Acetate excretion during growth of *Salmonella enterica* on ethanolamine requires phosphotransacetylase (EutD) activity, and acetate recapture requires acetyl-CoA synthetase (Acs) and phosphotransacetylase (Pta) activities

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This report shows that *Salmonella enterica* catabolizes ethanolamine to acetyl-CoA (Ac-CoA), which enters the glyoxylate bypass and tricarboxylic acid cycle for the generation of energy and central metabolites. During growth on ethanolamine, *S. enterica* excreted acetate, whose recapture depended on Ac-CoA synthetase (Acs) and the housekeeping phosphotransacetylase (Pta) enzyme activities. The Pta enzyme did not play a role in acetate excretion during growth of *S. enterica* on ethanolamine. It is proposed that during growth on ethanolamine, acetate excretion is necessary to maintain a pool of free CoA. Acetate excretion requires the *eut* operon-encoded phosphotransacetylase (EutD) and acetate kinase (Ack) enzymes. EutD function was not required for growth on ethanolamine, and an *eutD* strain showed only a slight reduction in growth rate. The existence of an as-yet-unidentified system that releases acetate was revealed during growth of a strain lacking Acs, the housekeeping phosphotransacetylase (Pta), and EutD. The functions of pyruvate oxidase (PoxB), Ack and STM3118 protein [a homologue of the *Saccharomyces cerevisiae* Ac-CoA hydrolase (Ach1p) enzyme] were not involved in the release of acetate by the *acs pta eutD* strain.

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INTRODUCTION

The enterobacterium *Salmonella enterica* can use ethanolamine and 1,2-propanediol as sources of carbon, energy, and, in the case of ethanolamine, nitrogen (Blackwell & Turner, 1978; Bobik *et al.*, 1999; Chang & Chang, 1975; Roof & Roth, 1988). Catabolism of ethanolamine and 1,2-propanediol by this bacterium is intriguing. The biochemistry for the conversion of either compound is straightforward (Fig. 1). In both cases, an alcohol function is converted to the corresponding aldehyde by either coenzyme B₁₂ (AdoCbl)-dependent deamination (ethanolamine to acetaldehyde) (Faust *et al.*, 1990) or dehydration (1,2-propanediol to propionaldehyde) (Bobik *et al.*, 1997). Acetaldehyde is oxidized to acetate, and propionaldehyde is oxidized to propionate, each of which is activated to its corresponding acyl-CoA derivative (Bobik *et al.*, 1997; Faust *et al.*, 1990; Leal *et al.*, 2003).

In *S. enterica*, ethanolamine and 1,2-propanediol are likely catabolized within a carboxysome-like structure (Havemann

et al., 2002; Havemann & Bobik, 2003; Kofoed *et al.*, 1999; Stojiljkovic *et al.*, 1995), hereafter referred to as the metabolosome. Ethanolamine catabolism is important to the lifestyle of *S. enterica* in the gut, as strains unable to grow on ethanolamine are attenuated in the mouse model of infection (Stojiljkovic *et al.*, 1995). Results from *in vivo* expression technology (IVET) experiments have also indicated that 1,2-propanediol is relevant to the survival of *S. enterica* as a pathogen (Conner *et al.*, 1998). Why these compounds are metabolized within metabolosomes remains an open question.

Genetic and biochemical analyses of *S. enterica* strains identified the chromosomal locus required for growth of this bacterium on ethanolamine (Roof & Roth, 1988, 1989). Sequence analysis of the ethanolamine utilization (*eut*) operon revealed 17 open reading frames, *eutSPQTDNMNEJGHAB-CLKR* (Kofoed *et al.*, 1999; Stojiljkovic *et al.*, 1995) (Fig. 2a). The *eutR* gene, located 3' to the operon and independently transcribed from it (Sheppard & Roth, 1994), encodes the EutR transcription activator protein, which becomes active in the presence of ethanolamine and coenzyme B₁₂ (Roof & Roth, 1988, 1992; Sheppard & Roth, 1994). Several of the *eut* genes (e.g. *eutSMNLK*) encode proteins with homology to cyanobacterial carboxysomal shell proteins (Kofoed *et al.*,

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Abbreviations: Ac-CoA, acetyl-CoA; Ac-P, acetyl phosphate; Ack, acetate kinase; Acs, acetyl-CoA synthetase; Pta, phosphotransacetylase.

