., Connett, G., Legg, J., Daniels, T., Carroll,
463 M., Jefferies, J., Clarke, S. C., Stoodley, P., Webb, J., and Faust, S. N. (2011) Nitric oxide-
464 mediated dispersal and enhanced antibiotic sensitivity in *Pseudomonas aeruginosa* biofilms
465 from the cystic fibrosis lung, *Arch. Dis. Child.* 96, A45.
- 466 11. Huynh, T. T., McDougald, D., Klebensberger, J., Al Qarni, B., Barraud, N., Rice, S. A.,
467 Kjelleberg, S., and Schleheck, D. (2012) Glucose starvation-induced dispersal of
468 *Pseudomonas aeruginosa* biofilms is cAMP and energy dependent, *PLoS ONE* 7, e42874.
- 469 12. Gjermansen, M., Ragas, P., Sternberg, C., Molin, S., and Tolker-Nielsen, T. (2005)
470 Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms, *Environ.*
471 *Microbiol.* 7, 894-904.
- 472 13. Rowe, M. C., Withers, H. L., and Swift, S. (2010) Uropathogenic *Escherichia coli* forms biofilm
473 aggregates under iron restriction that disperse upon the supply of iron, *FEMS Microbiol. Lett.*
474 307, 102-109.
- 475 14. Katrina, C., Robert, H., Nicolas, B., Mary, C., Stuart, C., Gary, C., Victoria, C., Thomas, D.,
476 Caroline, D., Martin, F., Bernadette, F., Luanne, H.-S., Johanna, J., Michael, K., Staffan, K.,
477 Julian, L., Sandra, P., Scott, R., Geraint, R., Rami, S., Caroline, S., Paul, S., Priya, S., Jeremy,
478 W., and Saul, F. (2014) Low Ddose nitric oxide as adjunctive therapy to reduce antimicrobial
479 tolerance of *Pseudomonas aeruginosa* biofilms in the treatment of patients with cystic
480 fibrosis: report of a proof of concept clinical trial, In *B38. Update in adult cystic fibrosis*, pp
481 A2843-A2843, American Thoracic Society.
- 482 15. Gasco, A., Fruttero, R., Sorba, G., Di Stilo, A., and R, C. (2004) NO donors: Focus on furoxan
483 derivatives, *Pure Applied Chemistry* 76, 973 - 981.
- 484 16. Feelisch, M., Schonafinger, K., and Noack, E. (1992) Thiol-mediated generation of nitric oxide
485 accounts for the vasodilator action of furoxans, *Biochem. Pharmacol.* 44, 1149-1157.
- 486 17. Sorba, G., Medana, C., Fruttero, R., Cena, C., Di Stilo, A., Galli, U., and Gasco, A. (1997) Water
487 soluble furoxan derivatives as NO prodrugs, *J. Med. Chem.* 40, 463-469.
- 488 18. Schönafinger, K. (1999) Heterocyclic NO prodrugs, *Il Farmaco* 54, 316-320.
- 489 19. Cena, C., Lolli, M. L., Lazzarato, L., Guaita, E., Morini, G., Coruzzi, G., McElroy, S. P., Megson,
490 I. L., Fruttero, R., and Gasco, A. (2003) Antiinflammatory, gastrosparring, and antiplatelet
491 properties of new NO-donor esters of aspirin, *J. Med. Chem.* 46, 747-754.
- 492 20. Boschi, D., Tron, G. C., Lazzarato, L., Chegaev, K., Cena, C., Di Stilo, A., Giorgis, M.,
493 Bertinaria, M., Fruttero, R., and Gasco, A. (2006) NO-donor phenols: A new class of
494 products endowed with antioxidant and vasodilator properties, *J. Med. Chem.* 49, 2886-2897.

