Acinetobacter baumannii quorum-sensing signalling molecule induces the expression of drug-resistance genes

YI DOU[1], FEI SONG[1], FENG GUO[1], ZENGDING ZHOU[1], CAILIAN ZHU[2], JUN XIANG[1] and JINGNING HUAN[1]

[1]Department of Burns and Plastic Surgery, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025; [2]The Ninth People's Hospital, School of Stomatology, Shanghai Jiaotong University, Shanghai Research Institute of Stomatology, Shanghai 200011, P.R. China

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Abstract. Quorum-sensing signalling molecules such as N-acyl homoserine lactones (AHLs) enable certain Gram-negative bacteria to respond to environmental changes through behaviours, such as biofilm formation and flagellar movement. The present study aimed to identify *Acinetobacter baumannii* AHLs and assess their influence on antibiotic resistance. A clinical isolate of *A. baumannii* strain S (AbS) was collected from the wound of a burn patient and high-performance liquid chromatography and tandem quadrupole or quadrupole time-of-flight high-resolution mass spectrometry was used to identify AbS AHLs. Antibiotic sensitivity was assessed in an AHL-deficient AbS mutant (AbS-M), and the expression of drug-resistance genes in the presence of meropenem in AbS, AbS-M and AbS-M treated with the AHL N-3-hydroxy-dodecanoyl-homoserine lactone (N-3-OH-C_{12}-HSL). AbS-M was more sensitive to meropenem and piperacillin than wild-type AbS, but resistance was restored by supplementation with N-3-OH-C_{12}-HSL. In addition, meropenem-treated AbS-M expressed lower levels of the drug-resistance genes *oxacillinase 51, AmpC*, *AdeA* and *AdeB*; treatment with N-3-OH-C_{12}-HSL also restored the expression of these genes. Overall, the results of the present study indicate that N-3-OH-C_{12}-HSL may be involved in regulating the expression of drug-resistance genes in *A. baumannii*. Therefore, this quorum-sensing signalling molecule may be an important target for treating multidrug-resistant *A. baumannii* infections.

Correspondence to: Dr Jingning Huan, Department of Burns and Plastic Surgery, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Er Road, Huangpu, Shanghai 200025, P.R. China
E-mail: huanjn194@sina.com

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Introduction

Staphylococcus aureus and *Pseudomonas aeruginosa* are the main bacteria that opportunistically infect patients with burns (1). However, recent reports (2-5) indicate that the proportion of infections caused by *Acinetobacter baumannii* is gradually increasing and, in some instances, already exceeds the number of infections caused by *P. aeruginosa*. *A. baumannii* is the most commonly detected Gram-negative organism infecting patients with burns (2-5). However, the emergence of multidrug-resistant *A. baumannii* complicates the clinical treatment of these infections (6-8).

Quorum sensing is a form of cell-cell communication that bacteria use to coordinate the expression of genes involved in certain behaviours, such as flagellar movement (9,10), virulence factor production (11,12), and secondary metabolite and biofilm production (9,13). Various quorum-sensing signalling molecules have been identified, including oligopeptides in Gram-positive bacteria and N-acyl homoserine lactones (AHLs) in some Gram-negative bacteria (14). *Acinetobacter* spp. also produce AHLs that possess quorum-sensing activity (15), and the *A. baumannii* AHL, N-3-hydroxy-dodecanoyl-homoserine lactone (N-3-OH-C_{12}-HSL), is known to affect its motility and biofilm formation (16,17).

Since quorum sensing allows bacteria to respond to environmental changes as a colony and thereby boosts survival, disrupting the quorum-sensing system may be a promising new strategy for the treatment of infections (18-20). It is important to investigate novel strategies for the inhibition of *A. baumannii* by targeting AHLs (16,17,21-23), but little is currently known about the types and functions of AHLs produced by *A. baumannii*.

All AHLs share a common homoserine moiety but can contain acyl side-chains of various lengths and degrees of saturation and with various groups at the third carbon position. AHLs generate characteristic fragment ions on electrospray ionization (ESI) at a mass-to-charge ratio (*m/z*) of 102, and the acyl side-chains generate the corresponding fragment ions at *m/z* $[M+H-101]^+$ (15,24-33).

