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Ogata, Seiya
Laboratory of Applied Microbiology, Faculty of Agriculture, Kyushu University

Koyama-Miyoshi, Yuko
Laboratory of Applied Microbiology, Faculty of Agriculture, Kyushu University

Hayashida, Shinsaku
Laboratory of Applied Microbiology, Faculty of Agriculture, Kyushu University

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Transfection and Transformation Systems for Pock-Forming and Thiostrepton-Producing *Streptomyces azureus*

Seiya Ogata, Yuko Koyama-Miyoshi and Shinsaku Hayashida

Laboratory of Applied Microbiology, Faculty of Agriculture,
Kyushu University 46-02, Fukuoka 812

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To establish a procedure for high frequency transfection and transformation of *Streptomyces azureus*, at first, the condition for the protoplast formation and regeneration of this strain were studied. The effective formation and regeneration of protoplasts of *S. azureus* were obtained by using mycelia grown in the presence of 0.4 % glycine. The effective transfection of S. *azureus* protoplasts with temperate phage SAt 1 DNA was obtained at 20 % (W/V) PEG 2000. The frequency of transfection under the optimal condition was about 1 per 5×10^6 DNA molecule (equal to 5×10^3 transfectants per μg DNA) and 1 per 5.3×10^3 regenerated protoplasts. The DNA transfection of a virulent phage SAvl was also succeeded in the protoplasts of S. *azureus*. The transformation of pock-cured strain AF-6 of *S. azureus* with pock-inducing plasmid DNA occurred under the same condition as transfection of phage SAtl DNA.

INTRODUCTION

This study was carried out with the aim to establish the transfection and transformation systems of an antibiotic (thiostrepton) -producing *Streptomyces azureus*.

Streptomyces species produce many industrally important antibiotics and physiologically active substances. The protoplasts of these microorganisms are useful in genetic studies and molecular breedings using protoplast transformation or transfection and fusion techniques. The basic conditions for handling *Streptomyces* protoplasts were deveroped largely by Okanishi *et al.* (1974) and adapted with minor modifications by many investigators (Shirahama *et al.*, 1981; Pigac *et al.*, 1982; Ogawa *et al.*, 1983). Therefore, as first step of this work, we demonstrated the protoplast formation and regeneration in S. *azureus*. It forms unique pocks with the production of phage tail-like particles in the lawn of plate cultures, as reported in previous paper (Ogata *et al.*, 1981). A plasmid, which is associated with pock formation, was isolated from this strain (Ogata *et al.*, 1983). The pock formation of this strain has common phenotypic features with "pocks" like *Streptomyces coelicolor* and others (Bibb et al., 1978; Thompson *et al.*, 1983), but there are some distinct differencies among them, as also described elsewhere (Ogata *et al.*, 1981). The protoplast formation and regeneration procedures will help genetic studies of pock-forming S. *azureus* involving fusion, transformation and transfection. In this

paper, the condition for the protoplast formation and regeneration of this strain are clearly described.

Recently some efficient transformation (Bibb et *al.*, 1978) and transfection (Krügel *et al., 1980* Suarez *et al., 1980,* Isogai *et al., 1980,* Toyama *et al., 1983)* systems for Genus *Streptomyces* were established using their protoplasts. So, it is anticipated that the bacteriophages will be used as cloning vectors in genetic manipulation for Genus *Streptomyces.* But their abilities as vectors depend on the characteristics of phage and phage-host systems. We isolated a temperate phage SAtl infectious to S. *azureus* (Ogata *et al.*, 1985)from soil sample. As the second stage of this work, condition for the efficient transfection system using the protoplasts from S. *azureus* and SAtl DNA is described in this paper. Furthermore, the transformation system using pock-inducing plasmid DNA is investigated as the last step of this work.

MATERIALS AND METHOD

Bacterial and phage strains

Streptomyces azureus ATCC 14921 and its phage SAtl (temperate) were used throughout this work. *Streptomyces endus* KCC S-0213, S. *coerulescens* KCC S-0360, S. *lavendulae* KCC *S-0263, S. rimosus* KCC *S-0073, S. griseus* NRRL B-2926, S. Zividans KCC S-0783, were also used. These bacterial strains were supplied by Dr. A. Seino (KCC Culture Collection of Actinomycetes). Also a pock-cured mutant AF-6 (Ogata *et al.*, 1983) of *S. azureus* and a virulent phage SAvl (isolated from soil) were used in the transformation experiment and transfection experiment, respectively.

