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Complementation System for *Helicobacter pylori*

Jinmoon Kim[1†], Sung-Whan Kim[1†], Sungil Jang[1], D. Scott Merrell[2], and Jeong-Heon Cha[1*]

[1]*Department of Oral Biology, Oral Science Research Center, BK21 Project, Research Center for Orofacial Hard Tissue Regeneration, Yonsei University College of Dentistry, Seoul 120-752, Republic of Korea*
[2]*Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814, USA*

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Previously Langford *et al.* (2006) developed the pIR203C04 complementation system for *Helicobacter pylori*, which can be used to complement and restore phenotypic effects in *H. pylori* mutant, and furthermore they used the complementation system *in vivo* experiments to animals without altering the ability of strain SS1 to colonize mice. In their previous study, the pIR203C04 was able to transform 26695, SS1, J99, and 43504 *H. pylori* strains by an electroporation method. However, in the present study using a natural transformation the pIR203C04 transformed only 26695 *H. pylori* but not SS1, J99, 7.13, and G27 *H. pylori* strains. Since the useful complementation system has a limitation of narrow selection among *H. pylori* strains, we redesigned the complementation system for the improvement. The same intergenic chromosomal site between *hp0203* and *hp0204* was utilized for the new complementation system because the insertion at the intergenic site didn't show any polar effects and disruption of other *H. pylori* genes. The genome sequence analysis showed that the intergenic regions among *H. pylori* strains may have too low homology to each others to do a homologous recombination. Thus, in addition to the short intergenic region, the fragments of the new complementation system included 3′ conserved parts of *hp0203* and *hp0204* coding regions. Between the fragments there are a chloramphenicol acetyltransferase cassette and multicloning sites, resulting in pKJMSH. DNA fragment of the interest can be cloned into the multicloning sites of pKJMSH and the fragment can be integrated at the intergenic region of *H. pylori* chromosome by the homologous recombination. Indeed, by the natural transformation, pKJMSH was able to transform all five *H. pylori* strains of 26695, SS1, J99, 7.13, and G27, which are common for the investigation of molecular pathogenesis. Thus, the new pKJMSH complementation system is applicable to most *H. pylori* wild-type stains.

***Keywords*:** *H. pylori*, intergenic region, complementation system

Helicobacter pylori is a Gram-negative microaerophilic, spiral-shaped bacterium (Marshall and Warren, 1984), which infects over 50% of the world's population, and causes one of the most common infections of mankind (EUROGAST study group, 1993; Matysiak-Budnik and Megraud, 1997). *H. pylori* is an etiological agent of gastritis, peptic ulcer (duodenal and gastric ulcers), and two types of gastric cancers (adenocarcinoma and mucosa-associated lymphoid tissue lymphoma) (Parsonnet *et al.*, 1991; Dunn *et al.*, 1997; Blaser 1998; Covacci *et al.*, 1999; Ernst and Gold, 2000). *H. pylori* is classified as class I carcinogen by World Health Organization because of the association of *H. pylori* and gastric cancer (Neugut *et al.*, 1996; Ernst and Gold, 2000). High prevalence and association of gastric cancer give the second most common cause of cancer-associated death (Neugut *et al*., 1996). It has been proposed that the virulence factors of the infecting strains are associated with the severity of the clinical outcome. Also, an extensive genetic diversity of *H. pylori* contributes important in pathogenesis.

