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染色體之fosA與運輸蛋白在碳青黴烯抗藥性克雷伯氏肺炎桿菌造成之磷黴素抗藥性

Transporter Genes and Chromosomal fosA Associated with Fosfomycin Resistance in Carbapenem-Resistant *Klebsiella pneumoniae*

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Transporter Genes and fosA Associated with Fosfomycin Resistance in Carbapenem-Resistant *Klebsiella pneumoniae*

本論文係 王宇平 (姓名) D07445002 (學號) 在國立臺灣大學 微生物研究所(系/所/學位學程)完成之博士學位論文，於民國 112 年 07 月 28 日承下列考試委員審查通過及口試及格，特此證明。

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中文摘要

碳青黴烯抗藥性克雷伯氏肺炎桿菌(carbapenem resistant *Klebsiella pneumoniae*)造成全世界臨床上極大的問題。磷黴素(fosfomycin)是少數的治療選擇之一。然而碳青黴烯抗藥性克雷伯氏肺炎桿菌對磷黴素的抗藥性也被觀察到。在本研究中，我們目標為找出碳青黴烯抗藥性克雷伯氏肺炎桿菌對磷黴素抗藥的機轉。我們蒐集台灣四家醫學中心 2010 年至 2018 年間對磷黴素抗藥性的克雷伯氏肺炎桿菌。我們將磷黴素抗藥性相關的基因擴增並且定序。並且利用碳水化合物攝取試驗以及基因突變試驗以確認基因突變與抗藥性的關聯。我們總共蒐集到了 40 株磷黴素抗藥性的碳青黴烯抗藥性克雷伯氏肺炎桿菌做分析，其中 14 株為低度抗藥性(最小抑菌濃度 256-512mg/dl)，26 株為高度抗藥性(最小抑菌濃度大等於 1024mg/dl)。其中位於染色體上 $fosA^{KP}$ 點突變 I91V 在 40 株碳青黴烯抗藥性克雷伯氏肺炎桿菌當中的 39 株被發現。基因突變試驗發現 I91V 使得克雷伯氏肺炎桿菌對磷黴素最小抑菌濃度增加 8 倍，並且是低度抗藥性菌株唯一被找到的機轉。在 26 高度抗藥性株中同時找到了額外的機轉。多種不同的基因突變導致 G3P 和 G6P 運輸蛋白的缺陷在其中 19 株被找到，其中有三種造成過去未被發現過的氨基酸替換。(glpT E299D, glpT D274V and uhpC A393V)。其中 7 株發現質體中帶有的 fosA3 磷黴素分解酶基因。在本研究中，我們發現染色體上 $fosA^{KP}$ 點突變 I91V 造成低度抗藥性，而額外的磷黴素運輸蛋白(19/26, 73.1%)缺陷及磷黴素分解酶(7/26, 26.9%)造成了高度抗藥性。

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Abstract

Infections caused by carbapenem-resistant *Klebsiella pneumoniae* (CRKP) are of significant clinical concern worldwide. Fosfomycin is one of the limited treatment options for CRKP. However, resistance to fosfomycin in CRKP has been observed. In this study, we aimed to investigate the fosfomycin resistance mechanism of CRKP. Fosfomycin-resistant *Klebsiella pneumoniae* isolates were collected from four medical centers in Taiwan from 2010 to 2018. The genes that contributed to fosfomycin resistance were amplified and sequenced. Carbohydrate utilization assays and mutagenesis studies were performed to determine the mechanisms underlying fosfomycin resistance. Forty fosfomycin-resistant CRKP strains were collected and used for further analysis. Fourteen strains exhibited low-level resistance (MIC = 256–512 mg/dl), while 26 strains showed high-level resistance (MIC ≥ 1024 mg/dl). Chromosomal $fosA^{KP}$ I91V was detected in 39/40 fosfomycin-resistant CRKP strains. We observed that amino acid substitution of chromosomal $fosA^{KP}$ I91V increased the MIC of fosfomycin by approximately 8 folds, and this was the only mechanism elucidated for low-level fosfomycin resistance. Among the 26 high-level resistance strains, $fosA^{KP}$ I91V combined with transporter deficiencies (18/26, 69.2%) was the most common resistant mechanism, and one strain showed transporter deficiency only. Plasmid-borne fosA3 accounted for 27.0% (7/26) of high-level resistance. Various G3P and G6P transporter gene mutations, including three novel single amino acid mutations (glpT E299D, glpT D274V, and uhpC A393V) were detected in 19 strains. No murA mutation was found in this study. Our study highlights the need for new therapeutic agents for CRKP infections in Taiwan.

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1 Introduction

1.1 Background

The emergence of multidrug-resistant bacteria has become a significant global clinical concern. Among these pathogens, *Klebsiella pneumoniae* stands out as one of the most critical culprits responsible for various healthcare-associated infections. The increasing prevalence of multidrug-resistant strains of *K. pneumoniae* poses significant challenges to healthcare systems worldwide. (Podschun and Ullmann, 1998;Juan et al., 2019;Nordmann and Poirel, 2019). As a common nosocomial pathogen, *K. pneumoniae* can cause a wide range of infections, such as urinary tract infections, respiratory tract infections, bloodstream infections, and surgical site infections. These infections often occur in healthcare settings, affecting vulnerable populations, including immunocompromised patients, elderly individuals, and those with underlying health conditions. Carbapenems are considered as the last resort of treatment of multidrug-resistant *K. pneumoniae*, but an increasing prevalence of carbapenem-resistant *K. pneumoniae* (CRKP) has been recorded worldwide (Chiu et al., 2013;Logan and Weinstein, 2017). More recent data from Taiwan Nosocomial Infections Surveillance Report showed that the incidence of CRKP increased from 9.4% to 34.4% in regional hospitals, and from 10.5% to 44.4% in medical centers between 2011 to 2020. Compared with susceptible strains, the CRKP infections cause significantly higher mortality, morbidity, and cost.

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1.2 Treatment options for CRKP

Currently, there are only limited treatment options for CRKP (Karampatakis et al., 2023). Colistin, tigecycline, fosfomycin and aminoglycosides are older antibiotics that can still be used to treat CRKP. These older agents have distinct antibacterial mechanisms compared to popular classes of antibiotics such as beta-lactams and fluoroquinolones, allowing them to maintain effectiveness against CRKP. However, they are not the primary choice due to several drawbacks. One significant concern with these older antibiotics is their association with higher adverse effects (Fritzenwanker et al., 2018). For example, colistin and aminoglycosides can cause nephrotoxicity and neurotoxicity, tigecycline is associated with severe gastrointestinal upset, and fosfomycin use can result in electrolyte imbalance. The undesirable adverse effects associated with these older antibiotics make them less preferable as treatment options. Additionally, there have been reports of the emergence of resistance to these older antimicrobial agents (Cheng et al., 2015; Fritzenwanker et al., 2018). The newer agents, such as ceftazidime plus avibactam and cefiderocol, have promising clinical results. However, these newer agents are relatively expensive and are not available in some countries. There have been concerning reports of the rapid emergence of resistance to these newer antimicrobial agents shortly after their introduction into clinical settings.

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1.3 Usage and mechanisms of fosfomycin

Fosfomycin is one of the few treatment options for CRKP. (Morrill et al., 2015) Fosfomycin is a phosphonic acid antibiotic discovered in a fermentation broth of *Streptococcus fradiae* in Spain (Gadebusch et al., 1992). Fosfomycin acts by inhibiting the enzyme N-acetylglucosamine enolpyruvyl transferase (murA), which is a critical initial step in the bacterial cell wall biosynthesis process that occurs in the cytoplasm. Fosfomycin is actively transported into bacterial cytoplasm by two uptake pathways, the glycerol-3-phosphate (G3P) transporter (glpT) and the glucose-6-phosphate (G6P) transporter (uhpT) systems.

The target of fosfomycin, murA, is the first cytoplasmic step of bacterial cell wall biosynthesis (Castañeda-García et al., 2013). MurA is responsible for producing the peptidoglycan precursor uridine diphosphate (UDP) N-acetylmuramic acid. The enzyme murA is involved in peptidoglycan biosynthesis by catalyzing the transfer of the enolpyruvyl moiety of phosphoenolpyruvate to the 3′-hydroxyl group of UDP-N-acetylglucosamine. Fosfomycin acts by binding irreversibly to the thiol group of a cysteine residue in the active site of murA. In *Escherichia coli*, this specific cysteine is Cys115. The binding of fosfomycin to this site leads to the inactivation of murA, preventing it from carrying out its catalytic function. By disrupting cell wall synthesis at this early stage, fosfomycin effectively hinders the formation of the peptidoglycan layer, leading to bacterial death.

GlpT is a G3P permease, a protein belonging to the organophosphate phosphate antiporter (OPA) family within the Major Facilitator Superfamily. This permease serves as a crucial entry mechanism for fosfomycin into bacterial cells, as the antibiotic mimics

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G3P. In *E. coli*, the expression of glpT is induced in the presence of G3P. The protein glpR acts as a repressor and blocks the transcription of glpT by binding to specific DNA sequences known as operators near the glpT promoter. However, when G3P is present, it interacts with glpR, reducing the repressor's affinity for the glpT operator. As a result, this interaction leads to the activation of glpT synthesis, allowing the uptake of G3P and fosfomycin into the bacterial cytoplasm.

UhpT is another member of the Major Facilitator Superfamily that functions as a phosphate transporter in bacterial cells. It facilitates the exchange of a cytoplasmic phosphate for a hexose phosphate. The uhpT transport system is under the regulation of several key regulatory components, namely uhpA, uhpB, and uhpC. These regulatory factors are essential for the high-level expression of the uhpT transporter. In the two-component regulatory system controlling the uhpT transport system, the integral membrane component uhpC serves as a sensor that detects an extracellular G6P. Upon sensing G6P, uhpC initiates a signaling cascade that leads to the activation of uhpB. uhpB is a sensor histidine kinase, which, in response to the signal from uhpC, undergoes a His-to-Asp phosphoryl transfer. This phosphorylation process activates uhpB, allowing it to carry out its regulatory function. The response regulator, uhpA, forms a complex with uhpB in its phosphorylated state. UhpA is a DNA-binding protein that recognizes specific regions in the uhpT promoter. Upon phosphorylation and formation of the uhpB-uhpA complex, uhpA's ability to bind to the uhpT promoter is enhanced. This enhanced DNA binding stimulates uhpT transcription, promoting the expression of the uhpT transporter.

Furthermore, the gene expression of the both uptake systems require the presence of cyclic adenosine monophosphate (cAMP). High levels of cAMP are required for the full expression of the fosfomycin transporters, glpT and uhpT. cAMP synthesis is reliant

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on the activity of the enzyme adenyl cyclase cyaA. The levels of cAMP are also controlled by the phosphotransferase enzyme, ptsI, which is a component of the phosphoenolpyruvate sugar phosphotransferase transport system. When there are mutations in either cyaA or ptsI, it results in a decrease in intracellular cAMP levels, leading to a reduced synthesis of fosfomycin transporters. As a consequence, the antibiotic uptake is diminished.

With Clinical and Laboratory Standards Institute (CLSI) breakpoints, i*n vitro* antimicrobial susceptibility test suggest that fosfomycin is active against many Gram-negative and Gram-positive pathogens, including multiple drug resistant strains. In previous *in vitro* studies, fosfomycin was active against most Enterobacteriaceae, including *Salmonella* species, *E. coli*, *Klebsiella species, Enterobacter* species, *Serratia* species, *Citrobacter* species, and *Proteus* species, regardless of extended-spectrum beta-lactamase or carbapenem resistance. Fosfomycin also exhibits considerable activity against Gram-positive pathogens such as *Enterococcus faecalis*, *Enterococcus faecium* (including vancomycin resistance Enterococcus), *Staphylococcus aureus* (including methicillin resistance *Staphylococcus aureus*), and *S. epidermidis*.

1.4 Resistance of fosfomycin

Fosfomycin was also found to be active against more that 90% of CRKP in previous antimicrobial susceptibility report (Endimiani et al., 2010;Falagas et al., 2010). Several clinical studies have reported its promising efficacy and low toxicity (Michalopoulos et al., 2010;Pontikis et al., 2014). However, fosfomycin-resistant CRKP has been observed, and is a problem in clinical settings (Yang et al., 2019). In some

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clinical settings where fosfomycin is commonly prescribed, the resistance rates among CRKP reported is ranging from 48.5 to 80% (Liu et al., 2020; Huang et al., 2021). The rapid spreading of fosfomycin resistance leads to further medical challenge for CRKP treatment.

