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Prevention of *Gordonia* and *Nocardia* Stabilized Foam Formation by Using Bacteriophage GTE7▽†

Steve Petrovski,§ Robert J. Seviour,‡ and Daniel Tillett*

La Trobe Institute for Molecular Sciences, La Trobe University, P.O. Box 199, Bendigo, Victoria 3552, Australia

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Most activated sludge treatment plants suffer from the presence of foams on the surfaces of their aeration reactors. These are often stabilized by hydrophobic mycolic acid-synthesizing actinobacterial species. A polyvalent *Siphoviridae* phage, GTE7, which lysed several *Gordonia* and *Nocardia* species, is described here. Its genome has a modular structure similar to that described for *Rhodococcus* phage ReqiDocB7. In laboratory-scale experiments, we showed that GTE7 prevents stabilization of foams by these *Gordonia* and *Nocardia* species.

The appearance of stable foams on the surfaces of aerobic reactors of activated sludge wastewater treatment plants is a common feature and an operational problem for which no reliable and universally applicable control strategy exists currently (3). Its cause is the overproliferation of hydrophobic bacterial populations, among which are the mycolic acid-producing *Actinobacteria*, the mycolata (3, 5, 11, 18, 19). This group includes the genera *Corynebacterium*, *Dietzia*, *Gordonia*, *Skermania*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* (5). Lytic phages for these mycolata can be isolated readily from activated sludge mixed liquor, and the suggestion has been made that these phages may provide a targeted, environmentally friendly, and safe method for controlling foam formation by reducing the causative bacterial cell numbers below the threshold needed for foam stabilization (14, 16, 21, 23). Laboratory-scale studies suggest that these values are host cell specific (14), and so before such an application can be undertaken, suitable phages need to be purified for each of the foaming bacteria and characterized.

Consequently, as part of attempts to assess the feasibility of this control strategy, we describe a novel *Gordonia* phage, GTE7, that also targets some *Nocardia* species. Phage GTE7 was isolated from a wastewater treatment plant in Bendigo (Victoria, Australia) based on its ability to form small (<1-mm diameter) plaques on lawn plates of *Gordonia terrae* (Ben601) after incubation at 30°C for 2 days. Transmission electron microscopy (TEM) (15) revealed that GTE7 belongs to the *Siphoviridae* family, possessing a characteristic long noncontractile tail (~438 nm) and isometric capsid head (~70 nm) (Fig. 1). The burst size of GTE7 was determined (15) as 83 ± 3 PFU/cell, with a latency period of 2 h in peptone-yeast extract calcium (PYCa) broth at 30°C. When GTE7 was screened for its ability to lyse 65 different mycolata strains (see Table S1 in the supplemental material), it generated lytic plaques on lawn plates of five *G. terrae* strains (Ben601, Ben602, Ben603, Ben604, and Gter34), two of *Gordonia malaquae* (A554 and A448), and one of *Gordonia australis* (18F3M), *Gordonia amicalis* (Ben607), *Nocardia nova* (Nnov47), and *Nocardia asteroides* (Nast23). In comparison to *Gordonia* phage GTE2 (16), GTE7 lysed a wider range of mycolata species, making it potentially more attractive as a biocontrol agent for foaming. The molecular basis for this broad-host-range phenotype is unknown, but it does suggest that GTE7 is binding to a widespread and conserved receptor(s). GTE7 phage DNA was isolated and sequenced as described previously (15). This genome is organized as a circularly permuted double-stranded DNA molecule of 74,431 bp with a G+C content of 56.8 mol%. At the DNA level, only 6% of the sequence shares similarity with that of the phage ReqiDocB7 genome (20), and the remainder (94%) shares no sequence similarity to any other DNA sequence in GenBank. The GTE7 phage genome has 102 putative open reading frames (ORFs) and one tRNA. The first 31 ORFs are transcribed in one direction, and the following 71 ORFs are transcribed in the opposite direction (Fig. 2), an arrangement similar to that of the *Rhodococcus* phage ReqiDocB7 genome (20). Only 13 ORFs could be annotated functionally against known protein sequences using the BLASTP algorithm (see Table S2 in the supplemental material).

The *Siphoviridae* genomes sequenced so far share an organization of the packaging and structural proteins (1, 6). They encode either one or two DNA packaging proteins (17). In the GTE7 phage, the second ORF (*terL*) shares identity with the large terminase subunit of the ReqiDocB7 phage, which is required for DNA packaging into the phage head (17). No small terminase could be identified in the GTE7 phage based on sequence homologies. In other phage genomes, the small terminase is expressed from a gene upstream of the large terminase gene. The gene upstream of *terL* (i.e., *terS*) appears to be a fused version of two genes in phage ReqiDocB7. Neither has been assigned a putative function. Based on its

* Corresponding author. Mailing address: Biotechnology Research Centre, La Trobe Institute for Molecular Sciences, La Trobe University, Bendigo, Victoria 3552, Australia. Phone: (613) 5444 7361. Fax: (613) 5444 7476. E-mail: d.tillett@latrobe.edu.au.

