Expression and characterization of a magnetosome-associated protein, TPR-containing MAM22, in *Escherichia coli*

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Abstract A magnetosome-associated protein, MAM22, contains a TPR domain (five TPR motifs and one putative TPR motif) that has been known to mediate protein–protein interactions. We expressed the *mam22* gene in *Escherichia coli* and found that the purified MAM22 was reversibly self-aggregated by NaCl. The structural model of MAM22 which has been proposed on the basis of the crystal structure of the N-terminal TPR domain of a human Ser/Thr protein phosphatase suggests the novel hydrophobic colloidal features of MAM22 with TPR motifs.

Key words: TPR motif; Magnetosome; *Magnetospirillum magnetotacticum*

1. Introduction

The most significant property of *Magnetospirillum magnetotacticum* is that it synthesizes the magnetic particles in the cell. The particle, termed a magnetosome, consists of a magnetite crystal with a diameter of 50–100 nm, is enclosed by lipids including glycolipids, sulfolipids, and phospholipids similar to those of the cytoplasmic membrane [1]. Recently, we determined the gene structure of the magnetosome-specific protein with a molecular mass of 22 kDa designated as MAM22 (DDBJ/EMBL/GenBank DNA database accession number D82942), and found that the protein contained TPR motifs [2]. The TPR motif has since been shown to be ubiquitous; it is present in a number of proteins that are functionally unrelated, and mediates a variety of different protein–protein interactions [3]. Although the physiological role of the magnetosome is generally considered to involve enabling the bacterium to orient itself along the lines of the earth's magnetic field [4], the function of the MAM22 localized in the magnetosome is as yet unclear. In the present study, we constructed the plasmid for expression of the *mam22* gene in *Escherichia coli*, purified the MAM22 protein, and found that the expressed MAM22 showed a tendency to be self-aggregated by NaCl. These properties may be related to the structural features of MAM22 with TPR motifs.

2. Materials and methods

2.1. Bacterial strain and growth condition

M. magnetotacticum (ATCC31632) was cultivated in a chemically defined medium [5] and harvested at the stationary phase by centrifugation at 10 000×*g* for 15 min.

2.2. Synthesis of oligonucleotide DNA for PCR amplification

Based on the nucleotide sequence of the *mam22* gene, two oligonucleotide primers (set A, 5′-GGG GGA-TCC-CAT-ATG-TCT AGC-AAG-CCG-TCG-3′; set B, 5′-GGG-GGA-TCC-AAA TCA-TAC-GGC-CTC-AGA-CCG-3′) were obtained from BEX Co. Ltd. (Tokyo, Japan).

2.3. Expression of MAM22 fusion protein in E. coli BL21(DE3)

The recombinant plasmid pET-MAM22 containing the *mam22* gene was constructed by cloning the PCR-amplified *mam22* into the *Nde*I/*Bam*HI site of expression vector pET-15b. For PCR amplification of the *mam22* gene, a sense primer with an integrated restriction site *Nde*I containing the start codon ATG, and an antisense primer with the restriction site *Bam*HI located downstream of the stop codon of the *mam22* gene were used. The 50 μl PCR reaction mixture contained 50 ng *M. magnetotacticum* genomic DNA, 2.5U LA Taq (Takara Biomedicals, Tokyo, Japan), 400 μM dNTPs, 2.5 mM $MgCl_2$, and 0.2 μM of each of the two primers. The temperature program was one cycle of 1 min at 94°C, 30 cycles of 20 s at 95°C, 1 min at 60°C, 2 min at 72°C, and one cycle of 10 min at 72°C. The PCR product was purified via Prep-A-Gene Matrix (Bio-Rad) according to the protocol of the manufacturer. After purification, the PCR products were ligated into a pGEM-T Easy vector (Promega, Tokyo, Japan). The recombinant plasmids obtained were then introduced into *E. coli* XL1-Blue MRF′ and cloned. The PCR product cloned into the pGEM-T Easy vector was sequenced to reveal that no mutation of *mam22* occurred. This plasmid was treated with *Nde*I and *Bam*HI, and the fragments thus obtained were ligated into the pET-15b expression vector (Novagen, Madison, WI, USA) linearized with *Nde*I and *Bam*HI.

pET-MAM22 was used to transform *E. coli* BL21(DE3). Genes cloned into pET-15b were transcribed by T7 RNA polymerase, the gene of which resides on the prophage DE3 integrated into the chromosome of BL21, and is under the control of the lacUV5 promoter. This promoter can be induced by addition of isopropyl IPTG [6].

