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David J. Westenberg
Dartmouth College

Mary Lou Guerinot
Dartmouth College

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Succinate Dehydrogenase (Sdh) from *Bradyrhizobium japonicum* Is Closely Related to Mitochondrial Sdh

DAVID J. WESTENBERG* AND MARY LOU GUERINOT

Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755

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The *sdhCDAB* operon, encoding succinate dehydrogenase, was cloned from the soybean symbiont *Bradyrhizobium japonicum*. Sdh from *B. japonicum* is phylogenetically related to Sdh from mitochondria. This is the first example of a mitochondrion-like Sdh functionally expressed in *Escherichia coli*.

In the *Bradyrhizobium japonicum*-soybean (*Glycine max*) symbiosis, the plant host provides the bacterial partner with the dicarboxylates succinate, fumarate, and malate as carbon and energy sources (5, 10, 11). Bradyrhizobial mutants that lack a C_4-dicarboxylate uptake system develop nodules that are no longer able to fix nitrogen, clearly demonstrating a critical role for dicarboxylates in the symbiosis (6). Because dicarboxylates are assimilated via enzymes of the tricarboxylic acid (TCA) cycle, we have focused our attention on succinate dehydrogenase, a TCA cycle enzyme that is necessary for growth on succinate as a sole source of carbon and energy. Sdh and the related enzyme fumarate reductase (Frd) have been studied in a number of systems, most notably in *Escherichia coli*, *Bacillus subtilis*, and beef heart mitochondria (1). Sdh catalyzes the oxidation of succinate to fumarate, and this reaction is coupled to the reduction of ubiquinone to ubiquinol. Ubiquinol then transfers electrons to the electron transport chain for aerobic respiration. All Sdh complexes contain two catalytic subunits: SdhA, which forms the catalytic site for succinate oxidation and covalently binds flavin; and SdhB, which binds three iron sulfur centers (1). Most Sdh enzymes contain one (SdhC) or two (SdhC and SdhD) additional subunits that serve to attach the catalytic subunits to the inner side of the cytoplasmic membrane (1). The membrane-spanning subunits are also proposed to be involved in interaction of the enzyme with quinones (4, 13).

In this article, we report the cloning and sequencing of the *sdhCDAB* genes from *B. japonicum*. In addition, we investigate the close phylogenetic relationship between *B. japonicum* Sdh and eukaryotic Sdh and describe the functional expression of *B. japonicum* Sdh in *E. coli*.

Characterization of the succinate dehydrogenase (*sdhCDAB*) operon from *B. japonicum*. A PCR product, amplified from *B. japonicum* genomic DNA with primers to regions of *sdhA* from *Paracoccus denitrificans* (PD1, 5′-TCGCACACGGTCG CGGCGCAAGGC-3′; and PD3, 5′-CCTTCGCCGCGCGCG CCTTC-3′) was used to identify a 5.2-kb *Eco*RV fragment that carried all four *sdh* genes in the gene order *sdhCDAB* (Fig. 1). There is an overlap between the last codon of the *sdhC* gene and the first codon of the *sdhD* gene, and there is a 103-base gap between the last codon of the *sdhA* gene and the first codon of the *sdhB* gene. The *sdhB* gene is followed by a rho-independent transcriptional terminator, consistent with *sdhB* as the final gene in the operon. The 5′ end of the *B. japonicum sdhCDAB* transcript was determined by primer extension analysis (7) to begin 56 bases before the predicted start codon of *sdhC* (Fig. 1). The −35 and −10 regions upstream of the transcriptional start bear some similarity to a proposed *B. japonicum* consensus promoter (3). Despite the similarity of the *B. japonicum* consensus sequences to those of *E. coli*, the *B. japonicum sdh* promoter was not functional in *E. coli* (data not shown). However, all four subunits could be synthesized when an *E. coli* promoter was placed upstream of the *sdhC* gene (see below), consistent with the four *sdh* genes forming an operon.

