Infection and Drug Resistance

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ORIGINAL RESEARCH

Study on the Role of *ampG* in the Regulation of Plasmid-Mediated *ampC* -Induced Expression in *Klebsiella pneumoniae*

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Objective: In this study, we constructed *ampG* knock-out and knock-in strains from a clinically isolated Kp1strain carrying *ampR-ampC* in its plasmid and compared them with the Kp NTUH-K2044 strain to investigate the relationship between *ampG* and *ampR-ampC*-induced expression.

Methods: We created the *ampG* gene deletion mutant strains Kp1-Δ*ampG* and Kp NTUH-K2044-Δ*ampG* with pKO3-km plasmid using homologous recombination technology. We constructed the Kp NTUH-K2044-RC and Kp NTUH-K2044-Δ*ampG*-RC drug resistance model strains with plasmid pACYC184. We constructed the *ampG* knock-in strains by introducing the *ampG* genes of Kp1, *Enterobacter cloacae* 029M, *Pseudomonas aeruginosa* PAO1, *Escherichia coli* ATCC25922, and *Salmonella typhimurium* LT2 into the *ampG* gene-deleted strains with carrier pet-30a. Real-time polymerase chain reaction (real-time PCR) was used to detect the relative expressions of *ampC* and *ampG* mRNAs.

Results: Compared with Kp1, the induction phenotype of the *ampC* of Kp1-Δ*ampG* strain disappeared, the *ampC* expression was reduced, and the minimal inhibitory concentration (MIC) values of cefoxitin and ceftazidime significant decrease from 128 μg/mL to 1 μg/mL. Based on Kp1, five strain were successfully constructed to complement the *ampG* genes from five knock-in strain, and all of the above complemented strains showed inducible expression of *ampC* and restored the expression of *ampG* to varying degrees, as well as restored resistance to the antimicrobial drugs cefoxitin and ceftazidime ($P < 0.05$). The *ampC* and *ampG* genes were barely expressed in Kp NTUH-K2044-Δ*amp*G-RC when compared with Kp NTUH-K2044-RC. The expressions of *ampG* and *ampC* in each knock-in strain were recovered, the induction phenotype of *ampC* was restored, and the MIC values of cefoxitin and ceftazidime were increased. ($P < 0.05$).

Conclusion: In this study, we found that *ampG* was an essential regulator for the plasmid-mediated *ampC*-induced expression in *K. pneumoniae*.

Keywords: *ampC* inducible expression, AmpG regulator, gene knockout, gene knock-in, *Klebsiella pneumoniae*

Introduction

The chromosome *ampR-ampC* module is required for the β-lactam antibiotic resistance in *Enterobacteriaceae*. The bla *ampC* gene is elevated in the presence of an inducer (β-lactam antibiotics). The outer membrane protein is encoded by *ampG*, which is a transmembrane protein that is involved in promoting the recycling of murein degradation products. It influences the production of *ampC* by transmitting information about changes in biological mediators to the cytoplasm and regulating the expression of related genes, both of which are critical in *ampC* expression.[1,2] Although the *ampR-ampC* gene is naturally absent on the *Klebsiella pneumoniae* chromosome, *K. pneumoniae* Kp1, which carries *ampR-ampC* in plasmids, was clinically isolated. It exhibits high levels of *ampC* induced expression (DHA-1 type), which has emerged as a critical element of bacterial drug resistance.[3]

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The *Klebsiella pneumoniae* plasmid-mediated extended-spectrum β-lactamases (ESBLs) and AmpC are the primary contributors to its multi-drug resistance. The *ampC* gene is naturally absent from its chromosomes. However, the *ampC* gene can be introduced via plasmid so as to achieve sustained high expression of *ampC*, which has become a crucial component of bacterial drug resistance. Without enzyme inhibitors, the production of AmpC results in the hydrolysis of the third-generation cephalosporins, monobactams, and cephamycins antibiotics. The main regulators of chromosome-mediated *ampC* expression are *ampC, ampD, ampR, ampG*, and *ampE*. The transmembrane protein AmpG, located on the outer membrane of the cell, plays a key role in the production of *ampC* by transmitting information about changes in biological mediators to the cytoplasm and regulating the expression of related genes. However, the role of AmpG in plasmid-mediated *ampC*-induced expression is unclear.

The majority of current studies on drug resistance in *K. pneumoniae* focus on the detection of drug resistance phenotypes and genotypes, while the functional analysis of drug resistance genes is rarely reported. The gene knockout technique is the most effective method for studying gene function.[4–6] In this study, we used gene knockout technology based on the temperature-sensitive, high-efficiency suicide vector plasmid pKO3-km to knock out the drug-resistant gene *ampG* in *K. pneumoniae*, construct the *ampG* gene deletion mutant strain, and knock in the *ampG* genes of five kinds of bacteria into the gene knockout strains. In the same genetic background, we took up a more comprehensive study of the differences between the *ampG* gene deletion strain and the wild-type strain so as to make a more accurate assessment and gather insights into gene function.

Materials and Methods

Materials

The strains and plasmids used in this experiment are listed in Table 1.

Methods

Construction of the Kp1-Δ*ampG*, Kp NTUH-K2044-Δ*ampG*, Kp NTUH-K2044-RC, and Kp NTUH-K2044-Δ*ampG*-RC strains.

We used PCR to amplify the upstream and downstream homologous arm fragments of the experimental strain *ampG*. Next, we used the gene splicing by overlap extension PCR (SOE-PCR) method to obtain the upstream and downstream homologous arm fusion fragments of *ampG* genes, which were cloned to the temperature-sensitive suicide vector pKO3-km after digestion to construct pKO3-km-Δ*ampG* recombinant plasmids. The plasmids were electroporated into competent *K. pneumoniae* Kp1 and Kp NTUH-K2044 strains, respectively.

