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Effects of 14-Alpha-Lipoyl Andrographolide on Quorum Sensing in *Pseudomonas aeruginosa*

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In *Pseudomonas aeruginosa*, the quorum-sensing (QS) system is closely related to 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 it has little inhibitory effect on its growth. However, its molecular mechanism remains elusive. Here we investigated the effect of AL-1 on QS systems, especially the Las and Rhl systems. This investigation showed that AL-1 can inhibit LasR–3-oxo-C_{12}-homoserine lactone (HSL) interactions and repress the transcriptional level of QS-regulated genes. Reverse transcription (RT)-PCR data showed that AL-1 significantly reduced the expression levels of *lasR*, *lasI*, *rhlR*, and *rhlI* in a dose-dependent manner. AL-1 not only decreased the expression level of Psl, which is positively regulated by the Las system, but also increased the level of secretion of ExoS, which is negatively regulated by the Rhl system, indicating that AL-1 has multiple effects on both the Las and Rhl systems. It is no wonder that AL-1 showed synergistic effects with other antimicrobial agents in the treatment of *P. aeruginosa* infections.

Pseudomonas aeruginosa is an opportunistic human pathogen responsible for severe infections in immunocompromised and cystic fibrosis (CF) patients (15, 30). Due to its frequent occurrence in hospital water-supplying pipes and its capacity to persist on medical devices, *P. aeruginosa* is a leading cause of life-threatening infections (48). In addition, *P. aeruginosa* is notorious for the vigorous development of biofilm, which adds difficulties in antibiotic therapy and makes wounds unable to heal (12). Biofilm formation is believed to be one of the major causes of persistent infections.

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

Exopolysaccharides (EPSs) are key matrix components of biofilms, as they contribute to the overall biofilm architecture and resistance (1, 31, 45). The Psl polysaccharide is an essential matrix component that is required for *P. aeruginosa* to initiate and maintain biofilms (13, 23, 32, 37). In *P. aeruginosa*, *pslA* to *pslL* are positively regulated by the Las system, according to work reported previously by Gilbert et al. (14).

P. aeruginosa has another important virulence component, called the type III secretion system (TS33), which is negatively regulated by QS. TS33 is a needlelike complex which secretes a number of cytotoxins, including ExoS, ExoT, ExoU, and ExoY (44). These products have been shown to have 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).

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 the 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 paniculata* Nees. We reported previously that 14-alpha-lipoyl andrographolide (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.

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TABLE 1 Strains and plasmids used in this study

Bacterial strain or plasmid	Description[a]	Source or reference
Strains		
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 the attB1 site	22
P. aeruginosa CIM46	PAO1 with E89 (*lacZ* EB::pPslA TRO) at the attB1 site	22
Escherichia coli DH5α	*recA1* and *endA1* cloning strain	Invitrogen
Plasmids		
pMS402	Reporter vector carrying promoterless *luxCDABE*; Kan^r Tmp^r	19
pKD-*lasR*	pMS402 containing the *lasR* promoter region	11
pKD-*lasI*	pMS402 containing the *lasI* promoter region	11
pKD-*rhlR*	pMS402 containing the *rhlR* promoter region	11
pKD-*rhlI*	pMS402 containing the *rhlI* promoter region	11
pKD-*pqsA*	pMS402 containing the *pqsA* promoter region	29
pKD-*pqsR*	pMS402 containing the *pqsR* promoter region	29
pKD-*pqsH*	pMS402 containing the *pqsH* promoter region	29

[a] *gfp*(ASV) encodes mutant Gfpmut3* proteins (unstable Gfp) with the C-terminal extension sequence RPAANDENYAASV.

This study aims to investigate how AL-1 inhibits *P. aeruginosa* PAO1 biofilm formation. Since LasR play a critical role in biofilm development, we tested the effects of AL-1 on LasR using an AHL-deficient strain. We then 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 reverse transcription (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 amount of biofilm matrix Psl. To elucidate the effect of AL-1 on Psl, we used Psl immunoblots. A β-galactosidase assay further suggested that AL-1 has effects on Psl at both transcriptional and translational levels. A complete understanding of the effect of AL-1 on the *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 (see Fig. S1 in the supplemental material), as we previously reported (25).

