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Letter

Isolation and characterization of a novel linear-plasmid phage from the sediment of the Mariana Trench

Yali Hao [a,1], Siyuan Wang [a,1], Mujie Zhang [a], Qingxue Tang [a], Canxing Meng [a], Liping Wang [b], Qilian Fan [a], Yaxian Yan [c], Xiang Xiao [a,d], Huahua Jian [a,d,*]

[a] State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, 200240, China
[b] Key Laboratory of Marine Genetic Resources, Third Institute of Oceanography, Ministry of Natural Resources, Xiamen, 361005, China
[c] Shanghai Key Laboratory of Veterinary Biotechnology, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, 200240, China
[d] Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Zhuhai, 519082, China

Dear Editor,

Temperate bacteriophages are widely distributed in bacteria isolated from different natural environments (Howard-Varona et al., 2017). The phages normally lead to lysogenic infection and merge their genetic components into the bacterial chromosome. Among the temperate phages, linear plasmid phages are atypical because of their capability to reside in host cells as linear dsDNA with covalently closed ends, rather than being integrated in host genomes in the form of prophages (Ravin et al., 2000; Ravin, 2011). The life cycle of N15, the first isolated linear plasmid phage, has been thoroughly investigated in its host *Escherichia coli* (Ravin, 2011). The formation of the linear plasmid prophage is attributed to a protelomerase that is encoded by the *telN* gene. The protelomerase acts on an inverted repeat site (*telRL*) on the phage genome, and then forms two covalently closed ends (*telR* and *telL*) (Ravin, 2003). Similar to phage λ DNA, N15 phage DNA has two 12-bp single-stranded cohesive ends (*cosR* and *cosL*), which are also responsible for the formation of the linear plasmid prophage of N15 (Ravin, 2015). Additionally, a multifunctional replication protein, RepA, which combines primase, helicase and DNA-binding activities, is indispensable for the lytic replication of N15 (Ravin, 2015).

To date, a total of 10 linear plasmid phages have been reported, with genome sizes and GC contents between 35.03 kb and 51.60 kb and 44.60%–65.29%, respectively (Supplementary Table S1). These phages have been classified into 3 genera (*Ravinvirus*, *Hapunavirus*, *Vhmlvirus*) in the families *Myoviridae* and *Siphoviridae*, according to the International Committee on Taxonomy of Viruses (ICTV) (https://talk.ictvonline.org). Except for xhp1 infecting *Actinomyces odontolyticus* (Shen et al., 2018), the host of all other isolated linear plasmid phages belongs to γ-Proteobacteria. The present study characterizes the temperate bacteriophage HMP1 from the deep-sea bacterium *Halomonas* sp. MT08-1, which was isolated from the sediments of the Mariana Trench at a water depth of 8636 m. Based on genome sequencing and phylogenetic analysis of conserved proteins, HMP1 has been proposed as a novel linear plasmid phage belonging to the genus *Hapunavirus* of the *Myoviridae*.

The sediment samples used in this study were obtained from the Mariana Trench in the western Pacific Ocean (11°11.6988′ N, 141°48.7008′ E, water depth of 8636 m) during the TS01 hadal trench cruise carried out by the *R/V Tan Suo Yi Hao* in August 2016. Initially, the samples were diluted with artificial seawater and plated on 2216E marine agar. Subsequently, single colonies were picked, and pure culture was obtained after three rounds of plate streaking, after which, the genomic DNA of the isolated strain was extracted and the 16S rRNA gene was amplified and sequenced. The BLAST comparison against the NCBI 16S rRNA gene sequence database indicated that the isolated strain belongs to the genus *Halomonas*, named *Halomonas* sp. MT08-1. To examine whether MT08-1 contains inducible temperate phages, mitomycin C (MMC) (1 μg/mL), a known inducer of lytic development for many temperate phages (Mya Breitbart et al., 2018), was added to MT08-1 cultures in the early stage of the exponential growth phase (51.5 hrs, OD_{600} = 0.2). We found that the MT08-1 growth was significantly inhibited after the addition of MMC, suggesting that bacterial lysis due to prophage induction may have occurred (Fig. 1A). Finally, the phage particles in the culture were purified and quantified by fluorescence microscopy (Supplementary Fig. S1). The results show that the abundance of phage particles in the MMC supplemented culture reached 10^9 viral-like particles (VLPs) mL^{-1} after 18 hrs of MMC induction, which was significantly higher than that of the control culture (Fig. 1B).

* Corresponding author.
E-mail address: jiandy@sjtu.edu.cn (H. Jian).
[1] Yali Hao and Siyuan Wang contributed equally to this work.

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The purified phage particles were further examined under transmission electron microscopy. The results show that the phage has a polygonal head and a sheathed tail, resembling bacteriophages belonging to *Myoviridae* (Fig. 1C). Notably, only one type of phage particle was observed in the MT08-1 culture, and this phage was designated HMP1. The head diameter, tail length, tail width and tail sheath width of HMP1 were 56.32 ± 5.9 nm (n = 17), 181.47 ± 14.02 nm (n = 17), 8.72 ± 1.66 nm (n = 18), and 21.81 ± 2.04 nm (n = 18), respectively. Next, the nucleic acids were extracted from the purified phage particles, and nuclease digestion analysis was performed.

