Quorum sensing plays an important role in heteroresistance regulation of Pseudomonas aeruginosa

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Abstract

Background: The prevalence and genetic mechanism of antibiotic heteroresistance attracted more and more research attentions. However, its non-genenic mechanism is still largely unknown. As the most important mechanism that enable individual bacteria to coordinate their behavior in populations, the influence of Quorum sensing (QS) on heteroresistance was disclosed here.

Results: We investigated the prevalence of heteroresistance in 170 clinical isolates of *P. aeruginosa* against 7 different antibiotics. The population analysis profiles showed that *P. aeruginosa* was frequently heteroresistant to meropenem (MEM), amikacin (AMK), ciprofloxacin (CIP) and Ceftazidime (CAZ), which was related to down-regulated expression of QS genes *lasI* and *rhlI*. Further loss-of-function studies revealed that deficiency of *lasI/ rhlI* facilitated heteroresistance of *P. aeruginosa* to all the above four antibiotics, which was abrogated by overexpression of *lasI/ rhlI* or treatment with 3-oxo-C12-HSL/C4-HSL. In addition, we found that the expression of *oprD* and efflux-associated genes was regulated by *lasI/ rhlI*, while downregulation of *oprD* and upregulation of efflux-associated genes was observed in heteroresistant subpopulation.

Conclusions: These data suggest that QS plays a crucial role in heteroresistance regulation, partially mediated by *oprD* and efflux-associated genes. Our findings highlight the new function of QS and provide new insight into the mechanism of heteroresistance in the context of non-genetic factor.

Introduction

Heteroresistance (HR) describes a phenotype in which a seemingly isogenic bacterial isolate exhibit a range of susceptibilities to a particular antibiotic[1]. Emerging evidence suggests that heteroresistance is very common in clinical pathogens such as *Klebsiella pneumonia*[2–4] *Streptococcus pneumonia*[5], *Mycobacterium tuberculosis*[6], *Staphylococcus aureus*[7], *Acinetobacter baumannii*[8, 9], *Pseudomonas aeruginosa*[10, 11] and *Helicobacter pylori*[12]. Heteroresistant subpopulation of resistant cells can rapidly replicate in the presence of a given antibiotic, whereas the majority population of susceptible cells is killed, ultimately leading to failure of antibiotic treatment and even inducing bacterial resistance[13].

Currently, the research on mechanism of bacterial HR mainly focuses on gene mutations. Amplification of chromatin fragments in gram-negative bacteria was reported be a common mechanism, balancing resistance and fitness costs in the presence of antibiotics by spontaneous tandem amplifications rather than by heritable mutations[14]. Besides, under the pressure of exposure to antibiotics, bacteria can generate subpopulations at a high fitness cost through point mutations, and compensatory secondary mutations in the absence of antibiotic, thus reducing the fitness cost and possibly restoring the resistance to the native populations[15, 16]. It should be pointed out that HR is generally believed to be a strategy for bacteria to adapt to the environment under the antibiotic pressure before resistance is acquired. During the short-term evolutionary course, whether a non-genetic mechanism that may arise less fitness cost and lead to the generation of heteroresistance is still unknown. Considering that HR was

a population biological behavior, quorum sensing (QS), the most mechanism for bacteria to coordinate the activities of individual cells, attracts our attentions.

Pseudomonas aeruginosa (*P. aeruginosa*), one of the main causes of nosocomial infections, with extremely high morbidity and mortality in cystic fibrosis patients and immunocompromised individuals[17, 18], is a representative bacterium for studying QS. *P. aeruginosa* possesses two main QS systems (*las* and *rhl*) which are responsible for the synthesis of the N-acyl homoserine lactone (AHL) signal molecules, N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL), respectively. The 3-oxo-C12-HSL and C4-HSL bind to and activate their cognate transcription factors LasR or RhlR respectively, inducing biofilm formation and expression of various virulence factors[19–21]. Unfortunately, *P. aeruginosa* is increasingly resistance to multiple antibiotics due to the abuse of antibiotics such as aminoglycosides, quinolones and β-lactams[22]. In recent years, HR among *P.aeruginosa* to carbapenems[23, 24], cefepime[25], colistin[10] has been reported, which may be correlated with upregulation of efflux-associated genes and reduced expression of the porin-encoding gene *oprD* in resistant subpopulation[26]. However, it is still largely unknown for the prevalence and underlying mechanism. Therefore, an extensive investigation of HR mechanism will provide a new insight to solve the thorny problems in clinical treatment and bacterial resistance of *P. aeruginosa*.

In this study, we focused on the role of QS system in HR of *P. aeruginosa*. We characterized the prevalence of HR in clinical *P. aeruginosa* isolates to several antibiotics and showed that the down-regulation of LasI/RhlI was associated with increased HR. Restoration of LasI/RhlI significantly suppressed HR in *P. aeruginosa*. We further provided evidence that efflux-associated genes and *oprD* were involved LasI/RhlI-inhibited HR. Our findings may provide new insight into the regulatory networks of heteroresistance, and offer novel potential targets for preventing antibiotic resistance.

