Mechanism of azithromycin inhibition of HSL synthesis in *Pseudomonas aeruginosa*

Jianming Zeng, Ni Zhang, Bin Huang, Renxin Cai, Binning Wu, Shunmei E, Chengcai Fang & Cha Chen

*Pseudomonas aeruginosa* is an opportunistic pathogen and a leading cause of nosocomial infections. Unfortunately, *P. aeruginosa* has low antibiotic susceptibility due to several chromosomally encoded antibiotic resistance genes. Hence, we carried out mechanistic studies to determine how azithromycin affects quorum sensing and virulence in *P. aeruginosa*. *lasI* and *rhlI* single and double mutants were constructed. We then undertook a quantitative approach to determine the optimal concentration of azithromycin and culture time that can affect the expression of HSLs. Furthermore, based on the above results, the effect on quorum sensing was analyzed at a transcriptional level. It was found that 2 μg/mL azithromycin caused a 79% decrease in 3-oxo-C12-HSL secretion during cultivation, while C4-HSL secretion was strongly repressed in the early stages. Azithromycin acts on ribosomes; to determine whether this can elicit alternative modes of gene expression, transcriptional regulation of representative virulence genes was analyzed. We propose a new relationship for *lasI* and *rhlI*: *lasI* acts as a cell density sensor, and *rhlI* functions as a fine-tuning mechanism for coordination between different quorum sensing systems.

Quorum sensing (QS) is an important global regulatory mechanism in bacteria that enables individual bacteria to coordinate their behavior in response to cell density. The QS system relies on self-generated signaling molecules to coordinate gene expression. Two QS systems, i.e., the las and rhl systems, have been identified in *P. aeruginosa* (PA). In the las QS system, the *lasI* gene product directs the formation of a diffusible extracellular signal, N-(3-oxododecanoyl)-L-HSL (3-oxo-C12-HSL or OdDHL), which interacts with LasR to activate a number of virulence genes. On the other hand, the *rhlI* gene product catalyzes the synthesis N-butyryl-L-HSL (C4-HSL or DHL). This diffusible signal, in conjunction with RhlR, activates the expression of other virulence genes. Although the two QS systems have distinct downstream targets, they are still hierarchically connected. It has been found that the las system positively regulates the expression of both rhlR and rhlI.

Approximately 600 genes are regulated by QS in PA, and most of the regulation occurs during the stationary phase of growth. The Las system mainly regulates toxins (exotoxin A and exoenzymes) and proteases (elastase, LasA protease and alkaline protease), and hemolysins (phospholipase and rhamnolipid) are regulated by the rhl system. 4-hydroxy-2-alkylquinoline (HAQ), and acyl homoserine lactone (AHL) are two well-characterized signaling molecules. HAQ is involved in cell-to-cell communication pathway, while AHL is used to regulate growth and gene expression. The regulation of gene expression allows the integration of cell density with virulence factor production. Virulence is regulated by both external factors and interaction with the QS systems. Previous studies have reported that sub-inhibitory concentrations of antibiotics not only inhibit QS but also act as signaling molecules for triggering virulence factor production.

PA is an opportunistic human pathogen and a leading cause of nosocomial infections, particularly in immunocompromised patients, including those with cancer, burns and cystic fibrosis. The emergence and rapid spread of multidrug-resistant *PA* (MDRPA) isolates that cause serious nosocomial infections is of great concern. The extensive and irrational use of antimicrobial agents has promoted resistance in *PA*. There are now...
a number of multidrug-resistant strains, which make clinical treatment difficult. The resistance mechanisms of PA are very complex; horizontal gene transfer (HGT) is an important mechanism. Azithromycin (AZM), a member of the macrolide class of antibiotics, is used to treat certain bacterial infections, which are primarily caused by gram-positive bacteria but also some gram-negative pathogens. Many clinical and experimental studies have shown the beneficial effects of AZM in patients with diffuse panbronchiolitis and cystic fibrosis, which are associated with PA infection.

A study by Henkel showed that AZM has the potential to inhibit QS signal molecules and attenuate the virulence of PA. Sub-MIC concentrations of AZM were found to inhibit the production of QS signals, swimming, swarming and twitching motilities, and biofilm formation in vitro. AZM affects QS by interfering with one of the following pathways: biofilm formation, translation due to its interaction with ribosomes, or increase in outer membrane permeability. Moreover, 2 μg/mL AZM (1/64th of the MIC) showed promising results on QS-dependent virulence factor production, biofilm formation, and oxidative stress resistance in PA.

The mechanisms of QS regulation have mostly been described qualitatively. However, the time course of HSL synthesis and the peak activity of this process are currently unclear, and a quantitative analysis of AZM-mediated inhibition of QS signals has yet to be performed. In this study, we applied HPLC-MS to assay both C4-HSL and 3-oxo-C12-HSL synthesis and the effect of the lasI and rhlI genes in both lasI and rhlI mutants and wild-type PAO1 following treatment with AZM. Additionally, the effect of AZM on the transcription of toxin, elastase and hemolysin genes was investigated to assess the transcriptional dysfunction due to reduced LasI. We also assessed the expression of the HAQ’s downstream effector genes (phnAB) after adding AZM.

