การโคลนและวิเคราะห์ยีนการเข้ารหัสต่อต้านซัลโมแนลลาจาก *Lactobacillus reuteri* KUB-AC5

Cloning and analysis of anti-salmonella substance encoding gene from *Lactobacillus reuteri* KUB-AC5

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บทคัดย่อ

เนื่องจากสารละลายใสที่ได้จากการเพาะเลี้ยง *Escherichia coli* DH5α ไม่แสดงกิจกรรมการยับยั้งเชื้อ *Salmonella* Enteritidis S003 สำหรับการโคลนโครโมโซมขนาด 1 – 2 กิโลเบส จาก *Lactobacillus reuteri* KUB-AC5 ซึ่งผลิตสารยับยั้งจุลินทรีย์ที่มีน้ำหนักโมเลกุลขนาด 4.7 กิโลดาลตัน ลงในตำแหน่งตัดจำเพาะ BamHI ของพาหะ pNZ307 พบเชื้อลูกผสม 2 โคลน ACE-C10 และ ACE-C182 ซึ่งแสดงกิจกรรมการยับยั้ง *Salmonella* Enteritidis S003 เป็น 10 ยูนิตต่อมิลลิลิตร พลาสมิดลูกผสม ที่สอดแทรกด้วยชิ้นดีเอ็นเอขนาด 1,115 และ 1,581 คู่เบส เมื่อวิเคราะห์ open reading frame พบว่าโปรตีนขนาด 6.5 กิโลดาลตัน จากชิ้นดีเอนเอ 1115 คู่เบส มีความเหมือนกับ hypothetical protein BSn5_11685 ของ *Bacillus subtilis* BSn5 เป็น 100% ซึ่งสามารถยับยั้ง *Erwinia carotovora* subsp. *carotovora* ขณะที่พบ 4 open reading frame จากชิ้นดีเอนเอ 1581 คู่เบส มีความเหมือนกับ hypothetical protein ของ *Coxiella burnetii* 'MSU Goat Q177 ที่ 32 % *Candida tenuis* ATCC 10573 ที่ 38 % และ *L. farciminis* KCTC 3681 ที่ 53 และ 59 % เห็นได้ว่าลำดับกรดอะมิโนจากโคลนทั้งสองโคลนไม่เหมือนกับแบคเทอริโอซินหรือสารยับยั้งคล้ายแบคเทอริโอซินอื่นๆที่ศึกษามาก่อน

คำสำคัญ : สารยับยั้ง *Lactobacillus reuteri*, การโคลนยีน, *Salmonella* Enteritidis

ABSTRACT

Escherichia coli DH5α and plasmid pNZ307 were appropriate host cell and plasmid vector for this cloning study, respectively. Since the supernatant did not show the inhibition activity against *Salmonella* Enteritidis S003, they were selected as the hosts for cloning of chromosomal DNA size 1-2 kb from *Lactobacillus reuteri* KUB-AC5, which producing antimicrobial substance molecular weight 4.7 kD into *Bam*HI restriction site of pNZ307 vector. Two recombinant clones, ACE-C10 and ACE-C182 which inserted DNA size 1115 and 1581 bp, exhibited inhibition activities against *Salmonella* Enteritidis S003 10 AU/ml. Only one open reading frame of 1115 bp was identical to hypothetical protein BSn5_11685 of *Bacillus subtilis* BSn5 with 100% similarity. While another 3 open reading frames of 1581 bp displayed low similarity to the hypothetical protein produced by *Coxiella burnetii* 'MSU Goat Q177 at 32 % *Candida tenuis* ATCC 10573 at 38 % and *L. farciminis* KCTC 3681 at 53 and 59 %. Based on their amino acid sequences, the two recombinant clones showed no similarity to bacteriocin or bacteriocin-like inhibition substance previously studied.

Keywords: antimicrobial substance, *Lactobacillus reuteri*, cloning, *Salmonella* Enteritidis