Media, reagents and enzymes

Bennett medium and agar (glucose, 1.0 %, meat extract, 0.1% ; yeast extract, 0.1% ; NZ-amine type A, 0.2 %; agar, 1.5 % (hard) or 0.7 % (soft) ; pH 7. 2), Rye flake agar (rye flakes, 1.0 %; glucose, 0.2 %; yeast extract, 0.1% ; $CaCO_3$, 0.3 %; agar, 1.5 %; pH 7.2) and MG-1 medium (glucose, 1.0 %, NZ-amine type A, 0.4 %; yeast extract, 0.2 %; beef extract, 0.2 %; glycine, 0.4 %; pH 7.2) were used for the growth of organisms and phages. PWP buffer was prepared as described by Shirahama *et al.* (1981) and modified R 3 medium (sucrose, 0.4 M; glucose, 1.0 %; NZ-amine type A, 0.4% ; yeast extract, 0.4%; KCl, 0.05 %; $MgCl_2 \cdot 6\ H_2O$, 40 mM; $CaCl_2 \cdot 2\ H_2O$, 15 mM; K_2HPO_4, 0.002 %: Tris-HCl, 0.025 M; agar, 1.8 % (hard), low melting point agar, 0.7 %; pH 7.2) was used for the regeneration of protoplasts.

PEG 2000 was purchased from Nakarai Chemicals Ltd. Egg white lysozyme was from Seikagaku Kogyo Co. Ltd.

Preparation and regeneration of protoplasts, and estimation of regeneration frequency

Spores (about 10^8 colony forming units (cfu)) were inoculated into 50 ml of Bennett medium and grown for 24 hr at 28°C on a rotaly shaker (250 rpm). This cultivated broth was transferred (5 % v/v) to MG-1 medium, and cultivation was further carried out under the same conditions as above for 24 hr.

Then the mycelia were harvested by centrifugation ($5,000 \times g$), suspended in PWP buffer supplemented with lysozyme (1 mg/ml), and incubated at 30°C, for 30-60 min with gentle shaking. After that the protoplast suspension was centrifuged at $200 \times g$ for 5 min to remove the intact mycelia and cell debris, and the protoplasts in the supernatant were pelleted by centrifugation at 500 xg for 10 min. The protoplasts precipitated were gently suspended in 1 ml of PWP medium. The number of protoplasts converted was estimated by microscopic counts of 10 random haemocytometer fields. The percentage of regeneration was estimated by comparing the colony forming units (cfu) on modified R 3 agar plates with the number of protoplasts present. The protoplast suspension was also diluted with water and plated on the same agar except for the Mg^{2+}, Ca^{2+} and sucrose to determine the number of the osmotically resistant cells (nonprotoplast cells) in the protoplast suspension.

Phase contrast microscopy

Samples were observed with a phase contrast microscope (Nippon Kogaku Kogyo Ltd.) and photographs were taken using Fuji minicopy film.

Electron microscopy

Electron microscopical specimens were prepared by the method of Kellenberger (Higashi *et al.*, 1973) but 0.3 M sucrose was added to osmium fixative to prevent the bursting of protoplasts. Samples were observed with JEM-100 B electron microscope (Japan Electron Optical Laboratory Ltd).

Preparation of phage particles and phage DNA

Preparation of phage particles and phage DNA were carried out as described in our previous paper (Ogata *et al.*, 1985).

Transfection

A 50 μl of 30 % (w/v) PEG2000 in medium PWP was added. The mixture was incubated at 32°C for 3 min. After being diluted with 5 ml of medium PWP, the protoplasts were centrifuged (1000 x g, for 10 min) to remove PEG 2000. The pellet of protoplasts was suspended in 1 ml of medium PWP.

Transfection assay

(1) Infective center assay after phage DNA transfection.

A portion of the transfection mixture was diluted appropriately with PWP and plated with spore suspension of S. *azureus* (abut 10^7 cfu/plate) on the modified R3 agar according to double layer method. The plaques occurred were counted after 40 hr-incubation at 28°C. This number reveals the number of transfectants.

