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Cloning and expression of the antimicrobial peptide from *Lactobacillus reuteri* KUB-AC5 and its characterization

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Abstract *Lactobacillus reuteri* KUB-AC5 was isolated from chicken intestine and played an important role as probiotic in *Salmonella* growth inhibition and promoting the growth of broiler chicken. Gene coding for antimicrobial peptide from the strain KUB-AC5 (AMP-AC5) and characterization of its product were cloned. Genomic library cloning of gene coding for AMP-AC5 based on the size of homogeneous peptide of 4721.95 Dalton purified by amberlite adsorption-desorption, gel filtration chromatography, reversed phase HPLC and cation exchange chromatography, was successfully cloned into pNZ307 and expressed in *Escherichia coli* DH5α to obtain the recombinant clone *E. coli* ACE-C46. Its recombinant plasmid pACE-C46 was subsequently subcloned into pSIP609/*L. plantarum* TLG02 to obtain an active recombinant clone *L. plantarum* ACLP-C46-F2.1 which had recombinant plasmid containing an open reading frame I-C46-F2.1 (153 nucleotides). It deduced amino acid sequence of "YMLYKFLAGLFHTSIDSIYWSVTFIAPALALITYIVCWPDS" (ID number 2253028) which showed no similarity to bacteriocin. The AMP from both the wild type and the recombinant strain exhibited similar characters in stability at wide pH range of 2-9, high temperature up to 121℃ and inhibition spectrum against both G+ and G- bacteria but not to lactic acid bacteria including closely related specie of *L. reuteri* resulting in a potential single AMP produced by the wild type. Furthermore, overexpression of *kac5* into the wild type provided the recombinant *L. reuteri* ACLR-C46-F2.1 which exhibited higher inhibitory activities than the wild type for 1.6 folds. The novel AMP named KAC5 would be promising for food and feed safety uses in the future.

Keywords: Antimicrobial peptide KAC5, *Lactobacillus reuteri* KUB-AC5, *kac5* gene cloning, Food grade vector system

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Introduction

Attempts to reduce antibiotic use in animal production have resulted from increments of antibiotics' microbial resistance and their residues in animal products which pass down the food chain and can be harmful to consumers. Recently, probiotics have received increasing attention as possible antibiotic growth promoters. Lactic acid bacteria (LAB) is a microbial group that is considered to have probiotic properties. These organisms grow and contribute beneficial functions in the intestinal tract (Fuller, 1989). One of the most important considerations in achieving the desired effect from using LAB as growth promotants is to ensure that they can provide inhibitory activity against pathogens such as *Salmonella* serovar Enteritidis and *S.* serovar Typhimurium which cause salmonellosis outbreaks (Schoeni *et al.*, 1995). We previously isolated LAB from the gastrointestinal tract of chickens. *Lactobacillus reuteri* KUB-AC5 exhibited effective inhibition activity against 10 and 2 strains of *Escherichia coli* and *Salmonella* sp. resistant to antibiotic uses, respectively (Nitisinprasert *et al.*, 2000). Nakphaichit *et al*. (2011) investigated the effect of *L. reuteri* KUB-AC5 on ileal microbiota at 21 and 42 days for one-day-old chicks fed with feed supplemented with 10^5 colony forming unit per gram (cfu/g) *L. reuteri* KUB-AC5 for 1 week. Results showed growth suppression of five pathogenic genera *Klebsiella*, *Chryseobacterium*, *Citrobacter*, *Aeromonas*, *Acinetobacter* and order *Campylobacterales* which were still high in the control. *L. reuteri* also enhanced population level and species diversity of lactobacilli and high concentration of 10^7 cfu/g supplementation reduced cell concentration of *S.* Enteritidis and *Clostridium perfringens* in the gastrointestinal tract (Nakphaichit *et al.*, 2019). Addition of strain KUB-AC5 might activate metabolites which affect microbiota in chicken gastrointestinal (GI) tract. *L. reuteri* is well known as an obligately heterofermentative LAB resident in the gastrointestinal tracts of humans and most other animals examined to date for example pigs, chickens, cattle, dogs, mice, rats and hamsters (Casas *et al.*, 2000), and has been suggested to be a unique universal enterobacteria. To date, only three kinds of antimicrobial substances (AMS) as reuterin, reutericyclin and reutericin have been found. Reuterin is produced specifically from glycerol by anaerobic resting cell condition and exhibits a broad spectrum AMS against both Gram positive and Gram negative bacterial strains including some pathogens, *E. coli*, *Salmonella*, *Shigella*, *Proteus*, *Pseudomonas*, *Clostridium* and *Staphylococcus* (Axelsson *et al.*, 1989; Talarico *et al.*, 1988), while reutericyclin plays an inhibiting role only against Gram positive strains such as *Lactobacillus* spp., *Bacillus subtilis*, *B. cereus*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Listeria innocua* (Gänzle *et al.*, 2000). Kabuki *et al.*

(1997) reported the existence of a bacteriocin, reutericin 6, produced by *L. reuteri* LA6. Reutericin was isolated from human infant feces which had an antibacterial spectrum on *L. acidophilus*, *L. gasseri* and *L. delbrueckii*. It showed unique lytic activity against *L. delbrueckii* (Toba *et al.*, 1991). So far, only a few AMS such as organic acid, short chain fatty acid, lactocidin and reuterin have been shown to display inhibition activity against Gram negative bacteria like *Salmonella* sp. (Axelsson *et al.*, 1989; Gilliland and Speck, 1977; Ouwehand, 1998; Silva *et al.*, 1987). *L. reuteri* KUB-AC5 was previously described as exerting antimicrobial properties against Gram negative bacteria, however, its characterization remains unknown. This study aimed to purify antimicrobial peptide from the strain KUB-AC5 and to clone its coding gene including characterization of its product.

