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ENVIRONMENTAL MICROBIOLOGY

The First *Siphoviridae* Family Bacteriophages Infecting *Bordetella bronchiseptica* Isolated from Environment

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Abstract *Bordetella bronchiseptica* is a well-known etiological agent of kennel cough in dogs and cats and one of the two causative agents of atrophic rhinitis, a serious swine disease. The aim of the study was to isolate *B. bronchiseptica* bacteriophages from environmental samples for the first time. A total of 29 phages from 65 water samples were isolated using the strain ATCC 10580 as a host. The lytic spectra of the phages were examined at 25 and 37 °C, using 12 strains of *B. bronchiseptica*. All phages were able to plaque on 25.0 % to 41.7 % of the strains. The selected phages showed similar morphology (*Siphoviridae*, morphotype B2), but variation of RFLP patterns and efficacy of plating on various strains. The partial genome sequence of phage vB_BbrS_CN1 showed its similarity to phages from genus *Yuavirus*. Using PCR, it was confirmed that the phages do not originate from the host strain, and environmental origin was additionally confirmed by the analysis of host genome sequence in silico and plating heated and unheated samples in parallel. Accordingly, this is the first isolation of *B. bronchiseptica* phages from environment and the first isolation and characterization of phages of *B. bronchiseptica* belonging to family *Siphoviridae*.

Keywords *Bordetella bronchiseptica* · Bacteriophage · Isolation · *Siphoviridae*

Introduction

Bordetella bronchiseptica is a gram-negative coccobacillus, a microbita of upper parts of the respiratory tract of pigs, dogs, cats, rabbits, rats, horses, and other animals [1]. At the same time, it is a well-known etiological agent of various animal diseases, including infectious tracheobronchitis, conjuctivitis, rhinitis, mandibular lymphadenopathy, and pneumonia. In dogs and cats, it is one of the main causes of infective tracheobronchitis, known as kennel cough. In pigs, it is a causative agent of two diseases—bronchopneumonia and atrophic rhinitis. In the latter, *B. bronchiseptica* produces potent dermonecrotic toxin causing the atrophy of nasal septum and turbinate, as well as nasal and facial bones. This impairs swine feeding and consequently causes poor progression with great economic losses [2]. The disease has more severe consequences in mixed infections of *B. bronchiseptica* and *Pasteurella multocida* [3]. *B. bronchiseptica* is also a frequently detected microorganism in upper respiratory infections in rabbits, commonly called "snuffles" [4]. It can also be a pathogen of other animals, such as guinea pigs and horses [5, 6], but rarely of humans, with exception of immunocompromised individuals [7].

Apart from the bacteriophages of human pathogens, which have been studied widely, the phages of animal pathogens and opportunistic bacteria, including *B. bronchiseptica*, have mainly been neglected [8–10]. Although *B. bronchiseptica* was discovered more than a century ago [11], there is no reference describing its bacteriophages, except for a few related temperate bacteriophages induced from clinical strains, belonging to family *Podoviridae* [12, 13]. *B. bronchiseptica*

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bacteriophage examination, however, is important from several aspects. For many multiple resistant bacteria, bacteriophages can be an alternative to conventional antibiotics [14], and *B. bronchiseptica* possesses innate or acquired antibiotic resistance to β-lactams, macrolides, tetracyclines, sulphonamides, and aminoglycosides, usually used for the treatment of animals with respiratory infections [15]. Furthermore, phage genomes can be new sources of genes for lytic enzymes, i.e., enzybiotics, as well as other valuable proteins [16]. The examination of *B. bronchiseptica* temperate phages can also explain potential prophage involvement in *B. bronchiseptica* virulence and the phenomenon of lysogenic conversion. Finally, their examination can also add to general knowledge of phage biology, as well as to their abundance and diversity in the environment.

The aim of this study was to isolate *B. bronchiseptica*-specific bacteriophages from environmental samples for the first time and to partially characterize them.

Materials and Methods

Samples Origin

A total of 65 samples from Serbia (59), Hungary (1), Georgia (1), Egypt (2), Turkey (1), and Switzerland (1) were collected during 2013 and 2014 including surface water from various origin (Table 1). Some of the municipal wastewater samples were taken repetitively from the same locations, but in different time intervals, so that all phages were obtained from geographically and/or chronologically different sources. The samples were taken aseptically and delivered to the laboratory in a short time. If this was not feasible, the samples were kept at +4 °C for no longer than 48 h.

Bacterial strains

Twelve strains of *B. bronchiseptica* were used in the study, including one reference strain (ATCC 10580), six strains of animal origin (973, L:594, BbChiot, CV1, LORD, and 3416), and five of human origin (R1, LEG, SEI, DEL, and S2). Besides *B. bronchiseptica*, 14 strains belonging to various taxa were used for phage lytic spectra determination: *Alcaligenes faecalis* (JR-22), *Acinetobacter baumannii* (ATCC BAA-747 and ATCC 19606), *Plesiomonas shigeloides* (ATCC 51903 and ATCC 14029), *Escherichia coli* (ATCC 13706), *Staphylococcus aureus* subsp. *aureus* (ATCC 11632), *Eneterococcus faecalis* (laboratory strain 36913 EXB-U53), *Salmonella enterica* subsp. *enterica* serovar Eneritidis (ATCC 13076), *Klebsiella pneumoniae* (laboratory strain 36913), *Providencia rettgeri* (ATCC 9250), *Providencia stuartii* (ATCC 49809), *Morganella morganii* subsp. *morganii* (ATCC 25829), and *Proteus mirabilis* (ATCC 35659). All bacteria were stocked in Luria–Bertani broth (LB) containing glycerol (*v*/*v* 10:1) at −70 °C. For the purpose of the experiments, they were cultivated overnight in LB at 37 °C.

