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Identification and Characterization of a Novel Fosfomycin Bacillithiol Transferase FosB8 in Paenibacillus illinoisensis

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Abstract

Objective

To characterize a novel fosfomycin resistance gene, *fosB8*, elucidate its enzymatic parameters, and analyze the genetic environment of *fosB8* in the clinical *Paenibacillus illinoisensis* strain WP77, as has been done with other fosfomycin resistance genes such as *fosA7* in *Salmonella* and *fosB* in *Staphylococcus aureus*.

Method

Species identification was conducted using the VITEK 6 system and Laser Desorption Ionization-Time of Flight Mass Spectrometry. The minimum inhibitory concentrations (MICs) were then determined by the agar dilution method, adhering to the guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI), ensuring standardized and reliable results. The genetic profile of strain WP77, as revealed by whole-genome sequencing, indicates a genome length of 6.64 Mbp with a single chromosome and a GC content of 47.2%. The analysis identified key chemotaxis genes such as *mcp*, *che*, and *mot*, and antibiotic resistance genes including *drr* and *emr*, conferring resistance to various antibiotics. *fosB8* was characterized through conjugation experiments, molecular cloning, bioinformatics analysis, and enzymatic kinetic assays.

Results

P. illinoisensis WP77 exhibited resistance to fosfomycin (512 mg/L). Finally, A novel functional fosfomycin resistance gene, designated *fosB8,* was identified in WP77. The gene encodes a putative fosfomycin bacillithiol transferase that exhibits 43% to 62% amino acid identity with known metallothiol transferases. Expression of fosB8 in a recombinant strain resulted in an 256-fold increase in the fosfomycin MIC. FosB8 exhibited a catalytic efficiency (kcat/Km) of $(7.9 \pm 0.8) \times 10^3$ M^{-1} s^{-1} toward fosfomycin. Genetic environment analyses indicated that *fosB8* is probably intrinsic ingenus *Paenibacillus*.

Conclusion

The study for the first time reported a novel fosfomycin bacillithiol transferase FosB8. Notably, FosB8 was confirmed to play an essential role in the resistancemechanism exhibited by *Paenibacillus illinoisensis* against fosfomycin. FosB8 represents the eighth characterized example of a FosB enzyme in the existing literature.

Introduction

The emergence of antimicrobial-resistant pathogens presents a significant challenge to clinical treatment, as it undermines the effectiveness of antibiotics and complicates the management of infections. The number of microorganisms developing resistance to advanced antibiotics is on the rise,

posing a significant threat to global public health. Given the lack of effective antimicrobial alternatives, clinicians are turning to the reapplication of certain older antibiotics, such as fosfomycin, which have proven effective in treating infections [1]. Fosfomycin is a common drug that plays a crucial role in managing infections like urinary tract infections (UTIs) and upper respiratory tract infections caused by various drug-resistant pathogens [2, 3]. Notably, the combination of fosfomycin and linezolid has proven highly effective in treating multi-drug-resistant Gram-positive bacterial infections [4]. However, fosfomycin resistance has emerged frequently among bacteria isolated from clinical settings in recent years [5, 6].

The most clinically significant fosfomycin resistance mechanisms involve fosfomycin inactivation enzymes. Fosfomycin bacillithiol transferase, as one of the major fosfomycin-inactivating enzymes, was widespread in Gram-positive microbes [7, 8], especially in *Staphylococcus aureus*. It can be located on chromosomes or plasmids to mediate the spread of drug resistance [9]. FosB enzymes are Mn^{2+} dependent thiol transferases that catalyze nucleophilic addition of bacillithiol (BSH) to the fosfomycin via ring opening of the epoxide motif, thereby inactivating fosfomycin [7]. Recently, *fosB* has been identified in many Gram-positive cocci and Gram-positive rods. The first fosfomycin resistance gene *fosB* was found on plasmids from Methicillin-Resistant Staphylococcus aureus in 2016 [10]. Subsequently, fosB has been identified in Bacillus cereus and Enterococcus faecium [11, 12, 13] as well.

Paenibacillus illinoisensis was first reported in 1997 by O Shida et al. [14]. It was rod-shaped Gram-positive or Gram-variable endospore forming aerobic or facultatively anaerobic bacteria, and closely related to genus *Bacillus* [14]. Paenibacillus illinoisensis has been detected in a variety of sources including soil, humus, compost, sewage, sediments, food, rhizosphere, insect larvae and clinical samples [15]. Previous research described that many *Paenibacillus* species function as plant growth-promoting rhizobacteria (PGPR) [16]. Nevertheless, descriptions of pathogenicity and antimicrobial susceptibility testing for the genus *Paenibacillus* are rare. Several cases have shown that *Paenibacillus* has been isolated from hospitals worldwide. [17, 18]. The first hospital isolate of *Paenibacillus* was obtained in 2003 from patients diagnosed with cerebral infarction. [19] As early as 1989, a case of bacteremia that involvement *P. alvei* was reported [20]. In addition, there are increasing reports of clinical infections caused by *Paenibacillus* [21]. Nearly all cases of *Paenibacillus* infections were associated with immunocompromised patients [22, 23, 24]. However, the major threat posed by *Paenibacillus* is its extensive resistance to current antibiotics. Previous reports detailed that *Paenibacillus* exhibits uniform resistance to ampicillin, norfloxacin, and clindamycin, but susceptible to vancomycin, amikacin and erythromycin [25, 26]. However, resistance information about other old antibiotics such as fosfomycin is not available yet. It is currently essential to characterize the resistance profile of *Paenibacillus* and investigate its molecular mechanisms of antimicrobial resistance to develop effective strategies to combating this genus.

Rare pathogens, notably *Paenibacillus*, are increasingly displaying widespread multidrug resistance, presenting a considerable danger to patients. However, few studies have elucidated the multidrug-resistant phenotype of *Paenibacillus illinoisensis* or its molecular mechanisms, limiting effective

treatment options. Consequently, urgent research is needed to clarify these resistance mechanisms to guide clinical strategies against *Paenibacillus* infections. This study aims to identify the resistance determinant of a novel fosfomycin bacillithiol transferase encoded by the multidrug-resistant clinical isolate *Paenibacillus illinoisensis* WP77 and to characterize its kinetic properties and genetic environment.