Materials and methods

Bacterial strains and culture conditions

L. reuteri KUB-AC5 and *S.* Enteritidis S003 were used as an AMP producers and indicator strains, respectively. Strain S003 is shown to be resistant to various antibiotics previously used in Thailand, hence we used this strain as an indicator in this study. Both AMP producer and indicator strains were kept at the culture collection of the Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University and maintained as frozen stocks at -80°C in the presence of 20% glycerol. Strains KUB-AC5 and S003 were grown in MRS broth (Difco) or basal medium (BSM) containing (%) sucrose, 2; yeast extract, 0.5; $K_2HPO_4.3H_2O$, 0.26; $(NH_4)_2SO_4$, 0.1; $MgSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.004 and Tween 80, 0.1 (Hamsupo, 2005), and in Nutrient broth or Nutrient agar (Merck) at 37°C, respectively. For other bacterial strains used for inhibition spectrum and cloning study, all lactic acid bacteria (LAB) and bacilli were cultivated in MRS and LB media, respectively while *E. coli* and *Salmonella* sp. were in NB medium. All were cultivated at 37°C for about 15 h with shaking at 200 rpm, except for LAB. A list of bacterial strains and their relevant characteristics used for molecular cloning is given in Table 1.

Plasmid vectors and primers

All plasmid vectors and their recombinant DNA are listed in Table 2. Primers used for gene localization are listed in Table 3.

Table 1. Characteristics of bacterial strains for molecular cloning

Bacterial strain	Characteristic	Reference
Lactobacillus reuteri KUB-AC5	Bacteriocin KAC5 producing strain and source of *kac5*	Nitisinprasert *et al.*, 2000
L. plantarum TLG02	WCF1 derivative, Δ*alr*, D-alanine auxotroph used as an expression host	Nguyen *et al.*, 2011
Escherichia coli MB2159	MC 1000 derivative, D-alanine auxotroph used as a cloning host	Strych *et al.*, 2001
E. coli DH5α	Cloning and expression host	Woodcock *et al.*, 1989
E. coli ACE-C46	Recombinant clone *E. coli* containing pACE-C46 harbored I-C46 fragment	This work
E. coli MB-C46	Recombinant clone *E. coli* containing pSIP609(*alr*) harbored I-C46 fragment	This work
E. coli MB-C46-F2.1	Recombinant clone *E. coli* containing pSIP609(*alr*) harbored I-C46-F2.1 fragment	This work
E. coli MB-C46-F2.2	Recombinant clone *E. coli* containing pSIP609(*alr*) harbored I-C46-F2.2 fragment	This work
L. plantarum ACLP-C46	Recombinant clone *L. plantarum* containing pSIP609(*alr*) harbored I-C46 fragment	This work
L. plantarum ACLP-C46-F2.1	Recombinant clone *L. plantarum* containing pSIP609(*alr*) harbored I-C46-F2.1 fragment	This work
L. plantarum ACLP-C46-F2.2	Recombinant clone *L. plantarum* containing pSIP609(*alr*) harbored I-C46-F2.2 fragment	This work
L. reuteri ACLR-C46-F2.1	Recombinant clone *L. reuteri* containing pSIP609(*alr*) harbored I-C46-F2.1 fragment	This work

Table 2. Plasmid DNA and derivatives

Plasmid	Characteristic	Reference
pNZ307	Size of 3103 bp, genotype consisting of *lacZ*, *lacF* and *amp*^{r}	Nizo the food researchers, 2007
Food grade vector pSIP609	pSIP609 derivative, *alr* replaced with *erm*, *gusA* reporting gene	Nguyen *et al.*, 2011
pACE-C46	pNZ307 derivative containing insert DNA fragment I-C46	This work
pSIP609-C46	pSIP609 derivative, *alr* replaced with *erm*, *gusA* replaced by I-C46 fragment	This work
pSIP609-C46+2.1	pSIP609 derivative, *alr* replaced with *erm*, *gusA* replaced by I-C46-F2.1 fragment	This work
pSIP609-C46+2.2	pSIP609 derivative, *alr* replaced with *erm*, *gusA* replaced by I-C46-F2.2 fragment	This work

Table 3. Primers and sequences

Primer	Sequences (5'-3')	Purpose
P 1		Cloning of I-C46-F2.1 fragment
Forward	**CATGCCATGG**ATGATTATGTTGAACGGTTTTG	
Reverse	**CGGCTCGAG**TGAATCAGGCCAACAAACAA	
P 3		Cloning of I-C46-F2.2 fragment
Forward	**CATGCCATGG**ATGGGAAGTCAAAGAACG	
Reverse	**CGGCTCGAG**TAAACCTTTCAATCGCTTGA	
P 5		Cloning of I-C46 fragment
Forward	**CATGCCATGG**AGCTGGTACCGGGGACAT	
Reverse	**CGGCTCGAG**AGAGCAAAGTAATTAATGCATAAA	

Determination of antimicrobial activity

Cell free supernatant (CFS) of the test strain was prepared by centrifugation at 5800 g for 10 min and the intracellular fraction was extracted from its cell pellet according to the modified method of Fujimoto *et al.* (2004) to determine antimicrobial activities against *S.* Enteritidis S003. To determine intracellular extract preparation, cell pellets of 12 ml culture solution were washed twice with 1 ml sterile 50 mM phosphate buffer saline (PBS pH 6.0) and re-suspended in 500 µl of 50 mM PBS pH 5.0 containing 5.7 mg/ml PMSF (phenyl methyl sulfonyl fluoride) and 0.3 g of zirconia beads (0.1 mm). The cells were broken using a minibead beater 3110BX (Biospec, USA) for 3 min at 4800 rpm and the intracellular fraction was separated using centrifugation at 9000 g for 10 min.

Antimicrobial activities were determined, according to the modified methods of Hoover and Harlender (1993) and Rojo-Bezares *et al*. (2007), for spot-on-lawn and well diffusion agar assay, respectively. Briefly, the suitable agar medium was overlaid with 5 ml of soft agar medium (0.75% agar) containing 10 µl of an overnight culture of each studied indicator strain. Ten (spot-on-lawn) or 80 µl (well diffusion) of each sample with serial two-fold dilution were spotted onto the overlaid surface and incubated overnight at 37°C to determine the antimicrobial activity. Activity by spot-on-lawn method was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units (AU) per milliliter. The clear zone around the wells containing 80 µl of each sample, and regarded as the inhibitory zone, was measured in millimeters for each diameter.

Antimicrobial activity of the recombinant *L. reuteri* ACLR-C46-F2.1 was determined by a 96-well microplate according to the modified method of

Daba *et al*. (1991). Each well reaction of the microtiter plate consisted of 100 µl of two-fold concentrated NB broth pH 5.0 containing each overnight indicator strain diluted to OD_{600nm} ~0.6 and 100 µl of sample with serial two-fold dilution. Growth of the indicator strain at 5 h was determined for optical density at 600 nm with a Bio-Rad microtiter reader model 450, Bio-Rad Laboratories, Hercules, CA, USA. Activity was defined as the reciprocal of the dilution after the last serial dilution giving an optical density less than 0.05 and expressed as AU/ml. The activity calculation formula was represented as (1,000/100) x (1/D)-150, where D and 150 are the last serial dilution and the inhibitory activity of control (MRS), respectively.

