Characterization of Novel Broad-Host-Range Bacteriophage DLP3 Specific to *Stenotrophomonas maltophilia* as a Potential Therapeutic Agent

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A novel *Siphoviridae* phage specific to the bacterial species *Stenotrophomonas maltophilia* was isolated from a pristine soil sample and characterized as a second member of the newly established *Delepquintavirus* genus. Phage DLP3 possesses one of the broadest host ranges of any *S. maltophilia* phage yet characterized, infecting 22 of 29 *S. maltophilia* strains. DLP3 has a genome size of 96,852 bp and a G+C content of 58.4%, which is significantly lower than *S. maltophilia* host strain D1571 (G+C content of 66.9%). The DLP3 genome encodes 153 coding domain sequences covering 95% of the genome, including five tRNA genes with different specificities. The DLP3 lysogen exhibits a growth rate increase during the exponential phase of growth as compared to the wild type strain. DLP3 also encodes a functional erythromycin resistance protein, causing lysogenic conversion of the host D1571 strain. Although a temperate phage, DLP3 demonstrates excellent therapeutic potential because it exhibits a broad host range, infects host cells through the *S. maltophilia* type IV pilus, and exhibits lytic activity in vivo. Undesirable traits, such as its temperate lifecycle, can be eliminated using genetic techniques to produce a modified phage useful in the treatment of *S. maltophilia* bacterial infections.

Keywords: *Stenotrophomonas*, bacteriophage, phage, phage therapy, antimicrobial resistance

INTRODUCTION

*Stenotrophomonas maltophilia* is a robust, non-sporulating, obligate aerobe Gram-negative bacillus (Brooke, 2012). The genus name *Stenotrophomonas* was originally chosen due to the perceived limited nutritional range of the bacterium (Palleroni and Bradbury, 1993), though many studies have since shown an impressive metabolic versatility (Chatelut et al., 1995; Berg et al., 1999; Hauben et al., 1999; Minkwitz and Berg, 2001; Svensson-Stadler et al., 2012). *S. maltophilia* is often found in close association with plant rhizospheres where they actively promote plant growth through the secretion of growth-promoting compounds (Ryan et al., 2009). This plant growth promotion is now being used commercially; *S. maltophilia* strains are used as biofertilizers due to their ability to fix nitrogen, produce growth-promoting plant hormones, and protect plant roots from phytopathogens (Rathi and Nandabalan, 2017). Additionally, the vast metabolic diversity
observed in *S. maltophilia* enables these bacteria to be used in bioremediation, from heavy metal detoxification of soils and waterways to the degradation of insecticides and volatile organic compounds such as benzene. However, their widespread use in biotechnology and agriculture is problematic due to their ability to cause disease in humans (Berg and Martinez, 2015).

*S. maltophilia* is an important multidrug resistant, opportunistic pathogen that is most commonly associated with pneumonia and bacteremia in immunocompromised patients. *S. maltophilia* has also been identified as the cause of soft tissue infections, osteomyelitis, meningitis, endocarditis, otitis and scleritis (Brooke, 2012). Several risk factors are associated with *S. maltophilia* infections in the general population, including malignancy, human immunodeficiency virus, cystic fibrosis (CF), intravenous drug use, surgical and accidental trauma, prolonged hospitalization, mechanical ventilation, indwelling catheters, corticosteroids, immunosuppressive therapy, and treatment with broad-spectrum antibiotics (Al-Anazi and Al-Jasser, 2014). One study estimated the nosocomial mortality rates attributed to *S. maltophilia* bacteremia at 16.7%, and the overall mortality rate of patients infected with *S. maltophilia* was 25% (Naidu and Smith, 2012). A recent study on the clinical outcomes of cancer patients with bloodstream infections (BSI) and pneumonia caused by *S. maltophilia* infections in Mexico City indicated that 31.6% died within the first month; 22.1% of the deaths were due to pneumonia and 9.5% were due to BSI (Velazquez-Acosta et al., 2018). A similar study on BSI mortality rates associated with 937 German intensive care units found *S. maltophilia* infections had the highest mortality rates (28.4%), followed next by non-albicans-Candida spp. (27.1%) and *Pseudomonas aeruginosa* (25.8%) (Schwab et al., 2018).

Due to a wide array of pathogenicity factors, *S. maltophilia* can be difficult to clear once an infection has been established. *S. maltophilia* pathogenicity mechanisms include swimming and twitching motility (from flagella and type IV pili, respectively), a DNA hypermutator mechanism, iron uptake transporters, biofilm formation, lipopolysaccharide (LPS), many extracellular enzymes encoded such as fibrolysin, lipases, esterase, DNase, RNase, proteases and lecithinase, type II protein secretion systems, host cell invasion, and quorum sensing signaling systems (Brooke, 2012). In addition, clinical *S. maltophilia* isolate culture supernatants applied to Hep-2, HeLa, or Vero cells resulted in cytotoxic effects such as intensive rounding, loss of intercellular junctions, and membrane blebbing followed by cell death within 24 h (Figueiredo et al., 2006). In some cases, endocytosis, hemolytic activity, and cell aggregation were observed, and the addition of protease inhibitors did not prevent cell cytotoxicity. LPS production was shown to be required for attachment to surfaces, and colonization and virulence in a rat lung model (McKay et al., 2003). As well, it was shown that an O-polysaccharide synthesis mutant was susceptible to complement-mediated cell killing (Brooke et al., 2008). *S. maltophilia* can form biofilms on many surfaces such as glass, plastics, implanted medical devices, and lung epithelial cells (Huang et al., 2006; Pomplio et al., 2008, 2010; Brooke, 2014). A study focusing on the biofilm formation of CF-derived bronchial epithelial cell monolayers revealed that all *S. maltophilia* isolates were able to form biofilms on bronchial epithelial cells (Pomplio et al., 2010). Type-I fimbriae genes have only been identified in clinical *S. maltophilia* isolates, which suggests they may play a direct role in the colonization of infected CF individuals (Nicoletti et al., 2011). *In vitro* tissue culture assays have indicated the *S. maltophilia* fimbriae 1 (SMF-1) protein is important for adherence to eukaryotic cells and glass, and anti-SMF-1 antibodies inhibit adherence to eukaryotic cells and glass if the antibodies were applied during early stages of infection (de Oliveira-Garcia et al., 2003). The ability of *S. maltophilia* isolates to form biofilms and invade host cells contributes to the prolonged infections noted in hematology and oncology patients, despite aggressive antibiotic treatments (Lai et al., 2006).

Innate antibiotic resistance in *S. maltophilia* infections is a major contributing factor to treatment failure. Patients who receive the wrong antibiotic at initial infection diagnosis have an increased risk of mortality compared to patients who received appropriate *S. maltophilia* antibiotic treatment initially (Velazquez-Acosta et al., 2018). *S. maltophilia* infections can be further complicated by the emergence of mutants with pleiotropic antibiotic resistance (Denton and Kerr, 1998). Intrinsinc resistance is attributed to mechanisms such as reduced membrane permeability (Mett et al., 1988), chromosomally encoded multidrug efflux pumps, and chromosomally encoded Qnr pentapeptide repeat proteins (Sanchez et al., 2008; Sanchez and Martinez, 2010; Chang et al., 2011). Additionally, antibiotic inactivating enzymes such as inducible L1 and L2 β-lactamases (Walsh et al., 1997; Crowder et al., 1998; Okazaki and Avison, 2008), a TEM-2 β-lactamase from a *Tn1* like transposon (Avison et al., 2000), and aminoglycoside inactivating enzymes (Okazaki and Avison, 2007; Vetting et al., 2008) have been identified in many *S. maltophilia* isolates. The L1 protein is a molecular class B zinc-dependent metallo-β-lactamase which hydrolyzes virtually all classes of β-lactams, including penicillins, cephalosporins and carbapenems (Avison et al., 2001), whereas the L2 protein is a molecular class A clavulanic acid-sensitive cephalosporinase (Chang et al., 2015). The drug of choice to treat *S. maltophilia* infections continues to be trimethoprim/sulfamethoxazole (TMP/SMX) (Chung et al., 2013; Wang et al., 2016), although resistance to TMP/SMX across clinical isolates is common (30.5%) (Ochoa-Sanchez and Vinuesa, 2017). However, higher rates of TMP/SMX resistance, ranging from 16% to nearly 80%, have been noted in patients with cancer, CF, and in several specific countries (Chang et al., 2015; Rhee et al., 2016). The majority of TMP/SMX resistance is due to the presence of a *sul* gene encoding a sulfonamide resistance protein located on a class 1 integron (sul1) (Barbolla et al., 2004; Chung et al., 2015), and also on insertion sequence common region (ISCR) elements (sul2) (Toleman et al., 2007) rather than through the overexpression of efflux pumps. A *dfr* gene encoding a dihydrofolate reductase associated with a class 1 integron has also been shown to cause some level of resistance against TMP/SMX (Hu et al., 2011), along with the activity from several RND family efflux pumps, although not to the same degree as the resident *sul* genes (Huang et al., 2013; Lin et al., 2015; Sanchez, 2015). Thus, research into alternative treatment options is critical due to increased
resistance to TMP/SMX noted with specific comorbidities and geographic locations.

Related to this high-level antibiotic resistance, *S. maltophilia* is also capable of tolerating biocides and harsh environments, which makes this bacterium difficult to eradicate from hospital settings. Nosocomial isolation sources for *S. maltophilia* include ultra-pure water, hemodialysis water, nebulizers, hand-washing soap, hospital antiseptic solution, chlorhexidine-cetrimide topical antiseptic solution, hypochlorite cleaners, triclosan, sodium dodecyl sulfate, and antiseptics containing quaternary ammonium compounds (Brooke, 2012; Kampmeier et al., 2017).

Non-clinical multidrug resistant isolates of *S. maltophilia* have also been recovered from environments such as soil, water, plants and food sources (Berg et al., 2005; Qureshi et al., 2005; Brooke, 2012; Lin et al., 2017; Furlan and Stehling, 2018; Kim et al., 2018), and have the potential to cause community-acquired infections (Falagas et al., 2009; Chang et al., 2014).

High levels of innate antibiotic resistance in *S. maltophilia* isolates highlights the need for alternative treatment options to combat these infections. The use of bacteriophages, or phages, as viruses specifically lytic toward bacteria, is being investigated as a viable option. Phage therapy requires the isolation and characterization of phages prior to their use as prophylactic or therapeutic agents. Phage therapy holds several advantages over traditional antibiotics (Sulakvelidze et al., 2001; Burrowes et al., 2011; Golkar et al., 2014). First, phages possess a narrow host range, only infecting a few strains within a species, enabling precise targeting of the treatment, and preventing disruption of the patient's normal microbiota. Second, phages are self-replicating, such that phage replication can significantly increase phage abundance at the site of the bacterial infection. Third, phages use a different mechanism of action to kill bacteria than chemical antibiotics, and so are virulent against antibiotic resistant bacteria. Fourth, few side effects have been reported during or after phage application, including immune responses, suggesting that higher organisms are adapted to the presence of phages within tissues or on body surfaces. In addition, the high abundance of phages in the environment make them relatively simple to isolate. As well, phages are relatively easy to manipulate through molecular approaches to create therapeutically enhanced phages which exhibit improved performance in vivo, and phage biology is becoming better understood such that the development of new therapeutic phages will progress more rapidly than it has in the past. Ideally, a therapeutic cocktail of multiple phages targeting several bacterial strains with different phage-receptors will be developed to increase the host range of the cocktail and protect against receptor-mutation mediated resistance (Burrowes et al., 2011; Golkar et al., 2014).

