Detection and identification of Lactobacillus bacteria found in the honey stomach of the giant honeybee Apis dorsata

Naser Tajabadi, Makhdzir Mardan, Mohd Abdul Manap, Mustafa Shuhaimi, Amir Meimandipour, Leila Nateghi

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Original article

Detection and identification of *Lactobacillus* bacteria found in the honey stomach of the giant honeybee *Apis dorsata*

Naser Tajabadi[1,4], Makhdzir Mardan[1], Mohd Yazid Abdul Manap[2], Mustafa Shuhaimi[3], Amir Meimandipour[5], Leila Nateghi[6]

[1]Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Food Technology, Faculty of Food Sciences and Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[4]Department of Honey Bee, Animal Science Research Institute of Iran, Karaj, Iran
[5]Department of Animal and Marine Biotechnology, National Institute of Genetic Eengineering and Biotechnology (NIGEB), Tehran, Iran
[6]Department of Food Science and Technology, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran

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Abstract – This is the first assay that describes the isolation and identification of strains and species of *Lactobacillus* from the honey stomach of the Asiatic giant honeybee, *Apis dorsata*. Samples of honeybees were collected from *A. dorsata* colonies in different bee trees, and *Lactobacillus* was isolated from honey stomachs using selective media. The isolates were Gram-stained and tested for catalase reaction. The 16S rRNA genes from extracted DNA of bacterial colonies were amplified with polymerase chain reaction using lactobacilli genus primers (27F and 1492R). All bacterial 16S rRNA genes were sequenced and deposited in GenBank. The 34 isolated strains yielded three distinct rRNA sequences of 15 different strains. *Lactobacillus* sequences isolated from the bees' honey stomachs were comprised of *Lactobacillus kunkeei* related-sequences (56%) with other abundant sequences being related to other *Lactobacillus* sp. (38%) and *Lactobacillus vermiform* (6%). These strains can be good candidates for potential application as probiotics in honeybees and also as natural food preservatives, which, in turn, may be useful in the food industry.

Apis dorsata **/ honey stomach /** ***Lactobacillus*** **bacteria / probiotics**

1. INTRODUCTION

Lactobacilli are dominant lactic acid bacteria (LAB) found in the gastrointestinal tract of humans, honeybees (Rada et al. 1997; Killer et al. 2010), and other animals (Reuter 2001). They are usually found together with *Bifidobacteria*, and are widely utilized as human and animal probiotics for the promotion of health (Reuter 2001). Lactic acid bacteria are usually found in rich, carbohydrate-containing substances. Honey is produced from nectar collected by foraging workers, which is temporarily stored in the honey stomach during flight between flowers and the hive. The honey stomach is a

The study was conducted at the Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.

Corresponding author: M.Y. Abdul Manap, myazid@putra.upm.edu.my
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development of the gullet or honey crop that can expand to a large volume (Olofsson and Vásquez 2008). The honey stomach, when filled with nectar and nutrients, has a microaerobic state and is at an optimal temperature of 35°C in the hive (Jones et al. 2004), and thus, it represents an optimal niche for the LAB. The primary routes by which LAB enters the honey stomach are likely to ingested pollen, and other floral matter, dust, air, and the honeybees' digestive tract (Lee and Kime 1984). Larvae may be sterile initially, but they are fed nectar and pollen by workers and, therefore, subject to nectar, pollen, and the nursing worker's flora before pupation (Lee and Kime 1984).

Several studies have focused on the intestinal microflora of honeybees (Gilliam et al. 1977; Gilliam 1997; Rada et al. 1997; Lyapunov et al. 2008; Yoshiyama and Kimura 2009), but only a few studies exist on LAB (Olofsson and Vásquez 2008, 2009), and on the micoflora of the honey stomach of the European honeybee, *Apis mellifera*, and of bumble bees, *Bombus* spp., Olofsson and Vásquez (2008) showed that sampling from different flowers over the course of 1 year (summer 2005 to summer 2006) profoundly altered dominant LAB in the honey stomach of *A. mellifera*.

