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Effects of Exogenous *N*–Acyl–Homoserine Lactones on Biofilm Formation and Motility in *Acinetobacter nosocomialis*

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One of the major factors contributing to drug resistance in *Acinetobacter nosocomialis* infections is biofilm development, which is facilitate by quorum-sensing (QS) systems. Quorum sensing by the LuxI and LuxR homologues, AnoI and AnoR, in *A. nosocomialis* plays a role in biofilm formation and motility of this pathogenic bacterium. The aim of this study was to evaluate the effects of exogenous *N*-acyl-homoserine lactones (AHLs) on the regulation of biofilm and motility of *A. nosocomialis* and *anoI*-deletion mutant. We found that *anoR* mRNA expression levels in the *anoI*-deletion mutant were increased in the presence of different types of AHLs compared with that in the absence of exogenous AHL. Among AHLs, C12-HSL appeared to exert the greatest stimulatory effect on biofilm formation and motility. Notably, the *anoI*-deletion mutant also exhibited significant decreases in expression of the biofilm- and motility-related genes, *csuC, csuD* and *pilT*, decreases that were attenuated by addition of exogenous AHLs. Combining the AHL C12-HSL with C6-HSL or C10-HSL exerted synergistic effects that restored the motility phenotype in the *anoI*-deletion mutant. Taken together, our data demonstrate that C12-HSL may act as an important signaling molecule in *A. nosocomialis* through regulation of biofilm formation and cell motility, potentially providing a new target for the control of A. *nosocomialis* infections.

Key Words: Quorum sensing, Exogenous AHLs, Biofilm formation, Motility, *A. nosocomialis*

INTRODUCTION

The genus *Acinetobacter* is a genetically multifarious group of aerobic, Gram negative, and non-fermentative bacteria found in numerous healthcare environments (1). *Acinetobacter nosocomialis* belonging to the *Acinetobacter calcoaceticus-baumannii* community is an opportunistic nosocomial pathogen (2). Its invasive and non-invasive diseases have been extensively studied in various nosocomial contexts, although previous studies suggest that this bacterium might be less virulent than its closely related counterpart, *A. baumannii*. The pathogenic mechanism that allows *A. baumannii* to thrive in the environment is the formation of a biofilm through attachment to both biotic and abiotic surfaces, which initiates processes that protect against various factors and enable the bacteria to survive in healthcare

environments (3). Communal behaviors of bacteria, such as virulence, biofilm formation and motility, are often modulated by regulating target gene expression via the quorum sensing (QS) system (4).

One of the best-known QS systems is the LuxI/LuxR two-component system (TCS) of *Vibrio harveyi* (5). Autoinducer, *N*-acyl-homoserine lactones (AHLs) of *V. harveyi* is synthesized by AHL synthase, LuxI using two substrates, *S*-adenosylmethionine (SAM) and an acylated acyl carrier protein (ACP). At a minimum threshold level, AHLs bind to the *N*-terminal region of LuxR and leads to form LuxR homodimers, which enables to regulate transcription of the target genes by binding to specific DNA sequences and thereby regulate transcription of the target genes. AnoI/AnoR is known as LuxI/LuxR homologous in *A. nosocomialis* (6, 7). Prior studies have reported AnoI/AnoR complex is responsible for up- or down-regulation of several target genes through AHLs mediated TCS.

Previous studies have reported that the QS system is involved in the biofilm-formation process and exogenous AHLs promote or inhibit biofilm formation in other bacterial strains (8). Biofilm formation is one of the major defense mechanisms that allow bacteria to withstand challenging environmental niches and survive (9). Biofilm development is a multistep process involving initial attachment, irreversible attachment, maturation, and dispersion (10, 11). Accumulation of bacterial cells internally in an extracellular matrix enables the bacteria to form a self-protecting growth pattern, prominently comprising exopolymers (EPs), which facilitate adherence to the solid surface (12, 13). Thus, understanding about the mechanism by which the QS system contributes to biofilm development could be beneficial in eradicating the infection at the initial stage, underscoring the importance of identifying effective ways to target biofilm formation by this pathogenic bacterium.