Several mechanisms of resistance have been reported. First, fosfomycin inhibits murA activity. Overexpression of murA through mutations such as Asp369Asn and Leu370Ile confers fosfomycin resistance. Moreover, evidence has demonstrated the significance of mutations at position Cys115 in conferring resistance to fosfomycin. Mutations at this site can alter the binding affinity between fosfomycin and murA, reducing the effectiveness of the fosfomycin. (Kumar et al., 2009; Takahata et al., 2010)

Second, fosfomycin enters the cell through two different uptake pathways: the G3P transporter (glpT) system and the G6P transporter (uhpT) system. The acquisition of mutations affecting GlpT and uhpT function decreases the antibiotic uptake into the bacterial cells and confers to fosfomycin resistance. The uhpT transport system is under the control of several regulatory components, including uhpA, uhpB, and uhpC. When any of these regulatory genes are inactivated or malfunctioning, it leads to a reduced expression of the uhpT transporter, resulting in fosfomycin resistance. (Takahata et al., 2010). In previous studies (Ballestero-Téllez et al., 2017), deletion of glpT, uhpT or both in *E. coli* or *S. aureus* confers to increased fosfomycin minimum inhibitory concentration (MIC).

Third, three major types of fosfomycin-modifying enzymes, namely fosA, fosB, and fosX, are described to contribute to resistance. Glutathione S-transferase homologues (fosA) are mainly found in gram-negative bacteria chromosome, including *K.*

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pneumoniae, and they reduce fosfomycin susceptibility (Ito et al., 2017). Transferring these chromosomal fosA genes via plasmid to *E. coli* result in high-level fosfomycin resistance. There are also several subtypes of plasmid-mediated fosA homologues reported to confer high-level resistance (Yang et al., 2019).

1.5 Bacteriophage therapy and importance of capsular types

Bacteriophage therapy is another emerging therapy against CRKP. Actually, bacteriophage therapy origins back to a century ago (Venturini et al., 2022). Since the discovery of penicillin, antimicrobial therapy become the standard therapy for bacterial infections in most part of the world. The use of phage therapy is limited in some Eastern European countries. In recent decades, the growing threat of antimicrobial resistance has raised the interest of bacteriophage therapy again. However, the phages often highly specific for the bacteria they targeted. For example, at least 81 capsular types have been identified in *Klebsiella pneumoniae,* but most of the bacteriophage only infected one capsular type of *Klebsiella pneumoniae* (Pan et al., 2017). Even multihost bacteriophage, such as ΦK64-1, can only infected about 10 capsular types of *Klebsiella*. Therefore, to utilize phage therapy against CRKP, it is very important to find out the prevalent capsular type of CRKP.

1.6 Previous works

In our previous studies (Hung, 2016; Chu, 2018; Chen, 2020; Wu, 2020), we found transporters deficiencies, plasmid mediated fosA3 gene and chromosomal fosA were related to fosfomycin resistance. However, our investigation encountered challenges in identifying all the resistant mechanism in certain CRKP strains. In this study, we aimed to complete to find all mechanisms of fosfomycin resistance in CRKP strains and the

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epidemiology data such as capsular types, MLST and carbapenem resistance profiles.

1.7 Research Aims

In this study, we aimed to study the fosfomycin resistance mechanisms of CRKP strains in Taiwan. We analyzed capsular type distribution of fosfomycin resistance CRKP and attempted to clarify to resistance mechanism. Chromosomal mutagenesis, plasmid transformation and sole carbohydrate growth assay were performed to confirm that the phenotype changes were caused by the genetics changes.

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2 Study Design and Methods

2.1 The bacterial strains

2.1.1 Clinical strains of CRKP

Clinical CRKP strains which caused infections were retrospectively collected from the National Taiwan University Hospital, Taipei Veterans General Hospital, National Cheng Kung University Hospital, and Linkou Chang Gung Memorial Hospital from January 2010 to August 2018. If multiple strains were isolated from a same patient, only one strain will be enrolled. The strains used in this study are listed in Table 1. CRKP was defined as strains with a MIC of ≥ 4 mg/L for imipenem or meropenem, based on the CLSI guidelines. All isolates were obtained from a single colony picked from a freshly streaked agar plate and stored at −80°C in LB broth with 15% glycerol until they were tested against fosfomycin.

2.1.2 Production of competent cells

Competent cells of *E. coli* DH10B and *K. pnuemoniae* NTUH-K2044 were prepared for mutagenesis study with different protocols. Preparation for *E. coli* DH10B competent cells was performed by following method: The *E. coli* DH10B strain was inoculated into a 20mL LB broth and incubated at 37°C overnight. From the overnight culture, 200μL of the bacterial suspension was transferred into a fresh 20mL LB broth and allowed to grow with shaking at 37°C until the optical density (OD) value reached approximately 0.5 to 1.0. Once the desired OD value was reached, the culture was placed on ice for 10 minutes. The bacterial culture was then centrifuged (3,500g) at 4°C for 15 minutes. The supernatant was removed, and the pellet was resuspended in 10mL of 100mM CaCl2. The resuspended culture was placed on ice for an additional 30 minutes. After the incubation, the bacterial suspension was centrifuged (3,500g) again at 4°C for 15 minutes.

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The supernatant was removed, and the pellet was resuspended in 10mL of 100mM $CaCl_2$. The final cell suspension was then distributed into Eppendorf tubes with 200μL each. The prepared competent cells were stored in a −80°C refrigerator for future use in the mutagenesis study.

Preparation for *K. pneumoniae* NTUH-K2044 competent cells was performed by following method: The *K. pneumoniae* NTUH-K2044 strain was inoculated into a 20mL LB broth and incubated at 37°C overnight. The overnight bacterial culture was centrifuged (10,000g) at 4°C for 15 minutes. The supernatant was removed, and the pellet was resuspended in 10mL of sterile double-distilled water. The bacterial suspension was then centrifugated (10,000g) at 4°C for 15 minutes. The supernatant was once again removed, and the bacterial pellet was mixed with 1mL of 10% glycerol. The final cell suspension was then distributed into Eppendorf tubes with 200μL each. The prepared competent cells were stored in a −80°C refrigerator for future use in the mutagenesis study.

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2.2 Antimicrobial susceptibility testing

Following the CLSI guidelines, the MICs of fosfomycin (Sigma-Aldrich, Saint Louis, MO, USA) were determined by the agar dilution method using Mueller-Hinton agar plates (BD, France) supplemented with 25 mg/L G6P (Sigma-Aldrich).

To measure the MIC, the bacterial isolate was subcultured from the stock into LB broth overnight. The next day, 1ml of bacterial suspension was collected and centrifuged (10,000 g) at room temperature for 15 minutes. The supernatant was carefully removed, and the bacterial pellet was washed and suspended in 1 mL of sterile saline. The bacterial suspension was once again centrifuged at room temperature for 15 minutes, and the supernatant was discarded. The bacterial pellet was washed and resuspended in 1 mL of sterile normal saline. The optical density of the bacterial suspension was measured at a wavelength of 600 nm (OD600), and diluted the bacterial solution to achieve an OD600 value of 0.1. A total of 3μL of bacterial liquid was spotted on Mueller-Hinton agar containing serial concentrations of fosfomycin. The inoculated plates were incubated in ambient air at 37°C for 16 to 18 h, *E. coli* ATCC 25922 was used as quality control for antimicrobial susceptibility testing. Fosfomycin susceptibility was categorized as follows: (Susceptible: Intermidiate 128mg/dL; Resistant). High-level fosfomycin-resistant *K. pneumoniae* strains were defined as those with MIC ≥ 1024 mg/dL.

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2.3 Sequence analysis of capsular types and fosfomycin resistance genes

The capsular types of the CRKP strains were determined using *wzc* genotyping and whole cps sequencing, as previously reported (Pan et al., 2015). Enzyme-mediated fosfomycin resistance genes (fosA, fosA3, fosB, fosC, fosX, fomA, and fomB), and fosfomycin-related genes (murA, glpT, glpR, uhpT, uhpA, uhpB, uhpC, ptsI, and cyaA) were amplified and sequenced; the primers used are listed in Table 2. Two fosfomycin-susceptible strains, NTUH-K2044 (Fang et al., 2004) (GenBank: AP006725.1) and MGH 78578 (GenBank: CP000647.1) were used as reference strains to identify amino acid substitutions, excluding polymorphisms, observed in the fosfomycin-susceptible strains. Next-generation sequencing was performed for strains in which the genes could not be amplified with the various primer pairs. The genomic DNA extraction for CRKP was performed by following method: The clinical CRKP strain was cultured in 3 ml of LB broth overnight. The next day, centrifuged the culture at room temperature (10,000 g) for 15 minutes to pellet the bacterial cells, the supernatant was removed. Added 160 µl of TE buffer and 40 µl of lysozyme solution to the bacterial pellet for suspension and incubated it at 37°C for 30 minutes. After incubation, added 20 µl of proteinase K and 150 µl of FX buffer to the mixture. Vortexed the sample for 20 seconds and incubated it in 60°C water bath for 40 minutes. Added 10 µl of RNase A to the mixture and incubated it for 10 minutes. Took out the sample from the water bath and added 150 µl of FX buffer and 200 µl of isopropanol. Vortexed the mixture immediately. Transferred the supernatant to a column and centrifuge it at room temperature (10,000 g) for 2 minutes to remove the supernatant. Washed the column twice by adding 500 µl of WS buffer, centrifuged it at room temperature (10,000 g) for 2 minutes, and removed the supernatant. Centrifuged the

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column at room temperature (10,000 g) for 2 minutes to remove any residual ethanol. Redissolved the DNA pellet with 200 µl of ddH2O at 70°C, and left it on the table overnight. The next day, centrifuged the column at room temperature (10,000 g) for 2 minutes to remove the supernatant. Adddc 600 µl of phenol/chloroform to the sample and inverted it vigorously until the specimen appeared white and turbid. Centrifuged the sample at 4°C (10,000 g) for 10 minutes and removed the supernatant. Added 8/10 volume of isopropanol and 1/10 volume of 3M NaOAc to the supernatant. Centrifuge it at room temperature (10,000 g) for 10 minutes, and carefully removed the supernatant to keep the DNA pellet. Added 1 ml of 70% ethanol to the DNA pellet and centrifuged it at room temperature (13,000 g) for 10 minutes. Removed the supernatant, placed the eppendorf containing the DNA pellet in a 70°C dry bath for 5 minutes, and then redissolved the DNA sample with 70°C 200 µl of ddH2O. The extracted DNA was send for next-generation sequencing. The genomes were sequenced using the Pacific Biosciences RS II platform (Menlo Park, CA, USA). Assembly of the data was performed using the hierarchical genome assembly process (HGAP) compiled specifically for quality trimming, de novo assembly, and polishing of PacBio data.

2.4 Analysis of carbapenem resistance gene

Most common carbapenemase genes (*bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{GES}$, *bla*$_{OXA\text{-}23\text{-}like}$, *bla*$_{OXA\text{-}48\text{-}like}$) were amplified for the presence of carbapenemase, the primers used are listed in Table 3. If the carbapenemase genes were not detected by PCR method, The Simplified Carbapenem Inactivation Method (Jing et al., 2018) was performed to identify carbapenemase production. A 1:10 dilution of an inoculum of the indicator *E. coli* ATCC 25922, adjusted to a 0.5 McFarland turbidity standard, was used to incubate on Mueller-Hinton agar plates. Plates were allowed to dry 10 mins. The test strain grown in LB broth

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was smeared evenly onto one side of an imipenem disk (10 μg; Oxoid, Hampshire, United Kingdom). The imipenem disk coated with the test strain was then placed on the Mueller-Hinton agar plates incubated with *E. coli* ATCC 25922. An imipenem disk without coating bacteria placed on a Mueller-Hinton agar plate was used as the control. All plates were incubated at 37°C for 16–18 h in ambient air. If the test strain exhibited carbapenemase activity, the carbapenemase produced by the test strain inactivated the imipenem on the disk. As a result, the indicator strain *E. coli* ATCC 25922 was able to grow around the imipenem disk on the Mueller-Hinton agar. The interpretation of the test results is based on the zone of inhibition observed on the Mueller-Hinton agar plates around the imipenem disk. A zone of inhibition, including satellite growth of colonies with a zone diameter ≤22 mm is considered a positive phenotype. On the other hand, a zone of inhibition with a diameter >22 mm indicates that the test strain does not produce carbapenemase and is considered a negative result.

2.5 Multilocus sequence typing

Multilocus sequence typing (MLST) is a nucleotide sequence-based method that is adequate for characterizing the genetic relationships among bacterial isolates. Primer pairs were designed for PCR amplification and sequencing of internal portions of seven housekeeping genes (gapA, InfB, mdh, pgi, phoE, ropB, and tonB) described from previous studies (Diancourt et al., 2005). The primers used are listed in Table 4. The MLST analysis was based on the Center for Genomic Epidemiology web server (http://www.genomicepidemiology.org/), in which web-based multi-locus sequence type (MLST) 2.0 (Larsen et al., 2012) were used for cluster sequencing types.