The giant honeybee, *Apis dorsata*, is native to the lowland and highland of Malaysia and plays a central role in the livelihood of the rural people who sell the honey for local consumption (Mardan and Kiew 1985). The rainforest of Malaysia is estimated to contain 40,000 species of vascular plants (Spjut 1985) and, therefore, offers tremendous potential to prospect for LAB. *Lactobacillus* populations existing in the honey stomach of the giant honeybee, *A. dorsata*, may benefit human health as well as food preservation. Currently, there is no documented study in the literature on *Lactobacillus* from the honey stomach of the giant honeybee *A. dorsata*. Thus, the main objective of this study was to detect and identify lactobacilli from the honey stomach of the giant honeybee, *A. dorsata*, native to the highland of Malaysia.

2. MATERIALS AND METHODS

2.1 Sample collection

One hundred worker honeybees of *A. dorsata* were collected from each colony from ten different bee trees in the highlands of Kedah state in north of Malaysia during the dry season between January and March. Samples were placed in distinct sterile tubes, each containing 10 mL normal saline (0.9% *w*/*v* NaCl, 0.1% *w*/*v* Tween 80, and 0.1% *w*/*v* Peptone) and immediately transported to the Food Biotechnology Laboratory at Universiti Putra Malaysia for further processing. Thirty honey-filled stomachs were acquired from bees of each colony with aseptic excision under luminal flow (Olofsson and Vásquez 2008).

2.2 Culture method

Ten percent (*w*/*v*) of honey stomach solutions were prepared in normal saline, and lactobacilli were isolated from the honey stomachs on MRS (de Man, Rogosa, and Sharpe) agar medium (Oxoid). The isolates were incubated for 3–4 days at 37°C (Olofsson and Vásquez 2008) under anaerobic conditions using anaerobic jars with Anaerocult A gas packs (Merck, Darmstadt, Germany). To acquire pure bacterial isolates, 100 colonies with different morphology were picked off and subcultured.

2.3 Biochemical screening

Using an initial screening of *Lactobacillus*, Gram-positive and catalase-negative Bacilli were chosen (Coeuret et al. 2003). The isolates were maintained as frozen stocks at −20°C in MRS broth supplemented with 15% (*v*/*v*) glycerol for further analysis (Olofsson and Vásquez 2008).

2.4 DNA extraction

DNA was extracted according to DNA extraction kit protocol (QIAGene) with some modifications as described by Ward et al. (1994). The purity of DNA was determined by using a spectrophotometer and the ratio of the readings at 260 and 280 nm (A260/A280).

2.5 PCR and program

In the present study, one set of primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), targeting the genus level of lactobacilli, was used (Lane 1991). Polymerase chain reactions (PCRs) were performed in 25 μL reaction volumes, containing 1× Taq Master Mix, 1.5 mM $MgCl_2$, 0.25 mM forward primer, 0.25 mM reverse primer, and 0.4 ng of genomic DNA. Temperature cycling conditions for PCR were as follows: an initial heating of 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 55 s, extension at 72°C for 1 min, and terminating with a 10-min final incubation at 72°C. In order to achieve a high degree of primer specificity during assays, the Eppendorf Mastercycler gradient PCR (Eppendorf, Hamburg, Germany) was utilized to improve primer-annealing temperatures (Shuhaimi 2003). PCR products were examined on ethidium bromide-stained agarose gels (Gel Electrophoresis Systems Major Science, Taiwan). After strengthening, the PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's directions.

The purified PCR products originating from isolates were sequenced by a sequencing company (First BASE Laboratories, Malaysia) using primers 27F and 1492R. For identification, the 16S rRNA sequences were BLAST-searched at (http://www.ncbi.nlm.nih.gov/). A maximum likelihood test procedure was applied to phylogenetic analysis and investigated new LAB (Sokal and Rohlf 1995). The neighbor-joining tree was bootstrapped 1,000 times and used from MEGA (4) package (Tamura et al. 2007).

