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A marine inducible prophage vB_CibM-P1 isolated from the aerobic anoxygenic phototrophic bacterium *Citromicrobium bathyomarinum* JL354

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A prophage vB_CibM-P1 was induced by mitomycin C from the epipelagic strain *Citromicrobium bathyomarinum* JL354, a member of the alpha-IV subcluster of marine aerobic anoxygenic phototrophic bacteria (AAPB). The induced bacteriophage vB_CibM-P1 had *Myoviridae*-like morphology and polyhedral heads (approximately capsid 60–100 nm) with tail fibers. The vB_CibM-P1 genome is ~38 kb in size, with 66.0% GC content. The genome contains 58 proposed open reading frames that are involved in integration, DNA packaging, morphogenesis and bacterial lysis. VB_CibM-P1 is a temperate phage that can be directly induced in hosts. In response to mitomycin C induction, virus-like particles can increase to 7 × 10^9 per ml, while host cells decrease an order of magnitude. The vB_CibM-P1 bacteriophage is the first inducible prophage from AAPB.

Temperate phages integrate their nucleic acid into host bacterial genomes in a process termed lysogeny[1,2]. The genetic material of a bacteriophage in host chromosome, which is referred to as prophage, can be passed to daughter cells during cell division. Prophages may be induced under certain circumstances (such as UV radiation or mitomycin C treatment), which activates a lytic cycle[1,3–5]. Thus, prophages are viewed as "dangerous molecular time bombs"[6]. DNA could be transferred among different hosts during phage infection through either the lytic cycle or the lysogenic cycle[1–5]. Previous studies showed that more than 50% of marine isolates harbor prophages, which play a significant role in shaping the phenotypic traits of their hosts[1,4,5].

Citromicrobium bathyomarinum strain JL354, a member of aerobic anoxygenic phototrophic bacteria (AAPB), was isolated from surface water in the South China Sea[7]. The type strain *C. bathyomarinum* JF-1 was first isolated from the deep-sea hydrothermal vent plume waters[8]. A complete prophage sequence was found from the preliminary genomic analysis of strain JL354. Two different phototrophic operons and a xanthorhodopsin-like protein co-exist in the same genome[7,9,10]. Horizontal gene transfer is thought to occur for phototrophic genes of phototrophic microorganisms. The objectives of this study were to 1) induce the prophage from *C. bathyomarinum* JL354 and 2) compare its genome to other homologous phages.

Results

Discovery of the vB_CibM-P1 phage by induction from *C. bathyomarinum* JL354. A novel Mu-like phage-related gene cluster was discovered within the genomic sequence of *C. bathyomarinum* JL354[7]. To determine whether this phage was active, mitomycin C was used to treat *C. bathyomarinum* JL354 in the exponential growth phase and flow cytometry was used to measure the induction of virus-like particles (VLPs). Within 30 hours, the quantity of VLPs increased up to three orders of magnitude (Fig. 1), while the number of bacterial cells decreased an order of magnitude. The induced VLPs had *Myoviridae*-like (i.e., T4-like) morphology and polyhedral heads (approximately capsid 60–100 nm) with tail fibers (Fig. 2).

To confirm whether the induced phage matched the vB_CIBM-P1 prophage observed in the *C. bathyomarinum* JL354 genome, the induced viral DNA was re-sequenced. Although there was host DNA contamination, the read-rich region mapped to the predicted prophage position (Zheng et al., unpublished data).

