Mechanisms for Rapid Evolution of Carbapenem Resistance in a Clinical Isolate of *Pseudomonas aeruginosa*

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Infections by *Pseudomonas aeruginosa* are difficult to cure due to its high intrinsic and acquired antibiotic resistance. Once colonized the human host, and thanks to antibiotic treatment pressure, *P. aeruginosa* usually acquires genetic mutations which provide bacteria with antibiotic resistance as well as ability to better adapt to the host environment. Deciphering the evolutionary traits may provide important insights into the development of effective combinatory antibiotic therapy to treat *P. aeruginosa* infections. In this study, we investigated the molecular mechanisms by which a clinical isolate (ISP50) yields a carbapenem-resistant derivative (IRP41). RNAseq and genomic DNA reference mapping were conducted to compare the transcriptional profiles and *in vivo* evolutionary trajectories between the two isolates. Our results demonstrated that *oprD* mutation together with *ampC* hyper-expression contributed to the increased resistance to carbapenem in the isolate IRP41. Furthermore, a *ldcA* (PA5198) gene, encoding murein tetrapeptide carboxypeptidase, has been demonstrated for the first time to negatively influence the *ampC* expression in *P. aeruginosa*.

Keywords: *P. aeruginosa*, carbapenem resistance, *oprD*, *ampC*, *ldcA*

INTRODUCTION

Pseudomonas aeruginosa, as an opportunistic human pathogen, is one of the leading causes of nosocomial infections worldwide (Vincent et al., 1995). Infections by *P. aeruginosa* are difficult to treat due to its intrinsic and acquired resistance to a wide range of antibiotics, leaving limited number of effective antimicrobial agents. Carbapenems are used in clinical practice to treat *P. aeruginosa* infections. However, carbapenem resistance of clinical *P. aeruginosa* isolates has been increasingly reported (Davies et al., 2011). The mechanisms of carbapenem resistance are usually multifactorial which include: (i) acquisition of carbapenemase encoding genes through horizontal gene transfer (Poole, 2011; Potron et al., 2015), (ii) deficiency or repression of the porin (OprD) for carbapenem (Davies et al., 2011; Poole, 2011), (iii) overexpression of *mexAB-oprM* efflux pump (Poole, 2011; Liu et al., 2013; Choudhury et al., 2015), and (iv) overexpression of the chromosomal gene (*ampC*) encoding the *P. aeruginosa* intrinsic cephalosporinase (Poole, 2011; Mirsalehian et al., 2014). Although these and other studies have described the associated mechanisms of carbapenem resistance among clinical isolates of *P. aeruginosa*, there is little information on the detailed molecular mechanisms leading to the evolutionary dynamics of clinical *P. aeruginosa* isolates from carbapenems susceptibility to resistance and the impact of each of these resistance mechanisms.

In this study, we obtained two *P. aeruginosa* clinical isolates, later demonstrated to belong to the same clone, from sputum samples of the same patient with acute exacerbation of chronic bronchitis before and after treatment with biapenem. The first strain was obtained soon after the patient was admitted to the hospital while the second strain was obtained 4 days after the antibiotic treatment. The first isolate ISP50 was carbapenem susceptible, whereas the second one IRP41 was carbapenem resistant. Therefore, our goal was to decipher the molecular mechanisms by which the carbapenem resistance had been evolved so rapidly in the clinical setting. Our experimental results demonstrated that an *oprD* null mutation combined with an elevated *ampC* expression are the major contributory factors for the conversion. Furthermore, we have shown for the first time that LdcA functions as a repressor on the expression of *ampC* in *P. aeruginosa*. These findings provide novel insights into the regulatory mechanism of *ampC* expression in *P. aeruginosa*.

MATERIALS AND METHODS

Basic Characterization of the Bacterial Strains

Bacterial strains and plasmids used in the study are listed in **Table 1**. Carbapenem-susceptible (ISP50) and resistant (IRP41) *P. aeruginosa* isolates characterized in this study were obtained from sputum samples of the same patient with acute exacerbation of chronic bronchitis before and after treatment with biapenem for 4 days (dosage at 0.3 g × 2/day) at the Nankai University Affiliated Hospital, Tianjin, China. The 16S rRNA encoding gene was amplified (primers listed in **Supplementary Table S1**) and sequenced to identify the species of these two isolates (Spilker et al., 2004). Random amplified polymorphic DNA (RAPD) typing was carried out using primer 272 as described previously (Mahenthiralingam et al., 1996). Antibiotic susceptibility test was conducted using the disk diffusion method according to the manufacturer's recommendations, and minimum inhibitory concentration (MIC) of antibiotics was determined by the two-fold serial dilution method. Susceptibility was interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2011–2018).

Plasmid Construction

For overexpression of *oprD*, a 1,802 bp *oprD*-containing fragment with its putative Shine-Dalgarno (SD) sequence was PCR amplified using ISP50 and IRP41 genomic DNA as templates (the used primers are displayed in **Supplementary Table S1**). The PCR products were digested with *Bam*HI and *Hin*dIII, and then ligated into a shuttle vector pUCP24, resulting in pUCP24-*oprD*$_{ISP50}$ and pUCP24-*oprD*$_{IRP41}$, respectively. Constructs of pUCP24-*ampC*, pMMB-*ampR*$_{ISP50}$, pMMB-*ampR*$_{IRP41}$, pUCP24-*ldcA*$_{ISP50}$, pUCP24-*ldcA*$_{IRP41}$ and pUCP24-*pbpC* were all generated using similar procedures.

For deletion of the *ldcA* gene, a 836 bp fragment immediately upstream of the *ldcA* start codon and a 913 bp fragment downstream of the *ldcA* stop codon were PCR amplified, digested with *Eco*RI-*Bam*HI and *Bam*HI-*Hin*dIII, respectively.

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source of reference
Strains		
DH5α	F⁻ φ 80d*lacZ*ΔM15 *endA1 recA1 hsdR17*(r$_K$⁻ m$_K$⁺) *supE44 thi-1 relA1* Δ(*lacZYA-argF*)U169 *gyrA96 deoR*	TransGen
S17-1	RP4-2 Tc::Mu Km::Tn7 Tp^r Sm^r Pro Res⁻ Mod⁺	Dr. Ramphal
ISP50	Clinical isolate from sputum samples of the a female patient with acute exacerbations of chronic bronchitis before treatment with biapenem, IMP^s	This study
IRP41	Clinical isolate from sputum samples of the a female patient with acute exacerbations of chronic bronchitis after treatment with Biapenem for 4 days, IMP^R	This study
Klebsiella pneumoniae ATCC 700603	*Klebsiella pneumoniae* ATCC 700603	Fuxiang Biotechnology
PA-NK41	KPC2 carbapenemase producing *P. aeruginosa*	This study
PA14	Wild type *P. aeruginosa* strain	Liberati et al. (2006)
PA14*pbpC*::Tn	PA14 with *pbpC* disrupted by insertion of Tn	Liberati et al. (2006)
PAO1	Wild type *P. aeruginosa* strain	Kaufman et al. (2000)
PAO1Δ*ldcA*	PAO1 with *ldcA* gene deleted	This study
PA14Δ*ldcA*	PA14 with *ldcA* gene deleted	This study
ISP50Δ*ldcA*	ISP50 with *ldcA* gene deleted	This study
PAO1Δ*ampR*	PAO1 with *ampR* gene deleted	This study
Plasmids		
pUCP24	Shuttle vector between *E. coli* and *P. aeruginosa*; Gm^r	West et al. (1994)
pMMB67EH-Gm	Shuttle vector between *E. coli* and *P. aeruginosa*; Gm^r	Long et al. (2019)
pEX18Tc	Gene knockout vector, Tc^r	Hoang et al. (1998)
pUCP24-*oprD*$_{ISP50}$	*oprD* gene from ISP50 in pUCP24, Gm^r	This study
pUCP24-*oprD*$_{IRP41}$	*oprD* gene from IRP41 in pUCP24, Gm^r	This study
pUCP24-*ampC*	*ampC* gene from ISP50 in pUCP24, Gm^r	This study
pUC18T-miniTn7T-*ampC*-His	C-terminal His-tagged *ampC* in pUC18T-miniTn7T, Tc^r	This study
pUCP24-*pbpC*	*pbpC* gene from ISP50 in pUCP24, Gm^r	This study
pUCP24-*ldcA*$_{ISP50}$	*ldcA* gene from ISP50 in pUCP24, Gm^r	This study
pUCP24-*ldcA*$_{IRP41}$	*ldcA* gene from IRP41 in pUCP24, Gm^r	This study
pMMB-*ampR*$_{ISP50}$	*ampR* gene from ISP50 in pMMB67EH-Gm, Gm^r	This study
pMMB-*ampR*$_{IRP41}$	*ampR* gene from IRP41 in pMMB67EH-Gm, Gm^r	This study
pEX18-*ldcA*	*ldcA* gene deletion on pEX18Tc, Tc^r	This study
pEX18-*ampR*	*ampR* gene deletion on pEX18Tc, Tc^r	This study

The two fragments were then ligated into pEX18Tc that was digested with *Eco*RI and *Hin*dIII, resulting in pEX18-*ldcA*. A pEX18-*ampR* was constructed by similar manipulations. Gene knockouts in *P. aeruginosa* were carried out by conjugal transfer of these plasmids followed by selection for single

crossovers and then double crossovers as previously described (Schweizer, 1992).

Multilocus Sequence Typing

Multilocus sequence typing (MLST) was performed following a previous description (Curran et al., 2004) with minor modifications to confirm the allelic profiles of the two isolates. Briefly, genomic DNA was isolated from overnight bacterial culture with a DNA purification kit (Tiangen Biotech, Beijing, China) for use as PCR template. The internal fragments of seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE*) were amplified by PCR and sequenced using primers described previously (**Supplementary Table S1**) (Curran et al., 2004). Gene sequences were then submitted to the *P. aeruginosa* MLST database[1] for assignment of allelic numbers. A sequence type (ST) was assigned to each isolate by combining the seven allelic numbers.