The most common methods for identifying AHLs involve a combination of thin-layer chromatography and biosensors (34,35). These methods are simple and inexpensive but are limited by the sensitivity of the biosensor and the use of

standard substances as references. In the present study, a clinical isolate of *A. baumannii* strain S (AbS) was collected from the wound of a burn patient and cultured. AHLs produced by AbS were subsequently analysed by high-performance liquid chromatography (HPLC) and either tandem quadrupole (TQ) or quadrupole time-of-flight (Q-TOF) high-resolution mass spectrometry (HRMS). The present study adds to the growing body of research on the quorum-sensing system of *A. baumannii* and may contribute to the development of novel antibacterial therapies that target AHLs for treating multidrug-resistant *A. baumannii* infection.

Materials and methods

Bacterial strains and growth conditions. A single nosocomial specimen of AbS was collected from the wound surface exudates of a patient admitted to the Department of Burns and Plastic Surgery at Ruijin Hospital (Shanghai Jiaotong University School of Medicine, Shanghai, China) in 2008. Antibiotic sensitivity was assessed according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) (36), which included using ATB test strips (BioMérieux, Marcy l'Etoile, France) and the Kirby-Bauer disk diffusion method with antibiotic discs from Oxoid, Ltd. (Thermo Fisher Scientific, Inc., Waltham, MA, USA). *Agrobacterium tumefaciens* strain KYC55 was used as a biosensor, and was kindly provided by Professor Jun Zhu (College of Life Sciences, Nanjing Agricultural University, Nanjing, China). *A. baumannii* was cultured statically in Luria-Bertani (LB) medium or Mueller-Hinton (MH) medium (Oxoid, Basingstoke, UK) at 37˚C; *A. tumefaciens* KYC55 was cultured statically in LB medium at 28˚C.

Preparation of AHL extract. *A. baumannii* and *A. tumefaciens* were stored at -80˚C in bacteria stock solution (Beyotime Biotechnology, Shanghai, China). *A. baumannii* were inoculated on LB agarose plates and incubated overnight at 37˚C. Individual colonies ($1x10^8$ colony-forming units (CFU)/ml) were selected and cultured in 15 ml LB medium at 37˚C.

For HPLC-MS, bacteria were cultured in 500 ml LB medium from the overnight LB agarose plates, and 500 ml bacterial liquid was collected at 8 h, as determined by the AHL activity curve. Bacterial samples were centrifuged (4,500 x g for 20 min) and supernatants were passed through a 0.22 μm filter. An equal volume of 100% ethyl acetate was added to the filtrate, and the ethyl acetate phase was collected for AHL extraction and dried in a vacuum centrifuge. The residue was the AHL extract and was then re-dissolved in 50 μl ethyl acetate.

Analysis of AHL activity. AHL activity was measured at 4, 8, 16, 24, 32, 40 and 48 h after seeding. At each time point, 3 bacterial liquid were collected and the optical density (OD) 600 was measured. AHL extracts in 50 μl ethyl acetate from 4, 8, 16, 24, 32, 40 and 48 h were added to cultures of *A. tumefaciens* KYC55 cultures, and β-galactosidase activity was measured to indirectly indicate AHL activity, as described previously (15,37). Following overnight incubation, the OD_{600} was measured and 0.8 ml Z buffer (in each litre containing: 16.1 g (Na_2HPO_4) $7H_2O$, 5.5 g (NaH_2PO_4) H_2O, 0.75 g KCl, 0.245 g ($MgSO_4$) $7H_2O$, 2.7 ml 2-mercaptoethanol, adjusted to pH 7.0 with HCl), 10 μl 0.05% sodium dodecyl sulphate, 15 μl chloroform and 0.1 ml ortho-nitrophenyl-β-galactoside (4 mg/ml) were added, with a final sample volume of 0.2 ml. The time (T) taken for the solution to turn yellow was recorded, and 0.6 ml 1 M Na_2CO_3 was added to terminate the reaction. The OD_{420} of supernatants was determined, and relative AHL activity was calculated as follows: Activity in Miller units=$(1{,}000xOD_{420})/(OD_{600}xTx0.2)$.