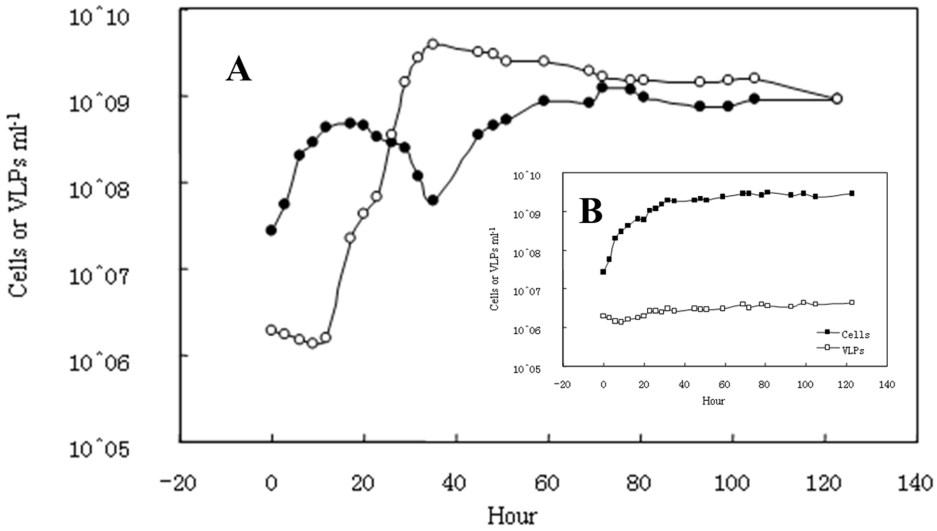

Figure 1 | Viral particle yield following mitomycin C induction of *C. bathyomarinum* JL354. Flow cytometry counts of JL354 cells and viral-like particles were performed with (A) a mitomycin C-treated culture and (B) a control culture without mitomycin C.

Genomic structure of phage vB_CibM-P1. The complete genomic sequence of phage vB_CIBM-P1 is ~38 kb, with a GC content of 66.0%, which is slightly higher than that of its host (65.0%). The vB_CIBM-P1 GC content is much higher than that of bacteriophage Mu (52.0%) from *E. coli* MH5361 and slightly higher than the prophage found in *Hoeflea phototrophica* DFL-43 (61.7%). Using PHACTS, a novel tool for predicting whether the lifestyle of a phage is primarily lytic or lysogenic, that- determined vB_CIBM-P1 was temperate[11].

In total, the vB_CIBM-P1 genome contains 58 predicted open reading frames (ORFs), representing 97% of the entire genome (Table 1 and Fig. 3). Forty-three ORFs have ATG start codons, whereas 13 ORFs and 2 ORFs start with GTG and TTG codons, respectively. No tRNA genes were detected by tRNAscan-SE[12]. Thirty-three ORFs yielded significant hits (E value $\leq$ 1e-5, coverage $\geq$ 75%) in the GenBank database, and 21 ORFs were assigned as unknown (Table 1).

Early expression region. The early expression region contains regulatory genes with associated lytic genes followed by two structural regions encoding for head and tail assembly (Fig. 3, Table 1)[13]. The genome starts with a *C*-repressor and *ner* (encoded by ORF 1 and ORF 2, respectively), which are involved in the regulation of lysogeny and lytic development. Similar to bacteriophage Mu, if the *C*-repressor bound, it would repress the lytic cycle and induce the lysogenic cycle instead; whereas if unbound, the lytic cycle would occur[13]. Transposases A and B, which are also located in this region at position 6661–9736, allow for integration (ORF 17) and transposition (ORF 18), respectively. ORF 17 encodes phage integrase, which is most similar to the integrase in *Roseomonas cervicalis* ATCC 49957.

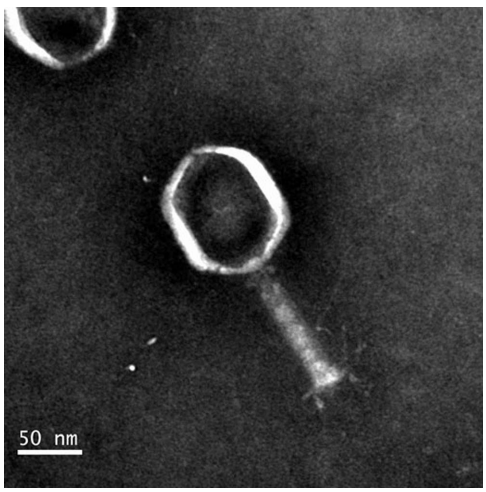

Figure 2 | Virus generated by mitomycin C induction of *C. bathyomarinum* JL354. Scale bars, 50 nm.