PAE-MHT Assay for Carbapenemase Test

The PAE-MHT assay was performed as described previously (Pasteran et al., 2011) with minor modifications. Briefly, an inoculum of *Klebsiella pneumoniae* ATCC 700603 was adjusted to a 0.5 McFarland turbidity standard (Mcfarland, 1907) followed by 10-fold dilution with sterile saline, and then inoculated onto the surfaces of Mueller-Hinton agar (Oxoid) plates (diameter, 10 cm) by swabbing. The plates were allowed to stand at room temperature. After 10 min, a disk of 10 μg imipenem/meropenem (Oxoid, United Kingdom) was placed in the center of each plate. Subsequently, one colony of each *P. aeruginosa* strain, grown overnight on LB agar plate, was inoculated onto the plate in a straight line from the edge of the disk to the periphery of the plate using a 1-μl loop (BOOPU Biological Technology, Changzhou, China). Presence of growth of the *K. pneumoniae* ATCC 700603 toward imipenem/meropenem disk was interpreted as carbapenemase positive.

Total RNA Isolation, RT-qPCR and RNAseq Analysis

Bacterial overnight culture was inoculated into fresh LB medium (1:50 dilution) and grown to an optical density of 1.0 (wavelength of 600 nm). Total RNA was extracted using an RNAprep Pure Cell/Bacteria Kit (Tiangen Biotech, Beijing, China) and reverse transcribed to cDNA with random primers and PrimeScript Reverse Transcriptase (Takara). The cDNA was mixed with indicated primers (**Supplementary Table S1**) and SYBR premix Ex Taq II (Takara), and then quantitatively amplified in a CFX Connect Real-Time system (Bio-Rad, United States). A 30S ribosomal protein encoding gene *rpsL* was used as an internal control.

For RNAseq, total RNA was extracted as described above, quantified and qualified by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States), NanoDrop (Thermo Fisher Scientific Inc.) and 1% agarose gel. Then, the rRNA

was depleted using Ribo-Zero rRNA Removal Kit (Bacteria, Illumina). The mRNA was then fragmented and reverse-transcribed. The double-strand cDNA was purified, ends repaired and ligated with adaptors. After 11 cycles of PCR amplification, the PCR products were cleaned, validated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States), and quantified by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, United States). The resulting libraries were subjected to sequencing using an Illumina HiSeq 2500 platform with a 2×150 paired-end configuration.

Sequence reads were mapped onto PAO1 reference genome (NC_002516.2) via software Bowtie2 (v2.1.0). Gene expression levels were analyzed with HTSeq (v0.6.1p1). Differentially expressed genes were identified by the DESeq Bioconductor package, with the fold-change larger than 2 and *P*-value no more than 0.05 as cutoff values.

DNA Isolation and Reference Mapping

Bacterial genomic DNA was extracted with DNA purification kit (Tiangen Biotech, Beijing, China). Fragments smaller than 500 bp were obtained from 200 ng genomic DNA by sonication (Covaris S220), followed by end treatment and adaptors ligation. Adaptor-ligated DNA fragments of about 470 bp were recovered using beads and then PCR amplified for six cycles, and the PCR products were cleaned up using beads, validated using a Qsep100 (Bioptic, Taiwan, China), and quantified by Qubit3.0 Fluorometer (Invitrogen, Carlsbad, CA, United States). Sequencing was carried out using a 2×150 paired-end (PE) configuration on an Illumina Hiseq instrument according to manufacturer's instructions (Illumina, San Diego, CA, United States). The data were aligned to the PAO1 reference genome (NC_002516.2) via software BW2 (version 0.7.12). Single nucleotide variation (SNV) or InDel mutation were detected using the software Samtools (version 1.1) and the Unified Genotyper module from GATK.

PCR and Sequencing of *oprD*, *ampR* and *ldcA*

The full-length *oprD* gene with its 105 bp upstream and 209 bp downstream region was amplified and sequenced using primers listed in **Supplementary Table S1**. The *oprD* gene sequence from each isolate was aligned with the *oprD* sequence of the reference strain PAO1[2]. Analysis of *ampR* and *ldcA* were conducted with similar procedures.

Western Blot Assay

Subcultured samples from equivalent number of bacterial cells of an optical density of 1.0 (wavelength of 600 nm) were separated on a 12% SDS-PAGE. The proteins were then transferred onto a PVDF (polyvinylidene difluoride) membrane and probed with a mouse monoclonal antibody against His-tag (Cell Signaling Technology, United States) and the RNA polymerase alpha subunit RpoA (Biolegend). Signals were generated using the ECL-Plus kit (Millipore) and detected by a Biorad imager (ChemiDocXRS).

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[1]http://pubmlst.org/paeruginosa/

[2]www.pseudomonas.com

Quantification of Biofilm

Biofilm production was measured as described previously (Li et al., 2013). Overnight culture of each bacterium was diluted 50-fold in LB broth and incubated in each well of a 96-well plate at 37°C. After 24 h, each well was washed with phosphate buffered saline (PBS, 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4) for three times, stained with 0.1% crystal violet, and then washed three times with PBS. Then, 0.2 ml of decolorizing solution [methanol (4): acetic acid (1): water (5)] was added into each well and incubated 10 min at room temperature. Each sample was then subjected to measurement for OD_{590} using Varioskan Flash (Thermo Scientific, Netherlands).

Statistical Analysis

Graphpad software was used to perform the statistical analyses. The real-time qPCR results were analyzed by Student's t-test (two-tailed).

RESULTS

Clinical Isolates ISP50 and IRP41 Belong to the Same Clone

Sputum samples from a patient with acute exacerbation of chronic bronchitis were collected before and after treatment with biapenem for 4 days (dosage at 0.3 g × 2/day). Two mucoid isolates were selected, one before the biapenem treatment which was sensitive to imipenem (ISP50), and the other one after the biapenem treatment which showed resistance against the imipenem (IRP41). PCR amplification of the 16S rDNAs as well as their sequence analysis indicated that both of them were *P. aeruginosa* (**Supplementary Figure S1A**). The MLST analysis of both isolates revealed an allelic profile for *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE* as 17, 5, 12, 3, 14, 4, 7, respectively, corresponding to the same sequence type, ST244. RAPD typing was further performed on the IRP41 and ISP50 isolates, which also suggested that they were of the same clone (**Supplementary Figure S1B**).

Isolate IRP41 Is Resistant to Carbapenem

Imipenem susceptibilities of IRP41 and ISP50 were found different based on the preliminary analysis with VITEK automatic microbe analysis instrument (data not shown). Therefore, we further examined the imipenem susceptibilities by both MIC and disk diffusion methods, with the results shown in **Table 2** and **Supplementary Figure S2**. Initial isolate (ISP50) was found to be susceptible to the imipenem (53.3 mm/diameter of inhibition zone), while later isolate (IRP41) was resistant to the imipenem (10.7 mm/diameter of inhibition zone), with a 32-fold increase in MIC of imipenem. Although both strains were susceptible to meropenem (another carbapenem antibiotic) according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2011–2018), IRP41 displayed a smaller diameter of inhibition zone (24 mm) compared to that of ISP50 (44 mm) (**Supplementary Figures S2A–D**). In addition, MIC

TABLE 2 | MICs (μg/ml) of indicated *P. aeruginosa* strains.

strains	Imipenem (μg/ml)[a]	Ampicillin (μg/ml)	Meropenem (μg/ml)[a]	Biapenem (μg/ml)[b]
ISP50	0.3125	ND	3.125	0.78125
IRP41	10	ND	6.25	6.25
ISP50/pUCP24	0.3125	ND	3.125	0.78125
IRP41/pUCP24	10	ND	6.25	6.25
IRP41/pUCP24-*oprD*$_{ISP50}$	0.625	ND	3.125	3.125
IRP41/pUCP24-*oprD*$_{IRP41}$	10	ND	6.25	6.25
IRP41/pUCP24-*ldcA*$_{ISP50}$	5	ND	3.125	3.125
IRP41/pUCP24-*ldcA*$_{IRP41}$	10	ND	6.25	6.25
PAO1	1.5625	625	3.125	1.5625
PAO1Δ*ldcA*	0.78125	1250	1.5625	0.78125
PA14	1.5625	312.5	1.5625	1.5625
PA14Δ*ldcA*	0.78125	1250	0.3906	0.3906

ND, not determined; [a]Clinical Laboratory Standards Institute (CLSI) susceptibility breakpoints: imipenem, meropenem ≤ 2 μg/ml, resistance breakpoints: imipenem, meropenem ≥ 8 μg/ml. [b] no CLSI breakpoint concentrations for biapenem have been established, but previous studies suggested that MIC ≤ 4 μg/ml is sensitive and MIC ≥ 16 μg/ml is resistant (Hoban et al., 1993; Hang et al., 2018).

of meropenem/biapenem in IRP41 showed two-fold/eight-fold increase compared to ISP50 (as displayed in **Table 2**).

Since the production of horizontally acquired carbapenem-hydrolyzing enzymes has been defined as a contributory factor in clinical *P. aeruginosa* isolates with carbapenem resistance (Potron et al., 2015), we tested if the IRP41 produced carbapenemase using a PAE-MHT assay. As the results shown in **Supplementary Figures S2E,F**, compared to the PA-NK41, which produces a KPC2 carbapenemase, no carbapenemase activity was observed in the IRP41 or ISP50 strain, suggesting that the carbapenem resistant phenotype of isolate IRP41 is not due to the presence of any horizontally acquired carbapenemases.