Identification of AHLs using HPLC-MS. AHLs of different structures contain the same homoserine lactone (HSL) ring, and this moiety generates characteristic fragment ions at *m/z* 102 (25). Based on this principle, AbS AHLs were identified by HPLC combined with either TQ or Q-TOF HRMS using a 1200 HPLC-6140 TQ MS or a 1260 HPLC-6538 Q-TOF HRMS (Agilent Technologies, Inc., Santa Clara, CA, USA), respectively. Resultant chromatograms were compared with those of standard substances to elucidate the structure of AbS AHLs. The test conditions were as follows: An Agilent Poroshell 120 SB-C18 chromatographic column (2.7 μm, 2.1x100 mm; Agilent Technologies, Inc.) was used with acetonitrile and water as the mobile phase. The initial acetonitrile concentration was 40%, which was increased to 100% after 30 min, with a z-flow rate of 0.3 ml/min and sample injection volume of 10 μl. Positive-ion ESI was conducted with the ion source at 350˚C. The dry N_2 flow rate was 8 l/min, and the air pressure of atomizing N_2 was 40 psi. The capillary voltage was 4,000 V. HPLC-TQ HRMS was performed with a precursor ion scan and daughter ion scan (collision energy, 15-30 units), while HPLC-Q-TOF HRMS involved MS1 and MS2 full scans (collision energy, 15-30 units).

Establishment of an AHL-deficient AbS mutant (AbS-M). An AbS mutant that is unable to produce AHLs was established using pKNG101.abaI::Km, as previously described (38); the pKNG101.abaI::Km plasmid was provided by Professor Philip N. Rather (Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, USA). This vector was transformed into *Escherichia coli* strain SM10, and the resultant SM10/pKNG101.abaI::Km was cultured with AbS in a filter-mating system in LB medium at 37˚C without antibiotics for 24 h, after which it was cultured and screened on LB agarose plates containing 10% sucrose without NaCl. Sucrose resistance indicates that the bacteria have lost the integrated pKNG101 plasmid and therefore streptomycin sensitivity. AbS-M was screened for kanamycin resistance, and Southern blotting was used to confirm that colonies with this phenotype had abaI::Km disruption, as previously described (38).

Antibacterial sensitivity of AbS and AbS-M. The minimum inhibitory concentration (MIC) of common antibacterial drugs (including, meropenem, piperacillin, ceftazidime, ciprofloxacin, sulfamethoxazole/trimethoprim and minocycline) against AbS, AbS-M and AbS-M supplemented with 10 μmol N-3-OH-C_{12}-HSL (AbS-M+HSL; #53727; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was assessed using the broth-micro-dilution method, according to the CLSI protocol. Briefly, overnight bacterial cultures were inoculated at $5x10^5$ CFU/ml in 1 ml MH medium containing

Table I. Primer sequences used for quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')	Product length (bp)
16S rRNA	F: ACGGTCGCAAGACTAAAACTCA R: GTATGTCAAGGCCAGGTAAGGT	108
OXA-51	F: CTATGGTAATGATCTTGCTCGTG R: TGGTGGTTGCCTTATGGTG	104
AmpC	F: TTATGCGGGCAATACACCA R: CTGACAGAACCTAGCTCAAAAATG	207
OXA-23	F: AAGGGCGAGAAAAGGTCATT R: TCCTGATAGACTGGGACTGCA	89
IMP-4	F: ATTCTCAATCCATCCCCACG R: CCTTTCAGGCAGCCAAACTAC	185
VIM-2	F: AACTCTTCTATCCTGGTGCTGC R: TGCGTGACAACTCATAAATCG	105
AdeA	F: AGTCGGAGGTATCATTGAAAAGG R: TGAACTTTGAGTCTTGCCACCT	162
AdeB	F: ATGCGTGAAATGGAACAACTG R: CCAAGACAAGGAAGACAACTAACA	145
AdeC	F: GCCATTCAATCAGCTTTTCGT R: GAGTTTATAGGTTGCAGCAGTCG	117
GAPDH	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA	440

bp, base pair; *OXA*, oxacillinase; F, forward; R, reverse; *AmpC*, AmpC type β-lactamase; *IMP*, IMP type metallo-β-lactamase; *VIM*, verona integron-mediated metallo-β-lactamase; *Ade*, *Acinetobacter* drug efflux; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

a range of concentrations (128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0 μg/ml) of the antibacterial drugs. Following 24 h incubation at 37°C, the MIC against bacterial growth was assessed by visual examination. Each antibiotic concentration was tested three times.

