Cefazolin and Imipenem Enhance AmpC Expression and Resistance in NagZ-Dependent Manner in *Enterobacter Cloacae*

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Abstract

Background: *Enterobacter cloacae* (EC) is a commonly occurring opportunistic pathogen and is responsible for causing various infections in humans. Owing to its inducible chromosomal AmpC β-lactamase (AmpC), EC is inherently resistant to the 1st- and 2nd- generation cephalosporins. However, whether β-lactams antibiotics enhance EC resistance remains unclear.

Results: In this study, we found that subinhibitory concentrations (SICs) of cefazolin (CFZ) and imipenem (IMP) are able to advance the expression of AmpC and improve its resistance towards β - lactams through NagZ in EC clinical isolate. Our work indicate that AmpC manifested a substantial upregulation in EC in response to SICs of CFZ and IMP. In *nagZ* knockout EC (Δ*nagZ*), we found that the resistance to β - lactam antibiotics was rather weakened and the effect of CFZ and IMP on induction of AmpC was completely abrogated. Ectopic expression of NagZ can rescue the induction effect of CFZ and IMP on AmpC and enhance resistance in Δ*nagZ*. More importantly, CFZ and IMP have the potential to bring about the target genes expressions of AmpR in a NagZ-dependent manner.

Conclusions: Our findings show that NagZ is a critical determinant for CFZ and IMP to promote AmpC expression and improve resistance and that CFZ and IMP should be used with caution since they may aggravate EC resistance. At the same time, this study further improves our understanding of resistance mechanisms in EC.

Background

Enterobacter cloacae (EC), *Enterobacter ludwigii, Enterobacter holmarchei, Enterobacter nimiparum, Enterobacter asperiae*, and *Enterobacter kobei* combinedly referred to as *Enterobacter cloacae complex* [1]. They are grouped within *Enterobacteriaceae* and have a wide-ranging prevalence [2]. Among *Enterobacter cloacae complex*, EC is isolated very frequently from clinical specimens obtained from humans as well as medical devices and takes on clinical significance to intensive care patients in the past decade, particularly to the patients put on mechanical ventilation [2]. The antibiotic-resistance characteristics of microorganisms, for instance, EC has been a point of focus of quite a large number of publications [3–5]. EC is inherently resistant to amoxicillin, ampicillin, 1st- and 2nd-generation of cephalosporin, and cefoxitin on account of the generation of inducible chromosomes AmpC β-lactamase (AmpC) [6].

NagZ, also known as the β-N- acetylglucosaminidase, is a crucial enzyme that takes part in peptidoglycan recycling, which has the potential to cleave GlcNAc-1,6-anhydroMurNAc-peptides into N-acetyl-β-d-glucosamine and 1,6-anhydroMurNAc-peptides (anhMurNAc) [7, 8]. NagZ inactivation has been demonstrated to reduce resistance to β-lactam antibiotics in *Pseudomonas aeruginosa* [9–11], *Stenotrophomonas maltophilia* [12], and *Yersinia enterocolitica* [13]. Moreover, within *Neisseria gonorrhoeae*, NagZ can also regulate the accumulation of biofilm [14], In *Pseudomonas aeruginosa*, anhMurNAc functions to improve AmpC expression by activating AmpR (a global transcriptional factor responsible for regulating hundreds of genes including *ampC*) [15, 16]. Despite these promising findings, the precise regulatory mechanism in *Enterobacter cloacae* is still rather unclear.

ampC genes are frequently found on the chromosomes of non-fermenting bacteria (such as *Pseudomonas aeruginosa*) and several family members of *Enterobacteriaceae* (for instance *Enterobacteriaceae*) [17, 18]. *ampC* overexpression renders these pathogens resistant to penicillin, the first and second-generation cephalosporins, and β-lactam/β-lactamase inhibitors [19], as well as carbapenems, especially with porin loss [20, 21]. The overproduction of AmpC is the major cause of EC's cephalosporin resistance [22]. It is reported that cefoxitin and cefotaxime can induce AmpC expression [1], which consequently fails antibiotics treatment, but the potential inducible mechanism is not unclear.

The precise objective of the current investigation is to screen whether other β-lactam antibiotics (except cefoxitin and cefotaxime) could induce AmpC expression and explore the induction mechanism. As discovered in this study, NagZ is a

key intermediate regulator in the process of AmpC induction expression by CFZ and IMP in EC. Our findings also suggest that CFZ and IMP should be used with caution, as they have the potential to exacerbate EC resistance and make therapy more challenging.

Results

SICs of CFZ and IMP Improve the Expression of Ampc and Improve Resistance to β-Lactam in EC Clinical Isolate

It was reported that cefoxitin and low concentration cefotaxime could improve AmpC expression [1]. To investigate whether other antibiotics can elicit the expression of AmpC, firstly, various antibiotics (including quinolones, β lactams and aminoglycosides) were tested for their minimum inhibitory concentration (MIC) against EC clinical isolate following the guideline outlined by the Clinical Laboratory Standard Institute (CLSI) [23]. The results are shown in Table S1. Later western blot assay was employed for determining whether or not the subinhibitory concentration (SIC, ≤1/4 MIC) of antibiotics induce AmpC expression. The findings (Fig. S1) demonstrate that CFZ, IMP, and cefoxitin have a strong induction impact on AmpC, whereas ceftriaxone, cefotaxime, ceftazidime, and cefepime have a modest induction effect. Other antibiotics, such as aminoglycosides and quinolones, showed no discernible effect on AmpC. Next, we explored the effect of induction of different concentrations CFZ and IMP on AmpC expression at mRNA level by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and protein level by western blot, respectively. CFZ and IMP have a dosage impact on AmpC induction, according to our findings (Fig. 1A-C). Finally, the AmpC generated by CFZ and IMP was tested using a nitrocefin hydrolysis assay to see if it had good β-lactamase activity. The results show that CFZ and IMP increase AmpC β-lactamase activity in a dose-dependent manner when compared to the control group. (Fig. 1D).

To investigate the role of CFZ and IMP in resistance, the inhibition zone and MICs of aztreonam (ATM), ceftriaxone (CRO), ceftazidime (CAZ), piperacillin (PIP), piperacillin-tazobactam (TZP), and cefoperazone-sulbactam (SCF) against EC clinical isolate treated with or without SICs of CFZ and IMP were estimated by employing broth microdilution method and disk diffusion technique (Kirby-Bauer method) in accordance with the CLSI guideline [23], as indicated in Table 1 and Fig. S2A, inhibition zones of those antibiotics against EC treated with SICs of CFZ and IMP are decreased obviously compared with control and the MICs of TZP, PIP, CRO, SCF, CAZ, and ATM manifest a substantial increase by SICs of CFZ and IMP.

Table 1
MICs for the EC clinical isolate treated with CFZ and IMP

Antibiotics	MIC(μg/ml)				
	control	CFZ(64μg/ml)	CFZ(256μg/ml)	IMP(0.0625μg/ml)	IMP(0.125μg/ml)
PIP	64	128	512	512	1024
TZP	32	128	256	512	1024
ATM	32	64	256	256	512
CRO	16	32	64	256	512
CAZ	16	64	128	128	256
SCF	4	8	32	32	128
Abbreviations. MIC: minimum inhibitory concentration; CFZ: cefazolin; IMP: and imipenem.					