Head, tail and tail fiber assembly. The head proteins have high identity to classical Mu or Mu-like bacteriophages, which suggests that heads may appear to be *Myoviridae*-like. From the structure and composition of the three phages (bacteriophage Mu, vB_CIBM-P1 and the Mu-like prophage from *Hoeflea phototrophica* DFL-43), the genes encoding head protein assembly are within the most conserved region (Fig. 3). ORF 37 and ORF 42 are the two identifiable head proteins found in the genome (Table 1). ORF 37 is most identical to a Mu-like phage in *Roseovarius* sp. 217, which is another marine alphaproteobacterial (AAPB) found in the euphotic zone. The final head assembly protein ORF 42 has the highest similarity (54%) with the Mu-like major head subunit of *Hoeflea phototrophica* DFL-43[14].

ORFs 50–54 form the major tail/tail fiber proteins, which are 9 kb in size and represent 24% of the genome. However, only the tail tape measure protein appears to be similar to the Mu-like tail protein, and the rest of the tail proteins have closer identity to *Siphoviridae* (e.g., Rhizobium phage 16-3)[15].

Discussion

The vB_CIBM-P1 genome is highly mosaic in nature, but it is a novel representative of marine Mu-like phages or myoviruses. The genome has 58 predicted ORFs, and 17% of the genome encodes for identifiable Mu-like genes. Forty percent of the genome encoding for predicted proteins has no match in GenBank database, indicating the novelty and lack of knowledge about the genomes of tailed phages. Additional data are needed to confirm this variability in genome structure.

Mu-like phages, in general, have few predicted and conserved tail proteins, which are supported by the hypothesis that tailed phages are genetic mosaics derived by multiple step-wise recombination exchanges that occur within a large gene pool[16–19]. Tail proteins are generally more variable due to their role as host-range determinants[17,18]. Consistent with other Mu-like phages, genes that contribute to tail morphology are quite diverse and may be a red-queen

