

Enterobacterial Common Antigen in Mutant Strains of *Salmonella*

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A good correlation was found to exist between the serologically determined presence of enterobacterial common antigen (ECA) and the amount of the rare sugar constituent D-mannosaminuronic acid. Strains classified by serological techniques as ECA⁺, ECA⁻, and ECA^{trace} were found to possess the expected amounts of mannosaminuronic acid in the ECA-enriched phenol-soluble PL-L fractions. This correlation provides strong evidence on the identity of the mannosaminuronic acid-glucosamine polymer with the ECA as defined by Kunin (J. Exp. Med. 118:565-586, 1963).

The enterobacterial common antigen (ECA) described by Kunin in 1963 (8) is a typical component of enteric bacterial cell envelope. Until recently, the presence of ECA was determined indirectly because the structure and composition of the antigen was not known. Passive hemagglutination (HA) has been the usual method for testing the ECA content of respective strains (9, 29). Immune precipitation in agar gels served as an alternative detection method (6, 20, 28), and recently bacterial agglutination has also been used, although the latter method is only applicable to enterobacterial R mutants (18). The recent isolation of ECA from *Salmonella montevideo* SH94 and the identification of its chemical structure as a polymer of N-acetyl-D-glucosamine and N-acetyl-D-mannosaminuronic acid (16, 17) made it possible to screen a number of strains for the presence or absence of ECA. To prove the validity of this approach, we have tested a number of strains, classified by the passive HA assay as ECA⁻, ECA⁺, or ECA^{trace}, for the presence of these ECA constituents. The excellent correspondence between the two methods supports the results on the chemical nature of ECA described above, which are important proof for the identity of the isolated ECA (12).

The strains used for this investigation are genetically related derivatives of three *Salmonella* serotypes (see Table 1); those mutants were obtained by slightly different procedures based on the recently acquired knowledge of the genetic determinants of ECA (12).

Three types of mutants defective in ECA have been detected in *Salmonella* and *Escherichia coli* (11, 13, 26). Both rff and rfe mutations block the synthesis of ECA. The rfe gene function is also required for the biosynthesis of the O-spe-

cific polysaccharide part of the cell wall lipopolysaccharide (LPS) in most but not all serotypes. *Salmonella* of group B (O antigens 4, 12) do not require the rfe function for O-chain synthesis: rfe mutants in this group are smooth (S), with complete LPS, but ECA⁻. By contrast, rfe mutants in both *S. montevideo* (group C, O antigen 6, 7) and *S. minnesota* (group L, O antigen 21) are rough (R), unable to synthesize complete LPS, and also ECA⁻.

In addition, some gene or genes in the rfb cluster, which is the main determinant of the O-specific polysaccharide, are required for ECA production in group B of *Salmonella* (14). Thus, *S. typhimurium* with a large part of its rfb genes deleted or replaced by the rfb genes of group C organisms are ECA^{trace} by the HA method. Although the immediate products of the rfe and the ECA-determining rfb genes have not been identified, strains with various combinations of rfe and rfb loci from groups B and C, respectively (abbreviated here as B-rfe, etc.), behave as if the C-rfe locus would contain the information which in group B is divided between the B-rfe and the B-rfb loci. Hybrid strains which are genotypically C-rfe⁺ are also ECA⁺, irrespective of the rfb locus, whereas B-rfe⁺ strains are ECA⁺ in combination with B-rfb⁺ but not with C-rfb⁻ (11, 14).

MATERIALS AND METHODS

The bacteria used in this study were previously described *S. montevideo*, *S. minnesota*, and *S. typhimurium* strains representing ECA⁺, ECA⁻, and ECA^{trace} categories; their properties are described in Table 1. For extraction of ECA, cultures were grown in a fermentor at 37°C at a constant pH of 7.2. The detailed conditions for growth and the medium used have been described previously (25).

