Engineering a *Bacillus subtilis* Expression-Secretion System with a Strain Deficient in Six Extracellular Proteases

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Received 25 February 1991/ Accepted 3 June 1991

We describe the development of an expression-secretion system in *Bacillus subtilis* to improve the quality and quantity of the secreted foreign proteins. This system consists of a strain (WB600) deficient in six extracellular proteases and a set of *sacB*-based expression vectors. With the inactivation of all six chromosomal genes encoding neutral protease A, subtilisin, extracellular protease, metalloprotease, bacillopeptidase F, and neutral protease B, WB600 showed only 0.32% of the wild-type extracellular protease activity. No residual protease activity could be detected when WB600 was cultured in the presence of 2 mM phenylmethylsulfonyl fluoride. By using TEM β-lactamase as a model, we showed that WB600 can significantly improve the stability of the secreted enzyme. To further increase the production level we constructed an expression cassette carrying *sacY*, a *sacB*-specific regulatory gene. This gene was placed under the control of a strong, constitutively expressed promoter, P43. With this cassette in the expression vector, an 18-fold enhancement in β-lactamase production was observed. An artificial operon, P43-*sacY*-degQ, was also constructed. However, only a partial additive enhancement effect (24-fold enhancement) was observed. Although degQ can stimulate the production of β-lactamase in the system, its ability to increase the residual extracellular protease activity from WB600 limits its application. The use of the P43-*sacY* cassette and WB600 would be a better combination for producing intact foreign proteins in high yield.

With the capability of secreting extracellular enzymes directly into the culture medium, *Bacillus subtilis* potentially can serve as an efficient expression host (4, 5, 9, 24, 25). The secreted foreign proteins usually remain in biologically active forms (20, 21), and the downstream purification is greatly simplified. However, at least two major limitations hinder the wide application of *B. subtilis* as a production microorganism. First, *B. subtilis* produces and secretes high levels of extracellular proteases which degrade the secreted foreign proteins (5, 41). It is well established that *B. subtilis* has six extracellular proteases: neutral protease A (45), subtilisin (also known as alkaline protease) (34, 42), extracellular protease (29, 37), metalloprotease (30), bacillopeptidase F (31, 43), and neutral protease B (36). The construction of a strain deficient in five proteases GP263 (30, 32), has eliminated over 99% of total extracellular protease activity. However, depending on the nature of the foreign proteins to be expressed in this strain, some of them remain unstable. Second, the lack of well-regulated inducible vectors also limits the wide application of the *B. subtilis* system. To overcome this problem, a few inducible vectors have been developed. Most of these vectors use either the *E. coli lac* system (16, 46) or the temperature-sensitive repressor from lambda (3) or φ105 (23) to regulate the expression of foreign genes. Some others (10, 39, 47, 48) are based on the regulatory region of a *B. subtilis* sucrose-inducible gene, *sacB*, which encodes the extracellular enzyme levansucrase. The regulatory region of *sacB* has been well characterized. Upstream of the promoter is a target sequence that is required for the action of both the *degQ* and *sacU* products (12). Both *degQ* and *sacU* products (1, 18, 28) can enhance *sacB* transcription. There is a terminatorlike structure between the *sacB* promoter and its ribosome-binding site (35).

A positive regulatory gene, *sacY* (2, 7, 49), which encodes an antiterminator protein, is responsible for mediating the sucrose-induced expression (6). The production of a foreign protein (β-lactamase) can be enhanced 17- to 23-fold by either introducing a mobile *degQ* cassette into the expression vector or using a *sacU*(Hy) strain, WB30 (39), which has the two major protease genes inactivated. However, no additive enhancement for β-lactamase production could be observed when the *degQ*-containing plasmid was transformed into WB30. Furthermore, both *degQ* and *sacU* can stimulate the production of extracellular proteases (12, 18). Even though WB30 is deficient in two proteases, a relatively high level of protease activity can still be detected. In this paper, we describe the development of an efficient expression-secretion system in *B. subtilis*. The first approach is the construction of an improved strain deficient in extracellular proteases. With our recent cloning and characterization of a novel protease gene, *nprB* (36), we describe the construction of a novel strain, WB600, deficient in six proteases. The second approach explores the possibility of achieving additive enhancement for the production of β-lactamase by the construction of an artificial operon carrying both *sacY* and *degQ* under the control of a strong, well-characterized promoter, P43 (38). A P43-*sacY* cassette was also constructed; this cassette offers the *sacY* enhancement effect without overproducing extracellular proteases.

