Genomic analysis of a novel active prophage of *Hafnia paralvei*

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Abstract

Little is known about the prophages in *Hafniaceae* bacteria. A novel *Hafnia* phage, yong2, was induced from *Hafnia paralvei* by treatment with mitomycin C. The phage has an elliptical head with dimensions of approximately 45 × 38 nm and a long noncontractile tail of approximately 157 × 4 nm. The complete genome of *Hafnia* phage yong2 is a 39,546-bp double-stranded DNA with a G+C content of 49.9%, containing 59 open reading frames (ORFs) and having at least one fixed terminus (GGG GCA GCG ACA). In phylogenetic analysis, *Hafnia* phage yong2 clustered with four predicted *Hafnia* prophages and one predicted *Enterobacteriaceae* prophage. These prophages and members of the family Drexlerviridae together formed two distinct subclades nested within a clade, suggesting the existence of a novel class of prophages with conserved sequences and a unique evolutionary status not yet studied before in *Hafniaceae* and *Enterobacteriaceae* bacteria.

Bacteria of the genus *Hafnia*, family *Hafniaceae*, including *Hafnia alvei* and *Hafnia paralvei*, are commonly found in food, in plants, and in the gastrointestinal tract of animals and humans [1, 2]. These are facultatively anaerobic, Gram-negative rod-shaped bacteria that are considered to be opportunistic pathogens of various animal species, including fish, mammals, and birds, and they are suspected of causing infections in humans [1–4].

Bacteriophages (phages) are viruses that can establish a lytic or lysogenic infection in bacterial cells. Virulent phages rapidly infect and lyse host bacteria in a lytic cycle. Unlike virulent phages, temperate phages can integrate their nucleic acid into the genome of the host bacterium, becoming part of the bacterial genome and staying dormant as a prophage [5]. A functional prophage, also called an active prophage, can be induced to enter a lytic phase under certain conditions such as treatment with mitomycin C or exposure to UV light [6, 7]. Prophages are of interest to researchers because of their influence on the characteristics of their host, including bacterial biofilm formation, virulence, and evolution [8–10].

Little is known about the prophages in *Hafniaceae* bacteria, as only two *Hafniaceae* prophages were found in a literature search. Edno5 is a spontaneously induced prophage of *Edwardsiella anguillarum* [11]. *Hafnia* phage yong1 is an *H. paralvei* prophage induced by mitomycin C at 37 °C [12]. Both of these active *Hafniaceae* prophages have myovirid-like morphology. Edno5 gene 47 encodes a protein with the predicted structure of an LRR ligase, resembling several confirmed toxins of pathogenic enterobacteria such as the *Shigella flexneri* effectors IpaH3 and IpaH1880. No ORFs associated with virulence factors or antibiotic resistance were identified in the *Hafnia* phage yong1 genome [12]. Only nine *Hafnia* phage genome sequences are available in public databases. Six of the nine *Hafnia* phages are *H. paralvei* phages, one is an *H. alvei* phage, and the hosts of the other two are *Hafnia* spp. No antibiotic resistance genes or virulence factor genes were found in the nine *Hafnia* phage genomes. In this study, an active prophage, *Hafnia* phage yong2, was induced by mitomycin C, and the complete genome of the phage was sequenced and analyzed.

*H. paralvei* LY-23, isolated from diseased turbot fish, was kindly provided by Professor Fengling Bai of Bohai...
University and deposited in the Marine Culture Collection of China under the number MCCC 1K06097. The draft genome sequence of *H. Paralvei* LY-23 was deposited in the GenBank database under accession number NZ_JAEMBX020000000.

Five mL of fresh LY-23 culture (1 × 10^7 CFU/mL) was treated with 100 μL of mitomycin C (Yingxin Laboratory, China) at a final concentration of 1 μg/mL for 4 h at 29 °C with shaking at 220 rpm. Lysates were centrifuged at 10,000 g for 10 min, and the supernatant was filtered using a 0.22-μm nitrocellulose filter (ANPEL Laboratory Technologies Inc., China). The filtrate was collected for morphological examination and genome sequencing.

