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Research Paper

Discovery of a novel Piscanivirus in yellow catfish (*Pelteobagrus fulvidraco*) in China

Xiaodong Zhang[a], Wenying Shen[a], Chuchu Xu[a], Yadi Wang[a], Hao Xu[a], Xiaoyu Liu[b], Yongwei Wei[b,*]

[a] School of Life Sciences, Shaoxing University, Shaoxing, Zhejiang 312000, China
[b] School of Medical Sciences, Shaoxing University, Shaoxing, Zhejiang 312000, China

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ABSTRACT

A bacilliform virus was isolated from yellow catfish in China. This virus can directly adapt in cultures of EPC cells. The virus particles, which were rod-shaped approximately 120 nm long and 20 nm wide, were visible in the cytoplasm of EPC cells. The full-length genome of this virus is 26,985 nt. The genome contains four open reading frames that encode polyprotein1ab, spike glycoprotein, M protein, and N protein. There was a putative slippery sequence ^{14861}UUUAAAC^{14867}, which could be modeled into an RNA pseudoknot structure. The predicted amino acid sequence of pp1ab, S, M, and N genes shares 8.7%–40.2% homology with those of the two known *Bafinivirus* strains—WBV and FHMNV. Based on the viral morphology, genome organization, and sequence homology, this newly identified bacilliform virus appears to be *Piscanivirus*. To the best of our knowledge, this is the first report of *Piscanivirus* in yellow catfish and *Piscanivirus* in China.

1. Introduction

Currently, the subfamily *Piscanivirinae*, family *Tobaniviridae*, order *Nidovirales* includes three species in two genera of *Bafinivirus* and *Oncotshavirus*. The first species of *Bafinivirus*, which was named as white bream virus (WBV), was isolated from white bream (*Blicca bjoerkna* L.; Teleostei, order *Cypriniformes*) in Germany (Granzow et al., 2001). Schütze then cloned the full genome of WBV and proposed the name *Bafinivirus* for this new nidovirus (Schütze et al., 2006). The second species fathead minnow nidovirus (FHMNV) of *Bafinivirus*, which was earlier mistaken as *rhabdovirus*, was isolated from fathead minnows (*Pimephales promelas*) in Arkansas, USA (Iwanowicz and Goodwin, 2002). This virus has also been found in muskellunge (*Esox masquinongy*) in wild (Faisal et al., 2016). The FHMNV can also infect spotfin shiner (*Cyprinella spiloptera*) and golden shiner (*Notemigonus crysoleucas*) in laboratory (Baird and Faisal, 2016). There is only one species, which was named as Chinook salmon nidovirus 1 (CSV), in the genus of *Oncotshavirus* (https://talk.ictvonline.org/taxonomy/). Although the genome sequence of CSV isolateed from *Oncorhynchus tshawytscha* in Canada is uploaded in GenBank, there is no any report on the phenotype of this virus.

Yellow catfish (*Pelteobagrus fulvidraco*) is a teleost fish belonging to the family *Bagridae*. It has become one of the most important freshwater aquaculture species in East Asia and South Asia owing to its excellent meat quality (Liu et al., 2018). In the present study, we identified a new *Piscanivirus* from yellow catfish in China. The virion of the isolated virus has a bacilliform shape. The genomic sequence of this virus was cloned and analyzed. The genome organization is similar to that of the established species WBV and FHMNV, containing four major open reading frames (ORFs) that encode polyprotein1ab, spike glycoprotein, M protein, and N protein. Based on the viral morphology, genome organization, and sequence homology, this newly identified virus isolated from yellow catfish appears to belong to the subfamily *Piscanivirinae*. To the best of our knowledge, this is the first report of *Piscanivirus* in yellow catfish and *Piscanivirus* in China.

2. Materials and methods

2.1. Sample collection and examination

In 2018, samples of yellow catfish, approximately 10 cm long, were initially collected from an open-air, earthen pond in a farm in the province Zhejiang, China.

* Corresponding author at: School of Medical Sciences, Shaoxing University, No.900, Chengnan Avenue, Shaoxing, Zhejiang province, China.
E-mail address: 1071246652@qq.com (Y. Wei).