For expression of the *mam22* gene, *E. coli* BL21 (pET-MAM22) was grown aerobically at 37°C in 300 ml LB medium [7] containing ampicillin (100 μg/ml). When the cultures reached an optical density of 0.6 at 600 nm, IPTG was added to the culture to achieve a final concentration of 1 mM. The cells (about 1 g wet weight) were harvested by centrifugation (10 000×*g* for 10 min) after 3 h incubation, and stored at −80°C until use.

2.4. Purification of MAM22 fusion protein in E. coli BL21(DE3)

The IPTG-induced cells harboring pET-MAM22 were suspended in 20 mM Tris–HCl buffer (pH 8.0), broken with sonication (20 kHz, 250 W) for about 15 min and centrifuged at 10 000×*g* for 10 min to

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Abbreviations: MAM22, magnetosome-specific 22 kDa protein from *Magnetospirillum magnetotacticum*; PAGE, polyacrylamide gel electrophoresis; TPR, tetratrico-peptide repeat; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside

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remove the unbroken cells and inclusion bodies. The supernatant, corresponding to cell-free extracts, was further centrifuged at 104 000×*g* for 1.5 h. The supernatant thus obtained was used as the soluble fraction, and the precipitated material obtained was used as the insoluble fraction. To the soluble fraction, 5 mM imidazole and 100 mM NaCl were added, then the suspension was centrifuged at 104 000×*g* for 1 h. The supernatant obtained was subjected to chromatography on a His-Bind resin (1.5×5 cm) equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 5 mM imidazole and 100 mM NaCl. After the column had been washed with 20 mM Tris–HCl buffer (pH 8.0) containing 60 mM imidazole and 100 mM NaCl, the proteins adsorbed on the column were eluted with 20 mM Tris–HCl buffer (pH 8.0) containing 200 mM imidazole and 100 mM NaCl. After dialysis against the 20 mM Tris–HCl buffer (pH 8.0), the N-terminal poly-His of the eluted protein was digested by thrombin as follows: 1 unit/20 μg target protein of thrombin was added and incubated for 2 h at 20°C in the presence of 20 mM Tris–HCl buffer (pH 8.4) containing 150 mM NaCl and 2.5 mM $CaCl_2$.

2.5. Determination of N-terminal amino acid sequence

The thrombin-treated MAM22 fusion protein was subjected to gas phase protein sequence analysis using Applied Biosystems model 478A to determine the N-terminal amino acid sequence.

2.6. Physical measurements

SDS–PAGE was performed by the method of Schöger and von Jagow [8]. The sample was treated with 4% SDS and 4% β-mercaptoethanol at 100°C for 2 min. The gels were calibrated with prestained SDS–polyacrylamide gel electrophoresis standards (broad range) (Bio-Rad, Tokyo, Japan).

2.7. DNA sequence and analysis

The cloned DNA fragment was sequenced on both strands by the dideoxy chain termination method after subcloning the appropriate overlapping restriction fragments into pUC119 vectors [9]. All DNA manipulations were carried out according to the method described in Sambrook et al. [7].

2.8. Search and analysis of TPR motif

The sequence data obtained from sequencing gels were compared and analyzed using the BLAST program [10]. The InterPro Search (http://oban.ebi.ac.uk:6600/pub/ipsearch.html) and ProfileScan Server (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html) were used for pattern and profile searches.

2.9. Reagents

All chemicals were of the highest grade commercially available.

