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Screening and Characterization of Secretion Signals from *Lactococcus lactis* ssp. *cremoris* LM0230

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Abstract A secretion signal sequence-selection vector (pGS40) was constructed based on an α-amylase gene lacking a secretion signal and employed for selecting secretion signals from *Lactococcus lactis* ssp. *cremoris* LM0230 chromosomal DNA. Six fragments were identified based on their ability to restore α-amylase secretion in *E. coli*, and among these, a fragment, S405, conferred the highest secretion activity (84%) in *E. coli*. Meanwhile, S407, which conferred poor secretion activity in *E. coli*, was quite active in *L. lactis*. The results suggested that the efficiency of a secretion signal depended on the host. All six fragments had an open reading frame (ORF) fused to the reporter gene, and the potential Shine-Dalgarno (SD) sequence and putative promoter sequences were located upstream of the ORF. Deduced amino acid sequences from the six fragments did not show any homology with known secretion signals. However, they contained three distinguished structural features and cleavage sites, commonly found among typical secretion signals. The characterized secretion signals could be useful for the construction of food-grade secretion vectors and gene expression in LAB.

Key words: Signal-sequence selection vector, secretion signal, *Lactococcus lactis* ssp. *cremoris* LM0230, α-amylase

Lactic acid bacteria (LAB) such as members of the genera *Lactococcus* and *Lactobacillus* are most commonly given generally recognized as safe (GRAS) status, and have been known as important microorganisms for the food industry as starter cultures for fermented foods, food preservatives, and flavor enhancers. In addition to these traditional roles, novel applications of LAB are rapidly expanding to the biotechnology area, such as production of heterologous proteins using LAB as hosts [6, 7]. Strong promoters and efficient secretion signals are requisites for overexpressing heterologous genes in LAB. There have been some studies conducted on screening and characterization of strong promoters from LAB, and as a result, a few strong promoters have been reported [3, 12]. LAB are known to secrete few proteins into the medium. The best known protein is PrtP, which is associated with cell wall in lactococci [5], and it is essential for providing an efficient growth in milk. Another protein is Usp45 [11] with unknown function.

The main mechanisms of protein secretion in bacteria can be divided into two. The general secretory pathway, by which proteins with a consensus N-terminal signal sequence are translocated, is called Sec-dependent secretion. The Sec-dependent secretion system requires a variety of proteins such as SecA, SecY, and SecE. The other secretion system is not dependent on the Sec proteins, but involves dedicated transmembrane translocators belonging to the family of the ABC-proteins. Proteins which are transported via the Sec-independent pathway do not contain the consensus N-terminal signal sequence found in proteins transported via the Sec-dependent pathway [3].

In bacteria, most proteins secreted via the Sec-dependent pathway are synthesized as precursors consisting of the mature proteins and N-terminal secretion signals [16]. Although the primary sequences are poorly conserved, all secretion signals display three distinguished domains in common: a positively charged amino-terminus (N-region, 5–8 amino acids); a hydrophobic core (H-region, 8–15 amino acids); a net hydrophilic profile (C-region, 5–8 amino acids) containing the cleavage site for signal peptidase [14-16]. The secretion signals of Gram-positive bacteria are longer than those of the Gram-negative

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bacteria [16]. Therefore, a secretion signal from a Gram-negative organism may not be able to direct an efficient secretion of a protein in a Gram-positive host [1, 10].

As an effort to develop food-grade expression/secretion vectors, a secretion-signal selection vector was constructed in this study. Secretion signals from *Lactococcus lactis* LM0230 were isolated using the vector, and the selected secretion signals were characterized.

