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Molecular cloning and functional research in drug-resistance of transposase gene in *Burkholderia pseudomallei*

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Transposons are widely used in genetic engineering research and play an essential role in microbial drug resistance research. Transposase is the key to constructing efficient transposons, but no studies have reported transposons in *Burkholderia pseudomallei.* In this study, a new transposase gene was cloned and identified from HNBp001 isolated from Hainan province. Blast and phylogenetic tree analysis showed that the gene had high homology with IS21 transposase of Burkholderia strains from other regions. The transposase gene deletion and over-expression strains of HNBp001 were successfully constructed. The biofilm detection result shows that the over-expression strain's biofilm production was higher than the other two strains. Although there was no significant difference in the amount of biofilm production, the biofilm synthesis rate of the over-expression strain was significantly faster. Wild and deletion strains' results were the same in drug resistance, while over-expressed strains changed compared with the other two strains. Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) revealed that there was no difference whether the transposase gene was knocked out or not. Two different sizes of proteins of over-expressed strain were significantly lower than that in the normal and knock-out strain. These results indicate that the cloned transposase genes play an essential role in the biofilm formation and drug resistance in HNBp001, but the specific mechanism remains to be further studied.

Key words: *Burkholderia pseudomallei*, transposase, biofilm, drug-resistance.

INTRODUCTION

Burkholderia pseudomallei, a facultative intracellular bacterium widely distributed in the soil of Southeast Asia and Northern Australia that causes melioidosis, commonly exists in tropical and subtropical regions (Jayasinghearachchi et al., 2023; Limmathurotsakul et al., 2016; Seng et al, 2019; Dance and Limmathurotsakul, 2018). As early as 2008, the Center for Disease Control and Prevention of the United States (CDC) listed it as a

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Class I pathogen (Peacock et al., 2008). In China, melioidosis cases are mainly distributed in Hainan, Guangdong, Guangxi, and Taiwan. A significant number of melioidosis cases were reported in Hainan (Zheng et al., 2019; Chen et al., 2015). The bacterial can infect the body directly through trauma or in aerosol form through the respiratory tract. Patients infected with *B. pseudomallei* do not have typical symptoms. Their clinical manifestations are similar to many diseases, such as fever, tuberculosis, etc., which can easily lead to misdiagnosis (Tang et al., 2018; Hautbergue and Wilson, 2012; Jacob et al., 2012; Gassiep et al., 2020). *B. pseudomallei* are naturally resistant to a variety of commonly used antibiotics. In addition, due to the non-standard use of broad-spectrum antibiotics, the drug resistance rate to these antibiotics keeps rising (Wiersinga et al., 2018). Multiple drugs and pan-drug resistance *B. pseudomallei* can be easily isolated in clinical practice, and this kind of strain occupies an increasing proportion of hospital infections (Chen et al., 2016; Chou et al., 2007). Some clinical studies show that this pathogen is sensitive to imipenem (antibiotic sensitive rate 10-100%), resistant to penicillins, aminoglycosides, and cephalosporins in varying degrees (Fen et al., 2021; Bandeira et al., 2013). Carbapenems, β-lactam, and cephalosporins are still the preferred drugs for the treatment of melioidosis. However, the isolation rate of drug-resistant strains to these antibiotics is increasing.

The acquisition of drug-resistance genes, mainly delivered by the transposase, is one of the leading causes of bacterial drug resistance. Transposons can jump freely in DNA and transfer resistance genes among bacterial chromosomes, plasmids, and phages, resulting in the diversification of resistance (Klein and O'Neill, 2018; Kelly et al., 2009). The research of Kelly and Beuzon found that the transposon Tn1721 of tetracycline resistance gene *(tetA*) and the transposon complex Tn3-△Tn1721 containing β-lactams antibiotic resistance gene (blatem-1) existed in salmonella (Pasquali et al., 2005; Pezzella et al., 2004).