Twenty phages specific to *S. maltophilia* have been isolated and characterized to date. Eleven phages (Sm1, IME13, IME15, S3, Sm14, ΦSMA5, DLP1, DLP2, DLP4, DLP5, DLP6) have been isolated and characterized specifically for their therapeutic potential. For example, the *Myoviridae* phage ΦSMA5 has an extensive host range successfully infecting 61 out of 87 strains tested (Chang et al., 2005), while Sm1 is the first *S. maltophilia* phage used in a murine model where it was found to provide 100% protection against *S. maltophilia* infection. The broad host range of ΦSMA5 and significant *in vivo* protection Sm1 provides in a murine model are promising examples of therapeutic phages that can be used for the treatment of antibiotic resistant *S. maltophilia* infections.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

Initial phage isolation was accomplished with five clinical *S. maltophilia* strains (D1585, D1571, D1614, D1576, D1568) from the Canadian *Burkholderia cepacia* complex Research and Referral Repository (CBCCRR; Vancouver, BC, Canada). An additional 22 *S. maltophilia* clinical isolates were obtained from the Provincial Laboratory for Public Health - North (Microbiology), Alberta Health Services for host range analysis. Strains SMDF92 and ATCC 13637 were gifted by Dr. Jorge Giron. Strains were grown aerobically at 30°C on Luria-Bertani solid media until single colonies were visible (16–36 h) or in LB broth with shaking at 225 RPM.

**Bacteriophage Isolation, Propagation, and Host Range**

The phage DLP3 (vB_SmaS_DLP_3) was isolated from soil collected in Edmonton, Alberta, Canada using host strain *S. maltophilia* D1571. No plants were associated with the soil sample. Approximately 10 ml of soil was mixed with 10 ml of LB broth, 1 ml of modified suspension media (SM) (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgSO4) and 100 µl of a D1571 overnight culture. The slurry was incubated overnight at 30°C with shaking at 225 RPM. The supernatant was filter-sterilized with a Millex-HA 0.45 µm syringe-driven filter (Millipore, Billerica, MA, United States) and stored at 4°C, as performed previously (Peters et al., 2015, 2017, 2019). A single plaque was picked to propagate a working-stock solution for analysis using top-agar overlays. Briefly, 100 µl of overnight D1571 culture and 100 µl DLP3 stock (≈10⁹ PFU/ml) were mixed and incubated for 5 min at room temperature then added to 3 ml of 0.7% LB top agar. The mixture was poured onto an LB plate and incubated for 18 h at 30°C. The top agar of plates showing confluent lysis was scraped into a 50 ml Falcon tube. A 3 ml aliquot of SM was added for each plate scraped and the slurry was shaken for 1 min followed by centrifugation (5 min at 10,000 x g) and filter sterilization. Host range analysis was performed on 29 *S. maltophilia* and 19 *Pseudomonas aeruginosa* strains using serially diluted DLP3 lysate into SM. A 10 µl aliquot of each concentration was spotted in triplicate onto a plate containing bacterial culture in a top-agar overlay and incubated overnight at 30°C.

**Electron Microscopy**

Phage lysate for electron microscopy was prepared using LB plates and top agar made with agarose and filter sterilized using a 0.22 µm filter. A carbon-coated copper
grid was overlaid with 10 µl of phage lysate for 2 min then stained with 4% uranyl acetate for 30 s. A Philips/FEI (Morgagni) transmission electron microscope (TEM) with charge-coupled device camera at 80 kV (University of Alberta Department of Biological Sciences Advanced Microscopy Facility) was used to obtain TEM images. The capsid diameter, tail length and tail width of ten virions were measured using ImageJ and averages calculated using Microsoft Excel.

**Phage DNA Isolation, Sequencing, and RFLP Analysis**

Genomic DNA was isolated from a high-titer DLP3 stock (10⁹ PFU/ml). Lysate was clarified by spinning 10,000 × g for 10 min and the supernatant was subsequently treated with 100 µl 100X DNase I buffer (1 M Tris–HCl, 0.25 M MgCl₂, 10 mM CaCl₂), 10 µl DNase I (Thermo Scientific, Waltham, MA, United States), 6 µl RNase (Thermo Scientific) and incubated 1 h at 37°C. A 400 µl aliquot of 0.5 M EDTA (pH 8.0), SDS (final concentration of 2%) and Proteinase K (final concentration of 400 µg/ml) was added followed by incubation at 55°C overnight. A 1/2 volume of 6 M NaCl was added and the solution was vortexed at high speed for 30 s followed by centrifugation at 17,900 × g for 30 min. The supernatant was transferred to a fresh tube with an equal volume of 100% isopropanol and stored at −20°C for at least 1 h to overnight. The DNA was pelleted with centrifugation at 17,900 × g for 20 min at 4°C followed by three 70% ethanol washes. The pellet was dried at room temperature and resuspended in nuclease-free water. Purity and concentrations of eluted DNA were checked with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and incubated 1 h at 55°C. A 400 µl aliquot of 0.5 M EDTA (pH 8.0), SDS (final concentration of 2%) and Proteinase K (final concentration of 400 µg/ml) was added followed by incubation at 55°C overnight. A 1/2 volume of 6 M NaCl was added and the solution was vortexed at high speed for 30 s followed by centrifugation at 17,900 × g for 30 min. The supernatant was transferred to a fresh tube with an equal volume of 100% isopropanol and stored at −20°C for at least 1 h to overnight. The DNA was pelleted with centrifugation at 17,900 × g for 20 min at 4°C followed by three 70% ethanol washes. The pellet was dried at room temperature and resuspended in nuclease-free water. Purity and concentrations of eluted DNA were checked with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, United States). DLP3 genomic (gDNA) DNA was sequenced using both Illumina and Pacific Biosciences technologies. A Nextera XT library was generated for paired-end sequencing on MiSeq (Illumina) platform using MiSeq v2 reagent kit. Restriction fragment length polymorphism (RFLP) analysis was used with 15 FastDigest (Thermo Scientific) restriction enzymes: EcoRI, XbaI, BamHI, HindIII, KpnI, Smal, Spnl, PstI, SacI, SalI, Apal, Clal, NdeI, Sphl, XhoI. Restriction reactions were set up using 1 µl FastDigest enzyme, 2 µl FastDigest restriction buffer, 1 µg of phage DNA and nuclease-free water to bring the final volume to 20 µl. Reactions were performed on a 0.8% (wt/vol) agarose gel in 1x TAE (pH 8.0).

**Bioinformatic Analysis of the DLP3 Genome**

A 96,852 bp contig assembled from the Illumina reads with SPAdes 3.8.0 was identified for further analysis. No gaps or ambiguous sites were found in the assembly, which has a mean coverage of 114 reads and Q40 of 93.8%. Prediction of open reading frames (ORFs) was accomplished with the GLIMMER plugin (Delcher et al., 2007) for Geneious (Kearse et al., 2012) using the Bacteria and Archaea setting, as well as GeneMarkS for phage (Besemer et al., 2001). Conserved domain searches were performed using CD-Search with the CDD v3.16 – 50369 PSSMs database (Marchler-Bauer et al., 2011). Phyre (Kelley et al., 2015), HHblits (Remmert et al., 2011; Zimmermann et al., 2018) and ITASSER (Yang and Zhang, 2015) were used to gain insights into possible functions of hypothetical proteins or to provide more support for putative functions. BLASTn and BLASTp were used to gain information on relatives based on genomic data and individual proteins, respectively (Altschul et al., 1997). The NCBI non-redundant protein sequence and nucleotide collection databases (update dates for both: 2018/08/26) were used for the BLASTp and BLASTn searches, respectively. BLASTp results above 1.00E-03 were annotated as hypothetical proteins. tRNAs were identified using the general trna model with tRNAscan-SE software (Schattner et al., 2005).

**D1585 pilT Deletion Construction**

A pilT clean deletion mutant of S. maltophilia D1585 was constructed using overlap-extension PCR and allele exchange as previously described (McCutcheon et al., 2018). Briefly, regions upstream and downstream of the pilT gene in D1585 were PCR amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs) with primer pairs UpF-HindIII (GGGCAAGCTTCTAGTACTCGCCGTTCACTG) and UpR-OE (CTCGAACAGGCCTTTGGAGCTTTTGTTCTTTACGG) for the upstream region, and DnF-OE (AACACAAAGCGTC CAAGCGCCGTTCCGTAGTAAGG) and DnR-XbaI (GGGCC TCTAGACCTAGCTTTGAGATCTGGC) for the downstream region. Overlap regions are italicized and restriction enzyme recognition sites are bolded. Following overlap-extension PCR, the deletion cassette was ligated into pEX18Tc and this plasmid, pD1585ΔpilT, was transformed into E. coli S17-1 for bacterial mating with D1585. Single crossover transconjugants were selected on LB agar containing 100 µg/mL tetracycline and merodiploid status was confirmed by colony PCR with pilT specific primers pilTF (GTTCCGTGAATCAGGAGGC) and pilTR (GGGGCGCATGTTCCAGGAAA). Positive merodiploids were grown in LB broth and plated on LB with 10% sucrose to select for double crossover ΔpilT mutants that were confirmed by colony PCR as above. For complementation, the D1585 pilT gene was cloned into pBR1MCS using pilTF and pilTR primers with tails for HindIII and XbaI restriction enzymes (Thermo Fisher) to create pD1585ΔpilT.

**Phage Plaquing Assays**

DLP3 plaquing ability on wildtype and mutant strains of D1585 and 280 was determined by spot assay on bacterial lawns as previously described (McCutcheon et al., 2018). Briefly, bacterial strains were grown in 1/2 LB supplemented with 35 µg/mL chloramphenicol at 30°C with aeration at 225 RPM for 18 h. 100 µL of overnight culture was used in a top agar overlay containing 35 µg/mL chloramphenicol and allowed to solidify. DLP3 lysate standardized to 10¹⁰ PFU/ml on S. maltophilia D1571 was ten-fold serially diluted in SM to 10⁵ PFU/ml and 5 µl of each dilution was spotted on the prepared plates in triplicate. Plates were incubated upright at 30°C and imaged.
after 16 h. Each experiment was repeated in biological and technical triplicate.

**Protein Isolation and Mass Spectrometry**

Isolation of DLP3 protein for SDS-PAGE analysis was accomplished following a protocol for the formation of ghost particles (Boulanger, 2009). Briefly, sterile DLP3 lysate (∼1 × 10⁹) was clarified twice with 10,000 × g centrifugations and treated with nucleases following the DNA isolation protocol described above. After the incubation, an equal volume of 10 M LiCl was added and the solution was incubated at 46°C for 10 min, followed by 10-fold dilutions into sterile Milli-Q water. The released DLP3 gDNA was digested with an addition of 10 mM MgCl₂ and 50 U of RNase-free DNaseI per 1 × 10¹² PFU. This solution was incubated overnight at 37°C, followed by ultracentrifugation at 28,700 × g for 1.2 h. The supernatant was discarded and pellets were resuspended with 100 µL SM. An aliquot of the sample was diluted in half with 2x Laemmli sample buffer (10% [v/v] beta-mercaptoethanol [BME], 6% [w/v] SDS, 20% [v/v] glycerol, 0.2 mg/ml bromophenol blue) and incubated 10 min at 99°C.

