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A comprehensive study of prophage islands in *Burkholderia pseudomallei* complex

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Introduction: Bacteriophages are known as predators of bacteria and key biological factors influencing genetic recombination through phage transduction in bacteria. Phage transduction is known as one of the most common genetic recombination events found in *Burkholderia pseudomallei*, a diverse bacterial species and the causative agent of a deadly tropical disease melioidosis. The main objective of this study was to catalog prophages or prophage islands that are common in *B. pseudomallei* genomes.

Methods: Various bioinformatic tools were used to identify prophages in 106 complete *B. pseudomallei* genomes, and complete and incomplete genomes in other species within the *B. pseudomallei* Complex (BPC). Temperate phages were spontaneously induced from selected *B. pseudomallei* and *B. thailandensis* strains, and further characterized by transmission electron microscopy and whole genome sequencing.

Results: Nine phage integration hotspots were identified in *B. pseudomallei* pan-genomes, eight of which were associated with tRNA gene-mediated site-specific recombination (tRNA-SSR) events. These genetic events occurred at various tRNA-genes including tRNA- Phenylalanine (anticodon GAA), - Methionine (CAU), - Proline (UGG), - Arginine (UCU), - Cysteine (GCA), - Arginine (CCG), - Serine (GGA), and – Selenocysteine (UCA) genes. Some of these events were also found in other related species within the *B. pseudomallei* Complex (BPC). We have demonstrated that lysogenic phages from select BPC strains could use *B. pseudomallei* strain Bp82 or 576mn as a host. These phages were classified into one of the two major groups, myoviruses or siphoviruses, based on their morphology and genomic composition.

Discussion: We have demonstrated that most *B. pseudomallei* strains are lysogenic, many containing at least one functional prophage in their genomes. Further investigation of the interactions between *B. pseudomallei*, bacteriophages, and other environmental and biological factors would provide a bigger picture of genomic diversity, potentially influence on survival of *B. pseudomallei* in the environment and its pathogenic specialization in hosts.

KEYWORDS

***Burkholderia pseudomallei*, melioidosis, prophage, temperate phage, tRNA-SSR**

Introduction

Burkholderia pseudomallei (*B. pseudomallei*), a Gram-negative soil bacterium, is the causative agent of melioidosis, a severe tropical disease with a high mortality rate. Melioidosis is endemic to most Asian countries, northern Australia, the Caribbean, and South America. *B. pseudomallei* is an opportunistic pathogen that can cause disease in both animals and humans from inoculation through skin abrasion, ingestion of contaminated food or water, or inhalation of contaminated dust or aerosol. One factor that influences the frequency and severity of *B. pseudomallei* outbreaks is its ability to survive in strictly environmental conditions for extended periods of time, including distilled water for at least 16 years (Pumpuang et al., 2011). *B. pseudomallei* has an open genome that promotes horizontal gene transfers (HGTs) or allow high frequency of recombination of mobile genetic elements (MGEs) to form genomic islands (GIs) which causes genetic diversity among the population (Kim et al., 2005; Tuanyok et al., 2008). This process in *B. pseudomallei* is not random, as many of these events are associated with specific recombination sites e.g., tRNA genes (Tuanyok et al., 2008).

In general, site-specific recombination is the insertion of foreign DNA into the bacterial genome, which occurs in conserved regions of DNA during chromosomal replication. Typically, this recombination results in the creation of a GI containing inserted genes from external sources, such as plasmids from other bacteria (Hallet and Sherratt, 1997). This system is not only used for insertion of foreign DNA, but is frequently used by temperate bacteriophages, the viruses which infect bacterial cells and enter a lysogenic life cycle leading to the insertion of phage DNA into the bacterial genome. The insertion sites used for site-specific recombination in the bacterial genome are highly similar to *attP* sequence, the bacteriophages' own insertion sequences, leading to the insertion of temperate bacteriophages, or prophages, into the genome at the same homologous sites (Gindreau et al., 1997). These prophages have a substantial effect on the phenotypes of the bacteria and increase the diversity of the species. Phage DNA can increase the virulence of a bacterial strain through phage-encoded virulence factors and the expression of antibiotic resistance genes as seen highly efficiently in *E. coli* O157:H7 (Marinus and Poteete, 2013). Prophages can also influence the evolution of microbial metabolic pathways. Our previous study on the genomic comparison of five *B. pseudomallei* strains, which causes various clinical presentations in melioidosis patients: K96243, 1710b, 1106a, MSHR668, and MSHR305, identified 71 different GIs. This study also reported the association between about 40-60% of GIs insertions and 13 tRNA locations. The term, "tRNA gene-mediated site-specific recombination" or "tRNA-SSR," which explained that the locations of these GIs are not random but specific to tRNA gene loci in *B. pseudomallei* genomes, was proposed (Tuanyok et al., 2008). This process resulted in locating GIs at the 3' end of tRNA genes and creating short, direct repeats of tRNA genes flanking GIs. In a follow-up study using a pan-genomic approach when 37 genomes were used, we found that gene order was highly conserved among strains, despite the high combination rate as previously observed (Spring-Pearson et al., 2015).

In order to understand the distribution of temperate phages and their impact on the survival and pathogenic specialization of *B. pseudomallei*, we identified the presence of tRNA-SSR in 106 complete *B. pseudomallei* genomes and genomes of other members within the *Burkholderia pseudomallei* complex (BPC), including *B. mallei* (Nierman et al., 2004), *B. thailandensis* (Brett et al., 1998), *B. oklahomensis* (Glass et al., 2006b), *B. humptydooensis* (Tuanyok et al., 2017), *B. singularis* (Vandamme et al., 2017), *B. savannae* and *B. mayonis* (Hall et al., 2022). The tRNA-SSRs were then identified as putative prophages due to lengths over 27,000 base pairs and containing phage biosynthesis genes. Some of these prophages were characterized for their functionality.

Materials and methods

tRNA-SSR identification

To identify the tRNA-SSRs, we first created a genome database by downloading all complete genomes of *B. pseudomallei* (N=106) and *B. thailandensis* (N=19), which were available from GenBank as of January 1st, 2023. Other complete and incomplete BPC species' genomes including *B. mallei* (N=108), *B. oklahomensis* (N=3), *B. humptydooensis* (N=4), *B. savannae* (N=1), *B. mayonis* (N=1), and *B. singularis* (N=2) were also acquired. All tRNA-SSR events were identified in each genome through local NCBI BLASTn (v 2.2.18) (Camacho et al., 2009) with the tRNA gene sequences from each species' reference strain (e.g., *B. pseudomallei* K96243, GenBank accession no. BX571965.1 &BX571966.1; *B. mallei*, CP000010.1 &CP000011.1; *B. thailandensis* E264, CP000086.1 &CP000085.1; *B. oklahomensis* C6786, CP009555.1 &CP009556.1; *B. humptydooensis* MSMB43, CP013380.1 &CP013382; *B. savannae* MSMB266, CP013417.1 &CP013418.1; *B. mayonis* BDU6, CP013386.1 &CP013387.1; and *B. singularis* LMG 28154, GCA_900176645.1) as the query. With a tabular output, the region between the tRNA genes and their 3' end direct repeat sequences were sorted and identified based on a length of at least 27,000 base pairs. These coordinates of the direct repeats were compiled into a table used to create BED files, then the BED files were used for extracting a multiFASTA file of each prophage genomes in every tRNA-SSR with the strain names, tRNA repeat, and start and stop points using BEDTools suite – getfasta version 2.30.0 (Quinlan and Hall, 2010). This allows for the sequences to be further analyzed. Supplementary Figure 1 demonstrates the identification of tRNA-SSR and the computational workflow for prophage prediction.

