A *Bacillus subtilis* secreted phosphodiesterase/alkaline phosphatase is the product of a Pho regulon gene, *phoD*

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A secreted phosphodiesterase/alkaline phosphatase, APaseD, was purified from a culture of *Bacillus subtilis* JH646MS. Its phosphodiesterase activity was reminiscent of an APase isolated and characterized previously. Immunoassay and N-terminal sequencing showed the two proteins to be identical. Using the first 20 amino acids of the mature protein, a BLAST search of GenBank was used to find an homologous sequence. An exact match was found but in a putative non-coding region. It was hypothesized that there was a base pair deletion in the *phoD* gene. A DNA fragment internal to the coding region was generated by PCR using template DNA from a strain which produced APaseD. The PCR fragment was cloned and used to interrupt the gene. Western blot analysis of the parent and the mutated strains showed that APaseD was missing in the mutant. Resequencing of the gene revealed a larger ORF encoding a protein similar in size to the 49 kDa APaseD estimated by SDS-PAGE. The promoter was then cloned, sequenced and used in *phoD-lacZ* promoter fusions which showed that the gene was phosphate-starvation-induced and dependent on PhoP and PhoR for expression.

Keywords: *Bacillus subtilis*, Pho regulon, phosphodiesterase

INTRODUCTION

Nutrient depletion is a stress frequently encountered by *Bacillus subtilis* in its natural environment, the soil. At the end of exponential growth, when nutrients become limiting, formerly inactive genes are induced which may help with the survival response. Such responses include antibiotic production (Marahiel *et al.*, 1987), development of competence (Albano *et al.*, 1987; Dubnau, 1991), degradative enzyme production (Ferrari *et al.*, 1986, 1988; Msadek *et al.*, 1993), flagellar synthesis (Gilman & Chamberlin, 1993), changes in respiratory/energy production pathways (Sun *et al.*, 1996), and the formation of a dormant endospore (Burbulys *et al.*, 1991). If the depleted nutrients are replenished before the commitment to sporulation is made then the cell may resume vegetative growth.

B. subtilis has an alkaline phosphatase (APase) gene family which is silent during vegetative growth but is turned on when phosphate starvation conditions are encountered

(Bookstein *et al.*, 1990; Hulett, 1993, 1996; Hulett *et al.*, 1990, 1991, 1994a, b). Limiting phosphate concentrations were determined to be 0.08 mM (Hulett & Jensen, 1988). The genes induced in response to phosphate deprivation include two regulator proteins and at least four APases. The proteins PhoP and PhoR form a two-component signal-transduction system which activates the APase genes. PhoR is a histidine kinase and PhoP is a response regulator which is phosphorylated by PhoR and is thought to activate the Pho regulon genes (Hulett, 1993, 1996; Hulett *et al.*, 1994a, b). Of the APase genes studied, *phoA* and *phoB* have been best characterized (Hulett *et al.*, 1990, 1991; Bookstein *et al.*, 1990). Together the products of these genes are responsible for 98% of the alkaline phosphatase activity during the Pho response (Hulett *et al.*, 1994b). However, other APase proteins have been isolated and may contribute to the phosphate starvation response.

Yamane & Maruo (1978a) isolated a protein, BC6, from the lysed cell pellet of *B. subtilis* strain 6060-BC6 (Table 1), which carries a mutation causing constitutive APase expression. A second protein, RAN1, was isolated from the culture fluid of a *B. subtilis* strain 6060-BC6 mutant, Ran1, which secreted APase. Antibody to RAN1 cross-

Abbreviation: APase, alkaline phosphatase.

The GenBank accession number for the sequence reported in this paper is U49060.

reacted with BC6, showing that they were likely to be the same protein. Further experiments by Yamane & Maruo (1978b) showed that the approximately 46 kDa BC6 had phosphodiesterase and APase activity in a ratio of 1.4:1.0. In our efforts to study the APase gene family in *B. subtilis* we were interested in characterizing BC6 further, especially its possible relationship to the Pho regulon.

An immunoassay study suggested that BC6 was the same protein as APaseD, an APase we purified from JH646MS (Hulett *et al.*, 1990). N-terminal sequencing of the first 20 amino acids of both proteins confirmed this. In this study we have identified the gene for APaseD and demonstrated that APaseD is a member of the Pho regulon.