(2) Free phage assay.

A portion of the transfection mixture was transferred to the modified R 3 liquid medium in L-tube and incubated at 28°C with gentle shaking. After appropriate intervals during incubation, the aliquots were diluted with 0.05 M Tris-hydrochloride buffer (pH7.2) containing 0.1 M Mg^{2+} and plated with spore suspension of *S. azureus* (about 10^7 cfu/plate) on the Bennett medium

according to the double layer method. The plaques were counted after 48 hr-incubation at 28°C.

Preparation of plasmid DNA

For the preparation of plasmid pSA 1 DNA of S. *azureus*, the mycelia from the cellophane-coated plate cultures were gathered, and then lysed following the methods of Chater et al. (1982) : 5 mg/ml of lysozyme was used instead of 2 mg/ml. The cleared lysate was centrifuged to obtain a clear nonviscous supernatant, and then used for the precipitation and isolation of plasmid DNA by CsCl-EtBr density gradient centrifugation (Maniatis *et al.*, 1982).

Transformation and its assay

The pock-transforming activity of purified pSA 1 DNA was examined following the methods of protoplast transfection described above; 1 μg/ml of extracted pSA 1 DNA was transferred into 2×10^8 protoplasts of pock-cured strain AF-6 per ml. The transformants were detected by the appearance of pocks in the lawn of strain AF-6 used as an indicator when the regenerated colonies were plated together with strain AF-6.

RESULT AND DISCUSSION

Formation and regeneration of protoplasts

Okanishi *et al.* (1974) showed that the formation of *Streptomyces*' protoplasts can be accomplished by using mycelia grown in the presence of subinhibitory concentration of glycine. They determined a suitable glycine concentration for the protoplast formation of certain *Streptomyces* species, but S. *azureus* could not grow enough in this condition. So, we examined the growth of mycelia and the formation and regeneration of protoplasts. Growth of the cells was decreased in accordance with increasing of the concentration of glycine (Table 1). In the presence of 0.2 % or more glycine the protoplasts were formed, but in the presence of 1.0% or more glycine, the large clump of mycelia formed during precultivation, therefore, the rate of formation and regeneration of protoplasts decreased, as shown in Table 1. The optimal forma-

Table 1. Effect of glycine (%) in MG-1 medium on growth of cells and formation and regeneration of protoplasts

Concentration of glycine (%) in MG-1 medium	Relative growth (%)	Protoplast formation (No./ml)	Protoplast regeneration (%)
0.2	102	$<10^4$	ND
0.4	92	7×10^6	45
0.6	85	2×10^8	45
0.8	69	2×10^8, 1×10^8	23, 12
1.0	59	ND	ND

