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I. Introduction

Brevibacillus choshinensis, a gram positive bacterium, has excellent ability to produce many kinds of proteins extracellularly. And the *Brevibacillus* Expression System based on this bacterium is well-suited for secretory production of heterologous proteins with high efficiency. Making use of this characteristic of the host bacterium, many successful records have been accumulated so far in this regard. This system has following merits :

- The host bacterium secretes proteins very efficiently.
- The host bacterium produces negligible amout of extracellular protease so that products remain unscathed in the culture medium.
- Proteins are produced as active forms.
- Easy to culture and sterilize the host bacterium.
- The host bacterium is amenable to genetic engineering.
- The host bacterium is guaranteed to be safe organism.

A part of our successful results are listed in Table 1. They cover enzymes, antigens, cytokines. All of them were produced at very high level and confirmed to have native biological activity. In addition to them, proteins from eubacteria, archaebacteria, eukaryotes, viruses, were successfully produced. Special merit of this sytem is that it exhibits very high efficiency in the production of eukaryotic secretory proteins. Usually they have some cystein residues intramolecularly and disulfide bonds must be formed at exact positions precisely, which makes it difficult to achieve efficient production using other bacterial expression systems based on intracellular expression. Meanwhile, this system is very good at secretory production of proteins with native structure irrespective of whether they have disulfide bonds or not.

Table 1. Some examples of successful production of heterologous proteins using *B. choshinensis* host-vector system.

Proteins	Origins	Production (g/L)	References
Enzymes			
α-amylase	*B. licheniformis*	3.7	
Sphingomyelinase	*B. cereus*	3.0	
Xylanase	*B. halodurans*	0.2	
CGTase	*B. macerans*	1.5	2)
Chitosanase	*B. circulans*	1.4	
Hyper thermo-stable protease	*A. pernix*	0.1	
Hyper thermo-stable nuclease	*P. horikoshii*	0.7	
PDI	human	1.0	3)
Antigens			
Surface antigen	*E. rhusiopathiae*	0.9	
Surface antigen	*T. pallidum*	0.8	
Cytokines			
EGF	human	1.5	4)
IL-2	human	0.6	5)
NGF	mouse	0.2	
IFN-γ	chicken	0.5	6)
TNF-α	bovine	0.4	
GM-CSF	bovine	0.2	
GH	flounder	0.2	

The host bacterium exhibits high transformation efficiency by electroporation so that it is easy to construct expression clones. There is a shuttle vector between *B. choshinensis* and *E. coli* as well as the one maintained only in *B. choshinensis*. Thus, either the 2 step construction method using the shuttle vector or 1 step one using the vector for only *B. choshinensis* can be selected.
Two kinds of culture media are recommended for the culture of transformants (components are shown in V-10.). Cultures are performed using test tubes or flasks fixed on an appropriate shaker. After culture, supernatant is separated from cells by centrifugation from which target protein can be purified. The unnecessity to conduct cell disruption makes the purification procedure simpler and easier.

II. Components

Brevibacillus Expression System (Cat.#HB100)

- Expression vector
  pNY326 DNA 10 μg (Cat.#HB111)
  pNCMO2 DNA 10 μg (Cat.#HB112)
- Control vector
  pNCMO2BLA DNA 1 μg (Cat.#HB113)
- Competent cells (for electroporation)
  Brevibacillus choshinensis Electro-Cells 100 μl×10 tubes (Cat.#HB115)

III. Storage

Expression vectors pNY326, pNCMO2 DNA: −20℃
Control plasmid pNCMO2BLA DNA: −20℃
Competent cells *Brevibacillus choshinensis* Electro-Cells: −80℃

IV. Overview of *Brevibacillus* expression system

The flow of experiments to produce the target protein using this kit is described below.

IV-1. Selection of the expression vectors

(1) pNCMO2

pNCMO2 is a shuttle vector between *B. choshinensis* and *E. coli*. At first, expression plasmids can be constructed by employing *E. coli* as the host, then transfered into *B. choshinensis* cells.
The pNCMO2 contains P2 promoter, which is one of 5 promoters driving transcription of cell wall protein (HWP) gene and does not work in *E. coli*. Therefore, it is suitable for cloning genes of which expressions are stressful for *E. coli*.
In *B. choshinensis* cells, P2 promoter works as very strong promoter, enabling efficient protein production.