Materials and Methods

Bacterial strains, plasmids and growth conditions.

Strains and plasmids applied in this study are listed in TableS1, *Paenibacillus illinoisensis* strain WP77 was collected in May 2022 from the secretion of an inpatient aged 80 with soft tissue infection at the Affiliated Hangzhou First People's Hospital of Westlake University in China. The initial identification of bacteria was conducted using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). Further identification of the isolate was performed using 16S rRNA gene sequence alignment and average nucleotide identity (ANI) analysis against reference genomes of closely related genera. The *E. coli* BL21(DE3) was used as the host strain for expression and purification of the FoB enzyme. *E. coli* DH5α was used as the recipient bacteria for routine cloning. Plasmids pUCP20 and pUCP24 were used as vectors for cloning and identification of candidate resistance genes. The pCold I vector was used for cold shock-induced expression and purification of histidine-tagged FosB. All the bacterial strains were cultured overnight in Luria-Bertani (LB) at 37°C, supplemented with the corresponding antimicrobial agents when required, unless noted otherwise.

Genomic sequencing and bioinformatics analysis of *Paenibacillus illinoisensis* strain WP77

The genomic DNA of strain WP77 was extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) and was subjected to High-throughput sequencing on the Illumina HiSeq 2500 platforms by the Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The raw data of Illumina sequence was filtered and processed by Trimmomatic v0.39 and Fastqc v0.12.1 and then optimized sequence was assembled with spades v3.15.3[27]. The ORFs in assembly scaffolds were initially predicted using the Prokka v1.14.6 [28] and Bakta v1.9.4 [29]. and further annotated using DIAMOND against the NCBI non-redundant (nr) protein databases and UniProtKB/Swiss-Prot (http://web.expasy.org/docs/swiss-prot_guideline.html). Resistance genes were determined using ResFinder and the Resistance Gene Identifier (RGI) software against the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/). Multiple sequence alignment based on amino acid sequences and an unrooted maximum likelihood phylogenetic tree reconstruction was accomplished by MEGA X [30]. Phylogenetic trees were visualized alongside bar graphs of allele frequencies using the interactive web platform iTOL[31]. Mobile genetic elements were identified via Integron-Finder and ISbrowser [32]. Conserved motif analysis in FosB8 sequence was achieved using the GeneDoc software [33]. Genomics

visualization was manipulated on Proksee online tool [34]. Sequence comparisons and map generation were performed using Easyfig [35].

Construction of expression plasmids and synthesis of the gene.

The sequence that encoding FosB5, flanked with NedI and BamHI restriction sites,was custom synthesized (Qingke) using the gene reported from the Methicillin-resistant *Staphylococcus aureus* (GenBank accession no. NG_054663.1) [36]. The gene encoding FosB8, along with candidate resistance genes and their upstream sequences, was amplified from the genome of WP77. The enzyme cleavage sites were introduced at the 5' and 3' ends of the corresponding primer (**TableS1**). Each gene was cloned into the pCold1 expression vector and shuttle vector pUCP20 to generate the corresponding plasmids. For protein expression and purification purposes, the recombinant plasmids pCold1::*fosB2* and pCold1::*fosB7* were transformed into *Escherichia coli* BL21(DE3) cells. Subsequently, colonies were selected on LB agar plates containing 100 μg/mL ampicillin. The plasmids pUCP20::*fosB3*, pUCP20::*fosB8*, and other candidate plasmids harboring resistance genes were transformed into *E. coli* DH5α. Subsequently, the recombinants were selected with 100 μg/mL ampicillin or 50 μg/mL gentamicin, were used for antibiotic susceptibility testing.

Antimicrobial susceptibility testing.

The minimum inhibitory concentrations (MICs) of ten selected antibiotics (fosfomycin, ampicillin, cefotaxime, meropenem, tetracycline, doxycycline, tigecycline, florfenicol and kanamycin cotrimoxazole) were determined using Mueller-Hinton Agar dilution method with 5% of defibrinated sheep's blood and incubated in aerobiosis at 37°C and read after 48 hours, the results were interpreted using criteria described for Bacillus strains [37] following the Clinical & Laboratory Standards Institute (CLSI) [http://clsi.org], and European Committee on Antimicrobial Susceptibility Testing (EUCAST) [http://www.eucast.org], *Staphylococcus aureus* ATCC 29213 was included as a control.

Expression and purification of FosB8 and FosB3.

The genes encoding FosB8 from *Paenibacillus illinoisensis* and FosB3 from *Staphylococcus aureus* were amplified and cloned into pCold1 vector as described above. Afterwards, a single colony of *E. coli* BL21(DE3) strain, harboring the pCold1::*fosB8* and pCold1::*fosB3* plasmid, was grown in a shaker/incubator at 37°C in LB broth which enriched with 100 μg/mL ampicillin. Upon reaching an optical density of 0.6 at 600 nm, protein expression was induced in the bacterial culture by the addition of 0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the culture was further incubate for 18 h at 15°C. Cells were harvested by centrifugation at 4,500 *g* at 4°C for 10 min, resuspended in buffer A (25 mM HEPES, pH 7.5), supplemented with 1 mM EDTA and 0.2 mM dithiothreitol (DTT), and lysed by sonication. The lysate was removed by centrifugation (20,000 *g*) for 30 min at 4°C. The centrifugation step was repeated, The supernatant was replenished with 1 μg/mL RNase A and loaded onto an gravity flow Ni-affinity column incubate at 4°C for 8 hour, then washed with buffer A (60 mM). Finally, the target-products was eluted with buffer B containing 500 mM imidazole. The his6-tag of FosB protein was

removed using TEV cleavase, following the instructions of the NEBExpress-kit (New England Biolabs: P8112S). The fractions were then analyzed by 12% SDS-PAGE. Fractions containing FosB were pooled, desalted and concentrated using Minipore ultrafiltration tube with a 10-kDa cutoff (Millipore, USA). And purified by affinity chromatography based on the instructions of the His-tag Protein Purification Kit (P2226, Beyotime, China). The protein concentration was determined using the theoretical extinction coefficient (ε280 = 33,000 cm^{-1} M^{-1}) and the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL). The purified FosB protein was stored in aliquots in 50% glycerol at 20°C.