The Effects of Induction of CFZ and IMP on AmpC Expression and Resistance Were Abrogated in ΔnagZ

NagZ, existing in Gram-negative bacteria and involved in the peptidoglycan recycling pathway, is identified as an exo-N-acetyl-β-glucosaminidase. In some Gram-negative bacteria, NagZ inactivation has been reported to arrest and reverse the

resistance to β-lactam antibiotics [24, 25]. As shown in Fig. S2A and Table 1, our data indicates the SICs of CFZ and IMP have the potential to improve the resistance of EC, so we speculated that NagZ may have an indispensable part in promoting resistance of EC. In an attempt to confirm the hypothesis, the knockout model of gene *nagZ* (Δ*nagZ*) was constructed by employing homologous recombination technology in EC clinical isolate (WT). As shown by RT-qPCR (Fig. 2A) and western blot (Fig. 2B), the *nagZ* gene was effectively knocked out. Next, we determined the effects of SICs of CFZ and IMP on the expression and activity of AmpC in Δ*nagZ*. The results, as shown in Fig. 2C-F, depict that the expression of AmpC was significantly downregulated, and the induction of AmpC by SICs of CFZ and IMP was completely abolished in Δ*nagZ* (Fig. 2C-E). At the same time, knocking down *nagZ* reduced AmpC's rising β-lactamase activity produced by CFZ and IMP SICs (Fig. 2F). Furthermore, antibiotic susceptibility tests revealed that EC resistance had been considerably reduced, while induction of CFZ and IMP had little impact on resistance in Δ*nagZ* (Table2 and Fig. S2B).

Table 2
The effect of nagZ on MICs for six anbiotics

Antibiotics	MIC(μg/ml)						
	WT	ΔnagZ	ΔnagZ+CFZ (4 μg/ml)	ΔnagZ+IMP (0.0625μg/ml)	ΔnagZ+NagZ	ΔnagZ+NagZ+ CFZ(256μg/ml)	ΔnagZ+NagZ+ IMP(0.125μg/ml)
PIP	64	2	2	1	32	256	512
TZP	32	2	1	0.5	32	128	512
ATM	32	1	1	0.5	16	256	512
CRO	16	0.5	0.5	0.25	32	64	1024
CAZ	16	0.5	0.5	0.25	16	64	128
SCF	4	0.25	0.125	0.0625	4	64	64

Abbreviations. Δ*nagZ*: *nagZ*-knockout *Enterobacter cloacae*; Δ*nagZ*+CFZ: Δ*nagZ* treated with CFZ; Δ*nagZ*+IMP: Δ*nagZ* treated with IMP; Δ*nagZ*+NagZ+CFZ: Δ*nagZ*+NagZ treated with CFZ; Δ*nagZ*+NagZ+IMP: Δ*nagZ*+NagZ treated with IMP.

Ectopic Expression of Nagz Rescues Induction Effect of CFZ and IMP on AmpC Expression and Resistance In Δ nagz

To investigate whether NagZ complementation rescues the expression of *ampC* and enhances resistance in Δ*nagZ* treated with or without SICs of CFZ and IMP, *nagZ* coding sequence (CDS) was cloned into the vector of pBAD33cm-rp4 (pBAD33-*nagZ*, *nagZ* overexpression vector). Later, the pBAD33-*nagZ* and the vector of pBAD33cm-rp4 (pBAD33, as control vector) were transformed into Δ*nagZ* by electroporator. RT-qPCR and western blot analyses were employed for detecting the availability of the pBAD33-*nagZ* vector (Fig. 3A, B). Next, the *ampC* expression was ascertained using western blot and RT-qPCR, the outcome showed that *ampC* levels of mRNA (Fig. 3C) and protein (Fig. 3D-E) were rescued by NagZ complementation in Δ*nagZ* in response to SICs of CFZ and IMP. Furthermore, NagZ was investigated in terms of its influence on the AmpC β-lactamase activity, and the result indicates that reduced activity of β- lactamase resulting from the elimination of *nagZ* was reversed by NagZ complementing in Δ*nagZ* (Fig. 3F). Additional confirmation of the significance of NagZ in resistance of Δ*nagZ* was acquired by measuring the inhibition zones and MICs of TZP, PIP, CRO, ATM, CAZ, and SCF. The findings demonstrate that NagZ overexpression may greatly reduce the inhibition zone and that SICs of CFZ or IMP can further reduce the inhibition zone (Fig. S2C). Consistent with the inhibition zone, NagZ complementation and SICs of CFZ or IMP can increase the MICs obviously (Table 2). To put it briefly, *ampC* expression and its β-lactamase activity are promoted by NagZ, which thereby enhances resistance in Δ*nagZ*.

CFZ and IMP Promote AmpC Expression Through the NagZ-AmpR-AmpC Pathway

Peptides from peptidoglycan degradation are transported by AmpG permease into the cytoplasm. In the cytoplasm, GlcNAc-1,6-anhydroMurNAc-peptides detach GlcNAc moiety with the aid of NagZ and forms 1,6-anhydroMurNAc-peptides (anhMurNAc) [24, 26]. Under normal physiological growth, AmpD cleaves anhMurNAc to generate free peptides and then synthesizes UDP-pentapeptides, which suppresses AmpR activity and represses AmpC transcription [27–29]. However, in the presence of inducers (such as β-lactams), AmpD cannot cleave the high amounts of anhMurNAc effectively. The accumulating anhMurNAc activates AmpR and increases AmpC transcription, which is also the main mechanistic step responsible for developing resistance to most β -lactams in *Pseudomonas aeruginosa* [30, 31]. Besides, several studies have shown that AmpR regulates the expression of a multitude of genes and is thus a global transcription factor (the genes regulated by AmpR include *oxyR, rsmA, rpoS, phoP*, and *grpE*) in *Pseudomonas aeruginosa* [30, 32]. Therefore, we hypothesize that, like *Pseudomonas aeruginosa*, there is a pathway in *Enterobacter cloacae* and that the induction of AmpC by SICs of CFZ and IMP is dependent on AmpR. To confirm our hypothesis, *Pseudomonas aeruginosa* and *Enterobacter cloacae* were both analyzed for their NagZ and AmpR protein sequence conservations. The AmpR sequences of the two species were identified (Fig. 4A) and the conservation of the NagZ sequence was as high as 67% (Fig. 4B). In addition, a high homology is additionally observed in the -35bp-0bp region (generally considered as transcriptional parameter zone of binding) for *ampC* among *Pseudomonas aeruginosa* and *Enterobacter cloacae* (Fig. 4C).

In an attempt to confirm if the induction of CFZ and IMP to AmpC is dependent on NagZ-mediated AmpR activation, we detected the effect of CFZ and IMP upon the expression of AmpR target genes in wild type EC and Δ*nagZ*. The outcome implies that CFZ and IMP are able to promote the AmpR target genes expression (for instance *oxyR, rsmA, grpE, rpoS*, and *phoP*) in wild type EC (Fig. 5A), while in the Δ*nagZ* strain, CFZ and IMP did not affect the expressions of AmpR target genes (Fig. 5B). In conclusion, these findings demonstrate that CFZ and IMP increase AmpC expression and resistance in *Enterobacter cloacae* in a NagZ-dependent manner.

Discussion

The development of new antibiotics is an imminent need as the number of multidrug-resistant bacteria grows, and medical institutions have recommended several priority antimicrobial pathogens, which include certain Gram-negative bacteria [23, 33]. A major area of concern is the growing resistance to β-lactam antibiotics, which currently are the most commonly utilized antibiotics for treating Gram-negative bacterial infections [34]. β -lactamase is an enzyme that cleaves the cyclic amide component of a β-lactam to render it inactive [35]. The inducible chromosomal AmpC β-lactamase (AmpC), which has broad-spectrum activity against β-lactam medicines, is one kind of lactamase that causes severe therapeutic difficulties [36]. Here, we found that CFZ and IMP could induce the expression of AmpC (the expression level was positively correlated with the dose of CFZ and IMP) and enhance resistance in clinical isolates of EC. This perhaps is the pioneer investigation, as per our knowledge, indicating that CFZ and IMP can induce AmpC β-lactamase expression in clinical EC isolate.

CFZ and IMP are β-lactam antibiotics, which have a broad spectrum of activity against bacteria, including aerobes and anaerobes [37, 38]. Furthermore, CFZ is a commonly used antibiotic for empiric therapy and postoperative infection prevention. Our findings imply that CFZ and IMP should be used with caution since they may exacerbate EC resistance.