Transposons are widely found in bacteria, yeast, and higher plants and animals and were first discovered in maize by McClintock, an American geneticist, in the 1940s (McCLINTOCK, 1950). Transposons are widely used in genetic engineering, including mutant library construction, transgene, gene function analysis, etc (Mougiakos and Beisel, 2021; Dance and Limmathurotsakul, 2018; Delaurière et al., 2009; Ivics et al., 2009). Transposons can be divided into retrotransposons and DNA transposons, in which retrotransposons can increase the copy number of the inserted sequences, while DNA transposons cannot (Pradhan and Ramakrishna, 2022; Feschotte and Pritham, 2007; Schulman, 2013). DNA transposons, which require the involvement of transposase which can combine with the specific sequence of IR region to open the double strand of IR region and insert into the new genome, are widely studied (Gorbunova et al., 2021; Trubitsyna et al., 2015). Frequent transposition will threaten the existence of the species. Typically, the activity of transposase is deficient (Surm and Moran, 2021; Vigil-Stenman et al., 2017). Highly active transposons generally need to be artificially optimized. In this process, the acquisition of high catalytic activity transposase is indispensable. Although the transposase is essential in constructing highly active transposon, some transposase genes were reported in *B. pseudomallei*, but no further functional research was conducted. In this study, a new transposase gene was cloned, and the transposase detected strain and over-expression strain of BPHN001 was also successfully constructed. Moreover, the influence of the transposase on biofilm formation, antibiotic resistance, and protein expression was detected by different assays. The results will provide valuable information for the transposase to dissect other molecular mechanisms and functional studies.

MATERIALS AND METHODS

HNBP001 strain was isolated from Hainan. The expression vector PET-42a-c(+) and knock-out vector PK 18mobsacb (TPR) were preserved by our laboratory. *Escherichia coli* BL21 (DE3) and S17-12PIR strains were purchased from Biyuntian Biotechnology Co., LTD.

Cloning and recombination vector construction of NovelGene_03572 transpoase gene

Total DNA was extracted according to the instructions of the bacterial Genomic DNA Extraction Kit (TianGen, China). According to the sequence of RNA libraries, design specific amplification primer, 03572 - OE - F: 5 '- CGCGGATCCATGATCAAG GACGTTCTAC- 3' (BamH I); 03572 - OE - R: 5 '- CCCAAGCTTTCAATCTCCAGCGAGCTTT - 3' (Hind III), total bacterial DNA as a template to amplify the NovelGene_03572 open reading frame (ORF). PCR amplification system: 2xTaqMaster Mix 12.5 μL, upstream primer 1 μL (10 μM), downstream primer 1 μL(10 μM), genomic DNA 1 uL (100 ng/μL), ddH_2O 9.5 μL. Amplification procedure: 94°C for 5 min; 94°C 30 s, 58°C 30 s, 72°C 1 min, 35 cycles; 72°C for 10 min. All amplified products were purified using a bulk agarose gel DNA Recovery Kit (TianGen, China). The DNA fragments were constructed into the prokaryotic expression vector PET42a-c (+) and sequenced by Invitrogen (Pudong, Shanghai, China). The verified sequence was constructed to PK 18mobsacb (TPR). The details of primers are shown in Table 1. The online tool ProtParam was used to obtain the sequence of 03572 transposase protein amino acid (http://web.expasy.org/protparam/). The amino acid sequences of 03572 transposase protein and other microorganism transposase proteins were aligned using CLUSTAL_X, and the phylogenetic tree was generated with the MEGA4.0 program using the NJ algorithm with 1000 bootstrap trials (Xiu et al., 2016).

The obtained of 03572 transposase gene overexpression and deletion strains

The recombinant plasmids PET42A-C(+)-03572 and PK18

Table 1. List of primers used for knock-out NovelGene_03572 transposase gene.

