Alginate Oligosaccharide-Induced Modification of the *lasI-lasR* and *rhlI-rhlR* Quorum Sensing Systems in *Pseudomonas aeruginosa*

Short title: OligoG CF-5/20 affects Quorum Sensing in *P. aeruginosa*

Alison A. Jack,[a*] Saira Khan,[a] Lydia C. Powell,[a] Manon F. Pritchard,[a] Konrad Beck,[a] Hina Sadh,[a] Lucy Sutton,[a] Alessandra Cavaliere,[b] Hannah Florance,[c*] Philip D. Rye,[d] David W. Thomas[a] and Katja E. Hill[a]

Advanced Therapies Group, Cardiff University School of Dentistry, Cardiff, UK[a]; School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, UK[b]; Department of Biosciences, College of Life and Environmental Sciences, Exeter University, Exeter UK[c]; AlgiPharma AS, Sandvika, Norway[d]

*Present address: Alison A. Jack, Cultech Ltd, Baglan Industrial Park, Port Talbot, West Glamorgan, UK; Hannah Florance, UK Centre for Mammalian Synthetic Biology, The King's Buildings, Edinburgh, UK.

[#]Address correspondence to Katja E. Hill, hillke1@cardiff.ac.uk

ABSTRACT

Pseudomonas aeruginosa plays a major role in many chronic infections. Its ability to readily form biofilms contributes to its success as an opportunistic pathogen and its resistance/tolerance to antimicrobial/antibiotic therapy. A low molecular weight alginate oligomer (OligoG CF-5/20), derived from marine algae, has previously been shown to impair motility in *P. aeruginosa* biofilms and disrupt pseudomonal biofilm assembly. As these bacterial phenotypes are regulated by quorum sensing (QS), we hypothesized that OligoG CF-5/20 may induce alterations in QS signalling in *P. aeruginosa*. QS regulation was studied using *Chromobacterium violaceum* CV026 biosensor assays that showed a significant reduction in acyl homoserine lactone (AHL) production following OligoG CF-5/20 treatment (≥2%; $P<0.05$). This effect was confirmed by liquid chromatography/mass spectrometry (LC/MS) analysis of C4-AHL and 3-oxo-C12-AHL production (≥2%; $P<0.05$). Moreover, quantitative PCR (qPCR) showed that reduced expression of both the *las* and *rhl* systems was induced following 24 h treatment with OligoG CF-5/20 (≥0.2%; $P<0.05$). Circular dichroism (CD) spectroscopy indicated that these alterations were not due to steric interaction between the AHL and OligoG CF-5/20. Confocal laser scanning microscopy and COMSTAT image analysis demonstrated that OligoG CF-5/20 treated biofilms had a dose-dependent decrease in biomass which was associated with inhibition of eDNA synthesis (≥0.5%; $P<0.05$). These changes correlated with alterations in extracellular production of the pseudomonal virulence factors pyocyanin, rhamnolipids, elastase and total protease ($P<0.05$). The ability of OligoG CF-5/20 to modify QS signalling in *P. aeruginosa* PAO1 which may influence critical downstream functions, such as virulence factor production and biofilm formation.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic and nosocomial human pathogen, which can cause extensive tissue damage through the production of virulence factors and toxins e.g. pyocyanin and proteases (1). The dynamic genome of *P. aeruginosa* is highly adaptable, enabling it to adjust to a wide range of environmental conditions (2, 3). This versatility allows it to colonise diverse physiological niches including the respiratory tract, genito-urinary tract and wounds.

The sodium alginate oligomer OligoG CF-5/20, produced from the brown seaweed *Laminaria hyperborea,* has been shown to potentiate (enhance) antimicrobial efficacy, perturb multidrug resistant bacteria (4-7) and inhibit biofilm formation in a broad range of organisms (8). Furthermore, it has also previously been shown to inhibit swarming and "twitching" motility, exhibiting a significant effect on bacterial flagella- and pilus-mediated chemotaxis (4, 5). Although OligoG CF-5/20 is known to have cation-chelating properties, the precise mechanism of action in mediating this diverse range of effects remains unclear.

Quorum sensing (QS) is a cell density-dependent communication system between local populations of bacterial cells, regulating and coordinating their gene expression using diffusible signalling molecules (9, 10). In Gram-negative pathogens (in particular *P. aeruginosa*), QS is regulated by acylated homoserine lactones (AHLs) produced by a transcriptional regulator based on the LuxR/LuxI-type QS system that was first characterized in *Vibrio fischeri* (11). The regulation of QS in *Pseudomonas* spp. is both subtle and complex (12) (**Fig 1**). *P. aeruginosa* has four QS systems, two acyl-homoserine lactone (AHL)- and one 2-heptyl-3-hydroxy-4-quinolone- mediated system known as the *Pseudomonas* quinolone signal (PQS) system and the more recently identified integrated QS system (IQS) (13). The AHL systems in *P. aeruginosa* are known as the *lasI-lasR* and *rhlI-rhlR* systems and the

transcriptional regulators LasR and RhlR regulate production of the signalling molecules (autoinducers) *N*-(3-oxododecanoyl)-L-AHL (3-oxo-C12-AHL) and *N*-butyryl-L-AHL (C4-AHL) respectively. The QS system in *P. aeruginosa* is regulated by an inter-linked, hierarchical mechanism where *lasR*/3-oxo-C12-AHL induces expression of *lasI,* as well as *rhlR*/*rhlI* and the PQS system. Disruption of the IQS signal can effectively paralyze the PQS and *rhl* QS systems (14). A number of additional regulators of this QS system exist, at both transcriptional and post-transcriptional levels, including the global activator GacA and regulator Vfr (13). In addition, QS also regulates key cellular processes such as promotion of eDNA release, RNA transcription and translation, cellular division and amino acid synthesis.

Global gene expression analysis of the QS systems in *P. aeruginosa* has shown that 6-10% of the genome is regulated through the *las* and *rhl* systems (12, 15). QS plays a role in swarming motility, biofilm development and expression of antibiotic efflux pumps (16) as well as virulence factor production. *P. aeruginosa* QS-activated virulence factors include proteases e.g. elastase, pyocyanin, lectins, rhamnolipids, and toxins. Such virulence factors can affect biofilm formation and maintenance, as well as swarming motility. Their regulation is complex, with numerous intrinsic and environmental factors involved such as cell-number, composition of the extracellular polymeric substance (EPS), matrix density and oxygen availability. However, the production of pyocyanin, proteases and rhamnolipids reflects optimal QS signalling (17). Pyocyanin is a blue secondary metabolite produced by *P. aeruginosa* evident in the sputum of infected cystic fibrosis patients (18). As a zwitter ion, at a physiological pH, it can readily penetrate biological membranes, inducing host cell necrosis and inflammation, both directly (e.g. IL-8) and indirectly via cellular damage (19). Importantly, in the context of biofilm persistence *in vivo*, pyocyanin induces the deposition of extracellular DNA (eDNA) which is a major component of biofilm EPS, being essential for

biofilm formation and stability (20). Production of both pyocyanin and eDNA is mediated by AHL and PQS molecules, as well as by flagella and type IV pili (21, 22).

The regulation of QS in *P. aeruginosa* is sensitive to, and modulated by, growth and environmental conditions, which impact significantly on the timing of *lasI, lasR, rhlI* and *rhlR* expression (9, 23). The complexity of this QS system in *P. aeruginosa* is thought to be one of the main factors responsible for its selective adaption and environmental versatility (24). The QS system also affords selective "fitness" advantages in human disease. For example, QS signalling molecules produced by *P. aeruginosa* are also recognised by *Burkolderia cepacia*, resulting in synergistic interactions in mixed-species biofilms (25), thereby potentially increasing the virulence of both species in the cystic fibrosis lung. Moreover, the expression of AHL and PQS molecules has been shown to affect the mammalian host-pathogen response (26) with 3-oxo-C12-AHL and PQS having anti-inflammatory and pro-apoptotic effects on murine fibroblasts and human lung epithelial cells at concentrations <10 μM (27).

Rhamnolipids are bacterial glycolipid surfactants, composed of a rhamnose glycosyl head and a 3-(hydroxyalkanoyloxy) alkanoic acid fatty acid tail. Rhamnolipid expression plays a crucial role in microbial motility, hydrophobic uptake and biofilm formation on host surfaces. Proteases (including the zinc-dependent metalloproteinase elastase) also play an important role in the pathogenicity of *Pseudomonas* spp. facilitating invasion and destruction of host tissue (28). Rhamnolipid production is regulated by the *P. aeruginosa* quorum sensing regulator, *rhlR*, whilst elastase and protease activities are regulated by the *lasIR* system.

QS inhibitors that impede QS pathways in microorganisms are an attractive target for antimicrobial therapy development. We hypothesized that the antibiotic susceptibility,

motility and biofilm-assembly modifications induced in *P. aeruginosa* by OligoG CF-5/20 might relate to alterations in the regulation of *lasI-lasR* and *rhlI-rhlR* and studied this *in vitro.*

RESULTS

OligoG CF-5/20 inhibits growth of *P. aeruginosa* PAO1 and reduces violaceum induction and inhibition of the *Chromobacterium violaceum* biosensor CV026. The effect of OligoG CF-5/20 on the growth of *P. aeruginosa* PAO1 was examined using growth curves. OligoG CF-5/20 at concentrations of ≥2% was found to significantly reduce the growth *P. aeruginosa* PAO1 (Minimum significant difference, MSD=0.154; P<0.01; **Fig 2A**). This growth curve data was used to determine the time-points (12, 18, 24 and 30 h) employed in the subsequent time-course study.

