



Natural quorum sensing inhibitors effectively downregulate gene expression of *Pseudomonas aeruginosa* virulence factors

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1 Natural Quorum Sensing Inhibitors
2 Effectively Downregulate Gene Expression
3 of *Pseudomonas aeruginosa* Virulence
4 Factors
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22 **ABSTRACT**

23 At present anti-virulence drugs are being considered as potential therapeutic alternatives and/or
24 adjuvants to currently failing antibiotics. These drugs do not kill bacteria but inhibit virulence
25 factors essential for establishing infection and pathogenesis through targeting non-essential
26 metabolic pathways reducing the selective pressure to develop resistance. We investigated the
27 effect of naturally isolated plant compounds on the repression of the quorum sensing (QS)
28 system which is linked to virulence/pathogenicity in *Pseudomonas aeruginosa*. Our results show
29 that *trans*-cinnamaldehyde (CA) and salicylic acid (SA) significantly inhibit expression of QS
30 regulatory and virulence genes in *P. aeruginosa* PAO1 at sub-inhibitory levels without any
31 bactericidal effect. CA effectively downregulated both the *las* and *rhl* QS systems with *lasI* and
32 *lasR* levels inhibited by 13- and 7-fold respectively compared to 3- and 2-fold reductions with SA
33 treatment, during the stationary growth phase. The QS inhibitors (QSI) also reduced the
34 production of extracellular virulence factors with CA reducing protease, elastase and pyocyanin
35 by 65%, 22% and 32%, respectively. The QSIs significantly reduced biofilm formation and
36 concomitantly with repressed rhamnolipid gene expression, only trace amount of extracellular
37 rhamnolipids were detected. The QSIs did not completely inhibit virulence factor expression and
38 production but their administration significantly lowered the virulence phenotypes at both the
39 transcriptional and extracellular level. This study shows the significant inhibitory effect of natural
40 plant derived compounds on the repression of QS systems in *P. aeruginosa*.

41

42 **Keywords**

43 Trans-cinnamaldehyde, salicylic acid, quorum sensing, quorum sensing inhibitor, *Pseudomonas*
44 *aeruginosa*

45 **INTRODUCTION**

46 A review commissioned by the UK government in 2014 (<https://amr-review.org/>) predicted that
47 there will be more deaths in the world due to antimicrobial resistance (AMR) than cancer by the
48 year 2050. Antibiotic usage creates an evolutionary stress response in the bacterial population
49 that, over time leads to the emergence of resistant strains. Extensive use of antibiotics coupled
50 with the diminished pipeline of new antibiotics has seen a rapid evolution of resistance that has
51 culminated in the development of multi-drug resistant pathogens that are extremely difficult to
52 treat.

53 *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen is prevalent in
54 immunocompromised patients suffering from cystic fibrosis (CF) and human immunodeficiency
55 virus (HIV). These bacteria are notorious biofilm producers. The biofilm provides a stratified
56 environment with the core being more anoxic with low bacterial growth and metabolic rates.
57 These metabolically inactive biofilm cells are resistant to the β -lactam antibiotics (Anwar and
58 Costerton 1990, Werner et al 2004) ciprofloxacin, tetracycline and tobramycin (Brown et al 1988).
59 The biofilm layer also acts as a diffusion barrier reducing the rate of antibiotic penetration,
60 preventing sufficient accumulation of antibiotics and allowing time for expression of resistance
61 genes (Jefferson et al 2005). In CF, *P. aeruginosa* forms biofilms and readily adapts to the lung
62 environment eventually leading to prolonged inflammation and chronic lung infections that are

63 very difficult to treat using conventional antibiotic methods. In addition, the presence of
64 inducible (MexXY) and constitutive (MexAB-OprM) efflux pumps and the poor permeability of
65 the outer membrane also contribute to the reduced susceptibility of *P. aeruginosa* to a broad
66 range of antibiotics (Aghazadeh et al 2014, Lopez-Causape et al 2017). Effective treatment of *P.*
67 *aeruginosa* is therefore becoming increasingly challenging with the bacterium showing resistance
68 to even the third and the fourth generations of carbapenems and cephalosporins (Luna et al
69 2013, Patel et al 2014). Therefore, it has become critical to find alternative therapies to
70 successfully clear *P. aeruginosa* infections.

71 *P. aeruginosa* produces a variety of virulence factors, in a coordinated system, that are reported
72 to enable host colonisation and adaptation (Valderrey et al 2010, Gellatly and Hancock 2013,
73 Sousa and Pereira 2014). These virulence factors include the production of biofilm, pyocyanin,
74 elastase and rhamnolipid and are under the control of a cell density dependent signalling
75 regulation known as quorum sensing (QS) (Stover et al 2000, Lee and Zhang 2014, Sousa and
76 Pereira 2014) The canonical QS system in *P. aeruginosa* includes the *las* and the *rhl* systems both
77 consisting of Lux-I type synthases (LasI and RhII) which produce specific acyl homoserine lactone
78 (AHL) molecules, N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C₁₂-HSL) and N-butanoyl-L-
79 homoserine lactone (C₄-HSL). At sufficiently high bacterial concentrations, these AHL molecules
80 then bind to the Lux-R type receptors (LasR and RhIR) to form transcriptional activation
81 complexes which regulate the transcription of various genes involved with virulence of *P.*
82 *aeruginosa* (Papenfort and Bassler 2016).

83 Interfering with this QS system through application of QS inhibitors (QSI) is a novel therapeutic
84 target that has shown to effectively reduce virulence in opportunistic pathogens. The disruption

85 of QS communication can be achieved through the enzymatic degradation of AHL molecules by
86 lactonases, acylases and oxidoreductases or by using small structural molecules that inhibit the
87 QS signal molecule from binding to its cognate regulatory protein (Morohoshi et al 2009, Kalia
88 2013, Kisch et al 2014, Gupta et al 2015). A synthetic derivative of a furanone, compound C30
89 (C30F), has been shown to suppress bacterial QS in mice lung models through interference with
90 AHL production (Wu et al 2004) and through attenuation of QS regulated production of virulence
91 factors (Henter et al 2003). In the recent past, a range of plant compounds have shown to be
92 effective as anti-QS and anti-biofilm agents (Musthafa et al 2010, Jayelakshmi et al 2016, Ouyang
93 et al 2016, Luo et al 2017). Kim et al. (2015), using an *in silico* approach, predicted that natural
94 gingerol could bind to the QS regulator LasR protein. They then demonstrated, using standard
95 assays, a decrease in production of several virulence factors and biofilm formation following
96 exposure to gingerol, consistent with interference of the binding of the cognate signal molecule,
97 3-oxo-C₁₂-HSL, to LasR. Moreover, access to crystal structure of LasI (Gould et al 2004) and LasR
98 (Bottomley et al 2007) along with the availability of computer-aided programs like structure
99 based virtual screening (SB-VS) and molecular docking have been useful in identifying more
100 compounds with potential anti-QS abilities.