Some studies indicate that metabolism and recycling of peptidoglycan take part in the expression of AmpC [39, 40], one of the key enzymes in peptidoglycan recycling is NagZ and its main function is to form anhMurNAc from GlcNAc-1,6-anhydroMurNAc-peptides by cleaving non reducing GlcNAc residues.[43] In the process of peptidoglycan metabolism, anhMurNAc promote the expression of AmpC by binding to the transcription factor AmpR. According to genetic research,

NagZ is required for the induction of AmpC β-lactamases in *Pseudomonas aeruginosa* [7]. In this study, our findings indicate NagZ is implicated in the regulation of CFZ and IMP to induce the AmpC expression and resistance in clinical EC isolates. In *nagZ* knockout EC, resistance to β- lactams and the expression of AmpC were reduced, and the inductive effects of CFZ and IMP on AmpC and resistance were totally abolished. While complementation of NagZ has the potential to rescue β-lactams resistance and the inducible effects of CFZ and IMP to AmpC in *nagZ* knockout model. Therefore, the data indicate that CFZ and IMP enhance AmpC expression and resistance in a NagZ-dependent manner in EC clinical isolate.

To further study how NagZ is involved in the regulation of CFZ and IMP to AmpC and resistance in *Enterobacter cloacae*, firstly, analysis of the homology of AmpR and NagZ between *Pseudomonas aeruginosa* and *Enterobacter cloacae* was carried out. The AmpR sequence of *Enterobacter cloacae* was determined to be similar to that of *Pseudomonas aeruginosa*, while the NagZ sequence had a 67 % homology with that of *Pseudomonas aeruginosa*. Then the upstream 35bp (transcription binding region) sequence of the *ampC* gene been aligned between *Pseudomonas aeruginosa* and *Enterobacter cloacae* and we found the sequences to be highly similar. Therefore, we hypothesized that the regulation of CFZ and IMP to AmpC and resistance is dependent on the NagZ hydrolysate (anhMurNAc). We tested the effects of CFZ and IMP on AmpR target gene expression in wild-type and NagZ deficient *Enterobacter cloacae* to confirm our hypothesis. The findings showed that CFZ and IMP have the potential to bring about the expression of target genes of AmpR in wild-type *Enterobacter cloacae*, while in *nagZ* knockout *Enterobacter cloacae*, the expression of AmpR target genes expression was downregulated, and CFZ and IMP had no obvious effect on the AmpR target gene expression. Here, CFZ and IMP have been confirmed to promote AmpC through NagZ-AmpR pathway. However, how NagZ affects the transcriptional activity of AmpR has not been known (for example, whether NagZ or its hydrolysate anhMurNAc can cause the conformational change of AmpR, as we known, the conformational change will activate the transcriptional activity of AmpR), so in the next research, we will work with scientists in the field of protein structure to study the effect of NagZ and its hydrolysate anhMurNAc on the structure of AmpR.

Conclusions

In conclusion, our study confirmed that NagZ is the key factor for CFZ and IMP to induce AmpC expression and enhance resistance in *Enterobacter cloacae* and that CFZ and IMP must be used carefully because they might aggravate the resistance of *Enterobacter cloacae*,which provides a new prospect for the treatment of multidrug-resistant *Enterobacter cloacae*. These prospects might include the use of NagZ inhibitors and β - lactam antibiotics to treat the infectious diseases caused by *Enterobacter cloacae.*

Methods

Bacterial Strains, Plasmids, Primers, and Antibiotics

The comprehensive data pertaining to the types of bacterial strains, primers, and plasmids involved in the investigation are presented in Table S2, S3, and S4 of supplementary materials.

Antibiotic Susceptibility Test

According to the CLSI guidelines [23], antibiotic susceptibility testing was conducted using broth microdilution and disc diffusion. *Enterobacter cloacae ATCC 13047* and *Escherichia coli* ATCC 25922 (Bio-kont Co. Ltd, Wenzhou, China) were employed as quality control organisms. The company of Wenzhou Kangtai (Bio-kont Co. Ltd, Wenzhou, China) procured all reagents used in antibiotic susceptibility tests. For CFZ and IMP treatment assays, the reagents were used at the initial stage.

Preparation of Anti-NagZ Antibody

As previously reported [41], anti-NagZ antibodies was prepared by immunizing rabbits. Briefly, molecular cloning was used to clone the EC *nagZ* coding sequence (CDS) into the pET28a vector (to form the pET28a-*nagZ* vector). The pET28a-*nagZ* was then converted into *E. coli* B21, which was capable of producing the NagZ recombinant protein. The recombinant protein was isolated using Ni-NAT and identified using electrophoresis before being used to immunize New Zealand rabbits (Dashuo. Co. Ltd, Chengdu, China). Finally, the antiserum was purified by the Ni-NAT column coupled with NagZ protein. Western blotting confirmed that this antibody has an excellent specificity. Shenggong Biotechnology Co., Ltd (Shanghai Sanguang Biotechnology Co., Ltd., China) provided the reagents as well as materials employed in the production of anti-NagZ antibodies. Table S4 shows the primers for amplification of *nagZ* CDS.

Assay of AmpC β-lactamase Activity

The activity of AmpC β-lactamase was ascertained using the nitrocefin hydrolysis technique. As reported earlier [1, 42], LB medium was utilized to culture the EC isolates for overnight at 37°C/250rpm. Sub-culturing of the overnight cultured bacterial suspension was carried out in a fresh LB milieu at a concentration of 1:100. When OD600 absorbance reaches 0.8, the organisms were collected, and 1ml protein lysate (Shanghai Sangguang Biotechnology Co., Ltd., China) was used to suspend the bacterial pellet. The samples were subjected to lysis through sonication using a microprobe three times on ice. The material was sonicated, then centrifuged at 10,000g for 10 minutes to obtain the supernatant. The protein quantification kit (Beyotime, Biotechnology, Shanghai, China) was employed for ascertaining the concentration of protein. For CFZ and IMP treatment assays, the reagents were utilized at the sub-culture stage. The assay of nitrocefin hydrolysis was carried out in 250µl buffer of phosphate (pH 7.0) with 50µg/ml nitrocefin (Sigma-Aldrich; Merck-KGaA, St. Louis, Missouri, USA) and 5µg total protein. The rate of nitrocefin hydrolysis was measured at 486nm every 5 minutes at ambient temperature. The nitrocefin extinction coefficient 20,500 M-1 cm-1 was employed for determining the activity of AmpC- β-lactamase.

RNA Extraction

EC isolate was cultured as described “AmpC β-lactamase activity assay”. The kit of the extraction of RNA (Sangon Biotech Co. Ltd, Shanghai, China) is utilized for extracting bacterial total RNA following the protocol outlined by the manufacturer. To describe in brief, the bacteria were gathered via centrifugation at 12000g for 2 minutes. TE buffer (100µl) containing 400µg/ml lysozyme (Sangon Biotech Co. Ltd, Shanghai, China) was employed for re-suspension of the bacterial pellet. After 5 minutes of incubation at room temperature, 900µl lysate was added and combined. The mixture was placed at ambient temperature for 3 minutes and 200µl chloroform (China Shanghai Sangon Biotechnology Co., Ltd.) was added. Then the mixture was subjected to vortex and centrifugation at 12000g/4℃ for 5 minutes. After that, 600µl of aqueous supernatant were collected and mixed with 200µl of 100% ethanol, which was then kept at ambient temperature for 3 minutes before being subjected to centrifugation at 12000g for 5 minutes at 4°C. The supernatant was thrown away, followed by washing the precipitate twice in 70% ethanol before drying naturally. Lastly, the precipitate was solubilized in an appropriate amount of ddH2O. For CFZ and IMP treatment assays, the reagents were employed at the sub-culture stage. NanoDropTM8000 spectrophotometer (Thermo Fisher Scientific, Waltham, Mass, USA) was employed for estimating the total RNA concentration. The total RNA was stored at -70 ℃ for determining genes expression levels.