***B. japonicum* Sdh activity and *sdh* gene expression.** Membranes prepared from *B. japonicum* cells (4) grown under iron- or heme-limited conditions (8) lack measurable Sdh activity (Fig. 2). This is consistent with the known properties of other Sdhs, which contain multiple iron sulfur centers and *b*-type cytochromes. *sdh* mRNA is undetectable in iron-deficient cells (Fig. 1B), consistent with Sdh abundance being controlled at the level of gene expression. Surprisingly, *B. japonicum* cells grown under oxygen-limited conditions contain elevated levels of Sdh enzyme (Fig. 2). This expression pattern is in contrast to that seen for *E. coli*, in which *sdhCDAB* expression is decreased under oxygen limitation (9). This discrepancy may reflect a requirement for *B. japonicum* Sdh under oxygen-limited conditions for metabolism of succinate, a major source of carbon and energy in the nodules.

The relationship of *B. japonicum* Sdh to mitochondrial Sdh. The predicted amino acid sequences of all available SdhA and SdhB subunits were aligned, and phylogenetic relationships (displayed as cladograms) were estimated (Fig. 3). All eukaryotic sequences form a single clade (indicated in Fig. 3 by the boxes) that also includes sequences from prokaryotes belonging to the α-subgroup of the proteobacteria (boldface in Fig. 3), including *B. japonicum*. This similarity is consistent with the endosymbiont theory of mitochondrial origin, which proposes that mitochondria evolved from symbiotic bacteria belonging to the α-subgroup of the proteobacteria (14). Therefore, *B. japonicum* Sdh may serve as an excellent model enzyme for analysis of mitochondrial complex II.

Several organisms contain both Sdh and Frd enzymes, and it is proposed that the duplicate sequences arose from a gene duplication (1). The Sdh and Frd sequences are generally separated into distinct clades, but this trend is not consistent. However, sequences in the mostly Sdh clade have the gene order *sdh*- or *frdCDAB* and sequences in the primarily Frd clade have the gene order *frd*- or *sdhABCD*. In this respect, gene order correlates well with the cladogram structure. Out-

* Corresponding author. Mailing address: Department of Biological Sciences, University of Missouri—Rolla, Rolla, MO 65409. Phone: (573) 341-4798. Fax: (573) 341-4821. E-mail: djwesten@umr.edu.