In our previous study, we demonstrated that AHLs are secreted by *A. nosocomialis* and exert a significant influence on biofilm and pellicle formation (7). In the current study, we constructed an *anoI*-deletion mutant of *A. nosocomialis* to further investigate the regulatory roles of the AHLs, C6-HSL, C10-HSL and C12-HSL, in biofilm formation and motility in *A. nosocomialis.* Here, we report the C12-HSL is the most prominent AHL, which plays a major role in regulating biofilm and motility in *A. nosocomialis*, whereas C6HSL and C10HSL may act as regulators in modulating other phenotypes through the QS mechanism.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains and plasmid used in this study are listed in Table 1. Wild-type (WT) *A. nosocomialis* (ATCC 17903) was obtained from American Type Culture Collection (ATCC; VA, USA) and an *anoI*-deletion mutant (*ΔanoI*) was constructed as a previously described method (7). All bacterial strains were grown in Luria-Bertani (LB) medium at 37°C. AHLs were

Table 1. Bacterial strains and plasmid used in this study

Strains and plasmids	Relevant characteristics[a]	Reference/source
A. nosocomialis ATCC17903	Type strain	(14)
ΔanoI	*A. nosocomialis* derivative, *ΔanoI::Km*^{r}	This study
DH5α *λ pir*	Plasmid replication	Laboratory collection
S17-I *λ pir*	Conjugal donor	Laboratory collection
pUC4K	pUC4 with *nptI*; Ap^r, Km^r	Laboratory collection
pHKD01	pDS132, multicloning sites; *ori*R6K, *sac*B, Cm^r	(15)
pOH01	pHKD01 with Δ*anoI::nptI*; Cm^r, Km^r	This study

[a]Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant

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exogenously added to the WT and *anoI*-deletion mutant as dictated by specified experimental requirements. Synthetic *N*-hexanoyl-homoserine lactone (C6-HSL), *N*-decanoyl-homoserine lactone (C10-HSL), and *N*-(3-hydroxy-dodecanoyl)-homoserine lactone (C12-HSL) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Construction of the deletion mutant *ΔanoI*

The *anoI*-deletion mutant was created from the *A. nosocomialis* WT strain by amplification of a DNA fragment following disruption of the open reading frame (ORF) of the target gene using an overlap extension-polymerase chain reaction (OE-PCR) method and a pair of specific primers, as listed in Table 2. Amplification of the mutated DNA fragment without the *anoI* gene was accomplished in two PCR steps using four PCR primers specific to upstream and downstream regions (~1 kb) of the coding region of *anoI*. To combine upstream and downstream regions of *anoI* with *nptI* (conferring kanamycin resistance) by overlap extension PCR, we designed ANOI02F and ANOI02R primers for amplification of the upstream region so as to contain an additional 25 nucleotides at their 5' end homologous to the downstream region and *nptI* (7). The fragments were amplified using *A. nosocomialis* WT genomic DNA. The resulting fragments were then mixed

