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Multiple Electrophoretic Forms of Methyl-Accepting Chemotaxis Proteins Generated by Stimulus-Elicited Methylation in *Escherichia coli*

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The *tsr* and *tar* genetic loci of *Escherichia coli* determine the presence in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of methyl-accepting chemotaxis proteins (MCPs) I and II, respectively, each of which consists of a distinct group of multiple bands. Synthesis of the *tsr* and *tar* products was directed in ultraviolet-irradiated bacteria by lambda transducing phages. The addition of appropriate chemotactic stimuli to these cells resulted in the appearance of additional, faster migrating electrophoretic forms of the Tsr and Tar polypeptides, which disappeared upon removal of the stimulus. The stimulus-elicited forms comigrated with component bands of the corresponding MCPs. These results indicate that methylation itself caused shifts in electrophoretic mobility and hence led to the observed MCP band patterns. The number of Tsr species suggested that there were at least three methylated sites on the Tsr polypeptide. The conclusion that methylation generates multiplicity was supported by the results of experiments in which the *tsr* product was synthesized in mutant bacteria defective in specific chemotaxis functions concerned with methylation or demethylation of MCPs. Thus, the presence of a *cheX* defect blocked the stimulus-elicited appearance of faster migrating forms of the *tsr* product; conversely, the presence of a *cheB* defect resulted in a pronounced shift toward these forms in the absence of a chemotactic stimulus.

The functions involved in chemotaxis in *Escherichia coli* and *Salmonella* have been defined by genetic, biochemical, and physiological experiments (8, 14, 25). A variety of genes have been described whose products are responsible for the transmission of information from ligand-binding proteins on the surface of the cell to the flagellar apparatus and for the modulation of the signal so that the cell can adapt to different ambient levels of attractants and repellents.

The products of three genes, *tar, tsr,* and *trg,* play central roles in the processing of transmembrane signals (10, 23, 26). The polypeptide products of these genes reside in the cytoplasmic membrane (19) and act as signaling elements responding in a parallel fashion to subsets of compounds that act as attractants or repellents. Thus, the *tsr* gene is responsible for signals generated by the attractant serine as well as a number of other compounds. The *tar* gene product is responsible for signaling from aspartate and a variety of other compounds. Finally, the *trg* gene appears to be responsible for signals generated by temporal changes in the concentrations of ribose and some other sugars. Stimulation by these effector substances also results in the methylation of the appropriate signaling polypeptides (4, 10, 23, 26, 27). Experiments involving attractant-stimulated methylation revealed a multiplicity of bands upon analysis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, the genetic studies done thus far are consistent with the conclusion that single genes code for the *tar* and *tsr* functions. It has been suggested that the multiple bands might represent different degrees of methylation of a single polypeptide (7). However, the precise nature of the banding pattern that results on SDS gels from methylation has not been resolved, and the relationship of the unmethylated peptide to the various methylated bands has not been clarified.

To understand this pattern, we determined the effect of the addition of attractants upon the distribution of [^{35}S]methionine-labeled bands seen after protein synthesis had been programed by specific lambda transducing phage that carried the *tar* and *tsr* genes. In this way, we could observe the effects of stimulus-elicited methylation upon the mobility on SDS-PAGE of the prelabeled polypeptide chains without recourse to methyl labeling. This pattern could be compared to the distribution of [^{3}H]methyl-labeled methyl-accepting chemotaxis proteins (MCPs) obtained under similar experimental conditions. Furthermore, by using high-resolution gels

cross-linked at relatively low levels, we were able to resolve hyperfine structure in the banding pattern and to observe complex changes in the patterns as a result of stimulation. These experiments provide an approach that could be useful in understanding the molecular mechanisms of transmembrane signal processing.

MATERIALS AND METHODS

Strains. *E. coli* 159 (λ *ind*) has been described (21). Strain constructions were accomplished by standard methods, using P1 *clr*ts *clm* (13). *E. coli* 159 (λ *ind*) *eda* was constructed by transduction into MS6103 [22; a *flaI* derivative of 159 (λ *ind*)] from RP477 (*flaI*$^+$ *eda*). Motile recombinants selected on tryptone swarm agar were tested for failure to grow on minimal agar containing glucuronic acid as the sole carbon and energy source. *che* mutations (*cheX239*, *cheB294*; see reference 15) were transduced into 159 (λ *ind*$^-$) *eda* from strains supplied by J. S. Parkinson. Selection was for Eda$^+$ recombinants, which were then tested for chemotaxis on tryptone swarm agar. Che$^-$ clones were further characterized on the basis of complementation tests with appropriate λ *fla* transducing phages (24). The λ *fla* hybrids used have been described previously (23, 24).