Construction of Secretion-Signal Selection Vector

To screen secretion signals functioning in lactococcal hosts for the secretion of heterologous proteins, a secretion-signal screening vector pGS40 (KCTC accession No. 10517BP) containing a reporter gene was constructed (Fig. 1). The reporter gene, the α-amylase gene from *Bacillus licheniformis* (NCBI accession No. X03236) lacking the initiation signals for transcription and translation along with the secretion-signal sequences, was introduced into a broad-host-range lactococcal expression vector, pMG36e [12], at *Pst*I and *Hin*dIII restriction sites after PCR amplification. Plasmid pMG36e contains the broad-host-range replicon of plasmid pWV01 derived from *Lactococcus lactis* ssp. *cremoris* Wg2, thus it replicates in various Gram-positive and negative bacteria including *E. coli* [2]. In addition, since promoter P32 contained in plasmid pMG36e is functional in both *E. coli* and *Lactococcus lactis*, it renders easy screening of secretion signals in the *E. coli* host system even when promoterless fragments were inserted [13]. Using the finally constructed secretion-signal selection vector pGS40 comprising of pWV01 replicon, promoter P32, and erythromycin resistance gene, it becomes possible to compare the efficiencies of cloned secretion signals in different hosts without subcloning.

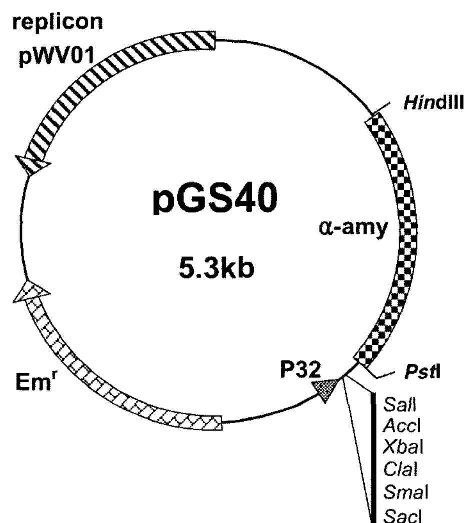

Fig. 1. Construction of the secretion-signal selection vector, pGS40.

α-Amylase gene (from *B. licheniformis*) lacking a secretion signal was inserted into *Pst*I and *Hin*dIII sites on pMG36e. Plasmid pGS40 contains P32 promoter from *L. lactis* MG1363 upstream of the truncated α-amylase gene, and the replication origin is derived from the broad-host-range plasmid, pWV01. Plasmid pGS40 contains the erythromycin resistance marker from plasmid pE194 [4].

Screening of Secretion Signals from *Lactococcus lactis* ssp. *cremoris* LM0230 Chromosomal DNA

Screening of secretion signals was carried out in an *E. coli* system. Chromosomal DNA from *L. lactis* ssp. *cremoris* LM0230 was digested with restriction enzyme *Pst*I. The digested fragments were ligated with pGS40, and the ligation mixture was introduced into *E. coli* MC1061 competent cells. α-Amylase-secreting transformants were identified after iodine staining (0.3% I_2+0.6% KI) of LB plates containing erythromycin (200 μg/ml) and 1% soluble starch. Clones with amylolytic activity formed clear halos around the colonies (Fig. 2A). Six recombinant plasmids carrying

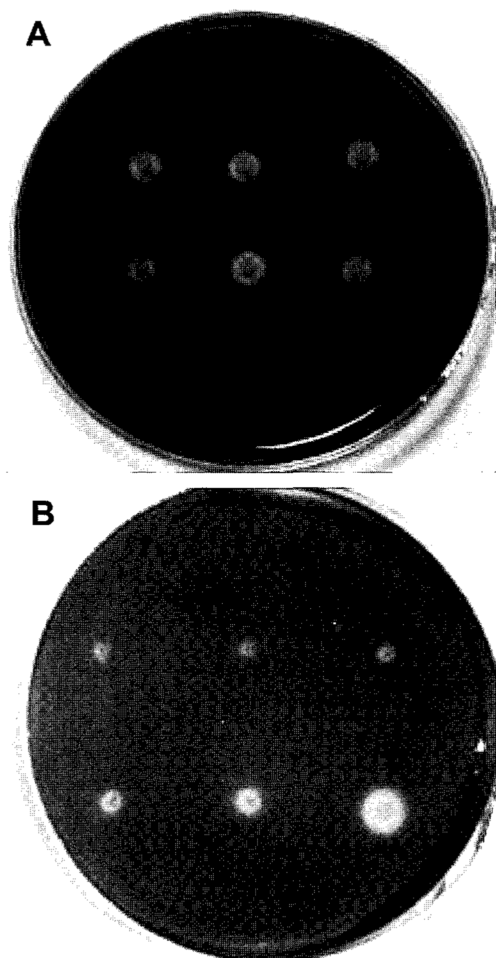

Fig. 2. The α-amylase activities of the *E. coli* and *L. lactis* transformants containing plasmids with screened putative secretion signals.