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2.6 Plasmid transformation

Nucleobond PC100 Plasmid Midiprep (Takara Bio, Mountain View, CA, USA) was used to purify the plasmid of fosA3-positive strains and controls. The target strain of CRKP was inoculate in 100ml LB broth overnight. The next day, the bacterial suspension was divided into four centrifuge tubes and centrifuged (10,000 g) at 4°C for 15 minutes. After centrifugation, the supernatant was removed. Then 8 mL of Buffer S1 and RNase A were added to each tube. Subsequently, 8 mL of Buffer S2 was added to each suspension, and the tubes were inverted 6-8 times to mix the contents. The mixture was then incubated at room temperature for 3 minutes. Next, pre-cooled 8ml Buffer S3 (4 °C) was added to the suspension. The lysate was gently mixed by inverting the tubes 6-8 times until a homogeneous suspension containing an off-white flocculate was formed. The suspension was then incubated on ice for 5 minutes to allow for proper lysis. The suspension was subjected to another centrifugation step (10,000g) at 4°C for 25 minutes. A filter was wetted with 2.5 mL of Buffer N2, and the supernatant was loaded onto the wet filter. The column was washed with 12 mL of Buffer N3, and the flowthrough was discarded. The elution of the plasmid DNA was performed using pre-heated Buffer N5 at 50°C. Room temperature isopropanol was added. The mixture was centrifuged (10,000) for 30 minutes at room temperature to separate the DNA pellet from the supernatant. The supernatant was discarded, and the DNA pellet was washed with 70% ethanol (room temperature) to remove residual impurities. Vortexed briefly and centrifuge at (10,000 g) for 10 min at room temperature. Dissolve the pellet in sterile deionized water. The plasmid yield was determined using UV spectrophotometry, and the plasmid integrity was confirmed by agarose gel electrophoresis.

Purified plasmids were transformed into competent *E. coli* DH10B cells through electroporation under the conditions of 2000 V/200 Ω/25 μF. After 1 h of recovery, the

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E. coli cells were cultured on plates with 16 mg/dL fosfomycin. Insertion of fosA3 in the *E. coli* cells that grew on the plates was confirmed using PCR amplification.

2.7 Sole carbohydrates growth assay

In sole carbohydrate growth assay, the functionality of transport proteins (glpT, uhpT) in *K. pneumoniae* was checked by observing bacterial growth on a minimal solid medium supplemented with G3P or G6P as a source of nutrition. The M9 minimal medium agar was supplemented with 0.2% (w/v) G6P or G3P as the sole carbon source. The recipes for G6P and G3P agar were listed in Table 1. CRKP strains to be tested are cultured overnight in Mueller-Hinton broth. The following day, the bacterial cells are collected by centrifugation at room temperature (10,000 g) for 15 minutes to remove the supernatant. The bacterial pellet is washed and suspended in 1 mL of sterile saline and then centrifuged again at room temperature (10,000 g) for 15 minutes. The supernatant was removed, and the bacterial pellet was washed and resuspended in 1 mL of sterile saline. The OD600 of the bacterial suspension was measured using a spectrophotometer, and diluted the bacterial solution to achieve an OD600 value of 1.0 (corresponds to approximately 10^9 colony-forming units (CFU) per mL of bacterial cells). The bacterial suspension is then serially diluted with sterile normal saline to obtain different concentrations as 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU/mL, with each dilution based on the measured OD600 value. Two microliters of each bacterial dilution are spotted onto minimal solid medium plates supplemented with different single sugars. The plates were then placed in a 37°C incubator for 16-24 hours to allow bacterial growth. NTUH-K2044 was used as the reference strain. If the colony was not formed after 1,000 fold dilution, the result is interpreted as negative phenotype.

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2.8 Site-Directed Mutagenesis

Site-directed mutations were generated in the fosfomycin-susceptible strain NTUH-K2044 (MIC = 32) using the pKO3-km plasmid (Link et al., 1997). The mutants were generated using a modified pKO3-Km vector that contained a temperature-sensitive origin of replication and markers for positive and negative selection for chromosomal integration and excision. The DNA fragments, including point mutation sites and designed flanking regions (glpT 274V, glpT 299D, uhpA ES86, uhpC 393V, and fosA91V) with NotI cutting site, were amplified using PCR. The primers used to amplify flanking regions was listed in Table 2. As shown in Figure S1, The amplified fragment was ligated with the pKO3-km plasmid using ligase. The resulting constructs pKO3-km were then electroporated into the competent cells *K. pneumoniae* NTUH-K2044. The transformants were cultured in LB medium containing kanamycin 50ug overnight at 30°C to select for bacteria containing the constructs. Subsequently, the selected strains with the plasmid were incubated at 43°C overnight. The higher temperature prevents the plasmid from replicating in the bacterial cells, therefore the plasmid was integrated into the bacterial chromosome by homologous recombination. The strain confirmed to have the plasmid inserted into the bacterial gene was then cultured on LB medium containing 5% sucrose. The pKO3-km plasmid carried the sacB gene, which makes the bacteria sensitive to sucrose. Therefore, culturing the bacteria in medium containing sucrose would stimulate the bacteria to remove the plasmid from the bacterial gene, leaving behind the desired gene modification. The final mutants were confirmed by PCR using appropriate combinations of primers.

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2.9 mRNA expression

For expression levels of the fosA, bacterial RNA and cDNA from the tested strains were obtained by RNeasy minikit (Qiagen, USA) and Prime Script RT master mix (TaKaRa, Japan). Clinical strains of CRKP were cultured in LB broth overnight. On the next day, 10 μl of the bacterial culture was taken and inoculated into 1000 μl of LB broth. The bacteria culture was allowed to grow for 2 hours until it reaches an optical density (OD) of 0.5. Then, centrifuged the bacteria culture at 10,000g for 15 minutes. The supernatant was removed and added 750 μl of Trizol Reagent to wash and suspend the bacterial pellet. Allowed it to stand for 5 minutes. Next, added 200 μl of chloroform to the Trizol-bacterial mixture, and thoroughly mix it. Let the mixture stand for 2-3 minutes, and then centrifuged (12,000g) at 4°C for 15 minutes. After centrifugation, distinct layers were visible: the upper transparent layer contained RNA, the middle layer contained proteins, and the lower purple layer contained DNA. Carefully transferred the upper clear layer (containing RNA) to a new 1.5ml tube. Then, added 0.5ml of isopropanol to the tube, mixed it thoroughly, and let it stand for 10 minutes. Afterward, centrifuged the tube (12000g) at 4°C for 15 minutes. Removed the supernatant, and added 1ml of 75% ethanol to the RNA pellet. Centrifuged it (7500g) at 4°C for 5 minutes after brief vortexing. the ethanol supernatant was removed, and let the RNA pellet air dry for 5-10 minutes. Finally, dissolved the RNA pellet with 50 μl of RNase-free H2O to obtain the purified RNA sample. 400 ng of the extracted RNA was used for complementary deoxyribonucleic acid (cDNA) synthesis, utilizing SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). The resulting cDNA was then subjected to quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using Power SYBR® Green Master Mix (Thermo Fisher Scientific) and an ABI 7900 Real-Time PCR system (Applied Biosystems). The

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expression of 23SrRNA served as the internal control group for normalization purposes. Data were calibrated against the baseline expression level of *K. pneumoniae* NTUH-2044, and fold change in expression was calculated by the comparative threshold cycle method.

2.10 Pulse Field Gel Electrophoresis

Pulse field gel electrophoresis is a common technique used for the separation of large DNA fragments and chromosomes. The target strain of CRKP was inoculated in 100ml LB broth overnight. The overnight bacterial culture was centrifuged (10,000g) at 4°C for 15 minutes. The supernatant was removed, and the pellet was suspended in SE buffer (75mM NaCl, 25mM EDTA, pH 7.5) and adjusted to a turbidity matching 0.12 absorbance at 620 nm. The cell suspension was mixed with molten 1% agarose solution at 54°C and dispensed into plug moulds. Once the agarose sets, it was removed from the moulds and transferred to tubes containing lysis buffer, where it was incubated in a water bath for 2 hours at 54°C. The lysis buffer was then decanted, and the agarose plug was washed four times with warm TE buffer (100mM Tris, 10mM EDTA, pH 7.4). Next, a 2 mm slice of the agarose gel containing the embedded DNA was cut using a scalpel blade. The slice was then mixed with an XbaI restriction enzyme mixture in an Eppendorf tube and incubated for 2 hours at 37°C. To create wells for electrophoresis, a 1% agarose gel was cast with a comb in place. The agarose plug slice with the DNA was removed from the tube and carefully placed into the well of the agarose gel. The wells were sealed with 1% agarose solution to prevent leakage during electrophoresis. The gel was electrophoresed for approximately 18 hours. After electrophoresis, the entire gel was transferred to a staining bath containing 1μg/ml ethidium bromide. The gel was de-stained and washed several times with distilled water and then placed on a UV transilluminator. The DNA bands were observed and photographed for documentation. The interpretation

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criteria of PFGE is shown in Supplement Table S1 (Tenover et al., 1995).

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3. Results

3.1 Antibiotic susceptibility and capsular types

In total, 108 CRKP strains were collected in our study. Nine strains were isolated from urine, 11 from sputum, 3 from pus, and the remaining 17 strains were isolated from blood cultures. Antibiotic susceptibility test revealed that 54 strains (50.0%) were susceptible to fosfomycin, 14 strains (13.0%) exhibited intermediate resistance, and 40 strains (37.0%) were resistant to fosfomycin. Among the 40 fosfomycin-resistant CRKP strains, 26 exhibited high levels of fosfomycin resistance (MIC > 1024 mg/dl), while 14 showed low levels of fosfomycin resistance (MIC between 256 and 512 mg/dl) (Table 5). As presented in Table 5, capsular typing results showed that K47 was the most dominant capsular type (52.5%, 21/40), followed by K64 (27.5%, 11/40) and K62 (7.5%, 3/25). Capsular types K5, K15, K24, K54, and KN2 were each identified in one strain (2.5%, 1/40). KPC was detected in 21 of 40 fosfomycin-resistant CRKP strains, and the remaining 19 strains fosfomycin-resistant CRKP strains had no carbapenemase detected by both genotype and phenotype assay. The MLST results revealed that among the 40 fosfomycin-resistant CRKP strains, 34 strains (85.0%) belonged to ST11, 3 strains (7.5%) belonged to ST378, and remaining 3 strains were ST15, ST 29 and ST 76 (2.5% each).

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3.2 Transporter genes

3.2.1 Transporter deficiencies exam

Genes of fosfomycin-targeting enzymes and transporters were examined using PCR amplification and sequencing (Table 6). We did not find any sequence changes or amino acid variations in *murA* in all 40 fosfomycin-resistant strains, while transporter gene mutations were observed in 19 strains. As growth impairment using a sole carbohydrate source among the 19 strains indicated the loss of function of transporter genes, we performed a growth assay using G3P and G6P to check the function of these transporters. All 19 strains had growth impairment on either G3P or G6P minimal medium agar, which was compatible with gene mutations (Table 6). Eight strains were not able to grow on G3P minimal medium agar only, three isolates could not grow on G6P minimal medium agar only, and growth impairment was observed on both G3P and G6P minimal medium agar in eight strains. No cyaA or ptsI gene mutation could be identified among 40 fosfomycin resistant CRKP.

3.2.2 Genotype and phenotype studies of glpT system

In the 15 strains that showed growth impairment on G3P minimal medium agar, an early stop codon was found in glpT of 5 strains (FO23, FO33, FO34, FO35, FO36), and an amino acid substitution in glpT (299D, 274V) were detected in 6 strains (FO27, FO28, FO29, FO30, FO31, FO32). Loss of small nucleotide fragment in glpT in 2 strains (FO25, FO26) and an insertion sequence added in the glpT coding region in one strain (FO22) were also detected. Mutations in glpR were not detected in this study. In the G3P growth assay, all these 15 strains with genetic deficiency had growth impairment (Figure 1).

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Among five strains with an early stop codon was found in glpT, there were three types of genetic change (FO23, Early stop codon 276; FO33, FO34, FO35, Early stop codon 293; FO36, Early stop codon 290). The G3P growth assay revealed all of the strains with three types of genetic change had only minimal growth on G3P agar, which indicated the function of G3P transporters system in these strains was loss. Among three strains with small fragments loss (FO25, 26) and insertion sequence added in the glpT coding region, The G3P growth assay showed the growth impairment was obvious for these strains on G3P agar. For 6 strains with an amino acid substitution in glpT (299D, 274V), the strains did not form colony after 10^3 folds dilution. However, we still observed partial growth on G3P. The G3P transportation system might still have partial function with the amino acid substitution in glpT (299D, 274V).

