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Molecular targets of 14-alpha-lipoyl andrographolide on quorum sensing in *Pseudomonas aeruginosa*

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Running title: Effects of AL-1 on quorum sensing of *P. aeruginosa*

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Abstract

In *Pseudomonas aeruginosa* (*P. aeruginosa*), quorum sensing (QS) system is closely related to the biofilm formation. We previously demonstrated that 14-Alpha-lipoyl andrographolide (AL-1) has synergistic effects on antibiofilm and antivirulence factors (pyocyanin and exopolysaccharide) of *P. aeruginosa* when combined with conventional antibiotics, while has little inhibitory effect on its growth. However, its molecular mechanism remains elusive. Here we investigated the effect of AL-1 on QS, especially the Las and Rhl system. It showed that AL-1 can inhibit LasR-3-oxo-C_{12}-HSL interactions and repress the transcriptional level of QS-regulated genes. RT-PCR data showed that AL-1 significantly reduced the expression of *lasR*, *lasI*, *rhlR*, and *rhlI*, in a dose-dependent manner. AL-1 not only decreased the expression of Psl which is positively regulated by Las system, but also increased the secretion of ExoS which is negatively regulated by Rhl system, indicating that AL-1 has multiple effects on both Las and Rhl system. It is no wonder that AL-1 showed synergistic effects with other antimicrobial agents in the treatment of *P. aeruginosa* infections.

64 **Introduction**

65 *P. aeruginosa* is an opportunistic human pathogen responsible for severe infections in
66 immune-compromised and cystic fibrosis (CF) patients (15, 30). Due to its large
67 occurrence in hospital water-supplying pipes and its capacity to persist on medical
68 devices, *P. aeruginosa* is a leading cause of life-threatening infections (48). In
69 addition, *P. aeruginosa* is notorious for the vigorous development of biofilm, which
70 adds difficulties in antibiotics therapy and makes the wounds unhealed (12). Biofilm
71 formation is believed to be one of the major causes of persistent infections.

72 Biofilm formation in *P. aeruginosa* is regulated by a complex network of signals
73 that includes QS, small RNAs, and nutritional cues (26). QS controls important
74 functions including biofilm formation and pathogenicity (53). *P. aeruginosa* has two
75 acylated homoserine lactones (AHLs)-based QS systems (Las and Rhl), and a
76 *Pseudomonas* Quinolone Signal (PQS, 2-heptyl-3-hydroxy-4-quinolone)-based
77 signaling pathway. The transcription factors LasR and RhlR interact with and are
78 activated by 3-oxo-C_{12}-HSL (*N*-3-oxo-dodecanoyl-homoserine lactone) and C_4-HSL
79 (*N*-butyryl-L-homoserine lactone), respectively. PqsR is LasR-RhlR homolog, which
80 responds to PQS (54). It has been reported that *P. aeruginosa* QS systems control up
81 to 11% of its genome (47, 55, 56). Of these QS systems, LasR-3-oxo-C_{12}-HSL system
82 is the dominant regulator because it is a turning-on system of the *P. aeruginosa* QS
83 cascade that triggers the successive activation of other QS systems, including
84 RhlR-C_4-HSL and PqsR-PQS systems (41).

85 Exopolysaccharides (EPS) are key matrix components of biofilms, as they
86 contribute to the overall biofilm architecture and resistance (1, 31, 45). Psl
87 polysaccharide is an essential matrix component that is required for *P. aeruginosa* to
88 initiate and maintain biofilms (13, 23, 32, 37). In *P. aeruginosa* PA2231
89 (*pslA*)-PA2242 (*pslL*) is positively regulated by the Las system, according to work
90 published by Kerrigan (14).

91 *P. aeruginosa* has another important virulence component called Type III
92 secretion system (TS33), which is negatively regulated by QS. TS33 is a needle-like
93 complex which secretes a number of cytotoxins, including ExoS, ExoT, ExoU, and

ExoY (44). These products have been demonstrated to show a cytotoxic effect *in vitro*. ExoS and ExoT are bifunctional proteins which have both N-terminal GTPase-activating protein (GAP) activity and C-terminal ADP ribosyltransferase (ADPRT) activity (16).

