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Isolation of *Stenotrophomonas maltophilia* virus Bf1: a biofilm inhibiting phage

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Isolation of *Stenotrophomonas maltophilia* virus

Bfi1: a biofilm inhibiting phage

BY

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ABSTRACT

The bacterium *Stenotrophomonas maltophilia* is an emerging infectious pathogen of global concern. Due to its drug-resistant nature, there are limited treatment options available. A potential option for combating *S. maltophilia* infections is phage therapy, the medicinal use of viruses to treat bacterial infections. *Stenotrophomonas* phage Bfi1 was isolated from a soil sample using *S. maltophilia* clinical strain S18202. Transmission electron microscopy provided evidence that this phage is a member of the *Siphoviridae* family. Host range analysis showed that the phage successfully infected and lysed 30% of the *S. maltophilia* strains tested. Genomic analysis revealed that the phage contains approximately 32.2-56.5 kbp dsDNA. This phage was assessed for its ability to affect biofilm formation. At an $\text{MOI} \geq 10^3$, the phage inhibited S18202 biofilm formation after 24 h incubation with the phage. To the best of our knowledge, this is the first time the effects of a bacteriophage on *S. maltophilia* biofilms have been studied.

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I. INTRODUCTION

***Stenotrophomonas maltophilia*: An Opportunistic Pathogen**

Stenotrophomonas maltophilia is a species with a complex taxonomic history. This species of bacteria was first described in 1943 by J.L. Edwards (Hugh and Ryschenkow, 1961). At the time, the species was named *Bacterium bookeri* (Hugh and Ryschenkow, 1961). Since its original characterization, it was reclassified as *Pseudomonas maltophilia* due to its physiological characteristics, including multitrichous flagella (Hugh and Ryschenkow, 1961). Later, the species was classified as a *Xanthomonas sp.* (Swings et al., 1983). The authors cited DNA-rRNA hybridization data, among other genotypic and phenotypic characteristics, as major influences for this reclassification. Because of the unique phenotypes of *P. maltophilia*, adding it to the taxonomic group, *Xanthomonas*, altered the definition of this genus (Palleroni and Bradbury, 1993). To resolve this conflict, a new bacterial genus, *Stenotrophomonas*, was named; this is where *S. maltophilia* is currently classified. This history is important as earlier publications use these different names in their work. Additionally, it demonstrates the relatedness of *S. maltophilia* to other bacterial pathogens, such as *Pseudomonas aeruginosa* or *Xanthomonas campestris*, a human and plant pathogen, respectively.

S. maltophilia is found throughout the world (Brooke, 2012). There is evidence that the species can live in a wide range of moist environments. It has been isolated from many environments, including plant roots and soil, river water, tap water, vertebrates, and invertebrates (Nakatsu et al., 1995; Denton et al., 2003; Hejnar et al., 2007; Romanenko, 2008; Berg, 2009). Its ability to live in diverse environments makes it a concern, because there are many sources from which an infection can be acquired. While nosocomial infections are common, a review of several studies from various health networks (United States, Australia, Taiwan, Canada,

Germany) estimated that 22 % of *S. maltophilia* infection cases are community-acquired (Falagas et al., 2009).

This bacterium is an opportunistic pathogen. This means it is not highly infectious, but it can cause disease in humans when the normal host barriers have been penetrated (Brooke, 2012). *S. maltophilia* causes diseases in patients with compromised immune systems, such as patients with cancer or cystic fibrosis (CF) (Chang et al., 2015). It has been the causative agent of bacteremia, biliary sepsis, meningitis, and urinary tract infections, among other infections (Nguyen and Muder, 1994; Papadakis et al., 1995; Vartivarian et al., 1996; Araoka et al., 2010). In general, these infections are life-threatening. In one hospital survey of *S. maltophilia* infections, the reported mortality rate was 60% (Nseir et al., 2006).

As a pathogen, evidence indicates that *S. maltophilia* is a growing concern. SENTRY Antimicrobial Surveillance Program studies show that over time the prevalence rates of *S. maltophilia* respiratory tract infections (RTIs) increased from 3.3%-3.5% during 1997-2004 to 4.4% during 2009-2012 (Gales et al., 2001; Hoban et al., 2003; Jones, 2010; Sader et al., 2014). These studies showed that *S. maltophilia* moved from the eighth most common cause of RTIs to the sixth in the United States. Additionally, of non-enteric Gram-negative bacilli, *S. maltophilia* ranks globally as the third most commonly isolated pathogen from any infection (Sader and Jones, 2005). These data demonstrate that *S. maltophilia* should not be overlooked, because a significant number of infections result from this pathogen with increasing frequency.

Use of Antibiotics

The primary treatment for *S. maltophilia* infections is antibiotic therapy. Currently healthcare professionals recommend trimethoprim-sulfamethoxazole (TMP-SMX) (Chang et al., 2015). This antibiotic is used primarily because resistance to TMP-SMX is not common. One surveillance study (2009-2012) found that 96% of US isolates and 98% of European isolates were susceptible to TMP-SMX (Sader et al., 2014). For patients with contraindications for TMP-SMX, the fluoroquinolone levofloxacin is recommended (Chang et al., 2015). There are concerns that the efficacy of levofloxacin is not as high as TMP-SMX. However, two retrospective studies reported no statistical difference in outcome between these two antibiotic approaches (Cho et al., 2014; Wang et al., 2014). These two antibiotics are currently considered to be effective therapies for *S. maltophilia* infections.

Despite the efficacy of levofloxacin and TMP-SMX, there are long-term concerns with antibiotic therapy. Worldwide susceptibility of *S. maltophilia* to levofloxacin has decreased from 83.4 % (2003-2008) to 77.3% (2011) (Farrell et al., 2010; Sader et al., 2013). Additionally, resistance to TMP-SMX has been identified (Toleman et al., 2007). As *S. maltophilia* infection rates increase, one can expect that antibiotic resistance will rise.

Antibiotic Resistance Genes of *S. maltophilia*

S. maltophilia is naturally resistant to many antibiotics. It has been suggested that this natural resistance may arise from selection pressures that arise from a plant commensal lifestyle. High levels of competition and exposure to natural antibiotics and secondary antimicrobial metabolites are strong evolutionary drivers that contribute to this resistance (Berg and Martinez, 2015).

Within the genome of *S. maltophilia*, the genes that facilitate the breakdown or removal of antibiotics play a major role in antibiotic resistance. L1 and L2 are β -lactamases encoded in the *S. maltophilia* genome (Crossman et al., 2008). L1 hydrolyses all β -lactams except aztreonam (a monobactam) (Paton et al., 1994). L2 hydrolyses all penicillins, all cephalosporins in the first, second and third generations, and aztreonam (Walsh et al., 1997). Several aminoglycoside-modifying enzymes have been described in *S. maltophilia* strains. These enzymes include AAC(6')Iz, APH(3')-IIc, AAC(6')-Iak, and AAC(6')-Iam (Li et al., 2003; Okazaki and Avison, 2007; Crossman et al., 2008; Tada et al., 2014). Drug efflux pumps also play a major role in antibiotic resistance. A recent analysis of the genome of *S. maltophilia* K279a identified four efflux pumps involved in antibiotic resistance: SmeABC SmeDEF, SmeIJK, and SmeYZ (Alonso and Martinez, 2000; Li et al., 2002; Crossman et al., 2008). These efflux pumps contribute to resistance of a broad range of antibiotics, including β -lactams, aminoglycosides, quinolones, tetracyclines, macrolides, chloramphenicol, novobiocin, and TMP-SMX (Wang et al., 2018).

While some resistance mechanisms involve the breakdown or effluxion of antibiotics, the *S. maltophilia* genome also contains genes whose encoded proteins resist the mechanisms of action of antibiotics. The *sul2* gene encodes for a dihydropteroate synthase which is not inhibited by sulfonamides. Strains that have class 1 integrons and insertion sequence common region elements that were linked to the gene *sul2* are resistant to TMP-SMX (Toleman et al., 2007). Sulfonamides, like sulfamethoxazole, work by binding to the active site of dihydropteroate synthase, which is involved in folic acid synthesis, converting *p*-aminobenzoate to dihydropteroic acid. The *sul2* codes for a dihydropteroate synthase that has a weak binding affinity for sulfonamides (Sköld, 2000). Variants of *dfr* genes, that code for dihydrofolate

reductase, are also associated with high levels of resistance to TMP-SMX (Hu et al., 2011). Dihydrofolate reductase is an enzyme involved in DNA synthesis. Trimethoprim binds to dihydrofolate reductase to inhibit its function. Variants in the *dhfr* genes lead to weaker binding by this enzyme to trimethoprim and consequently lead to resistance to this drug (Sköld and Widh, 1974).

The chromosomal *qnr* gene has been associated with quinolone resistance in *S. maltophilia*. Quinolones target DNA gyrase, an enzyme that relieves tension of supercoiling during DNA replication (Champoux, 2001). The antibiotic forms a stable complex with the DNA and DNA gyrase, preventing the progression of DNA replication (Hiasa and Shea, 2000). The *qnr* gene encodes a protein that can bind specifically to the gyrase holoenzyme in the DNA binding groove. This prevents the deleterious effects of the stabilized quinolone, DNA, and DNA gyrase complex (Hooper and Jacoby, 2015). By doing so, Qnr has demonstrated its importance in low-level resistance to quinolones (Sánchez and Martínez, 2010).