Identification of phage family for putative prophages

Every prophage underwent further analysis with PHASTER, a swift and specialized tool for phage sequence detection (Zhou et al., 2011; Arndt et al., 2016) to compare with previous BLASTn outcomes. Both BLASTn and PHASTER programs were utilized for predicting prophage regions. PHASTER offers the advantage of

providing a brief overview of phage genes within the predicted prophage regions. However, it may sometimes encounter difficulties in precisely identifying the start and stop nucleotides of the prophage regions. To address this limitation, BLASTn was employed as it excels in this aspect. In this process, each prophage was matched to the one it bore the closest resemblance to, based on the inserted sequence. The criterion for this matching required that over 80% of the proteins demonstrate substantial similarity in both the sequence and the query coverage to the target sequence. Subsequently, the affiliation of the identified prophage was traced within the GenBank database, and its confirmation was established through an in-depth analysis of the integrase gene. Lastly, the tRNA-SSR associated prophages identified were further annotated using Genome Annotation services via the Bacterial and Viral Bioinformatics Resource Center (BV-BRC; http://bv-brc.org). This included analyzing prophage features such as genes potentially involved in antimicrobial resistance phenotypes, metabolic or biochemical pathways, or special functions (specialty genes), using the BV-BRC's tools like the BLAT (BLAST-like alignment tool) (Olson et al., 2023).

Frequency of prophages in a local *B. pseudomallei* population

Conventional PCR was used to amplify six integrase genes known to be associated with prophages in 321 *B. pseudomallei* strains from 142 clinical and 179 environmental sources, collected from a local endemic area of southern Thailand (Kaewrakmuk et al., 2023), and 9 *B. thailandensis* strains. Each PCR assay contained a 10 µL reaction comprising 1X Chai Sahara Hot Start Master Mix (Chai Bio Inc., Santa Clara, CA, USA), 2 µM of each oligonucleotide primer, and 1 µL of 10 ng of genomic bacterial DNA. Thermocycling conditions, conducted in 8-well PCR strips thermal cycle, were as follow: hot start at 95 C for 5 minutes; 35 cycles of denaturation at 95°C for 45 seconds, annealing at 52°C for 45 seconds, and extension at 72°C for 1 minute; and final step to allow extension at 72 C for 5 minutes. Then, the PCR products were visualized by SYBR Safe stain in 2% agarose gel-electrophoresis running with 120 Volts 500 Amperes for 35 minutes. Details of PCR primers, expected amplicon sizes and their targeting genes are summarized in Supplementary Table 1.

Isolation of temperate phages

Temperate phages were isolated from three selected BPC strains including *B. pseudomallei* Bp82, and *B. thailandensis* E264 and TXDOH. Bacterial host strains *B. pseudomallei* Bp82 (Propst et al., 2010) and 576mn (Norris et al., 2017), the biosafe non-select agent strains and the representatives of serotype A and B, were used. Briefly, an overnight culture of each tested bacterial strain and each of the two host strains in LB broth was sub-cultured into a 3 mL fresh LB broth and grown at 37°C with shaking at 250 rpm for 5 hours. Cultures of both biosafe strains were supplemented with 0.8 µg/mL of adenine (Propst et al., 2010). A volume of 1 mL of overnight culture of each tested strain was centrifuged at 16,000 x *g* for 1 minute. The resulting supernatant was then filtered through a 0.45-micron (dia.) syringe filter into a sterile tube. Bacterial host soft agar was prepared by mixing 100 µL each of the fresh culture of Bp82 or 576mn with 4.5 mL of molten LB adenine soft agar (0.35% w/v) in 5 ml sterile tube. The mixture was then poured onto an LB agar plate supplemented with 0.80 µg/mL of adenine. Once the agar solidified, 100 µL of the filtered supernatant was added on top of the bacterial soft agar plate. The plate was briefly rotated to allow the phage solution to spread across the agar's surface and was left to dry for 10 minutes. After drying, the plate was inverted and incubated at 37°C in an incubator overnight. The plaque formations were subsequently observed.

Using conventional PCR targeting integrase genes to guide the identification of temperate phages

We used PCR to guide the identification of temperate phages present in *B. pseudomallei* Bp82. Conventional PCR assays were used to amplify three different phage integrase genes uniquely presented in different prophages. Each PCR had a 10 µL reaction containing 1X Chai Sahara Hot Start Master Mix (Chai Bio Inc., Santa Clara, CA, USA), 2 µM of each oligonucleotide primer, and 1 µL of phage solution diluted from each plaque. The primer sequences for each phage integrase assay are as follows: i) tRNA-SSR-Pro (UGG) assay targeting gene BP1026B_I2161; forward primer, 5'-GTGGCCGAAGTATGGAGATTAC-3', and reverse primer. 5'-CGATCCACGTCTGACCATTT-3'; ii) tRNA-SSR-Arg (CCG) assay targeting gene BP1026B_I13636; forward primer, 5'-TGCTGTCGAATGGGAATGG-3', and reverse primer, 5'-AACCGCGGTAAACGAACA-3'; and iii) tRNA-SSR-Phe (GAA) assay targeting gene BPSL0129; forward primer, 5'-AAG GTTGCGAGCGATTACTG-3', and reverse primer, 5'-GCCATG CGTGGTTGTAGAT-3'. Thermocycling conditions included hot start at 95°C for 5 minutes, and 35 cycles of denaturation at 95°C for 45 seconds, annealing at 52°C for 45 seconds, and extension at 72°C for 1 minute, and the final extension at 72°C for 5 minutes. PCR products were visualized by gel electrophoresis operated at 120 Volts and 500 Amperes for 35 minutes. Expected PCR product sizes for these three assays were 438, 362, and 676 bp, respectively.

Phage DNA extraction

A volume of 100 mL of filtered plate lysate phage suspension in SM buffer was subjected to filtration using a 0.22-micron (dia.) bottle-top vacuum filter to eliminate any potential residual bacterial cells. Subsequently, the resulting mixture underwent centrifugation at 25,000 × *g* for a duration of 60 minutes. Following this centrifugation step, the supernatant was discarded, revealing a noticeable yellow spot, which constituted the phage pellet. To resuspend the pellet, 1 mL of LB broth was added. To facilitate the extraction of phage DNA, 500 µL of concentrated lysate was combined with 6.25 of µL 1 M $MgCl_2$, 0.4 µL of DNase I

(1000 U/mL), and 5 µL of RNase A (20 mg/mL) was introduced into the mixture. This mixture was then incubated at 37°C for 60 minutes. Subsequent steps involved the destabilization of phage capsids and inactivation of DNase I by the addition of 25 µL of 10% SDS, 20 µL of 0.5 M EDTA (pH 8.0), and 1.25 µL of proteinase K (20 mg/mL). The incubation was continued at 55°C for another 60 minutes. DNA extraction was executed by introducing an equal volume of phenol-chloroform-isoamyl alcohol (PCI) in a ratio of 25:24:1, followed by vigorous mixing and centrifugation at 13,000 × *g* for 5 minutes. The resulting aqueous phase was carefully transferred to a new tube. The DNA extraction process was repeated twice using PCI and once using chloroform. To precipitate the DNA, an equal volume of absolute ethanol containing 50 µL of 3 M sodium acetate at pH 5.2 was added and incubated at -20°C overnight. The resulting DNA pellet was obtained by centrifugation at 13,000 × *g* for 10 minutes. Afterward, the pellet was washed twice with 1 mL of 70% ethanol and air-dried within the confines of a biosafety cabinet. Finally, the pellet was dissolved in 50 µL of DNase-free sterile water.