Primer name	Primer sequence (5' to 3')	Product length (bp)
3572-up-F	CGCGGATCCCATACATTCCCGTCTTCGGT（BamH I）	499
3572-up-R	TCCCCCGGGCGTCGAGCCTGGCTTGATTC（Sma I）	
3572-down-F	TCCCCCGGGACGATGGAAGACTGGTTAGGC（Sma I）	691
3572-down-R	CCCAAGCTTGCGGGACGATGTGGAGAT（Hind III）	

Source: Authors

MobSACb(TPR)-03572 were transformed into *E. coli* S17-12pir receptor cells according to standard transformation steps, respectively. It was cultured in solid LB medium (containing 50 mg/L kanamycin) at 37°C overnight. Positive colonies were screened by colony PCR. Then the s17-12pir colony containing pet42a-c(+)-03572 or PK18mobsacb (TPR)-03572 recombinant plasmid was co-cultured with HNBp001 strain, respectively. The overexpression strain was screened on the LB sodium medium containing Gm and Kan. The deletion strain was screened on LB sodium medium containing 15% sucrose and Gm and finally verified by PCR (The primers of overexpressed strain were 03572-OE-F, 03572-OE-R; the primers of deletion strain were: 3572-up-F, 3572-down-R).

The detection of biofilm formation

Single colonies cultured in LB liquid medium at 37°C, 200 rpm/min overnight were selected from HNBp001 wild strain, deletion strain, and overexpression strain. The bacteria were transferred to the new LB liquid medium at a ratio of 2 to 10%, respectively, cultured with the condition of 37°C, 200 rpm/min, until OD_{600}=0.5-1. 200 µL of LB liquid medium was added without antibiotics into a sterile 96-well flat bottom culture plate for each well, 4 µL bacterial liquid was added into each well containing 200 µL LB liquid medium, incubated aerobically in the incubator at 37°C for 24, 48, and 72 h. At least three multiple wells were set for each strain. After incubation, the culture medium was gently poured out and gently rinsed twice with 200 µL phosphate buffer saline (PBS) per well. The medium was placed upside down in a cool, ventilated place for drying and fixation. 200 µL methanol was added, fixed for 15 to 30 min, methanol was discarded and dried thoroughly. After being dyed with 150 to 200 µL 1% crystal violet solution in each well for 5 min, it was gently rinsed with PBS until no obvious color can be seen in the blank dyeing well, then placed in a cool and ventilated place upside down for drying. The dye was fully dissolved with 200 µL 95% ethanol per well. The absorbance value was measured at 590 nm with a microplate reader.

The detection of the minimal inhibitory concentration (MIC)

Single colonies were selected from HNBp001 wild strain, deletion strain, and overexpression strain, and added into a diluent, respectively, then thoroughly mixed. Then 50 µl suspension was transferred into a 9 ml LB liquid medium and mixed thoroughly. LB liquid medium containing different strain was transfused into the 96-well drug-sensitive culture plate, each well added 100 ul, then the plate was sealed and incubated at 37°C (Fahim et al., 2022). The results were observed within 24 h. If the bacteria still grow well, even though, cultured in the highest drug concentration, the result was defined as "Resistance". In the event that the bacteria did not grow in all drug concentrations including the minimum concentration, the result was defined as "Sensitive". In case the bacteria did not grow in a drug concentration, but when the concentration was lower than it, then the germ survive and the number of the concentration was recorded as the MIC for the antibiotic.

Protein expression analysis by SDS-PAGE

Single colonies were selected from HNBp001 wild strain, deletion strain, and overexpression strain, cultured in LB liquid medium at 37°C. When the OD_{600}=0.6 was reached, 500 µl of the bacterial solution was taken from each strain. The supernatant was discarded after centrifugation at 12000 rpm. The bacteria were suspended with 80 µL sterile PBS, and a 5x protein loading buffer was added. SDS-PAGE was performed to observe the protein expression in different strains.

RESULTS AND DISCUSSION

Cloning and phylogeny

A gene named NovelGene_03572 was selected from the RNA library. The specific primers were designed using the HNBp001 DNA as a template, and specific amplification of the NovelGene_03572 gene was carried out. The agarose gel electrophoresis result showed a specific band between 250 and 500 bp, which was basically the same as the target fragment (384 bp). The target fragment was verified by sequencing.

