Fucose sensing regulates bacterial intestinal colonization

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The mammalian gastrointestinal tract provides a complex and competitive environment for the microbiota4. Successful colonization by pathogens requires scavenging nutrients, sensing chemical signals, competing with the resident bacteria and precisely regulating the expression of virulence genes5. The gastrointestinal pathogen enterohaemorrhagic Escherichia coli (EHEC) relies on inter-kingdom chemical sensing systems to regulate virulence gene expression6–4. Here we show that these systems control the expression of a novel two-component signal transduction system, named FusKR, where FusK is the histidine sensor kinase and FusR the response regulator. FusK senses fucose and controls expression of virulence and metabolic genes. This fucose-sensing system is required for robust EHEC colonization of the mammalian intestine. Fucose is highly abundant in the intestine5. Bacteroides thetaiotaomicron produces multiple fucosidases that cleave fucose from host glycans, resulting in high fucose availability in the gut lumen6. During growth in mucin, B. thetaiotaomicron contributes to EHEC virulence by cleaving fucose from mucin, thereby activating the FusKR signalling cascade, modulating the virulence gene expression of EHEC. Our findings suggest that EHEC uses fucose, a host-derived signal made available by the microbiota, to modulate EHEC pathogenicity and metabolism.

The gastrointestinal tract is inhabited by trillions of commensal bacteria that play crucial roles in human physiology6. This fundamental relationship between the host and microbiota relies on chemical signalling and nutrient availability7, and invading pathogens compete for these resources through the precise coordination of virulence traits. EHEC colonizes the colon, leading to haemorrhagic colitis7. EHEC colonization depends on the locus of enterocyte effacement (LEE) pathogenicity island8. This pathogenicity island encodes a regulator for its own expression, ler, and a type III secretion system, a molecular syringe which injects effectors into the host cell, leading to the formation of attaching and effacing lesions on enterocytes. Attaching and effacing lesions are characterized by remodelling of the host cell cytoskeleton, leading to the formation of a pedestal-like structure beneath the bacteria9. LEE expression is regulated by an inter-kingdom chemical signalling system involving the host hormones adrenaline and/or noradrenaline and the microbial flora produced signal autoinducer-3 (AI-3)10. These signals are sensed by two histidine sensor kinases, QseC (ref. 3) and QseE (ref. 4), which initiate a signalling cascade that promotes virulence.

Histidine sensor kinases, together with response regulators, comprise a two-component system, which plays a major role in bacterial signal transduction. Upon sensing a signal, the histidine sensor kinases autophosphorylate and then transfers its phosphate to the response regulator. Subsequently, most response regulators bind DNA, promoting changes in gene expression11. The cognate response regulator for QseC is QseB and for QseE it is QseF (Fig. 1a). QseBC and QseEF repress expression of the z0462 and z0463 genes (Fig. 1b)11,12. QseF repressing of z0462/z0463 expression is direct, whereas QseF-mediated repression is indirect (Fig. 1c, d), in agreement with QseF being a σ54-dependent transcriptional activator12. QseF activates the expression of a repressor of z0462/z0463.

The z0462/z0463 genes are within a pathogenicity island (O-island 20 (O1-20))13, which is found in EHEC O157:H7 strains and enteropathogenic E. coli strains exclusively from the 055:H7 serotype (which gave rise to the O157:H7 serotype), but absent in all other E. coli strains whose genomes are currently publicly available. This pathogenicity island is organized in three transcriptional units (Supplementary Fig. 1). The genes z0462/z0463 encode for a putative two-component system: z0462 encodes a histidine sensor kinase with eight transmembrane domains that shares similarity (around 30% similarity at the amino acid level) with a glucose-6-phosphate sensor, UhpB; z0463 encodes a response regulator with a receiver and a DNA-binding