- 495 21. Hugo, C., and Williams, P. (2005) Pharmacological properties of furoxans and benzofuroxans:
496 recent developments, *Mini-Rev. Med. Chem.* 5, 57-71.
- 497 22. Fruttero, R., Crosetti, M., Chegaev, K., Guglielmo, S., Gasco, A., Berardi, F., Niso, M., Perrone,
498 R., Panaro, M. A., and Colabufo, N. A. (2010) Phenylsulfonylfuroxans as modulators of
499 multidrug-resistance-associated protein-1 and P-glycoprotein, *J. Med. Chem.* 53, 5467-5475.
- 500 23. Chegaev, K., Riganti, C., Lazzarato, L., Rolando, B., Guglielmo, S., Campia, I., Fruttero, R.,
501 Bosia, A., and Gasco, A. (2011) Nitric oxide donor doxorubicins accumulate into
502 doxorubicin-resistant human colon cancer cells inducing cytotoxicity, *ACS Med. Chem. Lett.*
503 2, 494-497.
- 504 24. Barraud, N., Schleheck, D., Klebensberger, J., Webb, J. S., Hassett, D. J., Rice, S. A., and
505 Kjelleberg, S. (2009) Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates
506 phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal, *J.*
507 *Bacteriol.* 191, 7333-7342.
- 508 25. Thomas, D. D., Ridnour, L. A., Isenberg, J. S., Flores-Santana, W., Switzer, C. H., Donzellie, S.,
509 Hussain, P., Vecoli, C., Paolucci, N., Ambs, S., Colton, C., Harris, C., Roberts, D. D., and
510 Wink, D. A. (2008) The chemical biology of nitric oxide. implications in cellular signaling,
511 *Free Rad. Biol. Med.* 45, 18-31.
- 512 26. Miller, M. R., and Megson, I. L. (2007) Recent developments in nitric oxide donor drugs, *Br. J.*
513 *Pharmacol.* 151, 305-321.
- 514 27. Schleheck, D., Barraud, N., Klebensberger, J., Webb, J. S., McDougald, D., Rice, S. A., and
515 Kjelleberg, S. (2009) *Pseudomonas aeruginosa* PAO1 preferentially grows as aggregates in
516 liquid batch cultures and disperses upon starvation, *PLoS One* 4, e5513.
- 517 28. Chua, S. L., Liu, Y., Yam, J. K. H., Chen, Y., Vejborg, R. M., Tan, B. G. C., Kjelleberg, S.,
518 Tolker-Nielsen, T., Givskov, M., and Yang, L. (2014) Dispersed cells represent a distinct
519 stage in the transition from bacterial biofilm to planktonic lifestyles, *Nat Commun* 5.
- 520 29. Sasnow, S. S., Wei, H., and Aristilde, L. (2016) Bypasses in intracellular glucose metabolism in
521 iron limited *Pseudomonas putida*, *MicrobiologyOpen* 5, 3-20.