Protein methylation. The methylation of proteins in the absence of protein synthesis was essentially as described previously (23) except that 0.5% glycerol replaced sodium lactate in the methylation buffer. L-[*methyl*-^{3}H]methionine with a specific activity of 60 Ci/mmol (New England Nuclear) was used for methyl labeling.

Phage-directed protein synthesis and SDS-PAGE. λ *fla* were used to program protein synthesis in UV-irradiated bacteria. Infection and labeling procedures have been described (21). [^{35}S]methionine with a specific activity of 900 Ci/mmol (Amersham) was used to label polypeptides.

SDS-PAGE was as described previously (21) except that a stock solution containing 44% acrylamide and 0.3% bisacrylamide was used to prepare 11% gels (6). This procedure gave greatly enhanced resolution of the bands of interest. Except where indicated in figure legends, gels were 10 cm long. All gels were treated for fluorography, using standard methods (1).

RESULTS

Effects of a serine stimulus upon the Tsr band pattern. Previous observations have indicated that the *tsr* locus determines the presence on SDS gels of a group of methylated bands, designated MCP I (23, 26). We determined the effect of the addition of serine upon the Tsr bands seen after protein synthesis had been programed by a lambda transducing phage (λ *fla*91) in UV-irradiated cells. In this way, we were able to see the effect of a stimulus upon Tsr species without recourse to methyl-labeling.

Figure 1, lanes 1 and 2, shows an SDS-PAGE analysis of the change in Tsr band pattern produced when λ *fla*91-programed cells were subsequently incubated with serine in the absence of further protein synthesis. It is clear that adaptation to this stimulus was accompanied by a redistribution of ^{35}S-labeled material, in particular by the appearance of the fastest migrating band, D4. It was also observed that the very faint uppermost band, D1, disappeared from the pattern (this is more easily seen in Fig. 2). The bands have been numbered in order of increasing

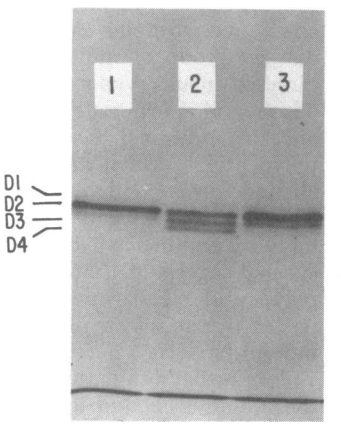

FIG. 1. *Effect of a serine stimulus upon the [^{35}S]methionine-labeled Tsr polypeptides whose synthesis was directed by λ fla91 in UV-irradiated bacteria. Cells were programed, harvested, resuspended in methylation buffer, and incubated for 15 min at 37°C in the absence (lane 1) or presence (lane 2) of 5 mM serine before final harvesting and lysis. A further sample of serine-stimulated cells was harvested, resuspended in methylation buffer without serine, and incubated for 5 min to allow deadaptation before harvest and lysis (lane 3).*

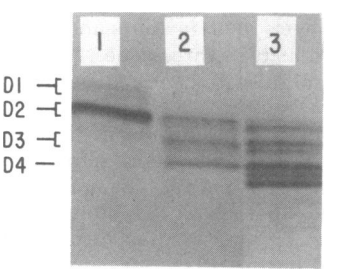

FIG. 2. *Comparison of λ fla91-programed Tsr species and serine-specific MCP. The [^{35}S]methionine-labeled samples in lanes 1 and 2 were prepared similarly to those in the corresponding lanes of Fig. 1. The sample in lane 3 was of polypeptides labeled with L-[methyl-^{3}H]methionine in the absence of protein synthesis after a serine stimulus. Samples were analyzed on a single 22-cm gel. Lanes 2 and 3 were aligned by reference to an intervening lane (not shown) which also contained Tsr species.*