The multidrug resistance is now a worldwide problem. Novel small molecule inhibitors for *P. aeruginosa* are urgently needed. Natural products are notable not only for their potent therapeutic activities, but also for the fact that they frequently possess the desirable pharmacokinetic properties required for clinical development (62). Many natural products have been widely used in clinic, a testimony to the remarkable ability of microorganisms to produce drug-like small molecules (4, 27, 61). We have developed a high-throughput synergy screening platform to realize the full potential of natural products (63).

Andrographolide (Andro) is extracted from an herb *Andrographis panicuoata* Nees. We have reported that AL-1, a derivative of Andro, inhibited biofilm formation and sensitized the bacterium *P. aeruginosa* to a variety of antibiotics for distinct synergistic effects (59). However, how this QS inhibitor exerts its effects on biofilm formation is still elusive.

This study aims to investigate how AL-1 inhibits the *P. aeruginosa* PAO1 biofilm formation. Since LasR play a critical role in the biofilm development, we test the effects of AL-1 on LasR using the AHL-deficient strain. Then we investigated the effects of AL-1 on the expression levels of seven QS-related genes (*lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, *pqsH*, and *pqsR*) of *P. aeruginosa* using luminescent reporters. The anti-QS activity of AL-1 was validated further by RT-PCR. Psl provides a hydrated scaffolding to stabilize the structure of the biofilm. This led to the hypothesis that AL-1 may decrease the biofilm matrix Psl. To elucidate the role of AL-1 on Psl, we used the Psl immunoblots. The β-galactosidase assay further suggested that AL-1 has effect on Psl both at transcriptional and translational level. A complete understanding of the AL-1 on *P. aeruginosa* biofilm matrix may help us in the development of novel therapeutics.

Materials and Methods

Reagents

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO). AL-1 was chemically synthesized (Fig.S1) as we have previously reported (25).

Bacterial strains and growth conditions

Pseudomonas putida F117 (pKRC12) (3) was kindly provided by Professor Jo Handelsman from the University of Wisconsin-Madison, USA. *P. aeruginosa* CIM45/46 (22) were provided by Professor Luyan Ma from the Institute of Microbiology, Chinese Academy of Sciences, China.

P. putida F117 (pKRC12) was grown at 28°C in Luria-Bertani (LB). All *P. aeruginosa* strains were grown at 37°C in LB without NaCl (LBNS) or Jensen's medium (24) and are listed in Table1. The plasmid pMS402 carrying a promoterless *luxCDABE* reporter gene cluster was used to construct promoter-*luxCDABE* reporter fusions of seven genes (*lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, *pqsH*, and *pqsR*) as reported previously (11, 29). Antibiotics were added as required at final concentrations of Trimethoprim (TMP) 300 μg/ml and Gentamicin (GEN) 25 μg/ml.

Inhibitory activity of AL-1 in reporter strains with LasR

We used *P. putida* strain F117 (pKRC12), an AHL-deficient strain that has been engineered to produce green fluorescent protein (GFP) upon activation of LasR by 3-oxo-C_{12}-HSL (3). *P. putida* F117 pKRC12 was grown overnight and diluted with LB media to achieve an optical density of 0.05 at 595 nm (OD_{595}), 100 μl aliquots of cells were added to the 96-well plates with DMSO or AL-1 preincubated with 3-oxo-C_{12}-HSL at a final concentration of 50 nM or 1000 nM for 30 min. Fluorescence was measured at regular intervals after 4 h using the EnVision plate reader (PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA).

Luciferase activity-based bioassay for QS inhibitors

A chemiluminometric assay was developed to study effects of AL-1 on expression

levels of genes. Using *lux*-based reporters which indicate the luciferase activity, gene expression in liquid cultures was measured as light production (in counts per second) in a Victor^3 multilabel plate reader (PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA). Overnight cultures of the reporter strains were diluted to an optical density of 0.2 at 620 nm (OD_{620}), and cultivated for an additional 2 h before use. The cultures were inoculated into parallel wells in a 96-well black plate with a transparent bottom. Fresh culture (5 µL) was inoculated into the wells containing a total of 95 µL medium plus other components (OD_{620} in the wells was ~0.07). Filter-sterilized mineral oil (60 µL) was added to prevent evaporation during the assay. Promoter activity was measured every 30 min for 24 h. Bacterial growth was monitored at the same time by measuring the OD_{595} in the Victor^3 multilabel plate reader.