An SDS-PAGE gel with a 4% stack and a 7.5% resolving portion was made with 40% 37:5:1 acrylamide/bis-acrylamide solution (Bio-Rad) and fresh 10% ammonium persulfate. The gel was loaded into a Mini-PROTEAN electrophoresis chamber (Bio-Rad) using 1x running buffer. A 6 µL aliquot of PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was used as a molecular weight standard and 6–12 µL of DLP3 ghost particles in 1x sample buffer was loaded into the remaining wells. The gel was run at 180 kV for 75 min and placed in Coomassie R-250 stain for 1 h with gentle rocking. The gel was destained over 2 h, with the destaining solution replaced every 30 min. The gel was placed in a 50 mL Falcon tube with Milli-Q to transport the gel for mass spectrometry analysis at the Alberta Proteomics and Mass Spectrometry (APM) facility located at the University of Alberta.

In-gel trypsin digestion was performed on the samples. The lane was cut into seven equal gel sections, destained twice in 100 mM ammonium bicarbonate/acetonitrile (ACN) (50:50), reduced (10 mM BME–100 mM bicarbonate) and then alkylated (55 mM iodoacetamide–100 mM acetonitrile). After dehydration, trypsin digestion (6 ng/µL) was allowed to proceed overnight at room temperature. Tryptic peptides were extracted from the gel using 97% water–2% acetonitrile–1% formic acid followed by a second extraction using 50% of the initial extraction buffer and 50% acetonitrile. Fractions containing tryptic peptides were resolved and ionized using nanoflow high-performance liquid chromatography (HPLC) (Easy-nLC 1000; Thermo Scientific) coupled to a Q Exactive Orbitrap mass spectrometer (MS) (Thermo Scientific). Nanoflow chromatography and electrospray ionization were accomplished by using a Pico- Frit fused silica capillary column (ProteoPepII; C18) with a 100-µm inner diameter (New Objective) (300 Å, 5 µm pore size). Peptide mixtures were injected onto the column at a flow rate of 3,000 nL/min and resolved at 500 nL/min using 75-min linear gradients of 4% to 40% (vol/vol) aqueous ACN with 0.2% (vol/vol) formic acid. The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution Orbitrap survey spectra using external mass calibration, with a resolution of 35,000 and m/z range of 400 to 2,000. The 15 most intensely multiply charged ions were sequentially fragmented by HCD fragmentation. After two fragmentations, all precursors selected for dissociation were dynamically excluded for 60 s. Data were processed using Proteome Discoverer 1.4 (Thermo Scientific). The UniProt *Stenotrophomonas* database and all DLP3 proteins were searched using SEQUEST (Thermo Scientific). Search parameters included a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 Da. Peptides were searched with carbamidomethyl cysteine as a static modification and oxidized methionine and deamidated glutamine and asparagine as dynamic modifications.

**Determination of DLP3 Lifestyle**

Top agar overlay plates showing confluent lysis of D1571 by DLP3 were used to obtain resistant colonies. Briefly, 3 ml of SM was added to the plates and a sterile glass rod was used to gently skim the agar. The SM was collected and placed into microcentrifuge tubes, then centrifuged at 5,000 × g for 5 min. The supernatant was discarded and 1 ml of fresh SM was added to resuspend the pellet, followed by centrifugation at 5,000 × g for 5 min. This wash step was repeated three times in total. Following the final wash centrifugation, the supernatant was removed and the pellet was resuspended in 500 µL LB broth. Cells were serially diluted with LB and plated on LB plates, then incubated at 30°C for 16 h. Single colony isolates were selected for further study and tested for superinfection resistance using overnight cultures of every isolate in a top agar overlay assay with DLP3. After an 18 h incubation at 30°C, the plates were observed for plaque development. Single colony isolates without plaque development were retained for analysis.

**Erm Functionality**

Triplicate minimal inhibitory concentration (MIC) experiments on *S. maltophilia* strains D1571 and D1571:DLP3 were conducted using established protocols (Wiegand et al., 2008) to study the functionality of the DLP3-encoded Erm. Overnight cultures were grown at 30°C in 5 mL LB. A 1:100 subculture was grown at 30°C to an OD₆₀₀ of 0.1 in Mueller-Hinton broth (MH) and used in 96 well plates containing an erythromycin dilution series (MP Biomedicals). Following a 16 h incubation, OD₆₀₀ absorbance was obtained using a Wallac 1420 VICTOR2 multilabel counter (PerkinElmer, Waltham, MA, United States) and values were averaged using Excel. Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, United States) to perform a two-way analysis of variance (ANOVA) with Sidak’s multiple comparisons.

**Growth Analysis of Wild Type D1571 and the DLP3 Lysogen**

Single colony triplicate overnight cultures of wild type D1571 and the lysogen D1571:DLP3 were grown in LB broth at
30°C with shaking. Subcultures (1:100) for each sample were performed using LB broth and each subculture was grown to an OD$_{600}$ of ~0.32 at 30°C with 225 RPM shaking. Subcultures were distributed in triplicate aliquots of 200 µL in 96 well plates; an LB broth control was included for each plate. The OD$_{600}$ was then obtained for each plate using a Wallac 1420 VICTOR$^2$ multilabel counter (PerkinElmer, Waltham, MA) at the following time points: 0, 2, 4, 6, 8 h. The OD$_{600}$ data were used to determine the growth rate (µ) with the established formula: \[ \log_{10} N - \log_{10} N_0 = (\mu/2.303) (t - t_0), \] whereby $N_0$ is the time zero ($t_0$) OD$_{600}$ reading and $N$ is the final OD$_{600}$ reading obtained at a specific time ($t$) in the experiment. Resulting data were analyzed with GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, United States) to graph the growth curve and growth rate. Statistical analysis of the growth curve and growth rate data were performed in GraphPad Prism 8 using two-way repeated measurement ANOVA with multiple comparisons (Sidak’s).

**Galleria mellonella Killing and Phage Rescue Assays**

*G. mellonella* infections were performed as previously described, with modifications (Seed and Dennis, 2008; Kamal et al., 2019). Single colony triplicate overnight cultures of wildtype strain D1571 and the DLP3 lysogen, D1571:DLP3, were grown aerobically at 37°C in LB for 19 h corresponding to approximately $2 \times 10^{10}$ CFU/mL. Cultures were standardized by OD$_{600}$, washed once in 1× phosphate-buffered saline (PBS, pH 7.4) and serially diluted tenfold in PBS. *G. mellonella* larvae were bred in-house at 30°C using artificial food (wheat germ: 264 g, brewer’s yeast: 132 g, beeswax: 210 g, glycerol: 132 g, honey: 132 g, water: 66 g) and larvae weighing approximately 250 mg were selected for experiments. Each experiment consisted of ten larvae per group and 5 µL aliquots of bacterial culture were injected into the rear left proleg of each larva using a 250 µL Hamilton syringe fitted with a repeating dispenser. Sterile PBS injected larvae were used as negative controls and showed 100% survival for the duration of the experiment. Colony counts on LB agar were used to determine the CFUs injected. Following injection, larvae were placed in a static incubator in the dark at 37°C and scored for death every 24 h until 120 h post-infection (hpi). Larvae were considered dead when they did not respond to touch with movement.

For DLP3 phage rescue trials, wildtype D1571 culture was prepared as described above and an inoculum of approximately $8 \times 10^8$ CFU/larvae was chosen. DLP3 lysate was used at $8.9 \times 10^{10}$ PFU/mL. Larvae were injected with 10 µL of DLP3 lysate dilutions to give MOIs of approximately 100 and 50 into the rear right proleg 1.5 hpi with D1571. Aliquots of 5 µL PBS and 10 µL SM were used in place of bacteria and phage, respectively, for negative controls. Each worm was therefore injected with 15 µL total volume. Larvae were incubated and scored for survival as above. Results from three separate trials were combined and survival at each timepoint was plotted using the Kaplan–Meier method with error bars for standard error using GraphPad Prism 8. Statistical analysis of survival differences was completed using the Log-rank (Mantel–Cox) test.

**RESULTS AND DISCUSSION**

**Isolation, Morphology, Host Range, and RFLP Analysis**

Bacteriophage DLP3 (vB_SmaS-DLP_3) was isolated from soil using clinical *S. maltophilia* strain D1571. Transmission electron microscopy (TEM) enabled the classification of DLP3 as a Siphoviridae of the B1 morphotype (Ackermann and Eisenstark, 1974) due to the long, non-contractile tail averaging 202.2 ± 5.7 nm and isometric capsid with a length and width of 92.8 ± 4.1 and 84.0 ± 2.8 nm, respectively (Figure 1). No tail fibers were observed in the TEM images. The host range of DLP3 against all 29 clinical *S. maltophilia* isolates reveals a broad tropism through the successful infection of 22 strains, although DLP3 is not capable of infecting the *P. aeruginosa* strains tested (Table 1). The restriction fragment length polymorphism analysis revealed DLP3 genomic DNA is resistant to the 15 restriction enzymes screened. These results suggest DLP3 contains modified DNA, although the exact types of modifications are currently undetermined.

**FIGURE 1** | DLP3 Siphoviridae morphology. Phage lysate was applied to a carbon-coated copper grid and stained with 4% uranyl acetate. Transmission electron micrographs were obtained at 180,000× magnification. A *S. maltophilia* D1571 pili is shown to be interacting with the baseplate portion of the DLP3 tail. The averaged measurements for tail length, capsid length and width from ten virions is 202, 92, and 84 nm, respectively.
**TABLE 1** | Host range analysis for *S. maltophilia* bacteriophage DLP3 on *S. maltophilia* and *P. aeruginosa* strains.

| *S. maltophilia* strain | Efficiency of plating |
|-------------------------|-----------------------|
| 101                     | ++                    |
| 102<sup>b</sup>         | ++ +                  |
| 103                     | ++                    |
| 152                     | −                     |
| 155                     | ++++                  |
| 174                     | +                     |
| 176                     | ++++                  |
| 213                     | +                     |
| 214                     | ++                    |
| 217                     | −                     |
| 218                     | +                     |
| 219                     | ++++                  |
| 230                     | + + + +               |
| 236                     | +                     |
| 242                     | ++                    |
| 249<sup>b</sup>         | +                     |
| 278                     | −                     |
| 280                     | + + +                 |
| 282                     | +                     |
| 287                     | ++                    |
| 446                     | −                     |
| 667                     | +                     |
| D1585<sup>a</sup>       | ++++                  |
| D1571<sup>a,b</sup>     | ++++                  |
| D1614<sup>a,b</sup>     | −                     |
| D1572<sup>a,b</sup>     | ++++                  |
| D1568<sup>a</sup>       | −                     |
| SMDP92<sup>b</sup>      | ++++                  |
| ATCC13637               | −                     |

**P. aeruginosa** strain

|                 |                  |
|-----------------|-----------------|
| PA01            | −               |
| HER1004         | −               |
| HER1012         | −               |
| 14715           | −               |
| Utah3           | −               |
| Utah4           | −               |
| 14655           | −               |
| 6106            | −               |
| pSHU-OTE        | −               |
| D1606D<sup>a</sup> | −               |
| D1615C<sup>a</sup> | −               |
| D1619M<sup>a</sup> | −               |
| D1620E<sup>a</sup> | −               |
| D1623C<sup>a</sup> | −               |
| ENV003<sup>a</sup> | −               |
| ENV009<sup>a</sup> | −               |
| FC0507<sup>a</sup> | −               |
| R285            | −               |
| 14672           | −               |

−, no sensitivity to phage; +, plaques at 10−2; ++, clearing at 10−2; ++++, plaques at 10−4; ++++, plaques at 10−6. <sup>a</sup>isolates from the Canadian Burkholderia cepacia complex Research Referral Repository. <sup>b</sup>Strain susceptible to phage DLP5.