Differential Expression of Genes Relevant to β-Lactam Resistance

To elucidate the mechanism of reduced susceptibility to carbapenem in isolate IRP41, we compared the global gene expression profiles between strains ISP50 and IRP41. Expression levels of 284 genes were altered comparing the two strains (**Table 3** and **Supplementary Table S2**). Among them, *ampC* gene, encoding the β-lactamase, displayed a 285-fold higher mRNA level in IRP41 than that in ISP50 (**Table 3**). To confirm the up-regulation, we further examined the mRNA levels by real-time qPCR. As shown in **Figure 1A**, consistent with the RNAseq result, the mRNA level of *ampC* was 413-fold higher in the strain IRP41 than that in ISP50. To further determine if the increased *ampC* expression contributed to the reduced susceptibility to carbapenem in IRP41, the *ampC* gene was overexpressed in the ISP50 strain background and the MIC of carbapenems was examined. As shown in **Supplementary Table S3** and **Supplementary Figure S3A**, overexpression of the *ampC* (1,239-fold increase compared to that in ISP50/pUCP24) reduced the susceptibility of ISP50 to imipenem/meropenem/biapenem, with 16/2/4-fold increase in MIC. These results suggested that increased expression of the *ampC* indeed contributed

TABLE 3 | mRNA levels of genes related to β-lactam resistance and biofilm in IRP41 compared to those in ISP50.

Gene ID	Gene name	Gene function	Fold change ($_{IRP41/ISP50}$)	P-value
PA4110	ampC	Beta-lactamase	285	6.42E−20
PA2233	pslC	Biofilm formation protein PslC	30	4.71E−09
PA2272	pbpC	Penicillin-binding protein 3A	8	1.49E−05
PA3719	armR	MexR antirepressor ArmR	5	0.001828
PA2234	pslD	Biofilm formation protein PslD	11	3.63E−06
PA2235	pslE	Biofilm formation protein PslE	16	8.15E−08
PA2236	pslF	Biofilm formation protein PslF	18	9.04E−08
PA2237	pslG	Biofilm formation protein PslG	8	4.15E−05
PA2238	pslH	Biofilm formation protein PslH	11	3.93E−06
PA2239	pslI	Biofilm formation protein PslI	10	7.32E−06
PA2240	pslJ	Biofilm formation protein PslJ	7	0.000124
PA2241	pslK	Biofilm formation protein PslK	5	0.002139
PA2242	pslL	Biofilm formation protein PslL	5	0.001121

to the decreased susceptibility of IRP41 to carbapenems, at least partially.

Besides the *ampC*, a *pbpC* gene, encoding penicillin binding protein 3a, displayed an eight-fold up-regulation in IRP41 (**Table 3**). Real-time qPCR confirmed the increased expression of the *pbpC* in IRP41 (**Figure 1B**). However, overexpression of the *pbpC* (**Supplementary Figure S3B**) in ISP50 (ISP50/pUCP24-*pbpC*) had no effect on the bacterial MIC against imipenem/meropenem/biapenem (**Supplementary Table S3**), although it restored the MIC of carbenicillin in PA14*pbpC*::Tn to that of wild type PA14 strain (**Supplementary Table S3**). These results indicated that the elevated *pbpC* expression did not contribute to the carbapenem resistance in IRP41.

Our RNAseq analysis also revealed that *armR*, encoding MexR antirepressor (ArmR), was up-regulated five folds in IRP41. It had been demonstrated that MexR represses the expression of *mexAB-oprM* efflux pump (Daigle et al., 2007) whose over-expression confers meropenem resistance in *P. aeruginosa* (Okamoto et al., 2002). However, RNAseq results showed no significant difference in the transcriptional levels of the *mexAB-oprM* between the two strains, and this is confirmed by real-time qPCR (**Figures 1C,D**). Further sequence analysis showed that no mutation happened in the *mexR*, *armR* or *mexR–mexA* intergenic region of the IRP41 and ISP50 strains (data not shown).
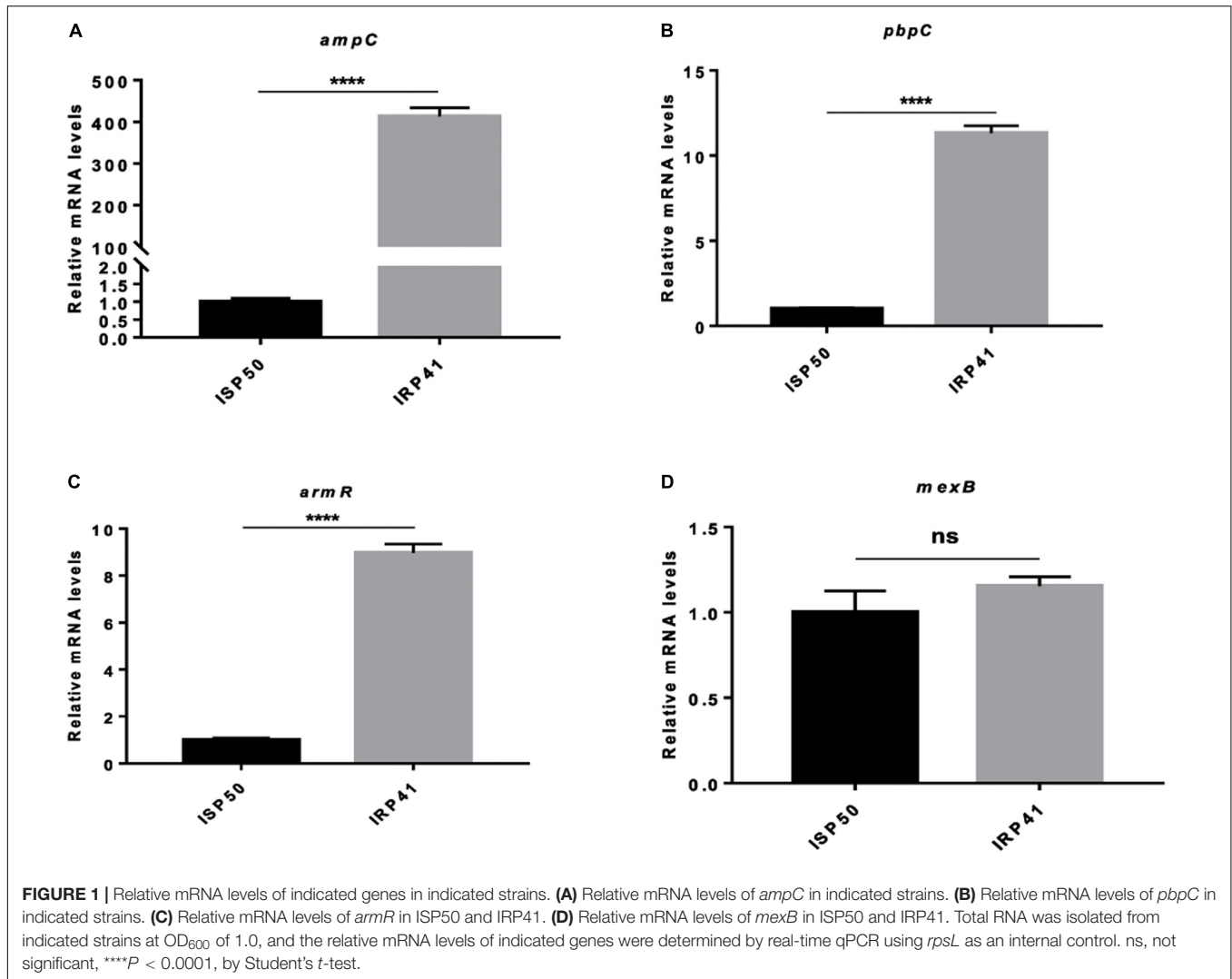
IRP41 Encodes an Inactive OprD

To further elucidate the molecular mechanisms of the decreased susceptibility to carbapenem in IRP41, genome reference mapping was performed to identify the accumulated mutations in the genomes of IRP41 and ISP50 in comparison with PAO1 reference genome (see text footnote 2). Between strains ISP50 and IRP41, there are 84 frameshifts (deletion/insertion) and 377 nonsynonymous single nucleotide variations (SNV) (including 17 early stops) (**Supplementary Table S4**). Among them, a G831A substitution in the *oprD* gene resulted in a premature termination of the OprD in the IRP41 strain. To confirm the

result, *oprD* genes were PCR amplified from the genomic DNA of strains ISP50 and IRP41. Sequence analysis of the amplicons revealed that *oprD* in ISP50 was the same as that of PAO1, while the *oprD* from IRP41 showed a G831A substitution, which confirmed the reference mapping results. To assess if the G831A substitution in *oprD* contributed to the reduced susceptibility of the IRP41 to carbapenems, the *oprD* gene from both ISP50 and IRP41 were expressed in the IRP41 strain background. As can be observed in **Table 2**, introduction of the *oprD*$_{ISP50}$ reduced the MIC of imipenem/meropenem/biapenem in IRP41, with 16/2/2-fold decrease, while the *oprD*$_{IRP41}$ did not. These results indicated that the G831A substitution in *oprD* indeed disrupted its function and lead to the reduced susceptibility to carbapenem in strain IRP41.

