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Characterization of Bacteriophage Specific to *Bacillus pumilus* from Ciapus River in Bogor, West Java, Indonesia

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***Bacillus pumilus* is a spore-forming bacteria that is rod-shaped, gram positive, and aerobic. *B. pumilus* produced pumilacidins, known to have toxic effects on epithelial cells. Antibiotics were usually used to treat the disease caused by bacteria. Antibiotic typing test of *B. pumilus* indigenous from sewage water showed that this isolate was resistant to ampicillin and clindamycin. An alternative way was by application of bacteriophages as biocontrol agents to reduce *B. pumilus* in environment. The aim of this study were to isolate and characterize *B. pumilus* bacteriophage isolated from Ciapus River in Bogor, West Java. Bacteriophages infecting *B. pumilus* were isolated from river water using the double agar overlay method. Phages were defined by plaque morphology, structure, host range, and characteristic of molecular weight protein phage. Phage FBa1, FBa2, and FBa3 had narrow host range and they were specific for infecting *B. pumilus*. Electron microscope observation showed that phage FBa1 had icosahedral head without tail (166.67 nm in diameter), so it is called phage-like particles. Characterization of phage FBa1 by SDS-PAGE showed five proteins band. Molecular weight of FBa1 proteins was 70.9, 54.9, 33.8, 28.3, and 21.4 kDa.**

Key words: *Bacillus pumilus*, bacteriophage, food poisoning

INTRODUCTION

Bacillus pumilus is a spore-forming bacteria that is commonly associated with foodborne illness caused by intoxication. From *et al.* (2007) reported a food poisoning outbreak caused by *B. pumilus*. In this case, *B. pumilus* was found as contaminant from reheated rice at a Chinese restaurant. Large numbers of *B. pumilus* strain was produce a complex of lipopeptides known as pumilacidins, a substance toxic to mammalian cells (From *et al*. 2007). This toxin can destroy cell membranes through production of selective cationic channels (Sheppard *et al*. 1991). The structural of pumilacidin is similar to surfactin (Naruse *et al*.1990). *Bacillus pumilus* could grow well at low temperatures (10-15 ^{o}C) and produce large amounts of a toxic metabolite. Food poisoning incident by *B. pumilus* was occurred after ingestion of foods containing large numbers of *B. pumilus* (10^5-10^7 cfu/g) followed by stomach cramps and diarrhea (Suominen *et al*. 2001). Parvathi *et al*. (2009) reported that *B. pumilus* had *ces*A and *ces*B genes encoded cereulide synthetase. Cereulide is a stable cyclic dodecadepsipeptide produced by some strains of *B. cereus*. This toxin has high toxicity to humans (Agata *et al*. 1995; Yokohama *et al*. 1999).

Multidrug resistant bacteria are a serious health problem these days. Antibiotics are not bacteria specific and thus can result in disruption in the balance of normal flora (Giuliano *et al*. 1987). Phage therapy seems to be a good option for this problem. Phage therapy is use of a specific bacteriophage to a bacterial cell. The introduction of phage therapy was seen in the early 1930s (Levin & Bull 2004). Bacteriophages (phages) are viruses that infect bacteria. They have the absolute requirement to infect host cells in order to multiply and survive. Phages are viruses that have a protein coat that encloses a nucleid acid (DNA or RNA). There are two types life cycle of phages, lytic and lysogenic phages. Lytic phage kill bacteria through a multiple-step process. The bacteria are destroyed through lysis, resulting in release many particles of phages. Lytic phage provides opportunity to control bacteria. Lysogenic phage do not immediately replicate in the bacterium they infect, but coexist within the bacterium as a prophage. When lysogenic bacteria are stress, the prophage can become activated and the virus killing the bacteria through lysis.

