

Efficiency of vB_BvS_ZagBV02 Phage in Controlling Antiseptic-Tolerant *Brevundimonas vesicularis*

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2 **Tolerant *Brevundimonas vesicularis***
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15 **Abstract**

16 *Brevundimonas vesicularis* has emerged as an opportunistic pathogen with reduced
17 susceptibility to antiseptics, posing a challenge for infection control. This study
18 investigated the genetic basis of antiseptic resistance and evaluated the lytic phage
19 vB_BvS_ZagBV02 as a potential antibacterial agent. PCR confirmed the presence of
20 the antiseptic-resistance gene (*qacE*) in *B. vesicularis*. Phage vB_BvS_ZagBV02
21 showed a siphovirus-like morphotype, with an icosahedral head measuring 57 nm and
22 a tail 142.8 nm in length. The phage vB_BvS_ZagBV02 adsorbed to host cells within
23 15 min, completed its infection cycle within 45 min, and had a burst size of
24 approximately 296 phage particles per infected cell. The phage remained stable at pH
25 3–9 and at temperatures up to 80°C, retained infectivity for 7 months at 4°C and under
26 freezing conditions and remained viable for 4 months at 37°C. Host range analysis
27 showed infection of another *Brevundimonas* strain but no activity against the other
28 tested bacteria. In planktonic culture, the phage reduced the bacterial growth at all tested
29 multiplicities of infection (MOIs), while on stainless steel surfaces complete
30 elimination of antiseptic-tolerant *B. vesicularis* was achieved when combined with
31 antiseptic treatment at MOI 1 and 10. Genome analysis showed that vB_BvS_ZagBV02
32 was assembled as a single contig of 42,502 bp with a GC content of 67.52% and
33 contained 62 predicted coding sequences, with no tRNA genes, antimicrobial resistance

genes, virulence-associated genes, or lysogeny-related genes. Comparative analyses using BLASTn, VIRIDIC, ViPTree, and tail protein phylogeny showed that vB_BvS_ZagBV02 is distinct from the selected reference phages and does not cluster closely with currently available genomes. These findings support the potential of vB_BvS_ZagBV02 as a phage-based approach for controlling antiseptic-tolerant *B. vesicularis*.

Keywords: *Brevundimonas vesicularis*, antiseptic resistance, bacteriophage vB_BvS_ZagBV02, phage therapy, genome characterization, infection control

1. Introduction

Antiseptics are chemical agents designed to inhibit or eliminate the growth of microorganisms such as bacteria, fungi, and viruses, on living tissues. They play a vital role in infection prevention particularly, in wound care, surgical preparation, routine clinical procedures, and personal hygiene. Common antiseptic agents include chlorhexidine, povidone-iodine, hydrogen peroxide, ethanol, quaternary ammonium compounds (QACs), and benzalkonium chloride. Despite their widespread use in infection prevention, the emergence of bacterial resistance to these agents has become an increasing concern in both healthcare and environmental settings [1-4].

Bacteria may become less susceptible to antiseptics through several mechanisms. These include intrinsic, acquired, environmental, or human-related factors. Intrinsic mechanisms include inherent structural features of the bacterial cell membrane that limit antiseptic uptake [5], the expression of efflux pumps that actively expel agents from the cell [6], and enzymatic degradation, where bacterial enzymes neutralize antiseptic compounds [7]. Acquired resistance arises through genetic mutations that alter antiseptic target sites [8] or via horizontal gene transfer, which enables bacteria to share resistance genes within populations. Furthermore, the misuse or overuse of antiseptics exerts selective pressure, favoring the survival of resistant strains [10]. Environmental factors also contribute, such as the application of sub-inhibitory concentrations of antiseptics [11], insufficient exposure times [12], and the presence of organic matter, which can significantly reduce efficacy [13]. Finally, human-related factors—including excessive use of antiseptics use, poor hygiene practices, and weak antimicrobial stewardship—further accelerate the spread of resistance [9].

In this context, *Brevundimonas vesicularis* requires attention as an emerging opportunistic pathogen. It is a Gram-negative, aerobic, non-fermenting bacterium that was once regarded as an environmental microorganism with limited clinical relevance. However, an increasing number of clinical reports have described its involvement in human infections, particularly in immunocompromised patients, individuals with chronic illnesses, and patients undergoing invasive medical procedures. *B. vesicularis* has been associated with bacteraemia, infective endocarditis, peritonitis, meningitis, and other systematic infections. Although it is still considered an uncommon clinical isolate, existing evidence underscores the clinical importance of this organism as an emerging opportunistic pathogen [14,15].

Reports indicate that *B. vesicularis* exhibits resistance to several antiseptics, including chlorhexidine [16], quaternary ammonium compounds [17], hydrogen peroxide [18],

and ethanol [19]. While its resistance mechanisms are not yet fully elucidated, they may involve efflux pumps, biofilm formation that shields cells from antiseptic agents [20], and genetic mutations that modify antiseptic target sites [21]. The antiseptic resistance of *B. vesicularis* has significant clinical implications, as infections caused by this bacterium may be difficult to control using standard antiseptic protocols.

Potential strategies to address this challenge include restricting antiseptic use to essential application, promoting improved hygiene practices, and developing novel antiseptics with enhanced activity against resistant strains. Another promising approach is bacteriophage (phages) therapy [59]. Phages are viruses that infect bacterial cells and, in the case of lytic phages, they disrupt cellular metabolism, ultimately leading to bacterial lysis. Phage therapy has been proposed as a viable alternative for managing bacteria resistant to antibiotics or antiseptics [22]. The high specificity, efficiency, safety, and adaptability of phages make them attractive candidates for therapeutic applications [23].

Therefore, the aim of this study is to investigate the mechanisms underlying bacterial resistance to antiseptics in *B. vesicularis*, isolate and characterize a lytic bacteriophage active against this bacterium and evaluate its potential use for controlling antiseptic-tolerant *B. vesicularis*.

2. Material and methods

2.1 PCR Detection of Antiseptic-Resistance Genes in *Brevundimonas vesicularis*

Polymerase Chain Reaction (PCR) analysis was used to detect antiseptic-resistance genes (*qacE* and *qacEΔ1*) in *Brevundimonas vesicularis* isolates. Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN) in accordance with the manufacturer's instructions. PCR amplification was performed using EmeraldAmp GT PCR Master Mix (Takara, Code No. RR310A) in a final reaction volume of 25 µl containing 12.5 µl of 2× premix, 4.5 µl of PCR-grade water, 1 µl of each primer (20 pmol), and 6 µl of template DNA. Primer sets targeting the *qacE* and *qacEΔ1* genes were used as described by [35]. The *qacE* primers generated a 341 bp amplicon, whereas the *qacEΔ1* primers generated a 361 bp amplicon (Table S1).

