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Genomic and evolutionary perspectives on bacteriophage ΦAfa-NA1 targeting MDR Gram-negative bacteria in diabetic ulcer

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Abstract. *Narulita E, Utami DAW, Izzaturrohmah L, Arrachmi HI, Febrianti RA, Ludfillah RZ, Widianto AH, Addy HS, Utami BR, Kuswati, Fikri K. 2025. Genomic and evolutionary perspectives on bacteriophage ΦAfa-NA1 targeting MDR Gram-negative bacteria in diabetic ulcer. Biodiversitas 26: 6014-6024.* The rise of Multidrug-Resistant (MDR) bacterial infections poses a significant challenge in clinical settings, particularly for diabetic ulcer patients who are highly susceptible to persistent infections. This study reports the isolation, characterization, and genomic analysis of a novel lytic bacteriophage, ΦAfa-NA1, specifically targeting MDR Gram-negative bacteria associated with diabetic ulcers in Indonesia. The bacteriophage was isolated from wound dressings and tested against four clinical isolates: *Alcaligenes faecalis* T17, *Pseudomonas* sp. FP1911, and the Gram-positive control *Streptomyces violaceoruber* S21. ΦAfa-NA1 exhibited strong lytic activity against three Gram-negative isolates but none against the Gram-positive control, confirming host specificity. ΦAfa-NA1 produced clear plaques with an average diameter of 3.0±0.5 mm on *Alcaligenes faecalis* T17, indicative of its potent lytic activity. Stability tests showed the phage retained >90% infective titer after 24 hours at -20°C and 4°C, and exhibited robust stability across a wide pH range (pH 6 to 11). One-step growth analysis revealed a short latent period of 21.7±8.1 minutes and a burst size of 27.7±1.5 PFU per infected cell. Genomic analysis showed ΦAfa-NA1 possesses a 78,105 bp double-stranded DNA genome with 45.8% G+C content encoding 83 predicted open reading frames. Comparative genomics and phylogenetic reconstruction confirmed its placement among T7-like Podoviruses, consistent with its obligate lytic lifecycle and genomic architecture. However, the potential for therapeutic application remains preliminary, as host range testing was conducted on a limited panel of single clinical isolates, and all assays were performed in vitro.

Keywords: Bacteriophage, diabetic ulcer, genome analysis, Gram-negative bacteria, phylogeny

INTRODUCTION

The global burden of diabetes mellitus is increasing rapidly, with the International Diabetes Federation (IDF) estimating that 643 million adults worldwide currently live with the disease, a figure projected to rise to 783 million by 2045 (IDF 2024). Indonesia is severely affected, ranking fifth globally with 21.3 million cases in 2024 (IDF 2024). High rates of complications compound this substantial prevalence: Indonesia ranks seventh for diabetes-related complications, where Diabetic Foot Ulcers (DFUs) contribute to a 32% mortality rate and account for 30% of all amputations (Indonesian Ministry of Health 2023). DFUs are complex, chronic wounds arising from impaired perfusion, tissue breakdown, and recurrent bacterial infections (Mayrovitz et al. 2023). The high risks of limb loss and mortality underscore the critical need for effective therapeutic interventions against the bacterial pathogens that exacerbate these chronic wounds.

DFU infections are typically polymicrobial, but are predominantly driven by Multidrug-Resistant (MDR) Gram-negative species. Common clinical isolates include *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and notably, *Alcaligenes faecali*s (Yang et al. 2024). This study focused on a panel of four bacteria, previously isolated from DFU patients, including the Gram-negative species: *Alcaligenes faecalis, Pseudomonas* sp., alongside the Gram-positive *Streptomyces violaceoruber*. *A. faecalis* is a notorious opportunistic pathogen frequently isolated in hospital settings, known to cause bacteremia and skin infections, and has developed extensive resistance to a range of antibiotics, including carbapenems and quinolones globally (Huang 2020; Samia 2024). Similarly, *Pseudomonas* species are a major clinical challenge due to their intrinsic and acquired MDR mechanisms, such as efflux pumps and

biofilm formation, complicating wound management (Qin et al. 2022). The prevalence of these MDR Gram-negative bacteria in DFUs highlights the urgent need for new treatment modalities.

The inclusion of *S. violaceoruber* in our panel is scientifically justified by the atypical and complex nature of chronic ulcers. *S. violaceoruber*, a soil bacterium, served as an essential Gram-positive negative control to validate the lytic specificity of the isolated bacteriophage towards the primary Gram-negative targets of the study (Sadigh-Eteghad et al. 2013; Atlaw et al. 2022).

To address the critical gap in therapeutic options created by increasing antibiotic resistance, bacteriophage therapy offers a promising alternative. Phages are viruses that specifically target and lyse bacteria, providing a mechanism that preserves the beneficial commensal flora (Fymat 2017). Phages possess several key therapeutic advantages: i) Ubiquity, being naturally abundant in diverse environments including human microbiota (Narulita et al. 2023); ii) Specificity, targeting only pathogenic bacteria; and iii) Lytic potency, functioning as obligate parasites that hijack bacterial machinery to produce progeny virions and induce cell lysis (Lin et al. 2017). The lytic cycle is ideal for therapy as it involves cell wall degradation and virion release, inherently avoiding host genome integration (Ranveer et al. 2024). This process is orchestrated by phage-encoded proteins essential for host recognition and cell lysis. Critically, phages exhibit a rapid co-evolutionary capacity, allowing them to circumvent or evolve countermeasures against bacterial resistance mechanisms, potentially, thus sustaining their lytic potency over time (Mavrich and Hatfull 2017; Naureen et al. 2020).

This study highlights the urgent need to characterize novel, locally sourced phages targeting common Indonesian MDR pathogens. The rich diversity of bacterial hosts in Indonesian environments offers a fertile ground for discovering unique phages adapted to these specific bacteria, which may possess novel genetic traits and lytic capacities. This local phage biodiversity is crucial for developing effective and targeted biocontrol agents against region-specific bacterial pathogens. We report the isolation, detailed biological characterization, and advanced genomic and evolutionary analysis of a novel bacteriophage, ΦAfa-NA1, specifically targeting MDR Gram-negative bacterial pathogens isolated from diabetic ulcers in Indonesia. This research provides a strong basis for assessing ΦAfa-NA's potential as an effective therapeutic agent.

MATERIALS AND METHODS

Bacterial rejuvenation

All strains were clinically isolated from diabetic ulcer patients and confirmed as multidrug-resistant isolates (Narulita et al. 2023).

Bacteriophage isolation and purification

The isolation protocol was adapted from Narulita et al. (2023). Bacteriophages were isolated from fifteen patient wound dressings, enriched using *Alcaligenes faecalis* T17 at an MOI of 0.1, and sterilized by filtration. The protocols were approved by the Ethics Committee of the Faculty of Dentistry, Universitas Jember (Approval Protocol No. 2669/UN25.8/KEPK/DL/2024).