101 A SB-VS experiment unlocked six drugs with LasR structural similarity including salicylic acid (SA),
102 nifuroxazide, and chlorzoxazone (Yang et al 2009). These compounds were able to significantly
103 inhibit QS gene expression and phenotypes in *P. aeruginosa*. In another study, molecular docking
104 results showed that a plant compound, trans-cinnamaldehyde (CA), was able to interact with the
105 LasI substrate binding sites by forming hydrophobic and π - π bonds with Phenylalanine- 27 and
106 105, Tryptophan-33 and a hydrogen bond with Arginine-30 in the LasI synthase (Chang et al

107 2014). Since QS is related to several virulence mechanisms in *P. aeruginosa*, therefore the ability
108 of compounds like SA and CA to interfere with the QS system can open the possibility of utilizing
109 these as effective anti-QS agents for controlling the pathogenic phenotypes of *P. aeruginosa*.
110 QS system allows bacteria to adapt to changing environmental conditions at the population level,
111 with the adaptation mediated at the transcriptional level via regulated expression of the QS
112 genes in response to metabolic and environmental stimuli (Wagner et al 2003, Scott and Hwa
113 2011). Understanding the transcriptional expression of the QS genes is therefore essential for
114 understanding the physiology of the cell under QS inhibitory conditions. The current information
115 on the ability of CA and SA to reduce QS activity in *P. aeruginosa* is very limited and has been
116 mostly acquired through crude estimations of virulence proteins or by using high throughput
117 microarray analysis for identifying changes to gene expression (Prithiviraj et al 2005, Yang et al
118 2009). Therefore, in this study we have used a very robust and MIQE (minimum information for
119 publication of quantitative real-time PCR experiments) compliant reverse transcription-
120 quantitative PCR (RT-qPCR) assay, a gold standard for low-medium throughput quantitative
121 expression analysis, to study the changes in transcriptomic profiles when *P. aeruginosa* is
122 subjected to CA and SA treatments at sub-inhibitory concentrations. To correlate the effects of
123 the gene expression on the phenotypic profiles following QSI treatment, the QS regulated
124 virulence factors rhamnolipid, elastase, protease and pyocyanin were estimated.

125

126 **Materials and Methods**

127 **Bacterial strains and media**

128 The fully sequenced and widely reported laboratory strain *P. aeruginosa* PAO1 (ATCC 15692) was
129 used in the study. Overnight cultures were prepared from -80°C frozen culture stocks in a nutrient
130 rich LB broth at 37°C under shaking conditions at 180 rpm. This culture was subsequently used
131 to inoculate proteose-peptone-glucose-ammonium-salts (PPGAS) medium (Zhang and Miller
132 1992). The bacteria were cultivated in PPGAS medium at 1/5th MIC levels of 2.27 mM CA and 3.62
133 mM SA in either single or combination treatments. A positive control for QS was included using
134 10 µM C30F (Skindersoe et al 2008). All experiments were carried out in biological triplicates. The
135 experimental compounds were purchased from Sigma-Aldrich, UK unless otherwise stated.

136 **Minimum Inhibitory Concentration (MIC) determination**

137 The MIC of the test inhibitors against *P. aeruginosa* PAO1 was determined using the resazurin
138 microtiter plate assay (Elshikh et al 2016) which used the redox indicator resazurin that changed
139 colour from blue to pink in the presence of viable cells. The MIC was determined as the
140 concentration at which there was no colour change following 4 hours incubation of the overnight
141 cells with 0.015% resazurin.

142 **RNA isolation and purity assessment**

143 The cell pellets were collected from different growth phase cultures by spinning them at 13000 x
144 g for 2-3 minutes at room temperature and the RNA extracted using JetGene RNA Purification Kit
145 (Thermo Fisher Scientific). The cells were lysed with occasional vortexing in a buffer solution with
146 1X TE buffer, 15 mg/ml lysozyme and 20 mg/ml proteinase K (Promega). The samples were then

147 transferred to a 2 ml Lysing Matrix A tube (MP Biomedicals) with β -mercaptoethanol containing
148 RLT buffer (provided in the kit) for enhanced lysis. The contents in the lysing matrix tubes were
149 then homogenized using the FastPrep™ FP 200 cell disrupter at speed 5.5 for 30 seconds. A
150 double DNA-digestion treatment was done to ensure that the RNA was free of any genomic DNA
151 (gDNA) contamination. The RNA isolated was quantified using the Nanodrop spectrophotometer
152 with A_{260}/A_{280} ratio of 1.8-2.1 being considered as pure. The integrity of the samples was checked
153 by agarose gel electrophoresis for presence of two sharp distinct bands representing 23S and 16S
154 rRNA. The integrity was further verified by analysing the samples in an Agilent 2100 Bioanalyzer
155 where RNA Integrity Number (RIN) values greater than 8 were observed for all samples. The RIN
156 is based on a numbering system from 1-10 with 1 being the most degraded and 10 being the
157 most intact. The RNA samples were aliquoted and stored at -80°C.

