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Research Article

The First Isolation of a Cyanophage-*Synechococcus* System from the East China Sea

Yan Zhang[1], Min Xu[1], Yijun Zhao[2] and Kai Cheng [1, 2]✉

1. College of Life Science, Central China Normal University, Wuhan 430079, China;
2. Key Laboratory of Fermentation Engineering (Ministry of Education),Hubei Provincial Cooperative Innovation Center of Industrial Fermentation, College of Resources and Environmental Engineering, Hubei University of Technology, Wuhan 430068, China.

A cyanophage strain and its host *Synechococcus* were isolated from the East China Sea. The host *Synechococcus* sp. SJ01 was characterized by its 16S rRNA, ITS, and *psbA* gene sequences as well as by its morphological appearance and pigmentation. The cyanophage, strain S-SJ2, was able to cause a lytic infection of the coastal *Synechococcus*. TEM of negative-stained specimens showed that the phage isolate has an isometric head with a diameter of 68 nm and a long tail with a length of 280 nm. The cyanophage-*Synechococcus* system from the East China Sea shares many properties with other marine cyanophage-*Synechococcus* systems worldwide.

Synechococcus sp.; Cyanophage; East China Sea

Synechococcus, some of which differ genetically and physiologically and display different environmental distribution patterns, are found in high concentrations in coastal waters (Scanlan D J, 2002; Waterbury J B, 1986). It has been estimated that in coastal waters, 80% of *Synechococcus* cells may encounter infectious cyanophage particles each day (Suttle C A, 1994). Although many *Synechococcus* isolated from coastal waters are resistant to cyanophages, a large number of cyanophages have been successfully isolated from different coastal environments (Suttle C A, 1994; Waterbury J B, 1993).

Cyanophages are viruses that can infect *Synechococcus* sp. and other prokaryotic algae. The isolation and characterization of cyanophages infecting marine *Synechococcus* strains began in the 1990s (Suttle C A, 1993; Waterbury J B, 1993; Wilson W H, 1993). Five marine cyanophages propagated on *Synechococcus* sp. strain WH7803 were isolated by Waterbury and Valois from different oceanographic areas, including open ocean and coastal waters (Waterbuty J B, 1986; Waterbury J B, 1993). Representatives of all three families of tailed phages were found among the seven *Synechococcus* phages isolated by Suttle, Chan and Wilson (Suttle C A, 1993; Wilson W H, 1993). The diversity of cyanophages in the environment is substantial, and many new cyanophages have been discovered over the past decade (Prangishvili D, 2006; Suttle C A, 2007; Van Etten J L, 2010). However, metagenomic data show that more than 70% of the genes in the oceanic viral fraction cannot be associated with known viruses, indicating that a substantial amount of viral diversity remains to be identified (Angly F E, 2006; Bench S R, 2007; Suttle C A, 2007).

Cyanophages infecting *Synechococcus* are ubiquitous in marine environments and are highly abundant in coastal waters, with concentrations of up to 10^8 cyanophages per liter (Suttle C A, 1993; Suttle C A, 1994; Waterbury J B, 1993). However, no previous reports have documented marine *Synechococcus* cyanophages in China. The purpose of this study was to isolate a cyanophage-*Synechococcus* system from the East China Sea; this goal is fundamental for studying phage-host interaction in the Chinese coastal ecosystem.

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✉ Corresponding author.
Phone: +86-27-67867915, Fax: +86-27-67867915,
Email: chengkaicn@163.com

MATERIALS AND METHODS

Location and sampling

Surface seawater samples were collected from the coastal waters of the East China Sea (33°44'38″N, 122°25'44″E) in August 2011 at a depth of 1 m. The water samples for the isolation of cyanophages were concentrated by the ultrafiltration method described by Chen *et al.* and stored in the dark at 4 °C until use (Chen F et al, 1996). The water samples for the isolation of *Synechococcus* were filtered through a 1.0-mm filter and stored at 15 °C until use.