Colonies were stained with iodine solution to expose halos. A, *E. coli* MC1061; B, *L. lactis* MG1363. The first row, from left to right; S401, S402, and S404, the second row; S405, S406, and S407.

*Pst*I-digested fragments with 1- 5 kb size (S401, 402, 404, 405, 406, and 407) were obtained (data not shown).

Secretion Efficiency of Cloned Fragments in *E. coli*

To measure the secretion efficiency of the selected secretion signals, *E. coli* cells were grown in LB broth, harvested, washed, and resuspended in 0.1 M of potassium phosphate buffer (pH 7.5). Cell suspension was ultrasonicated and centrifuged. After recovering the supernatant (i.e., intracellular fraction), the soluble periplasmic fraction was prepared as follows by applying the method of Witholt *et al.* [17]. The cell debris treated with 5 mg/ml of lysozyme for 30 min at 37°C in 0.1 M of potasium phosphate buffer (pH 7.5) supplemented with 10 mM of EDTA and 0.3 M of sucrose was centrifuged, and the periplasmic fraction released from the disrupted cell wall part of the cell debris was obtained as the supernatant from the centrifugation. α-Amylase activity of each fraction was determined by measuring the amount of reducing sugars released by the dinitrosalycylate (DNS) method [6]. Secretion efficiency is defined as: Secretion efficiency (%)=(α-amylase activity of periplasmic fraction)/(total α-amylase activity of extracellular, periplasmic, and intracellular fractions)x100.

The efficiencies of secretion signals were in the range of 12 to 84% (Table 1). Among the six fragments, S405 showed the highest secretion efficiency (84%). SDS-PAGE of protein extract combined with activity staining showed that a 55-kDa band from *E. coli* MC1061 cells harboring S405 corresponded to α-amylase, which insinuated that S405 successfully directed the secretion of α-amylase in *E. coli.*

Secretion Efficiencies of Cloned Fragments in *L. lactis*

To investigate the efficiency of secretion signals (S401, 402, 404, 405, 406, and 407) in lactococci, the plasmids containing the secretion signals were introduced into *L. lactis* ssp. *cremoris* MG1363 by electroporation. After transformation, the colonies were cultured on M17 agar medium containing erythromycin (5 μg/ml) and 1% soluble starch. A transformant containing the S407 fragment which showed poor secretion efficiency in *E. coli*, made a clear halo around its colony after the iodine staining (Fig. 2B). This result suggested that the efficiency of a secretion signal could be different depending on the host.

To confirm the secretability of S407 in *L. lactis*, the recombinant cells containing S407 were grown in M17 broth medium for 18 h at 30°C, then both the intracellular (including periplasmic) and extracellular α-amylase activities were assayed. The culture broth was centrifuged, and the supernatant and pellet were separated. The supernatant was used to determine the extracellular enzyme activity. The pellet was suspended in 0.1 M of potassium phosphate buffer (pH 7.5), ultrasonicated, and centrifuged to obtain the intracellular fraction. α-Amylase activities of the extracellular and intracellular fractions were measured by the DNS method. Since the α-amylase activity of *L. lactis* containing S407 was too low to measure, extracellular proteins of the two recombinant *L. lactis* strains harboring S407 and a negative-control plasmid pGS40 in broth were concentrated by ammonium sulfate precipitation. The α-amylase activity from the extracellular fraction of S407 was 73.7 U/mg protein. However, the α-amylase activity of the extracellular fraction from pGS40-containing *L. lactis* was still undetectable after the protein concentration. These results imply that S407 is capable of secreting the fused α-amylase, although the intracellular enzyme activity was too low to measure and this is possibly due to the low strength of the promoter in S407.

