



# Characterisation of a newly detected bacteriophage infecting *Bordetella bronchiseptica* in swine

Yibao Chen<sup>1,2</sup> · Lan Yang<sup>1,2</sup> · Erchao Sun<sup>1,2</sup> · Jiaoyang Song<sup>1</sup> · Bin Wu<sup>1,2</sup>

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## Abstract

A novel virulent bacteriophage, vB\_BbrM\_PHB04, infecting *Bordetella bronchiseptica* was isolated from wastewater collected at a swine farm in China. Phage vB\_BbrM\_PHB04 exhibited growth over a wide range of temperature and pH conditions and showed different efficiency of plating values and lytic spectra within the same strains at 25 °C and 37 °C. High-throughput sequencing revealed that vB\_BbrM\_PHB04 has a linear double-stranded DNA genome with 124 putative open reading frames. Overall, the genome of vB\_BbrM\_PHB04 showed very low similarity (the highest nucleotide identity 82%, 1% coverage) to other phage sequences in the GenBank database. Phylogenetic analysis indicated that vB\_BbrM\_PHB04 is a new member of the family *Myoviridae*. In addition, polymerase chain reaction-based detection of phage genes in phage-resistant *B. bronchiseptica* variants revealed no evidence of lysogenic activity of phage vB\_BbrM\_PHB04.

## Introduction

*Bordetella bronchiseptica* is a widespread Gram-negative pathogen that can be isolated from many different mammalian species. It is one of the primary causative agents of infectious respiratory diseases such as swine pneumonia and atrophic rhinitis [1]. In addition, *B. bronchiseptica* is often isolated in combination with other pathogens from swine suffering from pneumonia. Previous studies have shown that co-infection of viral and bacterial pathogens, including porcine reproductive and respiratory syndrome virus, porcine respiratory coronavirus, *Haemophilus parasuis*, *Pasteurella multocida*, and *Streptococcus suis*, exacerbates the severity

of respiratory disease in pigs [2]. Importantly, human *B. bronchiseptica* infections have also been reported [3, 4].

*B. bronchiseptica* virulence factors are regulated by the complex *Bordetella* virulence gene product BvgAS [5]. BvgAS is a two-component signal transduction system that regulates the expression of multiple virulence factors in response to changes in environmental conditions. Different virulence factors are expressed in the three different *B. bronchiseptica* phases: the bvg<sup>+</sup>, bvg<sup>i</sup>, and bvg<sup>-</sup> phases. In the virulent bvg<sup>+</sup> phase, which occurs at 37 °C in the absence of MgSO<sub>4</sub> and nicotinic acid, *B. bronchiseptica* can rapidly colonize the respiratory tract of its host through the expression of adhesins, autotransporters, and toxins. The avirulent bvg<sup>-</sup> phase occurs at growth temperatures of ~25 °C or in the presence of MgSO<sub>4</sub> or nicotinic acid. Finally, the intermediate (bvg<sup>i</sup>) phase is responsible for biofilm formation by *B. bronchiseptica* [5, 6].

With the increasing prevalence of multidrug-resistant bacteria, several reports have indicated the emergence of *B. bronchiseptica* isolates with decreased susceptibility to β-lactam antibiotics such as penicillins and cephalosporins [7]. To combat multidrug-resistant bacteria, phages offer a potential alternative to antibiotics. In the past, temperate *B. bronchiseptica* phages have been induced in clinical bacterial strains, but their phenotypic characteristics have rarely been described. Petrovic et al [8] isolated 29 lytic *B. bronchiseptica* phages from the environment and described their phenotypic characteristics; however, the genomes of these

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✉ Bin Wu  
wub@mail.hzau.edu.cn

<sup>1</sup> State Key laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, Hubei, China

<sup>2</sup> The Cooperative Innovation Center for Sustainable Pig Production, Huazhong Agricultural University, Wuhan 430070, Hubei, China

phages were only partially sequenced to determine whether they were lytic or temperate phages. Here, we isolated a novel lytic phage from wastewater collected from a swine farm in China and studied its phenotypic and genetic characteristics. To our knowledge, the complete genomes of only three temperate *B. bronchiseptica* phages have been sequenced [9].