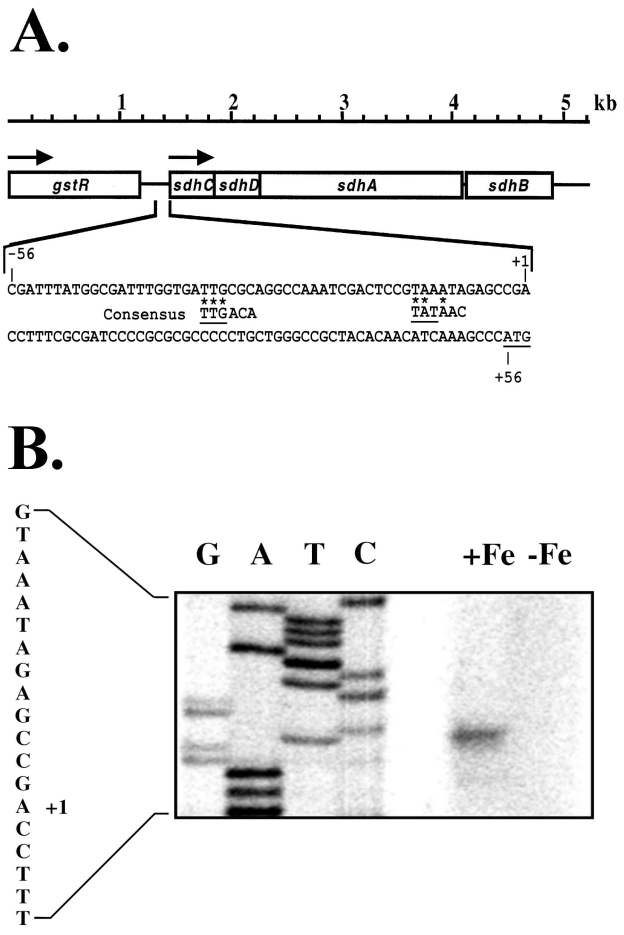

FIG. 1. (A) Physical map of the 5.2-kb *Eco*RV fragment encoding the entire *B. japonicum sdhCDAB* operon. The top line indicates the size of the DNA fragment in kilobase pairs. The second line represents the relative position of the genes coding for the individual subunits of the Sdh enzyme plus an additional incomplete open reading frame which encodes a protein with similarity to the *R. leguminosarum gstR* gene product. The arrows indicate the direction of transcription. The last line shows the nucleotide sequence of the promoter region and 5′ untranslated leader sequence with the start of transcription indicated as +1 and the start of translation for the *sdhC* gene underlined. The consensus *B. japonicum* "housekeeping" promoter sequence is indicated beneath the *sdhCDAB* sequence with the six critical bases underlined. An asterisk marks bases within the *sdhCDAB* sequence that match the consensus. (B) Determination of the *sdhCDAB* transcriptional start site by primer extension analysis. Twenty micrograms of total RNA from cells grown under either iron-sufficient or iron-deficient conditions was hybridized to a primer complementary to the beginning of the *sdhC* coding region. The DNA sequencing ladder was generated by using the same primer. The nucleotide sequence of the coding strand is indicated to the left of the DNA sequencing ladder.

lying sequences have the gene order *sdh-* or *frdCAB* (these enzymes have one C subunit that is equivalent to the CD subunits of other enzymes), indicating that the four-subunit enzymes may have evolved from the three-subunit enzymes. The *sdh* genes from two organisms, *Synechocystis* sp. and *Rickettsia prowazekii*, are not found in an operon.

The archaeal sequences form a heterogeneous group appearing in at least three separate clades. The methanogenic archaea appear in one clade; *Acidianus ambivalens*, *Sulfolobus acidocaldarius*, and *Aquifex aoelicus* appear in a second clade; and *Archaeoglobus fulgidis*, *Thermoplasma acidophilum*, and *Natronobacterium pharaonis* appear in a third clade. In some of the archaea, the genes do not form an operon (*Methanobacterium thermoautotrophicum*, *Methanococcus jannaschii*, and *A. aoelicus*), two have the gene order *sdhABCD* (*A. fulgidis* and *S. acidocaldarius*), and another has the gene order *sdhCDBA* (*N. pharaonis*). *M. thermoautotrophicum* also has the unique distinction of having two fumarate reductase enzymes, one of which is a thiol/fumarate reductase. The predicted amino acid sequences of the *M. thermoautotrophicum* Frds are very closely related, indicating a recent gene duplication leading to two genes with different specificities.

The diversity of Sdh and Frd structure at the level of subunit composition, gene order, and activity suggests that these enzymes have evolved rapidly. The mechanism for this rapid evolution is not readily apparent, but could involve lateral gene transfer and warrants further investigation.

Complementation of an *E. coli sdh frd* double mutant. The *B. japonicum sdhCDAB* operon under the control of a heterologous promoter (the IPTG [isopropyl-β-D-thiogalactopyranoside]-inducible *trc* promoter of plasmid pTrc99A) (2) complements *E. coli* DW35 (an *sdh frd* double mutant) (12) for growth on minimal succinate medium (Fig. 4). However, the doubling time is longer than the doubling time of DW35 transformed with the *E. coli sdhCDAB* operon (10 h versus 2 h). Even DW35 transformed with the *E. coli frdABCD* operon has a shorter doubling time in minimal succinate medium than the same strain transformed with *B. japonicum sdhCDAB* (Fig. 4). The doubling time was consistent over a wide range of IPTG concentrations (10 μM to 10 mM), indicating that insufficient expression levels are not the reason for slow growth (data not shown). This was confirmed by determination of abundance of membrane-associated flavin. (DW35 lacks membrane-associated covalent flavin—diagnostic for Sdh and Frd, the only membrane-associated enzymes with a covalent flavin.) Membrane-associated flavin indicates that the abundance of Sdh in the membrane of DW35 complemented with the *B. japonicum sdhCDAB* operon is equivalent to the abundance of Sdh in the membrane of DW35 complemented by the *E. coli sdhCDAB* operon (data not shown). The slow growth may simply reflect the respiration rate in the more slowly growing *B. japonicum*. The activity of respiratory enzymes may be optimized to the respiratory chain in which they operate and could act as a rate-limiting step when expressed in another bacterium.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned accession no. AF007569.