Molecular biological research on *H. pylori* pathogenesis is important to find out the mechanism of gastric disease development including gastric cancer. Scientists have tried intensively to develop animal models of *H. pylori* infection, and recently the animal models of mouse and gerbil are available for *H. pylori in vivo* research (Hirayama *et al.*, 1996; Arnold *et al*., 2011). However, only certain *H. pylori* strains, 7.13 for gerbil and PMSS1 for mouse, can be used for *in vivo* research. Also, the extensive genetic diversity of *H. pylori* makes the selection of *H. pylori* strain limited and difficult even for the *in vitro* research. Other limitation of research on *H. pylori* pathogenesis would be a lack of useful tools for molecular biology such as the complementation system. Previously Langford *et al.* (2006) developed the pIR203C04 complementation system for *H. pylori*. They complemented, and restored phenotypic effects in arginase mutant *H. pylori*, and furthermore used the pIR203C04 complementation system *in vivo* experiments to animals without altering the ability of strain SS1 to colonize mice. When they used an electroporation method to introduce the pIR203C04 complementation system, of *H. pylori* strains 26695, SS1, J99, and 43504 could be transformed. Another transformation method is a natural transformation that is the most common for *H. pylori* research because a special equipment is not required unlike the electroporation. Unfortunately, the natural transformation of pIR203C04 failed with G27 and 7.13 *H. pylori* strains and it gave us a rationale to redesign the complementation system to be appli-

† These authors contributed equally to this work.
* For correspondence. E-mail: Jcha@yuhs.ac; Tel: +82-2-2228-3061; Fax: +82-2-2227-7903

Table 1. Primer sequences

Primer	Sequence
IR1	GGgagctcAAGTCGCAAAGCAGTTATTGCAGCG (a)
IR2	AAAATATTAActcgagCCTggatccTTTATCTATACTTTACTTGG (b, c)
IR3	TATAGATAAAggatccAGGctcgagTTAATATTTTTTTAGTCTGT (c, d)
IR4	GGgtcgacTTGTGTGGGGGCAAAGGATTTGG (d)
HP 0203-F	GATTGACTTGGGGTTCAGCGTTGGTG
HP 0204-R	GTTTGAGCTTGCTAATGATAAGCGG

Restriction enzyme sites (a, *Sac*I; b, *Xho*I; c, *Bam*HI; and d, *Sal*I) were inserted in the primer sequences.

Table 2. Transformation of pIR203C04

DNA (method) \ Strain	26695	SS1	J99	7.13	G27	reference
pIR203C04 (Natural transformation)	7.0×10^{3}[a]	0	0	0	0	This study
pIR203C04 (Electroporation)	2.2×10^{-4}[b]	$< 10^{-10}$	$< 10^{-10}$	NA	NA	Langford *et al*. (2006)

[a] Transformation efficiency
[b] Transformation frequency; NA, not applicable

cable to more *H. pylori* strains containing G27 and 7.13.

Materials and Methods

H. pylori strains and culture conditions

All *H. pylori* strains (26695, G27, 7.13, SS1, and J99) were preserved at -80°C as stocks, grown, and cultured on antibiotic-supplemented horse blood agar plates under microaerophilic conditions created by an Anoxomat evacuation/replacement system (Sprial Biotech, USA) as previously described (Carpenter *et al.*, 2007; Jones *et al.*, 2009, 2011; Jang *et al*., 2010). For liquid cultures, brucella broth (Acumedia, USA) containing 10% fetal bovine serum (Invitrogen, USA) and 10 μg/ml vancomycin (AMRESCO, USA) was used and grown under the microaerophilic conditions.

Preparation of plasmid constructs

Chromosomal bacterial DNA was extracted using the Wizard Genomic DNA Purification kit (Promega, USA). PCR amplifications were carried out in a DNA thermal cycler (Biometra, Germany) using the Expand High Fidelity PCR System (Roche Applied Science, Germany) as follows: 1 cycle at 95°C for 2 min; 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; a final elongation step at 72°C for 10 min. Primer sequences are listed in Table 1. An QiaQuick Gel Extraction kit (QIAGEN, Germany) was used for the extraction of PCR fragments from 1% agarose gel. Restriction enzymes of *Sac*I, *Bam*HI, *Xho*I, and *Sal*I were purchased from Promega (USA). A ligation was performed using Rapid DNA Ligation kit (TaKaRa, Japan). For the transformation of pKJM and pKJMSH to *Escherichia coli*, DH5α strain was used. Plasmid DNA was isolated by Plasmid DNA Purification kit (iNtRON, Korea).

