Expression of Streptolysin O Gene in Bacillus subtilis

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Streptolysin O (SLO) is a hemolytic, extracellular protein produced by Streptococcus pyogenes. A hybrid gene consisting of the promoter and signal sequence fused to the region encoding the mature sequence of the slo gene was constructed to secrete SLO in Bacillus subtilis. To increase secretion of SLO into the culture supernatant, several SLO expression vectors containing various combinations of promoters and pre-pro sequences were constructed. B. subtilis harboring pP4 consisting of the P-43 promoter and the coding sequence of the pre-region of the alkaline protease gene that was fused to the pro-mature region of the slo gene secreted SLO into media. The degree of hemolytic activity was found to be about 40-fold higher in the genetically engineered B. subtilis strain than that of S. pyogenes. Recombinant SLO was reacted with patients' sera infected by group A streptococci.

Streptococcus pyogenes produces a thiol-activated cytolytic toxin, known as streptolysin O (SLO). Clinically, the measurement of antibodies to SLO has also been used to diagnose the nonsuppurative complications of group A beta-hemolytic streptococcal infections. Several methods have been developed to detect the anti-SLO titers in patients' sera, including the Rantz-Randall method, latex agglutination test, enzyme-linked immunosorbent assay, and turbidimetric immunosassay. Partially purified culture supernatants from S. pyogenes are used as a source for anti-SLO detection systems. Because of the poor molecular biological knowledge in S. pyogenes, the enhancement of the SLO production in S. pyogenes seems to be difficult. Kehoe et al. reported the cloning of the slo gene in E. coli, but the production of SLO in this host was rather low. They also reported that SLO has 571 amino acids (M, 63,645) and a pre-pro type enzyme.

For secretion of SLO, we have selected a Gram-positive bacterium, Bacillus subtilis, which undoubtedly represents an interesting host for genetic engineering because the genetical, physiological, and biochemical properties of this bacterium have been well documented. Moreover Bacillus subtilis has been genetically engineered to secrete high levels of bacterial proteins, protein A and \( \beta \)-lactamase. We describe here the construction and use of a series of plasmids containing various promoters and pre-pro sequences combinations fused to the region encoding the mature sequence of the slo gene. Recombinant SLO produced extracellularly by B. subtilis had the antigenicity and hemolytic activity of the original.

Materials and Methods
Bacterial strains and plasmids. Bacillus subtilis DB403 (trpC2, Δ aprE1, ΔrepE1, ΔrepI) was used as a host strain for SLO expression. Plasmid pUB110, which carried a kanamycin resistance gene and a unique BamHI site, has already been reported. Streptococcus pyogenes ATCC 10389 was used as the source for SLO and chromosomal DNA.

DNA manipulations. Plasmids preparations were made by the rapid alkaline sodium dodecyl sulfate method. Total DNA of S. pyogenes was extracted as described previously. Restriction enzymes and DNA modification enzymes were purchased from Toyobo Co., Ltd. (Osaka, Japan) and Takara Shuzo Co. (Kyoto, Japan) and were used as recommended by manufacturers. The other DNA manipulations were done by the standard methods described by Sambrook et al. Transformation. B. subtilis competent cells were prepared by the procedure of Spiizioni. Strains harboring SLO expression vectors were selected by a halo of lysis on blood agar plates as in the procedure of Kehoe et al. with a modification, kanamycin (5 µg/ml) being used instead of ampicillin. E. coli competent cells were purchased from Takara Shuzo Co.

Growth conditions. B. subtilis was grown on 4L-broth (4% tryptone, 2% yeast extract, 0.5% NaCl) with kanamycin (5 µg/ml). S. pyogenes was grown for 15 h at 30°C on Todd–Hewitt broth (Difco Laboratories, Detroit, U.S.A.) with 1% tryptone and 1% yeast extract.

PCR amplification. Amplification of the target DNA fragment was done with 30 cycles of 1 min at 92°C, 1 min at 55°C, and 2 min at 72°C.