Fig. 1. Acyl-CoA synthesis from ethanolamine and 1,2-propanediol. The first step in ethanolamine or 1,2-propanediol catabolism is catalysed by a coenzyme B_{12} -dependent enzyme that yields acetaldehyde or propionaldehyde, followed by an oxidation to the corresponding short-chain fatty acid, which is released as either acetyl- or propionyl-CoA. EutBC, coenzyme B_{12} -dependent ethanolamine ammonia-lyase (EC 4.3.1.7; Babior, 1982; Faust *et al.*, 1990); EutE, putative acetaldehyde dehydrogenase (Roof & Roth, 1989; Stojiljkovic *et al.*, 1995); PduCDE, 1,2-propanediol dehydratase (EC 4.2.1.28; Bobik *et al.*, 1997); PduP, propionaldehyde dehydrogenase (Leal *et al.*, 2003).

1999; Stojiljkovic *et al.*, 1995). In cyanobacteria and thio-bacilli, this multiprotein complex is involved in carbon dioxide fixation and concentration (Badger & Price, 2003; Baker *et al.*, 2000; Friedberg *et al.*, 1993). The function of the metabolosome encoded by the *eut* operon remains unclear. In *S. enterica*, a similar multiprotein complex is assembled during growth on 1,2-propanediol (Havemann *et al.*, 2002; Havemann & Bobik, 2003), and it was suggested that this structure might be used to contain propionaldehyde (Bobik *et al.*, 1999; Rondon *et al.*, 1995). Similarly, the metabolosome involved in ethanolamine catabolism was proposed to contain acetaldehyde (Rondon *et al.*, 1995).

At present, a good understanding of the physiology of ethanolamine catabolism is lacking. Work presented here provides insights into how *S. enterica* acquires carbon and energy from ethanolamine catabolism and reveals mechanisms that the cell uses to maintain a balance between acetyl-CoA (Ac-CoA) and free acetate (Fig. 2b).

On the basis of the data reported here, we propose that a fraction of ethanolamine is converted to acetate via the

energy-conserving phosphotransacetylase (EutD)/acetate kinase (Ack) pathway, with no apparent involvement of the housekeeping phosphotransacetylase (Pta) enzyme. Ac-CoA synthetase (Acs) and Pta are responsible for recapturing acetate for later use via the Krebs cycle and glyoxylate bypass.

METHODS

Bacterial strains, culture media and growth conditions. All bacterial strains used in this study were derivatives of *Salmonella enterica* serovar Typhimurium LT2. The genotypes of strains used in this work are listed in Table 1. Bacterial strains were grown on no-carbon E minimal medium (NCE) (Berkowitz *et al.*, 1968) supplemented with: ethanolamine (30 mM) as the source of carbon and energy; MgSO_4 (1 mM); L-methionine (0.5 mM); cyanocobalamin (150 μM); and trace minerals (1:100, v/v) (Balch & Wolfe, 1976). Luria-Bertani (LB) broth was used as rich medium. Growth curves reported here were performed at least twice in 125 ml sidearm flasks with vigorous shaking (200 r.p.m.) at 37 °C. All chemicals were purchased from Sigma.

Phage P22 transductions. All transductional crosses were performed as described by Davis *et al.* (1980) with phage P22 HT105/1 *int-210* (Schmieger, 1971; Schmieger & Bakhaus, 1973). Transductants were freed of phage as described by Chan *et al.* (1972).

PCR amplifications. Enzyplus (ENZYPOL) was used in PCR amplification during construction of the *eutD* deletion strain of *S. enterica*. All reactions were performed in an Eppendorf Mastercycler gradient PCR thermal cycler (Brinkmann Instruments). All primers were purchased from Integrated DNA Technologies.