Quantitative reverse transcription PCR (qRT-PCR)

P. aeruginosa PAO1 was grown in LBNS shaking at 37°C overnight, and diluted with LBNS media to achieve an optical density of 0.05 at 595 nm (OD_{595}). 0.5 mM AL-1 or DMSO was added. After 5 hours, total RNA was extracted using a total RNA miniprep kit (Axygen). Residual DNA was removed by DNase I treatment (Fermentas) as recommended. cDNA synthesis was performed using SuperScript III First-Strand Synthesis (Invitrogen) according to the manufacturer's protocol using random hexamers. qRT-PCR was performed with the SYBR Green qPCR Master Mix (Fermentas). To calculate the relative expression level of target genes, the expression level of 16S rRNA was used as an internal control. Primers are listed in Table S1. The data presented below are the results obtained from three independent experiments.

Immunoblotting of Psl polysaccharide extracts

Psl immunoblots were performed as described previously with the following changes (6). *P. aeruginosa* PAO1 was grown in LBNS shaking at 37°C overnight treated with 0.5 mM AL-1 or DMSO. Crude polysaccharide extracts were obtained by spin down 10 OD culture and resuspended in 100 µl of 0.5 M EDTA and boiling 5 min at 100°C.

The supernatant fraction was treated with Proteinase K (final concentration 0.5 mg/ml) for 60 min at 60°C, followed by Proteinase K inactivation for 30 min at 80°C. 5 µl of the sample was spotted onto a nitrocellulose membrane. Blocking with 10% non-fat milk in TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) for 1 h at room temperature. Psl were detected using α-Psl antibodies (1:25000 dilution) and 1:10000 diluted goat anti rabbit IgG conjugated secondary antibody (Thermo-Scientific). Add NBT and BCIP for detection.

β-Galactosidase assay

β-Galactosidase activity was measured as described by Miller (38) and is expressed in Miller units (MU). Cell lysates were assayed for both β-galactosidase activities, as well as for protein content by BCA protein assay (Thermo-Scientific). The data presented below are the results obtained from three independent experiments. The variance is indicated by error bars in the figures.

Western blotting analysis

P. aeruginosa PAO1 grown in LBNS medium overnight at 37°C diluted 1000-fold in fresh LB supplemented with 200 mM NaCl containing dimethyl sulfoxide (DMSO), AL-1 or nitrilotriacetic acid (NTA) for 6 h at 37ºC. The culture supernatant was collected by centrifugation, and the secretion proteins were concentrated by ultrafiltration. Proteins were separated by 12% SDS-PAGE, then blotted to polyvinylidene fluoride (PVDF) membrane using a Trans-Blot SD semidry transfer cell (Bio-Rad Laboratories, Hercules, CA), and subjected to immunodetection. Blocked with 5 % non-fat milk in PBS buffer overnight, the membrane was incubated in PBS buffer with an anti-ExoS polyclonal antibody (Accuarte & Scintific Corp., Westbury, NY) for 1 h. After washed with PBS buffer containing 0.3% (*v*/*v*) Trition X-100 for 3 times, the membrane was incubated in PBS buffer with an anti-chicken IgG (H+L) conjugated with alkaline phosphatase (AP) (SouthernBiotech, Birmingham, AL) for another 1 h. Washed for 3 times, membrane was incubated by AP reaction buffer (100 mM Tris bace, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$) for 5 min, and ExoS was

detected by the chromogenic method.

Statistical data analysis

The scientific statistic software Statistical Package for the Social Sciences (SPSS) version 17.0 was used to evaluate the significance of differences between groups. Each experimental value is expressed as the mean ± standard deviation (S.D.). $p < 0.01$ or $p < 0.05$ was taken to indicate statistical distinct significance or significance.

Results

Inhibitory activity of AL-1 in reporter strains with LasR

Our previous report showed that AL-1 can inhibit the biofilm formation and virulence factors of *P. aeruginosa*. As both of them controlled by QS system, we hypothesized that AL-1 interfered the Las system of *P. aeruginosa*. To understand whether AL-1 interacts with Las system, the 3-oxo-C_{12}-HSL sensor strain engineered with LasR transcriptional activator was used. In our experiment, AL-1 inhibited biosensor activity by nearly 20% at a concentration of 0.5 mM against 3-oxo-C_{12}-HSL ($p<0.05$) (Fig.1). This result demonstrated that AL-1 interferes with the Las system via inhibiting LasR-3-oxo-C_{12}-HSL interaction. To further evaluate the activity of AL-1, 3-oxo-C_{12}-HSL was added at the final concentration of 1 μM, inhibition was not detected, suggesting the competitive interaction between the AL-1 and 3-oxo-C_{12}-HSL for LasR binding was exist (Fig.1).