- 522 30. Hider, R. C., and Kong, X. (2010) Chemistry and biology of siderophores, *Nat. Prod. Rep.* 27,
523 637-657.
- 524 31. Duong, H. T. T., Jung, K., Kutty, S. K., Agustina, S., Adnan, N. N. M., Basuki, J. S., Kumar, N.,
525 Davis, T. P., Barraud, N., and Boyer, C. (2014) Nanoparticle (star polymer) delivery of nitric
526 oxide effectively negates *Pseudomonas aeruginosa* biofilm formation, *Biomacromolecules*
527 15, 2583-2589.
- 528 32. Wurst, J. M., Drake, E. J., Theriault, J. R., Jewett, I. T., VerPlank, L., Perez, J. R., Dandapani, S.,
529 Palmer, M., Moskowitz, S. M., Schreiber, S. L., Munoz, B., and Gulick, A. M. (2014)
530 Identification of inhibitors of PvdQ, an enzyme involved in the synthesis of the siderophore
531 pyoverdine, *ACS Chem. Biol.* 9, 1536-1544.
- 532 33. Oglesby-Sherrouse, A. G., Djapgne, L., Nguyen, A. T., Vasil, A. I., and Vasil, M. L. (2014) The
533 complex interplay of iron, biofilm formation, and mucoidy affecting antimicrobial resistance
534 of *Pseudomonas aeruginosa*, *Path. Dis.* 70, 307-320.
- 535 34. Lamont, I. L., Beare, P. A., Ochsner, U., Vasil, A. I., and Vasil, M. L. (2002) Siderophore-
536 mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*, *Proc.*
537 *Natl. Acad. Sci, USA* 99, 7072-7077.
- 538 35. Banin, E., Vasil, M. L., and Greenberg, E. P. (2005) Iron and *Pseudomonas aeruginosa* biofilm
539 formation, *Proc. Natl. Acad. Sci. U.S.A.* 102, 11076-11081.
- 540 36. Fang, Y.-I., Ohata, H., and Honda, K. (2009) Fluorometric determination of nitrite with 2,3-
541 diamionaphthalene by reverse phase HPLC under alkaline conditions, *J. Pharmacol.*
542 *Toxicol. Methods* 59, 153-155.
- 543 37. Tan, S. Y.-Y., Liu, Y., Chua, S. L., Vejborg, R. M., Jakobsen, T. H., Chew, S. C., Li, Y., Nielsen,
544 T. E., Tolker-Nielsen, T., Yang, L., and Givskov, M. (2014) Comparative systems biology
545 analysis to study the mode of action of the isothiocyanate compound iberin on *Pseudomonas*
546 *aeruginosa*, *Antimicrob. Agents Chemother.* 58, 6648-6659.
- 547 38. Barraud, N., Moscoso, J. A., Ghigo, J.-M., and Filloux, A. (2014) Methods for studying biofilm
548 dispersal in *Pseudomonas aeruginosa*, In *Pseudomonas Methods and Protocols* (Filloux, A.,
549 and Ramos, J.-L., Eds.), pp 643-651, Springer New York, New York, NY.