Bacterial strains and growth conditions. *Pseudomonas putida* F117(pKRC12) (3) was kindly provided by Jo Handelsman, from the University of Wisconsin—Madison. *P. aeruginosa* CIM45/46 (22) was provided by Luyan Ma, from the Institute of Microbiology, Chinese Academy of Sciences, China.

P. putida F117(pKRC12) cells were grown at 28°C in Luria-Bertani (LB) medium. All *P. aeruginosa* strains were grown at 37°C in LB medium without NaCl (LBNS) or in Jensen's medium (24) and are listed in Table 1. 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) of 300 μg/ml and gentamicin (GEN) of 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 the activation of LasR by 3-oxo-C_{12}-HSL (3). *P. putida* F117(pKRC12) cells were grown overnight and diluted with LB medium to achieve an optical density at 595 nm (OD_{595}) of 0.05, and 100-μl aliquots of cells were added to 96-well plates with dimethyl sulfoxide (DMSO) or AL-1 preincubated with 3-oxo-C_{12}-HSL at a final concentration of 50 nM or 1,000 nM for 30 min. Fluorescence was measured at regular intervals after 4 h by using the EnVision plate reader (PerkinElmer Life and Analytical Sciences, Wellesley, MA).

Luciferase activity-based bioassay for QS inhibitors. A chemiluminometric assay was developed to study the effects of AL-1 on expression levels of genes. Using *lux*-based reporters which indicate luciferase activity, gene expression in liquid cultures was measured as light production (in counts per second) with a Victor^3 multilabel plate reader (PerkinElmer Life and Analytical Sciences, Wellesley, MA). Cultures of the reporter strains grown overnight were diluted to an optical density at 620 nm (OD_{620}) of 0.2 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. A fresh culture (5 μl) was inoculated into the wells containing a total of 95 μl medium plus other components (the 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} with the Victor^3 multilabel plate reader.

qRT-PCR. *P. aeruginosa* PAO1 cells were grown in LBNS medium with shaking at 37°C overnight and diluted with LBNS medium to achieve an OD_{595} of 0.05. A total of 0.5 mM AL-1 or DMSO was added. After 5 h, total RNA was extracted by using a total RNA miniprep kit (Axygen). Residual DNA was removed by DNase I treatment (Fermentas), as recommended by the manufacturer. cDNA synthesis was performed by using SuperScript III first-strand synthesis (Invitrogen), according to the manufacturer's protocol, using random hexamers. Quantitative reverse transcription-PCR (qRT-PCR) was performed with SYBR green qPCR master mix (Fermentas). To calculate the relative expression levels of target genes, the expression level of the 16S rRNA gene was used as an internal control. Primers are listed in Table S1 in the supplemental material. The data presented below are the results obtained from three independent experiments.

Immunoblotting of Psl polysaccharide extracts. Psl immunoblots were performed as described previously (6), with the following changes. *P. aeruginosa* PAO1 cells were grown in LBNS medium with shaking at 37°C overnight and treated with 0.5 mM AL-1 or DMSO. Crude polysaccharide extracts were obtained by spinning down the culture to 10 OD units, resuspending the extracts in 100 μl of 0.5 M EDTA, and boiling the extracts for 5 min at 100°C. The supernatant fraction was treated with pro-

teinase K (final concentration, 0.5 mg/ml) for 60 min at 60°C, followed by proteinase K inactivation for 30 min at 80°C. Five microliters of the sample was spotted onto a nitrocellulose membrane. Blocking was done with 10% nonfat milk in TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween 20 [pH 7.6]) for 1 h at room temperature. Psl was detected by using anti-Psl antibodies (1:25,000 dilution) and 1:10,000-diluted goat anti-rabbit IgG-conjugated secondary antibody (Thermo-Scientific). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) were added for detection.