Recently, several potential phages were used as biocontrol of foodborne pathogens. Phage application as a food biocontrol had been used to decrease microbe contaminant on food. Phage FB4 could lyses EPEC K1.1 and this phage is expected

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as a biocontrol to prevent foodborne disease in Indonesia (Budiarti *et al*. 2011). It was reported that EPEC K1.1 produces extracellular protease enzyme to degrade mucin in gastrointestinal tract (Budiarti & Mubarik 2007). Phage FGCSSa1 isolated from sewage had potential for biocontrol of *Salmonella* spp. in foods (Carey-Smith *et al*. 2006). Oral application of phage FR38 in rat diet to reduce *Salmonella* P38 had no effect on body weight, blood chemistry, kidney and liver functions in rat (Sartika *et al*. 2012). Bacteriophage cocktail as a biocontrol agent of *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis were used in food matrices and their production (Spicigo *et al*. 2013). A cocktail of phages isolated from cattle feces reduced *Escherichia coli* O157:H7 populations in the gut of sheep (Callaway *et al*. 2008). Application of phage P100 on meat could reduced *Listeria monocytogenes* (Soni & Nannapaneni 2010). Cocktail of phages application in milk decreased contamination of *S. aureus* (Martínez *et al*. 2008). Many phages infecting *Bacillus* spp. have been isolated such as phages FWLBc1 and FWLBc2 which infected *B. cereus* (Lee *et al*. 2011), Tsamsa phage isolated from *B. anthracis* (Ganz *et al*. 2014), SPP1 phage infecting *B. subtilis* (Jakutytė *et al*. 2011), and phiAGATE phage infecting *B. pumilus* (Barylski *et al*. 2014). Nevertheless, *B. pumilus* phages have not been reported in Indonesia. The objectives of this research were to isolate and characterize *B. pumilus* bacteriophage isolated from Ciapus river in Bogor, West Java, Indonesia.

MATERIALS AND METHODS

Identification of Host Bacterium. Host bacterium was isolated from sewage water located in Cigudeg, Bogor, Indonesia. Haemolitic activity of this indigenous bacterium was determined using blood agar plates. Identification was performed using API 50CHB (bioMérieux, Marcy I`Etoile, France). The isolate was maintained aerobically on Tryptic Soy Agar (Difco), at 37 °C for 24 h. Antibiotics test with disk diffusion method was used to identify the susceptibility of the bacterium to ampicillin, amoxicillin, ciprofloxacin, and clindamycin (CLSI 2012). Antibiotic test was assessed by measuring the diameter (in millimeters) of the inhibition zone surrounding the disks.

Isolation and Purification of Phage. Isolation of phage from water samples was collected in Ciapus river, Bogor, West Java, Indonesia. Water sample of 4.5 mL was incubated overnight with 0.5 mL of Nutrient broth (Difco) and 0.5 mL of host bacteria culture (previously grown 24 h in Nutrient broth medium). An enrichment culture was used to multiply phages. The culture was centrifugated at 5000 rpm for 25 min and filtered through a 0.22 µm sterile filter. Stock solution of phage were stored in sterile tube at 4 °C. Soft agar (0.8%) was prepared and autoclaved. As much as 7 mL of soft agar was warmed in water bath at 42 °C before used as an agar overlay. 100 µL of phage stock solution (10^{-5} to 10^{-8} dilutions) was mixed with 100 µL *B. pumilus* culture which contain 10^8 CFU/mL, and incubated at 37 °C for 30 min. After incubation, it was mixed with 7 mL soft agar, and poured as an overlay onto the top of a solid Nutrient Agar (Difco) base plates. Plates were incubated at 37 °C for 24 h.

Plaques were purified according to methods in Goodridge *et al*. (2001). Plaques were purified by single-plaque isolation based on plaque morphology. Purified phage was diluted in saline-magnesium (SM) buffer and stored at 4 °C. SM buffer is a buffer used for storage of phage stocks (5 M NaCl, 1 M $MgSO_4$, 1 M Tris-HCl pH 7.5, 1% gelatin in distilled water). The suspension was mixed using vortex for 30 sec and then incubated at room temperature for 1-2 h. The suspension was centrifugated at 5000 rpm for 20 min. After centrifugation, the supernatant was filtered through a 0.22 µm sterile filter and transferred to sterile tubes. The stock solution of purified phage was stored at 4 °C.

