

Genome Sequence and Characterization of the *Tsukamurella* Bacteriophage TPA2^{∇†}

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The formation of stable foam in activated sludge plants is a global problem for which control is difficult. These foams are often stabilized by hydrophobic mycolic acid-synthesizing *Actinobacteria*, among which are *Tsukamurella* spp. This paper describes the isolation from activated sludge of the novel double-stranded DNA phage TPA2. This polyvalent *Siphoviridae* family phage is lytic for most *Tsukamurella* species. Whole-genome sequencing reveals that the TPA2 genome is circularly permuted (61,440 bp) and that 70% of its sequence is novel. We have identified 78 putative open reading frames, 95 pairs of inverted repeats, and 6 palindromes. The TPA2 genome has a modular gene structure that shares some similarity to those of *Mycobacterium* phages. A number of the genes display a mosaic architecture, suggesting that the TPA2 genome has evolved at least in part from genetic recombination events. The genome sequence reveals many novel genes that should inform any future discussion on *Tsukamurella* phage evolution.

A common problem in activated sludge systems is the formation of stable foams on the aerated reactor, leading to major environmental, operational, cosmetic, and health problems (12, 41, 42). These foams are typically stabilized by members of the mycolic acid-containing *Actinobacteria* (the Mycolata), although other hydrophobic filamentous bacteria, including “*Candidatus* *Microthrix parvicella*,” are also important (12, 24, 39). One potentially attractive approach for controlling such foaming events is the use of lytic bacteriophages targeting the problematic foam-stabilizing populations (46, 48). Similar “phage therapy” strategies have been proposed to treat infectious diseases (6) and decrease bacterial contaminants in food (50). Furthermore, Thomas et al. (46) showed that Mycolata lytic bacteriophages could be isolated readily from activated sludge, making the development of a phage-based foam control approach an environmentally safe and attractive option for this worldwide operational problem.

Tsukamurella is in the suborder *Corynebacterineae*, and on the basis of its mycolic acid-containing hydrophobic cell surface it has been categorized as a Mycolata (18). *Tsukamurella* spp. have been isolated from activated sludge foams (12, 31), arthropods (44), and soil (18) and more recently in opportunistic clinical infections (23, 43). Although *Tsukamurella* spp. are a problem in environmental and medical contexts, little attention has been directed toward the isolation and characterization of bacteriophages specific for members of this genus. To our knowledge, only one partially characterized *Tsukamurella* phage (TPA1, targeting *T. paurometabola*) has been reported in the literature (46). With the aim of developing a biocontrol approach to manage foaming within activated

sludge systems, we have sought to isolate and characterize new lytic phages for members of this genus.

More fundamentally, the ecological role of bacteriophages in activated sludge communities has received relatively little attention. This is surprising given the high numbers of bacteriophages found in this environment (10^7 to 10^9 per ml) and the importance of phages in regulating bacterial community biodiversity (33). Characterizing new phages from environments such as activated sludge will provide a greater insight into phage evolution and diversity, as most phage studies have been dominated by the use of a limited range of bacterial hosts (48).

In this paper, we report the isolation and characterization of two *Tsukamurella* phages (TPA2 and TPA3) from activated sludge plants. These phages, together with the phage TPA1 isolated by Thomas et al. (46), were characterized on the basis of their morphologies, host ranges, phage life cycle, burst sizes, and complete genome sequences. Annotation of these phage genome sequences reveals that TPA1 and TPA2 phages share some similarity with other *Corynebacterineae* phages (21), but their DNA sequences reveal many novel attributes.

MATERIALS AND METHODS

Bacterial strains used in study. The bacterial strains used in this study are listed in Table 1. All were grown on peptone-yeast-calcium (PYCa) (0.1% yeast extract [Oxoid, Adelaide, Australia], 1.5% peptone [Oxoid], 0.5% CaCl₂, 0.1% glucose) broth and agar (PYCa plus 1.4% agar [Oxoid]) at 30°C. All chemicals were obtained from Sigma (Sydney, Australia) unless otherwise noted.

Isolation and purification of phages. Bacteriophages were isolated from mixed liquor samples taken from activated sludge plants in Australia as follows. Samples (20 ml) were centrifuged ($3,000 \times g$ for 20 min) before being filtered through cellulose acetate membrane filters (0.2- μ m pore size) to remove bacterial cells. *Tsukamurella* phage enrichment was performed by pooling 1 ml of the six species of *Tsukamurella* listed in Table 1 ($\sim 10^6$ per ml) in 50 ml of PYCa broth in 300-ml flasks and incubating with 1 ml of filtered activated sludge supernatant. Flasks were left for 1 h at room temperature without shaking to encourage phage infection before a further incubation with shaking at 30°C for 2 days. Following enrichment, the remaining bacterial cells were removed by centrifugation and the supernatants filtered through 0.2- μ m cellulose acetate membrane filters. Individual lawn plates of each *Tsukamurella* species were prepared by swabbing, and 20- μ l aliquots of enriched supernatants were drop diluted onto each lawn

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TABLE 1. Strains used in this study