Resistance to polymyxins can be attributed to the gene, *spgM*. This gene encodes a phosphoglucomutase involved in lipopolysaccharide (LPS) synthesis. Bacterial mutants that lack *spgM* have less LPS when compared to SpgM⁺ cells (McKay et al., 2003). Polymyxin selectively binds to LPS and destabilizes the outer membrane enough to penetrate. Ultimately, the antibiotic causes lysis of the host by destroying the integrity of the cell's inner membrane (Yu et al., 2015; Malinowski et al., 2017). With less LPS, there are fewer targets for the antibiotic's action.

Biofilms of *S. maltophilia*

Along with possessing antibiotic resistance genes, *S. maltophilia* can form biofilms. A biofilm is an accumulation of microbial cells that are associated with a surface and enclosed in a

polysaccharide matrix (Donlan, 2002). Biofilms are formed when, individual, free-floating cells (referred to as planktonic cells), adhere to a surface. The cells replicate, form a monolayer, and begin to secrete a polysaccharide matrix (extracellular polymeric substance) outside of the cell (Gupta et al., 2016). *S. maltophilia* can form biofilms on a variety of surfaces. *S. maltophilia* can form biofilms on glass, plastics, and host tissue (Jucker et al., 1996; de Oliveira-Garcia et al., 2003).

Biofilms have been shown to facilitate antibiotic resistance in two ways. First, extracellular polymeric matrices act as a physical barrier. For example, extracellular matrix components in *P. aeruginosa* biofilms can impede penetration of the antibiotic ciprofloxacin into cells living within the biofilm (Suci et al., 1994). The biofilm acts to shield the cells within the structure.

The second mechanism of biofilm resistance relates to variation in the cell populations of a biofilm. Because cells in a biofilm have access to differing nutrient levels, there is heterogeneity in the cellular metabolic rates within the population (Burrowes et al., 2011). Some cells with little nutrient access exist in slow-growing or starved states. These are referred to as persister cells. Since antibiotics often target the pathways involved in actively growing and dividing cells, persister cells are not affected by these antimicrobials (Costerton et al., 1999).

Bacteriophages

The ability for *S. maltophilia* to persist, despite antibiotic treatments has led to a need for alternative therapies, and directed an interest in the research of bacteriophages. Often referred to as phages, they are defined as viruses that infect a bacterial host (Labrie et al., 2010). They usually come in two forms, lytic (or virulent) and lysogenic (or temperate). Upon infection by a

lytic phage, the virus takes over the host's machinery and replicates itself (Labrie et al., 2010). At the end of the lytic cycle, the host cell is lysed to release viral progeny (Labrie et al., 2010).

When a temperate phage infects their host, the phage DNA is incorporated into the genome of the host, becoming a prophage (Labrie et al., 2010). Inside the host, the virus can remain dormant and is transmitted vertically to host daughter cells (Labrie et al., 2010). Under certain conditions, the prophage can be activated via induction to enter into a lytic cycle and lyse the host cell (Labrie et al., 2010).

A third, less common form of phage infection is known as pseudolysogeny. During pseudolysogeny, the infection of the host is stalled. The viral nucleic acids do not replicate as it would in a lytic cycle or integrate as it would in a lysogenic cycle (Ripp and Miller, 1997). It is present in the host in an inactive state.

Lytic phages have drawn a lot of interest from researchers concerned with antibiotic resistance. Because the lytic cycle is inherently lethal to the bacterial host, physicians could potentially use phages to treat and prevent bacterial infections in humans. Prior to the discovery of antibiotics, phages were documented as being used in the treatment of infections (Abedon et al., 2011). This legacy has continued in several countries in Western Europe, including Georgia and Poland (Abedon et al., 2011).

Phage therapies may offer many advantages over antibiotic therapies. A major benefit is that phages have a limited effect on resident flora. Phages usually only infect organisms of the same species. The breadth of strains or species which can be infected by a virus is referred to as the viral host range. The fact that many phages are shown to have limited host ranges means the killing of off-target bacteria is minimized (Abedon et al., 2011). This results in a lower potential

for side effects brought about by dysbiosis, the disturbance to gut microbiota homeostasis (DeGruttola et al., 2016).

The abundance of phages is another benefit to be considered. Estimations by phage ecologists have determined that the number of phage particles found in soil is on average $1.5 \times 10^8 \text{ g}^{-1}$ (Ashelford et al., 2003). In aquatic environments, it has been estimated that there are approximately 100-300 phage strains ml^{-1} (Wommack et al., 1999). This means that there are potentially many undescribed phages in these habitats that have yet to be assessed for therapeutic use. In the case that a bacterial pathogen develops resistance to one phage, there are many other phages that may cause host lysis.

***S. maltophilia* Phages**

To the best of our knowledge, there have been six lytic and pseudolysogenic *S. maltophilia* bacteriophages described in the literature to date. Of the described lytic phages, four are myoviruses, phiSMA5 (Chang et al., 2005), Smp14 (Chen et al., 2007), and S3 (García et al., 2008), DLP6 (Peters et al., 2017), one is a podovirus, IME15 (Huang et al., 2012), and one is a siphovirus DLP2 (Peters et al., 2015). Each of these phages has a double stranded (ds) DNA genome. At the time of writing, these reported phages represent the entire library of phages with potential to be used in treatments for *S. maltophilia* infections.

The first lytic phage to be characterized was phiSMA5 (Chang et al., 2005). The phage was determined to have a dsDNA genome that is ~250 kbp and contains at least 25 proteins (Chang et al., 2005). Smp14 was the first phage to have its genome partially sequenced (Chen et al., 2007). The genome is estimated to be ~160 kbp and consists of at least 20 unique proteins. Phage S3 was found to have a genome of ~33 kbp (García et al., 2008). The DNA of phage S3

was resistant to digestion by restriction enzymes, which suggests that it might contain atypical bases or be enzymatically modified. IME15 was the first phage to have its full genome sequenced, with a genome of ~39 kbp. The second potentially lytic phage to have its whole genome sequenced was DLP2 with a genome of ~42 kbp in size (Peters et al., 2015). The final phage described was DLP6. This T4-like phage undergoes pseudolysogeny prior to lytic activation. The phage genome does not integrate into the host DNA, but proceeds into the lytic cycle following activation. The genome of DLP6 was sequenced and found to be ~168 kbp (Peters et al., 2017).

The first step in this thesis research was to isolate a *Stenotrophomonas* phage from an environmental sample. Upon isolation, the phage was characterized using molecular and microbiological techniques. At the time of writing, this thesis research provides the first assessment of a *Stenotrophomonas* phage's effect on its bacterial host's biofilm. Using an *in vitro* model of biofilm formation in the presence or absence of phage, we hypothesized that the presence of phage significantly inhibits biofilm formation by *S. maltophilia*

II. METHODS

Maintenance and Growth of Bacteria

S. maltophilia S18202 was grown and stored on LB (Luria-Bertani) agar. *S. maltophilia* cultures were prepared by growing them overnight in LB broth at 37°C with shaking at 235 rpm. Cultures were standardized to $OD_{600} \cong 1.0$. Dilutions of culture were added to fresh LB broth and incubated with agitation at 37°C until exponential growth was obtained (2h). Overnight cultures were used for phage isolation, plaque enumeration assays, biofilm assays, and host range spot tests.

Isolation of Bacteriophage

Soil samples were taken from landscaping mulch on the north side of the William G. McGowan Building on the DePaul University Lincoln Park campus, in Chicago, Illinois. A 10 g sample of the soil was added to 15 ml of a modified LB suspension medium (SM) (Peters et al., 2015).

After mixing for 1 h on a Stovall Belly Dancer (IBI Scientific) at room temperature (25°C), the sample was centrifuged at 12,000 x g at 25°C to remove excess soil debris. The supernatant was filter sterilized (0.45 µm pore size). One hundred microliters of an overnight culture of

S. maltophilia S18202 was added to the filtrate, and was incubated at 37°C overnight (Van Twest and Kropinski, 2009). The mixture was then centrifuged at 12,000 x g for 5 min at 25°C. The liquid supernatant was filter sterilized (0.45 µm pore size) and stored at 4°C. This preparation was used as a phage stock for plaque purification.

Plaque Purification

To purify the virus, a soft agar overlay of the phage sample was performed according to Kropinski et al. (2009) with modifications from Peters et al. (2015). In microfuge tubes, a 200 μ l preparation of *S. maltophilia* was added to 100 μ l of phage from a serial dilution of the phage stock. The virus and bacteria were incubated statically for 8-10 min at 25°C. The phage-bacteria mixture was added to 4 ml of molten soft LB agar (0.4% agar). This was gently swirled and poured over an LB agar plate. After the soft agar was allowed to solidify, the plates were incubated at 37°C overnight. A single plaque was picked from a plate with isolated plaques by touching a sterile glass pipette tip to it. The pipette tip was immersed in 500 μ l of SM with 20 μ l of chloroform for 1 h statically at 25°C. The chloroform was used to kill any living bacteria present in the sample. The plaque isolation and purification steps were repeated two subsequent times to obtain purified phage. The plaque purified stock was used to prepare a high titer stock.