Phage Quantification. Quantification of phage solution was measured by counting the number of Plaque Forming Unit (PFU) on the top agar. Phage quantification (plaque assay) was examined using double layer method as mentioned earlier. Stock solution of purified phage was serially diluted (10^{-1} to 10^{-8}) in 0.85% NaCl. Each dilution was subjected to plaque assay. Plaques were counted on the plate that contain 30-300 plaques and expressed as plaque forming unit per milliliter (PFU mL^{-1}).

Phage titer (PFU mL^{-1}) = number of plaques x 10 x reciprocal of counted dilution

Host Range Determination. Exponential phase cultures of the host bacteria (*B. pumilus, Proteus mirabilis, Photobacterium damselae, Salmonella* sp., EPEC K1.1) were prepared, and agar overlays were inoculated with a host (100 µL) and phage stocks (100 µL). The plates were incubated at 37 °C for 24 h and the plaques were observed for each host.

Transmission Electron Microscopy (TEM). Stock solution phage (5 µL) was dropped in to grid for 30 sec, and then dried up with filter paper. Uranyl acetate 2% solution (5 µL) was also dropped in to grid for 1 min, and then dried up with filter paper for 60 min. The grid placed in holder and left for perfect dry. Specimen was observed using transmission electron

microscope (TEM JEOL JEM-1010 model) at 80 kV, and phages were examined at 20000-80000 times magnification.

Structural Analysis of Phage Proteins. Stock of buffer sample (2 mL mercaptoethanol, 4 mL glycerol, 0.3 g Tris, 2 mL bromfenol blue 0.1%, pH 6.8) was prepared and added with 0.92 g Sodium Dodecyl Sulfate (SDS), homogenized with addition of aquades until the volume of buffer reached 20 mL. Stock phage were mixed with buffer sample (4:1), and boiled for 5-10 min. Sixty µL phage protein samples were loaded onto a SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel (12% acrilamide) at 20 mA, 50 volt, for 3.5 h. Silver staining was used for visualization of the result.

RESULTS

Identification of Host Bacterium. The bacterium was isolated from sewage water in Cigudeg, Bogor, West Java, Indonesia. Several tests were used to identify species of the bacterial isolate (Table 1). Our result showed that the isolate were Bacillus gram positive, and showed a clear zone around the colonies on blood agar. It was an indication that the isolate exhibited β-haemolytic. The isolate was identified as *B. pumilus* with 99.9% this biochemical identification was matched with identification noted on API 50CHB. The antibiotic test of *B. pumilus* showed that the isolate was resistant to ampicillin and clindamycin (Table 2).

Isolation and Purification of Phage. The phage isolate of indigenous *B. pumilus* was found in a river water sample collected from Ciapus river, Bogor, West Java, Indonesia. Samples were collected from river water because of phage is naturally part of environmental ecosystem. Treatment of *B. pumilus* with phage resulted in complete lysis of the bacterial cells. The lytic activity formed two types of plaque, haloing plaque and plaque without halo. Average plaque diameter was 0.5 to 2 mm after 18 h incubation at 37 °C (Figure 1).

Phage FBa1 produced one 2 mm diameter translucent plaque, FBa2 1 mm diameter translucent plaque, and FBa3 0.5 mm diameter translucent plaque. Three phages were successfully isolated and they had differences on plaque morphology (Table 3). FBa1 and FBa2 phage were formed plaque with halo, and FBa3 phage was formed plaque without halo. Purified phages were test on *B. pumilus* for three times. Plaques morphology were the same as plaques morphology of isolation step, but with differences in plaque size or diameter.

Phage Quantification. Natural phage number in water samples was too low to produce a quantifiable

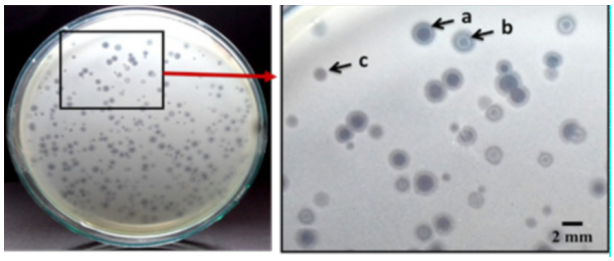

Figure 1. Morphology of plaques: FBa1, a. large plaque with halo; b. FBa2, medium plaque with halo; c. FBa3, small plaque without halo.

