Delineation of the virulence-related locus (*vrl*) of *Dichelobacter nodosus*

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***Dichelobacter nodosus* is the primary pathogen implicated in ovine footrot. In this paper we have delineated a 27 kb locus, termed the virulence-related locus (*vrl*), that was essentially specific for virulent *D. nodosus* isolates. The precise ends of this locus were mapped and the sequences of the junction regions from the virulent strain A198 were compared to corresponding sequences from the benign isolate C305. The left end of the *vrl* locus was located in a sequence similar to that of the small stable 10Sa RNA molecule of *Escherichia coli*, next to a phage-attachment-site-like sequence, which indicated that the *vrl* locus might have arisen by the integration of a phage. However, no attachment-like sequence could be found at the right end of the *vrl* locus. In the chromosome of the benign strain the sequences bordering *vrl* were not contiguous but were separated by about 3 kb. It was concluded that the divergence of the benign and virulent strains at this locus was a multi-step process. Several potential ORFs were identified at the junction regions but only one ORF, encoding a 126 kDa protein, was expressed in a T7 expression system in *E. coli*.**

Keywords: footrot, virulence, *Dichelobacter nodosus*, *att* site, small stable RNA

INTRODUCTION

Ovine footrot is an economically significant contagious disease, primarily affecting the feet of sheep (Stewart *et al.*, 1984). It is a mixed bacterial infection with the main causative agent being *Dichelobacter nodosus*, a strictly anaerobic Gram-negative rod (Beveridge, 1941; Dewhirst *et al.*, 1990). *D. nodosus* isolates are classified as virulent, intermediate, or benign depending on the severity of infection they can cause under optimal climatic and environmental conditions (Stewart, 1989; Stewart *et al.*, 1986). However, little is known about the genetic differences between benign and virulent strains. It is likely that virulence is dependent upon several factors, with extracellular proteases and type 4 fimbriae being impli-

cated in the disease (Depiazzi *et al.*, 1986, 1991; Elleman, 1988; Moses & Yong, 1989; Thomas, 1962).

The study of potential virulence factors in *D. nodosus* is hampered by the lack of a genetic system which could be used to analyse the effect of defined mutations on virulence. There are currently no transformation or conjugation methods available for this organism. Therefore, the analysis of genetic elements potentially involved in virulence is primarily carried out by cloning and characterizing DNA regions unique to benign or virulent strains of *D. nodosus*.

Differential genetic analysis of such isolates led to the isolation of three DNA fragments that were present in the reference virulent strain A198, but not in the benign strain C305 (Katz *et al.*, 1991). When used as DNA probes these fragments were found to be useful for the differential diagnosis of *D. nodosus* isolates (Katz *et al.*, 1991). In dot-blot hybridization experiments one of the plasmids, pJIR318, hybridized with DNA from virtually all virulent and intermediate isolates as well as 33% of the benign strains. The other two fragments, carried in pJIR313 and pJIR314B, hybridized with virtually all virulent strains,

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and with 36 % of the intermediate isolates but with only two (6 %) benign isolates (Katz *et al.*, 1991). These results suggest that the genetic loci carried by pJIR313 and pJIR314B may be involved in the virulence of *D. nodosus*. Moreover, the almost identical results obtained with these plasmids suggest that they are closely linked.

In this paper we present data showing that the inserts in plasmids pJIR313 and pJIR314B were derived from a genetic locus that encompasses about 27 kb of essentially virulent-specific DNA. The exact point of divergence between virulent and benign sequences was determined. Our findings indicate that the transition in this locus between virulent and benign strains can not be explained by a simple insertion or deletion event.