Table 1 Strains and Plasmids Used in This Study

Strains and Plasmids	Gene or Characteristic	Sources
Strains		
K. pneumoniae strains		
Kp1	producing plasmid-mediated DHA-1 AmpC	Stored in this laboratory
Kp NTUH-K2044	Clinical isolates collected from National TaiWan University Hospital (NTUH; serotype KI;Amp^r,Tet^s,$SacB^S$)	Donated by Jin-Town Wang
E.coli DH5a	φ80d/lacZ M15,recAl,endAl,gyrA96,thi-l,hsdRl7(r.k-.,m.k+.),supE44,relAl,deoR, (1acZrA-argF)U169	Stored in this laboratory
E.coli ATCC 25922	sensitive to all antibiotics	Stored in this laboratory
E.Cloacae 029M	*Enterobacter cloacae* with depressing constitutive hyperproduction of AmpC	Stored in this laboratory
P.aeruginosa PAO1		Stored in this laboratory
S.*typhimurium* LT2		Stored in this laboratory
Plasmids		
pACYC184-ampCR	*ampC*$^+$, *ampR*$^+$, Cm^R	Stored in this laboratory
pKO3-Km	Km^R	Donated by Jin-Town Wang
pet-30a	Km^R, Cm^R	Stored in this laboratory

We screened the *ampG* deletion mutant strains of *K. pneumoniae*, Kp1-Δ*ampG* and Kp NTUH-K2044-Δ*ampG* using homologous recombination technology and the temperature-sensitive characteristics of pKO3-km. Our laboratory team had earlier constructed the recombinant plasmid pACYC184-*ampCR* (*ampC+, ampR+*, Cm^R).[7] We then obtained the *K. pneumoniae* model strains Kp NTUH-K2044-RC and Kp NTUH-K2044-Δ*ampG*-RC carrying the exogenous *ampC* gene by electroporating the competent Kp NTUH-K2044 and NTUH-K2044-Δ*ampG* with plasmids carrying the *ampC* and *ampR* genes of the Kp1strain.

Construction of Δ*ampG::ampG* Knock-in Strains

Construction of the Pet-30a-*ampG* Recombinant Plasmid

The five bacterial DNAs were used as templates to amplify the *ampG* genes of each strain, and the corresponding primers are listed in Table 2. The 50 μL PCR reaction system was as follows: 5×buffer 10 μL, dNTP 4 μL, *ampG*-F/R 2 μL, bacterial genomic DNA template 2 μL, PrimeSTAR HS DNA Polymerase 1 μL, sterilized deionized water 29 μL. The reaction conditions were as follows: 98°C for 10s, 58°C for 5s, 72°C for 1 min 30s for a total of 30 cycles and then 72°C for 7 min. We used electrophoresis to detect the specificity and concentration of PCR products and the PCR products were purified and recovered with reagent kits for use later. We subjected the above-purified PCR products of the five bacterial *ampG* genes and plasmid pet-30a to double enzyme digestion. The *ampG* fragment enzyme digestion system (20 μL) contained 8 μL of gene product, 1 μL of *SacI* endonuclease, 2 μL of buffer, 1 μL of *EcoRI* endonuclease, and 8 μL of water, which was incubated at 37°C for 24 hours. The plasmid pet-30a digestion system (50 μL) contained 25 μL of plasmid, 2.5 μL of *SacI* endonuclease, 5 μL of buffer, 2.5 μL of *EcoRI* endonuclease, and 15 μL of water, which was incubated at 37°C for 24 hours. The byproduct of the enzyme digestion was isolated and recovered. The digested *ampG* gene fragment and pet-30a were ligated using T4DNA ligase and transformed into competent *E. coli* DH5a cells. Then it was coated on LB medium (containing km 50 μg/mL) and cultured overnight at 30°C. The monoclonal colonies were selected, and plasmids were extracted for double enzyme digestion and identification to obtain recombinant plasmid pet-30a-*ampG* for the construction of complementary strains.

Construction of Δ*ampG::ampG* Strain

We used the solid plate method to prepare the Kp1-Δ*ampG*, Kp NTUH-K2044-RC, and KpNTUH-K2044-Δ*ampG-RC* competent cells.[8] We obtained a total of 100 μL competent cells, which were placed along with 400 ng of pet-30a-*ampG* plasmid in an electric shock cup with a diameter of 0.2 cm. The cup was chilled on ice for 15 minutes, and electroporated with the parameters of 25 μF, 200 Ω, and 2.5 kV. It was immediately transferred into 800 μL LB liquid medium and incubated at 30°C for 3 hours. The transformed products were all coated onto the LB plates (Km, 50 μg/mL) and incubated overnight at 30°C. Monoclonal colonies were selected, and plasmids were extracted. Each recombinant plasmid was identified with primers as listed in Table 2. Positive clones were determined when the products obtained by PCR amplification were consistent with the *ampG* fragments of each strain. The obtained knock-in strains were named as:

Table 2 Primer Sequences of the *Klebsiella Pneumoniae ampG* Knock-in Strains

Primer	Sequence (5′→3′)	Fragment Length (bp)
PAO1-ampG-F	CGGAATTCATGACTCAGCAATCCTGGCGAGAG	1785bp
PAO1-ampG-R	GTAGAGCTCTCAGTGCTGCTCGGCGTTCTGG	
S.tyLT2 -ampG-F	CCGGAATTCATGTCAAGTCAATATTTACG	1476bp
S.tyLT2 -ampG-R	GTAGAGCTCTTACAGCAGGCGTGTTTTTC	
E.clo029M-ampG-F	CGGAATTCATGTCCAGTCACTACTTACGT	1476bp
E.clo029M-ampG-R	GTAGAGCTCTCATGTCATCTGCGTTTTCC	
E.coli- ampG-F	CGCGAATTCATGTCCAGTCAATATTTACGTAT	1476bp
E.coli- ampG-R	GTAGAGCTCTCATGTCATCTGCGTTTTCC	
Kp1-ampG-F	CGCGAATTCATGTCCAGTCATTACTTACGCATTTTTCAGCAGCCG	1476bp
Kp1-ampG-R	GTGGAGCTCTTAAATCAGGCGGGTTTTACGCAGCGCCAGATAGTCA	

Notes: The underlined GAATTC is the *EcoRI* restriction endonuclease; GAGCTC is the *SacI* restriction endonuclease.