Table 1. Influence of the secretion signals on the α-amylase activities of *E. coli* transformants.

Secretion signal	α-Amylase activity (U/ml) Total fraction	Periplasmic fraction	Secretion efficiency (%)
S401	2.9	2.0	69
S402	2.8	1.8	64
S404	23.6	4.2	18
S405	2.5	2.1	84
S406	23.7	5.0	21
S407	22.6	2.8	12

One unit of amylase activity is expressed by the amount of enzyme that produces 1 mol of glucose from soluble starch at 60°C for 1 min.

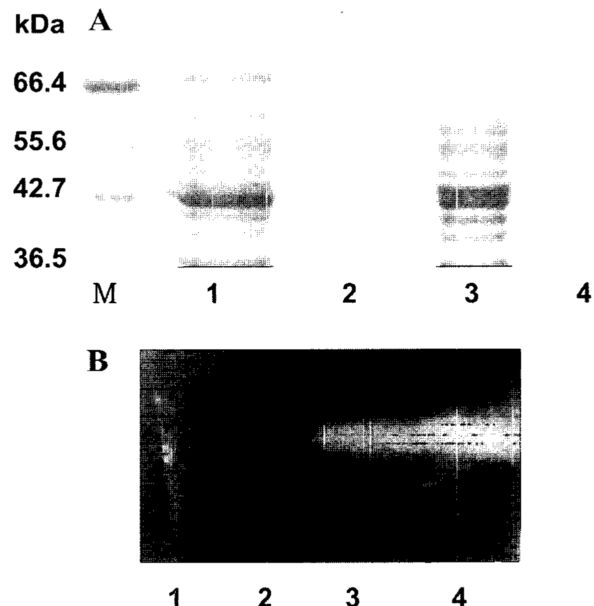

Fig. 3. SDS-PAGE of protein extracts from *E. coli* MC1061 cells harboring S405.

A, Coomassie brilliant blue staining; B, active staining. Lanes M, molecular weight marker; 1, total fraction of MC1061; 2, periplasmic fraction of MC1061; 3, total fraction of MC1061 harboring S405; 4, periplasmic fraction of MC1061 harboring S405.

S405

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AGACCATCAAAAACAAGTTGATAAATGGTTTAAATAATAAGATTATTATT

AACTTAAAAAAACTTTCTGACATTATAGTTAGAAGGTTTTTTATTATTTC

ATTCTAAGTTTATTATTTTTAGATTTATAACATTTATGTTAAAATATGCT

TGTAGAGTTAACTAAGAAAATCTCAGTGAAGTTTTCTTAGTTGATGATTT

ATCTATAAAATTAGGAGGAGAAAGAAATGAATCGTACATTTATTAAAGGC
          ******         M  N  R  T  F  T  K  G
TTTTTGGCGGGGAATGCACTTTTGTTAGCAGGAATTACTGCTGCA
 F  L  A  G  N  A  L  L  L  A  G  I  T  A  A
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S407

