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Colonial Opacity Variation in *Mycoplasma pulmonis*

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Colonial size and opacity variation were observed in four independently isolated strains of the murine pathogen *Mycoplasma pulmonis*. Selecting colonial opacity variants of similar size, we identified opaque and transparent stable variants. Opaque colony-derived broth cultures shed transparent colonies at a rate of about 1.2×10^{-8} per CFU per generation. The reverse conversion was about two orders of magnitude less frequent. Appearance of opacity and plating efficiency of each pure culture were altered by changing the serum source used to supplement the growth medium. Horse or sheep serum was most efficient at accentuating visualization of opacity differences. Fetal bovine serum was least efficient. In two *M. pulmonis* strains, each opacity variant showed a distinctive polypeptide profile, as displayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In the same strains, distinctive intrastrain differences were found by agarose gel electrophoresis to display the DNA fragments produced after digestion by several endonucleases. Each pure culture variant retained these differences in DNA even when grown in a medium supplemented with a serum which did not accentuate visualization of the opacity phenotype. Characterization of variants in 30 other *M. pulmonis* strains is in progress.

Colonial phenotypic variations have been noted in a variety of both gram-negative and gram-positive bacteria (reviewed in reference 3). One system which has been well characterized is *Neisseria gonorrhoeae*. The opacity of *N. gonorrhoeae* colonies appears related to a variety of biological factors, including virulence in chicken embryos, adherence to diverse eucaryotic cell types, and susceptibility to proteases or sera components (17). Although colonial morphology is often emphasized in the description of mycoplasmas (18), no studies exist concerning colonial phenotypic variants. While studying the murine pathogen *Mycoplasma pulmonis*, we observed colonial size and opacity variations. Selecting similarly sized colonies which showed obviously different opacities, we found that stable, pure cultures could be isolated and maintained. The characterization of each population revealed several biological and biochemical differences which should be critical in developing our understanding of the biology of *M. pulmonis*.

MATERIALS AND METHODS

Microorganisms and media. Four strains of *M. pulmonis* were used. Each isolate (designated AL-1 to AL-4) was obtained from different rats maintained at different laboratories. Except for initial studies, all cultures were derived from the cloning of a single opaque (op) colony of each strain. Transparent (tr) cultures were derived from light colonies shed from the original op colony. All strains were maintained in SP-4 broth (19) modified by replacing fetal calf serum with donor horse serum (K-C Biological, Lenexa, Kans.). This medium was also used as a solid medium with the addition of 0.8% Bacto-Agar (Difco Laboratories, Detroit, Mich.). In some experiments (as noted below), fetal bovine, porcine, or sheep serum (Flow Laboratories, McLean, Va.) was used as the serum source. All incubations were at 35°C in air.

Microscopy. A stereo microscope (Stereo Zoom 7; Bausch & Lomb, Inc., Rochester, N.Y.), equipped with a substage reflector having both a diffusing surface and a plane polished mirror, was used. Photography was carried out as described by Swanson (16).

Polyacrylamide gel electrophoresis. Polypeptide profiles of whole-cell preparations were obtained by using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as previously described (13).

DNA extraction and restriction enzyme analysis. DNA was extracted by a phenol extraction procedure. A cell pellet was obtained from 50 ml of a 48-h culture of each *M. pulmonis* culture. The pellet was suspended in a Tris (10 mM)–sodium EDTA (1 mM) buffer (pH 7.4), designated TE. Cells were lysed with 1% (vol/vol) SDS (final concentration) at 37°C. The lysate was extracted thrice with an equal volume of recrystallized phenol saturated with TE. The resulting aqueous phase was extracted once with a chloroform-isoamyl alcohol mixture (250:1) to remove residual phenol.

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After the addition of 0.3 M (final) sodium acetate, nucleic acids were precipitated from this solution with 3 volumes of ethanol at −40°C overnight. The precipitate was collected by centrifugation, resuspended in TE, treated with 25 μg of bovine pancreatic RNase (Calbiochem, La Jolla, Calif.) per ml, and then reextracted and precipitated as before.