Mechanisms of *ampC* Hyper-Expression in IRP41

AmpR is a transcriptional regulator encoded immediately upstream of the *ampC*, regulating the *ampC* expression (Kong et al., 2005). The genome reference mapping result revealed that the *ampR* sequence of IRP41 is identical to that of PAO1, while in strain ISP50 an "A" was substituted by "G" at 821th position of the *ampR* gene, resulting in an E274G substitution. These results were further confirmed by sequencing of the PCR amplicons. To test if the nonsynonymous mutation in *ampR* of ISP50 resulted in a lower level of *ampC* expression, both *ampR* genes from IRP41 and ISP50 were cloned and expressed in the background of ISP50. As shown in **Supplementary Figure S3C** and **Supplementary Table S3**, expression of the *ampR*$_{ISP50}$ conferred ISP50 strain a higher *ampC* mRNA level and increased MIC against ampicillin compared to that of *ampR*$_{IRP41}$, although it had no observable influence on the MIC of imipenem/meropenem/biapenem. The *ampR*$_{ISP50}$ and *ampR*$_{IRP41}$ were further introduced into PAO1Δ*ampR*, and their effects on MICs to imipenem, meropenem, biapenem and ampicillin in PAO1Δ*ampR* were examined. As shown in **Supplementary Table S3**, both *ampR*$_{ISP50}$ and *ampR*$_{IRP41}$ restored the MIC against ampicillin and biapenem in PAO1Δ*ampR* to that in wild type PAO1 strain, while in contrast to *ampR*$_{IRP41}$, the *ampR*$_{ISP50}$ conferred PAO1Δ*ampR* a repeatable two-fold increase in MIC against imipenem compared to that of wild type PAO1. Unexpectedly, no influence on MIC of meropenem was observed in PAO1Δ*ampR*. These results suggested that A821G SNV of *ampR* in fact confers slightly higher *ampC* expression-activating capacity than the wild type AmpR, thus resulting in higher, rather than lower *ampC* in ISP50.

ldcA (PA5198) encodes a cytosolic LD-carboxypeptidase which removes the terminal amino acid D-alanine from the tetra peptide of the peptidoglycan (Korza and Bochtler, 2005). Our genome reference mapping and PCR amplicons sequencing results revealed that a C was deleted at 445 bp of the *ldcA* gene in strain IRP41, leading to a frameshift mutation after R149 of the LdcA. To test if this frameshift mutation in *ldcA* contributed to the elevated expression of the *ampC* in IRP41, we expressed the *ldcA*$_{IRP41}$ or *ldcA*$_{ISP50}$ (same as *ldcA* of PAO1) in the IRP41 background. As shown in **Figure 2A**, the relative mRNA level of

FIGURE 1 | Relative mRNA levels of indicated genes in indicated strains. **(A)** Relative mRNA levels of *ampC* in indicated strains. **(B)** Relative mRNA levels of *pbpC* in indicated strains. **(C)** Relative mRNA levels of *armR* in ISP50 and IRP41. **(D)** Relative mRNA levels of *mexB* in ISP50 and IRP41. Total RNA was isolated from indicated strains at OD_{600} of 1.0, and the relative mRNA levels of indicated genes were determined by real-time qPCR using *rpsL* as an internal control. ns, not significant, ****$P < 0.0001$, by Student's *t*-test.
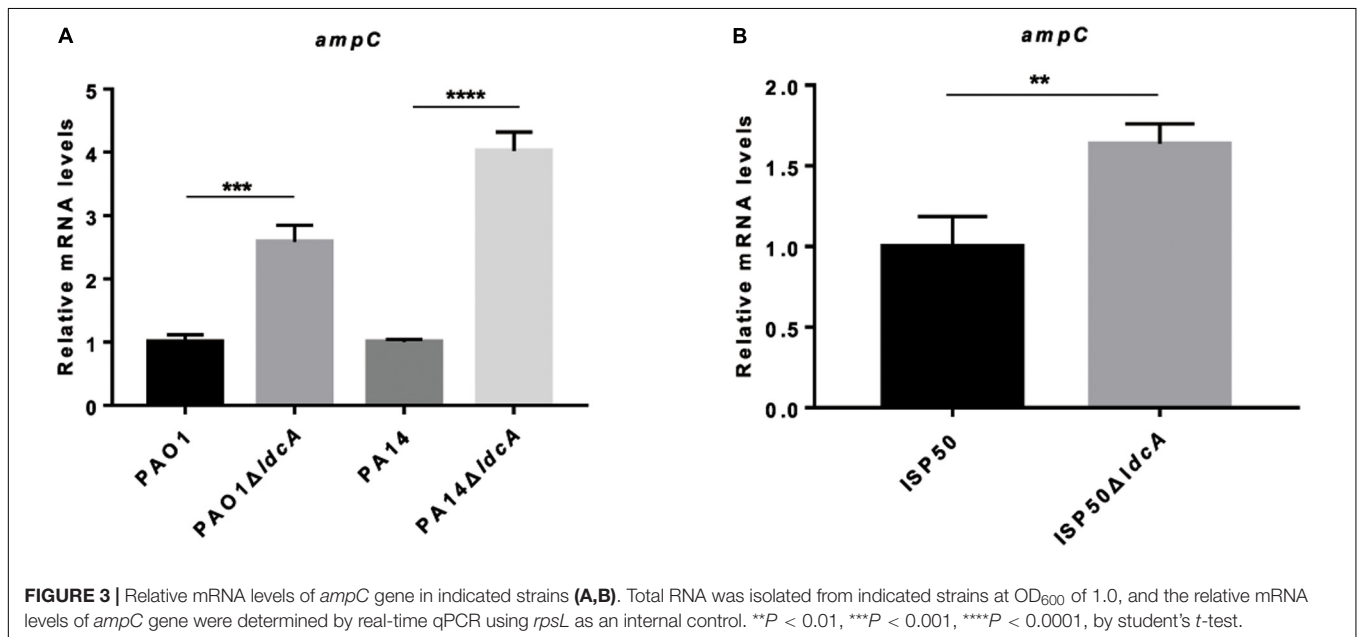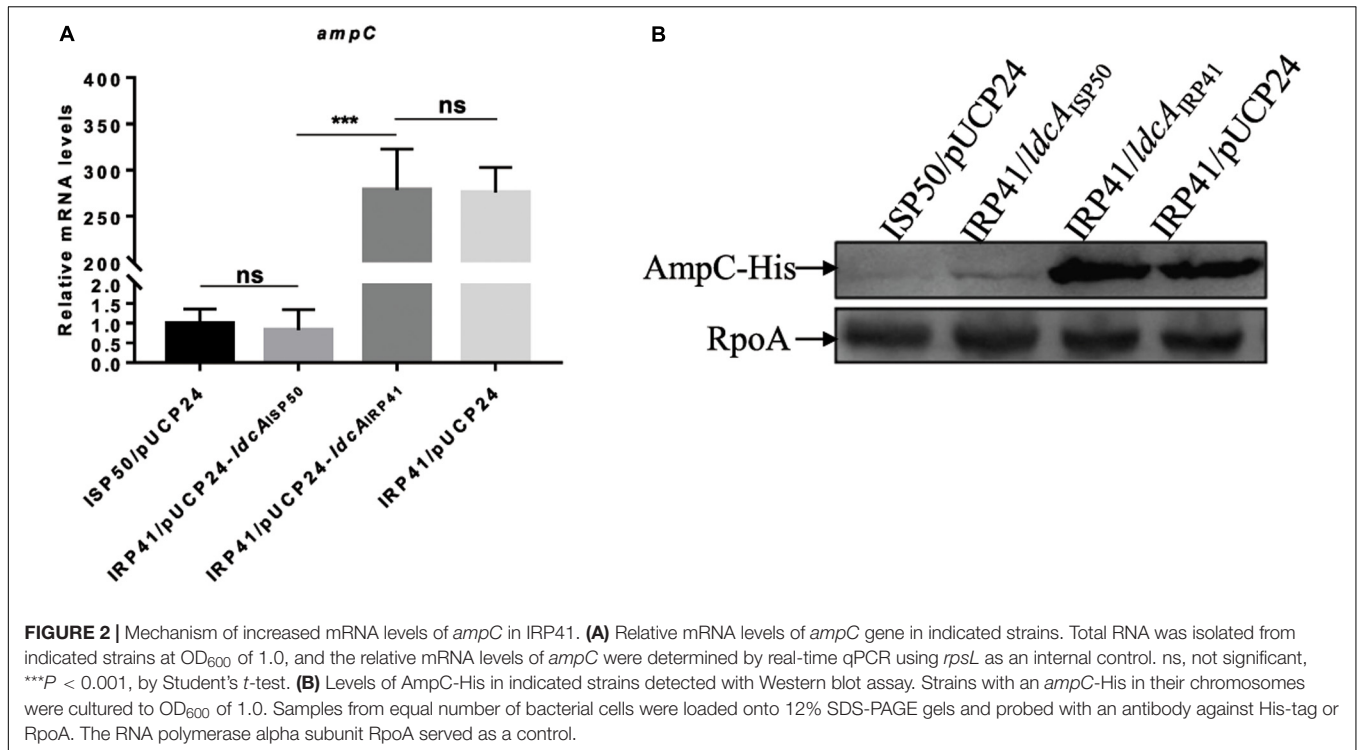
ampC in IRP41/pUCP24-*ldcA*$_{ISP50}$, but not in IRP41/pUCP24-*ldcA*$_{IRP41}$, was restored to that in ISP50/pUCP24, and the MIC to imipenem/meropenem/biapenem in IRP41/pUCP24-*ldcA*$_{ISP50}$ was decreased by two folds (**Table 2**). To confirm the expression level of *ampC* gene, a C-terminal His-tagged *ampC* driven by its native promoter was integrated into the genomes of ISP50 and IRP41. As shown in **Figure 2B**, the AmpC-His level was much higher in IRP41 than that in ISP50, and overexpression of *ldcA*$_{ISP50}$, but not *ldcA*$_{IRP41}$, reduced the amount of AmpC-His in the IRP41 to almost that of the ISP50. These results demonstrated that frameshift-mutation of the *ldcA* in IRP41 contributes to the increased expression of the *ampC* in IRP41.

To further confirm the effect of *ldcA* on *ampC* repression, the whole *ldcA* open reading frame was deleted in PA14 and PAO1 backgrounds, resulting in PA14Δ*ldcA* and PAO1Δ*ldcA*, respectively. Similar to previous report in *E. coli* (Templin et al., 1999), both of the *ldcA* deletion mutants showed a reduced growth rate and proneness to lysis during stationary growth phase (**Supplementary Figure S4**). Interestingly, the expression levels of *ampC* displayed 2.6-fold and 4.0-fold increase in

the Δ*ldcA* mutants compared to their parental strains PAO1 and PA14, respectively (**Figure 3A**). Accordingly, the MIC of ampicillin in PAO1Δ*ldcA* and PA14Δ*ldcA* displayed a two-fold and four-fold increase compared to their respective parental strains (**Table 2**). We further deleted the *ldcA* gene from the ISP50 strain. Consistent with the above observations, the expression level of *ampC* in the ISP50Δ*ldcA* increased 1.8-fold compared to that in the ISP50 strain (**Figure 3B**). The difference in fold increase of *ampC* mRNA levels between ISP50 and IRP41 (413 folds) vs. between ISP50 and ISP50Δ*ldcA* (1.8 folds) suggested that other factors also contributed to the increased expression of the *ampC* in IRP41.