A time-course study was undertaken using induction or inhibition of violaceum in the *Chromobacterium violaceum* biosensor strain CV026 as an indicator of QS signalling (C4-AHL and 3-oxo-C12-AHL respectively), following treatment with OligoG CF-5/20 (**Fig 2B, 2C and 2D**). Untreated controls showed distinct differences in *P. aeruginosa* PAO1 AHL production with time, which were maximal at 18 h for C4-AHL induction and 24 h for 3-oxo-C12-AHL inhibition. OligoG CF-5/20 treated samples showed a reduction in C4-AHL, particularly at 18 and 24 h which was significant from 0.2% OligoG CF-5/20 and at 30 h from 2% (**Fig 2B and 2C**). Measurement of zones of clearing indicated that OligoG CF-5/20 had less of an effect on 3-oxo-C12-AHL inhibition when compared to C4-AHL induction (zone of coloration). Violaceum inhibition was significantly reduced at the 24 h time point, at all OligoG CF-5/20 concentrations, in comparison to the control (P<0.05; **Fig 2B and 2D**).

Homoserine lactones C4-AHL and 3-oxo-C12-AHL can be detected using LC/Mass Spectrometry. For a more accurate determination of AHL concentrations, preliminary analysis of the AHLs, C4-AHL (Fig 3A) and 3-oxo-C12-AHL (**Fig 3B**) using LC/MS (**Fig 3C**) was undertaken from an initial time course following PAO1 growth at 18, 24 and 30 h. LC/MS demonstrated a time-dependent decrease in C4-AHL (**Fig 3D**) which was significantly different at 30 h ($P<0.05$). Conversely, levels of 3-oxo-C12-AHL were considerably lower (up to 6-fold) and did not demonstrate time-dependent decreases.

LC/Mass Spectrometry shows time-dependent decreases in AHL production following OligoG CF-5/20 treatment. A subsequent time-course of OligoG CF-5/20-treated PAO1 (grown at 12, 18, 24 and 30 h) demonstrated significant reductions in C4-AHL production at all time-points ≥2 % OligoG (**Fig 4A**), the exception being 18 h at 2% which was not significant. A similar significant reduction was seen for 3-oxo-C12-AHL (**Fig 4B**; $P<0.05$) in comparison to the untreated control (except for 18 and 24 h at 2%), although much lower overall levels were detected (up to 29.5 mg/L) compared to C4-AHL (up to 102.3 mg/L) (**Fig 4).**

OligoG CF-5/20 reduces extracellular virulence factor production in *P. aeruginosa* PAO1. As the biosensor analysis showed that OligoG CF-5/20 affected bacterial signalling, the production of virulence factors, regulated in *P. aeruginosa* PAO1 by quorum sensing, was investigated. OligoG CF-5/20 (≥ 0.2%) significantly reduced the amount of pyocyanin at all time points ≥18 h (**Fig 5A**; $P<0.05$). However, for rhamnolipid production a significant reduction was only observed at 18 h (for all OligoG CF-5/20 concentrations tested) or 24 h (at ≥2% OligoG CF-5/20; $P<0.05$) with no significant change seen at either 12 or 30 h (**Fig 5B**). In contrast, a significant reduction in total protease (**Fig 5C**) and elastase (**Fig 5D**) production was seen at ≥0.2% OligoG CF-5/20 and then only at the 24 h time point ($P<0.05$).

OligoG CF-5/20 reduces expression of quorum sensing genes. Phenotypic studies were confirmed by genotypic analysis using qPCR. Temporal expression of QS genes following OligoG CF-5/20 treatment was observed (**Fig 6**). Significant reductions in expression of *lasI, rhlI* and *rhlR* at 12 h (**Fig 6A**), *lasI*, *lasR and rhlR* at 18 h (**Fig 6B**) and *rhlR* at 24 h (**Fig 6B and C** respectively; $p<0.05$) were evident, which for *lasI, rhlI* and *rhlR* at 12 h and *lasR* at 18 h, were significant for all three concentrations of OligoG tested. No significant effect of OligoG CF-5/20 on AHL expression was detected by qPCR at the 30 h time point (Fig 6D).

CLSM shows that OligoG CF-5/20 reduces production of eDNA and behaves similarly to QS inhibitors against biofilms of *P. aeruginosa* PAO1. CLSM imaging of TOTO-1 nucleic acid-stained 24 h biofilms demonstrated that OligoG CF-5/20 (≥ 0.5%) induced a significant decrease in eDNA production after treatment ($P>0.05$) (**Fig 7 and 8**). This was evident in biofilms grown in the presence of OligoG (biofilm formation studies) and for 24 h biofilms subsequently treated with OligoG for 24 h (biofilm disruption studies). Although the CLSM imaging did not appear to show a dose-dependent decrease in eDNA production (**Fig 7**). A dose-dependent decrease was, however, evident at ≥2% OligoG in direct analysis of treated biofilm samples (**Fig 8**).

The structural alterations induced in biofilms by OligoG CF-5/20 were compared to the effects of the QS inhibitors, 2(5H)-furanone and N-decanoyl cyclopentylamide (C10-CPA) (29, 30) using LIVE/DEAD staining (**S1A Fig**) showing that the effects of OligoG resembled the inhibition induced by the other AHL-dependent quorum sensing inhibitors tested (**S1B and S1C Fig**).

Circular dichroism showed that OligoG CF-5/20 does not interact directly with AHLs. Circular dichroism (CD) spectroscopy rapidly determines protein and polypeptide secondary structure, and has previously been shown to give excellent comparability to ^{1}H NMR

spectroscopy in determining alginate M/G residue ratios (31). CD was used here to confirm that the effects of OligoG CF-5/20 were not due to simple physical interaction with the AHL molecules. The CD signal of OligoG CF-5/20 titrated with C4-AHL or 3-oxo-C12-AHL showed no substantial change (**S2 Fig**). The minima of the spectra around 210 nm, revealing the orientation of the alginate carboxy groups and thus directly indicative of the conformation of OligoG CF-5/20 (32), appeared unaffected by either of the two AHLs. The ellipticities recorded at 208 nm (after addition of AHLs at their maximum concentrations over ~1h) suggested that kinetic effects were not responsible for the absence a signal (insets in **S2 Fig**).

DISCUSSION

This study confirms that OligoG CF-5/20 affects global regulatory QS signalling in *P. aeruginosa* PAO1 as was hypothesised following the original observations on bacterial motility (4, 7). The biosensor strain *C. violaceum* CV026 demonstrated that OligoG CF-5/20 reduced C4-AHL and 3-oxo-C12-AHL production in *P. aeruginosa* PAO1, (as seen by QS induction and inhibition respectively) in a time- and dose-dependent manner. This was further confirmed by LC/MS and qPCR, and that OligoG CF-5/20 also had a significant effect on the production of other virulence factors such as pyocyanin, rhamnolipid, total protease, and elastase. The dose-dependent nature of the observed inhibition suggested that OligoG does not simply act as an AHL receptor antagonist by binding to the receptor, thereby effectively “blocking” all AHL binding. Furthermore, the CD analysis excluded the possibility that the observed alterations in QS signalling molecules and virulence factor expression were the result of simple, steric interactions between the oligosaccharide and the AHL signalling molecules in the biofilm system.

213 The LC/MS data demonstrated the complex, time-dependent nature of virulence factor
214 production by *P. aeruginosa* with optimal (maximum) production of both AHLs (C4-AHL and
215 3-oxo-C12-AHL) at 12 h (equivalent to late exponential/early stationary growth phase).
216 These findings are in keeping with previous studies, which showed that whilst AHL
217 production peaks during exponential growth, C4-AHL levels decrease as stationary phase is
218 attained (12). The finding here, that 3-oxo-C12-AHL levels remained relatively constant if
219 the medium was sufficiently buffered to avoid alkali-mediated lactonolysis, is consistent
220 with those of Yates et al (33).

221 Las and Rhl are regulated by the LuxR family of transcriptional regulators (lasR and
222 RhlR), making their expression extremely sensitive to environmental conditions e.g. hypoxia,
223 pH and hydrodynamic shear (which are important in biofilm infections). AHL production has
224 been shown to vary significantly under different environmental growth conditions,
225 especially under nutrient-limitation, with higher AHL expression observed in minimal or
226 diluted media, when compared to nutrient media (9). In addition, both *las* and *rhl* were
227 expressed earlier (in early to mid-log phase) in nutrient-limited media compared to early
228 stationary phase in nutrient media. Interestingly, these phenomena were unrelated to cell-
229 density, which is usually considered a pre-requisite for QS expression. Comparing 46
230 different experimental conditions, Duan and Surette (9) showed that the individual
231 dominance of the *las* and *rhl* system reflected environmental conditions. LasR mutants are
232 commonly found in both clinical and environmental isolates, indicating autonomous
233 regulation of these integrated systems (34, 35). Transcription of Las and Rhl may also occur
234 independently, permitting further "fine-tuning" of each system *in vivo*. This may, in part,
235 explain the independent (and distinct) responses to OligoG CF-5/20 treatment observed in
236 C4-AHL and 3-oxo-C12-AHL production in the time-course experiments.