Purification of AMP produced by L. reuteri KUB-AC5

Five percent (v/v) of 12-15 h MRS culture were inoculated into each 1 l of basal medium (BSM) and grown at 37℃ for 15 h. The cell free supernatant (CFS) was obtained by centrifugation at 5800 g for 10 min, heated to 70℃ for 35 min and further purified using the modified method of Cintas *et al.* (1995). Forty percent (w/v) of Amberlite XAD-16 matrix (Sigma) was added to 2 l of CFS and kept at 4°C with stirring for 2 h. Active AMP binding to the matrix was harvested using filtration through a plugged glass column, washed with 100 ml of deionized water to remove the brownish color, and then eluted with 100 ml of 70% (v/v) iso-propanol in deionized water adjusted to pH 2 with 50% phosphoric acid. The eluate showing antimicrobial activity by spot on lawn method was concentrated to 10 ml using an evaporator at 40°C, 50 bars (Buchi R-200/205, Switzerland). Only 6 ml of concentrated eluate was further subjected to gel filtration using a Sephacryl S-100 HR (Amersham Biosciences, Uppsala, Sweden) column (1 x 100 cm), equilibrated with 20 mmol citrate buffer pH 3. Active fractions exerting antimicrobial activity against *S.* Enteritidis S003 were combined and applied to reverse phase HPLC, Resource RPC (Amersham Biosciences, Uppsala, Sweden) column (3 ml) equilibrated with solution A (0.065% trifluoroacetic acid (TFA) and 2% (v/v) acetonitrile in deionized water). Active fractions were eluted with a gradient of 2% acetonitrile + 0.065% TFA (solution A) to 80% (v/v) acetonitrile + 0.05% TFA (solution B) at a constant flow rate of 1 ml/min. The gradient consisted of 100% A for 50 min, followed by a linear gradient from A (100-0%) to B (0-100%) for 45 min. Then, both unbound and eluted fractions were tested for their antimicrobial activities. Active fractions detected by the HPLC system (Thermo Separation Products, Inc., USA) were applied to a cation exchange SP-Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden) column of 1 x 10 cm equilibrated with 20 mM citrate buffer pH 3 as running buffer. Purified

peptide was detected using low pressure chromatography (Econo System, Bio-Rad, CA USA) and eluted in a linear gradient of 0-0.5 M NaCl in the same buffer at a constant flow rate of 1 ml/min for 30 min. Protein concentrations of all active fractions were assayed using the Lowry (Folin-Ciocalteau) method, according to Baines *et al.* (1999).

Determination of enzymatic sensitivity

Sensitivity of the AMP to proteolytic and other enzymes was tested by 1 mg/ml of the following enzymes: proteinase K, protease type XIII, chymotrypsin type II, trypsin type I, papain, pepsin A, α-amylase type X-A and lipase under their suitable conditions. Treated samples were determined for residual antimicrobial activity by the agar well diffusion method.

Determination of pH and temperature stability

Effects of pH were determined by adjusting the pH of purified and crude KAC5 to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 with NaOH or HCl. After 1 h, pH values of the sample mixtures were adjusted to their original pH and tested against the indicator strain for remaining antimicrobial activities by agar well diffusion spot-on-lawn method. Solution mixtures containing elution buffer (20 mmol citrate buffer pH 3 and 0.25 mol NaCl) and either NaOH or HCl contents used for pH adjustment which were performed as control treatments to remove the background due to the acid or base added.

Temperature effects were studied by heating to 37, 42, 70, 90 and 100°C for 30 min and at 121°C for 15 min. Remaining antimicrobial activities were determined by the agar well diffusion method.

Mode of action

Dilutions of overnight target cultures of *S.* Enteritidis S003 (10^4 and 10^8 cfu/ml) were prepared in a two-fold concentration of NB. Then, one volume of the purified AMP of 200 AU/ml was added to make the NB concentration one-fold. Elution buffer added instead of purified AMP solution was used as a control. The cultures were incubated at 37°C and their growth was determined using standard plate count assay at 1 h intervals for 4 h.

Determination of molecular mass and amino acid sequence of AMP

Molecular mass of AMP from the strain KUB-AC5 was analyzed using Voyager-RP matrix-assisted laser desorption and ionization time-of-flight mass

spectrometry (MALDI-TOF-MS, Perceptive Biosystems, Framingham, MA, USA) serviced by Bangkok Suvarnabhumi University (BSU), Thailand. A saturated α-cyano-4-hydroxycinnamic acid solution was used as a matrix (Ennahar *et al.*, 2001). To determine the N-terminal amino acid sequence, the AMP was blotted onto PVDF membranes. Automated Edman protein degradation was performed using a gas-phase sequencer (PPSQ-21, Shimadzu, Japan) facilitated by the Laboratory of Microbial Technology, Kyushu University, Japan.

Molecular cloning of gene coding for AMP and its nucleotide sequence analysis

The genomic library was performed using the modified method of Sambrook *et al.* (1989). Total genomic DNA isolated from *L. reuteri* KUB-AC5 was partially digested with Sau3AI to obtain 1 to 2 kb sized fragments, and later cloned into the BamHI site of *E. coli-Lactococcus lactis* shuttle vector pNZ307 and transformed to *E. coli* DH5α as a host cell. The recombinant clones were screened according to antibiotic resistance and antimicrobial activity against *S.* Enteritidis S003 using broth dilution assay. The positive clone was analyzed for its nucleotide sequence using the services of Microgen Co., Korea, and directly compared with non-redundant nucleotides in the GenBank database using the Basic Local Alignment Search Tool BLAST, (Rockville, MD, USA; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The open reading frame was investigated using the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/ gorf.html).