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INTRODUCTION

Bacteriocin, like nisin, produced by lactic acid bacteria (LAB) is a peptide which is accepted to use as a food preservative for dairy products, meat, fish and shrimp by the Food and Drug Administration, United States (Food and Drug Administration; FDA) (Muriana and Luchansky, 1993). Bacteriocin can be used in the form of pure or semi-purified compounds, or products containing ingredients derived from the fermentation of the microbial inhibitors (Chen and Hoover, 2003). *L. reuteri* KUB-AC5 isolated from the intestine of chicken exhibited inhibitory activity against the growth of Gram-negative bacteria causing disease in chickens (Nitisinprasert *et al.*, 2000). It exhibited antimicrobial substance (AMS) against 29 sero-types of *Salmonella* found in poultry farm. Its probiotic properties were carried out and resulted in toleration at wide pH range of 2-9, 3% bile salt and adhesion activity of 21.6%. When it was fed to newborn broiler chicks for the first week posthatch, it was demonstrated that the strain KUB-AC5 in the early posthatching period had a delayed effect on ileum microbiota, which resulted in the enrichment of potentially beneficial lactobacilli and the suppression of *Proteobacteria* including non-beneficial bacterial groups, genera *Klebsiella, Chryseobacterium*, *Citrobacter, Aeromonas, Acinetobacter* and order *Campylobacterales* (Nakphaichit et al. in press). Therefore, its AMS produced was further purified and characterized. Only low AMS concentration of 0.4 % (w/v) was obtained. By MALDI TOP analysis, a peptide of 4704.379 and 4721.95 daltons, designated “KAC5”, were detected. However, their structure could not be identified by Automated Edman protein degradation yet. Another way of molecular cloning approach of gene coding for AMS was proposed for this presentation, which aimed to investigate the suitable vector and host cell and to analyse a gene coding for AMS.

MATERIALS AND METHODS

1. Bacterial strains

Lactobacillus reuteri KUB-AC5 was used as a source of chromosomal DNA. It was grown at 37°C in MRS medium (Merck). *Escherichia coli* strain DH5α, TOP10 and their recombinants were grown at 37°C in Luria-Bertani (LB) medium (Merck) containing 100 µg/ml ampicillin .

2. Plasmid DNA

Plasmid pUC19 and pNZ307 were used as vector to prepare genomic library.

3. Growth determination of *Salmonella* Enteritidis S003

Salmonella Enteritidis S003 grown in Nutrient broth (NB) for 24 h was diluted to 7logCFU/ml. The cell pellet of 6logCFU obtained from 100 µl of the diluted culture was suspended in twice concentration of NB and then transferred to each well of microtiter plate containing 100 µl of test sample. The growth of *Salmonella* at 37°C for 24 h was followed by measuring the OD of 600 nm.

4. DNA manipulations

Total genomic DNA was isolated from a 24 h culture of *L. reuteri* KUB-AC5 by the method of Sambrook et al. (1989). To construct the genomic library, DNA partially digested with *Bsp*143I to obtain 1 to 2 kb fragments were ligated into *Bam*HI site of pNZ307 and transformed to *E. coli* DH5α. The recombinant clones grown on Luria Bertani agar containing 100 µg/ml ampicillin, 40 µg/ml X-gal and 100 µg/ml IPTG were screened according to ampicillin resistance, blue-white colony and antimicrobial activity against *Salmonella* Enteritidis S003.

5. Determination of antimicrobial activity

Each recombinant strain was grown in LB medium at 37°C for 22 h. Their supernatants were tested for antimicrobial activity by microtiter plate assay (Turcotte et al. 2003). The antimicrobial activity, expressed as AU (arbitary unit) per milliliter, was defined as the reciprocal of the highest twofold dilution showing 50% growth inhibition against *S.* Enteritidis S003 used as a indicator strain at 6 h.

6. Nucleotide sequence analysis

DNA sequencing was performed by the dideoxy-chain termination method using the service of 1st Base laboratory (Malaysia). Bioinformatics analysis was performed using data from GenBank.

RESULTS AND DISCUSSION

1. Effect of low pH to the growth of Salmonella Enteritidis S003

Since the antimicrobial substance KAC5 displayed the inhibition activity against *Salmonella* Enteritidis S003 at low pH. The NB growth medium of the target strain S003 was needed to adjust to low pH, at which would be able to inhibit the growth of most Gram negative bacteria including *Salmonella* as well (Arpa, 2007). Hence, growth of the strain S003 in NB medium adjusted to pH of 3, 4, 5 and 7 (LB,control) were studied as shown in Figure 1. After 6 h cultivation, the strain S003 at pH 5 was still growing while the growth at pH 3 and 4 were inhibited. However, the growths at pH 4 occurred during 6-24 h. Therefore, pH 5 was chosen to determine the inhibition activity at 6 h.