Bacteriophage titer measurement

Double-layer agar assays yielded homogenous, clear plaques with a diameter of 3.0±0.5 mm (mean±SD, n = 3 independent replicates). Serial 10-fold dilutions (to 10^{-8}) were plated via double-layer agar assay and incubated at 37°C for 24 hours. Plaques were enumerated, and the morphology was noted as clear with sharp edges and a diameter of ~3mm in the double-layer assay (Narulita et al. 2023).

Host range test

The host range was tested on three Gram-negative MDR clinical isolates: *A. faecalis* T17, *Pseudomonas* sp. FP1911, each represented by a single isolate, and one Gram-positive control *Streptomyces violaceoruber* S21 (Narulita et al. 2023).

Stability test

Stability test for temperature (-20°C, 4°C, 25°C, 37°C, 50°C, and 60°C) and pH (4, 5, 6, 7, 8, 11, 12, and 13) were carried out in triplicate (n = 3). The SM buffer used to control pH was specified. Stability was assessed by determining the post-incubation titer (PFU/mL) (Choi and Kim 2021).

One step growth

The assay was performed in triplicate (n = 3). A proper adsorption phase of 5 minutes was ensured before diluting out non-adsorbed phages. The latency period, eclipse period, and burst size were determined from the data, and plaque counts determined phage concentration (Kawasaki et al. 2016).

Lysis kinetics assay

The lysis kinetics assay was performed to measure bacterial killing over time by monitoring the decrease in host bacterial density via Optical Density (OD600) measurements. Briefly, *A. faecalis* cells were incubated with bacteriophage ΦAfa-NA1 at a defined MOI in the presence of 10 mM $CaCl_2$ and chloramphenicol to inhibit bacterial protein synthesis, thus isolating the initial lytic effect. OD600 was measured every 5 min for 30 min using a spectrophotometer. Bacterial lysis was indicated by a significant reduction in OD compared to a control culture containing only bacteria without phage (Zhang et al. 2024).

Genomic and phylogenetic analysis

The whole-genome sequence (GenBank accession number [PV550095.1]) was screened using PHASTEST to confirm the absence of known lysogeny, virulence, and antibiotic resistance genes. Annotation was performed using Pharokka v1.3.2 (Galaxy Europe platform https://usegalaxy.eu/) and Geneious Prime. Phylogenetic reconstruction used UPGMA-based trees from MEGA 11 on the large subunit terminase gene and the whole-genome sequence, and complementary analysis of five essential protein families (RNAP, DNAP, ligase, MCP, lysozyme).

RESULTS AND DISCUSSION

Bacteriophage ΦAfa-NA1 characteristics

During isolation, ΦAfa-NA1 required a bacterial host for infection (Table 1). *Alcaligenes faecalis* strain T17 was selected as the primary host from the panel of four diabetic ulcer-associated bacteria due to its confirmed multidrug antibiotic resistance (Risqiyah et al. 2022). Initial characterization via spot testing and double-layer plaque assays revealed clear plaques with mean diameters of ~3mm (Figure 1.A) and ~1mm (Figure 1.B), respectively. Titer measurements, performed in triplicate, demonstrated a mean infectivity of 5×10^{6}±0.6PFU/mL, with infectivity observed up to a 10^{-7} dilution.

The host range of ΦAfa-NA1 was assessed against four diabetic ulcer-associated pathogens. Lytic activity, confirmed by spot assay, was observed against all tested Gram-negative species: *A. faecalis* T17 and *Pseudomonas* sp. FP1911 (Figure 2). Crucially, ΦAfa-NA1 exhibited no lytic activity against the Gram-positive bacterium *Streptomyces violaceoruber* strain S21. This lytic specificity to Gram-negative bacteria is advantageous for phage therapy, as it minimizes non-target effects and preserves the commensal Gram-positive flora in the wound environment (Fymat 2017).

Temperature stability assays, conducted across the full range of tested conditions, confirmed ΦAfa-NA1's viability across clinically relevant conditions. Phage ΦAfa-NA1 retained >90% infectivity after 24 hours at -20°C and 4°C. Full stability was observed at -20°C and 4°C, supporting its suitability for long-term storage, approximately 80% at 25°C, and 50% at 37°C. At 25°C, only minor degradation occurred, retaining short-term efficacy. While accelerated degradation was noted at 37°C (body temperature), infectivity against the host bacterium persisted, demonstrating therapeutic relevance at the site of infection. Complete inactivation at ≥50°C occurred only at elevated temperatures 50°C and 60°C, establishing its upper thermal limits. These values represent means±95% confidence intervals from triplicate independent experiments (Figure 3).

Similarly, pH stability tests revealed the phage's robustness across a wide range of conditions (Figure 4). For pH exposure, samples across pH 4 to 13 were neutralized post-treatment using buffered neutralization media to prevent carryover effects before titer assays. Phage was stable between pH 6 and 11 (>85% infectivity) and inactivated outside this range; full statistical analyses are reported. ΦAfa-NA1 remained stable across neutral (pH 7), slightly acidic (pH 6), and alkaline conditions (pH 8, 11). Inactivation was observed only at extreme acidic (pH ≤5) and extreme alkaline (pH ≥12) conditions. The tolerance to a range of pH 6-11 is vital for a phage intended for diabetic ulcers, whose pH can fluctuate significantly, often becoming alkaline, a condition that necessitates robust phage stability for topical application (Mayrovitz et al. 2023).

One step growth

The one-step growth assay (n = 3 replicates) quantitatively characterized the lytic life cycle of ΦAfa-NA1. One-step growth was conducted at MOI 0.01 with an adsorption phase of 5 min, sampling every 5 min until the plateau. The results were plotted on a curve to determine the key kinetic parameters (Figure 5.A). The average eclipse period was calculated as 1.7±1.1, and the average latency period was determined to be 21.7±8.1 min. The calculated burst size was determined to be 27.7±1.5 PFU/infected cell. A short latency period and a high burst size are hallmarks of highly virulent lytic phages, confirming ΦAfa-NA1 as an obligately lytic agent with potential for rapid bacterial clearance (Lin et al. 2017).