Expression of drug-resistance genes in AbS, AbS-M and AbS-M+HSL treated with 0.125 μg/ml meropenem or AbS untreated with meropenem (AbS-U) for 24 h. A total of 45 μl 0.5 McFarland bacterial liquid (AbS, AbS-M and AbS-M+AHL) was added to 3 ml LB medium containing 0.125 μg/ml meropenem, with a final concentration of 10 μM AHL (N-3-OH-C_{12}-HSL). Alternatively, 45 μl 0.5 McFarland bacterial liquid was added into 3 ml LB medium without meropenem (AbS-U). The cultures were incubated at 37°C. After 24 h, 1 ml bacterial liquid was centrifuged (10,621 x g for 1 min) and supernatants were discarded. Total RNA was extracted using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol; concentration and purity were determined using an ultraviolet spectrophotometer. RNA was reverse transcribed into cDNA using the AMV First Strand cDNA Synthesis kit (New England Biolabs, Inc., Ipswich, MA, USA), according to the manufacturer's protocol. AbS-U, AbS, AbS-M and AbS-M+HSL cultures were incubated for 24 h and the expression levels of 16S rRNA, Oxacillinase (*OXA*)-*51*, AmpC type β-lactamase (*AmpC*), oxacillinase (*OXA*)-*23*, IMP type metallo-β-lactamase (*IMP*)-*4*, verona integron-mediated metallo-β-lactamase (*VIM*)-*2*, *Acinetobacter* drug efflux (*Ade*) *A*, *AdeB* and *AdeC* were assessed by quantitative polymerase chain reaction (qPCR) using a StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR-Green Master Mix (Thermo Fisher Scientific, Inc.); primers used are listed in Table I. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was measured as a reference, and gene expressions were calculated in terms of fold change using the comparative Cq method; relative mRNA expression was calculated using the $2^{-\Delta\Delta Cq}$ method (39). The experiments were repeated 3 times.

Statistical analyses. Data were presented as the mean ± standard deviation and analysed using Student's t-test, analysis of variance and least significant difference pot hoc test with SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Changes in AHL activity of AbS. AbS growth rate and AHL activity were measured periodically between 4 and 48 h incubation (Fig. 1). AHL activity increased from 5.00±1.00 Miller units at 4 h culture to a maximum of 279.33±27.59 Miller units at 8 h. Subsequently, the activity decreased to 28.67±4.16 Miller units at 16 h and plateaued. The AbS growth curve did not correlate with AHL activity after bacterial growth reached the log phase; the OD_{600} (bacterial growth) peaked at 0.90±0.01 after 24 h of culture and then plateaued.

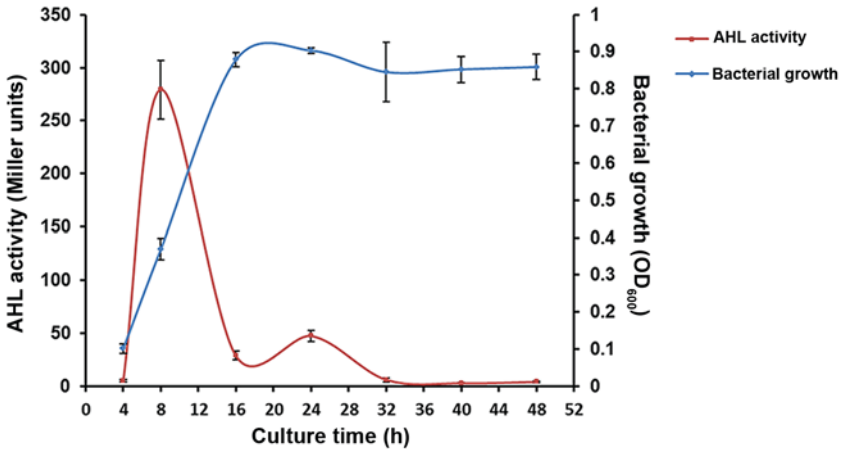

Figure 1. *Acinetobacter baumannii* strain S growth curve and corresponding measurements of AHL activity. AHL, N-acyl homoserine lactone; OD, optical density.

AHLs produced by AbS. AHLs that were extracted from AbS culture supernatants using ethyl acetate were screened using HPLC-TQ MS. As presented in Fig. 2, the precursor ion scan at *m/z* 102 detected 30 precursor ions, including those at *m/z* 282, 284 and 300 (each precursor ion represents one compound). MS2 spectrum analysis of these 30 ions confirmed that they could generate fragment ions at *m/z* 102, suggesting that they may be AHLs.