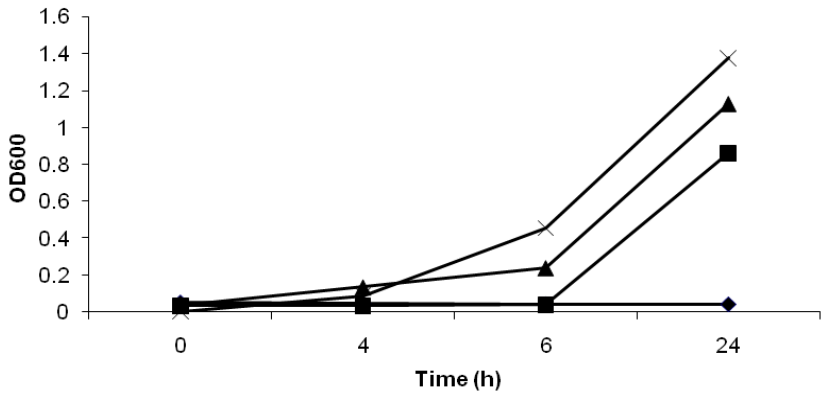


Figure 1 Growth of *Salmonella* Enteritidis S003 in NB medium at pH 3, ◆; 4, ■; 5, ▲ and LB, X.

2. Investigation of suitable *E. coli* strain and plasmid vector

Antimicrobial activity was a criterion to screen the recombinant clones producing AMS. The culture solution of various strains of *E. coli* containing different vectors or recombinant *E. coli* clones might produce some metabolites causing growth inhibition of the strain S003 used as the target strain. This would provide false positive in determination of inhibition activity. Two *E. coli* strains of TOP10 and DH5α were tested in this study. The effect of 22 h cell-free culture supernatant (CFS) of each strain grown in NB to the growth of *S.* Enteritidis S003 was determined at 0, 4, 6 and 24 h as shown in Figure 2. The OD of DH5α treatment was similar to the control treatment (LB medium). This result showed that the CFS of the strain DH5α did not affect the growth of the target strain. While the ones of Top10 treatment were only 0.04 and 0.8 at 6 and 24 h cultivation, respectively, resulting in growth inhibition of the target strain. Therefore, *E. coli* strain DH5α was selected and used as a host cell for further genomic library preparation.

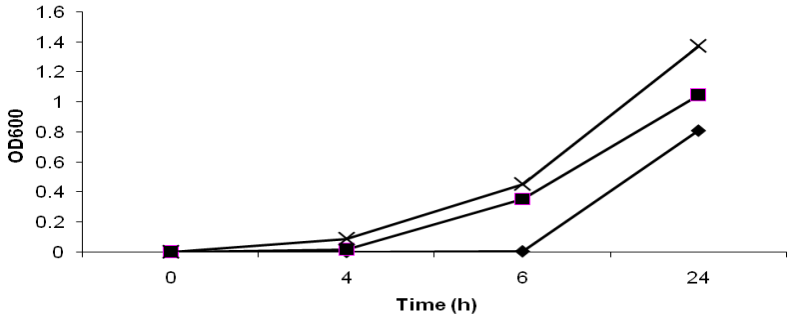


Figure 2 Growth of the target strain *Salmonella* Enteritidis S003 in NB containing CFS of *E. coli* DH5α, ■ ; Top10, ◆ and control (LB medium), X.

Plasmid pUC19 and pNZ307 were commonly used as cloning vectors when *E. coli* strain DH5α was used as a host cell. The CFS of pUC19 harbouring *E. coli* DH5α and pNZ307 harbouring *E. coli* DH5α were tested for their inhibition activities against a target strain at 0, 4, 6 and 24 h as shown in Figure 3. The CFS of both *E. coli* DH5α alone (control) and pNZ307 harbouring *E. coli*

DH5α affected the growth of target strain with the similar OD of 0.44-0.46 and 1.36-1.37 at the cultivation time of 6 and 24 h, respectively. While the CFS of pUC19 harbouring *E. coli* DH5α delayed the growth of *S.* Enteritidis with the OD of 0-0.9 during 6-24 h. Therefore, plasmid pNZ307 was chosen to use as a vector to perform genomic library.

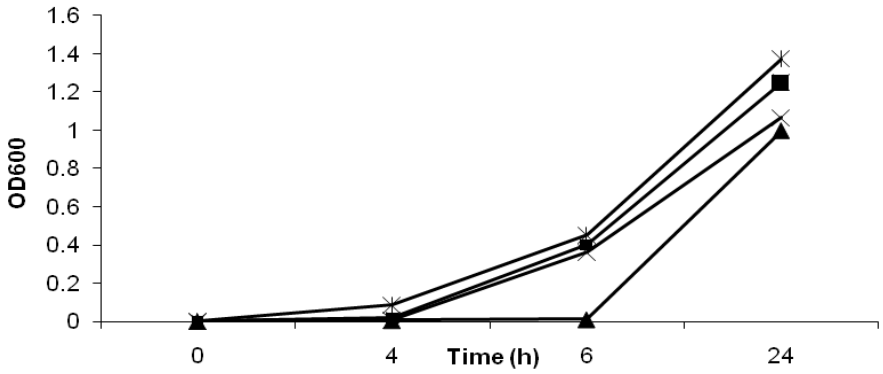


Figure 3 Growth of the target strain *Salmonella* Enteritidis S003 in NB containing CFS of pNZ307 harbouring *E. coli* DH5α, X ; pUC19 harbouring *E. coli* DH5α, ▲; *E. coli* DH5α. ■ and Control (LB medium), ✳.