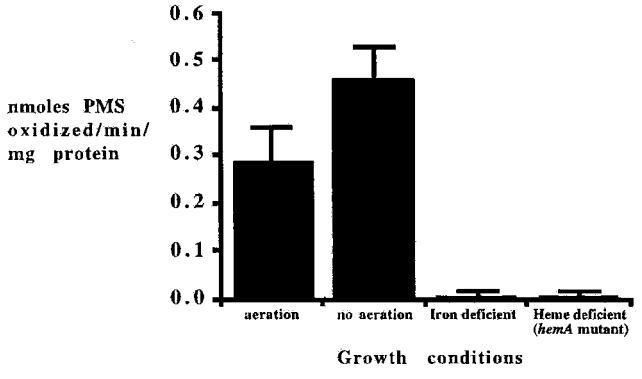

FIG. 2. Sdh activity of membranes prepared from *B. japonicum* cells grown under various physiological conditions. Membranes were isolated from cells grown under the described growth conditions and assayed for succinate-dependent reduction of phenazinemethosulfate (PMS). Each data point represents the average of at least three independent assays. Standard error bars are shown.

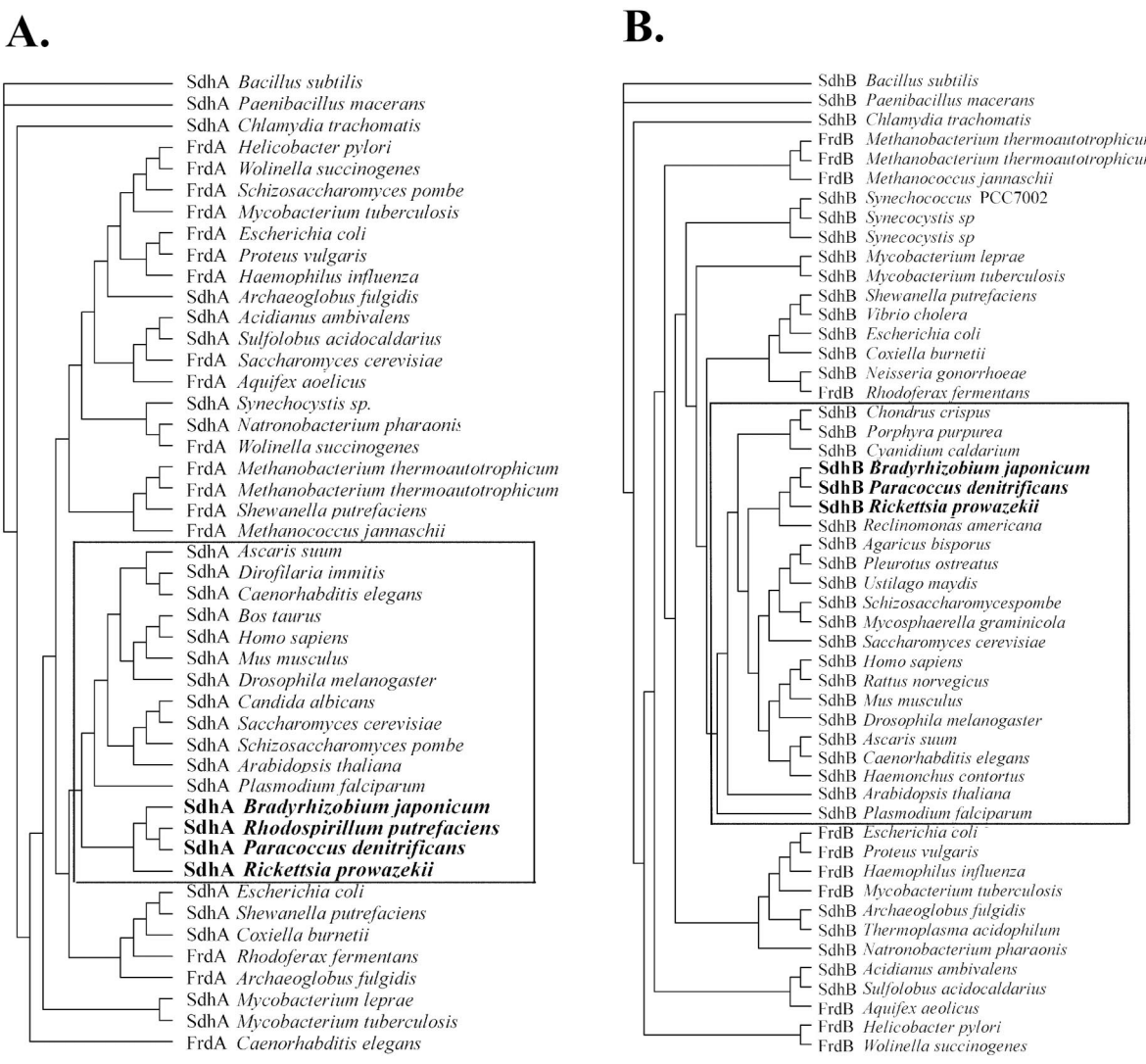

FIG. 3. Phylogenetic relationships of all publicly available, predicted SdhA or FrdA and SdhB or FrdB amino acid sequences. Sequences were aligned by using the GCG program PILEUP, distances were calculated by using PAUP, and trees were drawn by using neighbor joining. (A) The predicted SdhA or FrdA sequences used for the organism and subunit were as follows (with accession numbers in parentheses): *Acidianus ambivalens* SdhA (AJ005961), *Aquifex aeolicus* SdhA (AE000697), *Arabidopsis thaliana* SdhA (AJ001809), *Archaeoglobus fulgidus* SdhA (AE001057), *Ascaris suum* FrdA (D30650), *Bacillus subtilis* SdhA (P08065), *Bos taurus* SdhA (P31039), *Bradyrhizobium japonicum* SdhA (AF007569), *Caenorhabditis elegans* SdhA (Q09508), *Caenorhabditis elegans* FrdA (U23514), *Candida albicans* SdhA (Y10377), *Chlamydia trachomatis* SdhA (AE001330), *Coxiella burnetii* SdhA (P51054), *Dirofilaria immitis* SdhA (S78630), *Drosophila melanogaster* SdhA (Y09064), *Escherichia coli* FrdA (J01611), *Escherichia coli* SdhA (P10444), *Haemophilus influenzae* FrdA (P44894), *Helicobacter pylori* FrdA (O06913), *Homo sapiens* SdhA (P31040), *Methanobacterium thermoautotrophicum* FrdA (AE000910), *Methanobacterium thermoautotrophicum* Thiol:FrdA (AJ000941), *Methanococcus jannaschii* FrdA (Q60356), *Mus musculus* SdhA, *Mycobacterium leprae* SdhA (U00022), *Mycobacterium tuberculosis* FrdA (Q10760), *Mycobacterium tuberculosis* SdhA (AL021841), *Natronobacterium pharaonis* SdhA (Y07709), *Paenibacillus macerans* SdhA (Y08563), *Paracoccus denitrificans* SdhA (Q59661), *Plasmodium falciparum* SdhA (D86573), *Proteus vulgaris* FrdA (P20922), *Rickettsia prowazekii* SdhA (P31038), *Rhodoferax fermentans* FrdA (AB015757), *Rhodospirillum rubrum* SdhA (AB015756), *Saccharomyces cerevisiae* FrdA (JC5123), *Saccharomyces cerevisiae* SdhA (Q00711), *Saccharomyces cerevisiae* FrdA (JC5123), *Schizosaccharomyces pombe* SdhA (D89263), *Schizosaccharomyces pombe* FrdA (Z99292), *Shewanella putrefaciens* FrdA (Q02469), *Shewanella putrefaciens* SdhA (Y13760), *Sulfolobus acidocaldarius* SdhA (Y09041), *Synechocystis* sp. SdhA (D90906), *Wolinella succinogenes* FrdA (P17412), and *Wolinella succinogenes* FrdA (Y10581). (B) The predicted SdhB sequences used were as follows: *Acidianus ambivalens* SdhB (AJ005961), *Agaricus bisporus* SdhB (Y15942), *Aquifex aeolicus* FrdB (AE000695), *Arabidopsis thaliana* SdhB (P21915), *Archaeoglobus fulgidus* SdhB (AE001057), *Ascaris suum* SdhB (AB008568), *Bacillus subtilus* SdhB (P08066), *Bradyrhizobium japonicum* SdhB (AF007569), *Caenorhabditis elegans* SdhB (AB008569), *Chlamydia trachomatis* SdhB (AE001330), *Chondrus crispus* SdhB (P48932), *Coxiella burnetii* SdhB (P51053), *Cyanidium caldarium* SdhB (P48933), *Drosophila melanogaster* SdhB (P21914), *Escherichia coli* FrdB (P00364), *Escherichia coli* SdhB (P07014), *Haemonchus contortus* SdhB (X75822), *Haemophilus influenzae* FrdB (P44893), *Helicobacter pylori* FrdB (O06914), *Homo sapiens* SdhB (P21912), *Methanococcus jannaschii* FrdB (Q57557), *Methanobacterium thermoautotrophicum* FrdB (AE000937), *Methanobacterium thermoautotrophicum* Thiol:FrdA (AJ000942), *Mus musculus* SdhB, *Mycobacterium leprae* SdhB (S73040), *Mycobacterium tuberculosis* FrdB (Q10761), *Mycobacterium tuberculosis* SdhB (AL021841), *Mycosphaerella graminicola* SdhB (O42772), *Natronobacterium pharaonis* SdhB (Y07709), *Neisseria gonorrhoeae* SdhB (J03844), *Paenibacillus macerans* SdhB (Y08563), *Paracoccus denitrificans* SdhB (Q59662), *Plasmodium falciparum* SdhB (D86574), *Pleurotus ostreatus* SdhB (AB007361), *Porphyra purpurea* SdhB (P80477), *Proteus vulgaris* FrdB (P20921), *Rattus norvegicus* SdhB (P21913), *Reclinomonas americana* SdhB (P80480), *Rickettsia prowazekii* SdhB (3860614), *Rhodoferax fermentans* FrdB (AB015757), *Saccharomyces cerevisiae* SdhB (P21801), *Schizosaccharomyces pombe* SdhB (Z99091), *Shewanella putrefaciens* SdhB (Y13760), *Sulfolobus acidocaldarius* SdhB (Y09041), *Synechococcus* sp. strain PCC7002 SdhB (AF052290), *Synechocystis* sp. SdhB (D90909), *Synechocystis* sp. SdhB (D64003), *Thermoplasma acidophilum* SdhB (S34619), *Ustilago maydis* SdhB (P32420), *Vibrio cholera* SdhB (AJ231124), and *Wolinella succinogenes* FrdB (P17596). Note that the *Mus musculus* SdhA and SdhB sequences were derived by combining the sequences of several overlapping EST clones. The accession numbers for the various fragments are as follows: SdhA, W97337, W59232, AA048847, AA103522, AA028724, and W90791; and SdhB, AA050217 and AA109032.

