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Isolation, Characterization, and Genome Sequence Analysis of a Novel Lytic Phage, *Xoo-sp15* Infecting *Xanthomonas oryzae* pv. *oryzae*

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Abstract
Xanthomonas oryzae pv. *oryzae (X. oryzae)* is a bacterial pathovar of rice diseases all over the world. Owing to emerging antibacterial resistance, phage therapies have gained significant attention to treat various bacterial infections. Nevertheless, comprehensive research is needed for their use as a safe biocontrol agent. In this study, isolation and characterization of a novel phage *Xoo-sp15,* that infects *X. oryzae* was ascertained through experimental and bioinformatics analyses to determine its virulent potency and reliability. High throughput sequencing demonstrated that *Xoo-sp15* has a dsDNA genome with a total size of 157,091 bp and 39.9% GC content lower than its host (63.6%). Morphological and phylogenetic analyses characterized it as a new member of the Bastille-like group within the family *Herelleviridae*. In silico analysis revealed that it contains 229 open reading frames and 16 tRNAs. Additionally, this novel phage does not contain any resistant determinants and can infect nine *X. oryzae* strains. Therefore, *Xoo-sp15* has the potential to serve as a novel candidate for phage therapy.

Introduction

Xanthomonas oryzae pv. *oryzae (X. oryzae)*, a Gram-negative bacterium, has been reported as a causative agent of bacterial blight (BLB) in rice [1]. BLB is the most devastating rice disease, which causes serious threats to this food [2]. However, different strategies such as germicidal chemicals, different cultivation ways, antibiotics, and biocontrol agents have been utilized to control the BLB [3]. But still, these conventional approaches are hindered by various concerns. Therefore alternative methods are urgently required to reduce potential risk factors to control the pathogenicity of these microorganisms [4]. For the past few years, most of the studies have focused on bacteriophages as the most important entities for controlling bacterial diseases [5].

Bacteriophages are bacterial viruses that specifically infect and replicate within the host bacteria. It is not surprising that more than 10^{31} phage particles have existence in this biosphere [6] and so far needed to explore more to gain insight into their potential characteristics. Bacteriophages present huge diversity [7], and interestingly, many indigenous phages (environmental samples) of such

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characteristics play a significant role in various aspects of biotechnology [7]. Although the sequence data of phage genomes is increasing on daily basis, still, only a few are taxonomically categorized and well-characterized [4].

Phage therapies have been employed for bacterial control in different industries including water treatment, agriculture, food production, and the medical field. In order to control the *X. oryzae* few phages had been isolated and their basic properties have been studied in past [8, 9]. Unfortunately, existing biological control agents have not proved so efficacious as expected. It is necessary to find new phages to control *X. oryzae* and exploring their potential for the control of BLB. Since *X. oryzae* is a major bacterial pathogen of rice that causes BLB and severe threats to economies [4]. The current study isolated and characterized a novel bacteriophage, *i.e., Xoo-sp15,* with unique genome properties. Its detailed molecular characterization including morphology, host range, classification, functional proteins,and genome sequence analyses were determined through experimental and computational approaches. Based on the present study, it is proposed as a novel potential phage for treating *X. oryzae* infections and developing novel biotechnological tools.

Materials and Methods

Bacterial Strains and Culture Conditions

Nine typical *X. oryzae* strains [10], including YN1, YN7, YN11, YN18, YN24, GD414, HEN11, FuJ, and ScYc-6, were isolated from different regions of China and used as hosts for phage isolation as described in our previous study [4]. An *X. oryzae* strain PXO99A (derivative of philippine race 6 strain PXO99) was also used as a host. Besides *Xanthomonas* spp., we tested host specificity of the phage *Xoo-sp15* to some less related bacteria including two strains of *Ralstonia solanacearum* (Rs3224, Rs3211) and four strains of *Pseudomonas hibiscicola* (Ps3213, Ps3214, Ps3216, Ps323). *Ralstonia* and *Pseudomonas* both are the most important bacterial phytopathogen targeted for phage control. Three strains of *Bacillus Subtilis* (859, 210, 1655) were also utilized to check the host range of *Xoo-sp15*. A total of ten *X. oryzae* strains and three *Bacillus subtilis* species were cultured in nutrient broth (NB) at 28 °C and 37 °C respectively with shaking at 220 r.p.m. [11], whereas *P. hibiscicola*, *R. solanacearum* cells culture in casamino acid peptone glucose (CPG) at 28 °C with shaking at 220 r.p.m.