Table 1. Name of bacterial species

Bacterial species	Gram type
Streptomyces violaceoruer strain S21	Gram positive
Pseudomonas sp. FP1911	Gram negative
Alcaligenes faecalis strain T17	Gram negative

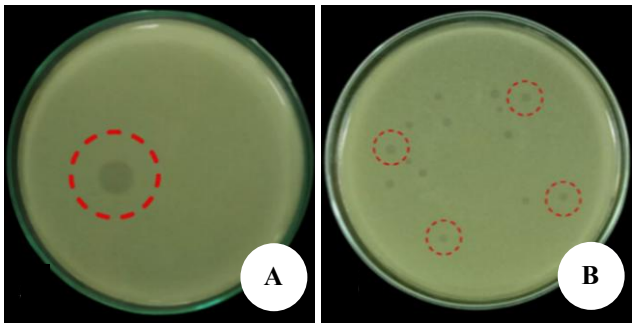

Figure 1. Plaque forming on the double layer media. A. Spot test, B. Plaque assay. Red circle shows clear plaque

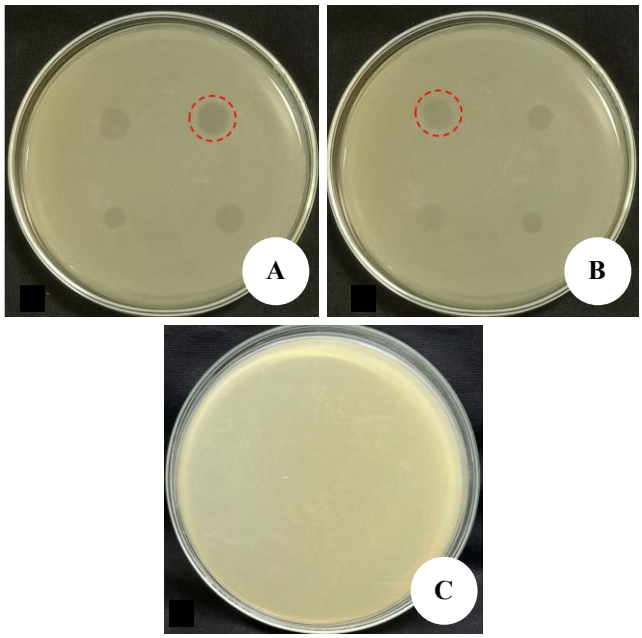

Figure 2. Host range test with the spot assay method on bacteria. A. *Pseudomonas* sp. FP1911, B. *Alcaligenes faecalis* strain T17, C. *Streptomyces violaceoruber* strain S21

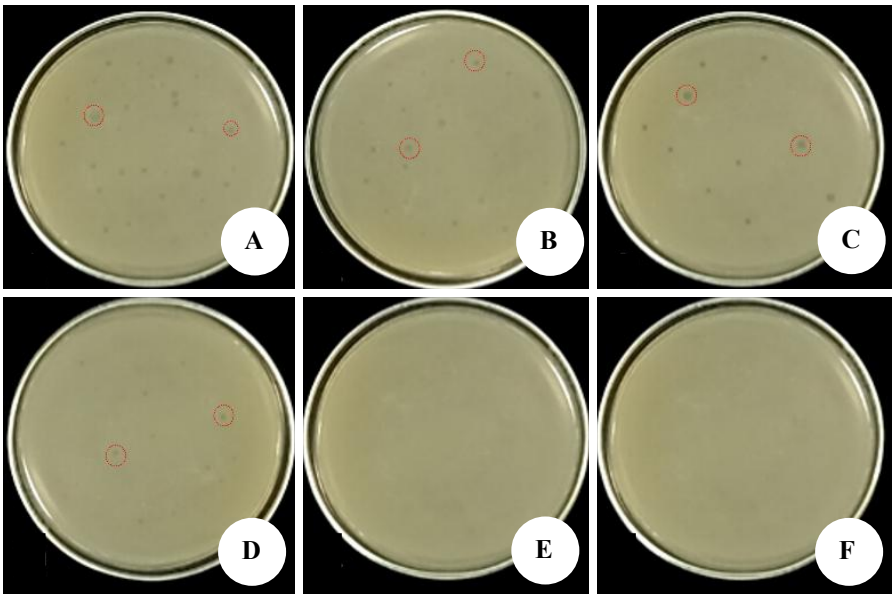

Figure 3. Temperature stability test. A. -20°C, B. -4°C, C. 25°C, D. 37°C, E. 50°C, F. 60°C

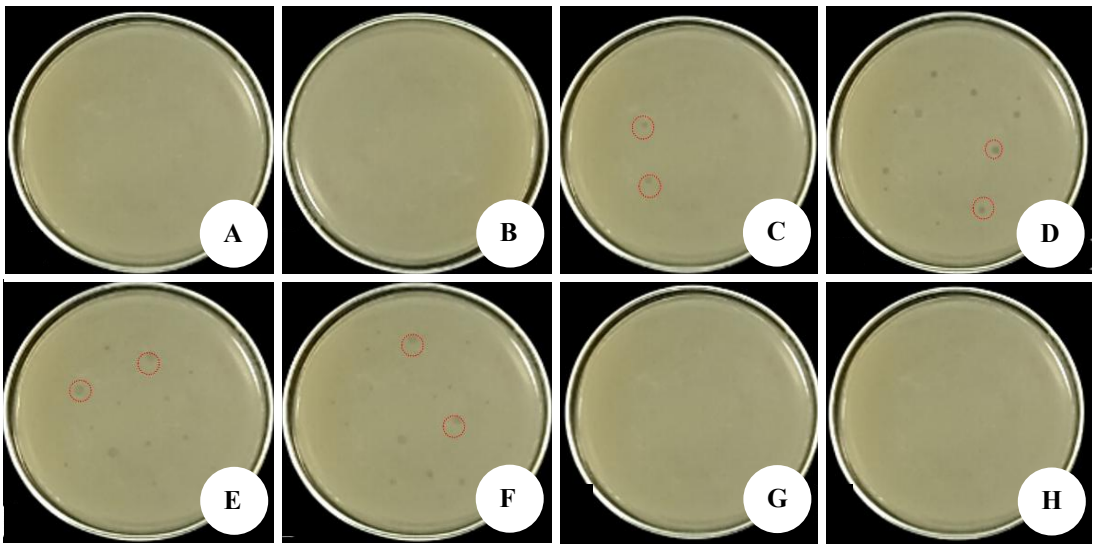

Figure 4. pH stability test. A. 4, B. 5, C. 6, D. 7, E. 8, F. 11, G. 12, H. 13

Lytic kinetic assay

The assay monitoring host density reduction over time, correctly termed the Lysis Kinetics Assay (Kawasaki et al. 2016), quantified ΦAfa-NA1's lytic efficacy against *A. faecalis*. Treatment groups exhibited a significant and rapid decline in OD_{600} following phage addition, indicative of effective host lysis. The control group, which contained only the bacterial host, showed increasing OD_{600} values consistent with normal bacterial growth (Figure 5.A). The rapid OD drop within the first 30 min confirms the phage's potent lytic capability, a desirable feature for initiating rapid treatment of acute infections (Zhang et al. 2024). The lysis kinetics assay employs chloramphenicol to inhibit bacterial protein synthesis, isolating initial phage-induced lysis without new virion replication. OD_{600} decline reflects bacterial lysis, not adsorption or replication kinetics, with limitations discussed.

Initially (5 min), treatment and control OD_{600} values were comparable (0.307 vs. 0.315), with only 2.54% lysis observed. Progressive OD_{600} reduction occurred in phage-treated cultures as contact time increased, reaching 0.122 by 30 min. Concurrently, control values rose to 0.399. Lysis efficiency increased from 20.49% at 10 min to 69.42% at 30 min, confirming ΦAfa-NA1's effective host lysis capacity (Table 2).