RT- qPCR Assays

Using a FastKing gDNA Dispelling RT SuperMix kit (Tiangen Biotech Co., Ltd. Beijing, China), cDNA was synthesized from 500ng of total RNA. SuperReal PreMix Color (SYBR Green) kit (Tiangen Biotech Co., Ltd. Beijing, China) was employed for Real-time fluorescence quantitative PCR (qPCR) assay in the light of the protocol outlined by the manufacturer. In qPCR assays, 16S was utilized as an internal control. The concentration of all primers was 0.25µM (the final concentration in each reaction) and the amplification efficiency of all primers ranged from 91% to 96 % (Table S4).

The Analysis of Protein Extraction and Western Blot

Bacterial culture and total protein preparation as described “AmpC β-lactamase activity assay” and 30 μg total protein was taken for carrying out western blot assay. For CFZ and IMP treatment assays, the reagents were employed at the sub-culture stage. Western blot analysis was carried out using the standard methodology as elaborated earlier [43]. The detailed attributes of antibodies employed in western blot assay are as follows: rabbit anti-NagZ (preparation by ourselves), mouse anti-DnaK (Abcam, Cambridge, MA, USA), rabbit anti-AmpC (Abnova Taipei, Taiwan, China), goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). SPOT-CCD camera was used to take images. In western blot assay quantitative analysis, image J was employed for the quantification of the intensity of protein bands, and the internal control employed was DnaK.

Construction of nagZ-knockout EC Model

Using a homologous recombination method and a suicide vector, the *nagZ*-knockout EC model was built using a previously described methodology [44]. Briefly, PCR was used to amplify two homologous arms DNA fragments of the *nagZ* gene. The fusion DNA fragment containing two homologous arms was procured through the fusion PCR. The fusion DNA fragment was cloned into the suicide plasmid pLP12 and identified through sequencing and PCR. And then recombinant plasmid with fusion DNA fragment was transformed into *Escherichia coli* β2163. *nagZ*-knockout EC organism was finally screened through co-culturing *Escherichia coli* β2163 with recombinant plasmid and wild-type *Enterobacter cloacae*. All the reagents and strains used in *nagZ*-knockout EC preparation were bought from Nuojing Biological Company (Knogen Biotech Co., Ltd, Guangzhou, China).

Preparation of EC Models of NagZ Complementation

nagZ CDS was cloned into a pBAD33cm-rp4 plasmid (Knogen Biotech Co., Ltd, Guangzhou, China), and authenticated by PCR and sequencing. An electroporator was then used to transform the recombinant plasmid containing *nagZ* gene (pBAD33-*nagZ*) into competent *Escherichia coli* β2163 (Knogen Biotech Co., Ltd, Guangzhou, China). Ultimately, a conjugation assay was used to transform the recombinant plasmid pBAD33-*nagZ* into *Enterobacter cloacae*. *nagZ* expression of pBAD33-*nagZ* was induced by 0.05% L-Arabinose (Sangon Biotech Co. Ltd, Shanghai, China). For antibiotic susceptibility test, L-Arabinose was initially used. While for extraction of RNA, β-lactamase activity of AmpC, and western blot asaays, L-Arabinose was applied at the stage of sub-culture.

Statistical Analysis

All data were presented as mean ± standard deviation. Statistical difference analysis among two groups was performed by GraphPad Prism5 using a Two-tailed t-test. P<0.01(**) was used as statistically highly significant. Each experiment was performed at least 3 times.

Abbreviations

EC: *Enterobacter cloacae;* MIC: minimum inhibitory concentration; SIC: subinhibitory concentrations; PIP: piperacillin; TZP: piperacillin-tazobactam; ATM: aztreonam; CRO: ceftriaxone; CAZ: ceftazidime; SCF: cefoperazone-sulbactam; CFZ: cefazolin; IMP: imipenem; Δ*nagZ*; *nagZ*-knockout *Enterobacter cloacae*; Δ*nagZ*+CFZ: Δ*nagZ* treated with CFZ; Δ*nagZ*+IMP: Δ*nagZ* treated with IMP; Δ*nagZ+*NagZ+CFZ: Δ*nagZ+*NagZ treated with CFZ; Δ*nagZ+*NagZ+IMP: Δ*nagZ+*NagZ treated with IMP.

Declarations

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Authors' contributions

XGY conceived this study and written manuscript. ZW contributed to design experiments and analyzed data. XY contributed to search literatures and review the manuscript. YZ and FW performed experiments. YX conceived this study and reviewed the manuscript. All authors have read and approved the final manuscript.

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Ethics approval and consent to participate

The protocols of human test subjects (clinical samples), animal subject research (for anti-NagZ antibody generation) and microbiological research were approved by the scientific research ethics committee of the Institutional Review Board (IRB) of the Clinical Medical College and the First Affiliated Hospital of Chengdu Medical College (Ethics Approval Number: CYFYLL2019-183). We confirm that all methods were performed in accordance with the relevant guidelines and regulations. For clinical *Enterobacter cloacae* isolates collection, all participants gave written consent after clearly explaining the nature and purpose of this scientific research, in the process of informed consent, sufficient time is provided for questions and answers.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request

Competing interests

All authors declare that they have no conflicts of interest.

References

1. Guerin F, Isnard C, Cattoir V, Giard JC. Complex Regulation Pathways of AmpC-Mediated beta-Lactam Resistance in Enterobacter cloacae Complex. Antimicrob Agents Chemother. 2015; 59(12):7753–7761.
2. Mezzatesta ML, Gona F, Stefani S. Enterobacter cloacae complex: clinical impact and emerging antibiotic resistance. Future microbiology. 2012; 7(7):887–902.
3. Ku YH, Lee MF, Chuang YC, Yu WL. Detection of Plasmid-Mediated beta-Lactamase Genes and Emergence of a Novel AmpC (CMH-1) in Enterobacter cloacae at a Medical Center in Southern Taiwan. Journal of clinical medicine, 2018; 8(1).
4. Davin-Regli A, Pages JM. Enterobacter aerogenes and Enterobacter cloacae; versatile bacterial pathogens confronting antibiotic treatment. Frontiers in microbiology. 2015; 6:392.
5. Perez LRR, Carniel E, Narvaez GA. High minimum inhibitory concentrations among derepressed AmpC-beta-lactamase-producing Enterobacter cloacae complex isolates for ceftolozane with tazobactam. Infection control and

hospital epidemiology. 2020; 41(5):631–633.