AL-1 depressed the expression of *lasI, lasR, rhlI,* and *rhlR* genes

Since AL-1 inhibits not only the production of protease and pyocyanin, but also the development of biofilms (59). LasR-3-oxo-C_{12}-HSL is at the top of the hierarchical regulatory in QS. We expected that AL-1 would affect the QS-related genes such as *las*, *rhl*, and *pqs*. The results showed that AL-1 decreased the expression of *lasI*, *lasR*, *rhlI*, and *rhlR* in a dose-dependent manner (Fig.2). The expression of *lasI* and *rhlI* genes were reduced more than three-fold, and expression of *lasR* and *rhlR* were decreased nearly two-fold when measured as the ratio of maximal levels of expression

in the presence of 1 mM AL-1. The inhibitory effects of AL-1 on *lasI*, *lasR*, *rhlI*, and *rhlR* could be reversed by exogenous 3-oxo-C_{12}-HSL at the final concentration of 1 μM (Fig.S2 in the supplemental material). These results further indicate that AL-1 can inhibit LasR-3-oxo-C_{12}-HSL interactions and repress the transcriptional level of *las* and *rhl* genes. However, the other tested genes (*pqsA*, *pqsH*, *pqsR*) were not significantly influenced by AL-1 even at 10 mM (data not shown). In all experiments, no significant effects were observed on the growth of the *P. aeruginosa* when treated with AL-1. The transcript levels of QS-related genes were also measured using qRT-PCR. Consistent with the data obtained from luciferase reporters, AL-1 treated strains *lasR*, *lasI*, *rhlR*, and *rhlI* transcripts decreased about 2.5-fold, 2-fold, 2-fold and 3-fold respectively, compared with the control, while the *pqsA*, *pqsB*, *pqsC*, *pqsD*, and *pqsE* were not influenced by AL-1 (Fig.S3 in the supplemental material).

AL-1 reduced the production of Psl polysaccharide

As AL-1 significantly reduces the production of EPS in *P. aeruginosa* (59) and Psl polysaccharide is the primary matrix structural polysaccharide, AL-1 may also inhibits the biofilm formation by decreasing production of Psl, which is the key biofilm matrix polysaccharide in *P. aeruginosa*. By using Psl anti-serum, it is easy to find that AL-1 did reduce the Psl production (Fig.3A). To further investigate the effect of AL-1 at *psl* gene transcriptional or translational level, we utilized *pslA* chromosomal transcriptional and translational *lacZ* fusion reporter strains. The result showed that AL-1 decreased *psl* expression at both transcriptional (Fig.3B) and translational level (Fig.3C) ($p<0.01$).

AL-1 increased secretion of T3SS proteins

Since TS33 is negatively regulated by QS, it might be also influence on the ExoS secretion. Western blot showed that AL-1 led to severe increase of ExoS at 1 mM (Fig.4). qRT-PCR was also performed, AL-1 can increase the level of *exoS*, *exoY*, and *exoT* by 2.5-fold, 1.6-fold, and 2-fold respectively (Fig.S3 in the supplemental material). Similar to the previous reports, ExoT was detected in the supernatant

(Fig.4). The anti-ExoS cross-reacts with the ExoT may be responsible for this phenomenon as previously described (8, 58).

Discussion

In many pathogenic bacteria, QS regulate a variety of physiological processes such as antibiotic biosynthesis, biofilm formation and production of virulence factors. In *P. aeruginosa*, the QS regulators LasR and RhlR control the expression of hundreds of genes (47), many of which encode central metabolic functions. Controlling the virulence of *P. aeruginosa* is one of the biggest issues in medicine. QS system has been used as an effective antimicrobial drug target by altering the tolerance of biofilms to antibiotics. The development of QS targeted antivirulence compounds is urgently needed.

Previously, several natural compounds have been reported to decrease the virulence and antibiotic-resistant biofilm formation of *P. aeruginosa* without affecting its growth. For example, furanones prevent AHLs from binding to the *luxR* homologues and eventually cause a rapid turnover of these proteins (33, 34). Baicalein significantly inhibits biofilm formation of *P. aeruginosa* at 20 μM without affecting its growth. Its action mode is to promote proteolysis of the signal receptor TraR protein at 4-40 mM (60) whereas PD12 and V-06-018 inhibit LasR dependent gene expression (40). However, the applications of these compounds have been hindered by either their low solubility or high toxicity (60). In the present study, we reported the efficient effects of AL-1 on QS related genes and biofilm development, which is a low toxic compound in animal experiments (LD_{50} of AL-1 was 1243 mg/kg/d) (7).