158 **Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

159 First strand cDNA was synthesized using Superscript™ Reverse Transcriptase II (Invitrogen). Each
160 reaction mix contained DNase treated RNA (500 ng), 20-250 ng random primers (Promega), 10
161 mM dNTPs and RNase free water to make to the reaction volume 15.6 μ l. The reactions were
162 heated at 65°C for 5 minutes before adding 5X strand buffer, 0.1 M DTT and RNase inhibitor
163 (RNase out™ Invitrogen) in final concentrations of 1X, 10 μ M and 40 units respectively. The
164 reactions were incubated at 25°C for 2 minutes before adding Superscript™ II Reverse
165 Transcriptase (200 units final concentration) (Invitrogen). The RT reactions were carried out at a
166 series of temperature starting with 25°C for 10 minutes, 42°C for 50 minutes and 70°C for 15
167 minutes. The first strand cDNA synthesis was performed for all the biological triplicates from each

168 time point. A negative reaction without reverse transcriptase was included in every run. All cDNA
169 samples were stored at -20°C prior to use.

170 The cDNA synthesised was then used as a template for real time PCR amplification using the
171 ROCHE LightCycler LC480 system with a SYBR-Green probe. Since PCR efficiency is highly
172 dependent on primer specificity therefore a qPCR calibration curve was generated from each
173 primer set using PAO1 gDNA. Only those primers which gave a calibration curve with a slope
174 value between -3.1 to -3.6 that translated into amplification efficiencies of 90-110% were used
175 for PCR quantification. The binding specificity of these primers were also validated post-
176 amplification by generating a melt curve for each primer set with the presence of a single sharp
177 peak eliminating the chances of any non-specific binding.

178 The qPCR 10 µl reaction mix each contained 2X SYBR Green master mix (1X), forward and reverse
179 primers (1 µM), cDNA template and molecular grade water. Negative controls in form of -RT (no
180 reverse transcriptase) and no template control NTC (no DNA template added) were included to
181 rule out any contamination during the preparation process. A positive control in the form of gDNA
182 was also included. The cut-off values for residual gDNA amplification and NTC were set at greater
183 than 35 and 40 cycles respectively. The cycling parameters were: initial denaturation at 95°C for
184 5 minutes, 40-50 cycles of denaturation at 95°C for 10 seconds, annealing at 59°C for 10 seconds,
185 extension at 72°C for 10 seconds.

186 **Reference gene validation**

187 A total of six candidate genes (*gyrB*, *proC*, *cysG*, *rpoD*, *rpoB* and *16S*) were analysed under
188 inhibitory conditions to assess for the most stable and reliable reference genes for this study. The

189 stability of the six candidate genes were validated under inhibitory conditions using three
190 independent software packages geNorm (Vandesompele et al 2002), NormFinder (Andersen et
191 al 2004) and BestKeeper (Pfaffl et al 2004). The geNorm algorithm measures the stability of the
192 genes based on pairwise variation between one candidate gene and the other genes and was
193 calculated using the online available tool RefFinder (Fu et al 2013). The NormFinder model
194 considers the intra- and inter- group variation to calculate the stability of the genes using a R-
195 based software excel package (MOMA, Aarhus University Hospital, Denmark). The BestKeeper is
196 a free excel based tool that correlated the coefficient of the candidate gene with a BestKeeper
197 Index to generate the most stable gene. The genes *rpoD* and *proC* were identified as most stable
198 for use as reference genes in this study by the three algorithms.

199 **Relative Gene Expression Data Analysis**

200 System (LC480 software, version 2) generated analysis was performed on the real-time PCR data.
201 The threshold values (Cq) values from each of the qPCR run was extracted from the LC480 system
202 using the second derivative maximum method (Rasumssen 2001). Data analysis was performed
203 by taking the arithmetic mean of the Cq values of the technical replicates and transferring it into
204 log values to generate the relative quantities (RQ). The RQ values of the target genes were then
205 divided by geometric mean of reference gene RQs (*rpoD* and *proC*) to give normalized relative
206 quantity value (NRQ). The NRQ value was then divided by the experimental calibration which in
207 the experiment was relative expression at early log (6-hour) and was set to 1. The output was the
208 calibration normalized ratio (CNRQ) which was used in extrapolating information on the
209 expression profile of the target genes.

210

211 **Production of virulence factors**

212 An overnight PAO1 culture was used to inoculate PPGAS medium and incubated for 24 hours
213 under continuous shaking at 37°C. The supernatant was collected, and filter sterilized for use in
214 the following assays:

215 **Protease:** The amount of LasA protease produced by PAO1 following incubation with and without
216 the inhibitors were estimated by adding 0.1 ml culture supernatant to a reaction mixture
217 containing 0.8% azocasein in 500 µl of 50mM K₂HPO₄ (pH 7) and incubating at 25°C for 3 hours.
218 The reaction was terminated by adding 0.5 ml of 1.5 M HCl and then keeping it on ice for 30
219 minutes. The precipitated protein was removed by centrifugation (10000 x g for 10 minutes).
220 NaOH (1N) was added to the supernatant in equal ratios and the concentration of acid soluble
221 azopeptides measured spectrophotometrically at 440 nm.

222 **Elastase:** The LasB elastase production was measured by adding 1 ml of the culture supernatant
223 to a 2 ml reaction buffer (100 mM Tris-HCl, 1 mM CaCl₂) containing the substrate elastin congo-
224 red and incubating for 3 hours at 37°C with shaking at 180 rpm. The reaction was terminated by
225 adding 2 ml of 0.7 M sodium phosphate buffer (pH 6) and placing it on ice for 15 minutes. The
226 absorbance of the supernatant was measured at 495 nm.

227 **Pyocyanin:** The pyocyanin concentration was estimated by adding 7.5 ml filtered supernatant to
228 4.5 ml of chloroform and vortexed until the colour changed to greenish blue. The samples were
229 centrifuged (10000 x g for 10 minutes) and 3 ml of the resulting blue coloured liquid was
230 transferred to a new tube containing 1.5 ml of 0.2 M HCl and shaken until the blue colour turned
231 to pink. The pink layer was transferred to a cuvette and the absorbance measured at 520 nm.

232 The concentration was calculated in $\mu\text{g}/\text{ml}$ by multiplying the absorbance by factor 17.072 (Essar
233 et al 1990).