Genomic and phylogenetic analysis of ΦAfa-NA1

Comprehensive genomic and phylogenetic analyses elucidated the evolutionary relationships and genetic diversity

of ΦAfa-NA1. Screening with PHASTEST confirmed the absence of known lysogeny, virulence, and antibiotic resistance genes, which is paramount for establishing the phage's safety profile for therapeutic use.

The ΦAfa-NA1 genome is a linear double-stranded DNA molecule of 78,105 bp with a G+C content of 45.8%. It encodes 83 potential Open Reading Frames (ORFs). The detailed, annotated genome map (Figure 6) displays all coding sequences and hypothetical proteins, offering a clear visualization of the genomic architecture and key functional elements.

Comparative genomic analysis (Figure 7) revealed substantial size variation among related podovirus genomes: T7 (39,938 bp), vB_AbaP_QDWS (70,466 bp), CASP1 (47,254 bp), and ΦAfa-NA1 (78,105 bp). The genomes were functionally classified into three gene classes: Class I (early transcription), Class II (DNA metabolism), and Class III (structural proteins). The homologous regions highlight conserved genetic elements for essential components like DNAP, RNAP, MCP, Lysin, and Ligase, suggesting functional conservation characteristic of the Autographiviridae (T7-like) family.

Phylogenetic reconstruction, performed using advanced bioinformatics methods (UPGMA) on the large subunit terminase gene and the whole-genome sequence (Figure 8), positioned ΦAfa-NA1 within the class Caudoviricetes, confirming its close evolutionary relationship with T7-like Podoviruses. Complementary analysis of five essential protein families (RNAP, DNAP, ligase, MCP, lysozyme) further resolved the evolutionary trajectories (Figure 9), showing close clustering with phages like T7 and ΦAfa-NA1. The phylogenetic positioning and gene clustering suggest genomic adaptations and evolutionary divergence in ΦAfa-NA1 that may confer its observed broad host range and stability (Wangchuk et al. 2021).

Discussion

The isolation and comprehensive characterization of bacteriophage ΦAfa-NA1 directly address the critical need for alternative antimicrobial agents against MDR Gram-negative pathogens in diabetic ulcer infections. The study provides a robust foundation for evaluating ΦAfa-NA1's potential as a phage therapeutic. In this study, ΦAfa-NA1 produced consistently clear, well-defined plaques (±3 mm diameter) on *A. faecalis* T17 lawns—a hallmark of productive lytic infection. Plaque clarity directly correlates with phage-induced host lysis efficiency. Mechanistically, this involves the temporal coordination of holins (membrane pore formers) and endolysins (peptidoglycan hydrolases) encoded within the phage genome. ΦAfa-NA1's endolysin likely targets the glycosidic β-1,4 bonds between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan, followed by amidase or endopeptidase activity that disrupts cross-linking (Legotsky et al. 2014). The resulting osmotic imbalance culminates in cell burst and virion release—a process visually captured as plaque formation (Cisek et al. 2017; Lin et al. 2017). Beyond plaque clarity, diameter measurements further illuminate ΦAfa-NA1's infection kinetics and taxonomic implications.

Plaque diameter variation (typically 1-7 mm among phages) reflects capsid size, diffusion kinetics, and burst size. ΦAfa-NA1's 3-mm plaques align with T7 like Podoviruses (Ul Haq et al. 2012). Jamal et al. (2016) demonstrated that smaller capsids diffuse faster through agar matrices, explaining the correlation between plaque size and infection kinetics. Crucially, plaque clarity confirms ϕΦAfa-NA1's obligately lytic lifecycle, lacking integrase or repressor genes characteristic of temperate phages—a prerequisite for therapeutic safety (Abedon et al. 2021). Quantifying phage density and host specificity complements these morphological insights, directly informing therapeutic applicability.

Table 2. Optical density and percentage of lysis

Time (Min)	Control	Treatment	% Lysis
5	0.315	0.307	2.54%
10	0.327	0.260	20.49%
15	0.373	0.247	33.78%
20	0.382	0.210	45.03%
25	0.389	0.156	59.90%
30	0.399	0.122	69.42%

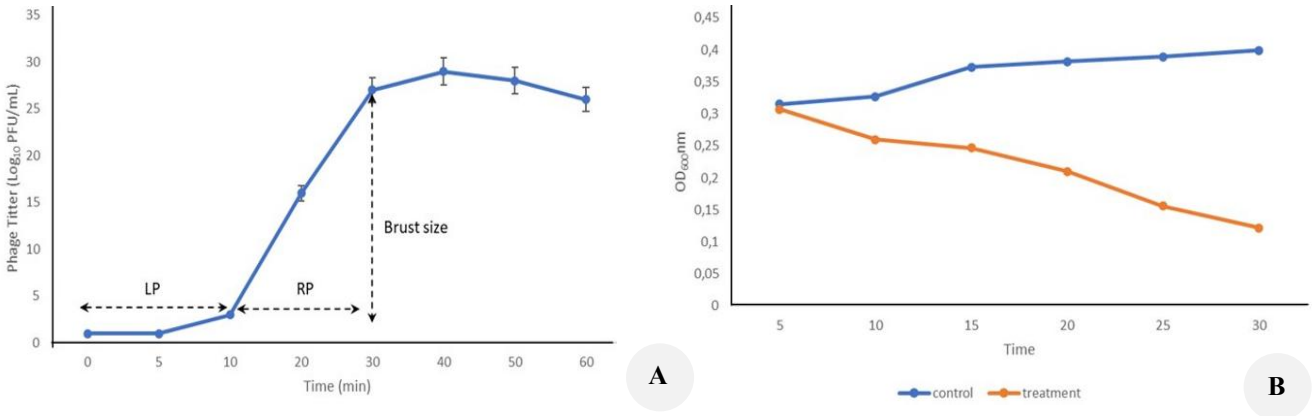

Figure 5. A. One-step growth curve. LP: Latent Period, RP: Rise Period, and burst size. The results show the mean of 3 experiments. Bars: Standard deviation. B. Lysis curve of *Alcaligenes faecalis* with the addition of bacteriophage MOI 0.01