Enzyme kinetics analysis of modification of fosfomycin.

Thiol transferase activity detection was conducted based on a previously reported method. Enzymatic reactions were performed in 20 mM ammonium formate buffer (pH 7.0) containing 0.1 mM $MnCl_2$, 1.3 mM fosfomycin, and 1 μM FosB8. Bacillithiol (BSH; 6.8 mg in 10 mL) was added to initiate the reaction. Thiol consumption was monitored throughout the reaction by titrating aliquots with DTNB, with the reaction deemed complete after 2 h. The reaction mixture was freeze-dried (to remove excess ammonium formate) and then resuspended in 2 mL of water. The solution was applied to a cation-exchange column (Isolute-SCX2 SPE, 0.5 g), eluted with water (10 mL) and dried. The resultant residue was resuspended in water (5 mL) and adjusted to pH 8.0 with 2% (v/v) aqueous ammonia, then purified further by anion-exchange chromatography using DEAE-cellulose (1 g, AX, fibrous fast flow, Sigma) packed in an SPE cartridge. The product was eluted with a 10–200 mM stepwise gradient of ammonium bicarbonate using 12 mL portions at 10–20 mM increments. Fractions containing the product (which eluted at 100–120 mM) were identified by spotting on a TLC plate and staining with ninhydrin or charring with 10% (v/v) ethanolic sulfuric acid. The steady-state kinetic parameters, kcat and Km were calculated using the Michaelis-Menten equations in GraphPad Prism 9 software.

Results Discussion

Identification and antibiotic susceptibility of the Paenibacillus illinoisensis isolate WP77.

P. illinoisensis WP77 was resistant to fosfomycin, β-lactams, erythromycin, and others, but susceptibility to rifampicin, tetracyclines, and meropenem. *P. illinoisensis* WP77 was isolated from the exudation of soft tissue in a patient with fracture and skin ulcer on the buttock. The patient was cured with meropenem and amikacin in 2022. In the clinical laboratory, WP77 was initially reported as non-susceptible to ampicillin, fosfomycin, chloramphenicol. WP77 was sensitive to amikacin, aztreonam, meropenem, apramycin, rifampicin, and azithromycin, while it was resistant to ampicillin, fosfomycin, chloramphenicol, erythromycin, vancomycin and fortimicin. Due to the infections caused by *Paenibacillus illinoisensis* being occasional cases, and the multidrug-resistant phenotype of *Paenibacillus illinoisensis* isolate in clinic had been rarely reported, previously. Therefore, the strain was further tested for its comprehensive antibiotic susceptibility using agar dilution method. As is shown in **Table S2**.The MICs of tested antibiotics illustrated that *P. illinoisensis* WP77 manifested antimicrobial resistance, as evidenced by the MIC of Fosfomycin(512 mg/L, R); Cefuroxime(256 mg/L, (R)); Gentamicin(2 mg/L (S));

Erythromycin(256 mg/L (R)); ampicillin(> 1,024 mg/L, (R)); cefotaxime, 128 mg/L (R); cefepime, 64 mg/L (R); ceftazidime, 256 mg/L (R); aztreonam(256 mg/L (R)); meropenem(0.25 mg/L susceptible (S)); chloramphenicol(2 mg/L (R)); trimethoprim(256 mg/L (R)). The organism exhibited susceptibility to rifampicin (MIC: 4 mg/L), tetracycline (MIC: 0.5 mg/L), minocycline (MIC: 0.5 mg/L), azithromycin (MIC: ≤1 mg/L), and ciprofloxacin (MIC: 0.5 mg/L). in summary, the MICs results were in line with those of the hospital laboratory tested above, which denoting additional the multidrug-resistant phenotype of strain WP77. Previous studies have reported that most clinical strains of *P. illinoisensis* showing pervasive resistance to β-lactams, polymyxin B and E, tobramycin and netilmicin, but are susceptible to ciprofloxacin and trimethoprim-sulfamethoxazole [38, 39]. Similarly, the multidrug-resistance phenotypes observed in WP77 in this study conformed to previous studies. An approach based on homology analysis of 16S rRNA sequences, displayed that WP77 was closely related to the genus *Paenibacillus* and showed high levels of sequence identity with *Paenibacillus xylanilyticus* XIL14 (97.38%) and *Paenibacillus illinoisensis* NRS-1356 (99.23%), but higher 16S rRNA gene sequence similarities were also discovered in other sub-type species (99.0%). Moreover, an accurate identification method based on average nucleotide identity, showing that strain WP77 revealed higher DNA-DNA similarity with type strain *P. illinoisensis*. (Fig. 1).

Overall genome characteristics of Paenibacillus illinoisensis WP77.

In recent years, the application of advanced sequencing technologies has led to numerous studies focusing on the identification and characterization of rare, clinically significant microbes isolated in hospital settings. However, genomic data on *Paenibacillus spp*. remain limited in existing databases. To uncover the antimicrobial resistance mechanisms of strain WP77, whole-genome sequencing was conducted.

Assemble data revealed that the WP77 genome comprised 6.64 Mbp in size. [GenBank accession number: GCA_044770055.1], with the average length of coding genes of 1106 bp, with a mean GC content of 47.2%. Annotation of the WP77 genome distinguished a total of 5980 protein-coding sequences and 1 oriC, 56 tRNA, 1 tmRNA, 9 ncRNA and 4 rRNA (Table 1 **and** Fig. 1). Comparative genomics analysis with the publicly available genome sequences of *Paenibacillus* species uncovered that genome of *P. illinoisensis* WP77 shared the highest sequence similarities with that of *P. illinoisensis* NBRC15959 (accession number GCA_004000925.1, at 95.60% identity and 99% coverage) but shown low sequence identity with *Paenibacillus xylanilyticus* LMG 21957 (accession number GCA_013359935.1, at 87.19% identity and 91% coverage) (Fig. 1). A value identical to that strain indicating WP77 is a type strain of *Paenibacillus illinoisensis* again, which is also consistent with the result obtained by genotyping based on 16S rRNA sequencing and housekeeping gene analysis (data not shown). All predicted genes were annotated in KEGG (Kyoto Encyclopedia of Genes and Genomes), ARDB (Antibiotic Resistance Genes Database), Nr (Non-Redundant Protein), and CARD (Comprehensive Antibiotic Resistance Database) databases.