Table 1 | Gene Annotation List for vB_CIBM-P1

ORF	Start	Stop	Size (aa)	Start codon	GC content	Predicted function	Significant matches to proteins in GenBank	% aa identity	PSI-BLAST E-value
orf1	432	1	144	ATG	0.63	Transcription regulation	putative phage repressor [Thioalkalivibrio sp. HL-EbGR7]	42/105 (40%)	2.00E-12
orf2	759	980	74	GTG	0.59	Transcription regulation	hypothetic protein		
orf3	1243	1118	42	ATG	0.57		hypothetic protein		
orf4	1514	1209	102	ATG	0.61		hypothetic protein		
orf5	1509	1748	80	ATG	0.63		hypothetic protein		
orf6	1849	2853	335	ATG	0.65	ParB protein, nuclease	chromosome partitioning protein parB nuclease [Bartonella tribocorum CIP 105476]	69/243 (28%)	4.00E-12
orf7	2853	3077	75	ATG	0.60		hypothetic protein		
orf8	3067	3762	232	ATG	0.66	DNA methyltransferase?	hypothetical phage protein (possible DNA methyltransferase) [Campylobacter phage CPt10]	52/203 (25%)	3.00E-10
orf9	3759	4121	121	ATG	0.67		hypothetic protein		
orf10	4121	4447	109	ATG	0.69		hypothetic protein		
orf11	4441	4668	76	GTG	0.67		hypothetic protein		
orf12	4671	4874	68	ATG	0.68		hypothetic protein		
orf13	4867	5100	78	ATG	0.62		hypothetic protein		
orf14	5097	5291	65	GTG	0.71		hypothetic protein		
orf15	5288	6214	309	ATG	0.66		Gp37Gp68 family protein [Gluconacetobacter diazotrophicus PAl 5]	153/306 (50%)	8.00E-83
orf16	6207	6668	154	ATG	0.68		hypothetical protein PPMP29_gp03 [Pseudomonas phage MP29]	40/107 (37%)	3.00E-13
orf17	6661	8628	656	ATG	0.68	Transposase A	Mu DNA binding I gamma subdomain [Xanthobacter autotrophicus Py2]	262/639 (41%)	7.00E-134
orf18	8699	9736	346	ATG	0.64	Transposase B	DNA transposition protein gpB [Bordetella petrii DSM 12804]	108/285 (37%)	4.00E-44
orf19	9733	9882	50	GTG	0.73		hypothetic protein		
orf20	9882	10205	108	GTG	0.65	Replication initiation protein DnaA	chromosomal replication initiator protein DnaA [Carboxydothermus hydrogenoformans Z-2901]	31/83 (37%)	5.00E-07
orf21	10253	10885	211	ATG	0.68	Modulation of host genes	Mu-like prophage protein gp16 [Fulvimarina pelagi HTCC2506]	62/168 (36%)	7.00E-24
orf22	10882	11232	117	ATG	0.67		hypothetic protein		
orf23	11453	11719	89	GTG	0.69		conserved hypothetical protein [Rhodopseudomonas palustris TIE-1]	31/74 (41%)	3.00E-10
orf24	11709	12068	120	ATG	0.63		conserved hypothetical protein [Roseomonas cervicalis ATCC 49957]	35/91 (38%)	2.00E-11
orf25	12129	12704	192	ATG	0.64	Lysozyme	phage lysozyme [Rhodobacter capsulatus SB1003]	51/158 (32%)	7.00E-31
orf26	12785	13381	199	ATG	0.66		hypothetic protein		
orf27	13390	13818	143	ATG	0.68		hypothetic protein		
orf28	13815	14249	145	ATG	0.65		hypothetic protein		
orf29	14246	14554	103	ATG	0.67		hypothetical protein HPDFL43_14842 [Hoeflea phototrophica DFL-43]	50/89 (56%)	5.00E-26
orf30	14554	15096	181	ATG	0.68	Transmembrane protein	putative ubiquitin-conjugating enzyme E2 transcript variant [Taeniopygia guttata]	55/178 (30%)	2.00E-20
orf31	15093	15671	193	ATG	0.63	Terminase small subunit	Mu-like phage gp27 [Fulvimarina pelagi HTCC2506]	76/187 (40%)	3.00E-37
orf32	15889	15683	69	TTG	0.65		hypothetic protein		
orf33	16006	16173	56	TTG	0.64		hypothetic protein		
orf34	16166	17872	569	ATG	0.67	Terminase large subunit	Mu-like prophage FluMu protein gp28 [Magnetospirillum magneticum AMB-1]	312/524 (59%)	0
orf35	17994	19565	524	ATG	0.67	Portal	Mu-like prophage FluMu protein gp29 [Hoeflea phototrophica DFL-43]	288/530 (54%)	0
orf36	20037	19786	84	ATG	0.62		hypothetic protein		
orf37	20050	21162	371	ATG	0.68	Head morphogenesis	bacteriophage Mu GP30-like protein [Roseovarius sp. 217]	189/374 (50%)	9.00E-110
orf38	21239	21730	164	ATG	0.63	Virion morphogenesis	virion morphogenesis protein [Roseovarius sp. 217]	78/171 (45%)	3.00E-40
orf39	21805	21939	45	ATG	0.71		hypothetic protein		
orf40	21936	23021	362	GTG	0.67	Protease	Mu-like prophage I protein [Hoeflea phototrophica DFL-43]	162/360 (45%)	7.00E-80
orf41	23023	23427	135	ATG	0.67	DNA mismatch repair	hypothetical bacteriophage protein [Pseudomonas aeruginosa]	72/134 (53%)	9.00E-40
orf42	23440	24339	300	ATG	0.62	Major head subunit	Mu-like prophage major head subunit gpT-like protein [Hoeflea phototrophica DFL-43]	160/299 (54%)	4.00E-115
orf43	24446	25132	229	ATG	0.70		hypothetic protein		
orf44	25136	25564	143	GTG	0.68		Mu-like prophage Flumu protein gp36 [Syntrophus aciditrophicus SB]	57/144 (39%)	1.00E-16
orf45	25561	26019	153	GTG	0.68		hypothetical protein RTM1035_05080 [Roseovarius sp. TM1035]	53/143 (37%)	2.00E-23
orf46	26016	26258	81	ATG	0.73		hypothetic protein		

