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Cloning and production of a novel bacteriocin, lactococcin K, from *Lactococcus lactis* subsp. *lactis* MY23

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Abstract

A gene encoding the antimicrobial peptide, lactococcin K, was isolated from *Lactococcus lactis* subsp. *lactis* MY23 then cloned and expressed in *Escherichia coli*. Because the expressed lactococcin K was formed as an inclusion body in recombinant *E. coli*, a fusion protein containing lactococcin K and maltose-binding protein (MBP) was produced in a soluble form. For high-level production of lactococcin K, we performed a pH-stat fed-batch culture to produce 43,000 AU lactococcin K ml^{-1} in 12 h.

Introduction

Lactic acid bacteria (LAB) produce antimicrobial proteins and peptides called bacteriocins, which are considered advantageous for organisms that survive and dominate in microbial ecosystems such as the digestive tract. The bacteriocinogenic LAB antagonize closely related strains or other bacterial species occupying the same ecological niche. Selective antimicrobial activities in the ecological niches of fermented foods have focused on the preservation of bacteriocinogenic LAB, which are indispensable in the production of certain fermented foods and beverages and have been used as preservatives in foods.

Kimchi, a traditional Korean dish of fermented vegetables, is a safe source for obtaining LAB. For this study, we selected a strain of LAB from kimchi that shows antimicrobial activity against an acne pathogen. This strain was identified as *Lactococcus lactis* subsp. *lactis* MY23 from which we cloned a gene encoding an antimicrobial peptide, *lcnK*. For over-production of this peptide, a fusion protein was introduced in *Escherichia coli* and then removed. A high production level of the antimicrobial peptide lactococcin K was achieved using a pH-stat fed-batch culture of *E. coli* cells harboring the gene. The potential use of the recombinant antimicrobial peptide is discussed.

Materials and methods

Strains and cloning

Chromosomal DNA was prepared from *Lactococcus lactis* subsp. *lactis* MY23 isolated from kimchi and had shown antimicrobial activity against *Propionibacterium acnes* ATCC 6919 as well as other Gram-positive pathogens.

Escherichia coli JM 105 and BL21 (DE3) were used as the hosts for cloning and expression, respectively. All genetic experiments were conducted as described in Sambrook *et al.* (1989). Strains and plasmids used in this study are listed in Table 1. LB medium was used for cloning,

Table 1. Bacterial strains and plasmids.

Strains and plasmids	Description	Reference
Strain		
Lactococcus lactis subsp. *lactis* MY23	Origin of lactococcin K isolated from Kimchi	This study
E. coli JM105	*lacIq* Δ *(lacZ)M15 proA+B+/thi rpsL (Strr) endA sbcB15 sbcC? hsdR4* (rK. mK+) Δ *(lac-proAB)* for DNA manipulation	Yanisch-Perron *et al.*
E. coli BL21(DE3)	rB. mB.; an *E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene for recombinant protein expression	Studier *et al.*
Plasmids		
pUC18	Cloning vector	Yanisch-Perron *et al.*
PEMBP	Expression vector containing maltose binding protein-tag	Bioprogen.
PBMY	pUC18 containing *L. lactis* subsp *lactis* MY23 chromosomal DNA fragment (4-Kb) by *Sau*3AI cut	This study
PBMYX	pBMY derivative with DNA fragment (1.8-Kb) by *Xba*I cut	This study
PTMY	pET21c containing *lcnK*	This study
PEMBPB	pEMBP containing *lcnK*	This study

and 50 μg ml^{-1} of kanamycin or ampicillin was used to select transformants. The prepared chromosomal DNA was partially digested with the restriction enzyme *Sau*3AI at 37 °C for 5 min and ligated into *Bam*HI-digested pUC18 plasmid. The *E. coli* transformants were screened for antimicrobial activity against *P. acnes*, and the plasmid was sequenced in the sequencing facility of the Korea Advanced Institute of Science and Technology (KAIST, Daejeon, Korea). A structural gene encoding an antimicrobial peptide (*lcnK*) was subcloned into a pET21c and pEMBPB, which yielded a pTMY and pEMBP plasmid, respectively (Figure 1).