Phage genome sequencing, genomic assembling and annotation

DNA samples were subjected to sequencing using Illumina sequencing techniques. Illumina sequencing libraries were created using the tagmentation-based and PCR-based Illumina DNA Prep kit, along with custom IDT 10 bp unique dual indices (UDI), targeting an insert size of 320 bp. No additional DNA fragmentation or size selection steps were included in the process. The Illumina sequencing was conducted using an Illumina NovaSeq 6000 sequencer in multiplexed shared-flow-cell runs, generating 2x151 paired-end reads. Following sequencing, demultiplexing, quality control, and adapter trimming were carried out using bcl-convert version v4.1.5, a software tool developed by Illumina Inc. in San Diego, California, USA. Genomic assemblies were performed utilizing Phage genome assembler v2021.03 workflow within the CPT Phage Galaxy platform, a web-based framework for biological computation hosted at the Center for Phage Technology (CPT), which was established by the Texas A&M University (Ramsey et al., 2020). Genome annotations were performed using online tools via BV-BRC as described above (Olson et al., 2023).

Morphology of phages

A 100 µL volume of phage sample containing a minimum of 10^8 plaque forming units/mL concentration was diluted with 900 µL of SM buffer. Subsequently, 10 µL of this phage solution was carefully applied to a glow-discharged copper grid stabilized with carbon and allowed to incubate for 5 minutes. Following this incubation period, the grid was briefly exposed to a 1% (w/v) uranyl acetate solution for 1 minute to enable staining. The prepared grids were then subjected to visualization by FEI -Tecnai G2 Spirit Twin Transmission Electron Microscope (Thermo Fisher Scientific Inc., Hillsboro, OR, USA) housed at the University of Florida's Interdisciplinary Center for Biotechnology Research. Phage sizes were determined based on the average measurements obtained from five independent phages, providing valuable insights into their structural characteristics.

Results

tRNA-SSR frequency in *B. pseudomallei*

The frequency of tRNA-SSRs within the pangenome of *B. pseudomallei* was examined, revealing a total of 185 occurrences across 106 complete genomes. These tRNA-SSR events were linked to 15 distinct tRNA gene sequences. Among them, 53 integrated DNA segments exhibited sizes exceeding 27,000 base pairs and were predicted to be potential prophages using the PHASTER tool (Zhou et al., 2011; Arndt et al., 2016). Notably, these prophage predictions were associated with specific tRNA-SSRs located at seven different tRNA genes such as tRNA-Phenylalanine (anticodon GAA), -Methionine (CAU), -Proline (UGG), -Arginine (UCU), -Cysteine (GCA), and -Arginine (CCG) on chromosome 1, and as well as tRNA-Selenocystiene (UCA) on chromosome 2. Frequencies of these tRNA-SSR events along with the identified prophages in *B. pseudomallei* pangenome are shown in Figure 1. Furthermore, in order to validate the prophage identity of the insertions at these tRNA genes, an analysis was conducted on the direct repeat sequences which are identical to 3'end sequence of tRNA genes, downstream of the integration sites. These short sequences were believed to be generated during phage transduction and consistently displayed identical length and sequence characteristics for each tRNA-SSR instance associated with each respective tRNA gene. This observation underscores the presence of similar prophages from the same phage group at each tRNA gene location. Supplementary Table 2 (2.1) summarizes the presence of these tRNA-SSR events and the associated direct repeats for each *B. pseudomallei* genome.

tRNA-SSRs in other members of the BPC

Our investigation extended to seven additional species within the BPC including *B. mallei*, *B. thailandensis*, *B. oklahomensis*, *B. humptydooensis*, *B. sevannae*, *B. mayonis*, and *B. singularis*. Within the genomes of *B. thailandensis*, we identified a total of 69 instances of tRNA-SSRs across 19 complete genomes. These occurrences were distributed among 19 distinct tRNA gene loci, with two of them—tRNA-Pro (UGG) in strain USAMRU Malaysia#20 and tRNA-Ser (GGA) in strain TXDOH—being associated with prophage elements. Similar prophage associations were observed in *B. oklahomensis* genomes, specifically at tRNA-Arg (UCU) in strain C6786, tRNA-Phe (GAA) in strain E0147, and tRNA-Arg (CCG) in strain FDAARGOS_900. Notably, in both *B. humtydooensis* strains MSMB121 and MSMB122, we found a putative prophage linked to tRNA-SSR at tRNA-Leu (UAG), a novel prophage location unique to *B. humptydooensis* among BPC species. For *B. savannae*, *B. mayonis*, and *B. singularis*, which had a limited number of available genomes, we identified two putative

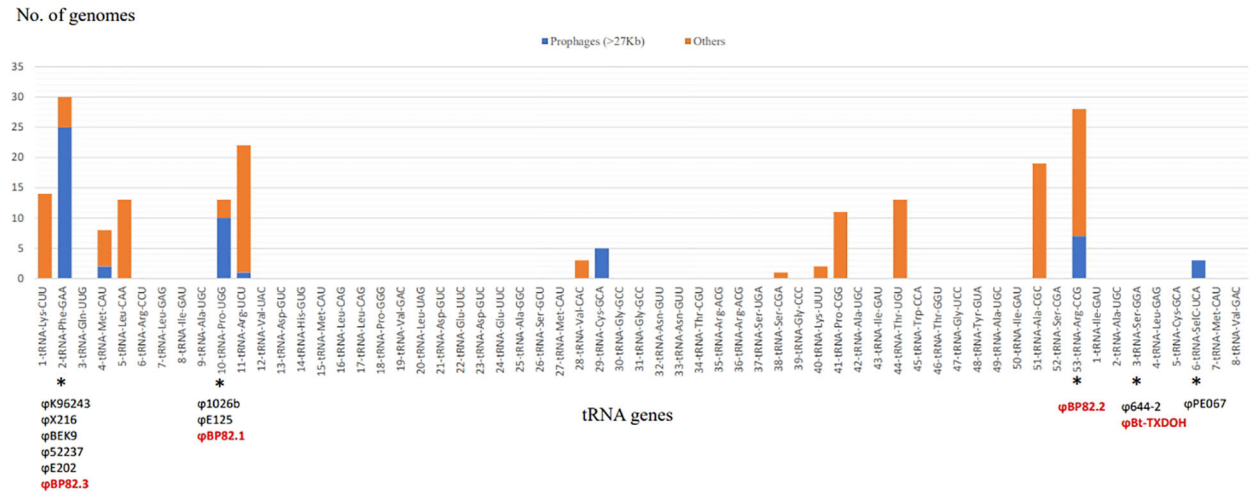

FIGURE 1
Distribution of tRNA-SSR sites in *B. pseudomallei* Genomes. The bar chart illustrates the count of genomes with either anticipated prophages (blue bars) or alternate exogenous DNA sequences (orange bars) associated with specific tRNA genes. Asterisks mark the prophage sites that have undergone characterization, highlighting the names of the temperate phages isolated for this research (red text) as well as those identified in prior research (black text).