In addition, we also examined the effect of *ldcA* mutation on MICs against piperacillin, ceftazidime, aztreonam, ciprofloxacin and tobramycin. Except for a two-fold decrease against ceftazidime and meropenem in PAO1Δ*ldcA*, no MICs change was observed for piperacillin, aztreonam, ciprofloxacin and tobramycin in *ldcA* mutants (**Table 4**). However, we didn't obtain the IRP41Δ*ldcA*, although deletion of *ldcA* in IRP41 strain had been tried for several times. We assume that it may

FIGURE 2 | Mechanism of increased mRNA levels of *ampC* in IRP41. **(A)** Relative mRNA levels of *ampC* gene in indicated strains. Total RNA was isolated from indicated strains at OD_{600} of 1.0, and the relative mRNA levels of *ampC* were determined by real-time qPCR using *rpsL* as an internal control. ns, not significant, ***$P < 0.001$, by Student's *t*-test. **(B)** Levels of AmpC-His in indicated strains detected with Western blot assay. Strains with an *ampC*-His in their chromosomes were cultured to OD_{600} of 1.0. Samples from equal number of bacterial cells were loaded onto 12% SDS-PAGE gels and probed with an antibody against His-tag or RpoA. The RNA polymerase alpha subunit RpoA served as a control.

FIGURE 3 | Relative mRNA levels of *ampC* gene in indicated strains **(A,B)**. Total RNA was isolated from indicated strains at OD_{600} of 1.0, and the relative mRNA levels of *ampC* gene were determined by real-time qPCR using *rpsL* as an internal control. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, by student's *t*-test.

be due to presence of some unknown factors to prevent the recombination in IRP41.

Since AmpR is the regulator of *ampC*, and its 274th amino acid is different between IRP41 and ISP50, we further compared the *ampC* expression levels and the MIC to ampicillin/imipenem/meropenem/biapenem between ISP50Δ*ldcA* expressing AmpR$_{ISP50}$ and AmpR$_{IRP41}$. As shown in **Supplementary Figure S3D**, consistent with that in the ISP50 strain, expression of AmpR$_{ISP50}$ in ISP50Δ*ldcA*

(ISP50Δ*ldcA*/pMMB-*ampR*$_{ISP50}$) resulted in a higher mRNA level of *ampC* compared to that of ISP50Δ*ldcA*/pMMB-*ampR*$_{IRP41}$, as well as a two-fold increase in MIC of ampicillin (**Supplementary Table S3**). Similar to that in ISP50 strain, overexpression of both AmpR$_{IRP41}$ and AmpR$_{ISP50}$ showed no influence on MIC of imipenem/meropenem/biapenem in the ISP50Δ*ldcA* strain. Again, these are consistent with the earlier observation that the AmpR point mutation in ISP50 confers a higher transcriptional activator activity on *ampC*.

Furthermore, as the expression level of *pbpC* is much higher in IRP41 than that in ISP50, we overexpressed *pbpC* in the ISP50Δ*ldcA* background to see if the elevated *pbpC* expression had any effect on the *ampC* mRNA levels. However, the mRNA level of *ampC* was not affected by the overexpression of *pbpC* in ISP50Δ*ldcA* (data not shown), consistent with such null effect observed in the ISP50 background (**Supplementary Table S3** and **Supplementary Figure S3E**).

IRP41 Is a Better Biofilm Former Than ISP50

Our RNA-seq result also revealed an increased expression of *pslD-pslL* operon (**Table 3**) which is involved in biofilm formation. Accordingly, we examined and compared the biofilm formation between IRP41 and ISP50 strains. As the results shown in **Figure 4**, the IRP41 strain produced more biofilm than the ISP50 strain.

DISCUSSION

In this study, we examined MICs of imipenem, meropenem and biapenem, three carbapenem antibiotics, for all the strains in **Table 2** and **Supplementary Table S3**. Compared to meropenem,

the contribution of AmpC overproduction and OprD deficiency to biapenem susceptibility in IRP41 resembles to that of imipenem. The difference among the susceptibility to imipenem, meropenem and biapenem between ISP50 and IRP41 may reflect their difference in penetration rate, stability to the hydrolysis by β-lactamase, PBP protein binding profile (Yang et al., 1995; Bonfiglio et al., 2002), β-lactamase deactivating capability and export by the efflux pumps (Chen and Livermore, 1994; Okamoto et al., 2002).

AmpC expression is regulated by AmpR, a LysR family transcriptional regulator (Kong et al., 2005). Previous studies reported that *ampR* gene point mutations were associated with increased *ampC* expression in both *Enterobacter cloacae* and *P. aeruginosa* clinical isolates (Kuga et al., 2000; Bagge et al., 2002). Our study presents E274G substitution in AmpR actually lead to a higher level of *ampC* expression. Besides the expression of *ampC*, AmpR regulates expression of hundreds of genes involved in diverse pathways such as physiological processes and metabolism (Balasubramanian et al., 2015). It has been demonstrated that *ampR* mutant displays an impaired growth under iron-limiting conditions and sensitive to many agents that affect cell growth (Balasubramanian et al., 2014, 2015). Thus, it is possible that restoration to wild type AmpR from E274G substituted AmpR of ISP50 provided the IRP41 strain

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TABLE 4 | MICs (μg/ml) of indicated *P. aeruginosa* strains.

Strains	TAZ (μg/ml)	Cipro (μg/ml)	Tob (μg/ml)	Pip (μg/ml)	AZT (μg/ml)	Mem (μg/ml)
PAO1/pUCP24	3.125	0.3125	8	31.25	1.953125	3.125
PAO1Δ*ldcA*/pUCP24	1.5625	0.3125	8	31.25	1.953125	1.5625
PAO1Δ*ldcA*/pUCP24-*ldcA*	3.125	0.3125	8	31.25	1.953125	3.125
ISP50/pUCP24	3.125	0.625	16	62.5	7.8125	3.125
ISP50Δ*ldcA*/pUCP24	3.125	0.625	16	62.5	7.8125	3.125
ISP50Δ*ldcA*/pUCP24-*ldcA*	3.125	0.625	16	62.5	7.8125	3.125

TAZ, ceftazidime; Cipro, ciprofloxacin; Tob, tobramycin; Pip, piperacillin; AZT, aztreonam; Mem, meropenem.
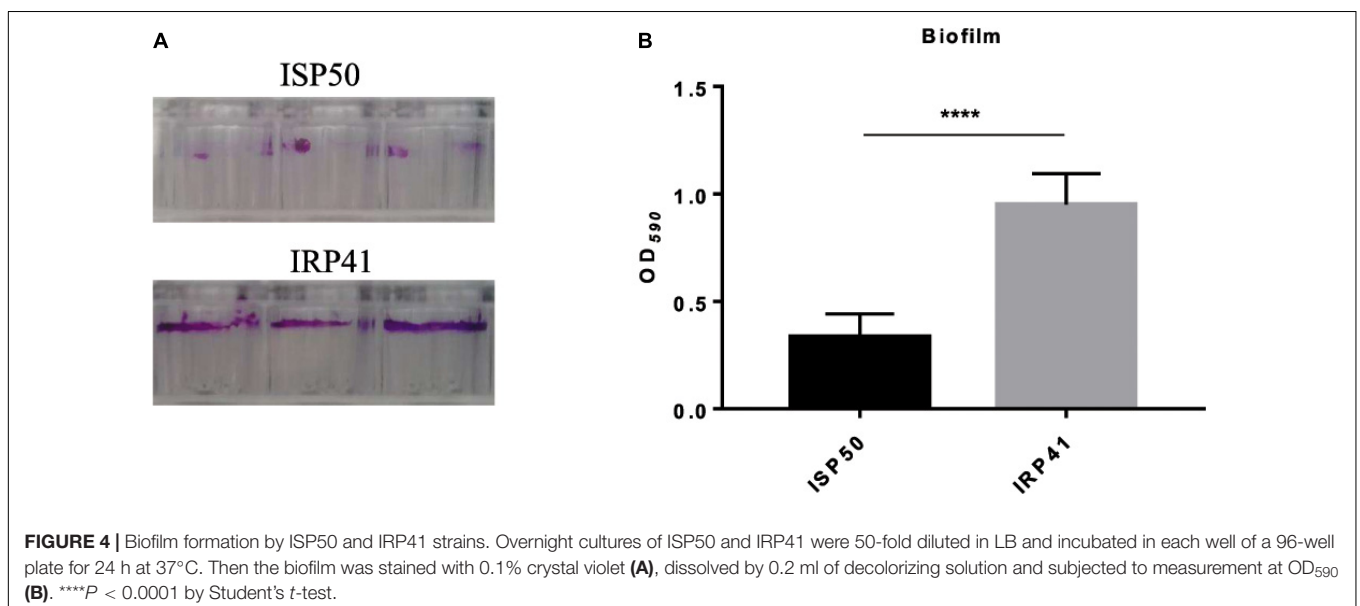

FIGURE 4 | Biofilm formation by ISP50 and IRP41 strains. Overnight cultures of ISP50 and IRP41 were 50-fold diluted in LB and incubated in each well of a 96-well plate for 24 h at 37°C. Then the biofilm was stained with 0.1% crystal violet **(A)**, dissolved by 0.2 ml of decolorizing solution and subjected to measurement at OD_{590} **(B)**. ****$P < 0.0001$ by Student's *t*-test.

a better adaption to the host environment. Also, an increased expression of the *pbpC* is neither responsible for the higher *ampC* expression nor the decreased susceptibility to carbapenem in IRP41 strain. The regulatory mechanism for the elevated *pbpC* expression in IRP41 and its functional roles remain to be investigated.

