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Discovery and Genomic Characterization of Cluster EE Bacteriophage Rira, Isolated Using *Microbacterium foliorum*

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Abstract

Microbacteriophage Rira was collected from a soil sample in Rockdale, Texas, and isolated on a *Microbacterium foliorum* NRRL B-24224 culture. The 16,971-bp double-stranded DNA genome contains 25 predicted protein-coding genes. Based on gene-content similarity, Rira was assigned to actinobacteriophage cluster EE, where it shares >98% nucleotide identity with bacteriophages Slizam and Stem. Comparison with the Texas isolates Loca and Godfather revealed conserved gene order and cohesive ends, with the largest difference in gene 13, which encodes a minor tail protein that is roughly half the length of its Loca and Godfather homologs and belongs to a different pham.

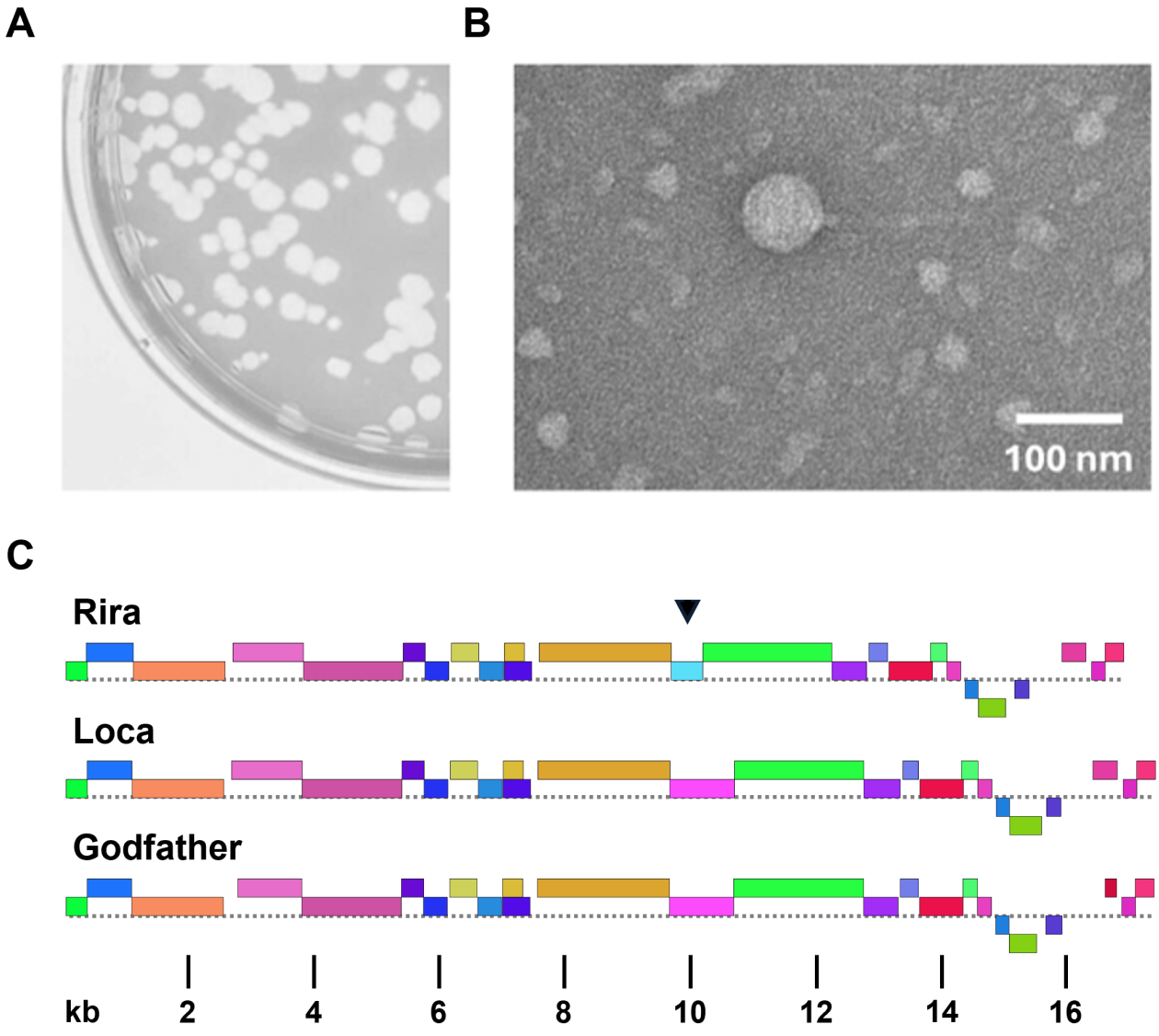


Figure 1. Plaque morphology, virion morphology, and genome comparison of bacteriophage Rira:

(A) Plaques formed by Rira on *Microbacterium foliorum* NRRL B-24224 after 48 h at 29 °C. (B) Transmission electron micrograph of Rira virions negatively stained with 1% uranyl acetate on a 300-mesh copper grid, showing a siphovirus morphology with a capsid diameter of 49.2 ± 0.6 nm and a tail length of 112.9 ± 1.0 nm (n = 6). Scale bar: 100 nm. (C) Genome map comparing Rira, Loca, and Godfather. Genes are represented as boxes above (rightward-transcribed) or below (leftward-transcribed) the dotted gray line and are colored by pham assignment. The arrowhead marks gene 13, which encodes a minor tail protein.

Description