electrophoretic mobility. Since an aim of this research is to map all Tsr and Tar species onto the MCP patterns seen in methylation experiments, the prefix D was chosen to denote Tsr bands. Incubation with the attractant aspartate, which is sensed through a parallel signaling pathway involving the analogous *tar* gene product, had no effect upon the unstimulated Tsr band pattern (data not shown). Also, the Tsr pattern seen in cells harvested immediately after programing was identical to that in lane 1 (not shown). To determine whether or not the serine-elicited shifts in the electrophoretic mobilities of the Tsr bands were reversible, the stimulus was removed by washing the cells (lane 3). Stimulus removal was accompanied by a reversal of the mobility shifts, with the virtual disappearance of band D4.

Tsr bands comigrate with the bands of MCP I. To find out if the multiple ^{35}S-labeled Tsr bands seen in λ *fla*91-programed cells could account for MCP I band multiplicity, we next compared the two patterns on the same gel. The data of Fig. 2 demonstrate that major serine-specific methyl-labeled bands (i.e., MCP I) did indeed comigrate with the λ *fla*91-programed species. The methylation experiment was performed in a Tar^+ strain; this explains the presence of a fast-migrating methylated band ahead of the lambda-encoded Tsr bands.

It should be noted that in the high-resolution gel of Fig. 2, bands D1, D2, and D3 of Fig. 1 were all resolved into closely migrating doublets, as were their methylated counterparts. This resolution was reproducibly obtained in these gels. The fastest migrating band, D4, has, however, never been seen to form a doublet. For the sake of simplicity the nomenclature of Fig. 1 will be used throughout this paper. Possible reasons for the doublet pattern will be considered in Discussion.

Identification of the unmethylated *tsr* product. Examination of Fig. 2 reveals that the only λ *fla*91-encoded species without a prospective methylated counterpart is the uppermost band, D1. This suggested to us that D1 might be the unmethylated form of the *tsr* product. To test this, we constructed a derivative of the host strain used in the programing experiments [159 (λ *ind*)] which now carried a defective *cheX* gene. The *cheX* gene of *E. coli* is thought to encode a carboxymethyl-transferase which transfers methyl groups from *S*-adenosylmethionine to the MCPs (2, 28). We expected, therefore, that expression of the *tsr* gene in the *cheX* background would enable us to identify the unmethylated form with certainty.

The result of this experiment is shown in Fig. 3. Comparison of lanes 1 and 3 reveals that in the *cheX* mutant the unstimulated Tsr pattern is very different from that seen in a wild-type host, with the D1 band being the predominant species in the mutant. This result indicates that D1 is the least methylated form of Tsr and is consistent with the notion that it is the unmodified *tsr* product. The presence of considerable levels of D2 in the mutant may arise as a result of leakiness of the *cheX* allele used (5, 16). Alternatively, there may be another methylation system capable of performing the D1 → D2 modification.

Comparison of lanes 3 and 4 of Fig. 3 shows that in the *cheX* mutant the addition of serine had little or no effect upon the pattern of Tsr bands. This confirms that the appearance of D3 and D4 in a stimulated cell is directly related to methylation of D1 and D2. We were able to restore *cheX* function to the *cheX* mutant in a programing experiment by coinfecting irradiated cells with λ *fla*91 and λ *fla*52Δ2. The latter phage carries a functional *cheX* gene (24). This "complementation" experiment resulted in a Tsr band pattern closely similar to that seen in the wild-type host cell with respect to the relative intensities of bands D1 and D2 (Fig. 4).

Effect of a loss of methylesterase function. To further extend our analysis of the methylation of the *tsr* product, we constructed a programing host derivative which carries a defective *cheB* gene. The *cheB* gene of *E. coli* is thought to encode a methylesterase which is responsible for the demethylation of the MCPs (29). The very low level of methyl labeling of the MCPs seen in *cheB* mutants has been attributed to the saturation of all available sites with methyl groups in the absence of a functional esterase (7).

In accordance with these ideas and with our hypothesis of the association between methylation and the mobility shifts of Tsr bands, we found that synthesis of the *tsr* product in a *cheB* mutant background was associated with unusually high levels of D3 and D4 in the absence of a serine stimulus (Fig. 5). Furthermore, addition of serine produced levels of D4 higher than any seen in a wild-type host. This high level of D4 persisted after the serine was removed; indeed, in this further period of incubation, the level of D3 increased at the expense of D2.