550 **Figures**



551

552 **Figure 1.** Structures of LL4254 (a), LL4212 (b) and LL4216 (c).

553

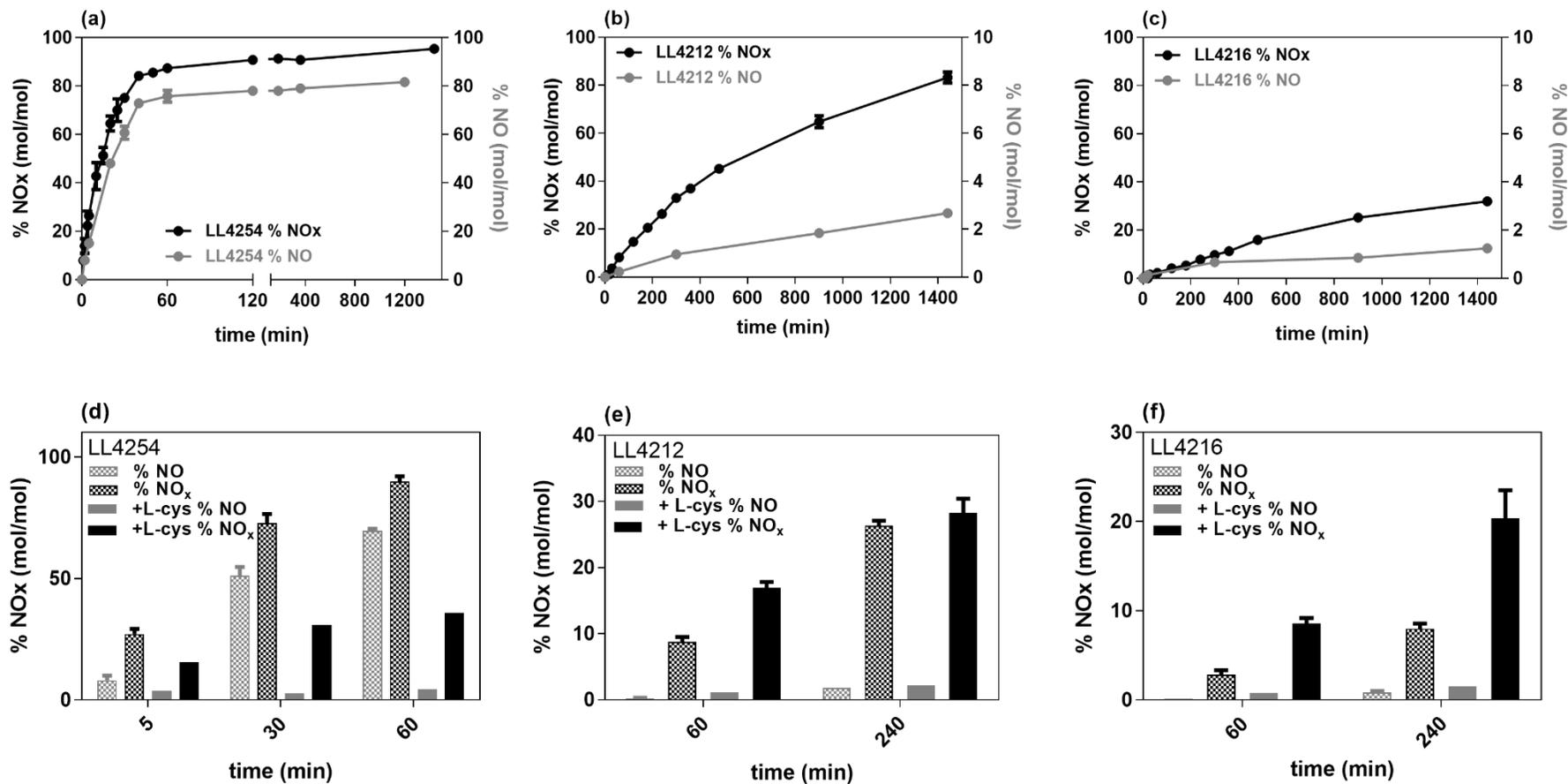


Figure 2. NO (gray circle) and NO_x (NO and NO₂⁻) (black circle) release kinetics of LL4254 (a, d), LL4212 (b, e) and LL4216 (c, f) in phosphate buffer at pH 7.4 in the absence of L-cysteine over time (a – c) and in the presence or absence of L-cysteine at selected time points (d – f). The results are expressed as percent (% mol/mol) of NO or NO_x released with respect to the quantity of parent furoxan compound. Bars or symbols represent data from three or more replicates and error bars represent standard deviation from the mean.