Phage Isolation

B. bronchiseptica-specific phages were isolated using in parallel a single layer method with Luria-Bertani and Tryptone Salt Agar with ATCC 10580 strain as a phage host. All samples were prepared for isolation by filtration through a filter with pore diameter 0.45 μm, except two wastewater swine farm samples, which were filtered previously through filter paper because of visible impurities. Bacteriophage presence was determined in 100 ml of sample according to U.S. EPA (2001) [17]. The plates were incubated 24–48 h at 37 °C and

Table 1 Water samples and isolated phages

Origin of samples	No. of samples	No. of isolated phages	Phage designation
River	12	4	vB_BbrS_RV1; vB_BbrS_RV2; vB_BbrS_RV3; vB_BbrS_RV4
Lakes	12	5	vB_BbrS_LK1; vB_BbrS_LK2; vB_BbrS_LK3; vB_BbrS_LK4; vB_BbrS_LK5
Fish pond water	6	5	vB_BbrS_FP1; vB_BbrS_FP2; vB_BbrS_FP3; vB_BbrS_FP4; vB_BbrS_FP5
Swamp	4	2	vB_BbrS_SW1; vB_BbrS_SW2
Creek	6	3	vB_BbrS_CR1; vB_BbrS_CR2; vB_BbrS_CR3
Canal	5	3	vB_BbrS_CN1; vB_BbrS_CN2; vB_BbrS_CN3
Animal water trough	12	2	vB_BbrS_TR1; vB_BbrS_TR2
Wastewater swine farm	2	0	–
Municipal wastewater	6	5	vB_BbrS_MW1; vB_BbrS_MW2; vB_BbrS_MW3; vB_BbrS_MW4; vB_BbrS_MW5
Total	65	29	

examined for plaques. Phage isolation assay for negative samples was repeated in order to confirm the negative results. From each plate with plaques, only one plaque was transferred into SM buffer (50 mmol L^{-1} Tris–HCl [pH 7.5], 100 mmol L^{-1} NaCl, 8 mmol L^{-1} $MgSO_4$, 0.01 % *w/v* gelatin) to avoid isolation of clonally identical phages. The phage suspensions were subsequently 10-fold serially diluted, and the plaques were re-isolated three times.

Phage Propagation and Purification

The phages were propagated on solid LB medium by double-agar overlay method. The plates with semi-confluent plaques on *B. bronchiseptica* lawns were soaked with SM buffer overnight at 4 °C, and the obtained phage suspensions were centrifuged to remove bacterial cells (13,000×*g* 15 min at 4 °C), filtered (pore diameter 0.45 and 0.22 μm), and finally precipitated with NaCl and PEG8000. The concentrated phage suspensions were purified by ultracentrifugation in discontinuous CsCl density gradient [18]. The viral bands were collected and dialyzed overnight against SM buffer at 4 °C. The plaque forming units per milliliter (PFU ml^{-1}) in the purified stocks were determined by preparation of serial 10-fold dilutions and double-agar overlay method in triplicate.

Phage Host Range

The analysis of phage lytic spectra was conducted using the spot method (10 μl of 10^6 PFU mL^{-1}) on the lawns of potential hosts, with parallel incubation at 25 and 37 °C for 24 h. Apart from 12 *B. bronchiseptica* strains, the other above listed bacterial species were included in the experiment and incubated at 37 °C. After incubation, presence or absence of lysis was detected, and the obtained lytic zones were characterized as clear, clear with halo or turbid, and no reaction [9]. Each phage was tested against each bacterial strain in duplicate and in three independent experiments.

Transmission Electron Microscopy

Droplet method was applied for transmission electron microscopy of phages [19]. In brief, suspensions were transferred over copper grids with Formvar film and carbon, and the phages were adsorbed for 5 min. In the next step, the excess of suspension was removed and fixed viral particles were contrasted with aqueous solution of uranyl acetate (0.5–1 % *w/v*) for 1 min. After the samples were dried, viruses were observed using Philips CM 100 transmission electron microscope (Philips, Netherlands) at acceleration voltage of 80 kV. Electron micrographs of phages were obtained by a GatanBioscan CCD camera and Digital Micrograph software 3.4. (Gatan Inc., USA). The resulting images were used for phage morphology assessment. At least 10 virions were examined per phage isolate; the phage head and tail length and width were measured. Eight phages originating from various samples were examined by TEM: vB_BbrS_RV4, vB_BbrS_FP2, vB_BbrS_CR1, vB_BbrS_LK3, vB_BbrS_MW2, vB_BbrS_SW1, vB_BbrS_CN1 and vB_BbrS_TR1. The obtained values were averaged and presented as mean ± SD.