In the positive ionization mode of ESI, extracted AHLs generated a quasi-molecular ion at *m/z* 300 and major fragment ions at *m/z* 102 and *m/z* 74 (Fig. 3A). The ion at *m/z* 102 was the most abundant, and in order to determine its structure, HPLC-Q-TOF HRMS was used to examine the elemental composition of this ion and related daughter fragment ions. The elemental composition at *m/z* 300 was $C_{16}H_{30}NO_4$, representing the $[M+H]^+$ ions of N-3-OH-C_{12}-HSL. The two major fragment ions were C_4H_8NO (*m/z* 102) and C_3H_8NO (*m/z* 74), both of which were derived from the HSL ring of N-3-OH-C_{12}-HSL, which was the only AHL molecule identified (Fig. 3B). The major fragmentation pathway is shown in Fig. 4. In addition, some low-abundance ions were detected in the MS2 spectrum (*m/z* 121, 97 and 83), and they contained only two elements, H and C. We hypothesized that these were derived from the fragmentation of carbon chains near the acyl group.

Since components from the culture media may interfere with AHL detection, HPLC-Q-TOF HRMS and tandem MS were used to scan for AHL molecules identified in previous screens. The present study also determined the elemental compositions of the 30 precursor ions and their corresponding daughter ions at *m/z* 102. The results revealed that only ions detected at *m/z* 300≥102 met the structural requirement for AHLs. The daughter ions at *m/z* 102 were derived from 29 candidate molecules that contained $C_5H_{12}NO$ and therefore could not be AHLs.

According to the composition and degree of unsaturation, the signal molecule at *m/z* 300 was inferred to be N-3-OH-C_{12}-HSL. HPLC-MS was then used to examine a commercially available N-3-OH-C_{12}-HSL, and the results confirmed that it was structurally identical to the N-3-OH-C_{12}-HSL detected in the present study.

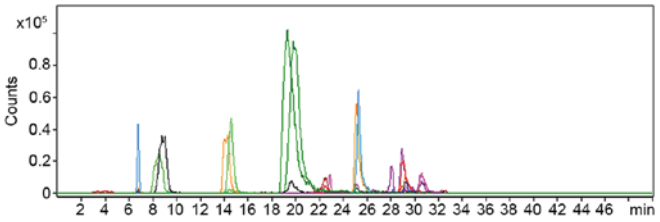

Figure 2. Ion chromatogram of the *Acinetobacter baumannii* strain S culture medium extract. A total of 30 precursor ions that could generate daughter ions at a mass-to-charge ratio of 102 were detected.

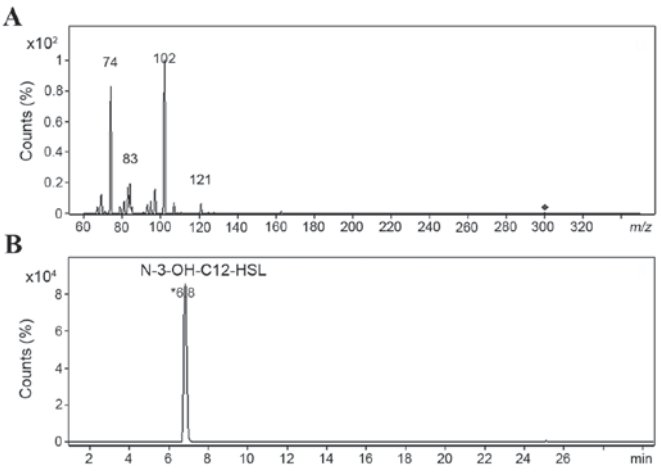

Figure 3. MS profiles of N-3-OH-C_{12}-HSL. (A) MS2 spectrum of N-3-OH-C_{12}-HSL (*m/z* 300). (B) Secondary ion chromatogram of N-3-OH-C_{12}-HSL derived from *Acinetobacter baumannii* strain S obtained using high-performance liquid chromatography and tandem quadrupole MS (*m/z* 300≥*m/z* 102). MS, mass spectrometry; *m/z*, mass-to-charge ratio; N-3-OH-C_{12}-HSL, N-3-hydroxy-dodecanoyl-homoserine lactone.

Activity of mutant AHL. AHLs were isolated from the supernatant of AbS and AbS-M cultures incubated for 8 h at 37˚C and the activity levels were analysed. AHL activity was significantly lower in AbS-M (12.67±1.53 Miller units) compared with wild-type AbS (255.67±16.01 Miller units) ($P<0.01$; Fig. 5).