Materials and Methods

Construction of P. aeruginosa PAO1 lasI and rhlI mutant strains. Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are shown in Table 1. PAO1 was a gift from Dr. Zhou Lin (Children’s Hospital of Chongqing Medical University). Strains were routinely grown in rich liquid or solid (15 g/L agar) Luria-Bertani medium (LB). The medium was supplemented with ampicillin (Amp, 60 μg/mL), chloramphenicol (C, 34 μg/mL) or gentamicin (GM, 30 μg/mL). Solid LB medium with 10% sucrose and without NaCl was used to select for plasmid excision from the chromosome in the gene allelic exchange experiments. Omission of NaCl from this medium was previously shown to improve sucrose counter-selection in Escherichia coli (E. coli).

DNA techniques. The Taq DNA polymerase, restriction endonucleases, DNA-modifying enzymes, Klenow fragment, DNA kination kit and DNA ligation kit used in this study were purchased from TaKaRa. The plasmid extraction kit and DNA gel purification kit were purchased from Guangdong Dongsheng Biotech Corporation. Antarctic phosphatase and T4 DNA ligase were purchased from NEB.

The PCR-amplified lasI gene upstream fragment A (lasI-P4: AATTCATAGTGCCGAGCTCCTGGCGTTTTCACTGACGGGGGAC, lasI-P2: CGGTGCGCTATCTGCGGTATAC) from PAO1 was ligated into pMD18T by TA cloning to construct pMD18B, and the PCR-amplified lasI gene downstream fragment (lasI-P1: TACAAGCTTTAAGAAGAAGCTGCGCATATGGC, lasI-P3: CTCAAGCTTCACCTCCCTACAATTAGAA, containing a HindIII site) was cloned into the HindIII site of pMD18B to create pMD18AB.

Two primers (rhlI-P1: TCCCGCCGGTTATATCACTGACGGGGGAC, rhlI-P3: TCCCGCCGGAGCATGACCAAGCCGTTGGTCG, containing a Smal site) were synthesized to amplify fragment C, and C was then digested with Smal and ligated into pUC19C. Fragment D was amplified with primers (rhlI-P4: AATTCATAGTGCCGAGCTCCTGGCGTTTTCACTGACGGGGGAC, rhlI-P2: GGGGTACCAAGAACGCCCAGAGGACGGTGAC, containing a KpnI site). The pUC19C vector and D were digested with KpnI, followed by ligation to obtain pUC19CD.

The pGSM-ΔlasI vector was derived from pMD18AB and pCVD442 in several steps. First, the GM sequence from stock solutions of AZM (10 mg/mL), different dilutions (5–200 mg/mL) were prepared. The minimal concentration of antibiotic resulting in no visible growth was taken as the MIC after 6 h of culture. For all further experiments, 1/64th of the MIC of AZM was used for all four strains.
Eleven cultures were simultaneously supplemented with AZM at a final concentration of 2 μg/mL. The cultures were harvested at 20 h according to their growth curves.

2.3 Determination of HSL concentrations

The HPLC-separated compounds were detected by electrospray ionization ion trap mass spectrometry (ESI-MS) using a Bruker Esquire-LC spectrometer (Bruker Daltonic, Germany) under positive-ion conditions. The analysis of two structurally distinct HSLs requires fast and selective analysis. The choice of + ESI mode was based upon the greater sensitivity for both HSL analytes; this was relatively straightforward because all the analytes demonstrated protonated \([M + H]^+\) species as the dominant pseudo-molecular ion.

Ten microliters of the sample was injected for HPLC-MS/MS analysis and introduced onto a ZORBAX SB-C18 column (4.6 × 100 mm, 2.1 μm). The HPLC system used 0.3% formic acid in water as mobile phase A, and mobile phase B was acetonitrile (HPLC grade). The working flow rate and gas were as follows: curtain, gas 1 and 2 were nitrogen, 60 L/h, respectively. The ion source potential was 5,500 Vm, and the source was held at 550 °C. Quantification was performed using Analyst 1.6 in Quantitate mode.

Table 1. Strains and plasmids used in this study.

| Strain or plasmid | Relevant genotype and/or phenotype | Source or reference |
|-------------------|-----------------------------------|---------------------|
| **PA strains**    |                                   |                     |
| PAO1              | Wild-type prototroph             | This lab            |
| PA-ΔJ231          | Δlas derivative of PAO1, Tcr      | ref. 1              |
| PA-ΔlasI          | ΔlasI derivative of PAO1          | This study          |
| PA-ΔrhlI          | ΔrhlI derivative of PAO1          | This study          |
| PA-ΔlasIrhlI      | Δlas derivative of PA-ΔrhlI       | This study          |
| **E. coli strains** |                                  |                     |
| DH5α              | F-, Φ80lacZΔM15, recA1, endA1, gvrA96, thi-1, hsdR17 (rK-·, M+) supE44, relA1, deoR, Δ(lacZYA-argF)/U169, Ap°, Km° | This lab            |
| SM10ApR           | thi-1, thr-1, leuB6, supE44, tnaA21, lacY1, recA::RP4-2::Tc::Mu mpir Km° | ref. 2              |
| **Plasmids**      |                                   |                     |
| pMD18T            | ori (ColE1) Ap'                   | TaKaRa              |
| pUC19             | ori (ColE1) Ap'                   | TaKaRa              |
| pCVD442           | 86K ori, mobRP4, bla, sacB        | ref. 3              |
| pDS132            | Derived from pCVD442, without IS1 sequences. bla gene replaced by the cat gene, Cβ | ref. 4              |
| pMD18-mob         | 2.2 kb mob fragment from pCVD442 TA cloned into pMD18T, Ap' | This study          |
| pMD18GM           | 0.83 kb aacCC1 fragment from pBBR-LuxAB cloned into pMD18T, GM° | This study          |
| pBBR-LuxAB        | luxAB, tra−, mob−, Gm°            | ref. 5              |
| pMD18AB           | pMD18 with 1.1 kb upstream of lasI and 1 kb downstream of it, Ap' | This study          |
| pUC19CD           | pUC19 with 1.1 kb upstream of rhlI and 0.95 kb downstream of it, Ap' | This study          |
| pG-ΔlasI          | 832 bp GM° fragment from plasmid LuxAB cloned into pMD18BA, GM°, Ap' | This study          |
| pGS-ΔlasI         | 1.8 kb sacB fragment from pCVD442 cloned into pG-ΔlasI, GM°, Ap' | This study          |
| pGSM-ΔlasI        | 2.2 kb mob fragment from pCVD442 cloned into pGSM-ΔlasI, GM°, Ap' | This study          |
| pG-ΔrhlI          | 832 bp GM° fragment from plasmid pBBR-LuxAB clones into pUC19CD, GM°, Ap' | This study          |
| pGS-ΔrhlI         | 1.8 kb sacB fragment from pCVD442 cloned into pG-ΔrhlI, GM°, Ap' | This study          |
| pGSM-ΔrhlI        | 2.2 kb mob fragment from pCVD442 cloned into pGSM-ΔrhlI, GM°, Ap' | This study          |