Effects of an aspartate stimulus upon the Tar band pattern. To confirm that our observations of the Tsr protein reflect general properties of MCPs, we determined the effect of the addition of aspartate upon Tar bands seen after protein synthesis had been programed by an appropriate lambda transducing phage (λ *fla*3Δ26) in the irradiated cells.

The results of this experiment are illustrated

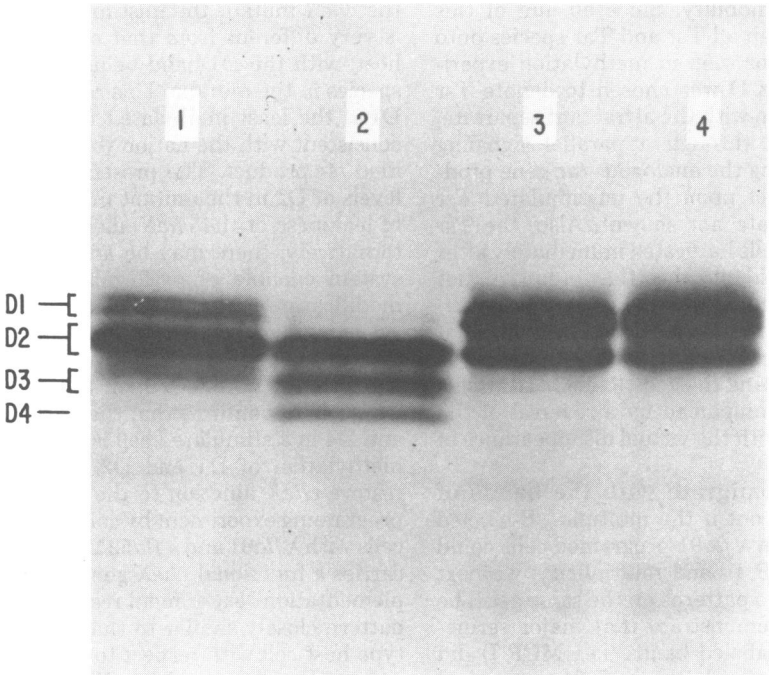

FIG. 3. *Tsr species encoded by λ fla91 in UV-irradiated bacteria defective in cheX function. The gel is of λ fla91-encoded protein in 159 (λ ind) (lanes 1 and 2) and in 159 (λ ind) cheX239 (lanes 3 and 4). Lanes: 1 and 3, no stimulus; 2 and 4, incubation with 5 mM serine. Samples were analyzed on a 22-cm gel. Shorter exposures of this gel revealed that in the cheX mutant the D1 component was resolved into a doublet, whereas the D2 component was not.*

in Fig. 6. The addition of aspartate was accompanied by the appearance of fast-migrating species (compare lanes 1 and 2). This effect was not seen in response to the addition of serine (not shown), a result which contrasts with previous observations of serine-stimulated methylation of MCP II (26). The aspartate-elicited Tar bands comigrated with major components of the MCP II (methyl-labeled) pattern (lane 3). Removal of the aspartate was accompanied by a reversion to the unstimulated pattern (not shown).

It will be recalled that the maximum number of *tsr*-encoded electrophoretic species, including doublets, was seven. It is apparent from Fig. 6, however, that the *tar* locus encodes at least 10 species. We have not attempted to impose any arbitrary nomenclature upon this pattern at this time. The extreme multiplicity of Tar forms will be discussed below.

DISCUSSION

There are two simple hypotheses which can explain the observation that the *tsr* and *tar* genes specify the multiple methylated band subsets of MCPs I and II, respectively. These hypotheses are: (i) that MCP band multiplicity reflects an equivalent multiplicity of unmethylated forms and (ii) that methylation itself in some way generates multiple bands from a primary unmethylated gene product. In the present study, we used the lambda programing system to specifically label the *tsr* and *tar* products independently of their methylation and determined the effects of addition and removal of appropriate specific stimuli. The conclusion which we have reached is that it is the second of the above hypotheses which is correct, namely, that methylation itself, apparently at multiple sites on the Tsr and Tar polypeptides, generates multiple electrophoretic forms.