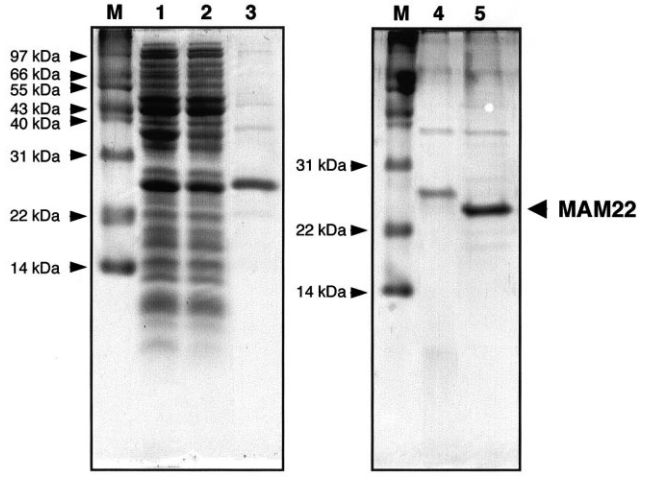

Fig. 1. SDS–PAGE analysis of MAM22 expression in *E. coli* and purification of the MAM22 fusion protein. Lanes 1 and 2 are total proteins and soluble proteins of IPTG-induced cells harboring pET-MAM22, respectively. Lanes 3 and 4, peak fraction of His-Bind resin column chromatography; lane 5, MAM22 fusion protein treated with thrombin; lane M, marker proteins.

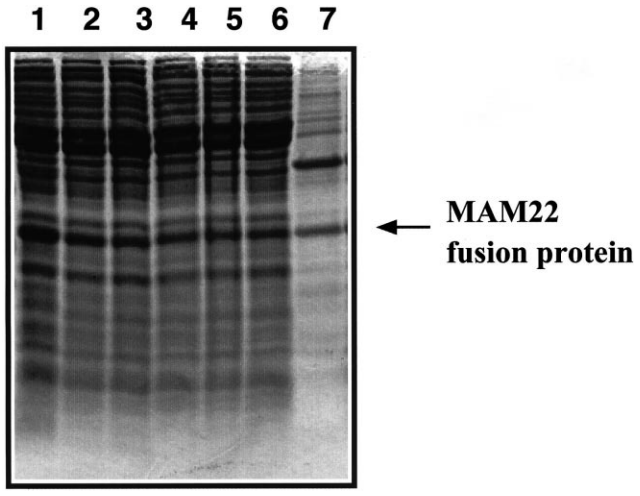

Fig. 2. Precipitation of MAM22 fusion proteins with NaCl. The cell-free extract was prepared from IPTG-induced cells harboring pET-MAM22 and then 2 M NaCl was added to yield final concentrations of 25 mM to 200 mM. After an addition of 2 M NaCl, the cell-free extract was centrifuged at 104 000×*g* for 1 h. The supernatants with equal amounts of total proteins were loaded on SDS–PAGE (lanes 1–6). The concentrations of NaCl in the cell-free extract are 0 M (lane 1), 25 mM (lane 2), 50 mM (lane 3), 75 mM (lane 4), 100 mM (lane 5) and 200 mM (lane 6), respectively. Lane 7 is the precipitate obtained from the cell-free extract in the absence of NaCl by centrifugation at 104 000×*g* for 1 h.

3. Results and discussion

3.1. Expression, purification, and characterization of MAM22

After the recombinant plasmid pET-MAM22 was transformed into *E. coli* BL21(DE3), the expression of the *mam22* was induced by addition of 1 mM IPTG. To investigate the expression of the *mam22* fusion gene, the total protein profiles of the whole cells and the soluble fraction were analyzed by SDS–PAGE for visualization using Coomassie Blue R-250. As shown in Fig. 1, the protein band of 25 kDa, which corresponded to the molecular mass of the histidine-tagged recombinant protein was highly expressed. The final preparation of the protein prepared by the method described in Section 2 was almost pure, and the molecular mass was determined to be about 23 kDa on SDS–PAGE. The N-terminal amino acid sequence determined with a gas phase protein sequencer was GSHMSSKPSD, which finding is consistent with the N-terminal amino acid sequence deduced from the *mam22* gene MSSKPSD. Therefore, it was concluded that the *mam22* gene was expressed in *E. coli*, and the histidine-tagged recombinant protein was solubilized in the cytoplasmic fraction. In the present study, about 4.6 mg MAM22 fusion protein was purified from the 1.25 g of cells cultured in 300 ml LB medium.

