

Optimized Recombinant DNA for the Secretion of Pediocin PA-1 in *Escherichia coli*

– Research Note –

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Abstract

To enhance the expression and secretion of pediocin PA-1 from heterologous bacterial hosts, the promoter and deduced signal sequence (PS) of an α -amylase gene from a *Bifidobacterium adolescentis* strain was fused with pediocin PA-1 structural and immunity genes (AB) and the resulting functions were evaluated in *Escherichia coli*. Two recombinant PCR products were created—one with just the deduced signal sequence and one with the sequence plus the Ser and Thr sequences that are the next two amino acids of the signal sequence. These two products, the PSAB (---AQA::KYY---) and PSABST (---AQAST::KYY---), respectively, were inserted into a TA cloning vector (yT&A) and named pPSAB, which was previously reported, and pPSABST. The two recombinant plasmid DNAs were transferred into *E. coli* JM109 and the transformants displayed antimicrobial activity, where the activity of *E. coli* JM109 (pPSAB) was stronger than that of *E. coli* JM109 (pPSABST), indicating that the ST amino acid residues were not necessary for secretion and might have even decreased the antimicrobial activity of recombinant pediocin PA-1.

Key words: pediocin PA-1, signal sequence, protein secretion, heterologous expression

INTRODUCTION

Pediocin PA-1 is produced from *Pediococcus* sp. and is a representative class IIa bacteriocin, which is a small, heat-stable, and non-modified protein that has a broad spectrum of antimicrobial activity with a strong anti-listerial activity (1). Because of these properties, this protein could be used as an antimicrobial agent or bio-preservative (2,3). Heterologous expression and secretion are important aspects to investigate to maximize the usefulness of the bacteriocin (4,5). To accomplish this goal, a gene cassette (*pedABCD*) for the expression and transportation of pediocin PA-1 should be cloned (6). However, the size (~3.6 kb) of the pediocin operon is quite large, which may affect the stability of the resulting plasmids. Another possible solution is to use signal sequences of secreted proteins from bacterial cells. In this respect, a promoter and signal sequence of an α -amylase (*amy59*) from *Bifidobacterium adolescentis*, which was previously identified by a Korean research group (7), may facilitate the expression and secretion of pediocin PA-1 from heterologous bacterial hosts. In the previous paper, the N-terminal of the amino acid sequence of *amy59* starting with Ser⁴⁵-Thr⁴⁶-Asp⁴⁷-Arg⁴⁸-Asp⁴⁹ was identified, which corresponded with the predicted cleavage site. The signal sequence (44 amino acids) also contained common features found in other signal peptides,

i.e., N (amino)-, H (hydrophobic)-, and C (carboxy-terminal) regions. The C region has a cleavage site that is recognized by the relevant signal peptidase. In this study, the promoter and deduced signal sequence of *amy59* gene was fused with the mature structural pediocin PA-1 gene (*pedA*) and immunity gene (*pedB*) and the secretion efficiency was evaluated in *Escherichia coli*. Additionally, the effect of Ser and Thr, the next two amino acids of the cleavage site of *amy59*, on cleavage and secretion was investigated.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* JM109 (Yeastern Biotech Co., Taipei, Taiwan) was grown in LB (Luria-Bertani) broth (10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract, pH 7.0) at 37°C. *E. coli* JM109 (yT&A), *E. coli* JM109 (pPSAB), *E. coli* JM109 (pPSABST), and *E. coli* JM109 [pPSABST(-)] transformants were selected on LB agar plates supplemented with 100 µg/mL of ampicillin and 0.8 mg/plate of X-Gal (5-bromo-4-chloro-3-indolyl β -D-galactopyranoside; Sigma Co., St. Louis, MO, USA) and IPTG (isopropyl-1-thio- β -D-galactopyranoside; Sigma Co.), respectively for blue/white colony selection. *Lactobacillus plantarum* NCDO 955 was grown in MRS