Expression of the antimicrobial peptide in E. coli

Actively growing *E. coli* cells harboring the pEMBPB plasmid were induced by adding IPTG at 1 mM. Cells were harvested by centrifugation and disrupted by sonication. After removal of the cell debris, the pH of the supernatant was adjusted to 7.0 by adding 5 M NaOH. The expressed fusion protein containing the recombinant peptide, maltose binding protein (MBP), and an enterokinase cleavage site was concentrated with an MBP Excellose Spin Kit (Bioprogen, Daejeon, Korea). The protein was then treated with enterokinase in Tris/HCl buffer (pH 7.0) to remove the MBP-tag.

Antimicrobial activity assay

To quantify the antimicrobial activity of peptides from *Lactococcus lactis* subsp. *lactis* MY23, we used a microtiter plate assay (Carbo *et al.* 1999) as follows; *Propionibacterium acnes* grown in an Actinomyces broth (Difco, 100 μl) was mixed with two fold serial dilutions of culture supernatants of *Lactococcus lactis* subsp. *lactis* MY23 (100 μl) and incubated anaerobically for about 40 h at 37 °C. Growth of *P. acnes* was measured turbidometrically at 550 nm using a microplate reader. One unit of antimicrobial peptide activity was arbitrarily defined as the amount of antimicrobial peptide that exhibited 50% growth inhibition of *P. acnes*, compared to growth without the addition of the antimicrobial peptide.

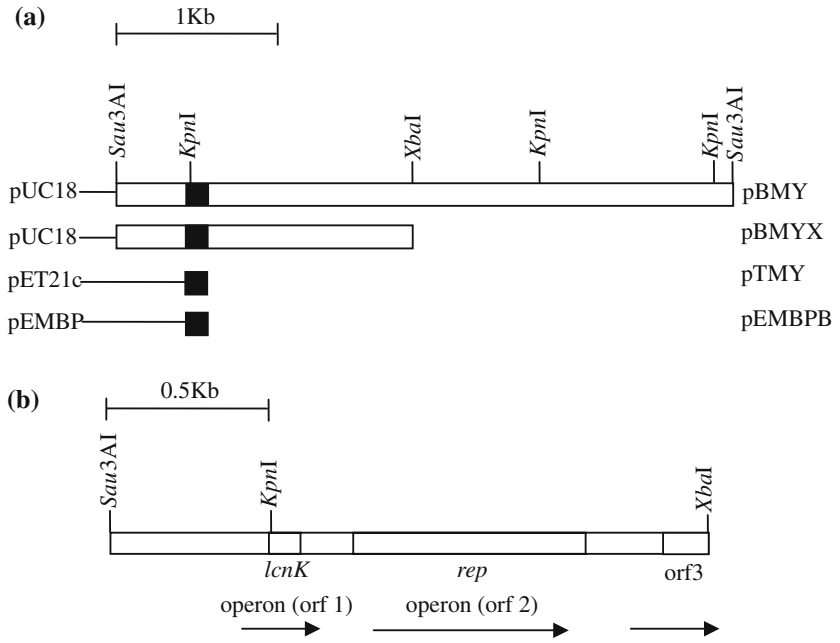

Fig. 1. (a) Schematic representation of the insert in pBMY and its derivatives. Black squares indicate the location of *lcnK*. (b) Location of *lcnK* operon in pBMYX. The arrows indicate the transcription direction of three operons.

To assay the inhibitory activity of the recombinant peptide, we used SDS-PAGE (Bhunia *et al.* 1987). One ml of culture sample was harvested and re-suspended in cracking buffer. The re-suspended solution was sonicated and loaded on a tricine/SDS-polyacrylamide gel (16%). The gel was fixed immediately for 2 h in 20% (v/v) 2-propanol and 10% (v/v) acetic acid in water and washed in distilled water for 6 h. This gel was subsequently placed on brain heart infusion (BHI) agar. After 15 min, an overlayer of 20 ml soft Actinomyces agar (Difco, 0.75%) containing 200 μl *P. acnes* culture as an indicator was poured on the surface of the agar plate. After 40 h at 37 °C, the plate was examined for zones of growth inhibition. As a reference, 10 μl peptide solutions of *L. lactis* subsp. *lactis* MY23 mixed in a 1:1 ratio with tricine–SDS sample buffer (Novex, San Diego, CA, USA) was also applied to the gel.