β-Galactosidase assay. β-Galactosidase activity was measured as described previously by Miller (38) and is expressed in Miller units (MU). Cell lysates were assayed for both β-galactosidase activities as well as protein content by a bicinchoninic acid (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. *P. aeruginosa* PAO1 cells grown in LBNS medium overnight at 37°C were diluted 1,000-fold in fresh LB medium supplemented with 200 mM NaCl containing DMSO, AL-1, or nitrilotriacetic acid (NTA) for 6 h at 37°C. The culture supernatant was collected by centrifugation, and the secretion proteins were concentrated by ultrafiltration. Proteins were separated by 12% SDS-PAGE and then blotted onto a polyvinylidene difluoride (PVDF) membrane by using a Trans-Blot SD semidry transfer cell (Bio-Rad Laboratories, Hercules, CA) and subjected to immunodetection. After being blocked with 5% nonfat milk in phosphate-buffered saline (PBS) overnight, the membrane was incubated in PBS with an anti-ExoS polyclonal antibody (Accurate Chemical & Scientific Corp., Westbury, NY) for 1 h. After being washed three times with PBS containing 0.3% (vol/vol) Triton X-100, the membrane was incubated in PBS with an anti-chicken IgG(H+L) conjugated with alkaline phosphatase (AP) (Southern Biotech, Birmingham, AL) for another 1 h. After being washed three times, the membrane was incubated with AP reaction buffer (100 mM Tris base [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 statistical 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 (SD). A P value of <0.01 or a P value of <0.05 was taken to indicate a statistically 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 are controlled by the QS system, we hypothesized that AL-1 interfered with the Las system of *P. aeruginosa*. To understand whether AL-1 interacts with the Las system, a 3-oxo-C_{12}-HSL sensor strain engineered with a 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 the LasR–3-oxo-C_{12}-HSL interaction. To further evaluate the activity of AL-1, 3-oxo-C_{12}-HSL was added at a final concentration of 1 μM, and inhibition was not detected, suggesting that the competitive interaction between AL-1 and 3-oxo-C_{12}-HSL for LasR binding did exist (Fig. 1).

AL-1 depressed the expression levels of the *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 pathway in QS. We expected that AL-1 would affect QS-related genes such as *las*, *rhl*, and *pqs*. The results showed that AL-1 decreased the expression levels of *lasI*, *lasR*, *rhlI*, and *rhlR* in a dose-dependent manner (Fig. 2). The expression levels of the *lasI* and *rhlI* genes were reduced more than 3-fold, and the expression levels of the *lasR* and *rhlR* genes were decreased nearly 2-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 a final concentration of 1 μM (see 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 levels of the *las* and *rhl* genes. However, the other tested genes (*pqsA*, *pqsH*, and *pqsR*) were not significantly influenced by AL-1, even at 10 mM (data not shown). In all experiments, no significant effects were observed for the growth of *P. aeruginosa* cells when treated with AL-1. The transcript levels of QS-related genes were also measured by using qRT-PCR. Consistent with the data obtained from luciferase reporters, the *lasR*, *lasI*, *rhlR*, and *rhlI* transcript levels in AL-1-treated strains decreased about 2.5-fold, 2-fold, 2-fold, and 3-fold, respectively, compared with the control, while *pqsA*, *pqsB*, *pqsC*, *pqsD*, and *pqsE* were not influenced by AL-1 (see Fig. S3 in the supplemental material).