Quantitative kinetic studies of MCP methylation have shown that adaptation to serine and aspartate stimuli is accompanied by an increase in the level of methylation of MCPs I and II, respectively (27). Under the same stimulus conditions, we found that ^{35}S-labeled Tsr and Tar polypeptides undergo shifts toward the anode in SDS-PAGE and comigrate with the corresponding methyl-labeled MCP bands. Furthermore, these effects upon electrophoretic mobility were reversed when the stimulus was removed, as were the changes in the level of methylation. These correlations are consistent with the idea that it is methylation itself which causes shifts

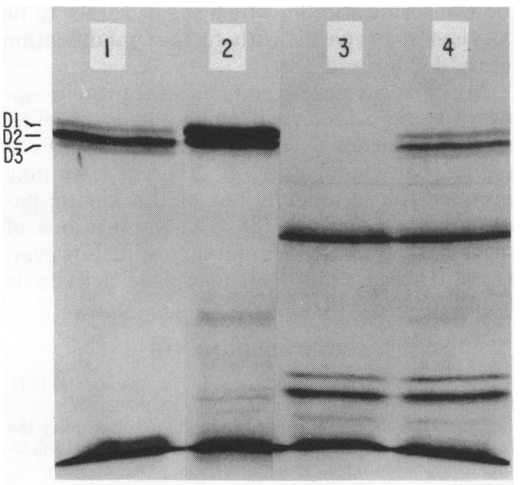

FIG. 4. *Restoration of cheX function by infection with λ fla52Δ2. The gel shows phage-encoded polypeptides in various combinations of irradiated bacteria and transducing phage. Lanes: 1, λ fla91 in 159 (λ ind); 2, λ fla91 in 159 (λ ind) cheX239; 3, λ fla52Δ2 in 159 (λ ind) cheX239; 4, λ fla91 and λ fla52Δ2, coinfection in 159 (λ ind) cheX239.*

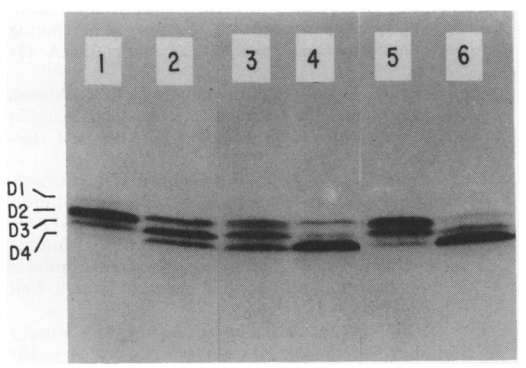

FIG. 5. *[^{35}S]methionine-labeled Tsr species encoded by λ fla91 in UV-irradiated bacteria defective in cheB function. An experiment similar to that described in the legend to Fig. 1 was performed in 159 (λ ind) (lanes 1, 3, and 5) and in 159 (λ ind) cheB294 (lanes 2, 4, and 6). Lanes: 1 and 2, no stimulus; 3 and 4, incubation with 5 mM serine; 5 and 6, removal of the serine stimulus.*

in mobility. Since the presence of the various forms depends upon the presence of the stimulus, it is clear that the multiple MCP bands do not simply reflect the existence of a corresponding set of unmethylated polypeptides.

The results of our experiments in which phage-directed synthesis of the *tsr* product occurred in mutants with defects in methylation or demethylation functions are entirely consistent with these ideas. Thus, in a mutant lacking the putative *cheX* methyltransferase, the appearance of D3 and D4 was blocked. Conversely, in a mutant lacking the methylesterase function (*cheB* product), almost all of the labeled *tsr* product was swept irreversibly into the fastest migrating band, D4. In the case of the Tsr pattern, it seems therefore that D1, D2, D3, and D4 represent increasing levels of methylation. The most economical hypothesis which fits our data is that D1 is the unmethylated form of Tsr and that methylation at one or more of three sites on the polypeptide generates D2, D3, and D4. Unexpectedly, the D2 band was found in the *cheX* mutant. This may be the result of leakiness in this allele of *cheX*, or it may reflect the existence of a second methylation activity. These alternatives are currently being tested.