2.6 Reference sequences used in phylogenetic analysis

The following bacterial 16S rRNA gene sequences were tested as out groups in phylogenetic analysis: *Lactobacillus apis* (AY667701), *Lactobacillus alvei* (AY667698), *Lactobacillus insectis* (AY667699), *Lactobacillus* sp.Bma5 (EF187242) (cluster I in Figure 1), *Lactobacillus kunkeei* YH-15(NR_026404) (cluster I in Figure 1), *Lactobacillus helveticus* (FJ915631), *Lactobacillus parabuchneri* (AB429372), *Lactobacillus kefiri* (AB429371), *Lactobacillus plantarum* (FM179607), *Lactobacillus buchneri* (AB205055), *Lactobacillus vermiforme* (M59295) (cluster V in Figure 1) (Vasquez et al. 2009).

3. RESULTS

From the MRS plates, 100 developed colonies were picked up for the limited biochemical tests, and of these, 34 colonies were subjected to sequence analysis. The isolated strains displayed a similarity from 73% to 99% with five closest database sequences in NCBI (Table I). Although no exact 16S rRNA similarity limits exist for defining specific taxa, such as genus and species, species definition in general requires sequence similarities greater than 98% (Stackebrandt and Goebel 1994). Phylogenetic analysis showed that *Lactobacillus* flora in the honey stomach of the Asiatic giant honeybee *Apis dorsata* was comprised of 15 different phenotypes, 5 of which (Mardan Taj-1, Yazid Naser-1, Yazid Mardan-1, Naser Faegheh-1, and Taj Arash-1) were related to *L. kunkeei* (clusters II in Figure 1 and Table I). Eight phylotypes (Yazid Taj-1, Naser Makhdzir-1, Taj Mahdi, Dilah Makhdzir-1, Faegheh Hadi-1, Taj KS164, Taj KS82, and Taj Mustafa-1) were classified into *Lactobacillus* sp. (cluster I in Figure 1 and Table I). Phylotype, Naser Mardan-1, was distant but most closely related to *L. vermiform* with a sequence similarity level of 73% (Table I) and the phylotype Adi Mardan-1 also belonged to *L. kunkeei* (clusters III in Figure 1 and Table I).

The results of this study displayed that lactobacilli in the honey stomach of the giant honeybee *A. dorsata* is dominated with the phenotype Mardan Taj-1 (Figure 1), which is related to species *L. kunkeei*. Thirteen out of 15 different honey stomach lactobacilli (Mardan Taj-1, Yazid Naser-1, Yazid Mardan-1, Adi Mardan-1, Naser Faegheh-1, Yazid Taj-1, Naser Makhdzir-1, Taj Mahdi, Dilah Makhdzir-1, Faegheh Hadi-1, Taj KS164, Taj KS82, and Naser Mardan-1; Figure 1), were found in the