Results

Prevalence of HR in clinical P. aeruginosa isolates

To investigate the prevalence of HR against different antibiotics covering major classes in clinical *P. aeruginosa* isolates, 170 strains were random selected to perform Population Analysis Profiles (PAPs). Base on the definitions of HR according to Hervé Nicoloff and Karin Hjort[14] as the presence of a subpopulation of cells capable of growing at concentrations of the antibiotic at least eight-fold higher than the highest concentration that does not affect the replication of the dominant population, and resistant subpopulations at frequencies of 1×10^{-7} or higher, 113 isolates (113/170, 66.5%) were heteroresistant to amikacin (AMK, Fig. 1A), 99 isolates (99/170, 58.2%) were heteroresistant to meropenem (MEM, Fig. 1B), 87 isolates (87/170, 51.2%) were observed heteroresistance for ciprofloxacin (CIP, Fig. 1C), and 69 isolates (68, 34.1%) were heteroresistant to ceftazidime (CAZ, Fig. 1D). Notably, 55 isolates (55/170, 32.4%) display a cross-HR to all four antibiotics above, 9 isolates (9/170, 5.3%) were resistant to multiple antibiotics had mentioned. In summary, a high frequency of HR in clinical *P.*

aeruginosa isolates was observed for AMK, MEM, CIP and CAZ (Table 1). However, we did not observe HR with FOS, PB, or GM, even though HR to PB has been described in previous studies with clinical isolates of *P. aeruginosa*. In addition, we also assayed the heteroresistance of PAO1, the most commonly used strain for research in laboratory. The result showed that PAO1 were also heteroresistant to MEM, AMK, CIP and CAZ (Supplementary Fig. 1).

Table 1
Heteroresistance (HR) of clinical P. aeruginosa isolates(n = 170)

Antibiotics	Resistance	Resistance Ratio(%)	HR	HR Ratio(%)
AMK	37	21.8	113	66.5
MEM	50	29.4	99	58.2
CIP	44	25.9	87	51.2
CAZ	15	8.8	58	34.1
FOS	10	5.8	0	0
PB	15	8.8	0	0
GM	12	7.0	0	0

The expression of LasI and RhlI is negatively correlated with HR of P. aeruginosa

To investigate the biological significance of QS in heteroresistant regulation, we first analyzed the correlation between LasI/RhlI levels and heteroresistant features. The 55 isolates that displayed to all four antibiotics were defined as heteroresistant positive, while 9 isolates that showed non-heteroresistant to any of these antibiotics were defined as heteroresistant negative. The expression of LasI and RhlI in heteroresistant positive group was obviously lower than that of negative group (Fig. 2A, B), indicated that QS may be negatively correlated with heteroresistant in *P. aeruginosa*.

Typically, after serial passages in antibiotic-free medium, some highly resistant subpopulations revert to the heterogeneous resistance phenotype displayed by the original population[14]. Thus the phenotypic stability, as well as the expression of LasI/RhlI during this course was tested in PAO1. As shown in Fig. 2C, PAP analysis was performed on the original PAO1 (ORI), resistant subpopulations (Enrich) after 40 generations with or without exposure to AMK or CIP (AB-sub40, Sub40 respectively) simultaneously. The result displayed that ORI and Sub40 were heteroresistant positive, while AB-sub40 became to resistant but heteroresistant negative strain (Fig. 2D and Supplementary Fig. 2A), indicated that resistance of subpopulations (Enrich) would be enhanced under the long-term antibiotics pressure, while almost restored to the level of native populations after 40 generations without exposure to antibiotics. More importantly, we found that in AB-sub40 group, the expression of LasI and its downstream gene *lasA*, as well as RhlI and its downstream gene *rhlA*, was higher than that of ORI and Sub40 group (Fig. 2E and

Supplementary Fig. 2B). This result further suggested that QS may be negatively correlated with heteroresistant in *P. aeruginosa*.

LasI and RhlI Inhibit HR of P. aeruginosa

In order to explore the effect of QS on *P. aeruginosa* heteroresistance, we next performed PAPs with PAO1 and *lasI* or *rhlI* single gene-deficient strains, *PAO1*Δ*lasI* and *PAO1*Δ*rhlI*. Compared with wild-type, subpopulations of *PAO1*Δ*lasI* and *PAO1*Δ*rhlI* showed an enhanced ability to grow at high concentration of AMK (Fig. 3A), indicated that deficiency of *lasI* or *rhlI* significantly promoted heteroresistant of *P. aeruginosa* against AMK. Similar phenomenon was also observed for MEM, CIP and CAZ (Supplementary Fig. 3).

To confirm the results from the loss-of-function study, gain-of-function analysis was performed using *lasI*-overexpression plasmid or extracellular signal N-(3-oxododecanoyl)-L-HSL (3-oxo-C12-HSL) for *PAO1*Δ*lasI, rhlI*-overexpression plasmid or N-butanoyl-L-homoserine lactone (C4-HSL) for *PAO1*Δ*rhlI*, respectively. For the *PAO1*Δ*lasI* strain, compared with control group (Δ*lasI*-pROp200 or Δ*lasI*-DMSO), transformation of *lasI*-overexpression plasmid or treatment with 3-oxo-C12-HSL caused an obvious decrease of subpopulations at 8 and 16 mg/L of AMK (Fig. 3B, C). For the *PAO1*Δ*rhlI* strain, overexpression of RhlI or treatment with C4-HSL also impaired the viability of subpopulations for AMK (Fig. 3D, E).