Localization of gene coding for AMP in L. plantarum

The recombinant pACE-C46 harboring *E. coli* ACE-C46, a source of gene coding for AMP, was amplified by PCR reaction using three primer sets of P1, P3 and P5 with the *Nco*I and *Xho*I recognition sites to obtain amplified DNA inserts of I-C46, I-C46-F2.1 and I-C46-F2.2, respectively. The PCR reaction contained approximately 50 ng of recombinant DNA pACE-C46; 10 μM of each primer, 5 μl; 2 mmol each dNTP, 5 μl; 1.25 U of *Pfu* DNA Polymerase (Thermo Scientific) and 10X *Pfu* buffer with $MgSO_4$ provided by the manufacturer with 5 μl of MilliQ water added to obtain the final volume of 50 μl. Amplification conditions consisted of initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 3 min, followed by a final extension step at 72°C for 10 min. The amplified PCR products and plasmid vector pSIP609 derivative were

purified using the NucleoSpin Plasmid Mini Kit (NucleoSpin® Plasmid/Plasmid (NoLid)) and further digested by *Nco*I and *Xho*I restriction enzymes. The DNA fragment obtained was ligated into the *Nco*I-*Xho*I site of gene coding for *gusA*-free pSIP609 and transformed into competent cell *E. coli* MB2159 prepared using the method of Sambrook and Russell (2001). The positive clone was screened using colony PCR reaction. In brief, a colony was suspended in 10 µl of PCR reaction using a sterilized toothpick. Amplification conditions consisted of denaturation at 95°C for 10 min followed by 30 cycles of 95°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 3 min, followed by a final extension step at 72°C for 10 min. The recombinant plasmid DNA of about 1 µg was transformed into the D-alanine auxotroph expression host *L. plantarum* TLG02 using an electroporation machine (Bio-Rad Gene Pulser II, USA) as described by Josson *et al.* (1989). The positive recombinant clones were screened using PCR reaction, and the antimicrobial activities of their intracellular extracts were assessed under an induction system following the modified method of Nguyen *et al.* (2011b). In brief, 1% of each wild type and recombinant clone was inoculated in 12 ml MRS broth and cultivated at 37℃ for 3 h to obtain optical density at 600 nm of approximately 0.3. Then, the expected bacteriocin was induced using peptide pheromone IP-673 at a final concentration of 25 ng/ml until 12 h at 30°C and determined for their antimicrobial activity by well diffusion assay.

Production of AMP from the recombinant L. plantarum

1% culture of each recombinant clone or *L. plantarum* harboring pSIP609 was inoculated into 120 ml of MRS broth and grown at 37°C for 3 h to obtain optical density at 600 nm of approximately 0.3. Then, peptide pheromone IP-673 was added to obtain a final concentration of 25 ng/ml. The culture reaction was incubated for 12 h at 30°C to harvest the cell pellets by centrifugation at 5800 g for 10 min. The intracellular fraction was prepared according to the modified method of Fujimoto *et al.* (2004). Cell pellets were washed by 10 ml sterile 50 mmol PBS (pH 6.0) once and resuspended into 5 ml of 50 mmol PBS (pH 5.0) containing 5.7 mg/ml PMSF (phenyl methyl sulfonyl fluoride) including an additional 0.3 g of zirconia beads (0.1 mm). The cells were broken using a minibead beater 3110BX (Biospec, USA) for 3 min at 4800rpm and centrifuged at 9000 g for 10 min to obtain the intracellular fraction. Its activity was determined by well diffusion assay. The CFS from the wild type *L. reuteri* KUB-AC5 (pH 5.0) and PBS (pH 5.0) containing 5.7 mg/ml PMSF were used as a positive and negative control, respectively.

Overexpression of the kac5 gene in wild type L. reuteri KUB-AC5

About 1 μg of the recombinant plasmid from the strain ACLP-C46-F2.1 was transformed into the wild type KUB-AC5 by the electroporation method following Josson *et al.* (1989), and screened for recombinant clones following the method of recombinant *L. plantarum* screening. To study time course fermentation, 1% of each test strain grown in MRS medium at 37℃ for 12 h was inoculated into 450 ml of MRS broth and grown at 37°C for 24 h. Each sample was collected at 2 h intervals to determine culture pH, antimicrobial activity and viable cell count using a standard plate count method (Wehr and Frank, 2004). Specific growth rate (μ) at the exponential phase was calculated using the equation: $\mu = (\ln x_{t2} - \ln x_{t1})/(t_2-t_1)$ where x_{t1} and x_{t2} are viable cell concentrations measured within the exponential phase of growth at times t_1 and t_2, respectively.

Results

Preliminary characterization of AMP from L. reuteri KUB-AC5

The CFS was treated with various proteolytic enzymes of proteinase K, protease type XIII, chymotrypsin type II, trypsin type I, papain and pepsin A including other enzymes of α-amylase type X-A and lipase. Only protease type XIII completely inactivated the inhibition activity of 200 AU/ml against the target strain *S.* Enteritidis S003. However, the activities were reduced to 50% by pepsin and α-chymotrypsin while still remained with other enzyme treatments. It indicated that inhibition activity occurred because the substance had a proteinaceous structure, possibly classified as antimicrobial peptide (AMP).

Two liters of CFS from the BSM culture were concentrated and partially purified using the amberlite adsorption-desorption technique, gel filtration and reversed phase chromatography to obtain a 2.4% yield as shown in Table 4. Interestingly, the brown compounds in the buffer at low pH 3.0 obtained from the gel filtration step were bound to C18 resin using reversed phase high performance liquid chromatography (RP-HPLC), and released the AMP as a clear solution which was purified using cation exchange chromatography SP-Sepharose Fast Flow. The AMP was eluted with 0.25 M NaCl for purification and yields of 38.84 folds and 0.4%, respectively. SDS-PAGE analysis confirmed only one homogeneous peptide band appeared (data not shown). The molecular mass of AMP analyzed using MALDI TOF mass spectrophotometry showed a peak of 4721.95 Dalton **(**Figure 1). About 900 pmol were applied into

the amino acid sequencer, but no amino acid peak was obtained. To confirm the peptide structure of KAC5, protease type XIII treatment was re-applied and resulted in complete inactivation of inhibition activity. This implied that AMP had a protein structure exhibiting inhibitory activity. Some amino acid modification might occur and affect amino acid sequencing. Nevertheless, the purified AMP was determined for mode of action at 10^4 and 10^8 cfu/ml *S.* Enteritidis S003 as bacterial target resulting in no survival cells detected within 1 and 4 h, respectively (Figure 2). This elucidated that AMP had bactericidal activity.