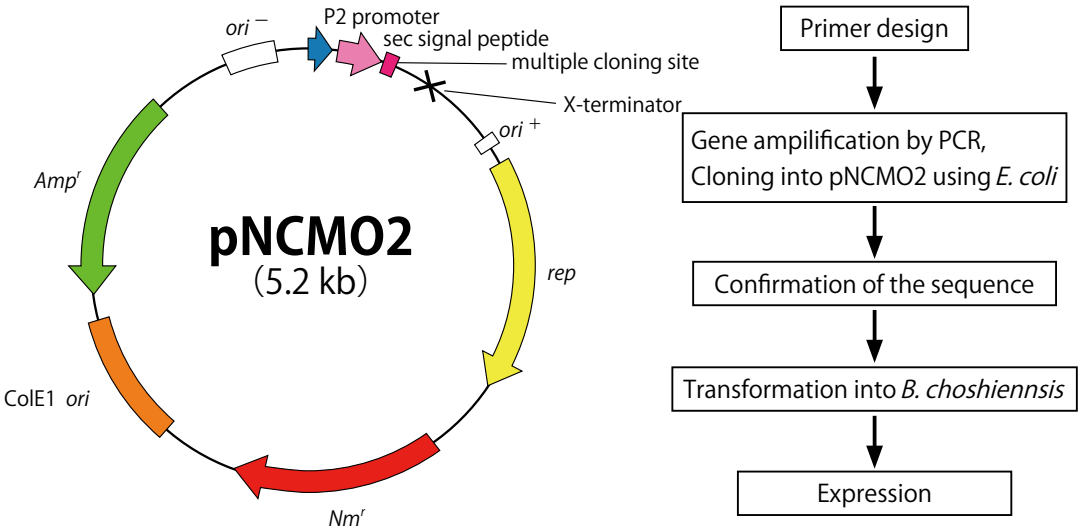


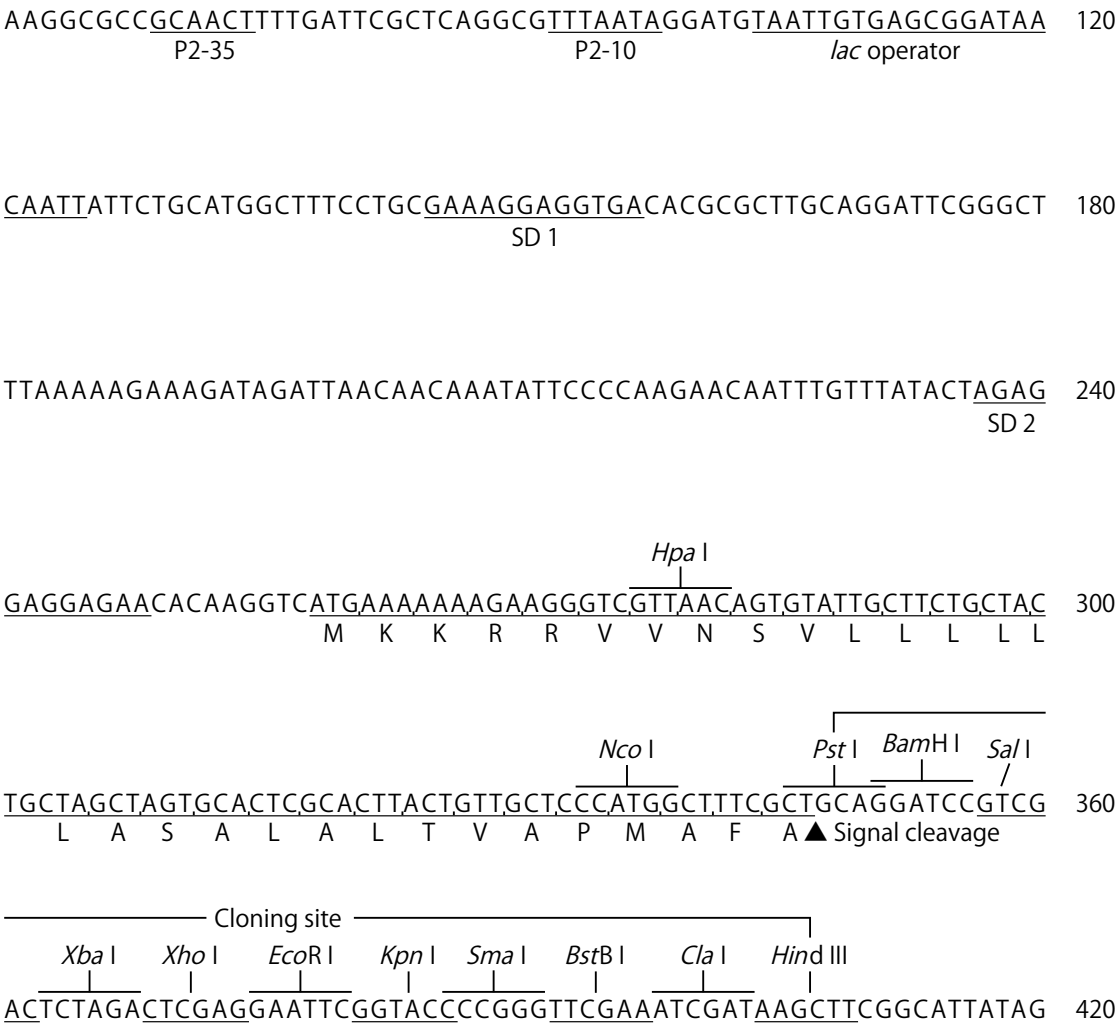
Fig 1. Map of pNCMO2

Because of the strong promoter activity of P2 promoter, it shows detrimental effect on the growth of the transformants. In that case, the use of pNY326 is recommended.