Synechococcus isolation and growth conditions

The filtered samples were enriched with sterilized artificial seawater medium (ASW) and incubated under a constant illumination of approximately 25 μE m^{-2} s^{-1} at 20 °C. After visible growth (based on color and turbidity) was evident in the enrichments, clonal isolates were obtained by repeated cycles (repeated five times) of colony isolation using a serial dilution method described by Throndsen and Rippka (Rippka R, 1988; Throdson J, 1969). The resulting isolate SJ01 was clonal and unialgal.

In vivo absorption spectra and pigment measurements of *Synechococcus*

Cultures in the exponential to early stationary phase were used for recording *in vivo* absorption spectra. An aliquot of the culture was transferred to a cuvette, and the *in vivo* absorption spectrum was measured from 400 to 750 nm using a Scinco S-3100 UV-VIS Spectrophotometer equipped with LabPro Plus Software. The A_{495}/A_{545} ratio is a measure of the amount of PUB (phycourobilin) relative to that of PEB (phycoerythrin) (Rocap G, 2002). The experiment was performed in triplicate.

Chlorophyll was estimated using a previously described method (Becker W E, 1994). The cells were resuspended in 90% methanol and allowed to stand in the dark for 24 hours (Becker W E, 1994). The suspension was cleared by centrifugation, and chlorophyll was estimated spectrophotometrically using the following equations: Chlorophyll a (μg L^{-1}) = (13.95×A_{665}) - (6.88×A_{650}), and Chlorophyll b (μg L^{-1}) = (24.96×A_{650}) - (7.32×A_{665}) (Rocap G, 2002), where A_{650} and A_{665} are the absorbance values of the sample at 650 and 665 nm, respectively. The experiment was performed in triplicate.

DNA extraction, PCR amplification, sequencing and phylogenetic analyses of SJ01

DNA was prepared from exponential-phase culture using a method modified from that described by Tillett and Neilan (Tillett D and Neilan B A, 2000). The extracted DNA (2 μL) was added to a 48 μL PCR mixture containing Taq DNA polymerase assay buffer (50 mmol/L KCl, 20 mmol/L Tris-HCl, pH 8.4), 5.0 mmol/L $MgCl_2$, 200 μmol/L deoxyribonucleoside triphosphate, 0.25 μmol/L each of the primers, and 2.0 U of Taq DNA polymerase (TaKaRa). The PCR primers that were used to amplify the 16S rRNA, ITS, and *psbA* genes of *Synechococcus* sp. SJ01 are shown in Table 1. The negative controls contained all of the above reagents, but sterile water was used as the template. The amplification products were verified by electrophoresis and then sequenced.

BLAST searches of the GenBank database (www.ncbi.nlm.nih.gov) were performed to identify closely related sequences. Neighbor-joining (NJ) trees were generated using the MEGA 4.0 program (http://www.megasoftware.net/) for all sequences, which included selected representative sequences from GenBank following alignment using ClustalX2. Bootstrap values were obtained with 1000 resamplings, and clades with bootstrap values greater than 50% were shown on the nodes of the branches.

Table 1 Primers used in this study

Species		Primer	Sequence	Source
Synechococcus SJ01	16S rRNA	27F1	5′-AGAGTTTGATCMTGGCTCAG-3′	Neilan B A, 1997
		409R	5′-TTACAA(C/T)CCAA(G/A)(G/A)(G/A)CCTTCC TCCC-3′	
	ITS	16S-1247f	5′-CGTACTACAATGCTACGG-3′	Rocap G, 2002
		23S-1608r	5′-CYACCTGTGTCGGTTT-3′	
	psbA	*psbA*-1	5′-AACATCATYTCWGGTGCWGT-3′	Lindell D, 2004
		psbA-2	5′-TCGTGCATTACTTCCATACC-3′	
	g20	CPS1	5′-GTAG[T/A]ATTTTCTACATTGA[C/T]GTTGG-3′	Sullivan M B, 2008
		CPS2	5′-GGTA[G/A]CCAGAAATC[C/T]TC[C/A]AGCAT-3′	
Cyanophage S-SJ2	*psbA*	*psbA*-1F	5′-AACATCATYTCWGGTGCWGT-3′	Huang S, 2012
		psbA-1R	5′-TCGTGCATTACTTCCATACC-3′	
	DNA*pol*	90Fa	5′-GAYACIYTIRYIYTITCIMG-3′	Huang S, 2012
		355Ra	5′-GGIAYYTGIGCIARRTTIGG-3′	