Table 1 | Continued

ORF	Start	Stop	Size (aa)	Start codon	GC content	Predicted function	Significant matches to proteins in GenBank	% aa identity	PSI-BLAST E-value
orf47	26259	27197	313	ATG	0.67		hypothetical protein HPDFL43_14907 [Hoeflea phototrophica DFL-43]	164/309 (53%)	2.00E-113
orf48	27758	27961	68	ATG	0.72		phage protein [Silicibacter sp. TrichCH4B]	30/64 (46%)	5.00E-12
orf49	27261	27590	110	ATG	0.65		hypothetical protein Rsph17025_1309 [Rhodobacter sphaeroides ATCC 17025]	48/109 (44%)	2.00E-21
orf50	27987	30326	780	GTG	0.64	Tail tape measure	Tail length tape measure protein [Polymorphum gilvum SL003B-26A1]	42/118 (36%)	5.00E-07
orf51	30323	30991	223	ATG	0.67		hypothetical protein Oant_0252 [Ochrobactrum anthropi ATCC 49188]	57/167 (34%),	6.00E-07
orf52	30975	31583	203	ATG	0.66	Tail fiber protein	hypothetical protein Oant_0253 [Ochrobactrum anthropi ATCC 49188]	75/205 (36%)	7.00E-23
orf53	31598	31999	134	ATG	0.69		hypothetic protein		
orf54	31984	34098	705	ATG	0.67	Tail fiber protein H	putative tail fiber protein H [Rhizobium phage 16-3]	187/677 (27%)	7.00E-47
orf55	34111	36042	644	GTG	0.65		hypothetical protein [uncultured phage MedDCM-OCT-S09-C299]	124/488 (25%)	2.00E-05
orf56	36076	36867	264	ATG	0.63		hypothetic protein		
orf57	36876	37052	59	GTG	0.61		hypothetic protein		

driven process[20]. If driven by a red-queen process, both phage tail and host entry receptors need to evolve at a highly consistent rate[20], which could potentially explain the high diversity and lack of conservation observed in this region.

Phylogenetic analysis of Mu-like phages. The major head gene was compared to many other Mu-like phages or prophages present in host genomes. The replicative nature of the genome in the phage vB_CIBM-P1 made it difficult to assign all sequences to a meaningful phylogeny due to their high mosaicism and variable genome structure[13]. Many Mu-like phages or prophages have been found in enteric bacteria, e.g., Gamma group (Fig. 4)[13]. It appears that AAPB hosts containing Mu-like prophages are mainly found in aquatic environments (freshwater or marine, Alpha group, Fig. 4). The vB_CIBM-P1 host *C. bathyomarinum* JL354 is a member of the Alpha group, with close identity to *Hoeflea phototrophica* DFL-43 (Fig. 4).

Bacteriophage of vB_CIBM-P1. Mu genome replication occurs by duplicative transposition into host chromosomes by random integration[13,19,21,22]. At the time of Mu genome packaging, the phage transposase cuts beyond the phage genome and can include approximately 1.8–3.0 Kb of host DNA[23,24]. In our re-sequencing data of the induced phage vB_CibM-P1, none of the reads contained host DNA fragments at either end of the phage genome. Additional experiments are needed to determine whether our phage has similar transposition properties as the Mu phage. Furthermore, our prophage could be induced by mitomycin C instead of high temperature (42°C), while previous studies showed that the Mu phage is usually induced by high temperature rather than mitomycin C[13,24]. All of the evidence indicates that the phage vB_CIBM-P1 is a novel myovirus.