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2.2. Cell lines and viral cell culture assay

Samples from different tissues of yellow catfish (liver, spleen, kidney, and intestines) were mixed, minced using a pair of scissors, and passed through a steel mesh to obtain a homogeneous suspension. After centrifugation at 1000 ×*g* for 20 min at 4 °C, the supernatant was collected. The supernatant was filter sterilized with a 0.20-μm syringe filter and inoculated into Epithelioma papulosum cyprini (EPC) cells, which were maintained in M199 medium supplemented with 10% fetal bovine serum (FBS) at 25 °C in a humidified 5% CO_2 incubator.

2.3. Electron microscopy

After two passages, negative stain electron microscopic (EM) examination of this virus in EPC cells was performed. Eighty percent confluent EPC cells in T75 flask were inoculated into the suspension that was harvested in the first passage, incubated for 1 h, and grown until notable CPE appeared. The cells were then removed from the growth vessel by scraping, rinsed with 0.1 mol/L phosphate buffer, centrifuged, and fixed in buffered 2.5% glutaraldehyde for 1 h. The specimens were post fixed in 1% osmium tetroxide, stained with 4% uranyl acetate, dehydrated through a graded series of alcohol and propylene oxide, embedded in a mixture of Epon substitute and Araldite, and cut into thin sections. The sections were stained with 4% uranyl acetate and Reynolds's lead citrate. A Hitachi Model H-7650 TEM was utilized to observe the viral particles.

2.4. Genomic cloning

The virus was grown in EPC cells, and then large scale purification was performed. Briefly, the virus was grown in EPC cells and harvested after 7 d of infection. After freezing and thawing three times, the infected EPC cells were centrifuged at 5000 ×*g* for 10 min. The clear supernatant was layered onto 4 mL of 40% sucrose cushion and ultracentrifuged at 22,000 ×*g* for 3 h at 4 °C. The pelleted virus particles were resuspended in TNE-buffer (10 mM Tris/HCl, 100 mM NaCl, and 1 mM EDTA; pH 8.0).

Both DNA and RNA were respectively extracted using the viral DNA or RNA isolation kits (Takara Bio Inc.), according to the manufacturer's instruction. The pellets were dissolved in 20 μL of ddH_2O. Random detection of unknown virus genomic DNA or RNA was performed by the method previously described by Allander (Allander et al., 2001). Amplified polymerase chain reaction (PCR) products were then analyzed by agarose gel electrophoresis. The distinct DNA bands were gel-purified using the Gel Extraction Mini Kit (WATSON Biotechnologies). The target DNA fragments were cloned into pMD19-T simple vector (Takara Bio Inc.)for sequencing.

The sequences obtained were subjected to homology search with BLAST. Each sequence was analyzed for nucleotide sequence homology (BLASTn) by searching the standard nonredundant databases and the high throughput genomic sequence database. The BLAST results showed that two fragments share high homology with Chinook salmon *Bafinivirus* isolate NIDO (GenBank No. KJ681496). The genomic sequence of the virus was cloned using a multiple fragment cloning strategy (there was a 20-bp overlapping sequence for each adjacent fragment). Primers were designed based on this strain; they are listed in Table 1. The reverse transcription -PCR (RT-PCR) was performed using reverse primers complementary to the viral genomic sequence and the M-MULV reverse transcriptase system (Takara Bio Inc.). Briefly, the viral RNA mixed with the negative sense primer was incubated at 70 °C for 5 min and cooled on ice for 2 min. Ten microliters of the RT-PCR reaction buffer mixture, containing 4 μL of 5× reaction buffer, 2 μL of 10 mM dNTP mix, 20 U of RNase inhibitor, and 40 U of M-MULV reverse transcriptase, was added, and the reaction mixture was incubated at 42 °C for 1 h. Finally, the reaction was heat inactivated at 70 °C for 10 min and chilled on ice. Using the first-strand cDNA as a template, the cDNA fragment was amplified using the 2× Master PCR Kit (Takara Bio Inc.). The amplification was performed for 35 cycles with denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and elongation at 72 °C for 1 min with an increment of 5 min after 35 cycles in a thermocycler. The PCR amplicons of expected size were excised and cloned into the pMD19-T vector. To obtain precise sequences, three independent positive clones were bi-directionally sequenced by Tsingke Biological Technology (Hangzhou, China). All cloned fragments were assembled using SeqMan of DNAstar 5.0 software.