HSL analysis by HPLC-MS/MS. Chemicals and standards. The standards N-butyryl-L-HSL (C4-HSL) and N-(3-oxododecanoyl)-L-HSL (3-oxo-C12-HSL) were purchased from Sigma. Methanol, acetonitrile (HPLC grade), and formic acid (MS grade) were purchased from Fisher Scientific (Loughborough, UK). Acetic acid and ethyl acetate (HPLC grade), and formic acid (MS grade) were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China), and ultra-pure water (>18 MΩ/cm) was obtained from a Milli-Q water Elga Maxima water purification system (Merck KgaA, Germany).

Growth curve. PAO1, PA-ΔrhlII and PA-ΔlasIrhlII were grown in 3 mL of LB medium at 37°C with shaking. The cultures were simultaneously supplemented with AZM at a final concentration of 2 μg/mL. The cultures were collected after 0, 2, 4, 6, 8, 10, or 12 h of growth and assayed at OD600.

Quantification of HSLs by HPLC-MS/MS. After clarification by centrifugation, 1 mL of the cell-free supernatant was extracted three times with an equal volume of ethyl acetate, which was supplemented with 0.2 M acetic acid. The combined organic phase was dried in an N2 stream, redissolved in 1 mL of methanol and stored at −20°C to prepare other stock solutions. Twenty microliters of each of these samples was added to 980 µL of methanol for a 50–fold dilution immediately prior to HPLC-MS/MS analysis. All samples were filtered through 0.22 µm nylon disk filters before HPLC-MS/MS analysis.

The HPLC-separated compounds were detected by electrospray ionization ion trap mass spectrometry (ESI-MS) using a Bruker Esquire-LC spectrometer (Bruker Daltonic, Germany) under positive-ion conditions. The analysis of two structurally distinct HSLs requires fast and selective analysis. The choice of + ESI mode was based upon the greater sensitivity for both HSL analytes; this was relatively straightforward because all the analytes demonstrated protonated \([M + H]^+\) species as the dominant pseudo-molecular ion.

Ten microliters of the sample was injected for HPLC-MS/MS analysis and introduced onto a ZORBAX SB-C18 column (4.6 × 100 mm, 2.1 μm). The HPLC system used 0.3% formic acid in water as mobile phase A, and mobile phase B was 50% acetonitrile in methanol. The gradient profile was as follows: isocratic for 3 min with 10% B, then a further gradient with 90% B over 2 min, followed by 10% B for 5 min, at a flow rate of 0.5 mL/min. All mass spectrometry (MS) experiments were conducted on a 4000 QTRAP hybrid triple-quadrupole linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbolon source used in positive ion electrospray mode. A Windows XP (Microsoft, Redmond, WA, USA) workstation running Analyst (version 1.6) was used for data acquisition and processing.

The working flow rate and gas were as follows: curtain, gas 1 and 2 were nitrogen, 60 L/h, and 60 L/h, respectively. The ion source potential was 5,500 Vm, and the source was held at 550°C. Quantification was performed using Analyst 1.6 in Quantitate mode.

| Strain or plasmid | Relevant genotype and/or phenotype | Source or reference |
|-------------------|-----------------------------------|---------------------|
| PAO1              | Wild-type prototroph             | This lab            |
| PA-ΔJ231          | Δlas derivative of PAO1, Tcr      | ref. 1              |
| PA-ΔlasI          | ΔlasI derivative of PAO1          | This study          |
| PA-ΔrhlI          | ΔrhlI derivative of PAO1          | This study          |
| PA-ΔlasIrhlI      | Δlas derivative of PA-ΔrhlI       | This study          |
transcription was performed with the PrimeScript RT reagent kit (TaKaRa, Dalian, Liaoning, China) using 1 μg of the indicated cells was extracted using the total RNA isolation reagent (Promega, Madison, WI, USA). Reverse transcription of the cDNA, and qPCR reactions were performed on ViiA™ 7 Dx system (Applied Biosystems, Foster, CA, USA). SYBR Green qPCR Master Mixes (ThermoFisher Scientific, Waltham, MA, USA) were used for qPCR terms of growth at 0, 2, 4, 6, 8, 10 and 12 h. Based on the data, 0–8 h is the exponential phase, followed by the early stationary phase. Growth comparisons to PAO1 were performed using paired t-test analyses.