Name of organism ^a	Culture collection number(s) and other synonyms ^b
<i>Tsukmurella inchonensis</i> ^T	DSMZ44067
<i>T. inchonensis</i>	NCTC10741
<i>T. paurometabola</i> ^T	Tpau37, ATCC 25938
<i>T. paurometabola</i>	NCTC107411
<i>T. paurometabola</i>	IMRU1283
<i>T. paurometabola</i>	DSMZ20162
<i>T. paurometabola</i>	IMRU1520, M334, DSMZ44119
<i>T. paurometabola</i>	IMRU1312, M343
<i>T. paurometabola</i>	IMRU1505, M337
<i>T. pseudospumae</i> ^T	N1176, DSMZ44118
<i>T. pulmonis</i>	DSMZ44142
<i>T. spumae</i> ^T	N1171, DSMZ44113, NCIMB139647
<i>T. spumae</i>	JC85
<i>T. tyrosinosolvens</i> ^T	DSMZ44234
<i>Nocardia asteroides</i>	Ben600
<i>N. asteroides</i> ^T	Nast23, DSMZ43757
<i>N. asteroides</i>	Noast4, UOCC131
<i>N. brasiliensis</i> ^T	Nbra42, DSMZ43758
<i>N. carneae</i> ^T	Ncar30, DSMZ 43397
<i>N. nova</i> ^T	Nnov47, ATCC 33726
<i>N. otitidisdiscariarum</i>	Noti14, AMMRL 19.11
<i>N. otitidisdiscariarum</i> ^T	Noti25, DSMZ43242
<i>N. otitidisdiscariarum</i>	Noti15, AMMRL 19.12
<i>N. transversalis</i> ^T	Ntra40, DSMZ43405
<i>Rhodococcus coprophilus</i> ^T	Rcop41, DSMZ43347
<i>R. coprophilus</i>	Rcop18, UOCC1259
<i>R. equi</i>	Requ10, UOCC702
<i>R. equi</i> ^T	Requ28, UOCC20307, DSMZ20307
<i>R. erythropolis</i>	Rery19, UOCC379
<i>R. erythropolis</i> ^T	Rery29, DSMZ43066
<i>R. fascians</i> ^T	Rlut36, DSMZ43673
<i>R. globerulus</i> ^T	Rglo35, DSMZ43954
<i>R. rhodnii</i> ^T	Rrho46, DSMZ43336
<i>R. rhodochrous</i>	Rrho3, UOCC2807
<i>R. rhodochrous</i> ^T	Rrho39, DSMZ43241
<i>R. rhodochrous</i>	Rrho11S, UOCC2808
<i>R. ruber</i> ^T	Rrub33, DSMZ43338
<i>R. tritumae</i> ^T	DSMZ44892
<i>Gordonia aichiensis</i> ^T	Raic22, DSMZ43978
<i>G. alkanivorans</i>	Ben606
<i>G. amarae</i> ^T	Gama44, DSMZ43392
<i>G. amarae</i>	Gama9, UOCC2810
<i>G. amictica</i>	Ben607
<i>G. australis</i>	18F3 M
<i>G. defluvi</i> ^T	J4, DSMZ44981
<i>G. desulfuricans</i> ^T	213E, NCIMB40816
<i>G. hydrophobica</i> ^T	N1123, DSMZ44015
<i>G. malaquae</i> ^T	A554, ATCC 35215
<i>G. malaquae</i>	A448
<i>G. rubropertincta</i> ^T	Grub48, DSMZ43197
<i>G. sputi</i>	Gobu31, DSMZ43979
<i>G. sputi</i> ^T	Gspu49, ATCC 29627
<i>G. sputi</i>	Gspu48, ATCC 336609
<i>G. terrae</i> ^T	Gter34, DSMZ43249
<i>G. terrae</i>	Ben601
<i>G. terrae</i>	Ben602
<i>G. terrae</i>	Ben603
<i>G. terrae</i>	Ben604
<i>Dietzia maris</i> ^T	Dmar27, DSMZ43672
<i>Streptomyces griseus</i>	Sgri05
<i>Millisia brevis</i> ^T	J82, DSMZ44463
<i>Mycobacterium chlorophenolicus</i> ^T	Mchl24, DSMZ43826
<i>M. smegmatis</i>	Msmel, UOCC422
<i>M. fortuitum</i>	Mfor21, UOCC120

^a T, type strain.

^b Culture collection numbers have been obtained from the following organizations: DSMZ, German Collection of Microorganisms and Cell Cultures; ATCC, American Type Culture Collection; UOCC, Australian Collection of Microorganisms (now ACM); AMMRL, Australian National Reference Laboratory in Medical Mycology; and NCIMB, National Collection of Industrial Bacteria. All culture numbers have been derived from the La Trobe University Bendigo culture collection.

plate and allowed to dry. Plates were incubated for 48 h to allow visualization of plaques. Single plaques were seen on plates inoculated with filtered mixed liquor from two activated sludge plants in Victoria, Australia. Plaques were purified through four rounds of dilution and reisolation to ensure that each plaque resulted from a single virion before further studies were undertaken. These phages were named TPA2 and TPA3.

Phage DNA isolation. *T. paurometabola* (Tpau37) was inoculated in PYCa broth and incubated overnight. One milliliter (~10⁷ CFU/ml) of *T. paurometabola* culture was added to 50 ml of PYCa broth and infected with the appropriate TPA phage using a multiplicity of infection (MOI) equal to 0.1 PFU/bacterium and incubated as described previously. The lysate was recovered by centrifugation at 3,000 × g for 20 min, and the supernatant was removed and filtered through a 0.2-μm filter to remove bacterial cells and cell debris. Non-phage nucleic acids were eliminated from the filtered supernatant (the titer was ~10¹⁰ PFU/ml) by nuclease digestion (final concentrations of 10 μg/ml DNase I, 10 μg/ml RNase A, and 5 mM MgCl₂; 30 min at room temperature). Phage virions were recovered by polyethylene glycol (PEG) precipitation (final concentrations of 10% [wt/vol] polyethylene glycol 8000 and 1 M NaCl; 4°C overnight) and collected by centrifugation at 13,000 × g for 15 min before being resuspended in 50 μl of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA).

Phage DNA was extracted from ~10¹⁰ PFU using SDS-proteinase K (final concentrations of 20 mM EDTA, 0.5% SDS, and 50 μg/ml proteinase K; 55°C for 1 h) in a total volume of 1 ml. After incubation, an equal volume of phenol-chloroform-isoamyl alcohol (29:28:1) was added and mixed by vortexing before centrifugation at 10,000 × g for 3 min. The upper aqueous phase was removed to a fresh tube containing 1 volume isopropanol and 0.1 volume 3 M sodium acetate (pH 5.2) and incubated at room temperature for 30 min. The precipitated phage DNA was recovered by centrifugation at 10,000 × g for 10 min. The DNA pellet was washed once with 70% ethanol, air dried, and finally resuspended in 50 μl of TE buffer.

Phage genome sequencing. Phage DNA for TPA1 and TPA2 was genome sequenced using the Roche GS FLX genome sequencer and titanium chemistry by Genoseq (UCLA, Los Angeles, CA). The pyrosequencing reads were assembled using the gsAssembler (Roche Applied Science, Indianapolis, IN). All resulting single contigs obtained for each phage had a minimum of 50 times read coverage.

DNA annotation. Geneious 4.0.4 (14) was used initially to identify all open reading frames (ORFs) longer than 30 codons in all six reading frames. The putative proteins encoded by the identified ORFs were screened using the NCBI database and the BLAST P algorithm with a significance cutoff E value of 10⁻⁴. The conserved domain database (CDD) (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) and Pfam database (<http://pfam.sanger.ac.uk>) were used to identify conserved motifs and make predicted protein family allocations (15). The presence of putative tRNA and tmRNA was screened for using ARAGORN (<http://www.acgt.se/online.html>) (25).