Isolation and purification of ECA. The procedure employed was recently described in detail (16). Briefly, bacterial cells were extracted by a combined hot phenol-water extraction (27) and PCP (phenol-chloroform-petroleum ether) fractionation (5). After dialysis and lyophilization, the aqueous phase of the phenol-water extraction was treated with the PCP mixture. The LPS precipitated when some drops of water were added to the phenol phase, while ECA remained in solution and was subsequently recovered by extensive dialysis and lyophilization. This fraction was resuspended in water and centrifuged at 105,000 $\times g$ for 4 h. The lyophilized supernatant is enriched in ECA and was used in several tests as the PL-L fraction. Further purification was achieved by column chromatography on diethylaminoethyl-cellulose (DE 32, Whatman) with stepwise elution with 0.5, 1.0, and 1.6 M ammonium acetate-methanol buffer (1). ECA is eluted in the middle fraction and, after dialysis and/or electrodialysis (4), was lyophilized.

Analytical methods. Hydrolysis of the ECA material was usually done in sealed ampoules with 4 N HCl at 100°C for 2 h. Amino sugars were separated and identified by high-voltage electrophoresis on SS 2043a paper (Schleicher & Schüll, Duren, Germany) at a field strength of 45 V/cm in a buffer containing pyridine, formic acid, acetic acid, and water (1:1.5:10:90, vol/vol) at pH 2.8 (21).

The dried chromatograms were stained with silver nitrate-NaOH. Determinations of total acetyl as well as of O- and N-acetyl were performed by the method of Fromme and Beilharz (3).

Serological methods. ECA antisera were prepared as described earlier (17, 29) by intravenous immunization of New Zealand White rabbits at 4-day

intervals with increasing amounts (0.25, 0.5, and 1.0 ml) of either living or heat-treated (100°C for 1 h) bacterial suspensions (10^{10} cells per ml) thoroughly washed with saline. *E. coli* and *Shigella boydii* type 3⁻ (F3140) were used as immunogens (29).

ECA determination by HA. Indirect HA was performed (22) with fresh human erythrocytes (RBC) of blood group A; they were thoroughly washed with saline and suspended to a concentration of 0.5% in saline. For sensitizing RBC with ECA, supernatants of heated cultures (1 h, 100°C) or amounts of 50 µg of antigenic material were added to 5 ml of RBC suspensions. After incubation at 37°C for 30 min, the excess antigen was washed off, and the sensitized RBC were resuspended in saline to give a 0.5% suspension. The RBC suspension was added to series of antiserum dilutions in microtiter plates as described in detail previously (13, 22).

Supernatants of ECA⁺ bacterial cultures could sensitize the RBC to give maximal HA titers (about 5,000, corresponding to a serum dilution of 1:5,000) even when small volumes (0.01 ml) were added to the 5-ml RBC suspension. In contrast, 1-ml volumes of the supernatants of heated ECA⁻ strains left the titer usually below 10. However, sometimes intermediate reactions were seen, so that the titer remained below the maximal value with larger amounts of the sensitizing supernatant. Such reactions were designated as ECA^{trace} (11, 14).

HA inhibition. To measure the inhibiting capacity of a substance, the HA inhibition (HAI) test was used. The inhibitor was dissolved in normal rabbit serum (diluted 1:50 with phosphate-buffered saline). Serial dilutions (0.25 µl) ranging from 250 to 0.25 µg of the inhibitor per ml were incubated at 37°C for 1 h with

TABLE 1. *Salmonella* strains used

Strain	No.	ECA	LPS ^a	rfa ^b	rfb	rfe	rff	Source or reference
<i>S. montevideo</i> group C	SH94	+	S 6,7	+	+	+	+	<i>S. montevideo</i> 129 (2)
	SH3465	-	S 6,7	+	+	B ^c	+	(11)
<i>S. minnesota</i> group L	F1114	+	S 21	+	+	+	+	(10)
	F1119(R5)	Trace	RcP ⁻	P3785	+	3786 ^d	+	(7, 10)
	SH5641	+	RcP ⁻	P3785	+	+	+	Recombinant from F1119 (7)
	F1122(R8)	Trace	RcP ⁻	P4277 E3787	+	3788 ^d	+	(7, 10)
	SH5644	+	RcP ⁻	P4277 E3787	+	+	+	Recombinant from F1122; (7)
	SH3786	Trace	S 21	+	+	+	4257	(13)
	SH5657	+	S 21	+	+	+	+	Recombinant from SH3786 (7)
<i>S. typhimurium</i> group B	SH5150	-	Ra	+	Deletion ^e	+	4270	(14)
	SH5177	Trace	Ra	+	Deletion	+	+	Recombinant from SH5150 (7)
	SH5178	-	S 4,12	+	+	+	4270	(7)
	SH5179	+	S 4,12	+	+	+	+	Recombinant from SH5177 (7)

^a LPS characteristics: S, smooth, complete LPS with O antigens as denoted (6,7; 21; or 4,12); R, rough incomplete LPS of chemotypes Ra, complete core, or RcP⁻, incomplete core (Lüderitz et al. [10]).