Figure 1 The two-component system FusKR of EHEC. a, The QseC/QseE signalling cascade. QseC senses AI-3 and adrenaline and noradrenaline. QseE senses adrenaline and noradrenaline and SO4 and PO4. QseE phosphorylates QseB (which activates flagella), KpdE (activates the LEE) and QseF. QseF only phosphorylates QseF. QseBC and QseEF repress the expression of z0462 and z0463. AE, attaching and effacing, b, qRT–PCR of z0462 in wild type (WT), ΔqseB, ΔqseC, ΔqseF and ΔqseE in DMEM media. Gene expression is represented as fold differences normalized to wild type. Error bars indicate standard deviations of AddCt values (n = 18 biological samples per strain; *P < 0.01, **P < 0.001, Student’s t-test), c, Electrophoretic mobility shift assay (EMSA) of z0463 with QseB and QseF, d, EMSA of qseE (positive control) with QseF. e, Autophosphorylation of Z0462 in liposomes (top panel), and a Coomassie gel of Z0462 (lower panel), f, Phosphotransfer from Z0462 (in liposomes) to Z0463 (ratio 1:1) (RR) (top panel), loading control Coomassie gel of Z0462 and Z0463 (lower panel).

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domain (Supplementary Fig. 2). Z0462 in liposomes is a functional histidine sensor kinase (Fig. 1e) and it transfers its phosphate to Z0463 (Fig. 1f). Hence, Z0462 and Z0463 constitute a cognate two-component system.

Transcriptomic studies (Supplementary Tables 4 and 5) indicated that Z0462/Z0463 act mainly as repressors of transcription. Z0462 and Z0463 represses LEE gene expression (Supplementary Fig. 3). Transcription of all LEE operons is increased in Δz0462 (deletion mutant of z0462) and Δz0463 (deletion mutant of z0463) and plasmid complementation restored the expression of ler to wild-type levels (Fig. 2a–d). Transcription of the LEE genes is activated by Ler14. The response regulator Z0463 directly represses ler (Fig. 2a, d and Supplementary Figs 6–8).

Expression of z0463 is increased by the mucus produced by intestinal HT29 cells. EHEC-infected undifferentiated HT29 cells were used as negative controls, because they do not produce mucus (Fig. 3a and Supplementary Fig. 9). Z0462 is a predicted hexose-phosphate sensor, hence, Z0462 may sense sugars in the mucus. Fucose is a major component of mucin glycoproteins, it is abundant in the intestine2, and fucose utilization is important for EHEC intestinal colonization3,4. In

As expected with the increased LEE transcription, both Δz0462 and Δz0463 secreted more EspB, a LEE-encoded protein (Fig. 2g), and formed more pedestals than wild type (Fig. 2h, i). Therefore, Z0462/Z0463 repress attaching and effacing lesion formation. However, expression of other genes encoding non-LEE-encoded type III secretion system effectors, not involved in attaching and effacing lesion formation, are activated by Z0462/Z0463 (Supplementary Fig. 4).

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Figure 2 | z0462/z0463 regulates LEE expression. a, qRT–PCR of LEE genes in wild type and Δz0462 in DMEM media (n = 18; error bars, s.d.; **P < 0.001). b, qRT–PCR of ler in wild type, Δz0462 and Δz0462+ (Δz0462 rescued with plasmid complementation of z0462) in DMEM media (n = 18; error bars, s.d.; **P < 0.001). c, qRT–PCR of LEE genes in WT and Δz0463 in DMEM media (n = 18; error bars, s.d.; **P < 0.001; ***P < 0.0001). d, qRT–PCR of ler in WT, Δz0463 and Δz0463+ (Δz0463 rescued with plasmid complementation of z0463) in DMEM (n = 18; **P ≤ 0.001, ***P < 0.0001; NS, not significant P > 0.05; Student’s t-test). e, Representation of the Ler and Z0463 regulation of the LEE operons. f, EMSA of ler with Z0463 with ler and kan (kanamycin) cold probes. Ratios represent hot:cold probe. g, Western blot of EspB in supernatants of wild type, Δz0462, Δz0462+, Δz0463 and Δz0463+ strains. BSA was added as a loading control. h, Fluorescent actin staining assay of HeLa cells infected with wild-type EHEC, Δz0462, Δz0462+, Δz0463 and Δz0463+, stained with fluorescein isothiocyanate–phalloidin (actin, green) and propidium iodide (bacterial and HeLa DNA, red). Original magnification, ×63. i, Quantification of fluorescent actin staining assay (n = 600 cells; ***P < 0.0001; P > 0.05 = NS; Student’s t-test; error bars, s.d.).
**Figure 3** | Z0462 and Z0463 is a fucose-sensing two-component system. 