Table 1. Strains and plasmids (PCR product) used in this study

Strain and plasmid	Characteristics	Source or reference
Strains		
<i>E. coli</i> JM109	F ⁺ <i>traD36 lacI^f Δ(lacZ)M15 proA⁺B⁺/e14 (mcrA) Δ(lac proAB) thi gyrA96 (Nal^r) endA1 hsdR17 (r_k⁻ m_k⁺) relA1 supE44 recA1</i>	Yeastern Biotech Co.
<i>Lb. plantarum</i> NCDO 955	Indicator sensitive to pediocin PA-1	
Plasmids (PCR product)		
Ped ⁺ plasmid	~9.5 kb; plasmid containing pediocin operon	(6)
pBESAF2	~8.2 kb; Ap ^r and Cm ^r ; <i>E. coli</i> - <i>Bifidobacterium</i> shuttle vector containing promoter and deduced signal sequence of bifidobacterial α-amylase gene	(7,8)
PSAB	~970 bp; PCR product (promoter and deduced signal sequence of bifidobacterial α-amylase gene::mature <i>pedA</i> and <i>pedB</i> genes)	(8)
PSABST	~970 bp; PCR product (promoter and deduced signal sequence of bifidobacterial α-amylase gene::mature <i>pedA</i> and <i>pedB</i> genes; containing ST amino acid residues in the junction)	This study
yT&A	~2.7 kb; Ap ^r ; TA cloning vector	Yeastern Biotech Co.
pPSAB	~3.7 kb; Ap ^r ; yT&A::PSAB	(8)
pPSABST	~3.7 kb; Ap ^r ; yT&A::PSABST	This study
pPSABST(-)	~3.7 kb; Ap ^r ; yT&A::PSABST (PSABST inserted in an opposite direction compared with pPSABST)	This study

Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

(De Man, Rogosa, and Sharpe) broth (Difco, Sparks, MD, USA) at 37°C without agitation.

Bacteriocin assay

E. coli JM109 transformants cultured in 5 mL of LB broth supplemented with 100 µg/mL of ampicillin (Sigma Co.) were washed with 0.1% (w/v) peptone (Difco) water twice and resuspended in the same solution. Aliquots (1 µL) of the cell suspensions were spotted on LB agar plates containing no antibiotics. The plates were incubated at 37°C for 18 hr and then soft agar (0.7%, w/v) seeded with *Lb. plantarum* NCDO 955 as an indicator was poured on the plates (deferred antagonism assay). The plates were incubated at 37°C for 18 hr and inhibition zones around the colonies were examined.

Construction of PSAB and PSABST

PSAB and PSABST were constructed by Polymerase Chain Reaction (PCR). PSAB consists of the promoter-deduced signal sequence (PS) of the bifidobacterial α-amylase gene and mature *pedA*-*pedB* genes (AB) of pediocin operon from *Pediococcus acidilactici* K10 (6). As described in a previous paper (8), the promoter-deduced signal sequence (~400 bp) was amplified by PCR using pBESAF2 as a PCR template, primer set PSamyF (5'-GCT CTA GAG CGG GCA TCG CCG AAT ATA CTC CC-3') and PSamyR (5'-GGC CTG TGC TGC GGT GCT GGC-3'), and Pyrobest[®] DNA polymerase (Takara Bio Co., Shiga, Japan). Mature *pedA*-*pedB* genes (~570 bp) were also amplified by PCR using Ped⁺ plasmid as a PCR template, primer set pedSF (5'-AAA TAC TAC GGT AAT GGG GTT AC-3', 5' phosphorylated) and pedABR (5'-CGG GAT CCC GAA AAA GCC GCA

AGG CGA GGG AGG TGC TGC GTT CGC GTT TGG GAC AAC GTT TAC TAT TGG CTA GGC CAC GT-3'; a putative terminator is underlined), and Pyrobest[®] DNA polymerase. Two PCR products were ligated by T4 DNA ligase (Takara Bio Co.) and purified using the QIAquick[®] PCR purification kit (Qiagen Co., Hilden, Germany). The ligated DNA PSAB was amplified by PCR using primer set PSamyF and pedABR and AccuPower[®] HL PCR premix (Bioneer Co., Daejeon, Korea) and purified using the QIAquick[®] PCR purification kit. PSABST was amplified by the same method used for PSAB amplification, where the reverse primer PSamyR(ST) (5'-GGT GCT GGC CTG TGC TGC GGT GCT GGC-3') was used instead of PSamyR. All the primers used in this study were synthesized from Bioneer Co. An overview of the construction of PSAB and PSABST is shown in Fig. 1.