3.2.3 Genotype and phenotype studies of uhpT system

Among 11 isolates with growth impairment on G6P minimal medium agar, an early stop codon was detected in uhpA and uhpT in one strain and six strains, respectively. Amino acid substitution was detected in uhpB (393V) in one strain. We were not able to amplify uhpA, uhpB, uhpC, and uhpT in two strains (FO26, FO40) using the primer pairs. Therefore, we designed primer pairs (Table 2) for the outer area of the uhpABCT operon to amplify and sequence the fragments. Results showed that FO40 had lost a 5154 bp-long fragment, including most of the uhpABCT operon and ilvNBL operon (Figure 2). Because we were unable to find suitable primer pairs, next generation sequencing revealed that FO26 (GenBank accession: JAHZSM000000000) had lost a large fragment (39058 bp), including the entire uhpABCT operon (Figure 3). In the G6P growth assay (Figure 4), significant G6P growth impairment was found among two strains with entire uhpT loss (FO26, FO40), one strain with insertion sequence in uhpT (FO22) and six

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strains with early stop codon at 69 (FO27, FO28, FO29, FO30, FO31, FO32). For the two strains with regulatory gene deficiency (FO24, uhpB 393V; FO39, uhpA early stop codon at 86), colony was not form after 10^3 folds dilution. One strain with uhpB Early stop codon at 446 had no growth impairment comparing with reference strain.

3.2.4 Mutagenesis studies for transporter system

To investigate whether transporter-related point mutations contribute to fosfomycin resistance, we conducted site-directed mutagenesis on four types of genetic deficiencies (glpT 274V, glpT 299D, uhpB 393V, uhpA early stop codon at 86) as shown in Table 7. For each NTUH-2044 mutant, we performed MIC tests and sole carbon growth assays. The results revealed that the MIC of fosfomycin for NTUH-K2044: uhpA ES86 mutant increased by 32-fold (1024 mg/dL), indicating significant resistance. Similarly, the other three mutants, NTUH-K2044: uhpB 393V, NTUH-K2044: glpT 274V, and NTUH-K2044: glpT 299D, also showed an increase in MIC above then 1024 mg/dL, suggesting elevated resistance levels.

Additionally, the sole carbon growth assays confirmed the growth impairment in the mutants (Table 7). Specifically, in Figure 5, the G6P growth assay of NTUH-K2044:uhpB393V was examined. Upon generating the uhpB393V mutation in NTUH-K2044, a significant growth impairment was observed compared to the reference NTUH-K2044 strain. However, it was noted that the growth on G6P agar was even poorer for FO24 (uhpB393V) and FO26 (Negative control, loss of entire uhpT system), indicating that there might be other contributing factors affecting G6P uptake. This impairment in growth further supports the role of the identified transporter-related point mutations in fosfomycin resistance.

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3.3 Enzyme-mediated resistance

3.3.1 Plasmid mediated resistance

We detected fosA3 in seven isolates (7/40, 24%) (Table 4). Next generation sequencing was performed for FO15 (Genbank accession: CP073002) to read the whole genome, and we wound the fosA3 of FO15 was located on plasmid. Primers were designed to exam the surrounding elements of fosA3. We found the flanking regions of the seven isolates were observed to be identical (Figure 6). Fragments with 2497 bp in total, which contained fosA3, orf1, orf2, and a truncated orf3, was located between two IS26 elements.

To further confirm the presence of fosA3 genes on the plasmid, we conducted a plasmid transformation study (Table 8). For this study, seven CRKP strains (FO15, FO16, FO17, FO18, FO19, FO20, FO21) with fosA3 detected, three randomly selected CRKP strains (FO22, FO26, FO38) without fosA3 detected, and NTUH-K2044 were used as donors in the plasmid transformation procedures. The recipient strain used for the plasmid transformation was *E. coli* DH10B.

After performing the plasmid transformation procedures, all seven CRKP strains carrying the fosA3 gene successfully transferred the plasmid to the recipient *E. coli* DH10B, as evidenced by their ability to grow on fosfomycin plates at a concentration of 16 mg/dL. A further PCR confirmation test was conducted, and the results were consistent. The MIC results for all seven CRKP recipient *E. coli* DH10B strains showed values above 1024 mg/dL. These findings indicate that the fosA3 gene was present on the plasmids.

On the other hand, the three CRKP strains without fosA3 detected, as well as the NTUH-K2044 strain, the recipient *E. coli* DH10B did not show growth on the fosfomycin plate after plasmid transformation. This result suggests that the plasmids from these

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strains did not carry the fosA3 or other fosfomycin resistant gene.

3.3.2 Chromosomal fosA mediated resistance

We named the chromosomal fosA in the genomes of *K. pneumoniae* as $fosA^{KP}$, as previously described (Ito et al., 2017). We designed suitable primer pairs to detect fosA and fosA3 that are able to distinguish $fosA^{KP}$ from the fosA3. There are 13 nucleotides differences between primer pairs of chromosomal fosA and fosA3 (Table 1). The chromosomal $fosA^{KP}$ is presented in most *K. pneumoniae*, including the fosfomycin susceptible strains. Among the 14 low-level fosfomycin-resistant strains, no mutations were detected in the murA gene, transporter genes, or in plasmid-mediated fosA3. The expression level of the chromosomal $fosA^{KP}$ was analyzed. However, upon comparison with the reference strain NTUH-K2044, there was no consistent increase observed in fosAKP mRNA expression among the 14 low-level fosfomycin-resistant strains (Figure 7).

We further used the fosA primers to amplify, sequence, and compare $fosA^{KP}$ with the reference strains. Compared with the reference strains (NTUH-2044, MGH 78578), mutation I91V was found in 39/40 (97.5%) fosfomycin-resistant CRKP. All 14 low-level resistant strains carried I91V mutation in $fosA^{KP}$. To further confirm the mutation is related to fosfomycin resistance, site-directed mutation study was performed to confirm whether this mutation contributed to fosfomycin resistance. The MIC of fosfomycin in the NTUH-2044 $fosA^{KP}$ I91V mutant was 8-fold higher (from 32 mg/dl to 256 mg/dl) than the wild type NTUH-2044 (Table 7).

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3.4 Analysis of clones

In addition, we observed that several strains shared the same resistance mechanism. To distinguish these strains, we analyzed the capsular types using the available nucleotide sequences and performed pulsed-field gel electrophoresis (PFGE) analysis. Among the 14 low-level fosfomycin-resistant strains with the fosA I91V mutation, we detected five different capsular types (K5, K47, K54, K64, and KN2). However, we could not distinguish six strains with capsular type K47 using the available methods. We examined all of the available nucleotide sequences related to fosfomycin resistance, but no difference could be identified among these six K47 CRKP strains. The results of MLST showed that all six strains belonged to ST11. So six strains shared the same gene deficiency of glpT D274V and uhpT ES 69 (FO27, FO28, FO29, FO30, FO31, FO32). FO30 had one fragment different, and FO29 had two fragments different from other strains of PFGE analysis (Figure 8). According to the interpretation criteria of PFGE (Table S1) (Tenover et al., 1995), FO30 was still considered the same clone, and FO29 was closely related to other five strains.

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4. Discussion

4.1 Interpretations

Treatment of CRKP continues to be a challenging task, with limited therapeutic options available. Fosfomycin has emerged as one of the few viable treatment choices. However, recent reports have indicated a high fosfomycin resistance rate among CRKP in clinical settings with fosfomycin was commonly prescribed, ranging from approximately 32% to 80% in different studies. (Kaase et al., 2014;Yang et al., 2019;Liu et al., 2020;Huang et al., 2021) Several studies have reported that the fosA family is the main mechanism of CRKP resistance, especially in East Asian countries. Among several variants of the fosA family, fosA3 was the most commonly reported subtype of plasmid-mediated fosA based on reports from Taiwan (Tseng et al., 2017), Korea (Lee et al., 2012), and China (Wang et al., 2020). Huang et al. (2017) and Tseng et al. (2017) reported that fosA5 was also prevalent among CRKP.

However, according to a previous literature (Ma et al., 2015), fosA5 showed high similarity to the chromosomal $fosA^{KP}$ of *K. pneumoniae*, which can be found in most of *K. pneumoniae* (Ito et al., 2017). In our current study, we designed primers that could differentiate between plasmid-mediated and chromosomal-mediated fosA in *K. pneumoniae* strains. Through our investigation, we observed that fosA5 was present even in fosfomycin-susceptible strains, including *K. pneumoniae* NTUH-K2044. As a result, the detection of fosA5 through amplification may not significantly contribute to plasmid-mediated high-level fosfomycin resistance. To further elucidate the genetic factors underlying fosfomycin resistance, we conducted an in-depth analysis of the surrounding genetic elements of fosA3 and performed plasmid transformation experiments. Our

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findings consistently supported the notion that only fosA3, and not $fosA^{KP}$, was located on the plasmid.

Chromosomal $fosA^{KP}$ is present in most *K. pneumoniae*, and $fosA^{KP}$ I91V has also been reported previously by Ito et al. (2017). However, the relationship between the mutation and MIC of fosfomycin was not studied. The baseline activity of Chromosomal fosA is not the same among different species. A previous study suggested that chromosomally-encoded fosA was associated with a higher MIC distribution in CRKP (Elliott et al., 2019). However, most *K. pneumoniae* harboring *fosA* still exhibited MIC below CLSI breakpoints (64 mg/dL), and the difference between resistant and susceptible strains was not clear. To the best of our knowledge, the genetic or functional mechanism underlying this phenomenon among fosfomycin resistant CRKP is not yet reported. Our study is the first to document that chromosomal $fosA^{KP}$ I91V is related to low-level fosfomycin resistance. Mutagenesis of fosA increased the MIC by 8-fold (from 32 to 256 mg/dL) in NTUH-K2044. Furthermore, genetic studies revealed that 39/40 of the fosfomycin-resistant CRKP contained chromosomal $fosA^{KP}$ I91V, and this is the only mechanism observed in low-level fosfomycin-resistant strains. This observation suggests that chromosomal $fosA^{KP}$ I91V alone might not be sufficient to confer high-level fosfomycin resistance in strains with very low intrinsic resistance. Interestingly, previous studies (Yang et al., 2019; Liu et al., 2020) on fosfomycin resistance mechanisms did not emphasize the significance of chromosomal $fosA^{KP}$. While the plasmid-mediated fosA genes have been the primary focus of fosfomycin resistance research, our study highlights the potential importance of considering chromosomally-encoded $fosA^{KP}$ in the context of treatment options. Even though these strains may initially show susceptibility to fosfomycin, the presence of chromosomal fosA could increase the risk of developing

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resistance over the course of treatment.

Aside from the chromosomal fosA mutation, transporter gene deficiencies were the most common mechanism identified among the high-level resistant strains in our study. Previous molecular study (Xu et al., 2017) showed deletion of glpT, uhpT in *S. aureus* increased fosfomycin 8-fold and 64-fold. With deletion of both transporter gene, the MIC of mutant increased more than 1024-fold. Another study (Ballestero-Téllez et al., 2017) revealed that the *E. coli* null mutant of glpT, uhpT and both increased fosfomycin 0-fold, 32-fold and 128-fold. Lack of glpT only did not have impact on MIC. However, highly resistant mutants appeared at 64mg/dL. Both studies showed deficiency of uhpT system has more impact on fosfomycin MIC.

Although transporter gene deficiency-related resistance has been well studied in *S. aureu* and *E. coli*, there are only limited reports on *K. pneumoniae*. Lu et al. (2016) reported that glpT and uhpT were found in 97% of fosfomycin-resistant extended spectrum β-lactamase (ESBL)-producing *K. pneumoniae*. Various amino acid substitutions were found in the study. However, most of the strains in their study exhibited intermediate resistance, and genetic studies were not performed to confirm this relationship. In the study conducted by Liu et al. (2020), only 3 of 48 strains harbored deletion or mutation of glpT. No gene deficiency or functional changes were found in the G6P transporter system. Furthermore, an antimicrobial susceptibility testing above 512 mg/dL was not performed. In our study, deficient transporters were associated with various kinds of gene mutations, including early stop codons, insertion in the coding region, and single amino acid mutations in transporters and their regulatory genes. The entire deletion of uhpABCT operon was also detected in two of the strains. In our study, we found that deficiencies in glpT or uhpT in the *K. pneumoniae* strains resulted in high

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levels of fosfomycin resistance, with minimum inhibitory concentrations (MIC) exceeding 1024 mg/dL. Both glpT and uhpT system deficiencies contributed significantly increased in fosfomycin MICs. Unlike previous studies conducted on *E. coli* and *S. aureus*, our study did not observe a different impact between glpT and uhpT system deficiencies in fosfomycin resistance. However, it is important to note that the baseline MIC of fosfomycin was relatively higher in *K. pneumoniae* compared to the two organisms. To investigate further differences, we could examine fosfomycin MICs above 4096 mg/dL, but this would require a substantial amount of expensive fosfomycin for testing. Additionally, the clinical relevance of such high MIC values may not be significant. Therefore, we did not conduct this specific study.