**Genomic Characterization**

The DLP3 genome is 96,852 bp long with a 58.3% global GC content. No low coverage or ambiguous regions were identified with the assembled contig, which has a mean coverage of 114 and a Q40 of 99.6%. NCBI non-redundant protein sequence database searches indicate that DLP3 shares a high identity to the *Siphoviridae* phage vB_SmaS_DLP_5 (DLP5) (Peters and Dennis, 2018). DLP5 is the type species of the new genus *Delepquintavirus* and based on the genomic similarities between DLP3 and DLP5, DLP3 is also a member of this new genus. Open reading frame calling with Glimmer and GeneMarkS identified a total of 148 protein coding domain sequences (CDS) covering 95% of the genome (Figure 2 and Table 2). DLP3 also encodes five tRNA genes with different specificities: Tyr (GTA), Sup (CTA), Ser (GCT), Ile (GAT), Glu (TTC). A total of 97 proteins could not be assigned functions due to lack of significant results from both BLASTp and CD-Search. The DLP3 genome with putative gene annotations has been deposited in GenBank under accession number MT110073.

The CD-Search did yield 37 DLP3 proteins with conserved domains predicted (Table 3). Three of the domains identified are domains of unknown function (DUF) which tended to be distributed throughout Gram-negative bacteria and to a lesser extent in Gram-positive bacteria, according to the species distribution for each DUF in pfam (Finn et al., 2014). There are six conserved domains (CD) identified which are involved in virion morphogenesis: phage portal protein superfamily, gp1; phage capsid family, gp6; phage tail proteins, gp10 and gp24;...
TABLE 2 | Genome annotations for DLP3 obtained from BLASTp and CD-Search data.

| CDS | Interval       | Length (AA) | Putative function                        | Species | Coverage (%) | E-Value | Identity (%) | Accession |
|-----|----------------|-------------|------------------------------------------|---------|--------------|---------|--------------|-----------|
| 1   | 48–1856        | 602         | Portal protein                           | DLP3    | 98           | 0       | 83           | ATS92275.1|
| 2   | 1856–3193      | 445         | ParB-like nuclease domain protein        | DLP3    | 100          | 0       | 75           | ATS92281.1|
| 3   | 3249–3864      | 201         | Serine protease                          | DLP3    | 98           | 1.00E-113| 80           | ATS92325.1|
| 4   | 3847–4017      | 56          | Hypothetical protein gp_005              | DLP3    | 100          | 3.00E-24| 77           | ATS92240.1|
| 5   | 4090–5307      | 405         | Hypothetical protein gp_006              | DLP3    | 100          | 0       | 79           | ATS92283.1|
| 6   | 5340–6272      | 370         | Major capsid protein                     | DLP3    | 100          | 0       | 93           | ATS92299.1|
| 7   | 6358–6624      | 88          | Hypothetical protein gp_008              | DLP3    | 100          | 3.00E-33| 65           | ATS92383.1|
| 8   | 6693–7436      | 247         | Ribonuclease E                           | DLP3    | 100          | 3.00E-113| 73           | ATS92315.1|
| 9   | 7457–8088      | 203         | Hypothetical protein gp_010              | DLP3    | 100          | 2.00E-121| 82           | ATS92324.1|
| 10  | 8068–8493      | 141         | Tail protein                             | DLP3    | 99           | 6.00E-71| 77           | ATS92347.1|
| 11  | 8490–8936      | 148         | Hypothetical protein gp_012              | DLP3    | 99           | 3.00E-82| 76           | ATS92342.1|
| 12  | 9012–9305      | 97          | Hypothetical protein gp_013              | DLP3    | 100          | 5.00E-50| 85           | ATS92376.1|
| 13  | 9316–9693      | 125         | Hypothetical protein gp_014              | DLP3    | 100          | 4.00E-48| 67           | ATS92360.1|
| 14  | 9693–10448     | 251         | Hypothetical protein gp_015              | DLP3    | 100          | 2.00E-152| 82           | ATS92314.1|
| 15  | 10465–10959    | 164         | Hypothetical protein gp_016              | DLP3    | 100          | 9.00E-83| 81           | ATS92333.1|
| 16  | 11016–11444    | 42          | Hypothetical protein gp_017              | DLP3    | 95           | 2.00E-15 | 83           | ATS92415.1|
| 17  | 11151–14915    | 1254        | Tape measure protein                     | DLP3    | 99           | 0       | 83           | ATS92270.1|
| 18  | 14917–16500    | 527         | Hypothetical protein gp_019              | DLP3    | 100          | 0       | 79           | ATS92277.1|
| 19  | 16500–17474    | 324         | Hypothetical protein gp_020              | DLP3    | 100          | 0       | 83           | ATS92295.1|
| 20  | 17474–19153    | 559         | Hypothetical protein gp_021              | DLP3    | 100          | 0       | 74           | ATS92276.1|
| 21  | 19150–19968    | 272         | Minor tail protein                       | DLP3    | 100          | 0       | 90           | ATS92307.1|
| 22  | 19968–20255    | 95          | Hypothetical protein gp_023              | DLP3    | 100          | 5.00E-62| 98           | ATS92377.1|
| 23  | 20252–20455    | 67          | Hypothetical protein gp_024              | DLP3    | 100          | 1.00E-38| 93           | ATS92399.1|
| 24  | 20445–22925    | 826         | Tail protein                             | DLP3    | 100          | 0       | 86           | ATS92271.1|
| 25  | 22925–23923    | 332         | Tail assembly protein                    | DLP3    | 100          | 0       | 86           | ATS92294.1|
| 26  | 23926–24102    | 58          | Tail assembly protein                    | DLP3    | 100          | 3.00E-30| 88           | ATS92246.1|
| 27  | 24110–25081    | 323         | Tail assembly protein                    | DLP3    | 100          | 2.00E-169| 70           | ATS92296.1|
| 28  | 25085–26107    | 340         | Tail protein                             | Salvo²  | 100          | 3.00E-76| 43           | AHB12229.1|
| 29  | 26183–26811    | 142         | Hypothetical protein gp_030              | DLP3    | 100          | 1.00E-91| 93           | ATS92345.1|
| 30  | 26611–27096    | 161         | Hypothetical protein gp_031              | DLP3    | 100          | 6.00E-87| 91           | ATS92334.1|
| 31  | 27096–27653    | 185         | Lysozyme                                 | DLP3    | 100          | 2.00E-120| 89           | ATS92330.1|
| 32  | 27655–28059    | 134         | Hypothetical protein gp_033              | DLP3    | 100          | 3.00E-80| 88           | ATS92325.1|
| 33  | 28110–28280    | 56          | Hypothetical protein gp_034              | DLP3    | 100          | 1.00E-11| 70           | ATS92240.1|
| 34  | 28329–28715    | 128         | DUF2500 containing protein              | DLP3    | 100          | 3.00E-77| 89           | ATS92350.1|
| 35  | 28687–28974    | 95          | Hypothetical protein gp_036              | DLP3    | 100          | 2.00E-57| 96           | ATS92380.1|
| 36  | 29077–29271    | 64          | Hypothetical protein                     | DLP3    | 100          | 0       | 93           | ATS92299.1|
| 37  | 29275–29574    | 99          | Hypothetical protein gp_041              | DLP3    | 100          | 2.00E-58| 96           | ATS92374.1|

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| CDS | Interval | Length (AA) | Putative function | Species | Coverage (%) | E-Value | Identity (%) | Accession |
|-----|----------|-------------|-------------------|---------|--------------|---------|--------------|-----------|
| 38  | 29634–30791 | 385 | Hypothetical protein gp_042 | DLP5 | 100 | 0 | 78 | ATS92287.1 |
| 39  | 30898–31506 | 202 | Hypothetical protein gp_043 | DLP5 | 100 | 2.00E-103 | 96 | ATS92341.1 |
| 40  | 31499–31951 | 150 | Phosphoglycerate kinase | DLP5 | 100 | 2.00E-25 | 79 | ATS92401.1 |
| 41  | 31951–32136 | 61 | Hypothetical protein gp_046 | DLP5 | 100 | 2.00E-14 | 85 | ATS92300.1 |
| 42  | 32197–33141 | 314 | Hypothetical protein gp_047 | DLP5 | 100 | 3.00E-138 | 50 | ATS92279.1 |
| 43  | 33255–34856 | 533 | Hypothetical protein gp_048 | DLP5 | 98 | 2.00E-156 | 64 | ATS92378.1 |
| 44  | 34856–35137 | 93 | Hypothetical protein gp_049 | DLP5 | 100 | 5.00E-35 | 90 | ATS92301.1 |
| 45  | 35139–36056 | 305 | Hypothetical protein gp_050 | DLP5 | 100 | 30.00E-86 | 80 | ATS92291.1 |
| 46  | 36060–36251 | 63 | Hypothetical protein BCC | DLP5 | 85 | 1.00E-19 | 78 | ATS92240.1 |
| 47  | 36389–36571 | 60 | Hypothetical protein gp_052 | DLP5 | 100 | 5.00E-128 | 93 | ATS92329.1 |
| 48  | 36568–37128 | 186 | Hypothetical protein gp_053 | DLP5 | 100 | 0 | 90 | ATS92282.1 |
| 49  | 37125–38414 | 429 | Helicase | DLP5 | 100 | 2.00E-86 | 80 | ATS92231.1 |
| 50  | 38481–38981 | 327 | Primase | DLP5 | 100 | 0 | 77 | ATS92297.1 |
| 51  | 40036–40812 | 258 | Hypothetical protein gp_057 | DLP5 | 100 | 3.00E-157 | 89 | ATS92312.1 |
| 52  | 40660–41075 | 71 | Hypothetical protein gp_058 | DLP5 | 100 | 4.00E-41 | 92 | ATS92395.1 |
| 53  | 41072–42430 | 452 | Superfamily II DNA or RNA helicase | DLP5 | 100 | 0 | 90 | ATS92280.1 |
| 54  | 42427–42828 | 133 | Transcriptional regulator | DLP5 | 100 | 6.00E-68 | 74 | ATS92353.1 |
| 55  | 42831–43208 | 125 | Hypothetical protein gp_061 | DLP5 | 100 | 1.00E-62 | 81 | ATS92362.1 |
| 56  | 43208–44425 | 405 | RecA | DLP5 | 100 | 0 | 90 | ATS92286.1 |
| 57  | 44425–44799 | 124 | Hypothetical protein gp_063 | DLP5 | 100 | 9.00E-83 | 94 | ATS92359.1 |
| 58  | 44799–45680 | 293 | Hypothetical protein gp_064 | DLP5 | 100 | 0 | 87 | ATS92304.1 |
| 59  | 45677–46174 | 165 | RuvC | DLP5 | 99 | 5.00E-111 | 93 | ATS92332.1 |
| 60  | 46152–46766 | 204 | Hypothetical protein gp_066 | DLP5 | 100 | 5.00E-108 | 78 | ATS92321.1 |
| 61  | 46848–47657 | 269 | Hypothetical protein gp_067 | DLP5 | 100 | 2.00E-153 | 83 | ATS92308.1 |
| 62  | 47840–47947 | 35 | Hypothetical protein gp_069 | DLP5 | 100 | 5.00E-13 | 94 | ATS92417.1 |
| 63  | 48028–48504 | 158 | Hypothetical protein gp_070 | DLP5 | 100 | 3.00E-96 | 91 | ATS92336.1 |
| 64  | 48839–49174 | 111 | Hypothetical protein gp_071 | DLP5 | 100 | 8.00E-60 | 82 | ATS92357.1 |
| 65  | 50468–51409 | 313 | Hypothetical protein gp_073 | DLP5 | 100 | 6.00E-127 | 61 | ATS92313.1 |
| 66  | 51397–51585 | 62 | Hypothetical protein | DLP5 | 100 | 2.00E-124 | 91 | ATS92328.1 |
| 67  | 51582–52145 | 187 | Hypothetical protein gp_074 | DLP5 | 100 | 3.00E-87 | 87 | ATS92343.1 |
| 68  | 52165–52959 | 264 | SPFH domain-containing protein | DLP5 | 100 | 7.00E-74 | 90 | ATS92365.1 |
| 69  | 53058–53210 | 50 | Hypothetical proteins | DLP5 | 100 | 7.00E-96 | 44 | ATS92288.1 |
| 70  | 53207–53653 | 148 | Hypothetical protein gp_077 | DLP5 | 100 | 7.00E-65 | 84 | ATS92368.1 |
| 71  | 53650–54000 | 116 | Hypothetical protein gp_078 | DLP5 | 100 | 8.00E-37 | 66 | ATS92369.1 |