The NovelGene_03572 transposase protein was blasted with 18 transposases from other microbial species, including *Caballeronia arationis, B. pseudomallei*, *Caballeronia* species *dw*_276, *Paraburkholderia atlantica*, *Trinickia soli*, *Burkholderia vietnamiensis*, *Caballeronia* spp. NCTM1, *Paraburkholderia caribensis*, *Paraburkholderia phymatum*, *Paraburkholderia steynii*, *Burkholderia thailandensis* E264, *Burkholderia* species SFA1, *Caballeronia* spp. GAFFF2 and *Cupriavidus* species AcVe19-1a. The results showed that their homology was 83% (Figure 1).

MEGA 4.0 software was used to construct phylogenetic trees by the neighbor-joining method. NovelGene_03572 transposase protein was highly similar to transposase subunit from *B. thailandensis E264*, IS21 family transposase from *B. pseudomallei.* The black squares represent the new genes cloned (Figure 2).

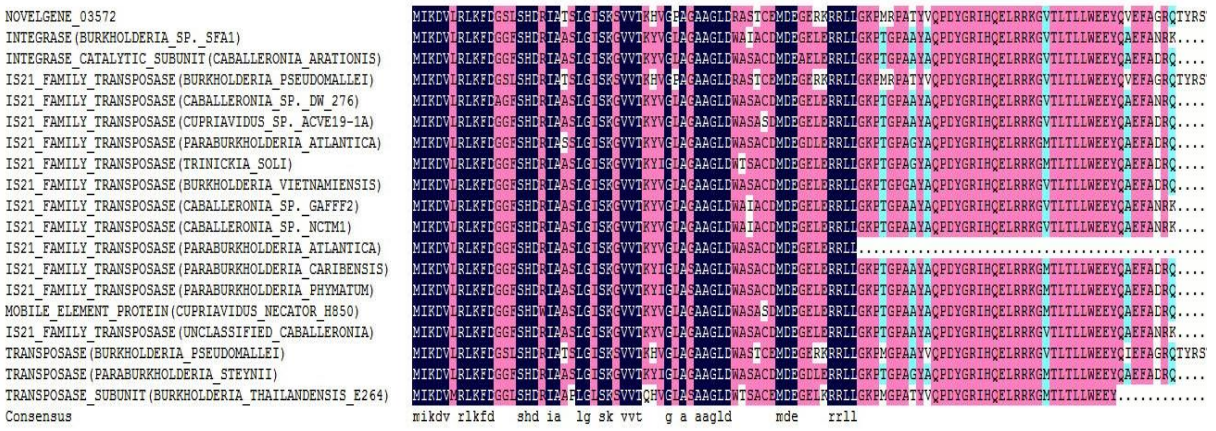

Figure 1. Protein comparison of NovelGene_03572 with 18 transposases from other microbial species.
Source: Authors

The construction of novel Gene_03572 transposase gene deletion and overexpression strains

The recombinant vector of PET42a-c(+)-03572 and PK 18mobsacb(TPR)-03572 were transformed into HNBp001, respectively. The overexpressed strains were screened by LB solid medium containing Kan (250 µg/mL) and Gm (100 µg/mL). Four colonies were selected from the plate for PCR with PET42a-c(+) universal primers. The results showed that No. 3 and No. 4 colonies had a specific band between 750 and 1000 bp. The amplification products' size was consistent with the target fragment (Figure 3a). The deletion strains were screened by LB solid medium containing Gm (100 µg/mL) and 15% sucrose. Colony PCR was performed by using 3572-up-F and 3572-down-R as primers. The length of the products were 1843 bp (wild strain) and 1190 bp (detected strain) (Figure 3b). The agarose gel electrophoresis result indicated that the PCR products met our expectations. We got the novelGene_03572 transposase gene deletion and overexpression strain in HNBp001.