**MATERIALS AND METHODS**

Bacterial strains, plasmids, and culture conditions. The *B. subtilis* strains used in this study were *B. subtilis* 168 (trpC2), DB104 (his nprR2 nprE18 aprΔ3) (14), WB30 (his nprR2 nprE18 aprΔ3 sacU*(Hy)200) (39), and DB428 (trpC2 ΔnprE ΔaprE Δepr Δbpf) (11). *Escherichia coli* DH5α (φ80lacZΔM15) (endA1 recA1 hisD1717 r− m− supE44 thi−I λ− gyrA relA1 F− Δ(lacZYA-argF)U169) was used for
routine transformation. pWB (39) is a pUB110 derivative carrying a sacB regulatory region and a modified sacB signal sequence which fuses in frame with the sequence encoding the mature portion of the E. coli TEM β-lactamase. pQL (39) is a pWB derivative carrying a P43-degQ cassette. pUB18 (39) is a derivative of pUB110, pE194 (13) was obtained from the Bacillus Genetic Stock Center, Ohio State University. E. coli Bluescribe plasmid (PBS; Stratagene) was used for routine subcloning. Standard DNA transformation was performed by the competent-cell method in B. subtilis (33) and E. coli (19). Modified Schaeffer medium (SG) (17) and tryptose blood agar plates (TBAB; Difco) were used to cultivate B. subtilis. Superrich medium (41) was used to culture B. subtilis for determining the secreted β-lactamase activity. For cells carrying pUB18, kanamycin was added to the medium at a final concentration of 10 μg/ml. L broth and YT medium were used for routine culture of E. coli.

**DNA manipulations.** Small-scale plasmid preparations were made by the rapid alkaline sodium dodecyl sulfate method (26), and large-scale plasmid preparations were made by CsCl-ethidium bromide centrifugation (26). Chromosomal DNA was prepared by the method of Doi (8). Restriction enzymes and DNA modification enzymes were purchased from Bethesda Research Laboratories, Pharmacia, and Boehringer Mannheim and were used as recommended by the manufacturers. Southern blotting and hybridization were performed as described previously (39).

**Enzyme assays.** The activity of the secreted β-lactamase was determined by using 7-(thienyl-2-acetamido)-3-[2-(4-N,N-dimethylaminophenylazo)]pyridinium methyl]-3-carboxymethyl acid (PADAC) as the substrate (39). One milliliter represents the hydrolysis of 1 nmol of PADAC per min at 37°C. The proteolytic activity was monitored by using resorufin-labeled casein (Boehringer Mannheim) as the substrate (43). One unit of protease activity is defined as the amount of enzyme required to increase 0.01 A₅₀₂₅ unit after a 1-h incubation at 37°C.

**Construction of pYQL, pYL, and pPY.** pYQL is a pQQL derivative with a pUB110 replication origin. It carries an artificial operon with sacY and degQ. Two synthetic oligonucleotides with the following sequences were synthesized as primers for the amplification of sacY through the polymerase chain reaction (PCR) (27): 5'-AAGGTACCTCTGA TTTCTATTCTAAAAAGGGGGA-3' (forward primer) and 5'-AAGGTACCTCTAGATGCCTTGGTTCGCGTGCGACTGACC-3' (backward primer). The forward primer carries a KpnI site at the 5' end and has 25 nucleotides which correspond to the sequence immediately upstream of the sacY ribosome-binding site. The backward primer covers the translation termination codon of sacY with the addition of three restriction enzyme sites, BglII, XbaI, and KpnI. A GeneAmp kit (Perkin-Elmer Cetus) was used for amplification under the following conditions: 93°C for 1 min, 52°C for 1 min, and 72°C for 2 min (30 cycles) with a programmable Dri-block (PHC-1; Techne). The PCR-amplified 900-bp sacY fragment was digested by KpnI and ligated to the KpnI-digested pQL to generate pYQL (Fig. 1). The sequence of the PCR-amplified sacY fragment and its orientation in pYQL were confirmed by DNA sequencing. To construct pYQL which carried the P43-sacY cassette upstream of the sacB-bla fusion, we deleted the degQ gene from the P43-sacY-degQ operon by a XbaI digestion of pYQL. The digested plasmid was then reclosed through a ligation reaction. The P43-sacY cassette was also installed into the polylinker region (between EcoRI and BamHI) of pUB18 to generate pPY. This P43-sacY cassette was obtained by digesting pYQL with EcoRI and BglII.

