

## Construction of carrier state viruses with partial genomes of the segmented dsRNA bacteriophages

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### Abstract

The cystoviridae are bacteriophages with genomes of three segments of dsRNA enclosed within a polyhedral capsid. Two members of this family,  $\Phi 6$  and  $\Phi 8$ , have been shown to form carrier states in which the virus replicates as a stable episome in the host bacterium while expressing reporter genes such as kanamycin resistance or *lacZ*. The carrier state does not require the activity of all the genes necessary for phage production. It is possible to generate carrier states by infecting cells with virus or by electroporating nonreplicating plasmids containing cDNA copies of the viral genomes into the host cells. We have found that carrier states in both  $\Phi 6$  and  $\Phi 8$  can be formed at high frequency with all three genomic segments or with only the large and small segments. The large genomic segment codes for the proteins that constitute the inner core of the virus, which is the structure responsible for the packaging and replication of the genome. In  $\Phi 6$ , a carrier state can be formed with the large and middle segment if mutations occur in the gene for the major structural protein of the inner core. In  $\Phi 8$ , carrier state formation requires the activity of genes 8 and 12 of segment S.

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### Introduction

There are many strategies for packaging viral genomes. Among the options for genomic packaging are the filling of preformed capsid structures with previously synthesized nucleic acid or material that is being synthesized during packaging (Black, 1988), the nucleation of capsid assembly around viral nucleic acid, or the association of genomic elements inside a membrane structure (Enami et al., 1991). Most RNA viruses chose the last two of these three options. Even among the dsRNA viruses, the yeast LA virus-like particle, the only dsRNA eukaryotic system where packaging has been approachable, appears to nucleate the capsid around a plus strand transcript (Wickner, 1993).

$\Phi 6$  is the first member of the cystoviridae to be discovered and it has been studied in great detail (Vidaver et al., 1973). A number of relatives of  $\Phi 6$  have been isolated recently (Mindich et al., 1999). Genomic packaging of these has been found similar to that of  $\Phi 6$ . Bacteriophage  $\Phi 8$  is the

most distant of the cystoviridae from  $\Phi 6$  in sequence and structure (Hoogstraten et al., 2000). We expected that the rules for packaging in  $\Phi 8$  might show some differences from those found for  $\Phi 6$ . The inner core or procapsid of  $\Phi 6$  can be assembled in cells of *Escherichia coli* carrying plasmids with cDNA copies of genomic segment L (Fig. 1). This segment codes for proteins P1, the major structural protein; P2, the polymerase; P4, the packaging NTPase; and P7, an accessory protein involved in both packaging and replication. The inner core particles can be purified and will package and replicate  $\Phi 6$  plus strand transcripts in vitro (Gottlieb et al., 1990). Using this system, it has been possible to work out the basic elements of the packaging of this genome (Mindich, 1999a). Plus strands of  $\Phi 6$  have an 18-base consensus sequence at their 5' ends. They have a *pac* sequence of approximately 200 nucleotides about 50 bases away from the consensus sequence. The *pac* sequences on each segment have no similarity to those on the other segments. The *pac* and consensus sequences are necessary and sufficient for packaging. Empty procapsids can bind and package plus strands of segment S. Plus strand copies of segment M are then packaged and finally those of segment L. Minus strand synthesis begins when all three plus strands are packaged. In vitro packaging shows fairly high stringency for the depen-