prophages associated with tRNA-SSR at tRNA-Arg (UCU). One was located in *B. savannae* MSMB266 genome, while the other was found in *B. mayonis* BDU6. Although tRNA-SSR events occurred in *B. singularis* LMG 28154 and TSV85, none of them exhibited a prophage association. Our analysis also encompassed 108 complete genomes of *B. mallei*, revealing a single type of tRNA-SSR event linked to tRNA-Pro (CGG), which is equivalent to BPSLt41 locus in *B. pseudomallei* K96243 and also known as BMA_tRNA_Pro-5 in *B. mallei* ATCC 23344, in most *B. mallei* strains (data not shown). This integration site, spanning approximately 4.6 kb, featured insertion sequence elements, specifically *IS*407A, as previously described (Tuanyok et al., 2008). These tRNA-SSR occurrences and their prophage associations in BPC genomes were summarized in Supplementary Tables 2 (2.1 to 2.7). Details of the 3'end tRNA-gene direct repeat sequences associated with tRNA-SSR events at each tRNA gene are presented in Supplementary Table 3.

Identification of tRNA-SSR and phage attachment sequences in publicly available phage genomes associated with BPC species

In this investigation, we focused on the identification of tRNA-SSR and phage attachment (*attP*) sequences within publicly accessible phage genomes associated with BPC species. We undertook this endeavor using 20 available BPC phage genomes, the majority of which are lysogenic phages that have been previously documented in various studies, as indicated in Table 1. Our research builds upon insights from previous genomic island research (Tuanyok et al., 2008) and our current findings have revealed that phage integrations are not random; rather, they exhibit a high degree of site-specificity. Among the 20 phage genomes we analyzed, 17 of them were found to have known *attP* sequences. Notably, 13 of these *attP* sequences were found to be identical to the 3' end sequences of five different tRNA genes. To illustrate: 1) the genomes of φE125, φ1026b, and φBP82.1 featured a 49 bp *attP* sequence identical to the 3' end sequence of tRNA-Pro (UGG); 2) φ52237, φE202, φX216, φBEK9, and φBp82.3 genomes contained a 45 bp *attP* sequence identical to the 3' end sequence of tRNA-Phe (GAA); 3) φBp644.2 and φBt-TXDOH genomes had a 30 bp *attP* sequence identical to the 3' end sequence of tRNA-Ser (GGA); 4) φE094 and φBP82.2 contained a 45 bp *attP* sequence identical to the 3' end sequence of tRNA-Arg (CCG), and 5) φPE067 possessed a 16 bp *attP* sequence, 5'-CGGCTGCCT TCCGCCA-3', identical to the 3' end sequence of tRNA-SelC (UCA) as described in (Hammerl et al., 2020). These tRNA genes are corresponding to BPSLt10, BPSLt02, BPSSt03, BPSLt53, and BPSSt06 locus tags annotated in *B. pseudomallei* K96243 genome (Holden et al., 2004). It's worth noting that φPE067 was previously identified to have a 19 bp *attP* sequence, 5'-CGGCTGCCTTCC GCCACAA-3', identical to a chromosomal region responsible for selenocysteine biosynthesis on chromosome 2 of *B. thailandensis* E067 (Hammerl et al., 2020). Among the remaining four phage genomes, two, namely φE131 and φE058, were identified in the same study to have an 18 bp *attP* sequence identical to a chromosomal region of *B. thailandensis* E058. The other two genomes, φE12-2 and φPK23, contained an 18 bp *attP* sequence identical to a genomic island repeat sequence associated with GI15 of *B. pseudomallei* K96243. Further details regarding these publicly available BPC phage genomes and their corresponding *attP* sequences are presented in Table 1. Building on previous findings of seven distinct tRNA gene loci associated with prophages and the identification of the 30 bp *attP* sequence of φBp644-2, which matches the 3'end sequence of tRNA-Ser (GGA), this study uncovered a total of eight primary sites of phage integration in *B. pseudomallei.* These genetic events have been observed at the tRNA- Phe (GAA), - Met (CAU), - Proline (UGG), - Arg (UCU), -

TABLE 1 Known or predicted *attP* sequences identified in *Burkholderia* phage genomes.

Phage	Group	Genome size (bp)	Lysogen or natural source	Propagation host	Associated tRNA-SSR	*attP*	*attP* sequence (5′ ->3′)	GenBank accession	References
φE125	Siphovirus	53373	*B. thailandensis* E125	*B. mallei* ATCC 23344	tRNA-Pro (UGG); BPSLt10	49 bp	CTGATTTGGGATCAGAGGGTCGTAGGTTCGAATCCTATC GCTCCGACCA	NC_003309	(Woods et al., 2002)
φ1026b	Siphovirus	54865	*B. pseudomallei* 1026b	*B. mallei* ATCC 23344	tRNA-Pro (UGG); BPSLt10	49 bp	CTGATTTGGGATCAGAGGGTCGTAGGTTCGAATCCTAT CGCTCCGACCA	AY453853	(DeShazer, 2004)
φBP82.1	Siphovirus	54921	*B. pseudomallei* Bp82	*B. pseudomallei* 576mn	tRNA-Pro (UGG); BPSLt10	49 bp	CTGATTTGGGATCAGAGGGTCGTAGGTTCGAATCCTA TCGCT CCGACCA	OM892879	This study
φ52237	Myovirus	37639	*B. pseudomallei* Pasteur 52237	*B. mallei* ATCC 23344	tRNA-Phe (GAA); BPSLt02	45 bp	TTGAAAATCCGCGTGTCGGTGGTTCGATTCCGCCCC AGGCCACCA	NC_007145	(Ronning et al., 2010)
φE202	Myovirus	35741	*B. thailandensis* E202	*B. mallei* ATCC 23344	tRNA-Phe (GAA); BPSLt02	45 bp	TTGAAAATCCGCGTGTCGGTGGTTCGATTCCGCCCCA GGCCACCA	NC_009234	(Ronning et al., 2010)
φX216	Myovirus	37637	*B. pseudomallei* E0237	*B. mallei* ATCC 23344	tRNA-Phe (GAA); BPSLt02	45 bp	TTGAAAATCCGCGTGTCGGTGGTTCGATTCCGCCCCA GGCCACCA	JX681814	(Kvitko et al., 2012)
φBEK9	Myovirus	37631	*B. pseudomallei* 9	Unknown	tRNA-Phe (GAA); BPSLt02	45 bp	TTGAAAATCCGCGTGTCGGTGGTTCGATTCCGCCCCA GGCCACCA	CP008753	N/A
φBp82.3	Myovirus	37637	*B. pseudomallei* Bp82	*B. pseudomallei* 576mn	tRNA-Phe (GAA); BPSLt02	45 bp	TTGAAAATCCGCGTGTCGGTGGTTCGATTCCGCCCCA GGCCACCA	ON135435	This study
φBp644.2	Siphovirus	48674	*B. pseudomallei 644*	*B. mallei ATCC 23344*	tRNA-Ser (GGA); BPSSt03	30 bp	TCGGGGGTTCGAATCCCCCTCTCTCCGCCA	CP000625	(Ronning et al., 2010)
φBt-TXDOH	Siphovirus	56453	*B. thailandensis* TXDOH	*B. pseudomallei* BP82	tRNA-Ser (GGA); BPSSt03	30 bp	TCGGGGGTTCGAATCCCCCTCTCTCCGCCA	OK095358	This study
φE094	Myovirus	37727	Rice field soil in Thailand	*B. thailandensis* DV1	tRNA-Arg (CCG); BPSLt53	45 bp	CTCCGAAGGCAGGGGTTGCTGGTTCGATCCCAGCCGG GCGCGCCA	MW072790	(Muangsombut et al., 2021)
φBP82.2	Myovirus	36280	*B. pseudomallei* Bp82	*B. pseudomallei* 576mn	tRNA-Arg (CCG); BPSSt53	45 bp	CTCCGAAGGCAGGGGTTGCTGGTTCGATCCCAGCCGG GCGCGCCA	OM304382	This study
φPE067	Myovirus	43649	*B. thailandensis* E067	Unknown	tRNA-SelC (UCA); BPSSt06	16 bp	CGGCTGCCTTCCGCCA	KT803877	(Hammerl et al., 2020)
φE131	Myovirus	44121	*B. thailandensis* E131	Unknown	None	18 bp	TCCGGTCCCCGGCACCAC	MH809532	(Hammerl et al., 2020)
φE058	Myovirus	44121	*B. thailandensis* E058	Unknown	None	18 bp	TCCGGTCCCCGGCACCAC	MH809533	(Hammerl et al., 2020)
φE12-2	Myovirus	36690	*B. pseudomallei* E12.2	*B. mallei* ATCC 23344	None	18 bp	AATTTGACATAAGGTAAA	NC_009236	(Ronning et al., 2010)