<Features of pNCMO2>

P2 promoter	A part of 5′ upstream region of cell wall protein (HWP) gene, which does not work in *E. coli*, and works as a strong promoter in *Brevibacillus*.
Sec signal peptide	Secretion signal sequence of HWP is modified to increase the secretion efficiency.
Multiple cloning site	11 restriction sites allowing insertion of target gene into the vector
Terminator	46 bp nucleotides sequence functioning as a terminator is introduced downstream of multiple cloning sites
rep	Gene involving in plasmid replication (pUB110 derived)
Ori	Replication origin allowing replication and maintenance of the plasmid in *Brevibacillus* (pUB110 derived)
Nm^r	Neomycin resistance gene, working as selection marker in *Brevibacillus*.
ColE1 *Ori*	Replication origin allowing replication and maintenance of the plasmid in *E. coli* (pUC derived)
Amp^r	Ampicillin resistance gene, working as selection marker in *E. coli*

Nucleotide sequence of the region from promoter to multiple cloning site on pNCMO2



P2 promoter is the main promoter which drives the synthesis of the cell wall protein. Lac operator sequence is introduced just upstream of the P2 promoter, which regulate the promoter activity in *E. coli*. There are 2 SD sequences (SD1 and SD2) which direct translation in the same frame.

(2) pNY326

pNY326 can replicate in *B. choshinensis* cells and its size (3.4 kb) is much smaller than that of pNCMO2 (5.2 kb). In addition, the promoter activity of the vector is substantially weaker than that of pNCMO2. Taken together, it can be maintained stably even if it expresses rather toxic proteins to the host cells. In some case, higher efficient production of protein can be achieved using this vector because of the more stable growth of the transformants. Even after repeated inoculation to scale up the culture, it still exhibits stable productivity. Thus, this vector is especially suited to culture on large scale. The vector can be maintained only in *B. choshinensis* so that only *B. choshinensis* can be used as the host. The construction of expression plasmid must be constructed in one step using *B. choshinensis* as the host. Because the transformation efficiency must be sufficiently high, the use of *Brevibacillus choshinensis* Electro-Cells (10^5 transformants/ μg DNA) is recommended.

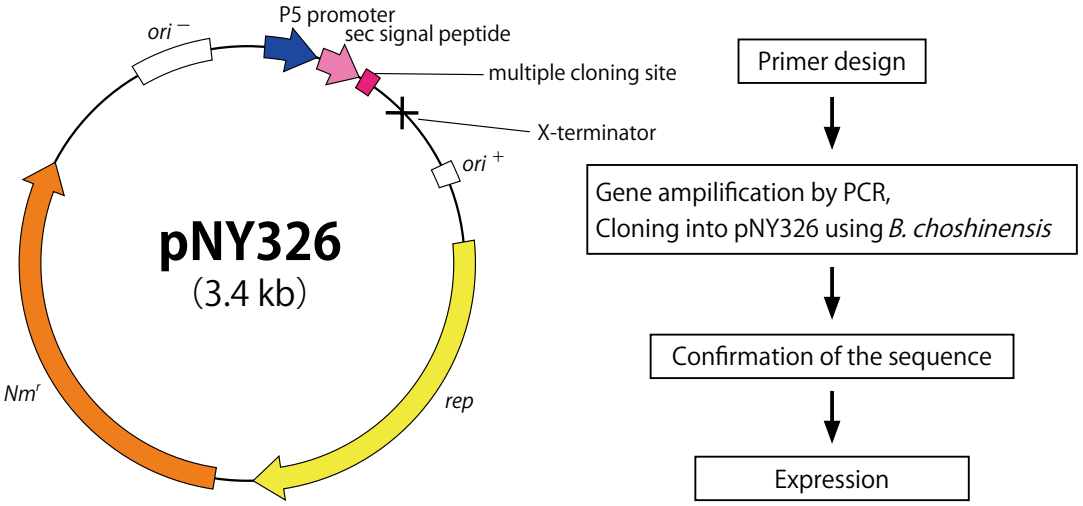
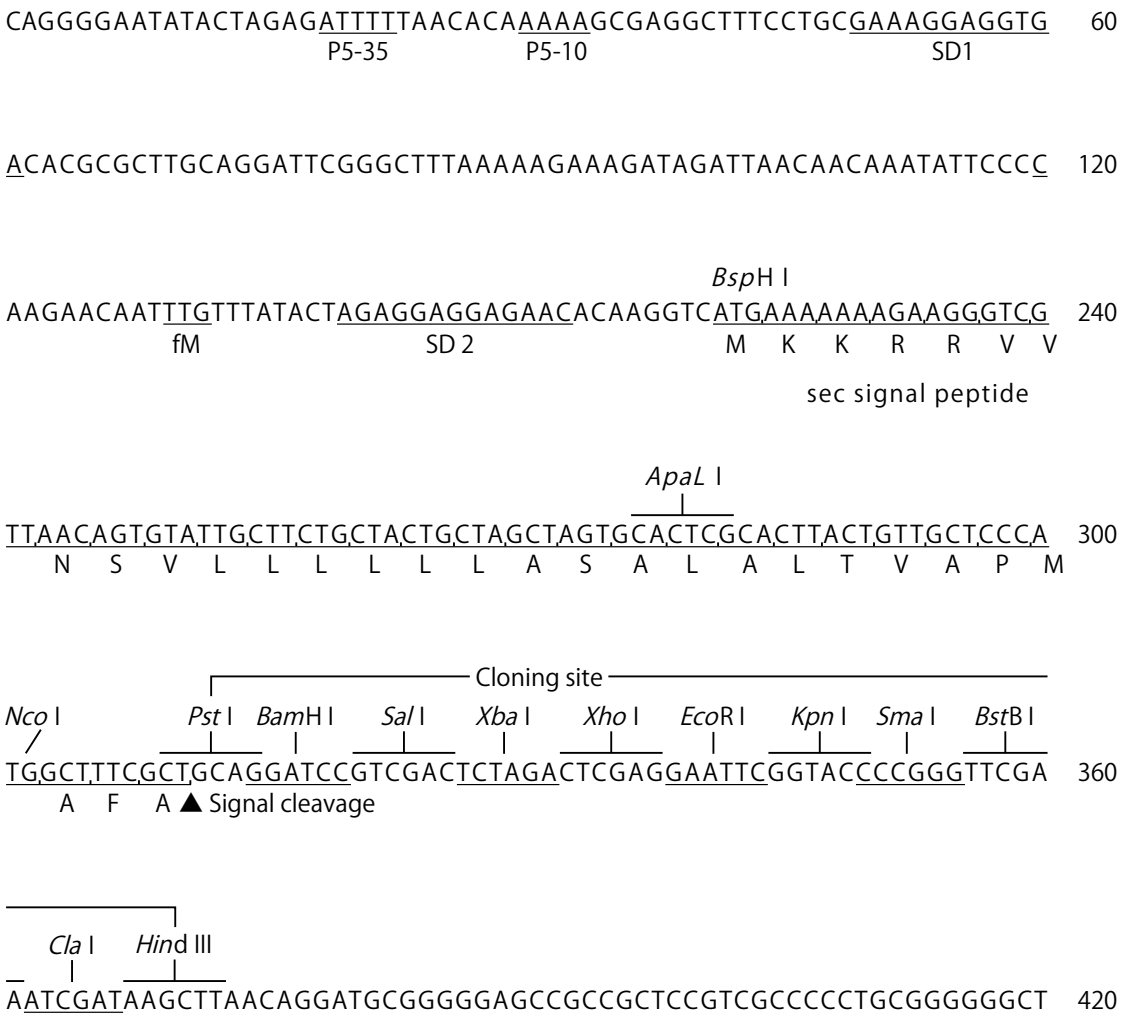