Table 1. Identification of host bacterium isolated from water in Cigudeg, Bogor

Identification test	Results
Gram staining	Gram-positive, rod shaped cells
Hemolytic activity	β-hemolytic
API 50 CHB	Positive: glycerol, L-arabinose, D-ribose, D-glucose, D-fructose, D-mannose, D-mannitol, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-saccharose (sucrose), D-trehalose, D-tagatose
	Negative: erythritol, D-arabinose, D-xylose, L-xylose, D-adonitol, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetylglucosamine, D-maltose, D-lactose, D-melibiose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate

Table 2. Results of antibiotic susceptibility test of *Bacillus pumilus*

Antibiotic	Disk content* (µg/mL)	Zone diameter interpretive criteria nearest whole* (mm)		Zone diameter of inhibition (mm)	Results
		S	R		
Ampicillin	10	≥ 29	≥ 28	23	R
Amoxicillin	20	≥ 20	≥ 19	22	S
Ciprofloxacin	5	≥ 21	≥ 15	27	S
Clindamycin	2	≥ 21	≥ 14	14	R

*Standards by CLSI (2012), S: sensitive, R: resistant.

Table 3. Phage isolates from Ciapus river water, Bogor

Phage isolate	Morphology of plaque	Diameter of plaque (mm)
FBa1	Translucent plaque, presence of a halo	2
FBa2	Translucent plaque, presence of halo	1
FBa3	Translucent plaque, absence of halo	0.5

Table 4. Phage quantification of FBa1, FBa2, and FBa3

Phage isolate	Plaque count	Titer (PFU/mL)
FBa1	102	10.2×10^8
FBa2	59	5.9×10^8
FBa3	85	8.5×10^8

Table 5. Host range of phage FBa1, FBa2, FBa3

Host bacteria	Phage isolate		
	FBa1	FBa2	FBa3
Bacillus pumilus	+	+	+
Photobacterium damselae	-	-	-
Proteus mirabilis	-	-	-
Salmonella sp.	-	-	-
EPEC K1.1	-	-	-

+: clear plaques; -: no plaque.

Figure 2. TEM images of phages negatively stained with 2% uranyl acetate. Phage FBa1 morphology is circular as phage-like particles. Phage diameter 166.67 nm. Magnification 25,000 times.

titer. Enrichment method was used to isolate phages and to produce quantifiable titer. Plaque count was showed in 10^{-6} phage dilution. The titer of FBa1 phage was 10.2×10^8 PFU/mL, FBa2 phage was 5.9×10^8 PFU/mL, and FBa3 phage was 8.5×10^8 PFU/mL (Table 4). High concentration of *B. pumilus*-phage in Ciapus river indicated that this river was contaminated with *B. pumilus*.

Host Range Determination. The host range of phage was defined by types of bacterial cells which it can lyse. The phages of FBa1, FBa2, and FBa3 were tested on exponential-phase cultures of other pathogenic bacteria i.e. *Photobacterium damselae*, *Proteus mirabilis*, *Salmonella* sp., and EPEC K1.1. FBa1, FBa2, and FBa3 phage isolates were lytic on *B. pumilus* but did not infect the other tested bacterial isolates (Table 5).

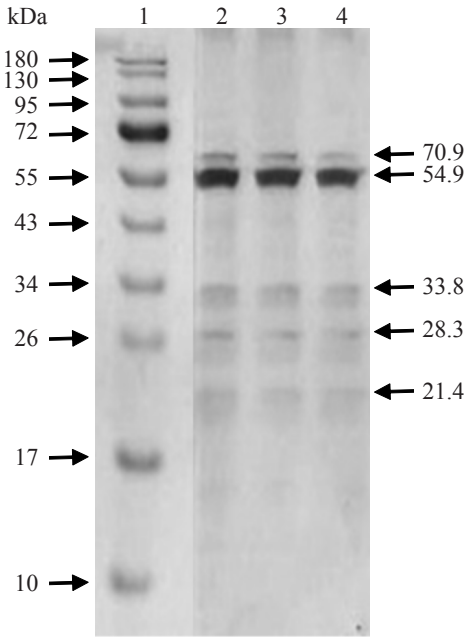

Figure 3. Molecular weight of phage FBa1 protein in SDS-PAGE gel. Lane 1, molecular size marker (PageRuler TM, Prestined Protein Ladder); lanes 2, 3, and 4, phage FBa1 protein.