ND : not done

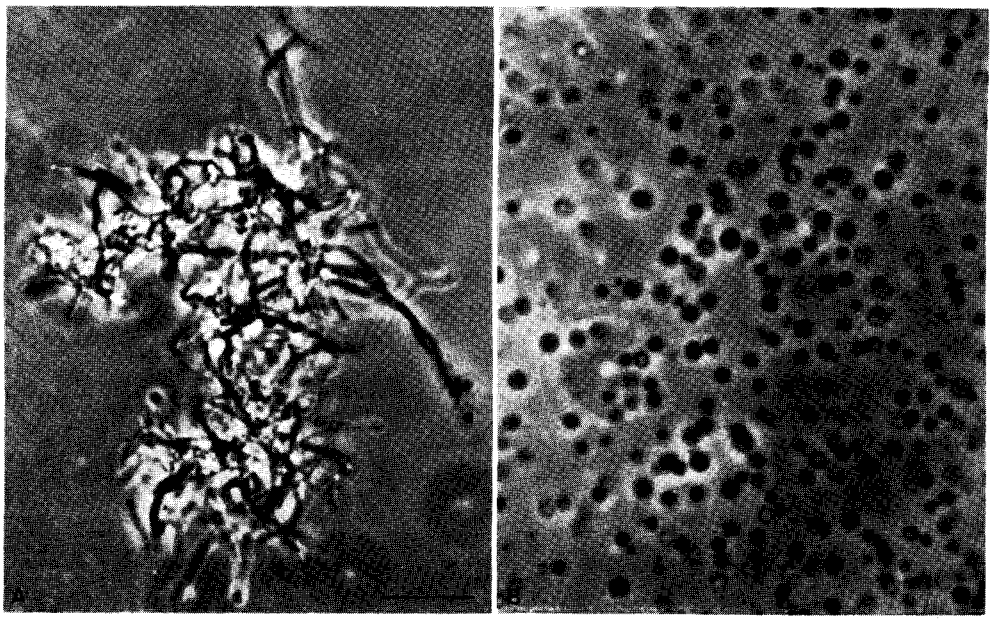

Fig. 1. Morphological changes in *Streptomyces azureus* during its protoplast formation observed with a phase contrast microscope.
Photographs were taken before (A) and after 60 min incubation (B). Scale indicates 10 μm.

tion and regeneration of protoplast of S. *azureus* were obtained by using mycelia grown in the MG-1 medium containing 0.4% glycine.

Figure 1 shows the morphological changes of S. *azureus*, under a phase contrast microscope. When the changes from mycelia to spherical cells (protoplasts) occurred, unicells were separated from the clump of mycelia. Almost all mycelia were converted to protoplasts in 60 min (Fig. 1-B). An electron microscope shows all cells became protoplasts with no cell wall (Fig. 2).

Effect of PEG 2000 concentration on the transfection frequency

Figure 3 shows the effect of the concentration of PEG2000 on the frequency of protoplast-transfection of S. *azureus* with SAt 1 DNA. The number of transfectants depended on PEG concentration. Optimal transfection was obtained with 20 % (w/v) PEGZOOO. No transfection was detected with less than 10 % PEGZOOO. When more than 20 % PEG2000 was used, the frequency decreased. This decrease may be due to the aggregation or fusion of the protoplasts.

Effect of DNA concentration on the transfection frequency

The effect of SAt 1 DNA concentration on the frequency of protoplast-transfection of S. *azureus* is shown in Fig. 4. The frequency of transfection

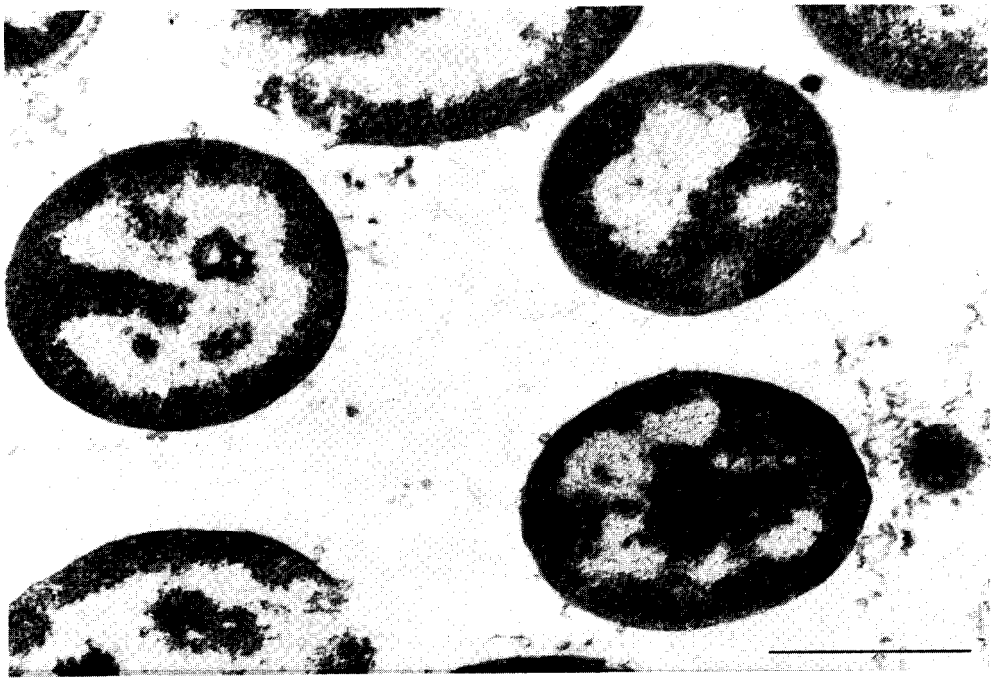

Fig. 2. Electron microphotograph of ultrathin section of *Streptomyces azureus* protoplasts.
Scale indicates 1 μm.

