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Heterologous Production of Pediocin PA-1 in *Lactobacillus reuteri*

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The recombinant DNA pLR5cat_PSAB, in which pediocin PA-1 structural and immunity genes (*pedAB*) fused with the promoter and deduced signal sequence of an α-amylase gene from a bifidobacterial strain were inserted in *Escherichia coli*-lactobacilli shuttle vector pLR5cat, was transferred to *Lactobacillus reuteri* KCTC 3679 and the transformant presented bacteriocin activity. The recombinant *L. reuteri* KCTC 3679 transformed with the shortened pLR5cat(S)_PSAB, where a nonessential region for the lactobacilli replicon was removed, also showed bacteriocin activity. The molecular mass of the secreted pediocin PA-1 from the recombinant bacteria was the same as that of native pediocin PA-1 (~4.6 kDa) from *Pediococcus acidilactici* K10 on a sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. In cocultures with *Listeria monocytogenes*, the recombinant *L. reuteri* KCTC 3679 effectively reduced the viable cell count of the pathogenic bacterium by a 3 log scale compared with a control where *L. monocytogenes* was incubated alone.

Keywords: Pediocin PA-1, *Lactobacillus reuteri*, heterologous production

Bacteriocins produced by lactic acid bacteria (LAB) have recently received significant attention owing to their potential use as a biopreservative and next generation antimicrobials [6, 13]. For that reason, many researchers have studied proteinaceous antimicrobial peptides and have identified and characterized various bacteriocins [1, 4, 5]. In particular, class II a bacteriocins, which are small, heat-stable, and nonmodified peptides that show strong antilisterial activity, have been extensively studied [4]. Pediocin PA-1 produced by *Pediococcus* sp. is a representative class II a bacteriocin and has been widely studied in detail [14]. Pediocin PA-1, which is composed of 44 amino acid residues and has two-disulfide bridges, is produced and exported by proteins encoded from the pediocin operon (*pedABCD*) [2, 8, 11]. Unlike nisin containing modified amino acid residues, didehydroalanine and didehydrobutyrine [1], pediocin PA-1 has no unusual amino acid residues and is a good target for heterologous production. In our previous reports, pediocin PA-1 was successfully secreted from a *Bifidobacterium longum* strain using a promoter and signal sequence of an α-amylase that originated from a *Bifidobacterium adolescentis* strain [10] and produced in *Escherichia coli* using overexpression vectors [9, 11].

L. reuteri has been shown to reside in the gastrointestinal (GI) tracts of humans and animals [16]. Since this species has beneficial effects on human health [7, 15], it has been a target bacterium for industrial applications. Specifically, this species has been shown to be capable of converting glycerol into reuterin (3-hydroxypropionaldehyde) [3], which has a broad range of antimicrobial activity and thus a wide range of potential commercial applications. Heterologous production of an antimicrobial peptide may be a good way to improve its antimicrobial capacity. Thus, in this study, structural and immunity genes of pediocin PA-1 fused with a promoter and deduced signal sequence of an α-amylase that originated from a *B. adolescentis* strain [10] were inserted in the *E. coli*–lactobacilli shuttle vector pLR5cat [12], and the production of pediocin PA-1 from the recombinant *L. reuteri* KCTC 3679 strain was evaluated.

The bacterial strains and plasmids used in this study are shown in Table 1. The *L. reuteri* KCTC 3679 strain was purchased from KCTC (Korean Collection for Type Cultures). *E. coli* was incubated in LB (Luria–Bertani) broth (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract, pH 7.0) at 37°C with vigorous shaking, and the *L. reuteri* strain was cultured in MRS (De Man, Rogosa, and Sharpe) broth (Difco, Sparks, MD, U.S.A.) at 37°C without shaking. One hundred μg/ml of ampicillin (Sigma Co., St. Louis, MO, U.S.A.) was used for *E. coli*, and 5 μg/ml of chloramphenicol (Sigma Co.) was used for *L. reuteri*, for antibiotic selection.

For heterologous production of pediocin PA-1 in *L. reuteri*, a recombinant DNA pLR5cat_PSAB (9.4 kb) was

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Table 1. Bacterial strains and plasmids used in this study.