Construction of SLO expression vectors. A pair of slo structure-specific primers: 5'-GAATTCCTGAATGATGTCGAGAAGACAAAC (N-SLO1) and 5'-GAATTCAGCTCACTTATCAAGTAACTC (C-SLO) was designed. These primers were used to generate a 1.8-kb EcoRI PCR fragment encoding pre-pro-mature SLO from S. pyogenes genomic DNA. The fragment was subcloned into the 4g10 EcoRI site. Two pUC19-derived vectors that contained the N-terminal and C-terminal regions of slo were constructed, because the intact slo gene subcloned into a high copy number plasmid was highly unstable. The 0.6-kb EcoRI–Eco1019I fragments of slo N terminal region was ligated to EcoRI- and Eco1019I-digested pUC19 to generate pUCSLO. The 1.2-kb Eco1019I–EcoRI fragment of slo C terminal region was ligated to Eco1019I- and EcoRI-digested pUC19 to generate pUCSLOC. The 0.2-kb BamHI–HpaI fragment carrying the spoO promoter from pSPTA51 was inserted into the BamHI–Smal site of pUCSLO to generate pOSLO. For pUBSLO construction, the 0.8-kb pOSALO BamHI–Eco1019I fragment and the 1.2-kb pUCSLOC Eco1019I–BamHI fragment were inserted into the BamHI site of pUB110.

The 5' end of the primer of the slo pro region, which contained a XbaI site, was designed. The amplified fragment digested with XbaI and Eco1019I was inserted into the XbaI–Eco1019I site of pUC18 to generate pSLON. A pair of apr promoter-pre-pro region specific primers was designed:

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Abbreviations: SLO, Streptolysin O; slo, gene coding for streptolysin O; apr, gene coding for alkaline protease.
the alkaline phosphatase-conjugated goat anti-human IgG (VECTOR, CA, U.S.A.) was added to know the secondary binding process.

**Enzyme immunoassay.** The culture supernatant was diluted to 1/500 with 0.1% Tween 20 in phosphate-buffered saline (PBS, Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), and 100 μl was distributed in 96-well microplates. After 1 h of incubation at 37°C, the plate was washed three times with 0.1% Tween 20 in PBS (TWEEN PBS). TSB (50 mM Tris, 150 mM NaCl, pH 7.4) containing 5% skim milk (w/v) was added and incubated for 1 h at 37°C. After three washes with TWEEN PBS, 100 μl of the sera of healthy donors and patients (diluted to 1/750) were added. The plate was incubated for 1 h at 37°C and washed three times with TWEEN PBS before 100 μl of the alkaline phosphatase-conjugated goat anti-human IgG (VECTOR, CA, U.S.A.) was added and incubated for an hour at 37°C. A color-developing reaction mixture was finally added as per the manufacturer's instructions. The alkaline phosphatase activity was measured by absorbance at 490 nm using a microplate reader (Bio-Rad Laboratories, CA, U.S.A.).

**Results and Discussion**

**Vector constructions.** To obtain secretion of large amounts of SLO into the medium, several SLO expression vectors were constructed. These SLO expression vectors contained the SLO expression cassette in the BamHI site of pUB110; the SLO expression cassette consisted of a promoter, ribosome-binding sequence, pre-pro sequence, and the mature SLO coding sequence. Figure 1 shows the SLO expression vectors with combinations of various promoters, SD sequences, and pre-pro sequences. Three well-characterized promoters that were active during vegetative growth or sporulation phase were used. The alkaline protease gene, apr, is expressed during the early stage of sporulation. An early sporulation-regulatory gene, spoA, had two promoters, a vegetative promoter and a sporulation-specific promoter. The P-43 promoter is a strong vegetative promoter. The SD sequences of slo, apr, and P-43 were used. The apr and slo pre-pro sequences were used for the secretion of SLO. Kimura et al. showed the deletion of the apr pro sequence yielded a mature but inactive alkaline protease. They proposed that the apr pro sequence was essential for guiding appropriate folding of the enzymatically active conformation of alkaline protease. However the slo pro sequence has not been studied yet.

**Hemolytic activities of B. subtilis strains harboring several SLO expression vectors.** The hemolytic activity of B. subtilis harboring several SLO expression vectors grown at 37°C in 4L-broth was measured. The culture supernatant were harvested at 1-h intervals. Figure 1 also shows hemolytic activity of B. subtilis harboring several SLO expression vectors. The hemolytic activity of S. pyogenes was 206. The culture supernatants from all B. subtilis strains harboring SLO expression vectors showed higher hemolytic activity than the culture supernatant of S. pyogenes. This indicates B. subtilis harboring SLO expression vectors produces and excretes SLO into the medium more than S. pyogenes. When the promoter was spoOA in various vectors, the culture supernatants from B. subtilis harboring pOAS (which contains apr in a presequence) was more highly hemolytic than that of pUBSLO (which contains slo in the presequence, Fig. 1). In addition, when the presequence had apr and the prosequence had slo with a spoOA promoter (pOAS.A.A, Fig. 1), the SLO secretion is slightly more than that of the pOAS vector that has the apr-slo pro sequence.
Concerning various promoters having the same pre-pro sequence, B. subtilis harboring pOAS containing the spoOA promoter is more highly hemolytic than that of pAS containing the apr promoter (Fig. 1). This indicates that the spoOA promoter is suitable for SLO expression. The apr and spoOA promoters were reported to be expressed mainly during sporulation phase, hence the hemolytic activity of B. subtilis was measured after growing in 2 × SG sporulation medium. 23) The culture of 4L-birth grown B. subtilis harboring pAS or pOAS showed 2-fold higher hemolytic activity than that of a 2 × SG sporulation- medium-grown culture (data not shown). Furthermore, the culture supernatants from B. subtilis harboring pPA containing the P-43 promoter showed higher hemolytic activity than pOAS, A containing the spoOA promoter (Fig. 1). Thus, the combination of P-43 promoter-SD sequence and the coding sequence of apr-pre region fused to a slo pro-mature region showed the highest hemolytic activity of all the SLO expression vectors (Fig. 1).