Construction of non-polar *eutD* deletion. The deletion of *eutD* was constructed as described by Datsenko & Wanner (2000), except that all manipulations were performed in *S. enterica*. The *cat*⁺ cassette in plasmid pKD3 was amplified using PAGE-purified primers with 50 bp of perfect homology for the 5' and 3' ends of *eutD*: *eutD5'*DEL (5'-TGACGGTCGCCCAAATTCAACAACGGTTGGGAGAGAAGC-CATGATCATTGAACGCGTGTAGGCTGGAGCTGCTTC-3') and *eutD3'*DEL (5'-CGTGTTCCTCTCATTAAGGGGTCCAGAACGGACCGTTCATTCAACCAGTGTGATATGAATATCCTCCTTA-3'). Construction of the deletion strain from this point was as described in the original protocol, except that the *S. enterica* strain JE6692 was used as the recipient. The presence of the deletion was confirmed using ABI PRISM non-radioactive BigDye cycle terminator DNA sequencing methodology (PerkinElmer Life Sciences) according to the manufacturer's instructions. DNA sequence was determined at the Biotechnology Center of the University of Wisconsin.

Determination of excreted acetate during growth on ethanolamine. Overnight cultures of *S. enterica* grown at 37 °C in LB were subcultured 1:100 into 20 ml NCE minimal medium containing ethanolamine as the source of carbon and energy. Samples (1 ml) were removed at intervals, and the optical density of the culture was determined using a Klett colorimeter furnished with a red filter. Samples were centrifuged for 5 min at 13 000 *g* in a Microfuge 18 centrifuge (Beckman Coulter). A 500 μl sample of the supernatant was filtered through a 0.45 μm Spin-X centrifuge tube filter (Corning) and acidified by the addition of 2.5 μl of a 5 M H_2SO_4 solution. Acidified samples were stored at -20 °C until used. HPLC was used to analyse the composition of the samples (see below).

HPLC analysis. Samples (200 μl) containing organic acids excreted by the strains of *S. enterica* used in these studies were resolved using a Waters HPLC system equipped with a model 600 solvent delivery system, a model 900 photodiode array detector, and a column heater. An Aminex HPX-87H organic acid analysis column (300 $\times$ 7.8 mm,

Fig. 2. *S. enterica* gene functions needed to convert ethanolamine to acetate. (a) Genes encoding proteins with a known or putative enzymic activity are indicated as such. Genes encoding proteins with homology to cyanobacterial carboxysome proteins are labelled with vertical arrows. Horizontal arrows above the operon identify the two transcripts encoded at this locus. *EutR* is the activator of transcription of the *eut* operon. (b) Pathways for acetate activation in *S. enterica*. Depending on the external concentration of acetate, *S. enterica* uses either of two independent pathways to convert acetate to Ac-CoA. Low concentrations (≤ 10 mM) of acetate require AMP-forming synthetase (Acs) activity (right); at ≥ 25 mM, acetate is converted to Ac-CoA by the acetate kinase (Ack) and phosphotransacetylase (Pta, *EutD*) enzymes (left).

Bio-Rads) was maintained at 45 °C and developed isocratically with 0.005 M H₂SO₄ at a flow rate of 0.6 ml min⁻¹. Separations were monitored at 210 nm. Under these conditions, acetate eluted 14.6 min after injection. The area under the peak corresponding to acetate was quantified using the Waters Millenium software package to determine the concentration of acetate in the sample.

RESULTS AND DISCUSSION

S. enterica undergoes acetate switch during growth on ethanolamine

As shown here, ethanolamine is an acetogenic carbon and energy source for *S. enterica*. During growth on ethanolamine, this bacterium excreted and recaptured acetate as it

would during the catabolism of other known acetogenic substrates (e.g. glucose, serine, aspartate, etc.). The acetate switch, as defined by A. Wolfe, is the physiological condition where acetate dissimilation equals its assimilation (Wolfe, 2005). During growth of wild-type *S. enterica* on ethanolamine, the acetate switch occurred at late exponential phase, with acetate being completely depleted from the medium by the time the culture reached early stationary phase (Fig. 3a).

Pta enzyme activity is not required for acetate excretion during growth on ethanolamine, but it is required for acetate recapture

It was previously reported that strains lacking the house-keeping Pta enzyme did not grow on ethanolamine (Kofoid

Table 1. Strains and plasmids

Unless otherwise stated, strains listed were constructed during the course of this work.