**Construction of WB600.** DB428 (11), a strain deficient in four proteases, with the chromosomal copy of nprE, aprA, epr, and bpf inactivated through internal deletions, was used as the starting material for the construction. The 3.6-kb PstI fragment carrying the entire mpr gene was isolated through colony hybridization by using an oligonucleotide probe of 30 nucleotides. The probe was designed according to the published sequence of mpr (30). A strain deficient in five proteases, WB500, was constructed by using the strategy described by Sloma et al. (30). The inactivation of mpr was confirmed by Southern blot hybridization. To inactivate the chromosomal copy of nprB, we replaced the wild-type gene by a truncated nprB. To facilitate the construction of WB600, a 3.5-kb PstI fragment carrying the entire nprB gene and its flanking sequence was isolated by colony hybridization with the cloned 2.4-kb nprB fragment (36) as a hybridization probe. The isolation of this 3.5-kb PstI fragment is necessary because the open reading frame of nprB is located 300 bp away from one end of the cloned 2.4-kb fragment and too short a flanking sequence would reduce the frequency of gene replacement. The 3.5-kb nprB fragment in pUB18 was named pNPRBL. This plasmid was digested by AvII, and
Determination of sporulation efficiency. The sporulation efficiency of WB600 in both synthetic (22) and SG (17) media was measured by the method of Ochi et al. (22). Spores were counted by plating the cells on a TBAB plate after heat treatment at 75°C for 20 min.

Preparation and purification of [35S]methionine-labeled β-lactamase. WB600(pYL) was cultured in 20 ml of synthetic medium containing Spizizen salts (33), 3% glucose, 0.01% thiamine, and 1 mg of the following amino acids per ml: tryptophan, arginine, proline, glutamic acid, and glutamine. When the cell density reached 100 Klett units, 1.5 mCi of [35S]methionine (800 mCi/mmol; Amersham) and sucrose (final concentration, 2%) were added. Supernatant was collected 3 h after induction, and β-lactamase was purified through Sephadex G-100 and DEAE-cellulose columns as described previously (40).

Half-life of β-lactamase. Each strain was cultured in 10 ml of superrich medium. When the cell density reached 100 Klett units, 25 μl (48,000 cpm/μl) of purified [35S]methionine-labeled β-lactamase and sucrose (final concentration, 2%) were added. A 400-μl sample from each culture was collected at each time point, and the proteins in the supernatant were precipitated by the addition of 1 ml of cold acetone. The samples were analyzed on an SDS–12% polyacrylamide gel. After fluorography, the protein band corresponding to the intact β-lactamase was excised and its radioactivity was determined by liquid scintillation counting.

RESULTS

The ability of SacY and DegQ to stimulate transcription originating at the sacB promoter makes them attractive agents for increasing production of desirable products in B. subtilis. A potential disadvantage of this system is that degQ also stimulates the production of extracellular proteases. We therefore adopted a two-track approach to maximizing the accumulation of secreted foreign proteins. First, we constructed a strain in which the genes for all six known extracellular proteases were disrupted. Second, we introduced into this strain a plasmid that codes for SacY and DegQ and expresses β-lactamase under control of the sacB promoter.