(Continued)

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TABLE 1 Continued

Phage	Group	Genome size (bp)	Lysogen or natural source	Propagation host	Associated tRNA-SSR	*attP*	*attP* sequence (5' ->3')	GenBank accession	References
φPK23	Myovirus	35343	Mountain soil, Thailand	*B. pseudomallei* CAM5 and 576mn	None	18 bp	AATTTGACATAAGGTAAA	MZ375754	Unpublished
φE255	Myovirus	37446	*B. thailandensis* E255	*B. mallei* ATCC 23344	None	Unknown	Unknown	NC_009237	(Ronning et al., 2010)
φST79	Myovirus	35430	Soil	*B. pseudomallei* P37	None	Unknown	Unknown	NC_02134	(Yordpratum et al., 2011)
φBt-E264.1	Siphovirus	44526	*B. thailandensis* E264	*B. pseudomallei* BP82	None	Unknown	Unknown	OP293079	This study

Cys (GCA), - Arg (CCG), - Ser (GGA), and – SelC (UCA) genes. Genomic locations of these tRNA-SSR events are illustrated in Figure 2.

Prophage gene features

All 63 prophages identified as being associated with tRNA-SSR events across multiple strains within the BPC species were further annotated for gene features. Their sizes ranged from 27 kb, with 37 protein-coding sequences (CDSs) in a prophage associated with tRNA-Phe (GAA) gene of *B. pseudomallei* BPs111, to 61 kb, with 81 CDSs in a prophage associated with tRNA-Pro (UGG) gene in *B. pseudomallei* 1710b. Details of these prophages, including sizes and genomic identifier - locus tags and their sequences are available in Supplementary Table 5.1 – 5.6, Supplementary Text 1, respectively. Based on the annotation findings (Supplementary Table 6.1), seven specialty genes encoding phage holins were identified. Five of them were found in the prophages associated with tRNA-Cys (GCA) gene in *B. pseudomallei* strain MSHR520, MSHR305, MSHR3763, MSHR4083, and BDP, while other two were found in the prophages associated with tRNA-Arg (UCU) gene in *B. pseudomallei* 3000015486 and *B. oklahomensis* C6786, as detailed in Supplementary Table 6.2. Furthermore, biochemical pathway analysis has shown that some prophage genes are involved in purine and pyrimidine metabolism, such as those encoding for DNA-directed DNA polymerases, enzyme commission (EC) 2.7.7.6 and 2.7.7.7). These genes were found in the prophages associated with tRNA-Arg (UCU) gene and tRNA-Leu (UAG) gene. Interestingly, a prophage gene (CDS: 32008.1054.peg.132) in a prophage of *B. humptydooensis* MSMB122 was annotated to encode a metabolic enzyme, 7-cyano-7-deazaguanine synthase (EC 6.3.4.20), responsible for metabolism of cofactors and vitamins. Details of these genes and their biochemical pathways are available in Supplementary Table 6.3. Notably, none of the prophage genes identified in this study are coded for virulence or antimicrobial resistance determinants.

Spontaneously induced temperate phages from selected BPC strains

Based on the analysis of prophage profiles, it was found that *B. pseudomallei* 1026b, a prototypic strain, harbored three potential prophages associated with tRNA-SSR sites at tRNA-Pro (UGG), tRNA-Arg (CCG), and tRNA-Phe (GAA), shown in a schematic diagram in Supplementary Figure 1B. In a previous study (DeShazer, 2004), a well-characterized temperate phage, φ1026b, was isolated from this particular *B. pseudomallei* strain, forming plaques on a *B. mallei* ATCC23344's lawn. It was established that this temperate phage was associated with a prophage caused by the tRNA-SSR at tRNA-Pro (UGG). Given that this strain contained two additional prophages, the question arose regarding their functionality, as only a temperate phage had been isolated in the previous study. To investigate this further, we used strain Bp82, a virulence-attenuated mutant of *B. pseudomallei* 1026b, to propagate the temperate phages from Bp82 using the host strain 576mn,