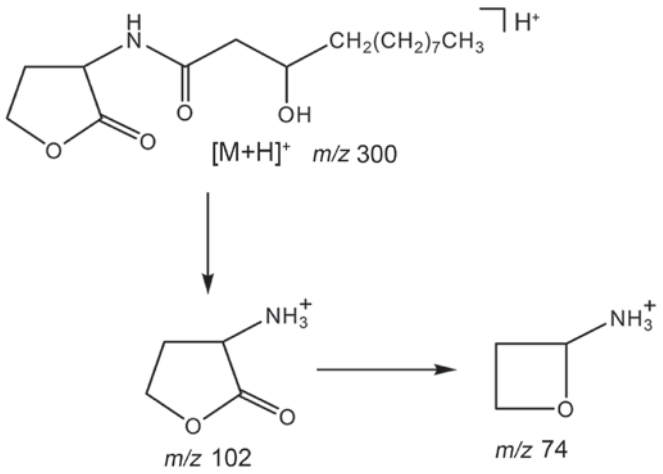

Figure 4. Major fragmentation pathway of N-3-hydroxy-dodecanoyl-homoserine lactone. *m/z*, mass-to-charge ratio.

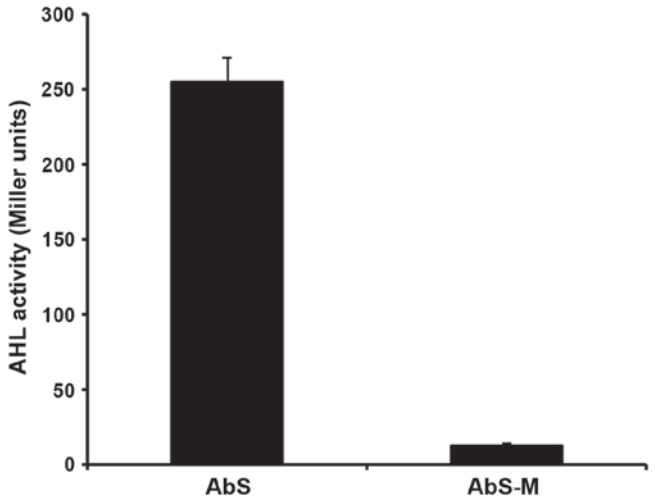

Figure 5. Activity of AHLs derived from AbS and AbS-M. The activity of AHLs derived from AbS-M (12.67±1.53) was significantly lower compared with AHLs derived from wild-type AbS (255.67±16.01) (P<0.01). AHL, N-acyl homoserine lactones; AbS, *Acinetobacter baumannii* strain S; AbS-M, AbS mutant.

Antibiotic sensitivity of AbS, AbS-M and AbS-M+HSL. AbS was sensitive to amikacin, cefuroxime, ceftazidime, imipenem, gentamicin, ciprofloxacin, sulfamethoxazole/trimethoprim, sulperazone, tazocin, cefepime, panipenem, meropenem, ampicillin, Unasyn (which is a combination of ampicillin and sulbactam) and piperacillin. Then the MIC of AbS, AbS-M and AbS-M + HSL cultures to meropenem, piperacillin, ceftazidime, ciprofloxacin, sulfamethoxazole/trimethoprim and minocycline was assessed (Table II). The MICs of meropenem and piperacillin against AbS-M (0.25 and 1 μg/ml, respectively) were lower than the MICs of these antibiotics against wild-type AbS (0.5 and 2 μg/ml, respectively). However, the addition of HSL to the AbS-M culture raised the MICs of meropenem and piperacillin to similar levels as wild-type AbS (0.5 and 2 μg/ml, respectively). By contrast, the MICs of ceftazidime, ciprofloxacin, sulfamethoxazole/trimethoprim and minocycline were similar for AbS, AbS-M and AbS-M + HSL.

Expression of drug-resistance genes in AbS-U, AbS, AbS-M and AbS-M + HSL treated with meropenem for 24 h. AbS, AbS-M and AbS-M + HSL were cultured for 24 h in LB medium supplemented with meropenem (0.125 μg/ml), and AbS untreated with meropenem (AbS-U) was additionally cultured. The mRNA expression levels of drug-resistance genes were assessed by qPCR (Table III). Meropenem treatment increased the expression of *OXA-51, AmpC, AdeA* and *AdeB* in all three bacterial cultures. The mRNA expression levels of these four genes were significantly lower in AbS-M compared with wild-type AbS; however, supplementation of AbS-M cultures with N-3-OH-C_{12}-HSL increased the mRNA expression of these four drug-resistance genes to higher levels compared with wild-type AbS and untreated AbS-M. The expression of *OXA-23, IMP-4, VIM-2* and *AdeC* could not be detected in any of the three strains.

