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Secretion of M2e:HBc fusion protein by Lactobacillus casei using Cwh signal peptide

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One sentence summary: The novel Cwh signal peptide selected from the Lactobacillus casei genome by using a bioinformatics approach was successfully used in the expression–secretion vector for heterologous protein secretion.

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ABSTRACT

The ability to serve as a delivery vehicle for various interesting biomolecules makes lactic acid bacteria (LAB) very useful in several applications. In the medical field, recombinant LAB expressing pathogenic antigens at different cellular locations have been used to elicit both mucosal and systemic immune responses. Expression–secretion vectors (ESVs) with a signal peptide (SP) are pivotal for protein expression and secretion. In this study, the genome sequence of Lactobacillus casei ATCC334 was explored for new SPs using bioinformatics tools. Three new SPs of the proteins Cwh, SurA and SP6565 were identified and used to construct an ESV based on our Escherichia coli–L. casei shuttle vector, pRCEID-LC13.9. Functional testing of these constructs with the green fluorescence protein (GFP) gene showed that they could secrete the GFP. The construct with CwhSP showed the highest GFP secretion. Consequently, CwhSP was selected to develop an ESV construct carrying a synthetic gene encoding the extracellular domain of the matrix 2 protein fused with the hepatitis B core antigen (M2e:HBc). This ESV was shown to efficiently express and secrete the M2e:HBc fusion protein. The identified SPs and the developed ESVs can be exploited for expression and secretion of homologous and heterologous proteins in L. casei.

Keywords: Lactic acid bacteria; Lactobacillus casei; expression and secretion vector; signal peptide

INTRODUCTION

Lactic acid bacteria (LAB) are a heterogeneous group of bacteria that are used in various biotechnological applications. With respect to human and animal health, many LAB are being used as mucosal delivery vehicles for therapeutic and prophylactic molecules because of their GRAS (Generally Regarded As Safe) status (Eijsink et al. 2002), their probiotic properties and the ease with which they can be engineered to express heterologous proteins (Ouwehand et al. 2002) to elicit an effective immune response (Lee et al. 2006). Up to now, a definite conclusion on the best cellular location for optimal immunization, i.e. intracellular, cell wall anchored or secreted, has yet to be reported. This seems to depend on parameters such as the bacterial type, the amount and localization of the antigen, the route of
administration, etc. (Wells and Mercenier 2008). Many expression and secretion systems for LAB to deliver protective antigens have already been developed (Kruger et al. 2002; Pusch et al. 2005). Such systems require the use of expression–secretion vectors (ESVs) with a signal peptide (SP) to translocate the protein after synthesis across the bacterial cell membrane. Several SPs have been identified and incorporated into ESVs (Hols et al. 1997; Hazebrouck, Pothelune and Azevedo 2007). The secretion efficiency of different SPs seems to be host specific. For this reason, homologous SPs are thought to drive protein secretion more efficiently than heterologous SPs (Mathiesen et al. 2008). The availability of the complete genome sequences of many Lactobacillus casei strains (Makarova et al. 2006) makes it easy to search for new and efficient SPs that can be used in the construction of new ESVs for this species and maybe other lactobacilli.

In this study, putative SPs were selected from the genome of L. casei ATCC334 (accession no. NC000423.1) using bioinformatics analysis. The selected SPs were tested for the secretion of a gene product encoding a green fluorescent protein (GFP) as a reporter protein and using the ESV based on Escherichia coli–L. casei shuttle vectors previously developed in our group (Panya et al. 2012; Suebwongsa et al. 2013). The SP with the highest efficiency was then used to drive the secretion of a synthetic gene coding for an M2e:HBc fusion protein. M2e is a highly conserved protein of the influenza A virus that has been incorporated as an antigen into many influenza vaccine formulations. The immunogenicity of M2e has been reported to be enhanced when fused with the hepatitis B core antigen (HBc) (De Filette et al. 2005). The final construct, pLC-Cwh:M2e:HBc, successfully expressed and secreted the fused antigenic protein.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains, plasmid vectors and oligonucleotide primers used in this study are listed in Table 1. Lactobacillus casei strains were grown statically in de Man Rogosa and Sharpe (MRS) medium (LabM, Heywood, Lancashire, UK) at 37 °C. Escherichia coli DH5α was cultured in Luria–Bertani (LB) broth (LabM) at 37 °C with shaking. Agarified media were prepared by adding 15 g l−1 bacteriological agar to the corresponding broth. When required, appropriate antibiotics (Sigma-Aldrich, St Louis, MO, USA) were added to the media as follows: erythromycin for L. casei at 2.5 μg ml−1, and ampicillin for E. coli at 100 μg ml−1.