Collectively, these data implicate that QS system may negatively regulate heteroresistant of *P. aeruginosa*.

Deficiency of lasI or rhlI favors the survival of PAO1 under the high concentration of antibiotics

We further tested and verified the advantage of relative growth rates for PAO1Δ*lasI* and PAO1Δ*rhlI* when exposure to AMK. The result showed that deficiency of *lasI* or *rhlI* favors the survival of PAO1 under the high concentration of antibiotics (Fig. 4A, B). Moreover, since the fitness cost has been reported to be important for reversible HR. We next determined the growth rate of PAO1 wild type, PAO1Δ*lasI* and PAO1Δ*rhlI*. Unlike exposure to high concentration of antibiotics, growth advantage of PAO1Δ*lasI* and PAO1Δ*rhlI* seem to be inapparent in the absence of antibiotic. (Fig. 4C). Considering the important role of QS in other functions for PAO1, we inferred that the downregulation LasI and RhlI causing resistance in HR cases are costly, which acted as drivers for the population to revert to susceptibility in the absence of antibiotic selection.

OprD and efflux-associated genes were involvled in QS-inhibited HR in P.aeruginosa

It has been reported that reduced expression of porin gene *oprD* and increased expression of efflux-associated genes were associated with carbapenem heteroresistance in *P. aeruginosa*[23]. We then tested the expression of these genes in PAO1 HR subpopulations (HSP) and native populations (NP) for different antibiotics. Consistent with the report, *oprD* was downregulated in HSP compared to NP for AMK, MEM and CAZ, while the expression of efflux-associated genes, including *mexA, mexB, mexE, mexX*

and mexY tended to increase in HSP for AMK, MEM and CIP (Fig. 5A, B, C and D). These results indicated that reduced expression of porin gene *oprD* and increased expression of efflux-associated genes was important for resistance of subpopulations.

The involvement of *oprD* and efflux-associated genes in QS-regulated heteroresistance was then addressed. Intriguingly, compared with wild-type, *PAO1ΔlasI* showed no difference for the expression of *oprD* but obviously increased expression of *mexX and mexY*, while in *PAO1ΔrhlI*, most of the efflux-associated genes were not up-regulated, but the expression of *oprD* was notably decreased (Fig. 5E, F). Taken together, these data implicate that inhibiting effect of QS system on heteroresistant of *P. aeruginosa* is mediated, at least partially, by *oprD* and efflux-associated genes.

Discussion

HR is a common resistance phenotype among clinical isolates that would have been missed from standard estimates of MICs. Thus, the high prevalence of *P. aeruginosa*-HR may have a crucial impact on clinical antibiotics treatment[13]. Heteroresistance of *P. aeruginosa* to carbapenems, colistin and cefepime has been reported. However, the comprehensive study of *P. aeruginosa*-HR is still in active demand. Here, we first detected *P. aeruginosa*-HR to several antibiotics that mostly used in clinical treatment. Our results displayed not only the high prevalence of *P. aeruginosa*-HR to carbapenems (MEM) and β-lactam (CAZ) antibiotics, but also to quinolones (CIP) and aminoglycosides (AMK) antibiotics. It is noteworthy that *P. aeruginosa*-HR to CIP, AMK and CAZ, to our knowledge, have not been reported yet. Heteroresistance might have contributed to treatment failure of these antibiotics.

Most recent publications in heteroresistance research have focused on the genenic mechanism. In this study, we identified QS as a non-genenic mechanism for heteroresistance based on the following evidence: in clinical isolated strains and hereditary stability model, heteroresistance was inversely associated with the level of LasI and RhlI; gain-of-function and loss-of-function studies showed that QS repressed heteroresistance, partially through regulating *oprD* and efflux-associated genes. Similarly, indoles, a chemical signal produced by the resistant subpopulations of *Escherichia coli* (*E. coli*), can also help the sensitive subpopulations to adapt to the antibiotics[27]. In Burkholderia cenocepacia, the resistant subpopulations secrete putrescine and YceI proteins to improve the resistance of sensitive subpopulations to polymyxin B[28]. These studies suggest that chemical signals involved in the inter action between bacteria may be an important mechanism for the regulatory networks of HR.

Hallmarks of heteroresistance are the resistant subpopulation increasing in frequency with antibiotic treatment and decreasing in frequency after removal from the drug[14]. Previous studies have shown that increased resistance of the subpopulations resulted from spontaneous tandem amplifications, typically including known resistance genes, but this procedure is costly and unstable, which may act as drivers for the population to revert to susceptibility in the absence of antibiotic selection these factors together[14]. In this study, we also found that after 40 passages in antibiotic-free medium, the highly resistant subpopulations revert to the heterogeneous resistance phenotype displayed by the original population.

This course was accompanied with the expression change of QS genes. Accordingly, the advantage of relative growth rates can be obviously observed in for *lasI* or *rhlI* deficiency strains when exposure to antibiotics. We speculated that in the presence of selective pressure, downregulation of QS genes may result in less fitness cost and population density restriction for *P. aeruginosa*. However, this hypothesis need to be further tested.