234 **Rhamnolipid extraction and purification**

235 The extraction of rhamnolipid was performed following the method of Smyth et al. (2010). The
236 culture supernatant (50 ml) from PAO1 grown in PPGAS medium for 24 hours was acidified to pH
237 2 and extracted with ethyl acetate three times. The organic solvent containing rhamnolipid was
238 dried with anhydrous MgSO_4 to remove residual water. Rhamnolipid was isolated from the ethyl
239 acetate solvent in the form of yellow gummy residue after removing the organic solvent in a
240 rotary evaporator. The rhamnolipid crude extract was then purified using solid phase extraction
241 by running the samples through Strata SI-1 Silica (55 μM , 70A) Giga tubes (Phenomenex). After
242 conditioning and removing the impurities from the column with chloroform, rhamnolipids were
243 eluted using chloroform and methanol in ratios of 5:0.3, 5:0.5 and 1:1.

244 **Rhamnolipid separation and analysis by high performance liquid chromatography-mass 245 spectrometry/mass spectrometry (HPLC-MS/MS)**

246 Analysis of the extracted rhamnolipid mixture was performed using a LCQTM quadrupole ion trap
247 with a negative electrospray ionisation (ESI) interface connected to a Thermo HPLC Spectra
248 system. A reverse phase C18 column with 5 μm particles was used to separate the rhamnolipids.
249 The parameters included desolvation gas at 65 units and source temperature 250°C, 20 μl
250 injection volume and 0.5 $\mu\text{l}/\text{min}$ flow rate. Two mobile phases were used: HPLC grade water (A)
251 and acetonitrile (B). The rhamnolipid congeners were resolved in a linear gradient mobile phase
252 starting with 70%A:30%B to 30%A:70%B over 50 minutes and then back to 70%A:30%B for 55

253 minutes with a final hold of 5 minutes. Tandem mass spectrometry was carried out using ESI in a
254 negative mode using collision induced dissociation (CID) at 35% peak within the MS range of 50-
255 800 m/z.

256 **Statistical analysis**

257 All statistical analysis was performed using the GraphPad prism v5.

258

259 **RESULTS**

260 **Growth phase dependent expression of QS genes**

261 The effect of the QSIs on the QS system of the fully sequenced laboratory strain *P. aeruginosa*
262 PAO1 (Stover et al 2000) was investigated by studying the transcriptional expression of the QS
263 synthase and regulatory genes. Both *lasR/lasI* and *rhlR/rhlI* systems were expressed in a cell
264 density dependent manner with expression levels increasing upon entering the stationary phases
265 of growth (Fig. 1B). Maximum expression levels for all genes was detected in the mid-late
266 stationary phase corresponding with highest cell density. In both *las* and *rhl* systems the
267 autoinducer synthase genes (*lasI* and *rhlI*) were expressed earlier and at much higher relative
268 concentrations in comparison to their cognate regulatory protein genes (*lasR* and *rhlR*). At high
269 concentrations, LasR and RhlR bind to their cognate N-acyl homoserine autoinducer molecules;
270 the bound complex is then a transcriptional regulator of several genes in *P. aeruginosa*.

271 The QS system regulates productions of most of the *P. aeruginosa* virulence factors including the
272 low molecular weight glycolipids rhamnolipids, that are under the direct regulation of the RhlR-

273 RhII system. The rhamnolipid biosynthetic genes display a differential expression profile where
274 *rhIA* and *rhIB* are expressed earlier relative to *rhIC*, which is only maximally expressed after
275 significant *rhIAB* expression (Fig. 2A-C). The products of *rhIAB* are responsible for the first step in
276 rhamnolipid biosynthesis, which produce mono-rhamnolipids. Mono-rhamnolipids are in turn
277 the substrate for the *rhIC* gene product to produce di-rhamnolipids. The differential sequential
278 expression pattern observed for the rhamnosyltransferases from this data is suggestive of a co-
279 ordinated regulation based on the substrate availability.

280 The other virulence-associated genes responsible for the production of the exoprotease LasA,
281 and elastase LasB, were also transcriptionally expressed in a cell density dependent manner with
282 maximum expression observed in mid-late stationary phase (Fig. 2D-E). The *las* regulated
283 virulence genes *lasA* and *lasB* were shown to be significantly upregulated during the mid-late
284 stationary phase with expression levels >300-fold relative to log phase levels ($p < 0.001$).

285

286 **Quorum Sensing Inhibitors (QSIs) effectively downregulate the QS regulatory** 287 **system**

288 Selectively interfering with QS systems is a novel strategy targeted at disarming virulent
289 opportunistic pathogens such as *P. aeruginosa*. In Gram negative bacteria, QS is typically
290 mediated by acyl-HSLs and rational analogues have been designed to specifically target these
291 systems. Several phenolic compounds have been shown to effectively disrupt QS systems in
292 Gram negative bacteria (Hossain et al 2017). In this part of the study, we investigated the anti-
293 QS abilities of naturally isolated plant compounds CA, SA and a synthetic furanone compound,

294 C30F, reported to attenuate virulence in *P. aeruginosa* (Fig. 3A) (Hentzer et al 2003, Yang et al
295 2009, Chang et al 2014). The minimum inhibitory concentration (MIC) of the test compounds CA
296 and SA were determined as 11.35 mM and 18.1 mM respectively. The use of the QSIs at the sub-
297 inhibitory concentrations (1/5th MIC) did not affect the growth phenotype of *P. aeruginosa* PAO1
298 (Fig. 3B). CA treatment resulted in a longer lag phase but reached similar optical densities to
299 untreated PAO1 within 6 hours of incubation. Since QS genes were significantly expressed in the
300 stationary phase (Fig. 1B), we tested the effect of the QSIs on the expression of QS associated
301 regulatory and virulence genes during mid to late stationary phase of growth, when the cell
302 density was at its highest.

303 CA at sub-inhibitory levels (2.27 mM) significantly ($p < 0.001$) reduced the expression of the QS
304 transcriptional regulatory genes *lasR* and *rhIR* (Fig. 3C). CA caused a 7-fold reduction ($p < 0.001$) in
305 *lasR* gene expression while the difference between the untreated and treated cells was even
306 higher in the LasR-controlled *rhIR* expression with a reduction of 19-fold being observed. CA also
307 effected a significant ($p < 0.001$) reduction in the AHL synthase gene expressions during the
308 stationary phase of growth. The downregulation in the *rhII* synthase gene following treatment
309 was 6-fold while in *lasI* synthase it was 13-fold during the late stationary phase.