3. Molecular cloning of gene coding for KAC5 in *E. coli*

Since the molecular weight of antimicrobial substance KAC5 was 4.7 kD. Partial digestion of chromosomal DNA to obtain 1-2 kb was therefore decided to prepare genomic library. One hundred eighty three recombinant clones obtained were screened by ampicillin resistance, blue-white colony and AMS activity. Two active clones “ACE-C10” and “ACE-C182” exhibited AMS activity of 10 AU/ml for 6 h as shown in Figure 4.

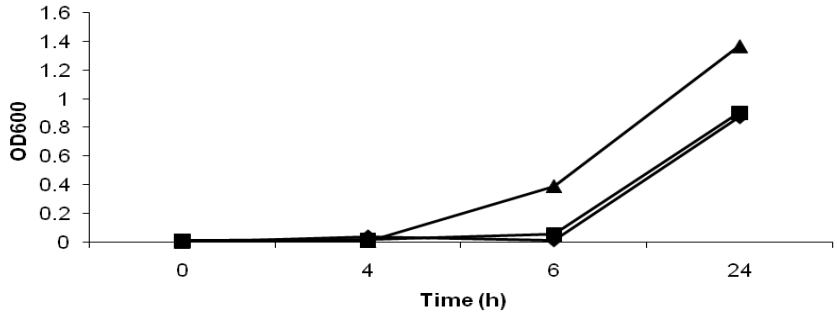
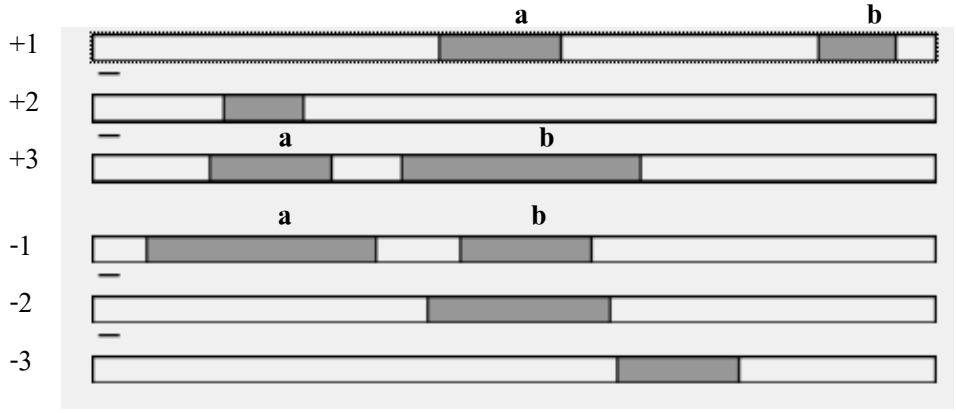


Figure 4 Growth of the target strain *Salmonella* Enteritidis S003 in NB containing CFS of ACE-C10,◆ ACE-C182, ■ and Control (pNZ307 harbouring *E. coli* DH5α), ▲.

4. Anlysis of gene coding for AMS from the recombinant clone ACE-C10

By Blastn analysis, the nucleotide sequences of 1115 bp insert DNA was similar to the promoter of the ampicillin resistance protein in the synthesis of proteins of plasmid pAm08CQ3205 and the ampicillin resistance protein gene of plasmid pESPBAC for 98%. By ORFinder program, 9

open reading frames (ORF) were obtained (Figure 5). No any ORF had the same size as KAC5 and showed similarity to any bacteriocin or bacteriocin like inhibition substance. However, one open reading frame (-1b) of 6.5 kD identitied to the hypothetical protein BSn5_11685 of *Bacillus subtilis* BSn5, protein sequence of BSn5_11685 were MRKEKIPHQA PFAIQAAQLL GRAIGAGLFA ITPAGERGMC CKAIKLGNAR VFPVTTL with strong inhibitory activity towards *Erwinia carotovora* subsp. *carotovora,* which causes soft rot disease in a wide variety of plantsfor (Yun Deng et al., 2011) at 100%. This might play a role in AMS activity. Purification and characteriazation of an active peptide is needed for further study.