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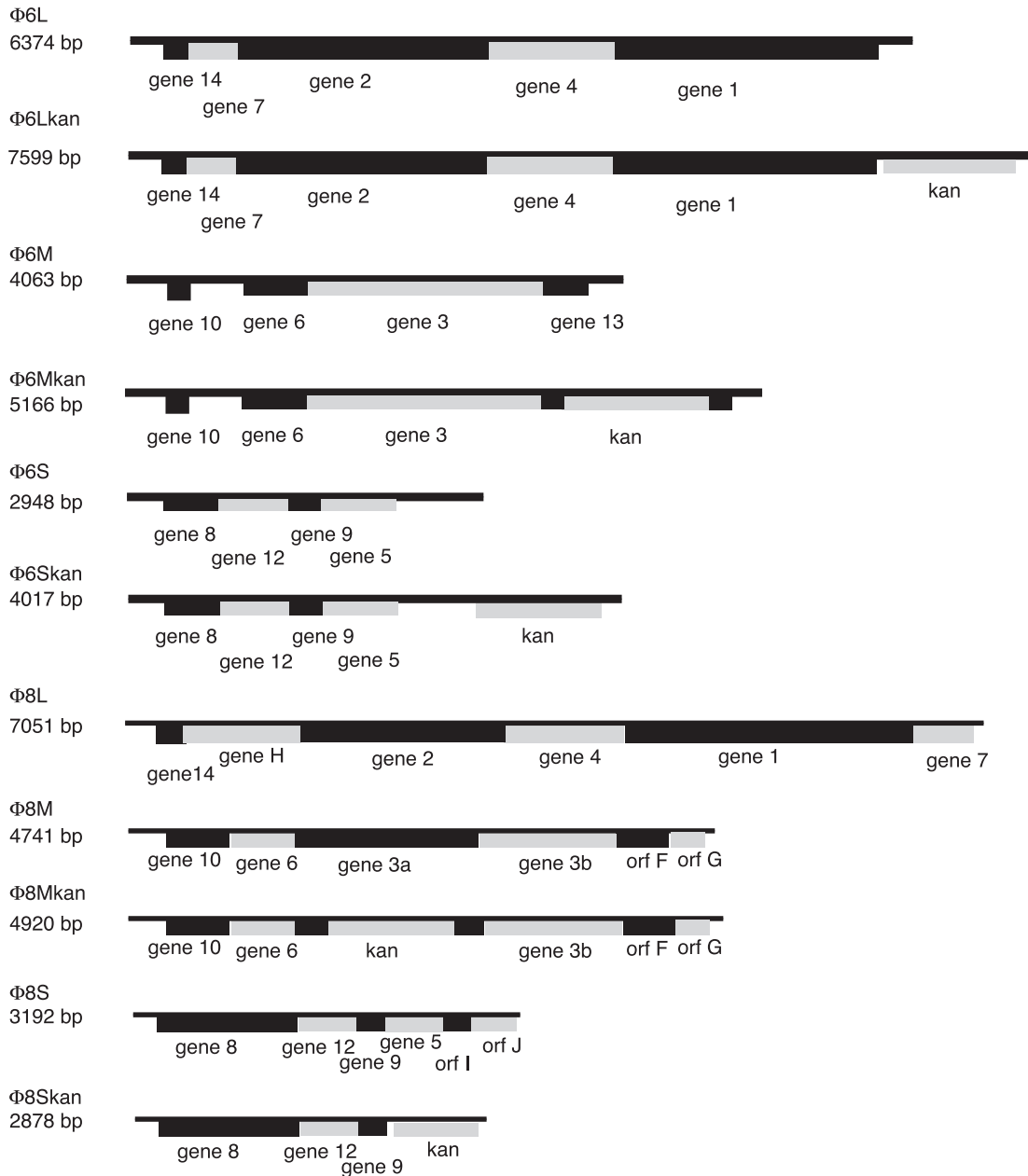


Fig. 1. Maps of the genomic segments of Φ6 and Φ8. Insertions of the *kan* gene in segments L, S, and M are also shown.

dence of M packaging on the prior packaging of S, but the plus strand of L can be packaged to a small extent after the packaging of S but without M (Qiao et al., 1995). A model for the genomic packaging has been proposed and is supported by several experimental findings (Mindich, 1999a).