Discussion

Quorum sensing affects bacterial biofilm formation (27,28), antibacterial drug sensitivity (29) and bacterial virulence (30), suggesting that inhibition of this system may be a useful therapeutic strategy in combating the emergence of antibiotic-resistant strains of pathogenic bacteria. The present study aimed to contribute to the growing body of literature on AHLs produced by clinical isolates of *A. baumannii*.

The present study found that although the activity of AHLs produced by AbS was positively correlated with bacterial density in the log phase of growth, AHL activity reduced as growth plateaued; this trend has been previously reported for other bacteria (31,32). N-(3-oxohexanoyl)-L-HSL produced by *Erwinia carotovora* was revealed to be unstable at pH >7-8, which is the pH of the stationary phase of bacterial growth (40). Another study demonstrated that, during growth plateauing, *A. tumefaciens* produces abundant levels of acyl-homoserine lactonases, which reduce AHL activity (32). Thus, AHL activity seems to be regulated by the growth rate, which allows bacteria to respond to their changing density.

In the present study, HPLC-MS with TQ and Q-TOF was used to successfully identify AHLs. This method is advantageous because it does not depend on biosensor sensitivity and reference substances, and thus may be preferable to the conventional methods used for AHL identification, which combine thin-layer chromatography with biosensors.

AHLs produced by *A. baumannii* have been proposed to vary depending on culture conditions (41). One previous study identified 3-OH-C_{12}-HSL and other AHLs of unknown structure in cultures of *A. baumannii* strain M2 (38), whereas another study identified C_6-HSL and C_8-HSL in cultures of *A. baumannii* strain 4KT (15). Furthermore, *P. aeruginosa* infections in patients with cystic fibrosis have been reported to produce different AHLs *in vivo* and *in vitro* (42). Thus, the AHL identified in the present study may differ from those identified previously from *A. baumannii*, owing to the particular strain and culture conditions used. Additional experiments are required to identify the range of AHLs produced by this organism.

The present study established an AHL-deficient AbS mutant that was used to determine whether AHLs affected antibacterial drug sensitivity of AbS. Antibacterial drug-sensitivity assays

Table II. Antibiotic sensitivity.

Antibiotic	Minimum inhibitory concentration (μg/ml)		
	AbS	AbS-M	AbS-M + HSL
Meropenem	0.5	0.25	0.5
Piperacillin	2.0	1.0	2.0
Ceftazidime	0.25	0.25	0.25
Ciprofloxacin	0.5	0.5	0.5
Sulfamethoxazole/ trimethoprim	0.25/4.75	0.25/4.75	0.25/4.75
Minocycline	0.5	0.5	0.5

Abs, *Acinetobacter baumannii* strain S; AbS-M, AbS mutant; HSL, homoserine lactone.

Table III. mRNA expression levels of multidrug-resistance genes in meropenem-treated cultures or untreated cultures.

Gene	AbS-U	AbS	AbS-M	AbS-M + HSL
OXA-51	0.13±0.02	1.09±0.13	0.68±0.04[a]	1.74±0.04
AmpC	0.12±0.03	0.94±0.11	0.60±0.04[a]	1.55±0.04
OXA-23	ND	ND	ND	ND
IMP-4	ND	ND	ND	ND
VIM-2	ND	ND	ND	ND
AdeA	0.08±0.04	1.17±0.17	0.59±0.08[a]	1.66±0.25
AdeB	0.09±0.08	1.08±0.16	0.51±0.09[a]	1.31±0.11
AdeC	ND	ND	ND	ND

[a]P<0.01 vs. Abs and AbS-M + AHLs. All data presented as the mean ± standard deviation. AbS-U, *Acinetobacter baumannii* strain S untreated; AbS, *Acinetobacter baumannii* strain S; Abs-M, AbS mutant; HSL, homoserine lactone; ND, not detected; *OXA*, oxacillinase; *AmpC*, AmpC type β-lactamase; *IMP*, IMP type metallo-β-lactamase; *VIM*, verona integron-mediated metallo-β-lactamase; *Ade*, *Acinetobacter* drug efflux.

revealed that the MICs of meropenem and piperacillin were lower in AbS-M compared with wild-type AbS; however, the MICs returned to wild-type AbS levels when AbS-M cultures were treated HSL. Although this AHL-mediated increase in MICs was not substantial, this finding is promising in that it confirms the association between AHLs and antibiotic resistance in *A. baumannii*.