Strain or plasmid	Relevant genotype	Source or reference*
<i>S. enterica</i> strains†*		
LT2	Wild-type	Laboratory collection
LT2 derivatives		
TR6583	<i>metE205 ara-9</i>	K. Sanderson (University of Calgary) via J. Roth (University of California – Davis)
TR6583 derivatives		
JE2236	<i>eutE18::Mud1-8‡</i>	Laboratory collection
JE4173	<i>aceB113::MudJ</i>	Laboratory collection
JE4561	<i>icd1::Tn10d(tet⁺)§</i>	Laboratory collection
JE6317	<i>pta103::cat⁺</i>	Laboratory collection
JE6692	pKD46	
JE7310	Δ <i>eutD1143</i>	
JE7437	Δ <i>eutD1143 pta103::cat⁺</i>	
JE7375	JE7310/pEUTD2	
JE7439	JE7437/pEUTD2	
JE7498	Δ <i>eutD1143 pta103::cat⁺ acs::kan⁺</i>	
JE7715	Δ <i>eutD1143 pta103::cat⁺ acs::kan⁺</i> Δ STM3118-2	
JE7758	Δ <i>acs2</i>	
JE7832	Δ <i>eutD1143 pta103::cat⁺ acs::kan⁺</i> <i>poxB1::cat⁺</i>	
JE8326	Δ <i>eutD1143 acs::kan⁺ ack408::Tn10</i>	
JE8507	Δ <i>eutD1143 pta103::cat⁺ Δacs2</i>	
JE8749	Δ <i>ack103</i>	Laboratory collection
JE8821	Δ <i>acs2 pta103::cat⁺</i>	
Plasmids		
pKD3	Source of FRT- <i>cat⁺</i> -FRT fragment	<i>E. coli</i> GSC†
pKD46	<i>araBp-gam-bet-exo, repA101(ts), oriR101</i>	<i>E. coli</i> GSC
pCP20	<i>ts-rep, {cI857}(lambda)(ts), Flp recombinase⁺</i>	<i>E. coli</i> GSC
pEUTD2	<i>eutD⁺</i> cloned into plasmid pBAD30	Brinsmade & Escalante-Semerena (2004)
pBAD30	Cloning vector, <i>bla⁺</i>	Guzman <i>et al.</i> (1995)

*GSC, Genetic Stock Center.

†All *S. enterica* strains used in this study were derivatives of *S. enterica* serovar Typhimurium LT2.

‡In the text, Mud1-8 is referred to as MudA.

§*Tn10d(tet⁺)* is an abbreviation of *Tn10 Δ 16 Δ 17* (Way *et al.*, 1984).

et al., 1999). In our hands, however, a *S. enterica pta* strain grew on ethanolamine as carbon and energy source at a rate similar to that measured for the wild-type strain, with both cultures reaching the same cell density (Fig. 3a, b). In addition, the kinetics of acetate excretion and recapture in the *pta* and wild-type strains was very similar (Fig. 3a, b). A strain lacking Acs enzyme activity depended on Pta to recapture excreted acetate. Unlike in the wild-type strain, acetate recapture in the *acs* strain was much slower, probably reflecting the kinetics of acetate recapture by the Pta enzyme (Fig. 3c). An *acs pta* strain excreted acetate but did not recapture any of it (Fig. 3d).

Acetate excretion during growth on ethanolamine requires Ack activity

Parallel studies with a strain carrying an in-frame deletion of the *ack* gene (lacked Ack) showed a factor-of-two increase in the doubling time of the *ack* strain (doubling time 3.9 h) relative to that of the wild-type strain (doubling time 2.1 h) (Fig. 3a vs Fig. 4). A more profound difference between the strains was observed when the acetate excretion/recapture pattern was determined. Unlike the wild-type strain, the *ack* strain excreted only a small amount of acetate (Fig. 3a vs Fig. 4), suggesting that acetate excretion proceeded via acetyl

Fig. 3. Acs and Pta activities are required for acetate excretion recapture. (a) *acs*⁺ *pta*⁺, strain TR6583; (b) *pta*, strain JE6317 (*pta103::cat*⁺). (c) Δ *acs2* *pta*⁺, strain JE7758. (d) Δ *acs2* *pta103::cat*⁺, strain JE8821. ■, Cell density; △, acetate concentration in the medium.