	Characteristics	Source/reference
Strains		
E. coli JM109	F' *traD36 lacI*^{q} *Δ(lacZ)M15 proA*$^{+}$*B*$^{+}$*/e14*$^{-}$ *(mcrA) Δ(lac proAB) thi gyrA96 (Nal*^{r}*) endA1 hsdR17 (*r_k^- m_k^+*) relA1 supE44 recA1*	Promega Co.
L. reuteri KCTC 3679	Recipient host for pLR5cat_PSAB and pLR5cat(S)_PSAB	KCTC[a]
Plasmids		
pPSAB1	Ap^{r}, Cm^{r}, a recombinant DNA harboring PSAB, 8.6 kb	[10]
pLR5cat	Ap^{r}, Cm^{r}, an *E. coli*–lactobacilli shuttle vector, 8.4 kb	[12]
pLR5cat_PSAB	Ap^{r}, Cm^{r}, a recombinant DNA harboring PSAB, 9.4 kb	This study
pLR5cat(S)_PSAB	Ap^{r}, Cm^{r}, a short form of pLR5cat_PSAB where a nonessential region was deleted from the recombinant DNA, 7 kb	This study

[a] KCTC, Korean Collection for Type Cultures.

constructed. PSAB (promoter and deduced signal sequence of a bifidobacterial α-amylase gene::structural and immunity genes of pediocin PA-1) [10] was liberated from pPSAB1 (8.6 kb) by restriction enzymes, *Sma*I and *Sal*I, and inserted into pLR5cat (8.4 kb) digested with the same restriction enzymes. The construction of pLR5cat_PSAB is outlined in Fig. 1. The ligation regions of recombinant DNA pLR5cat_PSAB was confirmed by restriction and nucleotide sequence analyses (data not shown). The PSAB fragment (~1 kb) was liberated from pLR5cat after treatment with *Sal*I and *Sma*I restriction endonucleases, which were used for insertion.

The size of pLR5cat_PSAB was not small, which suggests that the size should be shortened to improve the transformation efficiency and its stability. For that reason, pLR5cat_PSAB was treated with the *Kpn*I restriction enzyme and self-ligated, resulting in the removal of a 2.4 kb fragment, which is not an essential region for replication of the plasmid in lactobacilli, from pLR5cat_PSAB (Fig. 1). The shortened plasmid was confirmed by restriction