Phage isolation

Synechococcus phages were isolated with liquid dilution cultures rather than from plaques on solid media because lawn formation is erratic for many strains belonging to *Synechococcus* marine cluster A (Waterbury J B, 1993). Serial dilutions of the concentrated seawater samples were added to exponentially growing cultures of the host suspended in ASW growth medium in test tubes. The phage-host suspensions were incubated at 20 °C for 1 h with occasional agitation to allow the adsorption of the cyanophage. These tubes were incubated in constant illumination (approximately 25 μE m^{-2} s^{-1}) at 20 °C and were monitored daily for cell growth or lysis. The controls contained host cells but no seawater dilutions. The lysis of the host cells was usually observed for up to five weeks. Putative cyanophage lysates obtained by this method were passed through a 0.22 μm pore size filter, and the filtrate was stored at 4 °C in the dark. The purified cyanophage filtrates were then obtained with three successive serial dilutions in the liquid medium.

Phage purification

Phage purification was performed by sucrose density gradient centrifugation as described previously with the following modifications (Vidaver, 1973): both DNA and RNA were digested with 1 μg/mL Dnase I and Rnase A (TaKaRa); and five sucrose steps of 20%, 30%, 40%, 50%, and 60% were used. A visible band locating between the 40% and 50% steps was collected and washed to obtain highly purified phage particles.

TEM observation of *Synechococcus* and cyanophage

An ultra-thin section was used to observe the ultrastructure of *Synechococcus* sp. SJ01. One milliliter *Synechococcus* sp. SJ01 was centrifuged at 6000 g for 10 min, and 0.2 mL 2.5% glutaraldehyde was then added to the precipitate for postfixation. Fixation was performed by immersing the sample in 1% osmic acid for 4 h. The sample was then centrifuged at 6000 g for 5 min. PBS was added to the precipitate for washing, and the centrifugation and washing were repeated three times. Dehydration was performed by immersing the sample in 50% to 100% alcohol. The sample was then embedded in Spurr resin (ERL-4206). Ultra-thin sections were stained with uranyl acetate and lead citrate and then observed using a Tecnai G2 TEM.

A total of 20 μL of sucrose density gradient purified phage concentrate was transferred to 200 mesh formvar carbon-coated copper grids and then negatively stained with 2% sodium phosphotungstate (pH 7.0). The grids were viewed using a HITACHI 3H-7000FA TEM.

DNA extraction, PCR amplification and restriction enzyme digestion of the phage S-SJ2 genome

To estimate the size of the S-SJ2 genome, DNA was prepared from sucrose density gradient purified phages following the method of Wilson et al (Wilson, 1993). The DNA that was extracted from purified cyanophage was digested with the following restriction endonucleases: *Hin*d III, *Bam*H I, *Eco*R I, *Eco*R V, *Pst* I, and *Dpn* I. Extracted DNA (2 μL) was added to a 48 μL PCR mixture containing Taq DNA polymerase assay buffer (50 mmol/L KCl, 20 mmol/L Tris-HCl, pH 8.4), 5.0 mmol/L $MgCl_2$, 200 μmol/L deoxyribonucleoside triphosphate, 0.25 μmol/L each of the primers, and 2.0 U of Taq DNA polymerase (TaKaRa). The PCR primers that were used to amplify the g20, *psbA*, and DNA*pol* genes of cyanophage S-SJ2 are shown in Table 1. Negative controls contained all of the reagents, but sterile water was used as the template. The amplification products were verified by electrophoresis.

RESULTS

Morphology and pigmentation of the strain

The cells of *Synechococcus* sp. strain SJ01 averaged ca. 0.8 μm in width and 1.5 μm in length and were spherical to oval in shape (Fig. 1). No well-defined sheath layer or gas vacuoles were observed. Photosynthetic thylakoids of SJ01 were peripheral and oriented parallel to the cytoplasmic membrane. Several carboxysomes were observed in the central portion of the cells. The dividing cells showed transverse binary fission.