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)[a,b]	Target
For gene deletion in *A. nosocomialis*		
ANOI01F	TTTCTGAAGCCTTTAAGGTTGTT	*anoI*
ANOI01R	TACAAGTGCTTCCACTTATTTTTAATG	
ANOI02F	TTAAAAATAAGTGGAAGCACTTGTATGGTTTAAAGGTTAACTCTTGTCTCTC	
ANOI02R	GCAACACCTTCTTCACGAGGCAGACAGGATTTTGGGGAAGTTTGC	
For deleted gene confirmation		
AnoI-F	CTAATATTATTGGCTGTGCC	
AnoI-R	TCTACGGCTGAAAATCTTGA	
For qRT-PCR analysis		**Functions**
16S RNA_F	CGTGCTACAATGGTCGGT	16S ribosomal RNA
16S RNA_R	GTATTCACCGCGGCATTC	
*anoI*_qRT_F	GCTTATGTGGTCGCTCAAGATAG	AHLs binding gene
*anoI*_qRT_R	ACTGAGCCAACCGACATTT	
*anoR*_qRT_F	GACCCTACTGTGCATTG	AHLs receptor gene
*anoR*_qRT_R	ACTGAGCCCAACCGACATTT	
*csuC*_qRT_F	GTCAGTCCACAACAATGACA	pili structure gene
*csuC*_qRT_R	ACAACACATAGCCGAAAGCA	
*csuD*_qRT_F	ACTGTTCCAACGAAACGCAA	Pili structure gene
*csuD*_qRT_R	CGTCGGGTTAAATCGACACC	
*pilT*_qRT_F	ATGTCCATCGCCTCGTTTAT	Biofilm and motility gene
*pilT*_qRT_R	GCAACATTCGGTACTTCAAA	

[a]The oligonucleotides were designed using the *A. nosocomialis* ATCC17903 (GenBank Accession No. GCA_000248315.2) genome sequences
[b]Regions of oligonucleotides not complementary to the corresponding templates are underlined.

in equimolar amounts and again subjected to OE-PCR with specific primers. The resulting 3.1-kb DNA fragment was ligated into a *FspI*-digested pHKD01 vector to generate pOH01. The *E. coli* S17-1 strain harboring the pOH01 was used as a conjugal donor for transferring the plasmid to *A. nosocomialis* ATCC 17903. Transconjugants were created and isolated using a previously described method (15). Briefly, the donor and recipient strains were grown in LB until the optical density at 600 nm (OD_{600}) 0.8. The strains were mixed in equal ratio and spotted onto LB plate followed by incubation at 30 ℃ for 12 h. The bacteria were resuspended in LB broth and then plated on LB agar plates containing specific antibiotics to eliminate the donor strain and to select the merodiploid cells. The merodiploids were plated on LB agar containing 10% sucrose to excise the plasmid with the antibiotic resistance cassette from the chromosome. The deletion of the *anoI* gene was confirmed by PCR analysis.

Growth curves

A. nosocomialis WT and the *anoI*-deletion mutant were cultivated in LB broth overnight and diluted into the main cultures at a ratio of 1:100. The diluted cultures were incubated at 37°C, and optical density at 600 nm (OD_{600}) was measured at specific time points of incubation up to 24 h.

Biofilm formation assay

Biofilm formation was performed as previously described with slight modifications (7). Briefly, overnight subcultures of *A. nosocomialis* WT and *anoI*-deletion mutant were adjusted to an OD_{600} of 1, after which 50 µl was added to 5 ml of Mueller-Hinton (MH) medium in round-bottom polystyrene tubes (17′ 100 mm). The setup was incubated at 30°C without shaking for 48 h, then the pellicle formed in tubes was removed and the culture was carefully discarded without disturbing the biofilm attached to the surface of the tube. After air-drying tubes, 5 ml of 0.1% (w/v) crystal violet was added for 15 min to stain the biofilm, after which tubes were washed with sterile water and the crystal violet-stained biofilm was re-solubilized in 1 ml of 30% acetic acid. A 200-ml sample of the solution was then transferred to a 96-well microtiter plate and the absorbance in each sample at 595 nm was measured. Each experiment was independently repeated five times.

Motility test

Surface motility was examined on Mueller Hinton (MH) medium containing 0.25% Eiken soft agar (Eiken Chemical, Tokyo, Japan). *A. nosocomialis* WT and *anoI*-deletion mutant cultivated in MH broth overnight were diluted to adjust the OD_{600} to 1, and 5 µl of each sample was spotted onto the center of the motility plate, with (40 µM) or without exogenous AHLs. The plates were then incubated at 30°C for 18 h. Motility on the agar surface was observed and compared. Plates were prepared fresh for each surface motility experiment, and the test was repeated on at least three separate occasions.