Thermal cycling conditions consisted of an initial denaturation step at 94 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1.5 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. A negative control without template DNA was included in each run. Amplified products were analyzed by agarose gel electrophoresis in TBE buffer supplemented with ethidium bromide (0.5 µg/ml), and bands were visualized under UV light using a gel documentation system [36].

2.2 Isolation and Purification of *Brevundimonas vesicularis* Bacteriophage

Sewage water samples were collected from four locations (Meat Abo Ali, Elqaniat, Saft Al Hana, and Bahr Elbaqar) in Sharkia Governorate, Egypt, in sterile dark bottles. Bacteriophage isolation was performed using the enrichment method described by [24]. Briefly, samples were centrifuged at 6000 rpm for 20 min, and the supernatants filtered

through 0.45 µm membrane filters and used as phage suspensions. Fifty milliliters of each filtrate were mixed with an equal volume of double-strength nutrient broth, inoculated with 1 ml of a fresh logarithmic-phase *B. vesicularis* culture, and incubated at 37 °C for 24 h with shaking at 120 rpm.

Following incubation, bacterial cells were lysed by the addition of 1 ml chloroform, and the mixtures were centrifuged at 6000 rpm for 30 min. The resulting supernatants were filtered through 0.45 µm membrane filters and used as bacteriophage stocks. Phage presence was assessed using the spot test [24] and confirmed by plaque assay [25]. Individual plaques were purified by repeated single-plaque isolation using the double-layer agar technique for three to five rounds [26]. Purified phages were stored in SM buffer at 4 °C until use.

2.3 Morphological Characterization and Phage Titration

Bacteriophage titers were determined using the double-layer agar method as described by [24]. Briefly, 100 µl of appropriately diluted phage suspensions was mixed with 100 µl of an overnight *B. vesicularis* culture and added to 3 ml of semi-solid brain heart infusion (BHI) agar. The mixtures were immediately overlaid onto nutrient agar plates and incubated at 37 °C for 18 h. Plaque-forming units (PFU/ml) were calculated from plates containing 30–300 plaques, and all titrations were performed in triplicate.

High-titer phage stocks were prepared from plates showing complete lysis by overlaying the surface with 3 ml of sterile saline solution (0.9% NaCl) and incubating overnight at 4 °C. The saline containing phage particles was collected, centrifuged at 6000 rpm for 10 min, and filtered through sterile 0.45 µm filters, and stored at 4 °C.

Phage morphology was examined by transmission electron microscopy (TEM). Purified phage suspensions with high PFU/ml were placed onto carbon-coated Formvar films mounted on 200-mesh copper grids. Excess liquid was removed using filter paper, and the grids were negatively stained with sodium phosphotungstate. Phage particles were examined and imaged using a Hitachi H600A transmission electron microscope at the Faculty of Agriculture, Mansoura University, Egypt.

2.4 Effect of Temperature and pH on Phage Stability

The stability of the isolated bacteriophage under varying temperature and pH conditions was evaluated as described by [37]. For thermal stability analysis, aliquots of phage suspensions were incubated at 50, 60, 70, 80, 90, and 100 °C for a defined exposure period. For pH stability, phage suspensions were incubated in buffer solutions adjusted to pH values ranging from 3 to 14. Residual phage activity was determined by spot assay on the bacterial host lawn, and stability was expressed as the reduction in phage titer relative to untreated controls.

2.5 *In Vitro* Longevity of the Isolated Phage

The long-term stability of the isolated bacteriophage was assessed under different storage conditions, including refrigeration (4 °C), freezing temperature (0 °C) and at

37°C . Phage suspensions were stored under sterile conditions, protected from light and agitation. Phage titers were determined monthly over a seven-month period using the double-layer plaque assay method and expressed as PFU/ml.

2.6 Phage Adsorption Rate

The adsorption kinetics of the bacteriophage to its bacterial host were determined according to [27]. The multiplicity of infection (MOI), defined as the ratio of phage particles to bacterial cells, was set at 0.1. Phage suspensions were mixed with exponentially growing *B. vesicularis* cultures and incubated at 37 °C. Aliquots were withdrawn at 5-min intervals, and the concentration of unadsorbed phage particles was quantified using the double-layer agar technique.

2.7 One-Step Growth Curve

A one-step growth experiment was conducted to determine the latent period and burst size of the isolated bacteriophage as described by [28]. Phage particles were allowed to adsorb to the bacterial host at 37 °C for 15 min, followed by centrifugation at 10000 rpm for 10 min to remove unadsorbed particles. The resulting pellet was resuspended in 10 ml of nutrient broth and incubated at 37 °C. Samples were collected at regular intervals over 45 min, immediately diluted, and titrated using the double-layer agar method. All experiments were performed in triplicate.

2.8 Host Range of the Isolated Phage

The host range of the isolated bacteriophage was evaluated against a panel of bacterial strains using the spot assay method [29]. Briefly, 3 ml of nutrient soft agar inoculated with 100 µl of a fresh bacterial suspension of each tested strain was overlaid onto solidified nutrient agar plates. After solidification, 10 µl of the phage suspension were spotted onto the agar surface. Plates were incubated at 37 °C overnight, and host susceptibility was determined based on the presence of clear lytic zones or plaque formation.

2.9 Effect of Phage at Different MOI on Planktonic Growth of *B. vesicularis*

The effect of the isolated phage on the growth of *B. vesicularis* was monitored in BHI broth over 210 min by measuring optical density at 600 nm (OD₆₀₀). Briefly, 1 ml of fresh bacterial culture was added to 50 ml of BHI broth followed by 1 ml of phage suspension adjusted at various multiplicities of infection (MOI). OD₆₀₀ values were then recorded at regular intervals from 0 to 210 min [30].

2.10 DNA Isolation and Sequencing

The phenol-chloroform-isoamyl alcohol technique was used to extract DNA (ZKX). Briefly, 0.22 µm membrane filter was used to sterilize high titer phage lysate (10¹¹ PFU/ml), which was then mixed with lysis buffer and incubated for one hour at 56°C. After adding an equivalent volume of phenol, chloroform, and isoamyl alcohol, the mixture was centrifuged for ten minutes at 10,000 × g. To precipitate the DNA, the aqueous phase was collected, mixed with ice-cold isopropanol and sodium acetate, and then incubated at -80°C. Following centrifugation, the pellet was air-dried, cleaned with

90% and 70% ethanol, and then resuspended in nuclease-free water. The FLUOstar Omega Microplate reader (BMG LABTECH, Germany) was used to measure the concentration and purity of DNA.