OprD porin and efflux system could have contributed to heteroresistance in *P. aeruginosa*[10, 25], which was confirmed in our present study. Comparing with the dominant population, *oprD* downregulation was mainly performed in carbapenem heteroresistant subpopulation, efflux-associated genes overexpression mainly occurred in carbapenems, quinolones and aminoglycosides heteroresistant subpopulation, which may be related to the mechanism of *P. aeruginosa* resistance[29–31]. We further found that the expression of *oprD* and efflux-associated genes was under the control of QS, which was coincident with the previous reports that expression of MexAB-OprM was induced by C4-HSL[32]. These study results indicated that *oprD* and efflux-associated genes were not only the reason for antibiotic resistance in *P. aeruginosa*, but also played an important role in heteroresistance for bacteria to adapt to the environment under the temporary antibiotic pressure. However, it need to further verified that heteroresistance can act as the intermediate process of bacterial susceptibility to resistance with antibiotic pressure for a long period.

Conclusions

Pseudomonas aeruginosa is one of most important pathogens in clinical medicine. Besides its virulence, the acquisition or emergence of resistance toward antibiotic agents poses a major therapeutic challenge. Heteroresistance (HR) is a strategy for bacteria to adapt to antibiotic pressure that leading to failure of antibiotic treatment and even inducing bacterial resistance. Here, we identified a high prevalence of heteroresistance to MEM, AMK, CIP and CAZ among clinical isolates of *P. aeruginosa*, which was regulated by quorum sensing. Our findings highlight the new function of QS and provide new insight into the mechanism of heteroresistance in the context of non-genetic factor.

Methods

Bacterial strains and antimicrobial susceptibility testing

A total of 170 non-duplicate clinical *P. aeruginosa* isolates were obtained from two tertiary hospitals in Guangzhou from 2017 to 2018 as shown in this study. All isolates were stored at -80℃ and cultured onto blood agar plate before each experiment. All isolates were reconfirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). All bacterial strains and plasmids used in this study are listed in Table S1.

Antimicrobial susceptibility testing

Meropenem (APE-BIO, United States), Amikacin (BBI Life Science, United States), Ciprofloxacin (BBI Life Science, United States), Ceftazidime (Sigma-Aldrich, United States) Fosfomycin (APE-BIO, United States), polymyxin B (BBI Life Science, United States), and Gentamicin (BBI Life Science, United States) were freshly prepared for each experiment and filter sterilized using a 0.22 μm filter. Mueller-Hinton broth (Oxoid, United Kingdom) supplemented with calcium and magnesium (25.0 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+}) (CAMHB) and Mueller-Hinton II agar (Oxoid, United Kingdom) were used for susceptibility testing and all in vitro models. The breakpoints for above antibiotics were defined by CLSI-M100-S26. Quality control was monitored with P. aeruginosa strain ATCC 27853 and pandrug-resistant Escherichia coli stain 132A5.

Fitness cost measurements

Growth rates were analyzed in a biotek (synergy H1) as previously described[14]. Four colonies for each test strain were used as biological replicates. The overnight cultures were then diluted 1:1000 in 3ml of fresh Mueller-Hinton broth, followed by growing for 24h or 48h at 37℃. Absorbance (A600nm) was measured.

Population Analysis Profiles (PAPs)

HR for clinical *P. aeruginosa* isolates and PAO1 were determined by PAP according to a previously published protocol with several modifications[33]. Three colonies were used to start three independent overnight cultures in 3ml Mueller-Hinton broth. Twenty microlitres of gradient dilutions of an overnight culture (10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 CFU/mL) were plated on Mueller-Hinton agar plates supplemented or not with increasing amounts of antibiotic (2-fold increments). Breakpoint concentrations for *P. aeruginosa* from CLSI. Plates were incubated overnight at 37 °C and colonies were counted. The presence of a subpopulation of cells capable of growing at concentrations of the antibiotic at least eight-fold higher than the highest concentration that does not affect the replication of the dominant population was defined as HR. Quality control was monitored with *P. aeruginosa* strain ATCC 27853 and pandrug-resistant *E. coli* stain 132A5.

Stability of antibiotic resistance and heteroresistance.

Two clones isolated from the highest drug concentration plates were supplemented in 3 ml of Mueller-Hinton broth the same selection pressure overnight. MICs were determined by the broth microdilution method. The cultures were then grown for 2 additional overnight. The MICs after 40 generations in the absence of selective pressure were determined, and the resistance was deemed unstable if the MIC clearly decreased or reverted to that of the original parental isolate in at least one of the cultures.

Genes Expression Analysis

Total RNA was extracted using RNAiso Plus reagent (TaKaRa, Dalian, Liaoning, China). Reverse transcription (1 μg of total RNA) was performed with the PrimeScript RT reagent kit (TaKaRa, Dalian,

Liaoning, China). The cDNA was subjected to quantitative PCR (qPCR) on a ViiATM 7 Dx system (Applied Biosystems, Foster, CA, United States) using SYBR green qPCR master mixes (TaKaRa, Dalian, Liaoning, China). The expression levels of the target genes (*lasA*, *lasI*, *rhlA* and *rhlI* gene associated with QS expression and the *oprD*, *ampC*, efflux pumps gene associated with resistance machines) were normalized to the expression of an internal control gene (*rpoD*) using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers are listed in Table S2.