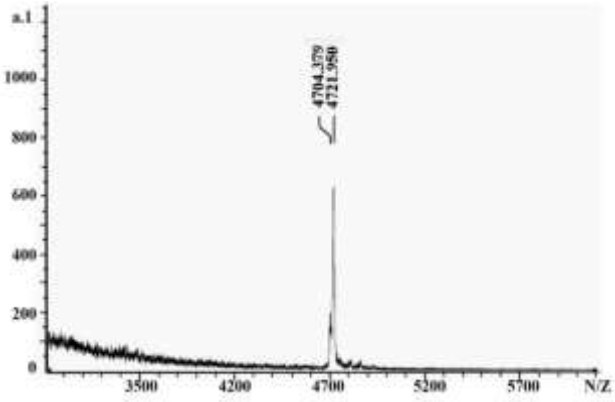

Figure 1. Mass spectrum of antimicrobial peptide KAC5 from MALDI TOF mass spectrophotometer

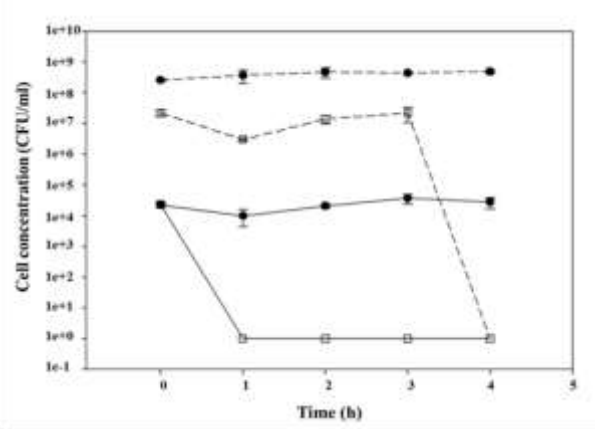

Figure 2. Nucleotide sequences and deduced amino acid sequence in open reading frame (ORF) +2 of *E. coli* ACE-C46, consisting of ORF-2.1 and ORF-2.2 at 116-268 bp and 401-1030 bp labeled in gray, respectively. When black arrow is expected site to cut with serine tyrosine kinase

Table 4. Purification of KAC5 produced by *L. reuteri* KUB-AC5

Purification step	Volume (ml)	Activity (AU/ml)	Protein (mg/ml)	Total activity (AU)	Specific activity (AU/mg protein)	Purification (fold)	Yield (%)
Supernatant	2000	200	1.67	400,000	119.76	1.00	100.0
Amberlite	6	3,200	168.33	19,200	19.01	0.16	4.8
Sephacryl S-100 HR	26	800	4.03	10,400	198.71	0.83	2.6
RPC-HPLC	24	800	0.26	9,600	3,076.92	12.85	2.4
SP-Sepharose Fast Flow	8	200	0.04	1,600	4,651.16	38.84	0.4

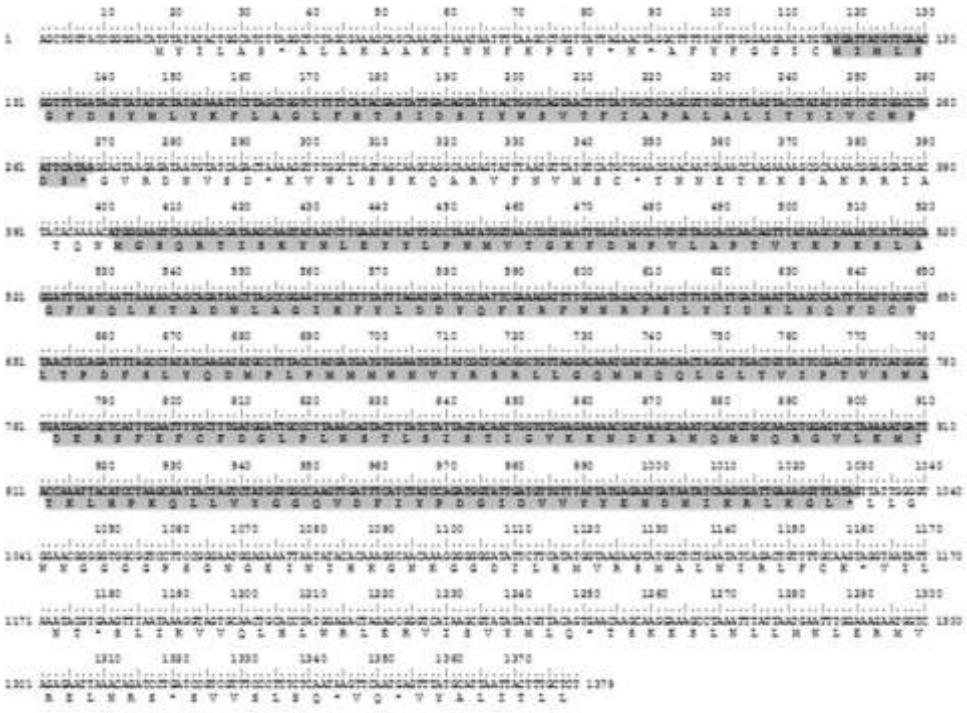

Figure 3. Mode of action of AMP-KAC5 against *S.* Enteritidis S003 at low concentration of 10^4 CFU/ml (_____) and of 10^8 CFU/ml (-------). Control, ●; KAC5 of 200 AU/ml addition, □□

Molecular cloning and sequencing of gene coding for AMP

The analysis of the amino acid sequence was not successful. The investigation of gene coding for AMP from the genomic library of *L. reuteri*

KUB-AC5 was performed and resulted in 183 clones. One positive clone, designated *E. coli* ACE-C46, exerted extracellular inhibitory activities by well diffusion assay of 10-15 mm against the indicator strain, while the negative control of the strain DH5α harboring plasmid pNZ307 showed no activity. The nucleotide sequence of the insert DNA, named I-C46 from the recombinant plasmid pACE-C46, contained 1384 base pairs. Its open reading frame (ORF) analyzed by the ORF Finder program revealed 1, 2, 1 and 2 ORF found in +1, +2, +3 and -3 strands, respectively. Based on the molecular mass of the AMP of about 4.7 kilo Dalton, only one possible ORF was found in the +2 strand, designated I-C46-F2.1 and I-C46-F2.2, comprising 153 and 630 nucleotides encoding 50 and 209 amino acids, respectively as shown in Figure 3. Using the NCBI BLAST analysis, the amino acid sequence of I-C46-F2.1 and I-C46-F2.2 showed low identity to the predicted protein accession no. ZP_05553516.1 of *L. coleohominis* 101-4-CHN and hypothetical protein LJP_0796 accession no. AEB93122.1 of *L. johnsonii* DPC 6026 at 35 and 44%, respectively. Localization of gene coding for AMP was planned for further study using the sub-cloning technique but unfortunately, the activities were lost after sub-cloning twice.

