Description of *Enterovibrio nigricans* sp. nov., reclassification of *Vibrio calviensis* as *Enterovibrio calviensis* comb. nov. and emended description of the genus *Enterovibrio* Thompson *et al.* 2002

Javier Pascual,^{1,2} M. Carmen Macián,³ David R. Arahal,^{2,3} Esperanza Garay^{2,3} and María J. Pujalte^{1,2}

Correspondence

María J. Pujalte
maria.j.pujalte@uv.es

¹Instituto Cavanilles de Biodiversidad y Biología Evolutiva (ICBiBE), Universidad de Valencia, Valencia, Spain

²Departamento de Microbiología y Ecología, Universidad de Valencia, Valencia, Spain

³Colección Española de Cultivos Tipo (CECT), Universidad de Valencia, Valencia, Spain

Eleven strains of halophilic, facultative anaerobes isolated from healthy and diseased *Dentex dentex* and *Sparus aurata* (bony fishes) cultured in Spanish Mediterranean fisheries have been studied by a polyphasic approach that included a wide phenotypic characterization, DNA–DNA hybridization and phylogenetic analysis using 16S rRNA, *recA* and *rpoD* gene sequences. All strains were phylogenetically related to *Enterovibrio* species and *Vibrio calviensis*. On the basis of sequence analysis and DNA–DNA hybridization data, eight of the strains were identified as *Enterovibrio coralii*. The remaining three strains formed a tight, independent clade in all sequence analyses and showed less than 70 % DNA–DNA hybridization with strains of the closest *Enterovibrio* species, from which they could be differentiated by several phenotypic traits. We conclude that these three strains represent a novel species in the genus *Enterovibrio* and we thus propose the name *Enterovibrio nigricans* sp. nov., with strain DAI 1-1-5^T (=CECT 7320^T =CAIM 661^T) as the type strain. In addition, we propose the reclassification of *Vibrio calviensis* Denner *et al.* 2002 as *Enterovibrio calviensis* comb. nov. (type strain RE35/F12^T =CIP 107077^T =DSM 14347^T =CECT 7414^T) and we provide an emended description of the genus *Enterovibrio*.

The genus *Enterovibrio* was proposed by Thompson *et al.* (2002) to accommodate a group of isolates obtained from larvae of *Scophthalmus maximus*, described as the novel species *Enterovibrio norvegicus*. The strains shared some phenotypic characteristics with *Vibrio* species but were phylogenetically closer to *Grimontia hollisae* (basonym *Vibrio hollisae*), although they shared only 94 % 16S rRNA gene sequence similarity. Three years later, a second *Enterovibrio* species was described, *Enterovibrio coralii*, isolated from diseased corals (Thompson *et al.*, 2005b). It

is also recognized, although no formal proposal has been made, that *Vibrio calviensis* (Denner *et al.*, 2002) shares greater phylogenetic relatedness with members of *Enterovibrio* than with *Vibrio* species, according to recent phylogenetic analyses (Thompson *et al.*, 2005b; Sawabe *et al.*, 2007). In fact, the generic assignment of *V. calviensis* was already considered somewhat provisional at the time of its description (Denner *et al.*, 2002).

During two surveys on bacteria associated with the internal organs of the cultured fish *Dentex dentex* (Company *et al.*, 1999) and *Sparus aurata* (Pujalte *et al.*, 2003), a group of strains isolated from the head kidney, unidentifiable by phenotyping, were found to be related to *E. coralii* by comparative analysis of 16S rRNA gene sequences. On initial isolation, the strains were considered as non-fermentative, so the taxonomic link with the genus *Enterovibrio* was surprising, and prompted a deeper characterization that has been performed through a wide phenotypic study, DNA–DNA hybridization and phylogenetic analysis using three housekeeping genes, the 16S rRNA gene, *rpoD* and *recA*.

Abbreviations: ML, maximum likelihood; MP, maximum parsimony; NJ, neighbour joining.

The GenBank/EMBL/DDBJ accession numbers for the *rpoD*, *recA* and 16S rRNA gene sequences determined in this study are AM942047–AM942062, AM942063–AM942078 and AM942722–AM942732, respectively, as detailed in Table 1.