Kp1-Δ*ampG* knock-in strains: Kp1-Δ*ampG*::Kp1; Kp1-Δ*ampG*:: 029M; Kp1-Δ*ampG*::PAO1; Kp1-Δ*ampG*::25922; Kp1-Δ*ampG*::LT2.

Kp NTUH-K2044-Δ*ampG*-RC knock-in strains: Kp NTUH-K2044-Δ*ampG*-RC::Kp1; KpNTUH-K2044-Δ*ampG*-RC::029M; Kp NTUH-K2044-Δ*ampG*-RC::PAO1; Kp NTUH-K2044-Δ*ampG*-RC::25922;Kp NTUH-K2044-Δ*ampG*-RC:: LT2.

The mRNA Expressions of *ampG* and *ampC* Detected by RT-PCR

We carried out RNA extraction as per the instructions of the Shanghai Sangon columnar bacterial total RNA extraction and purification kit. The reverse transcription was done according to the instructions of the Shanghai Sangon M-MuLV first-strand cDNA synthesis kit and stored at −20°C for further use. The real-time PCR reaction system and conditions were readied as per the usage instructions of the SYBR Premix Ex Taq II reagent manufactured by TaKaRa Company of Japan, the primers used are listed in Table 3, and three trials were performed in parallel for each gene. We calculated the average value of the obtained Ct value with 16SrRNA as the internal reference. We used the following formula to calculate the relative expressions of *ampC* and *ampG* genes: ΔΔCT value and relative content of $2^{-\Delta\Delta Ct}$. ΔCt=Ct target gene-Ct16SrRNA. We compared Kp1 and its clones with Kp1-Δ*ampG*, and Kp NTUH-K2044 and its clones with Kp NTUH-K2044-Δ*ampG-RC*.

We used analysis of variance (ANOVA) to statistically analyze the relative expressions of *ampG* and *ampC* in each experimental strain of *K. pneumoniae*. $P < 0.05$ indicated a statistically significant difference.

ampC Induction Experiment

Using the disk diffusion method,[9] the experimental strain was turned into a 0.5 M bacterial solution, which was evenly coated on M-H agar plates. Cefoxitin (FOX, 30 μg/tablet) was pasted as an in vitro inducer of *ampC* in the center of the plate, and Cefotaxime (CTX, 30 μg/tablet), Ceftazidime (CAZ, 30 μg/tablet), Cefepime (FEP, 30 μg/tablet), Piperacillin (PRL, 100 μg/tablet) and Cefoperazone/sulbactam (SCF, 105 μg/tablet) drug-sensitive paper sheets were pasted around it as indicators, with a distance of 20 mm between the center of each paper piece. The plate was incubated at 37°C for 18–20 hours. The result of the *ampC* induction experiment was deemed positive if any circle on the surrounding disk exhibited a truncation phenomenon on the side close to cefoxitin.

Minimal Inhibitory Concentration (MIC) Detection

The minimal inhibitory concentration (MIC) value was determined by the agar dilution method.[10] M-H agar plates containing cefoxitin (FOX), ceftazidime (CAZ), cefuroxime (CXM), and cefazolin (KZ) were prepared, and the detection range of the drug MIC concentration gradient was 1–1024 μg/mL.

Table 3 Primer Sequences Used in RT-PCR

Gene	Primer Sequence (5′-3′)	Product Length (bp)
E.coli -F	GCTTCTTATCGTGCTGTAT	126
E.coli-R	TAGTAAGCCAAGCGTTTT	
PAO1-F	CAACCACCAACTGCTCTCG	138
PAO1-R	CCACGGCGACAGGCAGAT	
S.ty LT2-F	TACTGCTCATTGTGCTTTAT	187
S.ty LT2-R	GCGAAACAGCGAAAGACG	
E.clo 029M -F	TGCTGCTGATTGTCCTTTAT	139
E.clo 029M -R	CGGAACAGCGTCAGGCG	
Kp1-F	TGTTCACCATTCCCACTTCG	115
Kp1-R	ATTCTGCTGCTCATCGTCCT	
ampC-F	ACGGTGTGACCAACGAGGT	134
ampC-R	CGGAATAAAGGCGACATAGG	
16SrRNA -F	ATGACCAGCCACACTGGAAC	241
16SrRNA -R	CTTCCTCCCCGCTGAAAGTG	

Results

Successful Construction of Kp1-Δ*ampG*, Kp NTUH-K2044-Δ*ampG*, Kp NTUH-K2044-RC and Kp NTUH-K2044-Δ*ampG-RC* Strains

The *ampG* genes in the experimental strain Kp1 and the wild-type strain Kp NTUH-K2044 were successfully knocked out using homologous recombinant technology and the temperature-sensitive suicide vector pKO3-km, and we constructed the *ampG* gene deletion strains Kp1-Δ*ampG* and Kp NTUH-K2044-Δ*ampG*. The Kp NTUH-K2044-RC and Kp NTUH-K2044-Δ*ampG-RC* clonal strains were obtained by recombinant plasmid pACYC184-*ampCR*.

Construction of Δ*ampG::ampG*

Construction of Pet-30a-*ampG*

The genomes of five bacteria were used as templates, and the *ampG* gene of each strain was amplified by PCR with the primers listed in Table 2. We successfully obtained the *ampG* gene fragments of Kp1, *S. typhimurium* LT2, *E. cloacae* 029M, *E. coli* ATCC25922 strains with a length of 1476 bp and the *ampG* gene fragment of PAO1 with a length of 1785 bp. After double enzyme digestion of the PCR products of the *ampG* gene of the five bacteria, we derived the recombinant plasmid pet-30a-*ampG* by linking the products to plasmid pet-30a-*ampG* through T4DNA ligase. The plasmids with positive clones were identified using double enzyme digestion. The recombinant plasmid with *ampG* fragments of Kp1, *E. cloacae* 029M, *E. coli* ATCC25922 and *S. typhimurium* LT2 had a length of 6898 bp. After digestion by *EcoRI* and *SacI*, two clear bands appeared. One was a linear vector with a length of 5422 bp, and the other was a target fragment with a length of 1476 bp. The recombinant plasmid with PAO1 had a length of 7207 bp. As expected, two clear bands appeared after digestion, with one being 5422 bp and the other being 1785 bp. The pet-30a vector was successfully connected to the *ampG* gene fragment.