**a.** qRT–PCR of z0463 in wild type in the presence of undifferentiated non-mucus-producing HT29 or differentiated mucus-producing HT29 cells. Error bar indicates standard deviation of ΔΔCt values (n = 18; ***P < 0.001; Student’s t-test). **b.** qRT–PCR of fucose-utilization genes in wild-type EHEC, Δz0462 and Δz0463 in DMEM (D_{0.5\text{min}} = 1.0) (n = 18; **P < 0.01; ***P < 0.001; Student’s t-test). **c.** Growth curves of wild type, Δz0462 and Δz0463 strains in M9 minimal-media with l-fucose as a sole carbon source. (n = 6; significance between generation times calculated through analysis of variance (ANOVA) P = 0.01, error bars too small to be visible on the graph). **d.** FusK autophosphorylation (in liposomes) in the presence of l-fucose, D-glucose or D-ribose (top panel), and Coomassie gel of FusK in liposomes (lower panel) (loading control). No signal indicates no activating sugar signal added. **e.** Quantification of FusK autophosphorylation. Phosphorylation represented as fold-change compared to absence of signal. Error bar, standard deviation of fold-change values. (n = 3; ***P < 0.001; P > 0.05 = NS; Student’s t-test). **f.** Schematic representation of the fusKR operon to z0461. MFS, major facilitator superfamily. g, qRT–PCR of z0461 in wild type and ΔfusK (also called Δz0462) (n = 18; **P < 0.01; Student’s t-test). **g.** Growth curves of wild type and Δz0461 in M9 medium with fucose as a sole carbon source (n = 6; significance between generation times calculated through ANOVA P ≤ 0.01, error bars too small to be visible on the graph). **h.** qRT–PCR of fusA, fusP and fusR in wild type and Δz0461 (n = 18, **P < 0.01; Student’s t-test).

**E. coli,** l-fucose utilization requires the fus genes and their activator FusR (ref. 17). Z0462/Z0463 repress the expression of the fus genes (Fig. 3b), and Δz0462 and Δz0463 grow faster with l-fucose as a sole carbon source compared to wild type (generation times of 92.4 min for wild type, 64 min for Δz0462 and 74 min for Δz0463) (Fig. 3c). Therefore, Z0462/Z0463 regulates fucose utilization, and this response is specific to fucose, with the mutants and wild type growing at similar rates with other carbon sources (Supplementary Fig. 10). Z0462 senses fucose, but not glucose or D-ribose (Fig. 3d, e). The concentration of fucose used (100 μM) is physiologically relevant to the mammalian intestine. Hence we renamed the Z0462 protein as FusK for fucose-sensing histidine kinase and its cognate response regulator, and Z0463 as FusR for fucose-sensing response regulator.

FusKR shares homology to the UhpAB two-component system. UhpAB senses glucose-6-phosphate and activates expression of the uhpT gene which encodes a hexose-phosphate major facilitator superfamily transporter19–21. FusKR represses transcription of the z0461 gene downstream of fusKR, which encodes a predicted major superfamily transporter (Fig. 3f, g and Supplementary Figs 3, 11). Δz0461 has decreased growth (lower concentration of cells) with fucose as a sole carbon source (generation times of 88.2 min for wild type and 96.6 min for Δz0461) (Fig. 3h), but grows with glucose similarly to wild type (Supplementary Fig. 12), indicating that z0461 is involved in optimal fucose import. Transcription of the fus operons is linked to fucose uptake, fucose yields fusulose-1-phosphate which is the inducer of the FusR activator of the fus operons17,21–23. Transcription of the fus genes is decreased in Δz0461 (Fig. 3i). Fucose induces FusKR, which represses z0461, decreasing fucose import and the intracellular levels of the fusulose-1-phosphate inducer of FusR. In further support of this indirect regulation of the fus genes, FusR does not bind to the fusPIKUR promoter region (Supplementary Fig. 13), in contrast to the direct regulation of the LEE (Fig. 2).