Cloning of PSAB and PSABST

The yT&A cloning kit (Yeastern Biotech Co.) was used for the cloning of PSAB and PSABST. The amplified PSAB and PSABST were ligated to the yT&A cloning vector and transferred into *E. coli* JM109 by the heat shock method according to the manufacturer's instruction. Briefly, competent *E. coli* JM109 cells stored at -75°C were thawed on ice. Chilled DNA sample was added to the cell solution and mixed by vortexing. The mixture was heat-shocked at 42°C for 45 sec and directly spread on LB agar plates supplemented with ampicillin, X-gal, and IPTG as described above. The resulting plasmids were named pPSAB and pPSABST, respectively. pPSABST (-) was constructed by inserting PSABST into the vector

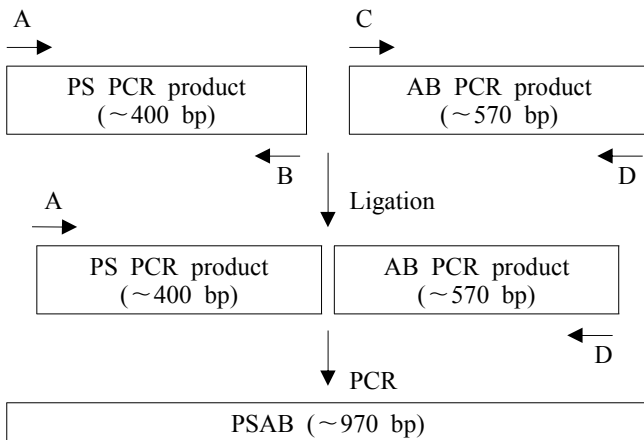


Fig. 1. Construction of PSAB. Primer A, PSamyF; primer B, PSamyR; primer C, pedSF (5' phosphorylated); primer D, pedABR. PS indicates promoter-deduced signal sequence of the bifidobacterial α -amylase gene and AB indicates *pedA-pedB* genes of pediocin operon. pBESAF2 and Ped⁺ plasmid were used as PCR templates. For construction of PSABST, PSamyR(ST) was used as primer B instead of PSamyR.

with an opposite direction compared with pPSABST. Universal primers M13-F and M13-R (Macrogen Co., Seoul, Korea) were used to analyze the nucleotide sequences of junctions of the recombinant DNAs.

RESULTS AND DISCUSSION

To express and secrete pediocin PA-1 from heterologous bacterial hosts, PSAB and PSABST were constructed as described in Materials and Methods and cloned into the yT&A cloning vector. The resulting plasmids, pPSAB and pPSABST, were confirmed by restriction profiles and nucleotide sequencing. PSAB or PSABST (~970 bp) was released by co-treatment with *EcoRI* and *SalI* restriction endonucleases, which restrict the boundaries of the inserts. PSAB and PSABST were shown to be correctly inserted into the yT&A cloning vector by nucleotide sequencing (data not shown).

The bacteriocin activities of *E. coli* transformants, *E. coli* JM109 (yT&A), (pPSAB), (pPSABST), and [pPSABST(-)], were examined. As shown in Fig. 2, *E. coli* JM109 (pPSAB) and *E. coli* JM109 (pPSABST) displayed bacteriocin activity whereas *E. coli* JM109 [pPSABST(-)] did not have antimicrobial activity, indicating that the signal sequence of the bifidobacterial α -amylase worked well in *E. coli* JM109. The case of *E. coli* JM109 [pPSABST(-)]'s activity might have been caused by the bifidobacterial promoter or terminator, because the transcription of PSABST in pPSABST(-) occurs in the opposite direction of the *lacZ* gene in the yT&A cloning vector (Fig. 2A). The bacteriocin activity of *E. coli* JM109 (pPSAB) was stronger than that of *E. coli* JM109