We had previously constructed Φ6 virions with reporter insertions such as *kan* or *lacZ* in the non-translated regions (nters) of the genomic segments (Mindich, 1999b). We found that these viruses could establish stable carrier state relationships with the host bacteria (Onodera et al., 1992). This work served to extend the findings of other laboratories that Φ6 could set up carrier states in *Pseudomonas syringae* HB10Y (Cuppels et al., 1979; Romantschuk and Bamford,

1981). During the course of this work, we found that a strain of Φ6 with *kan* in segment M could lose the S segment and continue to propagate. This seemed to be anomalous because we had shown that genomic packaging seemed to be serially dependent in that the packaging of the plus strand of L was dependent upon the prior packaging of M and that of M was dependent upon the prior packaging of S. Upon investigation, it was found that a mutation had occurred in the gene for the major structural protein P1 of the procapsid and that these procapsids were able to package the plus strands of M and L without the participation of S (Onodera et al., 1998). The plus strands of S were competed by those of segment M (Onodera et al., 1998). The mutation in gene

1 therefore caused a change in the packaging program of the virus. In the present report, we have used a new approach to forming virus constructs. We can electroporate plasmids that contain cDNA copies of the genomic segments into cells that express T7 or SP6 RNA polymerase. The cDNA copies are preceded by T7 or SP6 promoters. The plasmids are ColEI derivatives and do not replicate in pseudomonads. If one of the cDNA copies has the *kan* gene, a selection can be made for carrier state cells. If all three segments are introduced, one can select for the production of live virions. Using this system, it is possible to probe the limitations of the genomic packaging programs.

## Results

With the development of the plasmid electroporation system for the production of cystoviridae, it is now possible to investigate the consequences of special phage constructions with greater ease than was possible with our previous approaches that involved either in vitro packaging of purified procapsids or the acquisition of plasmid transcripts by virus constructs harboring deletions in particular genomic segments. It is now also easier to prepare carrier state systems. Since the ColEI plasmids used for the expression of the cDNA copies of the genomic segments do not replicate in pseudomonads, resistance to reporter drugs is dependent upon the formation of carrier states. Electroporating three plasmids containing cDNA copies of the genomic segments of either  $\Phi 6$  or  $\Phi 8$  into strains of *P. syringae* carrying plasmids that express T7 RNA polymerase resulted in the production of thousands of colonies resistant to kanamycin if the *kan* gene was incorporated into one of the genomic segments (Tables 1 and 2). Surprisingly, it became apparent that electroporating plasmids with cDNA copies of only S

Table 1  
Plasmids

Name	Characteristics	Source
pLM659	$\Phi 6S$ cDNA in pT7T319U	Gottlieb et al., 1992
pLM656	$\Phi 6M$ cDNA in pT7T319U	Gottlieb et al., 1992
pLM687	$\Phi 6L$ cDNA in pT7T319U	Gottlieb et al., 1992
pLM836	$\Phi 6S$ cDNA in pT7T319U with <i>kan</i> in 3'nt	this study
pLM779	$\Phi 6M$ cDNA in pT7T319U with <i>kan</i> in 3'nt	Onodera et al., 1993
pLM3191	$\Phi 6L$ cDNA in pT7T319U with mutation in gene 1	this study
pLM3192	$\Phi 6L$ cDNA in pT7T319U with mutation in gene 1	this study
pLM991	$\Phi 6L$ cDNA in pT7T319U with <i>kan</i> in 3'nt	this study
pLM2743	$\Phi 8S$ cDNA in pT7T319U	this study
pLM2622	$\Phi 8L$ cDNA in pT7T319U	this study
pLM2813	$\Phi 8M$ cDNA in pT7T319U with <i>kan</i> replacing gene 3a	this study
pLM3150	$\Phi 8S$ cDNA in pT7T319U with <i>kan</i> replacing gene 5	this study

Table 2

Results of electroporation into LM2691

Plasmids	Number of colonies	Verification of RNA content
$\Phi 6L$ , $\Phi 6Mkan$ , $\Phi 6S$	3000	three segments
$\Phi 6L$ , $\Phi 6M$ , $\Phi 6Skan$	10,000	not tested
$\Phi 6L$ , $\Phi 6Skan$	10,000	two segments
$\Phi 6L$ , $\Phi 6Mkan$	19	two segments
$\Phi 6Lkan$ , $\Phi 6S$	10,000	not tested
$\Phi 6Lkan$	0	
$\Phi 8L$ , $\Phi 8Mkan$ , $\Phi 8S$	1000	three segments
$\Phi 8L$ , $\Phi 8Skan$	5000	two segments
$\Phi 8L$ , $\Phi 8Mkan$	2	no phage RNA