The ΔfusK is not responsive to fucose, given that expression of ler is repressed by fucose in wild type, but not in ΔfusK (Fig. 4a). B. thetaiotaomicron produces multiple fucosidases that cleave fucose from host glycans, resulting in high fucose availability in the lumen2. B. thetaiotaomicron supplies mucin-derived fucose to EHEC, reducing ler expression, whereas in free fucose there is no change in ler expression whether B. thetaiotaomicron is present or not (Fig. 4b). Expression of ler is decreased when EHEC is grown in mucin compared to fucose (Fig. 4b), consistent with the increased expression of fusR in mucin (Fig. 3a).

**In vitro competitions between ΔfusK and wild type, and ΔfusK and Δler (which does not express the LEE) in the presence or absence of B. thetaiotaomicron, with either fucose or mucin as a sole carbon source were performed.** The competition index between ΔfusK and wild type was 1 (Supplementary Fig. 14a) both in the absence or presence of B. thetaiotaomicron during growth in fucose, indicating...
that, in the presence of free fucose, B. thetaiotaomicron does not affect the competition between δfusK and wild type, and that the growth advantage of δfusK in fucose is counteracted by decreased LEE expression in wild type. When these experiments were performed with mucin as a sole carbon source the competition index between wild type and δfusKΔfucR, which does not express the fus genes. The double mutant was outcompeted by the wild type with a similar competition index to the δfusK and wild type competition (Fig. 4c), indicating that fucose utilization does not play a major role in FusK-mediated intestinal colonization, and the burden of LEE overexpression by δfusK is a stronger determinant of its decreased fitness within the intestine. FusK repression of LEE expression in the mucus layer prevents superfluous energy expenditure. Once in close contact to the epithelial surface, the QseCE adrenergic-sensing systems are triggered to activate virulence both directly through the QseCE cascade, and indirectly by repression of fusKR (Supplementary Fig. 15). EHEC competes with commensal E. coli (γ-Proteobacteria), but not with B. thetaiotaomicron, for the same carbon sources (for example, fucose) within the mammalian intestine15,24–28. Commensal E. coli, however, are not found in close contact with the epithelium, being in the mucus layer, where it is counterproductive for EHEC to invest resources to utilize fucose, when EHEC can efficiently use other carbon sources such as galactose, hexorunates or mannose, which are not used by commensal E. coli within the intestine15. Additionally, in contrast to commensal E. coli, EHEC is found closely associated with the intestinal epithelium25. Therefore, EHEC can utilize nutrients exclusively available at the surface of the epithelial cells. Consequently, the decreased expression of the fus operon through fucose-sensing by FusKR (Fig. 3) may prevent EHEC from expending energy in fucose utilization in the mucus layer, where it competes with commensal E. coli for this resource, and focus on using other carbon sources, not used by that competitor. Thus, the colonization defect of δfusK results from its inability to correctly time virulence and metabolic gene expression.

METHODS SUMMARY

Strains and plasmids are listed in Supplementary Tables 1 and 2. Molecular biology techniques were performed as previously described26. Primers used in quantitative reverse transcription PCR (qRT–PCR) and cloning are listed in Supplementary Table 3. Construction of isogenic mutants was performed using a lambda-red method as previously described29. FusK liposome studies were performed as previously described30. Full Methods and any associated references are available in the online version of the paper.

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The important role FusK has in the metabolism and virulence of EHEC means it is also important for intestinal colonization. Competition assays in infant rabbits demonstrated that the wild type outcompeted δfusK tenfold (competition index of 0.12), which is statistically different (P = 0.039) from a control competition assay, in which the wild type (lacZ+) was competed against a ΔlacZ (competition index of 0.7) (Fig. 4c). Hence, a functional FusK is necessary for robust EHEC intestinal colonization. To determine whether the decreased ability of δfusK to colonize the mammalian intestine was due to uncontrolled expression of the LEE and/or fucose utilization, we performed competition experiments between wild type and δfusKΔfucR, which does not express the fus genes. The double mutant was outcompeted by the wild type with a similar competition index to the δfusK and wild type competition (Fig. 4c), indicating that fucose utilization does not play a major role in FusK-mediated intestinal colonization, and the burden of LEE overexpression by δfusK is a stronger determinant of its decreased fitness within the intestine.
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Author Information Microarray data are deposited in the Gene Expression Omnibus under accession number GSE34991. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to V.S. (vanessa.sperandio@utsouthwestern.edu).
METHODS