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| CDS  | Interval | Length (AA) | Putative function                  | Species | Coverage (%) | E-Value  | Identity (%) | Accession   |
|------|----------|-------------|------------------------------------|---------|--------------|----------|--------------|-------------|
| 76   | 55887–56930 | 347         | UDP-glucose 4-epimerase             | DLP5    | 100          | 0        | 83           | ATS92289.1  |
| 77   | 56962–57285 | 107         | Hypothetical protein gp_083        | DLP5    | 99           | 9.00E-56 | 78           | ATS92370.1  |
| 78   | 57355–58425 | 356         | Hypothetical protein gp_084        | DLP5    | 100          | 0        | 73           | ATS92290.1  |
| 79   | 58418–58939 | 173         | Hypothetical protein gp_087        | DLP5    | 45           | 6.00E-35 | 73           | ATS92388.1  |
| 80   | 58936–59283 | 115         | Hypothetical protein gp_086        | DLP5    | 86           | 1.00E-36 | 64           | ATS92361.1  |
| 81   | 59280–59456 | 58          | Hypothetical protein gp_088        | DLP5    | 88           | 4.00E-17 | 62           | ATS92405.1  |
| 82   | 59465–59737 | 90          | Hypothetical protein BV378_14040   | Nostoc sp. RF31Y | 97     | 8.00E-19 | 50           | OUL25853.1  |
| 83   | 59737–59862 | 41          | Hypothetical protein               | DLP5    | 100          | 2.00E-05 | 53           | ATS92403.1  |
| 84   | 59839–60128 | 89          | Hypothetical protein gp_089        | DLP5    | 89           | 3.00E-64 | 79           | ATS92383.1  |
| 85   | 60132–60314 | 60          | Hypothetical protein gp_090        | DLP5    | 98           | 7.00E-60 | 62           | ATS92399.1  |
| 86   | 60318–60737 | 139         | Hypothetical protein gp_091        | DLP5    | 98           | 3.00E-22 | 46           | ATS92382.1  |
| 87   | 60734–61210 | 158         | Hypothetical protein gp_092        | DLP5    | 100          | 0        | 65           | ATS92398.1  |
| 88   | 61207–61458 | 83          | Hypothetical protein gp_093        | DLP5    | 100          | 3.00E-100 | 53           | ATS92319.1  |
| 89   | 61455–61709 | 84          | Hypothetical protein               | DLP5    | 100          | 4.00E-35 | 66           | ATS92391.1  |
| 90   | 61709–62359 | 216         | Hypothetical protein gp_094        | DLP5    | 100          | 4.00E-31 | 70           | ATS92393.1  |
| 91   | 62362–62649 | 95          | Hypothetical protein gp_095        | DLP5    | 100          | 9.00E-96 | 78           | ATS92322.1  |
| 92   | 62646–62885 | 79          | Hypothetical protein gp_097        | DLP5    | 100          | 8.00E-108 | 82           | ATS92326.1  |
| 93   | 63161–63888 | 175         | Rhomboid membrane protein          | DLP5    | 100          | 3.00E-31 | 68           | ATS92385.1  |
| 94   | 63746–64342 | 198         | Hypothetical protein gp_098        | DLP5    | 98           | 3.00E-114 | 75           | ATS92320.1  |
| 95   | 64339–64593 | 84          | Hypothetical protein gp_099        | DLP5    | 98           | 6.00E-114 | 75           | ATS92320.1  |
| 96   | 64670–65308 | 212         | PchL family deacetylase            | DLP5    | 100          | 0        | 95           | ATS92298.1  |
| 97   | 65311–66252 | 313         | WcaG                               | DLP5    | 100          | 0        | 87           | ATS92291.1  |
| 98   | 66252–67298 | 348         | WecE                               | DLP5    | 100          | 0        | 87           | ATS92291.1  |
| 99   | 67295–67975 | 226         | Methyltransferase                  | DLP5    | 100          | 1.00E-139 | 82           | ATS92317.1  |
| 100  | 68043–68228 | 61          | Hypothetical protein gp_103        | DLP5    | 98           | 5.00E-20 | 62           | ATS92400.1  |
| 101  | 68225–68647 | 140         | Hypothetical protein gp_104        | DLP5    | 97           | 8.00E-56 | 68           | ATS92346.1  |
| 102  | 68647–70096 | 482         | N-acetyl-alpha-D-glucosaminyl l-malate synthase | DLP5    | 100          | 0        | 93           | ATS92278.1  |
| 103  | 70092–70403 | 103         | Hypothetical protein gp_106        | DLP5    | 100          | 1.00E-36 | 59           | ATS92372.1  |
| 104  | 70375–71229 | 284         | ParBc                              | DLP5    | 99           | 0        | 89           | ATS92305.1  |
| 105  | 71229–71879 | 216         | Hypothetical protein gp_108        | DLP5    | 99           | 5.00E-125 | 85           | ATS92318.1  |
| 106  | 71842–73755 | 637         | Terminase large subunit            | DLP5    | 100          | 0        | 91           | ATS92274.1  |
| 107  | 73767–74660 | 297         | Hypothetical protein gp_110        | DLP5    | 100          | 0        | 95           | ATS92303.1  |
| 108  | 74657–75067 | 136         | DUF3310 containing protein         | DLP5    | 100          | 1.00E-61 | 71           | ATS92351.1  |
| 109  | 75129–75371 | 80          | Hypothetical protein gp_112        | DLP5    | 100          | 8.00E-37 | 74           | ATS92390.1  |
| 110  | 75373–75765 | 130         | Hypothetical protein gp_113        | DLP5    | 92           | 7.00E-12 | 34           | ATS92355.1  |
| 111  | 75841–75939 | 32          | Hypothetical protein gp_114        | DLP5    | 100          | 7.00E-09 | 81           | ATS92418.1  |
| CDS  | Interval       | Length (AA) | Putative function              | Species | Coverage (%) | E-Value  | Identity (%) | Accession  |
|------|----------------|-------------|--------------------------------|---------|--------------|----------|--------------|------------|
| 112  | 75936–76253    | 105         | Hypothetical protein gp_115    | DLP5    | 96           | 6.00E-54 | 81           | ATS92371.1 |
| 113  | 76250–76504    | 84          | Hypothetical protein gp_116    | DLP5    | 100          | 2.00E-42 | 79           | ATS92387.1 |
| 114  | 76494–76859    | 121         | Hypothetical protein gp_117    | DLP5    | 98           | 6.00E-59 | 80           | ATS92358.1 |
| 115  | 76859–77011    | 50          | Hypothetical protein gp_118    | DLP5    | 100          | 1.00E-22 | 88           | ATS92412.1 |
| 116  | 77087–77335    | 82          | Hypothetical protein gp_119    | DLP5    | 100          | 2.00E-43 | 82           | ATS92389.1 |
| 117  | 77389–78528    | 379         | Hypothetical protein gp_120    | DLP5    | 70           | 2.00E-143| 94           | ATS92296.1 |
| 118  | 78528–78878    | 116         | Hypothetical protein gp_121    | DLP5    | 99           | 4.00E-71 | 90           | ATS92364.1 |
| 119  | 78904–79365    | 153         | Hypothetical protein gp_122    | DLP5    | 100          | 7.00E-78 | 75           | ATS92340.1 |
| 120  | 79301–79561    | 76          | Hypothetical protein gp_123    | DLP5    | 100          | 3.00E-33 | 76           | ATS92392.1 |
| 121  | 79558–79731    | 57          | Hypothetical protein gp_124    | DLP5    | 96           | 6.00E-23 | 78           | ATS92404.1 |
| 122  | 79604–79920    | 38          | Hypothetical protein           | DLP5    | 100          | 3.00E-59 | 87           | ATS92373.1 |
| 123  | 79920–80228    | 102         | Hypothetical protein gp_125    | DLP5    | 100          | 8.00E-153| 83           | ATS92316.1 |
| 124  | 80254–80988    | 244         | Hypothetical protein gp_128    | DLP5    | 96           | 1.00E-64 | 79           | ATS92356.1 |
| 125  | 80985–81350    | 121         | Hypothetical protein gp_127    | DLP5    | 96           | 2.00E-29 | 88           | ATS92402.1 |
| 126  | 81347–81523    | 58          | Hypothetical protein           | DLP5    | 96           | 2.00E-88 | 80           | ATS92337.1 |
| 127  | 81516–81698    | 60          | Hypothetical protein gp_128    | DLP5    | 96           | 2.00E-44 | 83           | ATS92367.1 |
| 128  | 81698–82180    | 160         | DUF1643 containing protein     | DLP5    | 100          | 0        | 92           | ATS92302.1 |
| 129  | 82180–82515    | 111         | Hypothetical protein gp_130    | DLP5    | 90           | 0        | 92           | ATS92302.1 |
| 130  | 82589–83485    | 298         | DNA ligase                     | DLP5    | 100          | 4.00E-65 | 74           | ATS92349.1 |
| 131  | 83482–83985    | 137         | Hypothetical protein gp_133    | DLP5    | 100          | 2.00E-47 | 77           | ATS92375.1 |
| 132  | 83895–84197    | 100         | Hypothetical protein gp_134    | DLP5    | 97           | 2.00E-124| 86           | ATS92338.1 |
| 133  | 84248–85459    | 403         | Hypothetical protein gp_135    | DLP5    | 100          | 3.00E-119| 85           | ATS92327.1 |
| 134  | 85456–86028    | 190         | Hypothetical protein gp_136    | DLP5    | 100          | 5.00E-93 | 86           | ATS92338.1 |
| 135  | 86122–88065    | 647         | Pyruvate phosphate dikinase     | DLP5    | 100          | 0        | 94           | ATS92273.1 |
| 136  | 88141–88527    | 128         | Hypothetical protein gp_138    | DLP5    | 98           | 7.00E-76 | 90           | ATS92348.1 |
| 137  | 88527–88688    | 113         | Hypothetical protein gp_139    | DLP5    | 100          | 5.00E-63 | 86           | ATS92366.1 |
| 138  | 88879–89238    | 119         | Transcriptional repressor      | DLP5    | 95           | 3.00E-71 | 88           | ATS92354.1 |
| 139  | 89228–89698    | 156         | Tyrosine phosphatase family protein | DLP5 | 95          | 5.00E-47 | 86           | ATS92354.1 |
| 140  | 89740–90759    | 359         | Hypothetical protein gp_142    | DLP5    | 98           | 0        | 77           | ATS92327.1 |
| 141  | 90756–91532    | 258         | Thymidylate synthase           | DLP5    | 98           | 8.00E-121| 72           | ATS92311.1 |
| 142  | 91618–92493    | 291         | Hypothetical protein gp_144    | DLP5    | 97           | 1.00E-124| 65           | ATS92306.1 |
| 143  | 92483–92737    | 84          | Hypothetical protein gp_145    | DLP5    | 97           | 3.00E-35 | 71           | ATS92386.1 |
| 144  | 92734–93231    | 165         | Hypothetical protein gp_146    | DLP5    | 96           | 1.00E-83 | 77           | ATS92335.1 |
| 145  | 93260–95482    | 740         | DNA polymerase I               | DLP5    | 100          | 0        | 85           | ATS92272.1 |
| 146  | 95794–96030    | 78          | Hypothetical protein           | DLP5    | 100          | 6.00E-52 | 87           | ATS92379.1 |
| 147  | 96134–96415    | 93          | Hypothetical protein gp_149    | DLP5    | 96           | 2.00E-61 | 85           | ATS92310.1 |