Spot Test

Spot testing was performed to screen the phages from soil samples collected from China by an enrichment method [12] with some modifications in the previous method. The 50 ml sterile water and 10 g soil sample were mixed and then shaken for 2 h at 28 °C to release the phages. After enrichment, culture samples were centrifuged at 13000 g for 10 min at 4 °C to remove the soil debris, and the supernatant was filtered with a 0.22 µm sterile filter to obtain the soil-free pure extract. A double layer plate method was applied to verify the *X. oryzae* phages in the extract. A 5 µl of soil extract was spotted on an NB medium plate and incubated for 48–60 h at 28 °C to form clear zones.

Isolation, Purification, and Propagation of Phage

The plaque assay technique was applied for the phage isolation, and all bacterial strains were inoculated into 5 ml of NB and incubated at 28 °C for 24 h. Subsequently, 100 µl bacterial culture was added into 10 ml of NB and incubated at 28 °C to the logarithmic phase. Later, 100 µl of soil extract was added into 900 µl of *X. oryzae* culture, mixed with 0.6% NB soft agar, and then overlaid onto 1.5% NB solid agar. Finally, the plate was incubated at 28 °C for 48–60 h. A sterile toothpick was used to collect the plaques and transferred to *X. oryzae* strain cultures. After incubation at 28 °C for 12–16 h, the bacterial cells were removed through centrifugation, and the supernatant was filtered with a 0.22 µm sterile filter to get the pure phage lysate. The experiment was performed in triplicate to confirm the results. Phage propagation was carried out using a double agar overlay technique. Pure *Xoo-sp15* stock was put in bacterial cultures ($OD_{600} = 0.2–0.6$) and incubated until the bacterial liquid became clear. Centrifugation was done to remove the cell debris, and the supernatant was collected for DNA extraction and sample preparation to examine through transmission electron microscopy (TEM).

Host Range Determination

The Host range of phage *Xoo-sp15* was determined through a spot testing assay. Phage suspensions (10^6p.f.u. (Plaque-Forming unit) ml^{-1}) were diluted in tenfold dilutions to a dilution of 10^{-6}. Subsequently, 200 µl of each *X. oryzae* bacterial culture ($OD_{600} = 0.6$) was homogenized with NB top agar (0.8%), and then 5 µl of each phage dilution was spotted onto the center of the plate. Plates were incubated at 28 °C for 36–48 h to detect the plaque since individual plaques could be detected on the plate in a proper titer of phage dilutions.

TEM Analysis

For TEM analysis, purified *Xoo-sp15* suspension (approximately 10^{11}p.f.u. m^{-1}) in 0.1 M ammonium acetate buffer was spotted onto the carbon-coated copper grids and negatively stained with 2% phosphotungstic acid (pH 6.8) for

20 s. The TEM image was captured at 200 kV in Wuhan Institute of Virology, Chinese Academy of Sciences (CAS), China.

Sequencing and In Silico Analyses of Phage

The DNA of *Xoo-sp15* was extracted according to $ZnCl_2$ precipitations [13] and subsequently purified genomic DNA was subjected to sequencing from Berry Genomics Biotechnology Co., Ltd (Beijing, China), who applied Illumina HiSeq 2500 paired-end sequencing technology with a read length of 150 bp. Filtered paired-end reads were assembled by employing an alignment tool SOAPdenovo2 [14]. The frequency of sequence reads was counted using an in-house python script to predict the genome termini [15]. Run-off sequencing with primers around the predicted termini was also performed to confirm the terminal sequence of the phage genome. A sequence similarity search was performed using BLASTN (https://blast.ncbi.nlm.nih.gov/) to compare the phage genome with other genomes against the nucleotide collection (nr/nt) database. Rapid Annotation Subsystem Technology (RAST, http://rast.nmpdr.org/) server was used to find putative ORFs, whereas BLASTp and PSI-BLAST algorithms against non-redundant protein database [16] were applied to verify the predicted ORFs. tRNAs detection was carried out using tRNAscan-SE v2.0 (http://lowelab.ucsc.edu/tRNAscan-SE/) [17]. The sequence alignment was generated using CLC Main Workbench, v7.7.3 (CLC Bio-Qiagen, Aarhus, Denmark) (https://www.qiagenbioinformatics.com/). A web tool RESFINDER v2.1(https://cge.cbs.dtu.dk/services/ResFinder/) [18] was employed to identify the antimicrobial resistance coding genes.