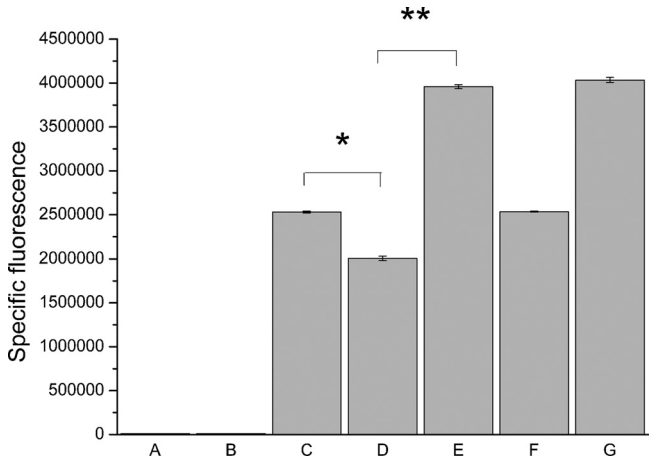

FIG 1 Specific fluorescence activity of *P. putida* F117(pKRC12) after 4 h when challenged with 0.5 mM AL-1 and induced with 50 nM 3-oxo-C_{12}-HSL or 1,000 nM 3-oxo-C_{12}-HSL. (A) DMSO only; (B) AL-1 only; (C) DMSO and induction with 50 nM 3-oxo-C_{12}-HSL; (D) AL-1 and induction with 50 nM 3-oxo-C_{12}-HSL; (E) AL-1 and induction with 1,000 nM 3-oxo-C_{12}-HSL; (F) 50 nM 3-oxo-C_{12}-HSL only; (G) 1,000 nM 3-oxo-C_{12}-HSL only. Values reported are the means of values from three replicates with the deduction of LB fluorescence. Error bars indicate standard deviations. *, statistical significance compared with controls ($P < 0.05$); **, distinct significance ($P < 0.01$) compared with controls.

AL-1 reduced the production of Psl polysaccharide. As AL-1 significantly reduces the production of EPS in *P. aeruginosa* (59), and the Psl polysaccharide is the primary matrix structural polysaccharide, AL-1 may also inhibit biofilm formation by decreasing the level of production of Psl, which is the key biofilm matrix polysaccharide in *P. aeruginosa*. By using Psl antiserum, it is easy to determine that AL-1 did reduce the level of Psl production (Fig. 3A). To further investigate the effect of AL-1 at the *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 levels at both transcriptional (Fig. 3B) and translational (Fig. 3C) levels ($P < 0.01$).

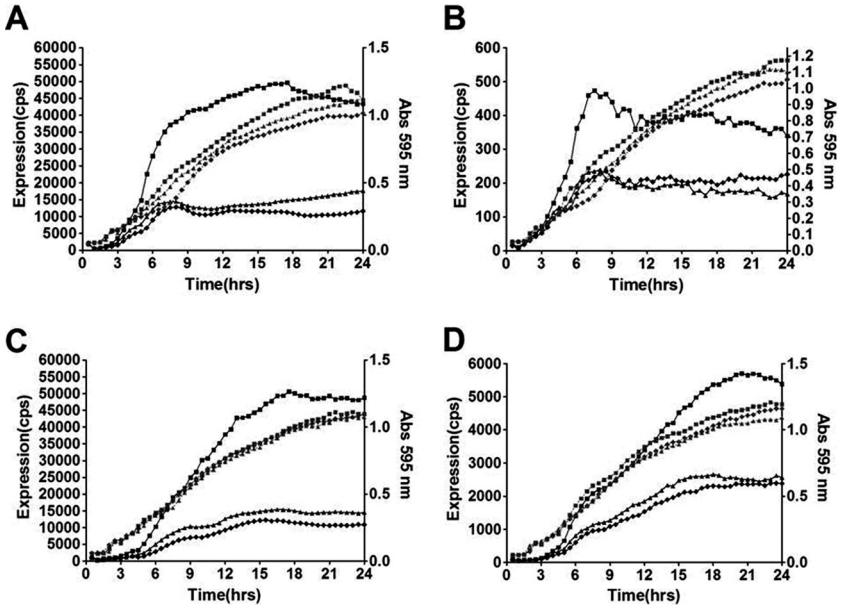

FIG 2 Inhibition of QS genes in *P. aeruginosa* PAO1 by AL-1. Expression profiles and corresponding growth curves are shown for *lasI* (A), *lasR* (B), *rhlI* (C), and *rhlR* (D). The black lines represent the expression levels of the promoters. The blue lines represent the growth of the strain. The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

AL-1 increased the secretion of T3SS proteins. Since the TS33 is negatively regulated by QS, it might also influence ExoS secretion. Western blotting showed that AL-1 led to a severe increase in the level of ExoS at 1 mM (Fig. 4). qRT-PCR was also performed: AL-1 treatment increased the levels of *exoS*, *exoY*, and *exoT* by 2.5-fold, 1.6-fold, and 2-fold, respectively (see Fig. S3 in the supplemental material). Similar to data from previous reports, ExoT was detected in the supernatant (Fig. 4). The anti-ExoS antibody cross-reacts with ExoT, which may be responsible for this phenomenon, as previously described (8, 58).