Natural Transformation for *H. pylori*

H. pylori was transformed with 1 μg plasmid by natural transformation as described earlier (Clayton and Mobley, 1997) with the following modification (Kim *et al.*, 2000, 2009). The *H. pylori* strain was struck and incubated for 3 days. Sweeps of the bacteria colonies from the initial plate were then restruck on a fresh Columbia blood agar plate without chloramphenicol antibiotics and incubated. After 24 h, these cells were inoculated as a circle on a fresh Columbia blood agar plate with four 1-inch scrapes. After 6 h, 1 μg DNA of pIC203C04 and pKJMSH was added to the *H. pylori* circle. After 24 h, the whole circle was restreaked onto Columbia blood agar containing 25 μg/ml chloramphenicol antibiotics and incubated for 3-5 days until transformants appeared. Transformation efficiency was determined by calculating the number of transformant colonies per microgram of DNA (CFU/μg of DNA).

DNA sequence analysis

The DNA sequences were analyzed using Vector NTI version 9.1 (Invitrogen, USA) and Sequencher 4.5 (Gene Codes Corp., USA). The accession numbers of GenBank for 26695, SS1, J99, and G27 were AE000511, DQ538311, AE001439, and CP001173, respectively. The DNA sequence of 7.13 strain was kindly provided by Richard M. Peek Jr. (Vanderbilt University, USA)

Results

Delivery of pIR203C04 complementation system to various *H. pylori* via natural transformation

To determine whether the pIR203C04 complementation system can transform 26695, SS1, J99, 7.13, and G27 *H. pylori* strains, the method of standard natural transformation was used. The

Table 3. Natural Transformation of pKJMSH

DNA \ Strain	26695	SS1	J99	7.13	G27
pKJMSH	8.7×10^{3}	1.9×10^{3}	0.3×10^{3}	4.1×10^{3}	1.5×10^{4}