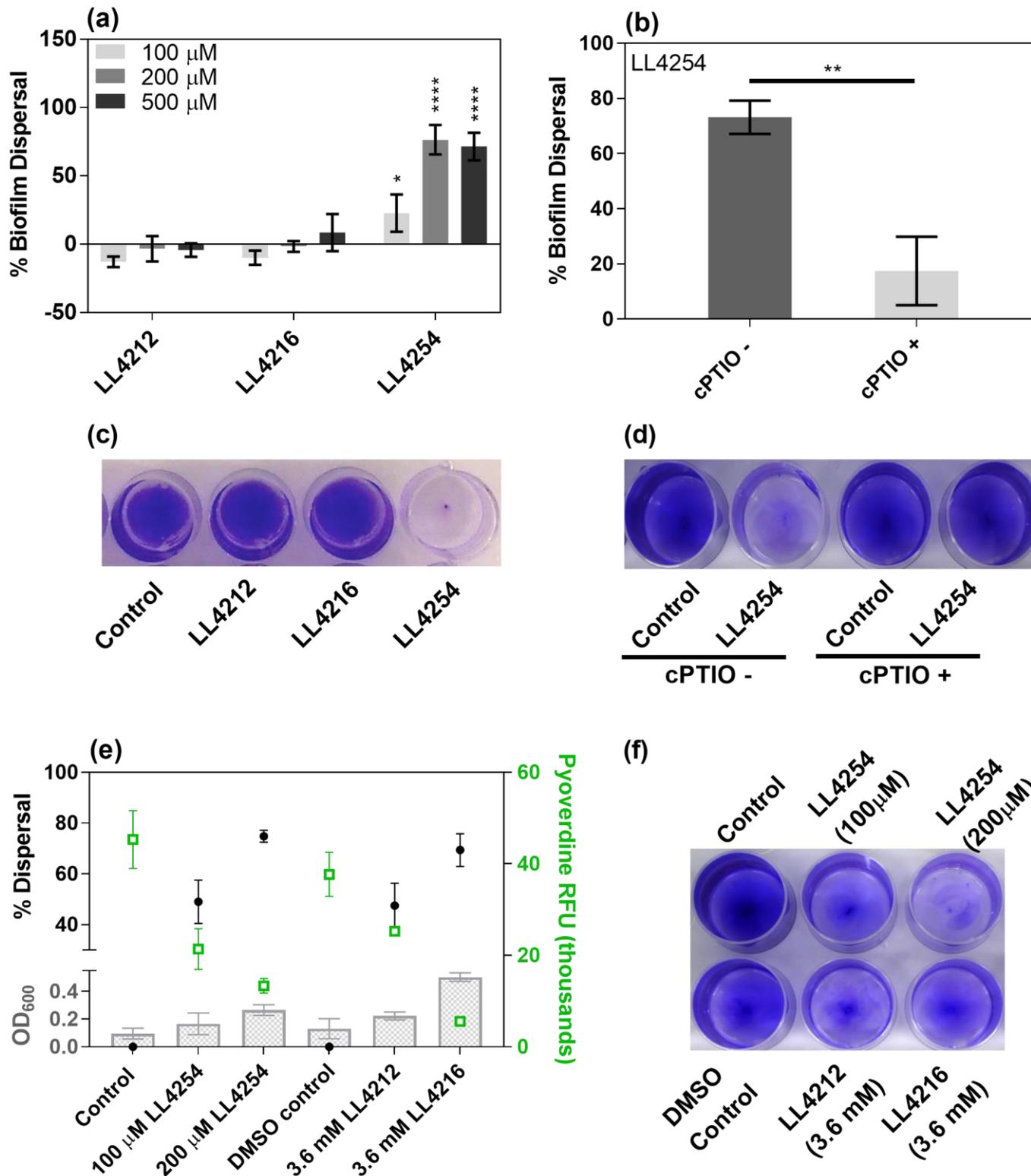


Figure 3. Dispersal of *P. aeruginosa* biofilms upon treatment with the furoxans for 1 h (a), or upon treatment with 200 μ M of LL4254 in the presence or absence of 0.5 mM cPTIO (b).

The extent of dispersal (black circle) corresponds to an increase in OD₆₀₀ (gray bars) and a decrease in pyoverdine fluorescence (green squares) (e). Bars or symbols represent data from three (a, b) or two (e) biological replicates whereas error bars represents the standard deviation from the mean – *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Photographs show CV stains of remaining biofilms following 200 μM of furoxan treatment (c, d) with or without cPTIO (d) or when treated with different concentrations of furoxans (f).