Comparative Genomic Analyses

An alignment of the *Xoo-sp15* phage with its homologous phages was conducted to determine the conserved and mutated sequence segments by Easyfig v2.1 [19]. The amino acid sequences of terminase large subunits were retrieved from the Genbank database https://www.ncbi.nlm.nih.gov/genbank/ and subsequently aligned through ClustalW [20] to determine the conserved domains and mutations. The phylogenetic tree was constructed to infer the evolutionary history of phages with a maximum likelihood method implemented in MEGA v5.0 [21] with 1000 bootstrap values.

GenBank Accession Number

The genomic sequence of phage *Xoo-sp15* was submitted to GenBank with the accession number MN364664.

Results and Discussion

Isolation and Host Range

Soil samples were collected from different rice-growing regions in China for the isolation of *X. oryzae* phages as *X. oryzae* is a major pathogen that causes BLB in rice. A total of 10 *X. oryzae* strains including YN1, YN7, YN11, YN18, YN24, GD414, HEN11, ScYc-6, PXO99A, and FuJ were used as indicator strains. Spot testing was carried out to screen the *X. oryzae* phages from soil samples and then plaque assay was performed for the isolation of *Xoo-sp15*.

Host specificity of *Xoo-sp15* was determined through spot testing technique. It has been seen that *Xoo-sp15* infect all nine strains of *X. oryzae* except strain FuJ. Moreover, *Xoo-sp15* was not able to infect any strain of *P. hibiscicola*, *R. solanacearum,* and *Bacillus subtilis*. Results showed *Xoo-sp-15* is specific to infect *X. oryzae*, and phage mixture can cover all *X. oryzae* strains. Furthermore, it is suggesting that *Xoo-sp15* can be formulated into cocktails and use as a biocontrol agent against *X. oryzae*.

Biological Characterization of Xoo-sp15

Xoo-sp15 forms clear and round plaques with a diameter of approximately 0.2 mm on NB plates after co-culturing with a bacterial lawn of PXO99A (Figure S1a). Morphological characteristics under electron microscope presented isometric, icosahedral head (diameter 51 ± 1.2 nm), and contractile tail (diameter 125 ± 1.5 nm) (Figure S1b). According to ICTV classification [22], *Xoo-sp15* is the largest myovirus (157-kb) belongs to the Bastille-like group. It is classified as the member of the subfamily *Bastillevirinae* of *Herelleviridae*, order *Caudovirales*, genus *Caeruleovirus*. However, previous *X. oryzae* phages reported, such as OP1, OP2, XP10, and Xop411 were from *Siphoviridae, Podoviridae, Myoviridae* families. Mostly, *X. oryzae* phages possessed dsDNA with an approximate size exceeds 40-kb [23].

General Features of Xoo-sp15 Genome

The phage genome was sequenced by Illumina HiSeq to investigate the distinctive and underlying features of *Xoo-sp15* phage. A de novo genome assembly based on 12,282,424 paired-reads (125 bp) yielded a single contig with a 99.50% genome coverage, while an average depth of sequencing was 9929.14 bp. Termini of the phage genome were predicted by examining the high-frequency reads [15]. Two high-frequency sequences (GGGTTAGGGTGG

TTGGGCCG and GGGCGTGCAAATAAGGTGCT) were repeatedly found accounting for 3235 and 2681 times, respectively, suggest that the phage genome has a fixed terminus for packaging.

Xoo-sp15 genome comprised 157,091 bp with 39.9% GC content and about 229 putative ORFs were found with a coding density of 89.94%. It has been observed that 61 gene products exhibit proteins impression with known functions, while 165 were hypothetical proteins. In addition, three gene products did not match with any viral or prokaryotic sequences available in the databases. Most of the ORFs were observed to transcribed on 195/229 position (negative-strand) and 34/229 position (positive strand). The complete annotation features are summarized in Table S1 and the organization of the *Xoo-sp15* genome including GC content, ORFs, and GC skew are shown in Figure S2.

The putative ORFs of the *Xoo-sp15* genome were mapped in a modular gene structure (Fig. 1). The phage genome was categorized into three modules: lysis, nucleotide metabolism and replication, and structuring and packaging based on functional proteins.