Preparation of High Titer Phage

A soft agar overlay plate with a confluent lawn of plaques (obtained after overnight incubation) was selected to make the phage stock. Ten milliliters of SM was added to the plate. The plate was gently agitated on a Stovall Belly Dancer for 1 h. The SM was then removed and added to microfuge tubes. The tubes were centrifuged at 12,000 x g at 25°C for 2 min and the supernatant was filter sterilized (0.45 μ m pore size). The purified high titer phage stock was stored at 4°C. This stock was used for the enumeration of the phage. For long-term storage of phage stocks, purified phage was prepared in 50% glycerol/SM and stored at -80°C according to Fortier and Moineau (2009).

Enumeration of Phage

Phage titer determination used the soft agar overlay technique and a serial dilution of the high titer purified phage stock (Kropinski et al., 2009). To a set of microfuge tubes, 200 μ l of *S. maltophilia* was mixed with 100 μ l of serially diluted phage stock. The samples were incubated statically for 8-10 min at 25°C. The mixture of phage and bacteria was transferred to a test tube containing 4 ml of molten soft LB agar. The tube contents were then poured over a plate of LB agar and allowed to solidify. The plates were incubated at 37°C overnight. The number of plaques was recorded and the original concentration of phage stock was determined according to Equation 1.

Equation 1

$$\frac{\text{plaque forming units}}{\text{ml}} = \frac{(\text{dilution factor} \times \# \text{ plaques per plate})}{\text{plating volume}}$$

Determining Plaque Size

A plaque assay was performed (see Enumeration of Phage). Plates were incubated overnight at 37 °C. The plates were then digitally photographed with a 50 mm ruler for reference. Images of plaques on a lawn of *S. maltophilia* S18202 were analyzed using tpsDig software (by F. James Rohlf, Stony Brook Morphometrics).

Transmission Electron Microscopy

The ultrastructure of the phage was analyzed using the Imaging Facility at the Loyola University Chicago Health Sciences Campus, Maywood, Illinois. Carbon-coated 200 mesh copper grids

(Ted Pella, Inc) were treated with 0.002% Alcian Blue in 0.03% acetic acid (Electron Microscopy Sciences) for 5 min then incubated with distilled water for 5 min to increase the hydrophilicity of the grids (Chattoraj et al., 1988). Grids were incubated with a high titer sample of phage (2×10^{11} pfu/ml) in SM for 1 min then stained with filtered 1% uranyl acetate for 1 min. A Philips CM120 transmission electron microscope (voltage = 80 kV) equipped with an AMT BioSprint camera was used to image the samples. Images of 15 phage particles were used to acquire measurements of phage ultrastructure. Tail length, tail width, and head diameter were measured using ImageJ (Schneider et al., 2012).

Phage DNA Analysis

S. maltophilia phage DNA was extracted using the Phage DNA Isolation Kit according to the manufacturer's instructions (Norgen Biotek Corporation) and stored at -20°C . The concentration of purified DNA was determined using a NanoDrop 2000c (Thermo Scientific). Phage DNA was subjected to restriction enzyme digestion (Table 1). Type II restriction enzymes were used due to their ability to digest dsDNA. To prepare each restriction enzyme digest, approximately 150-200 ng of DNA were used. The phage DNA digests were subjected to agarose gel electrophoresis at 80V for about 1 h and then visualized using a FlourChem HD2 system (Bio-Techne). Alphaview software (Bio-Techne) was used to analyze the DNA digests.

Table 1: Restriction enzymes used	
BglII	PvuII
ClaI	SalI
EcoRI	SmaI
KpnI	SphI
PstI	XhoI

Host Range Analysis

The host range analysis of the *S. maltophilia* phage was performed according to García et al. (2008) with the following modifications. The bacterial strains used for the tests are listed in Table 2. The clinical strains of *S. maltophilia* were kindly provided by Dr. Stanford Shulman (Northwestern University). Lawns (confluent growth) of each strain on LB agar plates were prepared using overnight cultures. Four 5 µl drops of phage (~10⁹ pfu/ml) were placed on top of each agar plate. Plates were incubated at 37°C overnight and the presence of clearings were recorded. Each bacterium was tested in two independent experiments.

Table 2: Bacterial strains used for host range tests	
<i>S. maltophilia</i>	
ATCC 13637	H2138
ATCC 17807	H43306
ATCC BAA-2423	H59296
F64644	S18202
F7221	X26332
<i>P. aeruginosa</i>	<i>E. coli</i>
ATCC 27853	ATCC 23922
ATCC 22580	<i>S. aureus</i>
ATCC BAA-47	ATCC 29213

Biofilm Assays

To determine if the purified *S. maltophilia* phage inhibited biofilm formation of *S. maltophilia* S18202, biofilm assays were performed according to Malinowski et al. (2017) with the following

modifications. After determining the phage titer (see Enumeration of Phage), a dilution was made to achieve a multiplicity of infection (MOI) of 10, 10^3 , or 10^5 . The MOI is a measure of the ratio of the number of plaque-forming units to number of colony-forming units (pfu/cfu). To each well of a 96-well polyvinyl chloride (PVC) microtiter plate (BD Falcon), 50 μ l of phage in SM (10mM MgSO₄, 50mM Tris-HCl pH 7.5, 100mM NaCl, 50% LB) diluted in LB broth were added. Fifty microliters of *S. maltophilia* culture were added to each well. Negative control wells contained 100 μ l of LB broth only (no cells or phage). Biofilm plates were incubated statically for 24 h at 37°C. Planktonic cells were removed to a new microtiter plate and the bacterial culture density was recorded using a spectrophotometer (OD₅₉₅). Adhered biofilms were washed twice with sterile distilled water (dH₂O), stained with 0.1% crystal violet for 10 min, washed three times with sterile dH₂O, and air dried overnight at room temperature with minimal light exposure. The crystal violet was thoroughly resuspended in 30% acetic acid and the amount of biofilm was recorded at OD₅₆₀.

When performing the experiments at the MOIs of 10 and 10^5 , each biofilm assay was performed once, with 5 and 4 replicates, respectively. For the experiment at the MOI of 10^3 , the biofilm assay was performed in two independent experiments, each with 5 replicates.

Statistical Analysis

In all the biofilm assays, we used a paired *t*-test to compare means of each treated subculture with the untreated subculture. The α -values were adjusted using the sequential Bonferroni test (Holms, 1979). Statistical analyses were performed using Microsoft Excel.

III RESULTS

Isolation of a *S. maltophilia* Phage

A *S. maltophilia* phage was isolated from soil acquired from a horticultural flower bed adjacent to McGowan North, on DePaul University – Lincoln Park campus. In soft agar overlays of *S. maltophilia* S18202 with phage after overnight incubation at 37°C, the phage produced clear plaques. The size of the plaques ranged from 0.5 – 2.0 mm in diameter (Figure 1). Some plaques had surrounding halos that were turbid in appearance (Figure 2). The plaque purified phage was named *Stenotrophomonas virus Bfi1* (Biofilm formation inhibitor 1).

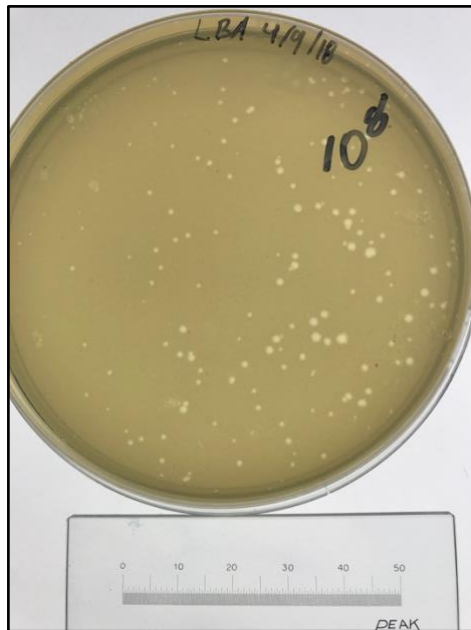


Figure 1: Phage plaques formed on a lawn of *S. maltophilia* S18202. The plate was incubated at 37°C overnight on LB agar with a 10⁻⁸ dilution of the phage stock. Scale bar = 50 mm.