As these transporters are not essential for *K. pneumoniae*, it is not surprising that deficient transporters with various gene mutations were frequently observed under fosfomycin selective pressure. Notably, we found that three single amino acid mutations (glpT E299D, glpT D274V, and uhpC A393V) were related to fosfomycin resistance. Although a previous biochemical study revealed that D274 and E299 are conserved sites in *E. coli* glpT essential to its function (Law et al., 2008), whether the site is essential to *K. pneumoniae* glpT was not reported. To confirm their role in resistance, we performed mutagenesis experiments by introducing these mutations into the transporter genes of NTUH-K2044. The results showed that these mutations led to a significant increase in fosfomycin MIC, with values surpassing 1024 mg/dL. Our findings suggest that the mutations at these specific sites in glpT and uhpC may be contributing factors to the observed fosfomycin resistance in *K. pneumoniae*. We did not identify any mutations in the cyaA and ptsI genes. These two genes play a significant role in regulating the production of cAMP, which serves as an essential intracellular second messenger

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molecule involved in the regulation of numerous physiological processes in bacteria. When mutations occur in cyaA and ptsI, they can lead to a reduced synthesis of cAMP, the impact of reduced cAMP synthesis extends beyond the specific mechanism of fosfomycin resistance. The altered levels of cAMP can have broader effects on bacterial physiology, including changes in metabolic pathways, signal transduction, and gene expression, which may influence bacterial growth, virulence, and other antibiotic resistance mechanisms. Actually, fosfomycin resistant that contributed to cAMP system deficiency is seldom reported in clinical strains.

We did not observe any murA mutations in the fosfomycin-resistant CRKP. This finding is in contrast to previous research conducted by Lu et al. (2016), in which variant murA modifications were detected in 70% (21/30) of fosfomycin-resistant ESBL-producing *K. pneumoniae* strains. Similarly, Liu et al. (2020) identified murA mutations in only one strain among 48 fosfomycin-resistant strains. MurA is a critical gene involved in cell wall synthesis, and losing its function can lead to a significant increase in biological fitness. Previous studies have shown that most strains with murA mutations also carry transporter gene mutations (Lu et al., 2016; Liu et al., 2020). However, in their studies, mutagenesis was not performed. As a result, the murA mutation observed in our study may be polymorphic, and other mechanisms might be responsible for the fosfomycin resistance observed in these strains.

In our previous study (Pan et al., 2015), we demonstrated that capsular type-specific bacteriophages and capsule depolymerases could be novel therapies for eradicating CRKP. Due to most phages can only infect limited capsular type *K. pnuemoniae*, investigating the capsular type is essential. We observed that K47 and K64 were the predominant capsular types among fosfomycin-resistant CRKP strains. Notably, K47 was

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not frequently detected in our previous research, which could be attributed to our specific focus on fosfomycin-resistant CRKP in this study. It is possible that K47 strains are more prevalent within the fosfomycin-resistant CRKP subset. Importantly, all of the K47 strains identified in this study were found to carry the KPC-2 gene, which has become the most common carbapenemase gene in Taiwan according to recent epidemiological studies. Therefore, it is still possible that there are changes in the endemicity of CRKP strains in the region. Considering these findings, it is crucial to acknowledge the significance of K47 coverage, especially in the context of developing therapies such as bacteriophage therapy development. Further exploration of novel capsular type-specific therapies may be warranted to address the changing epidemiology of *K. pneumoniae* and enhance our ability to combat multidrug-resistant infections effectively.

In our study, we observed the relationship between capsular typing and MLST in CRKP strains. Among the CRKP strains analyzed, a significant proportion belonged to the ST11 sequence type. Interestingly, we observed different capsular types, such as K64 and K47, among the ST11 strains. These findings are compatible with previous epidemiological studies conducted in China (Yang et al., 2020; Wei et al., 2022). A study by Liao et al. (2022) also reported marked variations in MLST and capsular types among hypervirulent *K. pneumoniae* strains. This suggests that the capsular type diversity is not solely determined by the genetic background (MLST sequence type) of the bacteria.

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4.2 Strengths and limitations

In our study, detailed gene examinations were performed, and serial functional assays and mutagenesis studies confirmed that the detected gene mutations contribute to the high-level fosfomycin resistance. Three single amino acid mutations (glpT E299D, glpT D274V, and uhpC A393V) were observed for the first time to contribute to *K. pneumoniae* fosfomycin resistance. A series of strains shared same gene deficiency. Although the clinical strains were collected from different patients, it is possible that these strains belonged to a clone. We also performed capsular typing, nucleotide sequences analysis, MLST, and PFGE analysis. Most of the strains could be differentiated by these methods. However, some of them, such as 6 strains of K47 with fosAI91V (FO02-07), and 4 strains of K47 with glpT D274V (FOS27, 28, 30, 32), could not be excluded as the same clone.

4.3 Conclusions

In summary, we observed that chromosomal $fosA^{KP}$ I91V was present in 39/40 fosfomycin-resistant CRKP and contributed to a low level fosfomycin resistance. The chromosomal $fosA^{KP}$ mutation was the primary mechanism responsible for low-level resistance in CRKP strains, and it was also prevalent in most high-level resistance strains. Another significant resistance mechanism among high-level fosfomycin-resistant CRKP strains was transporter deficiency. We identified various mutations in G3P and G6P transporter genes, including three novel single amino acid mutations (glpT E299D, glpT D274V, and uhpC A393V). Plasmid-mediated *fosA3* only accounted for 27% of the high-level resistant mechanisms. No murA mutation or cAMP regulatory system deficiency was found. Our findings suggest potential new therapeutic approaches against CRKP

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infections in Taiwan. Understanding the diverse resistance mechanisms involved can aid in developing targeted treatments and optimizing antibiotic regimens to combat CRKP infections effectively. These insights may contribute to better patient outcomes and the management of antibiotic resistance in healthcare settings.

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Table 1 Bacterial strains, plasmids, culture medium and antibiotics

Strains	Source
108 carbapenem-resistant *K.p* 40 fosfomycin resistant *K.p*（40/108）	National Taiwan University Hospital, Taipei Veterans General Hospital, National Cheng Kung University Hospital, and Linkou Chang Gung Memorial Hospital
E.coli DH10B	Invitrogen
E.coli ATCC25922	ATCC, Manassas, USA
K.pneumoniae NTUH-K2044	National Taiwan University Hospital
MGH 78578 （strain ATCC 700721）	ATCC, Manassas, USA

Plasmid		Source
pKO3-Km	pKO3 derived plasmid, with an insertion of kanamycin resistance cassette	Modified from (Chen, 2020)

Culture medium	Contents per liter	Source
Luria-Bertani（LB） agar	5g NaCl, 10g trypton, 5g yeast extract, 15g agar	Bio basic, Ortatio, Canada
Luria-Bertani（LB） broth	5g NaCl, 10g trypton, 5g yeast extract	Bio basic, Ortatio, Canada
Mueller-Hinton agar	2g beef extract, 17.5g acid digest of casein, 1.5g soluble starch, 17g agar	Becton Dickinson, Sparks, USA
Minimal Medium agar	Agar 15g, 5x M9 salt 200mL, 1M MgSO4 2mL, 1M CaCl2 100uL	Modified from (Chen, 2020)
G3P agar	Agar 15g, 5x M9 salt 200mL, 1M MgSO4 2mL, 1M CaCl2 100uL, 2g G3P	Modified from (Chen, 2020)

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G6P agar	Agar 15g, 5x M9 salt 200mL, 1M MgSO4 2mL, 1M CaCl2 100uL, 2g G6P	Modified from (Chen, 2020)

Antibiotics	**Usage**	**Sourse**
Fosfomycin disodium	Antimicrobial susceptibility test	Sigma-Aldrich, St louis, MO, USA
Kanamycin	Colony selection for pKO3-km	

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Table 2 Primers used for fosfomycin resistant genes

Primer	Sequence 5'-3'	
murA F	GCAAACTCAATGGCTTCTAAGC	*murA* gene sequencing
murA R	GAAGAAGATCGACAAGTGATGTG	
cyaA F	GTGACGGTTTTTGTTGAAATACTG	*cyaA* gene sequencing
cyaA R	CGCCTTATCCGGGCCAAAT	
cyaA IF	GCG GTT CGA CCC AGC ATA TC	
ptsI F	GGTTAAACTGATGGCTGAACTCG	*ptsI* gene sequencing
ptsI R	CAAACAAACCCATGATCTTCTCC	
glpT F	GCGACCATATTTGAAGTTGGTG	*glpT* gene sequencing
glpT R	CAGCGCCGAACTGGACAG	
glpR F	GCGAAAACGCGCATAAAGAC	*glpR* gene sequencing
glpR R	GCAGGACGCGGTACAGC	
uhpT F	CGGCGCGCTGGTTTTTAC	*uhpT* gene sequencing
uhpT R	GCGCTTTTTGCATCAGGC	
uhpA F	GTGAATCCGTAGGCCGGATAAG	*uhpA* gene sequencing
uhpA R	CTCCACAGGCAGAACCAGG	
uhpB F	CAACCTGCTGGAAAAGCTG	*uhpB* gene sequencing
uhpB R	GTTTATCGCTAATCGGCGCAG	
uhpC F	AATATCGTCAAGCACGCCAG	*uhpC* gene sequencing
uhpC R	GACGGCGTCCAGGTAAAAC	
IS6 FF	AGTGGCAGATCCCGCGATTC	gene sequencing between IS6
IS6 RR	GGTTTCATCCATGTGCCACG	
FosA3 F	ATGCTGCAGGGATTGAATCATC	*fosA3* gene sequencing
FosA3 R	TCAATCAAAAAAGACCATCCCC	
FosA F	ATGCTGAGTGGACTGAATCACCTG	fosA gene sequencing

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FosA R	TCACTCAGCAAAAAACACCATCC	
FosB F	TTGTTAAAAGGAATCAATCATCTTG	fosB gene sequencing
FosB R	TTAGTAAAACGTCACATGAGGTTTATC	
FosX F	ATGATCGAAGGCCTGTCCC	fosX gene sequencing
FosX R	TCATTGCGCAGCCTCCAAC	
FomA F	ATGACGCCCGATTTCTTGG	fomA gene sequencing
FomA R	TTACGCAGAAGCAGTCGTGG	
FomB F	ATGCTGGAAAACCTCACGATCC	fomB gene sequencing
FomB R	TCATTGCGCAGCCTCCAAC	
FosA frk F	CGACGCCATAGGCAAAGCC	fosA gene mutagenesis
FosA frk R	GACGCAGCCAAGCCTGAAG	
uhpC frk F	CCGGTATCGTCAGCGGCCGCTCCG	*uhpC* gene mutagenesis
uhpC frk R	CGGTCTCTTTGTCCTCTTCG	
uhpA frk F	AGACGGTGCGGTAAGATAG	*uhpA* gene mutagenesis
uhpA frk R	CGGTCTCTTTGTCCTCTTCG	
CP05 F1	CTCTCATCCATACTCTATGAC	FO alignment analysis
CP05 R1	GTCGGACCTGGCATAATG	
KP-wza-CF2	GGGTTTTTATCGGGTTGTAC	Capsular typing
KP-wzc-CR2	GCTTCCATCATTGCAAAATG	

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Table 3 Primers used for carbapenem resistant genes

Primer	Sequence 5'-3'	
KPC F	TGTCACTGTATCGCCGTC	*KPC* gene sequencing
KPC R	CTCAGTGCTCTACAGAAAACC	
IMP F	GGTTAAACTGATGGCTGAACTCG	*IMP* gene sequencing
IMP R	CAAACAAACCCATGATCTTCTCC	
VIM F	GCGACCATATTTGAAGTTGGTG	*VIM* gene sequencing
VIM R	CAGCGCCGAACTGGACAG	
NDM F	GCGAAAACGCGCATAAAGAC	*NDM* gene sequencing
NDM R	GCAGGACGCGGTACAGC	
OXA48 F	CGGCGCGCTGGTTTTTAC	*OXA-48-like* gene sequencing
OXA48 R	GCGCTTTTTGCATCAGGC	
OXA23 F	GTGAATCCGTAGGCCGGATAAG	OXA-23-like gene sequencing
OXA23 R	CTCCACAGGCAGAACCAGG	
GES F	ATGCGCTTCATTCACGCAC	GES gene sequencing
GES R	CTATTTGTCCGTGCTCAGG	