The results indicate that QS mutations, i.e., PAO1 deficient in lasI, had almost no influence on P. aeruginosa growth. Moreover, 2 μg/mL of AZM did not affect the growth of the strains compared with untreated controls (P > 0.05). The AZM MIC for P. aeruginosa PAO1 was determined to be 128 mg/mL, the same as described previously. Therefore, 2 μg/mL AZM (1/64th of the MIC) was added to all four strains in these experiments.

Six-point standard curves were generated for C4-HSL and 3-oxo-C12-HSL, and the standard mixture was reanalyzed after every sixth sample.

Recovery Test. The recovery of analytes from the medium was determined by adding a standard mixture of C4-HSL and 3-oxoC12-HSL at low (10 μg/mL) and high (100 μg/mL) concentrations to 3 mL of LB culture media. Each sample was extracted three times with an equal volume of ethyl acetate. The recovery was calculated by comparing the response ratios of spiked extracted medium with a standard mixture prior to extraction.

Precision was calculated from the relative standard deviation (RSD) of the replicates (n = 5), and accuracy was calculated by direct comparison of mean measured levels of spiked analytes with expected concentrations for unextracted standards.

Real-time quantitative RT-PCR. For real-time quantitative RT-PCR (qPCR) analyses, total RNA from the indicated cells was extracted using the total RNA isolation reagent (Promega, Madison, WI, USA). Reverse transcription was performed with the PrimeScript RT reagent kit (TaKaRa, Dalian, Liaoning, China) using 1 μg of total RNA. SYBR Green qPCR Master Mixes (ThermoFisher Scientific, Waltham, MA, USA) were used for qPCR detection of the cDNA, and qPCR reactions were performed on ViiA™ 7 Dx system (Applied Biosystems, Foster, CA, USA). The level of target genes was normalized to the expression of an internal control gene (rpoD), which yielded a 2−ΔΔCT value. Sequences for primers are listed in Appendix 1.

Statistical analysis. All experiments were carried out in triplicate to validate the reproducibility of the experiments. The results were analyzed statistically using repeated measures analysis of variance with SAS 9.2 software to calculate P values. P < 0.05 was taken as statistically significant.

Results

The AZM MIC for P. aeruginosa PAO1 was determined to be 128 mg/mL, the same as described previously. Therefore, 2 μg/mL AZM (1/64th of the MIC) was added to all four strains in these experiments.

HPLC-MS/MS Analysis. Using the experimental conditions described in the Materials and Methods section, the analogues of C4-HSL and 3-oxo-C12-HSL were separated. Active fractions were located in two single peaks (C4-HSL and 3-oxo-C12-HSL) with retention times of 5.09 min and 7.0 min, respectively. Two compounds with these retention times were obtained from P. aeruginosa PAO1 supernatants but were absent from PA-ΔlasIrhlI supernatants.

The MS parameters (precursor and product ions used for MRM transitions and corresponding optimized voltages) are listed in Table 2, which shows that common product ion fragments could be used for individual families of analytes, i.e., m/z 102 for HSLs. Our detection limits were 0.05 ng/mL and 50 ng/mL.

For the low concentration samples, the yields of C4-HSL and 3-oxo-C12-HSL were approximately 64.4% and 97.6%, respectively, while for the high concentration samples, the yields of C4-HSL and 3-oxo-C12-HSL were approximately 86.8% and 89.1%, respectively (Table 3).

Precision was calculated from the relative standard deviation (RSD) of the replicates (n = 5), and accuracy was calculated by direct comparison of mean measured levels of spiked analytes with expected concentrations for unextracted standards.

Table 2. Selected precursor and product ion m/z values, retention times and mass spectrometer parameters used for HSL analytes.

| Analytes       | Retention time (min) | Precursor ion (m/z) | Daughter ion (m/z) | Cone (v) | Collision Energy (v) |
|----------------|----------------------|---------------------|--------------------|----------|----------------------|
| C4-HSL         | 5.09                 | 172.1               | 102.1, 70.1        | 19       | 15                   |
| 3-oxo-C12-HSL  | 7.0                  | 298.2               | 102.1, 197.2       | 21       | 20                   |

Effect of mutation and AZM on P. aeruginosa growth. As shown in Fig. 1, bacterial growth curves indicated that there were no differences between PAO1 and the mutants deficient in lasI, rhlI and lasI/rhlI in terms of growth at 0, 2, 4, 6, 8, 10 and 12 h. Based on the data, 0–8 h is the exponential phase, followed by the early stationary phase. Growth comparisons to PAO1 were performed using paired t-test analyses (P > 0.05). The results indicate that QS mutations, i.e., PAO1 deficient in lasI, rhlI and lasI/rhlI, had almost no influence on P. aeruginosa growth. Moreover, 2 μg/mL of AZM did not affect the growth of the strains compared with untreated controls (P > 0.05) (Fig. 1).

Effect of lasI and rhlI mutations on QS signals. No C4-HSLs were detected in P. aeruginosa PAO1 in the early growth phase, which was between 2–4 h (Fig. 2). However, 3-oxo-C12-HSL was detected at 6 h, during
the exponential phase of growth, and secretion was maintained until 12 h; the concentration ranged from 89.6 ng/mL to 172.8 ng/mL (Fig. 3). A comparison of the different time points for the secretion of 3-oxo-C12-HSL showed no statistically significant differences, \( P > 0.05 \). The secretion of C4-HSL increased with time, with a maximum concentration of 512.2 ng/mL, which was stable from 10 h to 12 h, i.e., in the stationary phase. (A comparison of the different time points for the secretion of C4-HSL showed statistically significant differences, \( P < 0.05 \).)