6. Annavajhala MK, Gomez-Simmonds A, Uhlemann AC. Multidrug-Resistant Enterobacter cloacae Complex Emerging as a Global, Diversifying Threat. Frontiers in microbiology. 2019;10:44.
7. Votsch W, Templin MF. Characterization of a β- N -acetylglucosaminidase of Escherichia coli and Elucidation of Its Role in Muropeptide Recycling and β-Lactamase Induction *. Journal of Biological Chemistry. 2000; 275(50):39032–39038.
8. Cheng Q, Li H, Merdek K, Park JT. Molecular Characterization of the β-N-Acetylglucosaminidase of Escherichia coliand Its Role in Cell Wall Recycling. Journal of Bacteriology.2000; 182(17):4836–4840.
9. Acebron I, Mahasenan KV, De Benedetti S, Lee M, Artola-Recolons C, Hesek D, et al. Catalytic Cycle of the N-Acetylglucosaminidase NagZ from Pseudomonas aeruginosa. Journal of the American Chemical Society. 2017; 139(20):6795–6798.
10. Asgarali A, Stubbs KA, Oliver A, Vocadlo DJ, Mark BL. Inactivation of the glycoside hydrolase NagZ attenuates antipseudomonal beta-lactam resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2009; 53(6):2274–2282.
11. Zamorano L, Reeve TM, Deng L, Juan C, Moya B, Cabot G, et al. NagZ inactivation prevents and reverts beta-lactam resistance, driven by AmpD and PBP 4 mutations, in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2010; 54(9):3557–3563.
12. Huang YW, Hu RM, Lin CW, Chung TC, Yang TC. NagZ-dependent and NagZ-independent mechanisms for beta-lactamase expression in Stenotrophomonas maltophilia. Antimicrob Agents Chemother. 2012; 56(4):1936–1941.
13. Liu C, Li C, Chen Y, Hao H, Liang J, Duan R, et al. Role of Low-Molecular-Mass Penicillin-Binding Proteins, NagZ and AmpR in AmpC beta-lactamase Regulation of Yersinia enterocolitica. Frontiers in cellular and infection microbiology. 2017; 7:425.
14. Bhoopalan SV, Piekarowicz A, Lenz JD, Dillard JP, Stein DC. nagZ Triggers Gonococcal Biofilm Disassembly. Scientific reports. 2016; 6:22372.
15. Jacobs C, Huang LJ, Bartowsky E, Normark S, Park JT. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for beta-lactamase induction. Embo Journal. 1994; 13(19):4684–4694.
16. Dik DA, Fisher JF, Mobashery S. Cell-Wall Recycling of the Gram-Negative Bacteria and the Nexus to Antibiotic Resistance. Chemical Reviews. 2018; 118(12).
17. Herrmann L, Kimmig A, Rodel J, Hagel S, Rose N, Pletz MW, et al. Early Treatment Outcomes for Bloodstream Infections Caused by Potential AmpC Beta-Lactamase-Producing Enterobacterales with Focus on Piperacillin/Tazobactam: A Retrospective Cohort Study. Antibiotics. 2021; 10(6).
18. Gunter SG, Barber KE, Wagner JL, Stover KR. Fluoroquinolone Versus Nonfluoroquinolone Treatment of Bloodstream Infections Caused by Chromosomally Mediated AmpC-Producing Enterobacteriaceae. Antibiotics. 2020; 9(6).
19. Bastos MDS, Menegucci TC, Moreira RRB, Garcia LB, Cardoso CL, Tognim MCB. A rapid and simple method to detect ESBL in Enterobacter cloacae based on MIC of cefepime. Revista da Sociedade Brasilra de Medicina Tropical. 2015; 48(2):208–211.
20. Quale J, Bratu S, Gupta J, Landman D. Interplay of Efflux System, ampC, and oprD Expression in Carbapenem Resistance of Pseudomonas aeruginosa Clinical Isolates. Antimicrobial Agents and Chemotherapy. 2006; 50(5):1633–1641.
21. Piotr M, Piotr W, Dominika O, Anna S, Oksana K, Pawe S, et al. Altered Outer Membrane Transcriptome Balance with AmpC Overexpression in Carbapenem-Resistant Enterobacter cloacae. Frontiers in microbiology. 2016; 7:2054.
22. Nicolas MH, Honore N, Jarlier V, Philippon A, Cole ST. Molecular genetic analysis of cephalosporinase production and its role in beta-lactam resistance in clinical isolates of Enterobacter cloacae. Antimicrobial Agents & Chemotherapy.

1987; 31(2):295.

23. Wayne, PA. “2019CLSI.performance standards for antimicrobial susceptibility testing,” in CLSI Supplement M100, 29th Edn, 2019.
24. Ho LA, Winogrodzki JL, Debowski AW, Madden Z, Vocadlo DJ, Mark BL, et al. A mechanism-based GlcNAc-inspired cyclophellitol inactivator of the peptidoglycan recycling enzyme NagZ reverses resistance to beta-lactams in Pseudomonas aeruginosa. Chem Commun (Camb). 2018; 54(75):10630–10633.
25. Bouquet J, King DT, Vadlamani G, Benzie GR, Iorga B, Ide D, et al. Selective trihydroxylated azepane inhibitors of NagZ, a glycosidase involved in Pseudomonas aeruginosa resistance to beta-lactam antibiotics. Organic & biomolecular chemistry. 2017; 15(21):4609–4619.
26. Park JT, Uehara T. Peptidoglycan Recycling. Ecosal Plus. 2008; 3(1).
27. Johnson JW, Fisher JF, Mobashery S. Bacterial cell-wall recycling. Annals of the New York Academy of Sciences. 2013; 1277:54–75.
28. Kong KF, Schneper L, Mathee K. Beta-lactam Antibiotics: From Antibiosis to Resistance and Bacteriology. Apmis. 2010; 118(1):1–36.
29. Jacoby GA. AmpC beta-lactamases. Clinical microbiology reviews. 2009; 22(1):161–182.
30. Kong KF, Jayawardena SR, Indulkar SD, Del Puerto A, Koh CL, Hoiby N, et al. Pseudomonas aeruginosa AmpR Is a Global Transcriptional Factor That Regulates Expression of AmpC and PoxB ?-Lactamases, Proteases, Quorum Sensing, and Other Virulence Factors. Antimicrobial Agents & Chemotherapy. 2005; 49(11):4567–4575.
31. Lodge JM, Minchin SD, Piddock LJV, Busby SJW. Cloning, sequencing and analysis of the structural gene and regulatory region of the Pseudomonas aeruginosa chromosomal AmpC beta-lactamase. Biochemical Journal. 1991; 272(3):627–631.
32. Caille O, Zincke D, Merighi M, Balasubramanian D, Kumari H, Kong KF, et al. Structural and Functional Characterization of Pseudomonas aeruginosa Global Regulator AmpR. Journal of Bacteriology. 2014; 196(22):3890–3902.
33. team Ee. CDC publishes report on antibiotic resistance threats in the United States for the first time. 2013.
34. Fernandes R, Amador P, Prudêncio C. β-Lactams: Chemical structure, mode of action and mechanisms of resistance. Reviews in Medical Microbiology. 2013; 24(1).
35. Bush K. Bench-to-bedside review: The role of β-lactamases in antibiotic-resistant Gram-negative infections. Critical Care. 2010; 14(3):224.
36. Bork, Jacqueline, T., Heil, Emily, L., et al. Treatment options for extended-spectrum beta-lactamase (ESBL) and AmpC-producing bacteria. Expert opinion on pharmacotherapy. 2016.
37. Buckley MM, Brogden RN, Barradell LB, Goa KL. Imipenem/Cilastatin. Drugs. 1992; 44(3):408–444.
38. Saini S, Aparna, Gupta N, Mahajan A, Saini OP. Antibiotic susceptibility of bacterial isolates in gingivitis and periodontitis. Indian J Dent Res. 2003; 14(2):95–100.
39. Qian W, Fu Y, Liu M, Wang T, Zhang J, Yang M, et al. In Vitro Antibacterial Activity and Mechanism of Vanillic Acid against Carbapenem-Resistant Enterobacter cloacae. Antibiotics. 2019; 8(4).
40. Dominguez-Gil T, Molina R, Alcorlo M, Hermoso JA. Renew or die: The molecular mechanisms of peptidoglycan recycling and antibiotic resistance in Gram-negative pathogens. Drug resistance updates: reviews and commentaries in antimicrobial and anticancer chemotherapy. 2016; 28:91–104.
41. Arora S, Ayyar BV, O'Kennedy R. Affinity Chromatography for Antibody Purification; 2014.
42. Cavallari JF, Lamers RP, Scheurwater EM, Matos AL, Burrows LL. Changes to Its Peptidoglycan-Remodeling Enzyme Repertoire Modulate β-Lactam Resistance in Pseudomonas aeruginosa. Antimicrobial Agents & Chemotherapy. 2013; 57(7):3078–3084.