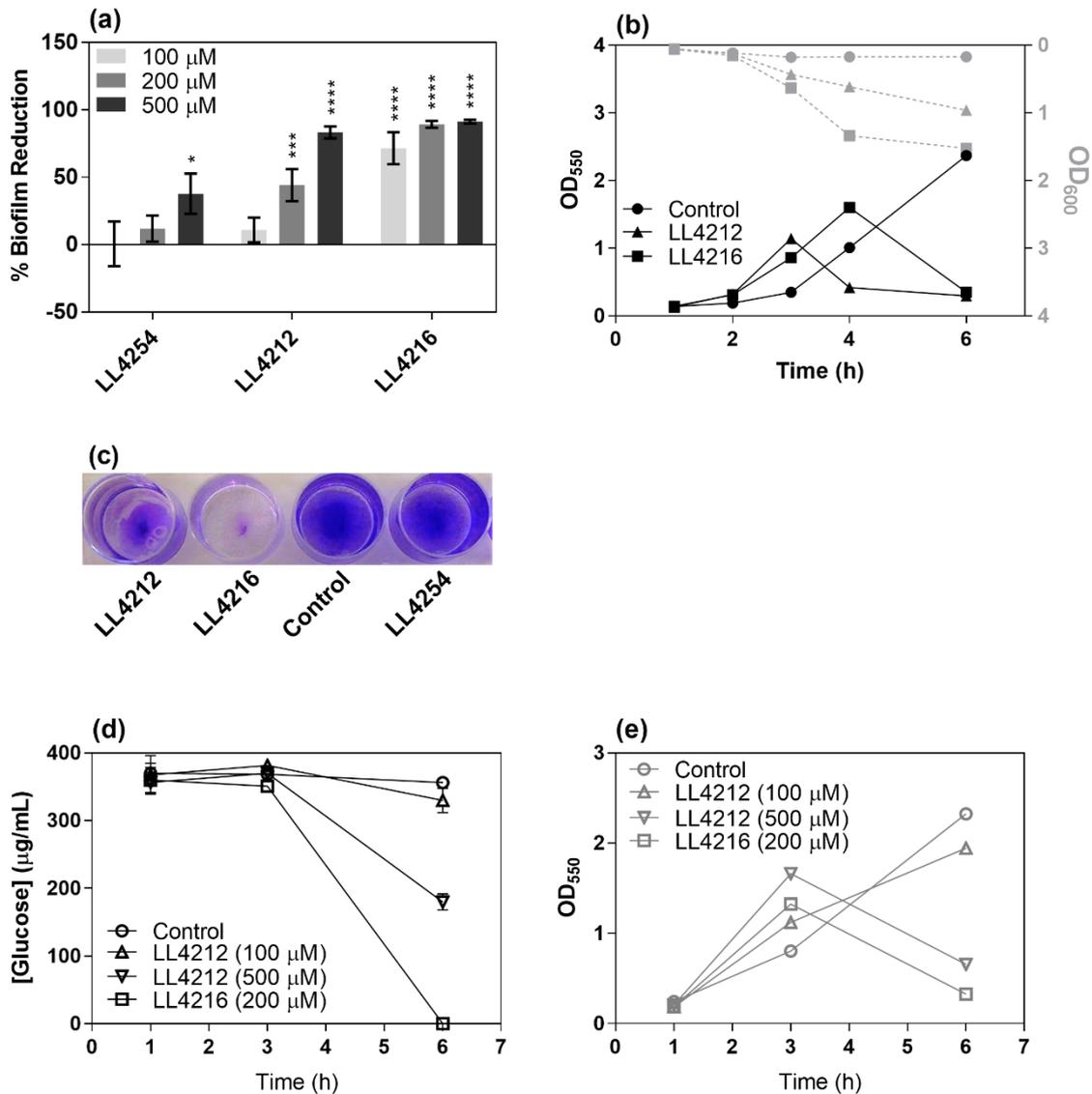


Figure 4. Reduction of *P. aeruginosa* biofilms upon treatment with furoxans for 6 h (a), with bars representing data from three biological replicates and errors bars representing standard deviation of the mean – *, $p \leq 0.05$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Photographs show CV stains of biofilm remaining after 6 h following 200 μM furoxan treatment (c). Biofilm formation and corresponding OD₆₀₀ changes over 6 h upon addition of 200 μM of LL4216 and 500 μM LL4212 (b). Glucose concentrations were quantified at t = 1 h, 3 h or 6 h after addition of furoxans (d), with corresponding changes in CV staining measured (e). Symbols

represent data from two biological replicates with error bars showing the standard deviation of the mean in (e), while one representative data set was plotted for (b) and (e).