Efficiency of Plating

The efficiency of plating was estimated using three *B. bronchiseptica* strains, lysed by all isolated phages at 37 °C: reference strain ATCC 10580 (the original host), 3416 and BbChiot (the alternative hosts). Series of 10-fold dilutions were applied to the lawn of bacteria, and the last dilution, which gave single plaques, was recorded. The titer obtained with alternative host was divided by the titer obtained with original host so that the results are expressed as relative values to the original host strain [20]. The experiment was carried out in duplicate in two independent occasions.

One-Step Growth Curve

One-step growth curve was performed as described by Nagayoshi et al. [21] with some modifications. An overnight culture was harvested by centrifugation (5000*g*, 5 min) and resuspended in LB broth (~10^5 CFU/ml). Phage vB_BbrS_CN1 was added to bacterial suspension at MOI ~10 and allowed to adsorb for 15 min with parallel incubation at 25 and 37 °C. After incubation, the mixture was washed with LB broth three times to remove any free virions (5000 g, 5 min). Thereafter, pelleted cells were resuspended in 100 ml of LB broth, and incubation was continued at 25 and 37 °C. Samples were taken, immediately centrifuged, and supernatant plated on LB agar to determine the phage titer at the beginning of the experiment and at 10-min intervals. Burst size, i.e., the number of released virions from cells, was determined as ratio of progeny phage to the initial number of infected cells. The experiment was performed in three independent occasions.

Phage DNA Extraction and Restriction Enzyme Digestion

The purified phage suspensions were treated with DNAse I and RNAse for 2 h at 37 °C. Thereafter, DNAse was inactivated at 65 °C for 1 h and proteinase K (50 μg ml^{-1}), SDS (0.5 %), and EDTA (20 mmol l^{-1}) were added to the phage suspension and incubated at 56 °C for 1 h in order to release DNA from virions. A standard phenol–chloroform method was used to extract phage DNA [18]. The DNA pellet was washed with 70 % ethanol, dried, and resuspended in TE overnight at 4 °C.

The phage DNA was digested using nine FastDigest endonucleases (Thermo Fisher Scientific, Inc.) according to

manufacturer recommendation: SmaI, HinfI, BamHI, XbaI, SalI, PvuI, BglII, EcoRV, and DraI. DNA fragments were visualized on agarose gels with ethidium bromide under UV light and documented by BioDoc system (Biometra, Germany).

Partial Genome Sequencing

The extracted and purified phage vB_Bbr_CN1 genomic DNA was partially sequenced by Ion Torrent method, with 50–150-fold coverage. De novo assembly was carried out with CLC Genomics Workbench 6.5 and Mira 4. Open reading frame (ORF) prediction was done using GenemarkS, Glimmer, and Rast [22–24]. The obtained sequence was analyzed by BLASTn algorithm (Basic Local Alignment Search Tool) [25] and used to create primers for the experiment performed in order to confirm the phage environmental origin.

Confirmation of Phage Environmental Origin

To confirm that the isolated phages originated from the samples and were not induced from bacterial host ATCC 10580, the presence of prophages in the host genome sequence (NZ_CM002881.1) was examined using the PHAST server (phast.wishartlab.com/). PHAST (PHAge Search Tool) is designed to identify, annotate, and graphically display prophage sequences within bacterial genomes [26]. The identified phage genome was subjected to theoretical digestion with endonucleases (http://tools.neb.com/REBsites/index.php) [27] and compared to obtained RFLP patterns of isolated phages. In addition, selected samples were heated at 100 °C for 20 min and plated in parallel with unheated samples to confirm the phage environmental origin. Finally, based on the partial genome sequence of vB_BbrS_CN1, a primer pair was created with expected product of 241 bp (Bbr-F 5′ TGACTTCATGGTTG CCGTTC 3′ and Bbr-R 5′ TCGGGAGCGTGATTTCAGTA 3′) and a PCR with host genomic DNA extracted using GeneJET Genomic DNA Purification Kit (Fermentas, Lithuania) was performed. A primer pair for housekeeping gene *recA* of *B. bronchiseptica* was designed (Bbr_recA-F: 5′ ATGGCGACAACGAGGTCGAA 3′ and Bbr_recA-R: 5′ CAGCAGGTCGGTCAGGTTGA 3′) and applied as a control of bacterial DNA quality, with the expected product of 263 bp. Thermal cycling conditions were as follows: an initial cycle of 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 20 s, and extension at 72 °C for 60 s, with a final 7-min extension at 72 °C. PCR products were analyzed on 2 % agarose gel with ethidium bromide. A positive control was phage DNA and a negative distilled water. In addition, DNAs of other isolated phages were used as templates, in order to confirm the presence of target genes in phage genomes, i.e., to examine their similarity.