Photographs of colonies of strain DAI 1-1-5^T, ML and MP trees based on 16S rRNA, *recA* and *rpoD* gene sequences and a concatenated dataset, growth responses and cellular fatty acid profiles of individual strains and detailed DNA–DNA hybridization results are available as supplementary material with the online version of this paper.

Table 1 shows the origin of the strains and/or sequences included in the study. Wild strains were isolated by direct streaking of a loop from *Dentex dentex* head kidney on tryptone soy agar (TSA) supplemented with 1% (w/v) NaCl, or from *Sparus aurata* head kidney on marine agar (MA) plates, incubated at room temperature (20–24 °C) for 2–7 days. Isolated strains were maintained as stab cultures in semisolid marine broth [MB, plus 0.2% (w/v) agar] and suspended in MB plus 20% (w/v) glycerol at –80 °C for long-term storage.

Methods for phenotypic characterization have been reported elsewhere (Macián *et al.*, 2001a). Strains *E. coralii* CAIM 912^T, *E. norvegicus* CECT 7288^T and *V. calviensis* DSM 14347^T were characterized along with 11 wild strains. All strains were routinely cultivated on MA or in MB and incubated at 20–22 °C.

Wild strains were Gram-negative, motile, oxidase-positive rods and showed, on initial characterization, no ability to ferment glucose anaerobically on Møller's decarboxylase medium plus 2% (w/v) NaCl. Later testing on OF medium plus glucose with half-strength artificial seawater revealed that all of them were able to ferment glucose under anaerobic conditions after 2–3 days incubation, but acid production was weak within the first 24 h, not allowing a clear identification of the strains as members of the *Vibrionaceae*. Growth on thiosulfate citrate bile sucrose (TCBS) medium, a selective medium routinely used for vibrio selection, was also slow and poor in most cases, and

gave rise to small, green colonies (i.e. not fermenting sucrose). None of the strains was able to hydrolyse gelatin, casein, starch or alginate. The two strains isolated from *Sparus aurata* produced a black/deep-brown, slightly diffusible pigment that accumulated in isolated colonies and peripheral mass growth after prolonged incubation on MA (Supplementary Fig. S1, available in IJSEM Online). All strains grew at 15–28 °C but not at 4 or 40 °C. They required NaCl for growth and thrived in media containing 0.9–6% (w/v) NaCl, but not with more than 8% total salinity. The strains grew well on several sole carbon and energy sources. Detailed information on phenotypic properties is displayed in Supplementary Table S1 and in the species description.

Isolation of genomic DNA was done according to Pitcher *et al.* (1989). Reference strains included the type strains of *E. norvegicus*, *E. coralii*, *V. calviensis*, *G. hollisae* and *Vibrio cholerae* (Table 1). Amplification and partial sequencing of genes coding for 16S rRNA, *recA* and *rpoD* were performed as described previously by Macián *et al.* (2001b), Thompson *et al.* (2005a) and Le Roux *et al.* (2005), respectively. Multiple sequence alignments were obtained using CLUSTAL_X (Thompson *et al.*, 1997). The lengths of the sequences used for further analysis were 786 nt (*recA*), 876 nt (*rpoD*), 1348 nt (16S rRNA) and 3010 nt (concatenated sequences). Phylogenetic analysis was performed using the program PAUP* version 4.0b10 (Swofford, 2002). Neighbour-joining (NJ; with Kimura's two-parameter

Table 1. Strains included in this study

Accession numbers given in *italics* were retrieved from public databases for the same strain or known equivalents. NA, No sequence available.