Effect of the novelGene_03572 transposase on biofilm formation in HNBp001

Biofilm formation experiment results show that, with the extension of incubation time, biofilm formation was gradually increased in all strains, including wild strain, deletion stain, and overexpression strain. The biofilm formation rate of the overexpression strain was significantly faster than the wild strain and deletion strain, and the biofilm formation quantity was higher than the other two strains after being cultured for 72 h (Figure 4). The results indicated that the novelGene_03572 transposase could affect the biofilm formation of HNBp001 to a certain degree.

Effect of NovelGene_03572 transpoase on drug resistance in HNBp001

The results showed no difference in drug sensitivity between wild and deletion strains. However, compared with wild and deletion strains, the minimal inhibitory concentration (MIC) of the overexpression strain against cefuroxime changed from resistance to 16 mg/L, the MIC against cefepime changed from 8 mg/L to resistance, the MIC against ciprofloxacin changed from 0.5 to 1 mg/L. MIC against meropenem changed from sensitive to 4 mg/L, and MIC against amikacin changed from 16 mg/L to resistance. Detailed results are shown in Table 2. In conclusion, the overexpression of the NovelGene_03572 transposase gene plays a vital role in developing drug resistance in the HNBp001 strain.

Protein expression analysis

SDS-PAGE result showed no difference in protein expression between the HNBp001 wild strain and transposase deletion strain. Interestingly, the protein expression in transposase overexpression strain had changed, two proteins were down-regulated, and the size of the two proteins was almost 37 and 130 kD, respectively (Figure 5). Dependent on the result, we suppose that the 03572 transposase genes do not express or the activity of the 03572 transposase protein is not high in normal conditions. Combined with the result of drug resistance, we have reason to believe the 03572 transposase protein can affect the drug resistance in the HNBp001 strain by down-regulating the two proteins, which were 37 and 130 KD, respectively.