For PA-ΔrhlI, C4-HSL was not detected, but 3-oxo-C12-HSL production was similar to that of PAO1; there was no significant difference between the two strains (\( P > 0.05 \)). For PA-ΔlasI, 3-oxo-C12-HSL was not detected, and C4-HSL levels were below the detection limits. For PA-ΔlasIrhlI, there was no C4-HSL or 3-oxo-C12-HSL secretion. In the QS system, the lasI gene product directs the formation of the diffusible extracellular signal 3-oxo-C12-HSL, and the rhlI product catalyzes the synthesis of C4-HSL 35; therefore, the las system positively regulates the expression of rhlI36.

**Effect of AZM on QS signals.** At 6 h, 8 h, and 10 h, C4-HSL levels in the PAO1 strain were 13.1 ng/mL, 183.2 ng/mL, and 308.4 ng/mL, respectively. With addition of AZM, the levels in the PAO1 strain decreased to 3.2 ng/mL, 119.7 ng/mL, and 197.6 ng/mL, respectively. However, at 12 h, C4-HSL increased from 512.2 ng/mL in the untreated group to 530.4 ng/mL in the AZM group (Fig. 2). With the addition of 2 μg/mL AZM, the HSL levels were noticeably lower than in the untreated controls, although there was no significant difference between the two groups (\( P > 0.05 \)).

In the presence of AZM, a significant reduction in 3-oxo-C12-HSL was observed for PAO1. At 6 h, 8 h, 10 h, and 12 h, 3-oxo-C12-HSL levels were 156.2 ng/mL, 172.8 ng/mL, 89.6 ng/mL, and 140.5 ng/mL, respectively (\( P < 0.05 \)) in the untreated group. In PAO1 treated with AZM, 3-oxo-C12-HSL levels were 58.3 ng/mL, 35.5 ng/mL, 17.6 ng/mL, and 49.4 ng/mL, respectively. The mutant strain PA-ΔrhlI also showed a reduction in 3-oxo-C12-HSL levels in the presence of AZM (\( P < 0.05 \)) (Fig. 3).

**Effect of AZM on QS genes.** Real-time polymerase chain reaction (RT-PCR) was used to assess representative genes from the AHL and HAQ signaling systems. The experiment was conducted after 6 h of growth with or without 2 μg/mL AZM (Fig. 4). Selected genes regulated by lasR (aprX, toxA and lasA) and rhlR (rhlA and rhlB), genes of the HAQ system (phnA and phnB) and a repressor of lasI and rhlI, qscR, were assayed for expression in PAO1, PA-ΔrhlI, PA-ΔlasI and PA-ΔlasIrhlI. The expression of the genes is reported as a ratio of the target to a reference (rpoD).

The reduction in lasI expression in PAO1 was observed to correlate with the protein expression; 3-oxo-C12-HSL was reduced by 65% with AZM, matching the 79% reduction in protein levels under the same conditions. In the PAO1 strain, although lasI (3-oxo-C12-HSL) levels were reduced, as assessed by HPLC-MS/MS analysis, the expression of other QS genes was induced after addition of AZM. Additionally, the expression of other virulence genes, except toxA, showed a stronger repression in PAO1 compared to the mutants when AZM was not added. In the PA-ΔrhlI, PA-ΔlasI and PA-ΔlasIrhlI strains, the expression of virulence genes was induced with addition of AZM. Among the mutants, repression of virulence genes of the AHL system was stronger in PA-ΔlasI than in PA-ΔrhlI. Additionally, the expression of phnA and phnB from the HAQ system was greater in PA-ΔrhlI (and PAO1) compared to PA-ΔlasI.

### Table 3. The recovery test of HSLs from LB media.

| Compound          | Spiked level (μg/L) | Mean recovery (%) | RSD (%) |
|-------------------|--------------------|-------------------|---------|
| C4-HSL            | 10                 | 64.4              | 3       |
|                   | 100                | 86.8              | 10      |
| 3-oxo-C12-HSL     | 10                 | 97.6              | 11      |
|                   | 100                | 89.1              | 8       |

**Figure 1. Growth curves of PA-ΔlasI, PA-ΔrhlI, PA-ΔlasIrhlI and their parent strain PAO1.** The relationship of OD\(_{600}\) to viable count was equivalent for all strains examined. Each point indicates the mean of the OD\(_{600}\) values. AZM indicates this strain was treated with 2 μg/mL of azithromycin.
Discussion

In this study, we constructed lasI and rhlI single and double mutant strains, which had a complete deletion of the coding domain. A selective and rapid method for the simultaneous analysis of the two main HSLs in PA was also developed. Levels of 3-oxo-C12-HSL in PAO1 and PA-ΔrhlI showed a significant decrease following addition of AZM, while the accumulation of C4-HSL was not greatly influenced by AZM in the PAO1 strain. At the gene level, addition of AZM was observed to increase the expression of virulence genes in all backgrounds.

The las and rhl systems are hierarchically connected and regulate the timing and production of multiple virulence factors. The las system positively regulates the expression of both rhlR and rhlI. When a strain is deficient in lasI, rhlI gene expression is repressed. Because of this, no 3-oxo-C12-HSL was detected for PA-ΔlasI, and
C4-HSL was below the detection limit. However, in PA-ΔrhlI, there was no C4-HSL detected, but 3-oxo-C12-HSL was produced normally. The lasI/rhlI gene mutation and AZM had no effect on PA growth.