Table 1
General features of the genome of *Paenibacillus illinoisensis* WP77

Characteristics	Chromosome
Size (Mb)	6.64
G + C %	47.2
Genes	6105
ORFs	6062
Protein-coding	5980
Hypothetical proteins	2059
Average ORF length (bp)	1106
Average protein length (aa)	369
ncRNAs	9
tRNAs	56
oriC	1
rRNAs	16S*1; 23S*1; 5S*2

With its multidrug resistance phenotype, we employed bioinformatics tools to investigate the molecular basis of antibiotic resistance in WP77. Predicted open reading frames (ORFs) were analyzed using BLAST against both the Comprehensive Antibiotic Resistance Database (CARD) and the NCBI non-redundant protein database. To our surprise, no known resistance genes emerged from these analyses. To further explore potential novel resistance mechanisms, we present detailed experimental results in detail later in this paper.

Phenotypic and genotypic characterization of antibiotic resistance of P. illinoisensis WP77

The in vitro susceptibility test displayed that *P. illinoisensis* WP77 exhibited resistance to several tested antimicrobials, in order to further study the molecular mechanism of drug resistance based on whole genome sequencing data, bioinformatics tools was engaged to reveal known resistance genes and gene variants linked to the *P.illinoess* WP77 resistance phenotype. However, no known antimicrobial resistance genes (ARGs) were identified within the coding sequences of the genome of *P. illinoess* WP77. Moreover, the conjugation transfer experiment showed that the bacteria did not successfully transfer the corresponding antibiotic-related mobile plasmid (data not shown). Hence, 13 unique ORFs, with diverse identities to the closest known resistance genes, were selected as candidate novel resistance genes for further assessment of their function (**Supplementary Table S3**).

As shown in **Table S4**, the presence of *fosB8* and *fosB2* in *S. aureus* increased the fosfomycin MIC by 128-fold and 256-fold, respectively, whereas MICs of other antibiotics remained no discernible trend change.

the minimal inhibit concentration (MIC) of Fosfomycin against RN4220-pAM401 was 1 μg/mL which was practically the same as that of the recipient strain RN4220(MICs of Fosfomycin 1μg/mL). However, transformants of *S. aureus* RN4220 that included the shuttle plasmid pAM401 with *fosB8* gene shown 128-fold increases in the MICs of Fosfomycin when compared with those for *S. aureus* RN4220 with pAM401 (Table 2). In addition, introduction of *fosB8* into BL21 exhibited 256-fold increase in fosfomyin MIC (> 256 μg/mL). The ASTs of tetracycline, gentamicin, erythromycin, roxithromycin, vancomycin, florfenicol, kanamycin and linezolid were not inhibited by the appearance of the novel *fosB* gene (data not shown). And others selected resistance gene were not displayed resistance to these antibiotics, The findings indicated that *fosB8* gene was functionally mediated resistance to Fosfomycin exclusively.

Table 2
Antimicrobial susceptibility test of Fosfomycin for *Paenibacillus illinoisensis* WP77 and transformed with *fosB*8.

Strains	**Characteristics**	**MIC[a](ug/ml)**	
		Fosfomycin with G6P(mg/L)	**Fosfomycin without G6P(mg/L)**
E. coli ATCC25922	Quality control strain	2	8
S. aureus ATCC 29213	Quality control strain	1	2
P. illinoisensis WP77	Contains original *fosB8*	1024	512
RN4220	Cloned recipient strain	1	4
RN4220/pAM401	Vector control	1	2
RN4220/pAM401-*fosB2*	Vector harboring *fosB2*	1024	1024
RN4220/pAM401-*fosB8*	Vector harboring *fosB8*	512	512
Fold change		512	256
BL21(DE3)	Cloned recipient strain	2	2
BL21/pCold1	Expression vector	2	1
BL21/pCold1/*fosB2*	Vector harboring *fosB2*	256	512
BL21/pCold1/*fosB8*	Vector harboring *fosB8*	256	512
Fold change		128	512

[a] The MICs were determined by the agar dilution method with and without 25 mg/L glucose-6-phosphate (G6P).

Characterization of fosB8 and comparative analysis with related FosB proteins.

Bioinformatics analysis of the genomics sequence WP77 identified the *fosB2*-like gene, which encodes a putative fosfomycin bacillithiol transferase. The gene is 420 bp in size and encodes a 140 amino acid protein, with a molecular weight of 16.17 kDa and a pI value of 6.381. The protein exhibited sequence identities of 45.3%, 54.2%, 43.1%, 52.1%, 62.2%, and 49.2% to FosB1, FosB2, FosB3, FosB4, FosB5, and FosB6, respectively. (Fig. 3). Thence, this novel *fosB2-like* was designated as *fosB*8 based on

nomenclature of Fosfomycin resistance gene, and due the eighth discovered member of the FosB family in congruence with the latest *fosB7* gene discovered by Sun L, etal [40, 41].

Phylogenetic analysis of the FosB8 with all known function characterized fosfomycin inactivate enzymes revealed that FosB8 has a close relationship with FosB2 and the next closest evolutionary relationship is FosD. It indicated that fosB8 is a novel lineage of the fosB family (Fig. 2)

To elucidate the structural mechanism underlying the fosfoymcin inactivating function of FosB8, a comprehensive multiple sequence alignment incorporating FosB8 and functionally characterized FosB proteins was conducted. (Fig. 3) it is proved that FosB8 share high sequence similarity to FosB2 and the corresponding protein owned the most typical traits in conserved sequence motifs and residues characteristic of the FosB enzymes family, such as two ATP-binding domains including Walker A and B motifs. It turns out that FosB8 contains 3 amino acid residues (N889, Y107 and E130) responsible for the catalytic destruction of the molecular structure of fosfomycin.

Likewise, in our pursuit of novel discoveries related to *fosB8*, we deposited the amino acid sequences of FosB8 into NCBI (National Center for Biotechnology Information) database and using BLASTp search against the non-redundant protein sequences database to find out the homologous sequences of *fosB*. 135 protein sequences with over 80% identity were from the genus *Paenibacillus*. Among the 135 sequences, the deduced protein sequence of fosB8 exhibited the highest amino acid sequence similarity (97.12% identity and 100% coverage) with uncharacterized metallothiol transferase protein (WP_095361530.1) encoded by *Paenibacillus sp.*

Kinetic characterization of FosB8 and antimicrobial susceptibility of recombinant strains.