## Materials and methods

### Bacterial growth conditions

*B. bronchiseptica* and *P. multocida* were cultured at 37 °C for 18–30 h in tryptic soy broth (TSB; Becton, Dickinson and company, MD, USA) or on tryptic soy agar (TSA; Becton, Dickinson and company, MD, USA) supplemented with 10% (v/v) sterile, defibrinated sheep blood (Jiulongbio, Zhengzhou, China). *Escherichia coli* was cultured at 37 °C for 12 h on TSA or TSB. All strains are available from the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, China (Table 1).

### Phage isolation

The conventional double-layer agar method was used to examine phage activity [10]. A sewage water sample (20 ml) was collected in Wuhan, China, and then sterilized by filtration through a 0.22- $\mu$ m membrane. A 5-ml aliquot of the filtrate was mixed with 10 ml of an exponential phase *B. bronchiseptica* 4715 culture, and the mixture was incubated at 37 °C for 8 h. The culture was then centrifuged at 12,000  $\times$  g for 10 min, and the resulting supernatant was filtered using a 0.22- $\mu$ m membrane. A 300- $\mu$ l aliquot of the filtered supernatant was then mixed with 500  $\mu$ l of *B. bronchiseptica* 4715, added to 6 ml of molten soft TSB (0.75% agar), supplemented with 10% sterile, defibrinated sheep blood and then poured onto the surface of a prepared TSA plate supplemented with 10% (v/v) sterile, defibrinated sheep blood. Plaques appearing after an overnight incubation at 37 °C were enumerated [10].

A single plaque was picked aseptically and re-suspended at 180 rpm in 6 ml of SM buffer (5.8 g of NaCl, 2.0 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 50 ml of Tris-HCl [pH7.4], 5.0 ml of 2% gelatin) for 3 h. The phage-containing SM buffer was collected and centrifuged at 12,000  $\times$  g for 30 s, and then the supernatant was filtered through a 0.22- $\mu$ m membrane. This phage preparation was subjected to serial 10-fold dilutions in sterile SM buffer. Phage isolation by the double-layer agar method was repeated four more times, and the isolates were stored at 4 °C.

**Table 1** Host range of phage vB\_BbrM\_PHB04

| Strains                                     | Efficiency of plating (EOP) |       | Isolation years |
|---|-----------------------------|-------|-----------------|
|   | 37 °C                       | 25 °C |                 |
| <i>B. bronchiseptica</i> 4715 (host)        | 1                           | 1     | 2008            |
| <i>B. bronchiseptica</i> 1099               | 1                           | 0.74  | 2005            |
| <i>B. bronchiseptica</i> 2782               | 1.82                        | 1.63  | 2006            |
| <i>B. bronchiseptica</i> 3814               | 0.26                        | 0.17  | 2007            |
| <i>B. bronchiseptica</i> 3814 $\Delta$ bvgA | 0.48                        | 0.46  | 2007            |
| <i>B. bronchiseptica</i> 4173               | 0.64                        | 0.42  | 2008            |
| <i>B. bronchiseptica</i> 7231               | 0.32                        | 0.14  | 2011            |
| <i>B. bronchiseptica</i> 5508               | –                           | –     | 2009            |
| <i>B. bronchiseptica</i> 5275               | –                           | –     | 2009            |
| <i>B. bronchiseptica</i> 6944               | –                           | –     | 2010            |
| <i>B. bronchiseptica</i> 6969               | –                           | –     | 2010            |
| <i>B. bronchiseptica</i> 7290               | –                           | –     | 2011            |
| <i>B. bronchiseptica</i> 7434               | –                           | –     | 2011            |
| <i>B. bronchiseptica</i> 8952               | –                           | –     | 2012            |
| <i>B. bronchiseptica</i> Bb01               | –                           | –     | 2014            |
| <i>B. bronchiseptica</i> Bb18               | –                           | –     | 2014            |
| <i>B. bronchiseptica</i> Bb24               | –                           | –     | 2016            |
| <i>B. bronchiseptica</i> Bb76               | –                           | –     | 2017            |
| <i>B. bronchiseptica</i> Bb77               | –                           | –     | 2017            |
| <i>P. multocida</i> capsular type A         | –                           | –     |                 |
| <i>P. multocida</i> capsular type D         | –                           | –     |                 |
| <i>E. coli</i> O157:H7                      | –                           | –     |                 |
| <i>E. coli</i> DH5 $\alpha$                 | –                           | –     |                 |

“–” indicates that no plaques were observed

### Electron microscopy

The phage filtrate was stained negatively with 2% uranyl acetate after addition of a drop of a phage suspension onto a grid surface, and then the excess stain was removed immediately. The morphology of the phages was examined under a 100-kV transmission electron microscope (HITACHI H-7650, Japan).