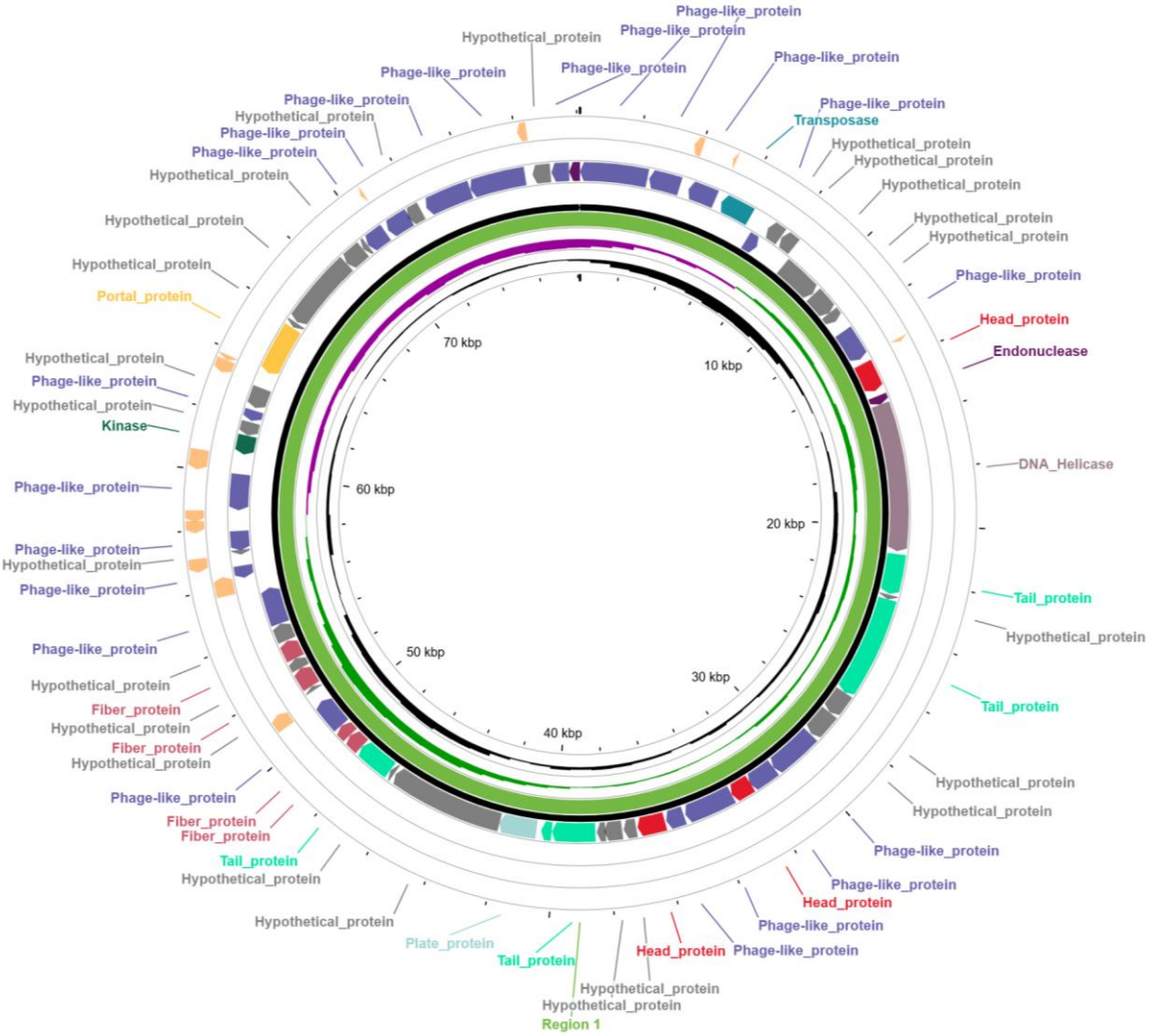

Figure 6. Circular map of bacteriophage ΦAfa-NA1

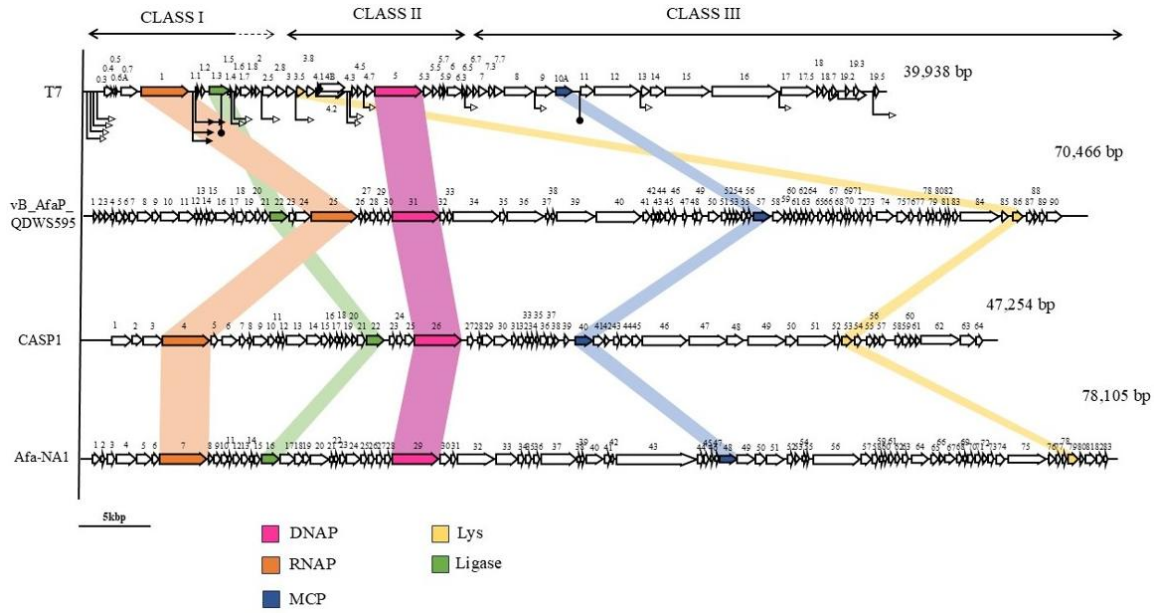

Figure 7. Comparative genomic analysis of four different bacteriophages: T7, vB_AbaP_QDWS, CASP1, and AfaNA1. Pink: DNA Polymerase (DNAP), Orange: RNA Polymerase (RNAP), Blue: Major Capsid Protein (MCP), Yellow: Lysin (Lys), Green: Ligase (Ligase)