310 The second inhibitor tested was the plant hormonal compound SA at sub-inhibitory
311 concentration of 3.62 mM. This also caused inhibition in QS gene expressions but unlike CA the
312 overall reductions were lower (Fig. 3C). The compound seemed to have a greater inhibitory effect
313 on the *las* QS circuit unlike CA which effectively repressed both *las* and *rhl* QS synthase and
314 regulatory genes. The down-regulation in QS transcriptional regulatory genes *lasR* and *rhIR* due

315 to SA treatment was 2-fold and 4-fold respectively. The transcript levels of the *lasI* synthase gene
316 was three times lower following SA treatment, while there was no significant reduction in
317 expression of the *rhlI* synthase gene in the stationary phase. The behaviour of the *lasR* and *rhlR*
318 regulatory genes determine the expression of virulence-related genes associated with the QS
319 mechanisms in *P. aeruginosa*, therefore these results suggest that SA would not produce a very
320 high down-regulation in QS regulated virulence gene expressions in comparison to CA.

321 Although CA and SA when used alone did show reduction in most QS gene transcripts but when
322 used in combination (PAO1+CA+SA) the results were inconclusive (Fig. 3C). The combination
323 treatment influenced the *lasI* synthase expression where it reduced the transcript level by 5-fold.
324 A similar reduction (3-fold) was also observed in the transcriptional regulator *rhlR* expression.
325 However, the combination treatment did not exert any significant effect on the expression levels
326 of the *lasR* and *rhlI* genes. These results suggest that the inhibitory effects of CA and SA on the
327 QS gene transcriptions were compromised when used in combination.

328 The positive control C30F produced an expected inhibitory effect on the *rhl* circuit of *P.*
329 *aeruginosa* during the mid-late stationary phase when used at a concentration of 10 μ M (Fig. 3C).
330 The *rhlR* transcript level was reduced 5-fold while the synthase gene *rhlI* was repressed by 2-fold.
331 The compound did not produce any significant inhibition on the transcription levels of the *lasRI*
332 genes.

333 **Trans-cinnamaldehyde significantly reduces expression of QS regulated**
334 **virulence factors**

335 After investigating the effect of the experimental inhibitors on the QS master genes- *lasRI* and
336 *rhIRI*, the study focused on investigating the inhibitory effects on the *las* and *rhl* QS regulated set
337 of virulence genes. The target genes selected were *las* controlled *lasA* protease and *lasB* elastase
338 and *rhl* regulated genes *rhlA*, *rhlB* and *rhlC* associated with rhamnolipid production (Fig. 4A). The
339 target gene expression was normalized using validated reference genes, *rpoD* and *proC*, across
340 all culture conditions.

341 The significant inhibition in *lasRI* expressions in *P. aeruginosa* PAO1 when subjected to CA as seen
342 before, affected the mRNA transcript levels of the *lasA* and *lasB* genes (Fig. 4B). The relative
343 expression data showed a 19-fold ($p < 0.001$) reduction in *lasA* gene expression while *lasB* showed
344 a 7-fold ($p < 0.001$) reduction when compared to the untreated cells during the mid-late stationary
345 growth phase. The compound was also effective in highly repressing the expression of the
346 rhamnolipid synthesis *rhIABC* genes during stationary phase (Fig. 4B). The reduction in transcript
347 level of *rhlA* was observed as greater than 100-fold ($p < 0.001$). A significantly high down-
348 regulation was also observed in *rhlB* ($p < 0.05$) expression while a 2-fold reduction ($p < 0.05$) was
349 observed in *rhlC* expression.

350 The ability of SA to repress the *las* QS genes *lasI* and *lasR* (Fig. 3C) consequently influenced the
351 virulence gene expressions of *lasA* and *lasB*. The transcript levels of *lasA* and *lasB* were reduced
352 by 4-fold ($p < 0.001$) and 2-fold ($p < 0.01$) respectively when treated with 3.62 mM SA (Fig. 4C). It
353 can be hypothesised that the inability of SA to produce an inhibitory effect on *rhlI* synthase gene

354 expression (Fig. 3C) meant there were enough signal molecules to drive the expression of the
355 *rhIABC* genes. Although a 3-fold ($p<0.001$) reduction was observed in *rhIA* gene expression, partly
356 due to the reduced expression of the *rhIR* regulatory gene, the overall inhibitory effect seemed
357 small as insignificant reductions in *rhIB* and *rhIC* gene expressions were observed with SA at the
358 concentration tested in this experiment (Fig. 4C).

359 The down-regulation in the *lasI* synthase gene when treated with both 2.27 mM CA and 3.62 mM
360 SA did not correlate in a mRNA reduction of the *las* regulated virulence genes *lasA* and *lasB* (Fig.
361 4D). However, the ability of the combination treatment to repress the QS regulatory *rhIR* gene
362 caused a significant down regulation ($p<0.001$) in the *rhIAB* genes. The combination treatment
363 exerted a 4-fold decrease in the *rhIA* gene and 6-fold decrease in the *rhIB* gene expressions
364 compared to the untreated samples. Interestingly, a minor up regulation of 2-fold was observed
365 in the *rhIC* gene.

366 The inability of C30F to reduce *las* regulated QS regulatory and synthase gene expressions was
367 only validated in the target gene expression analysis with no reduction being observed in *las*
368 controlled virulence *lasA* and *lasB* expressions (Fig. 4E). But C30F's ability to reduce *rhIRI* had a
369 consequential effect on the expression of *rhIAB* genes with reduction of 2-fold and 3-fold in *rhIA*
370 and *rhIB* gene expressions respectively at late-mid stationary phases.