### Thermolability and pH sensitivity

To examine these properties of the phage, we used a known method [10]. Briefly, stability of the purified phage at various temperatures (4, 20, 40, 50, 60, 70, and 80 °C) was assessed by incubation for 1 h. To evaluate the stability of the phage at different pH levels (3, 5, 7, 9, and 11), the purified phage particles were incubated at 37 °C for 1 h. Each assay was performed in triplicate, and then the samples were titered by the double-layer agar plate method.

## One-step growth curve

To analyze the infectivity and replication ability of phages, a one-step growth curve test was performed as previous described [10]. Briefly, phage vB\_BbrM\_PHB04 was mixed with the host strain in exponential phase at a multiplicity of infection (MOI) of 0.01 and incubated at 37 °C for 5 min. The mixtures were then centrifuged at 12,000 × *g* for 30 s to remove any unabsorbed free phage. The precipitate was washed with 37 °C TSB, then the suspension was transferred to 30 ml of TSB supplemented with 10% (v/v) sterile, defibrinated sheep blood followed by incubation at 25 °C and 37 °C. This time point was defined as *t* = 0 s. At 10-min intervals, a 0.5-ml sample was collected for a total of 200 min. The titration of phage particles was conducted by the double-layer agar method. The experiment was repeated three times.

## Host range determination

The bacterial strain susceptibility levels were detected using spotting methods as previously described [10]. In total, 19 *B. bronchiseptica* and other bacterial species (including *P. multocida* capsular type A, *P. multocida* capsular type D, *E. coli* O157:H7 and *E. coli* DH5α) were used for host range determination (Table 1). The tested bacterial strains were incubated at 25 °C and 37 °C for 16 h to allow cell lysis by the isolated phages. The efficiency of plating (EOP) values were determined by calculating the ratio of plaque-forming units (PFUs) of each phage-susceptible strain to PFUs of indicator strain (*B. bronchiseptica* 4715) by the double-layer agar method. This experiment was repeated three times.

## DNA extraction and analysis of genome sequences

The phages' genomic DNA was extracted using the phenol-chloroform protocol [11]. Briefly, purified phages were lysed by the addition of proteinase K (100 mg/ml), SDS (10%, wt/vol) and EDTA (0.5 mM, pH 8.0) and incubation at 56 °C in water for 2 h. The digested sample was mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) centrifuged at 12,000 × *g* (4 °C) for 10 min to remove any debris. This step was repeated three times. The supernatant was mixed with isoamyl alcohol kept at -20 °C overnight. Then the air-dried precipitate was washed three times with cold 75% ethanol. Finally, phages' genomic DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and was sequenced on an Illumina HiSeq 2500 sequencer with 2×100 bp read length. More than 6,200-fold coverage of phage genome was achieved by sequencing the cloned fragments. The short reads were assembled into the genome by means of SOAPdenovo (<http://sourceforge.net/projects/soapdenovo2/files/SOAPdenovo2/>) [12]. Protein-encoding

putative open reading frames (ORFs) were predicted using Glimmer [13, 14]. The final assembled sequence was searched against the current protein and nucleotide databases (<http://www.ncbi.nlm.nih.gov/>) by means of the basic local alignment search tool (BLAST). Protein BLAST (BLASTP) (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to identify putative homologies and proteins sharing similarities with the predicted phage proteins. Phylogenetic analysis of phage large terminase subunit sequences was performed using the ClustalW program in MEGA 6 [15].

