Functional analysis of the second methyltransferase in the bacteriophage exclusion system of *Lactobacillus casei* Zhang

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ABSTRACT

The antiphage ability is an important feature of fermentation strains in the dairy industry. Our previous work described the bacteriophage exclusion (BREX) system in the probiotic strain, *Lactobacillus casei* Zhang. The function of *L. casei* Zhang *pglX* gene in mediating 5'-ACRC^{me6}AG-3' methylation was also confirmed. This study aimed to further dissect the function of the BREX system of *L. casei* Zhang by inactivating its second methyltransferase gene (*LCAZH_2054*). The methylome of the mutant, *L. casei* Zhang $\Delta 2054$, was profiled by single-molecule real-time sequencing. Then, the cell morphology, growth, plasmid transformation efficiency, and stability of the wildtype and mutant were compared. The mutant did not have an observable effect in microscopic and colony morphology, but it reached a higher cell density after entering the exponential phase without obvious increase in the cell viability. The mutant had fewer 5'-ACRC^{me6}AG-3' methylation compared with the wildtype (1835 versus 1906). Interestingly, no significant difference was observed in the transformation efficiency between the 2 strains when plasmids without cognate recognition sequence (pSec:Leiss:Nuc and pG⁺host9) were transformed, contrasting to transforming cells with cognate recognition sequence-containing plasmids (pMSP3535 and pTRKH2). The efficiency of transforming pMSP3535 into the *LCAZH_2054* mutant was significantly lower than the wildtype, whereas an opposite trend was seen in pTRKH2 transformation. Moreover, compared with

the wildtype, the mutant strain had higher capacity in retaining pMSP3535 and lower capacity in retaining pTRKH2, suggesting an unequal tolerance level to different foreign DNA. In conclusion, *LCAZH_2054* was not directly responsible for 5'-ACRC^{me6}AG-3' methylation in *L. casei* Zhang, but it might help regulate the function and specificity of the BREX system.

Key words: *Lactobacillus casei* Zhang, bacteriophage exclusion system, phage infection, methyltransferase, transformation efficiency

INTRODUCTION

Bacteriophages are the most numerous organisms on Earth. They perpetuate by infecting specific bacterial or archaeal hosts (Sharma et al., 2017). Bacteriophages are generally considered harmful although they do not directly cause human diseases. Many phages carry antibiotic genes in their genomes and may facilitate the spread of antibiotic resistance genes between bacteria via horizontal gene transfer. Moreover, some of them can infect food starter cultures and decrease the efficiency of food fermentation, ultimately resulting in significant economic loss (Colavecchio et al., 2017).

In contrast, phage infection pressure has led to bacterial evolution of a variety of defense mechanisms that prevent bacterial extinction (Labrie et al., 2010). The main bacterial defense mechanisms include (1) virus receptor variation, (2) dormancy induction and programmed cell death, and (3) cell immunity (Makarova et al., 2012, 2013). Although some previous research has attempted to investigate the nature and components of these defense systems, our knowledge of their mechanisms of action remains incomplete, particularly for the newly reported Zorya and Thoeris defense systems (Doron et al., 2018). To minimize undesirable economic loss caused by phage infection, it would be desirable to select food starter strains that exhibit antiphage features. Thus, identifying the variety of such defense

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systems and gaining a thorough understanding of their molecular mechanisms would be of interest.

Epigenetic modifications such as DNA methylation allow bacteria to recognize genetic materials of self from nonself. In bacteria, DNA methyltransferases “mark” the DNA with specific methylation, and the most common prokaryotic methylation signatures are m5c, m4c, and m6a. Bacterial methyltransferases can be divided into 2 broad groups. Most methyltransferases are components of antiphage systems like restriction-modification systems or bacteriophage exclusion (BREX) systems, whereas orphan methyltransferases lack cognate restriction enzymes and have other cell functions (Marinus and Casadesus, 2009). The BREX system is widely distributed across prokaryotes, and it confers host resistance to phages and foreign plasmids by blocking phage absorption onto bacterial cell surface or phage replication (Goldfarb et al., 2015). Most BREX systems consist of 6 genes, including a Lon-like protease (coding gene *brxL*), an alkaline phosphatase-domain protein (coding gene *pglZ*), an RNA-binding protein (coding gene *brxA*), a DNA methylase (coding gene *pglX*), an ATPase-domain protein (coding gene *brxC*), and a hypothetical protein (coding gene *brxB*; Goldfarb et al., 2015). Gene variations have been reported in some BREX systems, some of which consist of more than one methyltransferase. For example, the BREX system of *Methanobrevibacter smithii* has 5 truncated *pglX* genes locating near the full-length *pglX* gene (Goldfarb et al., 2015). The *pglX* gene is essential for proper functioning of the BREX system. Deleting the *pglX* gene would abolish the antiphage effect and the modification of genomic DNA, whereas the phenotype could be rescued by gene complementation (Gordeeva et al., 2019). However, the functions of the truncated methyltransferase genes are unclear.