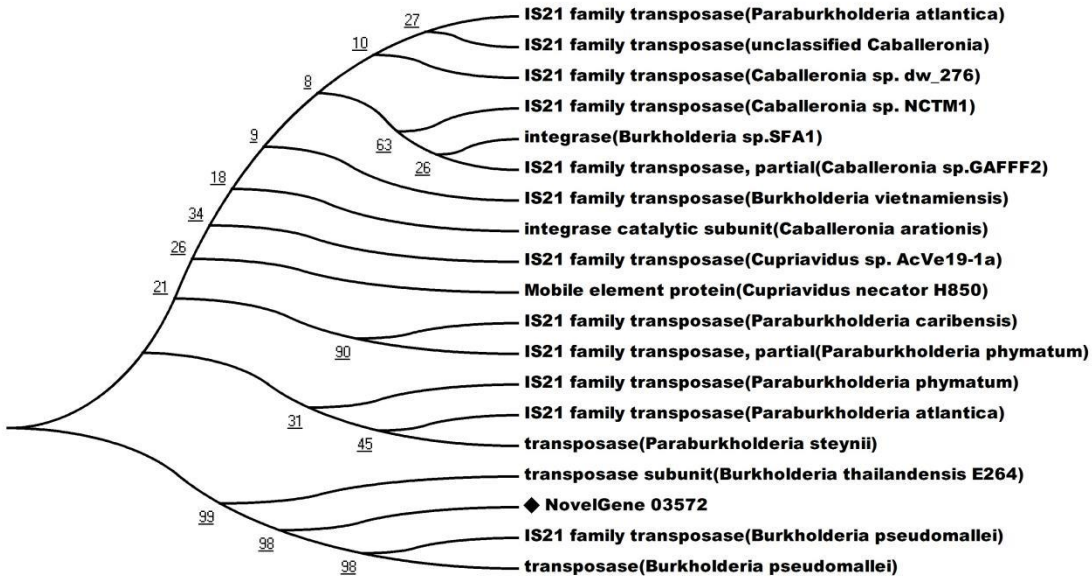

Figure 2. Phylogenetic relationships among the NovelGene_03572 transposase protein and other members of the transposases were estimated based on their amino acid sequences. Transposases amino acid sequences of the following proteins were used to make the tree: integrase [*Burkholderia sp*. SFA1] (BBQ02449.1), integrase catalytic subunit [*Caballeronia arationis*] (SAL06529.1), IS21 family transposase [*Burkholderia pseudomallei*] (WP_004536630.1), IS21 family transposase [*Caballeronia sp*. dw_276] (WP_213781838.1), IS21 family transposase [*Cupriavidus sp*. AcVe19-1a] (WP_209777234.1), IS21 family transposase [*Paraburkholderia atlantica*] (MPW09758.1), IS21 family transposase [Trinickia soli] (WP_102612997.1), IS21 family transposase [*Burkholderia vietnamiensis*] (WP_226103266.1), IS21 family transposase [*Caballeronia sp*. GAFFF2] (WP_250469771.1), IS21 family transposase [*Caballeronia sp*. NCTM1] (WP_250520593.1), IS21 family transposase [*Paraburkholderia atlantica*] (WP_227749239.1), IS21 family transposase [*Paraburkholderia caribensis*] (WP_252671094.1), IS21 family transposase [*Paraburkholderia phymatum*] (WP_244257842.1), Mobile element protein [*Cupriavidus necator* H850] (KAI3608382.1), IS21 family transposase, partial [unclassified *Caballeronia*] (WP_250520681), transposase [*Burkholderia pseudomallei*] (RAQ91269.1), transposase [*Paraburkholderia steynii*] (TCG03114.1), transposase subunit [*Burkholderia thailandensis* E264] (ABC34012.1).The neighbor-joining phylogenetic tree was constructed using the bootstrap method of MEGA 4.0 with 1000 replications, and the respective plant species of the above proteins are shown in the tree. The black square symbols indicate the NovelGene_03572 transposase protein.
Source: Authors

DISCUSSION

Antibiotics, one of the most significant medical discoveries, are vital in treating bacterial infections. Early antibiotics were mainly extracted from metabolic products by microbial fermentation. It becomes increasingly challenging to obtain antibiotics with development value through this method (Landecker, 2016). Researchers mainly focus on modifying existing known antibiotics to obtain new antibacterial drugs (Hutchings et al., 2019). At the same time, with the abuse of antibiotics in clinical practice, the effective concentration of antibiotics is getting higher and higher. Bacteria are becoming more and more resistant to certain antibiotics.

Moreover, some antibiotics even lose their effectiveness when targeting certain bacteria (Lee, 2019). Studies have shown that worldwide, almost 700,000 people died of infection related to drug resistance annually (Nicolaou and Rigol, 2018). This number is expected to reach 10 million by the middle of this century (O'Neill, 2014). It is urgent to develop new antibacterial drugs, clear the bacterial resistance mechanism, and find new antibacterial strategies. This study aims to clear the drug-resistance mechanism of B.p by finding a target protein of the 03572 transposase. Finally, two proteins which were down-regulated while the 03572 transposase over-expressed were found.

Drug resistance refers to the phenomenon that pathogenic bacteria are no longer sensitive to antibiotics or antibacterial drugs. The generation of drug resistance is one of the main reasons for the failure of clinical bacterial anti-infection treatment. Thoroughly studying the mechanism of bacterial resistance is the premise of developing new antibacterial drugs and optimizing clinical treatment plans. At present, the known mechanisms of bacterial drug resistance mainly include the following