## Lysogen screening

To test for possible lysogens, host *B. bronchiseptica* 4715 cells in exponential growth phase (approximately 10<sup>7</sup> colony-forming units/ml) were incubated with phage vB\_BbrM\_PHB04 at a multiplicity of infection (MOI) of 100 in SM buffer and the mixture was incubated for 10 min to allow adsorption of phages [16]. The conventional double-layer agar method was used to pick 40 random phage-resistant colonies. Single colonies were cultured with TSB supplemented with 10% (v/v) sterile, defibrinated sheep blood at 37 °C for 24 h and examined for phage vB\_BbrM\_PHB04 sensitivity by spot testing, applying 10 μl (10<sup>7</sup> pfu/ml) phage to visualize individual plaques. Genomic DNA was extracted from each of the bacteria resistance to phage using a commercial extraction kit (Roche, Switzerland). The PCR primers used in this study are described in Table S1. Primers were tested for specificity using the phage vB\_BbrM\_PHB04 genome as a positive control. This experiment was repeated three times.

## Results

### Morphology of phage vB\_BbrM\_PHB04

Phage vB\_BbrM\_PHB04 was isolated from its host, *B. bronchiseptica* strain 4715, which was isolated from a sewage sample from a swine farm in China. Transmission electron microscopy of the purified and concentrated phage particles showed that phage vB\_BbrM\_PHB04 had an isometric head (86.3 ± 1.3 nm in diameter) and a contractile tail (72.4 ± 3.2 nm long and 23.3 ± 1.8 nm wide) (Fig 1). Based on the current International Committee on Taxonomy of Viruses classification system, the phage was assigned to the family *Myoviridae*, order *Caudovirales*.

### Life cycle of phage vB\_BbrM\_PHB04

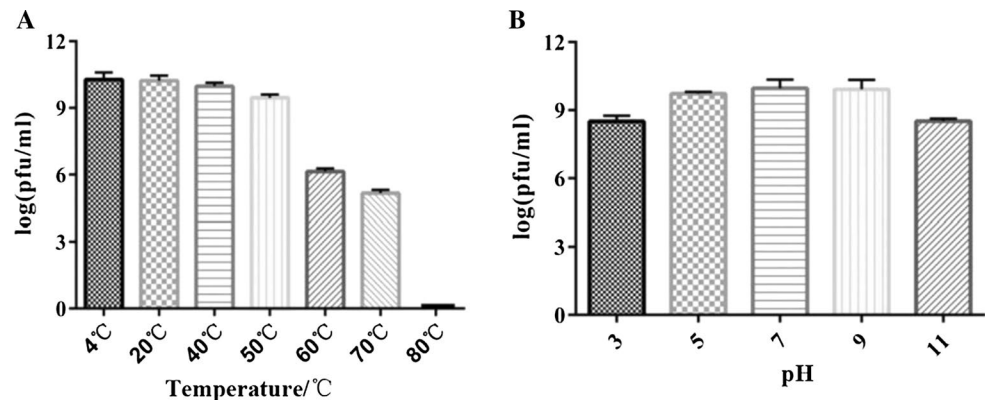
Phage vB\_BbrM\_PHB04 was stable at temperatures ranging from 4–50 °C, but a reduction in viral titers occurred at 60–70 °C, and titers were dramatically reduced at 80 °C (Fig 2A). The phage was stable over a broad pH range



**Fig. 1** Transmission electron micrograph of phage vB\_BbrM\_PHB04. Phage vB\_BbrM\_PHB04 has an isometric polyhedral head approximately  $86.3 \pm 1.3$  nm in diameter and a contractile tail approximately ( $72.4 \pm 3.2$  nm long and  $23.3 \pm 1.8$  nm wide). The number of measurements is 7 ( $n=7$ ) and the data are expressed as the means  $\pm$  SDs. Scale bar represents 100 nm. Magnification:  $\times 150,000$

(from 5.0–9.0), but the titer dropped by approximately 1.2 log at pH 3.0 and pH 11.0 (Fig 2B). A one-step growth experiment was conducted to determine the duration of the latent period and burst size of phage vB\_BbrM\_PHB04. The phage had a long latent period at both 25 °C and 37 °C (~130 min and ~140 min, respectively), with an average burst size of 51 and 40 phage particles, respectively, per infected cell after 180 min (Fig 3).