The Illumina DNA Prep Kit (Illumina, 20018705) and IDT for Illumina DNA/RNA UD Indexes Set B (Illumina, 20042666) were used to create indexed sequencing libraries. Five PCR cycles of library amplification were carried out. The Qubit 4 Fluorometer and Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, Q33231) were used for quantification. Using the High Sensitivity D1000 ScreenTape Assay (Agilent, 5067–5584 and 5067–5585), the library fragment size distribution was evaluated using the Agilent TapeStation 4200. The KAPA Library Quantification Kit for Illumina platforms (Roche, KK4824) was used to quantify libraries that were combined in equal amounts. The University of Nottingham used an Illumina MiSeq to sequence the pooled libraries using the MiSeq Reagent Nano Kit v2 (500 cycles, Illumina, MS-103-1003), producing 250 bp paired-end reads (LABTECH, Germany).

2.11 Genome Characterization and Bioinformatics Analysis of Phage vB_BvS_ZagBV02

2.11.1 Assembly, Annotation, and Safety Screening

The quality of the sequencing reads was assessed using Fastq Utilities [38]. As the reads showed acceptable quality, no trimming was performed. De Novo assembly was carried out in Qiagen CLC Genomics Workbench v20.0.3, yielding a single contig. The assembled genome sequence was compared against the NCBI database using BLASTn [39,40].

Coding sequences were predicted using Prodigal through the BV-BRC platform and PHOLD [41, 42]. Initial annotation was obtained using the BV-BRC auto-annotation pipeline and PHOLD, followed by manual curation based on BLASTp [39,40], InterProScan [43], and HHPred [44] to confirm the predicted functions and improve annotation of hypothetical proteins. Genes involved in the host lysis (endolysin, holin, and spanins) were further analyzed using DeepTMHMM and SignalP 6.0 [45, 46]. Transmembrane helices were predicted with DeepTMHMM, and potential polysaccharide depolymerases (DPOs) were identified using PhageDPO on Galaxy Docker platform [47]. Putative transfer RNA genes (tRNA genes) were identified using tRNAscan-SE [48] and ARAGORN [49].

Phage lifestyle and antimicrobial resistance gene content were assessed using PhageLeads [50]. Additional screening for antimicrobial resistance genes was carried out using Resistance Gene Identifier (RGI) within the CARD server (RGI v5.2.1, CARD v3.2.3) [51]. Lysogeny-related genes were assessed during manual genome curation. The curated genome annotation was visualized as a circular map on Proksee server [52].

2.11.2 Phylogenetic and Comparative Analysis

Comparative analysis of phage vB_BvS_ZagBV02 was performed using nucleotide- and proteome-based approaches. Whole-proteome comparison was carried out using the ViPTree server to examine the proteomic relationship of vB_BvS_ZagBV02 to reference phages [53]. Host prediction for vB_BvS_ZagBV02 was additionally

performed using PhageScope [64], applying default parameters, to complement the host-related signal obtained from the ViPTree proteomic tree. Reference genomes showing the highest similarity scores in ViPTree were selected for further analysis. Intergenomic similarity among vB_BvS_ZagBV02 and the selected reference phages were then assessed using the Virus Intergenomic Distance Calculator (VIRIDIC) under default settings [54].

Orthologous gene clusters shared between vB_BvS_ZagBV02, and the selected reference phages were identified using OrthoMCL in KBase in order to search for conserved proteins suitable for phylogenetic analysis [55]. Candidate proteins were screened based on annotation consistency, sequence length, and alignment quality, and only reliable homologs were retained for tree construction. Amino acid sequences of the selected marker protein were aligned in MEGA 11 using ClustalW. The best-fit amino acid substitution model was determined using the “Find Best DNA/Protein Models” function in MEGA 11. Phylogenetic analysis was then performed by the Maximum Likelihood method using the WG+G model, and branch support was evaluated with 1000 bootstrap replicates. Branch lengths were estimated as the number of substitutions per site, and the final tree was visualized in MEGA 11 [56].

2.12 Biofilm Reduction Assay

Stainless steel coupons (2 mm thick, 15 mm diameter). The coupons were prepared for treatment assays as described by Jan et al. (2011) [31]. The coupons were rinsed 5 times (5 min per rinse) with distilled water at 50 °C, followed by 5 rinses with distilled water at room temperature. They were then autoclaved for 20 min at 121 °C and placed individually into sterile microplate wells. A 300 µl aliquot of fresh bacterial culture was added to each well. The plates were incubated for 24, 48 and 72 h at 30 °C to allow for biofilm formation.

Following the incubation period, the cell suspensions were removed, and the coupons were rinsed with 9g/ L NaCl to remove non-adherent cells. To assess the effectiveness of phage treatment on antiseptic-tolerant *Brevundimonas vesicularis* biofilm, the coupons were treated with 300 µl of phage at different MOIs and incubated for 6 h at 30 °C. All tests were performed twice in triplicate.

After bacteriophage treatment, the coupons were rinsed three times with 9g/L NaCl solution and allowed to air-dry. Each coupon was then transferred to a new tube containing 5 ml of 0.02% Tween 80 solution. The recovered suspensions were centrifuged for 10 min at 7000 rpm at 25 °C. Finally, the cell pellets were resuspended in 1 mL of Tris – Saline (TS) buffer, and viable bacteria was enumerated using the micro-plate counting method [32].

2.13 Genome Sequence Accession Number

The complete genome sequence of phage vB_BvS_ZagBV02 was deposited in GenBank under accession number PZ288958.

2.14 Statistical Analysis

Each experiment was carried out in triplicate, with results expressed as the mean $\pm$ standard deviation (SD). GraphPad Prism 8.0.1 software was used for data analysis, including generating graphs and conducting one-way ANOVA. Statistical significance was defined as a p-value < 0.05 .

3.Results

3.1 Molecular Identification of *B. vesicularis*:

PCR was performed on two bacterial isolates to detect antiseptic resistance genes *qacE* and *qacEΔ1*. The PCR results revealed that the first isolate was positive for the *qacE* gene, whereas the second isolate was positive for the *qacEΔ1* gene (Fig.1). Based on these findings, the *qacE*-positive isolate, identified as *Brevundimonas vesicularis*, was selected for subsequent experiments because its specific bacteriophage was successfully isolated. The presence of the *qacE* gene suggests that this isolate possesses tolerance to antiseptics.