increased in proportion to the amount of DNA added up to 300 ng DNA (about 7.7×10^9 DNA molecules). The frequency of transfection under the optimal condition was about 1 per 5×10^6 DNA molecule (equal to 5×10^3 transfectants per μg DNA) and 1 per 5.3~ 10^3 regenerated protoplasts. This result is in good accordance with those of S. *lividans* (Kriigel *et al., 1980)* and S. *coelicolor* (Suarez *et al.,* 1980). Therefore, we judge that the protoplasts of S. *azureus* have transfection activity.

Time course of phage growth in protoplasts

The time course of phage growth in the protoplasts in R3 liquid medium after the transfection is shown in Fig. 5. The SAt 1 progenies appeared in the medium after 2 hr incubation and the number of free phages continued to increase for the following 4 hr. This result may indicate that the latent time for growth of phage SAt 1 is 2 hr.

Transfection of the protoplasts of various *Streptomyces* strains with phage SAt1 or phage SAv1 DNA

The transfection system established above was adapted to other *Streptomyces* organisms and a virulent phage SAv 1. Thus, experiments were carried out to understand whether or not their protoplasts can produce phage proge-

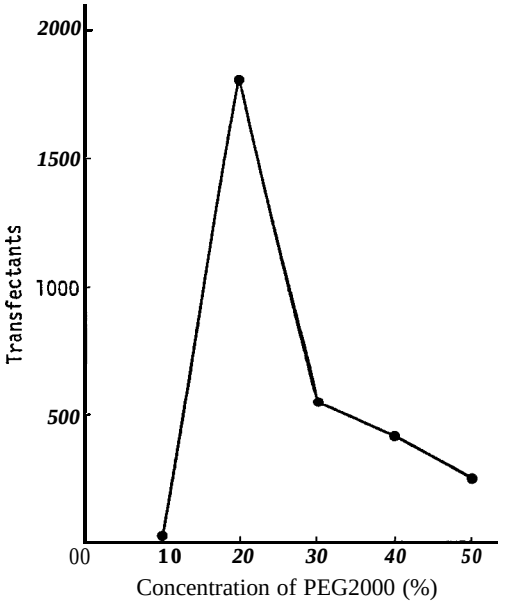

Fig. 3. Effect of PEG2000 concentration on the frequency of transfection of *Streptomyces azureus* protoplasts with phage SAt 1 DNA. About 8×10^6 cfu protoplasts of S. *azureus* were used for each transfection experiment.

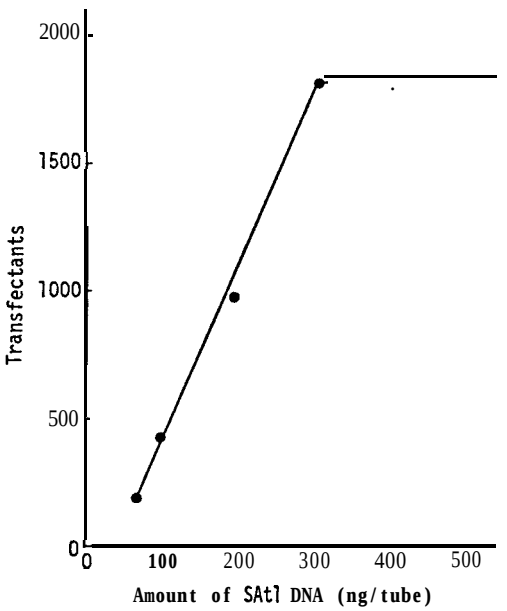

Fig. 4. Relationship between the amount of phage SAt 1 DNA and transfection frequency. About 8×10^6 cfu protoplasts of S. *azureus* were used for each transfection experiment.