In vivo absorption spectra indicated that this *Synechococcus* SJ01 absorbs light efficiently in the blue region, with two prominent peaks at 440 nm (chl a) and 492 nm (primarily due to PUB and carotenoids) (Fig. 2). On the basis of the PUB/PEB ratios, the *Synechococcus* sp. strain SJ01 was remarkably similar to the motile strain WH8102, which

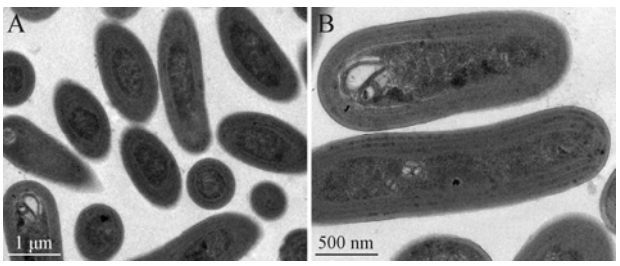

Fig. 1. Transmission electron micrographs of ultrathin sections of cells from strain SJ01. Scale bar, (A) 1 μm, (B) 0.5 μm. The cells of *Synechococcus* sp. strain SJ01 averaged ca. 0.8 μm in width and 1.5 μm in length and were spherical to oval in shape. Photosynthetic thylakoids were peripheral and oriented parallel to the cytoplasmic membrane.

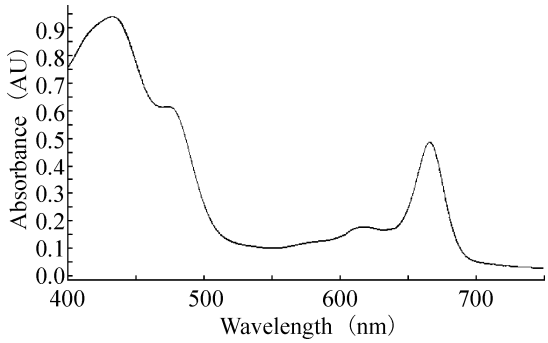

Fig. 2. *In vivo* absorption spectra of *Synechococcus* sp. SJ01 in the exponential to early stationary phase. The spectra were measured from 400 to 750 nm using a Scinco S-3100 UV-VIS Spectrophotometer equipped with LabPro Plus Software.

possessed phycoerythrin (PE) as the major light-harvesting pigment (Waterbury J B, 1986). Moreover, the chl b/a ratios of the *Synechococcus* sp. strain SJ01 was only 0.17, which differed from the chl b/a ratios of *Prochlorococcus* strains, which ranged from 0.20 to 1.41 (Rocap G, 2002).

Molecular identification of the strain

All of the PCR amplifications yielded single-band products, and the lengths of the 16S rRNA, ITS and *psbA* gene sequences were ≈400 bp, ≈1200 bp, and ≈750 bp, respectively. The *Synechococcus* isolate SJ01 is phylogenetically closely related to *Synechococcus* sp. WH8102 (Accession: BX569694.1), and the sequence identities between them were 99%, 94%, and 91% based on their 16S rRNA, ITS, and *psbA* gene sequences, respectively. In phylogenetic analyses of this strain, based on the 16S rRNA, ITS, and *psbA* sequences, SJ01 was always closely related to the members of Marine A *Synechococcus* clade III (Fig. 3). The sequencing results of 16S rRNA, ITS, and *psbA* sequences of *Synechococcus* SJ01 are shown in Supplementary material S1.

The morphology of the cyanophage

Using liquid dilution cultures and sucrose density gradient centrifugation, we isolated and purified a marine *Synechococcus* siphovirus, S-SJ2, infecting strain SJ01. The morphology of negatively stained S-SJ2, as observed with TEM, revealed an icosahedral capsid that was ≈68 nm in diameter and exhibited a long tail (≈280 nm long and ≈20 nm in diameter) (Fig. 4). S-SJ2 was morphologically similar to S-CBS4, a siphovirus infecting marine Synechococcus CB0101, which also has an isometric head (≈72 nm) and a long flexible tail (≈200 nm) (Huang S, 2012).