DNA manipulation

All DNA manipulation procedures were performed as described by Sambrook and Russell (2001). Plasmids from E. coli were isolated and purified using the HiYield plasmid mini Kit (RBC Bioscience, New Taipei City, Taiwan). Plasmids from L. casei were extracted as described by O’Sullivan and Klænhammer (1993). Genomic DNA from L. casei was extracted using the GenElute Bacterial Genomic DNA kit (Sigma–Aldrich). PCR amplification was performed using Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Amplicons were purified from agarose gels using the HiYield Gel PCR DNA Fragments Extraction kit (RBC Bioscience).

Analysis of candidate signal peptides

The nucleotide sequences of cell surface-associated proteins of L. casei ATCC334 were retrieved from the NCBI microbial genomes database (http://www.ncbi.nlm.nih.gov/genomes/1proks.cgi). The SignalP4.1 program (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the putative signal peptides. The likely location of the proteins was predicted with the programs PSORTB v 3.0 (http://www.psort.org/psortb/), SubLoc v 1.0 (http://www.bioinfo.tsinghua.edu.cn/SubLoc/pro-predict.htm) and Gpos-mPloc (http://www.csbio.sjtu.edu.cn/bioinf/Gpos-multi/). DNA sequences encoding SPs of cell surface-associated proteins with a clear SP cleavage site and an extracellular location were selected for the construction of ESVs.

Construction of an expression–secretion vector (ESV) for GFP

To determine whether the three selected SPs (i.e. CwhSP, SurASP and SP6565) could drive heterologous protein secretion in L. casei, an ESV based on each of these was constructed. SP strength was analyzed using the GFPuv–encoding gene as a reporter. Sequences encoding the three SPs were amplified from genomic DNA of L. casei ATCC334 under the following conditions: one cycle of 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 14 s; and 72 °C for 3 min. The lengths of the amplicons, CwhSP, SurASP and SP6565, were 129 bp, 105 bp and 126 bp, respectively. The gfpuv gene fragment, recovered as an KpnI/Spel-digested fragment from pGFPuv, was cloned into pCwh, pSurA and pSP6565 to generate pCwhSP-GFPuv, pSurSP-GFPuv and pSP6565-GFPuv, respectively. The Cwh-GFPuv fusion gene was obtained by double digestion of the pCwh-GFPuv with HindIII/Spel and cloned into HindIII/Spel-digested pLdh:Fro1, a vector containing the constitutive promoter (Pldh) and ribosome binding site (RBSsd) of the lactate dehydrogenase gene (GenBank accession no. D12591.1), resulting in pLdh:Cwh-GFPuv. Using the same procedure, pLdh:Sur-GFPuv and pLdh:SP6565-GFPuv were also obtained. The Ldh:Cwh-GFPuv DNA fragment obtained as a AatII/Spel-digested pLdh:Cwh-GFPuv was cloned into AatII/Spel-digested pRCEID-LC13.9 resulting in pLc-Cwh-GFPuv. The same strategy was followed to obtain the recombinant ESVs containing GFPuv with SurSP and RBSsd of the lactate dehydrogenase gene (GenBank accession no. D12591.1), resulting in pLdh:Sur-GFPuv and pLdh:SP6565-GFPuv. All three recombinant plasmids were verified by DNA sequencing (data not shown) and were independently electrotransformed into L. casei RCEID02. The preparation of competent cells and the electroporation protocol were as described elsewhere (Chassy and Flickinger 1987).