Quantitative real-time PCR analysis

Overnight subcultures of *A. nosocomialis* WT and *anoI*-deletion mutant were added to 10 ml of LB medium with (40 or 80 µM) and without AHLs (C6-HSL, C10-HSL and C12-HSL) and incubated under steady-state culture conditions until the OD_{600} 1.5. Total RNA was extracted using a High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. cDNA was synthesized from 1 mg of DNase-treated total RNA using Reverse Transcription Master Premix (Elpis Biotech Inc., Korea) as per the manufacturer's instructions. Quantitative RT-PCR was performed using a Bio-Rad CFX Real-Time PCR system with Bio-Rad CFX Maestro software (Bio-Rad, Hercules, CA, USA) and the indicated primers (Table 2).

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Data analysis and statistics

Data presented in this study represent the results from at least three independent experiments that yielded consistent results. The significance of differences between two groups was determined using unpaired Student's *t*-test, and differences among more than three groups were evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using Graph-Pad Prism Software (ver.5.01; GraphPad Software, San Diego, CA, USA).

RESULTS

Confirmation of the *anoI*-deletion mutant

The *anoI*-deletion mutant was confirmed by PCR of *anoI* gene (Fig. 1A) using the primer sets AnoIF and AnoR. 0.2 Kb PCR product of *anoI* gene was detected from WT, whereas none from *anoI* mutant. To determine whether deletion of *anoI* affected the growth of *A. nosocomialis*, we compared the growth in LB broth (Fig. 1B). The kinetics of growth of the *anoI*-deletion mutant were not different from those of the WT, suggesting that growth of these bacterial cells is not dependent on the product of the *anoI* gene. We also found that *anoI* mRNA was not expressed in the *anoI*-deletion mutant, further confirming deletion of the *anoI* gene (Fig. 1C).

Effects of exogenous AHLs on *anoR* expression

To study the impact of *anoI*-deletion in *A. nosocomialis*, we compared changes in the levels of *anoR* mRNA in the *anoI*-deletion mutant with those in the WT strain following treatment with different types of AHL. Under baseline