METHODS

Bacterial strains. The *D. nodosus* strains A198 (virulent) and C305 (benign) were obtained from J. Egerton, University of Sydney, Australia. Other isolates were from the Regional Veterinary Laboratories at Hamilton, Victoria (HA207, HA212, HA233, HA240, HA304, HA646, HA659, HA733 and HA734), and Wagga Wagga, New South Wales (WW849-4A and WW1100-1A), or from J. Egerton (VCS1008, VCS1040, and VCS1172). *D. nodosus* isolates were grown in an atmosphere of 10 % (v/v) H₂ and 10 % (v/v) CO₂ in N₂ at 37 °C on hoof agar (Thomas, 1958) or in TAS broth (Skerman, 1975, 1989). All *Escherichia coli* strains were derivatives of DH5 α (Bethesda Research Laboratories) or K38 (Russel & Model, 1984) and were cultured in 2 $\times$ YT medium (Miller, 1972) supplemented with ampicillin (100 μ g ml⁻¹) and/or kanamycin (40 μ g ml⁻¹). The *D. nodosus* A198 DNA library in λ GEM12 was obtained from E. Moses, Victorian Institute of Animal Science, Australia (Katz *et al.*, 1994). The host for the recombinant λ bacteriophage, LE392 (Sambrook *et al.*, 1989), was cultivated in LB broth supplemented with 1 % (w/v) maltose (Sambrook *et al.*, 1989).

DNA isolation and general molecular techniques. Genomic *D. nodosus* DNA was isolated as described by Anderson *et al.* (1984). Plasmid DNA from *E. coli* was prepared by previous methods (Birnboim & Doly, 1979; Holmes & Quigley, 1981). Oligonucleotides were synthesized with an automatic DNA synthesizer (Applied Biosystems, model 381A) and dissolved in glass-distilled water. General molecular techniques were as described by Sambrook *et al.* (1989). Exclusive radiolabelling of plasmid-encoded proteins was carried out using the T7 RNA polymerase/promoter system (Tabor & Richardson, 1985). The host *E. coli* strain K38 harbouring pGP1 and either the vector pTZ18U (Pharmacia), or recombinant plasmids, was used in this procedure.

Hybridization analysis. Dot-blots were prepared as previously described (Katz *et al.*, 1991) except that *D. nodosus* cells were grown on hoof agar for 4–7 d and the lysed cells were applied to the nylon membranes (Hybond N+, Amersham) with the aid of a dot-blot apparatus (Schleicher & Schuell). The membranes were washed in 2 $\times$ SSC, air-dried, and UV-crosslinked for 3 min. For Southern blots, genomic *D. nodosus* DNA (1–2 μ g) was digested with the appropriate restriction endonuclease and fractionated by electrophoresis through a 0.8 % (w/v) agarose gel. The DNA was denatured in 0.5 M NaOH, 1.5 M NaCl and transferred to a nylon membrane in 10 $\times$ SSC. Blots were hybridized with plasmid DNA, or DNA fragments, as before (Katz *et al.*, 1991), or using a DIG-labelling kit (Boehringer) according to the manufacturer's instructions. The λ library was screened as described previously (Katz *et al.*, 1994).

Construction of a *D. nodosus* C305 DNA library. DNA libraries from *D. nodosus* C305 were constructed by digesting 1–5 μ g of C305-derived DNA to completion with the appropriate restriction endonuclease, purifying the DNA by adsorption to glassmilk (Vogelstein & Gillespie, 1979), and ligating it with 1 μ g of pUC18 digested with the same enzyme. The ligation mixture was either used directly to transform RbCl-treated *E. coli* cells (Hanahan, 1983) or, after an ethanol precipitation step, to transform glycerol-treated cells by electroporation.

Nucleotide sequencing. All sequencing reactions were done using either a T7 DNA polymerase sequencing kit (Pharmacia) or an Automated Sequencing System 373A using a PRISM cycle sequencing kit (Applied Biosystems). The reported sequences were obtained independently from both strands either by constructing and sequencing overlapping subclones or by using specifically designed oligonucleotides as primers in the sequencing reactions.

RESULTS

Delineation of the *vrI* region

Hybridization analysis of pJIR313 (1.3 kb insert) and pJIR314B (0.6 kb insert) revealed that both fragments were present in only a single copy on the A198 genome (Katz *et al.*, 1991). Since preliminary sequence analysis showed that both plasmids contained incomplete ORFs it was initially postulated that these plasmids were derived from the same gene. However, chromosome walking experiments subsequently carried out using a λ GEM12 library showed that the fragments carried by pJIR313 and pJIR314B were separated by 9 kb of DNA, all of which had the same dot-blot hybridization specificity, with respect to virulent and benign *D. nodosus* isolates, as the original recombinant plasmids. Further chromosome walking experiments were carried out to determine the extent of this region, which was designated the virulence-related locus or the *vrI* region. One end of this locus was known, since pJIR314B was derived from a larger plasmid, pJIR314, and the adjacent sequences contained in pJIR314A hybridized to both virulent and benign isolates (Katz *et al.*, 1991). The other end was identified after four separate overlapping λ clones were isolated (Fig. 1). When appropriate subclones of these recombinant phages were constructed and tested in dot-blot hybridization experiments, the other end of the *vrI* region was shown to be located within pJIR744 (Fig. 1). Therefore, dot-blot experiments showed that the *vrI* locus consisted of about 27 kb of essentially virulence-specific DNA located between the regions carried on pJIR744 and pJIR314A (Fig. 1).