Bacteriophages infecting *Microbacterium* are genetically diverse and encode many genes of unknown function, and their continued characterization informs our understanding of phage evolution and biology (Hatfull, 2020; Jacobs-Sera et al., 2020). Our group has previously isolated and sequenced several microbacteriophages from central and north Texas (Adams et al., 2022; Hutchings et al., 2025; Lee et al., 2019; Ounsinegad et al., 2022; Suris et al., 2021). To extend this record of microbacteriophage diversity, we report the genome of microbacteriophage Rira, isolated by enrichment from a soil sample collected in Rockdale, TX (30.66544° N, 97.00423° W). The sample was submerged in peptone-yeast extract-calcium (PYCa) liquid medium and incubated while shaking for 2 h at 220 rpm and 29 °C. The supernatant was filtered through a 0.22-µm filter and incubated with the bacterial host strain *Microbacterium foliorum* NRRL B-24224 (Russell et al., 2019) in PYCa liquid medium for 4 days at 29 °C (Zorawik et al., 2024). The bacteria were pelleted, and the supernatant was passed through 0.22-µm filters. The filtrates were serially diluted 10-fold in phage buffer (10 mM Tris [pH 7.5], 10 mM $MgSO_4$, 68 mM NaCl, 1 mM $CaCl_2$, 10% glycerol) and incubated with *M. foliorum* in a soft-agar overlay on PYCa agar plates for 48 h at 29 °C. Bacteriophage replication produced clear plaques within the soft-agar overlay with a mean diameter of 3.89 ± 0.60 mm (n = 6; Fig. 1A), as measured using ImageJ v1.53m (Schneider et al., 2012). We isolated Rira by two rounds of single-plaque selection, followed by 10-fold serial dilution and plating on *M. foliorum* as before. We prepared high-titer lysates (2.5 × 108 PFU/mL) by flooding webbed plates with phage buffer overnight at 4 °C (Zorawik et al., 2024). After mounting on a 300-mesh copper grid and negatively staining with 1% uranyl acetate, transmission electron microscopy revealed a siphovirus morphology for Rira. ImageJ v1.53m (Schneider et al., 2012) was used to measure tail length (112.9 ± 1.0 nm) and capsid diameter (49.2 ± 0.6 nm) (n = 6; Fig. 1B).