Localization of gene coding for AMP by lactobacilli expression system

Expression of gene coding for AMP in the *E. coli* system was unsuccessful, possibly because the inhibition activity of AMP was specific against *E. coli.* Therefore, another choice of lactobacilli system was applied. Several expression systems for production of heterologous proteins in LAB have been developed using a food grade vector for applications in the food and feed industry. The food grade vector pSIP609 containing the alanine racemase gene was successfully used in the expression of gene coding for β-galactosidase, 2,5-diketo-D-gluconic acid reductase (2,5-DKG reductase) and nuclease A (Nguyen *et al*., 2011a and 2011b; Kaswurm *et al*., 2013). Therefore, it was decided to subclone three fragments of I-C46, I-C46-F2.1 and I-C46-F2.2 into pSIP609 and transform them into *L. plantarum* TLG02 as a host cell. Three DNA inserts of I-C46, I-C46-F2.1 and I-C46-F2.2 with the linker of *Nco*I and *Xho*I recognition sites at 5' and 3' end were amplified and first cloned into *E. coli* MB2159. Three clones of *E. coli* MB-C46, *E. coli* MB-C46-F2.1 and *E. coli* MB-C46-F2.2 containing recombinant DNA pSIP609-C46, pSIP609-C46-F2.1 and pSIP609-C46-F2.2, respectively, were obtained. About 1 µg of each pSIP609 derivative was subcloned into *L. plantarum* TLG02, resulting in the recombinant *L. plantarum* strains ACLP-C46, ACLP-C46-F2.1 and ACLP-C46-F2.2 containing insert DNA fragments of I-C46, I-C46-F2.1 and I-C46-

F2.2, respectively, and confirmed using PCR analysis. The antimicrobial activity of cell free supernatant (CFS) from each recombinant clone comparing to the wild type and the pSIP609 harboring *L. plantarum* strain used as positive and negative control, respectively were resulted no activity, except the wild type. However, when the activities of each intracellular fraction were determined for antimicrobial activity, the results indicated that only the strain ACLP-C46-F2.1 exhibited activities of 14.6 mm inhibition zone, and less activities than the wild type *L. reuteri* KUB-AC5 (22 mm). While the activities of the recombinant strains ACLP-C46, and ACLP-C46-F2.2 were similar to the negative control which possibly came from the metabolites produced by *L. plantarum* itself. Therefore, it was elucidated that the open reading frame I-C46-F2.1 encoded the active AMP. However, the size of the deduced peptide started from methionine was 6260.37 Daltons which was larger than the size of mature peptide (4721.95 Daltons). It possibly contained a prepeptide sequence at the N-terminal. A prepeptide sequence or leader sequence containing information specifying the choice of targeting pathway, translocation efficiency cleavage timing, and even post-cleavage functions was proposed by Hegde and Bernstein (2006), and the different roles of leader peptides (Oman and van der Donk, 2010). The most common function is a secretion signal. When the serine protease, which plays a role in the cleavage of the prepeptide at the N termini of serine sites, and PROTEIN CALCULATOR v3.3 analysis were possible to be mature peptides resulted in the sequence of "YMLYK FLAGL FHTSI DSIYW SVTFI APALA LITYI VCWPD S" (Figure 2) with molecular weight of 4731.59 Daltons which closely to 4721.95 Daltons of pure peptide analyzed by MALDI TOF technique. The deduced amino acid sequence (In submission process under ID number 2253028) showed no similarity to bacteriocins previously reported by NCBI BLAST analysis.

Characterization of KAC5

The AMP produced by the wild type and the recombinant *L. plantarum* ACLP-C46-F2.1 were characterized. Sensitivity of both sources to proteolytic enzyme gave similar results with complete inactivation of inhibition activity by protease type XIII. Stabilities of both sources were performed at pH values of 2-12 with similar results over a wide pH range of 2-9. Activity was lost at high pH of 10-12. The effect of temperature on the stability of AMP from both sources was tested at 37, 42, 70, 90 and 100°C for 30 min at 121°C for 15 min. At low temperatures of 37 and 42°C, both showed the same relative activities of 100%. Interestingly, when the temperature increased to 70-121°C, the activities still remained at 100%.

The inhibition spectrum of AMP against various strains of LAB and both Gram positive and Gram negative pathogenic bacteria was compared to AMP from the wild type as shown in Table 5. They displayed no inhibition activity against all LAB tested *Listeria innocua*, *Bacillus coagulans* and *B. circulans*. However, they exhibited inhibitory activity against Gram positive bacteria of *B. cereus* and *B. subtilis*, and Gram-negative bacteria of *E. coli*, *S.* Enteritidis and *S.* Typhimurium. The purified peptide showed high activities against the bacilli group.

Table 5. Inhibition spectrum of AMP-KAC5 from *Lactobacillus*

Target strain	Crude AMS-KAC5 Activity (mm)	
	wild type *L. reuteri* KUB-AC5	recombinant *L. plantarum* ACLP-C46-F2.1
Bacillus cereus JCM2125	21.5	11.0
Bacillus subtilis TISTR025	28.5	15.5
Bacillus subtilis JCM1465	26.0	18.0
Bacillus coagulans JCM2257	0.0	0.0
Bacillus circulans JCM2504	17.0	13.5
Listeria innocua ATCC33090	0.0	0.0
Escherichia coli E010	22.0	12.5
Salmonella Enteritidis S003*	22.0	14.6
Salmonella Typhimurium TISTR292	22.0	12.0
Enterococcus faecalis TISTR927	0.0	0.0
Lactobacillus sakei TISTR912	0.0	0.0
Lactobacillus plantarum TISTR541	0.0	0.0
Lactococcus lactis ATCC19435	0.0	0.0
Lactobacillus reuteri KUB-AC16*	0.0	0.0
Leuconostoc mesenteroides TISTR473	0.0	0.0
Pediococcus acidilactici TISTR953	0.0	0.0

*Bacterial strains from culture collection at the Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand. ATCC, American Type Culture Collection, Rockville, MD, USA; IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microoganisms, Wako, Japan; TISTR, Thailand Institute of Scientific and Technological Research.