**Fig. 2** Thermal and pH stability of phage vB\_BbrM\_PHB04. The data are expressed as the means  $\pm$  SDs (A: temperature; B: pH)

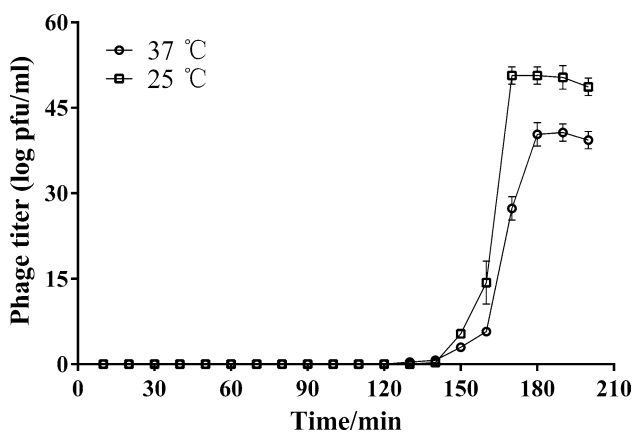


## Host range

Nineteen *B. bronchiseptica* strains, including mutant *B. bronchiseptica* strain 3814 $\Delta$ bvgA, along with four strains representing other bacterial species (including *P. multocida* capsular type A, *P. multocida* capsular type D, *E. coli* O157:H7 and *E. coli* DH5 $\alpha$ ), were included in a phage lysis assay to determine the lytic host range of phage vB\_BbrM\_PHB04. Seven of the *B. bronchiseptica* strains were sensitive to phage vB\_BbrM\_PHB04, while none of the other species were lysed (Table 1). For the seven sensitive *B. bronchiseptica* strains, the efficiency of plating (EOP) values differed slightly at different temperatures (25 °C vs. 37 °C) (Table 1). Interestingly, mutant strain 3814 $\Delta$ bvgA had higher EOP values than wild-type strain 3814 (Table 1).

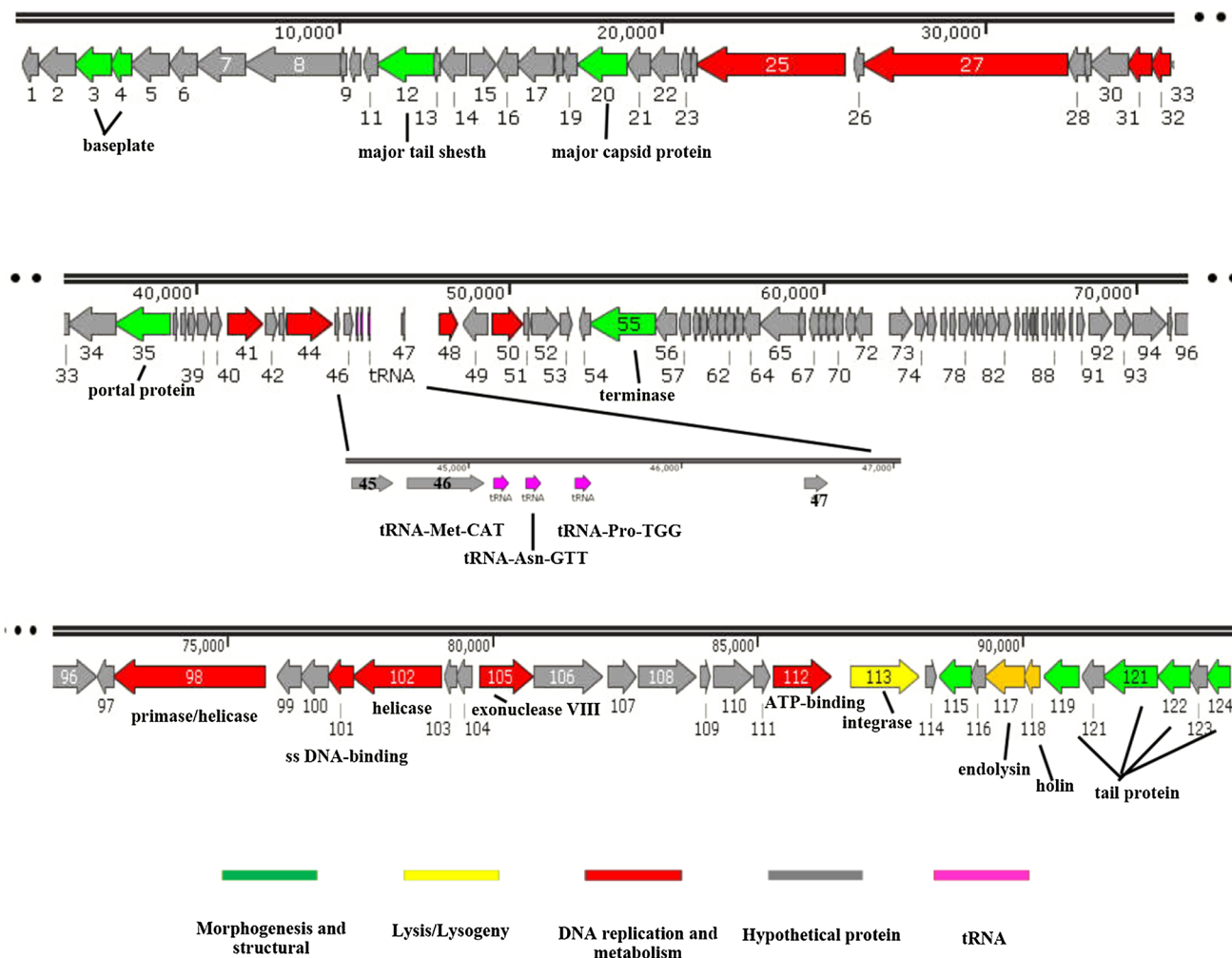
## Genome annotation and in silico analysis