Fig. 4. Ack activity is required for acetate excretion. Strain JE8749 (Δ *ack103*) carrying an in-frame deletion of the *ack* gene was used for this experiment. ■, Cell density; △, acetate concentration in the medium.

phosphate (Ac-P). Thus it appears that the slower growth rate of the *ack* strain may be due to an accumulation of Ac-P.

EutD phosphotransacetylase activity is not required for growth on ethanolamine

A strain lacking the EutD phosphotransacetylase enzyme showed a slight decrease in the rate of growth and the final cell density of the culture relative to the wild-type strain (Fig. 5a). Both defects were readily corrected by the wild-type allele of *eutD* (Fig. 5a). Similar results were obtained with a *pta eutD* strain (Fig. 5b), consistent with the idea that phosphotransacetylase activity is not central to ethanolamine catabolism in *S. enterica*.

We note that the growth behaviour of an *eutD* strain on ethanolamine was in sharp contrast to that of an *eutE* strain (Fig. 5a), indicating that unlike EutD, EutE function was required for growth of *S. enterica* on ethanolamine.

Acetate excretion during growth on ethanolamine requires EutD phosphotransacetylase activity

Although the absence of EutD activity only slightly affected growth of *S. enterica* on ethanolamine, in the absence of this

Fig. 5. The EutD/Ack pathway is needed for optimal growth on ethanolamine, but it is not essential. Differences in growth rate and cell yield between wild-type and *eutD* strains were corrected by introducing a wild-type allele of *eutD* on a plasmid (pEUTD2). This plasmid carried a wild-type allele of *eutD* under the control of the arabinose-inducible promoter in plasmid pBAD30 (Guzman *et al.*, 1995); 200 μ M L(+)-arabinose was added to the medium to induce *eutD* expression. Strains used: *eut*⁺, strain TR6583; *eutE*, strain JE2236 (*eutE18::MudA*); *eutD*, strain JE7310 (Δ *eutD1143*); *eutD pta*, strain JE7437 (Δ *eutD1143 pta103::cat*⁺); *eutD/peutD*⁺, strain JE7375 [Δ *eutD1143/pEUTD2* (*bla*⁺ *P*_{araBAD}-*eutD*⁺)]; *pta eutD/peutD*⁺, strain JE7439 [Δ *eutD1143 pta03::cat*⁺/pEUTD2 (*bla*⁺ *P*_{araBAD}-*eutD*⁺)].

enzyme acetate excretion was abolished during growth on ethanolamine (Fig. 6a; open triangles). Inactivation of the *pta* gene in an *eutD* strain did not affect growth rate, final cell density, or acetate excretion (Fig. 6b). Surprisingly, inactivation of the *acs* gene restored acetate excretion in the *pta eutD* strain, but no recapture was observed (Fig. 6c), consistent with the finding that Acs and Pta activities were needed for recapture (Fig. 3d). Based on these results, and others discussed above, we propose that ethanolamine catabolism proceeds via Ac-CoA, and that acetate is excreted via the EutD/Ack pathway.

Excretion of acetate by the *acs eutD pta* strain could be explained in at least three ways. One way would involve an Ac-CoA hydrolase, a second way would involve pyruvate oxidase (PoxB), and a third way would involve an unidentified system.

We used the protein sequence of *Saccharomyces cerevisiae* Ac-CoA hydrolase (Ach1p) (Buu *et al.*, 2003) to search for genes of *Salmonella enterica* encoding homologous proteins. The search yielded open reading frame STM3118 as the only gene possibly encoding a structural orthologue of Ach1p Ac-CoA hydrolase. We constructed an in-frame deletion of STM3118 in strain JE7498 (*eutD acs pta*) and studied the growth behaviour and acetate excretion pattern in the resulting strain (JE7715). In addition to growing on ethanolamine to wild-type levels, strain JE7715 excreted as much acetate as did its parent strain JE7498, indicating that STM3118 was not involved in acetate excretion in a strain lacking Acs, EutD and Pta activities (data not shown). This result does not rule out the existence of non-orthologous replacements of Ach1p Ac-CoA hydrolase in *S. enterica*, a possibility that has not been investigated. Inactivation of either *poxB* or *ack* did not prevent acetate excretion in strains JE7832 (*poxB acs pta eutD*) or strain JE8326 (*eutD acs*

ack) (data not shown). Results obtained with the *poxB* mutant suggested that PoxB was not responsible for the observed acetate excretion in the *acs pta eutD* strain JE7832. Results obtained with strain JE8326 suggested that acetate release in such a strain did not proceed via Ac-P. At present, the identity of the acetate-releasing system operating in the *acs pta eutD* strain remains unclear.