371

372

373 **QSIs reduce production of extracellular virulence factors at sub-MIC**
374 **concentrations**

375 **Biofilm formation:** The ability of *P. aeruginosa* PAO1 to form biofilm was assessed using a widely
376 employed *in vitro* model outlined by O'Toole (2011) with slight modifications. The biofilm growth
377 was visualized as a ring of biomass stained with crystal violet at the air-liquid interface. The
378 biofilm formation was evaluated both in the presence and absence of inhibitory compounds, by
379 measuring the absorbance of crystal-violet-stained-adherent-cells solubilized in ethyl acetate at
380 570 nm from stationary phase cultures (24-hour) (Fig. 5A). There were significant ($p<0.001$)
381 reductions in treated samples compared to the untreated PAO1. In comparison to CA, SA was
382 more effective in reducing the formation of biofilm in the microtiter wells with an absorbance
383 reduction of 54% compared to the untreated cells. CA was also effective, to a lesser degree, with
384 reductions of 26%. The combined use of CA and SA was the most effective of the treatment
385 methods with a reduction of 62%. The positive control C30F in comparison was the least effective
386 (24%) in reducing biofilm formation.

387 **LasA protease:** There were significant reductions in LasA protease activity in the QSI treated cells
388 as estimated from absorbance reading resulting from azo dyes released into the medium due to
389 proteolytic cleavage of the substrate azocasein. In the presence of SA, the OD₄₄₀ dropped from
390 0.3 to 0.1 to account for a 31% reduction ($p<0.05$) in absorbance reading while CA treatment
391 gave an even higher reduction of 65% ($p<0.01$). The combination QSIs (CA+SA) treatment
392 produced the highest reduction in protease production with a reduction of 80% ($p<0.001$)
393 absorbance being observed when compared to the untreated PAO1 (Fig. 5B).

394 **LasB elastase:** The elastase production was estimated through absorbance measurement of
395 congo red following cleavage of elastin-congo red substrate by the enzyme elastase produced by
396 *P. aeruginosa*. In presence of inhibitors CA and SA, the OD₄₉₅ decreased from 0.08 to 0.06 and
397 0.05 respectively giving subsequent reduction percentages of 22% (p<0.01) and 28% (p<0.05)
398 (Fig. 5C). The combination treatment was again the most effective in reducing absorbance with
399 reduction of 46%. However, like the protease assay, C30F was the least effective, confirming
400 earlier results of its reduced inhibitory effects on *lasA* and *lasB* gene expressions.

401 **Pyocyanin:** Pyocyanin production is regulated by the *rhl* QS via PQS, hence the measurement of
402 pyocyanin inhibition is also a good indicator of the effectiveness of the tested compounds as QS
403 inhibitors in *P. aeruginosa*. The pyocyanin concentration decreased from 3.1 µg/ml to 2.1 µg/ml
404 and 0.922 µg/ml in the presence of CA and SA respectively. When used together, the inhibitors
405 decreased the yield by 64% (1.1 µg/ml) (Fig. 5D).

406 **Rhamnolipid estimation:** The administration of the QS inhibitors caused a reduction in the yield
407 of rhamnolipid with CA and SA both producing a drop in crude weight from 1.72 g/l to 0.7 g/l
408 (approximately) (Fig. 6A). Rhamnolipids are produced as congeners containing one to two
409 rhamnose sugar moieties giving the compounds their distinctive properties (Chen et al 2010,
410 Chen et al 2013). The structural composition of rhamnolipid produced in the presence of the QSIs
411 was studied by analysing the purified crude sample using HPLC-MS/MS method. The inhibitor
412 treatment when used alone did not affect the composition of the rhamnolipid with the congener
413 profile resembling the untreated PAO1 sample (Fig. 6C). But in the combination treatment
414 (PAO1+CA+SA) only 2 rhamnolipid congeners were detected by the HPLC-MS/MS method (Fig.
415 6D) compared to 6 in the untreated sample. This combination treatment also effected the

416 maximum reduction in the relative amount of rhamnolipid obtained from a 50-ml culture
417 supernatant. The two predominant congeners identified in all the samples were Rha-Rha-C₁₀-C₁₀
418 (m/z 649) and Rha-Rha-C₁₀-C₁₂ (m/z 677). The mono-rhamnolipid detected in greatest abundance
419 was Rha-C₁₀-C₁₀. When the MS data for the two common di-rhamnolipid congeners were
420 compared to untreated sample, the combination treatment and CA treatment showed marked
421 differences (Fig. 6B). SA treatment did not show any noticeable difference in relative
422 quantification of the congeners although it resulted in a decrease in crude weight.

423

424

425 **DISCUSSION**

426 **Trans-cinnamaldehyde is more effective than salicylic acid in reducing expression** 427 **of QS genes**

428 This study is consistent with previous research showing that natural QSIs significantly modulate
429 transcriptional expression of QS regulatory and virulence-associated genes during stationary
430 phase in *P. aeruginosa* PAO1. CA effectively inhibited the expression of both *las* and *rhl* QS
431 systems. Both the regulatory proteins (LasR and RhIR) and the AHL synthases (LasI and RhII) were
432 significantly repressed with CA. Downregulation of both these QS systems correlated with
433 repression of their virulence associated genes. The exact mechanism of action is unknown for
434 these compounds. Several natural and synthetic antagonists have been described for LasR
435 however the relative instability of LasR::antagonist complexes has limited biochemical
436 characterisation *in vitro*. Recently (O' Reilly et al 2018) used potent agonists rather than known

437 antagonists to stabilise LasR *in vitro*. They were able to develop a focused library of agonists
438 based on previous tri-phenyl ligands, resulting in several new LasR::agonist complexes available
439 in/from the PDB. From these structures O' Reilly et al.(2018) determined an important
440 functional role for a flexible loop in the ligand binding domain (LBD), previously unknown, which
441 upon ligand binding promotes specific conformational changes that seals the ligand binding
442 pocket from solvent and directs the DNA binding domain (DBD) to form a transcriptional
443 activation complex. This suggest a plausible mechanism by which agonists stabilise and
444 antagonists destabilise LasR. These structures provide essential information for the fundamental
445 understanding of how LuxR type receptors bind to their cognate autoinducers.