The present study demonstrated that AL-1 affected the Las and Rhl systems. Recent research reveals that Las and Rhl systems are key areas to base infection treatments (20, 51). The Las system controls biofilm formation (10, 43), and the Rhl system is responsible for the production of rhamnolipids, pyocyanin, and elastase. Rhamnolipids play multiple roles in the establishment and maintenance of *P. aeruginosa* biofilms, while pyocyanin and elastase are related to the pathogenesis of *P.*

304 *aeruginosa*. LasR is a hierarchical regulator, co-regulated with the RhlR. This
305 'dense-overlapping regulon' makes exceptional adaptability of the QS response to
306 different environmental conditions (46). Considering that AL-1 can influence the Las
307 and Rhl systems, it could become an efficient compound for the treatment of *P.*
308 *aeruginosa* related infections.

309 EPS is an important constituent of the *P. aeruginosa* biofilm and is required for
310 bacterial cells to adhere to a substratum and maintain biofilm structure (35). *P.*
311 *aeruginosa* EPS was tested by using the phenol solution-sulfuric acid method as
312 previously described (9, 36). After treated with AL-1, *P. aeruginosa* EPS was
313 significantly reduced (59). *psl* cluster plays a role in biofilm development, so
314 immunobolotting assay was used to investigated the effect of AL-1 on *psl*. The result
315 showed that AL-1 can decrease the Psl production. β-Galactosidase activity also
316 suggested that *psl* transcription and translation are reduced by AL-1. It was suggested
317 that *psl* may be transcriptionally regulated by LasR. RsmA, a small RNA-binding
318 protein, is known to negatively regulate pathogenicity determinants such as motility,
319 AHLs and secondary metabolite production (5, 18, 42). Previous reports have
320 concluded that RsmA was acting as a translational repressor of *psl* (22). These results
321 strictly corroborate our data obtained with the qRT-PCR, *rsmA* is increased about
322 threefold with respect to the control (Fig.S3 in the supplemental material). It is
323 possible to postulate that the decreased *psl* in translational level may be due to the
324 increased level of *rsmA* by AL-1. RsmA is controlled by a complex regulatory system
325 including sensor kinases, response regulators and small RNAs *rsmZ* and *rsmY* (52).
326 LadS and RetS control biofilm and virulence phenotypes through two-component
327 regulatory system GacS/A, LadS promote the phosphorylation of GacA, then the
328 phosphorylated GacA activates the transcription of *rsmZ* and *rsmY*, the small RNAs
329 bind to *rsmA,* which eventually affects the biofilm formation and T3SS (17, 52, 56).
330 RetS exerts opposite effects in this system (52). Future studies should be done to find
331 out effects of AL-1 at the global regulatory networks.

332 The decrease in Psl expression mediated by AL-1 in the immunoblotting assay
333 appears to be far greater than the *psl* transcription and translation in β-Galactosidase

assay. It was speculated that AL-1 may have post-translational effects on Psl.

Meanwhile, AL-1 can increase the secretion of ExoS. This could be due to the effect of AL-1 on QS system. Interestingly, a previous study showed that treatment of *P. aeruginosa* with azithromycin (AZM) can inhibit the QS, but increase the expression of T3SS genes (49). The secretion of ExoS in an *rhlI* mutant showed that *exoS* was submitted to a negative RhlR-C_4-HSL-dependent control (2). Hogardt also reported that *exo*S is negatively regulated by the Rhl system (21). Mutations in T3SS genes result in enhanced biofilm formation in PAO1 (28). These provided evidence that AL-1 downregulates the *rhl* gene and possibly upregulates the type III effectors during the biofilm inhibition. RsmA exerted a negative effect on the synthesis of both 3-oxo-C_{12}-HSL and C_4-HSL (42). Mulcahy et al reported that RsmA is required for ExoS secretion (39). The increased ExoS secretion may be due to the elevated level of *rsmA* transcript by AL-1. Overall, T3SS and QS connected through both Rhl system and RsmA. As mentioned above, we think the potential benefits are outweighing the risk. The increased ExoS may be due to the exchanged lifestyle of *P. aeruginosa*. AL-1 inhibits the biofilm formation and makes *P. aeruginosa* planktonic, and the bacteria may express virulence factors such as T3SS effectors for self protection.