High-throughput sequencing of phage genomic DNA was performed using the Illumina HiSeq system according to the manufacturer's instructions. The assembly of the random library of sequences yielded a closed, circular genome by means of SOAPdenovo. The complete linear, double-stranded DNA genome of phage vB\_BbrM\_PHB04 consisted of 94,005 bp, with a GC content of 65.5% (Fig 4). BLASTn analysis revealed that the genome of phage vB\_BbrM\_PHB04 showed the highest nucleotide identity (82%, though only 1% coverage) to the genome sequence of *Burkholderia* phage strain KACC 11889. This low sequence similarity suggested that phage vB\_BbrM\_PHB04 is a novel species. The genome contained 124 putative open reading frames (ORFs), and tRNA prediction, conducted using tRNAscan-SE analysis, identified three tRNA encoding genes: tRNA-Met-CAT, tRNA-Asn-GTT, and tRNA-Pro-TGG (Fig 4). The predicted ORFs encoding proteins with known functions were classified into three groups: those associated with morphogenesis and structure, those associated with DNA replication and metabolism, and those associated with lysis or lysogeny (Fig 4).



**Fig. 3** One-step growth curves of phage vB\_BbrM\_PHB04 on *Bordetella bronchiseptica* 4715. The phage vB\_BbrM\_PHB04 had a long latent period at both 25 °C and 37 °C (~130 min and ~140 min, respectively), with an average burst size of 51 and 40 phage particles, respectively, per infected cell after 180 min. The data are expressed as the means±SDs

Proteins involved in structure and assembly were encoded by genes in the phage vB\_BbrM\_PHB04 genome. BLASTn analysis of these regions, including the baseplate (ORF3), major tail sheath (ORF12), major capsid protein (ORF20), and tail fiber protein (ORF119, ORF121, ORF122, and ORF124), showed low identity to the corresponding proteins in other phage (Fig 4; Table S1). Genes encoding DNA replication and regulation modules such as the helicase (ORF25), DNA primase (ORF98), ssDNA-binding protein (ORF101), and RecD-like DNA helicase (ORF112) were located in the middle of the phage vB\_BbrM\_PHB04 genome (Fig 4). However, all of these sequences showed only low levels of identity to genes encoding phage proteins of similar function. Additionally, we identified 96 genes of unknown function. With respect to lysis or lysogeny modules, ORF117 encoded a protein with 56% amino acid sequence identity to the endolysin protein of *Pseudomonas* phage LkO4 (Fig 4; Table S2). Holin, a protein that forms large holes in the cytoplasmic membrane of phage-infected bacteria



**Fig. 4** Phage vB\_BbrM\_PHB04 genome organization. Arrows represent predicted genes, and the direction of each arrow represents the direction of transcription. Different colors denote different phage gene functional groups

is encoded by ORF118, showed 42% amino acid sequence identity to the holin protein from *Pseudomonas* phage LkO4 (Fig 4; Table S2). In addition, the protein encoded by ORF8 contained domains from multiple families, including LT\_GEWL, SLT, MitE, PRK11619, and DUF3584 (Fig 4; Table S2). Proteins LT\_GEWL and SLT belong to the lysozyme-like superfamily, all members of which participate in the hydrolysis of  $\beta$ -1,4-linked polysaccharides.