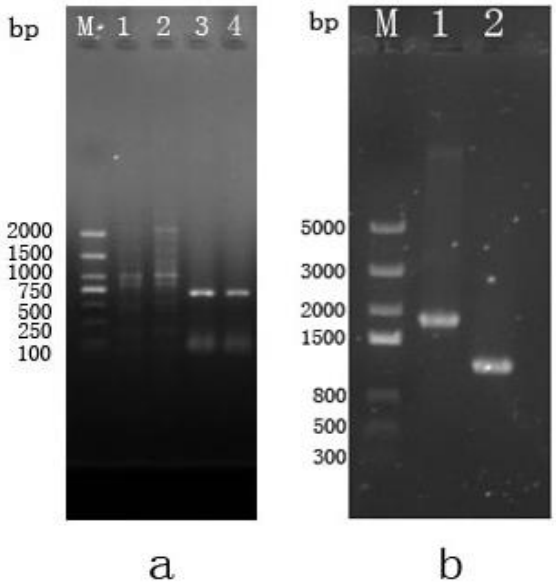

Figure 3. (a) Screening the 03572 transposase gene over expression strain by colony PCR. M: Marker (2000 bp DNA ladder); lane 1-4: Four single colony; (b) Screening the 03572 transposase gene deletion strain by colony PCR. M: Marker (5000 bp DNA ladder); lane 1: Wild strain colony (1843 bp); lane 2: Deletion strain colony (1190 bp).
Source: Authors

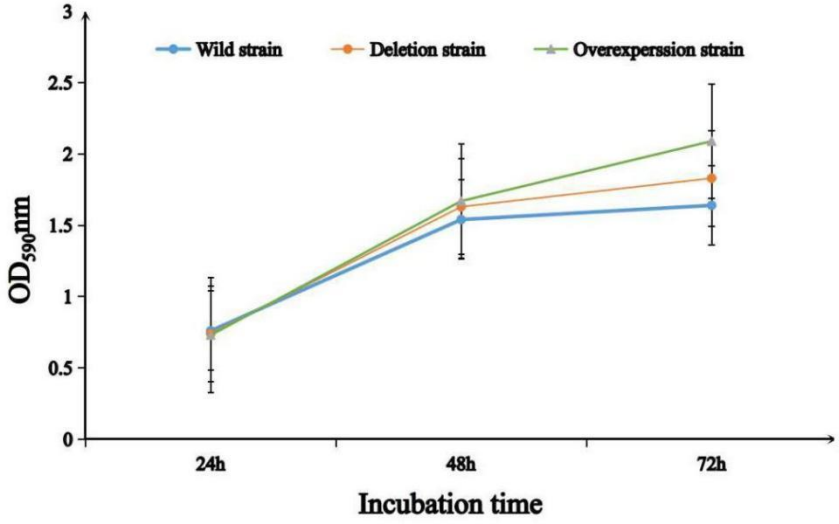

Figure 4. Detection of biofilm formation with the extension of incubation time in different strains.
Source: Authors

aspects: (1) Transmit the drug resistance gene to generations by the genome, plasmid, or transposase; (2) Reduce or inhibit drug activity by inhibiting the receptor affinity; (3) Produce hydrolytic enzymes to inactivate antibacterial drugs, for example, carbapenemase, erythromycin inactivation enzyme; (4) Change the structure of cell wall or cell membrane, decrease the number of antibacterial drugs into the cell, resulting in

Table 2. Drug resistance analysis in different strains.

Antibiotics	Minimum inhibitory concentration (mg/L)		
	Wild strain	Deletion strain	Overexpression strain
Cefuroxime	R	R	16
Cefepime	8	8	R
Ciprofloxacin	0.5	0.5	1
Meropenem	S	S	4
Amikacin	16	16	R