Transmission Electron Microscopy (TEM). Phage FBa1 was selected for characterization according to the morphological feature. Phage FBa1 was assigned to phage-like particles with 166.67 nm in diameter (Figure 2). It was a phage with icosahedral capsids without tail.

Structural Analysis of Phage Proteins. Capsids of many phage are constructed from multiple copies of the same protein(s). Analysis of phage FBa1 proteins by SDS-PAGE showed five protein bands (Figure 3). Molecular weight of phage FBa1 proteins was 70.9, 54.9, 33.8, 28.3, and 21.4 kDa.

DISCUSSION

Indigenous *B. pumilus* was isolated from sewage water in Cigudeg, Bogor, West Java, Indonesia. It indicated that water of their drainage system in the area was contaminated with *B. pumilus*. The *B. pumilus* isolate produced a toxin that lysed red blood cells in culture media. Hoult and Tuxford (1991) reported that *B. pumilus* produced hemolytic activity. Hemolysin toxin produced by *B. pumilus* caused diarrhea in human. Hemolysin is a toxin having

dermonecrotic and vascular permeability activities and causing fluid accumulation. It implicated in diarrheal illness.

Most studies on bacterial antibiotic resistance have focused on pathogenic microorganisms. Very limited information on antimicrobial susceptibility profiles of *B. pumilus* is available. In India, *B. pumilus* isolates were identified resistant to penicillin (Parvathi *et al*. 2009). In Turkey, Ozkocaman (2006) reported that *B. pumilus* was resistant to amikacin, tobramycin, gentamycin, penicillin, cefepim, cefotaxime, ampicillin-sulbactam, ceftazidime, piperacillin, imipenem, meropenem, aztreonam, cefoperazone-sulbactam, piperacillin-tazobactam, and tetracycline. Four antibiotics (ampicillin, amoxicillin, ciprofloxacin, and clindamycin) which had different mode of action were used to treat *B. pumilus* isolate in our study. Ampicillin acts as a competitive inhibitor of the enzyme transpeptidase, and amoxicillin had same potency but much better than ampicillin. Ciprofloxacin acts by binding to complexes DNA and gyrase or topoisomerase IV. Clindamycin acts as inhibitor in bacterial protein synthesis by inhibiting ribosomal translocation. Our results showed that *B. pumilus* isolate was resistant to ampicillin and clindamycin. Resistance to antibiotic would be a problem. This could solved by an alternative solution such as bacteriophage therapy.

Isolation of bacteriophage specific to *B. pumilus* was reported by Grilione and Carr (1960), but it has not been yet reported in Indonesia. Our study found phage isolate that can infect and kill *B. pumilus* through lysis. The lytic activity by phage formed plaque on the medium. The different of plaque size in this research may be caused by delay in adsorption. Delay in adsorption makes a lower adsorption rate and resulted in a smaller plaque size (Abedon *et al*. 2001). Plaque size was influenced by numerous factors, such as addition of sodium azide accompanied by an extended incubation period, reducing the agar concentration, condition of incubation, and log phase cells of host bacteria (Clokie & Kropinski 2009). In this study, log phase of host cell was used for optimum phage replication and produced large burst size.

The titer of FBa1 phage was higher than that of FBa2 phage and FBa3 phage. It means that FBa1 phage population in environment was higher than the other phages. Titer values could indicated phage generation time. The phage generation time was divided into three period, (i) diffusion of phage progeny to new host cells; (ii) the phage eclipse period; (iii) a period of progeny maturation (Abedon *et al*. 2001). The phage generation was controlled by the phage lysis time. The phage lysis time was defined by the infective phage particles released from the host bacteria. The longer lysis time resulted in larger burst size (Wang 2006). The FBa1 phage with larger burst size associated with longer lysis time. In the longer lysis time, FBa1 phage could continue to accumulate progeny virions before lysis. Larger burst size formed larger plaque size.