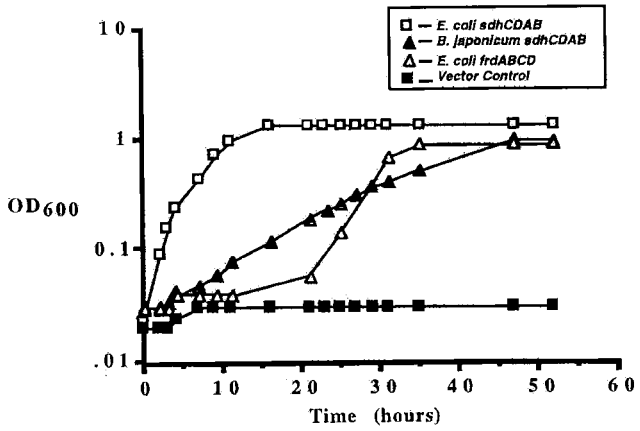

FIG. 4. Complementation of an *E. coli sdh frd* double mutant strain DW35. DW35 was transformed with plasmids carrying either the wild-type *E. coli sdhCDAB* operon (□), the wild-type *E. coli frdABCD* operon (△), the *B. japonicum sdhCDAB* operon (▲), or a vector alone as a control (■). OD_{600}, optical density at 600 nm.

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