Lactobacillus casei Zhang is a well-characterized probiotic strain that was isolated from koumiss collected in Inner Mongolia, China (Zhang et al., 2010). This strain has shown a wide range of beneficial functions (Xu et al., 2019; Hou et al., 2020). Our previous work has identified the BREX system of *L. casei* Zhang, verified the methyltransferase activity of the *pglX* protein, and identified the corresponding methylation recognition site (5'-ACRCAG-3'; Hui et al., 2019). The BREX system of *L. casei* is not a classical 6-gene system because it contains a second methyltransferase (*LCAZH_2054*), locating between the *pglX* and *pglZ* genes. This work aimed to characterize the function of *LCAZH_2054* by constructing a gene deletion mutant. The phenotype was characterized by single-molecule real-time (SMRT) sequencing and multiple plasmid transformation assays.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions

Strains and plasmids used in this study were listed in Table 1. *Lactobacillus casei* Zhang was provided by the Lactic Acid Bacteria Cell Collection of the Inner Mongolia Agricultural University, China. The bacterial host strain, *Escherichia coli* DH5 α , was used for plasmid cloning. The *L. casei* wildtype and mutant strains were propagated anaerobically in de Man, Rogosa, and Sharpe (MRS) broth at 37°C with or without antibiotics accordingly. Chloramphenicol (10 μ g/mL) or erythromycin (10 μ g/mL erythromycin) was used for selecting the *L. casei* mutants. Luria-Bertani broth was used to cultivate *E. coli* strains aerobically at 37°C. Chloramphenicol (10 μ g/mL) or erythromycin (250 μ g/mL) was used to select for plasmid-transformed *E. coli*.

Reagents Used for DNA Manipulations

Genomic DNA of *L. casei* was purified by using a DNA extraction kit (Qiagen). Plasmid DNA was isolated using the E.Z.N.A Plasmid Mini Kit (Omega Bio-Tek). Primers used in this study were listed in Table 1. Phusion High-Fidelity DNA polymerase (ThermoFisher) was used in PCR for vector construction, whereas rTaq DNA polymerase (TaKaRa) was used in PCR for verification of successful cloning. Fragments of DNA were purified by using the DNA purification Gel Extraction Kit (Omega) or Cycle-Pure Kit (Omega). Restriction enzymes and T4 DNA ligase were purchased from TaKaRa Ltd.

Construction of Gene Deletion Mutant

To inactivate the *LCAZH_2054* gene, a plasmid containing the homology arm of *LCAZH_2054* was first constructed. The upstream (~1 kb) and downstream (~1.1 kb) sequences of *LCAZH_2054* were amplified by PCR and cloned into the *Xho* I or *Pme* I and *Eco*53 KI or *Bgl* II restriction sites of pNZ5319 (Lambert et al., 2007), respectively, generating the vector pNZ5319–2054. One microgram of pNZ5319–2054 was transformed into wildtype *L. casei* Zhang cells to generate the *lox66*-P₃₂-cat-*lox71* replacement mutant. The target double-crossover clones, chloramphenicol resistance and erythromycin sensitive, were selected by replica plating and verified by PCR. To excise the selection marker (the P₃₂-cat sequence), 1 μ g of Cre expression vector pMSPcre (Xin et al., 2017) was transformed into the selected double-crossover clones. Cre protein expression was induced by nisin, and the cells were plated

Table 1. Strains, plasmids, and primers used in this study; underlined portions of primer sequences indicate restriction sites¹