2.5. Genome sequence analysis

The sequence analysis was performed using DNAstar 5.0 and fgenesV0 software. Multiple sequence alignments and phylogenetic tree analysis were performed using Jotun Hein Method in Mega7.0. *Tobaniviruses* used in the analysis were obtained from GenBank.

2.6. Syncytium formation assay

Monolayers of EPC cells in 6-well plates were infected with 2×10^4 $TCID_{50}$ of the isolated virus. After 24 h of infection, the cells were washed and incubated at 25 °C in M199 with 0.05 μg/mL L-1-tosylamide-2-phenylethyl chloromethyl ketone)-trypsin (TPCK). At 5 d post-infection, the cells were fixed with methanol and syncytia were visualized by Giemsa staining.

3. Results and discussion

The tissue samples from different organs of yellow catfish were mixed, minced, and passed through a steel mesh to obtain a homogeneous suspension. The filtered supernatant was inoculated into EPC cells following inoculation of EPC cell line, cytopathic effect (CPE) was observed at 72 h post inoculation of the first passage (Fig. 1A), indicating that this virus can directly adapt in cell culture. One week post inoculation, the supernatant was harvested and used for the infection of fresh EPC cells. Monolayers of infected and control EPC cell cultures were scraped from the dishes and fixed. All preparations were examined with an electron microscope. The results revealed that the virus particle is rod-shaped approximately 120 nm long and 20 nm wide in the cytoplasm (Fig. 1C). The virus was named as yellow catfish bacilliform virus (YCBV).

The concentrated viral particles purified from cell culture by ultracentrifugation, were subjected to DNA or RNA extraction. Random detection of unknown viral genomic DNA or RNA was performed. The amplified PCR products were then sequenced. The sequences obtained were subjected to homology search with BLAST. The BLAST results showed that two fragments share high homology with the Chinook salmon *Bafinivirus* isolate NIDO (GenBank No. KJ681496.1). The genomic sequence of the virus was then cloned using a multiple fragment cloning strategy. All cloned fragments were assembled using SeqMan of DNAstar 5.0. The sequence analysis was performed using DNAstar 5.0 and fgenesV0 software. The full-length genomic sequence of YCBV is 26,985 nt. The genome of YCBV contains four major ORFs that encode the polyprotein1ab (pp1ab; 7174 aa), spike glycoprotein (S; 1190 aa), M protein (225 aa), and N protein (165 aa). The terminal regions of 5′ untranslated region (UTR) and 3′ UTR were 845 and 193 nt in length, respectively. The genome organization is shown in Fig. 2A. The sequence of YCBV was deposited in the GenBank database (accession no. MH822145). The genome organization of YCBV appeared to be similar to that of WBV and FHMNV. There was a putative slippery sequence, ^{14861}UUUAAAC^{14867}, just upstream of the YCBV ORF1a translation stop codon. The sequence downstream of the slippery sequence could be modeled into an RNA pseudoknot structure (Fig. 2B), as observed in WBV (Schütze et al., 2006). The phylogenetic tree analysis of the full-long genome was performed by Jotun Hein Method in Mega 7.0. As shown in Fig. 2C, the phylogenetic tree of nine *Tobaniviruses* is

Table 1
Primers used for reverse transcription and amplification.

Purpose	Primers (5′-3′)	
	Forward	Reverse
Fragment 1	ATTGAATTACCAATAAACTAC	GAATATCGTTGTATTCAGTT
Fragment 2	TACACCACACAACAACCATC	GCTTGTTCTTGGAGTGTGTTG
Fragment 3	GATATCGAAGAGGAAGTAAC	GTTAACTGGTAGGGTGGTTA
Fragment 4	CAAAACCTTCCCGTAAACGC	GAACAACAATGCCAAGATCA
Fragment 5	GAACAATCGAAGATGTTCAAG	GGAAAGTCTTGGTTGGGAGG
Fragment 6	CAGGTTTCATTCCAGAAGGC	CTGTCAGAAAATCAAGGAC
Fragment 7	CGATTTAACAACAGAACACAC	GAGAAGATAACCGGGAATG
Fragment 8	CAACCCATCATTCCAACCAC	GTAAGATGTTGTAGCGAGTG
Fragment 9	CAATACGATCTCGACATGCTC	GTTACCATATGCTGTTGATG
Fragment 10	CTGAACCTGGCACAATAGTC	GTTGGATTGAGAATCTGTAG
Fragment 11	CTCGGACAACTTATCATAAC	GTGGTTGTTGGCGGTGTGTAG
Fragment 12	CGCTCAAATTAACACGACAC	GCATCGTAGTAAAAGGTTG
Fragment 13	CTACAACCACTAATCTTGAAC	GATGAAATCAGAGTGGACTG
Fragment 14	GTAACATCGCAGTGGCATTG	TTTTTAGATCTTATAGGATAG