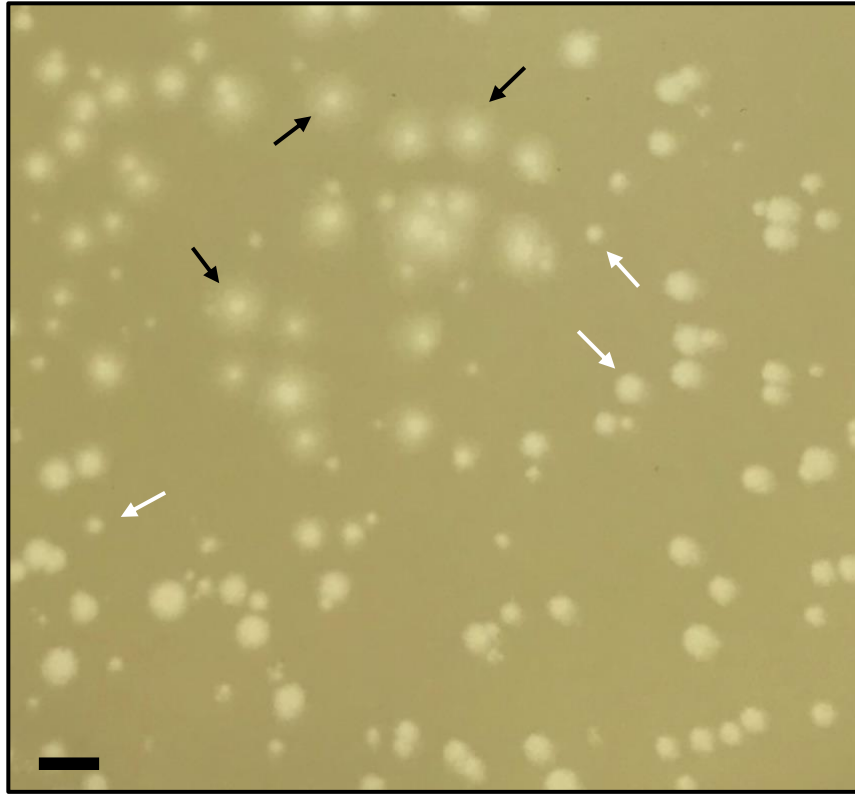


Figure 2: Plaque morphology of isolated phage Bfi1. Black arrows highlight plaques with turbid halos. White arrows highlight plaques without halos. Scale bar = 2 mm.

Bfi1 Ultrastructure

Ultrastructure morphological examination revealed that the phage has a head and an unsheathed flexible non-contractile tail (Figure 3). The head has an isometric icosahedral shape, with a diameter of 50.8 ± 5.7 nm. The tail length measures 197.0 ± 31.7 nm. The tail width measures 10.4 ± 1.7 nm.

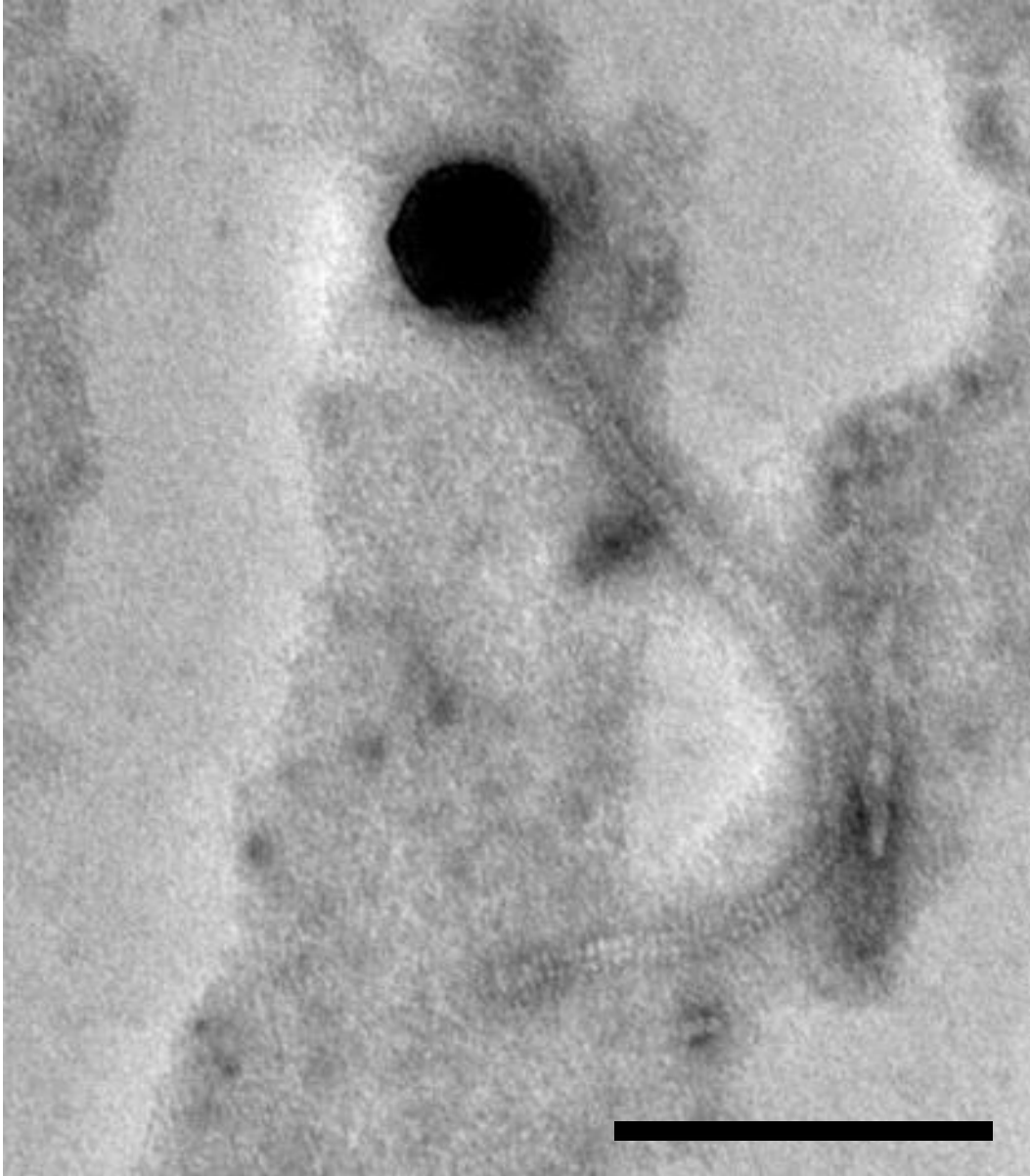


Figure 3: Transmission electron micrograph of *Stenotrophomonas* phage Bfi1, negatively stained with 1% uranyl acetate. Scale bar = 100 nm.

Phage Genome Analysis

Restriction enzyme (RE) digestion followed with agarose gel electrophoresis demonstrated that the *S. maltophilia* phage contains dsDNA. RE analysis of the DNA showed 5 of the 10 type II

restriction enzymes were able to digest the phage genome: EcoRI, KpnI, Sall, SmaI, and SphI (Figure 4).

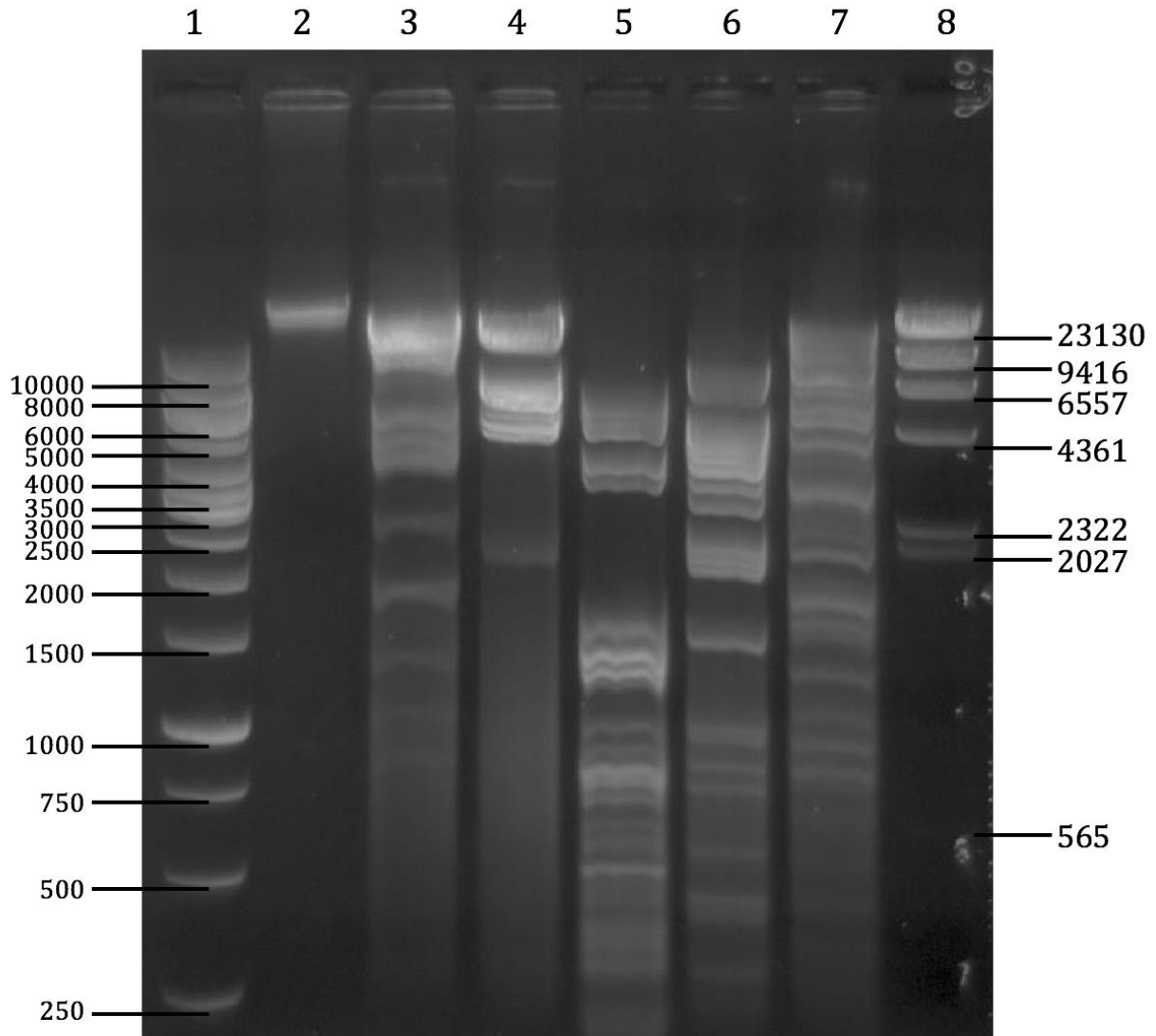


Figure 4: Agarose gel (1%) electrophoresis showing single RE digests of *Stenotrophomonas* phage DNA. Lanes: (1) 1 kbp marker, (2) uncut phage DNA, (3) EcoRI, (4) KpnI, (5) Sall, (6) SmaI, (7) SphI, (8) λ phage HindIII marker.