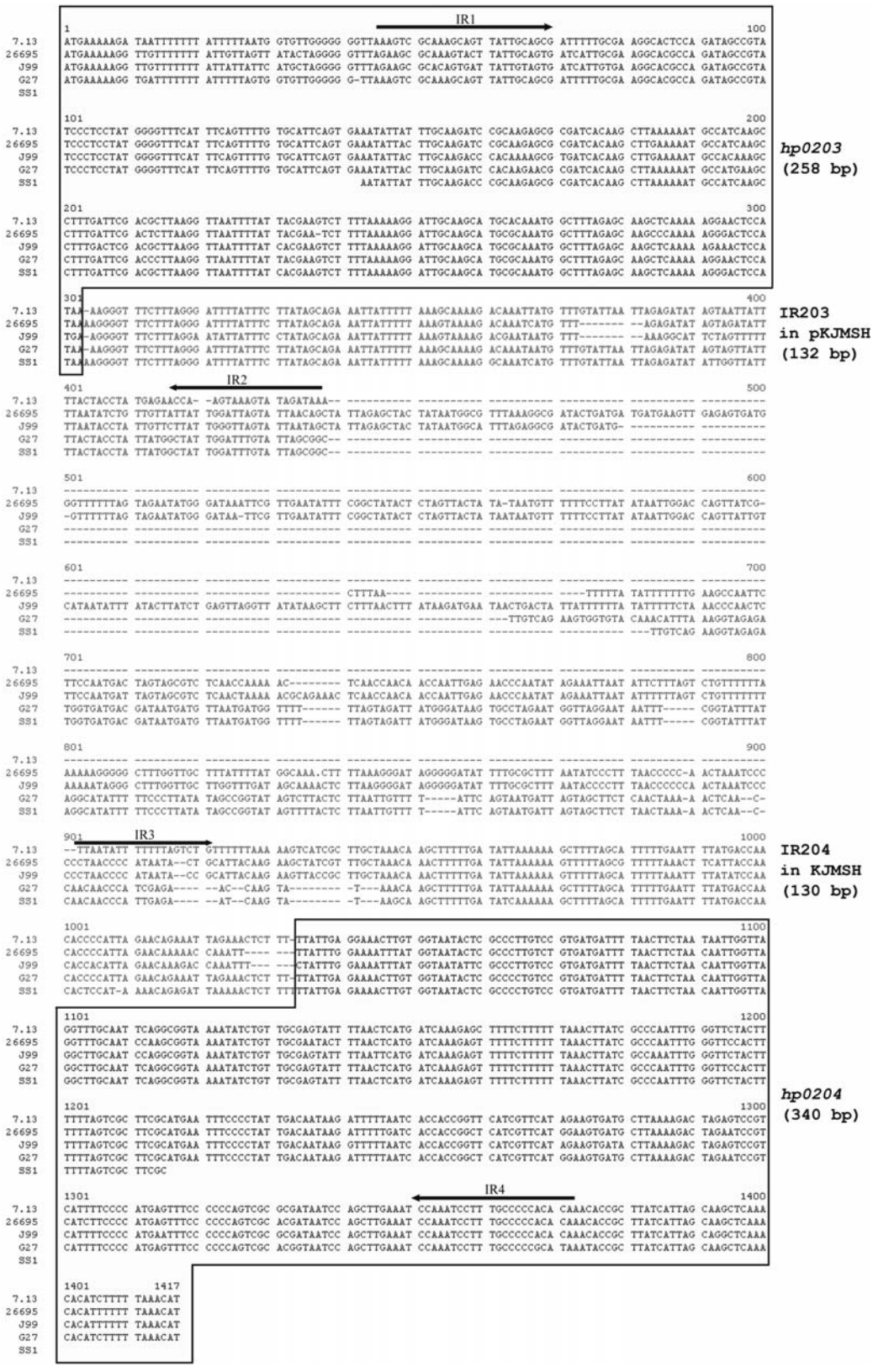

Fig. 1. Comparison of DNA sequences in *hp0203* and *hp0204* including intergenic region of *H. pylori* wild type strains of 7.13, 26695, J99, G27, and SS1. The structural genes of *hp0203* and *hp0204* are indicated with gray boxes. IR203 and IR204 were indicated on the right side. The arrows indicate primers used for construction of fragments in the pKJMSH. The sequence of SS1 started at 143 nucleotides and ended at 1215 nucleotides because there is no available sequence at the flanking regions. A dash means a missing nucleotide to match.

transformation efficiency was shown in Table 2. The natural transformation showed the different result from the previous result of the electroporation method (Langford *et al.,* 2006). Only 26695 *H. pylori* was successfully transformed by pIR203C04 via the natural transformation but the other SS1, J99, 7.13, and G27 *H. pylori* strains failed. It gave us a rationale to redesign the complementation system to be applicable to more *H. pylori* strains.

Variation of intergenic region in different *H. pylori*

To identify a problem why SS1, J99, 7.13, and G27 *H. pylori* strains failed to take up pIR203C04 by the natural transformation, the DNA sequence alignment of the intergenic region was performed (Fig. 1) because the system requires the homologous recombination. The intergenic region of pIR203C04 shares the perfect match with 26695 *H. pylori* because 26695 *H. pylori* genome was template to construct pIR203C04. However, except approximately 130-bp intergenic region near to *hp0204*, SS1, J99, 7.13, and G27 *H. pylori* strains showed low DNA sequence homology to that of 26695 *H. pylori*. Especially the intergenic regions between *hp0203* and *hp0204* of 7.13 and G27 are short because of the deletion. This made us postulate that the natural transformation was problematic due to the low homology between the intergenic regions of *H. pylori* genome and pIR203C04.