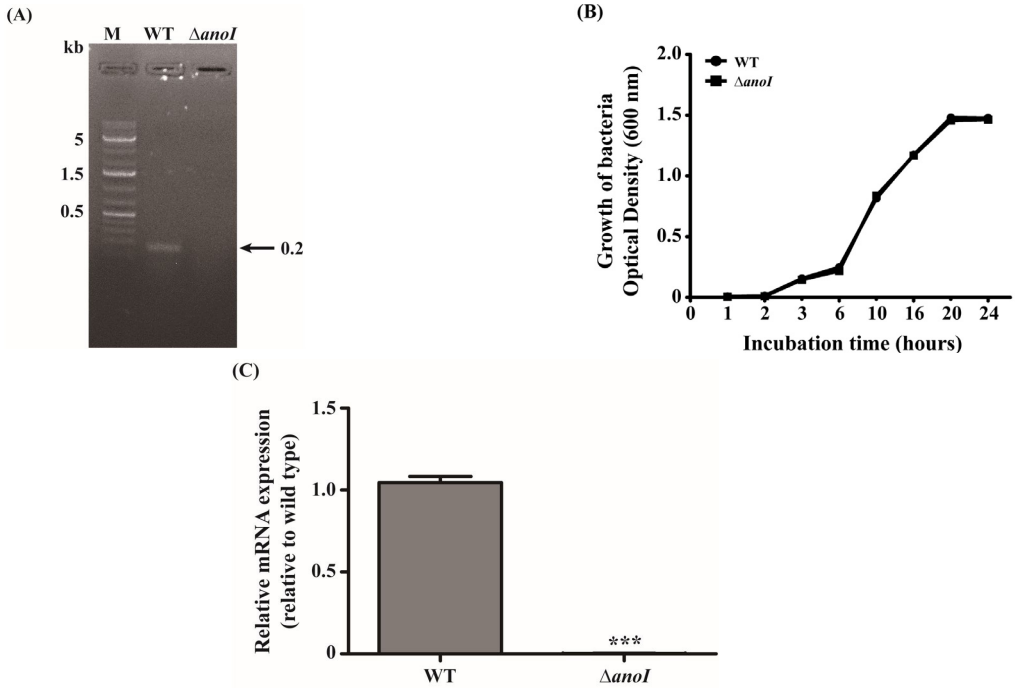

Fig. 1. Confirmation of the *anoI*-deletion mutant. (A) Gel image showing PCR analysis of the WT and *anoI*-deletion mutant (Δ*anoI*), confirming deletion of the target gene. (B) Representative growth curves for the WT and *anoI*-deletion mutant. (C) *anoI* mRNA expression was nearly absent in the *anoI*-deletion mutant. ***$P < 0.001$ versus WT.

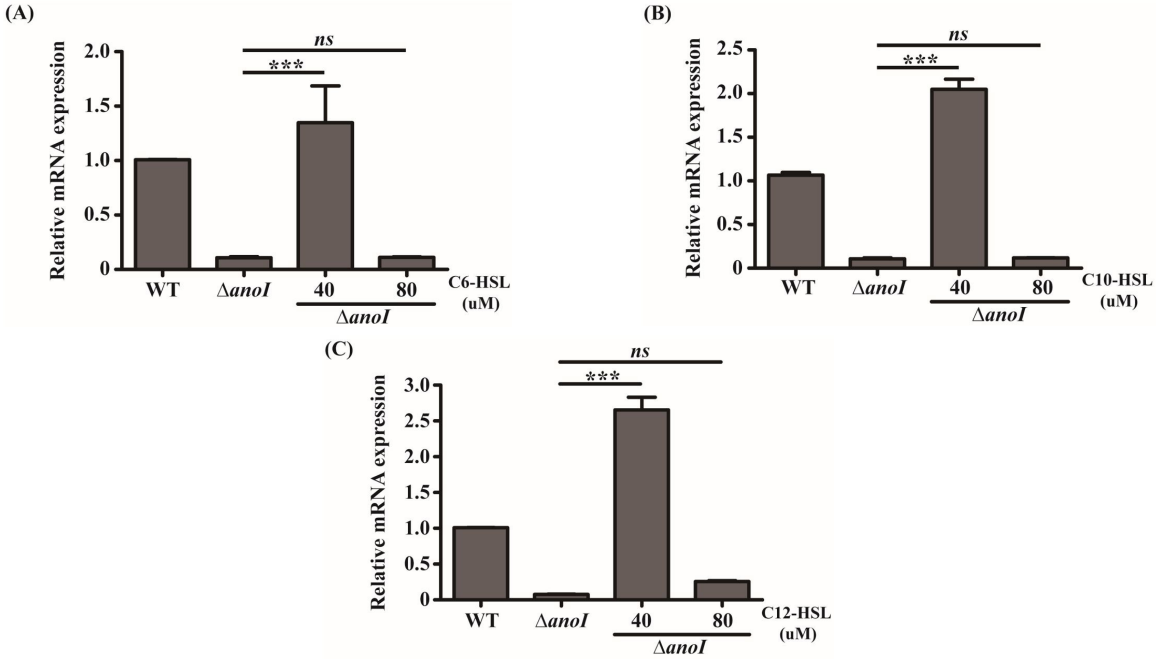

Fig. 2. Relative mRNA expression of *anoR* in the absence and presence of the indicated concentration (40 and 80 mM) and type of AHL. (A-D) *anoR* mRNA expression in the WT and *anoI*-deletion mutant following exposure to different concentrations of C6-HSL (A), C10-HSL (B) or C12-HSL (C). The differences among different sample groups were evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using Graph-Pad Prism Software. ***$P < 0.001$ versus the *anoI*-deletion mutant.