Fed-batch culture

The R/2 medium (pH 6.8) containing (per liter) 20 g glucose, 2 g $(NH_4)_2HPO_4$, 6.75 g KH_2PO_4, 0.85 g citric acid, 0.7 g $MgSO_4{\cdot}7H_2O$, and 5 ml of a trace metal solution, which contained (per liter of 0.1 M HCl) 10 g $FeSO_4{\cdot}7H_2O$, 2.25 g $ZnSO_4{\cdot}7H_2O$, 1 g $CuSO_4{\cdot}5H_2O$, 0.5 g $MnSO_4{\cdot}5H_2O$, 0.23 g $Na_2B_4O_7{\cdot}10H_2O$, 2 g $CaCl_2{\cdot}2H_2O$, and 0.1 g $(NH_4)_6Mo_7O_{24}$ were used for fed-batch culture. A seed culture was prepared in a 1 l flask containing 100 ml of R/2 medium and was then inoculated into a 5 l jar fermentor (KoBiotech, Incheon, Korea) containing 2.5 l of R/2 medium. The pH was kept at 6.8 by adding 28% (v/v) NH_4OH. The dissolved O_2 concentration was kept at 20% of air saturation by increasing the agitation speed and by mixing pure O_2. The nutrient feeding solution for fed-batch culture contained (per liter) 400 g glucose, 100 g yeast extract, and 20 g $MgSO_4{\cdot}7H_2O$. A nutrient feeding solution was added by using a pH-stat (with high limit) feeding strategy; when the pH rose to a value greater than its set point due to the depletion of glucose, the appropriate volume of the feeding solution was automatically added in order to increase the glucose concentration in the culture broth. Expression of the *lcnK* gene was induced by adding IPTG at 1 mM.

Results

Cloning and sequencing

Lactococcus lactis subsp. *lactis* MY23 was identified by 16S rRNA and showed antimicrobial activity against Gram-positive pathogens such as *Listeria monocytogenes*, *Propionibacterium acnes*, *Streptococcus mutans* and *Rothia dentocariosa*. To clone the gene encoding an antimicrobial factor from strain MY23, its chromosomal DNA was isolated because there was no plasmid in strain MY23. The chromosomal DNA was digested with *Sau3*AI and ligated into a *Bam*HI digest of pUC18 plasmid. The plasmid library was transformed into *E. coli* JM105, and the obtained transformants were screened for antimicrobial activity. The recombinant *E. coli* having the plasmid, referred to as pBMY (Figure 1), showed inhibitory activity against *P. acnes*. The pBMY plasmid was found to harbor a 4 kb genomic DNA fragment. The only restriction site for *Xba*I was in the middle of the 4-kb fragment of the pBMY plasmid. After digestions with *Xba*I to reduce the open reading frames (ORFs), a 1.8 kb fragment attached to pUC18 was ligated. The ligated product was a pBMYX plasmid. The *E. coli* harboring pBMYX showed inhibitory activity against *P. acnes*. The 1.8 kb fragment was sequenced and found to consist of two complete ORFs and an incomplete ORF. The ORF1 (69 bp) was determined as the antimicrobial peptide gene since the expression vector containing ORF1, pTMY, showed the inhibitory activity. The ORF1 sequence was previously known as an upstream region of the replication protein gene of *Enterococcus faecalis* (GenBank Accession No. AJ223161) not reported as a structural gene. A BLAST protein database homology search on the deduced peptide from ORF1 also did not show homology with any reported proteins. Therefore, the ORF1 was identified as the structural gene of the antimicrobial peptide encoding 22 amino acids and was named lactococcin K (Figure 2). The calculated molecular mass of lactococcin K was 2,700 Da.

Expression of antimicrobial peptide in E. coli

A gene encoding an antimicrobial peptide (*lcnK*) was expressed in *E. coli* BL21(DE3). Because the lactococcin K expressed by pTMY was formed as an inclusion body in recombinant *E. coli*, we created a fusion protein containing the peptide, maltose-binding protein (MBP), and an enterokinase cleavage site. The fusion protein containing

Fig. 2. Nucleotide sequence of the *lcnK* locus and the deduced protein sequence. The deduced amino acid sequence of lactococcin K and the N-terminal part of *rep* are shown below the nucleotide sequence. The putative ribosome-binding site (rbs) for *lcnK* is indicated in bold letters.

peptide and MBP were obtained in soluble form by expressing an *lcnK* gene with the pEMBPB plasmid. The MBP portion of the fusion protein was removed by the addition of enterokinase. We found that the resultant antimicrobial peptide also showed antimicrobial activity against Gram-positive pathogens such as *Listeria monocytogenes*, *P. acnes*, *S. mutans* and *R. dentocariosa*.