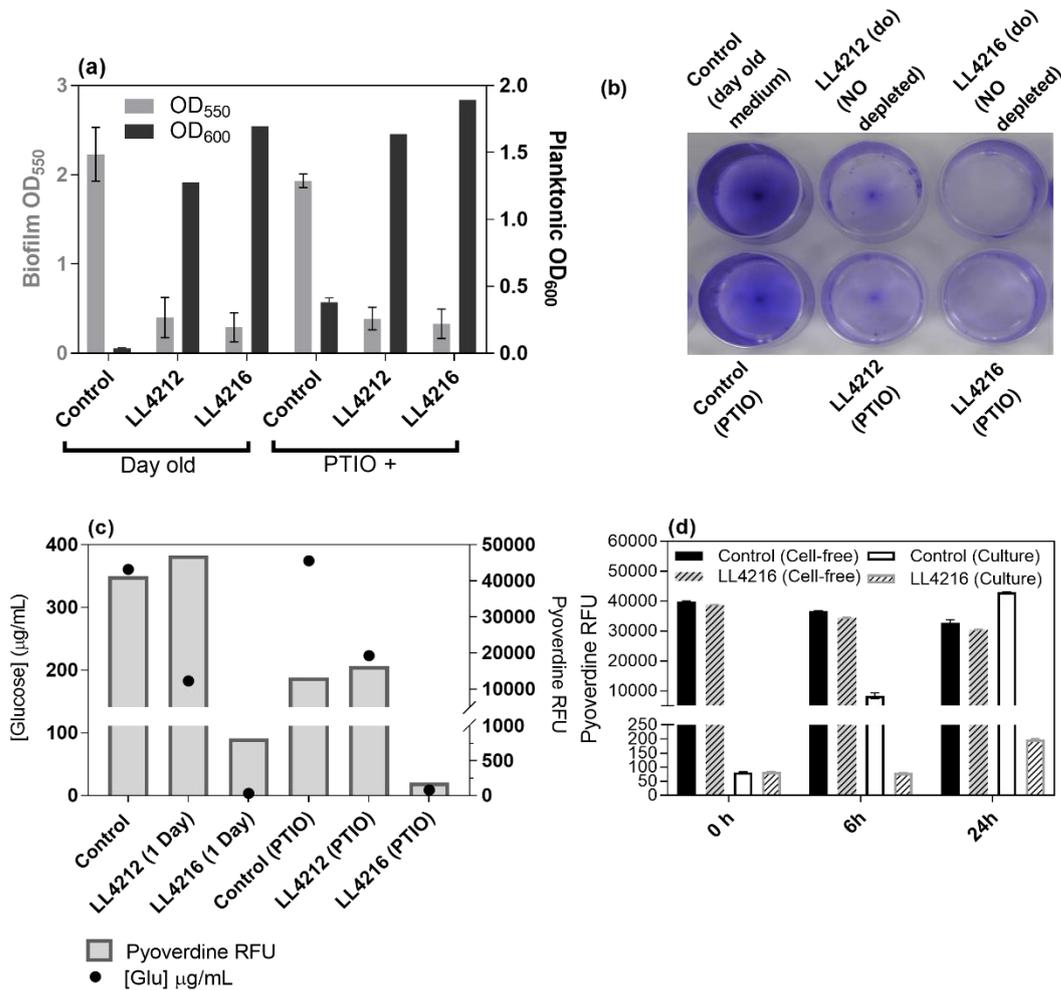


Figure 5. *P. aeruginosa* biofilm reduction upon treatment with 500 µM of furoxans in the presence of 0.5 mM cPTIO or NO-depleted furoxans in 1-day-old M9GC medium. Bars of OD₅₅₀ measurements represent data from three biological replicates while bars of OD₆₀₀ measurements represent data from one experiment. Errors bars represent standard deviation of the mean (a). Photograph show CV stains of biofilm remaining after 6 h following furoxan treatment (b). Changes in pyoverdine levels and glucose concentrations of samples treated as described in (a), with one representative data set plotted (c). Pyoverdine changes over time in cell-free medium or culture in the presence or absence of 500 µM LL4216 (d).