and L was sufficient for the production of large numbers of carrier state colonies for both  $\Phi 6$  and  $\Phi 8$  (Table 2). Electroporating cDNA copies of M and L resulted in a few carrier state colonies in  $\Phi 6$  and none in  $\Phi 8$ . In all cases, the production of the carrier state was confirmed by analysis of the dsRNA content of the cells (Fig. 2). Although we have found that the quantitation of the RNA content in the gels is not very precise, it can be seen that the two-segment phages seem to have equal numbers of segments. There does not seem to be a doubling of the content of either segment S or M in these particles.

Whereas the frequencies of carrier state formation for L and S were higher than the frequencies seen for L, M, and S, those for L and M were much lower. This suggested that a mutation was necessary for the maintenance of the L:M combination in  $\Phi 6$ , especially because we had seen previously that such a construction was dependent upon a mutation in gene 1 that codes for protein P1, the major structural protein of the inner core (Onodera et al., 1998). We prepared cDNA copies of segment L of two of the new carrier state cultures containing only L and M. Electroporation of these plasmids along with the cDNA copy of M with a *kan* insert gave high frequencies of carrier state colonies. Sequence analysis showed that gene 1 had new mutations in both cases. The plasmids designated as pLM3191 and pLM3192 showed base changes of G5706A and A5169C, respectively, which cause, again, respectively, amino acid changes in P1

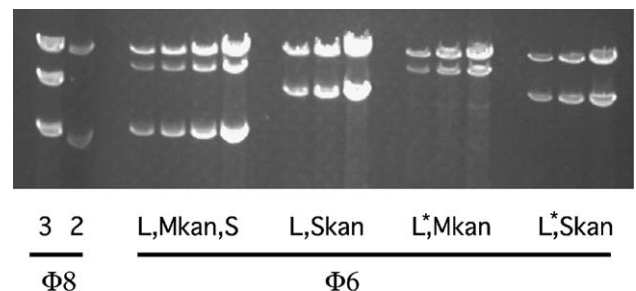


Fig. 2. Agarose gel electrophoresis of dsRNA isolated from carrier state cells. Combinations of plasmids listed in Table 1 were electroporated into strain LM2691 and selection for kanamycin resistance was used to isolate carrier state colonies. Increasing amounts of RNA were applied for each  $\Phi 6$  sample. L\* is derived from mutant pLM3191.

of E586K and T407P. The previous mutation called ERA12 was C3990G, resulting in a R14G change in P1 (Onodera et al., 1998). The three mutations are well separated and there is no particular pattern in the amino acid changes.

Electroporation of a plasmid with a cDNA copy of  $\Phi 6$  segment L and a *kan* insert did not result in any colonies (Table 2). This plasmid, pLM991, was found to be sufficient to produce carrier state colonies with normal S alone or with S and M segments.

$\Phi 8$  differed from  $\Phi 6$  in that its carrier states were dependent upon the presence of segment S. We examined the genes of  $\Phi 8$  S to determine what was required for carrier state maintenance. Constructs with specific deletions were prepared by cutting plasmid with appropriate restriction enzymes based upon the map of segment S (Hoogstraten et al., 2000). Gene 5 and orfs I and J are clearly not required because the *kan* insert replaces them (Fig. 1). Gene 9 is also not necessary. Removal of any part of genes 8 and 12 prevented the formation of the carrier state in  $\Phi 8$ . Protein P8 forms a shell between the inner core and the membrane of  $\Phi 6$ . In  $\Phi 8$ , P8 is a component of the membrane. There is no shell between the inner core and the membrane in the  $\Phi 8$  virion. Protein P12 is a nonstructural protein necessary for the assembly of the viral membrane. The roles of proteins P8 and P12 in the establishment or maintenance of the carrier state in  $\Phi 8$  are not clear at the present time.