Genomic DNA was extracted from the high-titer lysate using a modified zinc chloride precipitation method (Santos, 1991; Zorawik et al., 2024), and concentration was measured on a NanoDrop spectrophotometer. The Pittsburgh Bacteriophage Institute prepared sequencing libraries using the NEB Ultra II FS kit and sequenced them on an Illumina NextSeq 1000 instrument (XLEAP-P1 kit), producing 8,135 single-end 100-base reads. Raw reads were trimmed with cutadapt 4.7 (using the option: --nextseq-trim 30) (Martin, 2011) and filtered with skewer 0.2.2 (using the options: -q 20 -Q 30 -n -l 50) (Jiang et al., 2014) prior to assembly. The reads were assembled into a single contig using Unicycler v0.5.1 (Wick et al., 2017) and reviewed for completeness and genome termini using Consed v29 (Gordon & Green, 2013; Russell, 2018), yielding 14-fold coverage. The 16,971-bp double-stranded DNA genome contains 9-nucleotide 3′-single-stranded cohesive ends (5′-CCCGCCCCA-3′) and a 68.8% G+C content. A BLASTn query (Altschul et al., 1990) against the nonredundant nucleotide (nr/nt) database returned >98% nucleotide identity to the cluster EE bacteriophages Slizam (GenBank Accession No. PQ184819) and Stem (PQ244010) (Jacobs-Sera et al., 2020).

The genome was auto-annotated using GLIMMER v3.02 (Delcher et al., 2007) and GeneMark v2.5p (Besemer & Borodovsky, 2005), and manually refined using Phamerator (Cresawn et al., 2011) with the Actino_Draft database and PECAAN v20250130 (Rinehart et al., 2016). Default settings were used for all software. Putative functions were assigned to 18 of the 25 predicted protein-coding genes using PECAAN-integrated BLASTp searches against the Actinobacteriophage and NCBI non-redundant databases (Altschul et al., 1990) and HHpred searches against the PDB_mmCIF70, Pfam-A, UniProt-SwissProt-viral70, and NCBI_Conserved_Domains databases (Zimmermann et al., 2018). No tRNA or tmRNA genes were identified using Aragorn v1.2.41 (Laslett & Canback, 2004). Rightward-transcribed genes *1–19* encode virion structure and assembly proteins, terminase, and endolysin. Leftward-transcribed genes *20–22* encode an Lsr2-like DNA-bridging protein and two helix-turn-helix DNA-binding domains. Rightward-transcribed genes *23–25* encode a helix-turn-helix DNA-binding domain and an HNH endonuclease. Rira was assigned to cluster EE based on gene-content similarity of at least 35% to phages in the Actinobacteriophage Database, PhagesDB (https://phagesdb.org) (Pope et al., 2017; Russell & Hatfull, 2017).

The genome was compared with those of the cluster EE bacteriophages Loca (GenBank Accession No. ON260814) and Godfather (PP725414), previously reported by this laboratory (Ounsinegad et al., 2022; Hutchings et al., 2025) and isolated in Stephenville, TX, approximately 200 km northwest of the Rockdale collection site (Fig. 1C). Gene order and cohesive ends are conserved among the three genomes. The greatest difference among the three genomes is in gene *13*, which encodes a minor tail protein assigned to a different pham in Rira than in Loca and Godfather. In Loca and Godfather, this protein is 343 and 341 amino acids, respectively, while in Rira it is 168 amino acids and aligns only to the C-terminal region of both. This accounts for nearly the entire 504-bp difference in genome length between Rira and Loca. The shorter form of gene 13 is shared by Slizam and Stem and is present in 26 of the 153 sequenced cluster EE phages in PhagesDB, indicating that Rira's minor tail protein represents an established minority variant within the cluster rather than

a unique truncation. We identified no integrase or repressor gene, consistent with the lytic life cycle of cluster EE bacteriophages; Rira is predicted to be lytic.

Nucleotide sequence accession numbers

Rira is available at GenBank with Accession No. PZ319883 and Sequence Read Archive (SRA) No. SRX34536431.

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