METHODS

Bacterial strains and plasmids. These are listed in Table 1. Plasmids used in this study were constructed as follows.

pSE1. PCR was used to amplify the 389 bp promoter region of *phoD* from JH642 template DNA. The primers used for PCR were FMH208 (TTGAATTC¹⁶⁷⁴⁰GTAAGAGACAAGA-GCCTCT¹⁶⁷⁴⁰) and FMH209 (TTGGATCC¹⁷¹¹²ACGACTG-TCGTATGCCATTC¹⁶⁰⁹²). The numbering on the primers is based on the numbering in the sequence submitted to GenBank with accession number D30808 (Ogawa *et al.*, 1995). FMH208 contains an *EcoRI* site at its 5' end and FMH209 contains a *BamHI* site at its 5' end. The amplified DNA was then ligated to the pCRII vector provided in the TA cloning kit (Invitrogen).

pSE2. PCR was used to amplify a 292 bp fragment from the start of the 5' coding region of *phoD*. The template DNA was from JH642. The primers used for PCR were FMH210 (¹⁷⁰⁷⁷GAGAGAGGGGATCTTGAATG¹⁷¹⁰¹) and FMH211

(¹⁷³⁶⁹CCATTTCAGCGGATCTGGTG¹⁷³⁵⁰). The amplified DNA was then ligated to the pCRII vector from Invitrogen.

pSE3. Restriction digestion of pSE1 with *EcoRI* and *BamHI* released the 389 bp promoter region. This fragment was cloned into the *BamHI/EcoRI* sites of pDH32 to create pSE3, a *phoD-lacZ* promoter fusion for use in *lacZ* reporter gene assays.

pSE4. Restriction digestion of pSE2 with *EcoRI* released the 292 bp 5' coding region of *phoD*. This fragment was cloned into the *EcoRI* site of pJM103 to create pSE4. pSE4 was then used to create a mutation in the APaseD gene by Campbell-type integration of the plasmid.

General methods. Transformation of *E. coli* was done according to the method of Hanahan (1985). Transformants were selected for drug resistance and colour on LB plates containing 150 µg penicillin ml⁻¹ and 120 µg X-Gal ml⁻¹. Transformation of *B. subtilis* was done by the two-step transformation method of Cutting & Vander Horn (1990). Transformants were selected for drug resistance on Tryptose Blood Agar Base (Difco) plates containing 5 µg chloramphenicol ml⁻¹.

Media, growth conditions and enzyme assays. For phosphate starvation induction of APases and the *phoD* promoter fusions, the cells were cultured in low-phosphate defined medium (LPDM) as described by Hulett *et al.* (1990). APase activity was determined as previously described by Hulett *et al.* (1990); the units are µmol *p*-nitrophenol formed min⁻¹ at 37 °C. Specific activity was calculated by dividing the APase activity as assayed (units ml⁻¹) by the OD₅₄₀ of the culture (determined with a Hitachi U-2000 spectrophotometer). β -Galactosidase specific activity was determined according to Ferrari *et al.* (1986), and was expressed in units (mg protein)⁻¹.

Protein purification and N-terminal sequencing. The proteins BC6 and RAN1, from the APase constitutive strain 6060-BC6, were isolated by Yamane & Maruo (1978a). APaseD was