Digestion of the genome by type II restriction enzymes (EcoRI, KpnI, SalI, SmaI, and SphI) produced large, well-separated DNA bands (Table 3). Using the AlphaView software to analyze the DNA fragments, the size of the phage genome was estimated to be 32.2-56.5 kbp. This was done by adding the fragment sizes of each band from individual digests to get a total DNA size.

Table 3: Bfi1 single RE digest DNA sizes					
Band	DNA fragment length (bp)				
	EcoRI	KpnI	SalI	SmaI	SphI
1	19027	19027	7067	8083	9833
2	14377	7800	5800	5000	8833
3	6800	7000	4333	4700	7467
4	5550	6267	3825	4267	6467
5	4567	5700	1550	3725	5400
6	2833	2307	1447	3211	3625
7	1950	-	1340	2409	2648
8	1436	-	1048	2307	2027
9	-	-	936	2170	1858
10	-	-	801	1517	1583
11	-	-	736	1000	1330
12	-	-	656	881	1074
13	-	-	594	771	949
14	-	-	534	588	831
15	-	-	458	460	-
16	-	-	402	428	-
17	-	-	366	306	-
18	-	-	314	-	-
Total	56540	48101	32207	41823	53925

Host Range Analysis

The host range of this phage was assessed using available strains of *S. maltophilia*, *P. aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* (Table 4 and Figure 5). Of the

bacteria tested, 30% of the *S. maltophilia* strains (S18202, H2138, H43306) were infected by the phage (as indicated by phage generated clearings formed within the lawn of bacteria), and the remainder of the bacteria were resistant to infection (as indicated by the absence of clearings). These observations indicate that *S. maltophilia* phage Bfi1 has a moderate host range.

Table 4. Host range of Bfi1	
Bacterial strain	Infection by phage*
<i>S. maltophilia</i>	
ATCC 13637	-
ATCC 17807	-
ATCC BAA-2423	-
F64644	-
F7221	-
H2138	+
H43306	+
H59296	-
S18202	+
X26332	-
<i>P. aeruginosa</i>	
ATCC 27853	-
ATCC 22580	-
ATCC BAA-47	-
<i>E. coli</i>	
ATCC 23922	-
<i>S. aureus</i>	
ATCC 29213	-
* + phage infection, - no infection	

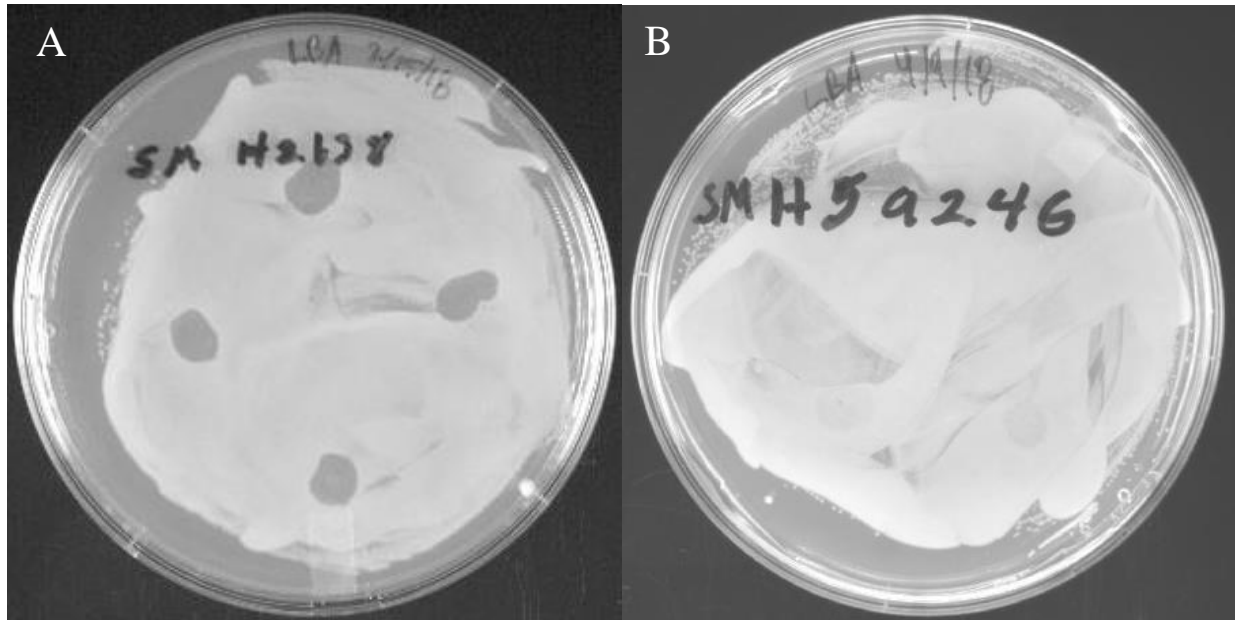


Figure 5: Bfi1 spot tests of *S. maltophilia* strains. (A) Strain H2138 shows infectivity and (B) strain H59269 shows no infectivity by the phage.

Phage Inhibition of *S. maltophilia* Biofilm Formation

Following a 24 h incubation of S18202 in a microtiter plate, liquid medium containing planktonic cells was removed and the cell density of the media quantified by spectrophotometry (OD_{595}). At an $MOI=10$ (actual $MOI \approx 5.1$) a significant inhibition of planktonic cell culture density was observed (Figure 6A). Inhibition of planktonic cell growth was also observed at an $MOI=10^3$ (actual $MOI \approx 1.8 \times 10^3$) and an $MOI=10^5$ (actual $MOI \approx 1.5 \times 10^5$) (Figure 6B, C).

The amount of biofilm formed on the microtiter plate surface was determined using the crystal violet assay. The difference in the amount of biofilm formed was not statistically significant at $MOI=10$ (Figure 7A). At $MOI=10^3$ and $MOI=10^5$, the amount of biofilm formed was significantly reduced in the phage treatment groups (Figure 7B, C).