Results

In this study, *B. bronchiseptica* phages were isolated for the first time from environmental samples. The phages were successfully isolated from 44.6 % samples (29 out of 65) (Table 1). The most successful phage isolation was from municipal wastewaters, since all samples were positive, while no single phage was isolated from swine farm wastewater. From all other types of samples, *B. bronchiseptica* phages were isolated, but with various success. In some samples, the number of bacteriophages was relatively high (more than 10 PFU/ml; the results are not shown).

From each positive sample, one plaque was isolated and 29 phages were further examined to determine their lytic spectra. On the original host lawn, all phages formed clear plaques, with diameter in range 0.5–2 mm, while on the lawn of alternative hosts the plaques were turbid (Table 2). Among 12 *B. bronchiseptica* strains, 5 strains were sensitive to bacteriophages including the original host, while 7 strains were not lysed by any phage. The lytic spectra of phages at 25 and 37 °C significantly differed, and besides the original strain, the phages were able to lyse 2 or 4 strains in addition. Accordingly, the phages possessed narrow or moderate activity spectra. Based on the host range, it was possible to observe only three different lytic groups at 37 °C: first group was able to lyse 25 % (16 phage isolates), while the phages of second and third group were able to lyse 33.3 % of the strains (second group comprises 11 and third group comprises 2 phage isolates). The results of lytic spectra obtained at 25 °C were more diverse than at 37 °C. In effect, six groups were differentiated: the first three groups were able to lyse 33.3 % of the strains (13 phage isolates in first, two in second, and one in third group), the phages of the fourth group were able to lyse 41.7 % (11 phage isolates) and the fifth and the sixth group were able to lyse 25 % of *B. bronchiseptica* strains (one phage isolate in each group).

Efficiency of plating (EOP) of *B. bronchiseptica* bacteriophages is shown in Table 3. According to the results, six phages showed higher EOP (10 and 100) on non-original host strains than on the original ATCC 10580. A large number of phages show equal EOP (1) for original and two alternative hosts. As expected, the most phages showed lower EOP in comparison to the original host, ranging from 0.001 to 0.1. One-step growth curve showed long latent period at both 25 and 37 °C (90 and 110 min, respectively), and more than double higher burst size at 37 °C (approx. 6 vs. 13) (Fig. 1).

Morphological characteristics of eight phages were examined, and since all these phages had highly similar morphology, only morphological details of four phages selected based on different RFLP and/or lytic activity are presented in Fig. 2 and Table 4. Based on their morphological properties, all phages appeared to be relatively uniform: they belong to order *Caudovirales*, as they have binary structure consisting of head

Table 2 Lytic spectra of *B. bronchiseptica* bacteriophages at 37 and 25 °C

Bacterial species	Label of strain	Lytic groups								
		37 °C			25 °C					
		I group	II group	III group	I group	II group	III group	IV group	V group	VI group
		vB_BbrS_CN1 vB_BbrS_CN2 vB_BbrS_CN3 vB_BbrS_CR2 vB_BbrS_CR3 vB_BbrS_FP2 vB_BbrS_FP3 vB_BbrS_LK4 vB_BbrS_MW3 vB_BbrS_MW5 vB_BbrS_RV1 vB_BbrS_RV2 vB_BbrS_RV3 vB_BbrS_SW1 vB_BbrS_SW2 vB_BbrS_TR1	vB_BbrS_CR1 vB_BbrS_FP1 vB_BbrS_FP4 vB_BbrS_LK1 vB_BbrS_LK2 vB_BbrS_LK3 vB_BbrS_LK5 vB_BbrS_MW1 vB_Bbrs_MW2 vB_BbrS_MW4 vB_BbrS_RV4	vB_BbrS_FP5 vB_BbrS_TR2	vB_BbrS_CN1 vB_BbrS_CR1 vB_BbrS_CR3 vB_BbrS_FP2 vB_BbrS_LK2 vB_BbrS_LK3 vB_BbrS_MW4 vB_BbrS_RV2 vB_BbrS_RV3 vB_BbrS_SW1 vB_BbrS_SW2 vB_BbrS_TR1 vB_BbrS_FP3	vB_BbrS_MW1 vB_BbrS_RV1	vB_BbrS_CN2	vB_BbrS_CN3 vB_BbrS_CR2 vB_BbrS_FP4 vB_BbrS_FP5 vB_BbrS_LK1 vB_BbrS_LK4 vB_BbrS_MW2 vB_BbrS_MW3 vB_BbrS_MW5 vB_BbrS_RV4 vB_BbrS_TR2	vB_BbrS_FP1	vB_BbrS_LK5
Bordetella bronchiseptica	L:594	–	–	+	–	+	+	+	–	–
	SEI	–	–	–	–	–	–	–	–	–
	LEG	–	–	–	–	–	–	–	–	–
	S2	–	+	–	+	–	+	+	–	+
	R1	–	–	–	–	–	–	–	–	–
	LORD	–	–	–	–	–	–	–	–	–
	973	–	–	–	–	–	–	–	–	–
	BbChiot	+	+	+	+	+	–	+	+	–
	CV1	–	–	–	–	–	–	–	–	–
	3416	+	+	+	+	+	+	+	+	+
	DEL	–	–	–	–	–	–	–	–	–
	10580[i]	++	++	++	++	++	++	++	++	++
Other species		–	–	–	–	–	–	–	–	–
No. of strains		3	4	4	4	4	4	5	3	3