^b Alleles: +, wild-type functional allele; P3785 etc., mutation 3785 in cistron rfaP, etc.

^c B^c, Wild-type genes from group B.

^d The mutations rfe-3786 and rfe-3788 may be identical (7).

^e Deletion his-809 covering part of the rfb and his clusters.

25 μ l of a serum dilution containing 2 to 3 HA units. Then 50 μ l of the sensitized RBC was added, and the plates were again incubated at 37°C for the same time. The lowest inhibitor concentration giving a total inhibition of HA was recorded after 1 h at room temperature.

Agar gel precipitations. Precipitations in agar gel were performed by the method of Ouchterlony (23); immunoelectrophoresis in sodium barbiturate buffer of pH 8.6 was by the microtechnique of Scheidegger et al. (24). For semiquantitative determination of ECA, the radial diffusion test of Mancini et al. (15) was used, substituting antiserum for the agar gel buffer, 1 volume to 3 volumes, respectively. The diameter of the precipitation area was measured after 24 h.

RESULTS

S. montevideo strains. In a previous investigation (16) the chemistry of ECA was studied by using as its source SH94, a *S. montevideo* wild-type strain which in preliminary experiments was shown to contain a rather large amount of ECA. We showed that ECA can be extracted from freeze-dried bacterial cells by a combination of the phenol-water and PCP procedures. ECA becomes finally enriched in the phenol phase, and this phenol-soluble material can be isolated in amounts corresponding to 0.3% of the dry weight of the bacteria (16). The same extraction method was applied to the *S. montevideo* derivative SH3465 (Table 1), which by the HA test had been characterized as ECA negative (12), and some (less than 0.2%) of phenol-soluble material was obtained. The material from the wild-type strain was a potent inhibitor (at <0.25 μ g/ml) in the HAI test, whereas material from SH3465 was unable to inhibit the ECA-specific HA reaction even in a very high concentration (>250 μ g/ml).

Equal amounts of hydrolyzed PL-L material from both strains were then analyzed by high-voltage electrophoresis. The material from SH94 contained large amounts of glucosamine (GlcN) and mannosaminuronic acid (ManNUA), whereas the SH3465 material showed only small amounts of GlcN (so little that it could have derived from contaminating LPS) and no ManNUA or its lactone (Fig. 1).

Second, we measured the acetyl content in the PL-L fractions of SH94 and SH3465. In ECA from *S. montevideo* SH94, all amino sugars have been shown to be *N*-acetylated, and the total acetyl content amounted to 13% of ECA dry weight (16). Table 2 shows a large difference between the strains in the acetyl content, mainly due to *N*-acetyl. These chemical results parallel the serological findings: SH3465 is ECA negative in the serological assay, and contains very few constituents typical to the isolated ECA from *S. montevideo* SH94 (no ManNUA, little GlcN and *N*-acetyl).