## Discussion

The rules for genomic packaging in  $\Phi 6$  are stringent when examined by *in vitro* packaging or by the behavior of *in vivo* packaging for the production of live virus (Mindich, 1999a). However, there is a low but significant level of *in vitro* packaging of S and L plus strand segments in  $\Phi 6$  (Qiao et al., 1995). It appears that *in vivo* packaging constraints can be significantly relaxed if the requirement is only for replication of the components in the carrier state. The packaging of L and S without M can be carried out without the need for new mutations. Perhaps the conditions in the host cell where the RNA concentrations are much higher than those in our *in vitro* reactions are sufficient to facilitate breaking of the rules that were established from the *in vitro* work. In the case of a normal infection, the presence of the M plus strand might be the sufficient condition for correct packaging behavior. There are limits to the relaxation though, because  $\Phi 6$  packaging without S requires mutations in gene 1, which directs the synthesis of P1, the protein that is responsible for the specificity of packaging. Maintenance of  $\Phi 8$  requires at least part of segment S containing genes 8 and 12.

It seems, then, that the carrier state is a simpler system in which to study viral replication than that of the whole virus. The only requirements are the proteins necessary for replication and transcription and stable maintenance. The

requirements for stable maintenance might be modified by the drug selection that we use. In the present study, we do not select for mutations that promote high efficiency of carrier state formation or higher stability of the carrier state. The kanamycin resistance selection makes this unnecessary. We have also found that mutations that lower polymerase activity increase carrier state stability (unpublished observations).

The finding that neither  $\Phi 6$  nor  $\Phi 8$  can dispense with packaging S without a suppressor mutation suggests that the binding sites for M and L do not appear unless the plus strand of S is packaged. Our working model is that the suppressor mutations result in the exposure of the binding site for M in the absence of S packaging. Binding studies have shown that this is true for the previously isolated mutant ERA12 (Onodera et al., 1998; Qiao et al., 2003). The mutations that we have so far isolated are localized in distant regions of the linear sequence of gene 1; however, they might either be neighbors in the tertiary structure or simply induce conformational changes in P1 that result in exposure of the proper binding sites.

$\Phi 8$  packaging seems to need not only the packaging properties of S but also some of its genes. Deleting genes 9 and 5 do not have consequences, but deletions in genes 8 and 12 do have consequences. Protein P12 is a morphogenetic protein in  $\Phi 6$  involved in viral membrane assembly (Johnson and Mindich, 1994). Its role in  $\Phi 8$  has not yet been studied. Protein P8 is a membrane protein in  $\Phi 8$ , but it is the structural element of a shell that exists between the inner core and the membrane of  $\Phi 6$  (Sinclair et al., 1976). In  $\Phi 6$ , it plays a role in shutting down transcription from the inner core (Tuma et al., 1999). In  $\Phi 8$ -infected cells, there is much less P8 than in  $\Phi 6$ . The protein may, however, still play a role in the control of transcription before membrane acquisition.

It might be that such a system utilizing carrier state formation would be of utility in the case of the reoviridae, where the attempts to employ reverse genetics have met with limited success. Chloramphenicol acetyl transferase has been used as a reporter gene in reovirus but not as a selective marker (Roner and Joklik, 2001). There are several drug resistance genes that could serve in eukaryotic cells. Geneticin (G418) has been used for the selection of carrier states in cytomegalovirus infections (Wolff et al., 1993). Simply infecting cells with virus while injecting either modified viral plus strands with antibiotic resistance or a nonreplicating/nonintegrating plasmid with a cDNA copy of such a segment might produce as carrier state infection. Persistent infection has been described for reovirus (Dermody, 1998), but this condition does not involve a stable equilibrium of the virus with the host cells but rather a horizontal transfer of virus to cells wherein either the virus or the cells show defects in the entry processes. However, a carrier state infection in rotavirus has been described wherein the propagation of infected cells was not affected by antibody treatment (Babiuk and Misra, 1979).