Strain	Origin	GenBank accession number		
		16S rRNA	<i>recA</i>	<i>rpoD</i>
<i>E. norvegicus</i>				
CECT 7288 ^T	<i>Scophthalmus maximus</i> larvae, gut, Norway (1997)	<i>AJ316208</i>	AM942075	AM942062
LMG 19840	As above	<i>AJ316207</i>	<i>AJ842349</i>	NA
LMG 19842	As above	<i>AJ437193</i>	<i>AJ842350</i>	NA
<i>V. calviensis</i> DSM 14347 ^T	0.2 µm-filtered Mediterranean seawater, France (1999)	<i>AF118021</i>	AM942077	AM942047
<i>E. coralii</i>				
CAIM 912 ^T	Bleached <i>Merulina ampliata</i> extract, Australia (2002)	<i>AJ842343</i>	AM942063	AM942051
8/13	<i>Dentex dentex</i> , head kidney, Castellón, Spain (1997)	AM942725	AM942064	AM942052
9/1a	As above	AM942726	AM942065	AM942053
9/1c	As above	AM942727	AM942067	AM942054
9/2a	As above	AM942728	AM942066	AM942055
9/2b	As above	AM942729	AM942068	AM942056
9/5b	As above	AM942730	AM942069	AM942057
10/6	As above	AM942731	AM942070	AM942058
11/2a	As above	AM942732	AM942071	AM942059
<i>E. nigricans</i> sp. nov.				
DAL 1-1-5 ^T	<i>Sparus aurata</i> head kidney, Alcanar, Spain (2000)	AM942722	AM942072	AM942048
DAL 1-1-4	As above	AM942723	AM942073	AM942049
8/6b	<i>Dentex dentex</i> , head kidney, Castellón, Spain (1997)	AM942724	AM942074	AM942050
<i>G. hollisae</i> CECT 5069 ^T	Human faeces, Annapolis, MD, USA	<i>AJ514909</i>	AM942076	AM942061
<i>V. cholerae</i> CECT 514 ^T	Human faeces	<i>X76337</i>	AM942078	AM942060

correction), maximum-parsimony (MP; heuristic search option) and maximum-likelihood (ML) analyses were done for each of the genes and the concatenated sequence. For ML, the optimal models of nucleotide substitution were estimated through the program MODELTEST 3.7 (Posada & Crandall, 1998; Posada & Buckley, 2004) using the Akaike information criterion (AIC). All bootstrap analyses were performed using 1000 replications. The different trees obtained with NJ are shown in Fig. 1. The trees obtained by using the MP and ML algorithms displayed the same topology as those obtained with NJ for each set of sequences in most cases (Supplementary Figs S2 and S3). Table 2 shows the percentages of pairwise sequence similarity within and between species.

Cellular fatty acid analyses of strains DAL 1-1-5^T, 8/6b and *V. calviensis* DSM 14347^T were performed by GLC at the DSMZ using a method described previously (Kämpfer & Kroppenstedt, 1996). Cells were harvested from cultures grown on MA plates incubated at 26 °C for 24 h. Results showed that strains DAL 1-1-5^T and 8/6b exhibited very similar profiles, whereas their close relatives differed mainly in the relative amounts of fatty acids common to all and in the presence or absence of some minor fatty acids (Supplementary Table S2). DNA G+C content was determined by HPLC at the DSMZ according to Mesbah *et al.* (1989).

DNA–DNA hybridization experiments were done by the hydroxyapatite method using microtitre plates as described by Ziemke *et al.* (1998) with a hybridization temperature (T_m) of 50 °C. DNA from strain DAL 1-1-5^T was labelled and assayed against DNA from the remaining wild strains and *E. corallii* CAIM 912^T. DNA from strain 8/13 was assayed against DNA from *E. corallii* CAIM 912^T and most of the other isolates (Supplementary Table S3).

Considering all the data obtained, it can be concluded that eight of the fish isolates belong to the species *E. corallii*, as they form a tight, independent clade with the type strain, *E. corallii* CAIM 912^T, in sequence analyses performed with the three genes employed in this study, 16S rRNA, *rpoD* and *recA* (Fig. 1 and Supplementary Figs S2 and S3). The maximal percentages of sequence difference within the species were 0.9 % (16S rRNA), 2.1 % (*recA*), 1.0 % (*rpoD*) and 1.2 % (concatenated sequences), which are considerably lower than the minimal interspecies percentage of sequence difference recorded for each of the datasets (2.4, 11.7, 12.4 and 7.7 %, respectively; Table 2). The strains in the clade shared more than 70 % DNA–DNA relatedness in hybridization experiments with strain 8/13, which had 97.6 % reassociation to the DNA of *E. corallii* CAIM 912^T (Supplementary Table S3). Some phenotypic features of these novel *E. corallii* strains do not fit the original species description: arginine dihydrolase was variable (six of eight strains positive) and indole production was positive in all strains (including the type strain CAIM 912^T, reported as negative in the original description). Additional information on phenotypic characteristics of the strains is given