Results below 0.01 were not included and the function was annotated as hypothetical. aStenotrophomonas phage DLP5, bXylella phage Salvo, and cBurkholderia cepacia complex.
TABLE 3 | The conserved domains found in the 148 DLP3 gene products.

| Gp   | Hit type       | PSSM-ID   | Interval   | E-Value          | Accession      | Short name       | Superfamily       |
|------|----------------|-----------|------------|------------------|----------------|------------------|-------------------|
| 1    | Superfamily    | 327517    | 29–471     | 2.89E-49         | c19194         | Phage_portal superfamily | –                 |
| 2    | Specific       | 214678    | 359–441    | 8.46E-06         | smart00470     | ParB             | c02129            |
| 3    | Superfamily    | 317012    | 5–114      | 7.39E-23         | c24270         | Peptidase_S78_2 superfamily | –                 |
| 6    | Superfamily    | 331903    | 20–307     | 5.06E-12         | c27082         | Phage_capsid superfamily | –                 |
| 10   | Superfamily    | 321796    | 6–137      | 7.98E-15         | c20299         | Phage_tail_S superfamily | –                 |
| 17   | Superfamily    | 331332    | 525–930    | 5.90E-15         | c26511         | Neuromodulin_N superfamily | –                 |
| 17   | Superfamily    | 333387    | 163–230    | 3.22E-03         | c28567         | HI1514 superfamily | –                 |
| 19   | Superfamily    | 316645    | 14–69      | 9.35E-03         | c16864         | DUF4302 superfamily | –                 |
| 21   | Superfamily    | 312753    | 188–264    | 8.54E-16         | c10710         | Phage_BR0599 superfamily | –                 |
| 21   | Superfamily    | 331404    | 18–261     | 5.62E-12         | c26583         | DUF2163 superfamily | –                 |
| 24   | Specific       | 316107    | 207–368    | 2.18E-13         | pfam13550      | Phage_tail_3     | c26145            |
| 28   | Superfamily    | 328935    | 62–184     | 1.13E-05         | c22861         | LamG superfamily | –                 |
| 31   | Superfamily    | 331815    | 6–185      | 4.73E-34         | c26994         | Glyco_hydro_106 superfamily | –                 |
| 38   | Superfamily    | 332389    | 92–371     | 4.11E-42         | c27568         | TIP49 superfamily | –                 |
| 41   | Specific       | 332243    | 13–46      | 5.11E-03         | c27422         | SecD superfamily | –                 |
| 49   | Superfamily    | 333705    | 167–405    | 5.02E-22         | c28885         | RecA-like_NTases superfamily | –                 |
| 51   | Superfamily    | 331610    | 31–322     | 7.81E-12         | c26789         | Toprim_N superfamily | –                 |
| 54   | Superfamily    | 331760    | 51–451     | 4.03E-38         | c26939         | DEXDc superfamily | –                 |
| 55   | Superfamily    | 322007    | 53–98      | 3.22E-04         | c302800        | HTH_MerR-SF superfamily | –                 |
| 57   | Specific       | 333705    | 77–272     | 2.57E-48         | c28885         | RecA-like_NTases superfamily | –                 |
| 60   | Superfamily    | 328743    | 1–140      | 6.73E-14         | c21482         | RuvC_resolvase superfamily | –                 |
| 69   | Specific       | 307341    | 23–207     | 2.15E-17         | pfam01145      | Band_7           | c19197            |
| 76   | Specific       | 224012    | 1–336      | 1.19E-76         | COG1087        | GalE             | c21454            |
| 78   | Specific       | 330522    | 59–126     | 2.19E-04         | c25701         | RuvB_N superfamily | –                 |
| 93   | Superfamily    | 328780    | 29–159     | 3.68E-14         | c21536         | Rhoiboid superfamily | –                 |
| 96   | Specific       | 308281    | 5–122      | 3.46E-11         | pfam02585      | PIG-L            | c00929            |
| 97   | Specific       | 223528    | 1–297      | 7.51E-40         | COG0451        | WcaG             | c25660            |
| 98   | Superfamily    | 327488    | 20–340     | 5.46E-56         | c18945         | AAT_I superfamily | –                 |
| 99   | Superfamily    | 327401    | 30–135     | 1.43E-04         | c17173         | AdoMet_MTases superfamily | –                 |
| 102  | Specific       | 223515    | 1–323      | 4.33E-03         | COG0438        | RfaB             | c28206            |
| 104  | Specific       | 214678    | 18–109     | 7.28E-17         | smart00470     | ParB             | c02129            |
| 104  | Specific       | 224392    | 34–187     | 5.36E-08         | COG1475        | SpoU             | c26722            |
| 108  | Superfamily    | 314594    | 17–68      | 1.07E-11         | c13237         | DUF3310 superfamily | –                 |
| 128  | Specific       | 311648    | 12–146     | 4.16E-44         | pfam07799      | DUF1843          | c01787            |
| 130  | Superfamily    | 325160    | 25–186     | 2.14E-25         | c12015         | Adenylation_DNA_ligase_ike superfamily | –                 |
| 130  | Superfamily    | 330238    | 112–270    | 1.28E-08         | c25417         | CDC9 superfamily | –                 |
| 133  | Superfamily    | 332389    | 206–341    | 8.78E-33         | c27568         | TIP49 superfamily | –                 |
| 133  | Superfamily    | 332204    | 43–150     | 8.52E-03         | c27383         | ERC4 superfamily | –                 |
| 135  | Superfamily    | 331842    | 16–471     | 0.00E+00         | c27021         | PtsP superfamily | –                 |
| 139  | Superfamily    | 330819    | 39–125     | 1.50E-10         | c25998         | CDC14 superfamily | –                 |
| 141  | Superfamily    | 330819    | 85–189     | 1.80E-05         | c25998         | CDC14 superfamily | –                 |
| 145  | Superfamily    | 322025    | 107–710    | 7.76E-64         | c02626         | DNA_pol_A superfamily | –                 |

laminin G, gp28; and tape measure protein domain, gp17. Nine proteins with domains involved in DNA replication and repair identified with the CD-Search include two ParB domains, gp2 and 104; two helicases, gp49 and 54; Holliday junction resolvase, gp60; RecA recombinase, gp57; topoisomerase primase, gp51; DNA ligase, gp130; and DNA polymerase A, gp145. The remaining CD results appear to be quite diverse in their functions such as the SpoVK family domain involved in sporulation (gp38) (Fan et al., 1992), protein-tyrosine phosphatases (gp139 and gp141) typically involved in signal transduction (Whitmore and Lamont, 2012), and a membrane-associated serine protease of the rhomboid family (gp93). One CD identified that is of particular interest is the glycosyltransferase domain of gp102. T-even bacteriophages have been shown to use glycosyltransferases for DNA modification by linking a glycosyl group to hydroxymethyl-cytosine, thus protecting the DNA against digestion by bacterial restriction systems (Bair and Black, 2007). Another role for glycosyltransferases within bacteriophages is highlighted by some Shigella phages which have been shown to seroconvert their host by modifying the...
O-antigen polysaccharides to prevent infection of the bacteria by other O-antigen receptor phages (Allison and Verma, 2000). The specific glycosyltransferase family is RfaB, a protein involved in the assembly of the LPS core of Escherichia coli K-12 (Pradel et al., 1992). This result suggests DLP3 may use the glycosyltransferase to modify the host LPS, similar to the seroconverting Shigella phages, though this has yet to be confirmed experimentally.

**Phage DLP3 Relatedness to Phage DLP5**

While characterizing the Siphoviridae phage DLP3 genome, it was evident through BLASTn and BLASTp searches that phages DLP3 and DLP5 are closely related. The genome size of DLP3 and DLP5 are similar (96,852 versus 96,542 bp), as is their GC content (58.3 versus 58.4%). BLASTn analysis of DLP3 against DLP5 revealed 81% identity over 90% of the DLP5 genome (0.0 E-value). A LASTZ alignment (Harris, 2007) of the phages using DLP5 as the reference sequence shows sequence identity >30% between their genomes, represented as a mustard yellow color in the consensus (Figure 3). BLASTn alignment between DLP3 to DLP5 gives a query coverage of 89% and 81% identity. Two small stretches with a breakdown in identity are observed between the DLP5 and DLP3 genomes, which is viewed as a breakdown in the alignment blocks of the dot plot around 30,000 and 60,000 bp (Figure 3). These stretches correspond to four genes (DLP05_037 to DLP05_039, plus DLP05_087) encoding hypothetical proteins with no significant matches in the NCBI database, no conserved domains, and no significant results when using structural prediction software such as HHpred and Phyre. Two additional genes, DLP05_045 and DLP05_068, which encode hypothetical proteins, are not present in the DLP3 genome. The DLP05_045 gene product shares 93% coverage with 36% identity (1.0E-08) to a Polaronomas naphthalenivorans hypothetical protein which contains no conserved domains, whereas the DLP05_068 gene product did not have similar sequences in the NCBI database or conserved domains predicted. Both phages encode five tRNAs, with four of the five tRNAs sharing the same specificity: Sup-CTA, Glu-TTC, Ser-GCT, Tyr-GTG. Phage DLP3 differs from DLP5 with respect to the fifth tRNA, which is Ile-GAT in DLP3 but Gly-TCC in DLP5. The amino acid usage of each phage does not explain the differences, as they each have the same usage rates for isoleucine (4.7%) and glycine (7.7%), but there are several nucleotide changes observed in this region when comparing DLP3 to DLP5.