Abbreviations

QS
Quorum sensing
P. aeruginosa
Pseudomonas aeruginosa
HR
Heteroresistance
AHL
N-acyl homoserine lactone
3-oxo-C12-HSL
N-(3-oxododecanoyl)-L-homoserine lactone
C4-HSL
N-butanoyl-L-homoserine lactone
PAPs
Population Analysis Profiles
MIC
Minimum inhibitory concentration
qPCR
quantitative polymerase chain reaction.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

The materials that support the findings of this studyare available from the corresponding author upon reasonable request.

Author contributions

Y.L., YY.L. and C.Z. performed most of the experiments; YF.L., D.L., R.X. and Q.X. discussed some results; B.H., C.C., and Y.L. analyzed and organized the data and wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Competing interests

No potential conflict of interest was reported by the authors.

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Figures

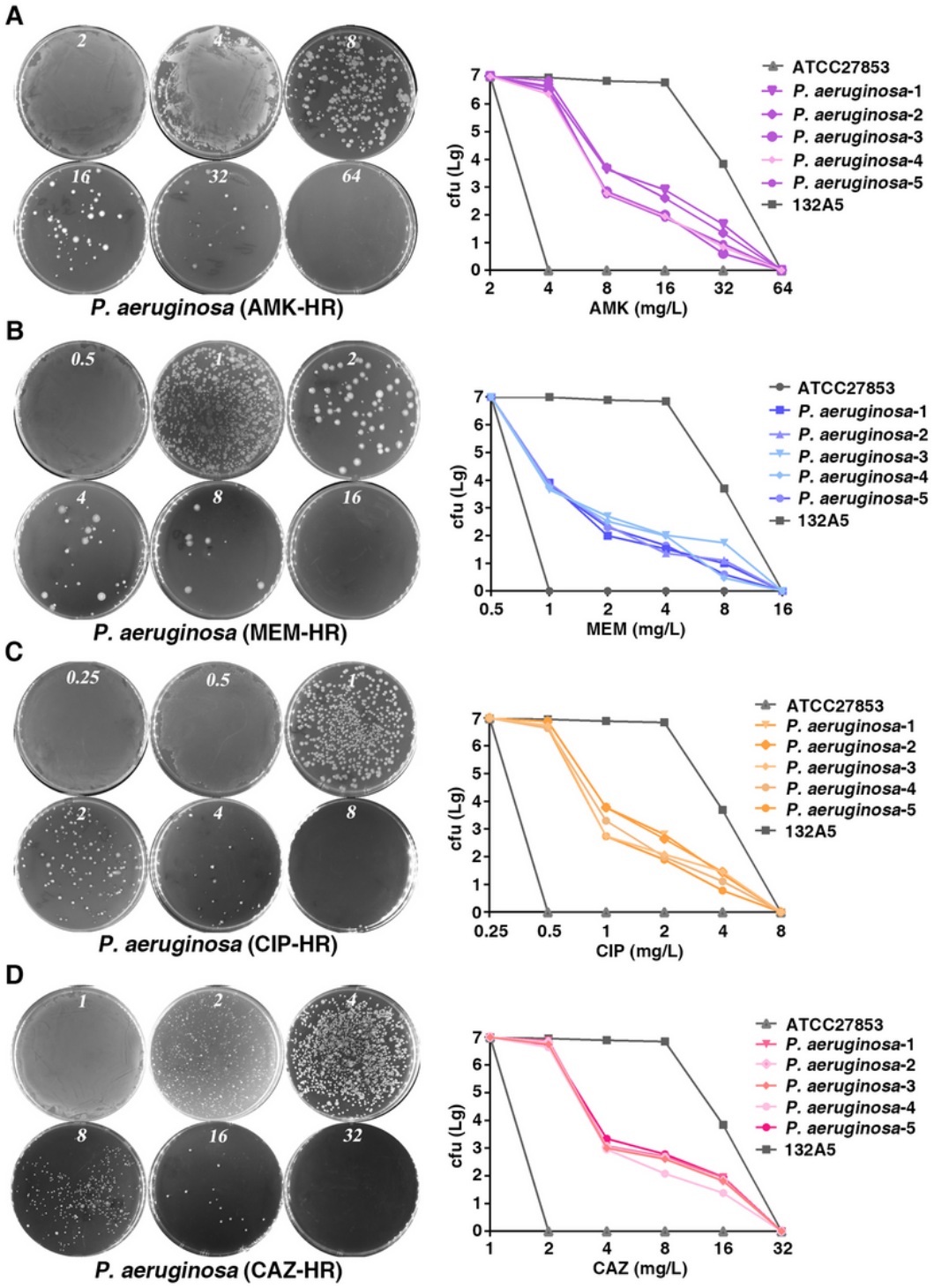

Figure 1

Prevalence of heteroresistance in clinical *P. aeruginosa* isolates. Population analysis profiles (PAPs) were performed in five clones for each *P. aeruginosa* isolate. *P. aeruginosa* sensitive strain (ATCC 27853) and resistant strain (132A5) were used as control strain. AMK, amikacin; MEM, meropenem; CIP, ciprofloxacin; CAZ, ceftazidime.