++ clear lysis; + clear lysis with halo or turbid zone;– no reaction; [i] phage original host ATCC 10580

Table 3 Efficiency of plating (EOP) of *B. bronchiseptica* bacteriophages on two alternative hosts

B. bronchiseptica alternative host strains	EOP					
	100	10	1	0.1	0.01	0.001
BbChiot	vB_BbrS_LK4	vB_BbrS_CN1 vB_BbrS_CR3 vB_BbrS_LK5 vB_BbrS_MW3	vB_BbrS_CN2 vB_BbrS_CN3 vB_BbrS_FP1 vB_BbrS_FP3 vB_BbrS_LK2 vB_BbrS_MW2 vB_BbrS_RV1 vB_BbrS_RV4 vB_BbrS_SW2 vB_BbrS_TR1 vB_BbrS_TR2 vB_BbrS_MW5	vB_BbrS_CR2 vB_BbrS_FP5 vB_BbrS_LK1 vB_BbrS_LK3 vB_BbrS_MW1 vB_BbrS_MW4 vB_BbrS_RV3 vB_BbrS_SW1	vB_BbrS_CR1 vB_BbrS_FP4 vB_BbrS_RV2	vB_BbrS_FP2
3416	–	vB_BbrS_FP1 vB_BbrS_LK5	vB_BbrS_CN3 vB BbrS_CR3 vB_BbrS_FP2 vB_BbrS_LK4 vB_BbrS_RV1 vB_BbrS_TR1 vB_BbrS_TR2	vB_BbrS_CN2 vB_BbrS_CR2 vB_BbrS_FP3 vB_BbrS_FP5 vB_BbrS_LK1 vB_BbrS_LK2 vB_BbrS_MW1 vB_BbrS_MW2 vB_BbrS_MW3 vB_BbrS_MW4 vB_BbrS_MW5 vB_BbrS_RV4	vB_BbrS_CN1 vB_BbrS_CR1 vB_BbrS_FP4 vB_BbrS_LK3 vB_BbrS_RV2 vB_BbrS_RV3 vB_BbrS_SW1 vB_BbrS_SW2	–

and tail [28, 29]. The phages have head of length approx. 72 nm and the width 52 nm. They possessed a long, non-contractile, and flexible tail of length approx. 160 nm and the width 12 nm. Based on all characteristics mentioned above, *B. bronchiseptica*-specific phages belong to the family *Siphoviridae* and possess the prolate head, corresponding to Bradley's group B2 [30].

The nucleic acids of selected phages were extracted and digested using nine restriction enzymes. The digestion of

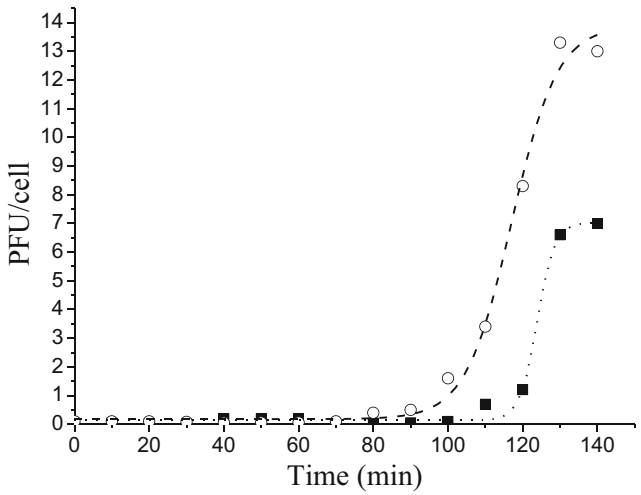

Fig. 1 One-step growth curve of phage vB_BbrS_CN1 at 25 °C (*square*) and 37 °C (*circle*)

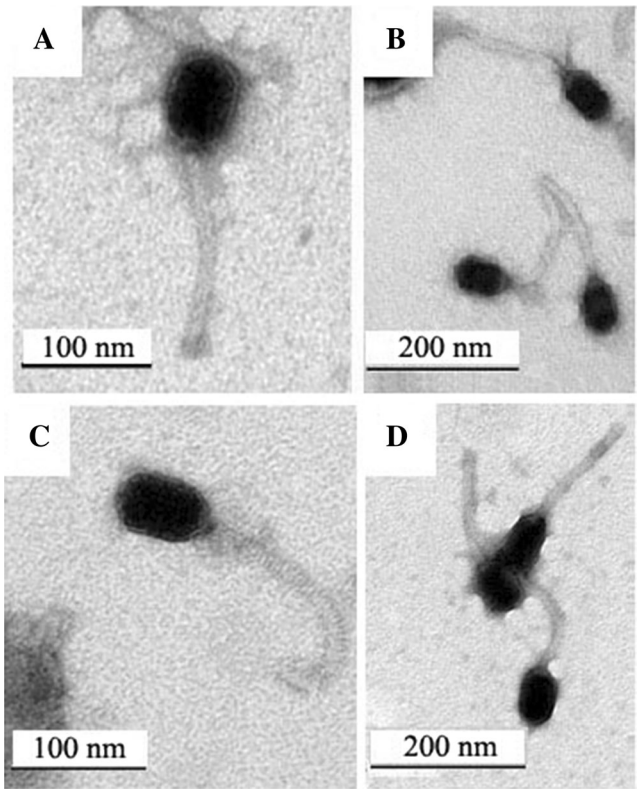

Fig. 2 Electron micrographs of selected *B. bronchiseptica* phages: **a** vB_BbrS_LK3, **b** vB_BbrS_FP2, **c** vB_BbrS_RV4, **d** vB_Bbr_CN1