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ATGCTCTTGCAACAATCGAAGGTGGTGTACCAATTGATACATTGAACGTT

GGTTCTATGGCTCACTCAACTGGTAAAACAATGCTCAACAAAGTTCTTTC

TATGGACAAAGATGACGTTGCTACTTTTGAAAAATTGCGTGACCTCGGAG

TTAAATTCGACGTACGTAAAGTTCCGGCTGATTCTAAATCAGACCTCTTT

GGTTTGATTAAAAAAGCTAACGTACAATAATCAGAATATGCTCGTCTGAT

ATTCTGATTAATAAAATTGAATATTAGGCAGCTAATTAATTAAGGAGATA
                                            ******
TAAAAAACATGGAATACGGTGTTTTATCTGTAATCTTGGTCATTCTCGTT
          M  E  Y  G  V  L  S  V  I  L  V  I  L  V
GCCTTCCTTGCTGGTCTTGAAGGTATCTTTGACCCCTGGCAATTCCAC
 L  F  L  A  G  L  E  G  I  L  D  P  W  Q  F  H
```

Fig. 4. Nucleotide sequences and deduced amino acid sequences of fragments S405 and S407.
Putative promoter sequences are underlined (-35 region, dotted line; -10 region, solid line); Shine-Dalgarno sequences are indicated with asterisks underneath the nucleotides; Putative TG-dinucleotide is indicated in bold.

Characterization of Secretion Signals

A sequencing primer (5'-TTGCAAGCGCGCTTCCAATG-3') complementary to the reporter gene was designed and used for the sequencing of the six secretion signals. The nucleotide sequences of secretion signals S405 (efficient in *E. coli*) and S407 (efficient in *L. lactis*) are shown in Fig. 4. All fragments had an open reading frame (ORF) fused to the reporter gene, and the putative promoter sequences were located upstream of the ORF. The homology of the putative promoters to previously described lactococcal promoters [3] was rather limited. No promoters had the perfect consensus -35 sequences (TTGACA) and -10 (TATAAT) sequences. The potential Shine-Dalgarno (SD) sequence and the translation start codon (AUG) were located.

To analyze S405 and S407, and predict cleavage sites, the Hidden Markov Model (HMM), a probabilistic model generally applicable to time series or linear sequences [9], was used (Fig. 5). The results of the HMM analysis confirmed the presence of three distinguished structural features for typical signal peptides [15]; an N-region containing five (S407) and eight amino acids (S405) of positively charged amino-terminus; an H-region containing from thirteen (S405) to fourteen amino acids (S407); a net hydrophilic profile (C-region, five and eight amino acids) containing the cleavage site for signal peptidase I.

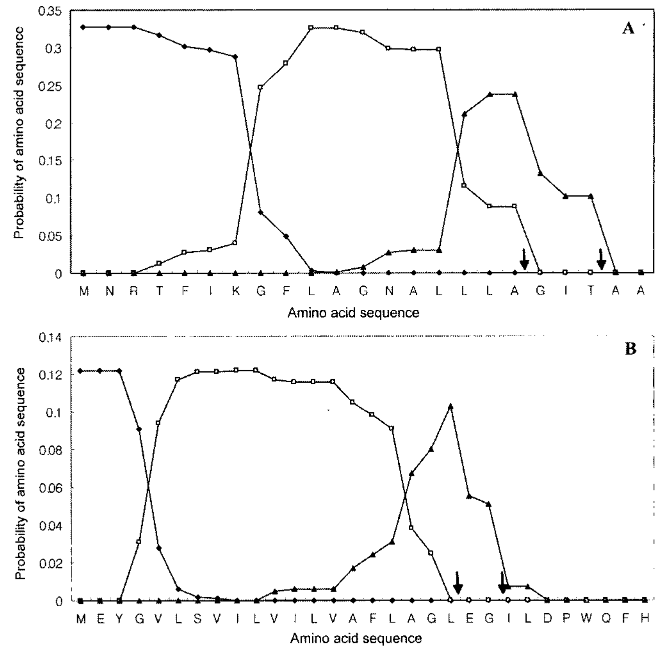

Fig. 5. HMM profile of peptides encoded by S405 and S407.
A, prediction of S405 in Gram (-) bacteria; B, prediction of S407 in Gram (+) bacteria. The probabilities of amino acids sequences were predicted according to Nielsen and Krogh [7]. Vertical arrows indicate potential cleavage site for Spase I. ◆: N-region probability; □: H-region probability; ▲: C-region probability.

In conclusion, a secretion signal-selection vector, pGS40, was constructed. By using pGS40, six fragments that restored the secretion of α-amylase were obtained from the chromosomal DNA of *L. lactis* ssp. *cremoris* LM0230. Among them, S405 and S407 were the most efficient signals in *E. coli* and *L. lactis*, respectively. The nucleotide sequences of six cloned fragments did not show any homology with known secretion signals. However, they contained three distinguished structural features for typical signal peptides. In particular, S407 having secretion ability in *L. lactis* is likely to be used as a secretion signal for the construction of the food-grade secretion vectors in the future.

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