Fig 2. Map of pNY326

<Features of pNY326>

P5 promoter	A part of 5′ upstream region of cell wall protein (HWP) gene, which works as a relatively weak promoter in *Brevibacillus*.
Sec signal peptide	Secretion signal sequence of HWP is modified to increase the secretion efficiency.
Multiple cloning site	11 restriction sites allowing insertion of target gene into the vector
Terminator	26 bp nucleotide sequence functioning as a terminator is introduced downstream of multiple cloning sites
rep	Gene involving in plasmid replication (pUB110 derived)
Ori	Replication origin allowing replication and maintenance of the plasmid in *Brevibacillus* (pUB110 derived)
Nm^r	Neomycin resistance gene, working as selection marker in *Brevibacillus*

Nucleotide sequence of the region from promoter to multiple cloning site on pNY326



P5 promoter exerts weak promoter activity in *Brevibacillus*. There are 2 SD sequences which direct translation in the same frame.

IV-2. Cloning into expression vectors

The expression vectors have secretion signal sequence which derives from *B. choshinensis* cell wall protein gene. DNA fragment encoding the target protein should be cloned into the MCS which is located at the 3' end of the signal sequence. Using two restriction sites, one is on the signal sequence and the other on MCS, the gene of interest can be cloned into the vector with correct orientation.

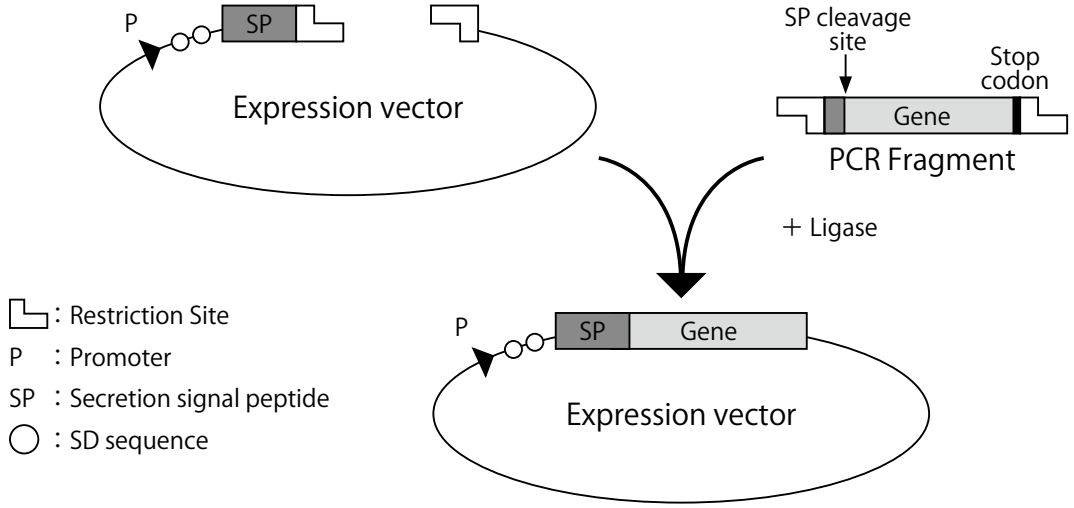


Fig. 3. Construction of *B. choshinensis* expression plasmid

IV-3. Transformation of *Brevibacillus*

The transformation of *B. choshinensis* cells can be done by electroporation. Selection marker is neomycin resistance. When the shuttle vector, pNCMO2, is used in combination with *E. coli* host, ampicillin resistance can be used as the selection marker.