Genome features of cyanophage S-SJ2

Agarose gel electrophoresis of the nucleic acid extracted from purified cyanophage S-SJ2 showed that the genome is less than 23 kb in length (Supplemental material, Figure S1).

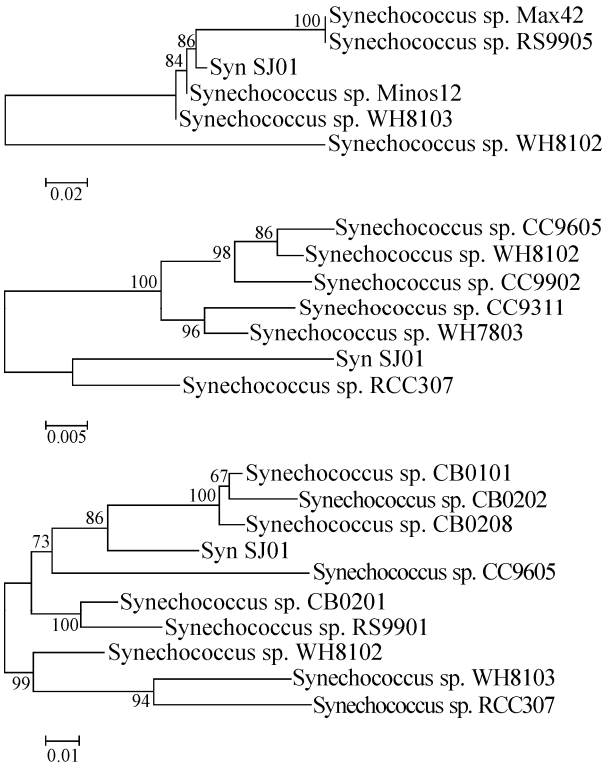

Fig. 3. Neighbor-joining phylogenetic tree of 16S rRNA (A), ITS (B), and *psbA* (C) genes from marine *Synechococcus* strains (with Jukes-Cantor correction). The tree was constructed from an alignment using Clustal X2. The bootstrap values were obtained through 1000 repetitions. The sequences were extracted from BLAST searches of GenBank.

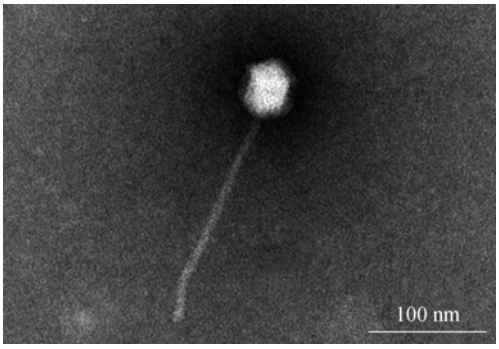

Fig. 4. Purified S-SJ2 particles visualized using a negative staining method. Scale bar, 100 nm. The phage particles were purified by liquid dilution cultures and sucrose density gradient centrifugation. The sample was negatively stained with 2% sodium phosphotungstate (pH 7.0) and observed at a magnification of ×100000.

DNA extracted from purified S-SJ2 could not be restricted with the restriction endonucleases *Hin*dIII, *Bam*HI, *Eco*RI, *Eco*RV, *Pst*I, and *Dpn*I. The g20, *psbA* and DNA*pol* sequences from S-SJ2 could not be amplified by any of the primer sets in this study. This result was consistent with observations made by Huang et al (Huang S, 2012).

DISCUSSION

The cyanobacterial picoplankton SJ01 isolated from a coastal area of the East China Sea was identified as *Synechococcus* sp. based on morphological features and 16S rRNA, ITS, and *psbA* sequence data. The strain was very similar to *Synechococcus* sp. WH8102, a member of clade III of the marine cluster A (MC-A) group. This group has been isolated from the euphotic zone from both open-oceans and coastal waters and has an elevated salt requirement for growth (Fuller N J, 2003). In contrast with the open-ocean strains, certain coastal strains of clade III of marine cluster A (e.g., the SJ01 and WH8103 strains) present a very high PUB. Their presence in the coastal environment may be more representative of physical oceanographic patterns (Ong L J, 1984; Vera T, 2009). Their occurrence in late summer coincides with periods of strong stratification (Palacios D M, 2004). Partensky *et al*. have stated that the vertical distributions of *Prochlorococcus* and *Synechococcus* populations tend to exhibit certain general features (Partensky F J, 1999). Typically, *Synechococcus* populations are approximately 10-fold less abundant than *Prochlorococcus* populations except in coastal waters (Mann N H, 2003). Due to its photosynthetic lifestyle and sheer abundance, *Synechococcus* contributes 20% of the primary productivity in certain coastal environments (Li W K W, 1994).