Restriction endonucleases were added (1 U of enzyme per 1 μg of DNA), and incubations were carried out as prescribed for each enzyme by the manufacturer. The following enzymes were used: *Alu*I, *Bgl*II, *Hpa*II, *Kpn*I, *Pst*I, *Sal*I, *Sau*3A, *Sma*I, *Sst*I (all from BRL, Bethesda, Md.), *Bal*I, *Bam*HI, *Bcl*I, *Dde*I, *Eco*RV, *Hae*III, *Hha*I, *Hin*fI, *Mst*II, *Pvu*I, *Taq*I, *Xmn*I (New England Biolabs, Beverly, Mass.), *Bst*I, *Eco*RI, *Xho*I (P-L Biochemicals, Milwaukee, Wis.), *Cla*I, and *Hin*dIII (Boehringer, Mannheim, Germany). Agarose gel electrophoresis was done by standard methods (6).

Biological characterization. Growth patterns of each culture were determined by using standard plate counting procedures. Rates of conversion from one phenotype to the other were determined by the formula $M = \ln 2\ [(M_2 - M_1)/(N_2 - N_1)]$, where M is the minority population and N is the viable count (described in reference 15). Filtration experiments were done with 0.45-μm Millex filters (Millipore Corp., Bedford, Mass.).

RESULTS

Occurrence of colony opacity variants. The initial observations were made on strain AL-2 during the course of investigating mutants resistant to the virus MVMP-1 (A. Liss et al., Can. J. Microbiol., in press). For these experiments, plates were incubated for 7 to 10 days, as compared to 3 to 5 days used routinely for colony counting assays. Accidentally, reflected light from the diffusing surface of the mirror on the stereo microscope was used to view colonies, and an opacity difference in colonies was seen. The extremes of a continuum of opacity shades were selected and designated op (dark colonies) and tr (light colonies).

Description of opacity variants. As shown in Fig. 1A, using the plane polished mirror, most colonies appeared similar, with a suggestion of the typical fried-egg appearance characteristic of most mycoplasmas. However, distinctive opacity differences were seen in the same colonies when they were illuminated with reflected light from the diffusing mirror (Fig. 1B). Opacity variation was most apparent with colonies grown on medium containing horse serum after 7 to 10 days of incubation. op colonies had rounded margins. Sizes varied, ranging from 0.2 to 1.2 mm in diameter. Occasionally, dark sectors were seen radiating from the center of the colonies. tr colonies also had a complete margin. Colony sizes ranged from 0.2 to 2.0 mm in diameter. More striking was the sectoring of tr colonies with op-like opacity. Similar observations were evident in each of the four *M. pulmonis* strains examined.

Pure culture characteristics. For more detailed studies, *M. pulmonis* AL-1 and AL-2 opacity variants were selected. Each opacity variant was purified by isolating single colonies and restreaking each colony at least three times. If the streaked colony developed >99% of the desired phenotype, a colony from this final plate was picked and propagated in broth medium. Broth cultures were passaged every 48 h. At each passage, a portion was removed and streaked onto solid medium, and colonial opacity was noted. Each phenotype was stable for at least five passages. Beyond this, op cultures began to produce tr colonies at a frequency higher than the tr cultures produced op variants (see below).

Growth rates of either pure culture appeared similar (data not shown). Each had a doubling time of approximately 4 h and reached a titer peak of 1×10^9 to 5×10^9 CFU/ml.

Cell clumping of both broth- and agar-grown cultures was estimated by comparing the fraction of CFU which passed through a 0.45-μm (pore size) filter. Broth cultures of op or tr showed no difference in fraction of filter-passing CFU ($n = 10$). The same was seen with single colonies of either phenotype ($n = 10$). The fraction of filter-passing CFU ranged from 0.02 to 0.05 of the original CFU titer.

Rate of appearance of alternate phenotype. The conversion of op to tr was followed. op and tr colonies appeared to be "pure." That is, when a single colony was picked, broken up by vortexing, and then plated out to check opacity, each colony ($n = 10$) appeared homogeneous. However, in broth culture, the op → tr conversion appeared at a rate of $1.2 \pm 0.07 \times 10^{-8}$ per CFU per generation (given as mean value ± standard error of the mean). The tr → op event occurred at a rate of $1.0 \pm 0.12 \times 10^{-10}$ per CFU per generation.