Bacterial strains, plasmids and growth conditions. Strains and plasmids are listed in Supplementary Tables 1 and 2. E. coli strains were grown aerobiocally at 37°C in DMEM (Gibco) or LB media unless otherwise stated. For studies involving fucose utilization, bacterial cultures were grown in M9 minimal media containing 0.4% t-fucose, t-glucose, t-rhamnose, t-galactose or t-xylate (Sigma) as the sole carbon source. For the co-culture experiments between EHEC and B. thetaiotaomicron, these strains were grown anaerobiocally at 37°C in DMEM buffer (lacking glucose and pyruvate) with or without fucose or free, at a 1:1 ratio of EHEC to B. thetaiotaomicron. Enumeration of EHEC was performed through serial dilution of these cultures in McConkey agar containing streptomycin (EHEC strain 86-24 is streptomycin resistant, whereas B. thetaiotaomicron is streptomycin sensitive). Enumeration of B. thetaiotaomicron was performed through serial plating in TYG medium supplemented with 10% horse blood in the presence of gentamycin (B. thetaiotaomicron is gentamycin resistant, whereas EHEC is gentamycin sensitive).

Recombinant DNA techniques. Molecular biology techniques were performed as previously described29. Primers used in qRT–PCR and cloning are listed in Supplementary Table 2.

Isogenic mutant construction. Construction of isogenic ΔfusK, ΔfusR, Δz0461, Δler and ΔfusΔfucR mutants was performed using a lambda-red mediated recombination method as previously described30. Primers used to construct these knockouts are depicted in Supplementary Table 3. Briefly, a PCR product used to knock out with FusK at a ratio of 20:1. Liposomes were reconstituted as described previously32. FusK presence in liposomes was confirmed by western blot using anti-Myc antibody (Invitrogen).

FusK purification and reconstitution into liposomes. FusK was cloned into ZeroBlunt TOPO, digested using XhoI and HindIII restriction enzymes and then cloned into pBAD/MycHisA, generating pARP11. pARP11 was subsequently transformed into TOP10 cells, generating the ARP04 strain. ARP04 strain was grown in LB to 

D600nm = 0.6 at 37°C, then protein expression was induced by addition of arabinose (final concentration of 0.2%) and overnight growth at 25°C. FusK was subsequently purified using nickel columns (Qiagen).

Nested deletion analyses. Transcriptional fusions of the ler promoter with promotorless lacZ have been described previously31. To integrate the transcriptional fusions into the chromosome, E. coli MC4100 was lysogenized with phage λ45, generating strains FS14 and FS16.

FusR purification and reconstitution into liposomes. FusR was cloned into ZeroBlunt TOPO, digested using XhoI and HindIII restriction enzymes and then cloned into pBAD/MycHisA, generating pARP11. pARP11 was subsequently transformed into TOP10 cells, generating the ARP03 strain. ARP03 strain was grown in LB media at 37°C until D600nm = 0.5 then protein expression was induced by addition of arabinose (final concentration of 0.2%) and growth at 37°C overnight. ARP03 strain was grown in LB media at 37°C until D600nm = 0.5 then protein expression was induced by addition of arabinose (final concentration of 0.2%) and growth at 37°C overnight.

FusR was subsequently purified using nickel columns (Qiagen).

Fluorescent actin staining (FAS) assay. Fluorescein actin staining (FAS) assays were performed as previously described37. Pedestal enumeration was performed in 600 infected cells. The Student t-test was used to determine statistical significance. A P-value of less than 0.05 was considered significant.

In vitro competition assays. Bacterial strains were grown for 18 h in LB at 37°C, resuspended in DMEM without glucose and inoculated 1:100 in DMEM (without glucose, without pyruvate) containing fucose or mucin as the sole carbon source. B. thetaiotaomicron was grown in TYG medium, resuspended in DMEM (without glucose) and inoculated at a 1:9 ratio. In vitro competitions were carried out anaerobiocally and samples were collected hourly for serial dilution and plating for the colony-forming units count. A competition index was determined by the ratio of ΔfusK to wild-type EHEC or the ratio of ΔfusK to Aler.