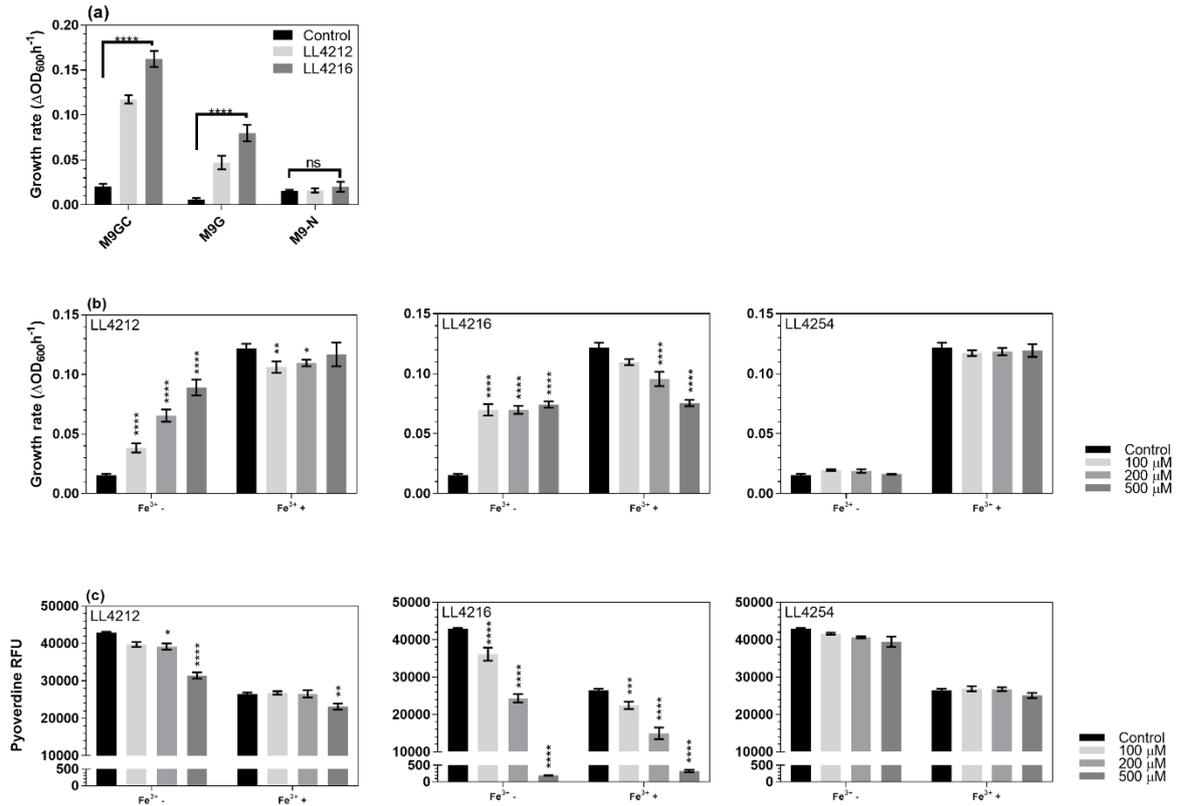


Figure 6. Growth rates in the absence or presence of 500 μM of LL4212, LL4216 and LL4254, calculated by changes in OD_{600} during the exponential growth phase, in M9 medium supplemented with different carbon or nitrogen sources (a). Growth rates (b) and pyoverdine fluorescence (c) at 24 h of *P. aeruginosa* inoculated statically at 37°C in M9GC (Fe^{3+} -) or M9GCFe (Fe^{3+} +) medium in the presence of 100 – 500 μM of the furoxans. Bars represent data from three biological replicates and errors bars representing standard deviation of the mean. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$