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or antibiotic resistance	Source
<i>B. subtilis</i> strains		
6060-BC6	<i>trpB3 metB5</i> (APase constitutive)	Marburg
JH644MS	<i>pheA1 trpC2 spo0A12 abrB15</i>	J. A. Hoch
JH642	<i>pheA1 trpC2</i>	J. A. Hoch
MH5117	<i>pheA1 trpC2 phoPA EcoRI mdh::Tet^r</i>	Hulett <i>et al.</i> (1994)
MH5124	<i>pheA1 trpC2 phoRA BalI::Tet^r</i>	Hulett <i>et al.</i> (1994)
MH5441	<i>pheA1 trpC2 amyE::phoD-lacZ Cm^r</i>	This study
MH5442	<i>pheA1 trpC2 phoPA EcoRI Tet^r amyE::phoD-lacZ Cm^r</i>	This study
MH5443	<i>pheA1 trpC2 phoRA BalI mdh::Tet^r amyE::phoD-lacZ Cm^r</i>	This study
MH5444	<i>pheA1 trpC2 phoDΩpSE4 Cm^r</i>	This study
<i>E. coli</i> strains		
DH5α	F ⁻ ϕ 80 <i>lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r_k⁻ m_k⁺)supE44 λ⁻ thi-1 gyrA relA1</i>	Lab. strain
Plasmids		
pCRII (3.9 kb)	Ap ^r Km ^r	Invitrogen
pDH32 (9.9 kb)	Ap ^r Cm ^r	D. J. Henner
pJM103 (3.7 kb)	Ap ^r Cm ^r	J. A. Hoch
pSE1 (4.3 kb)	Ap ^r Cm ^r	This study
pSE2 (4.2 kb)	Ap ^r Cm ^r	This study
pSE3 (10.3 kb)	Ap ^r Cm ^r	This study
pSE4 (4.0 kb)	Ap ^r Cm ^r	This study

isolated by the method of Hulett *et al.* (1990). Preparation of all three proteins for N-terminal sequencing was done as described by Matsudaira (1987). The only differences were that the gel was 7.5% (w/v) acrylamide and the staining was carried out for only 30 s. Each protein was sequenced at the Harvard Micro-Chemistry facility on a 470A protein sequencer equipped with a 120A on-line PTH-AA analyser (Applied Biosystems).

Western blot analysis. MH5444, MH5117 and JH642 (Table 1) were used to inoculate 1.5 ml of LB medium and the cultures were incubated at 37 °C for 3 h. Thirty microlitres of each LB culture was then used to inoculate 1.5 ml of LPDM. The LPDM cultures were incubated at 37 °C with vigorous aeration for 18 h. The amount of growth was recorded by OD₅₂₅ measurement. Using the formula OD₅₂₅ × 83 = µg protein ml⁻¹ culture, specific volumes of each sample were taken containing 50 µg or 100 µg of protein. The Western blot was then performed as described by Hulett *et al.* (1990). The primary antibody used was RAN1 anti-APase, and the secondary antibody was APase-conjugated goat anti-rabbit IgG (Bio-Rad).

Nucleotide sequencing. pSE1 and pSE2 were sequenced directly by using Sequenase and universal primers (United States Biochemical) according to the instructions of the manufacturer.

Computer searches. A BLAST search of GenBank was used to explore the *B. subtilis* genome for a match using the first 20 amino acids from APaseD as the search probe. The modified sequence was submitted to GenBank and has the accession number U49060.

RESULTS

N-terminal sequencing of BC6 and APaseD

An immunoassay using the RAN1 anti-APase antibody (Yamane & Maruo, 1978b) showed cross-reactivity between BC6, RAN1, and APaseD, suggesting that they are the same protein (data not shown). APaseD, RAN1 and BC6 were subjected to SDS-PAGE and blotted onto Imobilon membranes, from which the N-terminal sequence of each protein was determined. Nineteen of the first 20 amino acids from the mature APaseD protein were found to be identical to the mature BC6 protein isolated by Yamane & Maruo (1978a) (Fig. 1). The third amino acid in BC6 is a lysine, whereas in APaseD an asparagine was identified as the third amino acid sequenced. This asparagine/lysine discrepancy at the third amino acid can be accounted for by a single base substitution and could be due to the fact that the proteins were isolated from different strains. N-terminal sequencing of RAN1 showed that the mature RAN1 protein lacked at least five N-terminal amino acids found in APaseD and BC6. A ragged amino terminus in a concentration ratio of 8:4:2:1 was determined for RAN1 species lacking 5, 6, 7 or 8 of the N-terminal amino acids found in APaseD and BC6 respectively.

The N-terminal 20 amino acids of APaseD were then used to perform the BLAST sequence similarity search in the GenBank database using the program tblastn (Altschul *et al.*, 1990). A single match with 100% identity to the search query was identified as GenBank entry D30808 and was located on the 24° region of the *B. subtilis* chromosome (Ogawa *et al.*, 1995). However, the DNA which

would encode the amino acid sequence was within a non-coding region downstream of ORF17 and 5' of ORF18, a hypothetical gene. We hypothesized that a 1 bp deletion had disrupted the gene encoding APaseD in the organism used for the Japanese *Bacillus* genome sequencing project (Ogasawara *et al.*, 1995).