AmpD, a cytosolic peptidoglycan amidase, cleaves the peptide chain from *N*-acetyl-anhydromuramic acid peptides which are the inducer molecules for the *ampC* expression via binding to the AmpR (Langaee et al., 1998; Lister et al., 2009; Dhar et al., 2018). Mutations in AmpD and its two homologous proteins, AmpDh2 and AmpDh3, contribute to a stepwise de-repression of the *ampC* in the wild-type strain PAO1 (Juan et al., 2006). Similarly, *ampD* mutations leading to *ampC* de-repression have been reported in clinical isolates (Schmidtke and Hanson, 2008). However, some AmpC over-expressing *P. aeruginosa* strains do not exhibit mutation in *ampD*, *ampDh2*, *ampDh3*, *ampR*, or the *ampR–ampC* intergenic region (Wolter et al., 2007; Schmidtke and Hanson, 2008), suggesting that other undiscovered factors or pathways likely contribute to the upregulation of *ampC* expression. Besides, mutational inactivation of *dacB*, encoding penicillin-binding protein 4 (PBP4), has been reported to trigger a stable AmpC overproduction in *P. aeruginosa* (Moya et al., 2009). Mutation of the lytic transglycosylases encoding genes *sltB1* and *mltB1* in PAO1 resulted in a stable AmpC hyperproduction in the presence of β-lactam antibiotic (Cavallari et al., 2013; Juan et al., 2017). Inactivation of *mpl* (encoding cytosolic UDP-*N*-acetylmuramate:L-alanyl-γ-D-glutamyl-meso-diaminopimelate ligase) and *nuoN* (encoding NADH dehydrogenase I chain N) led to an increase in the expression of *ampC* (Tsutsumi et al., 2013; Juan et al., 2017). In the two isolates used in this study, no mutation was found in the above genes, except for the AmpR with E274G substitution in ISP50 which had no effect on the increased *ampC* expression in IRP41. These suggested the presence of unknown molecular basis driving AmpC hyperproduction in the IRP41 strain.

In the present study, inactivation of the *ldcA* contributes to the elevated expression of the *ampC* in *P. aeruginosa*. LdcA, encoding an LD carboxypeptidase, cleaves the D-alanine from *N*-acetyl-anhydromuramic acid tetrapeptides (anhNAM-P4) in *P. aeruginosa* (Korza and Bochtler, 2005), generating *N*-acetyl-anhydromuramic acid tripeptide (anhNAM-P3). Functional loss of the LdcA leads to the accumulation of anhNAM-P4, thus our observation suggests that the anhNAM-P3 is less potent inducer for the *ampC* expression than the anhNAM-P4. This is consistent with a previous study in which the anhNAM-P4 was shown to be a critical activator ligand for β-lactamase expression in *Stenotrophomonas maltophilia* (Huang et al., 2017). Results from previous studies implied anhNAM-P5 as the genuine AmpR-binding signal (Dietz et al., 1997; Lee et al., 2016) and further suggested that only muropeptides containing a terminal d-Ala-d-Ala motif (i.e., muropentapeptides) is capable of binding to the AmpR for *ampC* induction (Vadlamani et al., 2015; Dik et al., 2017). However, in a most recent report, anhNAM-P3 was demonstrated to function as an *ampC* inducer, albeit much less potent than the anhNAM-P5 (Torrens et al., 2019). As these studies were carried out under different conditions

(with or without antibiotic induction) using different mutant backgrounds, it is possible that different signaling pathways may have been involved in the AmpR mediated regulation of the *ampC* in the clinical strains analyzed in this study.

This is the first report linking *ldcA* to the regulation of *ampC* expression in *P. aeruginosa*. Interestingly, compared to the 400-fold increase of *ampC* mRNA in IRP41, the Δ*ldcA* mutants of PA14, PAO1 and ISP50 showed only 1.8–4 folds increases in the *ampC* mRNA compared to their parental strains. Such minor changes in the *ampC* expression, in addition to the growth defect of the *ldcA* mutant at stationary phase, may explain why LdcA was not identified as a repressor for the *ampC* in previous studies. One may argue that the observed 2–4 folds increase in MIC to ampicillin may be due to the reduced growth of the Δ*ldcA* mutant. However, we do not feel this is the case because the proneness to lysis happens during the stationary growth phase (OD_{600} about 2.0). In the case of IRP41, we postulate that there are other factors contributing to the increased *ampC* expression in addition to the *ldcA*. The mechanism underlying this observation is yet to be elucidated.

OprD has been reported to be the most prevalent cause of imipenem resistance among clinical isolates of *P. aeruginosa*. Insertion of various IS elements and point mutations with premature stops on the *oprD* gene had been associated with the carbapenem resistance among clinical isolates of *P. aeruginosa* (Hirabayashi et al., 2017). In this study, a G831A substitution in *oprD* resulted in loss of the OprD function. With the OprD inactivation and elevated *ampC* expression, the clinical isolate IRP41 displayed resistance to carbapenem. It has been demonstrated that derepression of AmpC in PAO1 has no obvious impact on the MIC of imipenem (Juan et al., 2006). While in another study, the loss of AmpC from PAO1 displayed a four-fold increase in susceptibility to imipenem (Masuda et al., 1999). Derepression of AmpC was associated with 4–8/8–64-fold increase in MIC of imipenem in an OprD$^+$/OprD$^-$ background, respectively (Livermore, 1992; Mushtaq et al., 2004). And also, mutational variants of the AmpC cephalosporinase provide *P. aeruginosa* with imipenem resistance (Rodriguez-Martinez et al., 2009). In our study, overexpression of *ampC* conferred ISP50 a 16-fold increase in MIC of imipenem. However, both AmpC and OprD are of PAO1-type in ISP50, which suggests that other unknown factors may contribute to the change in susceptibility to imipenem by the overexpression of AmpC.

MexAB-OprM is able to expel a wide variety of antibiotics (Kohler et al., 1999; Lister et al., 2009) and its expression is repressed by MexR (Poole et al., 1996). A 53-amino-acid long antirepressor, ArmR, could interact with and inhibit the MexR function, upregulating the *mexAB-oprM* expression (Wilke et al., 2008). However, in the present study, increased expression of *armR* in IRP41 did not cause altered expression level of *mexAB-oprM*. In fact, *mexAB-oprM* is expressed and contributes to antimicrobial resistance in wild type *Pseudomonas aeruginosa*, it is more difficult to detect *mexAB-oprM* overexpression in mutant cells than the other efflux pump encoding genes (e.g., *mexCD-oprJ*, *mexEF-oprN*). In addition, previous study has revealed MexR is a redox regulator which senses oxidative

stress inside bacterial cells to regulate *mexAB-oprM* expression (Chen et al., 2008). *nalD* encodes a second repressor of the *mexAB-oprM* (Morita et al., 2006). Mutations in *nalD* resulted in increased MexAB-OprM expression in lab and clinical isolates of *P. aeruginosa* (Sobel et al., 2005). However, no mutation occurred in the *nalD* gene of the IRP41 or ISP50 isolates. The mechanisms underlying these observations are under active investigation. It has been demonstrated that *nalC* (PA3721), encoding a TetR family repressor, negatively regulated *armR*, and S127P substitution in NalC impaired its *armR*-repressing capacity (Cao et al., 2004). Compared to ISP50, no mutations were observed in the intergenic region between *nalC* and PA3720, while a S127P substitution in NalC happened in IRP41 (**Supplementary Table S4**), which may be the cause to result in the increased *armR* transcriptional level in IRP41.

IRP41 is a better biofilm former than ISP50. However, the MICs against ciprofloxacin and levofloxacin in IRP41 are the same as ISP50 (data not shown), and the MICs against gentamicin and tobramycin display a two-fold decrease in IRP41 than that in ISP50 (data not shown). A previous study had shown that the Psl did not affect bacterial MIC to biapenem for planktonic cells (Murakami et al., 2017). Therefore, we assumed that the increased biofilm production did not contribute to the increased MIC to carbapenem in IRP41.

It is not necessary for clinical isolates processing a major resistance determinant against a certain antibiotic category to accumulate the other resistance mechanisms. However, the concomitant presence of multiple carbapenem resistance mechanisms has been observed in *P. aeruginosa* (Meletis et al., 2014). In our study, the overproduction of AmpC β-lactamase together with OprD deficiency leads imipenem-susceptible ISP50 to imipenem-resistant IRP41 derivative, as well as a reduced susceptibility to meropenem and biapenem.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI, under accession PRJNA635437.

ETHICS STATEMENT

We have a waiver from the ethics committee, exempting this study from the requirement to have ethics approval and written informed as the clinical strains used in the study come from the routine procedures of the clinical laboratory rather than the clinical trials.

REFERENCES

Bagge, N., Ciofu, O., Hentzer, M., Campbell, J. I., Givskov, M., and Hoiby, N. (2002). Constitutive high expression of chromosomal beta-lactamase in *Pseudomonas aeruginosa* caused by a new insertion sequence (IS1669) located in ampD. *Antimicrob. Agents Chemother.* 46, 3406–3411. doi: 10.1128/aac.46.11.3406-3411.2002

Balasubramanian, D., Kumari, H., Jaric, M., Fernandez, M., Turner, K. H., Dove, S. L., et al. (2014). Deep sequencing analyses expands the *Pseudomonas*

AUTHOR CONTRIBUTIONS

YJ and FB conceived and designed the experiments. CX, DW, XZ, HL, GZ, and TW performed the experiments. WW, FB, ZC, and YJ analyzed the data. YJ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01390/full#supplementary-material

FIGURE S1 | PCR results of ISP50 and IRP41 strains. **(A)** 16S rDNA gene amplification; **(B)** RAPD typing.