### Phylogenetic analysis

The amino acid sequences of the terminase large subunit proteins (encoded by ORF55) from phage vB\_BbrM\_PHB04 and other phages belonging to the *Myoviridae* family were aligned using MEGA6 software. A neighbor-joining tree generated from these sequences revealed that phage vB\_BbrM\_PHB04 clustered with phages such as *Bacillus* phage SPO1, *Bacillus* phage TsarBomba, and *Staphylococcus* phage K in the subfamily *Spounavirinae* (Fig 5). This clear phylogenetic distinction from other phages and the novel characteristics of the genome indicate that phage vB\_BbrM\_PHB04 is a new member of the family *Myoviridae*, order *Caudovirales*.

### Examination of lysogenic activity

The lysogenic activity of phage genes in phage-resistant bacteria was examined by PCR. In each round of screening, 40 random phage-resistant colonies were picked for further analysis and examined for vB\_BbrM\_PHB04 sensitivity by spot testing. The PCR results showed that the phage major capsid gene (ORF20), the terminase gene (ORF55), the

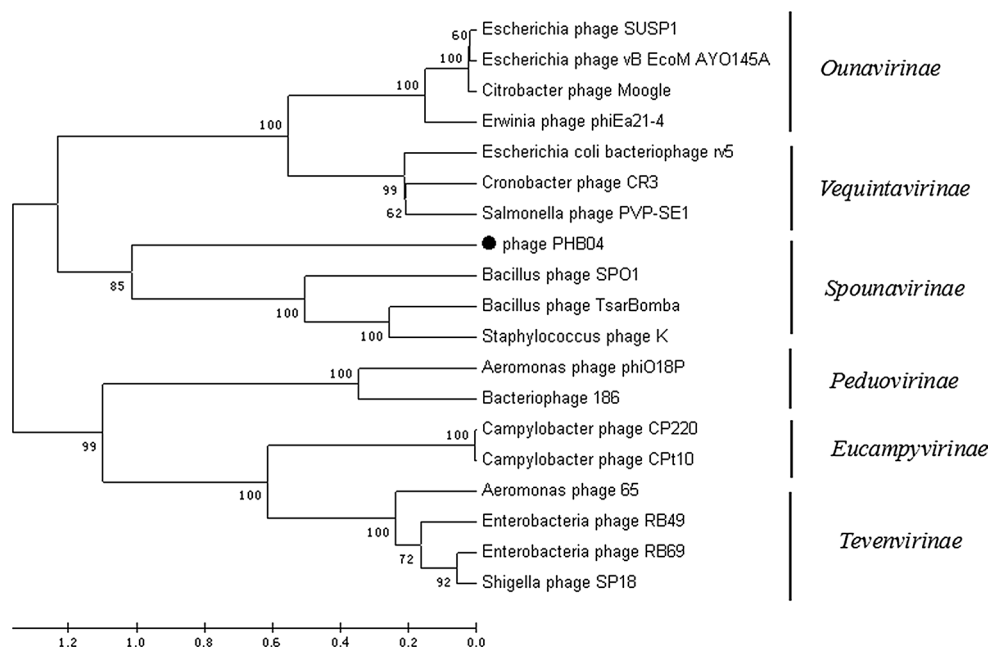
helicase gene (ORF102), and the integrase gene (ORF113) were all absent from the genomes of phage-resistant colonies, suggesting that phage vB\_BbrM\_PHB04 does not have lysogenic activity in host bacteria (the results are not shown).

### Discussion

In this study, we initially screened 17 domestic sewage samples for the presence of *B. bronchiseptica* phage, but failed to identify the phage in any of the samples (the results are not shown). Petrovic et al [8]. also reported a failed attempt to isolate *B. bronchiseptica* phage from samples collected from swine water troughs and farm wastewater. Interestingly, although we eventually isolated phage vB\_BbrM\_PHB04 from swine wastewater, the farm in question had no reported outbreaks or even suspected cases of bronchopneumonia or atrophic rhinitis in recent years. This may therefore indicate latent *B. bronchiseptica* infection of pigs.