Deletion of nprB in the chromosome. Transformation of linearized pANE to WB500 generated about 30 to 40 transformants on an SG plate containing 5 μg of erythromycin per ml and 5 μg of lincomycin per ml. These transformants showed no resistance to kanamycin (5 μg/ml) and hence should not carry any replicative form of pANE. For a quick confirmation of the replacement of the wild-type nprB by the in vitro-mutated nprB in these potential WB600 strains, we designed a pair of nprB-specific PCR primers. The forward and backward primers were 25 and 27 nucleotides long, respectively. One covered the sequence just upstream of the nprB translation initiation codon; the other covered the translation terminator region. PCR amplification of nprB from WB500 showed the expected 1.62-kb fragment (Fig. 3B). Screening of the potential WB600 clones indicated that they all showed a 2.2-kb fragment. The size of this fragment agrees with that of the truncated nprB carrying a 1.45-kb ery insert. This PCR method allows the rapid identification of WB600 strain. To further confirm the observation by the PCR method, chromosomal DNA from WB500 and WB600 was digested by PstI. Southern blot hybridization with either a labeled 3.5-kb nprB probe or a 1.45-kb ery probe showed the expected results as shown in Fig. 3A. The observation of two PstI fragments (2.3 and 1.8 kb) from WB600 hybridized

FIG. 2. Construction of pANE carrying a truncated nprB with an erythromycin marker.

then BamHI linker was added to generate pΔNPRBL. This deletion removes a 906-bp fragment which covers the “pre-pro” region and part of the sequence encoding the mature form of neutral protease B (Fig. 2). A 1.45-kb BamHI fragment carrying the erythromycin marker was obtained from pWB407 and inserted into pΔNPRBL to generate pΔNE (Fig. 2). pWB407 is a pUB18 derivative with a 1.45-kb TagI fragment from pE194. The HindIII site in pWB407 was converted into a BamHI site. pΔNE was linearized by BglII digestion, gel purified, and transformed into WB500. Transformants were selected on SG plates containing 5 μg of erythromycin per ml and 5 μg of lincomycin per ml. The replacement of the wild-type nprB gene by the truncated one was confirmed by PCR and Southern hybridization.

PCR amplification of nprB. Individual colonies were transferred via toothpicks to 1.5-ml microcentrifuge tubes containing 100 μl of sterilized water. The samples were boiled for 1 min, and 6.5 μl from each sample was used for amplification by mixing with 3.5 μl of PCR mix. The amplification conditions were the same as described above for the amplification of sacB. The entire 10 μl for each sample was loaded onto a 1% agarose gel for analysis.
with the nprB probe was due to the insertion of the ery cassette in nprB. This ery cassette carries a PstI site close to one end (Fig. 2). In the same way as B. subtilis 168, WB600 grew and sporulated normally in both the synthetic and the SG media (data not shown).

Protease activity in the supernatant of WB600 and other protease-deficient strains. B. subtilis 168(pUB18), DB428 (pUB18), WB500(pUB18), and WB600(pUB18) were cultured in superrich medium, and samples were collected at 8, 14, and 24 h after inoculation. By setting the protease level from B. subtilis 168 as 100%, DB428, WB500, and WB600 was found to have 3.1, 0.8, and 0.32% of protease activity, respectively (Table 1). With the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 2 mM during induction, no residual protease activity could be detected.

The effect of degQ and sacY on protease production in various protease-deficient strains. degQ is known to stimulate the production of various extracellular enzymes including levansucrase and proteases (12). As shown in Table 1, B. subtilis strains (168, DB428, WB500, and WB600) carrying pPQ (pUB18 carrying a P43-degQ cassette) showed a higher level of protease activity in the medium during the first 14 h of cultivation. Expression of both bpf and nprB could also be stimulated by degQ and sacU(Hy) (36, 44). However, no enhancement of protease production could be observed for WB600(pPY) (data not shown).