237 Swarming is a complex form of motility, and is consequently influenced by a large
238 number of different genes. Rhamnolipids are known to modulate the intricate swarming
239 motility patterns of *P. aeruginosa* (36). Therefore, it was perhaps unsurprising that, as
240 OligoG CF-5/20 was previously shown to affect swarming motility of *Proteus* and *P.*
241 *aeruginosa* (4, 7 respectively), that rhamnolipid production should also be affected by
242 OligoG CF-5/20. Importantly, QS regulation of rhamnolipids and swarming motility
243 contribute to *P. aeruginosa* biofilm dispersal, and therefore help to explain the dramatic
244 effect of OligoG CF-5/20 on both biofilm formation and disruption of established biofilms
245 previously described (4, 7). In support of this notion, a range of mini-Tn*5* insertion,
246 "swarming-negative" *P. aeruginosa* mutants exhibited impaired biofilm formation (37),
247 confirming the link between both phenotypes. The finding here, of more significant
248 inhibition of pyocyanin and rhamnolipid production by OligoG CF-5/20 (when compared to
249 the effects on elastase and total protease production) may relate to differential expression
250 of the different QS pathways. The three most characterised QS signalling systems in *P.*
251 *aeruginosa* are believed to be sequentially activated in "nutrient-rich" media, with LasR
252 sitting at the top of the temporal cascade, and AHLs (*las* and *rhl*) being released in early- and
253 PQS in late-exponential phases of growth (38).
254 Las and Rhl control both biofilm formation and expression of virulence factors in *P.*
255 *aeruginosa* (11). The LasR–3-oxo-C12-AHL complex activates transcription of target genes
256 including those encoding virulence factors such as elastase, proteases, and exotoxin. In
257 contrast, RhlR–C4-AHL activates target genes, including those encoding elastase, proteases,
258 pyocyanin, and siderophores (39). There appears to be a considerable overlap in the
259 virulence factors these regulons control (9). The finding that the inhibition of
260 pyocyanin/rhamnolipid production was more evident throughout the time-course of the

261 experiment than that of protease and elastase, may be a reflection of OligoG differentially
262 affecting the Rhl QS system, to a greater extent than the Las system.

263 The intrinsically high levels of antimicrobial resistance typically seen in *P. aeruginosa*
264 are due to its low permeability and multidrug efflux systems, four of which contribute
265 significantly to innate antibiotic resistance. Khan et al. (4) demonstrated that OligoG CF-
266 5/20 increased potentiation of antibiotics against MDR bacteria (up to 128 fold). The
267 authors established that this did not relate simply to permeabilisation of the pseudomonal
268 lipopolysaccharide cell-wall or targeting of the multi-drug efflux pump MexAB-OrpM,
269 suggesting that the QS inhibition observed here with OligoG CF-5/20, involves a mechanism
270 other than inhibition of AHL efflux pumps in *P. aeruginosa*. Instead, the OligoG CF-5/20-
271 induced reduction in AHL production more likely reflects an effect further "upstream" e.g.
272 on bacterial two-component system (TCS) signal transduction pathways (40) by which
273 means bacteria are able to detect and produce a response to environmental changes.

274 TCSs are comprised of an inner membrane-bound "sensor" generally a histidine kinase
275 (which detects environmental stimuli) and a response regulator (which modulates the
276 response). There are many TCSs in *P. aeruginosa*, and these are recognized to play a role in
277 regulating bacterial virulence, biofilm formation and antibiotic susceptibility; factors known
278 to be influenced by OligoG CF-5/20, although the precise links between TCSs and QS are still
279 poorly understood (41). At least three TCSs (BfiSR, MifR and BfmSR) are thought to be
280 involved in the activation of biofilm formation (42). The recently-published
281 BfmS/BfmR/RhlR TCS has been shown to be key to regulation of the *rhl* QS pathway in *P.*
282 *aeruginosa* (43) modulating expression of biofilm formation and virulence. Interestingly,
283 deletion of the sensor gene *BfmS* was shown to cause inhibition of the *rhl* QS system, with
284 *BfmR* playing a central role in biofilm maturation. In addition, it has also recently been

285 suggested that BfmRS is involved the development of virulence during bacterial adaptation
286 to the CF lung (43, 44). Interestingly, AlgR (another key *Pseudomonas aeruginosa*
287 transcriptional response regulator) also appears to play an essential role in bacterial
288 virulence and motility (45).

289 The chemical composition of the EPS represents a formidable "barrier" to diffusion and
290 contributes to resistance to antibiotic and antimicrobial therapy (46). The physical
291 disruption of the biofilm structure and alterations in eDNA distribution within the
292 pseudomonal biofilms (induced by OligoG) was, perhaps, unsurprising as QS and pyocyanin
293 have an important regulatory role in eDNA synthesis. Pyocyanin induces eDNA release, with
294 biofilms formed by QS mutants known to possess reduced eDNA compared to wild-type
295 biofilms and to be more susceptible to chemical disruption (21, 47). Our results further
296 confirm these findings, where OligoG treatment of *P. aeruginosa* PAO1 resulted in
297 significant decreases in pyocyanin and eDNA production

298 Virulence-targeted anti-bacterials, which effectively 'disarm' pathogenic bacteria, have
299 received considerable attention (48) although many have proved to be short-lived due to
300 issues with toxicity and the acquisition of bacterial resistance. Resistance to furanone in *P.*
301 *aeruginosa* can be selected for *in vitro*, as well as being found in clinical isolates (49). In
302 contrast to many of the previously described therapeutic modalities, OligoG, which targets
303 bacterial virulence as a QS antagonist, shows considerable promise. Phase I and Phase IIa
304 human studies failed to demonstrate toxicity. Moreover, extended *in vitro* serial passage in
305 the presence of OligoG, has failed to demonstrate the acquisition of bacterial resistance (4).

306 As QS inhibitors target specific pathogenicity traits such as virulence determinants,
307 there has been considerable interest in their use as use as novel anti-infective therapies (50)
308 both by screening for novel compounds (51) and by targeted synthesis of new ligands (52).

Similar to OligoG, the QS inhibitors, furanone and C10-CPA have previously been shown to impede AHL-mediated QS in *P. aeruginosa* leading to an altered biofilm architecture, reduced virulence factor production, as well as enhanced bacterial detachment and antibiotic susceptibility (53, 30 respectively). Predictably, the QS inhibition effects seen with OligoG appear to more closely resemble those of the C10-CPA tested here, interfering as it does with both the *las* and *rhl* QS systems, unlike furanone, which was predominantly found to perturb the *las* system. The dose-response effects and effects on bacterial growth observed in this study suggest that although OligoG CF-5/20 does not act as a true QS inhibitor, it does act as a QS antagonist, affecting signalling pathways in *P. aeruginosa*, with expression of Las and Rhl QS pathways altered in a dose-dependent manner following OligoG CF-/20 treatment. This also proposes a mechanistic rationale for the previously-described anti-biofilm properties of this novel antimicrobial agent that is currently in human clinical trials.

MATERIALS AND METHODS

Alginate oligosaccharides. The low molecular weight alginate oligosaccharide, OligoG CF-5/20 (Mn = 2800) used in the study was prepared, purified and characterized as previously described (4).

Growth curves. Overnight cultures of *P. aeruginosa* PAO1 grown in tryptone soya broth at 37^{O}C, 120 rpm were diluted (1:100) in Mueller-Hinton (MH) broth ± OligoG CF-5/20 (0.2%, 2% and 10%). The growth of *P. aeruginosa* PAO1 was monitored over 48 h, aerobically at 37^{O}C. Absorbance (OD_{600}) values taken every hour in a in a FLUOstar Optima plate reader

(BMG LABTECH). A one-way ANOVA using Tukey-Kramer post-test and the minimum significant difference generated.

***Chromobacterium violaceum* CV026 biosensor strain.** *C. violaceum* CV026 is unable to produce the purple pigment violacein without an external source of AHLs; therefore, violacein production is induced by AHLs that are C4-C8 in length, whereas inhibition of violacein production can also occur using AHLs of a longer carbon chain length (C10-C14). Both *P. aeruginosa* AHLs can, therefore, be detected using the *C. violaceum* CV026 strain: C4-AHL by induction of violacein production, and 3-oxo-C12-AHL by inhibition.

Acyl-homoserine lactone (AHL) extraction. Cell free supernatants were collected and equivalent volumes of ethyl acetate (acidified by supplementing with 0.5% formic acid) added. Following mixing for 30 s, the phases were allowed to separate and the top layer collected, this was repeated three times. The combined ethyl acetate fractions were evaporated and the precipitate was resuspended in 1 ml of distilled H_2O (54). Samples were used immediately or freeze-dried and stored at -20°C until required.

Screening of AHL extracts using CV026 induction and inhibition assays. AHL extracts were tested using the *C. violaceum* CV026 biosensor strain using a well-diffusion assay (55). *C. violaceum* CV026 was grown in LB for 16 h at 30°C supplemented with kanamycin (50 µg/ml). This overnight culture was incorporated into LB agar plates (1.2%) by dilution (1:100). In addition, induction plates also contained kanamycin (50 µg/ml) and inhibition plates both kanamycin and C10 AHL (50 nM) (17248, Sigma-Aldrich, Pool, UK). A well (6 mm) was made into the centre of each solidified agar plate. Test AHL extracts (or controls) were then added to the well (adjusting with dH_2O according to the dry weight of PAO1 culture used). The plates were then incubated at 30°C for 48 h. Distances of violaceum

355 induction or inhibition as determined by the extent of purple colouration or zone of
356 clearing, respectively, were then measured (mm).

357 **Cell-free culture supernatant.** Cultures of *P. aeruginosa* PAO1 were grown for 12, 18,
358 24 and 30 h and prepared as described previously for growth curves. MH broth was selected
359 as nutrient-limited media have been shown to enhance AHL production (9). Cells were
360 harvested (3900 *g*, 20 min, 4°C) and washed three times with ice cold 0.9% NaCl and dried
361 at 80°C for 24 h. In each case, differences in culture biomass (at OligoG CF-5/20
362 concentrations >2%) from cell-free culture supernatants, used for the screening of AHLs and
363 the extraction of all the virulence factors was corrected by normalisation according to dry
364 weight.