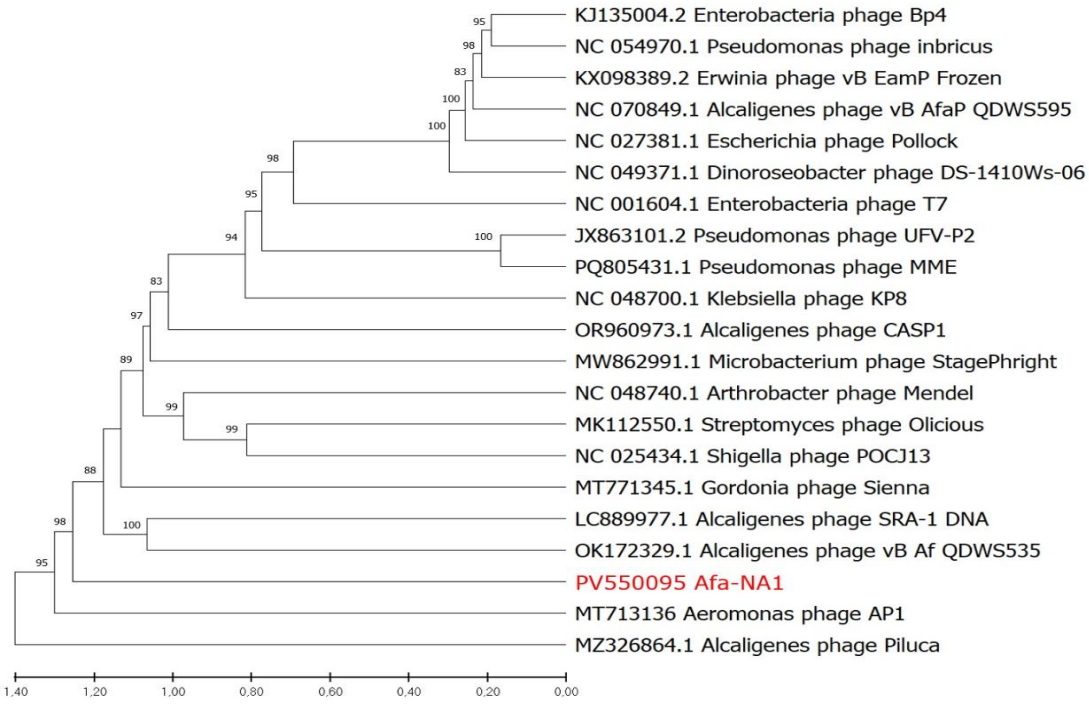

Figure 8. Phylogenetic tree of AfaNA1

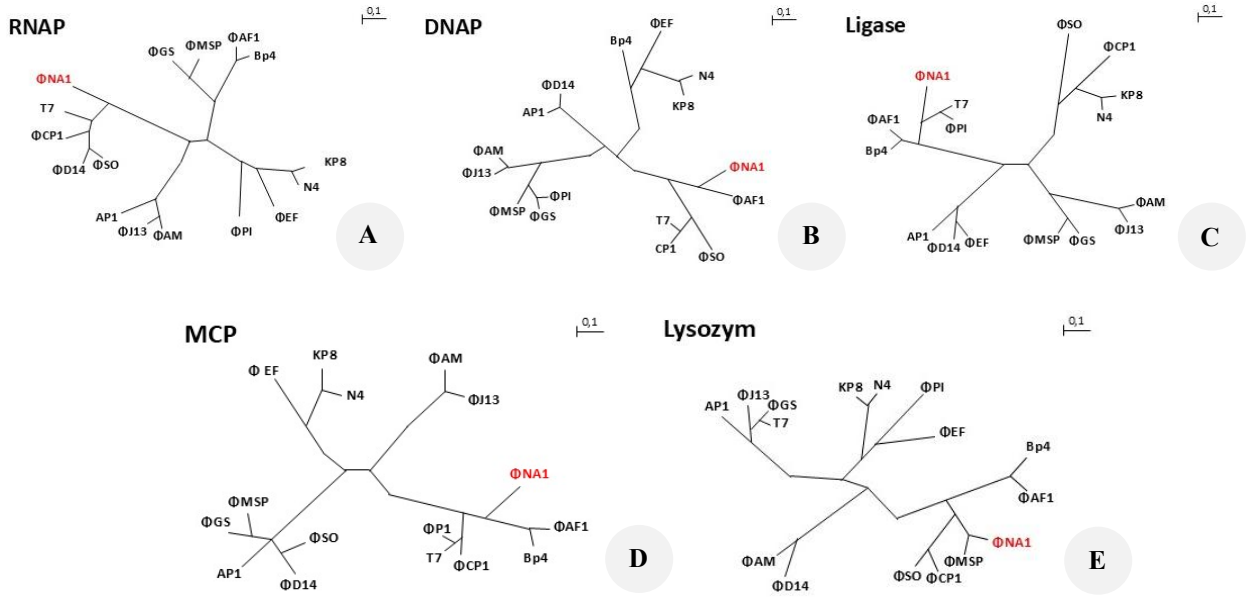

Figure 9. Phylogenetic tree with important genes. A. RNAP, B. DNAP, C. Ligase, D. MCP, E. Lysozyme

A titer of 5 × 10^6 PFU/mL indicates substantial virion yield, positioning ΦAfa-NA1 for practical applications. Titers exceeding 10^6 PFU/mL are clinically relevant, as phage cocktails require ≥10^7 PFU/mL doses for efficacy in deep-tissue infections (Lingga and Budiarti 2023). In this study, bacteriophage ΦAfa-NA1 was characterized for its lytic activity against three multidrug-resistant Gram-negative clinical isolates relevant to diabetic foot ulcer infections. Our host range assays, limited to single isolates per species, showed infectivity against *Pseudomonas* sp. FP1911 and *A. faecalis* T17, while no activity was observed against the Gram-positive *S. violaceoruber* S21 (Figure 2). Due to the small isolate panel, claims of a broadly applicable host range remain preliminary and warrant expanded testing across

multiple strains and species for therapeutic validation. The observed broad host range, encompassing three distinct MDR Gram-negative species (*Pseudomonas* sp. and *A. faecalis*), is a significant finding. Given this limited panel, we do not claim a broad host range. Future work will expand isolate diversity to assess therapeutic breadth across species and strains better. However, the ϕAfa-NA1 bacteriophage infects more than one species. Bacteriophages generally have a specific host range, meaning they are capable of infecting one or several strains of bacteria within a single species. However, there are bacteriophages that are capable of infecting more than one species of bacteria, such as the ϕAfa-NA1 bacteriophage. This demonstrates the biodiversity in the infection capabilities of bacteriophages and their potential influence on complex bacterial communities (Ross et al. 2016).

Bacteriophages with a multi-species host range can bind to and enter bacterial cells from different species, which is influenced by the interaction of phage surface proteins with specific receptors on the target bacteria. This ability allows bacteriophages to play a role in regulating diverse bacterial populations and can facilitate horizontal gene transfer, including antibiotic resistance genes, between different bacterial species (Göller et al. 2021). A broader host range also has implications for the use of bacteriophages in phage therapy, where bacteriophages with multi-species infection capabilities can provide greater effectiveness in treating mixed bacterial infections (Hyman and Abedon 2010; Ross et al. 2016).

In contrast, ΦAfa-NA1's inability to infect the Gram-positive *S. violaceoruber* strain S21 emphasizes the role of bacterial cell wall composition and receptor specificity in determining phage host range. Gram-positive bacteria differ significantly in their cell envelope architecture, primarily featuring thick peptidoglycan layers and lacking the outer membrane characteristic of Gram-negative bacteria. This structural difference likely impedes ΦAfa-NA1 binding and adsorption, restricting its infectivity. Such strict specificity towards Gram-negative hosts aligns with the known molecular mechanisms of phage receptor recognition, tail fiber interaction, and host adsorption, which are often highly adapted to particular bacterial surface molecules (Dunne et al. 2018).