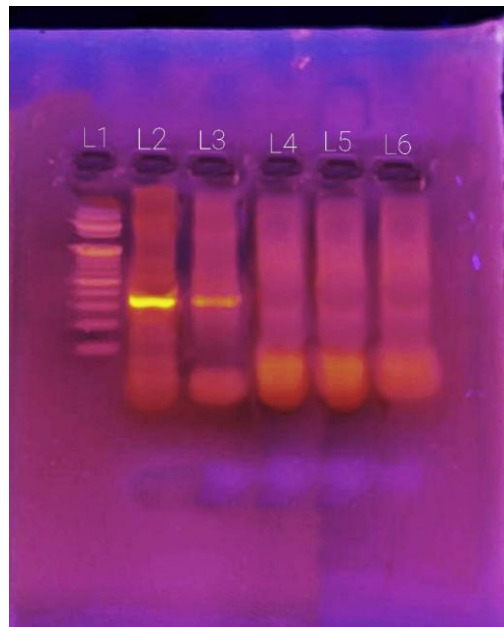


Fig.1: Gel electrophoresis analysis demonstrating specificity of PCR amplification for a *qacE* and *qacEΔ1* antiseptic-resistance genes; Lane 1: A gel pilot 100 bp DNA ladder (cat. no. 239045, QIAGEN, USA) is used to determine the size of the amplified DNA fragments. Lane 2: PCR amplicon from a bacterial isolate(*Brevundimonas vesicularis*) positive for *qacE* gene. Lane 3: PCR amplicon from another bacterial isolate positive for *qacEΔ1* gene . Lanes 4: control –ve. Lane 5-6: contained unrelated samples.

3.2 Bacteriophage Isolation and Morphological Characterization (TEM)

Bacteriophage infecting *B. vesicularis* were isolated from sewage water samples collected from four sites in Sharqia Governorate, Egypt. Phage Presence was first confirmed by spot and plaque assays. A single plaque was selected, purified, and designated as vB_BvS_ZagBV02 for further characterization. The plaque morphology of vB_BvS_ZagBV02 is shown in (Fig.2a).

Transmission electron microscopy (TEM) revealed the phage morphology, the phage had an icosahedral head with a diameter of approximately 57.0 nm and a long non-contractile tail measuring 142.8 nm (Fig.2b, c).

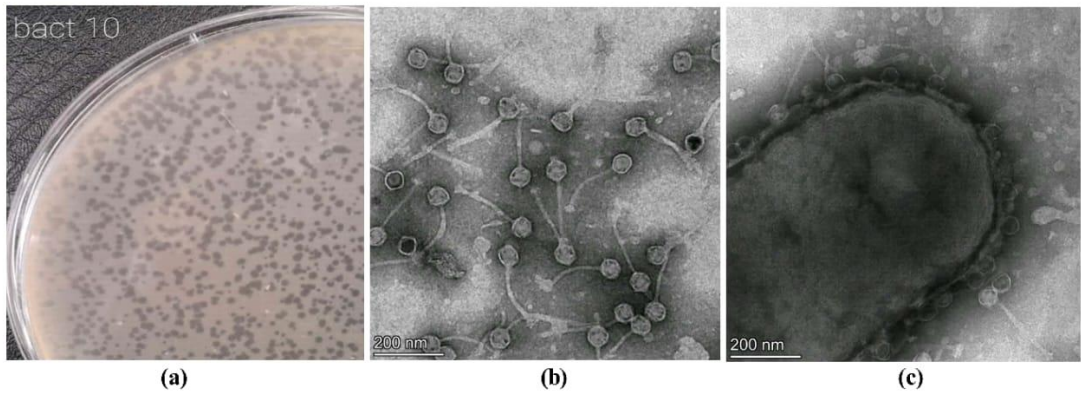


Fig.2 Morphological characteristics of *B. vesicularis* phage vB_BvS_ZagBV02 (a)Plaque morphology of vB_BvS_ZagBV02 on a double-layer agar. (b) Transmission Electron Micrograph showing multiple phage particles. (c) Higher magnification of TEM showing vB_BvS_ZagBV02 phage adsorption on *B. vesicularis* cell.

3.3 Effect of Temperature and pH on Phage Stability

The phage remained stable after exposure to temperatures up to 80 °C, with a gradual reduction in titer as temperature increased. No detectable lytic activity was observed after exposure to higher temperatures (Fig.3a). Regarding pH stability, vB_BvS_ZagBV02 phage remained stable over a pH range of 3 to 9. The phage showed the highest stability at pH 3 and pH 7. No detectable activity was observed at pH values above 9 (Fig.3b).

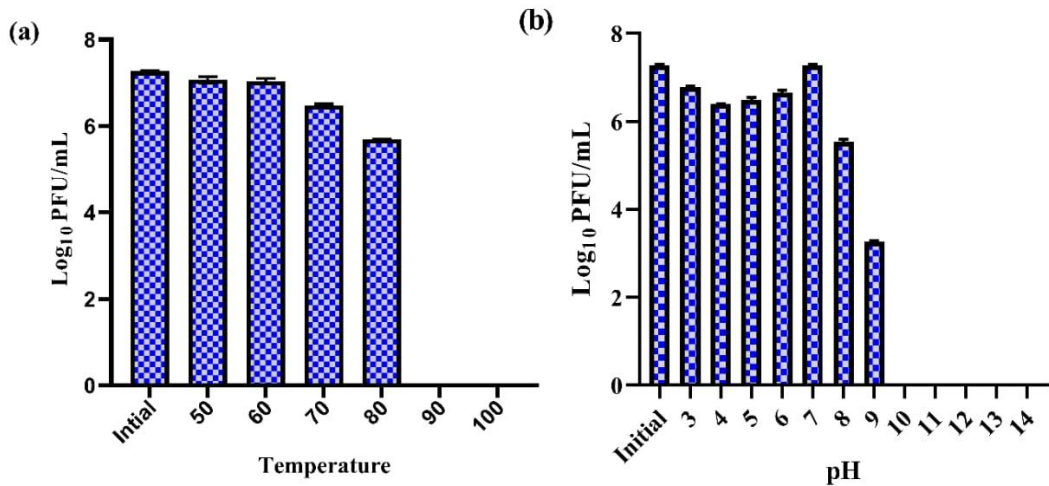


Fig.3 Stability of vB_BvS_ZagBV02 phage under different environmental conditions. (a) Thermal stability of the phage after exposure to different temperatures. (b) pH stability of the phage after exposure to different pH values The data are expressed as means $\pm$ SEM (n=3) which are significantly different at $P < 0.05$.