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Table 4 Primers used for MLST

Primer	Sequence 5'-3'	
rpoB F	GGC GAA ATG GCW GAG AAC CA	rpoB gene sequencing
rpoB R	GAG TCT TCG AAG TTG TAA CC	
gapA F	TGA AAT ATG ACT CCA CTC ACG G	gapA gene sequencing
gapA R	CTT CAG AAG CGG CTT TGA TGG CTT	
mdh F	CCC AAC TCG CTT CAG GTT CAG	*mdh* gene sequencing
mdh R	CCG TTT TTC CCC AGC AGC AG	
pgi F	CTG CTG GCG CTG ATC GGC AT	pgi gene sequencing
pgi R	TTA TAG CGG TTA ATC AGG CCG T	
phoE F	ACC TAC CGC AAC ACC GAC TTC TTC GG	phoE gene sequencing
phoE R	TGA TCA GAA CTG GTA GGT GAT	
infB F	ACT AAG GTT GCC TCC GGC GAA GC	infB gene sequencing
infB R	CGC TTT CAG CTC AAG AAC TTC	
tonB F	CTT TAT ACC TCG GTA CAT CAG GTT	tonB gene sequencing
tonB R	ATT CGC CGG CTG RGC RGA GAG	

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Table 5 Characteristics and detected mechanisms of fosfomycin-resistant CRKPs and reference strains

Strain	Specimen	Capsular type	MLST	Carbapenemase genes	MIC (mg/dl)	fosA enzyme		G3P transporter system		G6P transporter system			
						$fosA^{KP}$	fosA3	glpT	glpR	uhpA	uhpB	uhpC	uhpT
NTUH-2044		K1	ST23		32	I91							
MGH 78578		K52	ST38		32	I91							
Low level resistance													
FO01	Urine	K5	ST76		256	I91V							
FO02	Blood	K47	ST11	KPC-2	512	I91V							
FO03	Sputum	K47	ST11	KPC-2	256	I91V							
FO04	Blood	K47	ST11	KPC-2	256	I91V							
FO05	Blood	K47	ST11	KPC-2	256	I91V							
FO06	Sputum	K47	ST11	KPC-2	256	I91V							
FO07	Pus	K47	ST11	KPC-2	256	I91V							
FO08	Blood	K54	ST29		256	I91V							
FO09	Blood	K64	ST11		512	I91V							
FO10	Blood	K64	ST11		512	I91V							
FO11	Blood	K64	ST11		256	I91V							
FO12	Blood	K64	ST11		256	I91V							
FO13	Blood	K64	ST11		256	I91V							
FO14	Blood	KN2	ST11		512	I91V							
High level resistance													
FO15	Pus	K47	ST11	KPC-2	>2048	I91V	+						
FO16	Blood	K47	ST11	KPC-2	>2048	I91V	+						
FO17	Urine	K47	ST11	KPC-2	>2048	I91V	+						
FO18	Urine	K47	ST11	KPC-2	>2048	I91V	+						
FO19	Urine	K47	ST11	KPC-2	>2048	I91V	+						
FO20	Pus	K47	ST11	KPC-2	>2048	I91V	+						
FO21	Blood	K64	ST11		>2048	I91V	+						
FO22	Blood	K15	ST11		>2048	I91V		IS[$]					*IS*[%]
FO23	Blood	K24	ST15		>2048	I91		ES276					
FO24	Blood	K47	ST11	KPC-2	2048	I91V					A393V		
FO25	Blood	K47	ST11	KPC-2	>2048	I91V		LF[@]			ES446		
FO26	Urine	K47	ST11	KPC-2	>2048	I91V		LF[#]		D.	D.	D.	D.
FO27	Sputum	K47	ST11	KPC-2	>2048	I91V		D274V					ES69
FO28	Sputum	K47	ST11	KPC-2	>2048	I91V		D274V					ES69
FO29	Sputum	K47	ST11	KPC-2	>2048	I91V		D274V					ES69

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FO30	Sputum	K47	ST11	KPC-2	>2048	I91V	D274V				ES69
FO31	Sputum	K47	ST11	KPC-2	>2048	I91V	D274V				ES69
FO32	Sputum	K47	ST11	KPC-2	>2048	I91V	D274V				ES69
FO33	Sputum	K62	ST378		>2048	I91V	ES293				
FO34	Sputum	K62	ST378		>2048	I91V	ES293				
FO35	Sputum	K62	ST378		>2048	I91V	ES293				
FO36	Urine	K64	ST11		>2048	I91V	ES290				
FO37	Blood	K64	ST11		>2048	I91V	E299D				
FO38	Urine	K64	ST11		>2048	I91V	E299D				
FO39	Urine	K64	ST11		>2048	I91V		ES86			
FO40	Urine	K64	ST11		>2048	I91V		D.	D.	D	D.

MIC: Minimal inhibitory concentration; G3P: glycerol-3-phosphate;

G6P: glucose-6-phosphate; ES: Early Stop Condon; D.: Deletion

fosA3: Plasmid-borne fosA3 $fosA^{KP}$: Chromosomal fosA

$: Insertion sequence, *IS4-like in glpT coding region (+865)*

%: Insertion sequence, *IS4-like in uhpT coding region (+287)*

@: Loss of fragment, 1160^{th}-1172^{th} nucleotide deletion

#: Loss of fragment, 388^{th}-420^{th} nucleotide deletion

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Table 6 Deficiency in G3P and G6P transport systems and consequent carbohydrate utilization among the fosfomycin high-level resistant CRKP strains.

Strain	MIC	G3P growth	G3P System deficiency	G6P growth	G6P System deficiency
NTUH-2044	32	+		+	
FO15	>2048	+		+	
FO16	>2048	+		+	
FO17	>2048	+		+	
FO18	>2048	+		+	
FO19	>2048	+		+	
FO20	>2048	+		+	
FO23	>2048	-	glpT ES276	+	
FO33	>2048	-	glpT ES293	+	
FO34	>2048	-	glpT ES293	+	
FO35	>2048	-	glpT ES293	+	
FO36	>2048	-	glpT ES290	+	
FO37	>2048	-	glpT E299D	+	
FO38	>2048	-	glpT E299D	+	
FO24	2048	+		-	uhpB 393V
FO39	>2048	+		-	uhpA ES86
FO40	>2048	+		-	Loss of uhpA, ubpB, uhpC, uhpT
FO22	>2048	-	*IS4-like in glpT coding region*	-	*IS4-like in uhpT coding region*
FO25	>2048	-	glpT 1160^{th}-1172^{th} nucleotide loss	+	uhpB ES446
FO26	>2048	-	glpT 388^{th}-420^{th} nucleotide loss	-	Loss of uhpA, ubpB, uhpC, uhpT
FO27	>2048	-	glpT 274V	-	uhpT ES69
FO28	>2048	-	glpT 274V	-	uhpT ES69
FO29	>2048	-	glpT 274V	-	uhpT ES69
FO30	>2048	-	glpT 274V	-	uhpT ES69
FO31	>2048	-	glpT 274V	-	uhpT ES69
FO32	>2048	-	glpT 274V	-	uhpT ES69

MIC: Minimal inhibitory concentration; G3P: glycerol-3-phosphate; G6P: glucose-6-phosphate; ES: early stop codon; (+): growth; (-): no growth.

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Table 7 Minimum inhibitory concentration of fosfomycin in site-directed mutants of NTUH-K2044.

Strain	MIC to fosfomycin	G3P growth	G6P Growth
NTUH-K2044			
Wild type	32	+	+
glpT 299D	>1024	-	+
glpT 274V	>1024	-	+
uhpA ES 86	1024	+	-
uhpC 393V	>1024	+	-
fosA I91V	256	n/a	n/a

MIC: Minimal inhibitory concentration; G3P: glycerol-3-phosphate; G6P: glucose-6-phosphate; (+): growth; (-): no growth; n/a: not available.

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Table 8 Plasmid transformation result for fosA3-carried strains

	Detected fosA gene		Recipient *E. coli* can growth on fosfomycin plate
	fosA	fosA3	
NTUH-2044	+		-
FO15	+	+	+
FO16	+	+	+
FO17	+	+	+
FO18	+	+	+
FO19	+	+	+
FO20	+	+	+
FO21	+	+	+
FO22	+	-	-
FO26	+	-	-
FO38	+	-	-

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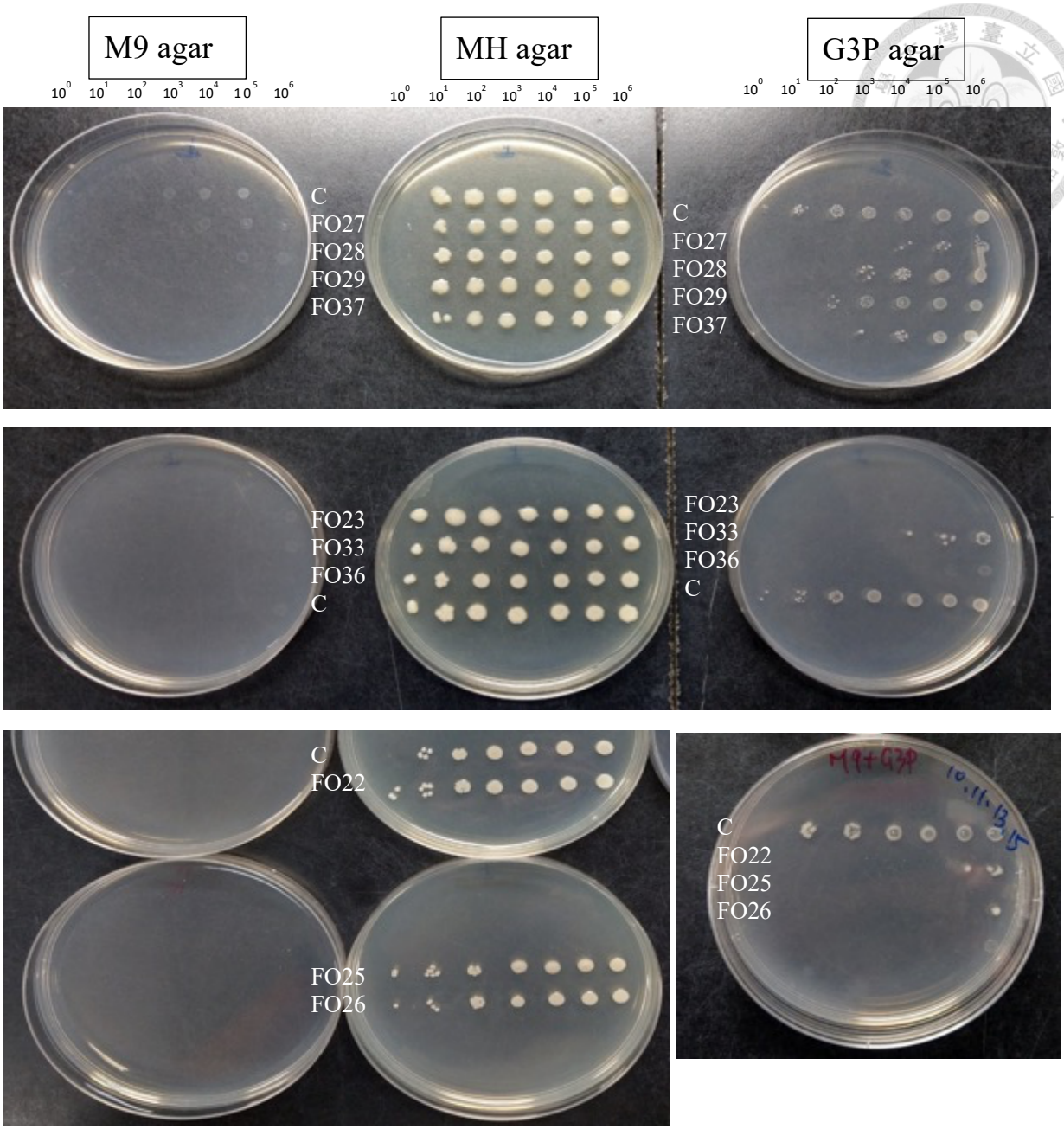

Figure 1 G3P growth assay

The G3P growth assay was performed for CRKP with glpT genotype deficiency using M9 salt medium supplemented with 0.2% G3P as the sole sugar source. The presence of growth indicates that the bacteria's transport protein is functioning normally, allowing the uptake of sugar for bacterial growth.