split into four major branches (each branch represents one subfamily): one encompassing *Oncotshavirus* YCBV, CSV and (Atlantic salmon bafinivirus, ASBV) and *Bafinivirus* (FHMNV and WBV), one containing *Torovirus* (bovine torovirus, BToV; equine torovirus, EToV; and porcine torovirus, PToV), and the *Pregotovirus* ball python nidovirus 1 (BPNV) and *Bostovirus* bovine nidovirus 1 (BNV) formed two separate clusters.

Multiple sequence alignments and amino acid sequence identity were performed using ClustalW in DNAstar 5.0. As shown in Table 2, the predicted amino acid sequence identity of the full length pp1ab, S, M, and N genes of YCBV is highly related to CSV and ASBV, with amino acid identity between 87.0% and 98.4% and medium homology (8.7%–40.2%) to the two known *Bafinivirus* strains WBV and FHMNV. The deduced amino acid sequences encoded by YCBV were less related to those of *other Tobaniviruses*, with identities between 7.4% and 18.4%. Strikingly, the S protein of YCBV shares the lowest homology (8.7%) with that of FHMNV and the N protein of YCBV has relative high sequence homology (18.4%) with that of BPNV. The inconsistent homology results indicate that genetic recombination might have occurred between *Tobaniviruses*.

The Amino acid homology of pp1ab, S, M and N proteins between CSV and ASBV is 99.4%, 98.3%, 97.1% and 100% respectively. The Amino acid homology between CSV and ASBV is higher than that between YCBV and CSV or YCBV and ASBV. The full-long genome sequence homology among YCBV, CSV and ASBV is 95.2% (YCBV/CSV), 95.4% (YCBV/ASBV) and 99.2%(ASBV/CSV). These results show that CSV and ASBV has a close evolutionary relationship.

Multiple sequence alignment of the full-long genomic sequences of YCBV, CSV and ASBV showed that the nucleotide acid sequence in region 1187-2214 nt is very different from each other (Supplementary Fig. 1). In this region, there are several deletions including 2-nt (AA) between 1187 and 1188 nt, 3-nt (GAA) between 1995 and 1996 nt, 3-nt (CGA) between 2005 and 2006 nt, 3-nt (ATC) between 2055 and 2056 nt, and 4-nt (TCGC) between 2213 and 2214 nt. There is one 2-nt (CT) insertion between 1223 and 1224 nt. The sequence homology in region 1187-2214 nt between YCBV and CSV is only 63.7%. However, the full-long genome sequence homology between them is 95.2%. The full-long genome sequence homology between CSV and ASBV is 99.2%. Of note, there is one fragment (543 nt) deletion in this region of ASBV (Supplementary Fig. 1). Taken together, sequence alignment of the full-long genomic results demonstrated that the nucleotide acid sequence in region 1187-2214 nt is highly variable.