below: they grew in TCBS agar as green colonies, from 0.9 to 7 % total salinity on MA (growth was variable at 8 % and negative at 9 % or more), they grew at 15–37 °C but not at 4 or 40 °C, the indole reaction in MB was strongly positive, Thornley's arginine dihydrolase test was negative in short incubations (up to 48 h) but turned positive for six of the eight strains after prolonged incubation, lysine decarboxylase, ornithine decarboxylase and the Voges–Proskauer reaction were negative. They reduced nitrate to nitrite. None of the strains was able to hydrolyse casein, gelatin, starch, alginate or lecithin; the only extracellular hydrolytic activities displayed were against Tween 80 and DNA (weak). Nutritional screening on basal medium agar plus sole carbon sources revealed a narrow range of compounds that sustained growth (Supplementary Table S1). This is also the first report of *E. corallii* strains isolated from fish, since the type strain was isolated from a diseased coral sample and to the best of our knowledge was the only known strain.

The remaining three fish isolates (DAL 1-1-4, DAL 1-1-5^T and 8/6b) constitute a well-defined clade whose sister group is *E. corallii* in all phylogenetic reconstructions done with *rpoD* and the concatenated sequences. The branching order in these six trees is also supported by high bootstrap values. The three 16S rRNA gene trees and the *recA* MP reconstruction show a somewhat different topology, since the clade formed by the three fish isolates merges with the pair *E. norvegicus*–*V. calviensis*, whereas, in the *recA* NJ and ML trees, the clade joins a group that includes *E. corallii*, *E. norvegicus* and *V. calviensis* (Fig. 1 and Supplementary Figs S2 and S3). These two topologies are generally less well supported by bootstrap analysis. In any case, and even if the relative order of evolution can not be completely ascertained, it is clear that the three fish isolates (DAL 1-1-4, DAL 1-1-5^T and 8/6b) and *V. calviensis* are members of genus *Enterovibrio*. In all the trees obtained, *G. hollisae* occupies a peripheral position. In terms of sequence similarity, *G. hollisae* has, on average, a larger number of differences from *Enterovibrio* species than the *Enterovibrio* sequences display among themselves (Table 2).

DNA–DNA hybridization assays (Supplementary Table S3) confirm that the three fish isolates (DAL 1-1-4, DAL 1-1-5^T and 8/6b) constitute a genomic species. All other strains, including CAIM 912^T, yielded values of 36–63 %, which are below the threshold for genospecies definition (Wayne *et al.*, 1987) and in the range of close congeneric species (for example, *Vibrio parahaemolyticus* and *Vibrio harveyi* show 47–59 % reassociation; Baumann & Baumann, 1981). The three fish isolates (DAL 1-1-4, DAL 1-1-5^T and 8/6b) could also be differentiated phenotypically from other *Enterovibrio* species (Table 3 and Supplementary Table S1). We therefore propose to recognize these three strains as members of a novel species, *Enterovibrio nigricans* sp. nov.

In the present work, the position of the type strain of *V. calviensis* as a member of the monophyletic group of

Fig. 1. Neighbour-joining trees based on partial 16S rRNA (a), *recA* (b) and *rpoD* (c) gene sequences of *E. nigrificans* sp. nov., *E. calviensis* comb. nov., *E. norvegicus*, *E. corallii*, *G. hollisiae* and *V. cholerae* and the concatenated dataset (d). Bars, 1 (a) or 5 (b–d) estimated substitutions per 100 base positions (note that the scale is not the same for all trees). Bootstrap values greater than 50 % confidence are shown at branching points (as percentages of 1000 resamplings). Sequence accession numbers are given in parentheses.

Table 2. Relatedness between members of the genera *Enterovibrio* and *Grimontia* as percentages of 16S rRNA, *recA*, *rpoD* and concatenated sequence similarity

Intraspecific variability (in bold) applies to *E. nigricans* sp. nov. (three strains), *E. coralii* (nine strains) and *E. norvegicus* (three strains, for 16S rRNA and *recA* only).