Overproduction of antimicrobial peptide

A pH-stat fed-batch culture of *E. coli* BL21(DE3) harboring pEMBPB was established to achieve high-level production of the antimicrobial peptide. Cells were induced with 1 mM IPTG at OD_{600} values ranging from 10 to 60 in the fermentor. Cells were harvested and disrupted by sonication. After digestion of the fusion protein with enterokinase, antimicrobial activity against *P. acnes* was estimated by SDS-PAGE. The specific activity, expressed as antimicrobial activity per cell density, was fairly constant at approximately 700 AU/OD_{600} indicating that the production of fusion protein is not related to cell density or the time of induction.

To maximize the production of recombinant antimicrobial peptide, IPTG induction was performed at dry cell weight = 18 g l^{-1}. After induction, the antimicrobial peptide was produced in 1 h (Figure 3). The antimicrobial activity of the culture reached approximately 43,000 AU ml^{-1} in 12 h; after this time, no further production was observed. We thus concluded that the recombinant lactococcin K exhibited a substantial inhibitory effect against *P. acnes* and could be effectively produced using *E. coli* as a soluble fusion protein form.

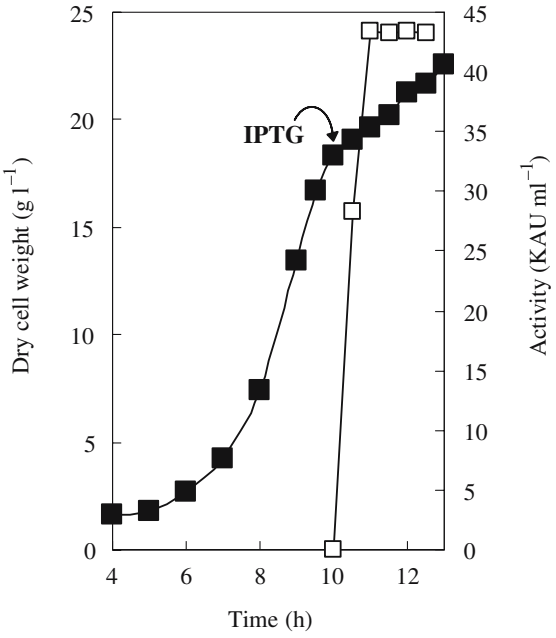

Fig. 3. Antimicrobial peptide production in a pH-stat fed-batch culture. Black squares indicate dry cell weight; white squares represent activity of the antimicrobial peptide. Arrow indicates the time of IPTG addition for the induction.

Discussion

During fermentation of kimchi, various LAB are indispensable at different stages of the ripening process. *Leuconostoc mesenteroides* has an important role in the formation of characteristic flavor and taste by producing many kinds of organic acids and carbon dioxide. Acid-resistant lactobacilli such as *Lactobacillus plantarum* and *L. brevis* become the dominant flora in the late stage after *Leuconostoc mesenteroides* cells disappear because of accumulated acids. These strains accelerate the deterioration of kimchi by producing more acids (Lee *et al.* 2005). Although the role of *Lactococcus lactis* subsp. *lactis* MY23 in kimchi fermentation is not clear, it may offer a potentially useful means of controlling spoilage microorganism growth based on the inhibition of other microorganisms by the antimicrobial peptide. The antimicrobial peptide of LAB could be used as a component of health care products such as oral and skin care products. Many antimicrobial peptides show antimicrobial effects against microorganisms related to human diseases such as acne, which is a disease of the pilosebaceous ducts (hair follicles; Jack *et al.* 1995). In the case of acne, the lipase produced by *P. acnes* can break down the oily sebaceous materials. The release of free fatty acids results in an inflammatory response causing erythema and swelling around the comedomes. It could be possible to prevent bacterial infection using an antimicrobial agent such as bacteriocin.

In summary, we have described gene cloning and expression of a novel antimicrobial peptide (*lcnK* gene; lactococcin K peptide) obtained from

kimchi, a traditional Korean dish of fermented vegetables. We were able to produce the recombinant lactococcin K in *E. coli*, which showed substantial inhibitory activity against acne-causing *P. acnes*. Lactococcin K may be useful as an antimicrobial ingredient in health care products such as oral and skin care products.

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