Sequencing the putative APaseD ORF

To explore the above hypothesis, we generated a PCR product from chromosomal DNA of JH642, a strain from which we had isolated APaseD. The PCR fragment included the short upstream coding region and extended 3' of the sequence encoding the N terminus of the APaseD protein (Fig. 1). Sequencing of the PCR product revealed an adenine missing at base pair 17207 (GenBank accession number D30808). Insertion of the deleted adenine revealed a new reading frame (Fig. 1). The newly discovered ORF connected the short upstream ORF, the DNA encoding the APaseD N terminus, and the entire second ORF. The 5' region of the ORF encoded 56 amino acids not found at the N terminus in the mature protein, suggesting that the preprotein of APaseD is a 62825 Da protein. This peptide contained eight basic amino acids followed by a hydrophobic region and is cleaved from the preprotein after an alanine residue, which is characteristic of Gram-positive signal sequences (Von Heijne *et al.*, 1989; Nagarajan, 1993). However, the positively charged domain is longer than in other signal peptides, making the prepeptide exceptionally long. The 56 kDa mature protein was similar in molecular mass to that of APaseD as estimated using SDS-PAGE by Hulett (1993) (49 kDa) and Yamane & Maruo (1978a) (46 kDa) and had an isoelectric point of 8.6.

In order to determine if this larger ORF actually encoded APaseD, we created a mutation in its 5' coding region. The 292 bp fragment used to sequence the 5' coding region of the new ORF was ligated into the PCRII vector (Invitrogen) and then the insert was subcloned into pJM103 at its *EcoRI* site using the *EcoRI* sites flanking the insertion in the pCRII vector. The resulting plasmid, pSE4, was transformed into *B. subtilis* strain JH642 and chloramphenicol resistance was selected for. Due to the sequence homology between the plasmid and the *phoD* region of the chromosome, it was forced to integrate into the *phoD* locus through Campbell-type recombination, thereby creating two incomplete copies of the putative *phoD* gene. The putative *phoD* mutant (MH5444), the *phoP* mutant (MH5117) and the parent strain (JH642) (Table 1) were then grown in low-phosphate defined medium (Hulett *et al.*, 1990) in order to induce the expression of *phoD*. Fig. 2 shows the results of the Western blot performed on the crude cell lysates of these strains following phosphate starvation induction. The parent strain showed RAN1 antibody cross-reaction with protein. The *phoD* and *phoP* mutant strains did not. These results suggested two things: firstly, that the APaseD protein was encoded in the proposed reading frame; and secondly, since no protein from the *phoP* mutant strain cross-reacted with RAN1, that PhoP is necessary for *phoD*

EcoRI															
GTAAGAGAACAAGAGCCTCTCTGCTGAAAAGCCGAAAAAGAAGGGCTTTTTCAGCAGATTGTTTTCGAAATAACTGAT															16817
AAAGAGCACTGAGTCATTCTGCGAAATGGCTCGGTGTTTTGCTTCTTTTAAAGATCATGTGAAGAAAAATAGGATGG															16895
GCGGCTTGCTTGCAAACGACAGGATATGCAGAAAACGCGTCATTACGCCAGCTTTCGGCATGTTTCAGCAGCAGGCT															16972
TGTGAGGGAACCGATTTTAATGTTTCAGCGCTTACAATCAGTTCACACTTCTTACAGTCGTTTAAACAATGATTCCAT															17051
AATGGAGACGATCAATGAGACCTGT															17076

Gene	Product	Promoter region
		-22 -10
<i>phoA</i>	APaseA	<u>TTAACAAAGTTTCCTTAACAT</u>
<i>phoB</i>	APaseB	<u>TTAACAGTTTAAAAATATAAT</u>
<i>phoD</i>	APaseD	<u>TTAACAATGATTTCCTATAAT</u>
<i>tuaA</i>	Teichuronic acid biosynthesis	<u>TTAACATCCGTCTGCTAAACT</u>

Fig. 3. Promoters of a number of Pho regulon genes have several common features. The -10 region has a consensus similar to sigma A promoters. There is either a poorly conserved sigma A sequence at -35 or none at all. However, 9 bp upstream of the -10 region (-22) there is a conserved TTAACA as shown here.