We tested a variety of medium components to see whether the op or tr phenotype could be altered (data not shown). The only significant changes (both in colony opacity seen and rate of op ⇄ tr) occurred with changes in serum source used to supplement the medium. Colonies from both op and tr cultures appeared to be of the same opacity (tr) when grown on fetal bovine serum-containing medium. However, although the plating efficiency (as compared to growth on horse serum) of both cultures was similar, tr colonies were about half the size of op colonies. With pig serum supplement, again opacity differences seemed less distinct. Plating efficiency of tr cultures was less (1 to 2 log titer loss) than that of op cultures. Sheep serum appeared equivalent to horse serum as a medium supplement capable

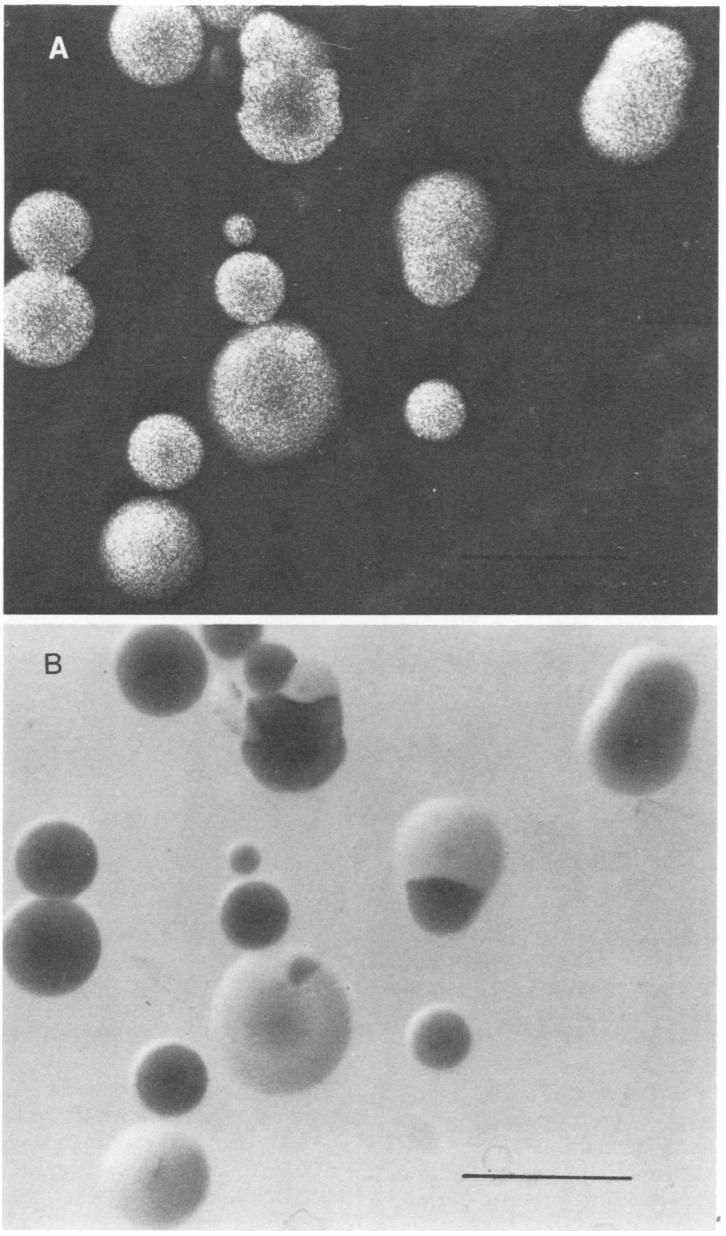

FIG. 1. Dissecting-microscopic appearance of op (dark) and tr (light) colony variants by two forms of illumination. When viewed with illumination from the polished mirror, colonies appear relatively the same (A); when the illumination is with a diffusing substage reflector, variations in opacity are seen (B). Bar, 0.5 mm.

of accentuating opacity differences. Plating efficiency was also equivalent.