§ Present address: Department of Primary Industries, Biosciences Research Division, Victorian AgriBiosciences Centre, 1 Park Drive, La Trobe University Research and Development Park, Bundoora, Victoria 3083, Australia.

‡ Present address: Department of Microbiology, La Trobe University, Bundoora, Victoria 3086, Australia.

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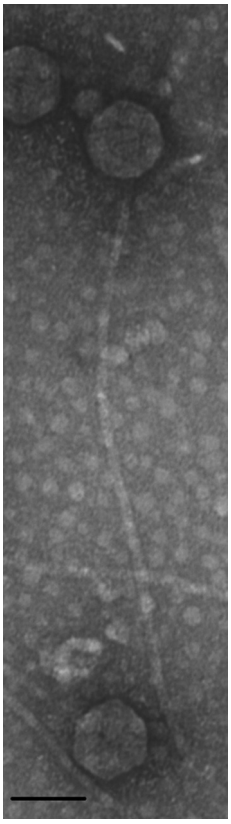

FIG. 1. Electron micrograph of GTE7. Bar = 70 nm.

location in phage GTE7, we hypothesize that this is the small terminase subunit.

The predicted amino acid sequences of *orf3* to *orf27* suggest that they encode the GTE7 phage structural proteins and many share a high level of identity with genes from phage ReqiDocB7 (20). The gene immediately downstream of *terL*, *orf3*, encodes the conserved motif DUF935, indicative of phage portal proteins. The major capsid protein is encoded by *orf9* based on its similarity with other major capsid proteins and the characteristic conserved domain pfam03864. The putative genes *orf4* to *orf8* probably encode other capsid-related proteins since in all other *Siphoviridae* phage genomes, the structural genes are clustered into functional domains (1). Thus, genes encoding the phage head morphogenesis proteins are followed by tail-encoding genes (1). This arrangement is also observed in phage GTE7. Genes transcribed downstream of the major capsid protein, *orf10* to *orf15*, encode proteins with no known function, but they share similarity to proteins in phage ReqiDocB7 (20). Again, it is hypothesized that these genes also encode phage structural proteins. The largest gene in the GTE7 phage genome is *orf19*, which shares identity to phage tape measure proteins (TMP) and contains the appropriate conserved domains found there (10). The genes upstream, *orf17* and *orf18*, appear to be involved in tail assembly, where *orf18* is translated via a conserved translational slippage mechanism, a common feature of phages (24). *Siphoviridae* genomes are typically arranged such that genes encoding the major tail protein precede the two tail assembly genes with translational slippage and are followed by TMP genes (1). Assuming the GTE7 phage fits this pattern, it seems reasonable to predict that its *orf16* encodes the major tail protein.

The remainder of genes (*orf20* to *orf27*) in this cluster may encode other GTE7 phage structural proteins, although their function is unknown. A conserved domain, PHA02579, identified in the predicted protein encoded by *orf25*, is often associated with phage proteins forming the virion baseplate (12).

Organization of the *Siphoviridae* phage lysis gene modules varies. All characterized phage lysis modules contain an endolysin and a holin gene (22). The GTE7 phage genome has three putative lysin genes downstream of the structural module, namely, *orf28*, *orf29*, and *orf40* (Fig. 2). The predicted protein of *orf28* has an amino acid sequence highly similar to that of an *N*-acetylmuramoyl-L-alanine amidase in a *Micromonospora* sp., with the pfam01510 motif characteristic of peptidoglycan recognition proteins (PGRP). The same motif is found in lysin proteins of other phages, including T7 and TPA2 (2, 9, 15). Immediately adjacent to *orf28* is another suspected lysin-encoding gene, *orf29*. Orf29 is closely related to LysA proteins in *Rhodococcus* phages ReqiPine5 and ReqiDocB7 (20). It contains the pfam01551 diagnostic of M23 peptidase proteins. Orf40 is closely related to the LysB of *Rhodococcus* phage ReqiDocB7, with the pfam01083 motif reported in cutinase enzymes. While some phage genomes may encode two lysin genes (13), it is unusual for a phage to produce three lysins. No gene encoding a holin protein could be identified in the GTE7 genome.