Identification of Δ*ampG::ampG* Knock-in Strain

The recombinant plasmids pet-30a-*ampG* derived from five bacteria were electroporated into competent *K. pneumoniae* strains Kp1-Δ*ampG* and Kp NTUH-K2044-Δ*ampG-RC*. Positive monoclonal colonies cultured overnight were selected for plasmid extraction. Each recombinant plasmid was identified with the primers listed in Table 2. We found that 1785 bp *ampG* fragment could be obtained in the strain knocked in with *ampG* of PAO1, and 1476bp *ampG* fragment was found in the strain knocked in with *ampG* of *E. coli* ATCC25922, *E. cloacae* 029M, *S. typhimurium* LT2, and Kp1, which was in line with expectations.

RT-PCR Detection Results for the mRNAs of *ampG* and *ampC*

The mRNAs of *ampG* and *ampC* were found in each knock-in strain, but the degree of expression was slightly different.

Compared with wild-type Kp1, the expressions of *ampC* and *ampG* significantly increased in Kp1-Δ*ampG* that knocked in with the *ampG* from itself ($P < 0.05$). In the strain knocked in with *ampG* of *E. cloacae* 029M, the expression of *ampC* increased ($P < 0.05$), and the level of *ampG* reached that in Kp1. In the strains knocked in with *ampG* of PAO1, *E. coli* ATCC25922, and *S. typhimurium* LT2, the expression of *ampC* was lower than that in Kp1 and the expression of *ampG* decreased as well ($P < 0.05$) (Figures 1A and B).

Compared with Kp NTUH-K2044-RC, the expression of *ampC* was slightly reduced in Kp NTUH-K2044-Δ*ampG*-RC knocked in with *ampG* of Kp1 and *E. cloacae* 029M, while the expression of *ampG* was significantly increased ($P < 0.05$). In the strains knocked in with *ampG* of PAO1, *E. coli* ATCC25922, and *S. typhimurium* LT2, the expression of *ampG* was also correspondingly reduced, and the expression of *ampC* decreased significantly ($P < 0.05$) (Figure 1C and D).

ampC Induction Experiment

The Kp1 strain is a DHA-1-producing *K. pneumoniae* with plasmid-mediated *ampC* expression, carrying the *ampR-ampC* gene. The drug susceptibility results showed that Kp1was resistant to cefoxitin, exhibiting an *ampC*-mediated resistance phenotype (Figure 2A). When the *ampG* gene was knocked out, Kp1-Δ*ampG* showed sensitivity to cefoxitin, and the *ampC*-induced phenotype disappeared (Figure 2B). The Kp1-Δ*ampG*, which knocked in with the *ampG* genes of