During the preparation of this manuscript, we learned that two other groups had also concluded, on the bases of two other experimental approaches, that the MCPs of *E. coli* are multiply methylated (1a, 3a). We also learned that results very similar to those reported here had been obtained for an MCP from *Salmonella*

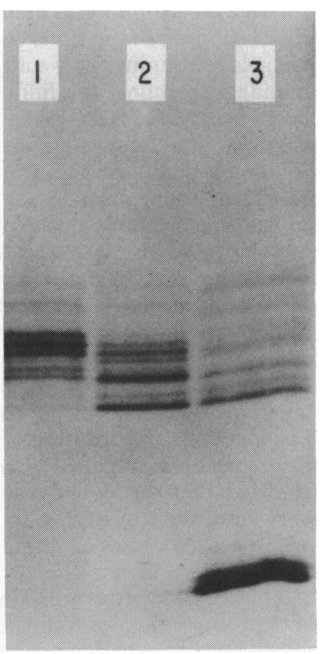

FIG. 6. *Tar species encoded by λ fla3Δ26: effect of an aspartate stimulus and comparison with aspartate-specific MCP. The experiment was analogous to that described for Tsr in the legends to Fig. 1 and 2. Lanes: 1, [^{35}S]methionine-labeled λ fla3Δ26-encoded species; 2, incubation with 5 mM aspartate; 3, polypeptides methyl labeled in response to a stimulus of 5 mM aspartate. Samples were analyzed on a 22-cm gel.*

typhimurium (1b). Thus, experiments in four laboratories, with two different species and three different experimental approaches, have all led to the same conclusion, namely, that multiple methylation events generate MCP band multiplicity.

At this point, it seems relevant to consider how the addition of a single methyl group might cause anodic shifts in electrophoretic mobility on SDS gels. The SDS-PAGE procedure for the separation of polypeptides according to molecular weight depends upon three factors: (i) that polypeptides bind a constant amount of SDS per unit mass, (ii) that the SDS masks the intrinsic charge of the polypeptide so that the charge/mass ratio of SDS-polypeptide complexes is constant, and (iii) that SDS-polypeptide complexes adopt a common configuration so that shape has a negligible effect in the electrophoretic separation (17, 18). The neutralization of negative charges in the Tsr and Tar polypeptides resulting from carboxymethylation of glutamyl residues (9, 30) might be expected to enhance SDS binding, resulting in increased charge/mass ratios relative to less methylated forms. Thus, each methylation could lead to a quantal increase in relative electrophoretic mobility. The increased resolution of the various methylated forms seen in gels containing a low concentration of bisacrylamide may reflect the finding that of the two forces operating in SDS-PAGE, namely, charge separation and molecular sieving, the former is of greater importance as polyacrylamide cross-linking decreases (20).

We recognize that we have not explained all of the complexities of the Tsr and Tar band patterns seen in our gels. In the case of Tsr we refer to the resolution of D1, D2, and D3 into doublets; in the case of the extreme complexity of the Tar pattern, we refer to almost the whole pattern. We cannot exclude the possibility that these proteins are unusually prone to gel artifacts, such as incomplete disruption of disulfide bonds or differential binding of SDS impurities.

It is noteworthy that the level of the D1 form of Tsr is very low, even in unstimulated cells. Furthermore, repeated cycles of serine addition and removal do not increase the level of D1 (unpublished data). Since this procedure might be expected to promote mixing of the ^{35}S-labeled Tsr material with the preexisting pool of Tsr, we believe that what we see in programing experiments is representative of the whole cellular pool. This apparent basal level of Tsr methylation may be caused by adaptation of the cell to ambient stimuli, such as temperature (12). Certainly it indicates that the transition from unmethylated Tsr (D1) to singly methylated Tsr (D2) may be relatively unimportant for adaptation to serine stimuli, which seems rather to be associated primarily with further modification of the D2 form of Tsr.

Multiple covalent modification of proteins appears to be a biological phenomenon of widespread occurrence (11), and in some cases it seems that modification at different sites may produce subtle modulations of the kinetic parameters of enzymes (3). The implications of methylation for MCP function will, however, remain obscure until an assayable activity is found for the proteins.

ACKNOWLEDGMENTS

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