To further characterization of FosB8 and for comparison with FosB2, we over expressed and isolated the proteins with the aim to present enzyme catalytic activity (Table 3). Compared with the control strain (pCold/BL21), the recombinant carrying *fosB8* (pCold-*fosB8*/E. coli BL21) and recombinant carrying *fosB3* (pCold-*fosB3*/E. coli BL21) exhibited 256- and 1024-fold increase in MIC levels of fosfomycin, respectively. However, no significant increase in the MIC level was observed in other tested antibiotics. As shown in Table 3, FosB8 efficiently catalyzed the bacillithiol (BSH)-dependent modification of fosfomycin, with a catalytic efficiency (kcat/Km) of $(7.9 \pm 0.8) \times 10^3\ M^{-1}\ s^{-1}$. Although this value was approximately 2.9-fold lower than that of FosB3 [$(22.6 \pm 5.6) \times 10^3\ M^{-1}\ s^{-1}$], FosB8 still conferred a substantial increase in the fosfomycin MIC. These results demonstrate that FosB8 possesses functional fosfomycin-modifying activity and provide biochemical evidence supporting its role in fosfomycin resistance.

Table 3

Kinetic parameters of fosfomycin antibiotics for the purified FosB8 modify enzyme[a].

Enzyme	MIC (ug/ml)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
FosB3	≥ 1024	232.4 ± 6.1	11 ± 3	(22.6 ± 5.6) × 10^3
FosB8	512	133.6 ± 2.9	17 ± 2	(7.9 ± 0.8) × 10^3

[a] The steady-state kinetic parameters for fosfomycin were determined in the presence of 40 mM bacillithiol and Mn^{2+} (~10 μM). Kinetic parameters are reported as the means ± standard deviations from three or four independent biological replicates.

Interestingly, the differences in catalytic efficiency between FosB8 and FosB3 were broadly consistent with their respective resistance phenotypes. FosB3 exhibited a higher kcat/Km and conferred a higher fosfomycin MIC than FosB8, suggesting that the efficiency of fosfomycin modification may contribute to the magnitude of resistance. However, the difference in MIC was considerably larger than that expected from the difference in catalytic efficiency alone, indicating that cellular fosfomycin resistance is likely influenced by additional factors, such as intracellular expression level, enzyme abundance, substrate availability, and the efficiency of fosfomycin uptake or detoxification. Therefore, although the catalytic efficiency of FosB8 is lower than that of FosB3, its activity appears sufficient to effectively protect bacterial cells from fosfomycin.

The absence of a substantial change in susceptibility to the other antibiotics further suggests that FosB8-mediated resistance is relatively specific to fosfomycin rather than resulting from a general increase in antibiotic resistance. Taken together, the concordance between the biochemical activity of purified FosB8 and the resistance phenotype of the recombinant strain supports FosB8 as a functional fosfomycin resistance determinant. These findings also suggest that BSH-dependent fosfomycin modification represents an important mechanism by which FosB8 contributes to fosfomycin resistance.

Genetic context and distribution of fosB8 in Paenibacillus.

To figure out whether the novel fosfomycin resistance genes located in mobile genetic elements, The 20 kbp region of *fosB8* gene along with the upstream and downstream was intercepted for comparative genomics analysis (Fig. 4). The genetic context of the *fosB8* gene in *Paenibacillus* strain WP77 differs from that of closely related *fosB-like* genes in three other *Paenibacillus* strains. The *fosB8* gene is chromosomally located, and no mobile genetic elements were detected in its surrounding genetic environment. On the upstream of *fosB8*, it shown four known gene *rcsC* (sensor histidine kinase), *sigK* (ECF RNA polymerase sigma factor), *nrdE* (ribonucleoside-diphosphate reductase 2 subunit alpha) and *nrdB* (ribonucleoside-diphosphate reductase subunit beta), none of these genes have a known association with antibiotic resistance. The *fosB8* in other *Paenibacillus* may potentially exert fosfomycin resistance too. Of note, the flanked region of *fosB8* gene displayed high identity with *Paenibacillus illinoisensis* 582 (Genbank acssion number: NZ_JACHVM010000007). Moreover, it shared high sequence

identity with *Paenibacillus xylanilyticus*W4 (CP044310) on the upstream region of *fosB*, but no sequence similarity is observed in the downstream region. These observations raise the possibility of horizontal acquisition; however, the origin and evolutionary history of *fosB8* remain uncertain. In summary, although *fosB8* was located on the chromosome in *P. illinoisensis* WP77, the presence of uneven GC content and potential mobile elements surrounding the *fosB* gene implied the potential capability of transmission. Moreover, the *fosB8* gene, which is widely present in *Paenibacillus* species, acts as a resistance reservoir, potentially conferring resistance when transferred to other species in the era of drug abuse. Therefore, additional studies are warranted to elucidate the origins and transfer of *fosB8*.

In conclusion, These findings are consistent with the possibility that fosB8 may represent an evolutionarily conserved resistance determinant that predates the clinical use of fosfomycin.

Conclusions

In this study, we discovered and characterized a novel fosfomycin metallothiol transferase gene, designated *fosB8* in *P. illinoisensis* for the first time. MIC test and enzymatic assay displayed that FosB8 exhibits efficient inactivation of fosfomycin. FosB8 displayed very low aminoacid identity with all known fosfomycin inactivated enzymes, the highest similarity is merely 62% with *fosB5*. Although *fosB8* was located on chromosome, the GC content and genetic context of the gene suggested that the transfer of *fosB8* remains to be uncertain. Notably, the gentetic environment of *fosB8* suggested that *P. illinoisensis* could function as a repository for fosfomycin resistance genes. It is crucial to further investigate the origins and iterations of *fosB8*, especially within animal-derived pathogenic bacteria, and the mechanisms of pathogen evolution. antimicrobial resistant *Paenibacillus*. In summary, this study presents the first complete genome sequence of *Paenibacillus illinoisensis*, revealing numerous unique genomic features. The whole-genome analysis provides a valuable foundation for future investigations into this species' genetic repertoire, including studies of its antibiotic resistance patterns, regulatory networks, and metabolic pathways.