conditions, expression of *anoR* mRNA was reduced in the *anoI*-deletion mutant compared with that in the WT strain, a finding clearly consistent with our previous report (7). However, exogenously applied AHLs especially 40 μM greatly stimulated the expression of *anoR* mRNA in the *anoI*-deletion mutant (Fig. 2A, B, C), hence, 40 μM was set as the standard concentration for subsequent experiments on AHLs in this study. When the mutant was incubated with 40 μM C6-HSL, C10-HSL, or C12-HSL, *anoR* expression was up-regulated at 1.3, 2.0, or 2.65 times compared to the untreated *anoI* mutant. The highest *anoR* expression difference was observed with C12-HSL (Fig. 2C).

Biofilm formation and motility tests

To examine the effects of exogenous AHLs on biofilm formation in the *anoI*-deletion mutant, we employed crystal violet staining. Crystal violet staining showed reduced biofilm formation by the *anoI*-deletion mutant compared with the WT strain. Notably, addition of exogenous AHLs to the *anoI*-deletion mutant increased biofilm development, as shown in Fig. 3A. Quantification of biofilm formation indicated that the greatest increase occurred in the presence of exogenous C12-HSL, which restored biofilm-formation ability to WT levels. Although the treatment of C10 or C6-HSL enhance the biofilm formation of *anoI*-deletion mutant, relative levels of biofilm was lower than C12-HSL treated *anoI*-deletion mutant (Fig. 3B). To test motility, we spotted overnight cultures of *A. nosocomialis* WT and the *anoI*-deletion mutant onto MH medium containing 0.25% Eiken soft agar, with or without exogenous AHLs. Motility was restored in *anoI*-deletion mutants treated with C12-HSL. In contrast, C6-HSL and C10-HSL show no effect on the motility of the *anoI*-deletion mutant, possibly indicating that suppression of the motility phenotype may reflect a certain threshold activity of a particular AHL. To better understand this relationship, we combined C12-HSL with C6-HSL or C10-HSL, and observed that each combination restored motility, suggesting a synergistic effect of AHLs. These data indicate that C12-HSL is the predominant AHL type involve in biofilm formation and motility phenotypes in *A. nosocomialis*.