FIGURE S2 | Carbapenem susceptibility and carbapenemase production of indicated strains. **(A,B)** Imipenem inhibition zones of indicated strains on a 6-cm disk. **(C,D)** Meropenem inhibition zones of indicated strains on a 6-cm disk. **(E,F)** PAE-MHT assay using *K. pneumoniae* ATCC 700603 as indicator. Carbapenemase production test on imipenem **(E)** and meropenem **(F)** on a 10-cm disk, 0: ISP50, 1: IRP41, 2: PA-NK41, a producer of KPC2 carbapenemase.

FIGURE S3 | Relative mRNA levels of indicated genes in indicated strains. **(A,C–E)** Relative mRNA levels of *ampC* in indicated strains. **(B)** Relative mRNA levels of *pbpC* in indicated strains. Total RNA was isolated from indicated strains at OD_{600} of 1.0, and the relative mRNA levels of *ampC* or *pbpC* gene were determined by real-time qPCR using *rpsL* as an internal control. ns, not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, by Student's *t*-test.

FIGURE S4 | Growth curves of indicated strains.

TABLE S1 | Primers used in this study.

TABLE S2 | Transcriptome analysis: differentially expressed genes by RNAseq without those listed in **Table 2**.

TABLE S3 | MICs (μg/ml) of indicated *P. aeruginosa* strains.

TABLE S4 | SNVs identified by genome reference mapping with elimination of SNVs with synonymous mutation and the same mutation between ISP50 and IRP41.

aeruginosa AmpR regulon to include small RNA-mediated regulation of iron acquisition, heat shock and oxidative stress response. *Nucleic Acids Res.* 42, 979–998. doi: 10.1093/nar/gkt942

Balasubramanian, D., Kumari, H., and Mathee, K. (2015). *Pseudomonas aeruginosa* AmpR: an acute-chronic switch regulator. *Pathog. Dis.* 73, 1–14. doi: 10.1111/2049-632x.12208

Bonfiglio, G., Russo, G., and Nicoletti, G. (2002). Recent developments in carbapenems. *Expert Opin. Investig. Drugs* 11, 529–544. doi: 10.1517/13543784.11.4.529

Cao, L., Srikumar, R., and Poole, K. (2004). MexAB-OprM hyperexpression in NalC-type multidrug-resistant *Pseudomonas aeruginosa*: identification and characterization of the nalC gene encoding a repressor of PA3720-PA3719. *Mol. Microbiol.* 53, 1423–1436. doi: 10.1111/j.1365-2958.2004.04210.x

Cavallari, J. F., Lamers, R. P., Scheurwater, E. M., Matos, A. L., and Burrows, L. L. (2013). Changes to its peptidoglycan-remodeling enzyme repertoire modulate beta-lactam resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 57, 3078–3084. doi: 10.1128/aac.00268-13

Chen, H., Hu, J., Chen, P. R., Lan, L., Li, Z., Hicks, L. M., et al. (2008). The *Pseudomonas aeruginosa* multidrug efflux regulator MexR uses an oxidation-sensing mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 105, 13586–13591. doi: 10.1073/pnas.0803391105

Chen, H. Y., and Livermore, D. M. (1994). In-vitro activity of biapenem, compared with imipenem and meropenem, against *Pseudomonas aeruginosa* strains and mutants with known resistance mechanisms. *J. Antimicrob. Chemother.* 33, 949–958. doi: 10.1093/jac/33.5.949

Choudhury, D., Das Talukdar, A., Dutta Choudhury, M., Maurya, A. P., Paul, D., Dhar Chanda, D., et al. (2015). Transcriptional analysis of MexAB-OprM efflux pumps system of *Pseudomonas aeruginosa* and its role in carbapenem resistance in a tertiary referral hospital in India. *PLoS One* 10:e0133842. doi: 10.1371/journal.pone.0133842

Curran, B., Jonas, D., Grundmann, H., Pitt, T., and Dowson, C. G. (2004). Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 42, 5644–5649. doi: 10.1128/jcm.42.12.5644-5649.2004

Daigle, D. M., Cao, L., Fraud, S., Wilke, M. S., Pacey, A., Klinoski, R., et al. (2007). Protein modulator of multidrug efflux gene expression in *Pseudomonas aeruginosa*. *J. Bacteriol.* 189, 5441–5451. doi: 10.1128/jb.00543-07

Davies, T. A., Marie Queenan, A., Morrow, B. J., Shang, W., Amsler, K., He, W., et al. (2011). Longitudinal survey of carbapenem resistance and resistance mechanisms in *Enterobacteriaceae* and non-fermenters from the USA in 2007-09. *J. Antimicrob. Chemother.* 66, 2298–2307. doi: 10.1093/jac/dkr290

Dhar, S., Kumari, H., Balasubramanian, D., and Mathee, K. (2018). Cell-wall recycling and synthesis in *Escherichia coli* and *Pseudomonas aeruginosa* - their role in the development of resistance. *J. Med. Microbiol.* 67, 1–21. doi: 10.1099/jmm.0.000636

Dietz, H., Pfeifle, D., and Wiedemann, B. (1997). The signal molecule for beta-lactamase induction in *Enterobacter cloacae* is the anhydromuramyl-pentapeptide. *Antimicrob. Agents Chemother.* 41, 2113–2120. doi: 10.1128/aac.41.10.2113

Dik, D. A., Dominguez-Gil, T., Lee, M., Hesek, D., Byun, B., Fishovitz, J., et al. (2017). Muropeptide binding and the X-ray structure of the effector domain of the transcriptional regulator AmpR of *Pseudomonas aeruginosa*. *J. Am. Chem. Soc.* 139, 1448–1451. doi: 10.1021/jacs.6b12819

Hang, Y., Chen, Y., Xue, L., Sun, S., Liu, L., Gao, J., et al. (2018). Evaluating biapenem dosage regimens in intensive care unit patients with *Pseudomonas aeruginosa* infections: a pharmacokinetic/pharmacodynamic analysis using Monte Carlo simulation. *Int. J. Antimicrob. Agents* 51, 484–487. doi: 10.1016/j.ijantimicag.2017.07.005

Hirabayashi, A., Kato, D., Tomita, Y., Iguchi, M., Yamada, K., Kouyama, Y., et al. (2017). Risk factors for and role of OprD protein in increasing minimal inhibitory concentrations of carbapenems in clinical isolates of *Pseudomonas aeruginosa*. *J. Med. Microbiol.* 66, 1562–1572. doi: 10.1099/jmm.0.000601

Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J., and Schweizer, H. P. (1998). A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212, 77–86. doi: 10.1016/s0378-1119(98)00130-9

Hoban, D. J., Jones, R. N., Yamane, N., Frei, R., Trilla, A., and Pignatari, A. C. (1993). In vitro activity of three carbapenem antibiotics. Comparative studies with biapenem (L-627), imipenem, and meropenem against aerobic pathogens isolated worldwide. *Diagn. Microbiol. Infect. Dis.* 17, 299–305. doi: 10.1016/0732-8893(93)90039-a

Huang, Y. W., Wang, Y., Lin, Y., Lin, C., Lin, Y. T., Hsu, C. C., et al. (2017). Impacts of penicillin binding protein 2 Inactivation on beta-Lactamase expression and muropeptide profile in *Stenotrophomonas maltophilia*. *mSystems* 2:17. doi: 10.1128/mSystems.00077-17

Juan, C., Moya, B., Perez, J. L., and Oliver, A. (2006). Stepwise upregulation of the *Pseudomonas aeruginosa* chromosomal cephalosporinase conferring high-level beta-lactam resistance involves three AmpD homologues. *Antimicrob. Agents Chemother.* 50, 1780–1787. doi: 10.1128/aac.50.5.1780-1787.2006

Juan, C., Torrens, G., Gonzalez-Nicolau, M., and Oliver, A. (2017). Diversity and regulation of intrinsic beta-lactamases from non-fermenting and other Gram-negative opportunistic pathogens. *FEMS Microbiol. Rev.* 41, 781–815. doi: 10.1093/femsre/fux043

Kaufman, M. R., Jia, J., Zeng, L., Ha, U., Chow, M., and Jin, S. (2000). *Pseudomonas aeruginosa* mediated apoptosis requires the ADP-ribosylating activity of exoS. *Microbiology* 146(Pt 10), 2531–2541. doi: 10.1099/00221287-146-10-2531

Kohler, T., Michea-Hamzehpour, M., Epp, S. F., and Pechere, J. C. (1999). Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. *Antimicrob. Agents Chemother.* 43, 424–427. doi: 10.1128/aac.43.2.424

Kong, K. F., Jayawardena, S. R., Indulkar, S. D., Del Puerto, A., Koh, C. L., Hoiby, N., et al. (2005). *Pseudomonas aeruginosa* AmpR is a global transcriptional factor that regulates expression of AmpC and PoxB beta-lactamases, proteases, quorum sensing, and other virulence factors. *Antimicrob. Agents Chemother.* 49, 4567–4575. doi: 10.1128/aac.49.11.4567-4575.2005

Korza, H. J., and Bochtler, M. (2005). *Pseudomonas aeruginosa* LD-carboxypeptidase, a serine peptidase with a Ser-His-Glu triad and a nucleophilic elbow. *J. Biol. Chem.* 280, 40802–40812. doi: 10.1074/jbc.M506328200

Kuga, A., Okamoto, R., and Inoue, M. (2000). ampR gene mutations that greatly increase class C beta-lactamase activity in *Enterobacter cloacae*. *Antimicrob. Agents Chemother.* 44, 561–567. doi: 10.1128/aac.44.3.561-567.2000

Langaee, T. Y., Dargis, M., and Huletsky, A. (1998). An ampD gene in *Pseudomonas aeruginosa* encodes a negative regulator of AmpC beta-lactamase expression. *Antimicrob. Agents Chemother.* 42, 3296–3300. doi: 10.1128/aac.42.12.3296