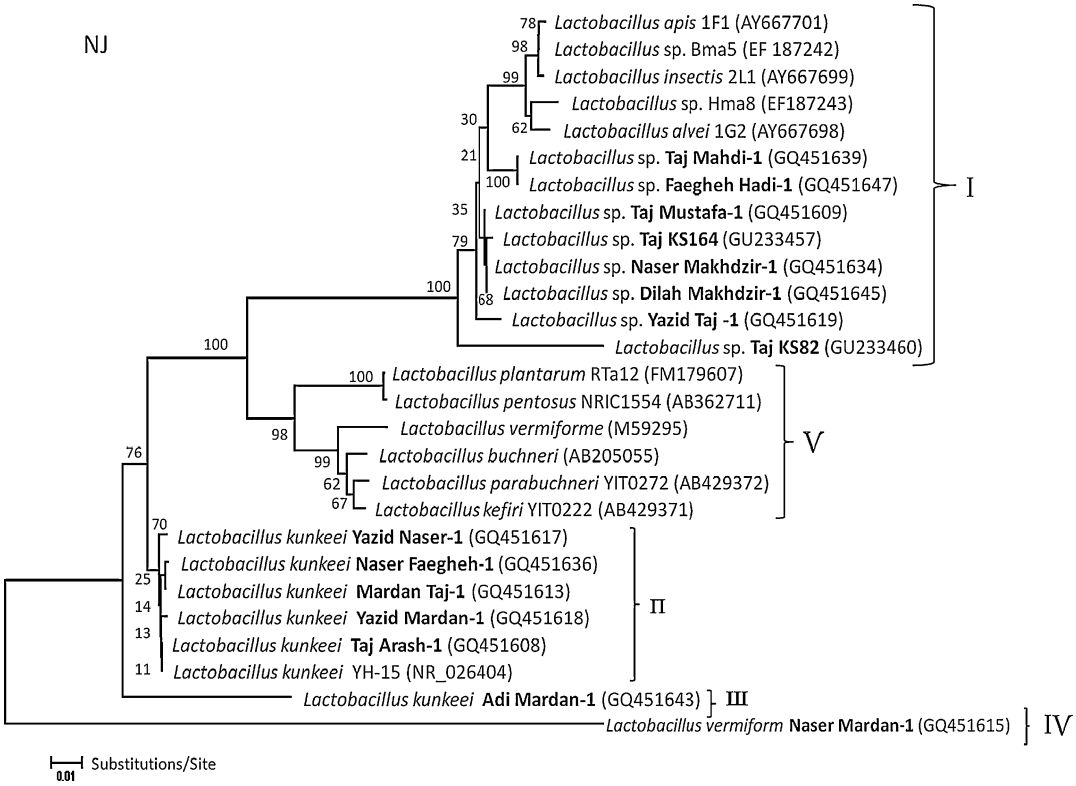

Figure 1. Phylogenetic tree based on a distance matrix analysis of 1,275 positions in the 16S rRNA gene. The phylogenetic tree was constructed by ClustalW using the neighbor-joining method within the MEGA (4) package (Tamura et al. 2007). Closely related type and reference strains are shown in parentheses together with accession numbers from GenBank. Bootstrap values based on 1,000 re-samplings display the significance of the interior nodes, and are shown at branch points. Cluster *I Lactobacillus* sp. group, cluster *II Lactobacillus kunkeei* group, cluster *III Lactobacillus kunkeei* group, cluster *IV* new *Lactobacillus* group, cluster *V Lactobacillus* as out group.

honey stomach of *A. dorsata* from different tall trees in the highland. But the other two *Lactobacillus*, (Taj Mustafa-1 and Taj Arash-1; Figure 1) which belonged to *Lactobacillus* sp. and *L. kunkeei*, respectively, were found in both the honey stomach and honeycomb (unpublished data) of *A. dorsata*.

Sequences of the 16S rRNA genes of the isolates representing different groups and possible new *Lactobacillus* strain and species were deposited in GenBank (NCBI) with accession numbers GQ451608, GQ451609, GQ451613, GQ451615, GQ451617, GQ451618, GQ451619, GQ451634, GQ451636, GQ451639, GQ451643, GQ451645, GQ451647, GU233457, and GU233460.

4. DISCUSSION

Previous studies have shown that honey produced by honeybees contains LAB that originates from the honey stomach (Olofsson and Vasquez 2008; Vasquez et al. 2009). The majority of LAB that exists in honey stomach have also been isolated from pollen and 2-week-old bee bread, which suggests a possible role of honey stomach LAB and its antimicrobial substances against honeybee diseases. Lactic

Table I. Bacterial phylotypes originating from honey stomach of honeybee *Apis dorsata*