Table 4 Morphological characteristics of phages

Phage	Phage particle dimensions (nm)			
	Head lenght	Head width	Tail lenght	Tail width
vB_BbrS_RV4	72.2 ± 1.5	50.6 ± 2.2	162.4 ± 3.9	11.8 ± 1.6
vB_BbrS_FP2	70.5 ± 3.5	49.4 ± 2.5	158.9 ± 1.9	12.6 ± 1.1
vB_BbrS_LK3	73.7 ± 2.6	54.9 ± 1.8	161.8 ± 4.9	13.1 ± 1.3
vB_BbrS_CN1	72.1 ± 1.3	53.4 ± 2.6	160.8 ± 3.1	12.8 ± 1.6

phage DNAs with BglII, PvuI, SalI, BamHI, XbaI, and DraI did not result in any visible fragment, due to the absence of restriction site or to the presence of modified bases. However, DNAs of all studied phages were cut by HinfI and SmaI. Both restriction endonucleases generated a great number of fragments (Fig. 3a, b), indicating the presence of many specific sites for these enzymes in phage DNAs. EcoRV gave patterns that allow genome size determination in phages vB_BbrS_CN1 and vB_BbrS_LK3 (Fig. 3c), which is estimated to be approx. 55 kbp. Using this enzyme, a faint band indicating a *pac* site was observed (Fig. 3c, arrow head).

By the genome examination of the strain ATCC 10580 by the PHAST server, only one phage was detected, highly related to a Cronobacter phage phiES15. However, the phage showed no similarity with any known phage when BLASTn was used. Its DNA is sensitive to all restriction endonucleases used in the study when theoretically digested, except for DraI and XbaI. Among enzymes used in the study, BamHI, DraI, and BglII are not sensitive to nucleotide methylation. Since BglII cannot cut DNA of any phage isolated in the study but can cut prophage DNA in the reference *B. bronchiseptica* strain in silico, it is clear that the isolated phages do not originate from the host strain. In contrast to unheated samples, no phage was isolated from heated samples, additionally confirming their origin from environmental samples and not from the bacterial host (Fig. 4).

The partial sequence of 2113 bp of phage vB_BbrS_CN1 (Access. No. KX015888; supplementary file 1) showed 40 % similarity with *P. aeruginosa* siphovirus phage LK04 (the result is not shown) belonging to genus *Yuavirus* when BLASTn was used. The PCR results with primer pair designed on the basis of vB_BbrS_CN1 sequence were negative since there were not annealing sites for the primers in total DNA of host strain (Fig. 5). The PCR was performed with DNAs of other selected phages and the product of the expected size was obtained.

Discussion

Bacteriophages are considered the most abundant entity in biosphere, whose estimated number is approx. 10^{30} with about 10^{8} species [31–33]. Up to date, many phages have been isolated, described, and their genomes have been sequenced. There are several reports on *B. bronchiseptica* phage isolation, but all of them were obtained from bacterial cultures by induction or spontaneous releasing, not from environmental samples. Rauch and Piccket [34] isolated 38 *B. bronchiseptica* specific phages from 48 strains and obtained three more as spontaneous virulent mutants of the isolates. Unfortunately, except from phage lytic spectra, other characteristics were not further examined. A temperate phage has also been noticed in a strain used for vaccine production [35]. The existence of this phage was only documented by