Figure 2

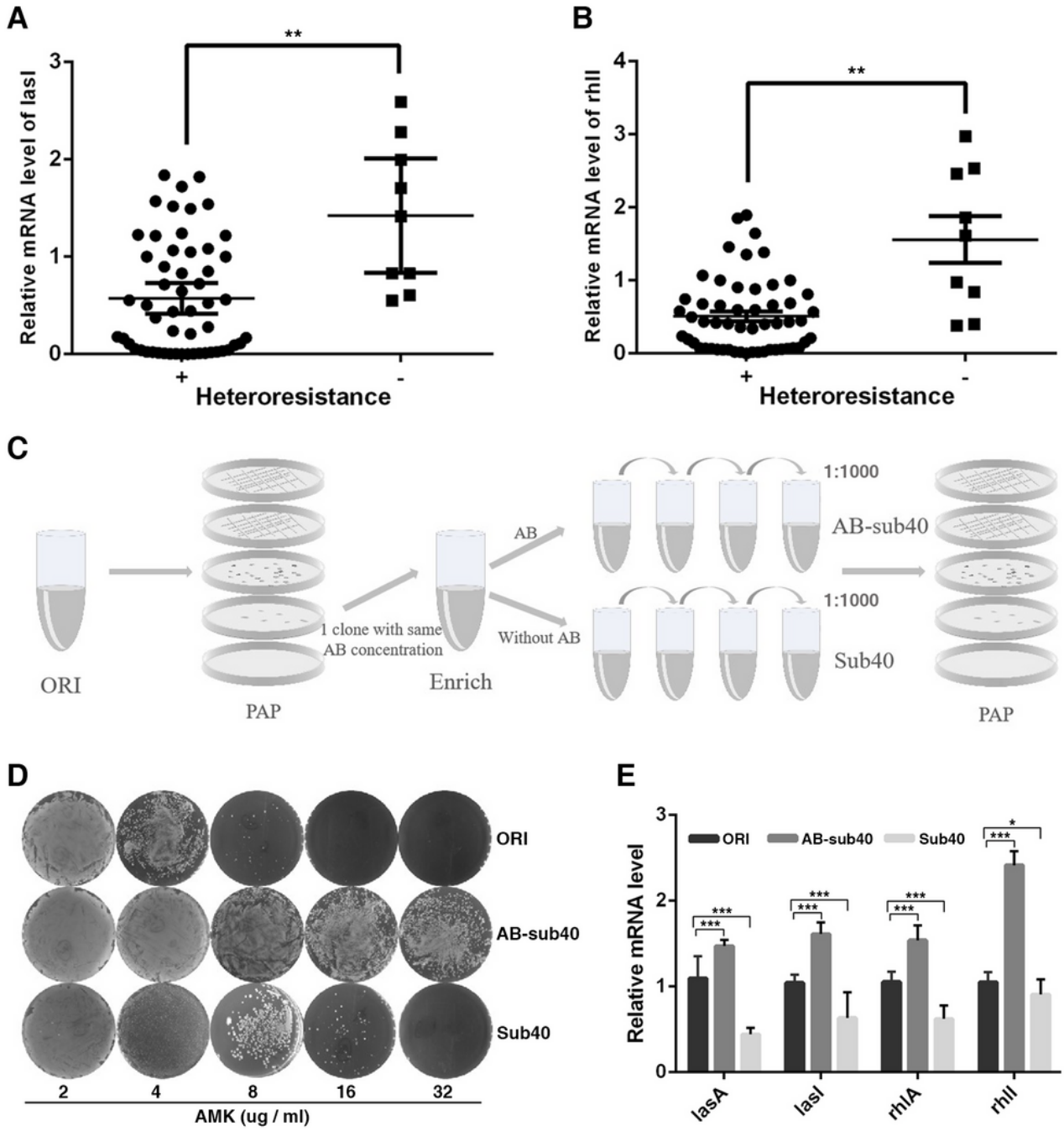

Figure 2

The expression of LasI and RhlI is negatively correlated with HR of *P. aeruginosa*. (A, B) The expression of LasI and RhlI was down-regulated in heteroresistance positive group compared with that of negative group. (C) A schematic outline of methods used to determine stability of HR. A resistant colony from the PAP test plates was re-streaked and grew on the same concentration of antibiotics (Enrich), followed by continuously passaging with or without antibiotic selection for 40 generations (1:1000 daily dilution). (D)

After serial passages in antibiotic-free medium, resistant subpopulations revert to the heterogeneous resistance phenotype displayed by the original population. (E) The expression of LasI and RhlI in original PAO1 (ORI), resistant subpopulations after 40 generations with or without exposure to antibiotics (AB-sub40, sub40 respectively).