446 We hypothesise that CA and SA act as QS antagonists. Previously, molecular docking studies have
447 suggested CA to interact with the LasI protein (Chang et al 2014). LasI synthase produces 3-oxo-
448 C₁₂-HSL which is the ligand for LasR. RhII is 47% homologous with LasI (, Chang et al 2014. Gokalsin
449 et al 2017) and a similar mechanism of action is interpreted in AHL production. In general, LuxI
450 synthases catalyzes the transfer of an acyl group bound to acyl carrier protein (ACP) from fatty
451 acid bio- synthesis to S-adenosyl-L-methionine (SAM) (Churchill and Chen 2011) which then
452 undergoes lactonization to form the N-acyl-homoserine lactone. CA is predicated to bind in the
453 SAM binding pocket of LasI, thus preventing SAM binding and subsequent 3-oxo-C₁₂-HSL
454 synthesis, In the absence of AHL, LasR will not dimerize and therefore cannot bind to DNA. These
455 interactions could modulate the QS autoinducer levels. The LasI::3-oxo-C₁₂-HSL complex
456 regulates the expression of many downstream genes including *lasI* and *rhII*. Since we showed CA
457 reduces *lasI* expression and previously CA has shown to reduce signal molecule concentration
458 (Chang et al 2014), we suggest that the intracellular concentration of autoinducer signal

459 molecules was not sufficient to trigger the activation of genes involved with rhamnolipid and
460 protease synthesis as shown in this study.

461 To date there is no crystal structure for RhlR, its inherent instability *in vitro* has proven intractable
462 to crystallisation and biochemical characterisation. Based on similarity, mechanistic
463 interpretations of LasR with AHL ligands and inhibitors are expected to extend to RhlR. However,
464 RhlR remains a viable QS target for developing targeted inhibitors, *lasR* mutants are frequently
465 isolated from cystic fibrosis patients suggesting the redundancy of LasR as a master regulatory in
466 chronic CF infections (Feltner et al 2016).

467 The reductions in *lasRI* and *rhIRI* expressions from CA treatments were correlated by assessing
468 the activity of *las* regulated elastase and protease and *rhl* regulated pyocyanin and rhamnolipid
469 productions. CA at sub-inhibitory concentrations caused a significant decrease in elastase (22%)
470 and protease (65%) activities. Pyocyanin, which is a good indicator of *rhlI* inhibition (Chang et al
471 2014), showed a decrease of 32% with CA. These reductions were comparable to other findings
472 with cinnamaldehyde as QSI in the literature (Brackman et al 2008, Brackman 2011). Although
473 CA was not able to abolish rhamnolipid production, the treatment caused a decrease in
474 rhamnolipid yield with the two main di-rhamnolipid congeners Rha-Rha-C₁₀-C₁₀ and Rha-Rha-C₁₀-
475 C₁₂ levels reduced by 59% and 34% respectively compared to that of untreated cells. The
476 inhibition at post-translational levels of these virulence factors complemented the RT-qPCR data
477 from this study where we observed significant reductions in *lasA*, *lasB*, *rhIA*, *rhIB* and *rhIC*
478 expressions following CA treatment.

479 SA unlike CA did not produce the same level of inhibition on the transcriptional profiles of the
480 *lasRI* and *rhIRII* genes with 2-4-fold reduction in mRNA levels being observed in the treated
481 samples compared to untreated controls. The binding affinity of SA to the LasR protein (Yang et
482 al 2009) possibly promoted conformational changes in the LasR-(3-oxo-C₁₂-HSL) complex thereby
483 causing a reduced expression of downstream genes. Due to the QS hierarchical arrangement,
484 *rhIR* expression can be regulated by *lasR*, hence the highest inhibition in QS regulatory expression
485 with SA was seen in *rhIR*. This was in agreement with a previously reported study where SA was
486 shown to reduce *rhIR* expression in *P. aeruginosa* (Yang et al 2009). The decreased expression in
487 *las* QS genes consequently repressed *lasA* and *lasB* levels supporting the findings of Prithviraj et
488 al. (2005) using SA. Since SA did not lead to an inhibitory effect on the overall *rhl* regulon,
489 significant downregulation was not observed in the *rhIB* and *rhIC* genes. El-Mowafy et al. (2014)
490 reported SA rich aspirin could cause significant downregulation in the *lasRI* and *rhIRI* expressions.
491 The findings however do not fully agree with the results from this study. The study with aspirin
492 (El-Mowafy et al 2014) used only one reference gene, *rpoD*, for data normalization along with a
493 higher concentration of the inhibitor thereby giving slightly different results. Although SA did not
494 show a profound effect at the transcriptional level, it seemed to be effective at the translational
495 level. This can be hypothesized from this study considering higher reductions in virulence
496 proteins elastase and protease were observed in the semi-quantitative assays following SA
497 treatment. Reduction of these proteases when *P. aeruginosa* were supplemented with SA had
498 been previously reported in a couple of studies with inhibition ranging between 40-80%
499 (Prithviraj et al 2005, El-Mowafy et al 2014). The choice of semi-quantitative assay and the
500 selection of growth medium were perhaps responsible for the large inhibitory range being

501 observed within the results published in the literature (Duan and Surette 2007). The choice of
502 media is very important as the production of secondary metabolites can be influenced by growth
503 limiting factors present in the medium. However, SA had a negligible effect on the *rhl* controlled
504 rhamnolipid production with HPLC results being similar to the untreated sample. This
505 complemented the qPCR findings where minimal reduction was seen in the rhamnolipid
506 biosynthesis gene expressions. Moreover, the unavailability of the RhIR crystal structure makes
507 it difficult to predict the possible interaction sites for these inhibitors.

508 **The combination treatment of CA and SA does not show significant inhibitory**
509 **effect on QS gene expressions**

510 Having ascertained the potential of CA and SA to repress QS regulated gene expressions and
511 virulence factor production when used separately, the effect of combination treatment was
512 investigated. Even though CA and SA have different QS targets, in the form of LasI and LasR
513 respectively, expression profiles suggested that the combination treatment was not very
514 effective at the transcriptional level. Noticeable downregulation was observed in *rhIR* which
515 subsequently affected the expression of the rhamnolipid genes, further supporting the view that
516 inhibitors targeting transcriptional regulators can be a potential drug target for reducing bacterial
517 virulence. At post-translational level, the combination treatment was successful in reducing the
518 *rhl* regulated production of pyocyanin and rhamnolipid. The HPLC-MS/MS analysis showed
519 negligible presence of rhamnolipids strengthening the idea that the effect of the combination
520 treatment was strongly at the translational level. A computational model study of LuxI/LuxR QS
521 suggested that LuxR competitive inhibitor, unlike LuxR non-competitive inhibitor, can display

522 antagonistic effects when used in combination with a LuxI inhibitor (Anand et al 2013).
523 Therefore, if an analogy is drawn with SA targeting LasR through competitive inhibition then
524 some of the inhibitory potential of LuxI-type inhibitor CA can be attenuated. However, the
525 mechanism by which this could happen is not known and was beyond the scope of current work.
526 A better understanding on how the inhibitors bind to the target proteins will help to elucidate
527 the lower inhibitory effects observed at expression levels with the combination treatment
528 especially when we consider that significant downregulation was observed with CA alone.