The putative genes *orf32* to *orf102* are transcribed in the opposite direction of the first 31 genes. Most gene products in this cluster have no known function based on their predicted peptide sequences compared to those in public databases. *orf37* encodes a DNA methylase, tentatively identified from the presence of a conserved pfam01555 motif and its encoded protein sequence similarity to the DNA methylase protein in *Rhodococcus* phage ReqiDocB7 (20). Orf47 is a predicted exonuclease from the presence of a pfam00929 domain (10). *orf50* and *orf97* are predicted to encode a putative helicase and primase, respectively. The predicted amino acid sequence of Orf50 possesses a conserved pfam00176 domain with no defined function, but which may be involved in DNA repair, recombination, and chromatin unwinding (4). Within the same cluster, *orf46* encoding the beta subunit of DNA polymerase III was identified from its high level of similarity to the DnaN protein sequence in *Rhodococcus* phage ReqiDocB7 and from the presence of the conserved domain cd00140, which is characteristic (8).

The remainder of this gene string has no known function, although *orf48*, *orf72*, and *orf102* encode proteins with conserved domains. Orf48 contains a pfam00176 domain characteristic of excisionase-type proteins. Genes *orf71* and *orf72* encode proteins with high similarities to those encoded by phage ReqiDocB7 genes (20). These were identified in ReqiDocB7 from the presence of conserved domains (20). In phage GTE7, *orf72* encodes a conserved domain pfam07728 characteristic of an ATP-hydrolyzing domain. The Orf71 predicted sequence is significantly similar to the protein vWFA, encoded by *Rhodococcus* phage ReqiDocB7, but no conserved domain was seen in this protein in phage GTE7. In phage ReqiDocB7, a von Willebrand factor type A (vWFA) con-