Lysis Module

L-alanoyl-D-glutamate peptidase encoded by an ORF98 and N-acetylmuramoyl-L-alanine amidase by ORF123 (a 38 kDa protein) were determined. They can degrade the host peptidoglycan and play a significant role in DNA synthesis by cleaving amide bonds [24]. Additionally, an ORF38 in the *Xoo-sp15* genome encodes lysis protein, which represents 95% similarity to the lysis protein of Bacillus phage Deep Blue. However, no holin was found in the phage genome, and the cleavage is proposed to be mediated by the lysins [25].

Nucleotide Metabolism and Replication

DNA replication-associated ORFs were studied in this module such as DNA helicase (ORF75 and ORF77), putative transcriptional regulator (ORF76), exonuclease (ORF73), primase (ORF69), resolvase (ORF63), putative integration host factor (ORF52), polymerase (ORF46), recombinase A (ORF41) and MreB-like (ORF217). Furthermore, the putative products (ORF15, 26, 34, 55, 60, 63, 86, 133, 134, and 136) possibly take part in DNA metabolism and replication. As the DNA replication process is semi-discontinuous, therefore it is predicted that primases are requisite for the DNA replication, repair, and transcription [26]. A putative integration host factor, ORF52, behaves as an activator of phage DNA replication [27].

In addition, PSI-BLAST showed that ORF43 of *Xoo-sp15* encodes putative DNA polymerase III subunits gamma and tau since tau and gamma, essential elements for replication [28]. ORF113 encoded HNH endonuclease, which is a key component for DNA packaging machines in bacteriophages [29].

A gene (39) that encodes the putative sigma factor is responsible for the modification of RNA polymerase to recognize the phage-promoter regions [30]. Additionally, ORF76 encodes putative transcriptional regulators, which regulate the gene expression of phage genes more as compared to host genes [30]. Noticeably, ORF217 encodes the MreB-like protein, which is reported as a key component in peptidoglycan synthesis [31]. An ORF89 encodes the chaperone domain of Endosialidase, which are tail spike proteins of bacteriophages for the degradation of host capsules [32].

A unique feature of *Xoo-sp15* phage is the presence of methylation proteins, i.e.,ORFs 50 and 51 are involved in the methylation modification and methyl transfer, respectively. The methylation enzyme is a restrictive endonuclease that plays a vital role in the host's defense against viral infections [33]. Interestingly, this feature is not reported in other traditional groups of phages that infect *X. oryzae.*

Structuring and Packaging

In the *Xoo-sp15* genome, structural and packaging gene clusters comprise terminase large subunits, portal protein, putative pro-head protease, major capsid protein, and major tail sheath.

In particular, a protein encoded by ORF122 showed similarity to the terminase large subunits of *Bacillus* phage Deep Blue. ORF110 encodes the portal protein, which facilitates the infusion of the phage genome into the host cell [34]. The proteins encoded by ORF107 and ORF109 include capsid protein and pro-head protease, respectively. The development of the procapsid structure initiates the morphogenesis of phages and the pro-head protease-activated for destroying the inner core and may create space for the genome [35]. Further six putative sub structural proteins of the contractile tail (ORFs 80, 90, 92, 93, 94, 96, 99, 100, 124) were identified. Phage tail fibers recognize the bacterial surfaces during the first step of viral infection [36]. ORF100 encodes the putative tail sheath protein, which shows the similarity to the sheath protein of *Bacillus phage Kioshi* (AXQ67752.1). Thus, proposing that sheath proteins are conserved in the sequence of many phage tail proteins.

In addition to the above-discussed proteins with possible functions, most of the ORF encoded by the phage is unknown, indicating that this phage has many exciting features of genetic resources that need to be further studied and excavated.

Xoo-sp15 presented more than 77% nucleotide genome similarity with Bacillus phages. BLASTn results showed that Deep Blue (AC# KU577463.1), JBP901(AC# KJ676859.1), pk16 (AC# KX495186.1), vBBceM Bc431v3