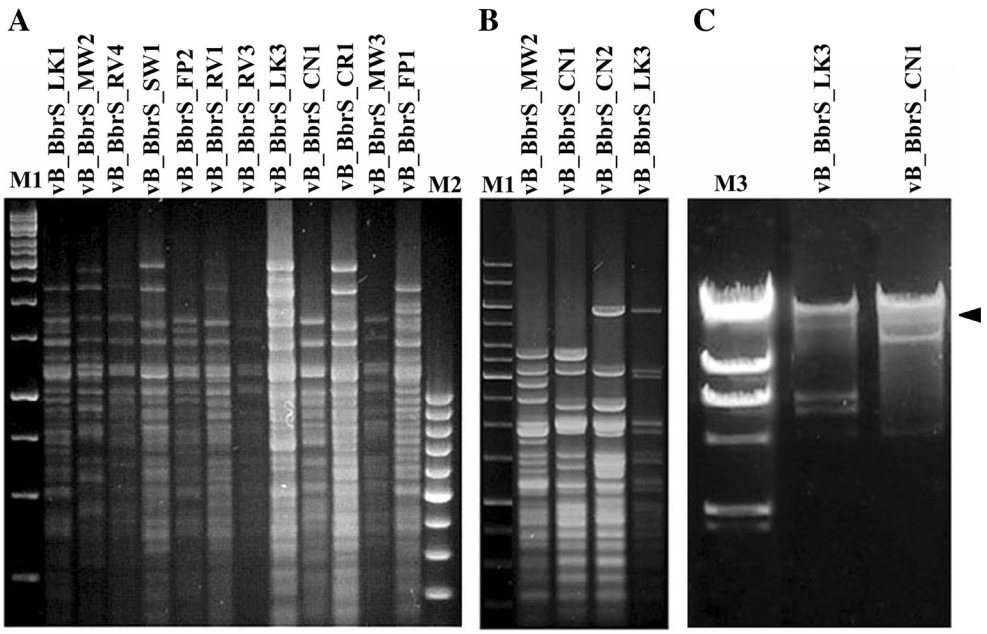

Fig. 3 RFLP profile of selected *B. bronchiseptica* phages DNAs digested with HinfI (**a**), SmaI (**b**), and EcoRV (**c**). *M1*: 1 kbp marker; *M2*: 100 bp marker; *M3*: Lambda DNA/HindIII marker

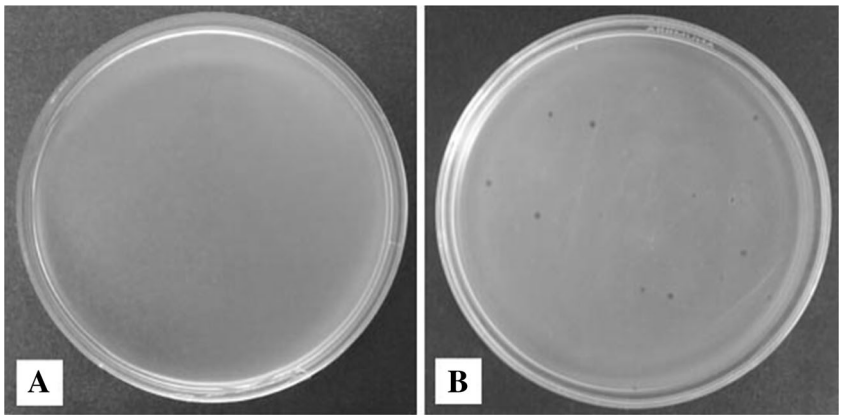

Fig. 4 Isolation of bacteriophages from a sample heated at 100 °C for 20 min (**a**) and the same unheated sample (**b**)

TEM, and according to a micrograph, it seems that it belongs to family *Siphoviridae*, morphotype B1. So far, only three highly related temperate *Podoviridae* (BPP-1, and its topic variants BMP-1 and BIP-1), identified in clinical isolates of *B. bronchiseptica*, have been described in more details and their genomes have been sequenced [12, 13].

For the first time, we isolated *B. bronchiseptica* bacteriophages from environment. The presence of *B. bronchiseptica* phages in water environment is very surprising, since phages are expected to inhabit environments in which their hosts exist [36], and *B. bronchiseptica* is not recognized as a water-related microorganism. It is also worth noticing that an enrichment procedure was not applied, but in some samples, the number of bacteriophages was relatively high (more than 10 PFU/ml). Although *B. bronchiseptica* is associated with swine, the phage isolation was unsuccessful from samples taken from swine water troughs and swine wastewater. In this regard, it must be taken into account that the samples from swine farm were filtered through a paper filter, and this pretreatment can cause a phage loss [9]. However, it is also possible that the phages were absent in the samples or that their number was very low.

The results of phage lytic spectra at 25 and 37 °C significantly differed. The obtained differences are not surprising, since the bacterium possesses BvgAS two-component signal transduction system, which regulates expression of genes responsible for virulence and motility. Growing the bacteria in rich media at 37 °C will induce Bvg^+ phase for which the expression of virulence genes such as filamentous hemagglutinin (FHA), fimbriae, and bifunctional adenylatecyclase/hemolysin (ACY) is typical. In the presence of millimolar concentration of modulators (nicotinic acid or $MgSO_4$) or at temperatures below 30 °C, BvgAS is negatively regulated (Bvg^- phase) and the bacterium exhibits decreased virulence, with upregulated flagellin expression [37, 38]. There is also a Bvg^i phase, i.e., interphase with upregulated FHA and fimbriae, and downregulated ACY, which is considered responsible for biofilm formation [38, 39]. The *B. bronchiseptica* phenotypic variation caused by temperature change, particularly in strains S2 and L: 594, probably had an impact on phage receptor expression as these two strains were more susceptible to a phage infection at 25 °C. It should also be stressed that Bvg^i and Bvg^- phases are temperature dependent and thus expected to be dominant in environmental and not in clinical samples. The broader phage activity spectra at 25 °C are in