Declarations

Ethics approval and consent to participate

The bacterial isolate WP77 was obtained from a residual clinical specimen collected during routine diagnostic procedures at the Affiliated Hangzhou First People's Hospital. This study focused solely on the microbiological characterization of the bacterial strain and did not involve human participants, identifiable personal data, or human tissue as research subjects. Therefore, the need for ethics approval and informed consent to participate was deemed unnecessary according to the institutional guidelines of the Affiliated Hangzhou First People's Hospital, School of Medicine, Westlake University, and relevant national regulations in China. All experimental procedures were performed in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Data availability.

The whole genome shotgun project of strain WP77 and the complete *fosB8* sequence has been archived in GenBank under accession numbers GCA_044770055.1 and OK335035, respectively.

Authors' contributions

The study was conceptualized and designed by S.W. D.Y. and W.L. Data acquisition was done by W.L. S.Z; D.L; Y.W; X.M; X.Z. and C.F. W.L. performed the experiments, analyzed and interpreted the data, and drafted the manuscript.

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Transparency declarations

The authors declare that they have no conflicts of interest relevant to the content of this manuscript. No financial support was received from any funder that had a role in study design, data collection, analysis, interpretation, or manuscript preparation.

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Figures

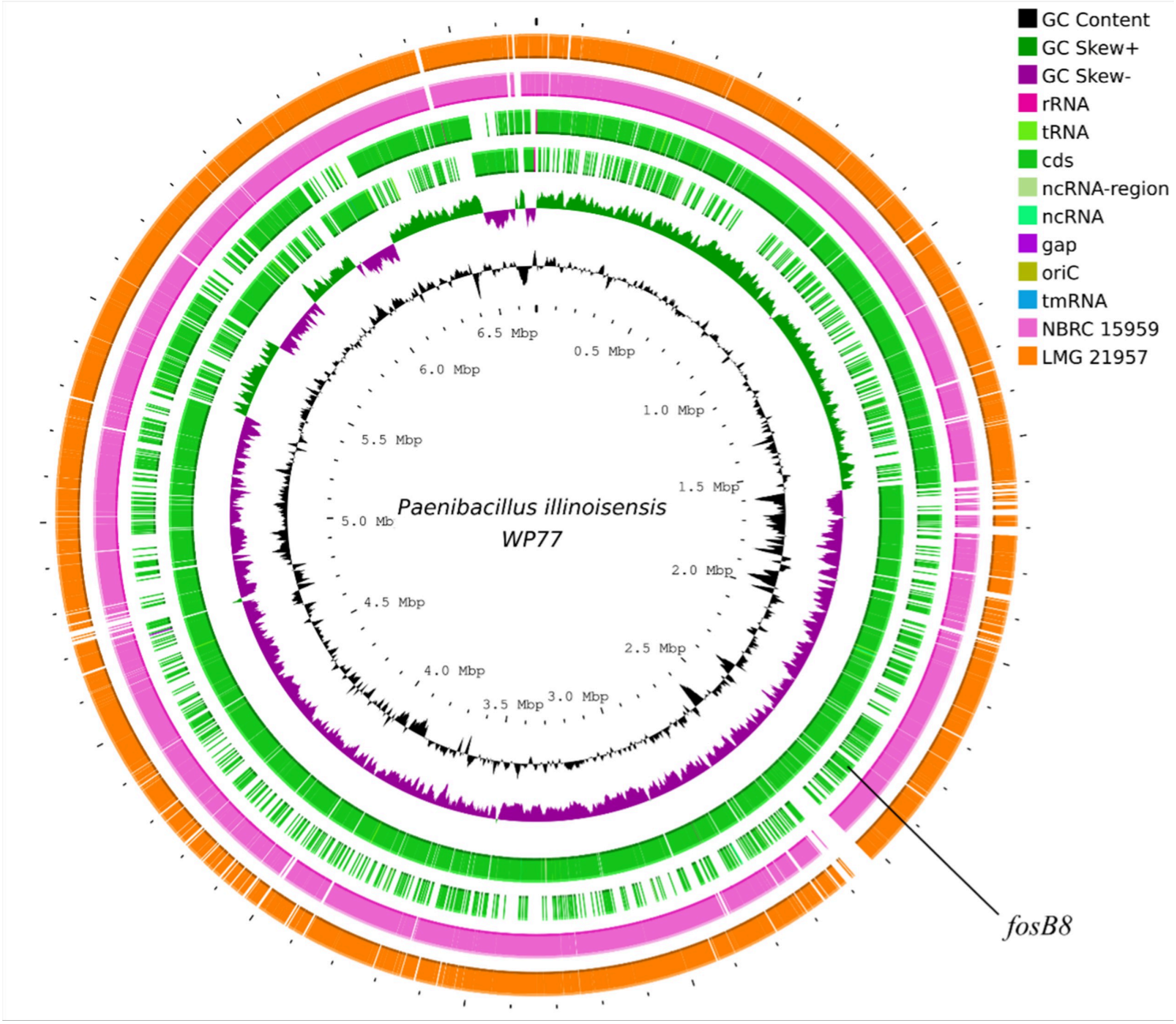


Figure 1

genome analysis of *Paenibacillus illinoisensis*

Circular representation of genome sequence of *Paenibacillus illinoisensis* WP77 (GenBank accession number: GCA_044770055.1). The black arrow indicates the genome location of *fosB8*. From outside to inside: circle 1, circular representation of the *Paenibacillus xylanilyticus* LMG21957 genome (GenBank accession number: GCA_013359935.1); circles 2, circular representation of the *Paenibacillus illinoisensis* NBRC15959 genome (GenBank accession number: GCA_004000925.1); circles 3 and 4, predicted ORFs encoded in the plus strand and Noncoding RNA, respectively; circles 5, GC skew maps; circles 6, GC content; circle 7, scale in Mb (each tick is 0.1Mb).