If the electroporation device is not available, another transformation method "Tris-PEG method" is applicable. However, the transformation efficiency of "Tris-PEG method" is very low compared to electroporation so that in that case it is recommended to use the completed plasmid which is constructed using *E. coli* as a host.

IV-4. Detection of the product and scaling-up

Pick the transformant harboring the expression plasmid and culture it in the liquid medium. After 48-64 hrs' culture, prepare culture supernatant by centrifugation of the culture broth. An aliquot of the supernatant should be subjected to SDS-PAGE analysis to confirm the production of the target protein. If the production is not sufficient, please try scaling-up. Large scale culture of *B. choshinensis* is not difficult.

V. Procedure

V-1. *Brevibacillus* strains

*S*tandard genetic engineering technique is applicable.

V-1-1. Genotypes

An essential gene for spore formation cascade is disrupted in *B. choshinensis* SP3 so that sterilization of the transformants is easily done. Furthermore, the trace activity due to intracellular protease gene (*imp*) and extracellular protease gene (*emp*) is nullified by gene disruption to secure the intactness of protein products.

V-1-2. Control DNA

pNCMO2-BLA : Positive control plasmid for secretory production which have the gene encoding *Bacillus licheniformis* α-amylase. The size of the protein is about 55 kDa and secreted to the amount of 0.1 g/l or more.

V-1-3. Storage

Short term storage (about 1 week)

Pick a single colony and spread it on MTNm plate (see V-10. Medium components). Place it overnight in the incubator kept at 30℃. Seal the plate and store it at room temperature (about 20℃).

Note) Do not store plates in a refrigerator !

Long term storage (longer than 1 month)

Pick a single colony and inoculate it into 2SYNm medium (see V-10. Medium components). Culture it overnight on a shaker. Transfer the broth to a freezing vial and add equal amount of LB medium containing 40 % glycerol. Store it at − 80℃.

V-2. *E. coli* host strains

The nucleotide sequence encoding lac operator is inserted in pNCMO2 to weaken the promoter activity in *E. coli*. For that reason, F factor integrated strains like JM109 have to be used as host strains. The genotype of JM109 is shown below for reference.

JM109 : *recA*1, *endA*1, *hsdR*17 thi, *hsdR*17 (rk− mk+), *e*14− (*mcrA*−), *supE*44, *relA*1, △ (*lac-proAB*) /F′ [*traD*36, *proAB*+, *laclq*, *lacZ*△M15]

V-3. Construction of expression plasmid using pNCMO2

Procedure and precaution for the construction of expression plasmids using pNCMO2 as the vector.

E. coli strains having *laclq* and *recA*- like JM109 are recommended as the host.

Insert DNA should be cloned in the vector in-frame at the downstream of secretion signal. Stop codon must be introduced at the 3′ end of the gene.

If target protein is prokayotic secretory protein, better result may be obtained using the protein's own secretory signal. In that case, pNY326 is selected.

V-3-1. Cloning into pNCMO2

<Amplification of the gene by PCR>

PCR primers should be designed to clone target gene into the site downstream of the secretion signal. Select appropriate 2 restriction sites available on the expression vector and introduce them into the primers' sequences. Be sure the orientation is correct. Then perform the amplification of the gene by PCR. Please set up optimal condition for PCR according to the characteristics of the gene and PCR enzymes. It is recommended to use high-fidelity PCR enzymes (PrimeSTAR® HS DNA Polymerase (Cat.#R010A) etc.).

<Construction of expression plasmids via ligation>

Both the PCR product and the vector (0.5-1.0 μg) should be digested with 2 species of restriction enzymes. The DNAs are subjected to electrophoresis on agarose gel. Extract and purify DNAs from the pieces of gel.

For ligation reaction, use 100ng of each DNA. Ligation can be done using, for instance, DNA ligation Kit <MightyMix> (Cat.#6023). Use 1/5 of the ligation mixture for the transformation of *E. coli* and the rest for the transformation of *B. choshinensis* after purifying it by ethanol precipitation. As for the procedure thereafter, refer to the corresponding part of V-4. pNY326.

<Transformation of *E. coli*>

Use *E. coli* host of which transformation efficiency is high. It is recommended to use *E. coli* JM109 Competent Cells (Cat.#9052) or *E. coli* JM109 Electro-Cells (Cat.#9022).

V-3-2. Analysis of pNCMO2 recombinants

After spreading 100-200 μl of the transformed mixture onto LB plates containing 50-100 μg/ml ampicillin, incubate the plates at 37℃ for 15-18 hr. Select 10-20 ampicillin-resistant colonies and inoculate them into 2 ml of LB liquid medium containing 50-100 μg/ml ampicillin.