The filtrate was collected and centrifuged at 35,000 g for 60 min. Phage sediment was washed and resuspended in phosphate-buffered saline (PBS) (Solarbio, China). A small amount of phage suspension was deposited on carbon-coated copper grids for 5 min and negatively stained with 3% uranyl acetate (Sigma-Aldrich, USA) for 25 s. The negatively stained grids were observed under a Hitachi-7650 transmission electron microscope.

Before DNA extraction, the phage suspension was pretreated with DNase and RNase (each 1 μg per mL) (TransGen Biotech, Beijing, China) at 37 °C for 2 h to remove nucleic acid contamination from the host. DNase was inactivated by incubating the mixture at 80 °C for 15 min. Phage DNA extraction was performed using a High Pure Viral Kit (Roche, product no. 1185882001, USA). The extracted phage DNA was used to construct a 2 × 300-bp paired-end DNA library using an NEB Next Ultra II DNA Library Prep Kit. An Illumina MiSeq (San Diego, CA, USA) sequencer was used to obtain paired-end reads for genome sequencing of the prophage. Low-quality (Q-value < 20) reads and adapter sequences were filtered out using Trimmomatic 0.36, and clean reads were assembled using SPAdes 3.13.0 software (http://cab.spbu.ru/software/spades/).

Phage termini were analyzed using a method reported elsewhere [14]. tRNAs in the prophage genome were searched using tRNAscan-SE software (http://lowelab.ucsc.edu/tRNAscan-SE/) [15]. A search for homologous sequences was performed using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [16]. Open reading frames (ORFs) were annotated using Rapid Annotation using Subsystem Technology (RAST) version 2.0 (selected domain, virus) (https://rast.nmpdr.org/rast.cgi) [17]. BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (E-value ≤ 10^-5) [16], HMMER (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) (E-value ≤ 10^-5) [18], and HHpred (https://toolkit.tuebingen.mpg.de/tools/hhpred) [19] (E-value ≤ 10^-4, probability of homologous sequences > 96%). PHASTER (PHAge Search Tool Enhanced Release) (http://phaster.ca/) [20] was used to find prophage sequences within bacterial genomes. Viral Proteomic Tree (ViPTree) version 1.9 (https://www.genome.jp/viptree/) [21] was used to generate a proteomic tree based on genome-wide sequence similarities computed by tBLASTx. A genome sequence comparison between *Hafnia* phage yong2 and the most similar prophage (present in the genome of *Hafnia paralvei* FDAARGOS_158) was performed using EasyFig 2.2. software [22]. Antibiotic resistance genes in the phage genome were identified using the platform CARD (https://card.mcmaster.ca/) [23] and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) [24]. Virulence factor genes in the phage genome were identified using VFDB (http://www.mgc.ac.cn/ VFs/main.htm) [25] and VirulenceFinder (https://cge.cbs.dtu.dk/services/VirulenceFinder/) [24].

The Pairwise Sequence Comparison (PASC) tool (http://www.ncbi.nlm.nih.gov/sutils/pasc/) was used to calculate pairwise sequence identity values [26]. Virus Intergenic Distance Calculator (VIRIDIC) (http://rhea.icbm.uni-oldenburg.de/VIRIDIC/) [27] was used to calculate nucleotide-based intergenic similarities. CRISPR loci in the *Hafnia paralvei* LY-23 genome and CRISPR spacers in the *Hafnia* phage yong2 genome were identified using the CRISPRs web server (https://crispr.i2bc.paris-saclay.fr/) [28, 29] and Integrated Microbial Genome/Virus (IMG/VR) version 3 (https://img.jgi.doe.gov/) [30].