Phage host range determination. All six *Tsukamurella* species were screened for lytic infection by TPA1, TPA2, and TPA3 phages. A drop dilution series of each purified phage was spotted onto swabbed lawn plates of each strain and incubated for 2 days. Additional actinobacterial strains (Table 1) were screened for their ability to support TPA2 lytic infection.

Single-step growth curve. Four replicas of a 1-ml volume of an early-exponential-phase culture of *T. paurometabola* (Tpau37) was infected with TPA2 at a multiplicity of infection (MOI) of 0.1 (2). After an adsorption period of 5 min at 30°C, the cells were washed twice in PYCa broth and resuspended in 1 ml of broth. This culture was diluted 1 in 100 to minimize the possibility of nonadsorbed phages infecting cells. All further steps were performed as described by Adams (2) except that the incubations were conducted at 30°C.

Electron microscopy. Virus particles were allowed to adsorb to Formvar-coated 200-mesh copper grids for 5 min. These were washed twice for 1 min in double-distilled water (ddH₂O) and negatively stained with 2% (wt/vol) uranyl acetate for 2 min. Excess liquid was absorbed onto filter paper, and the disc was allowed to air dry. Grids were examined under a JEOL JEM-100cx transmission electron microscope (TEM) at an accelerating voltage of 100 kV.

Nucleotide sequence accession number. The nucleotide sequence for TPA2 has been deposited in GenBank under accession number HM486077.

RESULTS AND DISCUSSION

Activated sludge samples from 10 different wastewater treatment plants in Australia were screened for *Tsukamurella* phages by enrichment and plaque plating onto lawn plates of six *Tsukamurella* species (*T. paurometabola*, *T. pulmonis*, *T.*

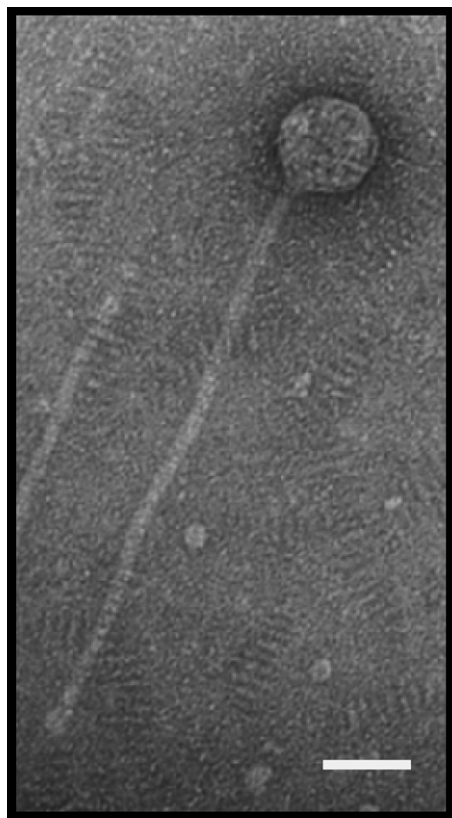


FIG. 1. Electron micrograph of TPA1. Scale bar, 50 nm.

tyrosinosolvens, *T. spumae*, *T. pseudospumae*, and *T. inchoensis*) (see Materials and Methods). Single plaques were observed on lawn plates of *T. paurometabola* from the activated sludge samples obtained from the Daylesford and Ballarat (Victoria, Australia) wastewater treatment plants. Both phages (TPA2 and TPA3) formed plaques of ~1 mm in diameter after 2 days incubation at 30°C. Restriction mapping of the TPA2 and TPA3 genomes suggested that these two phages were the same (see below), so only TPA2 was selected for genome sequencing, together with the *T. paurometabola* phage (TPA1), which had been isolated earlier by Thomas et al. (46). Subsequent sequence analysis showed that TPA1 and TPA2 were identical, and therefore this paper reports the characterization of the TPA2 genome.

TPA2 belongs to the *Siphoviridae* family, possessing the characteristic long, noncontractile tail (~340 nm) of members of the *Caudovirales* with a B1 isometric capsid (~58 nm) morphotype (1) (Fig. 1). The average burst size was determined to be 155 ± 5 particles per infective center, with a latency period of 4 h in PYCa broth at 30°C. The TPA2 phage displays a broad-host-range phenotype within the genus *Tsukamurella*. In addition to infecting *T. paurometabola* (Tpau37), TPA2 lysed a further six *T. paurometabola* strains (DSMZ20162, IMRU1520, IMRU1312, IMRU1505, IMRU1283, and NCTC107411), as well as members of four other *Tsukamurella* species, i.e., *T. pulmonis* (DSM44142), *T. tyrosinosolvens* (DSMZ44234), *T. pseudospumae* (N1176), and two strains of *T. spumae* (N1171 and JC85). Mock infection controls were performed using each

strain to ensure that lysis was not due to the release of prophages from the different hosts. The only *Tsukamurella* strain tested that was not lysed by TPA2 was *T. inchoensis* (DSMZ44067). Although clear lytic plaques were not observed, the TPA2 phage did induce a turbid lysis on *T. inchoensis* at a high MOI. Further investigations revealed that TPA2 was unable to propagate in this strain, suggesting that infected cultures of TPA2 generate a factor that can kill *T. inchoensis*. TPA2 appears to be restricted in its host range to members of the genus *Tsukamurella*, since it was unable to lyse any other Mycolata strain, including 20 different *Gordonia* spp., 15 different *Rhodococcus* spp., 10 different *Nocardia* spp., 3 *Mycobacterium* spp., *Dietzia maris*, *Millisia brevis*, and *Streptomyces griseus* (Table 1). The molecular basis for this host specificity remains to be determined.

Genomic features of TPA2. The genomic DNAs isolated from the TPA1, TPA2 and TPA3 bacteriophages were digested using a range of restriction enzymes. Several (EcoRI, HindIII, and BamHI) failed to digest DNA from any of these genomes. However, genomic DNA from all three was digestible by BglII, and each gave an identical restriction enzyme digest pattern (data not shown). Consequently, only TPA1 and TPA2 were selected for genome sequencing.

The genome sequences of TPA1 and TPA2 after Roche/454 pyrosequencing generated 30,440 and 27,589 reads, respectively, with a minimum of 50 times sequence coverage. The assembled genomes of TPA1 and TPA2 were identical despite their being isolated from separate activated sludge plants 9 years apart (46). The assembled sequence of TPA1 and TPA2 suggested a circularly permuted genome, which was further confirmed by BglII restriction endonuclease analysis (data not shown). While laboratory cross-contamination is a possibility, it is unlikely in this case given that TPA1 was recovered from a freezer stock only after TPA2 and TPA3 had been isolated and initially characterized. These results suggest not only that TPA-like phages may be widespread within activated sludge systems but that they may persist for many years within this environment. Since the two phages were identical at the DNA sequence level, all further discussion is based on TPA2.