Grow for 15-18 hr at 37℃ with shaking and isolate plasmid DNA.

Perform restriction enzyme analysis using appropriate amount of DNA. Usually the enzymes which were used for the construction of expression plasmid are employed. Confirm the existence of the insert DNA with agarose electrophoresis. If the existence of the insert could be detected, perform sequencing to confirm that the gene has no error introduced by PCR and is in frame with signal sequence.

V-3-3. Sequencing

For sequencing, following sequences are used as the forward and the reverse primers (the primer sequences are common in both pNCMO2 and pNY326).

Forward Sequencing Primer : 5'-CGCTTGCAGGATTCGG-3'
Reverse Sequencing Primer : 5-CAATGTAATTGTTCCCTACCTGC-3'

V-4. Construction of expression plasmids using pNY326

Procedure and precaution for the construction of expression plasmids using pNY326 as the vector.

B. choshinensis Electro-Cells are recommended to use for the transformation when pNY326 is used as the cloning vector, because competent cells with high transformation efficiency are needed.

Insert DNA should be cloned in frame with the signal sequence on the vector. Stop codon should be introduced at the 3' end of the gene. If target gene is a bacterial gene encoding secretory protein, it is recommended to try the original secretion signal as well as *B. choshinensis* cell wall protein signal. In that case, the restriction site of 5' end for the insert DNA is *Bsp*H I site which covers the initiation codon of cell wall gene. If transformants can not be obtained using pNCMO2 or higher stability is required, pNY326 should be selected.

V-4-1. Cloning into pNY326

<Amplification of the gene by PCR>

PCR primers should be designed to clone target gene into the site downstream of the secretion signal. Select appropriate 2 restriction sites available on the vector and introduce them into the 5′ ends of the primers. Be sure the orientation is correct. Then perform the amplification of the gene by PCR. Please set up optimal condition for PCR according to the characteristics of the gene and PCR enzymes. It is recommended to use high-fidelity PCR enzymes (PrimeSTAR® HS DNA Polymerase (Cat.#R010A) etc.).

<Construction of expression plasmid via ligation>

Both the PCR product and the vector (0.5-1.0 μg) should be digested with 2 species of restriction enzymes. The DNAs are subjected to electrophoresis on agarose gel. Extract and purify DNAs from the pieces of gel.

For ligation reaction, use 100 ng of each DNA. Ligation can be done using eg. DNA ligation Kit <MightyMix> (Cat.#6023). Use the entire ligation mixture for the transformation of *B. choshinensis* after ethanol precipitation.

V-4-2. Transformation of *Brevibacillus*

Competent cell of high transformation efficiency should be used as the host (It is recommended to use *Brevibacillus choshinensis* Electro-Cells).

As for the method of transformation, refer to the section V-5 Transformation of *Brevibacillus*.

V-4-3. Analysis of pNY326 recombinants

(1) Pick 10-20 colonies on the MTNm plates at random and inoculate them into 2 ml TMN liquid medium.
(2) Culture for 15-18 hr at 37℃ with shaking. Isolate plasmid DNA. Usually 1-2 μg of DNA can be recovered.
(3) Carry out restriction enzyme analysis using appropriate amount of DNA. Usually the enzymes which were used in the preparation of the vector and the insert DNA for the construction of expression plasmid are used. Confirm the existence of the insert by electrophoresis on agarose.
(4) If the existence of the insert was detected, perform sequencing to confirm the gene has no error introduced by PCR and is in frame with signal sequence (refer to the section V-3-3 for the primers of sequence).

V-5. Transformation of *Brevibacillus*

V-5-1. Equipments and reagents required

Competent cells for electroporation (*Brevibacillus choshinensis* Electro-Cells) (on ice)
Expression plasmid to express your protein
Positive control (pNCMO2-BLA)
Negative control (pNCMO2 or pNY326)
MTNm plates
MT liquid medium
Cuvette for electroporation
Culture tube
Sterilized microtube

V-5-2. Transformation by electroporation

(1) Place the tube containing competent cells on ice
(2) Add 5 μl DNA solution (200-500 ng)* to the competent cells and mix well by flipping the tube (Never vortex!).
(3) Transfer the mixture to the electroporation cuvette. Store the cuvette on ice for 10 min.
(4) Pulse the cells under the following condition :
Electroporator : Gene Pulser II (Bio-Rad)
Charging voltage : 7.5 kV/cm
Capacitance : 25 μF
Resistance : 1000 Ω
Gap of the cuvette : 2 mm
Electroporator : Electroporator 2510 (Eppendorf)
Charging voltage : 14 kV/cm
Gap of the cuvette : 1 mm
(5) Immediately add 1 ml MT medium and then transfer the mixture into a culture tube
(6) Incubate the tube at 30℃ with shaking at 120 rpm.
(7) Spread 100 μl of the mixture onto a MTNm plate. Centrifuge the remaining mixture (5000 rpm, 5 min, room temp.) to concentrate the cells. Spread the whole amount of cells onto another MTNm plate.
(8) Incubate plates for 15-18 hrs at 37℃. If the sizes of the colonies are small, extend the incubation further.
(9) Obtained colonies can be subjected to plasmid check or protein expression analysis.