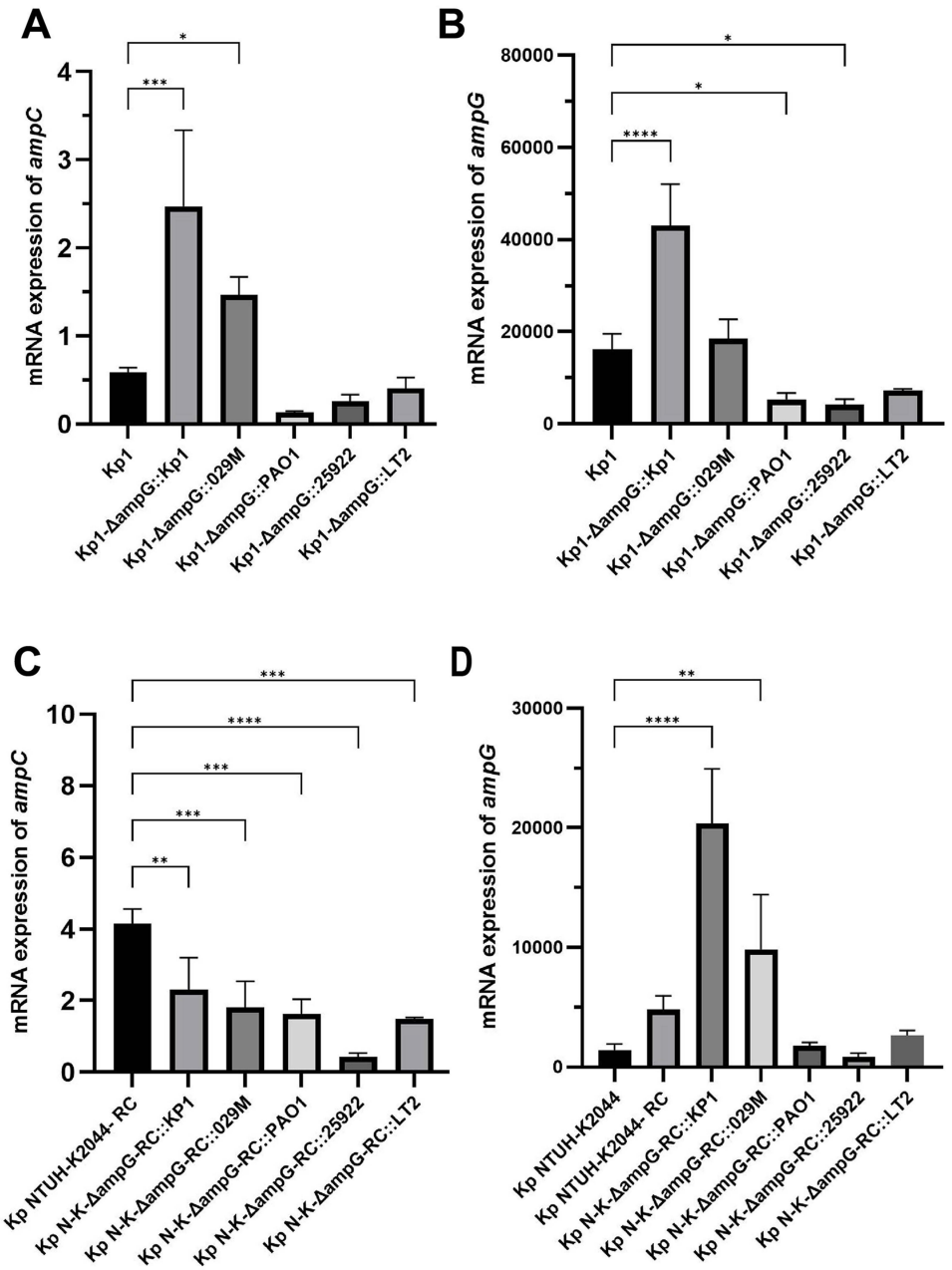

Figure 1 mRNA expression of experimental strains *ampG* and *ampC*. (**A**) mRNA expression of *ampC* in Kp1 and Kp1-ΔampG::ampG. (**B**) mRNA expression of *ampG* in Kp1 and Kp1-ΔampG::ampG. (**C**) mRNA expression of *ampC* in Kp NTUH-K2044 and Kp NTUH-K2044-ΔampG::ampG. (**D**) mRNA expression of *ampG* in Kp NTUH-K2044 and Kp NTUH-K2044-ΔampG::ampG.

Notes: ****Represents the comparison with the wild-type strain, *P*<0.0001; *** represents the comparison with the wild-type strain, *P*<0.001; **Represents the comparison with the wild-type strain, *P*<0.01; * represents the comparison with the wild-type strain, *P*<0.05.

Kp1 and *E. cloacus* 029M, showed drug resistance to cefoxitin, recovered induced phenotype, and truncation phenomenon (Figure 2C and D). In the strains knocked in with the *ampG* genes of *P. aeruginosa* PAO1, *E. coli* ATCC25922, and *S. typhimurium* LT2, the induced phenotype was not recovered, but they all showed drug resistance to cefoxitin (Figure 2E-G).

The Kp NTUH-K2044 is a non-*ampC*-producing strain that does not have the *ampR-ampC* gene. It was sensitive to all the drugs that were tested, and it did not exhibit the *ampC* induction phenomenon (Figure 2H). Also, we did not observe induction phenomenon in the *ampG*-knockout Kp NTUH-K2044 strain (Figure 2I). When the exogenous plasmid pACYC184-*ampRC* was introduced into the Kp NTUH-K2044 strain, it showed drug resistance to cefoxitin, and the