Endonuclease treatment of DNA. Total cellular DNA of op and tr cultures was treated with several endonucleases to ascertain the number of sites for each enzyme (Table 1). During this survey we noted that for several enzymes, differences in migration and/or number of bands could be detected between op and tr cultures of the same strain (Fig. 2). Comparison of op or tr cultures of two strains showed differences only with the enzymes giving intrastrain pattern differences. To determine whether the lack of opacity differences observed on the non-horse serum-supplemented agar (see above) actually reflected a selection of tr subpopulations, we compared op and tr cultures grown on horse serum-supplemented medium with a similar pair

TABLE 1. Analysis of *M. pulmonis* DNAs with different restriction endonucleases and electrophoresis on agarose gels

Restriction enzyme	Cleavage site	No. of bands[a] seen after cleavage			
		AL-1 op	AL-1 tr	AL-2 op	AL-2 tr
*Alu*I	A G/C T	>50	>50	>50	>50
Ball[b]	T G G/C C A	42	43	42	43
*Bam*HI[b]	G/G A T C C	9	10	9	9
*Bcl*I	T/G A T C A	40	40	40	40
*Bgl*II[b]	A/G A T C T	>50	>50	>50	>50
*Bst*I[b]	G/G A T C C	9	10	9	9
*Cla*I[b]	A T/C G A T	30	31	32	30
*Dde*I[b]	C/T N A G	>50	>50	>50	>50
*Eco*RI[b]	G/A A T T C	37	36	37	36
*Eco*RV	G A T A T/C	>50	>50	>50	>50
*Hae*III[b]	G G/C C	>50	>50	>50	>50
*Hha*I	G C G/C	45	45	45	45
*Hin*dIII	A/A G C T T	>50	>50	>50	>50
*Hin*fI	G/A N T C	>50	>50	>50	>50
*Hpa*II	C/C G G	45	45	45	45
*Kpn*I	G G T A C/C	4	4	4	4
*Mst*II	C C/T N A G G	0	0	0	0
*Pst*I[b]	C T G C A/G	15	0	15	15
*Pvu*I	C G A T/C G	1	1	1	1
*Sal*I	G/T C G A C	0	0	0	0
*Sau*3A	/G A T C	>50	>50	>50	>50
*Sma*I	C C C/G G G	0	0	0	0
*Sst*I	G A G C T/C	30	30	30	30
*Sst*II	C C G C/G G	1	1	1	1
*Taq*I[b]	T/C G A	>50	>50	>50	>50
*Xho*I	C/T C G A G	11	11	11	11
*Xmn*I[b]	G A A N N/N N T T C	47	48	45	45

[a] Refers to bands other than the single high-molecular-weight whole-chromosome band present when no enzyme was added.

[b] Enzymatic cleavages resulted in different patterns of restricted DNA fragments within a single strain.

grown on fetal calf serum supplement as to their DNA restriction pattern. In each instance, although opacity variation could not be observed on fetal calf serum supplement agar, the DNA from these cultures retained the sites characteristic of either an op or a tr culture. These data are shown for one enzyme, *Hae*III, in Fig. 3. Restriction patterns were reproducible for three different preparations of each DNA for each enzyme used. No plasmids (in undigested control preparations) were seen throughout these studies.

Polypeptide profile comparison. Opacity variation in gonococci (16, 17) has been found relative to possession of one or more outer membrane proteins. To ascertain whether this was also the case for mycoplasma membrane proteins, we compared polypeptide profiles by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 4, several intrastrain differences were apparent. However, interstrain polypeptide profiles were not exactly equivalent. These apparent differences were repeatable in four separate determinations made with cultures derived from four separate colonies of each phenotype from each strain.

DISCUSSION

Many references to the fried-egg appearance of mycoplasma colonies exist. Curiously, few reports exist concerning mycoplasma colony morphology variants. Recently, Valdivieso-Garcia and Rosendal (20) reported colony size variation in *Mycoplasma mycoides* subsp. *mycoides*. They reported a stable passage of large and small colonies, with each variant giving rise to the other. No further description or characterization was given.

Deeb and Kenny, in a comparison of *M. pulmonis* isolates from rabbits, noted colony variation (7). Using a medium containing agamma horse serum, they described a dark, granular colony as well as a white, smooth (also called tr) colony. Each phenotype was maintained by broth culture passage. Dark cultures were seen with lighter radiations. These observations were made by using a bright field. From the description in this paper, we conclude that Deeb and Kenny were describing the same colony opacity variation we describe in more detail here.