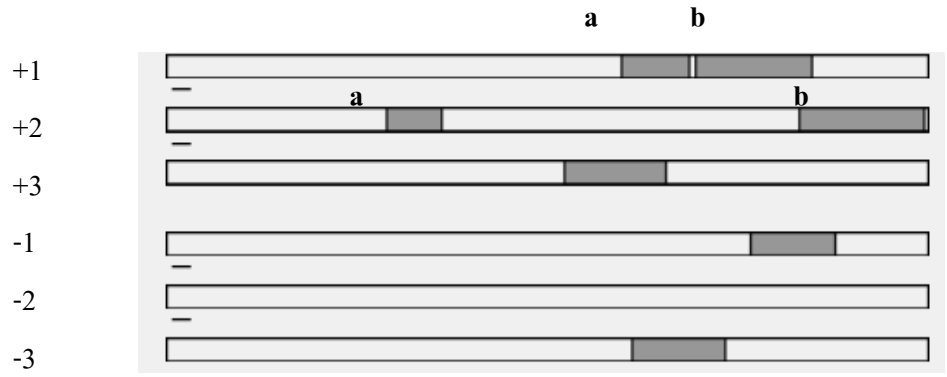


Frame -1b: MRKEKIPHQA PFAIQAAQLL GRAIGAGLFA ITPAGERGMC CKAIKLGNAR VFPVTTL

Figure 5 Open reading frame of insert DNA from the recombinant plasmid pACE-C10 and amino acid sequence of frame relating to hypothetical protein.

5. Anlysis of gene coding for AMS from the recombinant clone ACE-C182

By Blastn analysis, the nucleotide sequences of 1581 bp insert DNA identitied to the hypothetical protein gene of *L. farciminis* KCTC3681 for 59%. By ORFinder program, 7 open reading frames (ORF) were obtained (Figure 6). No any ORF had the same size as KAC5 and showed similarity to any bacteriocin or bacteriocin like inhibition substance. However, four open reading frame +1b, +2b, +3 and -1 of 10, 10.8, 3.9 and 7.4 kD displayed low similarity to the hypothetical protein produced by *L. farciminis* KCTC 3681 at 53%, *Candida tenuis* ATCC 10573 at 38%, *L. farciminis* KCTC 3681 at 59% and *Coxiella burnetii* 'MSU Goat Q177 at 32%, respectively. This might play a role in AMS activity. Purification and characteriazation of active peptide is needed for further study.



Frame +1b: MVIHQEINIK HDSMTDDVWS FIKDLVKQID EVNDFGLVSR SHQDSAWRDV YNEGYLHTPM NNEAIKEDYI NYVNEQSKI

Frame +2b MSMNSPKFSP KLFNTNKPTI KQPISIFPRH SQGMLKTKSL EAEVVYKKDF LHSIKDSKLH FTNCISELKD PDPSFPFLNK FNEFMH

Frame +3 MRPQYFFDDK YQLVSYLMER FKNPTPLKIQ KAMYFLWAFY AATYGNIDYS KESEFDEIKE YPKELFEAHF

Frame -1 MEIGCFIVGL FVLNNFGLNF GLFIDIIYIV LFYCFIIHWR MQITFIIDIA PCTILMTS

Figure 6 Open reading frame of insert DNA from the recombinant plasmid pACE-C182 and amino acid sequences of frames relating to hypothetical protein.

CONCLUSION

E. coli DH5α and plasmid pNZ307 were appropriate to be a host cell and plasmid vector for this cloning study because their supernatant did not show the inhibition activity, which may cause false positive. The AMS of *L. reuteri* KUB-AC5 produced antimicrobial substance of 4.7 kD. Therefore, chromosomal DNA of 1-2 kb was cloned into *Bam*HI site of pNZ307 and transformed to *Escherichia coli* DH5α. Two recombinant clones exhibited inhibition activities of 10 AU/ml against *Salmonella* Enteritidis S003. The nucleotide sequences of the insert DNA of 1115 and 1581 bp from the recombinant clone ACE-C10 and ACE-C182 identitied to *amp*^{r} gene and hypothetical protein gene of *L. farciminis* KCTC3681 for 98% and 59%, respectively. Only one open reading frame of 1115 bp showed 100% identity to hypothetical protein BSn5_11685 of *Bacillus subtilis* BSn5. While another 4 open reading frames of 1581 bp displayed low identity of 32-59% to the hypothetical protein produced by *Candida tenuis* ATCC 10573, *Coxiella burnetii* 'MSU Goat Q177 and *L. farciminis* KCTC 3681. Based on their amino acid sequences, they all showed no similarity to bacteriocin or bacteriocin-like inhibition substance previously studied.

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