43. Yang X, Zeng J, Zhou Q, Yu X, Zhong Y, Wang F, et al. Elevating NagZ Improves Resistance to beta-Lactam Antibiotics via Promoting AmpC beta-Lactamase in Enterobacter cloacae. Frontiers in microbiology. 2020; 11:586729.
44. Luo P, He X, Liu Q, Hu C. Developing Universal Genetic Tools for Rapid and Efficient Deletion Mutation in Vibrio Species Based on Suicide T-Vectors Carrying a Novel Counterselectable Marker, vmi480. PloS one, 2015; 10(12):e0144465.

Figures

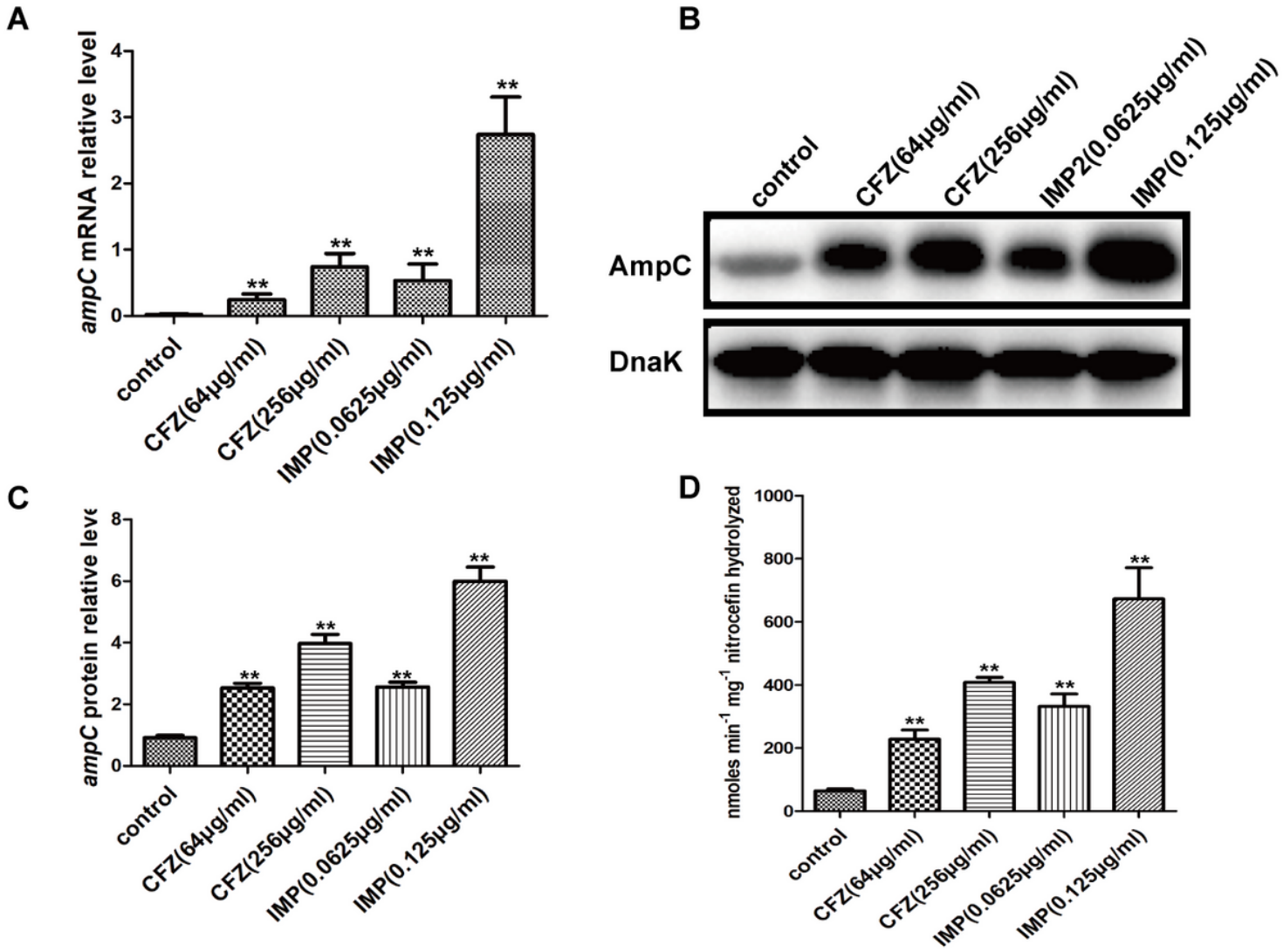

Figure 1

Effects of SICs of CFZ and IMP on AmpC expression and activity in EC clinical isolate. (A) Analysis of the ampC mRNA expression levels was made using RT-qPCR in EC clinical isolate treated with SICs of CFZ (64μg/ml and 256μg/ml) and IMP (0.0625μg/ml and 0.125μg/ml). (B) Western blot analysis of ampC protein expression in EC clinical isolate treated with SICs of CFZ and IMP. (C) Quantitative analysis of the findings of western blot (B) with image J software, DnaK was employed as an internal control. (D) Determination of the effects of SICs of CFZ and IMP on AmpC activity by nitrocefin hydrolysis assay. ** P<0.01 suggests high statistical significance