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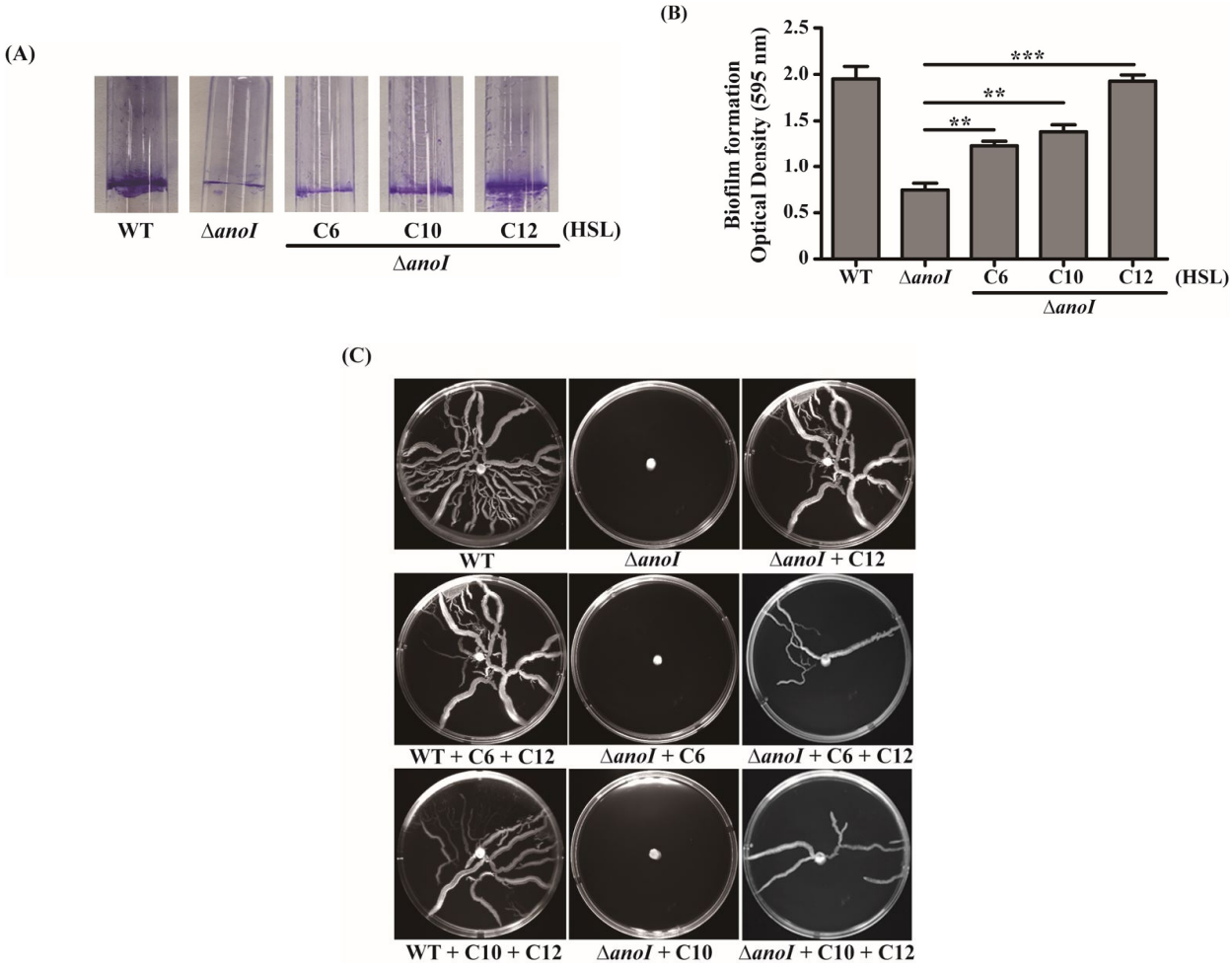

Fig. 3. Biofilm formation and motility. (A) Crystal violet staining of biofilm formed by the WT or *anoI*-deletion mutant in the presence (40 μM) and absence of exogenous AHLs. (B) Graphical representation of biofilm formation, quantified as absorbance at OD_{590} nm. The differences among different sample groups were evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using Graph-Pad Prism Software. (C) Motility assay of the WT in the absence of exogenous AHLs and of the *anoI*-deletion mutant in both the absence and presence of AHLs. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the *anoI*-deletion mutant.

Expression of *csuC*, *csuD* and *pilT*

To further understand the QS mechanism with respect to the genetic basis of biofilm formation and motility, we assessed the effects of exogenous AHLs on three biofilm related genes, *csuC*, *csuD* and *pilT*. The mRNA expression of these genes were significantly increased in *anoI*-deletion mutant treated with C6-, 10-, or C12-HSL compared to the mutant without AHLs. Among AHLs, C12-HSL induced the highest expression of *csuC*, *csuD* and *pilT* genes (Fig. 4). These data show that exogenous AHLs affect the expression of genes related to biofilm and motility and that C12-HSL is the most prominent QS molecule in *A. nosocomialis*.