Genomic analysis identified putative tail fiber proteins with lectin-like domains potentially binding to bacteria Lipopolysaccharide (LPS) O-antigens. While broad-host-range phages offer polyvalent utility, narrow specificity reduces off-target effects and resistance selection in commensals (Ross et al. 2016). This precision is advantageous for targeted ulcer therapy, where *A. faecalis* dominates polymicrobial communities. The translational potential of these traits is contingent upon biophysical resilience—a critical parameter we systematically evaluated under clinically relevant conditions.

Thermal and pH stability assays demonstrated retention of >80% infectivity within clinically relevant temperature and pH ranges. At subzero and refrigeration temperatures (-20°C/4°C), the phage exhibited near-complete stability (>90% viability after 6 months) due to suppressed enzymatic degradation and vitrification of capsid proteins (Narulita et al. 2025). Under ambient conditions (25°C), moderate degradation occurred (~20% viability loss at 7 days), yet retained infectivity remained suitable for tropical climate applications. At physiological temperature (37°C), accelerated degradation (~50% loss within 48 hours) still supported acute therapeutic utility given concurrent phage replication (Figure 3). Irreversible inactivation occurred at ≥50°C through capsid denaturation and DNA depurination.

Complementary pH tolerance spanned pH 6-11 (Figure 4), with optimal stability at neutral to slightly alkaline conditions (pH 7-8). Acidic environments (pH ≤5) induced capsid aggregation through protonation of surface proteins, while extreme alkalinity (pH ≥12) triggered dsDNA hydrolysis. Critically, the pH 6-8 stability range aligns with diabetic ulcer environments (pH 4.3-7.6; Das and Bal 2024), overcoming a key limitation of acid-labile therapeutic phages. Pre-adaptation through progressive exposure to acidic media could further enhance gastric survivability should oral delivery routes be explored. To decipher the molecular basis of ΦAfa-NA1's stability and lytic efficiency, we resolved its complete genomic architecture.

One-step growth analysis (Figure 5.A) revealed remarkably efficient replication kinetics characterized by a short latent period of 21.7±8.1 min and a burst size of 29 PFU/cell—surpassing CDHS-1 phage (15 PFU/cell; Nale et al. 2021) and rivaling Vp11 (32 PFU/cell; Tan et al. 2021). This exceptional efficiency stems from three synergistic factors: rapid adsorption (>80% within 5 min) mediated by Ca^{2+}-facilitated tail fiber binding to LPS receptors; a streamlined genome lacking metabolic "auxiliary" genes that accelerates replication; and optimized MOI (0.01) that prevents premature lysis through holin inhibition at high virion density ("lysis inhibition"). Complementary adsorption assays (Figure 5.B) quantified these dynamics, demonstrating 69.42% host lysis within 30 min following a distinct sigmoidal kinetic pattern: an adsorption phase (0-10 min) where lysis increased from 2.54% to 20.49%; an eclipse period (10-20 min) with minimal virion release and consequent lysis plateau; and a burst/reinfection phase (20-30 min) where lysis surged from 45.03% to 69.42% due to progeny virion release. Experimentally, $CaCl_2$ supplementation enhanced adsorption by neutralizing repulsive electrostatic forces between negatively charged phage capsids and bacterial membranes (Li et al. 2022), while chloramphenicol addition arrested host protein synthesis to isolate phage-mediated lysis from confounding bacterial growth (Canfield et al. 2021). The 69.42% lysis efficiency—exceeding typical therapeutic phage benchmarks (40-60%)—positions ΦAfa-NA1 as a prime candidate for high-efficacy phage cocktails. Collectively, these attributes position ΦAfa-NA1 as a compelling therapeutic candidate, contingent upon strategic development.

The physical stability of ΦAfa-NA1 is another major strength. Its stability over a wide range of clinically relevant temperatures (retaining infectivity up to 37°C) and pH (6-11) is essential for the successful formulation, storage, and in vivo efficacy of a topical phage therapeutic (Choi and Kim 2021). Furthermore, the kinetic analysis confirms the phage's obligately lytic nature, characterized by a rapid lytic cycle and high burst size. These properties

are essential for achieving rapid bacterial clearance and minimizing the risk of lysogeny or integration into the host genome, thereby enhancing the safety profile (Ranveer et al. 2024).

Whole-genome sequencing revealed that ΦAfa-NA1 possesses a linear dsDNA genome spanning 78,105 bp with 45.8% GC content—significantly larger than the T7 phage genome (39,938 bp) yet comparable to vB_AfaP_QDWS595 (70,466 bp). The genome encodes 83 predicted Open Reading Frames (ORFs) organized into three temporally regulated transcriptional classes (Figure 6), mirroring the "early-middle-late" expression cascade characteristic of T7-like Podoviruses (Kawasaki et al. 2016).

The genomic analysis places ΦAfa-NA1 as a Podovirus related to the well-studied T7-like phages, which are known for their lytic life cycles, safety, and history in therapeutic development (Taha et al. 2018). Crucially, the absence of lysogeny, virulence, and antibiotic resistance genes strongly supports its suitability for clinical application. The relatively large genome size of ΦAfa-NA1 (78,105 bp) compared to prototypical T7 (39,938 bp) suggests a significant evolutionary divergence and genomic adaptation. These genomic features, classified into three functional categories, provide a detailed genetic blueprint for understanding its lytic machinery, which is key for engineering or optimizing its therapeutic properties (Mavrich and Hatfull 2017). Mechanistic interpretations regarding endolysin target bonds and phage receptor usage are currently hypotheses based on domain annotations and comparative genomics. Direct biochemical assays or structural studies are necessary to confirm these mechanisms.

Class I (early-phase: 0-5 min post-infection) contains host takeover genes, including an RNA Polymerase (RNAP) that hijacks bacterial transcription machinery. Class II (middle-phase: 5-15 min) features DNA metabolism enzymes such as DNA Polymerase (DNAP), helicase, and DNA Ligase (DNAL), responsible for genome replication. Class III (late-phase: 15+ min) comprises structural proteins, including the Major Capsid Protein (MCP) and tail fibers, alongside lysis machinery components (holin and endolysin LYS).

Notably, DNAL occupies a Class I position—unlike its Class II localization in *Alcaligenes* phage CASP1—suggesting evolutionary adaptation in replication timing. As DNA ligase orchestrates Okazaki fragment joining during replication, its early expression may accelerate genome circularization or concatemer resolution in ΦAfa-NA1 (Doherty et al. 1996). Comparative genomics (Figure 7) revealed conserved synteny in core functional modules: DNA packaging (terL), capsid assembly (gp10), and lysis systems (hol-ly). However, significant divergence occurred in transcriptional regulators, indicating host-specific fine-tuning of infection dynamics through differential gene regulation. This gene-class divergence prompted phylogenetic investigation to resolve evolutionary relationships underpinning functional adaptations.