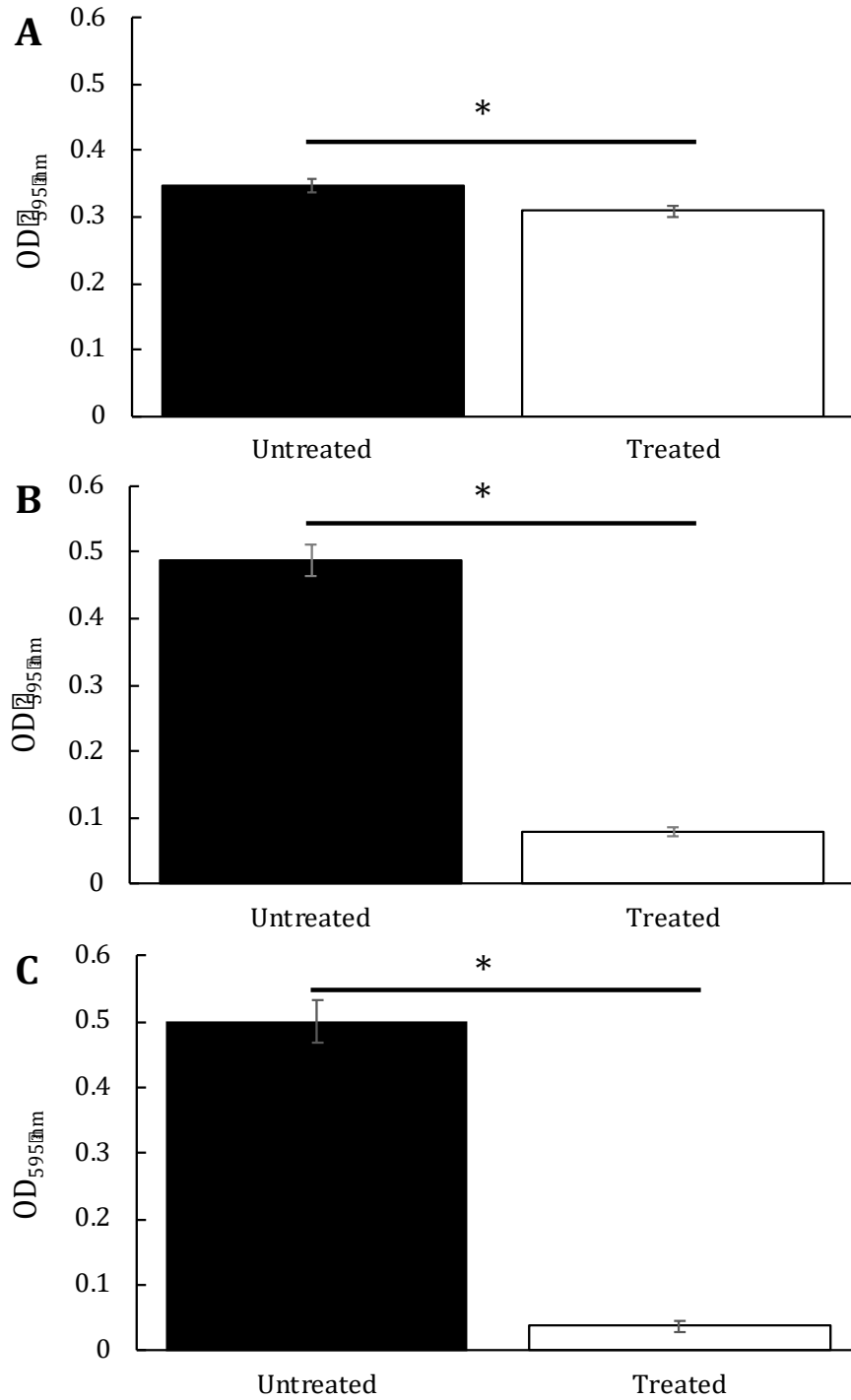


Figure 6: Planktonic cell culture density (OD₅₉₅) of *S. maltophilia* after 24 h treatment with phage Bfi1, (A) MOI=10 (B) MOI=10³ (C) MOI=10⁵. Vertical bars represent standard error.

(*) indicates statistical significance of paired *t*-tests adjusted using a sequential Bonferroni test for multiple comparisons. (A) $p = 2.1 \times 10^{-2}$, (B) $p = 5.7 \times 10^{-8}$, (C) $p = 1.1 \times 10^{-3}$

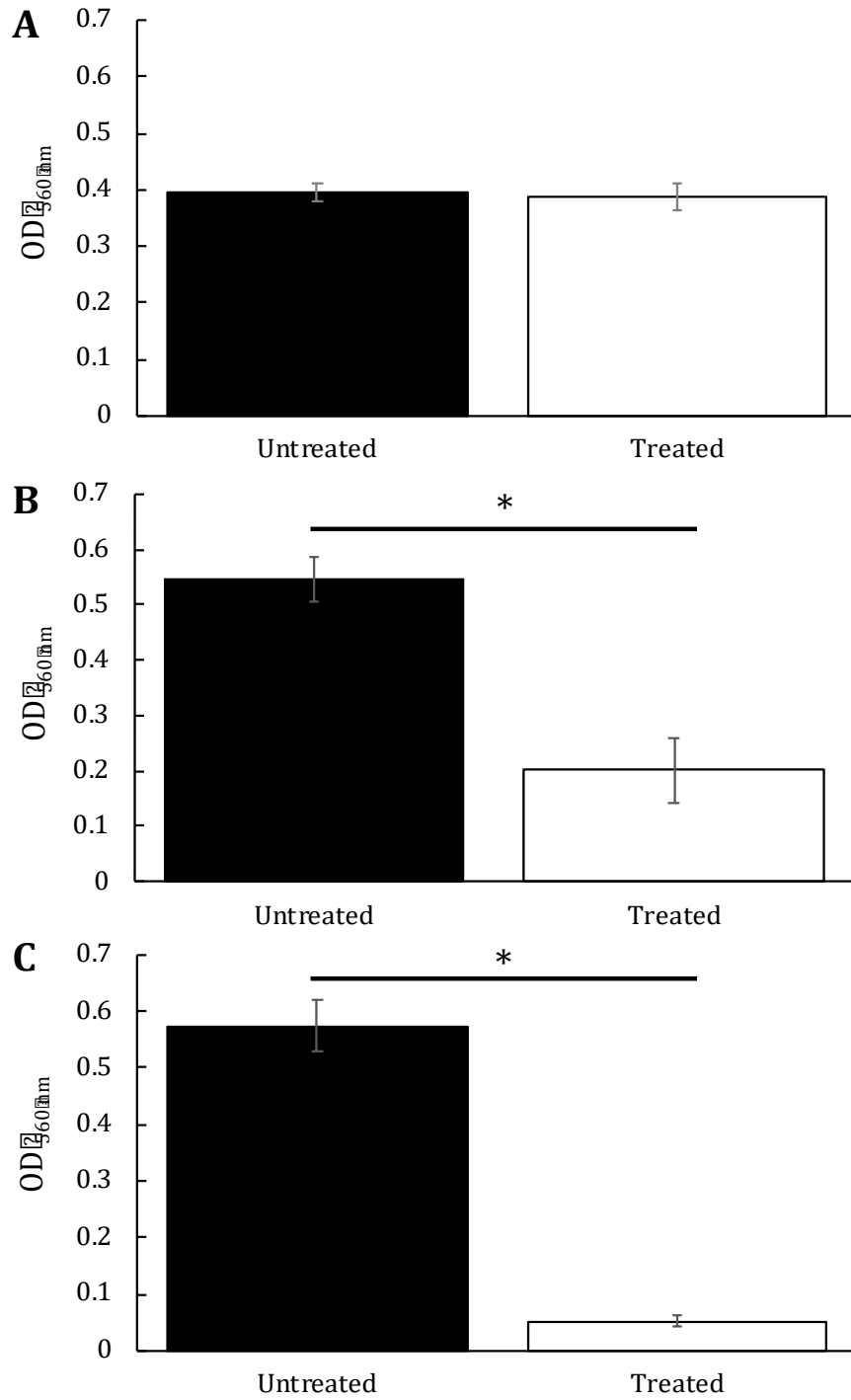


Figure 7: Amount of biofilm formed (OD₅₆₀) of *S. maltophilia* after 24 h treatment with phage Bfi1, (A) MOI=10 (B) MOI=10³ (C) MOI=10⁵. Vertical bars represent standard error.

(*) indicates statistical significance of paired *t*-tests adjusted using a sequential Bonferroni test for multiple comparisons. (A) $p = 0.75$, (B) $p = 5.9 \times 10^{-4}$, (C) $p = 1.2 \times 10^{-3}$

IV. DISCUSSION

S. maltophilia Bacteriophage Bfi1

S. maltophilia bacteriophage, Bfi1, was successfully isolated from a local soil sample. Initially, plaques were harvested and purified. The purified phage was further characterized using transmission electron microscopy, and its genome was analyzed using restriction enzyme digestion with agarose gel electrophoresis.

Plaque Morphology

Phage Bfi1 was able to form circular plaques, ranging from 0.5 – 2.0 mm in diameter (Figure 1). Plaque size is affected by a variety of factors including the virus's diffusivity, adsorption rate, latent period, and burst size (Abedon and Yin, 2009; Gallet et al., 2011). Diffusivity is a measure of the capability for a particle to be diffused. Adsorption rate is the rate at which plaque-forming units attach to susceptible host cells. The latent period is the infection time, between adsorption and host cell lysis. Burst size is defined as the number of new plaque-forming units produced when an infected cell lyses. The variation in plaque size within a single phage strain may be due to subtle differences in the genotypes that resulted from random mutations during replication. These differences could affect the burst size, adsorption rate, latent period and diffusivity of the phage.

Some phages can reduce biofilms by producing enzymes that break up extracellular polymers. These enzymes, called depolymerases have been shown to be important in biofilm penetration (Casey et al., 2018). One indicator that a phage may possess a depolymerase is through analysis of plaque morphology. The plaques of depolymerase-expressing phages are often surrounded by a large halo, which indicates depolymerase activity (Hughes et al., 1998).

Since there were halos surrounding some of the phage derived plaques (Figure 2), *S. maltophilia* phage Bfi1 may produce depolymerases.

Ultrastructure of Bfi1

Table 5 shows the ultrastructural diversity of bacteriophages. One third of the phages shown contain a tail. Tailed phages (Order: *Caudovirales*) are considered the most abundant phages found in nature (Ackermann, 2007). Of ~5500 phages documented using electron microscopy and submitted to the Félix d'Hérelle Reference Center for Bacterial Viruses, 96% of them were described as tailed phages (Ackermann, 2007). The phage tail is an appendage that is used in host receptor recognition, penetration of the cell wall and ejection of the virus genome into the host cell (Fokine and Rossman, 2014).

The *Caudovirales* order is divided into four families, *Ackermannviridae*, *Myoviridae*, *Podoviridae*, and *Siphoviridae* (Ackermann, 2009; Adriaenssens et al., 2018) Morphologically, *Ackermannviridae* and *Myoviridae* are indistinguishable, both possessing long sheathed contractile tails (Adriaenssens et al., 2018). *Podoviridae* possess short stubby non-contractile tails. Long unsheathed flexible non-contractile tails are a hallmark of the *Siphoviridae* family (Ackermann, 2009).

We compared the tail ultrastructure of 15 Bfi1 virions to the four families of *Caudovirales*. The tail length of Bfi1 is 197.0 ± 31.7 nm, which is much longer than the tail length of *Podoviridae* phages (20 nm) (King et al., 2012). Therefore, it is unlikely that Bfi1 belongs to *Podoviridae*. The tail width of Bfi1 is 10.4 ± 1.7 nm. This falls within the description of *Siphoviridae* phages, which have tail widths of 7-10 nm (King et al., 2012). The tail widths of *Myoviridae* and *Ackermannviridae* phages (16-20 nm) are much thicker than the tail of Bfi1

(King et al., 2012). When considering that Bfi1 also has a flexible tail (Figure 3), our observations lead us to conclude that Bfi1 is most likely a member of the *Siphoviridae* family within the *Caudovirales* order.