In the early growth phase (2–4 h) of the PAO1 strain, there were no HSLs detected; 3-oxo-C12-HSL was first detected at 6 h. 3-Oxo-C12-HSL was first secreted in the exponential growth phase and was maintained until the early stationary phase. These results are in agreement with a previous report on PAO1, in which 3-oxo-C12-HSL at a concentration of 0.8 μmol/L was detected at 70.5 h. Moreover, in this study, our detection limit was 0.05 ng/mL (0.17 nmol/L), more sensitive than previous reports of 0.2 μmol/L. Previous studies demonstrated that the expression of genes of the las system (lasR and lasI), which is affected by 3-oxo-C12-HSL, remains fairly constant throughout cultivation. In contrast, the secretion of C4-HSL increased over time and reached a maximum at 12 h. In another study, the secretion of C4-HSL reached the maximum concentration at 42.5 h.

At 2 μg/mL AZM, a concentration far below the MIC, the secretion of HSLs was inhibited through the blockade of QS. We observed that 2 μg/mL of AZM could decrease 3-oxo-C12-HSL secretion by nearly 79% and led to a noticeable reduction throughout the cultivation. The addition of 2 μg/mL AZM had a different effect on C4-HSL secretion, as it decreased C4-HSL secretion by 76% at t = 6 h and by 35% at t = 8–10 h, but secretion was not repressed at t = 12 h. Based on these results, we conclude that 2 μg/mL of AZM reduced 3-oxo-C12-HSL secretion during cultivation, but only repressed C4-HSL secretion in the early stages. In this study, both an appropriate model system with a known time course involving autoinducers as well as AZM inhibition of QS signals in PA were clearly demonstrated. The resistance mechanisms of PA are very complex, and HGT has been shown to be an important mechanism. Conjugation, one of the most important mechanisms of HGT in the environment, involves the direct transfer of genetic material from cell to cell, usually in the form of plasmids or transposons. It has been reported that HSLs have a positive effect on the conjugation of Ti plasmid transfer in Agrobacterium.

Figure 4. Comparison of gene expression with and without added 2 μg/mL azithromycin (AZM) in PAO1 (a) PA-ΔlasI (b) PA-ΔrhlI (c) and PA-ΔlasIrhlI (d). Series 1 (blue) denotes the untreated group and Series 2 (red) indicates the AZM group.
tumefaciens\(^{42}\). The basic model described here is intended to serve as a platform for further investigation into the effect of HSLs on conjugation in \(PA\) and inhibition of conjugation in \(PA\) by AZM.

Addition of AZM results in reduced HSLs at the protein level, while at gene level, AZM derepresses virulence genes. Transcriptional regulation of representative genes regulated by lasR and rhlR agreed broadly with previous findings. Stronger repression was observed in the wild-type strain compared to the mutants, and among the mutants, QS and virulence gene expression were observed only in the \(PA-\Delta rhlI\) strain. Our results validated the hierarchy of las activation on the rhl system\(^{47}\). Without addition of AZM, all the virulence genes except toxA were repressed, with a stronger effect observed in the wild-type than in the mutants. This could be due to the phase in which the gene expression study was performed. toxA has been shown to be produced mainly during the early exponential phase of growth when grown aerobically\(^{48}\). As observed by Linares et al.\(^{13}\), sub-inhibitory concentrations of antibiotics could be the reason for the increased expression of QS and virulence genes (Fig. 4a). Another explanation for this could be that addition of AZM and the subsequent reduction in lasI expression (relative mRNA levels were 99.1 and 34.8, respectively, with AZM and without AZM) can be an inactivating signal, which has been previously shown to enhance virulence\(^{49,32}\). We hypothesize a novel regulatory circuit where lasI acts as an initial sensor of the optimal cell density required for activating virulence genes, while activated rhlI acts as a repressor of all the other QS systems (Fig. 5). In support of this idea, in the current study we observed that phnAB expression is reduced in PA-\(\Delta lasI\) but not in PA-\(\Delta rhlI\) (and PAO1). It has been shown previously that the las and rhl systems regulate quinolone signals, where lasI provides information to the cells about the QS activation, and rhlI suppresses the HAQ system. One of the regulators of las and rhl system is the quorum-sensing control repressor (qscR) protein, which functions as a las-rhl antagonist, and lasI is known to positively influence qscR. In the present study, low levels of qscR were observed in the absence of lasI and rhlI. Expression of qscR was 6 times higher in the wild-type compared to the mutant strains (Fig. 4), suggesting a feedback mechanism dependent on the concentration of lasI and rhlI via the regulation of qscR. The relationship between QS and virulence is complex. It is known that QS systems activate virulence and that AZM interferes with the QS activation by preventing virulence. Because AZM targets ribosomes, the effect of transcriptional regulation on QS can be inferred as a consequence of the reduction of the autoinducer.