Syncytium formation is an important biological phenotype of several enveloped viruses. The FHMNV can cause syncytia in EPC cell line (Faisal et al., 2016). In this study, CPE caused by YCBV was clear, but no syncytium was observed. The furin cleavage site (RXXR) is associated with syncytium formation associated with CPE, and it is the best known among viruses in the families *Paramyxoviridae* and *Pneumoviridae*(Liu et al., 2014). The S glycoprotein of FHMNV has a putative furin cleavage site (RKKR; residues 803–806) (Batts et al., 2012). A similar putative furin cleavage site (RTRR; residues 801–804) has also been identified in the S protein of YCBV. The two amino acids in the middle of the cleavage site "RXXR" are variable. The variable amino acids in these two sites can lead to different phenotypes for fusion promotion. For example, the difference in the two amino acids in these two sites of metapneumovirus (family *Pneumoviridae*) resulted in different fusion promotion conditions. Trypsin is required for avian metapneumovirus subtype B, but not the subtypes A and C, for fusion (Wei et al., 2012). In fact, trypsin is not only essential for fusion promotion of human metapneumovirus, but also for viral growth in cell culture (Biacchesi et al., 2006; Schowalter et al., 2006). To determine the role of trypsin in YCBV fusion, we analyzed syncytium formation ability of YCBV in the presence or absence of trypsin. The infected cells were incubated in M199

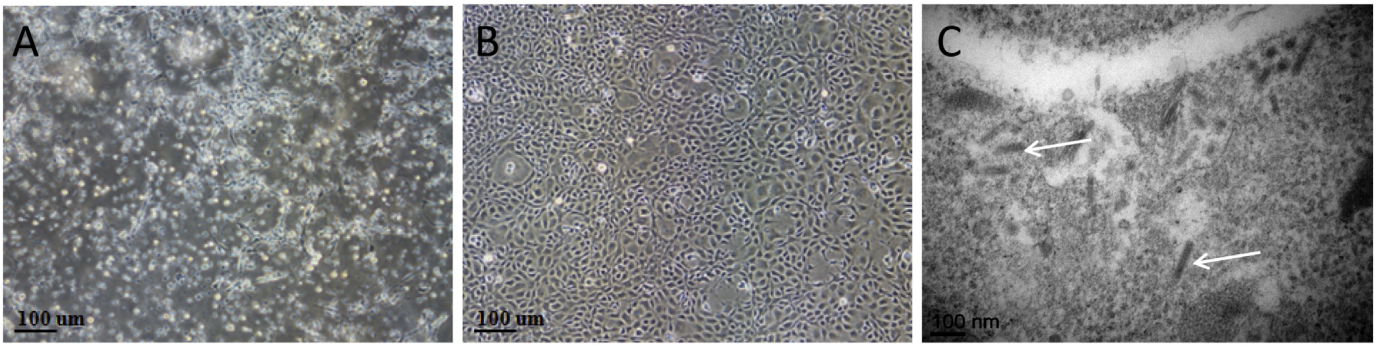

Fig. 1. (A) Phase contrast microscopy showing CPE in YCBV-infected cells at 72 h post-inoculation in the first passage, (B) uninfected EPC cells, (C) electron microscopic image of the YCBV isolate by phosphotungstic acid negative staining. Bar = 100 nm.