Our study showed that phage FBa1, FBa2, and FBa3 had specific activity to *B. pumilus*. It cannot infect *Photobacterium damselae*, *Proteus mirabilis*, *Salmonella* sp., and EPEC K1.1. A specific phage is known to be able to infect a narrow host range. Narrow host specificity means that non target bacteria will not be killed by them (Goodridge & Abedon 2003). Specificity interaction of phage with bacterial cell is determined by specificity of adsorption and dependent on the structural of reseptors on bacterial cell surface (Braun & Hantke 1977). The nature of reseptors contacting phages is largely defined by composition of host cell wall and surface structures (Rakhuba *et al*. 2010). The outer membrane of gram-positive bacteria differs in structure from the inner membrane and from the plasma membrane of gram-negative bacteria. Many phages are attracted to bacterial pili, flagella, capsular and slime polysaccharides as receptors. Among phages adsorbing to flagella, several agents have been reported including phage χ infecting *Salmonella*, *Serratia*, and *E. coli*, phage PBS7 specific to *B. pumilus* (Shade *et al*. 1967; Lovett 1972).

Phage FBa1 was assigned to phage-like particles. The phage-like particles are phage without tail and were more abundant than tailed phage (Ashelford *et al*. 2003). Phage FBa1 did not have tail to attach to *B. pumilus*. Phage FBa1 may be have the same mechanism of adsorption as phage PBP7 that specific to *B. pumilus* as discribed by Lovett (1972). Phage FBa1 in our result were different from previously discovered of phiAGATE. Phage phiAGATE infecting *B. pumilus* by virions phiAGATE had tail morphology and assumed that phage was a member of *Myoviridae* family (Barylski *et al*. 2014). This different morphology between FBa1 and phiAGATE might caused by different water sample collection. Phage phiAGATE was isolated from water sample in litoral and pelagic zone of lake, and phage FBa1 was isolated from river water. Ashelford *et al*. (2003) reported that different sample types might harbor different population of bacteriophage.

Molecular weight of phage proteins was analyzed using SDS-PAGE. Five bands were observed at SDS-PAGE gel, they may correlated to structural and functional protein of phage FBa1. Three bands

were observed at 70.9, 54.9, and 33.8 kDa; they may correlated to structural proteins of phage FBa1. Capsid proteins of phage SPP1 infecting *B. subtilis* was reported between 47.5 and 32.5 kDa (Vinga *et al*. 2006). While 28.3 kDa protein was similar to purified protein of LysB4. LysB4 (28 kDa) is an endolysin from the *B. cereus*-infecting bacteriophage B4 (Son *et al*. 2012), and 21.4 kDa protein may correlated to endolysin of phage. Molecular weight of endolysin of phage BtCS33 infecting *B. thuringiensis* was 24 and 11 kDa (Yuan *et al*. 2012).

Lytic phage produced holin and endolysin to degradate bacterial cell wall (Hanlon 2007). The holin-endolysin is essential for host lysis (Young 2002). Holin forms a hole in the cell membrane, and endolysin passes through the hole and destroys the peptidoglycan structure. Two gene products were determined to be the possible lysin, an N-acetylmuramoyl-L-alanine amidases and/or L-alanoyl-D-glutamate peptidase. The endolysin have two domains connected by a short linker: the N-terminal catalytic domain is responsible for cell lytic activity and the C-terminal cell wall binding domain that recognizes and binds a specific substrate in the cell wall of target bacteria (Fischetti 2008).

Several advantages of using phage as a biocontrol agent is its high specificity to target host bacterium, self-replication because phage will multiply as long as there is still a host present, low toxicity because phage consist mostly of nucleic acids and proteins, phage are relatively cheap and easy to isolate, and phage can add on food processing (Sillankorva *et al*. 2012). Indigenous phage FBa1, FBa2, FBa3 isolated from Ciapus river water, Bogor were capable to specifically lyse *B. pumilus*. Our study showed that these phage isolates had potency for biocontrol of *B. pumilus* in the environment.

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