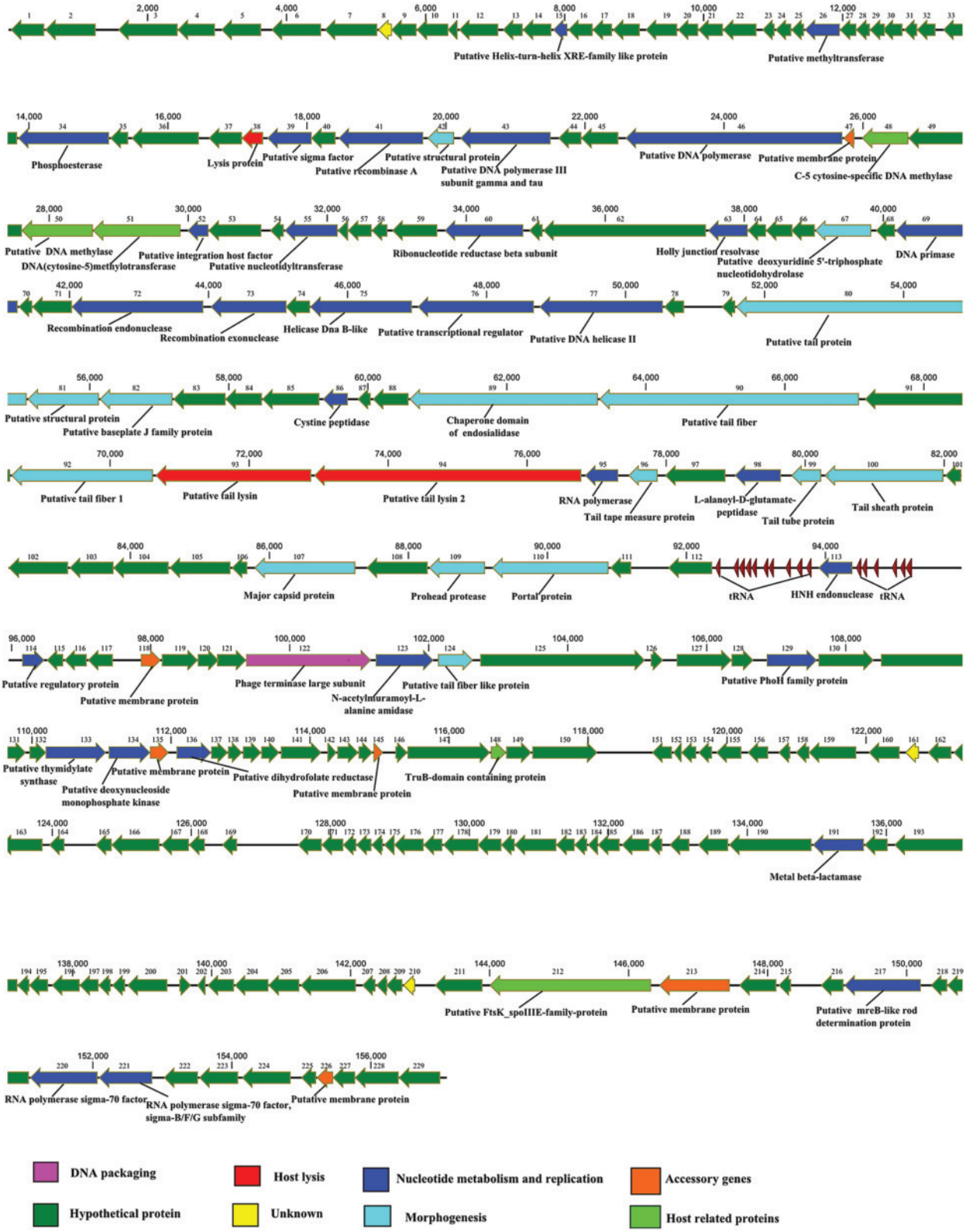

Fig. 1 Graphical representation of the *Xoo-sp15* genome. The 229 ORFs are depicted and the direction of transcription is indicated by arrows. Proposed modules are based on hypothetical functions predicted through bioinformatic analysis. The genome map was drawn using CLC Main Workbench, version 7.7.3 (CLC Bio-Qiagen, Aarhus, Denmark)

(AC# JX094431.1), BCP8-2 (AC# KJ081346.1), Bcp1(AC# KJ451625.1), and Bacillus virus BM15(AC# NC_042140.1) share highest similarities (Table S2). These all-homologous phages isolated from different regions around the world suggested a more complex evolutionary relationship among these phages. Moreover, the Easyfig alignment tool (Figure S3) demonstrated the highly homologous regions, with no significant rearrangements, thus indicatingthat a high level of nucleotide identity, which lacks significant rearrangements. *Xoo-sp15* genome was further examined using RESFINDER v2.1 for the presence of resistance determinants, but no such determinants were identified.