The findings here provide basic data about the time course of AZM and the AZM effect on the synthesis of HSL. These results can further our understanding of antibiotic resistance and the interrelationship between QS and virulence. Here, we propose that QS and virulence can subsist individually, and regulated by the growth phase and the environment.
References

1. Fuqua, C., Winans, S. C. & Greenberg, E. P. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu. Rev. Microbiol.* **50**, 727–751 (1996).
2. Bjarngård, T. et al. *In vitro* screens for quorum sensing inhibitors and *in vivo* confirmation of their effect. *Nat Protoc* **5**, 282–293 (2010).
3. Pearson, J. et al. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *P Natl Acad Sci USA* **91**, 197–201 (1994).
4. Gambello, M. J. & Iglewski, B. H. Cloning and characterization of the *Pseudomonas aeruginosa* lasR gene, a transcriptional activator of elastase expression. *J Bacteriol* **173**, 3000–3009 (1991).
5. Fuqua, C., Parsek, M. R. & Greenberg, E. P. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* **35**, 439–468 (2001).
6. Glessner, A., Smith, R. S., Iglewski, B. H. & Robinson, J. B. Roles of *Pseudomonas aeruginosa* las and rhl Quorum-Sensing Systems in Control of Twitching Motility. *J Bacteriol* **181**, 1623–1629 (1999).
7. Müller, M. M., Hörmann, B., Syldatk, C. & Hausmann, R. *Pseudomonas aeruginosa* PAO1 as a model for rhamnolipid production in biofilm systems. *Appl Microbiol Biol* **87**, 167–174 (2010).
8. Wagner, V. E., Bushnell, D., Passador, L., Brooks, a. I. & Iglewski, B. H. Microarray Analysis of *Pseudomonas aeruginosa* Quorum-Sensing Regulons: Effects of Growth Phase and Environment. *J Bacteriol* **185**, 2080–2095 (2003).
9. Pearson, J. P., Pesci, E. C. & Iglewski, B. H. Roles of *Pseudomonas aeruginosa* las and rhl Quorum-Sensing Systems in Control of Elastase and Rhamnolipid Biosynthesis. *Genes** **179**, 5756–5767 (1997).
10. Jimenez, P. N. et al. The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. *MMBR* **76**, 46–65 (2012).
11. Xiao, G. et al. MrVR, a key *Pseudomonas aeruginosa* pathogenicity LTTR-class regulatory protein, has dual ligands. *Mol Microbiol* **62**, 1689–1699 (2006).
12. Naala, Y. et al. Quorum-Sensing Antagonistic Activities of Azithromycin in *Pseudomonas aeruginosa* PAO1: a Global Approach. *Antimicrob Agents Chemother* **50**, 1680–1688 (2006).
13. Linares, J. F., Gustafsson, L., Baquero, F. & Martinez, J. L. Antibiotics as intermicrobial signaling agents instead of weapons. *P Natl Acad Sci USA* **103**, 19484–19489 (2006).
14. Haas, N. Diffuse panbronchiolitis and cystic fibrosis: East meets West. *Thorax* **49**, 531–532 (1994).
15. Jaffe, A., Francis, J., Rosenthal, M. & Bush, A. Long-term azithromycin may improve lung function in children with cystic fibrosis. *The Lancet* **351**, 420 (1998).
16. Ratjen, F. et al. Effect of azithromycin on systemic markers of inflammation in patients with cystic fibrosis uninfected with *Pseudomonas aeruginosa*. *Chest Journal* **142**, 1259–1266 (2012).
17. Carr, R. R. & Nahata, M. C. Azithromycin for improving pulmonary function in cystic fibrosis. *Ann Pharmacother* **38**, 1520–1524 (2004).
18. Gillis, R. J. & Iglewski, B. H. Azithromycin retards *Pseudomonas aeruginosa* biofilm formation. *J Clin Microbiol* **42**, 5842–5845 (2004).
19. Henkel, M. et al. Kinetic modeling of the time course of N-butyryl-homoserine lactone concentration during batch cultivations of *Pseudomonas aeruginosa* PAO1. *Appl Microbiol Biol* **97**, 7607–7616 (2013).
20. Ichimiya, T. et al. The influence of azithromycin on the biofilm formation of *Pseudomonas aeruginosa* *in vitro*. *Chemotherapy* **42**, 186–191 (1996).
21. Imperi, F. et al. Inhibition of quorum sensing in *Pseudomonas aeruginosa* by azithromycin and its effectiveness in a murine model of infection. *J Med Microbiol* **60**, 300–306 (2011).
22. Tateda, K. et al. Azithromycin inhibits quorum sensing in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **45**, 1930–1933 (2001).
23. Philippe, N., Alcaraz, J. P., Coursange, E., Geiselmann, J. & Schneider, D. Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. *Plasmid* **51**, 246–255 (2004).
24. Wayne, P. et al. Clinical and Laboratory Standards Institute: Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-. *CLSI* document M27-A3. *CLSI 2008a* (2008).
25. Nalca, Y. et al. Kinetic modeling of the time course of N-butyryl-homoserine lactone concentration during batch cultivations of *Pseudomonas aeruginosa* PAO1. *Appl Microbiol Biot* **78**, 186–192 (2010).
26. Ichimiya, T. et al. The influence of azithromycin on the biofilm formation of *Pseudomonas aeruginosa* *in vitro*. *Chemotherapy* **42**, 186–191 (1996).
27. Imperi, F., Leoni, L. & Visca, P. Antivirulence activity of azithromycin in *Pseudomonas aeruginosa*. *Antivirulence activity of azithromycin in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **49**, 1377–1380 (2005).
28. Bala, A., Kumar, R. & Harjai, K. Inhibition of quorum sensing in *Pseudomonas aeruginosa* by azithromycin and its effectiveness in urinary tract infections. *J Med Microbiol* **60**, 300–306 (2011).
29. Tateda, K. et al. Azithromycin inhibits quorum sensing in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **45**, 1930–1933 (2001).
30. Way, N. Clinical and Laboratory Standards Institute: Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-. *CLSI* document M27-A3. *CLSI 2008a* (2008).
31. Naala, Y. et al. Quorum-sensing antagonistic activities of azithromycin in *Pseudomonas aeruginosa* PAO1: a global approach. *Antimicrob Agents Chemother* **50**, 1680–1688 (2006).
32. Charlon, T. S. et al. A novel and sensitive method for the quantification of N-3-oxoacetyl homoserine lactones using gas chromatography–mass spectrometry: application to a model bacterial biofilm. *Environ Microbiol* **2**, 530–541 (2000).
33. Torri, C. A. et al. Simultaneous quantitative profiling of N-acetyl-L-homoserine lactone and 2-alkyl-4 (1H)-quinoline families of quorum-sensing signaling molecules using LC-MS/MS. *Anal Bioanal Chem* **399**, 839–850 (2011).
34. Chen, F., Chen, C. C., Riadi, L. & Ju, L. K. Modeling rhl Quorum-Sensing Regulation on Rhamnolipid Production by *Pseudomonas aeruginosa*. *Biotechnol Prog* **20**, 1325–1331 (2004).
35. Viretta, A. U. & Fussenegger, M. Modeling the Quorum Sensing Regulatory Network of Human-Pathogenic *Pseudomonas aeruginosa*. *Biotechnol Prog* **20**, 670–678 (2004).
36. De Kerval, T. R. Quorum sensing in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol* **11**, 279–288 (2009).
37. Wagner, V. E., Bushnell, D., Passador, L., Brooks, a. I. & Iglewski, B. H. Microarray Analysis of *Pseudomonas aeruginosa* Quorum-Sensing Regulons: Effects of Growth Phase and Environment. *J Bacteriol* **185**, 2080–2085 (2003).
38. Schmidberger, A., Henkel, M., Hausmann, R. & Schwartz, T. Expression of genes involved in rhamnolipid synthesis in *Pseudomonas aeruginosa* PAO1 in a biofilm cultivation. *Appl Microbiol Biot* **97**, 5779–5791 (2013).
39. Henkel, M. et al. Kinetic modeling of the time course of N-butyryl-homoserine lactone concentration during batch cultivations of *Pseudomonas aeruginosa* PAO1. *Appl Microbiol Biot* **97**, 7607–7616 (2013).
40. Wolak, K. I. Horizontal DNA transfer between bacteria in the environment. *Acta Microbiologica Polonica* **52**, 233–243 (2003).
41. Piper, K. R., von Bodman, S. B. & Farrand, S. K. Quorum-sensing factors of Agrobacterium tumefaciens regulates Ti plasmid transfer by autoinduction. *Nature* **362**, 448–450 (1993).
43. Sio, C. F. et al. Quorum quenching by an N-acyl-homoserine lactone acylase from Pseudomonas aeruginosa PAO1. *Infect Immun* **74**, 1673–1682 (2006).
44. Sacha, P. et al. Metallo-beta-lactamases of Pseudomonas aeruginosa—a novel mechanism resistance to beta-lactam antibiotics. *Folia Histochemica et cytobiologica* **46**, 137–136 (2008).
45. Warnes, S. L., Highmore, C. J. & Keevil, C. W. Horizontal transfer of antibiotic resistance genes on abiotic touch surfaces: implications for public health. *MBio* **3**, e00489–12 (2012).
46. Qian, F. [Conjugation of Plasmodium falciparum Pf625 to Pseudomonas aeruginosa ExoProtein A with different chemical linkers]. Zhongguo ji sheng chong xue yu ji sheng chong bing za zhi = Chinese journal of parasitology & parasitic diseases **29**, 254–257 (2011).
47. Duan, K. & Surette, M. G. Environmental regulation of Pseudomonas aeruginosa PAO1 las and Rhl quorum-sensing systems. *J Bacteriol.* **189**, 4827–4836 (2007).
48. Frank, D. W. & Iglewski, B. H. Kinetics of toxA and regA mRNA accumulation in Pseudomonas aeruginosa. *J Bacteriol* **170**, 4477–4483 (1988).
49. Schuster, M., Lostroh, C. P., Ogi, T. & Greenberg, E. P. Identification, Timing, and Signal Specificity of Pseudomonas aeruginosa Quorum-Controlled Genes: a Transcriptome Analysis. *J Bacteriol* **185**, 2066–2079 (2003).