Based on the deduced amino acid sequence of MAM22, there were no transmembrane regions in the molecule. However, MAM22 fusion proteins were found in the insoluble fraction as shown in Fig. 2, lane 7. In order to investigate why the MAM22 fusion proteins were precipitated in the cell-free extracts, we prepared the cell-free extract in the presence of various concentrations of NaCl, then compared the protein composition of the respective soluble fractions. As shown in Fig. 2, lanes 1–6, the soluble MAM22 fusion proteins in the

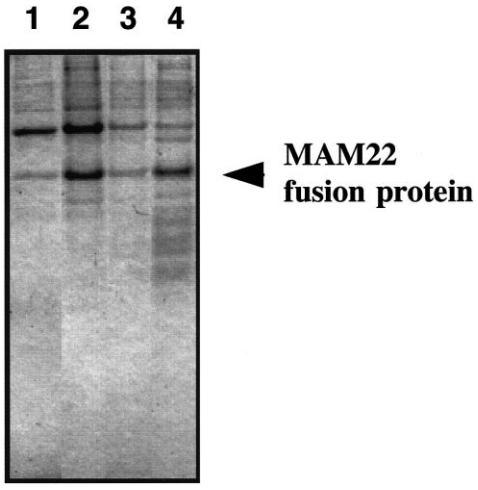

Fig. 3. Reversible solubilization of MAM22 fusion proteins precipitated with NaCl. The cell-free extracts were prepared in the absence of NaCl and in the presence of 0.1 M NaCl, respectively and centrifuged at 104 000×*g* for 1 h. The precipitates obtained containing the membrane-bound proteins and MAM22 fusion proteins were suspended in the sample buffer for SDS–PAGE and loaded on the gel (lane 1, 0 M NaCl; lane 2, 0.1 M NaCl). To investigate the reversible solubilization of MAM22 fusion proteins, the precipitates obtained were suspended in 20 mM Tris–HCl buffer (pH 8.0) with no detergents, respectively and centrifuged at 104 000×*g* for 1 h. The supernatants obtained were loaded on SDS–PAGE (lane 3, 0 M NaCl; lane 4, 0.1 M NaCl).

cell-free extract appeared to be decreased in proportion to the concentration of NaCl in the buffer used for the preparation of the cell-free extract. About 50% of the MAM22 fusion proteins was precipitated with 0.1 M NaCl. In addition, as shown in Fig. 3, the MAM22 fusion proteins precipitated with 0.1 M NaCl were dissolved in 20 mM Tris–HCl buffer (pH 8.0). These results indicate that although MAM22 fusion proteins were precipitated with NaCl, the precipitate could be reversibly dissolved in Tris–HCl buffer (pH 8.0).

3.2. Structural model of TPR motifs of MAM22

During sequencing of the *mam22* gene, we noticed an additional G, between G679 and G680, which was missed in the sequence published previously [2]. When the complete, corrected MAM22 sequence was analyzed by several computer programs (described in Section 2), we found that MAM22 contains a TPR domain, which consists of five TPR motifs and one putative TPR motif (Fig. 4). The TPR motif was first identified as a tandem-repeated degenerate 34 amino acid sequence in the cell division cycle genes [11,12]. It is now realized that >50 proteins present in organisms as diverse as bacteria and humans contain TPR motifs [3]. Multiple sequence alignments of the TPRs present in those more than 50 proteins reveal a highly degenerate sequence. Although there is no position characterized by an invariant residue, a consensus sequence pattern of small and large hydrophobic residues has been defined [3]. Small hydrophobic residues are commonly observed at positions 8, 20, and 27 within the motif. Position 32 is frequently a proline, and large hydrophobic residues are also located at characteristic positions.