Based on the single AMP obtained from purification process and similar physicochemical characteristics and inhibition spectra of the AMP from both wild type *L. reuteri* KUB-AC5 and recombinant *L. plantarum* ACLP-C46-F2.1, it was concluded that the AMP from both sources were coded by *I-C46-F2.1* and named as KAC5.

Maximization of KAC5 production by subcloning of the I-C46-F2.1 gene into the wild type L. reuteri KUB-AC5

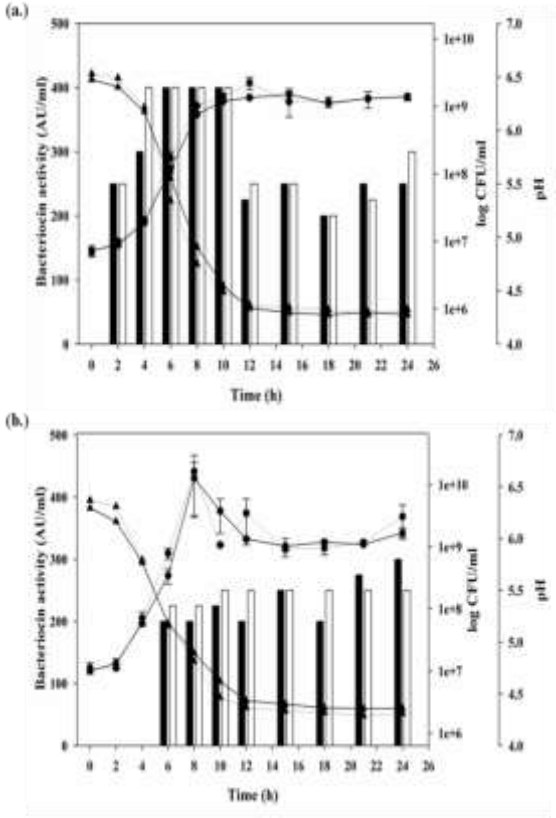

Figure 4. Time course of the bacterial growth and antimicrobial peptide KAC5 production by the recombinant strain (a) and wild type *L. reuteri* KUB-AC5 (b). ……, induction condition;_____, non-induction condition; ●, log CFU/ml; ▲, pH; black bar, AMP activity under inducible condition; gray bar, AMP activity under non-inducible condition

About 1 μg of pSIP609-C46-F2.1 from the strain ACLP-C46-F2.1 was transformed into the wild type KUB-AC5 and screened by colony PCR assay. Its transformation efficacy was 5.50 x 10^3 cfu/g of DNA, while efficiency of pSIP609(*alr*) was 3.32 x 10^3 cfu/g of DNA. One active clone No. 35 named *L. reuteri* ACLR-C46-F2.1 exerted extracellular antimicrobial activity of 250 AU/ml under induction condition at 12 h. Four treatments of induction and non-induction fermentation of the wild type *L. reuteri* KUB-AC5 and the recombinant strain ACLR-C46-F2.1 were performed as shown in Figure 4. The pH of all treatments showed similar patterns with decreased to 4.2. However,

specific growth rates of the wild type under induction and non-induction of 0.85-1.21/h were higher than the recombinant strain of 0.74-0.77/h. Bacteriocin production of all treatments showed growth association. Considering antimicrobial activity, both induction and non-induction fermentation of the wild type strain exhibited activities of 200-300 and 225-250 AU/ml, respectively while the recombinant strain displayed similar activities of 200-400 AU/ml during fermentation .However, AMP activities of the recombinant strain decreased during 12-24 h while the one of the wild type were stable. The CFS of the recombinant strain reached maximum antimicrobial activities of 400 AU/ml at 4 and 6 h under non-induction and induction, providing AMP productivities of 100 and 66.7 AU/ml.h, respectively. While the wild type reached its maximum activity at 10 and 24 h and exhibited lower activities of 100-150 AU/ml providing AMP productivities of 25 and 12.5 AU/ml.h under non-induction and induction, respectively. Thus, the peptide pheromone, IP-673, had no effect on maximum activities but might affect AMP productivity. In addition, the AMP productivity of the recombinant strain was higher than the wild type.

Discussion

The AMP produced by *L. reuteri* KUB-AC5 was successfully purified using minimal growth medium (BSM) to avoid several proteins from meat extract, yeast extract and peptone. Using several purification steps based on the characteristics of size, polarity and non-polarity, only one AMP with a low molecular weight of 4.7 kilo Daltons was obtained. To date, only a few AMPs or bacteriocins have been produced by *L. reuteri.* Reutericin 6, as a cyclic bacteriocin of 5.6 kilo Daltons, was produced by *L. reuteri* LA6 isolated from the feces of a human infant and exhibited inhibition activity against *L. delbrueckii* subsp. *bulgaricus* JCM1002^{T} (Kabuki *et al.*, 1997; Kawai *et al.*, 2001), while reutericyclin of only 349 Daltons from *L. reuteri* LTH2584 exhibited a broader inhibitory spectrum, especially for *B. subtilis* (Gänzle *et al.*, 2000). This suggested that the molecular weight of AMP KAC5 (4.7 kilo Daltons) was different from both previously found. The molecular mass of AMP from the wild type analyzed by MALDI TOF mass spectrophotometer could be a guideline to obtain gene *I-C46-F2.1* coding for AMP KAC5. Its stability at high temperature and wide pH range, as well as resistance to various proteolytic enzymes, indicated that KAC5 might have a circular peptide character which was more stable than linear peptides whose free ends are targeted by exopeptidases that weaken the stability of the molecule (Martin-Visscher *et al.*, 2011). Unsuccessful amino acid sequencing by Edman

degradation would support this potential circular character of KAC5. Currently, aspects of circular bacteriocin biosynthesis mechanisms are little known or understood. It is clearly shown that specific features in the mature peptide sequence are essential for circularization to occur. The N-terminal ends of circular bacteriocins, which are likely to contribute to the process which consist mainly of aromatic and/or hydrophobic residues with no C-terminal extension (Gabrielsen *et al.*, 2014). KAC5 contained tyrosine and methionine which are hydrophobic residues at the N-terminal and may be involved in cyclization reaction. However, this reaction is complicated. The VirB2 peptide is processed by the removal of a 47-residue N-terminal extension by a general signal peptidase, and further circularized in rapid succession either by an unknown enzyme or the same peptidase (Lai *et al.*, 2002). Therefore, circular form of KAC5 require extensive further study.