Transmission electron microscopy revealed that *Hafnia* phage yong2 has an elliptical head with dimensions of approximately 45 × 38 nm and a long noncontractile tail of 1279x. The phage genome is a double-stranded DNA with a length of 39,546 bp and a G+C content of 49.9%. The complete genome sequence of *Hafnia* phage yong2 was deposited in the GenBank database under the accession number MZ516527. In PHASTER analysis, the complete genome sequence of *Hafnia* phage yong2 was found within a contig (106,366 bp, GenBank accession number NZ_JAEMBX020000000) of the draft genome sequence of *H. Paralvei* LY-23 (nt 6,207-45,782), flanked by attL (AGC GCG ACA ), with R = 10^7 > 100, was identified using tBLASTx. A genome sequence comparison between *Hafnia* phage yong2 and the most similar prophage (present in the genome of *Hafnia paralvei* FDAARGOS_158) was performed using EasyFig 2.2. software [22]. Antibiotic resistance genes in the phage genome were identified using the platform CARD (https://card.mcmaster.ca/) [23] and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) [24]. Virulence factor genes in the phage genome were identified using VFDB (http://www.mgc.ac.cn/ VFs/main.htm) [25] and VirulenceFinder (https://cge.cbs.dtu.dk/services/VirulenceFinder/) [24].
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One ORF (encoding RecA recombinase) was most similar to its homolog in *Escherichia coli* (97% identity), one ORF (encoding a hypothetical protein) was most similar to a gene from *Enterobacter hormaechei* (75% identity), one ORF (encoding a hypothetical protein) was most similar to a gene from *Lelliottia amnigena* (39% identity), and two ORFs had no significant hits (Fig. 1b). The predicted ORFs were organized into six modules: structure, DNA packaging, replication and regulation, lysis-related, lysogeny-associated, and unknown function.

The functional module for DNA packaging contains ORF 1 (encoding the terminase small subunit) and ORF 2 (encoding the terminase large subunit). The structure module includes genes encoding head-to-tail joining proteins (ORFs 3 and 7), a portal protein (ORF 4), a capsid fiber protein (ORF 6), baseplate assembly proteins (ORFs 10 and 20), a tail sheath protein (ORF 11), a tail tube protein (ORF 12), a tail assembly protein (ORF 13), tail proteins (ORF 16, 17, 18, and 21), a baseplate wedge protein (ORF 19), a tail-collar fibre protein (ORF 22), and a tail fiber assembly protein (ORF 23).

The replication and regulation module of *Hafnia phage yong2* contains genes encoding an ATP-dependent Clp protease (ORF 5), a serine acetyltransferase (ORF 24), a RecA protein (ORF 26), a 3'-5' exoribonuclease (ORF 30), a 30S ribosomal protein subunit S22 family member (ORF 31), an HD phosphohydrolase (ORF 35), a YfdQ family protein (ORF 36), an asf family protein (ORF 43), a PerC transcriptional activator (ORF 45), a DNA N-6-adenine-methyltransferase (ORF 48), the endodeoxyribonuclease RusA (ORF 49), an antitermination protein (ORF 51), the transcription regulatory protein MotA (ORF 52), and an adenine-specific DNA methyltransferase (ORF 53). The presence of the ribosomal protein S22 gene (ORF 31) may be beneficial for protein synthesis in *H. paralvei* LY-23, as ribosomal protein S22 has been reported to be a component of the small ribosomal subunit [31]. The ATP-dependent Clp protease proteolytic subunit encoded by phage yong2 may contribute to the host, as this protein helps to maintain protein homeostasis in bacteria, especially under conditions of stress [32]. The YfdQ family protein encoded by ORF36 may participate in regulation of host replication, as proteins of this family have been found to be associated with regulation of the initiation of bacterial chromosomal replication [33]. Inducible prophages have been shown to mediate generalized transduction, and DNA recombination is important for horizontal gene transfer (HGT). As a mitomycin-C-inducible prophage containing genes encoding the recombinase proteins RecA and RusA, phage yong2 might play a role in gene transfer. Both RecA and RusA are involved in homologous genetic recombination. RecA promotes homologous pairing and strand exchange with a homologous duplex by forming a helical nucleoprotein filament, which is essential for recombination [34]. RusA is an endonuclease that can cleave Holliday junctions and correct defects that arise during genetic recombination [35]. The recA gene of *Hafnia phage yong2* might have originated from *E. coli*, as it shares the highest similarity with the *E. coli recA* gene.