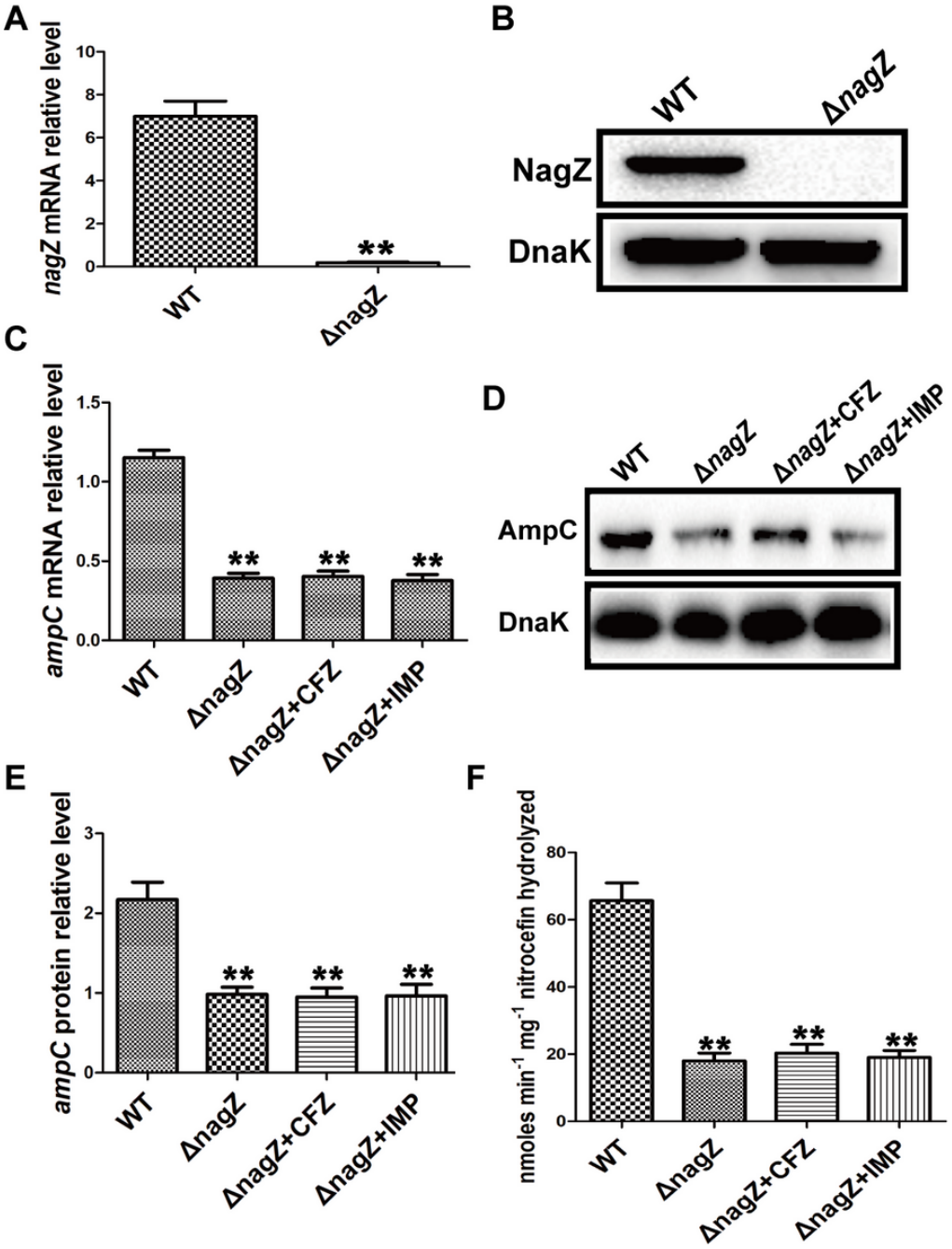

Figure 2

The role of NagZ in ampC expression and resistance in EC. RT-qPCR (A) and Western Blot (B) verified that nagZ-knockout EC clinical isolate (ΔnagZ) was successfully constructed. (C-D) RT-qPCR and Western Blot were employed for the determination of the role of NagZ in the expression of AmpC induced by subinhibitory concentration CFZ (256μg/ml) and IMP (0.125μg/ml) in EC clinical isolate (WT), ΔnagZ, ΔnagZ treated with CFZ (ΔnagZ+CFZ) and ΔnagZ treated with IMP (ΔnagZ+IMP). (E) Western blot Quantitative analysis (D), The internal control employed was DnaK. (F) Nitrocefin hydrolysis assay in WT, ΔnagZ, ΔnagZ+CFZ, and ΔnagZ+IMP was employed to examine the role of NagZ in AmpC β-lactamase activity. ** $P<0.01$ indicates high statistical significance.

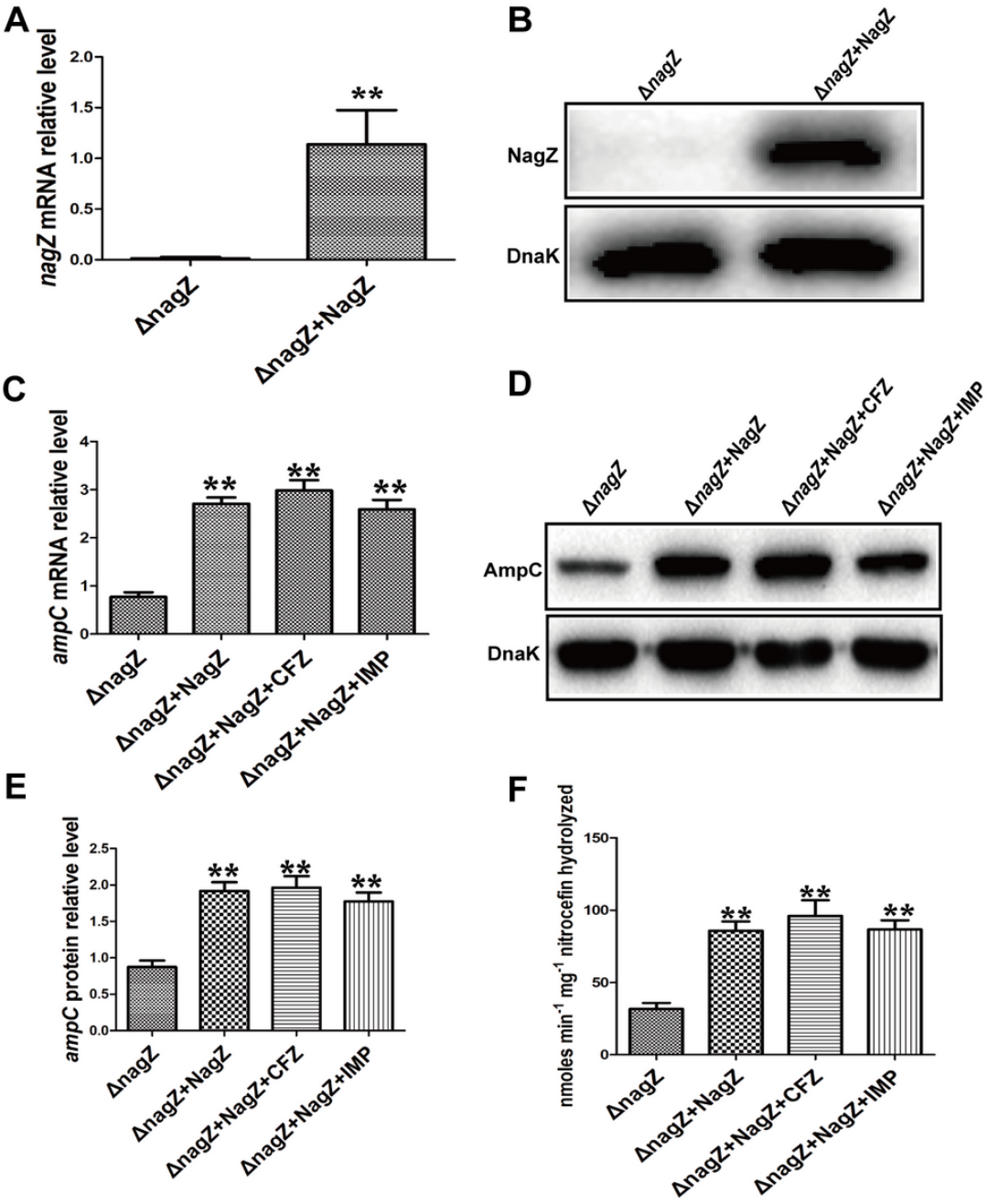

Figure 3

NagZ complementation can rescue induction of AmpC and resistance by subinhibitory concentration CFZ (256μg/ml) and IMP (0.125μg/ml) in ΔnagZ. RT-qPCR (A) and western blot (B) verified that the NagZ complementation model was successfully generated. (C) mRNA expressions of ampC were identified using RT-qPCR in ΔnagZ, ΔnagZ complemented with NagZ (ΔnagZ+NagZ), ΔnagZ+NagZ treated with CFZ (ΔnagZ+NagZ+CFZ) and ΔnagZ+NagZ treated with IMP (ΔnagZ+NagZ+IMP). (D) Western blot was employed for the determination of ampC protein expressions in ΔnagZ, ΔnagZ+NagZ, ΔnagZ+NagZ+CFZ, and ΔnagZ+NagZ+IMP strains. (E) Western blot Quantitative analysis (D), and the internal control employed was DnaK. (F) nitrocefin hydrolysis assay in nagZ, ΔnagZ+NagZ, ΔnagZ+NagZ+CFZ and ΔnagZ+NagZ+IMP was used for the analysis of AmpC β-lactamase activity. ** P<0.01 indicates high statistical significance.