Fine mapping of the ends of the *vrI* region

If it is assumed that the difference between the virulent and benign *D. nodosus* strains in this region is due to the insertion or deletion of *vrI* DNA, the sequences bordering each *vrI* end should be juxtaposed in benign strains. Southern blots revealed that pJIR314A, which contained the right end of *vrI*, hybridized to a 2 kb *Eco*RI fragment from the benign strain C305; this fragment was subsequently cloned. The resultant plasmid, pJIR787, showed

Fig. 1. Delineation of the *vrl* region. A restriction map of the *vrl* region, as deduced from the λ clones, is shown at the top. The relative positions of the inserts in pJIR313, pJIR314B and the isolated λ clones are indicated, as well as those subclones used to demonstrate the virulence specificity of this region (see below). The extent of the virulence-related locus is indicated by the open box at the bottom. Restriction sites for *Bam*HI (B), *Eco*RI (E), *Eag*I (G), *Hind*III (H), *Sst*I (S) and *Xho*I (X) are indicated. The orientation of the map is consistent with the large ORF found at the right end of *vrl* being transcribed from left to right. The virulence specificity of subclones of the *vrl* region is shown in the bottom section. Dot-blot of virulent (V) and benign (B) *D. nodosus* isolates were probed with labelled DNA of the plasmid indicated. The benign isolates tested were, from top to bottom: HA646, HA304, C305, WW849-4A, HA733, HA734, HA233 and VCS1040. The virulent isolates tested were A198, HA659, VCS1008, VCS1172, WW1100-1A, HA240, HA212 and HA207.

Fig. 2. Restriction maps of recombinant plasmids and the PCR fragment containing the *vrl* junctions. pJIR744 and pJIR314A are the initial isolates containing the left and right ends of *vrl*; pJIR880 is a subclone of pJIR744. Plasmid pJIR787 contains the region corresponding to the right end of *vrl* from the benign *D. nodosus* strain C305. The positions of the primers used to amplify the region corresponding to the left border are indicated, as is the resultant PCR fragment.

some restriction site similarity to pJIR314A (Fig. 2). Analysis indicated that the right junction between the virulence-related locus and the chromosome was located 300–500 nt from the right end of pJIR787. However,

pJIR787 had no restriction site identity with pJIR744 (Fig. 2), the plasmid containing the left end of *vrl*. Moreover, the pJIR744 insert did not hybridize with pJIR787 DNA, indicating that pJIR787 contained the

Fig. 3. Alignment of sequences at the left end of the *vrI* region (A198) with corresponding sequences from the benign isolate (C305). The point of divergence is indicated by an open arrowhead. Included in this alignment is the *E. coli* 10Sa RNA gene (EC *ssrA*). Nucleotides conserved in A198 and C305 are in capitals, as are the *E. coli* residues identical to the *D. nodosus* sequences. Sequences similar to *att* sites are shaded and regions of similarity to the pseudouridine loop of tRNAs are overlined by a bar. The end of the mature 10Sa RNA is marked by an arrow and the end of the precursor form by an asterisk. The 14 nt boxes following the *att* sites are boxed and the inverted repeats are overlined with arrows in the A198 sequence.

sequences bordering the right end of *vrI* but not the left end. It was concluded that in strain C305 these bordering sequences were not contiguous.

Southern blots of C305 DNA probed with pJIR744 revealed hybridizing 2.7 kb *EcoRI* and 8 kb *BamHI* fragments (results not shown). Despite screening over 2000 recombinant colonies from both *EcoRI* and *BamHI* libraries of C305 DNA constructed in pUC18, no clones hybridizing to pJIR744 were isolated, which suggested that this region may not be stably maintained on a multicopy plasmid in *E. coli*.