Table 5: Overview of bacteriophage families		
Family	Description of Ultrastructure	Nucleic Acid
<i>Myoviridae</i>	long sheathed contractile tail length: 80-455 nm; width: 16-20 nm	dsDNA linear
<i>Ackermannviridae</i>	long sheathed contractile tail length: 80-455 nm; width: 16-20 nm	dsDNA linear
<i>Siphoviridae</i>	long unsheathed flexible non-contractile tail length: 65-570 nm; width: 7-10 nm	dsDNA linear
<i>Podoviridae</i>	short non-contractile tail length: 20 nm; width: 8 nm	dsDNA linear
<i>Tectiviridae</i>	isometric, double capsid	dsDNA linear
<i>Corticoviridae</i>	isometric capsid	dsDNA circular
<i>Plasmaviridae</i>	enveloped, no capsid, pleomorphic	dsDNA circular
<i>Sphaerolipoviridae</i>	isometric capsid	dsDNA linear
<i>Inoviridae</i>	long filaments or short rods	ssDNA circular
<i>Microviridae</i>	conspicuous capsomers, isometric capsid	ssDNA circular
<i>Leviviridae</i>	isometric capsid	ssRNA linear
<i>Cystoviridae</i>	enveloped, spherical	dsRNA segmented

(Ackermann, 2009; King et al., 2012; Adriessens et al., 2018)

***S. maltophilia* Phage Bfi1 Genome**

The *Caudovirales* order is additionally unique in that their dsDNA is linear (Ackermann, 2009). This appears to be a result of the virion structure. To package DNA into a capsid and eject the genome from it, the DNA must be threaded through a narrow passage in the head portal. This passage is too small to accommodate two parallel dsDNAs simultaneously (as would be needed in the case of a circular genome) (Casjens and Gilcrease 2009). As *S. maltophilia* phage Bfi1 appears to be a member of the *Caudovirales* order, we can infer that the phage in this study is likely to have a linear genome.

The type II restriction enzyme digestions of the *S. maltophilia* phage Bfi1 genome indicate that the phage genome is dsDNA, as these enzymes are only able to cut this type of nucleic acid (Figure 4). Restriction enzyme analysis determined the size of the genome to 32.2-56.5 kb (Table 3). A more accurate determination of the size would result from fully sequencing the genome. Genomic sequencing can be done using a shotgun cloning protocol (Lynch et al., 2010).

Without genomic sequencing, this DNA analysis did not generate enough information for us to conclusively identify the novelty of this phage. According to the International Committee on the Taxonomy of Viruses (ICTV), the major basis for distinguishing a new species of virus requires evidence that its genome sequence identity be less than 95% similar to its closest taxonomic relative (Adriaenssens and Brister, 2017). Until the genome of this phage is sequenced, the Bacterial and Archaeal Virus Subcommittees within the ICTV will not be able to affirm the novelty of this phage.

Taken together, the ultrastructure and genome analyses of *S. maltophilia* phage Bfi1 support its classification as a siphovirus within the *Caudovirales* order.

S. maltophilia Siphoviruses

At the time of writing, Bfi1 is the sixth *Siphoviridae* phage to be characterized that infects *S. maltophilia*. Table 6 describes all *S. maltophilia* siphoviruses described in the literature.

Phage	Head Diameter (nm)	Tail Length (nm)	Tail Width (nm)	Genome size (kbp)	Reference
S1	61.4 ± 1.35	129.2 ± 1.3	9.93 ± 0.66	40.287	García et al., 2008
S4	87.5 ± 1.5	201.87 ± 1.22	10.7 ± 0.24	~200	García et al., 2008
DLP1	~70	~175	NA	42.887	Peters et al., 2015
DLP2	~70	~205	NA	42.593	Peters et al., 2015
DLP5	NA	NA	NA	96.542	Peters and Dennis, 2018
Bfi1	50.8 ± 5.7	197.0 ± 31.7	10.4 ± 1.7	32.2-56.5	This study

*NA, not available

García et al. (2008) reported two temperate siphoviruses that have a *S. maltophilia* host, S1 and S4. S1 was discovered by induction of a lysogen using mitomycin C. S4 was acquired from sewage samples. Peters et al. (2015) identified two *S. maltophilia* siphoviruses, DLP1 and DLP2. DLP1 was isolated from river sediment DLP2 was isolated from soil. DLP1 has a unique plaque development; at high titers (10^{10} pfu/ml) no plaques form. At lower titers, plaques were turbid with no distinct borders. DLP2 produces plaques with distinct borders. Neither phages contain any recognizable lysogeny-associated proteins in its genome. However, due to the irregular plaque formation, DLP1 is likely a lysogenic phage. Based on this data, DLP2 might be lytic, but this has not been confirmed experimentally. As of the writing of this thesis, there is not much information available on DLP5, but it was shown to be lysogenic (Peters and Dennis, 2018).

In comparing Bfi1 to other siphoviruses, we can see that our phage appears similar to others in tail size and length, while it has a smaller head diameter (Table 6). There is a large range of genome sizes for *S. maltophilia* siphoviruses, but Bfi1 falls within this range. With the current data, it is not possible to determine if Bfi1 is lytic or lysogenic. However, it is possible that this represents the first lytic *S. maltophilia* siphovirus to be described.

Lysogenic abilities can be assessed by characterizing the phage genome. Lysogeny-associated proteins, such as integrases and lytic cycle repressors are often readily identifiable (Casey et al., 2018). Lysogeny can also be tested experimentally by taking colonies that acquire phage resistance and testing the host genome for the presence of a prophage.

Host Range of Bfi1

The process of host cell lysis involves the adsorption of the phage to the host receptor (Labrie et al., 2010). After adsorption, the phage genome is ejected into the host. Replication of the phage genome and virion structure occurs during the latent period. Lysis occurs at the end of infection and viral progeny are released into the surrounding media (Labrie et al., 2010). The progeny viruses start the infection process over in neighboring host cells. Eventually enough cells are killed that a clearing is visible to the naked eye. The exact timing of this process, can be determined by performing a one-step growth assay. This can be useful information when looking at lytic activity of a phage and is an important feature of phage characterization.

The first step in the phage infection cycle, adsorption, is the initial point of contact between virus and host and dictates host range specificity (Silva et al., 2016). *Caudovirales* phages recognize hosts using their tail structures, which have phage receptor-binding proteins (RBPs) which can recognize specific peptide sequences or polysaccharide moieties (Silva et al.,

2016). A phage fails to effectively infect a strain when the host receptors are inaccessible or non-complementary to the phage RBPs (Silva et al., 2016). Of *Siphoviridae* phages studied with Gram-negative bacterial hosts, 16 recognized proteinaceous receptors and 3 required a combination of proteins and sugar moieties (Silva et al., 2016).

The moderate host range of 30% for *S. maltophilia* phage Bfi1 is based on only 10 strains of *S. maltophilia* (Table 4). Because the number of strains tested in this research was relatively small, it may not be reflective of the true host range of this phage. Ideally, we would develop an expansive *S. maltophilia* collection, containing a variety of pathogenic strains which would represent a breadth of genetic diversity within this species.

The host ranges of other *Stenotrophomonas* lytic phages are highlighted in Table 7. These host ranges could all be described as moderate, ranging from 30% to 70% of strains tested. It should be remarked that DLP2 formed plaques on two *P. aeruginosa* strains as well (Peters et al., 2015). The ability of the mentioned phages to infect hosts from different taxonomic orders is not typical.

Phage	Family	Host Range	Strains Tested	Reference
phiSMA5	<i>Myoviridae</i>	70%	10	Chang et al., 2005
Smp14	<i>Myoviridae</i>	56%	87	Chen et al., 2007
S3	<i>Myoviridae</i>	46%	26	García et al., 2008
DLP2	<i>Siphoviridae</i>	33%	27	Peters et al., 2015
DLP6	<i>Myoviridae</i>	48%	27	Peters et al., 2017
Bfi1	<i>Siphoviridae</i>	30%	10	This Study

The limited host range of *S. maltophilia* phages might be attributed to the considerable diversity of this host species. In comparing the genome of ATCC BAA-2423 (K279a, a

pathogenic strain) to R551-3 (a plant endosymbiotic strain), approximately 85% of the R551-3 strain's 4,175 genes were homologous to the pathogenic strain (Ryan et al., 2009). This leaves hundreds of divergent genes between these two strains. Even between pathogenic isolates there is considerable diversity. A study of 139 *S. maltophilia* isolates from the same hospital found that there was considerable phylogenetic and phenotypic variability between isolates (Valdezate et al., 2004). If host susceptibility can be blocked by modifications to a single receptor gene, then the diversity within the species *S. maltophilia* may explain the limits to the host range of these *Stenotrophomonas* phages.

The type of host receptor that is recognized by *S. maltophilia* phage Bfi1 has not been determined. Understanding the host receptors needed for phage infection of *Stenotrophomonas maltophilia* may be informative in understanding why the host range is moderate. This could be tested by developing a mutant library of S18202 and determining which gene(s) is/are necessary for phage infection.