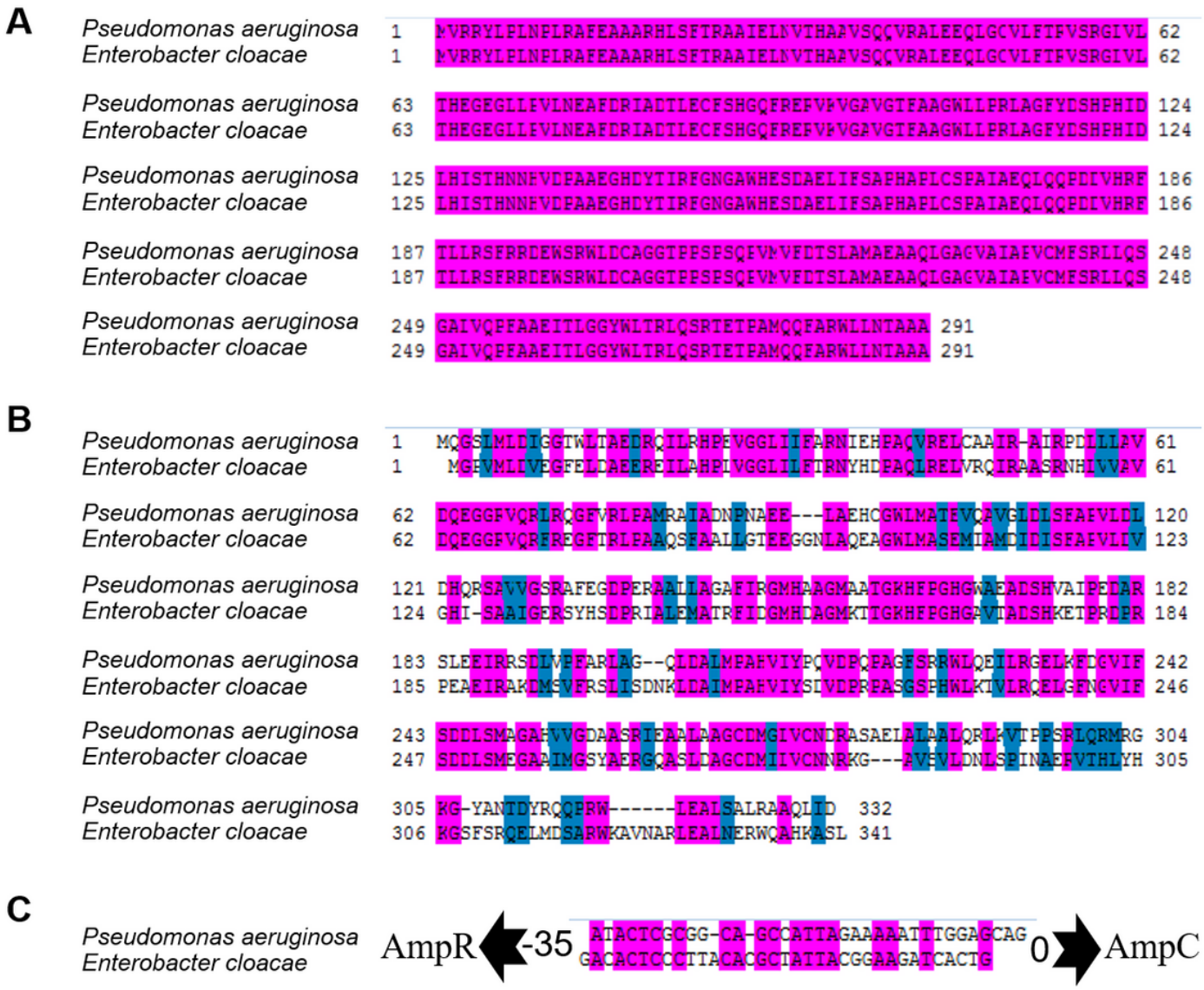

Figure 4

NagZ and AmpR sequence conservative analysis among Enterobacter cloacae and Pseudomonas aeruginosa. NagZ (A) and AmpR (B) amino acid sequence alignment among Pseudomonas aeruginosa and Enterobacter cloacae, the identical sequences are indicated using hot-pink, those marked by dark blue means the same class of amino acids with respect to their structure or function and marked by white means different types of amino acids. C: The transcription binding region nucleotide sequence alignment of AmpC (about -35bp) among Pseudomonas aeruginosa and Enterobacter cloacae, the conservative sequences are marked by hot-pink, the nucleotide sequences with differences between Enterobacter cloacae and Pseudomonas aeruginosa marked in white.

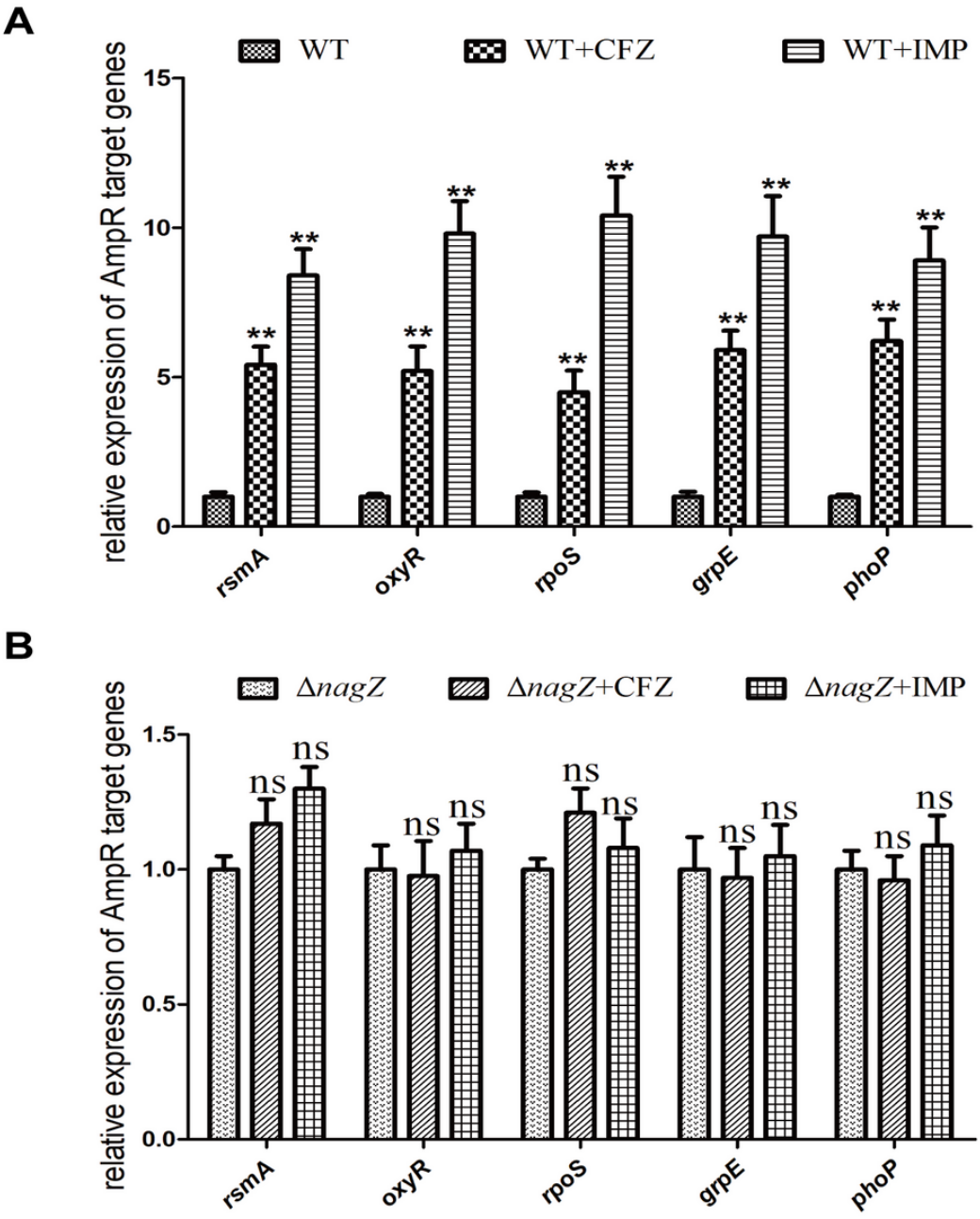

Figure 5

Influence of NagZ upon AmpR target genes expression, including rsmA, oxyR, grpE, rpoS, and phoP. mRNA expression of rsmA, rpoS, oxyR, grpE, and phoP was determined by RT-qPCR in EC (A) and ΔnagZ (B) treated with or without CFZ and IMP. ** P<0.01 indicates high statistical significance.

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