Determination of the secreted GFP by measurement of fluorescence intensity

To determine the secretion efficiency, the fluorescence intensity of culture supernatants derived from recombinant L. casei RCEID02 harboring pLC-Cwh:GFP, pLC-Sur:GFP and pLC-Sp6565:GFP was determined in triplicate. Cultures of the above three recombinant constructs were incubated at 37 °C for 20 h with shaking at 200 rpm until an optical density of 3.0 at 600 nm (OD600) was reached. Supernatants of the cultures were harvested and the fluorescence intensity was measured with a fluorometer (Cary Eclipse, Victoria, Australia), as previously described (Wu and Chung 2006). As a negative control, the culture supernatant of L. casei RCEID02 harboring the expression vector without signal peptide (pLC-GFPuv) was used.
Table 1. Bacterial strains, plasmid vectors and oligonucleotide primers used in this study.

| Materials | Relevant characteristics | Source or reference |
|-----------|--------------------------|---------------------|
| **Bacterial strains** | | |
| E. coli DH5α | Transformation host | Taylor, Walker and McInnes et al. (1993) |
| L. casei RCEID02 | Plasmid-free strain of L.casei TISTR1341 and protein expression host | RCEID |
| L. casei ATCC334 | Source of signal peptide-encoding genes | ATCC |
| **Plasmids** | | |
| pGEM-T Easy vector | Ap<sup>r</sup>, M<sub>13</sub> ori, T-overhang cloning vector | Promega |
| pGFPuv | Ap<sup>r</sup>, plasmid containing the GFPuv gene from *Aequorea victoria* | Clontech |
| pCwh | Ap<sup>r</sup>, M<sub>13</sub> ori, pGEM-T-derived vector containing a promoter-less part of the cwh gene (cwhSP) encoding the Cwh signal peptide (SP) | This study |
| pSur | Ap<sup>r</sup>, M<sub>13</sub> ori, pGEM-T-derived vector containing a promoter-less part of the SurSP gene encoding SurSP signal peptide | This study |
| pSP6565 | Ap<sup>r</sup>, M<sub>13</sub> ori, pGEM-T-derived vector containing a promoter-less part of the SP6565 gene encoding SP6565 signal peptide | This study |
| pCwh:GFPUv | Ap<sup>r</sup>, M<sub>13</sub> ori, pGEM-T-derived vector containing a promoter-less part of the cwh gene encoding Cwh:GFPUv fusion | This study |
| pSur:GFPUv | Ap<sup>r</sup>, M<sub>13</sub> ori, pGEM-T-derived vector containing a promoter-less part of the Sur gene encoding Sur:GFPUv fusion | This study |
| pSP6565:GFPUv | Ap<sup>r</sup>, M<sub>13</sub> ori, pGEM-T-derived vector containing a promoter-less part of the SP6565 gene encoding SP6565:GFPUv fusion | This study |
| pLdh-Pro1 | Ap<sup>r</sup>, M<sub>13</sub> ori, pGEM-T-derived vector containing the promoter of the lactate dehydrogenase (Ldh) gene and transcription terminator (TT) | This study |
| **Oligonucleotide primers** | | |
| CwhSP-F | ACGTAAGCTT CATGGTAGATGCAAAGAA (Hind III) Signal peptidase (SP) region | |
| CwhSP-R | ATTAGGTACC GCACCCGATTTGTAATTG (Kpn I) | |
| SurASP-F | GGGCAAGCTT AATGAAATTCAATAAAGT (Hind III) Surface antigen encoding SurASP | |
| SurASP-R | ATTTGGTACC GCGATACTGTCGCTTGTA (Kpn I) | |
| SP6565-F | ACGTAAGCTTATGAAGCATTTGAGACG (Hind III) Surface protein 6565 encoding gene | |
| SP6565-R | GACAGGTACC ACCACGTACCTTTTTGT (Kpn I) | |
| Cwh-ex-F | GCCCGGGCCCCGGGATGATGAGATGCAAA (Xmal) | Signal peptidase (SP) region of the cwh gene (YP.805583.1) |
| Cwh-ex-R | AAGGCGGGCCCCGGCAGCGCGATTT (Xmal) | |
| M13 (-40) forward | GTTTCCCCAGCTCAGGAC | Primer for sequencing of cloned fragments in TA cloning vectors |
| M13 (-48) reverse | AGGGATAACAAATTTCCACAGAGA | |
| Ldh:M2e:Hbc-F | GGGAAAATAGGGCGACAGGAAATGTTG | Ldh promoter and M2e:Hbc |
| Ldh:M2e:Hbc-R | TGGTTTCTGGCAAGGTGCAAGATTG | |
| M2e:Hbc:TT-F | TCAGTTGAATTTGTGTCACTTCCGGCAT | M2e:Hbc |
| M2e:Hbc:TT-R | GCCTGATGCCGATTTCCTCCTTA | M2e:Hbc and transcription terminator |