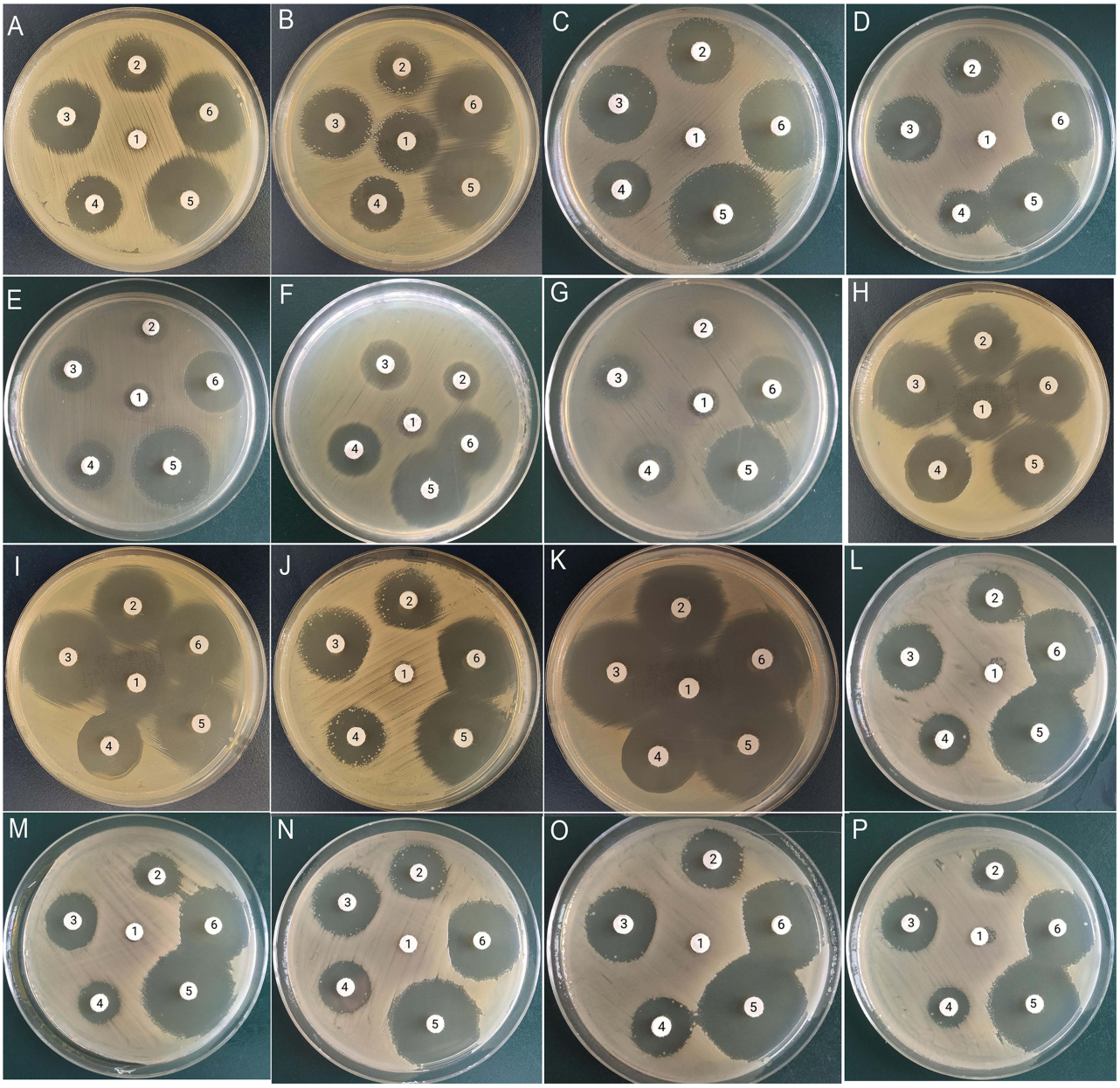

Figure 2 *ampC* induction experiment. 1: cefoxitin; 2: ceftazidime; 3: cefotaxime; 4: piperacillin; 5: cefepime; 6: cefoperazone/sulbactam; (**A**) Kp1; (**B**) Kp1-ΔampG strain; (**C**) Kp1-ΔampG::Kp1strain; (**D**) Kp1-ΔampG::029M strain; (**E**) Kp1-ΔampG::PAO1 strain; (**F**) Kp1-ΔampG::25922 strain (**G**) Kp1-ΔampG::LT2 strain; (**H**) Kp NTUH-K2044 strain; (**I**) Kp NTUH-K2044-ΔampG strain; (**J**) Kp NTUH-K2044-RC strain; (**K**) Kp NTUH-K2044-ΔampG-RC strain; (**L**) Kp NTUH-K2044-ΔampG-RC::Kp1strain; (**M**) Kp NTUH-K2044 -ΔampG-RC::029M strain; (**N**) Kp NTUH-K2044-ΔampG-RC::PAO1 *ampG* strain; (**O**) Kp NTUH-K2044-ΔampG-RC::25922 strain; (**P**) Kp NTUH-K2044-ΔampG-RC::LT2.
Notes: Using disk 1 as the inducer, if the induced phenotype exists in the experimental strain, there is a circle truncation phenomenon between disk 1 and the rest of the disks.

ampC induction experiment was positive (Figure 2J). When *ampR-ampC* was introduced into its *ampG* knockout strain, the strain regained sensitivity to cefoxitin, and the *ampC* induction experiment was negative (Figure 2K). In Kp NTUH-K2044-Δ*ampG*-RC that knocked in with the *ampG* genes of Kp1, *E. cloacae* 029M, *P. aeruginosa* PAO1, *E. coli* ATCC25922, and *S. typhimurium* LT2, they all showed drug resistance to cefoxitin, recovered *ampC*-induced phenotypes, and truncation phenomena (Figure 2L-P).

MIC Values of Each Experimental Strain

Kp1was resistant to cefoxitin, ceftazidime, cefuroxime, and cefazoline. When the *ampG* gene was knocked out, Kp1-Δ*ampG* became sensitive to the above antibacterial drugs, and the MIC value decreased, cefuroxime was reduced from

Table 4 Phenotype Detection of AmpC in the Experimental Strain

Strains	Gene		AmpC Induction Experiment	MIC Value of the Antibiotics (μg/mL)			
	ampR-ampC	*ampG*		CXM	KZ	CAZ	FOX
Kp1	+	+	+	512	512	128	128
Kp1-ΔampG	+	-	-	4	2	1	1
Kp1-ΔampG::Kp1	+	+	+	64	>1024	128	128
Kp1-ΔampG:: 029M	+	+	+	512	>1024	128	128
Kp1-ΔampG::PAO1	+	+	-	64	>1024	128	128
Kp1-ΔampG::25922	+	+	-	64	256	32	32
Kp1-ΔampG:: LT2	+	+	-	64	>1024	64	64
Kp NTUH-K2044	-	+	-	2	1	<1	4
Kp NTUH-K2044-RC	+	+	+	512	512	128	128
Kp NTUH-K2044-ΔampG	-	-	-	1	0.5	<1	4
Kp NTUH-K2044-ΔampG-RC	+	-	-	2	1	<1	<1
Kp NTUH-K2044-ΔampG-RC::Kp1	+	+	+	512	>1024	128	128
KpNTUH-K2044-ΔampG- RC::029M	+	+	+	512	>1024	128	128
KpNTUH-K2044-ΔampG-RC::PAO1	+	+	+	512	>1024	128	128
Kp NTUH-K2044-ΔampG- RC::25922	+	+	+	512	>1024	128	128
Kp NTUH-K2044-ΔampG-RC:: LT2	+	+	+	512	>1024	128	128

512 μg/mL to 4 μg/mL, cefazolin was reduced from 512 μg/mL to 2 μg/mL, and ceftazidime and cefoxitin were both reduced from 128 μg/mL to 1 μg/mL. When the *ampG* genes of the five strains were knocked in, all Kp1-Δ*ampG::ampG* strains showed drug resistance and increased MIC values, resistance to all four antimicrobials returned to the Kp1 resistance level, with resistance to cefazolin increasing to 1024 μg/mL, twice the Kp1 resistance level.

Kp NTUH-K2044 and Kp NTUH-K2044-Δ*ampG* strains were sensitive to cefoxitin, ceftazidime, cefuroxime, and cefazoline. When *ampR-ampC* was introduced, Kp NTUH-K2044-RC showed resistance to various antibacterial drugs with an increased MIC value, MIC values consistent with Kp1 resistance levels, while Kp NTUH-K2044-Δ*ampG-RC* was sensitive with a decreased MIC value. The NTUH-K2044-Δ*ampG-RC::ampG* strains that were knocked in with the *ampG* genes of five bacteria showed resistance to drugs, and the MIC value was increased, cefuroxime increased from 2 μg/mL to 512 μg/mL, cefazolin increased from 1 μg/mL to >1024 μg/mL, and ceftazidime and cefoxitin both increased from <1μg/mL to 128μg/mL (Table 4).