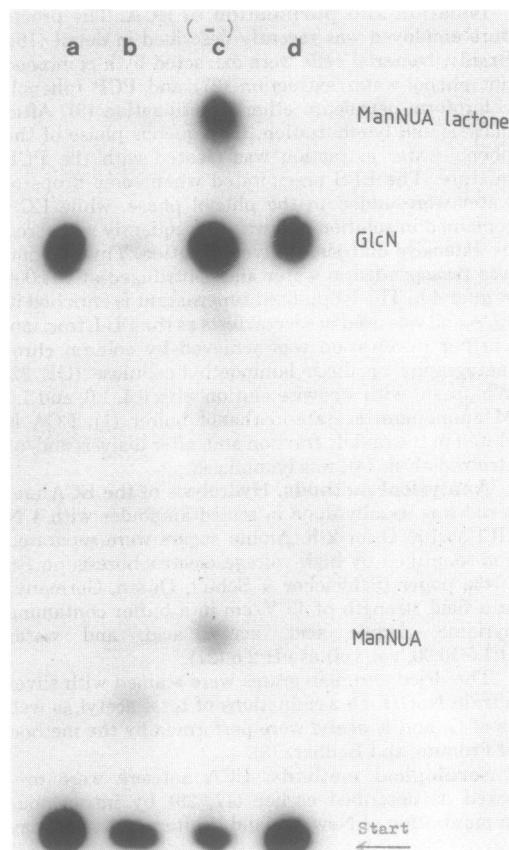


FIG. 1. High-voltage paper electrophoresis of hydrolysates of PL-L fractions of two *S. montevideo* strains. Lanes a and d, Standards of Glc and GlcN (10 μ g each); lane b, PL-L fraction of the ECA⁻ strain *S. montevideo* SH3465 (150 μ g of hydrolysate); lane c, PL-L fraction of the ECA⁺ strain of *S. montevideo* SH 94 (150 μ g of hydrolysate). Conditions: 3 kV, 1 h, pH 2.8, Trevelyan staining.

S. minnesota rfe and rff mutants. *S. minnesota* strains with known genetic defects were tested by HA for their ability to coat RBC for ECA-specific agglutination. Only *rfe*⁺ *rff*⁺ strains—the wild-type strains F1114, as well as the *rfe*⁺ *rff*⁺ derivatives SH5641, SH5644, and SH5657 of *rfe* or *rff* mutants (Table 1)—were positive in this test. The *rfe* and *rff* mutants showed an ECA^{trace} reaction (large amount of supernatant needed to sensitize RBC) (11, 12). This series of *S. minnesota* mutants was extracted with the phenol-water-PCP procedure to yield the corresponding PL-L fractions. Stained high-voltage electropherograms of hydrolyzed portions of these fractions clearly showed a substance with the migration value of the ManNUA lactone in all the ECA-positive strains (F1114, SH5641, SH5657). Trace

amounts of this substance were also detectable in the *rff* mutant SH3786 but not in the *rfe* mutant strains F1122 and F1119. Additionally, all tested fractions also contained GlcN in different amounts; the *rfe* mutants (F1122, F1119) had relatively little of this amino sugar compared with the *rff* mutant SH3786. Nevertheless, in all of the *rfe* or *rff* mutants the amount of GlcN was less than that in the *rfe*⁺ *rff*⁺ strains.

In Table 3 the PL-L fractions obtained from the wild type (F1114), the *rff* mutant (SH3786), and the *rff*⁺ recombinant (SH5657) are compared for yield, serological activity in HA, in the HAI and precipitation reactions, and in their content of ManNUA. These data show a good correlation between all these tests: the content of ManNUA is high in the strains serologically classified as ECA⁺, and low (but detectable) in the *rff* mutant which is serologically ECA^{trace}.

The PL-L from the *rfe* mutants (F1119 and F1122) and their ECA⁺ *rfe*⁺ recombinant derivatives (SH5641, SH5644) were subjected to a semiquantitative ECA precipitation assay by radial diffusion. Table 4 shows results obtained with equal amounts of the PL-L fractions compared with the PL-L fraction from *S. montevideo* SH94. The precipitation areas produced by the ECA of the mutant strains are significantly smaller than the precipitation areas of the cured strains. The PL-L fraction from *S. montevideo* SH94 seems to contain somewhat more ECA-specific material than the fractions from the ECA⁺ *S. minnesota* strains.

TABLE 2. Comparison of the acetyl content of the PL-L fractions from the *S. montevideo* strains SH94 (ECA⁺) and SH3465 (ECA⁻)

PL-L from	Lyophilized PL-L material (%) with:		
	Total acetyl	O-acetyl	N-acetyl
SH94, ECA ⁺	12.7	0.7	12.0
SH3465, ECA ⁻	1.0	0.2	0.8

The PL-L fraction from one of these ECA⁺ recombinant strains (*S. minnesota* SH5641) was purified further. The ECA activity was eluted with 1.0 M ammonium acetate-methanol buffer from the diethylaminoethyl-cellulose column as was the case with ECA isolated from *S. montevideo* SH94 (16). The isolated material was compared with the similarly purified ECA from *S. montevideo* SH94 in immunoelectrophoresis. Both gave an identical precipitation pattern with the *E. coli* O14 antiserum (17).