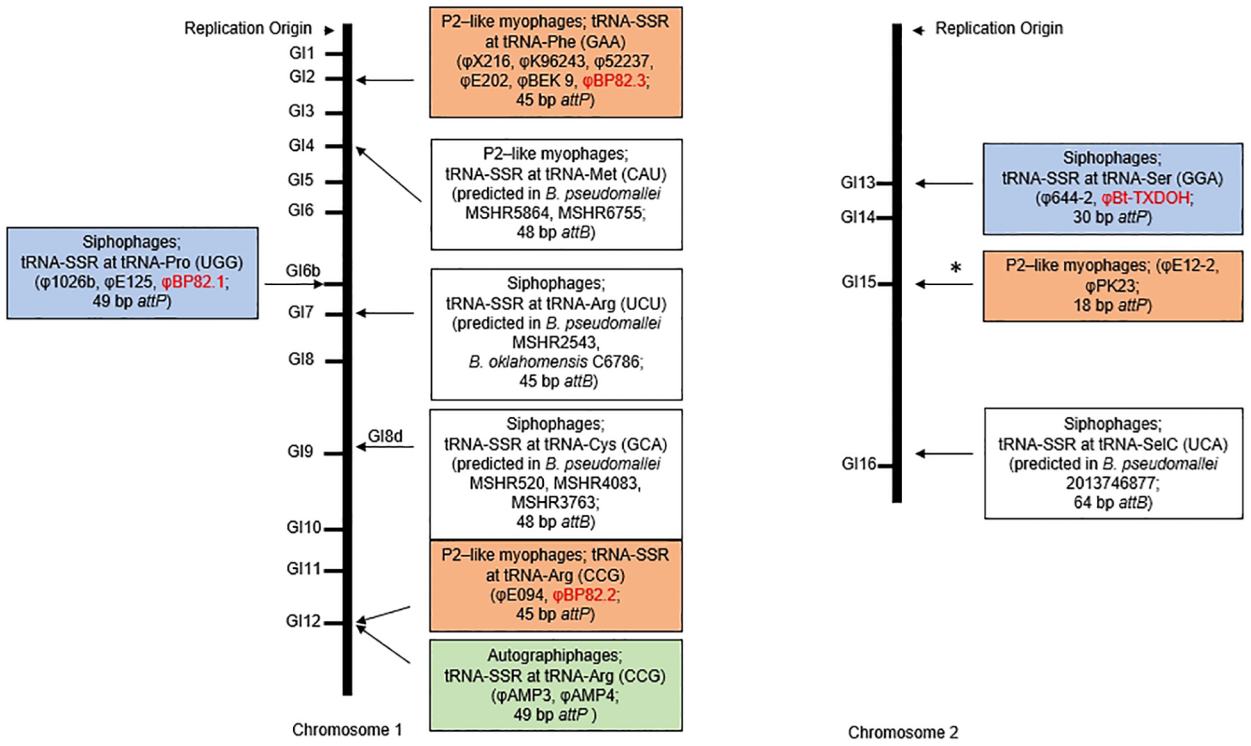

FIGURE 2
Illustrating the chromosomal locations of the prophages identified in *B. pseudomallei* genomes. This graphic depicts the chromosomal positions of prophages in *B. pseudomallei* genomes, corresponding to genomic island (GI) sites on *B. pseudomallei* K96243's two chromosomes. There are eight sites with either predicted or confirmed prophages linked to tRNA-SSR sites at specified tRNA genes. An additional site correlates with GI15 (denoted by an asterisk). The annotations in each box detail the phage group (myovirus in orange, siphovirus in blue, autographivirus in green), the type of tRNA-SSR, and names of recognized phages or predicted prophages in BPC strains. Names of the temperate phages investigated in this study are in red text.

which is also a virulence-attenuated strain suitable for a BSL-2 laboratory setting. Subsequently, we employed PCR assays targeting specific integrase genes associated with each tRNA-SSR to guide the isolation of distinct phages. As a result of these efforts, three different phage types were successfully isolated, named φBP82.1, φBP82.2, and φBP82.3. Transmission electron microscopy (TEM) was utilized to visualize the morphology of these phages. Notably, φBP82.1 exhibited characteristic similar to a lambda-like tailed phage belonging to the siphovirus group, while both φBP82.2 and φBP82.3 were identified as a P2-like phage with a contractile tail, classified within the myovirus group. TEM images of these three phages are presented in Figure 3.

Furthermore, we successfully isolated temperate phages from two different *B. thailandensis* strains, TXDOH (also known as 2003015869 (Glass et al., 2006a)), and E264 (Brett et al., 1998). In the case of TXDOH, it contained a putative prophage with an approximate size of 56.4 kb, identified to be associated with a tRNA-SSR at tRNA-Ser (GGA) located on its chromosome 2. This tRNA gene corresponds to the BTH_II2014 locus on chromosome 2 of *B. thailandensis* E264 and the BPSSt03 locus on chromosome 2 of *B. pseudomallei* K96243. From TXDOH, we isolated a temperate phage named φBt-TXDOH, which formed plaques on a *B. pseudomallei* Bp82 culture, while another host strain, 576mn, did not yield any plaques. Further analysis confirmed the morphology of φBt-TXDOH as a lambda-like tailed phage, classifying it within the siphovirus group.

In contrast, *B. thailandensis* E264, a reference laboratory strain used in many studies, did not show any prophages associated with tRNA genes based on its tRNA-SSR profile. Surprisingly, we identified two distinct temperate phage types from this strain. One of them, designated φBt-E264.1, exhibited characteristics resembling a lambda-like tailed phage and belonged to the siphovirus group. The other, named φBt-E264.2, was a P2-like phage with a contractile tail, categorized within the myovirus group. Both φBt-E264.1 and φBt-E264.2 were isolated, with φBt-E264.1 originating from the lawn of Bp82 and φBt-E264.2 from 576mn. Detailed TEM images of these three phages from *B. thailandensis* can be found in Figure 3.

Genomic analysis of the temperate phages

In this research, we conducted the sequencing of five distinct temperate phages, φBP82.1, φBP82.2, φBP82.3, φBt-TXDOH, and φBt-E264.1, utilizing Illumina technology. We have attempted to sequence φBt-E264.2, but were unsuccessful due to its low yield in its host strain (*B. pseudomallei* 576mn). The specific details of these sequenced genomes and their corresponding GenBank accession

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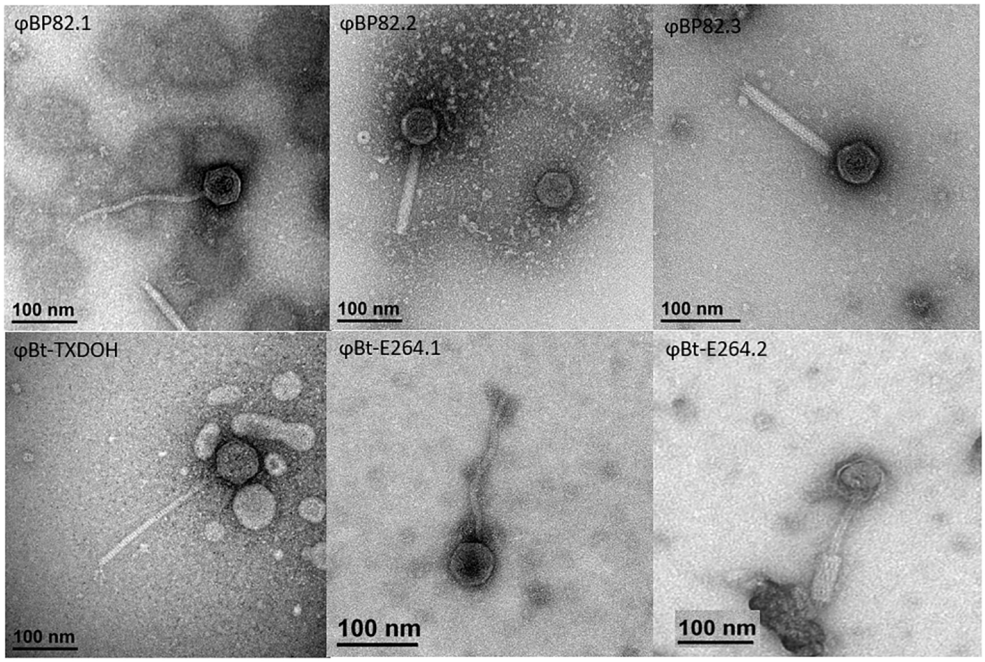