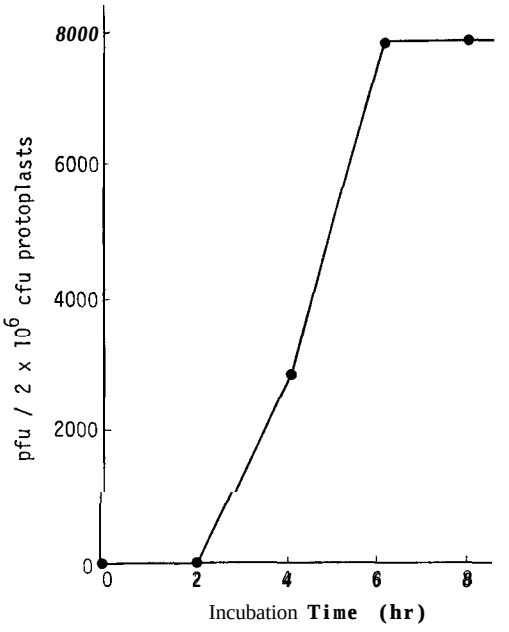

Fig. 5. Time course of development of phage SAt 1 after transfection of ***Streptomyces azureus*** protoplasts. 2×10^6 cfu protoplasts contained about 400 transfectants.
pfu: plaque forming units.

Table 2. Transfection of phage SAt 1 DNA and phage SAv 1 DNA on the protoplasts of various ***Streptomyces*** species.

Organism		Phage SAt 1		Phage SAv 1	
		DNA Transfection	Phage Infection	DNA Transfection	Phage Infection
Streptomyces azureus	ATCC 14921	+	+	+	+
S. viridochromogenes	NRRL B-1511	−	−	+	+
S. viridochromogenes	KCC S-0094	−	−	+	+
S. viridochromogenes	KCC S-0265	−	−	+	+
S. griseus	NRRL B-2926	−		+	+
S. lividans	KCC S-0783			+	−
S. ***endus***	KCC S-0213			−	−
S. ***rimosus***	KCC S-0073	−	−	−	−
S. ***coerulescens***	KCC S-0360	−		−	−
S. ***labendulae***	KCC S-0263	−		−	

+: production of phage progenies, −: no production

nies by infection of phenol-extracted phage DNA.

As shown in Table 2, phage SAt 1 had a very narrow host range and could infect only one strain, *S. azureus*. The DNA transfection of phage SAt1 was also limited only on the protoplasts of S. *azureus*. From this results, we judge that the narrow host range of phage SAt 1 is not due to the absence

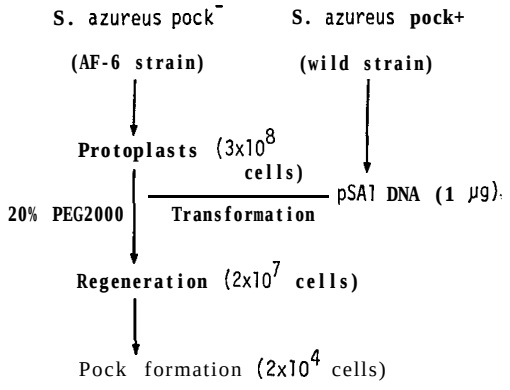

Fig. 6. Transformation of ***Streptomyces azureus*** AF-6 (pock- strain) protoplasts with pock-inducing pSA 1 plasmid DNA.

of receptors on the cell surface of various *Streptomyces* strains used. On the other hand, phage SAv 1 had a wide host range and could infect *S. azureus*, 3 strains of S. *viridochromogenes* and S. *griseus*. The DNA transfection of phage SAv 1 was occurred on the protoplasts of these organisms. Phage SAv 1 could not infect intact cells of S. *lividans*, but its DNA transfection was succeeded in the protoplasts of S. *lividans*. This result implies that insensitivity of phage SAv 1 to intact cells of S. ***lividans*** is due to the absence of phage receptors on the cell surface of S. ***lividans.*** Therefore, in the case of DNA transfection using protoplasts, the phenol-extracted DNA penetrated into the cells could produce phage progenies.

These results indicate that the transfection system descibed here should help genetic studies of pock-forming S. *azureus* and other *Streptomyces* organisms.

Transformation of protoplats with pock-inducing plasmid DNA

As shown in in Fig. 6 the frequency of transformants representing pock formation were 10^{-3} of the regenerated colonies, under the same condition as transfection of phage DNA. Almost all colonies which were transformed continued to harbor pock-forming activity even after several subculturings. This result indicates that the transformation system described here should be very helpful for the genetic studies of pock-forming S. *azureus* and other *Streptomyces* organisms.

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