DISCUSSION

In many pathogenic bacteria, QS systems regulate a variety of physiological processes, such as antibiotic biosynthesis, biofilm formation, and the production of virulence factors. In *P. aeruginosa*, the QS regulators LasR and RhlR control the expressions of hundreds of genes (47), many of which encode central metabolic functions. Controlling the virulence of *P. aeruginosa* is one of the most important issues in medicine. QS systems have been used as effective antimicrobial drug targets

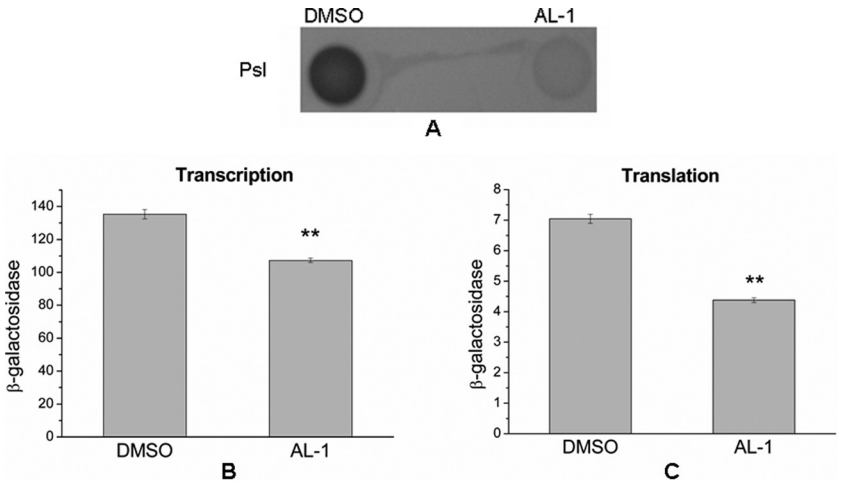

FIG 3 (A) Effects of AL-1 on production of Psl. The concentrations of AL-1 were 0.5 mM. (B and C) Transcriptional (B) and translational (C) *lacZ* fusion constructs assayed for β-galactosidase activities showed a deregulation of *pslA* mediated by AL-1 compared with DMSO. Data represent the means of data from duplicate β-galactosidase activity assays from three separate experiments, and activity is expressed as Miller units. **, a *P* value of <0.01 was taken to indicate a statistically distinct significance.

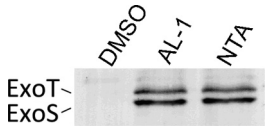

FIG 4 Effects of AL-1 on the T3SS effector ExoS. *P. aeruginosa* PAO1 cells were grown in the presence of 1 mM AL-1 and 10 mM NTA. The same volume of DMSO was added to the culture as a negative control.

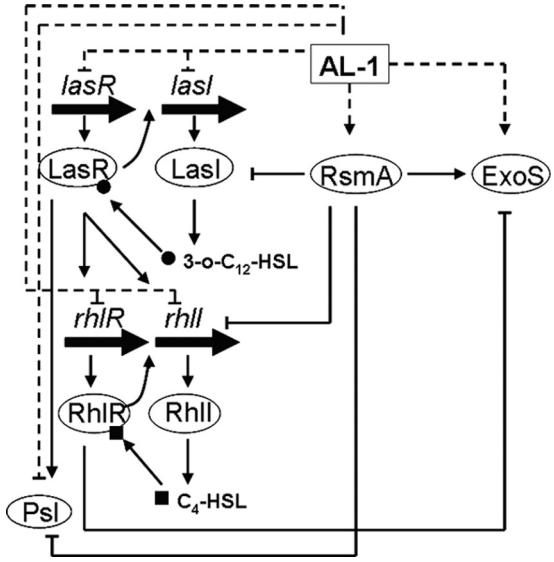

FIG 5 Proposed model of the effect of AL-1 on QS-related genes and *exoS*. Dashed lines indicate the effects of AL-1, and solid lines indicate the QS network. Arrowheads, activation; flat arrowheads, repression.

by altering the tolerance of biofilms to antibiotics. The development of QS system-targeted antivirulence compounds is urgently needed.

Previously, several natural compounds were 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* homologs and eventually cause a rapid turnover of these proteins (33, 34). Baicalein significantly inhibits the biofilm formation of *P. aeruginosa* at 20 μM without affecting its growth. Its mode of action is to promote the proteolysis of the signal receptor TraR protein at 4 to 40 mM (60), whereas PD12 and V-06-018 inhibit LasR-dependent gene expression (39). However, the applications of these compounds have been hindered by either their low solubility or their 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 (the 50% lethal dose [LD_{50}] of AL-1 was 1,243 mg/kg of body weight/day) (7).