Because the intergenic regions of 7.13 and G27 may not be enough for the homologous recombination, the conserved 3′ parts of coding regions of *hp0203* and *hp0204* were included in the fragments of newly designed construct. To construct the new complementation system, the PCR amplicons with a template of 7.13 chromosomal DNA using primer sets of IR1 and IR2, and of IR3 and IR4 (Fig. 2A) were gel-extracted and used as a template for PCR of Splicing by Overlapping Extension (SOE) ing with primers of IR1 and IR4. The PCR SOEing amplicon was cloned into pGEM T-easy vector and the resulting plasmid was digested with *Sal*I and self-ligated to make *Pst*I site unique in the construct, resulting in pKJM (Fig. 2B). The pIR203C04 was digested with *Bam*HI and *Xho*I restriction enzymes to get a DNA fragment of a *cat* containing multicloning sites. The *Bam*HI and *Xho*I sites of the pKJM were used to clone the DNA fragment containing a *cat* with multicloning sites, resulting in pKJMSH.

Delivery of the pKJMSH complementation system to various *H. pylori* strains via natural transformation

To determine whether the new pKJMSH complementation system can transform 26695, SS1, J99, 7.13, and G27 *H. pylori* strains, the method of standard natural transformation was used. The transformation efficiencies to 26695, SS1, J99, 7.13, and G27 *H. pylori* strains were 8.7, 1.9, 0.3, 4.1, 14.9×10^3 CFU/μg of DNA, respectively. The correct integration location at the intergenic site was confirmed by PCR with two sets of primers; HP0203-F and IR2, and IR3 and HP0204-R. The primers of HP 0203-F and HP0204-R are aligned at