*When constructed plasmids as the control plasmids are used, the amount of DNA should be reduced to 10-100 ng.

V-6. Expression of proteins by *Brevibacillus* recombinants

After the construction of expression clone is completed, small-scale expression check can be carried out. The general protocol for confirmation of expressed protein is shown below.

V-6-1. Experimental outline

Once a positive clone has been identified, expression check can be carried out using positive and negative controls. Following recombinants are used as the controls.
B. choshinensis SP3/pNCMO2-BLA (Positive control for secretory expression)
B. choshinensis SP3/pNCMO2 or pNY326 (negative control)

The production of the protein is variable among colonies depending on the nature of proteins. Thus, 6-10 colonies should be picked at random (including both large and small colonies) to carry out expression check. If the plates are left over for several days, the potential of protein production may decrease. In that case, do transformation once more to obtain fresh transformants.

V-6-2. Culture media

TM and 2SY are recommended as the basic media for expression check. The protein productivity may vary depending on the medium. Because the balance between growth and protein productivity is an important factor for final production rate, it is recommended to try both the media.

V-6-3. Culture for secretory production

Check the expression condition by conducting the expression trial using positive control, *B. choshinensis* SP3/pNCMO2-BLA, which expresses *B. licheniformis* α-amylase (about 55 kDa). By culturing the transformant and the negative control simultaneously and performing the same processing, the expression of target protein can be confirmed. Expression protocol is as follows.

(1) Pick single colonies and inoculate them into 3 ml of 2SYNm and TMNm in culture tubes (16 mm in diameter). Grow them for 48-64 hrs at 30-33℃ with shaking at 120 rpm. During the culture, check the protein production every 24 hrs.
(2) After the completion of the culture, centrifuge the broth (5000×g, 5 min) to separate supernatant from cells. Cells should be resuspended in the same volume of PBS.
(3) Supernatant and cellular fraction should be subjected to SDS-PAGE analysis (CBB staining or western blotting) or activity measurement.

V-7. SDS-PAGE analysis

Protocol for SDS-PAGE analysis of the protein is as follows.

V-7-1. Sample preparation

Add 10 μl of 5XSDS-PAGE loading buffer to 40 μl of culture supernatant and the cell suspension.
Mix and boil for 10 min to make samples for electrophoresis.

V-7-2. Control

Use following samples as the control.
a. molecular size marker
b. standard of your protein
c. sample from background control (prepared from the culture of the cells harboring only vector)
d. sample from positive control (prepared from the culture of *B. choshinensis* SP3/pNCMO2-BLA which secretes *B. licheniformis* α-amylase)

V-7-3. Analysis of expressed proteins

It can be confirmed to produce target protein by comparing the position and the density of the specific band with those of the standard protein, which was electrophoressed in parallel with the protein by SDS-PAGE. If the protein production is low, the product is insoluble, or the product is masked by other protein, the protein detection by SDS-PAGE may be difficult. In those cases, western blot analysis using specific antibody, functional evaluation (e.g. specific activity), or protein purification by special technique may be useful for detection of protein production.

V-8. Optimization of protein expression

In this system, protein production level is 100 mg/l or more, though the production exceeded 1 g/l in some of the cases. If the productivity of target protein is substantially low or no product can be detected, refer to the guideline described below.

V-8-1. In the case of low expression

a) Try using both the vectors, pNCMO2 and pNY326. The former has stronger promoter activity but does not always exhibit higher productivity. Sometimes pNY326 is the better choice because of the low promoter activity which reduces the growth stress caused by the extremely efficient production of proteins.
b) Select another type of culture medium. The productivity changes substantially depending on the composition of culture media.
c) Compare the plasmid copy number with the control. If remarkable reduction of the copy number is detected, use pNY326 as a vector, change the culture medium, or increase the concentration of the antibiotic (400 mg/l).
d) There is the case that the protein is not suitable for secretory production. In that case, try intracellular production.
   If the production is little, if any, try to produce the protein intracellularly.

V-8-2. In the case of no expression

It is recommended to do the same trial as that described in the V-8-1, "In the case of low expression". If no improvement is obtained, following possibilities are conceivable.

a) Check the secondary structure of mRNA. A strong palindrome structure in the sequence may occur bad effect on the efficiency of translation. In that case, change the sequence to remove the stacking region.
b) If the amino acid sequence around the signal cleavage site is improper, it may exert some influence on the secretion of the protein. In that case, try to introduce some tag sequences for purification or detection to the N-terminal of the protein, if the addition of some extra amino acids produces no substantial effect on its activity.

V-9. Purification of expressed proteins

Purification methods are variable according to the target proteins. If the protein is secreted, the proteins can be easily obtained from supernatant by removing the bacteria, allowing easier purification. After this step, continue purification with ordinary methods (e.g. ion exchange, hydrophobic, affinity chromatography etc.).

V-10. Medium components

2SY liquid medium

Components :

Glucose	20.0 g/L
Bacto Soytone (Becton Dickinson)	40.0 g/L
Bacto Yeast Extract (Becton Dickinson)	5.0 g/L
$CaCl_2 \cdot 2H_2O$	0.15 g/L

＊Autoclave the mixture of glucose and $CaCl_2$ separately from the rest of the components. Mix them after autoclaving.