3.4 In Vitro Longevity of Phage vB_BvS_ZagBV02:

The phage vB_BvS_ZagBV02 retained its infectivity for up to 7 months when stored at 4°C and under freezing conditions, although phage titer gradually decreased over time in both cases. At 4 °C, the reduction in phage titer over the 7-month period was 1%, 2%, 3%, 30%, 49%, 51% and 53%, respectively. Under freezing conditions, the corresponding reduction rates were 1%, 1%, 11%, 11%, 19%, 28% and 38%. In contrast, phage infectivity declined more rapidly at 37°C, and no detectable activity was observed after 4 months (Fig.4).

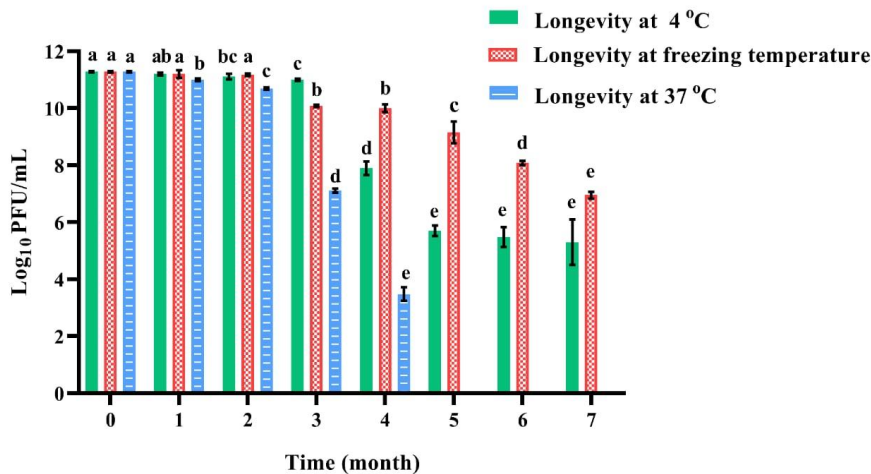


Fig.4 In vitro longevity of phage vB_BvS_ZagBV02 under different storage conditions. Phage titer was monitored monthly during storage at 4°C, under freezing conditions, and at 37°C. The data are expressed as means $\pm$ SEM (n=3) which are significantly different at $P < 0.05$.

3.5 Host Range of *B. vesicularis* vB_BvS_ZagBV02 Phage

The host range analysis showed that phage vB_BvS_ZagBV02 had a narrow host range. The phage was able to infect another *Brevundimonas* strain, *Brevundimonas diminuta*, but did not infect the other tested bacterial stains (Table 1).

Table 1: Host range of phage vB_BvS_ZagBV02 against selected bacterial strains

Bacterial host	Spot test
<i>E.coli</i> ATCC 8739	-ve
<i>Listeria innocua</i> ATCC 33090	-ve
<i>Enterobacter aerogenes</i> ATCC 13048	-ve
<i>Listeria monocytogenes</i> ATCC 13932	-ve
<i>Bacillus subtilis</i> ATCC 6633	-ve
<i>Pseudomonas aeruginosa</i> ATCC 27853	-ve
<i>Salmonella typhimurium</i> ATCC 14028	-ve
<i>Staphylococcus aureus</i> ATCC 25923	-ve
<i>Brevundimonas diminuta</i>	+ve

3.6 Adsorption Rate and One-Step Growth Curve of Phage vB_BvS_ZagBV02

The phage vB_BvS_ZagBV02 adsorbed rapidly to *B.vesicularis* cells, and most adsorption occurred within 15 minutes. The adsorption rate constant (K) was estimated to be approximately 2.0×10^{-10} mL/min (Fig.5a).

The latent period was approximately 20 min, followed by a rise period of about 30 min. The complete infection cycle was completed within 45 min. The burst size was estimated to be 296 phage particles per infected cell (Fig.5b).

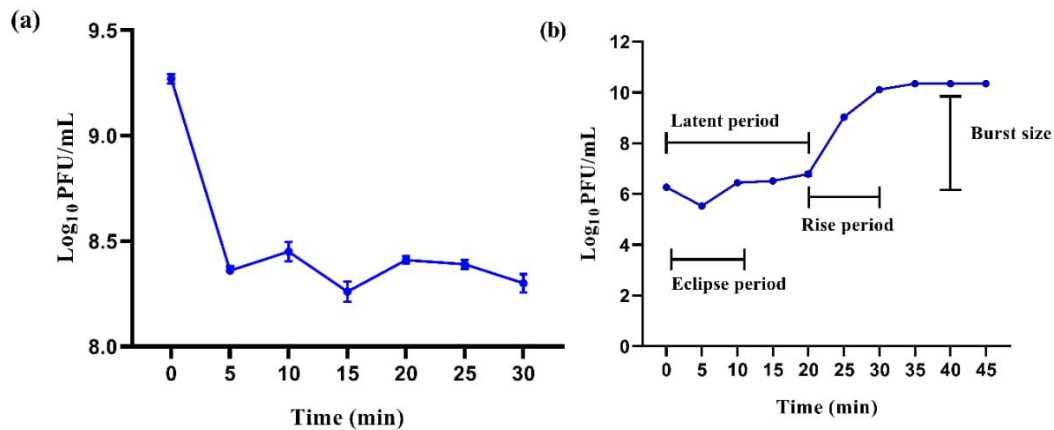


Fig.5 Adsorption rate and one -step growth curve of phage vB_BvS_ZagBV02.(a) Adsorption profile of vB_BvS_ZagBV02 on *B.vesicularis* cells over time. (b) One-step growth curve showing the latent period, rise period, and burst size of vB_BvS_ZagBV02 during infection of *B.vesicularis*. The data are expressed as means $\pm$ SEM (n=3) which are significantly different at $P < 0.05$.

3.7 Effect of Phage vB_BvS_ZagBV02 at Different MOIs on Planktonic Growth of *B. vesicularis*

Phage vB_BvS_ZagBV02 reduced the planktonic growth of *B. vesicularis* at MOIs of 0.1, 1, and 10 (Fig.6). At all the tested MOIs, the treated cultures showed lower OD values than the untreated control throughout the experiment. At the final recorded time point (210 min), the control culture reached an OD of 0.90 ± 0.01 , whereas the OD values of phage-treated cultures were 0.50 ± 0.01 , 0.45 ± 0.02 , and 0.50 ± 0.01 at MOIs of 0.1, 1, and 10, respectively.