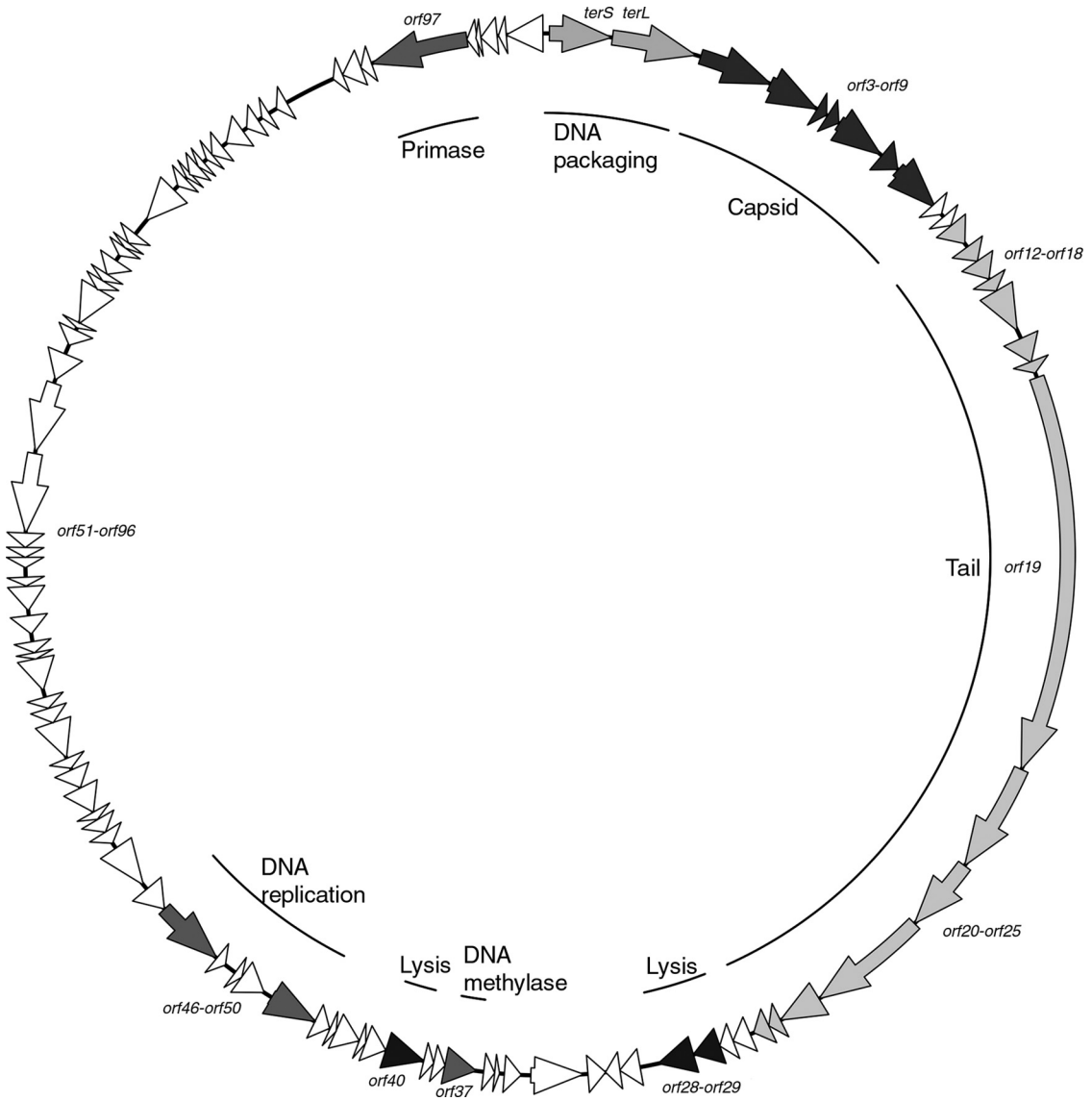

FIG. 2. Circular map of the GTE7 genome. Arrows represent the putative genes and the direction in which they are transcribed. Modules are shaded in similar colors, and the inner circle indicates the genes encoded within the modules.

served domain was identified, suggesting that these genes evolved along a shared lineage. The gene *orf102* encodes a COG4951 domain, commonly encountered in protein sequences of unknown function in bacteria.

The increasing availability of genome sequences of phages targeting the *Actinobacteria* is revealing novel insights into their evolution. *Mycobacterium* phages are the largest such group whose genomes have been sequenced (7). Of 70 complete sequences now available for *Mycobacterium* phages, several phylogenetically related groups are recognizable (7). Genome sequence data from *Tsukamurella* phage TPA2 (15) and *Rhodococcus* phage ReqiPine5 (20) showed that both can be assigned to the same group based on similarity, despite their lack of DNA sequence identity (7). However, *Gordonia* phage GTE2 (16) could not be assigned, and it has been grouped with phages called singletons, sharing no relationship with any other known phage (7). While phage GTE7 also fails to cluster with other groups, its genome has a high level of similarity to *Rhodococcus* phage ReqiDocB7. This suggests that the ReqiDocB7 and GTE7 phages may share an evolutionary lineage, despite clear differences in their host ranges. Phages GTE7 and ReqiDocB7 form a novel group, and perhaps the *Mycobacterium* phage grouping should be extended to include it.

The hosts that GTE7 infects all have hydrophobic mycolic acid-containing cell surfaces and stabilize foaming in laboratory-scale experiments (14). The ability to control a bacterium responsible for foam stabilization targeted by the GTE7 phage was pursued. Bacterial hosts producing scum (14) were excluded. The optical density at 600 nm (OD_{600}) of cultures were all adjusted to ~1.0 prior to addition of phage GTE7 (multiplicity of infection [MOI]= 0.3).

The OD_{600} of cultures exposed to phage GTE7 after 24 h of incubation at 30°C fell to an OD_{600} of ~0.7, and the mycolata CFU/ml was reduced by at least 1,000-fold (data not shown).

TABLE 1. Influence of GTE7 on production of stable foams by selected mycolata strains under laboratory conditions

Culture	Foaming score[a]	
	Without phage	In the presence of GTE7[a]
Gordonia aichiensis (Raic22)[b]	Stable foam (>15 cm)	Stable foam (>15 cm)
Gordonia terrae (Gter34/Ben601/Ben602)	Intermittent stable films	Fragile bubbles (1 cm)
Gordonia amicalis (Ben607)	Fragile bubbles (1 cm)	Fragile bubbles (1 cm)
Nocardia asteroides (Nast23)	Stable films (>8 cm)	Fragile bubbles (1 cm)

[a] Foaming scores are in accordance with the scale from Petrovski et al. (14).
[b] This culture was used as a negative control. GTE7 does not lyse this host.

The phage had no impact on foam stabilization by *Gordonia aichiensis* (Raic22), which is not a host for the GTE7 phage. The reduction in OD_{600} was lower than expected, probably because considerable amounts of cell debris remained after lysis. Unlike similar GTE2 phage data (16), these results (Table 1) suggest that all GTE7 phage host bacteria retained some capability for foaming, but foam stability was markedly reduced upon phage exposure (Table 1). This difference in response is not surprising since the GTE7 host bacteria produce surfactants that markedly reduce foaming threshold values (14). Thus, any surfactant produced by these bacteria in culture would persist over into the foaming assay. Unstable foam formation is still a desirable operational outcome since it will dissipate rapidly and hence not accumulate in activated sludge reactors.

Nucleotide sequence accession number. The genome sequence for *Gordonia* phage GTE7 has been deposited in GenBank under accession number JN035618.

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