Discussion

Nosocomial infections are frequently caused by the gram-negative bacillus *K. pneumoniae*. The primary source of drug resistance in *K. pneumoniae* is its ability to produce ESBLs and AmpC. Drug resistance in gram-negative bacilli against new broad-spectrum β-lactam antibiotics is mostly attributable to AmpC β-lactamases, commonly known as cephalosporinase.[11] AmpC expression was previously thought to be minimal and to be mediated only by the chromosome. However, plasmid-mediated *ampC* has increased, most often with sustained high expression, in recent years. Clinical anti-infection treatment has been complicated by the detection of *K. pneumoniae* strains with *ampC*-induced expression.[12,13]

AmpC encoded by structural gene *ampC*, and its expression is controlled by the amp complex operons *ampC, ampR, ampD, ampG*, and *ampE*.[14] There is an activator, *ampR*, and an inhibitor, *ampD*. When no β-lactam antibiotic is present to function as an inducer, *ampD* blocks the production of *ampR*, resulting in low expression of *ampC*. In the presence of the inducer, *ampR* may no longer be inhibited by *ampD*, resulting in high production of *ampC*. This kind of expression is linked to the synthesis and degradation of peptidoglycan in the cell wall and requires the involvement of the *ampR* regulator. Furthermore, *ampG* plays a vital role as a transporter in the murein cycle.[15] In earlier experiments, the AmpG topology of 20 strains of DHA-1-producing *K. pneumoniae* with plasmid-mediated *ampC* expression was predicted using

the Expasy online software SOSUI, and it was determined that AmpG is a transmembrane protein with 12 transmembrane structures, with its N and C terminals exposed outside the membrane. However, more research and confirmation are required to establish the role of *ampG* in the control of plasmid-mediated *ampC*-induced expression.

Knocking out the target gene and investigating its role by comparing pre- and post-knockout strain phenotypic alterations is the most direct method for studying gene function. To test whether the AmpG regulator can control the plasmid-mediated *ampC*-induced expression in *K. pneumoniae*, we chose two different *K. pneumoniae* strains and knocked out the *ampG* gene. The *ampRC* gene was found on the plasmid of the DHA-1-producing clinical strain Kp1, which had been previously isolated in the laboratory. Professor Wang Jintang of the National Taiwan University generously provided the other strain, known as Kp NTUH-K2044. No *ampRC* gene was located on any of the chromosomes or plasmids in the genome of the strain, making it a perfect model strain for studies on the mechanism of *ampC*-mediated drug resistance in *K. pneumoniae*.

The research on gene knockouts now relies heavily on homologous recombination strategies based on effective suicide vector systems. In this study, we selected a temperature-sensitive suicide vector, pKO3-km. The first round of recombination occurred in culture at 43°C, and the second round of recombination occurred at 30°C. Finally, the positive clonal strains were screened. The positive strains were screened out by resistance, with continuous bacterial recombination induced in fresh medium. The purpose was clear, and the positive rate was high. The unique capsule structure of *K. pneumoniae* results in a low transformation efficiency of plasmids. To address this issue, we used clones in solid medium to directly prepare competent cells, which increased the transformation efficiency of *K. pneumoniae*. The gene knockout strains constructed with this method do not leave any traces, which lays a foundation for further double and multiple gene knockouts and the construction of knock-in strains.

The Kp1strain carries the *ampC* gene and has a positive *ampC*-induced phenotype. When its *ampG* gene is knocked out, *ampC* is not expressed, and the *ampC*-induced phenotype is negative. The MIC of β-lactam antibiotics is significantly reduced. (Table 4) It can be seen that the *ampG* gene plays an important role in the expression of *ampC*. This conclusion is consistent with the findings of Daniel et al in 2008 that the loss of *ampG* in *N. gonorrhoeae* caused a significant increase in the release of peptidoglycan fragments,[16] and the reports of Schmidt et al, in which nitrosoguanidine was used for chemical-induced mutation of *E. coli* SN0301, so as to obtain an *ampG* mutant strain, which led to a loss of strain inductivity.[17]

The *ampRC* gene was absent in the Kp NTUH-K2044 wild-type strain, with no expression of *ampC*. The *ampC* induced phenotype was negative, and the strain was sensitive to cephalosporin antibiotics. When *ampG* gene was knocked out, the above indicators of the strain Kp NTUH-K2044-Δ*ampG* did not change. The pACYC184-a*mpRC* expression vector that was constructed earlier in the laboratory was introduced into KpNTUH-K2044-Δ*ampG*. Although the recombinant Kp NTUH-K2044-Δ*ampG-RC* obtained the exogenous *ampRC* gene, no *ampC* expression was found, the *ampC* induction experiment was negative, due to the knockout of *ampG*. Nevertheless, when the recombinant strain Kp NTUH-K2044-RC obtained the exogenous *ampRC* gene, the mRNA expression of *ampC* was 4.145 ± 0.415 ($P < 0.05$), and the *ampC* induction experiment was positive. Antibiotic resistance also increased significantly.(Table 4) It may be due to the high copy number of the pACYC184-*ampRC* plasmid and the resulting high expression of the *ampRC* gene, which significantly increases bacterial drug resistance. The above findings show that *ampG* is a necessary regulator for plasmid-mediated *ampC*-induced expression.

In order to further explore the function of *ampG* in *ampC* induced expression, we introduced the *ampG* genes of *K. pneumoniae* Kp1, *E. cloacae* 029M, *P. aeruginosa* PAO1, *E. coli* ATCC25922, and *S. typhimurium* LT2 into Kp1-Δ*ampG* and Kp NTUH-K2044-Δ*ampG-RC* strains, respectively. By detecting the differences in levels of *ampC* and *ampG* in each strain, the induced phenotype of *ampC*, and the change in MIC values, we analyzed the compensatory effect of *ampG* derived from different bacterial sources on *ampG* of *K. pneumoniae*. The expressions of *ampC* was observed in each knock-in strain of Kp1-Δ*ampG::ampG*, and the expression level was slightly different compared with that of the Kp1 wild-type strain. The expression of *ampC* in Kp1-Δ*ampG*::Kp1 increased most significantly ($P < 0.05$), when its own *ampG* gene was knocked into Kp1-Δ*ampG*, the expression of *ampC* was significantly increased to 1.47 ± 0.203 in Kp1-Δ*ampG*::029M ($P < 0.05$).