Comparative Southern hybridization analysis of C305 and A198 DNA showed that the sequences hybridizing to pJIR314A and pJIR744 were about 3 kb apart in the C305 chromosome (results not shown). Therefore, PCR could be used to obtain significant quantities of the junction fragment for further analysis. To this end, subclones of pJIR744 were generated and tested for their dot-blot hybridization pattern, using virulent and benign *D. nodosus* isolates as before. A *XbaI* subclone, pJIR880 (Fig. 2), was found to contain the junction site. Based on sequence data obtained from this plasmid, and from pJIR314A, two primers were designed to amplify the corresponding

region in C305. The sequence for primer #1129, ACCGTAAGCGACATTAACACAG, was 129 nt from the left end of the pJIR880 insert and the sequence of primer #1169, CGCCCGAACCCATTACCCGTGA, was 322 nt to the left of the *EcoRI* site of pJIR314A (Fig. 2). PCR analysis using these primers on C305 DNA as template yielded a product of about 3.5 kb, as expected. This product hybridized to both pJIR880 and pJIR314A probes in Southern blots.

Sequence analysis of the junction regions

The exact endpoints of *vrI* were determined by sequencing the A198- and C305-derived DNA fragments corresponding to the left and right junctions and comparing the resulting sequences. For the left *vrI* junction, the pJIR880 insert was completely sequenced and found to be 1487 nt long. The 3.5 kb PCR fragment was used to obtain relevant sequence from the corresponding C305 region; in all 329 nt, starting from primer #1129, were obtained and compared to the sequence from pJIR880 (Fig. 3). The sequences were identical for the first 156 nt (pJIR880, nt 167–322; PCR fragment, nt 1–156) then the sequences diverged. Therefore, the left end of the *vrI* region was

Fig. 4. Sequence alignment of the right end of *vrl* (A198) with the corresponding region of C305. Identical nucleotides are in capitals and the point of divergence is marked by an open arrowhead. The amino acid sequences of the identified ORFs are shown and their orientation is indicated. Inverted repeat sequences are marked by arrows and direct repeats by double-headed arrows.

located 322 nt from the left *Xho*I site in pJIR880. The mol% G+C content in the *vrl* sequence was higher (57%) than in the sequences conserved in A198 and C305 (43% and 44%, respectively) and was very low (35%) in the C305 sequence located to the right of the junction.

Since the right-hand end of the *vrl* region was contained in pJIR314, the complete nucleotide sequence of this insert (1591 nt) was determined. From pJIR787, which harboured the related region from C305, about 600 nt on either side of the junction were fully sequenced. Comparison of the sequences showed that the right junction was located 505 bp from the common *Eco*RI site, with the overlap having eight mismatches (Fig. 4). At this end, there were no significant differences in the mol% G+C content between *vrl* (54%) and non-*vrl* sequences (50–51%).

Identification of ORFs

In the pJIR880 sequence three potential ORFs of more than 100 codons were identified, namely ORF172 (nt 599–1115), ORF146 (nt 855–1293), and on the opposite strand ORF139 (nt 1155–738) (Fig. 5a). In addition, a small reading frame (ORF58') starting at nt 1309 was still open at the end of pJIR880. All three ORFs were located in the *vrl* portion of the sequence. With the exception of ORF172 they had upstream sequences similar to the consensus *D. nodosus* ribosome-binding site (Dewhirst *et al.*, 1990). No significant ORFs were found in the C305 sequence.

At the right junction both the virulent and the benign

sequences contained two incomplete ORFs which were convergent in pJIR314 (ORF277' and ORF1130) but had the same orientation and overlapped by 4 nt in pJIR787 (ORF172' and ORF240') (Fig. 5b). The ORFs from the non-*vrl* region, ORF172' and ORF277', were almost identical up to the junction site and continued past this site for 51 and 4 codons in pJIR314 and pJIR787, respectively (Fig. 4). Of the eight sequence differences in the common portions of pJIR314A and pJIR787, only three resulted in a change in the amino acid sequence. To find the start of the rightmost ORF within the *vrl* region, sequence analysis was continued. This putative gene, ORF1130, extended through pJIR590 into the adjacent clone, pJIR589 (Fig. 5). ORF1130 was preceded by a potential ribosome-binding sequence, AGGAGG, and another incomplete open reading frame, ORF42', which overlapped ORF1130 by 11 nt. In total, 4462 bp of the right end of the *vrl* region were sequenced. No sequences similar to *E. coli* promoters were found. None of the ORFs identified showed significant similarities with entries in the databases. However, examination of the amino acid sequence of the predicted ORF1130 product revealed similarity to the seven motifs associated with the DEAH superfamily of ATP-dependent helicase-related proteins (Gorbalenya *et al.*, 1989).