Why is EutD, and not Pta, function required for ethanolamine catabolism?

One plausible answer to this question may lie in part in the kinetic differences between these enzymes. Although the *S. enterica* Pta enzyme (SePta) has not been isolated and characterized, its close relative from *Escherichia coli* (EcPta) has (Brinsmade & Escalante-Semerena, 2004; Shimizu *et al.*, 1969; Suzuki, 1969). The EutD enzyme has a sevenfold higher affinity for CoA than does EcPta (46 vs 320 μ M), a 23-fold higher affinity for Ac-P than does EcPta (0.13 vs 3 mM), and a 10-fold higher specific activity than that reported for EcPta (3044 vs 333). Relative to EcPta (and, by extrapolation, to SePta), EutD is a very efficient enzyme (Brinsmade & Escalante-Semerena, 2004; Shimizu *et al.*, 1969; Suzuki, 1969). The high affinity of EutD for Ac-CoA and its presumable location within the ethanolamine metabolosome is proposed to prevent a non-physiological build-up of Ac-CoA, leading to a release of CoA and acetate and the concomitant production of ATP via substrate-level phosphorylation. Acetate excretion may help the cell minimize the negative effect of intracellular acetate accumulation on the pool of glutamate and other unidentified ions (Roe *et al.*, 1998; Russell & Diez-Gonzalez, 1998). Relief from the toxic effects of acetate during growth on ethanolamine would be reached when the acetate switch is flipped towards acetate assimilation.

Fig. 6. EutD phosphotransacetylase activity is required for acetate excretion during growth on ethanolamine. Lack of Acs activity in an *eutD* strain triggers acetate excretion. (a) *eutD*, strain JE7310 (Δ *eutD*1143); (b) *eutD pta*, strain JE7437 (Δ *eutD*1143 *pta*103::*cat*⁺); (c) *eutD pta* Δ *acs*, strain JE8507 (Δ *eutD*1143 *pta*103::*cat*⁺ Δ *acs*2). ■, Cell density; △, acetate concentration in the medium.

Fig. 7. Model for ethanolamine degradation in *S. enterica*. The size of the ethanolamine metabolosome is not to scale.

Growth on ethanolamine proceeds via the glyoxylate bypass and the Krebs cycle

A strain lacking isocitrate dehydrogenase [JE4561 *icd1::Tn10d(tet⁺)*] did not grow on ethanolamine as the carbon and energy source on medium supplemented with glutamate, indicating that catabolism of ethanolamine proceeds via the tricarboxylic acid (Krebs) cycle. Similarly, a strain lacking malate synthase (AceB) and isocitrate lyase (AceA) activities (strain JE4173) failed to grow on ethanolamine, indicating that the glyoxylate bypass is also required for the utilization of ethanolamine as the carbon and energy source.

A working model

Fig. 7 summarizes our interpretation of the data reported in this paper. We postulate that in wild-type *S. enterica*, a portion of the ethanolamine-derived pool of Ac-CoA is converted to acetate and excreted into the medium for later use, with the remaining Ac-CoA entering central metabolism. Acetate excretion may be needed to regenerate free CoA. As the Acs level increases, exogenous acetate is recaptured by Acs and Ack/Pta and is catabolized via the TCA

cycle and glyoxylate bypass, increasing the amount of carbon and energy available for growth. Elegant studies of *acs* expression indicate that the Acs level is very low in low-density cultures (Browning *et al.*, 2004), a fact that would explain the kinetics of acetate accumulation and recapture from the environment. The very efficient EutD phosphotransacetylase, in conjunction with acetate kinase, regenerates free CoA, a process that appears to also involve an as-yet-unidentified acetate-releasing system. It is an enigma why in the absence of EutD phosphotransacetylase the house-keeping Pta phosphotransacetylase does not compensate for EutD. We propose that the absence of EutD may trigger the diversion of acetaldehyde to ethanol, avoiding the accumulation of Ac-CoA. This and other ideas are under investigation.

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