Effects of P43-sacY and P43-sacY-degQ on the production of β-lactamase. To study the effect of sacY on β-lactamase production, we transformed pYL into DB104. As shown in Table 2, the β-lactamase activity in the supernatant from DB104(pYL) was 18-fold higher than that of DB104(pWB) (pWB is same as pYL except that it does not carry the P43-sacY cassette). The enhanced production of β-lactamase mediated by P43-sacY was comparable to that mediated by P43-degQ. Since degQ and sacY act on different target sequences in the sacB regulatory region, it would be interesting to see whether additive enhancement for β-lactamase production can be achieved by constructing an artificial operon carrying both sacY and degQ under the control of P43. Using the β-lactamase activity from DB104(pWB) as a reference, a 24-fold enhancement for the secreted β-lactamase activity from DB104(pYQL) could be observed (Table 2). Transforming pYQL or pYL to WB30 showed the same 23- to 24-fold enhancement of β-lactamase production.

Production of β-lactamase by using various protease-deficient strains as host. To determine whether protease-deficient strains can improve the stability and yield of β-lactamase, cells carrying pWB were cultured in superrich medium containing 3% glucose and 2% sucrose. β-Lactamase activity was reported to decrease when DB104(pWB) was cultured under identical conditions for 14 h (39). As shown in Fig. 4A, the use of DB428 and WB500 could prolong the stability of the secreted β-lactamase for up to 30 h of cultivation. The activity dropped to a very low level after 50 h of cultivation. The use of WB600 further improved the stability of the secreted β-lactamase; about 80% of the β-lactamase activity was retained after 50 h. To increase the expression level, we used the P43-degQ cassette. Significant enhancement of β-lactamase production was observed in DB428 and WB500 (Fig. 4B). However, a notable decline in

**TABLE 1. Extracellular protease activity from various B. subtilis strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>PMSF (mM)</th>
<th>Protease activity (U/ml) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>8 h</td>
</tr>
<tr>
<td>168</td>
<td>pUB18</td>
<td>0</td>
<td>8,670</td>
</tr>
<tr>
<td>DB428</td>
<td>pUB18</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>WB500</td>
<td>pUB18</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>WB600</td>
<td>pUB18</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>WB600</td>
<td>pUB18</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>168</td>
<td>pPQ</td>
<td>0</td>
<td>9,356</td>
</tr>
<tr>
<td>DB428</td>
<td>pPQ</td>
<td>0</td>
<td>564</td>
</tr>
<tr>
<td>WB500</td>
<td>pPQ</td>
<td>0</td>
<td>144</td>
</tr>
<tr>
<td>WB600</td>
<td>pPQ</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

* Data presented in this table are the average values of two independent enzyme assays.

* Time (hours after cultivation) when samples were collected for protease activity determination.

**TABLE 2. Effects of the P43-sacY-degQ artificial operon and its derivatives on the production of β-lactamase in DB104 and WB30**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Plasmid</th>
<th>Relative β-lactamase sp act</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB104</td>
<td>nprR2 nprE18 aprΔ3</td>
<td>pWB</td>
<td>1</td>
<td>39</td>
</tr>
<tr>
<td>DB104</td>
<td>nprR2 nprE18 aprΔ3 sacU(Hy)200</td>
<td>pWL</td>
<td>17</td>
<td>39</td>
</tr>
<tr>
<td>WB30</td>
<td>nprR2 nprE18 aprΔ3 sacU(Hy)200</td>
<td>pWB</td>
<td>24</td>
<td>39</td>
</tr>
<tr>
<td>WB30</td>
<td>aprΔ3</td>
<td>pQL-1</td>
<td>23</td>
<td>39</td>
</tr>
<tr>
<td>DB104</td>
<td>aprΔ3</td>
<td>pYL</td>
<td>18</td>
<td>This study</td>
</tr>
<tr>
<td>DB104</td>
<td>aprΔ3</td>
<td>pYQL</td>
<td>24</td>
<td>This study</td>
</tr>
<tr>
<td>WB30</td>
<td>aprΔ3</td>
<td>pYL</td>
<td>25</td>
<td>This study</td>
</tr>
<tr>
<td>WB30</td>
<td>aprΔ3</td>
<td>pYQL</td>
<td>23</td>
<td>This study</td>
</tr>
</tbody>
</table>

* The specific β-lactamase activity of DB104(pWB) (0.7 mU/ml/Klett unit) was set as 1.
the β-lactamase activity was observed after 6 h of cultivation. The use of WB600 could improve the production. A higher level of β-lactamase activity and prolonged stability (up to 12 h after cultivation) were observed (Fig. 4B).