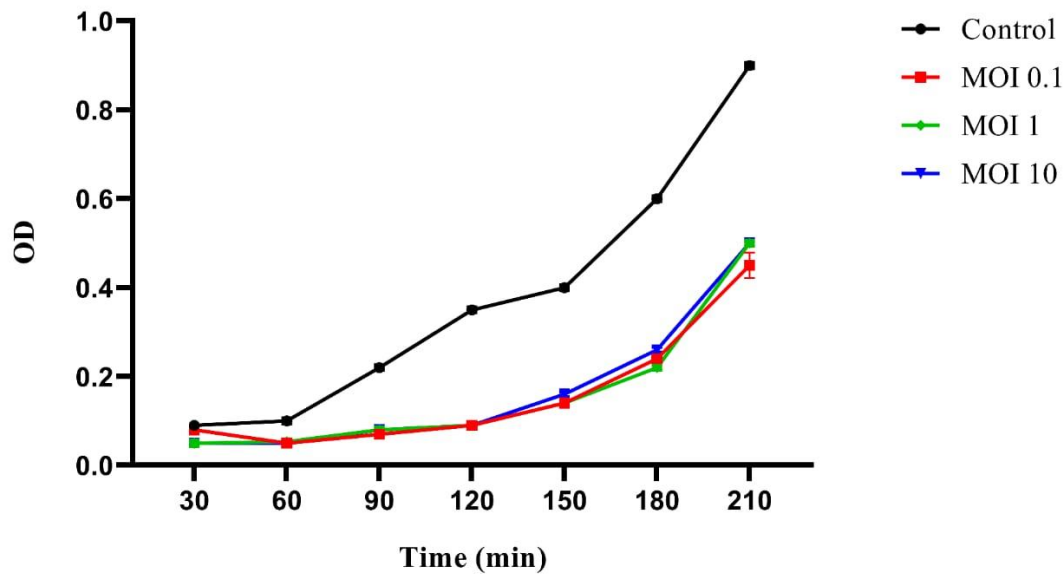


Fig. 6 Effect of phage vB_BvS_ZagBV02 on the planktonic growth of *B. vesicularis* at different multiplicities of infection (MOIs). Bacterial growth was monitored by measuring optical density at 600 nm over time in the untreated control and in cultures treated with phage at MOIs of 0.1, 1, and 10. The data are expressed as means $\pm$ SEM (n=3) which are significantly different at $P < 0.05$.

3.8 Genomic Characterization of vB_BvS_ZagBV02 Phage

3.8.1 Genome Assembly, Annotation and Safety Evaluation

De novo assembly of phage vB_BvS_ZagBV02 generated a single contig of 42,502 bp with a GC content of 67.52%. The genome sequence was deposited in GenBank under accession number PZ288958. Genome annotation predicted 62 coding sequences, of which 31 were assigned putative functions, while the remaining 31 were annotated as hypothetical proteins. No tRNA genes were detected (Table S2). Functional annotation showed that the genome encoded proteins involved in several major modules, including head and packaging functions (5 proteins), tail-associated proteins (6 proteins), DNA, RNA, and nucleotide metabolism (9 proteins), lysis-related proteins (4 proteins), connector proteins (2 proteins), immune-related proteins (2 proteins), and one infection-associated protein, in addition to two other proteins with predicted functions (Fig.7). A putative depolymerase-related domain was predicted in the tail protein encoded by ORF21, with a prediction score of 83%.

The predicted lysis cassette included an endolysin encoded by ORF25 containing a cysteine endopeptidase domain, suggesting a gamma-D-glutamyl-L-meso-diaminopimelic acid endopeptidase activity. The adjacent ORF28 encoded a type II holin with two transmembrane domains (TMD) and a length 85 amino acids. ORF31 encoded an Rz-like spanin, which was predicted to function as an inner membrane spanin (i-spanin) with one TMD at the N-terminus.

Genome safety screening did not detect any antimicrobial resistance genes, virulence-associated genes, or lysogeny-related genes.

BLASTn analysis showed that the closest match to vB_BvS_ZagBV02 was MAG: *Caudoviricetes* sp. isolate MSP1735 genomic sequence (OR221364.1), with 25% query coverage and 77.08% nucleotide identity. The remaining matches showed only very limited query coverage.

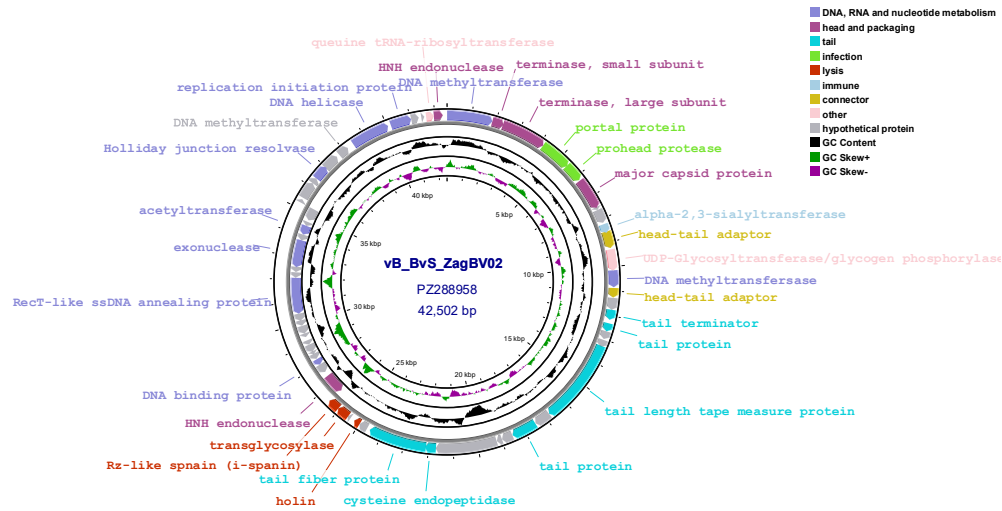


Fig.7 Circular genome map of *Brevundimonas vesicularis* vB_BvS_ZagBV02. Coding regions are color-coded according to the predicted functional categories: purple (head and packaging), turquoise (tail-associated proteins), blue (DNA, RNA, and nucleotide metabolism), red (lysis-related proteins), yellow (connector proteins), light blue (immune-related proteins), and green (infection-associated protein), pink (others proteins), and grey (hypothetical protein). The inner rings display GC content (black) and GC skew (green for positive, purple for negative).