365 **Quantitation of extracellular virulence factors.** Pyocyanin was extracted from the
366 culture supernatant (700 µl) using chloroform in the ratio of 3:2 and re-extracted with 150
367 µl of 0.2 M HCl and the absorbance read at 520 nm (17). Rhamnolipids were extracted from
368 culture supernatant with ethyl acetate in a 1:1 ratio, vortexed for 15 sec and centrifuged
369 (10,000 *g*, 4°C, 5 min). The upper layer was removed and ethyl acetate extraction repeated
370 (x3) for each sample. The combined upper layer was left to evaporate overnight. Then 900
371 µl of orcinol reagent (0.19% orcinol in 53% H_2SO_4) was added to the precipitate, and
372 incubated at 80^{O}C for 30 min, before reading the absorbance at 420 nm (51). Protease
373 activity was determined using 2% azocasein solution prepared in 50 mM phosphate buffer
374 saline (PBS), pH 7. The substrate and culture supernatant were incubated at 37°C in 1:1
375 ratio for 1 h in a reaction volume of 400 µl. The reaction was stopped by the addition of 500
376 µl of 10% trichloroacetic acid and centrifuged at 8000 *g* for 5 min to remove residual
377 azocasein. The absorbance was read at 400 nm (17). Elastase extraction employed, 200 µl
378 elastin Congo red solution (5 mg/ml in 0.1 M Tris-HCl pH 8; 1 mM $CaCl_2$) which was

379 incubated with 600 µl of cell-free, culture supernatant at 37˚C for 3 h at 200 rpm. The
380 mixture was then centrifuged at 3000 g for 10 min and the absorbance read at 490 nm (17).
381 **High performance liquid chromatography triple quadrupole mass spectrometry**
382 **(LC/MS).** AHLs were extracted as described above and freeze-dried until required. Freeze
383 dried samples were reconstituted in 200 µl of acetonitrile (ACN) with 0.1% acetic acid and
384 7.2 ng/ml of the internal standard umbelliferone. Samples were vortexed, centrifuged (16),
385 (100 *g*, 4°C, 10 min) and supernatants filtered through 0.4 µm syringe filter (Phenomenex,
386 UK) this was performed twice to increase metabolite extraction. Samples were kept on ice
387 throughout the extraction procedure prior to being run on the liquid chromatography Triple
388 quadrupole mass spectrometer (LC-QQQ-MS). Samples (5 µl) were loaded onto a C18 XDB
389 Eclipse (1.8 µm, 4.6 x 50 mm) reverse phase column (Agilent Technologies, Palo Alto, USA).
390 Samples were quantified using a 1200 series HPLC (Agilent Technologies, USA) coupled to a
391 6410B enhanced sensitivity triple quadruple (QQQ) mass spectrometer (Agilent
392 Technologies, USA). For detection using positive ion mode, mobile phase A comprised of 5
393 mM ammonium acetate in water modified with 0.1% acetic acid and B was acetonitrile
394 containing 0.1% acetic acid. The column was equilibrated in 2% B, before increasing in a
395 linear fashion to 100% over 6 min; with 100% B being maintained for a further 2 min before
396 column re-equilibration. The column temperature was maintained at 35°C for the duration
397 with a flow rate of 0.3 mL/min. Source parameters were as follows: temperature, 350 °C,
398 gas flow, 10 L/min; nebuliser, 35 psi; and capillary voltage, 4 kV. Data were analysed using
399 Agilent MassHunter QQQ Quantitative Analysis software (Version B.07.00). Peak areas were
400 normalized to the internal standard umbelliferone and concentrations calculated using
401 standard concentration curves, offset against blank values (the average peak areas for the
402 blanks).

403 **RNA extraction for real time PCR (qPCR).** RNA was extracted from 24 h cultures of *P.*
404 *aeruginosa* PAO1 grown at 37°C in MH broth +/- OligoG CF-5/20 (0.2, 2 and 10%). Cultures
405 were harvested (2000 *g*, 10 min), resuspended and adjusted to 1.0 x 10^8 CFU/ml in PBS and
406 centrifuged (12,000 *g*, 2 min) and re-suspended in 0.5 ml RNA later and stored at -20°C until
407 required. Cells were pelleted (12,000 *g*, 2 min) and re-suspended with lysis buffer (RLT
408 buffer, QIAgen, Crawley, UK) containing 1% (v/v) β-mercaptoethanol. Cell debris was
409 pelleted via centrifugation (12,000 *g*, 2 min), resulting supernatants were removed into
410 fresh tubes, phenol:chloroform:isoamyl alcohol (25:24:1) was used to acquire total nucleic
411 acid. Total RNA was recovered after DNase I treatment using the RNeasy® Mini Kit
412 (QIAGEN) according to the manufacturer's instructions. Gel electrophoresis was used to
413 check the purity and integrity of the total RNA and RNA concentration was measured
414 spectrophotometrically and an additional purity check using the absorbance ratio of
415 260/280 nm (NanoVue, GE Healthcare, Little Chalfont, UK) and standardised to 300 ng/ml.
416 Reverse transcription reactions for cDNA synthesis included total RNA (300 ng) template, 1
417 µl of 50 µg/ml random primer and molecular grade water was added to give a final reaction
418 volume of 10 µl. RT-qPCR was performed in triplicate using NanoScript2 RT-Kit (primer
419 design, UK) and a final annealing step of 5 min at 65°C, after which point the samples were
420 cooled on ice. Annealed samples were then added to the extension mix; 4 µl of 4 x
421 nanoScript2 Buffer, 1 µl dNTP mix (10 mM), NanoScript2 enzyme at 1.5 µl (Primer Design,
422 Southampton, UK), and 2.5 µl molecular grade water and a final volume of 20 µl was
423 incubated at 25°C for 5 min and then at 42°C for 20 min.

424 **Real-time PCR (qPCR) for analysis of gene expression.** RT-qPCR for analysis of the
425 expression of QS genes was carried out using the primers presented in Table 1. Primer
426 specificity was tested on genomic DNA. RT-qPCR was performed in triplicate with three

replicate samples, using an ABI 7000 instrument (Life Technologies, UK). Each reaction contained 2 µl cDNA, 12.5 µl (x2) of SYBR-Green PCR master mix (PrecisionPlus Mastermix; Primer Design, Southampton, UK), 10 mM of each primer and made up to 25 µl with highly purified water (Qiagen). The thermal cycler profile comprised of initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 15 sec, primer annealing at 58°C (15 s), and extension at 72°C (30 s). A final extension at 72°C for 2 min was performed, followed by cooling at 4°C. A dissociation step at 60°C was used to generate a melting curve for verification of the amplified product. After RT-qPCR, the threshold was adjusted according to the amplification curves of all evaluated genes. Comparison between groups was made based on the cycle number at which both the target and the average of endogenous control genes (*rpsL* and *proD*) attained threshold cycle (Ct) fluorescence. Analysis of relative gene expression was achieved according to the $^{\Delta\Delta}$CT method (56).

eDNA determination of *Pseudomonas aeruginosa* PAO1 biofilms treated with OligoG CF-5/20 using a nucleic-acid specific cell impermeable fluorescent TOTO-1® stain. The effect of OligoG on formation of 24 h *P. aeruginosa* PAO1 biofilms was tested. For this, adjusted *P. aeruginosa* PAO1 cultures (10^7 CFU/ml) were diluted (1:0) in MH broth +/-OligoG CF-5/20 (0.5, 2, 6% w/v) and then incubated in Whatman 96-well glass-bottomed plates at 37°C for 24 h with gentle agitation prior to staining. The effect of OligoG on established 24 h biofilms was also tested to look at its effect on biofilm disruption. For this, biofilms were grown without OligoG treatment using adjusted *P. aeruginosa* PAO1 cultures (10^7 CFU/ml), diluted (1:0) in MH broth. After 24 h incubation, half the supernatant was removed and replaced with 100 µl fresh MH broth ± OligoG CF-5/20 (0.5, 2, 6% w/v) and the samples incubated for a further 24 h before staining. After OligoG treatment, the supernatant was

450 removed and biofilms stained with TOTO®-1 (Thermofisher) for 25 mins. Biofilm samples
451 were imaged using a Leica TCS SP5 confocal system with a x63 lens.

452 For fluorescence determination of eDNA, biofilms were homogenised by vigorous
453 pipetting and the resulting supernatant filtered (0.2 µm). Culture purity was confirmed by
454 plating a loopful of supernatant onto non-selective blood agar. Supernatants were stained
455 with TOTO®-1 at room temperature for 35 min and fluorescence excitation/emission
456 measured at ~514/533 nm on a FLUOstar Optima plate reader (BMG LABTECH) (47).

457 **Synthesis of N-decanoyl cyclopentylamide (C10-CPA).** Decanoyl chloride (1 mol eq;
458 0.544 ml; 0.500 g; 2.6 mmol) was added dropwise to a stirring solution of cyclopentylamine
459 (2 mol eq; 0.513 ml; 0.443 g; 5.2 mmol) in anhydrous dichloromethane (5 ml) under
460 nitrogen atmosphere. The reaction was stirred for 6 h and then the solvent evaporated
461 under reduced pressure. The residue was re-dissolved in 20 ml of diethyl ether and washed
462 with water, 5% $NaHCO_3$, 0.2 M HCl and saturated NaCl solution. The organic layer was dried
463 over $MgSO_4$ and concentrated to furnish the N-cyclopentyldecanamide as a white solid and
464 confirmed by hydrogen-1 nuclear magnetic resonance (H-NMR), carbon-13 NMR and
465 electrospray ionization MS (30).