Isolates[a]	Sequence lengths and number of identical[b]	Most closely related type strain[c]	Sequence lengths and similarity[d]	Accession numbers[e]
Mardan Taj-1	[11–1,061] (10)	*Lactobacillus kunkeei* YH-15 (Y11374)	1,102 (95.6)	GQ451613
Yazid Naser-1	[13–1,115] (1)	*Lactobacillus kunkeei* YH-15 (Y11374)	1,136 (97)	GQ451617
Yazid Mardan-1	[10–1,100] (1)	*Lactobacillus kunkeei* YH-15 (Y11374)	1,118 (97)	GQ451618
Naser Faegheh-1	[9–1,076] (1)	*Lactobacillus kunkeei* YH-15 (Y11374)	1,139 (93)	GQ451636
Taj Arash-1	[6–917] (4)	*Lactobacillus kunkeei* YH-15 (Y11374)	919 (99)	GQ451608
Adi Mardan-1	[11–797] (2)	*Lactobacillus kunkeei* YH-15 (Y11374)	800 (98)	GQ451643
Naser Mardan-1	[522–917] (2)	*Lactobacillus vermiform* (M59295)	1,146 (73)	GQ451615
Naser Makhdzir-1	[20–1,114] (3)	*Lactobacillus* sp Bma5 (EF187242)	1,148 (96.3)	GQ451634
Yazid Taj-1	[38–1,032] (1)	*Lactobacillus* sp Bma5 (EF187242)	1,124 (88)	GQ451619
Taj Mustafa-1	[22–1,104] (4)	*Lactobacillus* sp Bma5 (EF187242)	1,111 (97.3)	GQ451609
Taj Mahdi-1	[22–1,099] (1)	*Lactobacillus* sp Bma5 (EF187242)	1,133 (95)	GQ451639
Dilah Makhdzir-1	[19–1,084] (1)	*Lactobacillus* sp Bma5 (EF187242)	1,134 (94)	GQ451645
Faegheh Hadi-1	[24–1,100] (1)	*Lactobacillus* sp Bma5 (EF187242)	1,138 (94)	GQ451647
Taj-KS164	[1–1,003] (1)	*Lactobacillus* sp Mardan Yazid-1 (GQ451614)	1,043 (96)	GU233457
Taj-KS82	[3–796] (1)	*Lactobacillus* sp Naser Makhdzir-1 (GQ451634)	798 (99)	GU233460

[a] The identity of 16S rRNA gene sequences were generated from isolates

[b] The number of identical sequences found are shown in parentheses, and the sequence lengths are shown in brackets

[c] GenBank accession numbers are shown in parentheses; taxonomic connection was established by comparing the sequence in the National Center for Biotechnology Information (NCBI)

[d] The similarity to the closest type strain sequence is exhibited as a percentage inside parentheses

[e] GenBank accession numbers for this study are shown in the latest column

acid bacteria from the honeybee stomach mainly belong to the genera *Lactobacillus* and *Bifidobacterium*. *Lactobacillus*, an important category in LAB, is prevalently found as commensal bacteria and is utilized as a probiotic for humans and animals (Ouwehand et al. 2002). Thus, detailed studies of dominant *Lactobacillus* bacteria existing in the honey stomach and honey are important in protecting honeybees against pathogens and for human health. Furthermore, *Lactobacillus* which originates from the honey stomach can be selected and used as a food preservative and food probiotic supplement for human consumption. In the present study, classical cultivation methods coupled with the 16S rRNA sequencing provided data to describe the bacterial diversity and phylogenetic relationships of *Lactobacillus* present in the honey stomach of *A. dorsata*. Our results point to novel lactobacilli that were found to be composed of 15 different phylotypes. The most striking result to emerge from the data is that amongst 34 isolates, *L. kunkeei* (YH-15) related sequences were the predominant lactobacilli in honey stomach, followed by *Lactobacillus* sp. Bma5. Similar results were obtained by Olofsson and Vasquez (2008) who identified *L. kunkeei* type strain YH-15 as the most frequently LAB in honey stomach and fresh honey of *A. mellifera*. It has been reported that *L. kunkeei* type strain YH-15, which was originally isolated from wine production, inhibited alcoholic fermentation by *Saccharomyces bayanus* and *Saccharomyces cerevisiae* (Huang et al. 1996)