TPA2 has a circularly permuted, double-stranded DNA (dsDNA) genome consisting of 61,440 bp, with a G+C content of 69.1 mol%. This is within the 67 to 74 mol% G+C range for *Tsukamurella* spp. (18), suggesting that TPA2 is adapted to its host. A comparison between the *in silico* and the experimentally determined restriction digest patterns indicated that the TPA2 genome was correctly assembled and lacked any EcoRI, HindIII, and BamHI sites (data not shown). At the DNA level, 30% of the TPA2 genome shares identity with a number of sequenced mycobacteriophages (21), while the remaining 70% of the genome sequence appears to be unique so far. Analysis of the TPA2 genome revealed 78 ORFs larger than 90 nucleotides, with no tRNA or tmRNA detected. These ORFs are numbered consecutively except in the case of the highly conserved large terminase (*terL*), lysin (*lysA*), and Holliday junction resolvase (*ruvC*) genes. Thirty-three of the ORFs are located on one strand and 45 on the opposite strand (Fig. 2). A total of 44 ORFs showed significant identity with previously reported ORFs, and only 15 (19%) of these could be assigned predicted functions. Thirty-four ORFs exhibited no significant identity to any hypothetical protein (Table 2).

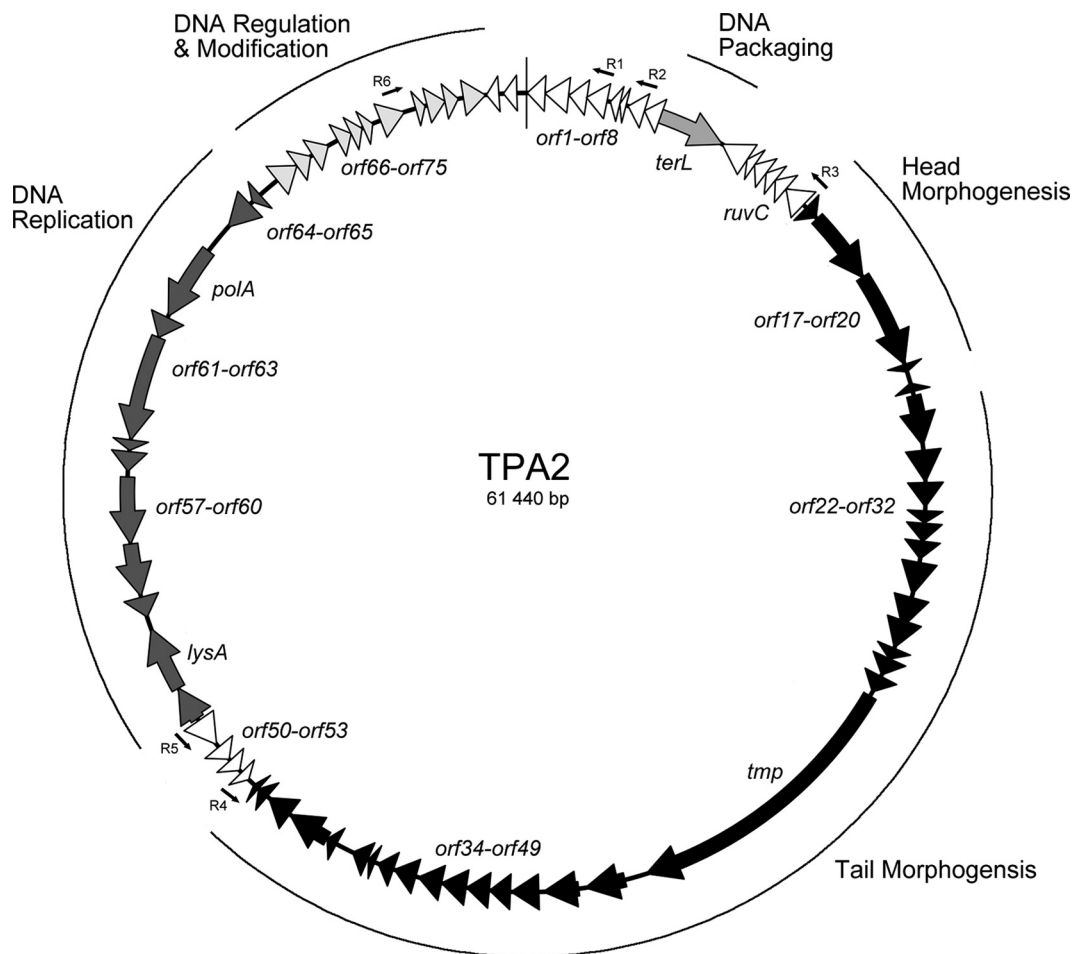


FIG. 2. Circular map of the TPA2 genome. The arrows represent the putative genes and the directions in which they are transcribed. Modules are shaded in similar tones, and the outer circle indicates the genes encoded within the modules. Arrows represent the repeat regions R1 to R6.

The phage genome is modularly organized, consisting of DNA packaging, head and tail morphogenesis, cell lysis, DNA replication, modification, and regulation modules (Fig. 2). In the absence of an obvious origin of replication, the ORFs were ordered from the putative large terminase (*terL*) gene. However, since *orf8* overlaps *terL* and is part of a putative operon of unknown function transcribed in the opposite direction to *terL*, the ORFs were numbered consecutively from the large noncoding region located before *orf1* in the transcriptional direction of *terL*.

Sequence repeats. A large number of repeat structures were observed in the TPA2 genome. Of these one is a near-identical 58-bp or 59-bp repeat (R1 to R6) located throughout the TPA2 genome (Table 3). Five of these repeat sequences (R1 to R5) occur on one strand, and one (R6) occurs on the opposite strand (Fig. 2). Four of the repeat sequences (R2, R4, R5, and R6) overlap the beginnings or ends of putative genes. Repeats R4 and R5 occur in regions overlapping the translational starts of ORFs, while R6 overlaps the end of a putative gene and R2 encompasses the start of one gene and stop of another, overlapping gene. The location of this overlap corresponds to the direction in which the repeat occurs (i.e., R6 overlaps the end of a putative gene and occurs in the opposite orientation to

repeats that overlap the start of genes). Such a repeat structure raises the possibility that they may be involved in antisense translation regulation. Expression of the ORF containing R6 would produce an RNA that would bind with high affinity to the RNA transcripts containing R1 to R5. Furthermore, the repeat structures carry a sequence highly similar to the sigma -35 (TTGACA) and -10 (TATAAT) sequences (Table 3). To the best of our knowledge, such structures have not been observed in other bacteriophages and warrant further study.