466 **Confocal laser scanning microscopy imaging of *Pseudomonas aeruginosa* biofilms**
467 **treated with QS inhibitors.** Overnight cultures of *P. aeruginosa* PAO1 grown in tryptone
468 soya broth (TSB) were adjusted to 10^7 CFU/ml and 10 µl of adjusted cultures added to 90 µl
469 of MH broth in glass-bottomed 96 well plates. Biofilms of *P. aeruginosa* PAO1 were grown
470 (24 h) whilst being treated (rocking gently) with known AHL quorum sensing inhibitors of
471 2(5H)-Furanone, (283754, Sigma-Aldrich, Pool, UK) at (1.25 µg ml^{-1} and 2.5 µg ml^{-1} (14.9 and
472 29.7 µM respectively) (29, 30) and N-decanoyl cyclopentylamide (C10-CPA) at 100 and 250
473 µM (30). Untreated and OligoG-treated biofilms were used as controls. Planktonic

474 cells/supernatant was removed before staining the biofilms with 6 % LIVE/DEAD® BacLight™
475 bacterial viability kit (Invitrogen, Paisley, UK) in PBS, incubating in the dark (10 min) and
476 imaging with a Leica TCS SP5 confocal system using a x 63 lens.

477 **Circular dichroism (CD) spectroscopy.** To evaluate whether AHLs influence the
478 conformation of OligoG CF-5/20, CD spectra were recorded on an Aviv 215 instrument (Aviv
479 Biomedical Inc., Lakewood, NJ) from 260 to 200 nm, 1 nm band-width, using a 0.5-cm quartz
480 cell at 37°C. OligoG CF-5/20 was dissolved in 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, at a
481 concentration of 0.5 mg/ml and either C4-AHL or 3-oxo-C12-AHL (Sigma-Aldrich, Pool, UK,
482 09014 and 09945) was added stepwise from 1mg/ml stock solutions. Buffer baselines and
483 the intrinsic AHL spectra were subtracted, and spectra were corrected for dilution. Data are
484 presented as mean-residue-weight ellipticities $[\Theta]_{MRW}$ assuming M_r = 194 g/mol for the
485 OligoG CF-5/20 monosaccharides.

486 **Statistical analysis.** Microsoft Excel was used to perform statistical analysis including
487 one-way ANOVA using the Tukey-Kramer post-test and the minimum significant difference
488 (MSD) was calculated using the Tukey-Kramer method (57). $P<0.05$ was considered
489 significant.

490

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500
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Table 1. Genes and primers used for qPCR in this study.

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Forward primer			Reverse primer			Product size (bp)	Reference
			BP	GC	TM	BP	GC	TM		
lasI	TGTTCAAGGAGCGCAAAGG	ATGGCGAAACGGCTGAGTT	19	52.6	62.4	19	52.6	63	244	58
lasR	AGCGACCTTGGATTCTCGAAG	CGAAGAACTCGTGCTGCTTTC	21	52.4	63	21	52.4	62.5	226	58
rhlI	TGCTCTCTGAATCGCTGGAA	GTTTGCGGATGGTCGAACTG	20	50	59.1	20	55	59.83	154	58
rhlR	TTGCTGAGCGTGCTTTCC	AGGATGATGGCGATTTCCC	19	52.6	62.6	19	52.6	62.1	228	58
**rpsL*	CCTCGTACATCGGTGGTGAAG	CCCTGCTTACGGTCTTTGACAC	21	57.1	62.8	22	54.5	63.1	148	59
**proD*	GGGCGAAGAAGGAAATGGTC	CAGGTGGCGTAGGTAGAGAA	20	55	63.1	20	55	58	178	60

*Reference/endogenous control genes; BP,primer length (basepairs); GC, G-C content of primer; TM, melting temperature of primer

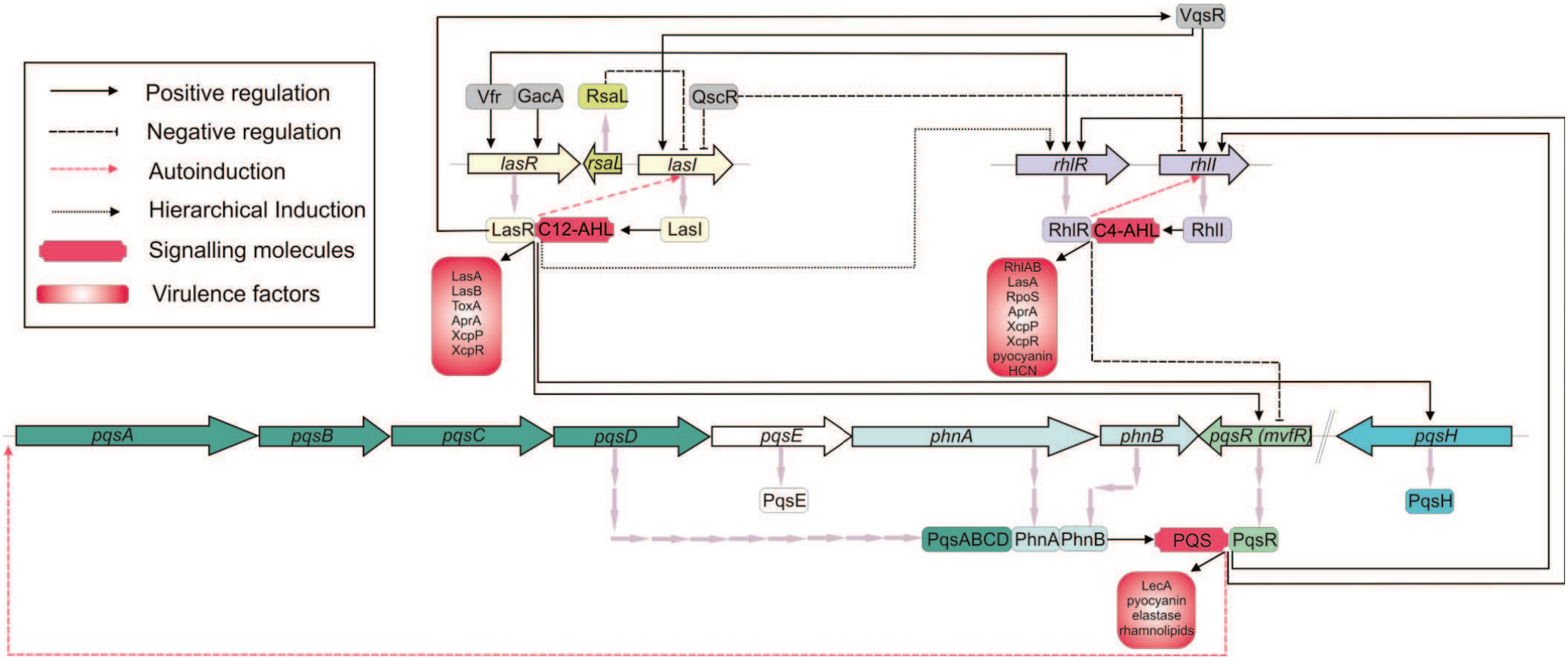

FIG 1 Schematic diagram of the *Pseudomonas aeruginosa* virulence regulatory network showing the three major QS signalling pathways namely, the acyl homoserine lactone Las and Rhl operons and the 2-heptyl-3-hydroxy-4-quinolone *Pseudomonas* quinolone signal (PQS) operon. Differences in culture biomass (at ≥2% OligoG) were corrected according to dry weight.