Left lane: M9 minimal agar without sugar supply, no growth was observed

Middle lane: MH agar, the growth pattern was similar in all isolates

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Right lane: G3P agar. Compared with reference strain NTUH-K2044, growth impairment was observed among CRKP with glpT genotype deficiency

C: Control strain (Reference strain NTUH-K2044)

doi:10.6342/NTU202303850

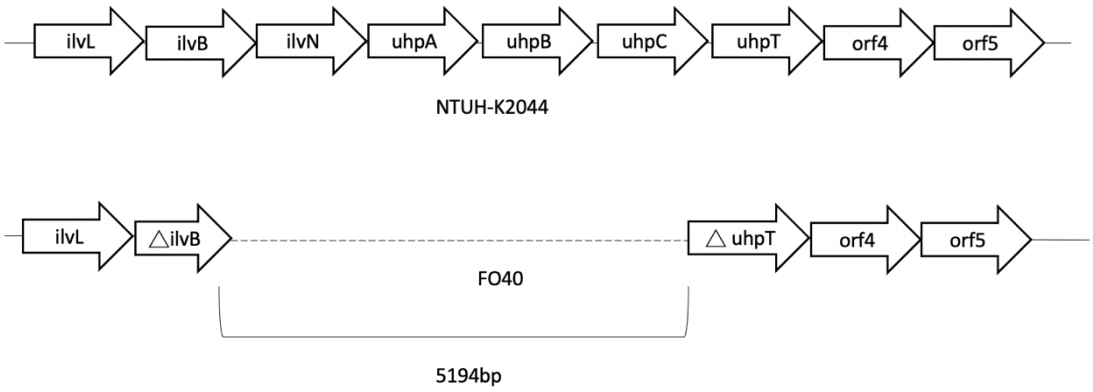

Figure 2 Flanking region of uhpABCT operon of NTUH-2044 and FO40

Previous primer pairs cannot detect the uhpT, uhpA, uhpB, and uhpC in FO40. We tried to design primer pairs from the outer region according to reference strain NTUH-K2044. After several trials, we found 5194bp loss between gene ilvB and uhpT.

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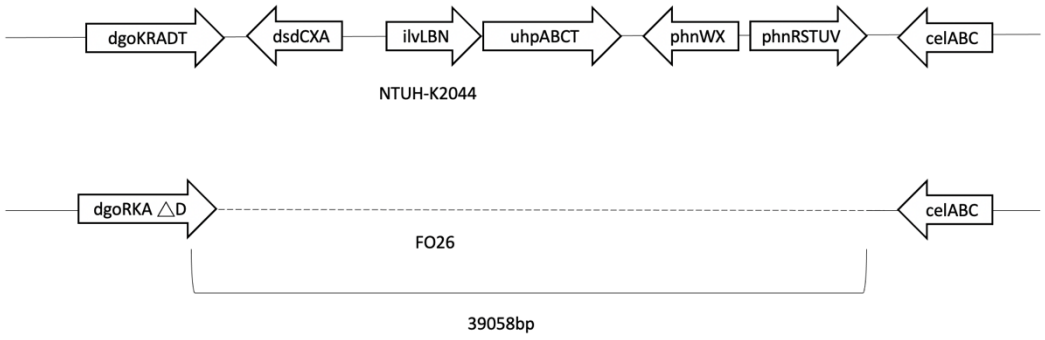

Figure 3 Flanking region of uhpABCT operon of NTUH-2044 and FO26

The next generation sequence result showed comparing with NTUH-K2044, FO26 has large genome loss (39058bp).

doi:10.6342/NTU202303850

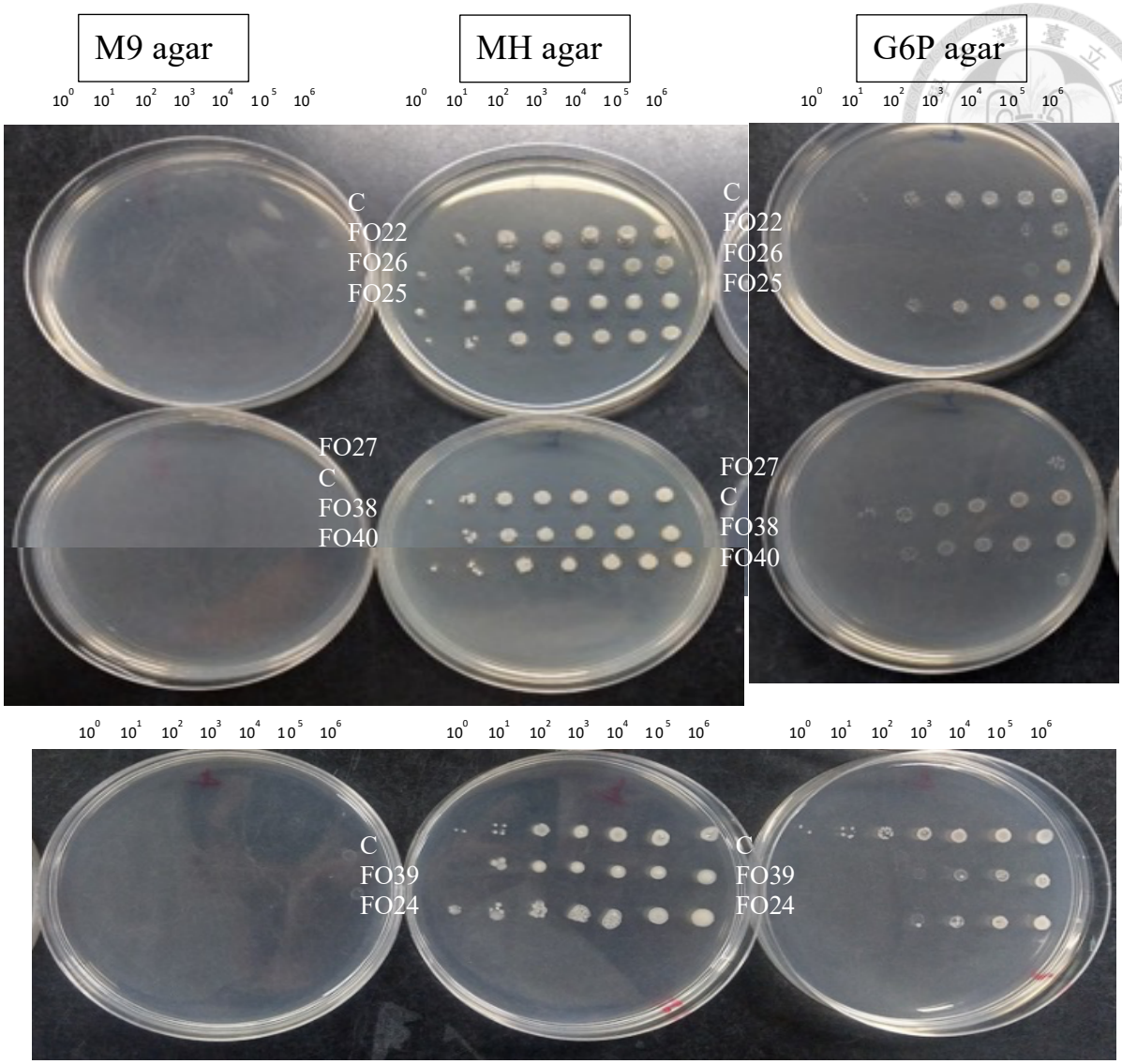

Figure 4 G6P growth assay

The G6P growth assay was performed for CRKP with ulpT, uhpA, uhpB, uhpC genotype deficiency using M9 salt medium supplemented with 0.2% G6P as the sole sugar source. The presence of growth indicates that the bacteria's transport protein is functioning normally, allowing the uptake of sugar for bacterial growth.

Left lane: M9 minimal agar without sugar supply, no growth was observed

Middle lane: MH agar, the growth pattern was similar in all isolates.

Right lane: G6P agar. Compared with reference strain NTUH-K2044, growth impairment was observed among CRKP with glpT genotype deficiency

C: Control strain (Reference strain NTUH-K2044

doi:10.6342/NTU202303850

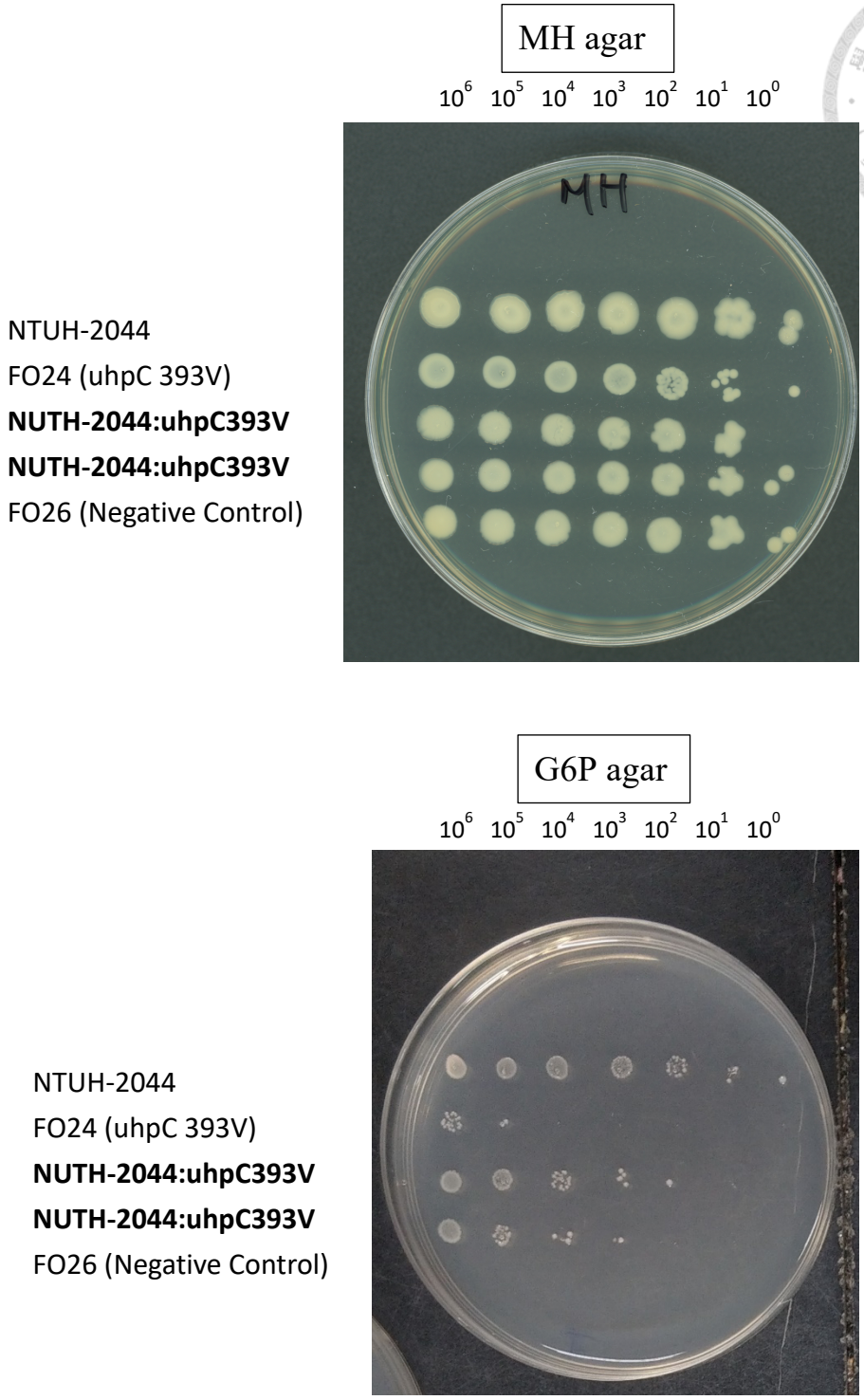

Figure 5 G6P growth assay for mutant

The G6P growth assay was performed for mutant NUTH-2044:uhpC393V. Upon generating the uhpB393V mutation in NTUH-K2044, a significant growth impairment was observed compared to the reference NTUH-K2044 strain.