Species	1	2	3	4	5
16S rRNA					
1. <i>E. nigricans</i>	99.9–100				
2. <i>E. coralii</i>	96.7–97.6	99.1–100			
3. <i>E. norvegicus</i>	96.8–97.2	95.2–96.3	100		
4. <i>E. calviensis</i>	97.5–97.6	96.5–97.2	97.3–97.4	—	
5. <i>G. hollisae</i>	96.0	95.1–96.1	95.1–95.8	95.1	—
recA					
1. <i>E. nigricans</i>	98.8–100				
2. <i>E. coralii</i>	86.0–87.0	97.9–100			
3. <i>E. norvegicus</i>	83.6–84.2	87.2–88.0	99.6–99.9		
4. <i>E. calviensis</i>	84.9–84.7	87.4	88.0–88.3	—	
5. <i>G. hollisae</i>	80.9–82.3	82.1–82.2	80.6–80.7	81.0	—
rpoD					
1. <i>E. nigricans</i>	98.9–100				
2. <i>E. coralii</i>	87.0–87.6	99.0–100			
3. <i>E. norvegicus</i>	76.6	75.1–76.3	—		
4. <i>E. calviensis</i>	80.0–80.6	78.5–80.4	78.9	—	
5. <i>G. hollisae</i>	74.7–74.9	76.7–78.1	73.8	76.1	—
Concatenated					
1. <i>E. nigricans</i>	99.3–100				
2. <i>E. coralii</i>	91.8–92.3	98.8–100			
3. <i>E. norvegicus</i>	88.8–89.0	88.6–89.2	—		
4. <i>E. calviensis</i>	90.1–90.3	90.0–90.6	90.8	—	
5. <i>G. hollisae</i>	87.3–87.7	87.6–88.1	86.7	87.2	—

Enterovibrio is clear in all analyses (individual genes and concatenated sequences) and with all algorithms used (NJ, MP, ML). In fact, *V. calviensis* relates directly to the type species of the genus, showing a position always closer to *E. norvegicus* than to *E. coralii* or *E. nigricans* sp. nov. Sequence similarity values for each of the sequence datasets confirm that *V. calviensis* should be considered a member

of the genus *Enterovibrio* (Supplementary Table S2). Moreover, the results of the phenotypic characterization of the type strain done in the present study (see Supplementary Table S1) show some discrepancies with the original description of *V. calviensis* (such as the inability to grow with glucose and *N*-acetyl D-glucosamine) and altogether support the transfer of *V. calviensis* to the

Table 3. Differentiation between *E. nigricans* sp. nov. and other *Enterovibrio* species

Data were obtained in this study for the following strains: *E. nigricans* sp. nov. 8/6b, DAL 1-1-4 and DAL 1-1-5^T, *E. coralii* 8/13, 9/1a, 9/1c, 9/2a, 9/2b, 9/5b, 10/6, 11/2a and CAIM 912^T, *E. norvegicus* CECT 7288^T and *E. calviensis* comb. nov. DSM 14347^T. The response of individual strains to a large set of nutritional tests can be found in Supplementary Table S1. v, Variable between strains.

Characteristic	<i>E. nigricans</i>	<i>E. coralii</i>	<i>E. norvegicus</i>	<i>E. calviensis</i>
Pigment production	v	—	—	—
Indole production	—	+	+	—
Growth on/at:				
4 °C	—	—	+	+
37 °C	—	+	+	—
7 % salinity	—	+	+	+
L-Alanine	—	+	+	+
D-Mannitol	—	+	+	+

genus *Enterovibrio* and an the emended description of the genus. Therefore, we also propose the new combination *Enterovibrio calviensis* comb. nov. and an emended description of the genus.

Description of *Enterovibrio nigricans* sp. nov.

Enterovibrio nigricans (ni'gri.cans. L. part. adj. *nigricans* blackish).