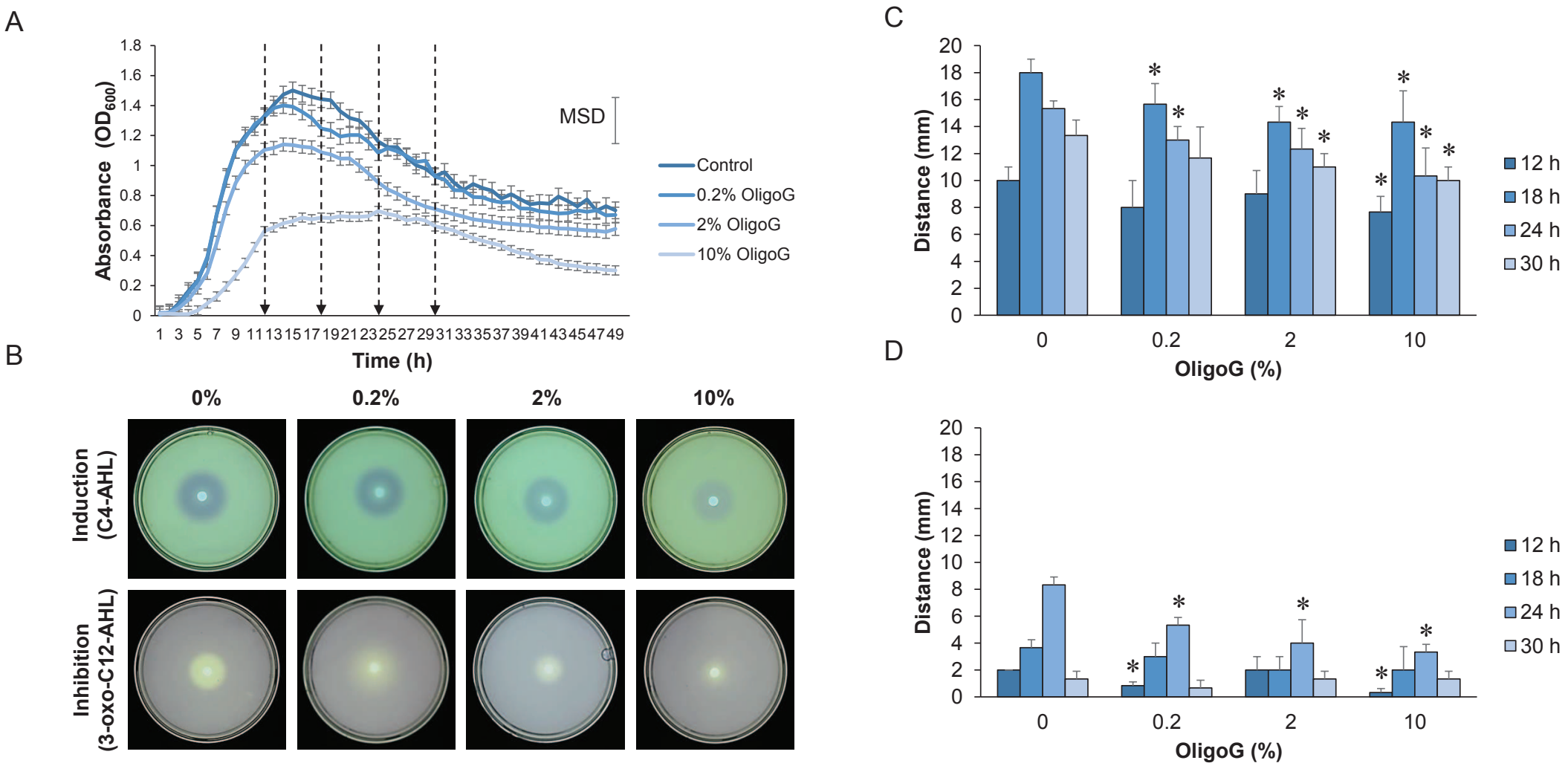

FIG 2 Effect of OligoG CF-5/20 on the growth of *P. aeruginosa* PAO1 and the production of signalling molecules using the biosensor *Chromobacterium violaceum* CV026. (A) Growth curves of *P. aeruginosa* PAO1 treated with OligoG CF-5/20 showing four specific sampling times (12, 18, 24 and 30 h) for AHL extractions (arrows). Well-diffusion time-course assay detecting AHLs from (B) 24 h or (C) and (D) 12, 18, 24 and 30 h extracts of *P. aeruginosa* cultures treated with OligoG (0.2, 2 and 10 %). (B) and (C) Induction (zone of colouration) or (B) and (D) Inhibition (zone of clearing) of violacein synthesis in *C. violaceum* CV026 showing changes in C4- and 3-oxo-C12-AHL production following OligoG treatment (n = 3 ± standard deviation; * P<0.05). MSD, minimum significant difference. Differences in culture bio-mass (at ≥2% OligoG) were corrected according to dry weight.

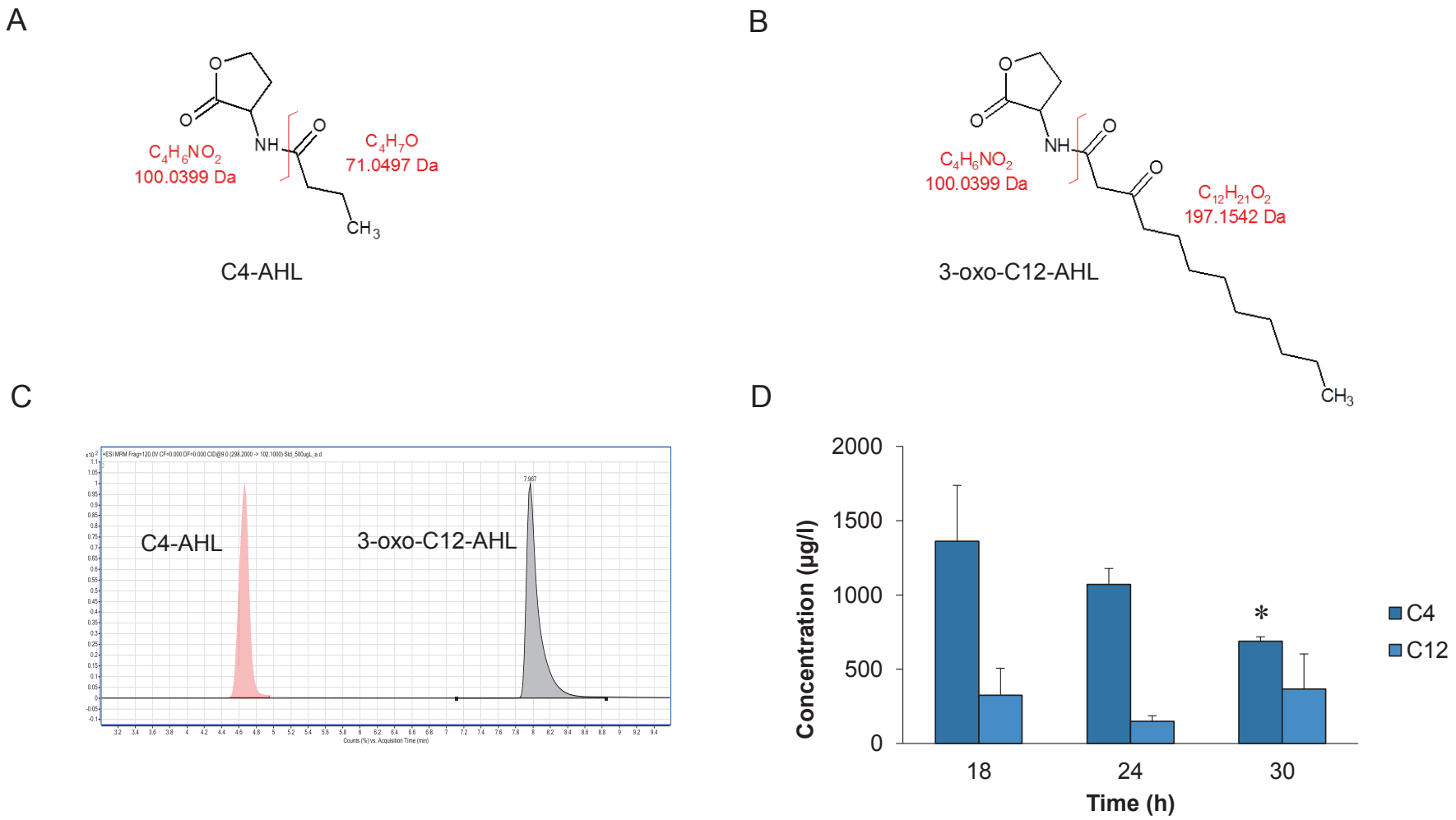

FIG 3 Method development for detection and quantification of acyl homoserone lactones (AHLs). (A) Structure of C4-AHL. (B) Structure of 3-oxo-C12-AHL. (C) C4-AHL and 3-oxo-C12-AHL LC/Mass spectrometry peaks. (D) Initial time course showing LC/MS quantification of AHL concentrations (µg/L) from *P. aeruginosa* PAO1 grown in Mueller Hinton (MH) broth at different time points (18, 24 and 30 h). (n = 3 ± standard deviation; * P<0.05). Differences in culture bio-mass (at ≥2% OligoG) were corrected according to dry weight.

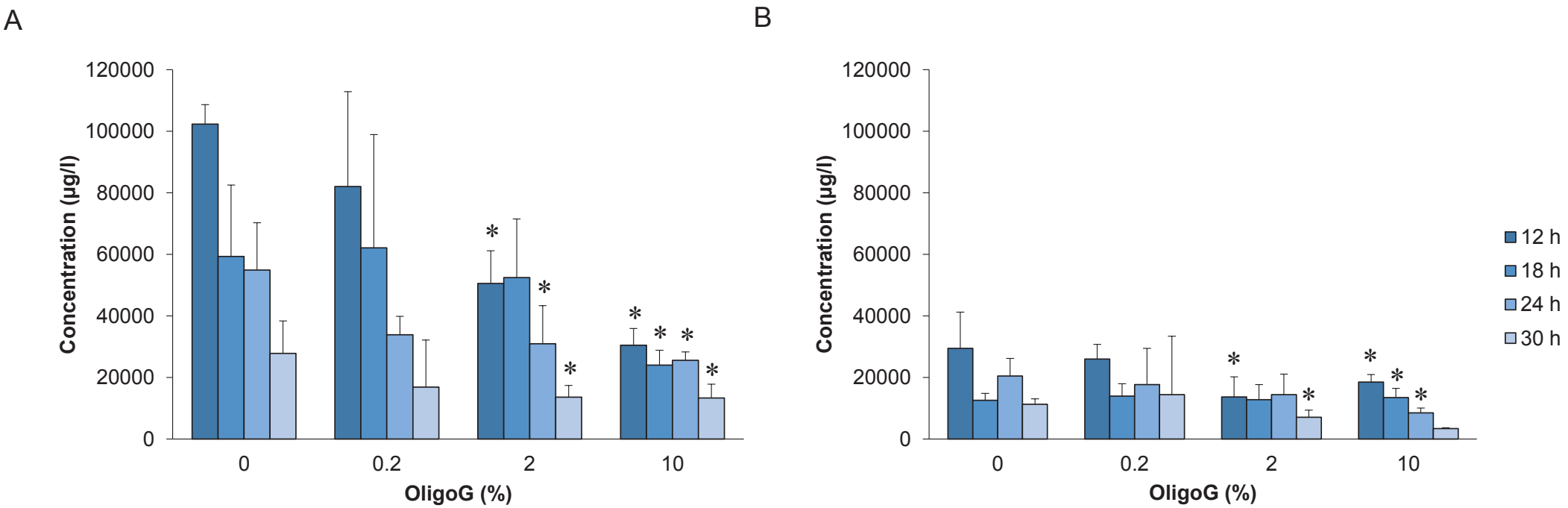

FIG 4 Effect of OligoG CF-5/20 on AHL concentrations (µg/L) determined by LC/MS at different time points (12, 18, 24 and 30 h) in *P. aeruginosa* PAO1 grown in Mueller Hinton (MH) broth ±OligoG (0.2, 2 and 10%). A) C4-AHL. B) 3-oxo-C12-AHL. (n = 3 ± standard deviation; * P<0.05). Differences in culture bio-mass (at ≥2% OligoG) were corrected according to dry weight.