APaseD is Pho regulated

In order to confirm that APaseD expression is Pho regulated, we amplified by PCR a 398 bp fragment which contained the promoter region of *phoD*. This fragment was then cloned (pSE1), sequenced and used to make *phoD-lacZ* promoter fusions for expression studies. The sequence agreed with the NCBI database, which showed that 9 bp upstream of the putative -10 (TATAAT) was the sequence TTAACA which has been observed in the promoter regions of *phoA*, *phoB* and *tuaA* (Hulett *et al.*, 1994a), all of which have been characterized as Pho-regulated genes (Fig. 3). *phoD* promoter expression studies were then undertaken in order to conclusively determine whether PhoP controls *phoD*. For this purpose the promoter fragment was subcloned from pSE1 into the integration vector pDH32. The resulting plasmid, pSE3, was linearized at the *Pst*I site and used to transform the wild-type *B. subtilis* strain (JH642), the *phoP* mutant strain (MH5117) and the *phoR* mutant strain (MH5124), (Table 1). A single copy of the linearized DNA was integrated

into the chromosomes at the *amyE* locus. The transformants were selected for chloramphenicol resistance and screened for the loss of α -amylase synthesis on starch plates. Each strain was then grown in low-phosphate defined medium (Hulett *et al.*, 1990), which is designed to maximize vegetative APase production in *B. subtilis*. Samples were taken every hour for 12 h and assayed for APase and β -galactosidase activity. Fig. 4 shows the results of these assays. Neither enzyme was expressed in any strain during the first 6 h while phosphate concentrations were above 0.1 mM. MH5441, the wild-type strain with the *phoD-lacZ* fusion, did show some β -galactosidase and APase activity initially because the cells had been grown overnight under phosphate-limiting conditions before being transferred to fresh LPDM at the beginning of the assays. During exponential growth the specific activity of these enzymes decreased as culture density increased, without additional enzyme synthesis. When the strains entered the stationary phase because of phosphate depletion, only the wild-type strain, MH5441, produced β -galactosidase and APases. Neither the *phoP* mutant strain (MH5442) nor the *phoR* mutant strain (MH5443) expressed activity. From these data we conclude that the *phoD* gene is Pho regulated, requiring both PhoP and PhoR for its induction.

DISCUSSION

The natural depletion of nutrients from the soil leads to the activation of *B. subtilis* genes directed at overcoming the deficiency (Msadek *et al.*, 1993). Phosphate limitation leads to the activation of Pho regulon genes (Hulett, 1993, 1996; Hulett *et al.*, 1990, 1991, 1994a, b; Bookstein *et al.*, 1990). APaseA and APaseB are the two major Pho-regulated alkaline phosphatases produced under these conditions, and together they account for 98% of the

Fig. 4. The *phoD* promoter is phosphate starvation induced. Strains were grown in LPDM according to Hulett *et al.* (1990). Growth is represented by the filled symbols in (a) and (b). (a) β -Galactosidase specific activity [units (mg protein)⁻¹] is represented by the open symbols. (b) APase specific activity (units ml⁻¹ per OD₅₄₀ unit) is represented by the open symbols. The strains are: ■, □, MH5444 (*amyE::phoD-lacZ*); ◆, ◇, MH5442 (*phoP*Δ, *amyE::phoD-lacZ*); and ●, ○, MH5443 (*phoR*Δ, *amyE::phoD-lacZ*).

alkaline phosphatase specific activity expressed (Hulett *et al.*, 1994b). However, APases other than APaseA and APaseB are also induced during the phosphate starvation response and their role must not be discounted. Yamane & Maruo (1978a) had previously isolated two proteins, BC6 and RAN1, which had both APase and phosphodiesterase activity. Antibody was made against RAN1 and an immunoassay of RAN1 and BC6 showed cross-reactivity, which suggested that the two isolated proteins were the same although RAN1 was secreted and BC6 was cell bound.