Researchers investigating the antibiotic resistance of bacteria in biofilm think that bacterial biofilms may make slow or incomplete penetration of antibiotics (50). However, if the antibiotic is permeate in the biofilm, some of the bacterial may differentiate into a protected phenotype and the altered chemical microenvironment within the biofilm also makes the antibiotic less effective (50). AL-1 has synergistic effect with traditional antibiotics, the underlying mechanism may be mediated by the markedly reduced biofilms formation.

In summary, AL-1 inhibits *P. aeruginosa* PAO1 biofilm formation by repressing QS system (Fig.5). Clearly AL-1 is an interesting compound due to its action mode and synergistic effects with antibiotics, and may address the potential use of old-generation antibiotics in the treatment of chronic *P. aeruginosa* infections.

Acknowledgements

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Table 1. Strains and plasmids used in this study

Bacterial Strains	Description	Reference
P. aeruginosa PAO1	Nonmucoid *P. aeruginosa* prototroph	Laboratory stock
Pseudomonas putida F117 pKRC12	AHL-deficient derivative of *P. putida* IsoF, Δ*ppuI*. pKR-C12: pBBR1MCS-5 carrying P_{lasB}-*gfp* (ASV)-P_{lac}-*lasR*; based on components of the *P. aeruginosa las* quorum-sensing system; GEN^R	(3)
P. aeruginosa CIM45	PAO1 with E88 (*lacZ*::pPslA TRO) at attB1 site	(22)
P. aeruginosa CIM46	PAO1 with E89 (*lacZ* EB::pPslA TRO) at attB1 site	(22)
Escherichia coli DH5α	*recA1* and *endA1* cloning strain	Invitrogen

Plasmids	Description	Reference
pMS402	reporter vector carrying promoterless *luxCDABE*, KAN^RTMP^R	(19)
pKD-*lasR*	pMS402 containing *lasR* promoter region	(11)
pKD-*lasI*	pMS402 containing *lasI* promoter region	(11)
pKD-*rhlR*	pMS402 containing *rhlR* promoter region	(11)

pKD-*rhlI*	pMS402 containing *rhlI* promoter region	(11)
pKD-*pqsA*	pMS402 containing *pqsA* promoter region	(29)
pKD-*pqsR*	pMS402 containing *pqsR* promoter region	(29)
pKD-*pqsH*	pMS402 containing *pqsH* promoter region	(29)

Figure legends

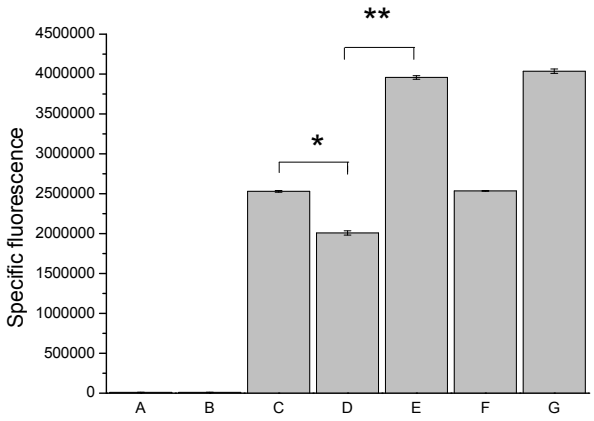


Fig.1 Specific fluorescence activity of *P. putida* F117 pKRC12 after 4 h when challenged with 0.5 mM AL-1 and induced with 50 nM of 3-oxo-C_{12}-HSL or 1000 nM of 3-oxo-C_{12}-HSL, (A) DMSO only, (B) AL-1 only, (C) DMSO and induced with 50 nM 3-oxo-C_{12}-HSL, (D) AL-1 and induced with 50 nM 3-oxo-C_{12}-HSL, (E) AL-1 and induced with 1000 nM 3-oxo-C_{12}-HSL, (F) 50 nM 3-oxo-C_{12}-HSL only, (G) 1000 nM 3-oxo-C_{12}-HSL only. Values reported are the mean of three replicates with the deduction of LB fluorescence. Error bars indicate the standard deviation.

* denotes statistical significance ($P < 0.05$) or ** denotes distinct significance ($p < 0.01$) compared with controls.