Strain, plasmid, and primer	Strain and plasmid description, primer sequence	Reference, strain and plasmid source, and restriction enzyme site
Strain		
<i>Lactobacillus casei</i>		
Zhang	Wildtype strain	This study
Zhang $\Delta 2054$	<i>LCAZH_2054</i> gene mutant strain	
<i>Escherichia coli</i>		
DH5 α	Subcloning host	Novagen
XL1-Blue	Subcloning host	Novagen
Plasmid		
pNZ5319	Cm ^r Em ^r ; containing <i>lox66</i> -P ₃₂ - <i>cat</i> - <i>lox71</i> cassette for multiple gene replacement	Lambert et al., 2007
pNZ5319- <i>2054</i>	Cm ^r Em ^r ; pNZ5319 derivative containing homologous regions up- and downstream of <i>LCAZH_2054</i>	This study
pMSPcre	pMSP3535 derivative, expresses Cre under <i>PnisA</i> control	Xin et al., 2017
pMSP3535	Erm ^r	Bryan et al., 2000
pTRKH2	Erm ^r	O'Sullivan and Klaenhammer, 1993
pSec:Leiss:Nuc	Cm ^r	Ribeiro et al., 2002
pG ⁺ host9	Erm ^r	Biswas et al., 1993
Primer		
2054upF	CCGCTCGAGATCAGCCACTTCACAGCG	<i>Xho</i> I
2054upR	AGCTTTGTTTAAACAAGTCCTTAATGACACCC	<i>Pme</i> I
2054downF	GGGTTTGAGCTCATTCACTTAGGGGGTTGATTTTTT	<i>Eco53K</i> I
2054downR	GAAGATCTGTTGAGGATCGCCCTTTT	<i>Bgl</i> II

¹Chloramphenicol resistance, Cm^r; erythromycin resistance, Erm^r.

on erythromycin-containing MRS agar. The P₃₂-cat excision was verified by PCR, and the Cre expression vector was cured by growing the P₃₂-cat-PCR-negative cells in the absence of erythromycin.

To transform *L. casei* Zhang or its derived strains, 200 μ L of preculture was statically incubated at 37°C in 5 mL of MRS broth containing 10 g/L glycine and 0.75 M. When the optical density at 600 nm (OD₆₀₀) reached 0.3 to 0.6, bacterial cells were kept on ice for 30 min and collected by centrifugation for 10 min at 6,000 $\times$ g at 4°C. The cell pellets were then washed twice by ice-cold wash solution (1 M sucrose, 3.5 mM MgCl₂) and resuspended in 40 μ L of wash solution. One microgram of plasmid was mixed with the competent cells and kept on ice for 10 min, followed by electroporation with a BioRad Genepulser (BioRad, 2,000 V, 25 μ F, 400 Ω). Then, 1 mL of SMRS (MRS with 0.1 M MgCl₂ and 0.5 M sucrose) was added to the cuvette, and the electroporated cells were recovered at 37°C for 2 h before plating on chloramphenicol- or erythromycin-containing MRS.

Analysis of Methylome by SMRT Sequencing Technology

The total genomic DNA of the mutant strain, *L. casei* Zhang $\Delta 2054$, was extracted. SMRTbell libraries were prepared as described previously (Hui et al., 2019). Sequencing was performed on a PacBio RS II platform using standard protocols for short and long insert libraries. The bacterial methylome was analyzed

by the PacBio SMRT Portal analysis platform. The RS_Modification_and_Motif_Analysis.1.4 protocol was selected to identify the positions and motifs of methylation in the genome.

Cell and Colony Morphology

Pure cultures of the wildtype and mutant strains isolated from MRS plates were picked based on colony morphology, and the cell morphology of the wildtype and mutant strains was observed after staining with the Gram stain.

Bacterial Growth

The wildtype and mutant strains were cultivated in MRS broth. Bacterial growth curves were constructed by recording the optical density (600 nm), pH, and viable counts at 2-h intervals over a 24-h period. All analyses were performed in triplicate.