TISTR: Thailand Institute of Scientific and Technological Research; ATCC: American Type Culture Collection; RCEID: Research and Diagnostic Center for Emerging Infectious Diseases, Khon Kaen University; Ap<sup>r</sup>: ampicillin resistance; Em<sup>r</sup>: erythromycin resistance; Ori: origin of replication. Underlined nucleotides show sequences in the primers to introduce restriction enzyme sites (indicated in parentheses).
Figure 1. Diagram depicting the construction of pLC-Cwh:GFPuv.
Construction of recombinant L. casei expressing M2e:HBc fusion protein in a secrated form

To construct the recombinant L. casei expressing the secreted M2e:HBc fusion protein, the CwhSP fragment with Xmal flanking recognition sequences was amplified using pLC-Cwh:GFP as the template. The CwhSP-coding region was inserted upstream of the M2e:HBc gene in pLC-M2e:HBc, a vector containing a gene cassette composed of the ldh-derived promoter, the fusion gene encoding M2e:HBc and the transcription terminator (TT) of Lactococcus lactis subsp. cremoris amino peptidase N (pepN) gene (GenBank accession no. M87840.1).

Figure 2 shows the expression cassette of pLC-Cwh:M2e:HBc. The recombinant plasmid pLC-CwhSP:M2e:HBc was verified by DNA sequencing (data not shown) and was electrotransformed into L. casei RCEID02. The transformants were checked for secretion of the M2e:HBc fusion protein.

Determination of the secreted M2e:HBc fusion protein by western blot analysis

Transformants containing pLC-Cwh:M2e:HBc were cultured at 37 °C for 20 h with shaking at 200 rpm until an OD600 of 3.0 was reached. The supernatant and cell pellet were collected. The supernatant was concentrated 10-fold using centricon with a molecular weight cut-off of 10 kDa (Pall Life Sciences, NY, USA). The total cell extract and concentrated supernatant was electrotransferred into L. casei RCEID02. The transformants were checked for secretion of the M2e:HBc fusion protein.

RESULTS

Selection of the candidate signal peptides

Seventy-seven deduced amino acid sequences of L. casei ATCC334 genes encoding cell surface-associated proteins were retrieved from the NCBI database and analyzed with the SignalP 4.1 program to predict likely SP sequences (data not shown). The program generates a parameter called the D score, which is used to discriminate signal peptide from non-signal peptide sequences. It also indicates the presumptive location of the signal peptide cleavage site. Six proteins with a D score above the cut-off value of 0.450 contained good predictions for SP sequences (Table 2). The D score of these proteins and the likely cleavage sites are summarized in Table 2. Cellular location of each of the six proteins was predicted using three different programs. Five, three and one of six proteins were predicted to be extracellular by Gpos-mPLoc, PSORTb and SubLoc programs, respectively. Based on this bioinformatics analysis, SPs of the proteins encoded by the ORFs YP_805583.1, YP_805328.1 and YP_806565.1 were selected. These were designated as CwhSP, SurSP and SP6565, respectively.

Secretion of GFPuv in recombinant L. casei using different SP-containing ESV

To determine the secretion efficiency of the selected sequences, ESVs based on each SP with the GFPuv gene as a reporter were constructed and designated pLC-Cwh:GFPuv, pLC-Sur:GFPuv and pLC-SP6565:GFP, respectively. These ESVs were independently transformed into L. casei RCEID02, resulting in the recombinant strains RCEID02:CwhSP, RCEID02:SurSP and RCEID02:SP6565. The fluorescence intensity of culture supernatants of these strains, and that of L. casei RCEID02 pLC-GFPuv (used as a negative control), was determined. The highest fluorescence intensity value was shown by the CwhSP (164.80), followed by that of SurASP (118.80) and SP6565 (113.49) (Fig. 3).