3.8.2 Phylogenetic and Comparative Genomic Analysis

In the ViPtree proteomic analysis, vB_BvS_ZagBV02 was positioned on a separate branch and did not group closely with *Caulobacter* phages. Instead, it was located within a broader proteomic neighborhood within the host group Actinomycetota (Fig. 8a). Intergenomic comparison using VIRIDIC supported this observation. Phage vB_BvS_ZagBV02 showed very low pairwise intergenomic similarity values with all selected reference genomes and did not cluster with any of them. In contrast, several of the reference phages formed clear high-similarity groups among themselves (Fig.8b). PhageScope host prediction assigned vB_BvS_ZagBV02 to Pseudomonadota, differing from the Actinomycetota-associated neighborhood observed in the ViPTree proteomic tree. A phylogenetic tree based on the amino acid of the tail protein showed a similar pattern. In this analysis, vB_BvS_ZagBV02 formed a separate branch from the tail proteins of the selected reference phages, further supporting its divergence from the compared genomes (Fig. 8c).

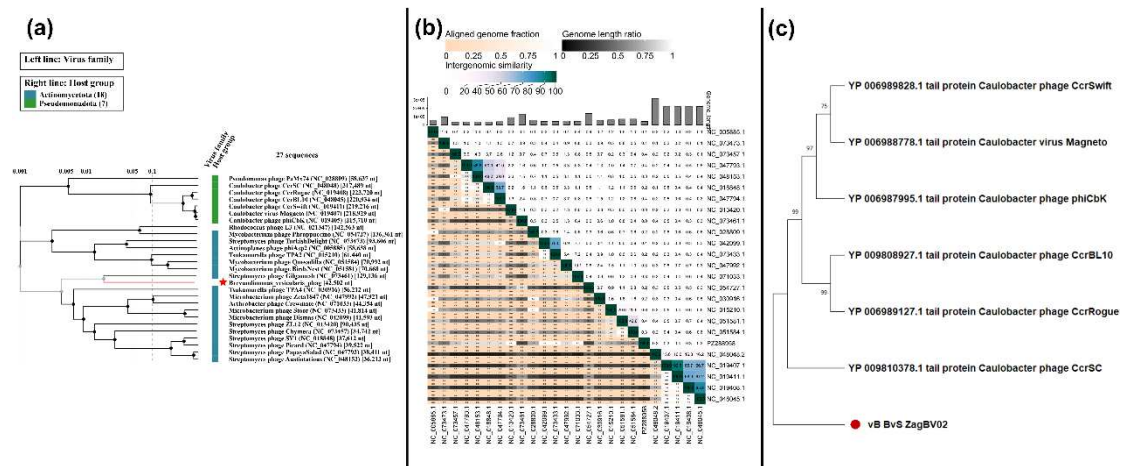


Fig.8 Phylogenetic analysis of phage vB_BvS_ZagBV02. (a) Whole-proteome comparison of phage vB_BvS_ZagBV02 using the ViPTree server. The position of the isolated phage in the proteomic tree is denoted with a red star. (b) VIRDIC heatmap showing pairwise genome similarity of the isolated phage (PZ288958) with the reference genomes of highest tBLASTx score (S_G score). (c) Maximum Likelihood phylogenetic tree based on the amino acid sequence of the tail protein of phage vB_BvS_ZagBV02 and selected reference phages.

3.9 Efficacy of Phage-Antiseptic Combination on Stainless Steel

Since *B. vesicularis* was originally isolated from sterilized stainless steel in the Intensive Care Unit (ICU) of Zagazig University Hospital, Sharqia, Egypt, the effect of phage treatment was evaluated on stainless steel coupons under specific conditions. Before the application assay, the possible effect of Cavicide on phage viability was examined by mixing Cavicide with isolated phage and incubating the mixture overnight. Phage activity was then checked by spot test and plaque assay. No reduction in phage viability was observed, as the phage titer remained unchanged at 10^{11} PFU/ml after exposure to Cavicide.

The treatment with Cavicide alone reduced the viable count of *B. vesicularis* but did not eliminate the bacterium completely, confirming its reduced susceptibility to the tested antiseptic (Fig.9). In contrast, combined treatment with Cavicide and vB_BvS_ZagBV02 phage produced a greater reduction in bacterial count. The reduction rate was 31.5% with Cavicide alone and 70% with the Cavicide-phage combination at MOI 0.1. No viable bacteria were detected after treatment with the Cavicide-phage combination at MOI 1 or MOI 10.

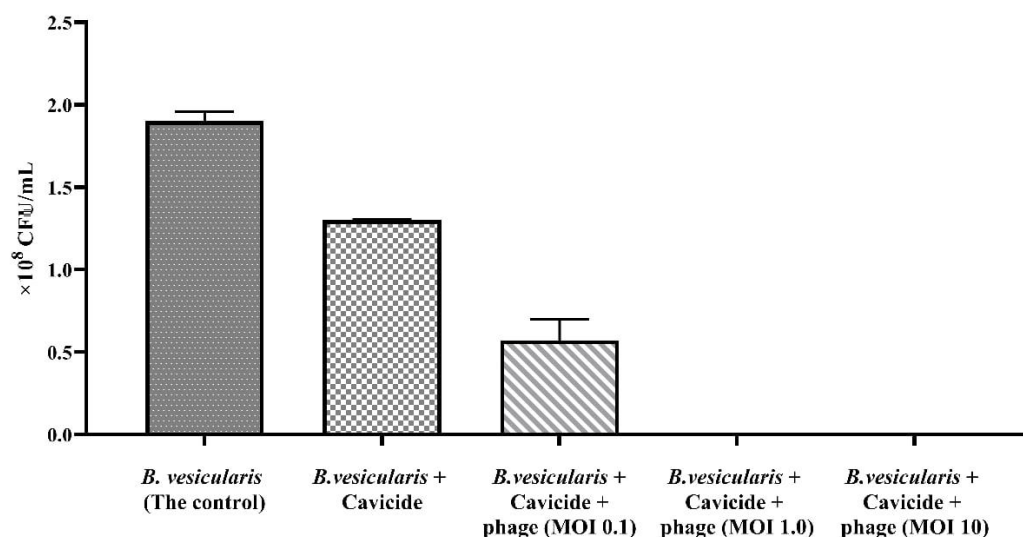


Fig.9 Effect of Cavicide alone and in combination with phage vB_BvS_ZagBV02 on antiseptic-tolerant *B.vesicularis* recovered from stainless steel surfaces. Bars represent viable bacterial counts after treatment with Cavicide alone and with Cavicide-phage combinations at MOIs of 0.1, 1, and 10, compared with the untreated control. The data are expressed as means $\pm$ SEM (n=3) which are significantly different at $P < 0.05$.