529 With antibiotics fast losing their efficacy, alternative strategies are imperative. The sole use of QS
530 inhibitors is unlikely to completely eradicate the bacterial infection and there would be legitimate
531 concerns around potential toxicity of high concentrations of cinnamaldehyde where maximum
532 permissible levels in foodstuffs have already been determined (Shreaz et al 2016). However, since
533 the inhibitors reduce the virulence phenotypes and weaken the bacterial biofilms, this would
534 allow the host innate immunity and externally administered antimicrobial compounds to function
535 more effectively. Synergistic enhancement of antibiotics by administration of sub-inhibitory
536 quorum quenching compounds is a potentially exciting future development but little is known
537 about such effects at the molecular level. Our system provides a suitable model system for future
538 studies aimed at elucidating these mechanisms and should contribute to extending the useable
539 life span of current drugs.

540

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551

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709

710

711 **Figure 1** *Las* and *rhl* QS regulatory genes in *P. aeruginosa* PAO1 are differentially
712 expressed in a cell density dependent manner.

713 (A) Growth of *P. aeruginosa* in phosphate limited media (PPGAS). (B) Transcriptional expression
714 of QS regulatory genes *lasR*, *lasI*, *rhIR* and *rhlI*. Expression levels were quantified by RT-qPCR,
715 relative mRNA levels for target genes were normalised to the geometric mean of two reference
716 genes (*rpoD* and *proC*) and values plotted are the mean calibrator normalised ratios to log phase
717 (6h). Vertical bars represent S.D± (n=3). Data was analysed using one-way ANOVA followed by
718 Dunnett's Multiple Comparison test (**p<0.01, ***p<0.001).

719

720 **Figure 2** QS regulated virulence associated genes are highly expressed in stationary phase
721 in *P. aeruginosa* PAO1.

722 Relative transcript levels of virulence associated rhamnolipid biosynthetic genes (A) *rhIA*, (B) *rhIB*
723 (C) *rhIC* and exoprotease (D) *lasA* and elastase (E) *lasB*. Relative mRNA levels for target genes
724 were normalised to the geometric mean of two reference genes (*rpoD* and *proC*) and values
725 plotted are the mean calibrator normalised ratios to log phase (6h). Vertical bars represent S.D±
726 (n=3). Data was analysed using one-way ANOVA followed by Dunnett's Multiple Comparison test
727 (*p<0.05, **p<0.01, ***p<0.001).

728

729 **Figure 3** **Quorum Sensing Inhibitors (QSIs) significantly reduce expression of *las* and *rhl***
730 **QS systems in *P. aeruginosa*.**

731 (A) Molecular structure of the natural QSIs used in this study, Salicylic acid (SA), trans
732 cinnamaldehyde (CA) and positive control furanone C30 (C30F). (B) Growth of *P. aeruginosa* with
733 QSIs at sub-MIC concentrations (SA: 3.62 mM, CA: 2.27 mM and C30F: 10 μ M). (C) Relative
734 expression of QS regulatory genes *lasR*, *lasI*, *rhlR* and *rhlI* with combinations of QSI treatments.
735 Relative mRNA levels for target genes were normalised to the geometric mean of two reference
736 genes (*rpoD* and *proC*). Vertical bars represent S.D \pm (n=3). Data was analysed using two-way
737 ANOVA followed by Bonferroni post-tests (**p<0.01, ***p<0.001).

738

739 **Figure 4** **Trans-cinnamaldehyde (CA) significantly reduces gene expression of virulence**
740 **associated genes in *P. aeruginosa* PAO1.**

741 (A) The schematic representation of the genetic location of *las* and *rhl* QS systems in *P.*
742 *aeruginosa*. Effect of QSIs at the following concentrations of: (B) 2.27 mM CA (C) 3.62 mM SA (D)
743 2.27 mM CA + 3.62 mM SA and (E) 10 μ M C30F on the transcriptional expression of virulence
744 associated genes *lasA*, *lasB*, *rhlA*, *rhlB* and *rhlC* in *P. aeruginosa* PAO1. Gene expression was
745 quantified at 24h for both treated and untreated cells, relative mRNA levels for target genes were
746 normalised to the geometric mean of two reference genes (*rpoD* and *proC*). Vertical bars
747 represent S.D \pm (n=3). Data was analysed using two-way ANOVA followed by Bonferroni post-tests
748 (*p<0.05, ** p<0.01, ***p<0.001).

749 **Figure 5** QSIs quantitatively reduce production of extracellular virulence factors in *P.*
750 *aeruginosa*.PAO1

751 QS regulated phenotypes (A) biofilm formation and extracellular factors (B) protease (C) elastase,
752 and (D) pyocyanin were significantly disrupted by QSIs. The percentage reductions mentioned
753 were calculated against the untreated PAO1. Error bars represent S.D± (n=3). Data was analysed
754 using one-way ANOVA followed by Dunnett's Multiple Comparison test (*p<0.05, **p<0.01,
755 ***p<0.001).

756

757 **Figure 6** QSIs significantly reduce *rhl* regulated rhamnolipid production *P. aeruginosa*.

758 (A) The QSIs reduces the rhamnolipid crude yield significantly compared to the untreated PAO1
759 cells. (B) % reduction in the two main RL congeners Rha-C₁₀-C₁₀ and Rha-Rha-C₁₀-C₁₀ relative to
760 untreated *P. aeruginosa* PAO1. RL congeners were studied by HPLC-MS/MS. The HPLC
761 chromatograms of (C) untreated *P. aeruginosa* PAO1 and (D) *P. aeruginosa* PAO1 treated with
762 trans-cinnamaldehyde (CA) and salicylic acid (SA).

763