The *vrl* region appears to have inserted into a 10Sa RNA gene

Comparison of the DNA sequences with entries in the GenBank and EMBL databases revealed significant simi-

Fig. 5. Comparison of genetic maps at the left (a) and right (b) ends of *vrl* and the corresponding regions in C305. The black boxes indicate the sequenced regions. The ORFs identified, and their orientation, are shown as open arrows. Incomplete ORFs are indicated by the broken arrows.

larity between the left junction and small stable 10Sa RNA sequences from *E. coli* (Chauhan & Apirion, 1989), *Alcaligenes eutrophus* (Brown *et al.*, 1990), and *Mycobacterium tuberculosis* (Tyagi & Kinger, 1992). The similarity in all five sequences was optimal around the site where the precursor RNA is cleaved to form the mature 10Sa RNA (Chauhan & Apirion, 1989). Interestingly, this region showed strong similarity to the pseudouridine arm of tRNA genes from different organisms (Tyagi & Kinger, 1992) and to the integration site of several prophages, *attL* (Kirby *et al.*, 1994). The similarity with the other 10Sa RNA genes extended 6 nt beyond the 3' end of the mature 10Sa RNA and the *D. nodosus* sequences were identical for another 4 nt (Fig. 3). In pJIR880, another sequence similar to *attL*, *attL2*, was found in the *vrl* region, some 300 nt downstream of the first site. Both *att* sites were followed by sequences that were identical in 13 out of 14 positions. The distance between these 14 nt boxes and the *att* sites was 16 nt for *attL* and 62 nt for *attL2*. In addition, the left end of *vrl* was marked by a 26 bp inverted repeat. None of these sequence motifs could be found at the right end of *vrl*. The only significant structures in the *vrl* portion of pJIR314 were two 19 bp inverted repeat sequences and two 21 bp direct repeats (Fig. 4). The position of the inverted repeat, about 50 bp

downstream of the last *vrl* ORF, could indicate that it functions as transcription terminator.

Expression of *vrl*-encoded genes

T7-expression studies were carried out to determine if any of the ORFs were expressed in *E. coli*. For ORF1130, a 7.6 kb *Hind*III subclone of λ R29 was cloned downstream of the T7 promoter in pTZ18U in both orientations. The resultant plasmids were pJIR903, where the T7 promoter was upstream of ORF1130, and pJIR902, which was in the opposite orientation. T7-directed expression of pJIR903 resulted in the specific labelling of a protein larger than 100 kDa, as would be expected from ORF1130 (Fig. 6). No other induced protein was observed using this plasmid or pJIR902. To examine the left end of *vrl* a 9.4 kb *Eco*RI fragment of λ R82 was cloned and tested in a similar experiment. Several induced proteins of 12–40 kDa were observed. While some of these proteins correlated with the sizes of ORFs found in the pJIR880 sequence, none of these proteins were observed in T7 expression experiments using pGEM7zf(–) recombinants containing only the insert present in pJIR880 (data not shown).

Fig. 6. Identification of ORF1130 gene product. The proteins were overexpressed from the plasmids indicated using the T7 protein expression system (Tabor & Richardson, 1985) and labelled with [35 S]methionine. Whole-cell extracts of *E. coli* were separated by electrophoresis on a denaturing 12% (w/v) polyacrylamide gel containing 0.1% SDS. U, T7 polymerase uninduced; I, T7 polymerase induced. The positions of protein standards are shown on the left. The band corresponding to ORF1130 is indicated by the arrow.