Recently, the crystal structure of the N-terminal TPR domain of a human Ser/Thr protein phosphatase, PP5, was re-

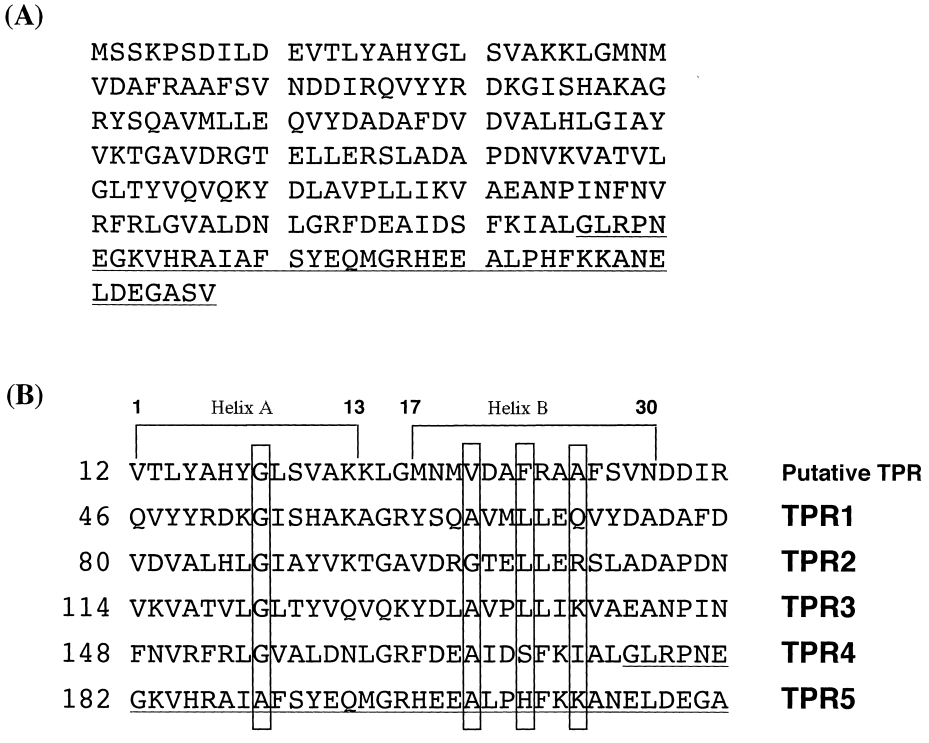

Fig. 4. Amino acid sequence deduced from *mam22* gene (A) and TPR motifs found in MAM22 (B). The underlined amino acid sequence was corrected in the present study. By computing analyses with the InterPro Search (http://oban.ebi.ac.uk:6600/pub/ipsearch.html) and ProfileScan Server (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html), five TPR motifs and one putative TPR motif were found in MAM22. A TPR motif is composed of a pair of antiparallel α-helices (A and B).