Plasmid Transformation Efficiency

To compare the plasmid transformation efficiency between strains, competent cells were prepared when the OD₆₀₀ of the bacteria reached 0.3. Then, 1 μ g of pMSP3535 (Bryan et al., 2000), pTRKH2 (O'Sullivan and Klaenhammer, 1993), pSec:Leiss:Nuc (Ribeiro et al., 2002), or pG⁺host9 (Biswas et al., 1993) was electroporated into the competent cells. After cell recovery in culture medium without antibiotics, cells were

spread on erythromycin-containing MRS agar. The transformation efficiency was expressed as the number of transformants per microgram of DNA. All analyses were performed in triplicate.

Plasmid Stability

The stability of the plasmids (pMSP3535 and pTRKH2) was monitored after being transformed into the wildtype and mutant strains. Plasmid-transformed cells were subcultured in MRS broth with or without erythromycin every 12 h for 96 h. After the parallel passage, cells were plated on MRS agar with or without erythromycin, respectively. The plasmid stability was calculated as the percentage of the number of colony-forming units observed after passage in erythromycin-containing MRS broth divided by the number of colony-forming units grown in MRS broth without antibiotics (Mosrati et al., 1993). All analyses were performed in triplicate.

Statistical Analysis

All data were expressed in mean $\pm$ standard deviation. Significant difference between groups was evaluated by Student's *t*-test at the confidence level of 0.05.

RESULTS

Construction of *L. casei* Zhang $\Delta 2054$

Our previous work analyzed the *pglX* gene in the BREX system in *L. casei*. This study inactivated the second methyltransferase-coding gene of the BREX system, *LCAZH_2054*. The upstream 1,013-bp and downstream 1,095-bp fragments of the *LCAZH_2054* were amplified from the genomic DNA of the wildtype strain. The fragments were gel purified and cloned into the plasmid pNZ5319, which contained the Cre-lox homologous recombination cassette. The resultant pNZ5319-2054 plasmid was transformed into the wildtype cells and the *LCAZH_2054* gene was replaced by a DNA fragment containing the *lox* sites and the chloramphenicol antibiotic resistance biomarker gene after homologous recombination. Then, the Cre expression plasmid was electroporated into the chloramphenicol-resistant transformants to express the Cre enzyme, mediate the *lox* site recombination, and excise the chloramphenicol resistance gene. The mutant strain *L. casei* Zhang $\Delta 2054$ (Figure 1) was then ready for further experiments after the Cre expression plasmid was cured by continuous subculturing in MRS broth without antibiotic.

Methylome Analysis of *L. casei* Zhang $\Delta 2054$

The methylation of the genomic DNA of *L. casei* Zhang $\Delta 2054$ was analyzed by using the PacBio SMRT sequencing technology. A total of 158,738 reads were obtained, and the mean read length was 10,228 bp. The average reference coverage was up to 408.17-fold. The sequencing quality met the data analysis requirements. The genomic sequencing analysis revealed 6-methyladenine (5'-ACRC^{me}AG-3') in both the wildtype and the mutant strains. However, a lower level of methylation was observed in the mutant strain (1835 versus 1906 in the wildtype strain; Figure 2). Therefore, *LCAZH_2054* might have partial influence on genome methylation.

Biological Characteristics of Wildtype and Mutant Strains

The microscopic appearance, colony morphology, and the bacterial growth of the wildtype and mutant were compared. The Gram-stained cells of both strains were similar, which were arranged in short rods, pairs, or chains (Figure 3a, 3b). The colony morphology of both strains was also highly similar, appearing as milky white opaque colonies with smooth surface (Figure 3c, 3d).

The wildtype and mutant strains exhibited similar pattern of growth, as shown by the growth curves plotted based on cell density (OD₆₀₀; Figure 4a), acidity of culture medium (Figure 4b), and viable count (Figure 4c). However, the OD₆₀₀ of the mutant strain was

Figure 1. The PCR validation of *Lactobacillus casei* Zhang $\Delta 2054$, using primers 2054upF and 2054downR. The expected PCR fragment from the mutant type is about ~2.1 kb (lanes 1–3), indicating the length of upstream and downstream homology arms, whereas the unexpected band from the wild type is ~4.3 kb (lane 5), indicating the length of homology arms and the *LCAZH_2054* gene. Lane M1: 1 kb marker; lane M2: DL 5000 marker.