The combination of WB600 and the P43-sacY cassette in the production of β-lactamase. Since P43-sacY could stimulate sacB expression to a level which is comparable to the stimulation mediated by P43-degQ, and it did not stimulate protease production, we studied the effect of using WB600 and the P43-sacY cassette to produce β-lactamase. Figure 4B shows that WB600(pYL) produced a higher level of β-lactamase with prolonged stability. Even when the samples were collected after 40 h of cultivation, they retained over 95% of the peak activity.

Half-life of β-lactamase. The determination of β-lactamase activity in the culture medium showed that there was a net balance between the biosynthesis and degradation of β-lactamase. To study the effect of protease on the stability of secreted proteins, we determined the half-life of β-lactamase by adding [35S]methionine-labeled β-lactamase to the culture during induction and monitoring the decay of the intact, labeled β-lactamase. The labeled β-lactamase was 80% pure after chromatography on a DEAE-cellulose column. As shown in Fig. 5, the estimated half-life of β-lactamase in B. subtilis 168(pWB), DB428(pWB), WB500(pWB), WB600(pWB), and WB600(pYL) was 1.5, 21, 27, 41, 6, and 40 h, respectively.

FIG. 5. Stability of the secreted β-lactamase in the culture of the wild-type B. subtilis 168(pWB) (+), WB600(pQI) (○), DB428(pWB) (△), WB500(pWB) (●), WB600(pYL) (▲), and WB600(pWB) (□). All these cells were cultured in superrich medium in the absence of PMSF. The degradation of β-lactamase in the culture of WB600(pWB) in the presence of 1 mM PMSF, which was added at the time of induction, is also shown (■).

DISCUSSION

Since DegQ and SacY act at different target sequences in the regulatory region of sacB to stimulate transcription and since the mechanisms to achieve transcription stimulation are different (positive transcription stimulation versus anti-termination), an additive enhancement for the production of β-lactamase would be expected for pQI which carries the artificial sacY-degQ operon. However, only partial enhancement (an increase from 17- to 24-fold stimulation) could be achieved. This suggests that the transcription process or another posttranscriptional step(s) such as secretion is a rate-limiting step and that the expression system has reached the maximum limit of one of these processes. A direct quantitation of bla mRNA from strains carrying either pQI or pQ1 can determine whether the rate-limiting step is at the transcriptional level.

WB600 has an extracellular protease level that is equivalent to 0.32% of the wild-type level after 24 h of cultivation. Because this residual protease level increases with time, the observed protease activity can be due to either the presence of some other uncharacterized minor extracellular proteases or the gradual release of the intracellular proteases into the medium because of cell lysis. The inhibition of the residual protease activity by the addition of PMSF indicates that this protease activity is contributed by at least one serine protease. Purification of the minor protease from the supernatant of WB600 to homogeneity confirmed that it is a serine protease with a molecular mass much larger than that of the
major intracellular serine protease 1 (15). This suggests the existence of a seventh extracellular protease in *B. subtilis*. Cloning and characterization of this protease are in progress. Although neutral protease B is produced at a low level (as shown by comparing the protease level of WB600 with that of WB500), the experimental data indicate that \( \beta \)-lactamase is quite sensitive to the NprB-mediated digestion. Monitoring the protease profiles of DB428(pFQ), WB500(pFQ) and WB600(pFQ) showed that the protease activity increased with time up to 14 h after cultivation and then decreased. This is true for the protease-deficient strains but not for *B. subtilis* 168, the wild-type strain. The cause of the decrease in protease activity at this late stage is unknown. Even though the degQ-enhanced protease production in WB600 is low, the half-life of \( \beta \)-lactamase drops significantly from 41 to 6 h (Fig. 5). Application of degQ to this system will not be appropriate until a strain is available that is 100% free of extracellular proteases.

**ACKNOWLEDGMENTS**

We thank Ariff Damji for the Southern blot analysis and the *Bacillus* Genetic Stock Center for pE194. This work was supported by a strategic grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) and an establishment grant from the Alberta Heritage Foundation for Medical Research (AHFMR).

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