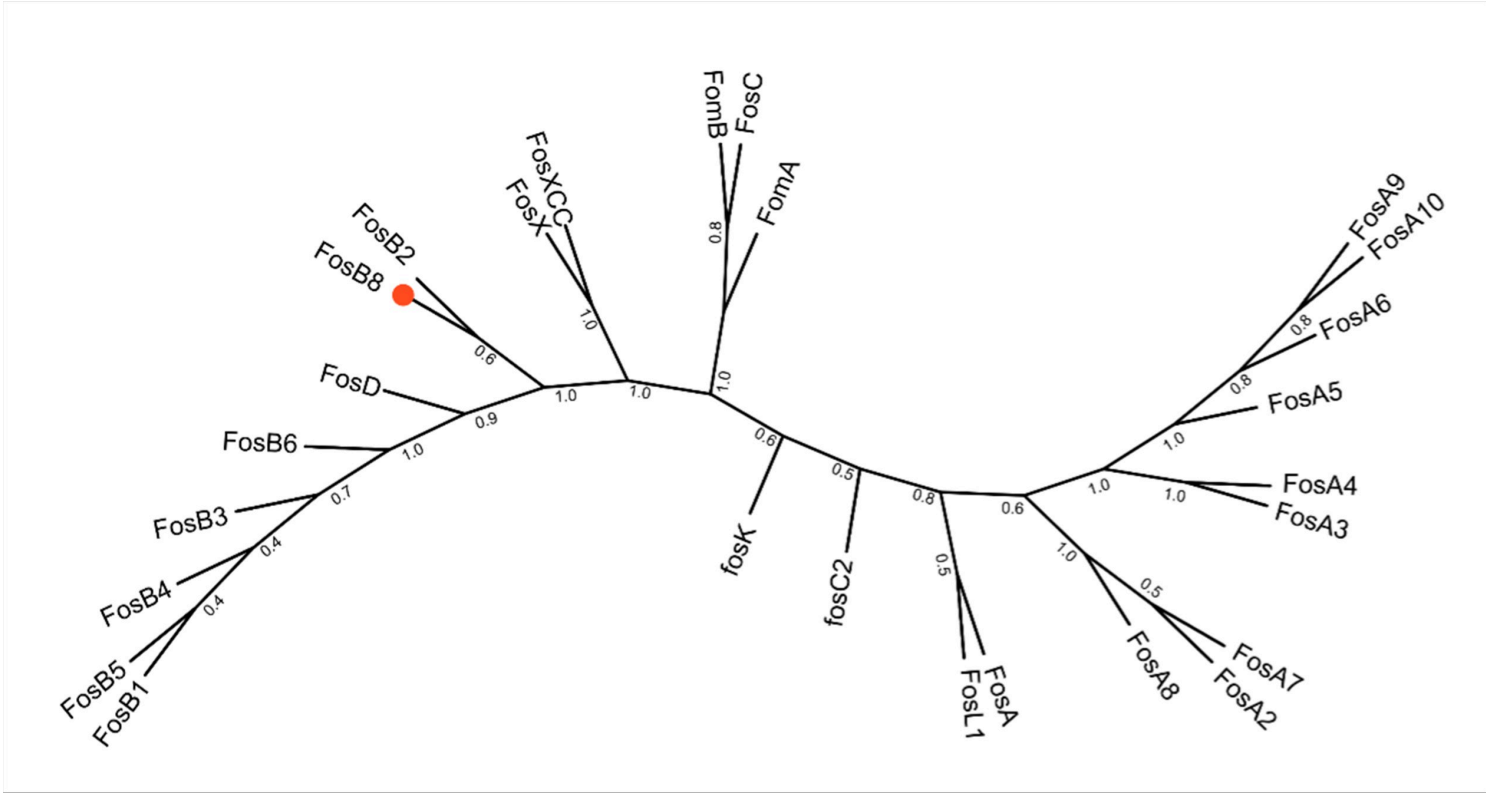


Figure 2

Phylogenetic Tree analysis of FosB8 and all other known fosfomycin resistance proteins.

GenBank accession numbers are list in FIG2. The bootstrap values are shown at the nodes of the tree. The scale bar represents a 10% amino acid sequence difference. FosB8 from this study is highlighted with a red filled circle.

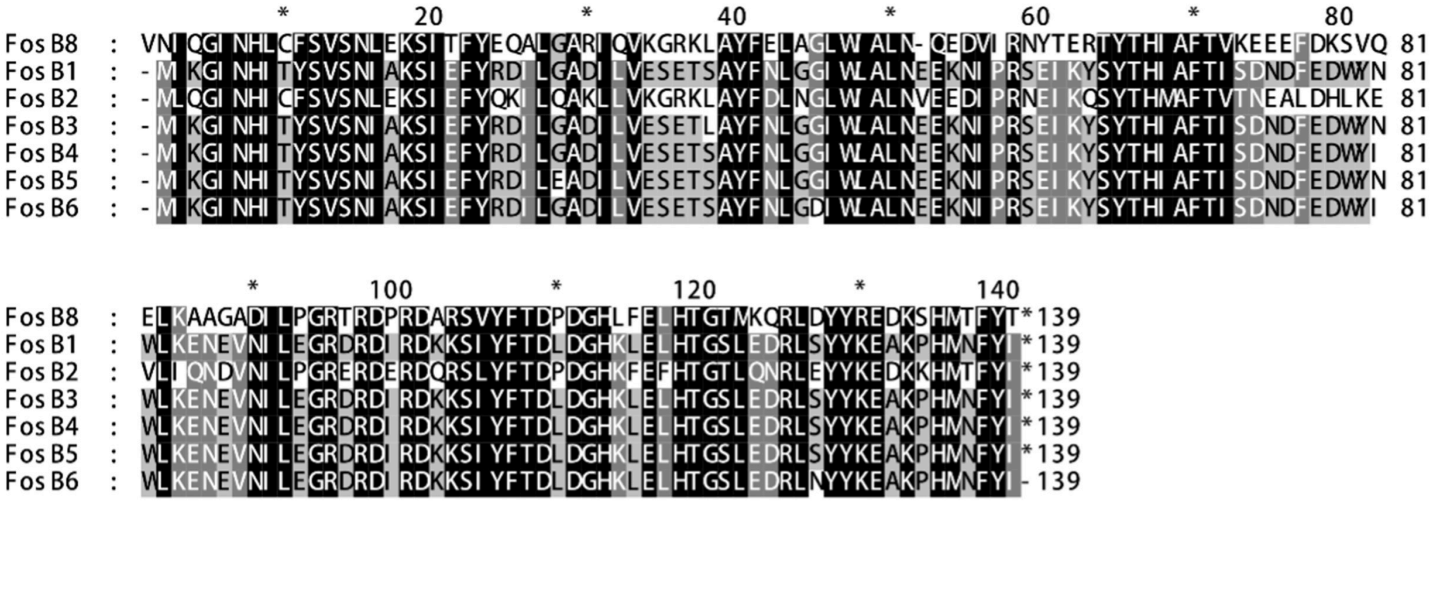


Figure 3

Multiple sequence alignment of Amino acid residual to analysis FosB proteins.