The lysogeny-associated module in the phage yong2 genome contains genes encoding integrase (ORF 28), repressor protein LexA (ORF 29), cI (ORF 38), and cII (ORF40). Integrase, the cI repressor protein, and the cII protein control the entry of phages into the lysogenic or lytic cycle in host bacteria. The cI repressor participates in the molecular switch from the lysogenic state to the lytic state [36, 37].
During the lysogenic state, cl binds to multiple operator sites by formation of dimers and higher-ordered oligomers in the C-terminal oligomerization domain, preventing phage gene transcription [38]. During the lytic state, cl is bound as a monomer to a RecA-ssDNA-ATP filament, resulting in its inactivation [38]. The regulatory protein cII is involved in the establishment of lysogeny [39]. It is a transcriptional activator that activates the pE promoter (necessary for initial synthesis of repressor Cl), the pI promoter (needed for synthesis of the integration protein Int), and the paQ promoter (which inhibits lytic gene expression) [40, 41]. The SOS response system is a DNA repair method that is regulated by RecA-LexA. LexA is a transcriptional repressor that binds as a dimer to specific operator sites to repress some SOS response genes [42]. When bacterial DNA is severely damaged, DNA replication may be interrupted and more single-stranded DNA regions emerge. Single-stranded DNA and ATP can activate the RecA protein, which then acts as a coprotease to stimulate LexA autoproteolysis, and this process is similar to cl autoproteolysis [43]. Typically, DNA damage can induce the bacterial SOS pathway, which triggers some prophages to enter the lytic state, and the induced prophages begin expressing phage genes. Interestingly, Hafnia phage yong2 harbors a RecA gene (ORF 26) and a LexA gene (ORF 29), suggesting that it might not only escape from the damaged host genome but also be involved in SOS repair of DNA.

The phage yong2 genome possesses adjacent genes encoding holin (ORF 55) and endolysin (ORF 56), respectively. The holin-endolysin system is generally required for efficient host lysis [44, 45]. Holin is a small membrane protein that lyses the bacterial cell membrane, and endolysin is an enzyme that hydrolyzes the bacterial cell wall.

Prophages are not only important genetic elements of the bacterial genome but are also vectors for horizontal gene transfer between bacteria [46]. Numerous prophages are carriers of antibiotic resistance genes or virulence factor genes [47–49]. In fact, many virulence factors, including cholera toxin, diphtheria toxin, and Shiga toxin from pathogenic bacteria are encoded by prophages [46]. In this study, no known antibiotic resistance genes or virulence genes were found in the genome of Hafnia phage yong2.

In a BLASTn search against the NCBI database, the top 67 hits obtained with the yong2 sequence as a query were from bacterial genomes. The genome sequence of yong2 was most similar to that of H. alvei strain FDAARGOS_1038 (identity, 95.82%; query coverage, 70%; E-value, 0.0; nt 1-17,575 of yong2; nt 2,555,902-2,573,484 of H. alvei strain FDAARGOS_1038. and Enterobacteriaceae bacterium bta3-1 (identity, 94.96%; query coverage, 55%; E-value, 0.0; nt 1-17,572 bp in yong2 and nt 920,111-937,696 in bta3-1), H. paralvei strain FDAARGOS_158 (identity, 94.88%; query coverage, 59%; E-value, 0.0; nt 1-17,572; in yong2 and nt 2,749,097-2,766,668 in FDAARGOS_158). Using PHASTER (http://phaster.ca/), five intact prophages were predicted in the genome of H. alvei strain FDAARGOS_1038 (region 1 position, nt 140,610-187,233; region 2 position, nt 490,709-526,998; region 3 position, nt 542,762-595,244; region 4 position, nt 1,836,605-1,909,748; region 5 position, nt 2,508,973-2,590,655), three intact prophage were found in the genome of Enterobacteriaceae bacterium bta3-1 (region 1 position, nt 904,818-945,707; region 2 position, nt 2,258,061-2,289,176; region 3 position, nt 4,466,231-4,516,604), and two intact prophages were predicted in the genome of H. paralvei strain FDAARGOS_158 (region 1 position, nt 2,730,674-2,773,856; region 2 position, nt 4,412,711-4,456,607). Analysis of the genome sequences of Hafnia phage yong2 and the above predicted prophages showed that phage yong2 has homology to the prophages in these bacteria (identity, 94.52%-97.67%), indicating that there is strong sequence conservation among prophages of some Enterobacteriaceae and Hafniaceae bacteria.