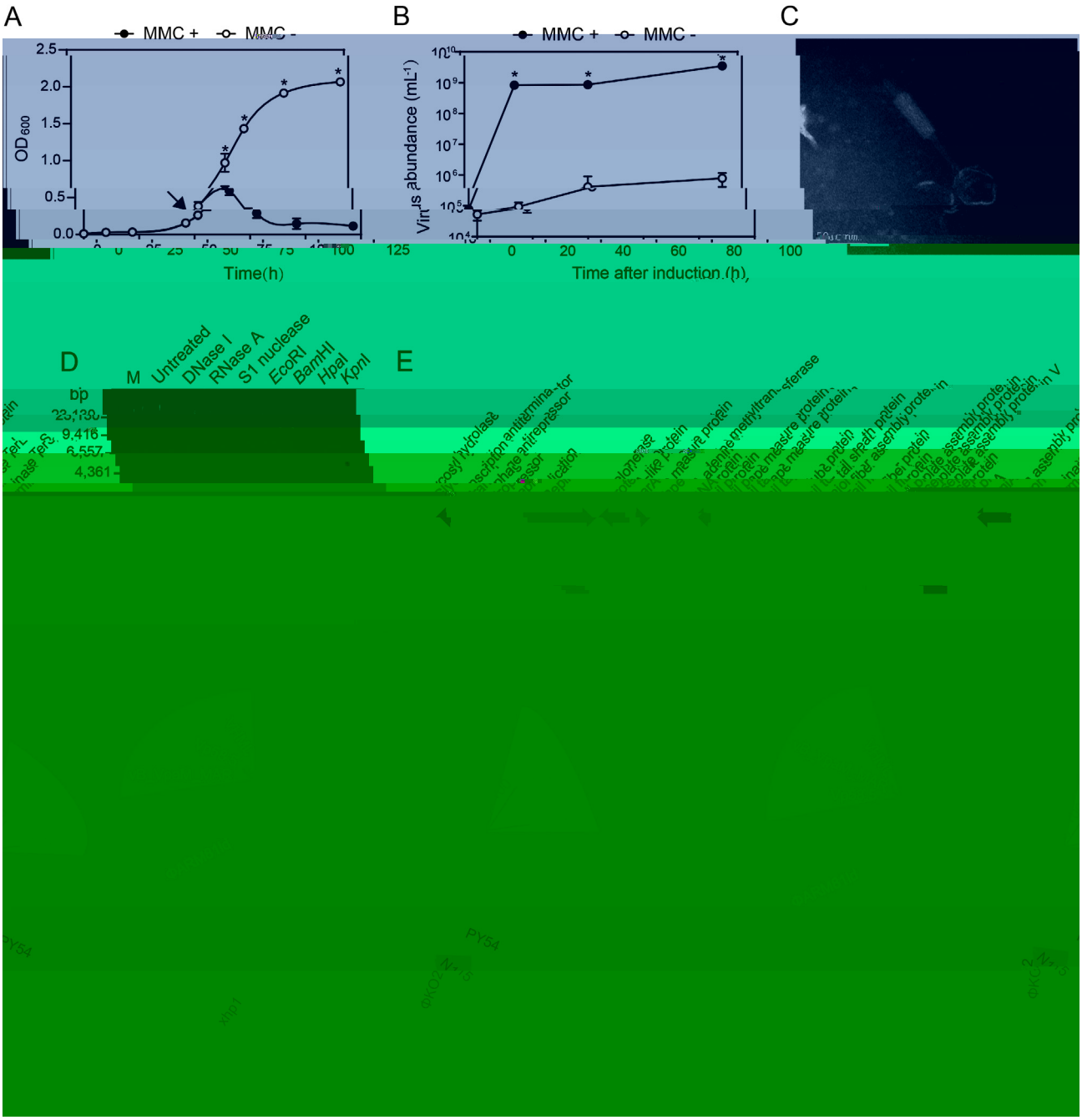

Fig. 1. **A**–**B** Induction and quantification of bacteriophages from the hadal bacterium *Halomonas* sp. MT08-1. In the prophage induction experiment, one subculture (solid circle) was treated with mitomycin C (MMC), while the other subculture (hollow circle) served as a control. Bacterium growth was detected at OD_{600}, and time points at which MMC was added are indicated by arrows. For each treatment, three independent cultures were tested, and the error bars indicate standard deviations of the means (n = 3). Significant changes are marked with asterisks ($P < 0.01$, two-sided unpaired Student's *t*-test). **C** Electron microscopic images of phage HMP1 particles induced from *Halomonas* sp. MT08-1. Scale bars are shown in the bottom left. **D** Nuclease digestion analysis of HMP1 nucleic acids. The nucleic acids derived from the purified phage particles were digested with different nucleases. The reaction products were separated electrophoretically in a 0.8% agarose gel. M, λ DNA/HindIII marker. **E** Genomic maps depicting predicted proteins encoded by HMP1. The arrows depict the location and direction of predicted proteins in the phage genomes, and the coloured arrows indicate different functional categories of genes, as indicated in the colour panels at the bottom of figure. **F**–**G** Phylogenetic analysis of RepA (**F**) and protelomerase (**G**) of all isolated linear plasmid phages. The phylogenetic trees are based on the alignments of amino acid sequences of RepA and protelomerase using MAFFT, filtered with trimA1 and constructed by the IQ-Tree, with LG + G4 model and 1000 bootstrap replicates. The bootstrap support values are indicated in each node. The taxonomy for different virus genera according to the ICTV is assigned different background colours. The phage names in blue and orange colours indicate the *Myo* and *Sipho*-type phage tails, respectively. The experimental details are presented in the supplementary information.

Obviously, the nucleic acids of HMP1 can be completely digested by DNase I and fragmented by the restriction enzymes *Eco*RI, *Bam*HI, *Hpa*I, and *Kpn*I (Fig. 1D). In contrast, neither RNase A nor S1 nuclease can degrade the HMP1 genome. These results indicate that the genetic material of HMP1 is dsDNA.

Subsequently, the complete genome of HMP1 was sequenced and deposited in the National Omics Data Encyclopedia (NODE) database under the accession number OER181318. The HMP1 genome has a length of 38,130 bp, with a GC content of 59.7%. A total of 52 open reading frames (ORFs) were predicted, 26 of which encoded hypothetical proteins with unknown functions (Fig. 1E and Supplementary Table S2). Generally, five functional modules could be identified in the HMP1 genome, namely, transcriptional regulation (ORF6, ORF10, ORF11); DNA replication (ORF12, ORF14, ORF15); tail tube (ORF24, ORF25, ORF26, ORF27, ORF30, ORF31); tail fiber and baseplate (ORF34, ORF39, ORF40, ORF41, ORF42, ORF43); and head and packaging (ORF49, ORF51, ORF52). Remarkably, the HMP1 genome shared high synteny and gene similarity with phage ΦHAP-1 (Mobberley et al., 2008), indicating that HMP1 may also belong to the linear plasmid phage (Supplementary Fig. S2). Accordingly, both the protelomerase-encoding gene and a 120-bp inverted repeat between the *parA* and *telN* genes were identified in the HMP1 genome (Supplementary Fig. S3). Furthermore, the presence of the HMP1 prophage as a linear plasmid within the host cells was experimentally verified by restriction enzyme digestion analysis of the MT08-1 plasmid (Supplementary Fig. S4). It should be noted that *in silico* analysis of the restriction enzyme cutting sites in the HMP1 genome (Supplementary Fig. S5) was well consistent with the results of the enzyme digestion experiment (Fig. 1D), thus further validated the sequence of HMP1 virion DNA.

To further investigate the taxonomy and classification of HMP1, the average nucleotide identity (ANI) was calculated by comparing the genome of HMP1 with other linear plasmid phages (Supplementary Fig. S6), and they all showed an ANI value below the standard threshold (95%) for prokaryotic virus at species rank (AdriaenssensandBrister, 2017). As previously mentioned, HMP1 shared the highest ANI (92.46%) with ΦHAP-1, which was isolated from the surface water of the Gulf of Mexico (Mobberley et al., 2008). Meanwhile, apparent discrepancies between the HMP1 and ΦHAP-1 genomes were noticed in the regions encoding prophage repressors, major capsids, and tail fibers (Supplementary Fig. S2), indicating that these two phages may have different viral particle structures and regulatory mechanisms. In contrast, the virion of HMP1 has a significantly larger capsid diameter, while a shorter tail length and width than ΦHAP-1 (Supplementary Fig. S7). Phylogenetic analysis based on the protelomerase and RepA proteins, which were conserved among linear plasmid phages, further confirmed that HMP1 and ΦHAP-1 are closely related (Fig. 1F and G). Clearly, the 11 isolated linear plasmid phages, including HMP1, could be well separated in the phylogenetic tree due to their different tail types (*Myo* and *Sipho*) and isolation environments (Fig. 1F and G and Supplementary Table S1). Specifically, marine-derived HMP1, ΦHAP-1 and VP882 were in the same clade (*Hapunavirus*), while the pathogenic bacteria infecting phages PY54, N15 and ΦKO2 were clustered together. Three *Vibrio* phages, VHML, vB-VpaM-MAR, and VP58.5, were highly similar and formed the *Vhmlvirus* clade. Similar results were obtained by the whole genome-based phylogeny analysis, but meanwhile demonstrated a significant differentiation between HMP1 [illegible]
)Na1417.3(p)p6.2(b)-9.base rea i12.1dMnuic tac-599.74f HMPdF