Table 2. Main characteristics of signal peptides (SPs) from the genome of L. casei, as predicted from bioinformatics analysis.

| Signal peptide | Protein                | Predicted signal peptide function | Predicted cellular location |
|----------------|------------------------|----------------------------------|-----------------------------|
| YP_805583.1    | Cell wall-associated   | D score* 0.696                   | VSA-ST                     |
| (CwhSP)        | hydrolase              |                                   | Extracellular               |
| YP_805328.1    | Surface antigen        | D score* 0.864                   | VFA-DT                     |
| (SurSP)        |                        |                                   | Extracellular               |
| YP_806565.1    | Cell surface protein   | D score* 0.798                   | VHA-DD                     |
| (SP6565)       |                        |                                   | Extracellular               |
| YP_805464.1    | Cell surface protein   | D score* 0.758                   | VGA-TT                     |
| YP_805465.1    | Cell surface protein   | D score* 0.834                   | VLA-LQ                     |
| YP_805466.1    | Cell surface protein   | D score* 0.777                   | VFA-SE                     |

*The D cut-off value for cell surface associate protein is 0.450.
DISCUSSION

Engineered LAB strains constitute bacterial systems that may serve as alternative mucosal delivery vehicles for a variety of biomolecules (Wells and Mercenier 2008). Recombinant LAB can be used as vaccine vehicles to elicit both secretory IgA and systemic antibody responses against the delivered antigen (Neutra and Kozlowski 2006). In this study we constructed recombinant L. casei cells expressing M2e:Hbc in a secreted form with the use of novel SPs derived from the L. casei genome. Several SPs derived from cell surface-associated proteins and secreted proteins of LAB have been identified and used to construct distinct ESVs (Wu and Chung 2006; Mathiesen et al. 2008). Of these, the SP of Usp45Sp from Lactococcus lactis (Daniel et al. 2011) is the most widely used heterologous SP for protein secretion in LAB species (Dieye et al. 2001; Mathiesen et al. 2008). Previous studies in Gram-positive bacteria have shown that the secretion efficiency depends not only on the SP but also on the protein to be secreted and on bacterial host (Dieye et al. 2001; Mathiesen et al. 2008). Nonetheless, studies on the SP functionality for heterologous protein secretion in Lactobacillus plantarum WCFS1 found that the homologous SPs have similar or higher secretion efficiencies than those of heterologous origin (Mathiesen et al. 2008). Aimed to express and secrete M2e:Hbc in L. casei, we screened for homologous SPs using a bioinformatics approach. In our hands, the best SP predictor is SignalP 4.1 (Petersen et al. 2011). This program has been successfully used to select efficient SPs from the protein pool of L. plantarum WCFS1 (Mathiesen et al. 2008). The SubLoc, PSORTb and Gpos-mPLoc programs were also used to predict the localization of the proteins produced. Using these programs, three putative SPs (CwhSP, SurASP and SP6565) were selected from cell surface-associated proteins of L. casei ATCC334 and used for construction of ESVs with GFP as reporter protein. The reason that the SubLoc program predicts CwhSP and SP6565 as periplasmic might be due to the fact that this program uses a dataset based on that of Reinhardt and Hubbard (1998). This dataset contains a small number of sequences of both extracellular and periplasmic groups for training the neural network, which may result in a less accurate prediction. Based on these ESV constructs, it was found that all three SPs can function for GFP secretion in L. casei RCEID02, and that the construct using CwhSP provides the highest GFP secretion. Previous studies found that an efficient signal peptide in Gram-positive bacteria generally contains the consensus sequence Vla-Ala↓Ala or Ala-X-Ala↓Ala as the SP cleavage site (Nielsen et al. 1997; Mathiesen et al. 2008). In this study, all three selected SPs have the consensus motif Val-X-Ala at position −3 to −1 relative to the putative cleavage site. However, none of the selected SPs contained Ala at position +1. In addition, the most efficient SP, CwhSP, harbors Ser at position +1, which indicates Ala at this position is not required. CwhSP was further used to construct an ESV containing M2e:Hbc. Recombinant L. casei carrying this construct expressed and secreted M2e:Hbc successfully, demonstrating the usefulness of bioinformatics to predict and select novel SPs for L. casei. However, this approach does not guarantee that the SPs selected will function, since protein secretion depends on the SP itself, the genetic background of the host strain and the target protein. In addition, secretion efficiency may be affected by the extent to which protein production levels and rates are adapted to the capacity of the translocation machinery, the influence of SP sequence on mRNA stability and its translation efficiency (Mathiesen et al. 2008). For these reasons, to maximize the production of a secreted protein in Lactobacillus, and before optimization and balancing of other factors,