FIGURE 3
Transmission electron microscopy depictions of six characterized temperate phages. The upper section displays phages φBP82.1, φBP82.2, and φBP82.3, which were naturally induced from *B pseudomallei* Bp82 and purified from plaques on *B pseudomallei* 576mn lawn cultures. The lower section shows phages φBt-TXDOH, φBt-E264.1, and φBt-E264.2, originating from *B thailandensis* strains TXDOH and E264. Phages φBt-TXDOH and φBt-E264.1 were extracted from *B pseudomallei* Bp82 lawn cultures, whereas φBt-E264.2 was obtained from *B pseudomallei* 576mn lawns. Phages φBP82.1, φBt-TXDOH, and φBt-E264.1 belong to the siphovirus group. In contrast, phages with contractile tail, φBP82.2, φBP82.3, and φBt-E264.2 are P2-like viruses classified under the myovirus group.

numbers can be found in Table 1. Subsequently, we performed a comparative analysis of these genome sequences with known temperate phages within the context of *B. pseudomallei*, employing Easyfig (Sullivan et al., 2011), shown in Figure 4. Our investigation unveiled pronounced dissimilarities between the genomes of the two phage families, characterized by variations in their sizes. Notably, siphophages φBP82.1, φBt-TXDOH, and φBt-E264 exhibited genome sizes ranging from 44.5 kb to 56.5 kb, while myophages φBP82.2 and φBP82.3 possessed genome sizes of approximately 36.3 kb and 37.6 kb, respectively.

The presence of prophages in *B. pseudomallei* strains from a local endemic area of melioidosis

Investigating this, we employed PCR assays targeting six distinct phage integrase genes, each uniquely associated with a prophage genomic location. This study encompassed 321 *B. pseudomallei* strains and 9 *B. thailandensis* strains originating from southern Thailand. These prophages were associated with tRNA-genes such as tRNA-Phe (GAA), -Pro (UGG), -Arg (CCG), -Arg (UCU) and -Ser (GGA), and the prophage associated with the temperate phage φBt-E264.1 in *B. thailandensis* E264, as described above. Our findings revealed that 165 (51.4%) of the *B. pseudomallei* strains tested yielded positive results in at least one of the PCR assays, with 47 of them exhibiting positivity in two different assays. Specifically, 108, 45, and 31 strains tested positive in the PCR assays targeting prophages associated with tRNA-SSR at tRNA-Phe (GAA), -Pro (UGG), and -Arg (CCG), respectively. Interestingly, 19 *B. pseudomallei* strains tested positive for the integrase gene BTH_II1011, associated with the prophage linked to φBt-E264.1 in *B. thailandensis* E264. Additionally, 31 strains tested positive in two PCR assays targeting prophages at tRNA-Phe (GAA) and tRNA-Pro (UGG), while none of the *B. pseudomallei* strains displayed positive results in PCR assays targeting prophages associated with tRNA-SSR at tRNA-Arg (UCU) or Ser (GGA). Furthermore, among the nine *B. thailandensis* strains included in our study, four of them tested positive in the PCR assay targeting a functional prophage linked to tRNA-Ser (GGA) in *B. thailandensis* TXDOH and φBt-TXDOH. The remaining *B. thailandensis* strains displayed positivity in various other PCR assays, except for the one targeting the prophage at tRNA-Pro (UGG). PCR results are summarized in Supplementary Table 4.

Discussion

It is well-recognized that *B. pseudomallei* is one of the most successful bacterial pathogens in causing severe infections in humans and many animal species, and indeed it has remarkable ability to survive in diverse environments. The acquisition of genetic materials through horizontal gene transfer and the resulting genomic plasticity significantly contribute to adaptability

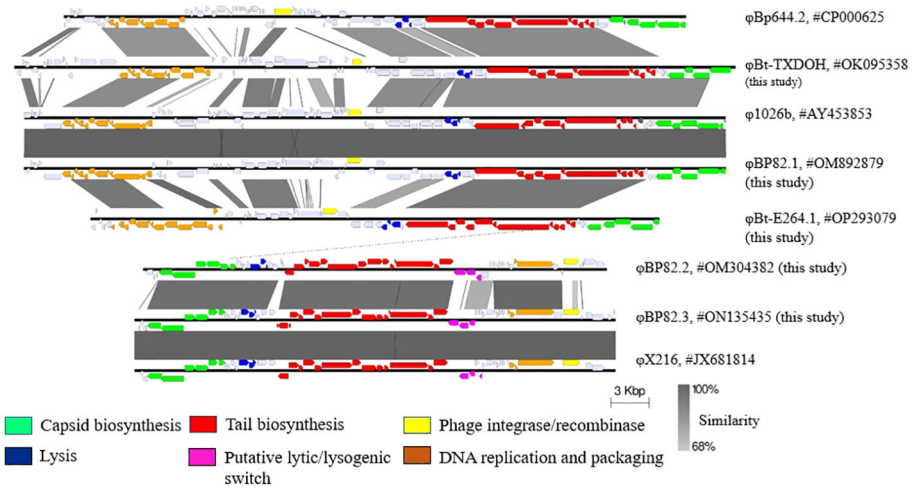

FIGURE 4
Genomic comparison of newly sequenced temperate bacteriophages. This analysis encompasses the genomes of five recently sequenced phages: φBt-TXDOH, φBP82.1, φBt-E264.1, φBP82.2, and φBP82.3, alongside known phages from the siphovirus and myovirus groups. The gene sequences associated with the formation of phage capsids and tails, as well as those involved in DNA replication and packaging, exhibit similarities within each family group. In contrast, genes implicated in the lytic/lysogenic cycle transition and phage integrase functions show variability, particularly when linked with distinct integration sites. Unexpectedly, φBP82.3 genome is almost identical to the genome of φX216 as previously described (Kvitko et al., 2012), even though both phages were induced from different *B pseudomallei* strains. We noted that the capsid biosynthesis genes in φBp644.2 and φBt-E264.1 are different to those in other siphoviruses being compared.

and pathogenicity in bacteria. This process enable not only *B. pseudomallei* but also other pathogenic bacteria to thrive in various ecological niches and contributes to their success as a pathogens. In the current study, we identified tRNA-SRR as a major mechanism that shaped up individual *B. pseudomallei* genomes and this mechanism was also found in its closely relative species within the BPC. We believe that phage transduction and the lysogenic state could have contributed to the fitness of *B. pseudomallei*. Here are some major points to discuss:

tRNA-SSR is a major genetic recombination mechanism that shapes up strains' diversity within *B. pseudomallei* and in other BPC species