Although temperate phages are widely found in marine bacteria[2,4,6], this study is the first to report a temperate phage isolated from a marine aerobic anoxygenic phototrophic bacterium. With the increasing number of bacterial genomes in the GenBank database, more marine prophages will be potentially found and characterized, which will improve our understanding of the evolution and function of phage-host interactions. Comparison of phototrophy and closely related non-phototrophy genomes may provide clues to how temperate phages influence the evolution of photosynthetic genes.

Methods

Sequence analysis. The draft genome sequence of *C. bathyomarinum* JL354 is available under the GenBank accession number NZ_ADAE00000000. The JL354 genome was manually analyzed for the presence of a putative prophage. First, the genome was scanned for phage-related genes. When a phage-related gene cluster (ZP_06862281-ZP_06862290) was encountered, the surrounding genes were also examined. Putative prophage fragments were re-annotated by performing a PSI-BLAST search against the GenBank database for further analysis[25]. The beginning and end of a specific prophage genome were estimated based on the annotations of surrounding genes. The complete prophage genome was used to predict lifestyle with the Phage Classification Tool Set (PHACTS) (http://www.phantome.org/PHACTS/)[11].

A tRNA search was performed in the prophage genome using tRNAscan-SE (http://lowelab.ucsc.edu/tRNAscan-SE)[12]. Homologous prophages from *Hoeflea phototrophica* DFL-43 (NZ_ABIA00000000) and bacteriophage Mu (NC_000929) were used for comparative genomic analysis with the prophage found in *C. bathyomarinum* JL354.

Nearly complete major head (>900 bp) gene was used to construct a phylogenetic tree. All sequences collected from the NCBI database were aligned using Clustal X, and phylogenetic trees were constructed using the neighbor-joining and maximum-likelihood algorithms in MEGA software 5.0[26]. The phylogenetic trees were supported by bootstrap for resampling test with 1000 and 100 replicates using neighbor-joining and maximum-likelihood algorithms respectively.

Induction of the *C. bathyomarinum* JL354 prophage with mitomycin C. *C. bathyomarinum* JL354 was grown in rich organic (RO, containing 1.0 g yeast extract, 1.0 g Bacto Peptone and 1.0 g sodium acetate per liter artificial seawater with vitamins and trace element.)[8] medium at 28°C with a shaking speed of 180 rpm throughout the induction experiment. The induction process and sampling were performed according to the protocol described by Chen *et al.*[3,27]. Briefly, 10 ml of JL354 culture in exponential growth phase was transferred to 200 ml of fresh RO

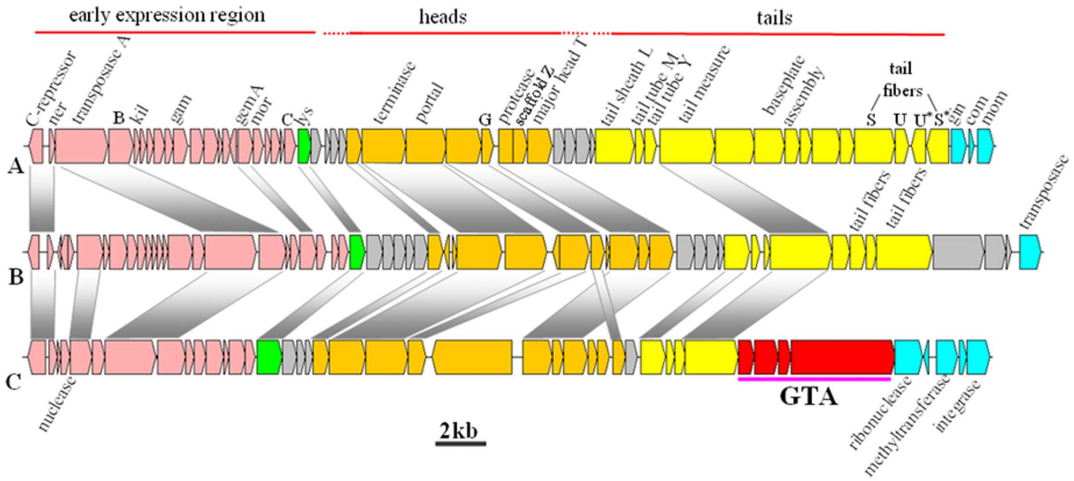

Figure 3 | Comparison of bacteriophage Mu (A), vB_CIBM-P1 (B) and prophage in *Hoeflea phototrophica* DFL-43 (C). Pink, early expression genes; orange, heads; yellow tails; red, GTA-like region; green, lysozyme genes; light gray, putative proteins.