Expression and secretion of the M2e:Hbc fusion protein by L. casei RCEID02

The CwhSP was selected for constructing the ESV containing the M2e:Hbc fusion gene, i.e. pLC-Cwh: M2e:Hbc. Western blot analysis of the supernatant from L. casei cells containing pLC-Cwh: M2e:Hbc showed a prominent band with an apparent molecular weight of around 27.6 kDa (Fig. 4, lane 6), which presumably corresponded to the expected fusion protein. The supernatant from an intermediate construct lacking CwhSP showed no band (Fig. 4, lane 4). The M2e:Hbc fusion protein was detected in total cell extracts of both L. casei carrying pLC-M2e:Hbc (Fig. 4 lane 3) and pLC-Cwh: M2e:Hbc (Fig. 4 lane 5). No protein band was detected in either total cell extracts (Fig. 4, lane 1) or supernatant (Fig. 4, lane 2) of the negative control strain.

Figure 3. Mean fluorescence intensity (MFI) of culture supernatants from the recombinant L. casei RCEID02 harboring different SP-containing constructs. The supernatant from L. casei RCEID02 harboring pLC-GFPuv was used as a negative control.

Figure 4. Western blot analysis of M2e:Hbc fusion protein expressed in recombinant L. casei RCEID02. Lanes 1 and 2 = total cell extracts and supernatant from L. casei RCEID02 containing pRCEID-LC13.9; lanes 3 and 4 = total cell extracts and supernatant from L. casei RCEID02 containing pLC-M2e:Hbc; lanes 5 and 6 = total cell extracts and supernatant from L. casei RCEID02 containing pLC-CwhSP: M2e:Hbc.
it might be worthwhile selecting an optimal SP for each individual protein.

Besides conferring only short-term immunity, a major drawback of the current influenza virus A vaccines is that they confer only short-term subtype-specific protection. Thus, keeping vaccines up-to-date with influenza A antigens requires constant monitoring of the subtypes of the circulating viruses (Johansson and Brett 2007). This inconvenience has led to much discussion about the development of a universal influenza A vaccine, able to provide protection against all virus subtypes (Du, Zhou and Jiang 2010). Such a vaccine would require identification of a viral component conserved through all subtypes of influenza A virus for eliciting a broad-spectrum immunity (Stanekova and Vareckova 2010). The extracellular domain of the M2 protein (M2e) of the influenza virus is one such conserved component (Ebrahimim and Tebianian 2011). However, this is a short peptide of only 24 amino-acid residues. In this study, the M2e-encoding gene was fused with the gene coding for the hepatitis B core antigen (Hbc) protein in order to increase its immunogenicity. In a previous study, strong immunogenicity and full protection were obtained in mice after either intraperitoneal or intranasal administration of the M2e:Hbc fusion protein (De Filette et al. 2005). Considering their GRAS status, the use of L. casei as an antigen delivery vehicle can confer great advantage for the expression of the M2e:Hbc fusion protein.

As a conclusion, in this study, we used a bioinformatics approach to select potential SPs from the L. casei ATCC2334 genome. The three selected SPs, which include those of Cwh, SurA and 6565 proteins, proved to direct secretion of the GFP in L. casei. CwhSP, which showed the highest secretion efficiency, was then used to develop an ESV construct expressing and secreting the fused M2e:Hbc protein. This SP and the ESV developed may further serve for the expression and secretion of a variety of homologous and heterologous proteins in L. casei and surely in other LAB species.

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Conflict of interest. None declared.

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