Infant rabbit infection studies. Litters of 3-day-old infant rabbits were infected as described previously36. Individual rabbits were orogastrically inoculated (approximately 5 × 108 colony-forming units per 90 g of animal's weight) with 1:1 mixtures of wild-type (ΔlacZ) EHEC and the ΔfusK of ΔfusKΔfucR. The animals were killed 2 days postinoculation and colonic tissue samples removed and homogenized before microbiological analysis. The number of wild-type and fusK mutant cells present in the tissue homogenate was determined by serial dilution and plating on media containing streptomycin and 5-bromo-4-chloro-3-indoly-l-β-D-galactoside (X-gal) as previously described36. Competition indexes were calculated as the ratio of ΔfusK to wild type or ΔfusKΔfucR to wild type in tissue homogenates divided by the ratio of ΔfusK to wild type or ΔfusKΔfucR to wild type in the input. The competition index was compared to the competition index value obtained when otherwise isogenic lacZ+ (wild type) and ΔlacZ strains were given to rabbits. Differences in cis were compared using the Mann–Whitney test, where a P-value of less than 0.05 was considered significant. All animal experiments performed were approved by the IACUC offices of University of Texas Southwestern Medical Center and Brigham and Women's Hospital. (ΔlacZ n = 2 litters (6–11 animals) and ΔfusKΔfucR, n = 3 litters (11 animals)).

β-galactosidase activity assays. The bacterial strains FS14, FS15 and FS16 were transformed with pFusR or empty vector (pBAD/MycHisA) and grown in aerobic in DMEM containing 0.2% arabinose at 37°C to D600nm = 0.8. The cultures were diluted 1:100 in Z buffer (60 mM NaHPO4, 7 mM KH2PO4 and 50 mM β-mercaptoethanol) and assayed for β-galactosidase activity by using o-nitrophenyl-β-D-galactopyranoside as substrate as previously described36.

Electrophoretic mobility shift assay (EMSA). EMSAs were performed using purified FusR–Myc–His and radio-labelled probes. Primers were end-labelled using [γ32P]ATP and T4 polynucleotide kinase (NEB) and subsequently used to generate radio-labelled DNA probes. EMSA experiments were run on a 6% polyacrylamide gel, excised and purified using a QiaGen gel extraction kit. To test the ability of FusR to bind directly to its target promoters, increasing amounts of FusR (0 to 4.35 ng) were incubated with end-labelled probe (10 ng) for 20 min at 4°C in binding buffer (50 mM NaHPO4, 0.5 ng poly(denosinonic-dideoxyctydilic) (ddIC) acid, 6 mM HEPES, pH 7.5, 5 mM EDTA, 3 mM DTT, 300 mM KCI and 25 mM MgCl2). A sucrose solution was used to stop the reaction37. The reactions were run on a 6% polyacrylamide gel for 6.5 h at 180 V. The gels were dried under vacuum and EMSAs were visualized by autoradiography. DNase I footprinting. DNase I footprinting was performed as previously described38. Briefly, primer Zer-299F (Supplementary Table 2) was end-labelled using [γ32P]ATP and T4 polynucleotide kinase (NEB) and used in a PCR with cold primer Ler-299F (Supplementary Table 2) to generate probe LerFP. The resulting end-labelled probe was used in a binding reaction (described in the EMSA subsection) for 20 min at room temperature. At this time, a 1:10 dilution of
DNase I (NEB) and the manufacturer-supplied buffer were added to the reaction and digestion proceeded for 7 min at room temperature. The digestion reaction was stopped by addition of 100 μl of stop buffer (200 mM NaCl, 2 mM EDTA and 1% SDS). Protein extraction was performed using phenol–chloroform and DNA was precipitated using 5 M NaCl, 100% ethanol and 1 μl glycogen. The DNase reactions were run in a 6% polyacrylamide-urea gel next to a sequencing reaction (Epicentre). Amplicon generated using primers Ler-299FP-F and Ler-18FP-R (radiolabelled) was used as a template for the sequencing reaction. The footprint was visualized by autoradiography.

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