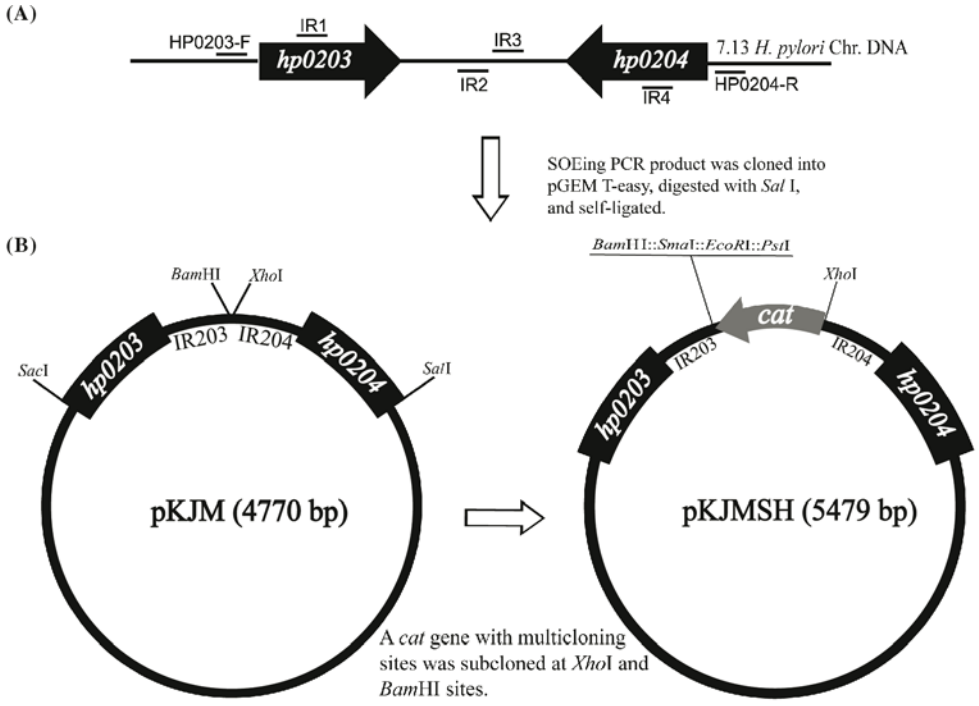

Fig. 2. Construction of pKJMSH complementation system. (A) Wild-type 7.13 *H. pylori* chromosome region of *hp0203* and *hp0204* genes including the intergenic regions IR203 and IR204. The intergenic region was divided into two fragments for cloning purposes indicated by primers of IR2 and IR3. (B) A 400-bp amplicon using primers of IR1 and IR2 and a 480-bp amplicon using primers of IR3 and IR4 were cloned by SOEing PCR into pGEM T-easy vector. (C) The DNA fragment containing a *cat* with multicloning sites (MCS) was from the pIR203C04 by digestion of *Bam*HI and *Xho*I restriction enzymes. The cat with MCS was cloned at *Bam*HI and *Xho*I sites of the pKJM, resulting in pKJMSH. Arrow indicates a direction of transcription for the coding regions.

hp0203 and *hp0204* coding regions, respectively, but outside from the construct fragments (Fig. 2). All five wild-type *H. pylori* used in this study were able to incorporate the pKJMSH complementation system at the intergenic site of *H. pylori* gene. When the transformation efficiencies of 26695 with pIR203C04 and pKJMSH were compared, pIR203C04 has approximately 2.5-fold better transformation efficiency. The results suggested that by natural transformation, pKJMSH can transform *H. pylori* strains of 26695, SS1, J99, 7.13, and G27 which are common for the investigation of molecular pathogenesis. Thus, the pKJMSH complementation system is applicable to more *H. pylori* wild-type stains than the pIR203C04.

Discussion

Langford *et al.* (2006) developed the pIR203C04 complementation system that is useful for identifying functions of specific genes *in vitro* and even *in vivo* studies. Their data using this system convinced us to apply this system to G27 for *in vitro* experiment and to 7.13 for *in vivo* gerbil experiment. G27 *H. pylori* strain is one of the common strains for *H. pylori in vitro* research, and 7.13 *H. pylori* strain is a gerbil-adapted strain for the gerbil infection. However, as this study indicated, when the natural transformation is used, their complementation system is limited to use for narrow range of *H. pylori* strains; only 26695 *H. pylori* among five common strains can be used. Because Langford *et al.* (2006) used 26695 *H. pylori* chromosomal DNA to construct the intergenic region of pIR203C04, the perfect match of intergenic region between pIR203C04 and *H. pylori* chromosomal DNA may increase the possibility of homologous recombination event and thus only 26695 *H. pylori* can be transformed by the natural transformation. Even though we redesigned the complementation system of pIR203-C04 for application to wider range of *H. pylori* strains containing 7.13 and G27, the same intergenic chromosomal site in *H. pylori* was utilized because it has been proved that the insertion to the intergenic site can be done without the polar effects and disruption of other *H. pylori* genes. Thus, to improve the efficiency of homologous recombination to various *H. pylori*, the conserved regions among these *H. pylori* strains were selected and employed to construct the new complementation system. The fragments of the new pKJMSH complementation system contain some intergenic regions and the conserved 3′coding sequences of *hp0203* and *hp0204* because the intergenic regions of 7.13 and G27 chromosomal DNA share too low homology with the intergenic fragments of other *H. pylori* strains to do the homologous recombination especially because the intergenic region near to *hp0204* in 7.13 and G27 *H. pylori* genomes was deleted mostly and showed poor homology. Since their coding regions have the similar homology to the fragments of pKJMSH, the different transformation efficiency may reflect the competency of each *H. pylori* strain for the natural transformation. It has been known that G27 has high transformation efficiency and it was the case in this study. The transformation efficiencies of pIR203C04 to 26695 *H. pylori* was an approximately 2.5-fold higher than that of pKJMSH probably because the fragment of pIR203C04 has higher homology to the longer region of 26695 *H. pylori* genome.

The transformants were selected by the resistance to chloramphenicol antibiotics, and therefore the transformants were able to express the *cat* gene that is located between the fragments of pKJMSH. It suggests that the gene integrated at the intergenic region in all five *H. pylori* strains has no problem to express. Therefore, the new pKJMSH complementation system may keep all strong points of pIR203C04 with better selection for *H. pylori* strains.

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