Analysis of the TPA2 genome also reveals 95 pairs of inverted repeats ranging in size from 16 to 53 bp (see the supplemental material). These sequences occur within both putative genes and intergenic regions. Their large number and wide distribution throughout the genome suggests that they may have a functional role. Interestingly, all the repeats identified are inverted, with none occurring in a direct orientation. Inverted repeat structures are associated with replication origins (30) and transposable elements, neither of which could be identified in the genome of TPA2. The functional role (if any) of these repeats remains to be experimentally determined.

In addition to the inverted repeats, six palindrome sequences were identified. The smallest is 22 bp long, and the largest is 49 bp long (Table 4). Four of the palindromes occur

TABLE 2. Summary of ORFs and gene products of TPA2

ORF ^a	No. of amino acids (% identity) ^b	Predicted function (conserved motif) ^c	Match ^d	E value ^e
1	146	Unknown		
2	193	Unknown		
3	146	Unknown		
4	172	Unknown		
5	79	Unknown		
6	53	Unknown		
7	139	Unknown		
8	140	Unknown		
<i>terL</i>	554 (51)	Large terminase (Pfamn03237)	Gp7, <i>Mycobacterium</i> phage Rosebush	1e-145
10	240	Unknown		
11	79	Unknown		
12	118	Unknown		
13	95	Unknown		
14	113	Unknown (Pfam05305)		
<i>ruvC</i>	192 (40)	RuvC resolvase (cd00529)	<i>Mycobacterium</i> phage Pipefish	6e-28
16	114 (42)	Unknown (Pfam07098)	Gp6, <i>Mycobacterium</i> phage Nigel	1e-19
17	611 (52)	Putative structural protein	Gp12, <i>Mycobacterium</i> phage Qyrzula	4e-164
18	793 (52)	Structural protein (Pfam04233)	Gp12, <i>Mycobacterium</i> phage Pipefish	0.0
19	64 (53)	Putative structural protein	Gp13, <i>Mycobacterium</i> phage Pipefish	8e-12
20	72 (0)	Unknown		
21	414 (69)	Unknown	Gp2, <i>Mycobacterium</i> phage Cooper	6e-167
22	260 (45)	Unknown	Gp13, <i>Mycobacterium</i> phage Orion	5e-53
23	199 (34)	Unknown	Gp16, <i>Mycobacterium</i> phage Pipefish	8e-17
24	101 (59)	Unknown	Gp15, <i>Mycobacterium</i> phage Cooper	1e-21
25	151 (49)	Unknown	GTE5p031, <i>Gordonia terrae</i> phage GTE5	5e-34
26	147	Unknown		
27	268 (67)	Major tail protein	Gp21, <i>Mycobacterium</i> phage Pipefish	2e-102
28	256 (50)	Putative tail assembly	Gp23, <i>Mycobacterium</i> phage Pipefish	1e-64
29	234 (48)	Putative tail assembly	Gp23, <i>Mycobacterium</i> phage Qyrzula	3e-45
30	97 (68)	Unknown	Gp20, <i>Mycobacterium</i> phage Nigel	2e-06
31	147 (51)	Tail component (Pfam04883)	Gp25, <i>Rhodococcus</i> phage ReqiPine5	5e-29
32	145 (49)	Unknown	Gp27, <i>Mycobacterium</i> phage Pipefish	1e-29
33	2,386 (36)	Tape measure protein (Pfam10145)	TMP, <i>Mycobacterium abscessus</i>	2e-102
34	331 (49)	Unknown	Gp30, <i>Mycobacterium</i> phage pipefish	3e-96
35	292 (49)	Unknown	Gp30, <i>Mycobacterium</i> phage Colbert	3e-69
36	227	Unknown		
37	171	Unknown		
38	188	Unknown		
39	209 (59)	Unknown	Gp36, <i>Mycobacterium</i> phage Rosebush	3e-59
40	196 (65)	Unknown	Gp32, <i>Mycobacterium</i> phage Nigel	3e-70
41	184 (45)	Unknown	Gp38, <i>Mycobacterium</i> phage Phlyer	9e-39
42	133 (37)	Unknown	Gp39, <i>Mycobacterium</i> phage Rosebush	2e-13
43	58 (44)	Unknown	Gp37, <i>Mycobacterium</i> phage Cooper	6e-04
44	137 (46)	Putative tail fiber	Gp43, <i>Mycobacterium</i> phage Pipefish	3e-23
45	88 (50)	Unknown	Gp44, <i>Mycobacterium</i> phage Pipefish	3e-12
46	364 (36)	Putative tail fiber	Gp6, <i>Mycobacterium</i> phage Jasper	4e-15
47	218	Unknown		
48	94 (65)	Putative regulator	Gp45, <i>Mycobacterium</i> phage Pipefish	2e-18
49	50 (56)	Unknown	Gp46, <i>Mycobacterium</i> phage Pipefish	6e-04
50	83	Unknown		
51	113	Unknown		
52	131	Unknown		
53	244 (32)	Unknown	Gp41, <i>Mycobacterium</i> phage PBI1	3e-18
54	294 (27)	Putative holing	<i>Rhodococcus opacus</i>	1e-04
<i>lysA</i>	536 (59)	Lysin (Pfam01510, Pfam08310)	<i>Nocardia facinica</i>	4e-103
56	169 (46)	Unknown	Gp48, <i>Mycobacterium</i> phage Phlyer	8e-26
57	435 (39)	Unknown	Gp47, <i>Mycobacterium</i> phage Cooper	1e-82
58	549 (56)	Helicase (Pfam04851) (Pfam00271)	Gp50, <i>Mycobacterium</i> phage Rosebush	4e-167
59	161 (40)	Unknown	Gp51, <i>Mycobacterium</i> phage Cooper	4e-15
60	86	Unknown		
61	870 (51)	Primase (Pfam09250)	Gp52, <i>Mycobacterium</i> phage Phaedruss	0.0
62	178 (47)	Unknown	Hypothetical, <i>Jonesia denitrificans</i>	7e-19
63	626 (52)	DNA polymerase I (Pfam01612, Pfam00476)	Gp54, <i>Mycobacterium</i> phage Cooper	6e-174
64	259	Unknown		
65	85	Unknown		

Continued on following page

TABLE 2—Continued

ORF ^a	No. of amino acids (% identity) ^b	Predicted function (conserved motif) ^c	Match ^d	E value ^e
66	202	Unknown		
67	136	Unknown		
68	150	Unknown		
69	124	Unknown		
70	99	Unknown		
71	85	Unknown		
72	208 (27)	Unknown	Gp65, <i>Mycobacterium</i> phage Rosebush	2e-07
73	79	Unknown		
74	151	Unknown		
75	112 (63)	Putative endonuclease	Gp85, <i>Mycobacterium</i> phage Scoot17C	7e-22
76	182 (46)	Unknown	Gp72, <i>Mycobacterium</i> phage Phaerdrus	1e-29
77	102	Unknown		
78	110 (50)	Metallophoesterase (COG4186)	Gp66, <i>Rhodococcus jostii</i>	1e-17

^a ORFs were numbered consecutively, with the exceptions of *terL*, *rvuC*, and *lysA*.