2SY Nm liquid medium

Add neomycin solution (stock sol. 50 mg/ml) to 2SY liquid medium to the concentration of 50 μg/ml.

TM liquid medium

Components :

Glucose	10.0 g/L
Polypeptone	10.0 g/L
Meat extract	5.0 g/L
Yeast extract	2.0 g/L
$FeSO_4 \cdot 7H_2O$	10 mg/L
$MnSO_4 \cdot 4H_2O$	10 mg/L
$ZnSO_4 \cdot 7H_2O$	1 mg/L

Adjust the pH to 7.0

＊Autoclave glucose separately from the rest of the components. Mix them after autoclaving.

TM Nm liquid medium

Add neomycin solution (stock sol. 50 mg/ml) to TM liquid medium to the concentration of 10 μg/ml.

MT liquid medium

Components :

Glucose	10.0 g/L
Polypeptone	10.0 g/L
Meat extract	5.0 g/L
Yeast extract	2.0 g/L
$FeSO_4 \cdot 7H_2O$	10 mg/L
$MnSO_4 \cdot 4H_2O$	10 mg/L
$ZnSO_4 \cdot 7H_2O$	1 mg/L
$MgCl_2$	4.1 g/L

Adjust the pH to 7.0

＊Autoclave glucose separately from the rest of the ingredients. Mix them after autoclaving.

MTNm plates

Add 7.5 g of agar to 500 ml MT liquid medium and autoclave. Allow the medium to cool to about 50℃ before adding neomycin solution (stock sol. 50 mg/ml) to the concentration of 10 μg/ml. Mix the medium by swirling and dispense it to plates.

VI. Appendix

VI-1. Transformation of *Brevibacillus* by Tris-PEG method

Highest efficiency of transformation can be obtained by electroporation but if the necessary device is not available, transformation can be done by Tris-PEG method. However, the transformation efficiency is as low as 10^1-10^2 transformants/μg plasmid DNA so that the use of the method should be limited to the case that premade pNCMO2-based expression plasmid is used.

VI-1-1. Preparation of solutions

Phosphate buffer : dissolve 1.905 g of KH_2PO_4 and 0.852 g of Na_2HPO_4 into distilled water to make 100 ml solution.
PEG solution : dissolve 40 g of PEG6000 into 50 ml of phosphate buffer. Add H_2O up to 100 ml.
TP medium : Mix phosphate buffer with the same volume of concentrated (×2) TM medium.
MT medium : TM medium containing 20 mM $MgCl_2$.
50 mM Tris-HCl buffer (pH7.5)
50 mM Tris-HCl buffer (pH8.5)
TE buffer : 10 mM Tris-HCl (pH7.5), 1 mM EDTA

VI-1-2. Transformation

(1) Inoculate *Brevibacillus* cultured overnight cultured into 5 ml TM medium and continue the culture on a shaker.
(2) Collect the cells in log-phase (OD_{660} = 2.0-3.0) by centrifugation (5,000×g, 5 min., at room temp.) using 50 ml tube.
(3) Rinse the cells with 5 ml, 50 mM Tris-HCl buffer (pH7.5) and collect them by centrifugation.
(4) Suspend the cells in 5 ml, 50 mM Tris-HCl buffer (pH8.5) and shake them on a shaker for 60 min. at 37℃.
(5) Collect the cells by centrifugation (5,000×g, 5 min, at room temp.) and suspend them in 0.5 ml TP medium.
(6) Prepare plasmid solution containing about 1 μg DNA in 50 μl TE buffer plus 50 μl TP medium. Add the solution to the cell suspension and mix them. Add 1.5 ml PEG solution and mix them quickly.
(7) Shake it for 10 min at 37℃ and then collect the cells by centrifugation (3000×g, 10 min, at room temp).
(8) Add 1 ml MT medium to suspend the cells and shake it for 1 hr at 37℃.
(9) Spread 0.1 ml/plate of the suspension onto MTNm plate and incubate them overnight at 37℃. Pick the colonies resistant to Nm and analyze them afterward. (as for the procedure after this step, refer to "V-4-3. Analysis of pNY326 recombinants").

VII. References

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VIII. Related products

E. coli JM109 Competent Cells (Cat.#9052)
E. coli JM109 Electro-Cells (Cat.#9022)
DNA Ligation Kit <Mighty Mix> (Cat.#6023)
PrimeSTAR® HS DNA Polymerase (Cat.#R010A)
pNC-HisT DNA (Cat.#HB121)
pNC-HisF DNA (Cat.#HB122)
pNC-HisE DNA (Cat.#HB123)
pNI DNA (Cat.#HB131)
pNI-His DNA (Cat.#HB132)

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These products (Cat.#HB100 and Cat.#HB115) include a genetically "Living Modified Organism (LMO)" defined in "The Cartagena Protocol on Biosafety". The supplied *Brevibacillus choshinensis* Electro Cells in these kits contain a part of 2 μm plasmid DNA derived from *Saccharomyces cerevisiae*.

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