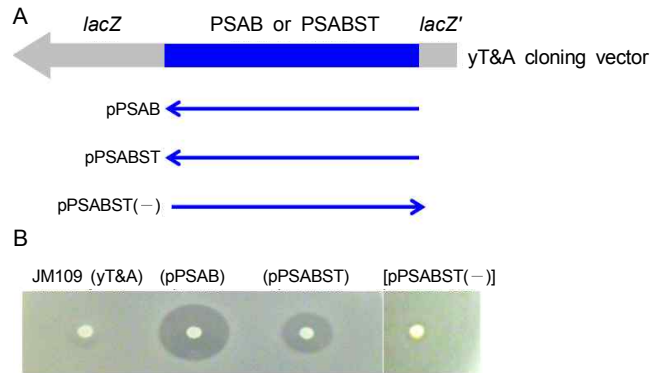


Fig. 2. Antimicrobial activities of *E. coli* JM109 transformants. A, structures of recombinant DNAs; B, antimicrobial activities of transformants. Antimicrobial activity was assessed using the deferred antagonism assay described in materials and methods (indicator: *Lb. plantarum* NCDO 955).

(pPSABST), indicating only the signal sequence (---AQA) was enough for the secretion of the recombinant pediocin PA-1 and the ST amino acid residues, which were the amino acids next to AQA, were not necessary for secretion. Indeed, these residues might have decreased the bacteriocin activity of pediocin PA-1 due to the extension of the N-terminal with uncharged amino acid residues, which is important for binding to the target bacterial cell membrane (9).

To additionally investigate whether the *lac* promoter of *lacZ* gene can improve the bacteriocin activity, the *E. coli* JM109 transformants were incubated with or without IPTG as a transcription inducer. Theoretically, the transcription levels of PSAB and PSABST should be increased when the transformants are cultured with IPTG, which would result in an increase in bacteriocin activity. As expected, the bacteriocin activities of transformants with IPTG were stronger than those of transformants without IPTG (Fig. 3). These results indicate that recombinant pediocin PA-1 could be expressed using the bifidobacterial promoter itself and this expression could be improved by the *lac* promoter induced with

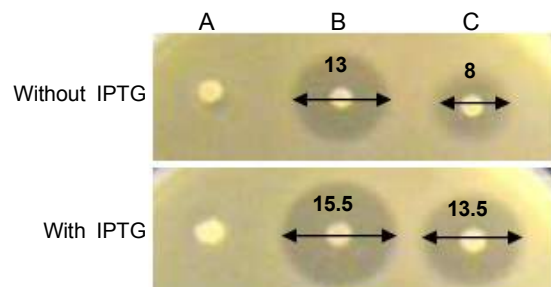


Fig. 3. Antimicrobial activities of *E. coli* JM109 transformants with or without IPTG induction. A, *E. coli* JM109 (yT&A); B, (pPSAB); C, (pPSABST). The diameters of inhibition zones are presented in mm.

IPTG.

Bacteriocins and other beneficial proteins that are produced by recombinant bacteria, such as antigenic proteins that function in human intestines to induce immunological responses, need to be secreted from the host cell before they can affect their useful functions. Because of this, appropriate transportation machinery is necessary for the secretion of foreign proteins from recombinant host cells. The pediocin operon consists of four genes *pedA*, *pedB*, *pedC*, and *pedD*. *pedA* and *pedB* are the structural and immunity genes, respectively, of pediocin PA-1. *pedC* and *pedD* genes are responsible for the transportation of pediocin PA-1 (6). In addition, the pediocin operon (~3.6 kb) is necessary for the exportation of pediocin PA-1 out of the cells; however, the size of the operon is too big to be easily inserted into plasmid vectors for the production of pediocin PA-1 and might have an influence on the plasmid stability. To overcome this problem, a bifidobacterial promoter and signal sequence for the expression and secretion of pediocin PA-1 was evaluated in *E. coli* and shown to function well in the host in this study.

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