The LPS did not seem to interfere with either the serological or chemical assays for ECA. Both of the tested ECA⁺ and ECA⁻ strains included smooth forms with complete LPS and rough forms with an incomplete core (chemotype RcP⁻) (see Table 1).

S. typhimurium rff and *rbf* deletion mutants. As described by Mäkelä et al. (14), *S. typhimurium* strains lacking certain *rbf* genes due to a chromosomal deletion in segments of the *his* and the *rbf* gene clusters give an ECA^{trace} reaction with the HA test, suggesting that they

TABLE 4. Comparison of the PL-L fractions from ECA⁺ *S. montevideo* and various *S. minnesota* mutants and their recombinants in the single radial diffusion technique (15).

Strain	Precipitation area ^a diam (mm)
<i>S. montevideo</i> SH94, ECA ⁺	14.5
<i>S. minnesota</i> F1122, ECA ^{trace}	6.0
<i>S. minnesota</i> F1119, ECA ^{trace}	6.5
<i>S. minnesota</i> SH5644, ECA ⁺	11.0
<i>S. minnesota</i> SH5641, ECA ⁺	10.0

^a The diameter of the well was 5 mm, receiving 5 µg of a 0.1% solution of the PL-L fractions. Twenty-five percent of the barbiturate buffer of the 1% agar gel was substituted by ECA antiserum prepared against *S. boydii* type 3⁻ (F3140) heat-killed whole cells. The diameter of the precipitation area was measured after 24 h.

TABLE 3. Comparison of the PL-L fractions from ECA⁺ and ECA^{trace} smooth strain of *Salmonella*

Strain	Description	ECA	Yield of PL-L (mg)	Antiserum titer obtained in HA ^a	Inhibitory amt in HAI ^b (µg/ml)	Precipitation in IE ^c	ManNUA ^d
F1114	Wild type	+	35.5	5,120	7.8	+	+
SH3786	<i>rff</i> defective	Trace	10.7	1,280	250	-	Trace
SH5657	<i>rff</i> ⁺ recombi-	+	28.5	5,120	3.9	+	+
	nant from SH3786						

^a HA, Indirect ECA-specific hemagglutination; the data indicate titer of the antiserum obtained when RBCs (5 ml of a 0.5% suspension) were coated with 25 µg of the PL-L fractions. Antiserum was as in footnote c.

^b HAI method as in Table 2.

^c IE, Immunoelectrophoresis. ECA antiserum was rabbit antiserum against heat-killed whole cells of *S. boydii* type 3⁻ (F3140).

^d The presence of ManNUA was determined by high-voltage electrophoresis at pH 2.8.

contain little ECA material. Upon subsequent transfer, these strains accumulate secondary *rff* mutations and become ECA⁻. Recombinants of the double mutant whose *his* and *rfb* regions have been replaced with functional *his*⁺ *rfb*⁺ regions by conjugation still contain the *rff* mutation; they are smooth but ECA⁻. Therefore, the new *rff* mutations by themselves appear to prevent ECA synthesis, but do not affect LPS.

To see whether the amount of chemically determined ECA would also in these strains correspond to their serologically determined ECA content, we isolated the PL-L fractions from a family of isogenic strains. The genotypes, *rfb*⁺ *rff*⁺, *rfb*⁺ *rff*, *rfb* *rff*⁺, and *rfb* *rff* were derived by conjugation from SH5150, which contains a *his-809* deletion that extends into *rfb* region as well as a *rff* mutation (14). The yield of the PL-L fractions, their serological activity in HA and HAI, and the ManNUA content determined by high-voltage electrophoresis are shown in Table 5. Two of the strains show no ECA activity and no ManNUA—they both contain the *rff* mutation either alone or together with the *rfb*-deletion. Some GlcN is found in these strains as in all other ECA⁻ strains, although in smaller amounts than in ECA⁺ strains. The yield of the PL-L material is low in all of the strains except the ECA⁺ *rfb*⁺ *rff*⁺ strain SH5179. The small amount of PL-L material extracted from SH5177, the *rfb* *rff*⁺ strain, is however active in both the HA and the HAI tests. This strain, therefore, contains trace amounts of ECA; correspondingly, some ManNUA could be detected by high-voltage electrophoresis.