Next, the effects of temperature on the SLO secretion was examined using B. subtilis harboring pPA. The hemolytic activities of 30℃-grown cells and 37℃-grown cells were 8745 and 1829, respectively. The culture supernatant of 30℃-grown cells had 4–5 fold higher hemolytic activity than that grown at 37℃. The result has at least two possible interpretations. The protease secreted by B. subtilis was assumed to be higher at 37℃ than at 30℃, which might have been degraded the secreted SLO. In addition, it is presumed that secretion and expression efficiencies of SLO might be temperature-dependent. The hemolytic activity of the culture supernatant from B. subtilis harboring pPA grown at 30℃ was about 40-fold higher than that of S. pyogenes. Our host strain DB403, which is lacking in neutral, alkaline, and extracellular protease activities, still contains at least three extracellular proteases. 24) Our unpublished results showed higher hemolytic activity by using a 3 extracellular proteases-deficient mutant than a 2 extracellular proteases-deficient mutant. Therefore, using a strain that is deficient in six extracellular proteases for SLO expression host might show higher hemolytic activity than the parent DB403.

**Immunoblot analysis**
Immunoblot analysis was done using the culture supernatants from S. pyogenes and B. subtilis harboring pUB110, pUBSLO, pAS, pOAS, and pPA. Figure 2 shows that secreted recombinant SLO from B. subtilis harboring pUBSLO and pPA have the same molecular mass as that of S. pyogenes. No comparable bands were seen in pUB110 control lanes. A serum from a healthy person who was not infected by streptococci did not react with any proteins in the culture supernatant of S. pyogenes and B. subtilis harboring pPA (data not shown). Recombinant SLO
produced by *B. subtilis* harboring pAS and pOAS had a molecular mass higher than native SLO produced by *S. pyogenes* and *B. subtilis* harboring pUSBLO and pPA. Possible explanations for this observation are *B. subtilis* harboring pAS and pOAS accumulate a fusion protein consisting of the pro peptide of alkaline protease and the pro-mature peptide of SLO into medium. *S. pyogenes* and *B. subtilis* harboring pUSBLO and pPA accumulate pro-mature peptide of SLO in the medium. Several bands in each lanes can be observed. The reason for the low accumulation of secreted proteins in the culture supernatant had generally been ascribed to the action of a protease from the host strain. Bhakdi *et al.*\(^\text{16}\) reported two hemolytically active forms of SLO (69 and 57 kDa) and one form derived from partial proteolytic degradation in *S. pyogenes*. Though the molecular size of the pro-SLO was calculated to be 60,151 from DNA sequence, immunoblot data showed a molecular mass higher than that. Similarly, the molecular weight of recombinant SLO from pAS and pOAS was calculated to be 69,161 from DNA sequence, but immunoblot data showed 80 kDa.

**Enzyme immunoassay using normal and patients’ sera**

The sera of 10 patients and 10 normal donors reacted with the culture supernatant of *B. subtilis* harboring pPA (Fig. 3). Statistical analysis by t-test, showed the difference in both patients and the normal group is significant ($p < 0.01$). Even when the sera of healthy donors and patients were diluted to 1/1500, the difference was significant ($p < 0.01$, data not shown). The result of the enzyme immunoassay suggested that antibodies in sera of patients infected by group A streptococci reacted with recombinant SLO.

To summarize, an effective production system for SLO by *B. subtilis* was proposed by a recombinant technique. Higher production of SLO in *B. subtilis* might be useful in clinical investigations that includes the ELISA system and other anti-SLO detection systems.

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**References**