medium. After the subculture reached OD_{600} = 0.25, it was split into two flasks (100 ml in each); one was treated with mitomycin C (final concentration, 0.5 μg ml^{-1}) and the other served as the control. After incubation for 30 min, the control and mitomycin C-treated cells were washed twice, centrifuged at 7,500 × *g* for 10 min, and resuspended in 100 ml fresh RO broth. Samples (0.5 ml) for viral and bacterial counting were fixed with glutaraldehyde (final concentration: 2.5%) for 15 min in the dark and then stored in liquid nitrogen for flow cytometry analysis[28,29].

Viral particles and bacterial count. Virus and bacterial counts were determined by an Altra Epics II flow cytometer (Beckman Coulter), which was equipped with an external quantitative sample injector (Harvard Apparatus PHD 2000). The bacterial and viral particles were identified and counted[28]. SYBR Green I (Molecular Probes) was used as a nucleic acid stain for bacteria identification in red fluorescence versus green fluorescence plots. Virus samples were analyzed separately from bacteria samples. Briefly, once thawed at 37°C, samples were diluted in 0.02-μm filtered TE (Tris-EDTA, pH = 8) buffer as needed and heated for 10 min in the dark at 80°C after staining with the DNA dye SYBR Green I, and then cooled for 5 min prior to analysis[28–30]. Viruses were discriminated on the basis of their green DNA-dye fluorescence versus 90° angle light scatter[28–30].

Purification of induced phage. Phage particles in lysates were harvested and purified as described by Chen *et al.*[3,27] with the following modifications. Phage lysates were treated with RNase A (final concentration: 2 μg ml^{-1}) and DNase I (final concentration: 2 μg ml^{-1}) at room temperature for 1 h. One liter of induced phage lysate was centrifuged at 12,000 × g for 15 min in a Hettich Rotina 38R centrifuge. Supernatants were filtered through a 0.45-μm pore size filter (type HA; Millipore) to remove host cells and cellular debris. Phage particles in the filtrate were treated with polyethylene glycol 8000 (final concentration: 100 g l^{-1}) overnight at 4°C and