DISCUSSION

Differential hybridization experiments using the virulent and benign *D. nodosus* strains, A198 and C305 respectively, resulted in the isolation of three recombinant plasmids, pJIR313, pJIR314B and pJIR318, carrying gene regions which were present only in the virulent strain (Katz *et al.*, 1991). Other studies have shown that pJIR318 represents a repeated region, designated *vap*, in the A198 genome (Katz *et al.*, 1992, 1994). In this study we have characterized the chromosomal region from which the inserts of pJIR313 and pJIR314B were derived. Subsequent hybridization analysis showed that the gene regions they represented were separated by about 9 kb and that they were part of the 27 kb *vrl* locus. It seems likely that this locus has been derived from the integration of a phage, plasmid or transposon, as such elements are known to be potential carriers of virulence determinants (Levin & Eden, 1990). Unfortunately, the lack of a genetic exchange system prevents the direct genetic analysis of *vrl* and the determination of its precise involvement in virulence in *D. nodosus*.

To test the hypothesis that *vrl* is an integrated genetic element, we analysed the ends of the *vrl* region and the 'integration site' in a benign strain. This analysis was somewhat hampered by the lack of isogenic strains, since all *D. nodosus* strains are field isolates with unknown evolutionary relationships. We chose the benign strain C305 to analyse the integration site, as this was the strain used in the isolation of the plasmids pJIR313 and pJIR314B (Katz *et al.*, 1991). It was anticipated that the sequences bordering the *vrl* locus would be adjacent in strain C305. However, our results showed that these bordering sequences were separated by about 3 kb of DNA found in both virulent and benign strains. This finding can either be explained by a displacement mechanism by which one region displaced the other in the insertion process, or by postulating that both regions inserted independently at the same site. The latter notion is supported by the identification, at the left end of *vrl*, of an *attL* site with high similarity to a site found in the gene for the small stable 10Sa RNA, *ssrA*, in *E. coli* (Kirby *et al.*, 1994). Moreover, the upstream region shares significant similarity with the *E. coli ssrA* gene (Fig. 3). This suggests that both the *vrl* region and the insert in C305 may be derived from the integration of a phage in a small stable RNA gene similar to the situation found in *E. coli* (Kirby *et al.*, 1994). The lack of the concomitant attachment site, *attR*, at the right end of *vrl* implied that the end of the *vrl* region was not identical with the end of the putative prophage. This conclusion is supported by the almost identical mol% G+C content on both sides of the junction in A198 and C305. The second attachment site could be outside the sequenced region or could have been deleted since the insertion event took place.

The function of the small stable RNA is not well understood. It is implicated in gene regulation (Kirby *et al.*, 1994) and in interaction with DNA-binding proteins (Retallack *et al.*, 1994). The *ssrA* transcript is processed within the *attL* site (Fig. 3) to give the mature RNA (Chauhan & Apirion, 1989). Excision of the prophage results in an altered 3' end of the transcript which is sufficient in *E. coli* to affect its function (Kirby *et al.*, 1994; Retallack *et al.*, 1994). Against this background it seems possible that *vrl* does not itself encode a virulence function but rather its insertion at *ssrA* activates such a function located somewhere else on the chromosome. In this context we noted that in A198 the *attL* site was followed by an inverted repeat (Fig. 3) that could function as a factor-independent transcription terminator. Such a structure is also found near the end of the *E. coli ssrA* gene (Kirby *et al.*, 1994) but is absent after excision of the prophage (Retallack *et al.*, 1994) and is absent from C305 (Fig. 3).

The significance of the second *attL*-like sequence near the left end of *vrl* is not clear. Although the sequence could be fortuitous it could also be an indication that more complex genetic processes lead to insertion of the *vrl* region in A198, e.g. the integration of two phages either independently or by one being carried by another at the time of integration. The notion of two different genetic elements at this site is strengthened by the significant

difference in the mol% G+C content of the sequence between *attL* and *attL2* (51%) and the sequence to the right of *attL2* (61%). The importance of the direct repeats following the *attL* sites in A198 (Fig. 3) is also not known. Similarly, within the *att* region of the P4 family of *E. coli*, conserved sites were identified (Kirby *et al.*, 1994), which have varying distances from the *att* sequences. These sites were assumed to be protein-binding sites implicated in integration and/or excision of the phage or regulation of transcription (Kirby *et al.*, 1994). Although there is no similarity between the A198 repeats and the conserved sites of the P4 family they could serve similar functions.

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