Secretion of Recombinant Pediocin PA-1 by *Bifidobacterium longum*, Using the Signal Sequence for Bifidobacterial α -Amylase

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A recombinant DNA, encoding the chimeric protein of the signal sequence for bifidobacterial α -amylase mature pediocin PA-1, was introduced into *Bifidobacterium longum* MG1. Biologically active pediocin PA-1 was successfully secreted from the strain and showed bactericidal activity against *Listeria monocytogenes* and the same molecular mass as native pediocin PA-1.

As a consequence of recurring and serious listeriosis outbreaks (5, 11), *Listeria monocytogenes* has become a focus of bacteriocin researchers. The search for bacteriocin-producing lactic acid bacteria has been directed towards substances whose targets are *Listeria* spp., and consequently, a large number of antilisterial bacteriocins have been revealed (2, 7, 12). Particularly, class IIa bacteriocins (pediocin-like bacteriocins) are highly active against *Listeria* spp. and are the most promising candidates for food biopreservatives (4). Pediocin PA-1, produced by *Pediococcus* spp., is a representative class IIa bacteriocin and has been heterologously produced by other genera to inhibit food-borne pathogens or food spoilage bacteria (6, 14, 17). However, to the best of our knowledge, study of the heterologous production of pediocin PA-1 in bifidobacteria has been absent. Bifidobacteria, the main microflora of the human intestinal tract (9), have been used as representative probiotics. Because bifidobacteria are generally recognized as safe and colonize the large intestine, they could be promising candidates as hosts for expression of beneficial foreign proteins/peptides for human use (8, 16). In this study, pediocin PA-1 was heterologously expressed in *Bifidobacterium longum* MG1 and secreted from the strain by using the signal sequence for bifidobacterial α -amylase. The recombinant *B. longum* MG1 efficiently killed *L. monocytogenes* in a coculture.

Escherichia coli JM109 was grown in LB medium at 37°C. *B. longum* MG1 was grown in MRS medium (Merck, Darmstadt, Germany) supplemented with 0.05% (wt/vol) L-cystein HCl (Sigma Co.) at 37°C without agitation. *Lactobacillus plantarum* NCDO 955 was grown in MRS medium at 37°C without agitation. *L. monocytogenes* KFRI 799 was grown in BHI medium at 35°C and on Oxford Listeria selective agar for the selective enumeration. Agar plates were made by adding 1.5% agar to broth media. Antibiotics (Sigma Co.) were added as selective agents with appropriate concentrations (chloramphenicol, 2 μ /ml for *B. longum* and 20 μ /ml for *E. coli*; ampicillin, 100 μ /ml). MRS agar plates spread with *B. longum* were anaero-

bically incubated in an atmosphere generation system (GasPak system, Oxoid, Basingstoke, Hampshire, England).

Plasmids and PCR product used in this study are summarized in Table 1. Primers PSamyF (5'-GCTCTAGAGCGGG CATCGCCGAATATACTCCC-3') and PSamyR (5'-GGCCT GTGCTGCGGTGCTGGC-3') were used to amplify a 400-bp fragment containing a promoter and deduced signal sequence of the bifidobacterial α -amylase gene. A recombinant DNA, pBESAF2, was used as the template. Primers pedSF (5'-AAATACTACGGTAATGGGGTTAC-3') and pedABR (5'-CGG GATCCCGAAAAAGCCGCAAGGCGAGGGAGGTGCTG CGTTTCGCGTTTGGGACAACGTTTACTATTGGCT AGGC CACGT-3') were used to amplify a 570-bp region containing mature *pedA* and *pedB*. PedB (immunity protein) could be used for the bifidobacterial strains sensitive to pediocin PA-1. A putative bifidobacterial transcription terminator is underlined (15). Ped⁺ plasmid was used as the template. The two PCR products were ligated by T4 DNA ligase (Takara Bio, Shiga, Japan) and designated PSAB. PSAB was amplified by using primers PSamyF and pedABR and cloned into the yT&A cloning vector. The recombinant DNA, named pPSAB, was introduced into *E. coli* JM109. The *E. coli* transformant exhibited strong antimicrobial activity against an indicator, *L. plantarum* NCDO 955 (data not shown). This result indicates that the promoter and signal sequence for bifidobacterial α -amylase work well in the host strain. For the construction of the *E. coli*-*Bifidobacterium* shuttle vector, a 4.9-kb fragment containing pMG1 and the chloramphenicol acetyltransferase gene was liberated from pBESAF2 digested by the SphI and XbaI restriction enzymes and ligated with pPSAB digested by the same enzymes. The recombinant DNA, named pPSAB1 (Fig. 1A), was introduced into *B. longum* MG1 by using an electroporation method (1). A negative control DNA, named pYMGCAT, was also introduced in the host strain. As expected, *B. longum* MG1 transformed by pPSAB1 exhibited antimicrobial activity against *L. plantarum* NCDO 955, whereas the host strain transformed by pYMGCAT did not exhibit this (Fig. 1B). These results indicate that the bifidobacterial promoter and signal sequence work well in their own genus. Approximately 90% of pPSAB1 was stably maintained in *B. longum* MG1 over 20 successive subcultures without an antibiotic press, and the re-