**Acknowledgements**

The authors thank B. Iglewski (Professor of Microbiology and Immunology and Director of International Programs at the University of Rochester Medical Center) for the gift *P. aeruginosa* PAO-JP1 as the control strain, Professors Kan Biao and Huang Zheng for the gift of plasmids, and Dr. H. Dechun for the mass spectrometric analysis and identification of C4-HSL and 3-oxo-C12-HSL. Project supported by the National Nature Science Foundation of China. (Grant No. 81071397, 81271909) and Natural Science Foundation of Guangdong Province, China. (Grant No. S2013010012970).

**Author Contributions**

J.Z. data collection, data management, statistics, wrote first draft and reviewed final draft. N.Z. data collection, data management, statistics, reviewed first draft and reviewed final draft. B.H. study design, statistics, data management, review first draft, and wrote final draft. R.C. data collection, statistics and final draft. B.W. data collection and reviewed final draft. S.-M.E. data collection and reviewed final draft. C.F. reagents and reviewed final draft. C.C. study design, scientific and statistics advisory, wrote first draft, reviewed final draft, and research funding.

**Additional Information**

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zeng, J. et al. Mechanism of azithromycin inhibition of HSL synthesis in *Pseudomonas aeruginosa*. *Sci. Rep.* **6**, 24299; doi: 10.1038/srep24299 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/