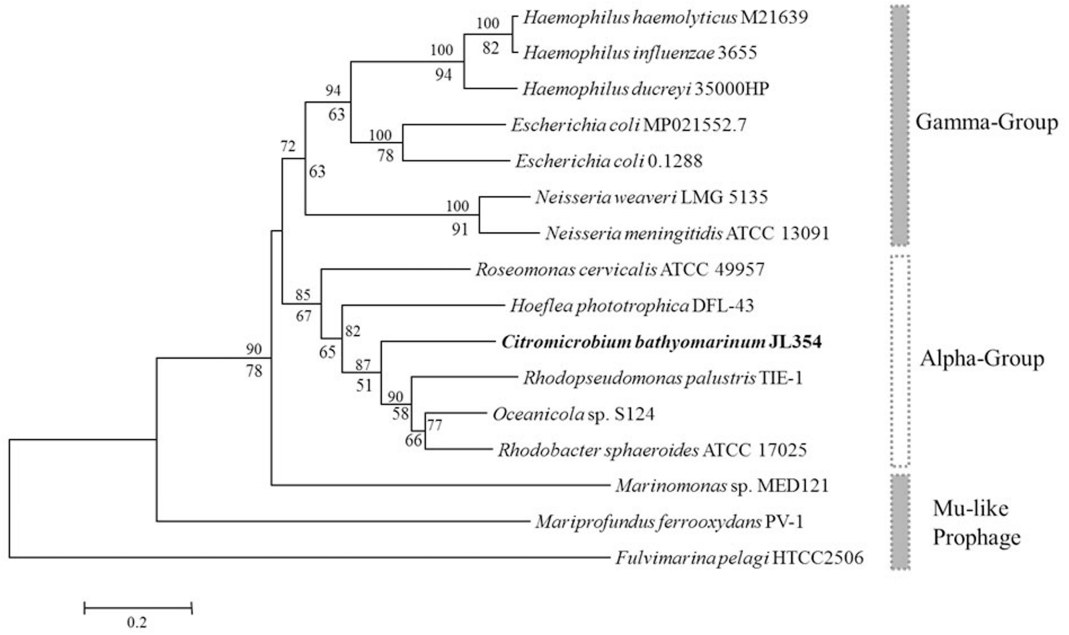

Figure 4 | Neighbor joining phylogenetic tree based on the phage major head gene sequences. The Mu-like prophage is commonly found in the *Neisseria*, *Escherichia* and *Haemophilus* genera. It has been recently found in marine bacteria genomes. All bacteria had complete Mu-like prophage sequences in their genomes. *Haemophilus haemolyticus* M21639, NZ_AFQR00000000.1; *Haemophilus influenzae* 3655, NZ_AAZF00000000.1; *Haemophilus ducreyi* 35000HP, NC_002940.2; NZ_ AQCL00000000.1; *Escherichia coli* 0.1288, NZ_AMVJ00000000.1; *Neisseria weaveri* LMG 5135, NZ_AFWQ00000000.1; *Neisseria meningitidis* ATCC 13091, NZ_AEEF00000000.1; *Roseomonas cervicalis* ATCC 49957, NZ_ADVL00000000.1; Hoeflea phototrophica DFL-43, NZ_ABIA00000000.2; *Citromicrobium bathyomarinum* JL354, NZ_ADAE00000000.1; *Rhodopseudomonas palustris* TIE-1, NC_011004.1; *Oceanicola* sp. S124, NZ_AFPM00000000.1; NC_009428.1; Marinomonas sp. MED121, NC_009654.1; *Mariprofundus ferrooxydans* PV-1, NZ_AATS00000000.1; *Fulvimarina pelagi* HTCC2506, NZ_AATP00000000.1. Bootstrap percentages (>50) from neighbor joining (above) and maximum likelihood (below) are shown in the tree. The scale bar represents 20% amino acid substitution.

precipitated by centrifugation at 12,000 × g for 90 min. Pellets were re-suspended with 6 ml of SM buffer (10 mM NaCl, 50 mM Tris, 10 mM $MgSO_4$, and 0.1% gelatin) and incubated overnight at 4°C. The phage suspension was mixed with CsCl (final concentration, 0.6 g ml^{-1}) and centrifuged for 24 h at 200,000 × *g* using an S55A rotor in a Micro-Ultracentrifuge CS-GX series centrifuge. Visible viral bands were extracted and then dialyzed (MWT = 30 KD) twice in SM buffer overnight at 4°C. CsCl-purified phage lysates were stored at 4°C until further analysis. Virus morphology was examined by transmission electron microscopy (JEM 2100 HC).

Extraction of phage DNA. Purified phage were treated with a proteinase K cocktail (100 μg ml^{-1} proteinase K, 50 mM Tris, 25 mM EDTA, and 1% SDS) at 55°C for 3 h. The phage DNA was extracted using phenol/chloroform/isoamyl alcohol (25 : 24 : 1 by volume)[3,27]. The DNA was then ethanol-precipitated, re-suspended in TE buffer (10 mM Tris, 1 mM EDTA), and re-sequenced with an Agilent 2100 Bioanalyzer (Ion Torrent PGM, Invitrogen, China). One library with average sizes of 350 bps was constructed. After quality control, total 26,064 reads (4,314,206 bps) were used to assemble the viral genome.

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Author contributions

Q.Z., R.Z. and N.J. conceived and designed the experiments; Q.Z., Y.X., R.A.W., Y.W. and T.L. analyzed the data. All of the authors assisted in writing the manuscript, discussed the results and commented on the manuscript.

Additional information

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