Figure 3

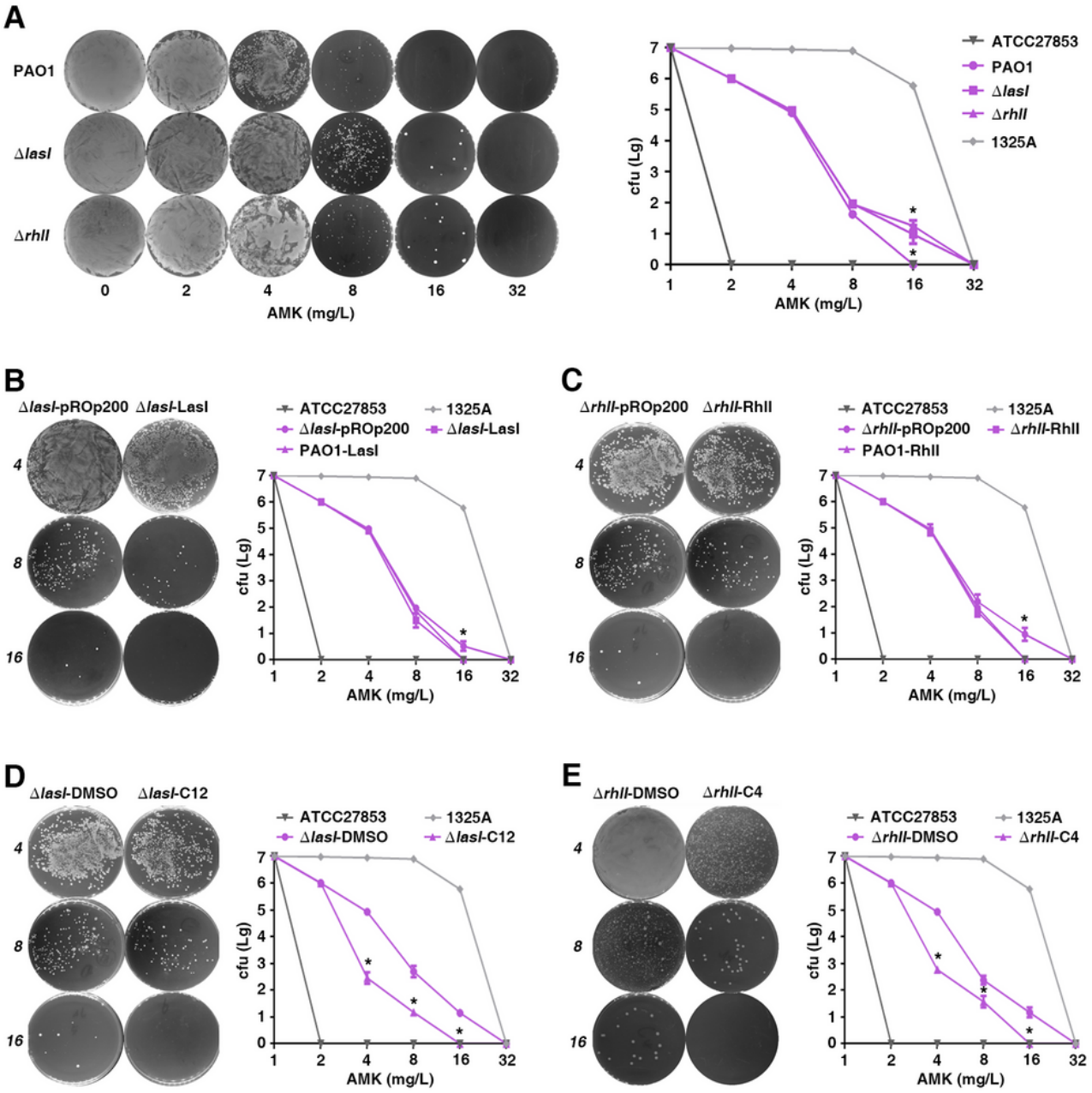

Figure 3

The quorum sensing system of *P. aeruginosa* inhibits heteroresistance. (A) Deficiency of *lasI* or *rhlI* in PAO promoted heteroresistance. (B, C) Over expression of *lasI* or *rhlI* inhibited heteroresistance. (D, E) Exogenous 3-oxo-C12-HSL (C12) or C4-HSL (C4) inhibited heteroresistance. PAPs was performed in PAO1Δ*lasI* and PAO1Δ*rhlI* treated with 40 μM of 3-oxo-C12-HSL or C 4-HSL respectively.

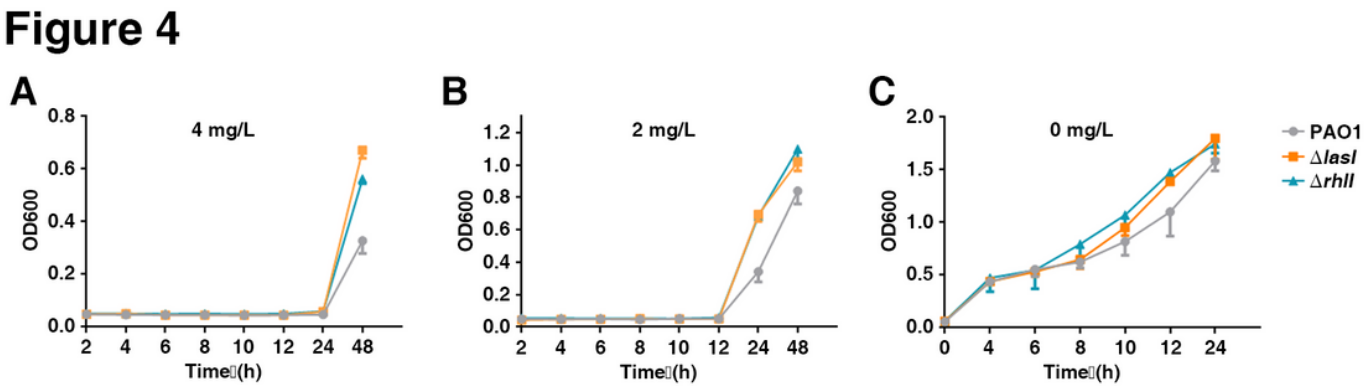

Figure 4

The growth rate of PAO1, PAO1Δ*lasI* and PAO1Δ*rhlI* in the presence or absence of antibiotic selection. (A, B, C) PAO1, PAO1Δ*lasI* and PAO1Δ*rhlI* were treated with indicated concentrations of AMK.