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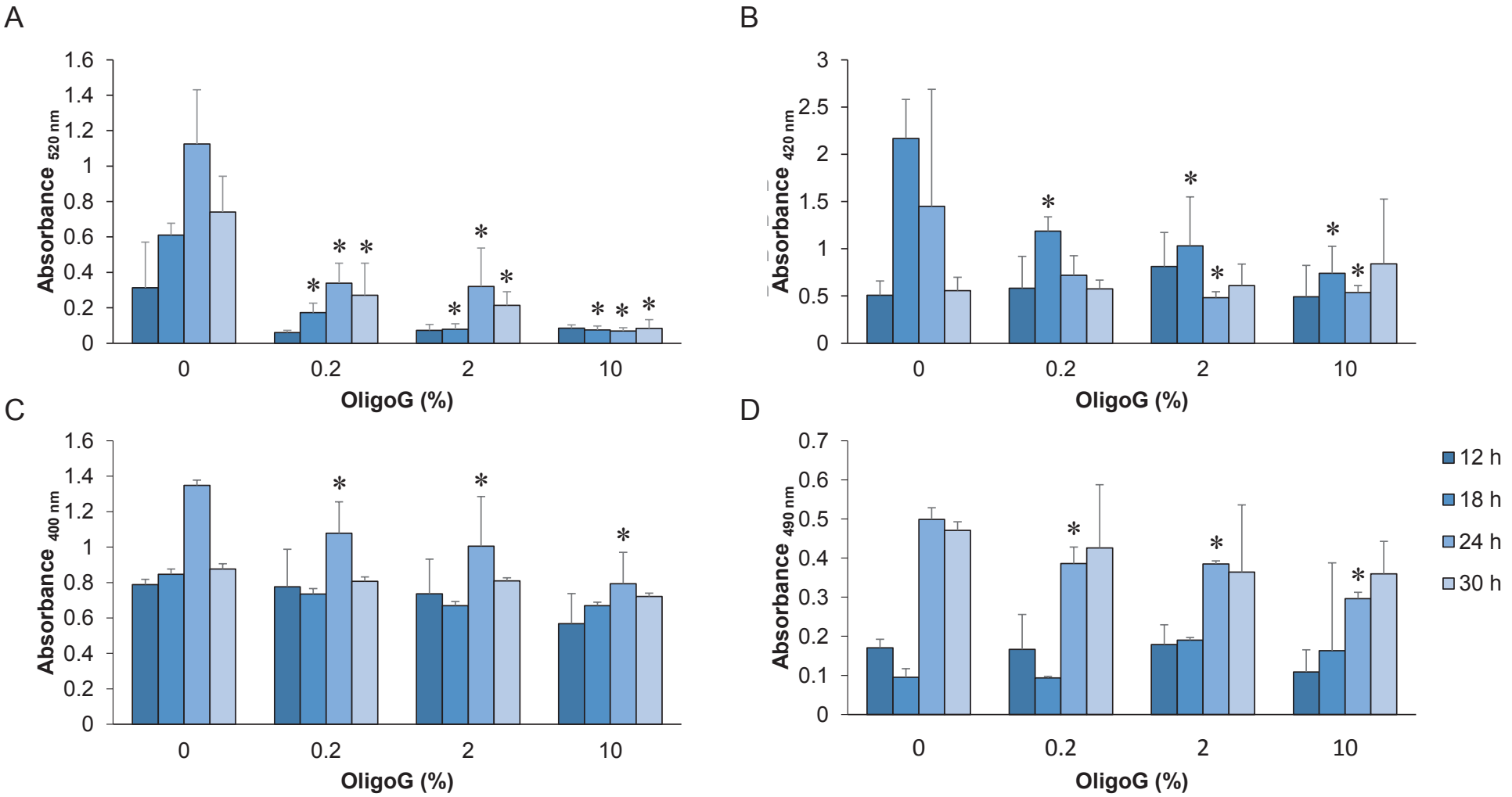

FIG 5 Extracellular virulence factor production by *P. aeruginosa* from 12, 18, 24 and 30 h cell free culture supernatants treated with OligoG CF-5/20 (0.2, 2 and 10 %). (A) pyocyanin. (B) rhamnolipids. (C) total protease. (D) elastase. (n = 4 ± standard deviation; * P<0.05). Differences in culture bio-mass (at ≥2% OligoG) were corrected according to dry weight.

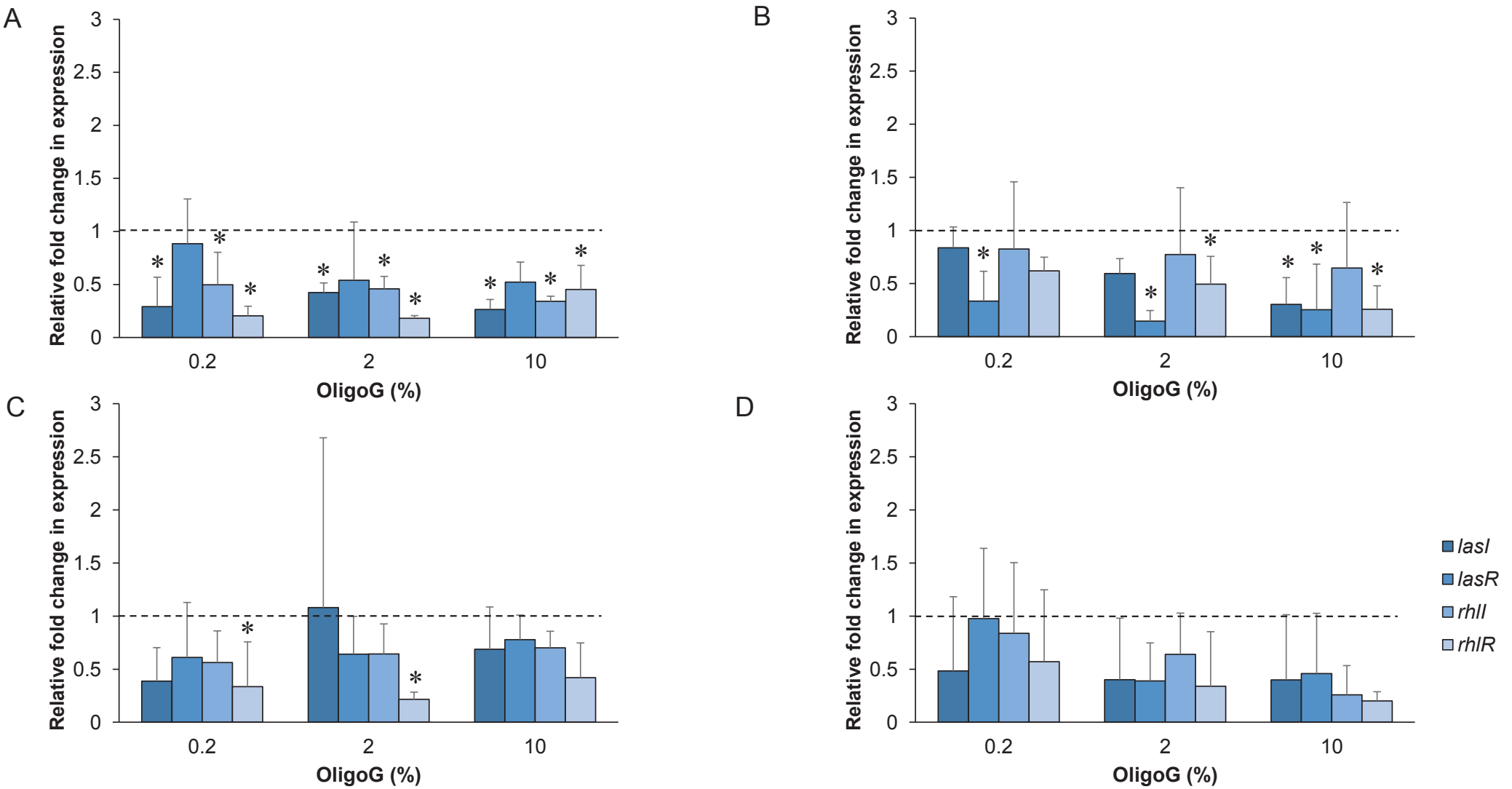

FIG 6 Relative fold change in gene expression compared to untreated control using quantitative PCR of *lasI/R, rhlI/R* genes from 12, 18, 24 and 30 h cultures of *P. aeruginosa* PAO1 treated with OligoG CF-5/20 (0.2, 2 and 10%). (A) 12 h. (B) 18 h. (C) 24 h. (D) 30 h. (n = 3 ± standard deviation; * P<0.05). Differences in culture bio-mass (at ≥2% OligoG) were corrected according to dry weight.