Figure 2. Comparison of 5'-ACRCAG-3' methylation sites of *Lactobacillus casei* Zhang $\Delta 2054$ and *L. casei* Zhang. From outside to inside, the first circle is methylation site of *L. casei* Zhang $\Delta 2054$ (in purple), and the second circle is methylation site of *L. casei* Zhang (in green).

consistently higher than that of the wildtype after the bacterial growth entered the exponential phase (Figure 4a). In contrast, the wildtype strain showed a consistently higher viable count compared with the mutant strain after the bacterial growth entered the stationary phase (Figure 4c).

Plasmid Transformation Efficiency of Wildtype and Mutant Strains

As part of the BREX system, the *LCAZH_2054* gene might be involved in blocking the uptake of foreign DNA. So, the plasmid transformation efficiency of the wildtype and mutant strains were tested. Four plasmids, namely pMSP3535, pTRKH2, pSec:Leiss:Nuc, and pG⁺Host9, were included in the assays (Figure 5). The 2 strains exhibited different plasmid transformation performance, and the efficiency of transformation also varied between plasmids. The transformation efficiency of pMSP3535 in the mutant strain was significantly lower than the wildtype strain ($P < 0.001$), and such trend was reversed for pTRKH2 ($P < 0.01$). For pSec:Leiss:Nuc and pG⁺Host9, no significant difference was observed in the transformation efficiency between the 2 strains.

Plasmid Stability in the Wildtype and Mutant Strains

The function of the BREX system is to recognize self from nonself genetic material by degrading foreign DNA. Thus, the stability of 2 plasmids (pMSP3535 and pTRKH2) was monitored along a 96-h period after the transformation (Figure 6). The number of plasmid-containing cells continuously decreased with time for both plasmids. However, the rate of plasmid loss was higher for pMSP3535 compared with pTRKH2. On the other hand, pTRKH2 was more stable in the wildtype compared with the mutant cells beyond 24 h of transformation, and the difference in stability was maximum at 96 h. Such differences were not observed for pMSP3535.

DISCUSSION

Lactobacillus casei is an important member of lactic acid bacteria. It has the ability to adapt to a wide variety of environments (Wu et al., 2009; Bao et al., 2016) and is thus widely distributed across numerous ecological niches, such as yogurt, cheese, and the digestive tract of humans and animals. *Lactobacillus casei* is often used as a starter culture or an additive in the food industry (Dantas et al., 2016). Phage infection of lactobacilli is one problem for the food industry, as this will cause the failure of fermentation and significant economic loss (Samson and Moineau, 2013). Moreover, phage infection is one mechanism that renders genetic elements mobile, which not only worsens the problem

Figure 3. Gram stain appearance and colony morphology of *Lactobacillus casei* Zhang wildtype (a, c) and mutant $\Delta 2054$ (b, d).

Figure 4. Growth curves of wildtype *Lactobacillus casei* Zhang and mutant $\Delta 2054$ constructed based on optical density at 600 nm (OD₆₀₀; a), pH (b), and viable count (c). Error bars represent SD.

of spreading of antibiotic resistance genes but also leads to the acquisition of these genes by lactobacilli. The BREX system is a bacterial antiphage mechanism that helps resist the invasion of foreign DNA, and it plays an important role in protecting the cells. The probiotic

strain, *L. casei* Zhang, was previously found to contain an atypical BREX system that has 2 genes coding for 2 putative methyltransferases. One of these was the *plgX* gene that was previously characterized to exhibit functional methyltransferase activity (Hui et al., 2019).

Figure 5. Plasmid transformation efficiency of wildtype *Lactobacillus casei* Zhang and mutant $\Delta 2054$. Difference in plasmid transformation efficiency was evaluated with Student's unpaired 2-tailed *t*-test. **, ***Double and triple asterisks represent a significant difference of $P < 0.01$ and $P < 0.001$, respectively. Error bars represent SD.

This study further analyzed the function of the second methyltransferase-coding gene (*LCAZH_2054*) in the BREX system.