Besides the genomic similarities observed between DLP3 and DLP5, they also share morphological similarities. The DLP3 and DLP5 phages have the same measurement averages for their head width (84 nm) and length (92 nm), as well as tail length (202 nm). The host range of DLP3 versus DLP5 is significantly different, with DLP3 exhibiting a broad host range infecting 22 S. maltophilia strains, whereas DLP5 only infects six (Table 1). Of the six strains DLP5 is capable of infecting, 10D1571, D1614, D1576, SMDP92, DLP3 is only unable to infect the DLP5 host strain D1614. The virion morphogenesis proteins of DLP3 range in sequence identity to the DLP5 equivalents from 43.8% (gp28) to 93.2% (gp6), with all but gp28 having sequence identity greater than 70%. The gp28 proteins of DLP3 and DLP5 are related to the Xylella phage Sano tail fiber protein. The annotation of tail fiber for the Sano protein was based solely on synteny to the Burkholderia phage BcepNazgul255. There are no shared conserved domains identified between these two proteins, and the Bcep Nazgul protein is 1,270 amino acids long whereas the DLP3 and DLP5 gp28 proteins and the Sano tail fiber protein are only approximately 340 amino acids long; thus, we consider the assignment of tail fiber function to the Sano phage protein to be incorrect.

The gp28 proteins of DLP3 and DLP5 may play a role in host range due to the variability observed between these proteins. The DLP5 gp28 protein shares 62.5% identity with the Sano tail fiber, while DLP3 only has 39.6% identity to the Sano protein and 41.8% identity to the DLP5 protein. The first 242 N-terminal amino acids of the DLP3 and DLP5 gp28 consensus show a high degree of variability at 24.5% pairwise identity. The remaining 104 amino acids from 243 to the C-terminal have high pairwise identity at 88.5%. Variability in the N-terminal region was also observed with a MUSCLE alignment using all three proteins from DLP3, DLP5 and Sano (Supplementary Figure S1). The first 258 N-terminal amino acids of the protein consensus share only 35.4% pairwise identity, which increases to 77.8% over the remaining 104 amino acids. The alignment shows at least nine insertion/deletion events have occurred within the DLP3 gene resulting in gaps and insertions in the translated DLP3 protein compared to the Sano and DLP5 proteins. Further investigation into gp28 of DLP3 and DLP5 as a host recognition protein is currently underway to help elucidate whether this protein plays a role in the host range of the Delequintavirus phages.

**Receptor Identification**

Three Siphoviridae phages, DLP1, DLP2 and DLP4, previously isolated on S. maltophilia strain D1585, were found to bind the type IV pilus as their cell surface receptor across their host ranges (McCutcheon et al., 2018; Peters et al., 2019). Despite isolating DLP3 on strain D1571, we assessed DLP3 plaquing ability on the previously constructed D1585 and 280 ΔpilA mutants lacking the major pilin subunit. Similar to the three previously characterized phages, S. maltophilia strains lacking type IV pili are also resistant to infection by DLP3, shown by an absence of cell lysis at high titer (Figure 4). Complementation with the endogenous genes restores phage infection to wildtype levels. Unfortunately, the pilA gene is undetectable in the incompletely assembled D1571 genome, which prevented construction of pilA mutants in this background. In contrast to host range data collected when DLP3 was initially isolated from the environment (Table 1), DLP3 efficiency of plating on D1585 and 280 is much lower than initially documented, showing phage activity at 10⁵ PFU/mL compared to plaque formation at 10⁶ PFU/mL (Figure 4). We attribute this reduced plaquing ability on some hosts to the repeated propagation of DLP3 on strain D1571. It is hypothesized that propagating DLP3 on a single host has selected for phages optimized to that host over time, resulting in the differences observed.

An additional mutant was constructed in strain D1585, ΔpilT, lacking the retraction ATPase required for depolymerization of the pilus (Burrows, 2005). This mutation results in hyperpiliated, non-motile cells having numerous non-functional pili projecting...
FIGURE 3 | Genomic alignment of DLP3 to DLP5 using the Large-Scale Genome Alignment Tool. Identity is indicated by color: mustard yellow; >30%, green; deletion in DLP3, red; deletion in DLP5.

FIGURE 4 | DLP3 requires a functional type IV pilus to infect host strains D1585 and 280. Phage DLP3 is capable of infecting wildtype *S. maltophilia* strains D1585 and 280, infecting both at 10⁹ PFU/mL, whereas phage infection is blocked in the ΔpilA and ΔpilT mutants. Complementation with the endogenous genes restores phage infection to wildtype levels. DLP3 plaquing ability on strain D1571 is shown for titer calculation, however, no mutants were constructed in this background. Images are representative of three biological replicates, each with three technical replicates.

from the cell surface. Similar to the ΔpilA mutants, D1585 ΔpilT is resistant to DLP3 phage infection. This strain grows poorly in liquid, as observed in the speckled overlay lawn, however, this phenotype, as well as susceptibility to phage, is restored by complementation with the pilT gene (Figure 4). These results indicate that DLP3 uses the type IV pilus as a cell surface receptor and requires a functional pilus capable of retraction to reach the cell surface for successful host infection. This is the fourth documented *S. maltophilia* phage to use the type IV pilus as its receptor, however, we have yet to identify the receptor for the highly similar phage DLP5. Minor differences in the genomes of these two phages likely explains the significant differences observed in phage host range due to the binding of different receptors.

Analysis of DLP3 Structural Proteins

To further investigate DLP3 morphology, phage structural proteins were analyzed by HPLC-MS and screened against DLP3 proteins and the *Stenotrophomonas* database in UniProt. The SEQUEST results from searching all DLP3 proteins identified 21 (Table 4), though only 11 proteins were classified as virion morphogenesis using BLASTp and CD-Search data (Supplementary Figure S2). The most abundant protein isolated was the major capsid protein (gp6), which is the main structural component of a bacteriophage capsid (Hendrix and Johnson, 2012). The second most abundant protein found was the portal protein (gp1), which forms the entry site for phage DNA to be packaged into the capsid by the large terminase. The portal protein also functions like a DNA sensor, measuring the amount of DNA packaged into the capsid and signaling the large terminase to end genome-packaging once full (Lokareddy et al., 2017).

Gene products gp14 and gp18 were also identified. Both gene products are hypothetical proteins without conserved domains. Also, they do not have significant results using HHpred; therefore, their structural function is unknown (Table 4). Phyre2 analysis of these proteins only suggested a putative function for gp14, which showed structural identity to the major tropism determinant P1 (MTD-P1) of *Bordetella* phage BPP-1 (38.7% confidence, 25% identity). The MTD-P1 protein in BPP-1 is responsible for phage receptor binding (Miller et al., 2008) and uses specific variable residues for target recognition similar to
an antibody (Miller, 2006). Another abundant protein identified is gp24, which is predicted to be a structural component of the DLP3 tail with a phage-tail_3 family (PF13550) conserved domain. The tape measure protein (gp17), which is responsible for setting the tail length (Belcaid et al., 2011), is the largest protein identified and the sixth most abundant, though the ∼130 kDa band was faint after fully destaining (Supplementary Figure S2). The remaining DLP3 proteins identified and ordered by relative abundance are gene products gp19, gp97, gp27, gp61, gp20, gp28, gp2, gp21, gp25, gp9, gp133, gp12, gp57, gp5, and gp3. Some of the proteins identified are surprising, such as gp2 (Par-B like nuclease domain protein) and gp5 (RecA), but based on the results obtained by screening the Stenotrophomonas protein database, it is evident that there were proteins present from the bacterial cell; thus, there may also be DLP3 proteins present that are not structural in function (Table 4 and Supplementary Table S2).

**Identification of Erythromycin Resistance Factor Erm(45)**

Annotation of the DLP3 genome revealed the presence of a methyltransferase domain in the gene product encoded by 12 Hypothetical protein gp_013 and ICU331. Phage DLP5 has been previously shown to cause lysogenic conversion of *S. maltophilia* strain D1571 (Peters and Dennis, 2018). Therefore, we sought to further investigate the characteristics of the D1571:DLP3 lysogen relative to wildtype *S. maltophilia* strain D1571.

**Lysogenic Conversion of D1571 by Temperate Phage DLP3**

Stable DLP3 lysogens of *S. maltophilia* strain D1571 have been isolated and due to the presence of ParB domains in two of the DLP3 proteins, DLP3 may lysogenize its host as a phagemid. The closely related phage DLP5 was also found to encode two proteins with ParB domains and was successfully isolated as a phagemid from the lysogenized strain D1614 (Peters and Dennis, 2018). Blastn and PHAST analysis (Altschul et al., 1997; Zhou et al., 2011) of GenBank-deposited *Stenotrophomonas* genomes indicates that phage DLP3 (or phages closely related to DLP3) has also lysogenized *S. maltophilia* strains ABB550, FDAARGOS_325 and ICU331. Phage DLP5 has been previously shown to cause lysogenic conversion of *S. maltophilia* strain D1571 (Peters and Dennis, 2018). Therefore, we sought to further investigate the characteristics of the D1571:DLP3 lysogen relative to wildtype *S. maltophilia* strain D1571.

**FIGURE 5** | Erythromycin resistance of D1571:DLP3 lysogen compared to wild type control D1571. Minimum inhibitory concentration assay was completed in biological and mechanical triplicates. Two-way ANOVA with Sidak’s multiple comparisons test was performed on the MIC data. Statistical significance is represented as: ****P < 0.0001; **P < 0.01; and *P < 0.05.