Three families of cyanophages, Myoviridae, Siphoviridae, and Podoviridae, have been reported. According to its morphological characteristics, S-SJ2 was very similar to S-CBS4, a siphovirus infecting marine *Synechococcus* CB0101, which was isolated from Chesapeake Bay (Huang S, 2012). All six of the restriction endonucleases tested failed to digest cyanophage DNA into clearly definable band patterns. This result was consistent with observations made by Wilson et al. (Wilson W H, 1993) and Lu et al (Lu J, 2001). Many cyanophages or bacteriophages that are resistant to digestion by certain endonucleases have been described previously (Lu J, 2001). This resistance to endonuclease digestion could result from either an absence of restriction sites or, more likely, the presence of modified bases, such as those resulting from the methylation of cyanophage DNA. To date, the genome sequences of 28 cyanophages (17 myoviruses, 6 podoviruses, and 5 siphoviruses) isolated from marine ecosystems have been reported (Huang S, 2012). Based on different gene markers, such as the g20 gene encoding viral capsid assembly protein for cyanomyovirus, the viral DNA polymerase gene for cyanobacterial podoviruses, as well as the core photosystem II *psbA* gene for both of these viruses, an extensive genetic diversity of cyanobacterial myoviruses and podoviruses has been found in various marine ecosystems (Huang S, 2012). However, the highly variable genomes of cyanobacterial siphoviruses suggest that it is impossible to identify a specific gene marker for this group of cyanophages (Huang S, 2012). The phage family *Siphoviridae* often contains temperate members with the capability of integrating into the host genome or entering a lysogenic lifestyle. For this reason, it is difficult to determine the lytic cycle and burst size of siphovirus S-SJ2 (Huang S, 2012). Like most previously reported unidentified cyanophage isolates, S-SJ2 was isolated without complete genome sequencing and identification, despite the fact that marine cyanobacteria have been used to isolate phages for two decades. (Wang K, 2011). The discovery of a novel cyanophage isolated on marine cyanobacteria substantially increases the likelihood that marine environments harbor many more novel phage types, especially those found on local cyanobacteria hosts that we are capable of culturing (Sabehi G, 2011). Given the dominance of *Synechococcus*-phage systems, it is unsurprising that many recent studies have found that phage-bacteria interactions strongly influence global biogeochemical cycles (Weitz J S, 2013).

Cyanophage-*Synechococcus* systems have been described from diverse habitats worldwide, including coastal and open-ocean environments. Since the early 1990s, more than 350 cyanophages infecting *Synechococcus* have been isolated, and 210 of these isolates have been further characterized (Marston M F, 2003; Wilson W H, 1993). Due to the importance of *Synechococcus*-phage interaction in the coastal ecosystem, the isolation and characterization of the *Synechococcus*-phage system has become an important topic in aquatic virology and marine environmental research (Singh P, 2012). Our study describes the first isolation of a cyanophage-*Synechococcus* system from the coastal ocean in China. The system shares many properties with other marine cyanophage-*Synechococcus* isolates, and additional details remain to be investigated. However, the successful establishment of a culturable cyanophage-*Synechococcus* system from the coastal ocean of China would be helpful for further research into phage-host interaction in the Chinese coastal ecosystem.

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Author contributions

Yan Zhang carried out all the experiments. Min Xu performed the genetic analysis. Yijun Zhao provided finical and equipment support. Kai Cheng conceived and designed.

Supplementary materials

The supplementary materials are available on the website of Virologica Sinica: http://www.virosin.org.

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