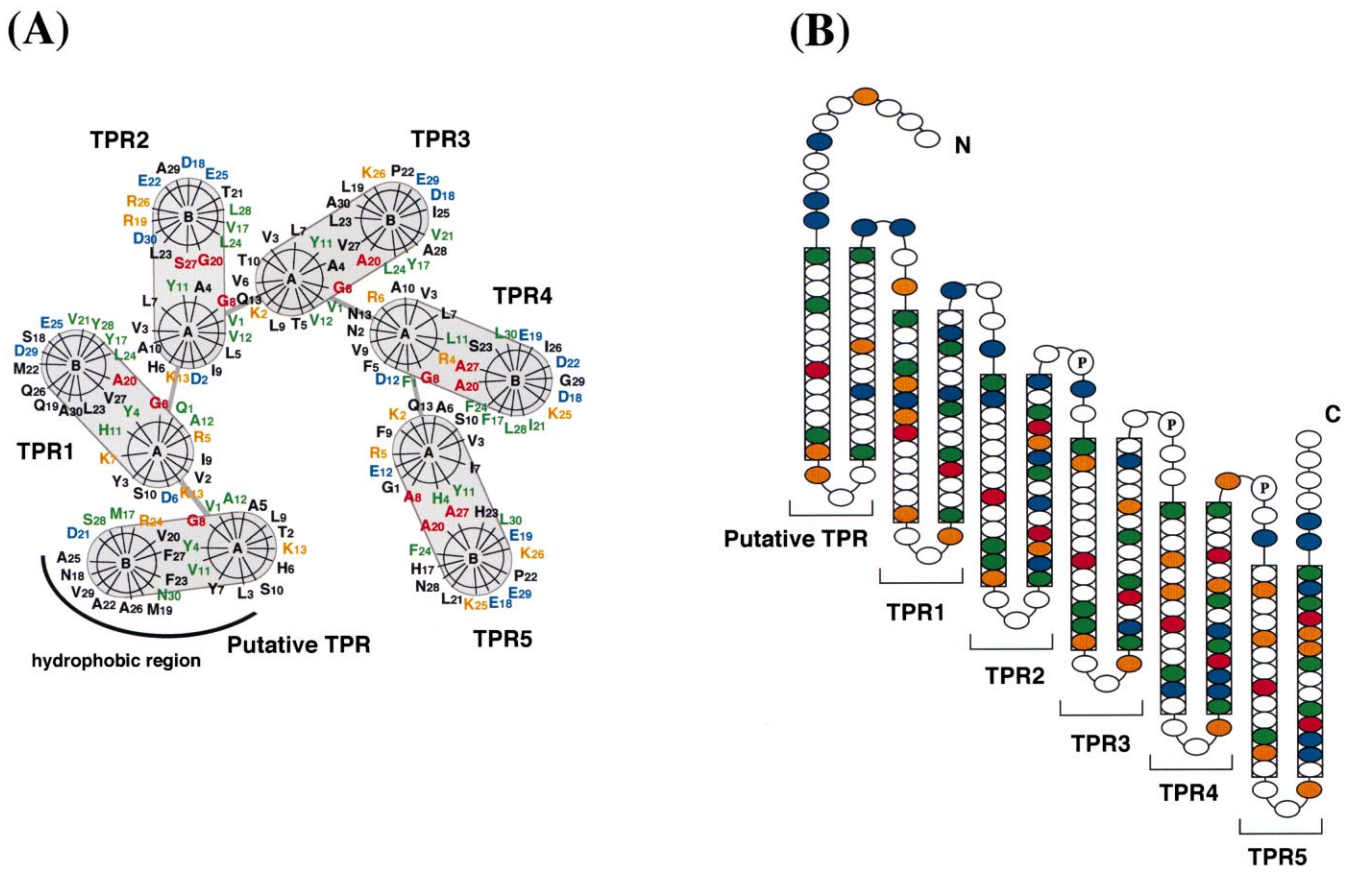

Fig. 5. A proposal for the structure of MAM22. A: View parallel to the helix axis of MAM22. A and B indicate helix A and B of TPR motif respectively. The red and the green residues correspond to the conserved small and large hydrophobic residues. The blue and the orange letter indicate negatively and positively charged residues, respectively. Outside of MAM22 superhelix is expected to have negative charge. B: Predicted secondary structure of MAM22. α-Helix is indicated by a rectangle. The red, green, blue and orange circles indicate the residues that are described in A.

ported [13]. The authors proposed a structural model for the tandem-arranged TPR motifs. This structural model includes a TPR motif continuous groove on the inside of the TPR helix, which groove is ideally suited to accept the α-helix of a target protein. According to these structural features of TPR motifs, we propose the structural model of MAM22 (Fig. 5). The five TPR motifs plus one putative TPR motif containing MAM22 form the 6/7 turn of the superhelix. The outside of the helix B of TPR1–5 motifs consists of negatively charged residues (D and E) and positively charged residues (R and K), although the outside of the helix B of the putative TPR motif consists of hydrophobic residues. These structural features suggest that MAM22s interact with each other via the distribution of charges. Therefore, addition of NaCl may drown out these negative/positive charges to induce aggregation of MAM22 by hydrophobic interaction between the putative TPR motifs. It should be noted that these properties are very similar to those of hydrophobic colloids, which have a tendency to be coagulated with NaCl.

On the other hand, the inner surface of the superhelix consists of hydrophobic residues, suggesting that the inside may form a hydrophobic cradle that could contact with target proteins, including MAM22. Therefore, it is tempting to speculate that the magnetosome protein MAM22 may have a functional role as a receptor for the cytoplasmic proteins that are transferred in the magnetosome, or may interact with proteins that are present in the contiguous magnetosome particle or cytoplasmic membrane, and result in holding the magnetosome chain structure. Although we have not identified any potential cytoplasmic proteins which interact with MAM22 by affinity chromatography with an immobilized MAM22, our study opens a new avenue for investigation of the physiological roles, and furthermore, 3D structure of magnetosome-associated protein, MAM22.

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