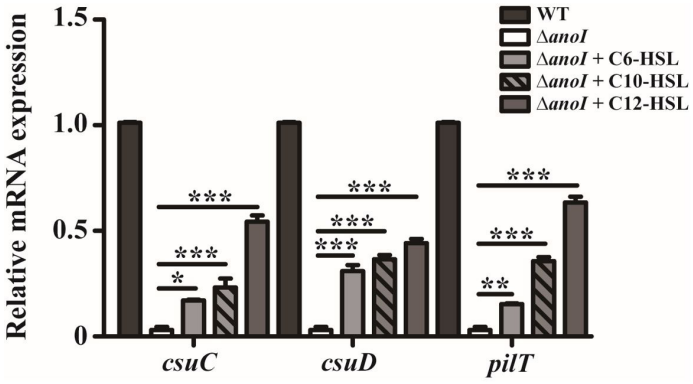

Fig. 4. Relative mRNA expression of various genes related to biofilm formation and motility. Expression of *csuC*, *csuD* and *pilT* genes in the WT and *anoI*-deletion mutant strain in the presence (40 μM) and absence of exogenous AHLs, measured by qRT-PCR. The differences among different sample groups were evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using Graph-Pad Prism Software. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ versus the *anoI*-deletion mutant.

DISCUSSION

In this study, we found that different types of AHLs differentially affect QS gene activity in the *A. nosocomialis* strain in an *anoI*-deleted setting, exerting effects on biofilm formation and motility. Previous studies have reported that when local AHL levels are high, AHLs diffuse back into cells, resulting in expression of the *anoR* gene (16). Hence, to study the regulation of biofilm formation and motility in *A. nosocomialis*, we used different types of AHLs—specifically, C6-HSL, C10-HSL and C12-HSL—that have been identified in other *Acinetobacter* strains. In this study, we found that expression of the *anoR* gene in the *anoI*-deletion mutant requires induction by exogenous AHLs (Fig. 2), with a concentration of 40 μM exerting the greatest stimulatory effect (17).

The QS system makes a major contribution to biofilm formation and motility, both of which are important virulence factors. In this study, biofilm formation was enhanced by addition of exogenous AHLs, with C12-HSL causing the greatest increase in biofilm formation. In the case of motility, only exogenous C12-HSL, or a combination of C12-HSL with other AHL types, restored motility (Fig. 3C) (18). The inability of C6-HSL and C10-HSL to support motility was correlated with their limited induction of *csuC*, *csuD* and *pilT* genes. These relationships may indicate that exogenous AHL types have an abrupt threshold below which they are unable to activate certain genes or operons to express a certain phenotype (19, 20).

It has been reported that genes such as *csuC*, *csuD* and *pilT*, are known to be involved in biofilm formation and motility. In the current study, these genes exhibited increased mRNA expression upon addition of exogenous AHLs in the QS-deficient mutant, *Δanol* (Fig. 4). This result is similar to that reported for *Hafnia alvei* and *Sinorhizobium meliloti* (8, 21), in which the QS genes, *luxI* and *sinI*, respectively, were deleted. Previous studies on the activity of AHLs in the QS system (8, 22, 23) reported that different AHLs may regulate different phenotypes in bacteria. We found that among the exogenous AHLs tested, the C12-HSL depicted increased mRNA expression of *csuC*, *csuD* and *pilT* genes in the *anoI*-deletion mutant (Fig. 4) indicating that C12-HSL could be the key AHL for biofilm and motility in *A. nosocomialis*.

Conclusions

The AHL-dependent QS system plays a significant role in biofilm formation and motility in *A. nosocomialis*. In this study, we showed that addition of exogenous AHLs to an *A. nosocomialis* strain lacking the QS gene, *anoI,* rescued the mutant

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phenotype and restored *anoR* expression levels. Among the different AHLs tested in this study, C12-HSL exerted a significant impact on biofilm formation and the expression of various biofilm and motility-related genes. Notably, C12-HSL was the only AHL that rescued motility in the *anoI*-deletion mutant strain, but the combination of C12-HSL with C6-HSL or C10-HSL was able to restore motility owing to possible synergistic effects of the AHLs. This suggests that the motility phenotype in *A. nosocomialis* might be regulated only by C12-HSL, as it may be the most abundant AHL produced by this bacterium. Further studies focusing on the regulation of AHLs in *A. nosocomialis* will be important in understanding the QS system, particularly as it relates to the potential to target *anoI/R* gene with QS inhibitors, as to design better strategies to control the spread of this infection in the environment.

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CONFLICT OF INTEREST

Authors declare no conflict of interests in this paper

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