R: Resistance; S: Sensitive.
Source: Authors

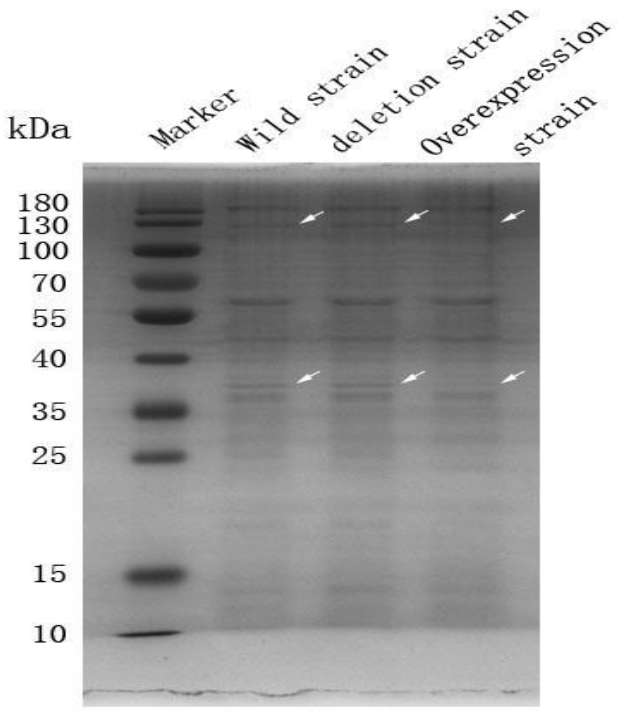

Figure 5. Protein expression analysis in different strains by SDS-PAGE. M: Marker (180 kDa protein ladder); lane 1: Total protein of wild strain; lane 2: Total protein of deletion strain; lane 3: Total protein of overexpression strain. Arrows: down-regulated proteins.
Source: Authors

drug resistance, such as the formation of biofilm (Al-Bayati and Samarasinghe, 2022); (5) Use the efflux pump to transport antibacterial agents to the cells to reduce the damage to the cells (Ogawara, 2019).

The formation of biofilm is one of the primary methods for the establishment of microbial resistance (De Silva and Heo, 2022; Rather et al., 2021; Dai et al., 2021; Pibalpakdee et al., 2012). The exopolysaccharides (EPS), a critical matrix component of biofilms, is negatively charged and can combine with the positively charged amino to prevent hydrophilic antibiotics from entering cells (Ma et al., 2022). In addition, some specific genes are expressed in the biofilm formation process. For example, pvrR and tolA genes are highly expressed in the biofilm formation process in *Pseudomonas aeruginosa* (Drenkard and Ausubel, 2002; Whiteley et al., 2001). Previous studies have shown that the amount of biofilm can affect the drug resistance of bacteria. In this study, we found that the overexpression of 03572 transposase could not significantly up-regulate the amount of biofilm in the HNBp001 strain. Still, its synthesis rate was substantially faster than wild and

deletion strains. We believe that the quicker the biofilm is formed, the earlier the osmotic barrier can be established which enhances bacterial resistance.

Drug sensitivity assay results showed that the deletion of the 03572 transposase gene did not affect the resistance of the HNBp001 strain. Still, its overexpression could affect the MIC of 5 antibiotics: cefuroxime, cefepime, ciprofloxacin, meropenem, and amikacin. SDS-PAGE also showed that the overexpression of the 03572 transposase gene could affect the protein expression of HNBp001, which significantly down-regulated the expression of two proteins (37 and 130 KD). Still, there is no difference in the protein expression between wild and deletion strains. Therefore, we speculated that the 03572 transposase gene was not expressed or the 03572 transposase protein was inactive in normal conditions. When certain conditions or substances stimulate the strain, the transposase expression is activated, the transposase's content reaches a certain level, and the transposition occurs, which makes the genes related to drug resistance up-regulated or down-regulated, thus resulting in the generation of drug resistance. Here, we only know that two proteins are down-regulated, and the drug resistance changed after 03572 transposase gene over-expression. No evidence exists to prove whether these two proteins are drug resistance or biofilm formation-related proteins; thus, further studies are needed.

Conclusion

The NovelGene_03572 gene, cloned from the HNBP001 strain, was identified as a transposase gene by Blust and phylogeny analysis. Deleting the NovelGene_03572 gene did not affect biofilm formation and drug resistance. However, the overexpression of the NovelGene_03572 gene can accelerate the generation of biofilm and affect the resistance to cefuroxime, cefepime, ciprofloxacin, meropenem, and amikacin, and down-regulate the expression of two proteins (37 and 130 KD). According to the results obtained, it was shown that the new transposase gene plays a very important role in drug resistance in *B. pseudomallei*. This study laid a foundation for in-depth studies on drug resistance in *B. pseudomallei* and provided a new idea for constructing a transposon suitable for *B. pseudomallei*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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