Gene coding for KAC5 was successfully expressed in both *L. plantarum* TLG02 and *L. reuteri* KUB-AC5. However, secretion of KAC5 from the recombinant *L. plantarum* ACLP-C46-F2.1 and *L. reuteri* ACLR-C46-F2.1 were different. The recombinant strain ACLP-C46-F2.1 produced KAC5 under an induction system and could not be secreted from the cells. This finding was similar to Jiménez *et al.* (2015) and Karlskas *et al*. (2014) who reported that secretion of enterocin A and nuclease A by recombinant strains *Lactobacillus* sp. and *L. plantarum*, respectively harbored the recombinant pSIP vector and needed their own signal peptide. Kaswurm *et al*. (2013) cloned dkr gene coding for 2,5-diketo-D-gluconic acid reductase from *Corynebacterium glutamicum* by *L. plantarum* TLG02/pSIP609 system, resulting in intracellular heteropeptide expression of 26.2 and 30.2 U/litre by induce and non-induce condition, respectively, while expression and secretion of extracellular chitosanase (CsnA) and beta-mannanase (ManB) from *B. licheniformis* and *B. subtilis*, respectively, in *L. plantarum* TLG02 using their own native signal peptides which was 50 kU/litre for ManB and 79 kU/litre for CsnA, respectively (Sak-Ubol *et al.*, 2016). Therefore, it could be concluded that protein expression by *L. plantarum* TLG02 required native signal peptide for secretion. The recombinant *L. reuteri* ACLR-C46-F2.1 obtained in this study did not need a peptide pheromone IP-673 for its expression and could secrete KAC5 as an extracellular product. Previous studies by Eijsink *et al*. (1996) and Nilsen *et al*. (1998) presented that secretion of enterocin A and enterocin B by *E. faecium* CTC492 and sakacin P by *L. sakei* LTH673, respectively, occurred as a result of their own signal peptides. Therefore, a signal peptide for the lactobacilli system would be needed. The mature peptide KAC5 proposed contained 41 amino acid residues which were different from 10 globular circular bacteriocins, ranging from 58 to 70 amino acids. There are cationic and amphiphilic molecules that kill bacterial

cells by insertion into the membrane, causing increased permeability and loss of barrier functions. However, leaders of the circular bacteriocins reflect remarkable differences in length and sequence, as well as the absence of conserved motifs, hindering a consensus sequence between their cleavable sites (Montalbán-López *et al.*, 2012). The proposed leader sequence of KAC5 was typical with different lengths and sequences to others. Considering the expression level by the pSIP system in different bacterial host cells, Jiménez *et al*. (2015) reported that the gene encoding enterocin A from *E. faecium* T136 cloned into the pSIP system and expressed in different LAB, resulting in higher productivity of antimicrobial substance in *L. sakei* and *L. casei* than the wild type T136 at 2.7 and 4.9 folds when pSIP411 was used as a vehicle. However, when *L. plantarum* NC8 was used as a host cell, lower productivities than the wild type at 4.8 folds were obtained. In addition, inhibition activities from these three hosts were lower than the wild type when pSIP409 used as a vector. The inhibitory activity of the recombinant strain ACLP-C46-F2.1 by similar vehicle series was lower than the wild type KUB-AC5. However, the vector pSIP609 harboring *lacLM* was successfully expressed beta-galactosidase by cloning into *L. plantarum* as proposed by Nguyen *et al.* (2011b), while expression of the *kac5* gene into the wild type strain KUB-AC5 via pSIP609 provided the recombinant *L. reuteri* ACLR-C46-F2.1 exerting higher activities than the wild type. Therefore, gene expression level depends to a considerable extent on gene carrier and the host used.

The peptide KAC5 displayed high stabilities at wide pH range of 2-9 and at high temperature of up to 121 ℃ as well as wide inhibition spectrum against Gram-positive and Gram-negative bacteria. The most intriguing aspect was lack of inhibition against the growth of LAB, even in closely related species of *L. reuteri*. This was different from the definition of bacteriocin characters that proposed as peptides or proteins exhibiting bactericidal activity against closely related species to the producer strain (Lindgren and Dobrogosz, 1990). In addition, the inhibition spectra of most bacteriocins of LAB did not inhibit Gram-negative bacteria, *E. coli* and *Salmonella* sp. However, their combination with acid, EDTA or other AMS could enhance their inhibition activities. Bacteriocin producing *Pediococcus acidilactici* K10 isolated from kimchi greatly inhibited *E. coli* O157:H7 when it was mixed with organic acid (Moon *et al.*, 2002). Some bacteriocins of LAB contributed to inactivation of Gram-negative bacteria in food when applied in combination with a chelating agent like EDTA (Scannell *et al*., 1997; Shefet *et al*., 1995), exposure to hydrostatic pressure (Kalchayanand *et al*., 2004), and low pH combined with 3% NaCl (Gänzle *et al*., 1999). In our study, KAC5 alone was still active

against Gram negative bacteria and showed bactericidal activity against *S.* Enteritidis, similar to reuterin (Mohamadi *et al*., 2005).

Bacteriocin production of both the wild type and recombinant strains was well clarified as a growth association which similar to enterocin P production by the recombinant *L. lactis* (Liu *et al.*, 2011) or pediocin production by the recombinant *L. lactis* NZ9000 (Liu, 2014). Although, they showed similar growth patterns, and their rate of growth and AMP productivities were different. The KAC5 productivity of the recombinant *L. reuteri* ACLR-C46-F2.1 started at early logarithmic phase of 2 h incubation, resulting in a higher productivity rate compared to the wild type which showed up at midlog phase of 6 h. This might be due to high copy number of the recombinant plasmid affecting high expression of gene coding for KAC5. By contrast, decrease of KAC5 activity at later stationary stage could be due to extracellular proteases produced during fermentation which similar to salivacin production proposed by Therdtatha *et al.* (2016) and enterocin A produced by the *Pichia pastoris* X-33EA, *Kluyveromyces lactis* GG799EA, *Hansenula polymorpha* KL8-1EA, and *Arxulaaden inivorans* G1212EA (Borrero *et al.*, 2012). Therefore, the expression of bacteriocin in its host cell, *L. reuteri* KUB-AC5, using a vehicle without an antibiotic as a selective marker at a suitable time would be valuable for food and feed applications in the future.

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