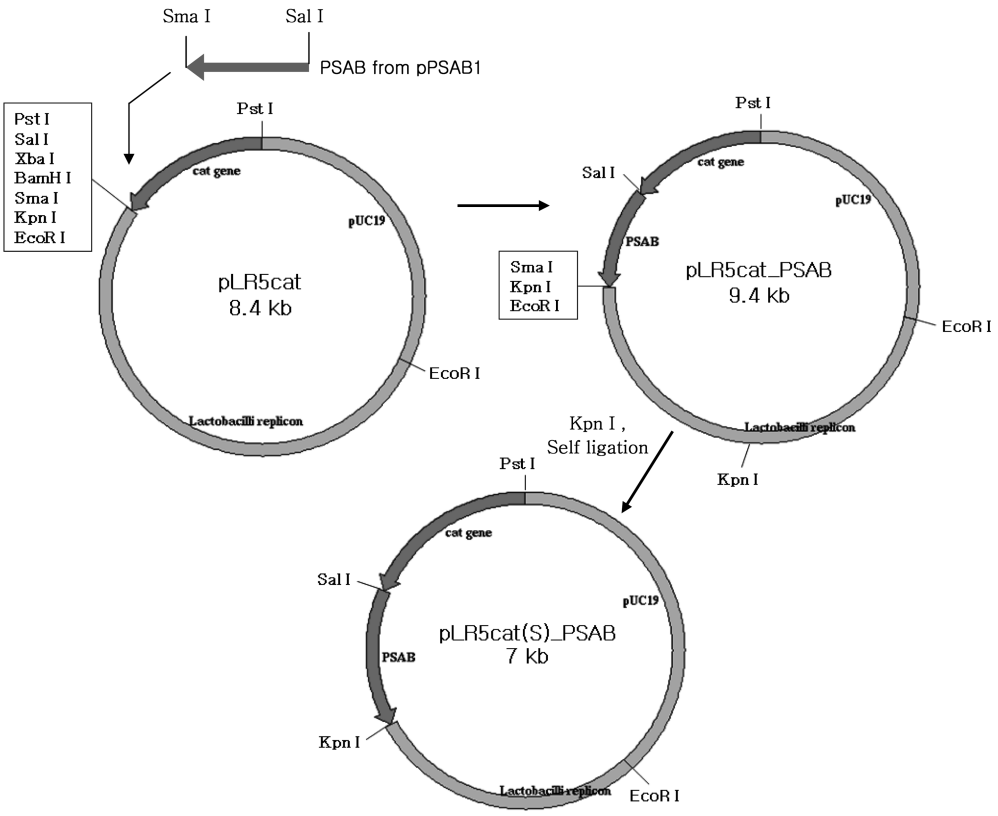

Fig. 1. Outline of the construction of pLR5cat_PSAB and pLR5cat(S)_PSAB.

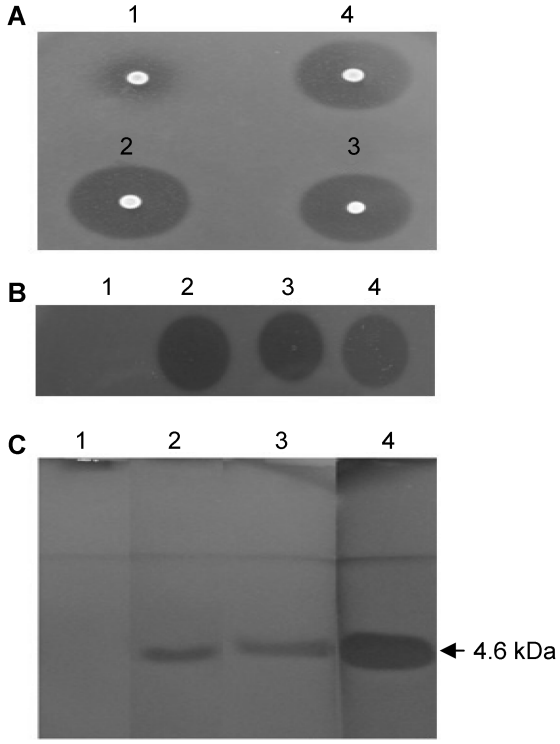

Fig. 2. Antimicrobial activities of cells and culture supernatants and the bioassay after tricine SDS–PAGE.
A. Antimicrobial activities of cells. **B**. Antimicrobial activities of culture supernatants. **C**. Activity staining of a tricine gel; 1, *L. reuteri* KCTC 3679 (-); 2, KCTC 3679 (pLR5cat_PSAB); 3, KCTC 3679 (pLR5cat(S)_PSAB); 4, *Pediococcus acidilactici* K10. *L. acidophilus* was used as an indicator for **A** and *L. monocytogenes* was used for **B** and **C**.

profiles (data not shown) and named pLR5cat(S)_PSAB (7 kb).

The recombinant DNAs pLR5cat_PSAB and pLR5cat(S)_PSAB were transferred into electrocompetent *L. reuteri* KCTC 3679. The preparation method for the electrocompetent cells was described in a previous paper [12]. The recombinant DNAs from *L. reuteri* KCTC 3679 transformants were confirmed by plasmid preparation (data not shown). The antimicrobial activities of the transformants were tested using an agar diffusion method. One μl of an overnight culture was spotted on a MRS agar plate and cultured for 10 h, which was overlaid with soft agar [0.7% (w/v)] that included the indicator strain *Lactobacillus acidophilus*. The plate was incubated overnight and inhibition zones around the colonies were examined.

The cultures of two transformants presented antimicrobial activities against the *L. acidophilus* strain, which was used as an indicator to exclude inhibition by the presence of acids (Fig. 2A).

To investigate whether the recombinant pediocin PA-1 was secreted or not from the recombinant *L. reuteri* KCTC 3679 strains, the bacteria were cultured for 13 h in 50 ml of MRS broth, centrifuged at 7,000 *xg* for 10 min, and the supernatants were filtered with 0.2-μm membrane filters (Millipore, Billerica, MA, U.S.A.). The filtrates were freeze-dried and diluted with ultrapure water for further studies. To test the bacteriocin activity of the concentrates, 5 ml of soft agar that included the indicator strain *L. monocytogenes* was poured on a MRS agar plate and then 1 μl of each concentrate was spotted on it. The plate was incubated at 37°C overnight and inhibition zones were examined. Based on this test, the freeze-dried culture supernatants showed antimicrobial activities against *L. monocytogenes*, indicating the recombinant pediocin PA-1 was successfully secreted from the transformants (Fig. 2B).