In the PASC classification search, Hafnia phage yong2 shared the highest pairwise nucleotide sequence similarity, as low as 22.38%, with the most closely related phage in the current database (October 8, 2021). In the VIRIDIC analysis, the highest intergenomic similarity to the most closely related phage was as low as 1.6%. This is far below the 70% nucleotide sequence identity threshold for establishing a new genus [50, 51], and yong2 should therefore be considered a member of a new genus. The sequences of Hafnia phage yong2, 114 classified phages belonging to the class Caudoviricetes), the five predicted prophages from bacterial strains that are homologous to Hafnia phage yong2, and all nine Hafnia phage genome sequences in the NCBI database were used to construct a proteomic tree (Supplementary Fig. S1) based on genome-wide sequence similarities, computed using tBLASTx. The genomes, with the shortest evolutionary distance from yong2, were selected and used as reference sequences to construct a more detailed proteomic tree (Supplementary Fig. 2a). In the proteomic trees (Supplementary Fig. S1 and Fig. 2a), Hafnia phage yong2 clustered with the four predicted Hafnia prophages and one predicted Enterobacteriaceae prophage. These six prophages and phages of the family Drexleviridae formed independent branches and were nested within a clade. Hafnia phage yong2 did not cluster with any other Hafnia phages. The results suggested that, in the families Hafniaceae and Enterobacteriaceae (belonging to the order Enterobacterales), there is a class of prophages with conserved sequences and unique evolutionary status that has not been studied before, yong2 is the first of the class to be induced and analyzed.

A genome comparison of yong2 and the most closely related prophage, which was identified via PHASTER in the genome of H. paralvei strain FDAARGOS_158, is shown.
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in Fig. 2b. Thirty of the 59 ORFs (50.8%) in yong2 show homology to those found in the predicted prophages. The left part of the genome, containing structural protein genes, is much more conserved than the highly divergent right part containing regulatory genes.

CRISPR loci are normally composed of discontinuous direct repeats separated by short stretches of DNA sequences called spacers, and these associate with cas genes (CRISPR-associated) [52]. CRISPR/Cas immune system is a defense strategy against foreign nucleic acids such as phage genomes of bacteria and archaea [53]. No CRISPR locus was found in the draft genome of *H. paralvei* LY-23 when compared to the CRISPR database. Five matching sequences between the *Hafnia* phage yong2 genome and viral spacer sequences of *Hafnia paralvei* strain FDAARGOS_158 (region 1 position, nt 2,730,674-2,773,856). The homologous regions are represented by green bars, with their color depth reflecting the degree of sequence similarity.
were found within the CRISPRs of *Hafnia* strains and *Obesumbacterium proteus* (belong to genus *Obesumbacterium*, family *Hafniaceae*), using a BLAST search of the CRISPR database and the viral spacer database of IMG/VR (E-value, 10^{-6}; GTGGTCTAGGAGGTCTGCTGTAATATGGAC (nt 32,736-32,767), GACAGACCCGAGCTCCTTCGGGGTCTGTT (nt 2670-2701), AATCAGATTGGCCCA GGGGGGCTTTATAAAGT (nt 8847-8878), GCACATCGA TGGAGAACGAAAGATTTGTA (nt 38,225-38,256), and GGCGCAGTGCTTAAAGTGTTGCGTGTGAG (nt 33,7238,225-38,256)). These sequences show similarity to the viral spacers of *H. paralvei* PCM_1211 (Bit Score, 59.0; E-value, 10^{-7}; identity, 100%), *H. paralvei* PCM_1133_RAQU, *H. paralvei* GTA-HAF03, *H. paralvei* PCM_1218, *H. paralvei* CITHA-6 (Bit Score, 59.0; E-value, 10^{-7}; identity, 100%), *H. paralvei* PCM_1192 (Bit Score, 59.0; E-value, 10^{-7}; identity, 100%), *Obesumbacterium proteus* PCM_1214 (Bit Score, 59.0; E-value, 10^{-7}; identity, 100%), and *H. paralvei* PCM_1223 (Bit Score, 55.4; E-value, 10^{-6}; identity, 96.77%). These results suggest that these *Hafniaceae* strains were infected by related phages in the past.

In summary, a novel active *H. paralvei* prophage, *Hafnia* phage yong2, was successfully induced by mitomycin C treatment. In a BLASTn search, the top 67 hits sharing the most sequence similarity with phage yong2 were all bacteria, and this was due to a similarity to prophages in these bacterial genomes (identity, 94.52-97.67%). Bioinformatics analysis revealed the existence of a novel class of prophages with conserved sequences and unique evolutionary status not yet studied before in *Hafniaceae* and *Enterobacteriaceae* bacteria.

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**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

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**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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