N-terminal sequencing of APaseD, RAN1 and BC6 revealed that they were in fact the same protein. Further analysis showed a single amino acid difference (asparagine to lysine) among the first 20 amino acids of APaseD and BC6, which could result from a strain difference affecting a single base pair. Sequence analysis of the secreted protein, RAN1, revealed a ragged N terminus, indicating that the protein preparation analysed contained protein species lacking between five and eight amino acids found at the N terminus of mature APaseD or BC6. These data suggest the mutation in the *B. subtilis* RAN1 strain results in proteolysis of APaseD that allows secretion of the protein into the medium. Although these data reveal no information concerning the mechanism for the cell association of APaseD or BC6, they do imply that one or more of the five N-terminal amino acids of the mature APaseD is required for cell association.

The N-terminal sequences allowed us to identify the gene encoding APaseD. DNA 5' of the coding region for the mature enzyme indicated that APaseD is translated as a preprotein having 56 additional amino acids which share certain characteristics of secretory signal peptides. The uncharged residues in position -3 and -1 relative to the cleavage site and the central hydrophobic region are typical of other signal peptides (Von Heijne & Abrahmsen, 1989; Nagarajan, 1993) but the charged N terminus is exceptionally long and highly charged. This N-terminal domain is longer than most complete signal sequences, which are about 35 amino acids long. This raises the question of whether the translocation and maturation of APaseD might not involve a novel process. Certainly, whole cell lysates of phosphate-starved cells contain several cross-reacting proteins of discrete sizes. This is reminiscent of the multiple processed lengths of *Bacillus licheniformis* 749C penicillinase (Lampen *et al.*, 1984) and the subtilisin pre-pro-protein maturation (Vasanthan *et al.*, 1984). It leads us to believe that these cross-reacting proteins are processed forms of APaseD; this hypothesis is supported by the fact that the cross-reacting species are all absent in the *phoD* mutant.

Two lines of evidence suggested that *phoD* was a Pho regulon gene. First, we isolated it from phosphate-starved cells. Second, we noted that the promoter region contained a conserved 6 bp sequence (TTAACA), located 9 bp 5' of a putative -10 consensus (TATAAT) that is found in the promoters of three Pho regulon genes, *phoA*, *phoB* and *tuaA*. Assignment of *phoD* to the growing list of Pho regulon genes is justified based on: (1) the induction of gene expression during phosphate starvation of the

culture; (2) the absence of APaseD protein in a *phoP* mutant; and (3) the absence of transcription of *phoD* in either a *phoP* or a *phoR* mutant.

The amino acid sequence of APaseD showed no similarity to any protein in the databases available and is quite different from that of other APases. APases previously cloned and sequenced are conserved in their active site and core structure (Hulett *et al.*, 1991). APaseD is a basic protein, as are APaseA and APaseB of *B. subtilis*, but there is no cross-reaction between APaseA and APaseB with the RAN1 antibody using the purified protein (unpublished data) or crude whole-cell lysates of *phoD* mutants which contain APaseA and APaseB (Fig. 2). Also, antibodies to APaseA or APaseB do not cross-react with APaseD (unpublished data).

The role of APaseD in the Pho response may now be explored using the *phoD* mutant. Earlier data (Yamane & Maruo, 1978a) and data presented here suggest that APaseD is a secreted but cell-associated protein that has phosphodiesterase and APase activity. We propose that the positively charged APaseD may be associated with the negatively charged cell wall. In this negatively charged wall there are anionic polymers, teichoic acids, which contain many phosphodiester bonds (Archibald *et al.*, 1993). When *B. subtilis* is grown in low-phosphate media teichoic acids are replaced by teichuronic acids, which do not contain phosphate (Grant, 1979; Mauël *et al.*, 1991). Teichuronic acid synthesis is Pho regulated (Soldo *et al.*, 1995). Thus, we hypothesize that the Pho-regulon-induced APaseD may function to cleave the phosphodiester bonds of teichoic acids while teichuronic acid biosynthesis genes are induced to replace the teichoic acid polymers with teichuronic acids. The product of the diesterase reaction may provide the substrate for a phosphomonoesterase (APaseD, APaseA or APaseB) resulting in the release of inorganic phosphate for cell growth. However, it is likely to be more complicated since just as there are multiple phosphomonoesterases in *B. subtilis*, there are surely multiple secreted proteins with diesterase activity (Le Hegarat & Anagnostopoulos, 1973) and more specifically with teichoicase activity (Kusser & Fiedler, 1982, 1983).

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