^b Percent identity is based on the best match when a BLAST P analysis was performed.

^c Predicted function is based on amino acid identity, conserved motifs, N-terminal sequencing, and gene location within functional modules.

^d The most closely related gene (only if named) and the name of the organism.

^e The probability of obtaining a match by chance as determined by BLAST analysis. Only values of less than 10⁻⁴ were considered significant.

in intergenic regions, suggesting that they may function as *rho*-independent transcriptional terminators. The downstream flanking sequences do not display the T-rich region typical of *rho*-independent transcriptional terminators in *Escherichia coli* (26); however, it has been observed that this T-rich region is not required for transcriptional termination in other bacteria such as the Gram-positive *Streptomyces lividans* (13, 32).

DNA packaging gene region. The protein encoded by the ninth ORF (*terL*) is highly similar to the putative large terminase from the *Mycobacterium* phage Rosebush (21). The terminase enzyme is essential for DNA packing of the phage genome into the phage head (38). It normally functions as a complex with a small terminase subunit (8), which determines the specificity of DNA binding, while the large terminase subunit mediates cleavage of the phage DNA packaged into the prohead (17). Typically, the genes encoding the small terminase are located immediately upstream of those encoding the large terminase subunit and transcribed in the same direction. The putative gene upstream of *terL*, *orf8*, is transcribed in the opposite direction to *terL* and appears to be part of an unrelated operon. Furthermore, an examination of the complete genome reveals no ORF with any identity to known or putative small terminase genes, suggesting that either TPA2 utilizes an

alternative DNA packaging mechanism or the small terminase has no similarity to any previously described one.

Structural gene region. The genes encoding the structural proteins are located from *orf17* to *orf47*. SDS-PAGE analysis of TPA1 phage proteins revealed 10 structural proteins (45). The major protein of ~35 kDa was previously extracted and N-terminally sequenced (ATFPLVKGTRLRATRINSCG) by Thomas (45). This sequence is identical to that of the protein encoded by *orf27* of the TPA2 genome. The N-terminal methionine is absent, a situation seen with other phage proteins and caused by the activity of a host cell methionine aminopeptidase (28).

The phage head morphogenesis module (Fig. 2) appears to be encoded by the genes *orf17* to *orf20*. All four ORFs are expressed in the same orientation and share high levels of amino acid identity with *Mycobacterium* phages and the *Rhodococcus* phage ReqiPine5. The gene *orf18* contains a pfam04233 motif, which is indicative of proteins involved in viral head morphogenesis of dsDNA phages (4). It is typical for the head protein genes in phage genomes to be clustered together and to precede the tail protein genes (7). This suggests that *orf17* and *orf19* are likely to be involved in viral head structural formation despite their products lacking identity to any known

TABLE 3. Repeats R1 to R6 located within the TPA2 genome

Repeat	Sequence ^{a,c}	Coordinates	ORF(s)	Direction
R1	AGTTGACAAGTACCACCACTGTAGGFAAAGTGGTTTCACGT-AGGCGGGAAGGACCCGCC	1702-1645	<i>orf4</i>	←
R2	CGTTGACAACACTACCACCAAGTAGGFAATGTCTCTCTGTCCAGGGAAGGCGGGAACGACC	2962-2903	<i>orf7^b</i> , <i>orf8^c</i>	←
R3	GGTTGACAATCACCACCAGAGTAGGFAACTTCAGACACGCCAGGCGGGAACGACCCGCC	7573-7515	<i>orf15</i> , <i>orf16^d</i>	←
R4	AGTTGACTTTCACCACCACAGTAGGFAAAGTCATTCCCGTGGCGGGAATGACCCGCC	38539-38480	<i>orf50^b</i>	←
R5	AGTTGACAACCACCACCAAGTAGGFAATGTCTCTCTGTCCAGGCGGGAACGACCCGCC	40168-40110	<i>orf53^b</i>	←
R6	AGTTGACAAGCACCACCACAGTAGGFAATGTGGTTCGT-AGGCGGGAACGACCCGCC	58403-58460	<i>orf72^c</i>	→

^a Repeats R1 and R6 are 58 bp long, whereas R2 to R5 are 59 bp. Shaded nucleotides indicate differences in comparison to the consensus.

^b Overlaps the start of the gene.

^c Overlaps the end of the gene.

^d Repeat is present in an intergenic region.

^e Sequences in boldface indicate the start codons and those underlined indicate the stop codons of a gene. The boxed sequences represent putative -35 and -10 promoter regions.

TABLE 4. Palindrome sequences found within the TPA2 genome

Palindrome	Coordinates	Sequence	Melting temp (°C)	Location within TPA2
P1	14252–14300	AGGGCGGGCGGCGTGGACCACCACAGGCCCGGTCTACGC CGCCCGCCCT	84	Intergenic (<i>orf21-orf22</i>)
P2	20738–20777	GGCCGCGCGCGATGTCGAAGCTCAGATGTCGCGCGCGGCC	79	Start of <i>tmp</i>
P3	42728–42763	GAGAAGGCCCGTCCGCGGGGAAGCGACGGGCCTTCTC	76	Intergenic (<i>lysA-orf56</i>)
P4	47408–47429	GACGGCGTCCGCCGACGCCGTC	68	End of <i>orf61</i>
P5	59817–59851	GAGAACCCCGCCGTCCTGAGTTCGCGGGGTTCTC	69	Intergenic (<i>orf75-orf76</i>)
P6	60852–60875	TCGTGCCTACCGCGGTAGACACGA	57	Intergenic (<i>orf77-orf78</i>)

bacteriophage structural proteins. The function of *orf20* is unknown, and it displays no identity to any other gene.