and therefore known as a spoilage organism (Edwards et al. 1998). *L. kunkeei* is present on grapes during visits by honeybee foragers (Bae et al. 2006; Huang et al. 1996). The presence of 50% to 80% water content in collected nectars facilitates the fermentation of honey by yeasts, and it is believed that *L. kunkeei* prevent the growth of yeasts and their spoilage-related effects on honey (Olofsson and Vasquez 2008; Snowdon and Cliver 1996). The presence of other LAB which may have a similar function, may contribute to the flavor, aroma, and texture of honey because these characteristics are due, in part, to the LAB metabolites (Olofsson and Vasquez 2008; Mato et al. 2006; Steinkraus 1995). Eight strains (Yazid Taj-1, Naser Makhdzir-1, Taj Mahdi, Dilah Makhdzir-1, Faegheh Hadi-1, Taj KS164, Taj KS82, and Taj Mustafa-1) of *Lactobacillus* sp. Bma5 with the sequence similarity level of 88–97.3% were the second most frequently found lactobacilli in the honey stomach of *A. dorsata*. *Lactobacillus* sp. Bma5, which was previously isolated from the honey stomach of *A. mellifera*, is classified as *Lactobacillus acidophilus* DSM 20079 species with 91% similarity (Olofsson and Vasquez 2008). *L. acidophilus*, which is generally considered as safe, has been isolated from the gastrointestinal tract of human and animals, and their probiotic effects were well characterized. In the present study, *Lactobacillus* sp. Yazid Taj-1, with only 88% level of similarity with *Lactobacillus* sp. Bma5, was classified as a new taxon.

The isolates of Naser Mardan-1 corresponded to *L. vermiforme* with only 73% homology, so they could also represent a new taxon. This is the first time that *L. vermiforme* has been reported in the honey stomach. This strain was isolated during the alcoholic fermentation and after malolactic fermentation has been completed in South African brandy base wines (Du Plessis et al. 2004). Since malolactic fermentation is important in winemaking for its role in deacidification, flavor modification, and microbial stability, it can be hypothesized that isolates related to *L. vermiforme* may function similarly to *L. kunkeei* in the honey stomach. Taj-KS164 and Taj-KS82 strains were associated with *Lactobacillus* sp. Mardan Yazid-1 and *Lactobacillus* sp. Naser Makhdzir-1 with the similarity levels of 96% and 99%, respectively. These two latter strains have been isolated from the honey stomach of *A. dorsata* in our previous work (unpublished data).

Overall, in the present study, the predominant lactobacilli species found in the honey stomach of *A. dorsata* were similar to those reported in previous work that examined the honey stomach of *A. mellifera*. However, there are some differences in *Lactobacillus* sp. strains found in these studies such as *L. vermiforme*, *Lactobacillus* sp. Mardan Yazid-1, and *Lactobacillus* sp. Naser Makhdzir-1 detected in the honey stomach of *A. dorsata* versus Hon2, Hma2, Biut2, Bma5, and Hma8 strains related to *Lactobacillus* genus from the honey stomach of *A. mellifera* (Olofsson and Vasquez 2008). By using molecular techniques, Mrazek et al. (2008) evaluated the influence of geographic location, season, age, and part of the digestive tract on the bacterial diversity of the intestinal microflora of honeybees. They reported that nutrition habits were the strongest factor affecting the insect microflora. Moreover, Olofsson and Vasquez (2008) showed that the honey stomach flora varies with the sources of nectar and the presence of other bacterial genera. Another possible reason for variation among lactobacilli isolated strains in our study and previous work is probably using of 16S rRNA sequencing associated with classical cultivation method in this study. By contrast, Olofsson and Vasquez (2008) extracted DNA directly from collected honeybee samples and indirectly from pure culture.

In conclusion, the results of the present study demonstrate novel lactobacilli in the honey stomach of *A. dorsata*, which was dominated by phylotypes most closely related to *L. kunkeei* and other abundant groups related to *Lactobacillus* sp. Our study provides an outline of lactobacilli present in the honey stomach of *A. dorsata* and indicates the suitability of 16S rRNA sequence analysis in this study.

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Détection et identification de bactéries *Lactobacillus* du jabot de l'abeille géante *Apis dorsata*

***Apis dorsata* / jabot / *Lactobacillus* / probiotiques**

Nachweis und Identifizierung von *Lactobacillus* Bakterien im Honigmagen der Riesenhonigbiene *Apis dorsata*

***Apis dorsata* / honigmagen / *Lactobacillus* Bakterien / probiotische Lebensmittel**

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