Our phylogenetic analyses place ΦAfa-NA1 within the T7-like Podoviridae family, consistent with genomic features and lifecycle characteristics typical of these phages. However, evolutionary divergence suggests unique adaptations requiring future functional validation. The tree reveals the possible evolutionary proximity of "PV550095 Afa-NA1" to other *Alcaligenes* phages and potentially certain *Aeromonas* phages (Figure 8). This clustering presents a notable paradox: despite ΦAfa-NA1 belonging to Siphoviridae and AP1 to Straboviridae—distinct taxonomic families—their conserved terminase architecture suggests Horizontal Gene Transfer (HGT) of DNA packaging machinery between evolutionarily distant phage lineages. The terminase complex (TerS-TerL), which mediates genome recognition and ATP-driven DNA translocation into procapsids, exhibits functional interoperability across phage taxa, enabling this unexpected conservation of a critical molecular machine (Wangchuk et al. 2021).

Complementary phylogenies of essential genes (Figure 9) uncovered deeper evolutionary patterns. For replication-associated genes (RNAP, DNAP, DNAL), ΦAfa-NA1 consistently clustered with T7 and ΦAF1 within Class I/II regions, indicating shared ancestry in host takeover strategies. Conversely, structural genes (MCP, LYS) showed tight grouping with podophages (T7, ΦPI) in Class III, reflecting conserved virion assembly principles. Topological analysis of the MCP tree (Figure 9.D) placed ΦAfa-NA1 basal to T7, suggesting retention of ancestral capsid features lost in its more derived relative. Meanwhile, LYS clustering with *Bacillus* phage Bp4 (Figure 9.E) implies convergent evolution in endolysin specificity toward *Alcaligenes* peptidoglycan. The substantial genetic distance (>1.00 substitutions/site) from close relative vB_AfaP_QDWS595 highlights niche adaptation—where ΦAfa-NA1's terminase may recognize unique pac sites in *A. faecalis* T17 DNA, while its RNA polymerase potentially exploits strain-specific sigma factors to optimize transcriptional regulation (Mavrich and Hatfull 2017). Genomic predictions required functional validation through kinetic studies, where we quantified replication and adsorption dynamics.

ΦAfa-NA1 emerges as a highly promising therapeutic candidate against *A. faecalis* infections, integrating four critical attributes essential for clinical translation. First, comprehensive genomic screening confirmed its safety profile, revealing no virulence factors, toxin genes, or antibiotic resistance determinants. Second, the phage exhibits exceptional biophysical stability, maintaining viability across diabetic ulcer pH ranges (4.3-7.6) and temperatures (4-37°C) without requiring cryoprotectants—addressing key formulation challenges in resource-limited settings. Third, its therapeutic potency is evidenced by a high burst size (29 PFU/cell) and rapid adsorption kinetics (>80% within 5 m), enabling efficient bacterial clearance. Finally, narrow host specificity minimizes dysbiosis risks in polymicrobial diabetic wounds while precisely targeting MDR strains.

The observed genomic plasticity—particularly the adaptive repositioning of DNA Ligase (DNAL)—further suggests an inherent capacity to counter emerging bacterial resistance through evolutionary fine-tuning. To fully leverage this potential, future research should prioritize four strategic directions: (i) Structural characterization of receptor-binding proteins to enable synthetic biology applications like host-range engineering; (ii) Efficacy

validation in physiologically relevant *A. faecalis* biofilms using porcine diabetic ulcer models; (iii) Development of lyophilized formulations ensuring tropical climate stability; and (iv) Exploration of Phage-Antibiotic Synergy (PAS) with β-lactams, where preliminary evidence suggests phage predation may resensitize resistant bacteria to conventional antibiotics. Collectively, these advancements could position ΦAfa-NA1 as cornerstone of next-generation antimicrobial strategies against resistant diabetic wound infections.

ΦAfa-NA1 represents a phylogenetically distinct yet functionally conserved podophage with high therapeutic potential against MDR *A. faecalis*. Its genome reveals a tapestry of evolutionary divergence (terminase) and conservation (lytic genes), enabling both host adaptation and efficient lysis. Combined with biophysical resilience and rapid kinetics, ΦAfa-NA1 addresses critical gaps in diabetic ulcer management. As antibiotic pipelines stagnate, this phage exemplifies the promise of evolution-guided phage therapeutics against neglected antimicrobial-resistant pathogens.

Overall, while ΦAfa-NA1 exhibits promising functional traits for phage therapy, the data underscore the need for cautious interpretation, further quantitative analyses, and expanded host testing to substantiate therapeutic claims. Future work will focus on comprehensive phenotypic validation, mechanistic studies, and in vivo efficacy assessments. This study presents the isolation and in vitro characterization of bacteriophage ΦAfa-NA1, targeting multidrug-resistant Gram-negative bacterial isolates associated with diabetic foot ulcers. Our findings demonstrate ΦAfa-NA1's lytic activity against a limited panel of clinical strains, with quantified plaque morphology, kinetic parameters, and genomic features consistent with T7-like Podoviruses. Importantly, clinical safety and therapeutic efficacy cannot be asserted at this stage. While genomic analyses confirm the absence of known toxin and antibiotic resistance genes across multiple databases, further validation through in vivo studies, expanded host range screening involving diverse clinical isolates, and biofilm disruption assays remains essential.

In addition, regulatory approval will require rigorous assessment of phage stability, reproducibility, safety, and efficacy in relevant animal models and clinical trials under Good Manufacturing Practice standards. Therefore, ΦAfa-NA1 represents a promising candidate for further investigation, forming a foundational step toward potential phage therapy development but necessitating comprehensive preclinical and clinical validation.

In conclusion, the comprehensive characterization of bacteriophage ϕAfa-NA1 establishes it as a highly promising biocontrol agent against multidrug-resistant (MDR) Gram-negative bacteria, specifically *A. faecalis* and *Pseudomonas* sp. isolates, commonly found in diabetic ulcers. Exhibiting strong lytic capacity (titer $5x10^6$ PFU/mL, short latent period, high burst size) and robust stability across relevant pH (6-11) and temperature (≤37°C) ranges, ϕAfa-NA1 is well-suited for topical wound-care application. Its genomic profile—a 78,105 bp dsDNA genome with no virulence, lysogeny, or antibiotic-resistance genes—confirms its obligately lytic nature and therapeutic safety, further supported by its phylogenetic placement among T7-like Podoviruses. While current limitations include a small host-range panel and lack of in vivo or biofilm data, the collective findings strongly underscore ϕAfa-NA1's translational potential as a safe, efficient, and targeted phage-based biotherapeutic candidate for chronic wound infections, necessitating future studies to confirm its clinical efficacy and expand its host verification.

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