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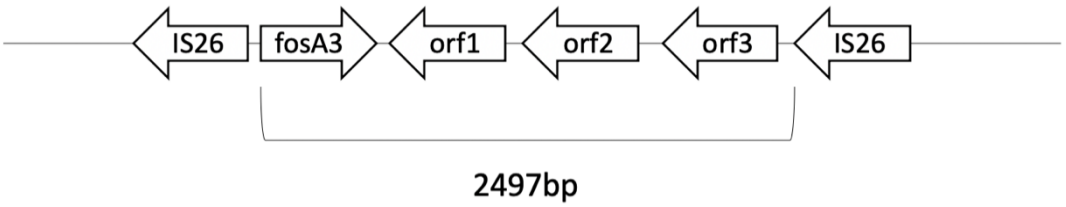

Figure 6 FosA3 flanking region of FO15

Fragment with 2497 bp in total, which contained *fosA3*, *orf1*, *orf2*, and a truncated *orf3*, was located between two IS26 elements. The fragment was presented in plasmid exclusively.

doi:10.6342/NTU202303850

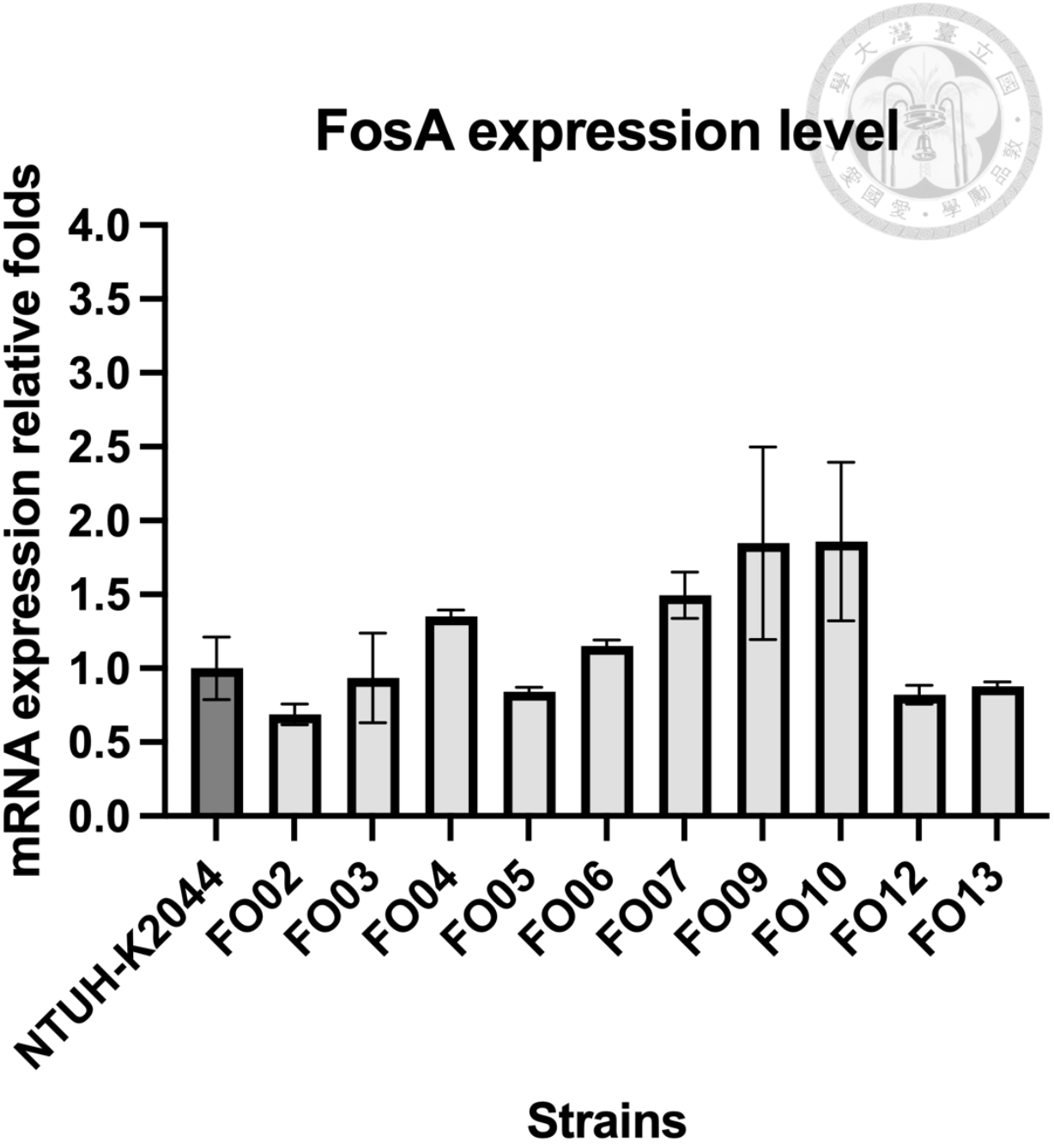

Figure 7 mRNA expression level between low-level fosfomycin resistant CRKP strains

Upon comparison with the reference strain NTUH-K2044, there was no consistent increase observed in fosAKP mRNA expression among the 14 low-level fosfomycin-resistant strains

doi:10.6342/NTU202303850

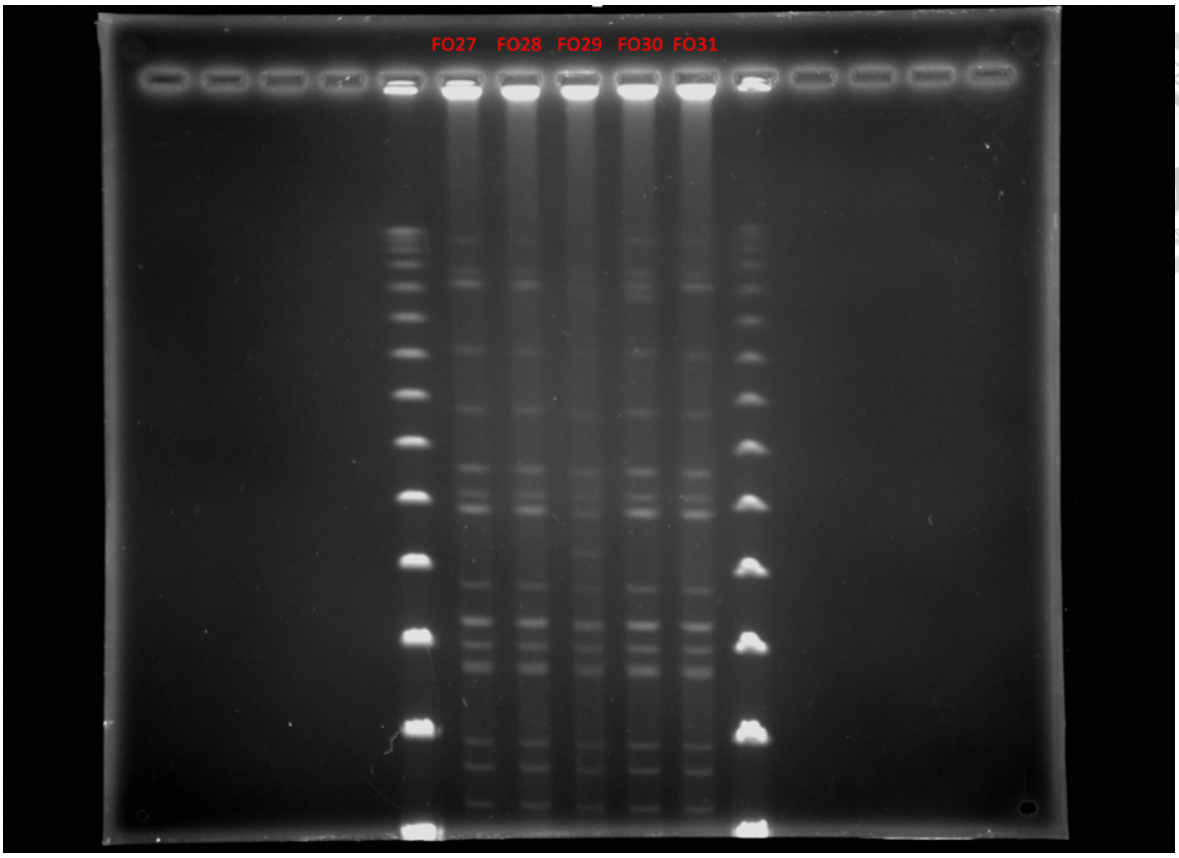

Figure 8 PFGE analysis of FO27, FO28, FO29, FO30, FO31

We performed PFGE analysis from 5 strains (FO27, FO28, FO29, FO30, FO31). FO30 had one fragment different, and FO29 had two fragments different from other strains of PFGE analysis. According to the interpretation criteria of PFGE (Table S1), FO30 was still considered the same clone, and FO29 was closely related to the other five strains.

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Declaration for publications incorporated in the dissertation

The previous publication "Transporter Genes and fosA Associated With Fosfomycin Resistance in Carbapenem-Resistant *Klebsiella pneumoniae*" is incorporated in this dissertation

I declare that:

I contributed greater than 50% of the content in the publication and is the "primary author"

The publication is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in the thesis

The detail of publications below is accurate

My academic supervisor has agreed to the material incorporated in my thesis

All co-authors of the publications have reviewed the information below and have agreed to its veracity

Publication detail: Wang Y-P, Chen Y-H, Hung I-C, Chu P-H, Chang Y-H, Lin Y-T, Yang H-C and Wang J-T (2022) Transporter Genes and fosA Associated With Fosfomycin Resistance in Carbapenem-Resistant *Klebsiella pneumoniae*. Front. Microbiol. 13:816806. doi: 10.3389/fmicb.2022.816806

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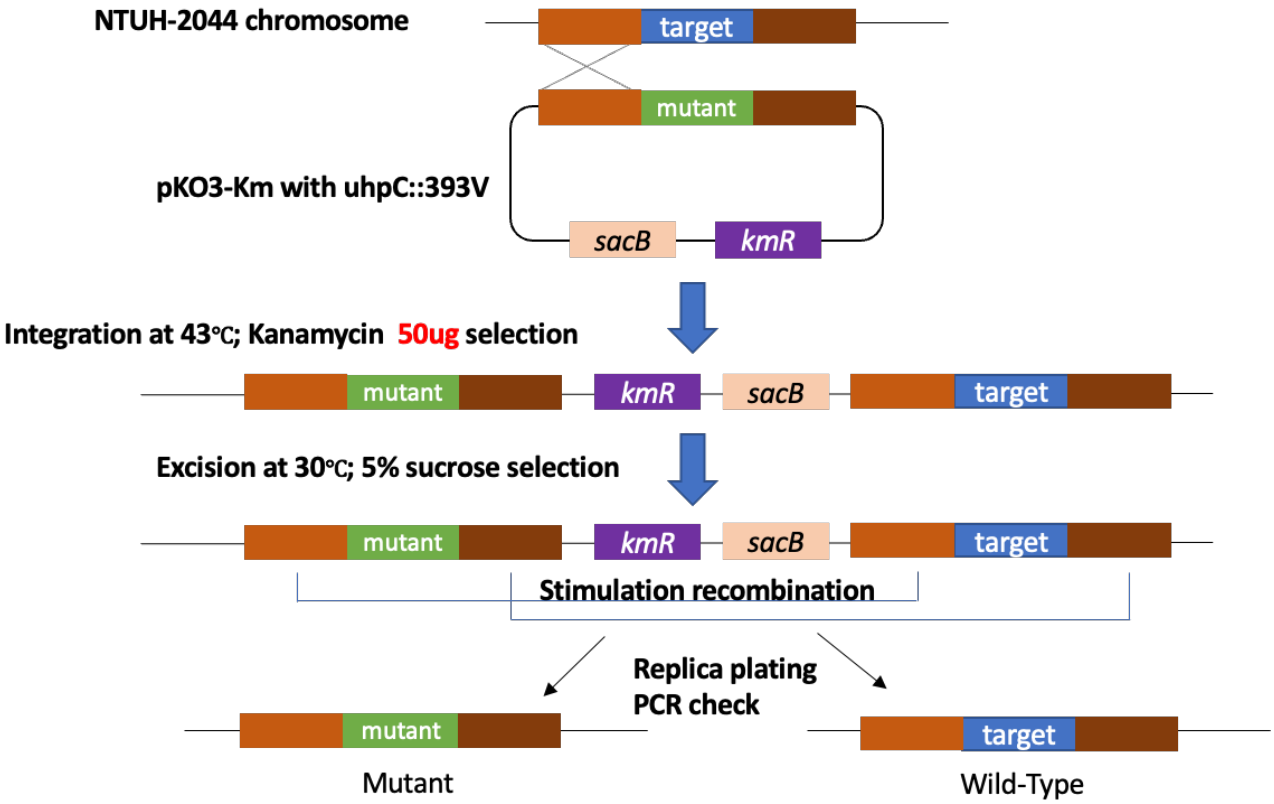
Supplementary data

Figure S1. Construction of mutants

The origin of replication of the plastid pKO3-km is temperature-sensitive, which is unable to replicate at 43°C. Under the circumstance, pKO3-km was inserted into the bacterial chromosome. In addition, the sacB gene will convert sucrose into levans, which is toxic to the bacteria. Taking advantage of this characteristic, a 5 % sucrose medium is used for negative selection to stimulate the bacteria to expel the plastids from the bacteria.



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Supplement Table S1. Interpretation criteria for PFGE

Category	No.of genetic differences compared with outbreak strain	Typical no. of fragment differences compared with outbreak pattern
Indistinguishable	0	0
Closely related	1	2-3
Possibly related	2	4-6
Different	3	7

(Tenover et al., 1995)

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