4. Discussion

Antiseptics and disinfectants are widely used to reduce microbial contamination on skin, instruments, and environmental surfaces, and they remain essential for infection control in health care and other settings [20, 60-62]. However, the emergence of bacterial resistance or reduced susceptibility to these compounds has become an increasing concern because it can reduce the effectiveness of routine decontamination procedures[20]. Several mechanisms have been linked to this phenotype, including genetic mutation, horizontal gene transfer and biofilm formation [34].

In this study, *Brevundimonas vesicularis* carried the *qacE* gene, while *qacEΔ1* was not detected. This agrees with previous reports describing the distribution of *qac* genes among Gram-negative bacteria and supports the view the antiseptic tolerance in this isolate may have a genetic basis [57,58]. At the same time, the presence of *qacE* alone doesn't fully explain the whole resistance phenotype, and other factors may also contribute.

To study this problem, a biological approach based on phage therapy was investigated [59]. A lytic bacteriophage, designed as vB_BvS_ZagBV02 was successfully isolated and characterized. Transmission electron microscopy (TEM) showed that vB_BvS_ZagBV02 phage had an icosahedral head and a long non-contractile tail, indicating a siphovirus-like morphotype. The phage also showed favorable biological

properties, including rapid adsorption, a latent period of about 20 min, a rise period of about 30 min, and a burst size of 296 phage particles per infected cell. These findings suggest efficient replication in the host bacterium. In addition, vB_BvS_ZagBV02 remained stable at temperatures up to 80 °C and across a pH range of 3–9, and it retained infectivity for prolonged periods under refrigerated and freezing conditions. Its host range was narrow, as it infected only another *Brevundimonas* strain among the tested bacteria. This narrow specificity may be useful for targeted control because it limits activity against non-target organisms.

The genome analysis supported the biological characterization of phage vB_BvS_ZagBV02. The genome was assembled as a single contig of 42,502 bp with a GC content of 67.52%, and annotation predicted 62 coding sequences, half of which remained hypothetical proteins. This is not unusual in phage genomes and reflects the limited functional information available for many phage genes in current databases [63]. The annotated genome showed the expected modular organization of a lytic tailed phage, including genes related to head and packaging functions, tail structure, lysis, and nucleotide metabolism. The predicted lysis cassette, which included an endolysin, a type II holin, and an Rz-like spanin, is consistent with the lytic behavior observed experimentally and with the multistep lysis system commonly required for phages infecting Gram-negative hosts [33]. No tRNA genes, antimicrobial resistance genes, virulence associated genes, or lysogeny related genes were detected. This genomic profile supports the lytic and apparently safe nature of vB_BvS_ZagBV02 and is favorable for further therapeutic or biocontrol consideration [50].

Comparative genomic and proteomic analyses indicated that vB_BvS_ZagBV02 is distinct from currently available reference genomes. BLASTn showed only partial similarity to the closest available sequence and low query coverage, suggesting that only a limited region of the genome matched known phages. This result was supported by the VIRIDIC analysis, in which vB_BvS_ZagBV02 showed very low inter genomic similarity values with all selected reference phages and did not cluster with any of them. This clear separation in the heat map indicates that the genome of vB_BvS_ZagBV02 is distinct within the comparison set. Since VIRIDIC is specifically designed for nucleotide-based inter genomic comparison of prokaryote-infecting viruses, this result strongly supports the genomic distinctness of vB_BvS_ZagBV02 [54]. The ViPTree analysis showed the same general trend at the proteome level, placing vB_BvS_ZagBV02 on a separate branch rather than within a close reference cluster [53]. The phage was positioned within a broader proteomic neighborhood dominated by phages associated with hosts from Actinomycetota, whereas PhageScope suggested a different host-related signal. These differences most likely reflect the limited representation of closely related phages in current databases and the different principles used by these tools for prediction. Therefore, the host-related output of these in silico analyses should be interpreted with caution. Notably, neither the ViPTree proteomic clustering nor the PhageScope host prediction matched the experimentally confirmed host of vB_BvS_ZagBV02, *Brevundimonas vesicularis* (class Alphaproteobacteria,

phylum Pseudomonadota). This is not unexpected: proteome- and sequence-based host prediction tools infer host association from patterns of similarity to reference phages with known hosts, rather than from direct experimental evidence, and their accuracy drops sharply for phages infecting under-represented host taxa such as *Brevundimonas*. The experimentally confirmed host range of vB_BvS_ZagBV02 (Section 3.5) therefore remains the most reliable indicator of its true host specificity. The tail protein phylogenetic tree also placed vB_BvS_ZagBV02 on a separate branch, in agreement with the VIRIDIC and ViPTree results. Taken together, these analyses support the view that vB_BvS_ZagBV02 is a genomically distinct lytic phage with limited similarity to currently available genomes.

The practical relevance of these findings was further demonstrated in the application assay on stainless steel surfaces. Since *B. vesicularis* had originally been recovered from stainless steel in a hospital setting, this model was directly relevant to the source of the isolate. Cavicide alone reduced the bacterial load but did not eliminate the organism completely, which is consistent with its reduced susceptibility to the antiseptic. In contrast, the combination of Cavicide with vB_BvS_ZagBV02 resulted in complete elimination of detectable bacteria at MOI 1 and MOI 10. In addition, Cavicide did not reduce phage viability under the tested conditions, which supports the compatibility of the two agents. The planktonic growth assay also showed inhibition of bacterial growth at all tested MOIs, with no marked difference among them. Taken together, these findings suggest that vB_BvS_ZagBV02 can enhance antiseptic-based control of *B. vesicularis*, and that an MOI of 1 may already be sufficient under the tested conditions.

Overall, the present study shows that vB_BvS_ZagBV02 combines favorable biological activity, a suitable genomic profile, and promising efficacy in combination with antiseptic treatment. These findings support the use of phage-based approaches as a potential strategy for controlling antiseptic-tolerant *B. vesicularis* in contaminated clinical environments.

Supplementary Materials

Supplementary materials associated with article include Table S1, which represents the oligonucleotide primers sequences of *qacE* and *qacE ΔI* gene; Table S2: Annotation table showing the open reading frames of phage vB_BvS_ZAGBV02 (PZ288958).

Author Contributions

G.E-D, A.A, A.E-B, A.E-S conceptualized the work; O.E.M performed the experiments; G.E-D, A.A, A.E-B, and O.E.M analyzed the results. Z.K.H performed the bioinformatics analysis; O.E.M and Z.K.H wrote and edited the manuscript. G.E-D, A.A, A.E-B, A.E-S edited and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Declarations

Ethical Approval

This study did not involve human participants, human tissues, or animals. Therefore, ethical approval was not required.

Availability of Data and Materials

The datasets generated and analysed during the current study are available from corresponding author on reasonable request.

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