We have examined four strains of *M. pul-*

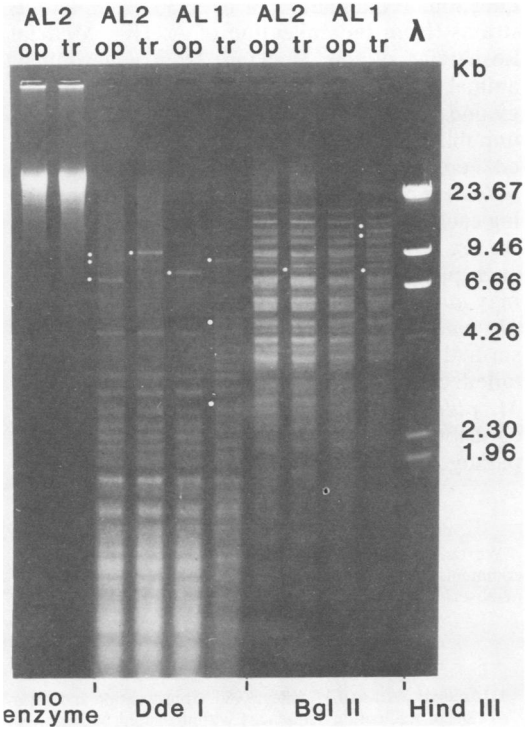

FIG. 2. Cleavage pattern of DNA from *M. pulmonis* AL-1 and AL-2. DNA from pure op and tr variant broth cultures was mixed with *Dde*I, *Bgl*II, or no enzyme. For molecular weight determinations, a *Hin*dIII digest of lambda bacteriophage DNA is also shown on this agarose (0.7%) slab gel electropherogram. Slab gels were run in a Tris-acetate buffer (6) at 60 mA of current for about 4 h. Gels were then stained with ethidium bromide (1 μm final concentration) and photographed under UV illumination. Dots denote some band differences within a single *M. pulmonis* strain. Kb, Kilobase.

monis and found colonial opacity and size variation in every one. Characterization of two strains showed that opacity was a stable characteristic. op cultures gave rise to tr colonies at a rate suggestive of a mutation rate for a single genetic event. For some reason, the shedding of op colonies from tr cultures was several orders of magnitude less frequent.

Growth rate comparisons revealed no significant differences between op and tr cultures of two *M. pulmonis* strains. However, in broth culture, tr cultures were not viable for as long as op cultures.

The mechanism for the stable opacity variation is still undefined. One factor involves the source of the serum used to supplement the medium. Horse serum was critical in enhancing the difference in opacity. No obvious phenotypic opacity variation occurred with fetal bovine serum. However, pure culture opacity variants had different plating efficiencies depending on serum source. The highest plating efficiency of pure tr was on horse serum, and the lowest was on pig serum. Plating efficiency of op cultures was less affected by serum source.

A potentially significant correlation between opacity and membrane polypeptide profiles was observed. Within a single strain, variation was predominantly in high-molecular-weight polypeptides. However, comparison of an opacity variation between strains also showed variation in polypeptide profile.

Digesting cellular DNA of a mycoplasma with restriction endonucleases helps define specific nucleotide sequences (i.e., the cleavage site of each enzyme) in that cell. Comparisons of DNA from several strains of a single species have been used to establish the existence of minor