To confirm the molecular mass of recombinant pediocin PA-1, an SDS–PAGE using a tricine gel was performed as described in a previous paper [9]. The molecular mass of the recombinant pediocin PA-1 was the same as that of native pediocin PA-1, indicating that the signal sequence from the bifidobacterial α-amylase worked well in a *Lactobacillus* sp (Fig. 2C).

To confirm the bactericidal effect of pediocin PA-1 from recombinant *L. reuteri* KCTC 3679 against *L. monocytogenes*, cocultures were performed. All experimental data were presented as mean ± standard deviation (SD) of triplicate measurements. SPSS v. 12.0 (Statistical Package for Social Science Software; SPSS Co., Chicago, IL, U.S.A.) was used to perform Duncan's multiple range tests for determining significance of difference at $p<0.05$. Overnight cultures of *L. reuteri* KCTC 3679, *L. reuteri* KCTC 3679 (pLR5cat_PSAB), and *L. reuteri* KCTC 3679 (pLR5cat(S)_PSAB) were separately inoculated in MRS broth containing *L. monocytogenes*, which was cultured at the level of the early stationary phase. The mixed cultures were incubated for 8 h and samplings for enumeration of *L. monocytogenes* cells were done at every 2 h. The viable cell counts of *L. monocytogenes* were determined by using Listeria Isolation Medium (Oxford agar, LAB M, Bury, Lancashire, U.K.). In the coculture experiments, recombinant *L. reuteri* strains producing heterologous pediocin PA-1 were shown to effectively inhibit *L. monocytogenes*. The viable cell counts of *L. monocytogenes* reached 8.5 log scale after 8 h of incubation when the bacterium was inoculated alone. When the bacterium was cocultured with *L. reuteri* KCTC 3679, the viable cell counts was not significantly different from that of the former case. The bacterium cocultured with recombinant *L. reuteri* KCTC 3679 (pLR5cat_PSAB) or (pLR5cat(S)_PSAB) producing pediocin PA-1 was significantly inhibited by 2.44 and 2.82 log scale, respectively, compared with the control where the *L. monocytogenes* was incubated alone (Fig. 3). These results indicate that the heterologously produced pediocin PA-1 from the *L. reuteri* KCTC 3679 strain plays a key role in inhibiting the pathogenic bacterium *L. monocytogenes*.

Bacteriocins from LAB are one of the most important weapons to effectively inhibit pathogenic or spoilage bacteria.

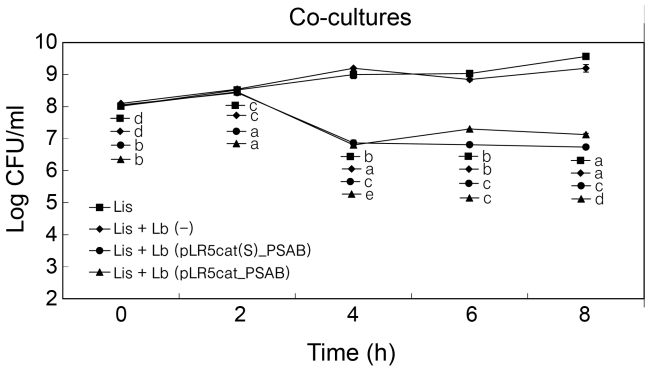

Fig. 3. Cocultures of recombinant *L. reuteri* with *L. monocytogenes*. Viable cell counts of *L. monocytogenes* were determined at different time points. Values are mean ± SD (n=3). Means having different letters are significantly different by Duncan's multiple range tests (p<0.05).

For that reason, many bacteriocins from LAB have been identified and characterized; however, most of these bacteriocins belong to similar categories. Thus, to expand the number of potential applications of the bacteriocins, methods must be developed for modifying these peptides. In this study, we combined pediocin PA-1, a strong antilisterial bacteriocin, with *L. reuteri*, a commercially important bacterium for probiotics, and produced a value-added probiotic *L. reuteri*. This recombinant *L. reuteri* producing pediocin PA-1 presented increased antimicrobial function.

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