Immediately downstream of the phage head morphogenesis-encoding region is a putative tail morphogenesis region. This region (*orf22* to *orf49*, with the exception of *orf36* to *orf38*) shares a high level of amino acid identity with similar regions from a range of *Mycobacterium* phages (19), the *Rhodococcus* phage ReqiPine5 (accession no. GU580943), and the *Gordonia terrae* phage GTE5 (accession no. AY846870). These genes are transcribed in the same direction and are flanked by putative *rho*-independent transcriptional terminators (Table 4).

The major tail protein appears to be encoded by the *orf27* gene, whose product has been confirmed by N-terminal sequencing to be a structural protein (45). On the basis of amino acid identity, we predict that an additional four structural proteins (encoded by *orf31*, *orf33*, *orf44*, and *orf46*) are involved in tail assembly. The protein encoded by *orf31* (15.8 kDa) contains a pfam04883 domain, which is common to tail component proteins in tailed phages. Furthermore, *orf33* encodes the largest protein (249 kDa) within the TPA2 genome and contains both the pfam10145 (found in tape measure proteins [TMPs]) and pfam06737 (lytic transglycosylase) motifs. The lytic transglycosylase domain located at the C terminus of this TMP also contains a peptidoglycan hydrolase domain, a feature seen in the *Mycobacterium* phage TM4 (37). The genes *orf31* and *orf33* encode conserved motifs specifically found in phage tail proteins.

The putative genes *orf28* and *orf29* display no significant identity to any known proteins. These two ORFs are located between the genes encoding the major tail protein (*orf27*) and the tape measure protein (*orf33*). Interestingly, between the major tail and tape measure protein genes of bacteriophages λ and μ is a single ORF that expresses two separate proteins via a programmed translational frameshift (49). A similar phenomenon has been noted in some *Mycobacterium* phages, such as *Mycobacterium* phage Tweety (36). No translational frameshift was identified in this region of TPA2; however, *orf28* and *orf29* overlap by 169 bp, and they have their own initiation codons. This intriguing structure suggests that TPA2 may be using an alternative mechanism to achieve coding overlap in this region.

Lastly, two ORFs (*orf44* and *orf46*) within this putative operon show identity at the amino acid level to genes encoding tail fibers in other bacteriophages (21). In addition to the major tail protein encoded by *orf27*, Thomas (45) detected nine minor structural proteins. Five of these proteins could be identified in the genome of TPA2 with sizes consistent with the

proteins identified by Thomas (45). The identities of the remaining structural genes remain unknown, but it is possible that some are components of larger proteins that have undergone posttranslational cleavage (9).

Host cell lysis gene region. Phage lysis modules typically consist of endolysin and holin genes that together are responsible for bacterial lysis and release of phage progeny (11). Within the TPA2 genome we could identify only the putative endolysin gene (*lysA*), with no ORF displaying identity to any known holin protein. At the amino acid level this putative endolysin shares high identity with proteins found in *Mycobacterium* phages and several hypothetical proteins thought to belong to the esterase and lipase family, encoded in the genomes of *Nocardia facinica* and *Corynebacterium* and *Rhodococcus* species. Close examination of the putative LysA amino acid sequence reveals two overlapping conserved motifs in the protein center with identity to the amidase 2 (Pfam01510) and the peptidoglycan recognition protein (PGRP) superfamily. These motifs suggest that this protein is most probably a zinc amidase involved in cleaving the amide bond between *N*-acetylmuramyl and L-amino acid residues in the bacterial peptidoglycan. This enzyme may have a structure and biological function similar to those in the T7 phage lysozyme homologue (10, 22). The C terminus of the putative endolysin contains three conserved 54-amino-acid repeat sequences with identity to a repeat structure seen in the *Corynebacterium glutamicum* and *Corynebacterium efficiens* genomes. These repeats belong to the pfam08310 family and are hypothesized to anchor proteins into the bacterial cell wall (3).

No lysin B (*lysB*) gene was identified in TPA2, which suggests that *lysA* may be sufficient for lysis or that if present its product is significantly different from previously identified lysin B proteins. Some *Mycobacterium* phages have been shown to encode a mycolylarabinogalactan esterase responsible for the release of the mycolic acid during cell lysis (34).

The identification of a holin-encoding gene remains unresolved. Holin genes are generally found adjacent to the lysin genes in phages (29) and typically contain two transmembrane domains. The putative gene *orf54* and *lysA* overlap and are expressed in the same direction. *In silico* analysis of the *orf54* protein sequence reveals two transmembrane domains, further suggesting that *orf54* encodes a novel holin; however, this has not been shown experimentally.

DNA replication, modification, and regulation region. The majority of genes involved in bacteriophage DNA replication and regulation appear to be located between *orf48* and *orf1*. Some exceptions exist (e.g., *ruvC*). The gene denoted *ruvC*

(between *orf14* and *orf16*) appears to encode a Holliday junction resolvase with high identity to products of genes observed in *Mycobacterium* phage genomes. Holliday endonucleases resolve Holliday junction structures formed during homologous recombination events (40). Such enzymes are frequently encountered in bacterial genomes, but similar *ruvC* genes have also been identified in phage genomes, including *Lactococcus lactis* phage bIL66 and a number of *Mycobacterium* phages (5). It has been speculated that the phage *ruvC*-like gene products are more similar to general branch-cutting endonucleases, such as those encoded by the T4 and T7 phage genomes (47), than to typical bacterial Holliday junction resolvases (40). The genes flanking *ruvC*, *orf14* and *orf16*, encode products with no known function. However, conserved motifs (pfam05305 and pfam07098, respectively) were detected in their amino acid sequences. Both motifs occur in bacterial proteins of unknown function.

The first gene in this cluster, *orf48*, encodes a putative regulator that shares identity with gene products in mycobacterium phages that may also have a similar function. The genes following *orf48*, which include *orf49* to *orf53* encode products of unknown function. Transcribed in the opposite direction to *lysA* are eight putative genes (*orf56* to *orf63*) organized into what appear to be two operons, since these appear to contain *rho*-independent transcriptional terminators located after *orf56* and between *orf61* and *orf62* (Table 4). A further two ORFs (*orf64* and *orf65*) are transcribed in the same direction as *orf56* to *orf63* but are separated by a 750-bp noncoding region. The putative genes in this cluster share identity with genes from *Mycobacterium* phages. Presumed functions can be assigned to only three of the gene products (*orf58*, *orf61*, and *orf63*). Genes encoding a putative helicase (*orf58*), primase (*orf61*), and DNA polymerase I (*orf63*) can be identified on the basis of their amino acid sequences. The products of *orf60*, *orf64*, and *orf65* have no significant similarity to products of any sequenced gene.