This study first constructed the *L. casei* Zhang $\Delta 2054$ mutant, followed by profiling the methylome of the mutant by SMRT sequencing and performing plasmid transformation assays. Both the wildtype and mutant strains exhibited the same type of m6A methylation, 5'-ACRC^{m6}AG-3', utilizing the same methylation recognition sequence and bases (Zhang et al., 2015). However, one mild difference between the 2 strains was the decrease in the number of methylation detected in the genome of the mutant strain (1907 versus 1846 in the wildtype). Ouellette et al. (2015) disrupted the methyltransferase gene, *HVO_A0006*, of *Haloferax volcanii* H26. The resultant mutant not only showed a reduced level of m6A methylation but also exhibited changes in its methylation recognition sequence. Thus, the study concluded that *HVO_A0006* was associated with m6A methylation in *H. volcanii* H26. The current study was only found a slight decrease in m6A methylation in the *L. casei* Zhang $\Delta 2054$ mutant contrasting to abrogation of 95.65% of methylation in the case of deleting the other methyltransferase gene (*pglX*) in *L. casei* Zhang in our previous work (Hui et al., 2019), suggesting that the m6A methylation in *L. casei* Zhang was mainly responsible for the action of the *pglX* gene and *LCAZH_2054* may have played a less direct role in such a process. The mild difference could even represent some level of technical variation in SMRT sequencing.

Generally, DNA methyltransferases in bacteria play important roles in regulating basic cellular biological processes (Vasu and Nagaraja, 2013). Methyltransferases not only participate in the antiphage activity, but some can also act as cell cycle regulators to control basic processes such as chromosome replication, transcription, and repair (Reisenauer et al., 1999). In

Caulobacter crescentus and other α -proteobacteria bacteria, such as *Rhizobium meliloti*, *Agrobacterium tumefaciens*, and *Brucella abortus*, DNA methylation could regulate cell cycle and chromosomal DNA replication (Marczynski, 1999; Collier et al., 2007; Mohapatra et al., 2014). Mutant strains of *Salmonella typhimurium* lacking adenine methyltransferases showed weakened growth, which proved the role of methyltransferase in cell growth regulation (Aloui et al., 2011). However, abrogating DNA methylation in *Helicobacter pylori* did not affect cell and DNA replication, as the bacterial growth was not altered under normal culture conditions (Kumar et al., 2018). Herein, inactivating the *LCAZH_2054* gene did not severely affect the growth of the mutant strain. Yet, it is interesting to note that, in comparison with the wildtype strain, the mutant strain reached a higher cell density after entering the exponential phase without obvious increase in cell viability. The cellular mechanism behind the changes in bacterial growth behavior and the exact molecular role of the *LCAZH_2054* gene in the mutant cells remain to be further elucidated.

Plasmid transformation efficiency and plasmid stability are 2 important indices for assessing the function of methyltransferases (Vasu and Nagaraja, 2013). This study found that the efficiency of transforming pMSP3535 into the *LCAZH_2054* mutant strain was significantly lower than the wildtype strain, whereas an opposite trend was seen in the transformation efficiency of pTRKH2. However, no difference was observed in the plasmid transformation efficiency for another 2 tested plasmids, pSec:Leiss:Nuc and pG⁺host9. The divergent response between the 2 groups of plasmids was likely due to the presence of methylation recognition sequence 5'-ACRCAG-3', which was found in pMSP3535 and pTRKH2 but not pSec:Leiss:Nuc and pG⁺host9. Yet, it is unclear why transforming pMSP3535 and pTRKH2

Figure 6. Plasmid stability after transformation of (a) pMSP3535 and (b) pTRKH2 into wildtype *Lactobacillus casei* Zhang and mutant $\Delta 2054$ cells. Error bars represent SD.

into the mutant cells yielded opposite responses. Similarly, the stability of pMSP3535 and pTRKH2 also varied after being transformed into the mutant cells. These results suggested that *LCAZH_2054* might be involved in regulating the specificity of the function of the BREX system. We speculate that cryptic unidentified sequences might be encoded in these 2 plasmids to regulate downstream responses. The mechanism of functional regulation of the BREX system merits further elucidation.

CONCLUSIONS

By using a combination of gene inactivation technique, SMRT sequencing, and multiple plasmid transformation assays, this work preliminarily explored the function of *LCAZH_2054* in the BREX system of *L. casei* Zhang. Our data suggested that *LCAZH_2054* was not directly responsible for 5'-ACRC^{m6}AG-3' methylation in *L. casei* Zhang, but it might help regulate the function and specificity of the BREX system. The exact role of this gene in regulating the BREX system requires further investigation.

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