A previous report regarding the influence of AHLs on bacterial drug resistance mainly focused on their influence on biofilm formation (9). Previous studies have also described multiple mechanisms of drug resistance in *A. baumannii*, including the production of β-lactamases (43), which can be divided into four categories: Extended spectrum β-lactamases (44-46), metallo-β-lactamases (47,48), AmpC enzyme (49) and oxacillinases (50,51). However, the present study sought to determine the influence of AHLs on the expression of drug-resistance genes and revealed that in the presence of meropenem AbS expressed *OXA-51* and *AmpC*, but not *OXA-23*, *IMP-4*, or *VIM-2*. *OXA-51* was previously demonstrated to be strongly expressed in *Acinetobacter* spp. and may be the main drug-resistance gene (52,53), whereas *AmpC* is often found in *A. baumannii* strains from China (54,55). The present study found that the mRNA expression levels of *OXA-51* and *AmpC* were significantly lower in AbS-M compared with wild-type AbS, but the levels recovered upon supplementation of the AbS-M culture with an AHL extract. These results indicate that AHLs may strengthen drug resistance by moderating the expression of drug-resistance genes.

In addition to producing β-lactamases, *A. baumannii* expresses efflux pump genes *AdeA, AdeB and AdeC*, which confer resistance to β-lactam antibiotics, aminoglycosides, erythromycins, quinolones, tetracyclines, chloramphenicol and trimethoprim (43,56-62). The present study found that *AdeA* and *AdeB* were expressed by AbS in the presence of meropenem. It was not unexpected that *AdeC* was not detected, since this gene is not essential for efflux pump activity (59). The mRNA expression levels of *AdeA* and *AdeB* were significantly lower in AbS-M than in wild-type AbS, and the expression of both *AdeA* and *AdeB* was recovered with AHL supplementation. Results from the present study indicated that AbS AHLs promote the expression of *OXA-51, AmpC*, *AdeA* and *AdeB* in the presence of meropenem, suggesting that AbS produces AHLs to enhance antibiotic resistance. Furthermore, upregulation of *AdeB* expression has been reported to be associated with the emergence of pan-resistant *A. baumannii* (57). Thus, AHLs may promote the emergence of meropenem-induced multidrug- and pan-resistance.

The present study has some limitations that should be noted. Although a mutant strain AbS was designed to be deficient in AHL, subsequent experiments with this mutant may have been influenced by the presence of *abaI* homologues; AbaI is similar to the LuxI family of autoinducer synthases (37). In addition, it is well known that AHLs can be degraded by N-acylhomoserine lactone-lactonase (32). Therefore, we cannot rule out the possibility of AHL degradation due to lactonolysis. Lastly, the present study did not determine whether the mutation in AbS-M specifically reduces the transcription of *abaI* or whether it causes a generalized reduction in transcription.

In Gram-negative bacteria, AHL receptor systems include the cytoplasmic LuxR receptor and the transmembrane LuxN receptor (19). Inactivation of suppressor of division inhibition (SdiA), a bacterial homolog of LuxR, hampers the expression of the efflux pump drug-resistance genes *acrA* and *acrB*, which are responsible for bacterial multidrug resistance, and AHLs may interact with SdiA to enhance the expression of *acrA* and *acrB* (63). Similar systems may exist in *A. baumannii*, and the interaction of AHLs with such systems may be able to induce the expression of drug-resistance genes. However, the MIC of the antibiotics ceftazidime, ciprofloxacin, sulfamethoxazole/trimethoprim and minocycline did not differ between the presence and absence of AHLs, suggesting that drug-resistant phenotypes may be produced by a diverse range of factors and genes. Conversely, exposure to meropenem for 24 h was perhaps insufficient to induce significant phenotypic alterations, and additional experiments are required to rule out longer-term

changes to genes encoding resistance to these antibiotics. However, results from the present study are notable, since to the best of our knowledge no previous study has addressed the mechanisms underlying the influence of AbS AHLs on the expression of drug-resistance genes.

In the present study, the quorum-sensing system of AbS was demonstrated to involve N-3-OH-C_{12}-HSL, which induced the expression of drug-resistance genes *OXA-51, AmpC*, *AdeA* and *AdeB* in the presence of meropenem. Loss of AHL production in AbS-M resulted in reduced mRNA expression of these four drug-resistance genes, while treatment with N-3-OH-C_{12}-HSL restored their expression. Thus, AHL-mediated induction of *AdeA* and *AdeB* expression could in turn lead to multidrug resistance in *A. baumannii*. These results highlight a new direction for the development of drugs targeting *A. baumannii*, particularly pan-resistant strains.

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