Amino acid sequence alignment of FosB8(UED96306), FosB1(WP_011276918), FosB2(WP_000938987), FosB3(WP_016922374) Enterococcus, FosB4(WP_053032028), FosB5(WP_085562382), FosB6(WP_085562381). The alignment was obtained using M_{EGA} X. The black background indicated fully conserved residues; grey background indicated strongly similar residues. Numbers on the right correspond to the amino acid residues in each full-length protein.

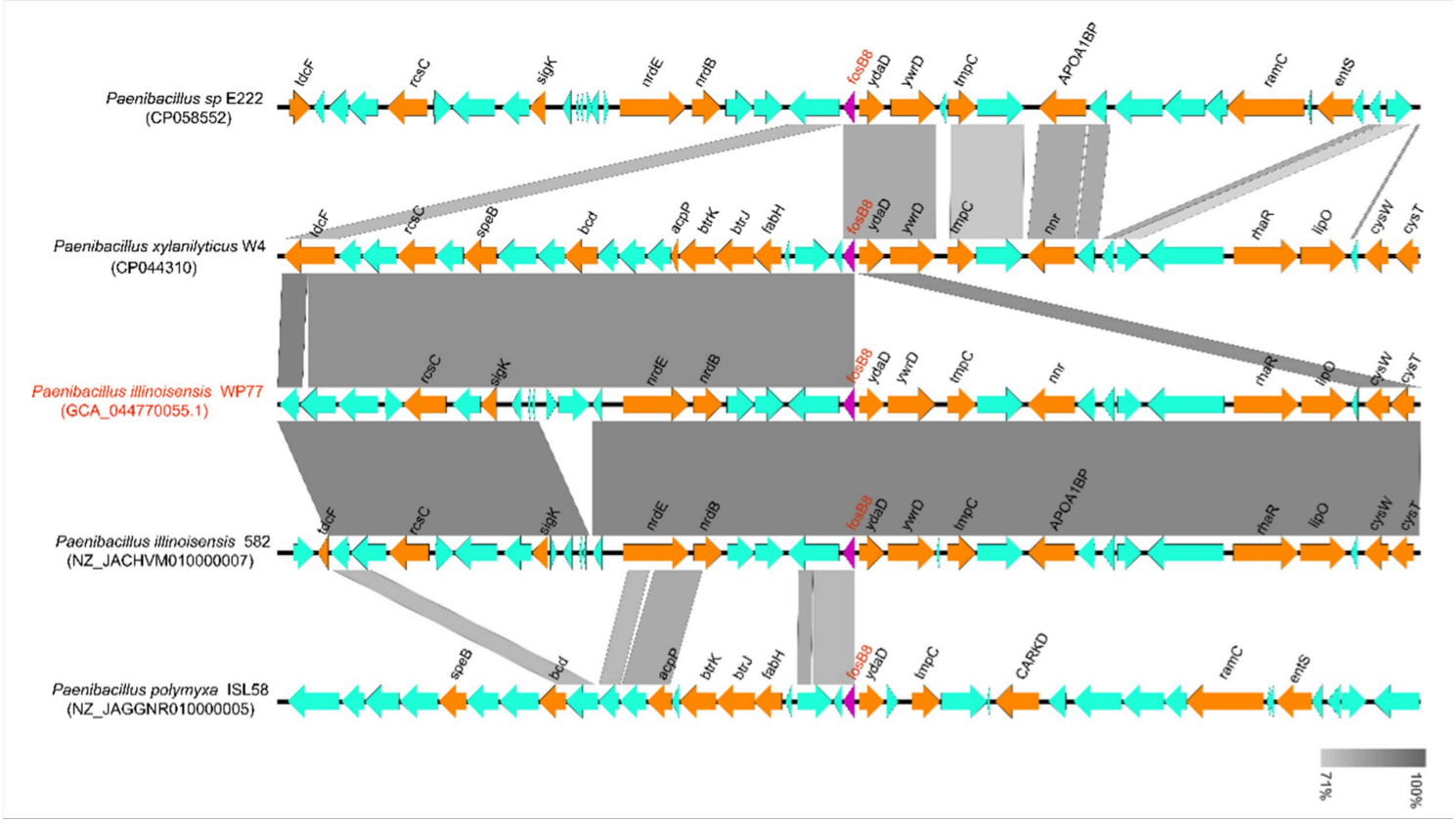


Figure 4

Schematic representation of structural comparison between *fosB8* of flanked region and four *fosB*-carrying regions in genomes of *Paenibacillus*strains.

Regions of >71% homology in flanked region are marked by dark grey shading. ORFs are shown as arrows drawn to scale to indicate the direction of transcription. The *fosB8*gene is coloured in violet and the other genes with known function are coloured in orange. The ORFs encoding hypothetical proteins are coloured in light blue. *rcsC*, Sensor histidine kinase RcsC; *sigK*, ECF RNA polymerase sigma factor SigK; *nrdE*, Ribonucleoside-diphosphate reductase 2 subunit alpha; *nrdB*, Ribonucleoside-diphosphate reductase subunit beta; *fosB8*, Fosfomycin Metallothiol transferase FosB; *ydaD*, General stress protein 39; *ywrD*, Glutathione hydrolase-like YwrD proenzyme; *tmpC*, Membrane lipoprotein TmpC; *nnr*, Bifunctional NAD(P)H-hydrate repair enzyme Nnr; *rhaR*, HTH-type transcriptional activator RhaR; *lipO*,

Membrane lipoprotein TmpC; *cysW*, Sulfate transport system permease protein CysW; *cysT*, Sulfate transport system permease protein CysT.

Supplementary Files

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- fosBsubmentarytables.docx