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TABLE 1. Plasmids (PCR product) used in this study

Plasmid	Characteristics ^a	Source or reference
Ped ⁺ plasmid	Plasmid containing pediocin operon	10
pBESAF2	Ap ^r , Cm ^r ; <i>E. coli</i> - <i>Bifidobacterium</i> shuttle vector containing a promoter and deduced signal sequence originated from α -amylase gene of <i>Bifidobacterium adolescentis</i> INT-57	13
PSAB (PCR product)	Bifidobacterial promoter and deduced signal sequence::mature <i>pedA</i> and <i>pedB</i>	This study
yT&A	Ap ^r , TA cloning vector	Yeastern Biotech Co.
pPSAB	Ap ^r , yT&A::PSAB	This study
pYMGCAT	Ap ^r , Cm ^r ; <i>E. coli</i> - <i>Bifidobacterium</i> shuttle vector; yT&A::pMG1 ^b and CAT gene originated from pBESAF2	This study
pPSAB1	Ap ^r , Cm ^r ; <i>E. coli</i> - <i>Bifidobacterium</i> shuttle vector; pPSAB::pMG1 and CAT gene	This study

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

^b pMG1 resides in *B. longum* MG1 and was used as a bifidobacterial replicon.

combinant host strain still showed antimicrobial activity against *L. plantarum* NCDO 955 (data not shown).

To investigate the influence of bacteriocin production on the growth of *B. longum* MG1, two transformants, *B. longum* MG1(pPSAB1) and MG1(pYMGCAT), were cultured at 37°C for 3 days, and the optical density at 600 nm, pH value, and bacteriocin activity of the neutralized culture supernatant were measured periodically. *B. longum* MG1(pYMGCAT) did not show antimicrobial activity against *L. monocytogenes* KFRI 799 until 72 h, whereas *B. longum* MG1(pPSAB1) showed the activity after 24 h and it reached 64 arbitrary units/ml (3) at 72 h. Although the two transformants showed a difference in bacteriocin activity, meaningful differences did not appear in the optical density at 600 nm and pH value (data not shown). These results indicate that the production of recombinant pediocin PA-1 in *B. longum* MG1 has little influence on the growth of the host strain.

To confirm secretion of the recombinant pediocin PA-1 from *B. longum* MG1(pPSAB1), exported proteins from the transformant were precipitated with ammonium sulfate, followed by a well diffusion assay (3) and tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The precipitate resuspended with distilled and deionized H₂O

showed antimicrobial activity against *L. monocytogenes* KFRI 799, whereas a precipitate from *B. longum* MG1(pYMGCAT) did not exhibit the activity (data not shown). The molecular mass of the antimicrobial peptide was the same as that of native pediocin PA-1 on a tricine SDS-PAGE gel (Fig. 1C). These results indicate that the recombinant pediocin PA-1 is expressed in and secreted from *B. longum* MG1(pPSAB1).

To investigate bactericidal activity of *B. longum* MG1 (pPSAB1) against *L. monocytogenes*, a coculture was performed in MRS broth. The transformant reduced cell counts of *L. monocytogenes* by 7 log compared with the control (only *L. monocytogenes* was inoculated), whereas *B. longum* MG1 (pYMGCAT) reduced by 3 log (Fig. 2). These results indicate that recombinant pediocin PA-1 from *B. longum* MG1 (pPSAB1) plays a significant role in the reduction of cell counts of *L. monocytogenes*. The secretion of the recombinant pediocin PA-1 in a probiotic *Bifidobacterium* sp. has a significant value, and regular intake of the improved probiotics could efficiently prevent several food-borne pathogens, including *L. monocytogenes*, from causing disease in human intestine.

In conclusion, a representative class IIa bacteriocin, pediocin PA-1, was expressed in and secreted from a *B. longum* strain by using the promoter and signal sequence for bifidobac-

FIG. 1. Genetic map of pPSAB1 (A), antimicrobial activities of *B. longum* MG1 and its transformants (B), and bioassay after tricine SDS-PAGE of exported proteins of the transformants (C). (B): deferred antagonism assay (3) against *L. plantarum* NCDO 955; lane 1, MG1; lane 2, MG1(pYMGCAT); lane 3, MG1(pPSAB1). (C) *L. monocytogenes* KFRI 799 was used as an indicator; lane 1, exported proteins from MG1(pYMGCAT); lane 2, exported proteins from MG1(pPSAB1); lane 3, native pediocin PA-1.

FIG. 2. Changes of cell counts of *L. monocytogenes* during coculture with *B. longum* MG1(pPSAB1) or MG1(pYMGCAT). Lis, *L. monocytogenes* KFRI 799; MG1, *B. longum* MG1. Each point represents the average cell count from triple experiments.

terial α -amylase. To the best of our knowledge, this is the first report on heterologous production of bacteriocin in *Bifidobacterium* sp. and meaningful consequences for human health. However, for the application of the recombinant bacterium to humans, the plasmid vector should be changed into food-grade one.

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