Again, LPS as such did not influence the ECA reactivity. Although the *rfb* deletion strains were rough, the *rfb*⁺ *rff* derivative was smooth but completely ECA⁻.

DISCUSSION

The results showed good correlation between the presence or absence of ManNUA and the serologically determined ECA phenotype. The

correlation extended to strains in which the serological behavior suggested the presence of trace amounts of ECA: they had reduced amounts of the ECA-containing material (the PL-L fraction), and this contained little ManNUA. This analysis, together with a similar analysis comparing ECA⁺ strains of *Enterobacteriaceae* with ECA⁻ strains of other gram-negative families (12), provides strong evidence supporting the identity of the ManNUA-GlcN polymer with the ECA as defined by Kunin (8).

ManNUA is a rare constituent of carbohydrate structures in gram-negative bacteria; in the family of *Enterobacteriaceae*, it has been found only as constituent of the K7 and the K56 capsular polysaccharides of *E. coli* (19; H.-C. Fleming, diploma work, University of Freiburg, Freiburg, West Germany, 1972). Its detection can serve, therefore, as a good indicator for the presence of ECA; however, these observations need to be confirmed by serological assays. GlcN or N-acetyl determinations in the semipurified ECA material (the PL-L fractions) of our strains agreed with the ManNUA determination. They would be less conclusive alone, because GlcN and N-acetyl-D-glucosamine are also present in other cell wall materials, most notably in LPS which easily contaminates the ECA material.

The correlation between the chemically and the serologically determined ECA content was consistent for the three types of mutants (*rfb*, *rfe*, and *rff*) possessing the altered ECA genes. Although the biochemical function of the first two is unknown, the *rff* genes probably determine enzymes of the N-acetyl-D-mannosaminuronic acid biosynthesis pathway (H. Nikaido, personal communication). Thus far, we have no direct information with regard to those factors that produce the ECA^{trace} phenotype, although we were able to correlate its presence with the presence of small amounts of ManNUA. The ECA^{trace} phenotype of the *S. minnesota* *rff* mutant SH3786 (Table 4) could easily be explained by assuming that the *rff* mutation is leaky and allows some synthesis of ECA. The same expla-

TABLE 5. Comparison of PL-L fractions obtained from the *S. typhimurium* strains

Strain	<i>rfb</i>	<i>rff</i>	LPS	Yield (%) ^a	Titer obtained in HA ^b	Inhibitory amt in HAI ^c (μg/ml)	ManNUA ^d
SH5120	Deletion ^e	—	R	3.9	10	250	—
SH5177	Deletion	+	R	3.5	640	125	Trace
SH5178	+	—	S	3.5	10	250	—
SH5179	+	+	S	18.5	2,560	62.5	+

^a Amount related to the water-phase material.

^b HA, Indirect ECA-specific hemagglutination; the data indicate titer of the antiserum obtained when RBC (a 0.5% suspension) were coated with 50 μg of the PL-L fractions per ml. ECA antiserum was rabbit antiserum against heat-killed whole cells of *S. boydii* type 3⁻ (F3140).

^c Method as in Table 2.

^d The presence of ManNUA was determined by high-voltage electrophoresis at pH 2.8.

^e Deletion *his-809*; see Table 1.

nation may apply to the ECA^{trace} phenotype of the *S. minnesota rfe* mutant strains F1122 and F1119: the *rfe* mutation probably occurs in the same *rfe* allele in both these strains (7). However, the third type of ECA^{trace} strains were *S. typhimurium* derivatives with a complete lack of certain *rfb* genes, either because of chromosomal deletion or because of replacement by *rff* genes of group C origin, as in the strain SH4146 described in detail by Mäkelä and Mayer (11). In this strain it is unlikely that trace ECA production occurred because of an incompletely blocked gene. When the trace reactivity was first discovered in these strains, we considered the possibility that it was not a true ECA reactivity at all but instead a serological cross-reaction given by a related compound (11). The correlation of serologically determined ECA and ManNUA in these strains argues against the cross-reaction hypothesis. We suggest that the ECA production in strains lacking the *rff* function can be attributed to a replacement by an as yet unidentified gene. Such a function could, for example, be to turn on the function of the *rff* or *rfe*; in this case group C could differ from group B by not requiring this activation, or by containing the regulatory gene(s) in the *rfe-rff* gene region.

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