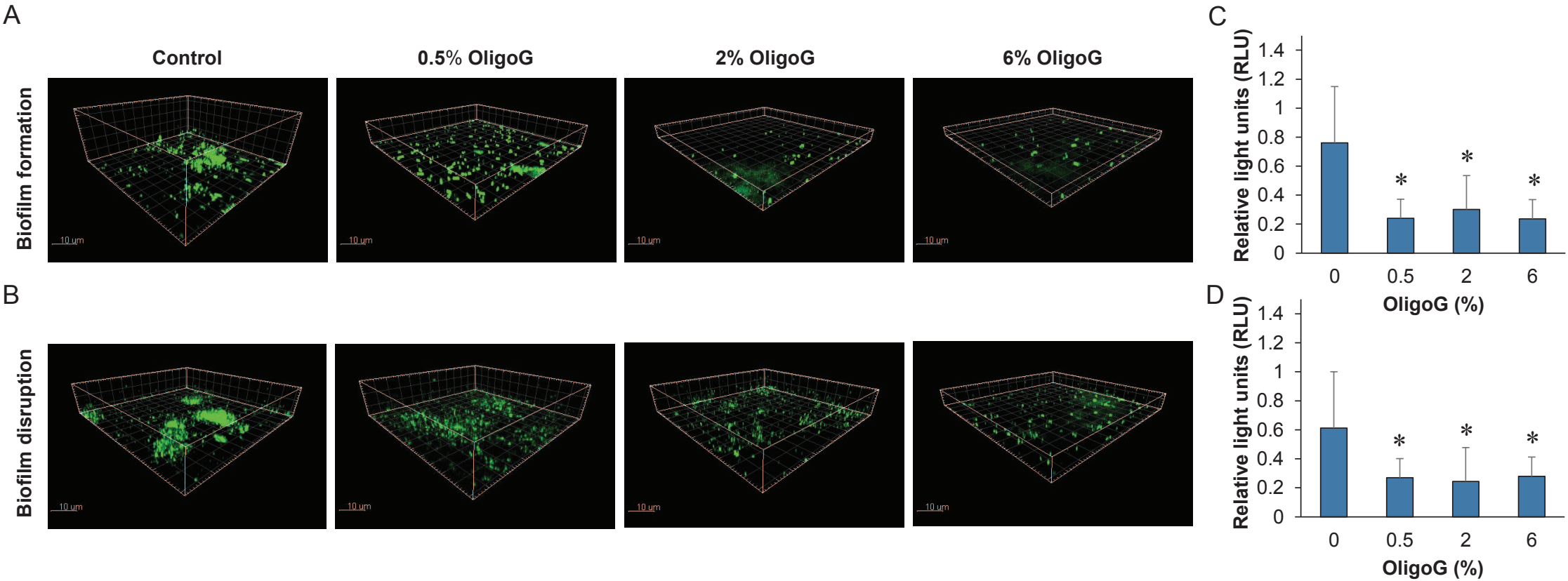

FIG 7 CLSM of *Pseudomonas aeruginosa* PAO1 biofilms treated with OligoG CF-5/20 (0.5, 2 and 10%) and stained with nucleic acid specific TOTO-1 (green). (A) Biofilm formation assay: Biofilms grown for 24 h in the presence of OligoG. (B) Biofilm disruption assay: 24 h established biofilms subsequently treated for 24 h with OligoG shown with corresponding fluorescence intensities in (C) and (D). (n = 3 ± standard deviation; * P<0.05).

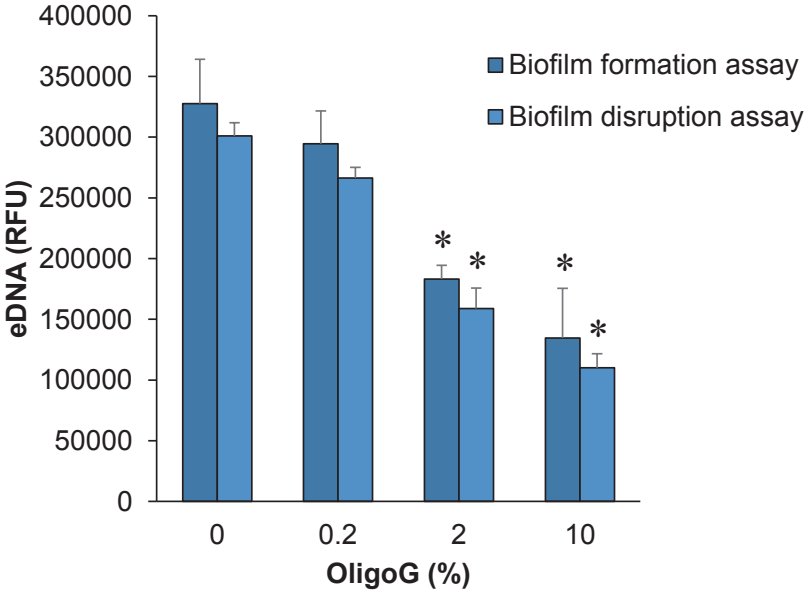

FIG 8 Determination of eDNA concentration. Effect of OligoG (0.2, 2 and 10 %) on relative eDNA concentration in *P. aeruginosa* biofilms. Biofilm formation assay: biofilms grown for 24 h in the presence of OligoG and Biofilm disruption assay: 24 h established biofilms subsequently treated for 24 h with OligoG. (n = 3 ± standard deviation; * P<0.05). Differences in culture bio-mass (at ≥2% OligoG) were corrected according to dry weight.

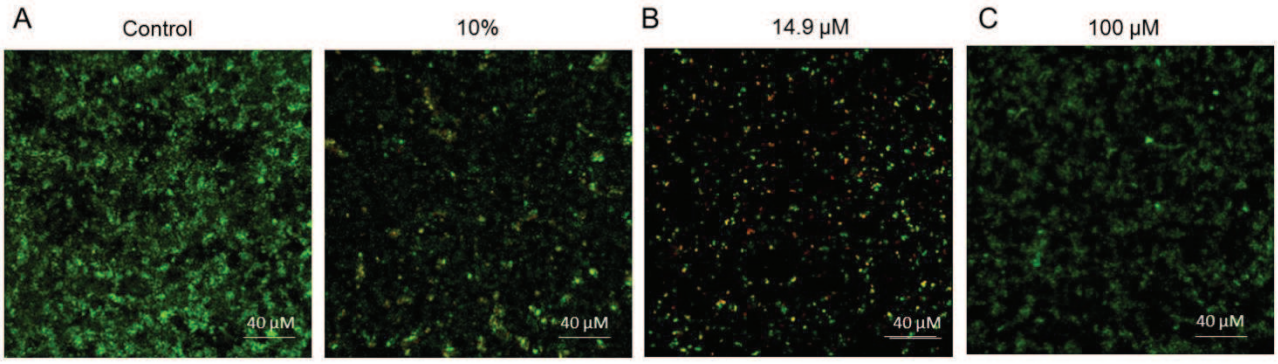

FIG S1 Confocal laser scanning microscopy of *Pseudomonas aeruginosa* PAO1 24 h biofilms treated with quorum sensing inhibitors. (A) OligoG CF-5/20, 0 and 10%. (B) 2(5H)-furanone, 1.25 µg/mL (14.9 µM). (C) N-decanoyl cyclopentylamide (C10-CPA), 100 µM, (n=3).

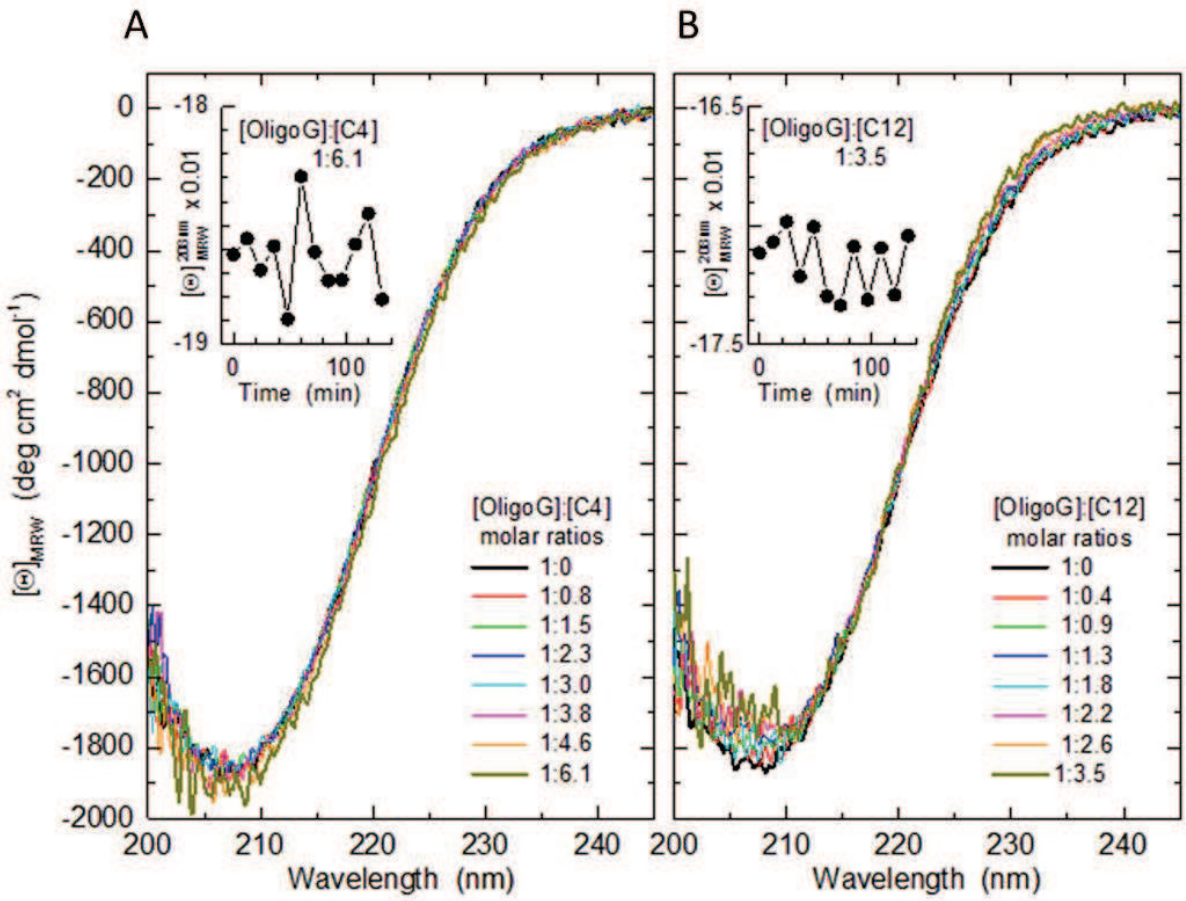

FIG S2 Circular dichroism spectra showing the effect of OligoG (at a range of molar ratios) on homoserine lactones. (A) C4-AHL and (B) 3-oxo-C12 AHL. Insets show a time course recorded at 208nm after addition of AHLs at their maximum concentration.