The protein encoded by *orf58* has two conserved motifs (Pfam04851 and Pfam00271) found in DNA helicases. The N-terminal portion of the protein has a type III restriction enzyme *res* subunit motif (Pfam04851) that is characteristic of ATP-dependent helicase proteins. The center of this protein possesses a helicase motif (Pfam00271), whose function is to unwind the phage DNA duplex by utilizing energy from nucleoside triphosphate hydrolysis. Two putative ATP-binding sites were also identified within the amino acid sequence.

The N-terminal region of the putative primase (Orf61) possesses three conserved motifs involved in nucleotide binding, primase-nucleotide binding, and polymerase-nucleotide binding. This region also encodes a primase polymerase domain (primpol; Pfam09250) commonly seen in archaeal plasmids and phages. The central region of Orf61 has an additional ATP-binding motif, together with Walker A and Walker B motifs, both of which are related to the 5'-3' DNA helicase found in plasmids and utilize ATP (27). This region is highly conserved and characteristic of the p-loop nucleoside triphosphatase (NTPase) superfamily (16).

A putative DNA polymerase I is encoded by *orf63*. The region corresponding to the N-terminal protein region encodes a 3'-5' exonuclease motif (Pfam01612), and that corresponding to the C-terminal region encodes a DNA polymerase A motif

(Pfam00476), as well as a DNA-binding motif. This gene appears to encode the DNA polymerase responsible for phage DNA replication. The gene cluster from *orf66* to *orf76* is transcribed in the opposite direction to *orf56* to *orf65*. The majority of gene products in this cluster display no significant identity to known proteins (Orf66, Orf67, Orf68, Orf69, Orf70, Orf71, Orf73, Orf74, and Orf76). Two of the genes (*orf72* and *orf76*) share similarity to genes of unknown function in *Mycobacterium* phages, while Orf75 appears to be an endonuclease of unknown function.

The final gene cluster (*orf77* to *orf8*) is transcribed in the same direction and appears to form a large operon. A putative *rho*-independent transcriptional terminator is located between *orf77* and *orf78* (Table 4), suggesting that *orf78* may be separately regulated. The product of *orf78* is a putative metallophoesterase based on its transcribed amino acid sequence and the presence of the COG4186 motif. The remainder of putative genes in this cluster encode products of unknown function (Table 2).

Evolutionary events that contributed to the TPA2 genome.

The transcribed products of the TPA2 genome share amino acid similarities with proteins found in bacterial members of the *Corynebacterineae* and to *Mycobacterium* and *Corynebacterium* phages. Interestingly, some proteins share high amino acid identity but display only low BLAST E values, since their genes appear to be chimeric. An excellent example of this apparent chimeric gene structure is seen in the tape measure protein Orf33. The first 1,280 amino acids share a 36% identity with a tape measure protein from *Mycobacterium abscessus* ATCC 19977 (accession no. YP_001702534). The next 270 amino acids are most similar to the tape measure protein in *Rhodococcus* phage ReqiPine5 (accession no. ADD81132), followed by 150 amino acids with no identity to any known protein. The subsequent 100 amino acids share 39% identity with the G5 domain from *Bifidobacterium gallicum* (accession no. ZP_05966447), and finally, the last 580 amino acids are 39% identical to gp23 of the *Mycobacterium* phage Nigel (accession no. YP_002003862). A further example is the *lysA* gene, encoding the putative phage endolysin. Its gene product is split into two sections, with the first 154 amino acid sequence sharing 46% identity with that of a hypothetical protein from *Mycobacterium* phage TM4 (accession no. AAD17596), followed by a sequence of 30 amino acids of unknown function. The final 374 amino acids share 59% identity with a hypothetical protein from *Nocardia farcinica* (accession no. YP_120121). These mosaic gene structures were also noted for other genes in the TPA2 genome (data not shown), suggesting that this arrangement is a common feature of TPA2 genome evolution.

Other complex recombination events appear to have occurred within TPA2. Evidence for this is apparent when its genome sequence is compared with that of the *Mycobacterium* phage Rosebush. This phage encodes 90 putative proteins, gp1 to gp90 (35). In TPA2, *terL* encodes a terminase most closely related to gp7 in phage Rosebush. Immediately adjacent to *terL* is *orf10* of no known function, although its closest relative is gp71 from the *Mycobacterium* phage Rosebush. This region of sequence similarity occurs over the center portion of the protein only, which suggests that the genes within the TPA2 genome, despite their conserved modular arrangement, can recombine and form new modular arrangements.

Comparative phage genomics reveals that many phages appear to be mosaics of each other (36). The genome sequence of TPA2 reported here also reveals such a chimeric structure, with both individual genes and operonic modules displaying a striking mosaic architecture. Interestingly, TPA2 has a gene organization similar to that of *Mycobacterium* phage Rosebush and can be grouped into the cluster B division constructed by Hatfull et al. (20). Although this cluster B is subdivided into four subclusters, it is difficult to allocate TPA2 to any of these because of the lack of nucleotide sequence identity with those currently located there. Any such identity is shared with all the mycobacteriophages in cluster B.

Conclusions. This report describes the first complete genome sequence of a *Tsukamurella* phage. Bioinformatic analysis reveals that TPA2 is novel lytic phage, with only 30% of its genome being related to other phages at the DNA level, and that it has many previously unreported features. This phage targets a wide range of *Tsukamurella* species. It may be possible to use the TPA2 phage for the biocontrol of *Tsukamurella* stabilized foams in activated sludge plants or other environments where *Tsukamurella* spp. are problematic.

This study expands our understanding of the ecology of phages in activated sludge. While it is known that bacteriophages play an important role in controlling the population densities of their bacterial hosts (46a), their impact on the community structure of activated sludge systems is largely unknown. The repeated isolations of this phage more than 9 years apart suggest that it is probably widespread and not ephemeral but is an important member of the activated sludge phage metapopulation. Such a persistence demonstrates the need to better understand phage ecology within such systems and understand how populations may change with plant operational conditions. The genome sequence described here will enable specific targeted quantitative PCR methods to be developed for this purpose. Equally important is understanding the role that TPA2 and other phages might have in promoting gene transfer within activated sludge communities. The evidence presented here suggests that this is likely to be substantial, but the genomes of more activated sludge phages clearly need to be analyzed before this can be elucidated. This work is currently in progress.

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