**TABLE 4** | Mass spectrometry protein results with the DLP3 protein database.

| Gp          | Function                                | Score | Coverage (%) | Unique Peptides | Peptides | PSMs | AAs | MW [kDa] | calc. pI |
|-------------|-----------------------------------------|-------|--------------|----------------|----------|------|-----|---------|---------|
| 1           | Hypothetical protein gp_015             | 189.02| 56.57        | 12             | 12       | 282  | 251 | 27.4    | 4.92    |
| 2           | Minor tail protein gp_019               | 54.48 | 19.54        | 10             | 10       | 123  | 527 | 58.2    | 6.27    |
| 3           | Tape measure protein gp_020             | 45.75 | 21.85        | 28             | 28       | 78   | 1254| 131.9   | 6.39    |
| 4           | Tail assembly protein gp_021            | 33.34 | 28.48        | 7              | 7        | 46   | 323 | 35.4    | 7.05    |
| 5           | Major capsid protein                    | 30.84 | 27.47        | 7              | 7        | 69   | 324 | 35.8    | 6.61    |
| 6           | Hypothetical protein gp_010             | 22.78 | 30.99        | 10             | 10       | 52   | 313 | 34.8    | 5.99    |
| 7           | ParB-like nuclease domain protein gp_011| 11.39 | 28.68        | 9              | 9        | 19   | 272 | 30.5    | 7.33    |
| 8           | Hypothetical protein gp_012             | 11.28 | 20.45        | 8              | 8        | 19   | 445 | 49.3    | 6.96    |
| 9           | Hypothetical protein gp_013             | 8.01  | 31.03        | 6              | 6        | 11   | 203 | 22.5    | 5.94    |
| 10          | Tail protein gp_014                     | 7.74  | 24.71        | 10             | 10       | 24   | 340 | 37.2    | 8.73    |
| 11          | Hypothetical protein gp_015             | 7.56  | 14.85        | 9              | 9        | 30   | 559 | 63.5    | 5.25    |
| 12          | Hypothetical protein gp_016             | 6.45  | 27.45        | 7              | 7        | 31   | 204 | 22.3    | 9.00    |
| 13          | Tail assembly protein gp_017            | 4.15  | 23.80        | 6              | 6        | 15   | 332 | 35.5    | 5.15    |
| 14          | Hypothetical protein gp_018             | 2.37  | 78.35        | 4              | 4        | 7    | 97  | 9.6     | 9.47    |
| 15          | Tail protein gp_019                     | 2.24  | 9.95         | 2              | 2        | 3    | 201 | 22.7    | 4.67    |
| 16          | RecA                                    | 2.03  | 15.56        | 5              | 5        | 7    | 405 | 43.5    | 6.46    |
| 17          | Hypothetical protein gp_006             | 0.00  | 6.67         | 2              | 2        | 4    | 405 | 43.4    | 5.39    |
| 18          | ATPase family protein                   | 0.00  | 10.67        | 4              | 4        | 9    | 403 | 44.6    | 5.80    |

Results are ordered by score.
A specific domain identified is the AdoMet_MTases superfamily comprised of class I S-adenosylmethionine-independent methyltransferases. The class I family is the largest and most diverse, with members targeting a range of substrate specificities such as small molecules, lipids, nucleic acids, and different target atoms for methylation (Struck et al., 2012). Members of this family are known to maintain structural similarity even when the amino acid sequence diverges to as little as 10% (Schubert et al., 2003). Further investigation into ggp69 with HHblits revealed high sequence identity to Erm(45) of Staphylococcus fleurettii (HHblits: 100% probability, 2.4e-104 E-value). Researchers identified Erm(45) as the enzyme responsible for increased erythromycin resistance in some strains of S. fleurettii (Wipf et al., 2015). The erm gene (erythromycin ribosome methylase) encodes a methylase enzyme that provides macrolide resistance by methylating the erythromycin target-site on the ribosome (Prunier et al., 2002). Together, these findings suggested that the DLP3 encoded methyltransferase is an erythromycin resistance protein similar to Erm(45) and could cause an increase in erythromycin resistance of the host cell during lysogeny.

To test the functionality of the DLP3 Erm protein, minimum inhibitory concentration (MIC) assays of the wild type D1571 and lysogen D1571:DLP3 were completed. The results show a statistically significant difference in erythromycin resistance with DLP3 lysogeny over a wide range of concentrations tested (Figure 5). There are differences noted between the wild type and lysogenized strains at lower concentrations of erythromycin, with wild type having a statistically significant increase in optical density at lower concentrations (6 and 34 µg/ml; $P < 0.05$, 12 and 23 µg/ml; $P < 0.01$). The cause of the decreased resistance in the lysogen noted at lower concentrations of erythromycin is currently unknown. This trend is reversed at higher concentrations of erythromycin, with the lysogen having significantly higher OD<sub>600</sub> readings at 144 and 188 µg/ml erythromycin concentrations compared to wild type control ($P < 0.0001$). The increased resistance noted at higher erythromycin concentrations indicates lysogenic conversion of D1571 by lysogenized DLP3 and confirms the functionality of the predicted Erm protein. Fortuitously, S. maltophilia strains are already highly resistant toward macrolides, and macrolides such as erythromycin are not considered to be a frontline treatment option.

**Differing Growth Characteristics of DLP3 Lysogen in vitro**

While working with the D1571:DLP3 lysogen and wild type D1571 for the erythromycin resistance assay, it was evident that the lysogen exhibited a faster growth rate as compared to the wild type strain. This difference in growth rate was also observed when growing single colony isolates of the lysogen and wild type on an LB agar plate, with the lysogen having larger single colonies after a 16 h incubation at 30°C as compared to the wild type D1571 (data not shown). To investigate this phenotypic difference further, a growth curve was conducted in mechanical and biological triplicates for the lysogen and wild type over 8 h. The plotted growth curve clearly shows the lysogenized strain exhibiting a faster growth rate than the wild type strain, with significance found following a two-way repeated measures ANOVA with multiple comparisons at 2 h, $P < 0.05$; 4 h, $P < 0.0001$; and 6 h, $P < 0.01$ (Figure 6A). This observation was confirmed by converting the OD<sub>600</sub> data into growth rate ($μ$) and plotting the resulting data (Figure 6B). A two-way repeated measures ANOVA with multiple comparisons revealed significant ($P < 0.0001$) differences between 0- to 2-h and 2- to 4-h time intervals for the lysogen and wild type D1571. The increased growth rate is only observed during the lag and early exponential phase of growth and disappears in the 4- to 6-h and 6- to 8-h growth rate intervals. The cause of the growth

![FIGURE 6](image-url)
rate differences observed with DLP3 lysogenization is currently unknown, though DLP3 does encode many hypothetical and moron genes which may be expressed to produce this phenotype.

**Virulence of D1571:DLP3 and DLP3 Rescue in G. mellonella**

Based on the differing growth characteristics of D1571:DLP3 lysogen and the wildtype strain, we examined if the increased growth rate of the lysogen observed in vitro affected the virulence of strain D1571 in vivo using the G. mellonella larvae infection model. G. mellonella have been used as a non-mammalian eukaryotic model for assessing the virulence of many bacterial pathogens, including S. maltophilia (Betts et al., 2014; Tsai et al., 2016; Melloul et al., 2018), as well as a model to study the efficacy of novel antimicrobial compounds and phages (Seed and Dennis, 2009; Kamal and Dennis, 2015; Cutuli et al., 2019).

Injection of G. mellonella larvae with S. maltophilia D1571 results in dose-dependent killing, with the lethal dose for this strain greater than $10^7$ CFU per larva (Figure 7A). Coinciding with the faster growth rate observed in vitro, the D1571:DLP3 lysogen was more virulent than the wildtype D1571 strain, resulting in significantly lower survival for G. mellonella larvae injected with $10^7$ ($P < 0.01$) or $10^6$ ($P < 0.001$) CFU over 120 h (Figure 7A). This increased virulence may be due to the faster growth rate of D1571:DLP3, however, CFU were not recovered from the larvae following infection.

Despite the DLP3 lysogen showing increased virulence in vivo, we sought to determine if DLP3 could rescue G. mellonella from wildtype D1571 infection. An inoculum of approximately $10^6$ CFU per larva was chosen to allow for phage rescue at a multiplicity of infection of at least 100, as DLP3 does not propagate higher than $10^{10}$ PFU/mL. Larvae were injected with DLP3 lysate at 1.5 h post-infection with D1571 and survival...
monitored over 120 h. Compared to treatment with SM buffer, significantly more larvae survived when given phage at a MOI of 50 or 100 (Figure 7B, P < 0.01), with an average survival at 120 h of 53% or 47% for worms treated at an MOI of 100 or 50, respectively, compared to 17% survival of untreated larvae. Attempts to concentrate DLP3 to a titer greater than 10^{12} PFU/mL without causing melanization of larvae following phage injection were unsuccessful, however, we expect that treatment at a higher MOI would increase the survival of infected larvae. Increased survival may also occur with repeated phage injections over the course of the experiment, however, this was not tested. Overall, this preliminary investigation using G. mellonella indicates the potential of DLP3 as a therapeutic for the treatment of S. maltophilia infections.

CONCLUSION

The novel temperate phage DLP3 isolated from a soil sample enabled the identification of a second member of the newly established Delepquintavirus genus. DLP3 has a genome size of 96,852 bp and a GC content of 58.4%, which is significantly lower than the host strain D1571 which has a GC content of 66.9%. DLP3 encodes two proteins with ParB conserved domains enabling the stable lysogenization of the host strain D1571. Lysogenization by DLP3 leads to a growth rate increase during the lag and early exponential phase of growth for the host when compared to the wild type strain. DLP3 also encodes a functional Erm protein, allowing for the lysogenic conversion of the host D1571 strain which is observed though increased resistance to erythromycin at 144 and 188 µg/ml concentrations. Despite the temperate lifestyle of this phage, DLP3 is capable of lytic activity in vivo.

Like DLP3, many of the S. maltophilia temperate phages characterized to date feature moron genes that encode virulence factors or antibiotic resistance proteins that could cause lysogenic conversion of their host (Hagemann et al., 2006; García et al., 2008; Peters and Dennis, 2018; Peters et al., 2019). Genetically modifying these phages by removing adverse genes, such as those encoding virulence factors or lysogeny proteins, will enable the creation of safe and highly targeted therapeutic agents (Lynch et al., 2010; Dedrick et al., 2019). Modified phages have been used in a case study against Mycobacterium abscessus (Dedrick et al., 2019) and a clinical trial (NCT04191148) sponsored by Locus Bioscience treating urinary tract infections with their modified crPhage cocktail. The genetically modified phage therapy examples set the precedence for other phages, such as DLP3, to be genetically modified and included in therapeutic phage cocktails. To this end, further study of DLP3 receptor binding proteins will possibly allow for the modification of other S. maltophilia phages showing therapeutic potential to target a wider host range.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in GenBank under accession number MT110073.

AUTHOR CONTRIBUTIONS

DP isolated phage DLP3, performed experiments to characterize the genome and viral particle, wrote the original manuscript draft and edited the manuscript. JM performed experiments to characterize the virus, wrote parts of the manuscript, and edited the manuscript. JD conceived of the research, directed the experiments, funded the research, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01358/full#supplementary-material

FIGURE S1 | MUSCLE multiple sequence alignment of gp28 proteins from phages DLP3 and DLP5, and tail fiber protein of Xylella phage Sano. Note the increased amino acid conservation toward the C-termini of the proteins versus the N-termini as observed through protein alignment.

FIGURE S2 | SDS-PAGE gel of DLP3 ghost particles (L) compared to a PageRuler Plus Pre-stained Protein Ladder (Thermo Scientific). Approximate masses are labeled on the right of the gel in kDa. Protein bands labeled using mass spectrometry results from SEQUEST scan of DLP3 and Stenotrophomonas protein databases. Stacking and resolving portions of the gel were 4 and 7.5%, respectively. The gel was stained with Coomassie R-250.

TABLE S1 | Bacterial strains and plasmids used in this study.

TABLE S2 | Mass spectrometry protein results using the Stenotrophomonas mass spectrometry results from SEQUEST scan of DLP3 and Stenotrophomonas protein databases. The datasets generated for this study can be found in GenBank under accession number MT110073.
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