Phage vB\_BbrM\_PHB04 was assigned to the family *Myoviridae* based on its morphological characteristics. In addition, phylogenetic analysis of the phage-encoded large terminase subunit protein revealed a close phylogenetic relationship between phage vB\_BbrM\_PHB04 and phages belonging to the *Spounavirinae* subfamily. Liu et al [9]. reported on three temperate phages isolated from *B. bronchiseptica* that were assigned to the family *Podoviridae*, while Petrovic et al [8]. isolated 29 *B. bronchiseptica* phages assigned to the family *Siphoviridae*. Based on its morphology and genome features, phage vB\_BbrM\_PHB04

**Fig. 5** Phylogenetic tree analysis of the phage-encoded large terminase subunit (ORF55) of phage vB\_BbrM\_PHB04. The amino acid sequences of the large terminase subunits were compared using MEGA6 in the analysis, and phylogenetic tree was generated using the neighbour-joining method and 1000 bootstrap replicates



was confirmed as a new member of the family *Myoviridae*, order *Caudovirales*.

One-step growth curve analysis revealed a significant difference in the duration of the latent period and burst size of phage vB\_BbrM\_PHB04 at 25 °C vs. 37 °C, which agrees with the findings of Petrovic et al [8], who determined that the latent period and burst size of phage vB\_BbrS\_CN1 is dependent on temperature. In addition, the EOP values of phage vB\_BbrM\_PHB04 showed slight differences in phage host range at the two temperatures. *B. bronchiseptica* displays phenotypic variation in response to temperature change, and this phase variation may have an impact on phage receptor expression. Moreover, phage vB\_BbrM\_PHB04 had higher EOP values in mutant *B. bronchiseptica* strain 3814Δ**avgA** than in wild-type strain 3814. We hypothesize that the loss of the virulence gene regulatory protein in the mutant strain resulted in enhanced vB\_BbrM\_PHB04 adsorption and subsequent infection.

The genome sequence of phage vB\_BbrM\_PHB04 showed the highest degree of nucleotide sequence identity to the genome sequence of *Burkholderia* phage strain KACC 11889. Functional predictions were possible for 27 of the predicted ORFs, while the remaining ORFs exhibited limited amino acid sequence identity to other hypothetical proteins, indicating that the phage vB\_BbrM\_PHB04 genome could be unique. In general, genome recombination occurs frequently between bacteria and phages and is conducive to phage adaptation to the host bacterium. The protein encoded by ORF113 showed 45% amino acid sequence identity to the putative integrase family protein of *Pseudomonas* phage PPpW-3. We know that temperate phage genomes can be integrated into bacterial genomes or plasmids and that prophages confer immunity that protects host bacteria against infection by other phages. In addition, the integrase gene is usually associated with lysogenic activity. To determine whether phage vB\_BbrM\_PHB04 is temperate, we designed four pairs of primers targeting essential phage genes. The results showed that none of the 40 phage-resistant bacterial clones were positive for the essential phage genes, suggesting that phage vB\_BbrM\_PHB04 does not have lysogenic activity.

Although no known lysogenic factors such as an excisionase, or anti-repressor proteins were identified in the genome of phage vB\_BbrM\_PHB04, the large number of predicted proteins with no known function may carry out lysogen-related functions. Phages containing lysogen-related genes, but not being temperate themselves, have been described previously. For example, while phage UAB\_Phi20 contains all the elements needed for a lysogenic cycle, phage PPpW-3 only contains an integrase gene; however, both are lytic phages [16, 17].

We did not consider the spontaneous release of temperate phage or prophage induction from the host strain for

life cycle of phage vB\_BbrM\_PHB04 assay. Moreover, an endolysin-encoding gene was identified in the phage vB\_BbrM\_PHB04 genome. Future work should therefore be conducted to express the endolysin, and to examine potential applications for this enzyme in the treatment of multidrug-resistant *B. bronchiseptica*.

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## Compliance with ethical standards

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors. I would like to declare on behalf of my co-authors that we have no conflict of interest.

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