The corresponding *ampC* induction phenotype of the above two knock-in strains recovered, and these strains changed from being sensitive to being resistant to cephalosporins. The MIC values significantly increased, which returned to the resistance level of Kp1.

There was a statistically significant ($P<0.05$) reduction in *ampG* expression compared to Kp1 in the knock-in strains Kp1-*ampG*::P AO1, Kp1-*ampG*::25922, and Kp1-*ampG*::LT2. The induction of *ampC* failed to occur, whereas cefoxitin resistance was seen. After gene knock-in, previously sensitive bacteria become resistant to cephalosporin drugs. These results indicate that these strains produced *ampC*, but it was a low output. The yielded *ampC* was not enough to produce an induced phenotype. Therefore, it manifested as cefoxitin resistance.

We performed a Basic Local Alignment Search Tool (BLAST) comparison between the *ampG* gene in the above strains and the *ampG* gene in *K. pneumoniae* to further explore the reasons for the above induction phenomenon. We found that the *ampG* gene of *K. pneumoniae* had 84% homology with that of *E. cloacae*, 82% homology with *S. typhimurium* LT2, and no homology with that of *E. coli* ATCC25922 and *P. aeruginosa* PAO1. Although the *ampG* gene of *S. typhimurium* LT2 had 82% homology with that of *K. pneumoniae*, the expressions of *ampC* and *ampG* still did not reach the expression level of wild-type, and the induced phenotype was not restored.

The *ampG* genes of Kp1 and *E. cloacae* 029M could completely compensate for the deletion of the *ampG* gene in Kp1, while the *ampG* genes of *E. coli* ATCC25922, *S. typhimurium* LT2, and *P. aeruginosa* PAO1 could partially make up for the deficiency. We speculate that this discrepancy may be due to the difference between the *ampG* origins of *K. pneumoniae* and the above strains. Therefore, it only partially compensated for the *ampG* gene deletion background of *K. pneumoniae*, manifesting as resistance to cefoxitin without the corresponding induction phenotype. In 2010, Zhang et al confirmed that *ampG* was an essential gene for *ampC* induced expression in *P. aeruginosa*, and the *ampG* gene of *E. coli* only partially compensated for the production of *ampC*.[18] In their study in 2012, using Xanthomonas str. 17, Yang et al reported that *ampG* was a key gene required for *ampC*-induced expression in *Xanthomonas*.[19] The overexpression of *ampG* gene could lead to an increase in *ampC* expression, and the *ampG* gene of *E. coli* only partially compensated for its *ampG* deletion. Our findings are consistent with the above conclusions that the *ampG* gene of *E. coli* can only partially compensate for the *ampG* gene of *K. pneumoniae*.

When the *ampG* genes of five strains were knocked into the Kp NTUH-K2044-Δ*ampG-RC* strain, RT-PCR showed *ampC* expression in each strain. Compared with Kp NTUH-K2044-RC, the *ampC* and *ampG* expression levels were significantly increased when the *ampG* of Kp1 and *E. cloacae* 029M were knocked in ($P < 0.05$). In contrast, when the *ampG* of *P. aeruginosa* PAO1, *E. coli* ATCC25922, and *S. typhimurium* LT2 were knocked in, the *ampC* expression decreased ($P < 0.05$) when compared with Kp NTUH-K2044-RC. The corresponding *ampG* expression was lower as well. However, despite the *ampC* expression of the above knock-in strains being lower than that of Kp NTUH-K2044-RC, it was sufficient to restore the *ampC* induced phenotype, and these strains showed resistance to cefoxitin. The MICs of cefuroxime and cefazolin increased to 512 ug/mL and > 1024 ug/mL, and the MICs of ceftazidime and cefoxitin both reached 128 ug/mL.

Since plasmid-mediated *ampC* has an inducible mechanism, certain powerful cephalosporin antibiotics regularly used in clinical practice frequently promote high expression of *ampC*, leading to the development of drug-resistant strains. Additional drug-resistance genes, such as extended-spectrum β-lactamases or drug-resistant integrators, may be expressed alongside *ampC* in clinical strains, making their genetic environment even more complicated.[20]

Conclusion

In summary, the role of *ampG* in plasmid-mediated *ampC* induced expression was validated by the knockout of the *ampG* gene in this experiment. The study of the *ampG* gene will help provide new insights for the development of novel gene therapies. In the future, knockout of the *ampG* gene in *ampC*-producing strains will result in drug sensitivity to antibiotics and undoubtedly become a new target for drugs.

Abbreviations

ATCC, American Type Culture Collection; CAZ, Ceftazidime; CLSI, Clinical and Laboratory Standards Institute; CXM, Cefuroxime; ESBLs, Extended-spectrum β-lactamases; FEP, Cefepime; FOX, Cefoxitin; I, Intermediate; Km, Kanamycin; Kp, *Klebsiella pneumoniae*; KZ, Cefazolin; MIC, minimum inhibitory concentration; PCR, polymerase chain reaction; R, resistant; RT-PCR, Real-time PCR; S, Susceptible; SCF, Cefoperazone/sulbactam; PIP, Piperacillin;

CTX, Cefotaxime; *E.cloa* 029M, *E.cloacae* 029M; *S.ty* LT2, *S.typhimurium* LT2; *E.coli* 25922, *E.coli* ATCC25922; PA O1, *P.aeruginos*a O1.

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Disclosure

The authors report no conflicts of interest in this work.

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