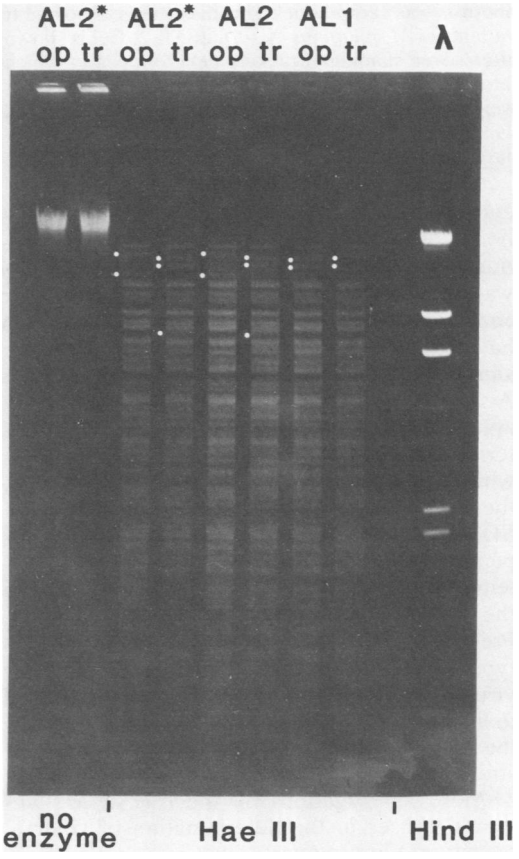

FIG. 3. Slab gel conditions were the same as those described in the legend to Fig. 2. Starred strains indicate cultures grown in fetal bovine serum rather than in horse serum-containing broth. The enzyme *Hae*III was used for digestion. Dots denote some band differences within strains.

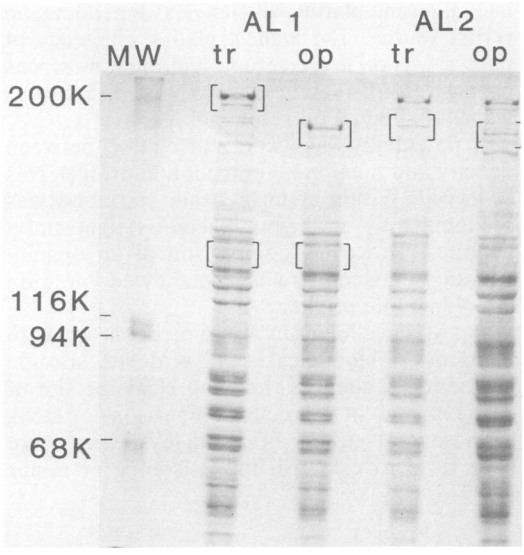

FIG. 4. SDS-polyacrylamide (7.5%) gel electrophoresis was carried out on broth cultures of op and tr variants of *M. pulmonis* AL-1 and AL-2. Gel is shown after silver staining. Brackets denote areas in which intrastrain differences can be seen. MW, Molecular weight standards whose weights are given to the left in daltons. Electrophoretic and silver staining procedures are described in reference 13.

evolutionary drift within that species (1) or taxonomic divisions (2). For *M. pneumoniae*, variation of cell DNA in response to two enzymes was reported not to exist (4). With several other enzymes, *Mycoplasma hyorhinis* was shown to have some intraspecific variation (5). Here we show variation between *M. pulmonis* strains. Also, within the same strain, colonial opacity appears to be related to unique restriction patterns. We cannot yet explain the gene alteration which results in the colonial opacity phenotype, the restriction endonuclease pattern, and the SDS-polyacrylamide gel electrophoresis polypeptide profile observed. In several other systems (11, 12, 14), colonial variation is linked to the host being lysogenized by a latent virus. Preliminary attempts to link the opacity phenotype with a lysogenic state of the *M. pulmonis* virus have failed. Initial observations appeared to link one colonial phenotype with sensitivity to the *M. pulmonis* virus. This linkage held true for strains AL-1 and AL-2 but not for AL-3 and AL-4. More investigations on the role of *M. pulmonis* viruses in the determination of colonial opacity are in progress.

Intrastrain variation in *M. pulmonis* virulence (10), membrane polypeptide composition (8), and serological response (9) have been previously reported. In no case was colonial phenotype characterized. The biological significance of *M. pulmonis* opacity variation remains an open question. Examination of over 30 *M. pulmonis* strains (from the collection of A. Hill, Medical Research Council, England) from a variety of animal sources also showed colonial morphology and opacity variation (F. Bober and A. Liss, unpublished data). Thus, the phenotypes reported here appear to be a property of many *M. pulmonis* cultures. We are currently characterizing each phenotype in more detail. Additionally, we are attempting to define other parameters of each pure culture to ascertain whether opacity may in some way correlate with the virulence of *M. pulmonis*. Although apparently first described in 1967, this rediscovery and more detailed characterization of colonial variation in *M. pulmonis* will be the start of a significant contribution to the understanding of this murine pathogenic mycoplasma.

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