Host receptor recognition is not the only component of host range specificity, but it is a major avenue to acquire resistance against viral infections (Rodriguez-Valera et al., 2009). Bacterial hosts can employ a variety of other mechanisms to block phage infection. These include: superinfection exclusion systems, restriction-modification systems, Argonaute proteins, CRISPR-Cas systems, abortive infection systems, and toxin-antitoxin systems (Dy et al., 2014).

S. maltophilia phage Bfi1 was tested against *P. aeruginosa*, a related pathogen (Williams et al., 2010). Based on observations by Peters et al. (2015) we know that there are some phages that infect both *S. maltophilia* and *P. aeruginosa*. There are limited examples of phages with this wide of a host range, so we did not expect to observe plaque formation by Bfi1 on *P. aeruginosa*. Regardless of our expectations, it is medically relevant to test this because *P. aeruginosa* and

S. maltophilia are often found together in polymicrobial communities (Berg et al., 2005). Most notably these bacteria can coinfect the CF patient's lungs (Graff and Burn, 2002).

Other organisms that are phylogenetically related to *Stenotrophomonas maltophilia*, may also be susceptible to phage Bfi1, including those of the *Xanthomonas* and *Xylella* genera (Williams et al., 2010). Both these genera harbor debilitating plant pathogens of agricultural significance, such as black rot (affecting cruciferous vegetables like cabbage and broccoli) (Williams, 1980) and Pierce's disease (a lethal grapevine disease) (Hopkins and Purcell, 2002). It may be significant to consider the effect of Bfi1 on these pathogens due to their economic and agricultural harm.

Phage-Biofilm Interactions

To the best of our knowledge, this is the first time the effects of a phage on *S. maltophilia* biofilms have been studied. From the biofilm assays, we demonstrated that the phage, at an MOI=10³, inhibits the development of biofilms when it is introduced simultaneously with planktonic cells in culture (Figure 7B). This effect is even greater at MOI=10⁵ (Figure 7C). At MOI=10 there was only a small effect on the planktonic cell culture of the bacteria (Figure 6A). There is no effect on the *S. maltophilia* biofilms at MOI=10 (Figure 7A). One explanation is that at a lower titer the host has enough time to begin to form phage-resistant biofilms. A higher titer of phages kills more cells before biofilm formation is underway, whereas a lower titer of phages allows more cells to begin to establish biofilms on the polyvinyl chloride surface.

Potential Use in Therapy

At the time of writing this thesis, no *S. maltophilia* phages have been used in any form of therapy. There is still much more information needed to determine if this phage should be recommended therapeutically. First, it will be necessary to confirm that the phage is lytic. At this point, the phage has not been definitively shown to be lytic or lysogenic. This is a significant consideration because lysogenic phages are not considered good candidates for phage therapy. Lysogenic phages can convert hosts into lysogens, preventing these hosts from undergoing immediate lysis and making the host phage-resistant (Casey et al., 2018).

Lysogens can also result in other phenotypic changes to their host that may enhance host virulence. For example, the Liverpool Epidemic Strain of *P. aeruginosa* contains multiple prophages that have been shown to confer enhanced virulence to their host (Salunkhe et al., 2005). In the same way, the *Vibrio* phage CTXphi carries the cholera toxin, which is required for the pathogen, *Vibrio cholerae*, to trigger toxin-mediated epidemic cholera (Waldor and Mekalanos, 1996).

A similar but separate concern in identifying phages useful in therapy is the horizontal gene transfer of virulence factors such as toxins (Pirnay et al., 2015). Genome sequencing may also allow us to rule out phages that carry toxins or antibiotic resistance factors. Even if a phage is not lysogenic, it may be risky to introduce a genetic element that could exacerbate an infection. Many bacteria, including *S. maltophilia*, are naturally competent and able to take up DNA from their environment (Berg and Martínez, 2015). Therefore, there is a risk that virulence genes could be acquired from a phage without lysogenic conversion.

Phage Resistance of Biofilms

The steps towards effective therapies require evidence of the efficacy of a phage treatment. This research has shown that biofilm formation is significantly affected by *S. maltophilia* phage Bfi1. However, the biofilm experiments in this thesis were performed *in vitro*, and in a patient, *S. maltophilia* biofilms may respond differently to phage treatment. Research on other bacterial pathogens indicate that biofilms may demonstrate resistance to phage. An *in vivo* mouse study using *P. aeruginosa* strain PAK demonstrated that a phage treatment 2 h post-infection resulted in 100% survival. However, this survival rate dropped down to 20% at 6 h post-infection (Debarbieux et al., 2010). The data in this study suggest that immature biofilms may be more susceptible to phage than fully developed mature biofilms. Therefore, it is important to design a therapy that anticipates the different stages of a biofilm. Future research with the *S. maltophilia* phage Bfi1 can address this by assessing the phage's effect on a mature biofilm, or through a time-course study of biofilm development.

There are a few reasons to explain why a biofilm might confer viral resistance. In some cases, biofilms cause phage resistance by preventing phage from reaching the host cells. Between the lytic T7 phage and its host *E. coli* biofilm, the protection from phage was due to prevention of phage transport into the biofilm and through competitive inhibition of the phage receptor by curli polymers (an amyloid fiber network) (Vidakovic et al., 2017). In other words, the phage was adhering to the extracellular polymers instead of adsorbing to the cells.

Phase variation observed during biofilm maturation may also be involved in phage resistance. Phase variation involves changes of protein expression within a bacterial population. As the biofilm-forming phenotypes will involve changes in the proteins expressed on the cell surface, the proteins required for adsorption may not be present on cells within a biofilm. As an

example of phase variation resistance, *Bordetella* species is 10^6 times more susceptible to phage BPP1 when pertactin, an adhesion protein, is expressed (Liu et al., 2002). This protein is expressed in much higher quantities during the virulent phase, when certain adhesins, toxins, and secretion systems are activated (Liu et al., 2002).

Phage Therapy

The research on phage therapy for treatment of bacterial infections has grown substantially in recent years. *S. maltophilia* phage Bfi1 may be useful in such treatments. While it may not demonstrate a broad host range, this phage could be tested in combination with other approaches to treat *Stenotrophomonas* infections.

Using multiple phages in a phage cocktail (termed polyphage therapy) may offer benefits that a monophage treatment cannot. Use of phage cocktails can solve two difficulties presented to clinicians: the limited host range and the development of phage resistance. By combining multiple phages that each have a different host range, there are greater chances that a strain of bacteria will be susceptible to one or more of the phages in the cocktail. For example, Alves et al. (2015) reported that a phage cocktail *in vitro* led to 100% inhibition of *Pseudomonas* PAO1 after 24 h. In contrast, *Pseudomonas* PAO1 began re-growing after 8 h following each monophage treatment. Phage resistance can evolve naturally among host bacteria through genetic mutation, but using multiple phages should reduce the possibility that bacterial mutants become multi-phage resistant.

It is important to acknowledge that polyphage therapy is not a perfect solution. In a study by Gu et al. (2012), the authors demonstrated the development of phage-resistant mutants of *Klebsiella pneumoniae* strain K7, even when treated with a three phage cocktail. It should be

noted that regrowth of *K. pneumoniae* from individual phage treatments was detected between 6-8 h, while regrowth from the phage cocktail occurred at 26 h. Although phage-resistance remains a concern in polyphage therapy, it is greatly reduced.

Another consideration, which was highlighted earlier, is that not all phages are innocuous. Some bacterial viruses can enhance the virulence of their hosts. This same concern exists and could be heightened in polyphage treatments. For example, one phage may confer resistance to other phages in a cocktail. This concern can be alleviated by requiring substantial characterization of prospective phages prior to their application. Bioinformatic techniques, which allow for rapid identification of undesirable genetic elements, can begin to eliminate phages from use that possess such characteristics (Chan et al., 2013).

Another emerging approach to phage therapy is the combination of phages with antibiotics to enhance phage virulence. The addition of a low dosage of the cephalosporin, cefotaxime with *Escherichia* phage phiMFP results a seven-fold increase in burst size compared to the phage by itself (Comeau et al., 2007). This phenomenon, referred to as phage-antibiotic synergy (PAS), has been demonstrated in phages of *P. aeruginosa* and *Burkholderia cepacia* complex, as well (Knezevic et al., 2013; Kamal and Dennis, 2015). It is difficult to identify the benefit and drawbacks for this approach currently, due to the limited data available.

In the United States, the pathway for phage therapy development includes a series of steps that have not yet been surmounted. The current process for the development of conventional medicinal products may not be compatible with timely phage therapy development (Pirnay et al., 2015). But most experts can agree that phages used in therapies require a complete characterization of their physiology, genetics, and pharmacological potential (Forde and Hill, 2018). Only after these steps, should the production and regulation of a therapy be considered.

Conclusion

There are still important research questions that must be addressed before we can definitively recommend this phage for such a therapy. We need to show that the phage is lytic, that its genome does not carry virulence factors, and demonstrate that it can be useful in treating biofilms, either by itself or in combination with other phages or antibiotics. With a growing prevalence of *S. maltophilia* infections, and the steady rise of antibiotic resistance, there is an urgent need for alternative treatments of this pathogen.

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