The homologous phages of *Xoo-sp15* and the other traditional *X. oryzae* phages (OP1, OP2, Xop411) were selected at the protein level of the terminase large subunits to infer the evolutionary history (Fig. 2). The maximum likelihood (ML) method in MEGA v5.0 was used to generate the phylogenetic tree with 1000 bootstrap values *Xoo-sp15* was clustered with *Bacillus* phages, representing more similarity with *Bacillus* phages as compared to *X. oryzae* phages. These results assisted the comparative analysis results shown in Figs S3. The tree represents that *Xoo-sp15* is a novel bacteriophage that forms a distinct branch of the family *Herelleviridae.*

A phage selected as a biocontrol agent should ideally be a virulent phage instead of a temperate one, and it should have no side effects on crop health. However, since mostly predicted ORFs of *Xoo-sp15* are hypothetical proteins, it is not known whether the phage is virulent or temperate. This phage most probably has minimum detrimental impacts on the environment as it is isolated directly from nature. However, the side effects of *Xoo-sp15* need to be determined by field trials.

Codon Usage and tRNAs

The *Xoo-sp15* genome has a significantly lower GC content than its host (39.9% v. 63.6%).The lower GC content of phage *Xoo-sp15* suggests an adaptive approach to optimize the gene expression of the viral genome. Interestingly, it seems like a beneficial feature for the activation of gene transcription because virulent phages have lower GC content as compared to their hosts [37]. The *Xoo-sp15* genome was found to have 16 tRNA genes with anti-codons for Ser, Cys, Asn, Gly, Asp, Glu, Arg, Ser, Gln, His, Pro, Tyr, Leu, Ile, Leu, and Met located around a region at position 92,417–95,244 bp of the genome (Fig. 3a). Aforementioned, out of sixteen phage-encoded tRNAs eleven contained codons abundantly found in the phage as compared to the host. Presumably, the tRNAs encoded bythe *Xoo-sp15* genome would counter a deficiency of codon usage in the host during translation. The presence of tRNA genes in bacteriophage genomes may relate to a remarkable contrast in codon usage and GC content between phages and their hosts [38].

Moreover, the codon usage of phage *Xoo-sp15* exceeded that of the host on 11 predicted tRNAs present in the phage (Fig. 3b). Thus proposed that *Xoo-sp15* could supply specific tRNAs on its own during their deficiency, potentially representing a strategy for translational efficiency [39]. For optimal translation, lytic phages encode many tRNA genes and have fast replication [40]. *Xoo-sp15* is unique in this

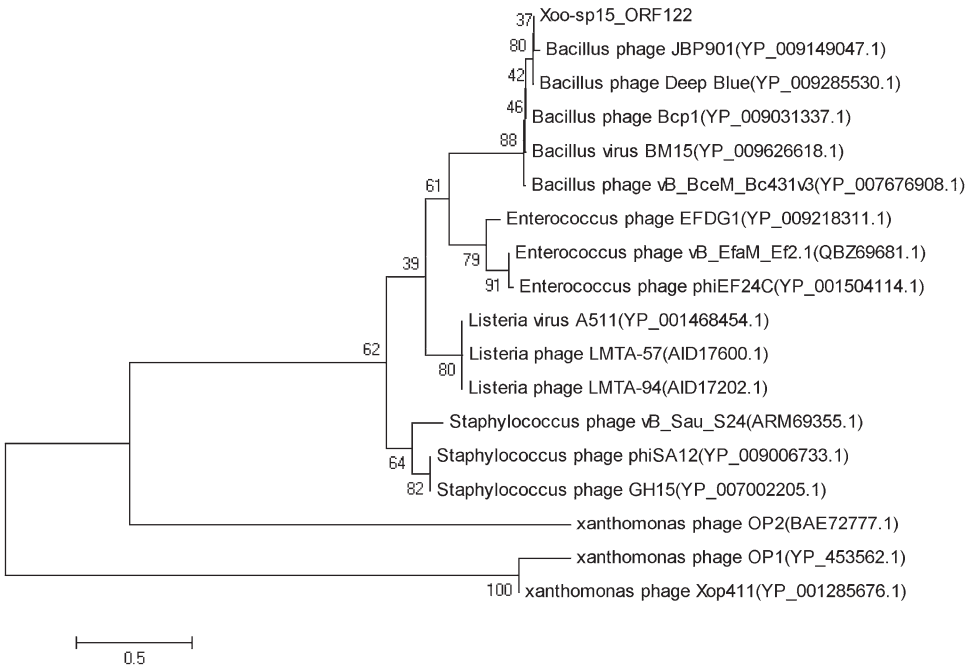

Fig. 2 Phylogenetic relationship between Xoo-sp15 and other selected phages. Tree was constructed based on amino acid sequences of the terminase large subunits using the maximum likelihood method with 100 bootstrap replicate in Mega v5.0