## Materials and methods

### Bacterial strains and plasmids

*P. syringae* HB10Y is the host for  $\Phi 6$  (Vidaver et al., 1973), while HB10Y derivatives LM2489 or LM128 are hosts for both  $\Phi 8$  and  $\Phi 6$ . LM2691 is LM128 with plasmid pLM1086, a derivative of the broad host range vector pRK290 (Ditta et al., 1980) that constitutively expresses T7 RNA polymerase (derived from plasmid pAR1219; Davanloo et al., 1984). LM128 is a derivative of HB10Y that is permissive for P type plasmids. *E. coli* strain JM109 (recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,  $\lambda$ -,  $\Delta$ (lac-proAB)) [F', traD36, proAB, lacI<sup>q</sup>,  $\Delta$ M15] (Yanisch-Perron et al., 1985) was used for the propagation of all plasmids. Plasmid pT7T319U is an expression vector with both *lac* and T7 promoters. cDNA copies of genomic segments were inserted into this plasmid so that transcription initiated by T7 polymerase started at the first nucleotide of the viral plus strand. The gene for kanamycin resistance derived from transposon Tn903 was inserted into plasmid pUC4K and ultimately into plasmid pLM778 (Onodera et al., 1993). The *kan* gene was inserted into the 3' non-coding region of plasmids as shown in Fig. 1. Plasmid constructs used for producing carrier states are listed in Table 1 and the cDNA inserts are diagrammed in Fig. 1. The sequences of the oligonucleotide primers and the details of the plasmid constructions are available from the authors upon request.

### Media

The media used were LC and M8 (Sinclair et al., 1976). Ampicillin plates contained 200  $\mu$ g of ampicillin per milliliter in LC agar.

### Enzymes

All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, were purchased from Promega, New England Biolabs, and Stratagene.

### Reverse genetics for the modification of the viral genome

The simplest means of virus construction is to electroporate host cells with T7 or SP6 promoter plasmids that contain cDNA copies of the genomic segments along with a plasmid that codes for either T7 or SP6 RNA polymerase. Using ColeI-based plasmids such as pT7T319U that are transcribed but do not replicate in pseudomonads, we can produce hundreds of infective centers. If the T7 or SP6 polymerase plasmid is resident in the host strain, we can produce tens of thousands of infective centers with wild-type constructions. Although the 5' sequence of the  $\Phi 8$  plus strands, GAAA, is more compatible with the specificity of SP6 polymerase, it is possible to use T7 RNA polymerase as well, although with somewhat lower efficacy. We have used

the T7 polymerase in this study because most of our constructions were on plasmids with T7 promoters.

If one of the genomic segments has a gene for kanamycin resistance *kan* inserted into either its non-coding region or replacing one or more genes, then electroporation can result in the establishment of a carrier state in which the viral genome is replicated in cells without lysis and with the expression of the drug resistance so as to form stable colonies.

### Electroporation

An overnight culture of LM2691 was used to inoculate 1 l of LB that was incubated at 28 °C until the cell density reached about 10<sup>9</sup> cfu/ml. The culture was chilled for 15 min and spun at 2400  $\times$  g for 15 min at 4 °C. The pellet was suspended in cold water, recentrifuged, and resuspended in one-half volume of cold water. The cells were spun again and resuspended in 20 ml of 10% glycerol. The cells were then spun down and resuspended in 2 ml of 10% glycerol. One microliter of each of the plasmids (1  $\mu$ g) was added to 70  $\mu$ l of the competent cells. The mixture was left on ice for 1 min. The cells were then subjected to electroporation at 21  $\mu$ F, 2500 V, and 200  $\Omega$ . The cells were then transferred to 700  $\mu$ l of SOC medium, incubated at 28 °C for 2 h, and plated on LB plates containing 40  $\mu$ g/ml kanamycin. The plates were incubated at 28 °C. Alternatively, the electroporated cells could be mixed with untreated host cells and plated for plaque production.

### RT-PCR

L segment dsRNA was isolated from sodium dodecyl sulfate cell lysates of carrier state cultures by first treating with DNase, then phenol extraction and electroelution of the RNA from agarose gels. The RNA was then denatured and annealed to primer and incubated with AMV reverse transcriptase. The products were then subjected to PCR with Pfu turbo DNA polymerase (Stratagene) (Mindich et al., 1999). The resulting DNA products were cloned into pT7T319U and sequenced at the Molecular Resources Facility of the University of Medicine and Dentistry of New Jersey.

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