* denotes statistical significance ($P < 0.05$) or ** denotes distinct significance ($p < 0.01$) compared with controls.

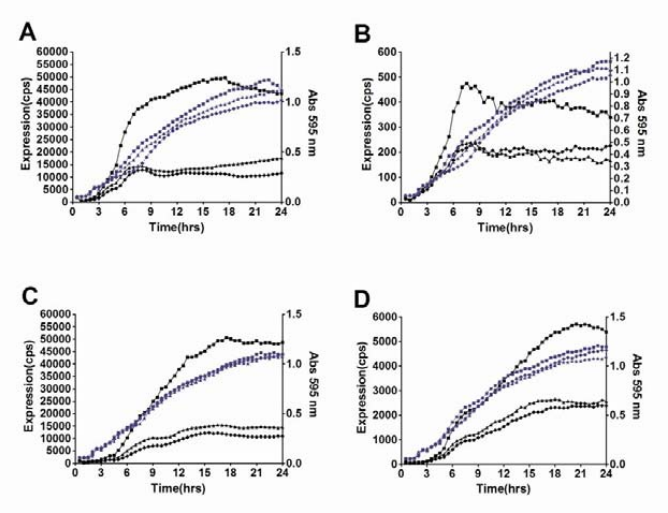
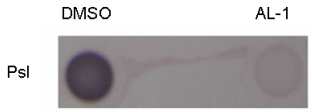
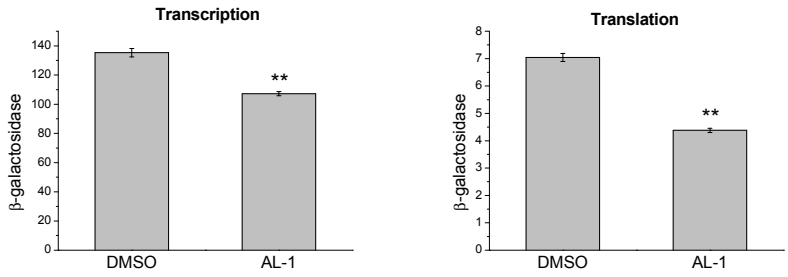


Fig.2 Inhibition of QS genes in *P. aeruginosa* PAO1 by AL-1. Expression profiles and corresponding growth curves are shown for (A) *lasI*, (B) *lasR*, (C) *rhlI* and (D) *rhlR*. The black lines represent the expression of the promoters. The blue lines represent the growth of the strain. The data from the control (without drug) (■), 0.5 mM AL-1 (▲) and 1 mM AL-1 (□) are shown by squares, triangles and diamonds, respectively. The assays were independently repeated at least three times, and the data shown are representative of comparable results (cps, counts per second).



A



B C

Fig.3 (A) Effects of AL-1 on the production of Psl. The concentration of AL-1 were 0.5 mM (B) Transcriptional and (C) translational *lacZ* fusion constructs assayed for β-galactosidase activities show deregulation of *pslA* by AL-1 compared with DMSO. Data represent the means of duplicate ß-Gal activity assays from three separate experiments, and activity is expressed as Miller units.

**$p<0.01$ was taken to indicate statistical distinct significance.

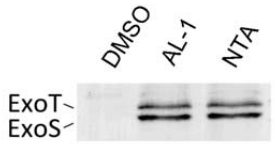


Fig.4 Effects of AL-1 on T3SS effector ExoS. *P. aeruginosa* PAO1 was grown in the presence of 1 mM AL-1 and 10 mM NTA. The same volume of DMSO was added to the culture as a negative control.

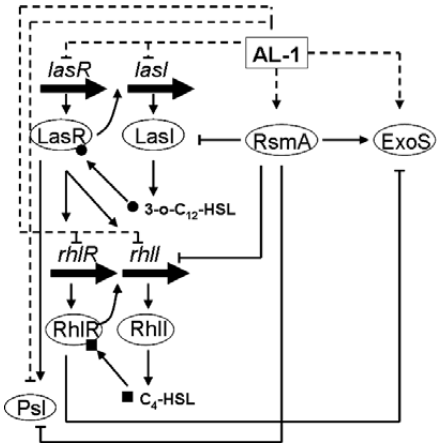


Fig.5 Proposed model of the effect of AL-1 on QS related genes and *exoS*, dash lines indicate the effects of AL-1, solid lines indicate the QS network. Arrow heads, activation, flat arrow heads, repression.

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