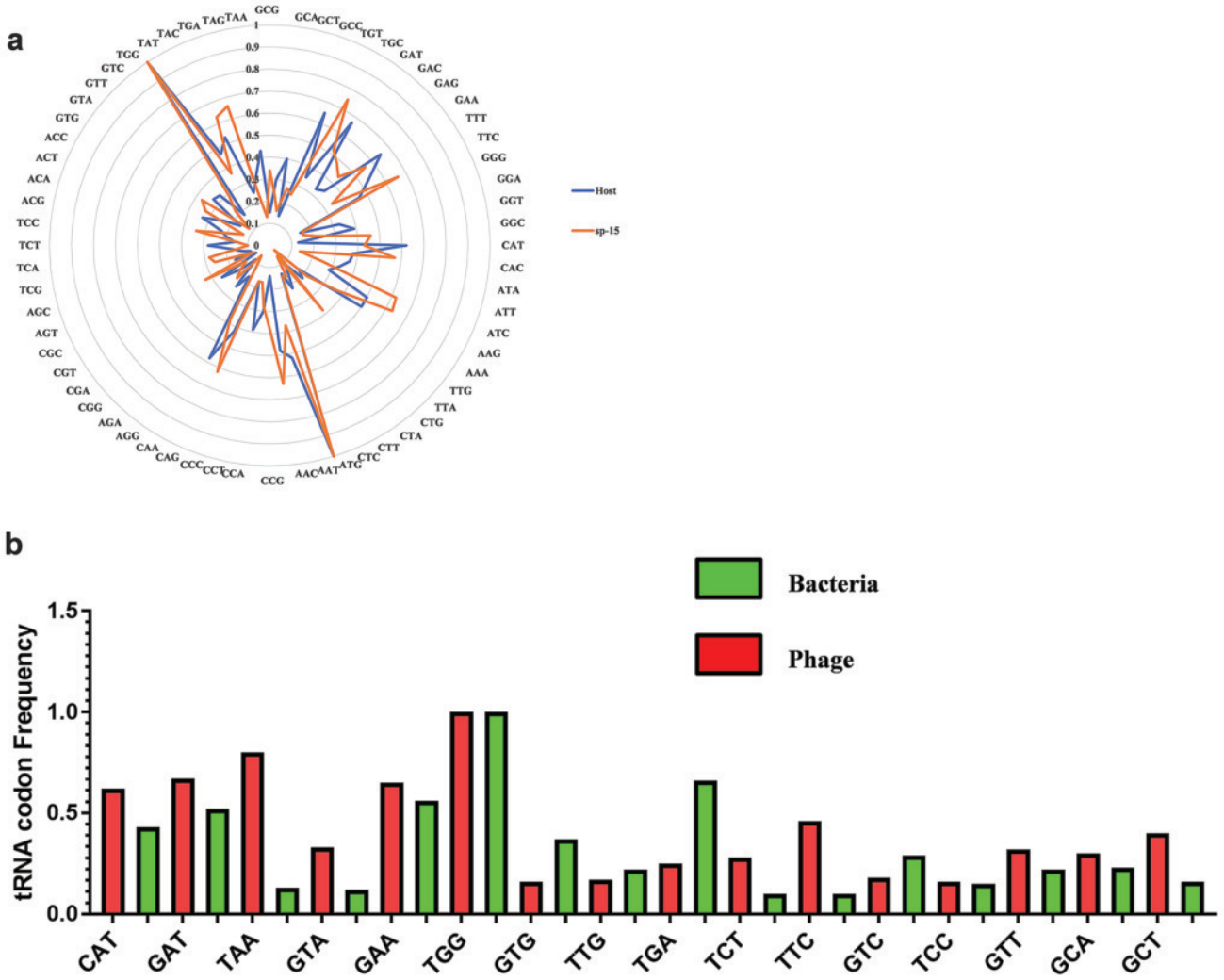

Fig. 3 Comparison of codon usage and tRNAs between Xoo-sp15 and its host. **a** Rose plot show the possible association between tRNAs and codon usage in phage and its host. The frequency scale is represented at the center of the rose plot. **b** Eleven tRNAs present in phage genome tend to correspond to codons that are highly used by the phage genes, while rare in the host genome

perspective in that it has a vast number of tRNAs, while other *X. oryzae* phages (OP1, OP2, Xp10, Xop411) did not presentany sequence of tRNA.