We have established a connection between tRNA-SSR events and a minimum of 15 tRNA gene loci within the pangenome of *B. pseudomallei*. Among these, seven loci are particularly noteworthy: tRNA-Phe (GAA), tRNA-Met (CAU), tRNA-Pro (UGG), tRNA-Arg (UCU), tRNA-Cys (GCA), tRNA-Arg (CCG), and tRNA-SelC (UCA). This association is drawn from an in-depth analysis of 106 complete genomes of *B. pseudomallei*, revealing the presence of potential prophages within these tRNA loci. Furthermore, our investigation uncovered an additional tRNA-SSR event involving tRNA-Ser (GGA), which is linked to a functional prophage in *B. thailandensis* TXDOH. This discovery was made through the examination of the temperate phage φBt-TXDOH. Conclusively, these eight tRNA genes are identified as the hotspots for phage integration (Figure 2). Historically, our understanding of the tRNA gene's involvement in phage integration began in 2002 when Woods and colleagues (Woods et al., 2002) demonstrated that the temperate siphophage φE125, spontaneously produced by *B. thailandensis* E125, integrated its genome into *B. mallei* ATCC 23344. This integration occurred at a short 49 bp homologous 3' end sequence of the tRNA-Pro (UGG) gene. A follow-up study by DeShazer (2004) revealed a similar case with the temperate siphophage φ1026b from *B. pseudomallei* 1026b, which shared a genome sequence with φE125 and utilized the same *attP* sequence, a 49 bp homologous sequence at the 3' end of the tRNA-Pro (UGG) gene. A few years later, Ronning and colleagues conducted an analysis of 37 prophages and prophage-like elements from six different *Burkholderia* species (Ronning et al., 2010). Among them, five temperate phages (φ52237, φE12-2, φ644-2, φE202, and φE255) were spontaneously isolated from lysogenic *B. pseudomallei* and *B. thailandensis* host strains. Three of these phages were found to have attachment or integration sites (*attP*) associated with 3' end sequences of tRNA genes. Specifically, φ52237 and φE202 featured a 45 bp *attP* identical to the 3' end sequence of tRNA-Phe (GAA), while φ644-2 possessed a 30 bp *attP* sequence identical to that of tRNA-Ser (GGA).

In addition, a recent study by Hammerl and colleagues characterized three temperate phages (φE067, φE131, φE058) from lysogenic *B. thailandensis* strains E067, E131, and E058 (Hammerl et al., 2020). Upon analysis, φE067 was found to have a 16 bp *attP* sequence identical to tRNA-SelC (UCA) in *B. pseudomallei* K96243. The cumulative findings from our research and others have suggested that these eight tRNA locations serve as hotspots for phage

integrations in *B. pseudomallei*, and some of these hotspots in other BPC species also harbor prophages. Charactering these prophages in these species warrants further investigation.

Multiple phage types can be spontaneously induced from functional prophages in a single strain of *B. pseudomallei*

This study has established that three phages, φBP82.1, φBP82.2, and φBP82.3, were naturally released from corresponding prophages within *B. pseudomallei* Bp82, a strain derived from *B. pseudomallei* 1026b lacking the *purM* gene (Propst et al., 2010). We employed PCR techniques, focusing on unique integrase genes in each tRNA-SSR from these prophages, to confirm their presences in many small plaques formed on a *B. pseudomallei* 576mn bacterial lawn. Further examination revealed that φBP82.1 is a match for φ1026b, a previously identified siphophage in *B. pseudomallei* 1026b, and the two other phages were developed from the two suspected prophages associated with tRNA-SSR events. Moreover, the study extended to explore the prevalence of such prophages among *B. pseudomallei* strains from clinical and environmental sources in a region of southern Thailand, with over half of the strains testing positive for at least one prophage. These findings indicate a widespread lysogenic state within the *B. pseudomallei* population. The study also noted the commonality of prophages linked to specific tRNA-SSR events, although some associated with certain tRNA genes were absent in the Thai strains, raising the question of geographic specificity of prophage presence. Despite their rarity, the prophage associated with tRNA-Arg (UCU) was identified in BPC strains from diverse geographical locations, including *B. pseudomallei* MSHR2543 and *B. savannae* MSMB266 from Australia and *B. oklahomensis* C6786 from the USA. These prophages are classified within the siphovirus group based on their genomic composition. However, due to the unavailability of these strains in public repositories, it was not possible to confirm their functionality in relation to the tRNA-SSR. Interestingly, a rare prophage associated with tRNA-Ser (GGA) in *B. pseudomallei* 644 (Ronning et al., 2010) was present in several *B. thailandensis* strains, and we successfully induced φBt-TXDOH from a functional prophage correlating with this tRNA-Ser site. This finding suggests potential avenues for further research into the distribution of this prophage among *B. thailandensis* populations and the biological significance of its temperate phage, since they infected *B. pseudomallei* BP82 in our experiments.

During the peer review of this article, a question was raised about whether the presence of prophages contribute to virulence, antimicrobial resistance, and or fitness of *B. pseudomallei*, particularly since the genomes analyzed originated from diverse sources, including human and animal cases, as well as the environment. To date, no virulence or antimicrobial resistance genes have been identified in the 63 prophages from the genomes of *B. pseudomallei* and other BPC species. Notably, a higher frequency (43/91, 47.3%) of prophages was observed in strains from human and animal cases compared to environmental strains (2/10, 20%), according to Supplementary Table 5.1. It is important to note that most genomes used in this study were from the clinical *B. pseudomallei* strains and two of them, 1026b and BGR, contained up to three prophages. Furthermore, the presence of specialty genes, such as those encoding holins (phage-encoded membrane proteins involved in bacterial cell lysis in the final stage of the bacteriophage reproductive cycle (Abeysekera et al., 2022)), in some prophages are of particular interest. These genes are notably present in two rare prophages: one associated with the tRNA-Cys (GCA) gene found only in Australian *B. pseudomallei* strains, and the other was associated with tRNA-Arg (UCU) in *B. oklahomensis* C6786 (as detailed in Supplementary Table 6.2). Additionally, we have identified several prophage genes involved in biochemical pathways, particularly in purine and pyrimidine metabolism in *B. humptydooensis* MSMB121, MSMB122, and *B. savannae* MSMB266, as well as in folate biosynthesis in a prophage of *B. oklahomensis* C6786 (refer to Supplementary Table 6.3). These genes could enhance the fitness of the bacterial host strains. We believe that the possession of prophage genes could confer benefit to their bacterial hosts at certain points, which warrants further investigation. These studies would include mutagenesis of prophage genes, phage transduction, and virulence determination in animal models.

In summary, the data presented here displays that there is a clear association between specific tRNA genes and the presence of prophages, and that this presence is not random. Additionally, due to the high number of tRNA-SSR events in BPC genomes associated with various MGEs including prophages, we conclude that these MGEs contribute to an increase in diversity of the BPC species similar to other bacterial species that have an open-genome and not related to BPC genomic plasticity.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, OM892879 https://www.ncbi.nlm.nih.gov/genbank/, ON135435, https://www.ncbi.nlm.nih.gov/genbank/, OM304382 https://www.ncbi.nlm.nih.gov/genbank/, OK095358, https://www.ncbi.nlm.nih.gov/genbank/, OP293079.

Author contributions

PK: Data curation, Investigation, Methodology, Writing – review & editing, Formal analysis. II: Data curation, Investigation, Methodology, Writing – original draft, Formal analysis. PA: Investigation, Methodology, Writing – review & editing. MA-R: Investigation, Methodology, Writing – review & editing. JK: Resources, Writing – review & editing. AT: Funding acquisition, Supervision, Writing – original draft, Writing – review & editing, Conceptualization, Formal analysis, Project administration.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbrio.2024.1339809/full#supplementary-material

SUPPLEMENTARY TEXT 1
DNA sequences of tRNA-SSR associated prophages in *B. pseudomallei* and other BPC species.

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