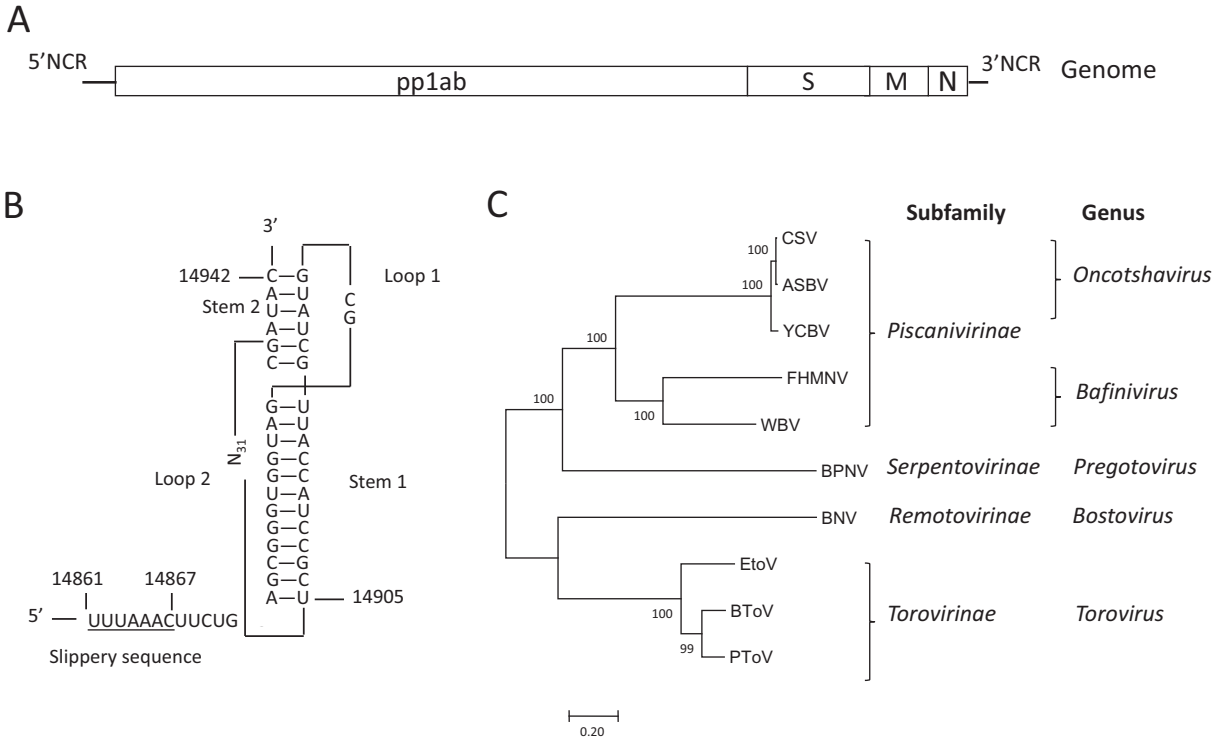

Fig. 2. Genetic characterization of YCBV. (A) Genome organization of YCBV. (B) Model of the YCBV ribosomal frameshifting element is proposed to consist of a putative pseudoknot structure comprising two stems, two loops, and a 'slippery' sequence (^{14861}UUUAAAC^{14867}, underlined). (C) The phylogenetic tree analysis of complete genome sequences of YCBV and other *Tobaniviruses*. The phylogenetic trees were generated by Jotun Hein Method using Mega 7.0 software. The phylogenetic analysis was based on the complete genome sequences, using the neighbor-joining method with 1000 bootstrap replicates. The bootstrap values based on the consensus tree were plotted at the main internal branches to show the support values. The sequences of other *Tobaniviruses* were obtained from GenBank. Bovine *Torovirus* (BtoV, No. LC088095), equine *Torovirus* (EtoV, No. MG996765), porcine *Torovirus* (PtoV, No. JQ860350), ball python nidovirus 1 (BPNV, No. KJ541759), bovine nidovirus 1 (BNV, No. KM589359), Chinook salmon *Bafinivirus* (CSV, No. KJ681496), Atlantic salmon bafinivirus (ASBV, No. KY130432) , white bream virus (WBV, No. DQ898157), and fathead minnow nidovirus (FHMNV, No. GU002364).

Table 2
Protein amino acid sequence identity (%) between YCBV and other *Tobaniviruses.*

Virus	pp1ab	S	M	N
FMNV	30.9	8.7	26.2	21.8
WBV	32.0	40.2	23.3	16.8
BToV	17.0	8.8	9.4	11.0
EToV	16.8	12.6	13.3	9.4
PToV	17.0	11.2	14.6	7.4
BPNV	15.2	9.2	11.6	18.4
BNV	16.5	9.1	11.7	9.6
ASBV	97.1	93.6	96.7	87.0
CSV	96.1	94.6	98.4	87.0

with TPCK-trypsin. Nonetheless, syncytia were not observed (data not shown). This indicates that the entry mechanism of YCBV might be different from that of FHMNV.

Currently, only two species in the genus *Bafinivirus,* the subfamily of *Piscanivirinae* have been identified. Although the genome sequence of species CSV isolated from *Oncorhynchus tshawytscha* in Canada is in GenBank, there is no any report on the phenotype of this virus. To facilitate a better taxonomic understanding of the subfamily of *Piscanivirinae*, more studies are needed to gain further information about the poorly characterized members.

In conclusion, we identified a new bacilliform virus isolated from yellow catfish collected in China. Based on the viral morphology, genome organization, and sequence homology, this newly identified bacilliform virus appears to be a new member in the subfamily *Piscanivirinae.*

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103924.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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