The present study demonstrated that AL-1 affected the Las and Rhl systems. Recent research revealed that the Las and Rhl systems are key areas for 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. aeruginosa*. LasR is a hierarchical regulator coregulated with the RhlR. This "dense-overlapping regulon" makes exceptional adaptability of the QS response to different environmental conditions (46). Considering that AL-1 can influence the Las and Rhl systems, it could become an efficient compound for the treatment of *P. aeruginosa*-related infections.

EPS is an important constituent of the *P. aeruginosa* biofilm and is required for bacterial cells to adhere to a substratum and maintain biofilm structure (35). *P. aeruginosa* EPS was tested by using a phenol solution-sulfuric acid method, as previously described (9, 36). After being treated with AL-1, the amount of *P. aeruginosa* EPS was significantly reduced (59). The *psl* cluster plays a role in biofilm development, so an immunoblotting assay was used to investigate the effect of AL-1 on *psl*. The result showed that AL-1 can decrease the level of Psl production. β-Galactosidase activity also suggested that the levels of *psl* transcription and translation are reduced by AL-1. It was suggested previously that *psl* may be transcriptionally regulated by LasR. RsmA, a small RNA-binding protein, is known to negatively regulate pathogenicity determinants such as motility, AHLs, and secondary metabolite production (5, 18, 42). Previous reports concluded that RsmA was acting as a translational repressor of *psl* (22). These results strictly corroborate our data obtained by qRT-PCR: the level of *rsmA* was increased about 3-fold with respect to the level of the control (see Fig. S3 in the supplemental material). It is possible to postulate that the decreased *psl* translational level may be due to the increased level of *rsmA* mediated by AL-1. RsmA is controlled by a complex regulatory system, including sensor kinases, response regulators, and the small RNAs *rsmZ* and *rsmY* (52). LadS and RetS control biofilm and virulence phenotypes through the two-component regulatory system GacS/A, LadS promotes the phosphorylation of GacA, the phosphorylated GacA then activates the transcriptions of *rsmZ* and *rsmY*, and the small RNAs bind to *rsmA*, which eventually affects biofilm formation and the T3SS (17, 52, 56). RetS exerts opposite effects in this system (52). Future studies should be done to find out the effects of AL-1 on the global regulatory networks.

The decrease in the Psl expression level mediated by AL-1 in the immunoblotting assay appears to be far greater than *psl* transcription and translation in β-galactosidase assays. It was speculated that AL-1 may have posttranslational effects on Psl.

Meanwhile, AL-1 can increase the level of secretion of ExoS. This could be due to the effect of AL-1 on the QS system. Interestingly, a previous study showed that the treatment of *P. aeruginosa* with azithromycin (AZM) can inhibit the QS system but increase the expression levels of T3SS genes (49). The secretion of ExoS in an *rhlI* mutant showed that *exoS* was submitted to negative RhlR–C_4-HSL-dependent control (2). Hogardt et al. also reported that *exoS* is negatively regulated by the Rhl system (21). Mutations in T3SS genes result in enhanced biofilm formation in strain PAO1 (28). These data provided evidence that AL-1 downregulates the *rhl* gene and possibly upregulates the type III effectors during 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 previously that RsmA is required for ExoS secretion (40). The increased level of ExoS secretion may be due to the elevated level of *rsmA* transcripts mediated by AL-1. Overall, the T3SS and the QS system were connected through both the Rhl system and RsmA. As mentioned above, we think that the potential benefits outweigh the risk. The increased ExoS level may be due to the exchanged life-style of *P. aeruginosa*. AL-1 inhibits 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 biofilms thought that bacterial biofilms may cause a slow or incomplete penetration of antibiotics (50). However, if the antibiotic can permeate the biofilm, some of the bacteria may differentiate into a protected phenotype, and the altered chemical microenvironment within the biofilm also makes the antibiotic less effective (50). AL-1 has a synergistic effect with traditional antibiotics; the underlying mechanism may be mediated by the markedly reduced biofilm formation.

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

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