Gram-negative, motile rods or coccobacilli. Cells are 1.0–1.5 µm long and 0.4–0.7 µm wide. Chemo-organotrophic and facultatively anaerobic, able to ferment glucose without gas production under anaerobic conditions. Oxidase-positive. Some strains reduce nitrate to nitrite, but not to N₂. Able to grow on seawater-based media such as MA, on which some strains produce a black/deep-brown, slightly diffusible pigment. Colonies are round, with entire margins and a bright surface. They also grow on TSA supplemented with 1 % NaCl. They do not swarm or luminesce. Growth on TCBS agar is scarce and slow, producing green (non-sucrose-fermenting) colonies. Growth is observed at 15–28 °C but not at 4 or 37 °C. Requires NaCl for growth. Media containing 0.9–6.0 % total salinity sustain good growth; no growth is observed at 0.4 or 7 % or more salinity. In O/F medium, acid is produced under anaerobic conditions from D-mannose, sucrose (weakly) and melibiose (weakly and only strain 8/6b). The following carbohydrates are not fermented: D-ribose, D-xylose, L-arabinose, D-fructose, D-galactose, trehalose, L-rhamnose, maltose, cellobiose, lactose, amygdalin, salicin, D-mannitol, D-sorbitol and *myo*-inositol. Under aerobic conditions, strain 8/6b, but not DAL 1-1-4 or DAL 1-1-5^T, produces acid from D-fructose, D-galactose, lactose, melibiose, D-mannitol and D-sorbitol. None of the known strains is able to hydrolyse casein, gelatin, starch, alginate or lecithin. Tween 80 and DNA are degraded, the latter weakly. Thornley's arginine dihydrolase test is variable. Lysine decarboxylase, ornithine decarboxylase, indole and Voges-Proskauer tests are negative. Carbon sources that support growth are D-glucose, D-mannose, *N*-acetyl D-glucosamine, D-gluconate and L-glutamate. The use of D-ribose, D-fructose, fumarate, acetate, lactate and glycerol is variable among strains. No growth is observed on L-arabinose, D-xylose, trehalose, D-galactose, L-rhamnose, maltose, cellobiose, sucrose, lactose, melibiose, salicin, amygdalin, D-mannitol, D-sorbitol, *myo*-inositol, D-glycerate, D-saccharate, D-glucuronate, D-galacturonate, pyruvate, propionate, butyrate, citrate, *trans*-aconitate, 2-oxoglutarate, succinate, malate, 3-hydroxybutyrate, glycine, L-leucine, L-serine, L-threonine, L-alanine, L-arginine, L-tyrosine, L-ornithine, L-citrulline, L-aspartate, L-histidine, L-lysine, L-sarcosine or putrescine. The major cellular fatty acids are 16:1ω7c and/or iso-15:0 2-OH (43–50 % in known strains), 16:0 (18–19 %) and 18:1ω7c (11–15 %). Minor amounts (1–5 %) of the following fatty acids are also detectable: 12:0, 12:0 3-OH, 14:0, 14:0 3-OH/iso-16:1 I, iso-16:0 and 16:1ω9c.

The type strain is DAL 1-1-5^T (=CECT 7320^T =CAIM 661^T), isolated from the head kidney of *Sparus aurata* cultured on the Spanish Mediterranean coast. The type strain produces black pigment, is negative for arginine dihydrolase and positive for nitrate reduction and has a DNA G+C content of 47.9 mol%.

Description of *Enterovibrio calviensis* comb. nov.

Enterovibrio calviensis (cal.vi.en'sis. N.L. masc. adj. *calviensis* pertaining to the Bay of Calvi, Corsica, from where the type strain was isolated).

Basonym: *Vibrio calviensis* Denner *et al.* 2002.

The description is the same as given for *Vibrio calviensis* by Denner *et al.* (2002), with the following modifications: D-glucose, D-fructose and *N*-acetyl D-glucosamine, but not D-mannose, are used as sole carbon and energy sources for growth. The type strain designated in the original publication is RE35F/12^T =DSM 14347^T =CIP 107077^T. However, in the abstract, it is cited twice as RE35/F12 (Denner *et al.*, 2002). According to StrainInfo (<http://www.straininfo.net>), other equivalences of the type strain are ATCC BAA-606^T and CCUG 48319 B^T. As part of this study, the type strain has been also deposited at the Spanish Type Culture Collection as CECT 7414^T.

Emended description of the genus *Enterovibrio* Thompson *et al.* 2002

The description is the same as that provided by Thompson *et al.* (2002) with the following modifications: arginine dihydrolase activity, indole production, nitrate reduction and susceptibility to the vibriostatic agent O/129 may be variable among species. The main fatty acids are 16:1ω7c/iso-15:0 2-OH (32–50 % in known strains), 16:0 (15–21 %) and 18:1ω7c (11–23 %). Other fatty acids detected (up to 5 %) in all species, even under different growth conditions, are 16:1ω9c, 18:1ω9c, 12:0, 14:0, 14:0 3-OH/iso-16:1 I, 12:0 3-OH, 18:0 and iso-16:0. The G+C content of the DNA is 47–50 mol%.

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