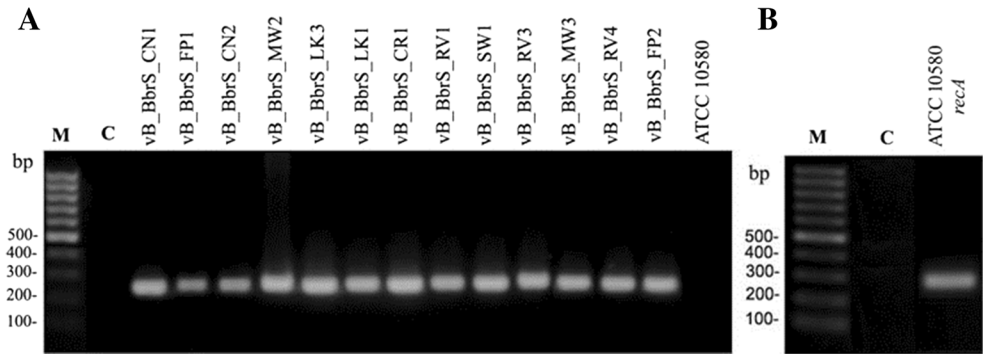

Fig. 5 PCR products: **a** obtained with primer pair Bbr-F and Bbr-R, created based on vB_BbrS_CN1 partial DNA sequence. All phages gave the expected product of 241 bp, while the product is absent when host DNA was used as a template; **b** positive control of bacterial DNA quality with primer pair Bbr-*recA*. *M*: 100 bp marker; *C*: a negative control with distilled water

accordance with their environmental origin. However, other reasons for the temperature difference in lytic spectra should not be neglected, including the possibility that the function of some phage proteins could be affected by temperature (receptor ligands, repressor protein, etc.). One-step growth curve pointed to the long latent period and generally small burst size at both temperatures, indicating that optimal growth conditions for *B. bronchiseptica* were not achieved and/or that phages predominantly formed lysogens, rather than entering a lytic cycle.

Considering the phage relative abundance in the environmental samples, it was expected that the phages were not specific for *B. bronchiseptica*. Surprisingly, inclusion of the other species in lytic spectra determination showed that the phages were not able to lyse any of the examined species, including members of classes Betaproteobacteria (family *Alcaligenaceae*) and Gammaproteobacteria (families *Enterobacteriaceae* and *Moraxelaceae*) (Table 2). These results indicate the specificity of the phages, at least for the genus *Bordetella*, although the possibility that they infect other species or strains, particularly of enterobacteria not included here into lytic spectra examination should not be abandoned.

The obtained EOP results clearly indicate that for some phages, alternative *B. bronchiseptica* hosts were more appropriate for infection. The reason for this can be the presence of more phage receptors on bacterial cells that enhanced adsorption and subsequent infection. However, the presence of a contaminating temperate phage spontaneously released or induced from propagation strain in the purified lysates or prophage induction from the strains used for assay after infection with examined *B. bronchiseptica* phages cannot be excluded.

Restriction digestion of extracted nucleic acid revealed that all bacteriophages possessed dsDNA within the virions. Taking into account the results of RFLP profiles, it can be noted that the phages were very similar, although some of them showed considerable variations in patterns when HinfI and SmaI enzymes were used. Additionally, as the product of the expected size was obtained in all examined phages by the PCR method with primers created based on vB_BbrS_CN1 partial sequence, it is obvious that the phage genomes are highly related. Using EcoRV, it was possible to determine genome size of vB_BbrS_LK3 and vB_BbrS_CN1, which is approx. 55 kbp. The presence of faint, i.e., submolar band in RFLP pattern, here obtained with EcoRV, is considered to be diagnostic of headfull packaging [40].

According to the PHAST server and in silico digestion by restriction endonuclease, the isolated phages in the study are distinctive from the prophage existing in *B. bronchiseptica* ATCC 10580 and are newly isolated. It is interesting to note that the phages are morphologicaly similar to those belonging to *Yuavirus*. According to the similarity of the phage vB_BbrS_CN1 sequence and morphology to member of this genus, there is a possibility that phages are able to form lysogens and thus inappropriate for application as an antimicrobial agent as whole virion. However, its lysine could be of interest for preparation of enzybiotics, so the phage genome should be further examined to detect the gene for endolysin.

The PCR with primer pair designed on the basis of vB_BbrS_CN1 sequence confirmed that phage has environmental origin since in total DNA of host strain there were not annealing sites for the primers. Additionally, phage environment origin was confirmed since in contrast to unheated samples, no phage was isolated from heated samples.

In summary, this is the first isolation of *B. bronchiseptica* bacteriophages from environmental samples in which they have proven to be relatively frequent and abundant. This is also the first time that the phages from family *Siphoviridae* specific for *B. bronchiseptica* are described in more details. All examined phages were highly related, showing similar morphology, but with certain variation in lytic spectra, EOP and RFLP patterns. Based on vB_BbrS_CN1 similarity to *Yuavirus* members that possess integrase, the phage is probably not useful for application as antimicrobial agent, but the future examination should be directed toward their endolysin characterization. More important, genome sequence and phage ability to form stable lysogeny can reveal its possible involvement in bacterial virulence, i.e., in the phenomenon of lysogenic conversion of their hosts.

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