Lee, M., Dhar, S., De Benedetti, S., Hesek, D., Boggess, B., Blazquez, B., et al. (2016). Muropeptides in *Pseudomonas aeruginosa* and their role as elicitors of beta-lactam-antibiotic resistance. *Angew. Chem. Int. Ed. Engl.* 55, 6882–6886. doi: 10.1002/anie.201601693

Li, K., Xu, C., Jin, Y., Sun, Z., Liu, C., Shi, J., et al. (2013). SuhB is a regulator of multiple virulence genes and essential for pathogenesis of *Pseudomonas aeruginosa*. *mBio* 4:e419-13. doi: 10.1128/mBio.00419-13

Liberati, N. T., Urbach, J. M., Miyata, S., Lee, D. G., Drenkard, E., Wu, G., et al. (2006). An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2833–2838. doi: 10.1073/pnas.0511100103

Lister, P. D., Wolter, D. J., and Hanson, N. D. (2009). Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin. Microbiol. Rev.* 22, 582–610. doi: 10.1128/cmr.00040-09

Liu, Y., Li, X. Y., Wan, L. G., Jiang, W. Y., Li, F. Q., and Yang, J. H. (2013). Efflux system overexpression and decreased OprD contribute to the carbapenem resistance among extended-spectrum beta-lactamase-producing *Pseudomonas aeruginosa* isolates from a Chinese university hospital. *Microb. Drug Resist.* 19, 463–468. doi: 10.1089/mdr.2013.0010

Livermore, D. M. (1992). Interplay of impermeability and chromosomal beta-lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 36, 2046–2048. doi: 10.1128/aac.36.9.2046

Long, Y., Fu, W., Li, S., Ren, H., Li, M., Liu, C., et al. (2019). Identification of novel genes that promote persister formation by repressing transcription and cell division in *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 74, 2575–2587. doi: 10.1093/jac/dkz214

Mahenthiralingam, E., Campbell, M. E., Foster, J., Lam, J. S., and Speert, D. P. (1996). Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J. Clin. Microbiol.* 34, 1129–1135. doi: 10.1128/jcm.34.5.1129-1135.1996

Masuda, N., Gotoh, N., Ishii, C., Sakagawa, E., Ohya, S., and Nishino, T. (1999). Interplay between chromosomal beta-lactamase and the MexAB-OprM efflux system in intrinsic resistance to beta-lactams in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 43, 400–402. doi: 10.1128/aac.43.2.400

Mcfarland, J. (1907). The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *J. Am. Med. Assoc.* 49, 1176–1178. doi: 10.1001/jama.1907.25320140022001f

Meletis, G., Vavatsi, N., Exindari, M., Protonotariou, E., Sianou, E., Haitoglou, C., et al. (2014). Accumulation of carbapenem resistance mechanisms in

VIM-2-producing *Pseudomonas aeruginosa* under selective pressure. *Eur. J. Clin. Microbiol. Infect. Dis.* 33, 253–258. doi: 10.1007/s10096-013-1952-3

Mirsalehian, A., Kalantar-Neyestanaki, D., Nourijelyani, K., Asadollahi, K., Taherikalani, M., Emaneini, M., et al. (2014). Detection of AmpC-beta-lactamases producing isolates among carbapenem resistant *P. aeruginosa* isolated from burn patient. *Iran J. Microbiol.* 6, 306–310.

Morita, Y., Cao, L., Gould, V. C., Avison, M. B., and Poole, K. (2006). nalD encodes a second repressor of the mexAB-oprM multidrug efflux operon of *Pseudomonas aeruginosa*. *J. Bacteriol.* 188, 8649–8654. doi: 10.1128/jb.013 42-06

Moya, B., Dotsch, A., Juan, C., Blazquez, J., Zamorano, L., Haussler, S., et al. (2009). Beta-lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. *PLoS Pathog.* 5:e1000353. doi: 10.1371/journal.ppat. 1000353

Murakami, K., Ono, T., Viducic, D., Somiya, Y., Kariyama, R., Hori, K., et al. (2017). Role of psl Genes in Antibiotic Tolerance of Adherent *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 61:e002587-16. doi: 10.1128/aac.02587-16

Mushtaq, S., Ge, Y., and Livermore, D. M. (2004). Doripenem versus *Pseudomonas aeruginosa* in vitro: activity against characterized isolates, mutants, and transconjugants and resistance selection potential. *Antimicrob. Agents Chemother.* 48, 3086–3092. doi: 10.1128/aac.48.8.3086-3092.2004

Okamoto, K., Gotoh, N., and Nishino, T. (2002). Alterations of susceptibility of *Pseudomonas aeruginosa* by overproduction of multidrug efflux systems, MexAB-OprM, MexCD-OprJ, and MexXY/OprM to carbapenems: substrate specificities of the efflux systems. *J. Infect. Chemother.* 8, 371–373. doi: 10.1007/s10156-002-0193-7

Pasteran, F., Veliz, O., Rapoport, M., Guerriero, L., and Corso, A. (2011). Sensitive and specific modified Hodge test for KPC and metallo-beta-lactamase detection in *Pseudomonas aeruginosa* by use of a novel indicator strain, *Klebsiella pneumoniae* ATCC 700603. *J. Clin. Microbiol.* 49, 4301–4303. doi: 10.1128/jcm. 05602-11

Poole, K. (2011). *Pseudomonas aeruginosa*: resistance to the max. *Front. Microbiol.* 2:65. doi: 10.3389/fmicb.2011.00065

Poole, K., Tetro, K., Zhao, Q., Neshat, S., Heinrichs, D. E., and Bianco, N. (1996). Expression of the multidrug resistance operon mexA-mexB-oprM in *Pseudomonas aeruginosa*: mexR encodes a regulator of operon expression. *Antimicrob. Agents Chemother.* 40, 2021–2028. doi: 10.1128/aac.40.9. 2021

Potron, A., Poirel, L., and Nordmann, P. (2015). Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology. *Int. J. Antimicrob. Agents* 45, 568–585. doi: 10.1016/j.ijantimicag.2015.03.001

Rodriguez-Martinez, J. M., Poirel, L., and Nordmann, P. (2009). Extended-spectrum cephalosporinases in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53, 1766–1771. doi: 10.1128/aac.01410-08

Schmidtke, A. J., and Hanson, N. D. (2008). Role of ampD homologs in overproduction of AmpC in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 52, 3922–3927. doi: 10.1128/aac.00341-08

Schweizer, H. P. (1992). Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable oriT and the counter-selectable Bacillus subtilis sacB marker. *Mol. Microbiol.* 6, 1195–1204. doi: 10.1111/j.1365-2958.1992.tb01558.x

Sobel, M. L., Hocquet, D., Cao, L., Plesiat, P., and Poole, K. (2005). Mutations in PA3574 (nalD) lead to increased MexAB-OprM expression and multidrug resistance in laboratory and clinical isolates of *Pseudomonas aeruginosa*.

Antimicrob. Agents Chemother. 49, 1782–1786. doi: 10.1128/aac.49.5.1782-1786.2005

Spilker, T., Coenye, T., Vandamme, P., and LiPuma, J. J. (2004). PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. *J. Clin. Microbiol.* 42, 2074–2079. doi: 10.1128/jcm.42.5.2074-2079.2004

Templin, M. F., Ursinus, A., and Holtje, J. V. (1999). A defect in cell wall recycling triggers autolysis during the stationary growth phase of *Escherichia coli*. *EMBO J.* 18, 4108–4117. doi: 10.1093/emboj/18.15.4108

Torrens, G., Hernandez, S. B., Ayala, J. A., Moya, B., Juan, C., Cava, F., et al. (2019). Regulation of AmpC-Driven beta-Lactam resistance in *Pseudomonas aeruginosa*: different pathways, different signaling. *mSystems* 4:e00524-19. doi: 10.1128/mSystems.00524-19

Tsutsumi, Y., Tomita, H., and Tanimoto, K. (2013). Identification of novel genes responsible for overexpression of ampC in *Pseudomonas aeruginosa* PAO1. *Antimicrob. Agents Chemother.* 57, 5987–5993. doi: 10.1128/aac.01291-13

Vadlamani, G., Thomas, M. D., Patel, T. R., Donald, L. J., Reeve, T. M., Stetefeld, J., et al. (2015). The beta-lactamase gene regulator AmpR is a tetramer that recognizes and binds the D-Ala-D-Ala motif of its repressor UDP-N-acetylmuramic acid (MurNAc)-pentapeptide. *J. Biol. Chem.* 290, 2630–2643. doi: 10.1074/jbc.M114.618199

Vincent, J. L., Bihari, D. J., Suter, P. M., Bruining, H. A., White, J., Nicolas-Chanoin, M. H., et al. (1995). The prevalence of nosocomial infection in intensive care units in Europe. results of the european prevalence of infection in intensive care (EPIC) study. EPIC international advisory committee. *JAMA* 274, 639–644. doi: 10.1001/jama.274.8.639

West, S. E., Schweizer, H. P., Dall, C., Sample, A. K., and Runyen-Janecky, L. J. (1994). Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* 148, 81–86. doi: 10.1016/0378-1119(94)90237-2

Wilke, M. S., Heller, M., Creagh, A. L., Haynes, C. A., McIntosh, L. P., Poole, K., et al. (2008). The crystal structure of MexR from *Pseudomonas aeruginosa* in complex with its antirepressor ArmR. *Proc. Natl. Acad. Sci. U.S.A.* 105, 14832–14837. doi: 10.1073/pnas.0805489105

Wolter, D. J., Schmidtke, A. J., Hanson, N. D., and Lister, P. D. (2007). Increased expression of ampC in *Pseudomonas aeruginosa* mutants selected with ciprofloxacin. *Antimicrob. Agents Chemother.* 51, 2997–3000. doi: 10.1128/aac.00111-07

Yang, Y., Bhachech, N., and Bush, K. (1995). Biochemical comparison of imipenem, meropenem and biapenem: permeability, binding to penicillin-binding proteins, and stability to hydrolysis by beta-lactamases. *J. Antimicrob. Chemother.* 35, 75–84. doi: 10.1093/jac/35.1.75