Discussion

Owing to the diversified genetic characteristics of *X. oryzae*, Phage therapies turned out to be more challenging to control bacterial pathogenicity. Isolation and characterization of novel phages, particularly lytic phages, are needed for phage research. In the past, Phages that infect *X. oryzae*, such as Xp10, Xop411, OP1, OP2, and Xoo-sp2, have been isolated, and basic characteristics (morphology, host ranges, and classification), and their genomics, proteomics, and host interactions have been studied. Kuo et al. [41] have found Xf, a filamentous phage of *X. oryzae*, belongs to the family *Inoviridae*, while Yuzenkova et al. [42] reported phage Xp10 fromthe *Siphoviridae* family, encodinga single-subunit of RNAP with approximately 44-kb genome size. Moreover, Phage Xop411 share almost similar morphology with phage Xp10 despite OP1 proteins. However, it is entirely different from phage OP2 in genome organization, size, sequence, and morphology [43]. The OP1, lytic *X. oryzae* bacteriophage, possesses a dsDNA genome with an approximate size of 44-kb. It shares 63 to 99% sequence identity with Xp10 proteins [8]. Additionally, OP2 phage is circularly permuted and terminally redundant. Its genome size is 47-kb with 60.9% GC content [44]. Moreover, Zhaoxia D et al. [4] isolated phage Xoo-sp2, according to morphology it belonged to *Siphoviridae* family. Genomic analysis of phage showed it consists of a linear double stranderd DNA molecule with a

genome size of about 60-kb while encoding 79 ORFs. Comparative analysis of phage Xoo-sp2 represented its similarity with *Pseudomonas* and *Stenotrophomonas* phages. In contrast, the phage in this study has double-stranded DNA with an isometric, icosahedral head and contractile tail, a genome size of 157-kb with 39.9% GC content, and 16 tRNA, which are characteristics of the family *Herelleviridae*. Comparative genetics showed its similarity with *Bacillus* phages. Soil samples for the isolation of Xoo-sp2 and *Xoo-sp15* were obtained from different provinces of China. It showed that *X. oryzae* phages present abundantly in the soil of different rice growing regions of China [4]. *Xoo-sp15* can infect all the *X. oryzae* strains except Fuj while Xoo-sp2 can infect all strains except PXO99A. Host spectrum of both phages were also checked against other bacterial species like *Bacillus subtilis*, *Ralstonia solanacearum* and *Pseudomonas hibisicola*. Results showed both phages are specific to *X. oryzae* [4].

Previously reported *X. oryzae* phages belong to the family *Siphoviridae* due to the abundance of these in the environment [23]. Phages from the family *Siphoviridae* typically have an icosahedral head and contractile/non-contractile tails with double-stranded DNA and a genome size of approximately 50-kb with a GC content of ~ 52% [23]. However, members of the *Herelleviridae* family present variable differences [22] from these three families, including *Myoviridae* (long contractile tails), *Podoviridae* (short non-contractile tail), and *Siphoviridae* (long non-contractile tail) in the order *Caudovirales.*

Conclusion

Although results showed *Xoo-sp15* has the potential as an alternative biological antibacterial agent in further studies on its use in phage therapy. Specifically, advantageous properties are, (i) lack of the genes related to virulence or antibiotic resistance, (ii) absence of lysogenization specific genes (iii) efficient infection of many *X. oryzae* strains, (iv) presence of a large number of tRNAs. However, the main limitation of phage application is its narrow host range. We need to isolate more phages to use them in phage cocktails for field experiments. Furthermore, a key factor in efficient phage application is the effective phage preparations that can be readily stored and transported. Therefore, further efforts are required to achieve better prevention or treatment effects on BLB with phages.

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Author Contributions D.P, H.Q., and Y.T. proposed the idea and designed the experiments. Z.D. and J.L performed the experiments. A.N performed the computational analyses, drafted and wrote the manuscript. R.A.T and N.A critically read, analyzed and revised the manuscript.

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Declarations

Conflict of interest All the authors declared that there are no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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