Figure 5

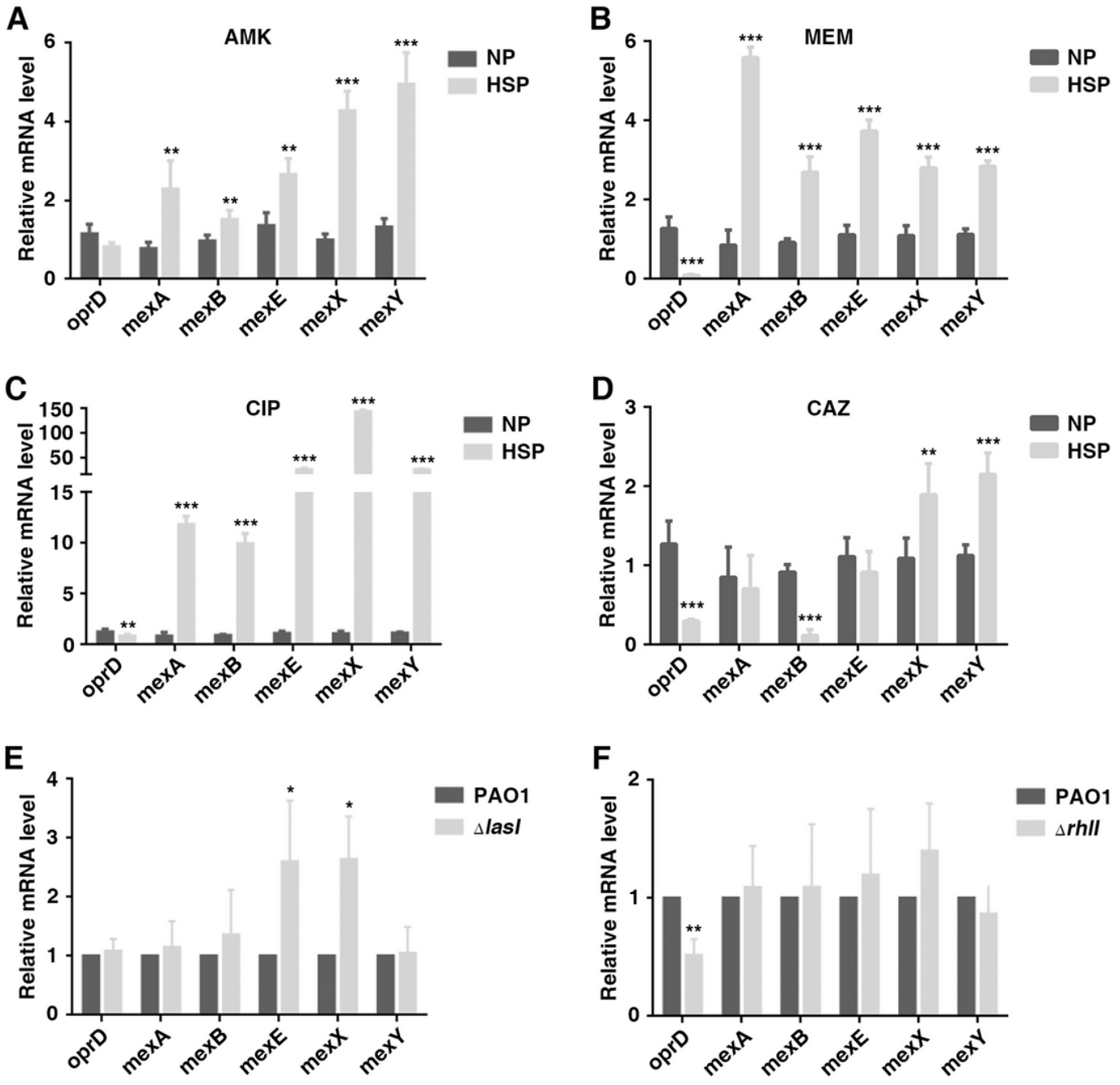

Figure 5

OprD and efflux-associated genes were involvled in QS-inhibited HR. (A, B, C, D) The expression of OprD and efflux-associated genes in native population (NP) and resistant subpopulation (HSP) of AMK, MEM, CIP or CAZ. A resistant colony growing on the highest concentration of PAP test plates was re-streaked on the same concentration of indicated antibiotics, followed by real-time PCR analysis. (E, F) The expression of OprD and efflux-associated genes was regulated by LasI and RhlI. PAO1, PAO1Δ*lasI* and PAO1*ΔrhlI* were cultured in LB for 6 h before qPCR analysis. The *rpoD* gene was used as an internal control.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformation.pdf