

Full Length Research Paper

# Cell division inhibitor *minC* from *Lactobacillus Acidophilus* VTCC-B-871 and detection of antimicrobial activities in functional study

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The *minC* gene of *Lactobacillus acidophilus* VTCC-B-871 was identified based on the genome data of *L. rhamnosus* strains in genebank, and antimicrobial activities other than cell division inhibitor was detected. After cloning and sequencing, there was 99.1% homology between *minC* gene of *L. acidophilus* VTCC-B-871 and *L. rhamnosus* GG using Blast search. By analysis, the result also showed the close relation between these strains that was necessary in the identification of these probiotics. Besides, using pET21a(+) vector for the over-expression of *minC* protein in *Escherichia coli* BL21(DE3)plysS under 2 mM Isopropyl  $\beta$ -D-1-thiogalactopyranoside within 5 h, the filamentation occurred and showed that *minC* was functional across species. Especially, the study detected successfully, the antimicrobial activities on *Pseudomonas aeruginosa* ATCC 27853 in the cultures containing glucose, saccharose and manitol. The antimicrobial agent still showed the activities after treated with temperature at 100, 90, 80, 70, and 60°C in 15 min, but loss activity after protease K treatment at 10 mg/ml for 30 min. The characterization pointed the antimicrobial agent seemed as colicin in *E. coli*. The present work was the first reported in characterizing the *Lactobacillus minC* homolog that might be useful in probiotic classification and antibiotic production.

**Key words:** Antimicrobial activities, cell division inhibitor *minC*, comparative analyses, *Lactobacillus acidophilus* VTCC-B-871, morphology changes.

## INTRODUCTION

*MinC* was one of cell division inhibitors in *Escherichia coli* (*E. coli*), *Neisseria gonorrhoea* (Szeto et al., 2001) and *Bacillus subtilis* (Stahlber et al., 2004). In *E. coli*, the Min system was composed of the *minC*, *minD* and *minE* proteins encoded by the *minB* operon (De Boer et al., 1988, 1989). Most previous studies showed the effects of Min system in cell division, and only one study showed that *minC* was a nanorecorder (Bhomkar et al., 2011). However, there was no study on the oscillation of *minC* causing the alteration in secondary products in organisms that will produce and improve many products in the pharmaceutical field. *MinC* is dynamically related to the

FtsZ and other proteins (De Boer et al., 1989). Colicins are bacterial antibiotic toxins produced by *E. coli* cells and are active against *E. coli* and closely related strains. To penetrate the target cell, colicins bind to an outer membrane receptor at the cell surface and then translocate their N-terminal domain through the outer membrane and the periplasm. Once fully translocated, the N-terminal domain triggers entry of the catalytic C-terminal domain by an unknown process. Colicin K uses the Tsx nucleoside-specific receptor for binding at the cell surface, the OmpA protein for translocation through the outer membrane, and the TolABQR proteins for the transit through the periplasm (Benedetti et al., 1991; Aurélie et al., 2010). Colicins also interacted with FtsH (Mathieu et al., 2011). Here, we initiated studies to identify *minC* gene in *L. acidophilus* VTCC-B-871. That would be the way to

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identify *L. acidophilus* and *rhamnosus*, probiotics commonly used in healthcare (Kaur et al., 2001). Besides, the functional study on secondary product producing of the host, the secondary product was antimicrobial agents on pathogens. Until now, the pathogens as *Candida albicans*, *Salmonella typhi*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were the causes of infectious diseases.

For the above reasons, the study focused on the *minC* gene from *L. acidophilus* VTCC-B-871 to support the identification of *Lactobacillus* probiotics and to improve the secondary product in host. Firstly, *minC* gene in *L. acidophilus* VTCC-B-871 should be cloned and identified, followed by function study as cell division and antimicrobial activities.

## MATERIALS AND METHODS

### Plasmids, bacterial strains, growth conditions

The pGEM-T vectors used for molecular cloning and *E. coli* JM109, BL21(DE3)pLysS were purchased by Promega. The pET21(a+) used for over-expression was purchased by Novagen. *L. acidophilus* VTCC-B-871 was purchased by Vietnam type culture collection (VTCC). *E. coli* JM109 was used as a host to clone *Lactobacillus minC* gene. *E. coli* BL21(DE3)pLysS was used as an expression strain. *Lactobacillus* strain were grown on deMan, Rogosa and Sharpe (MRS) for 72 to 96 h at 30°C. *E. coli* strains were grown in Luria-Bertani (LB) for 18 to 24 h at 37°C, with shaking at 200 rpm. When required, antibiotics were added to media in the following concentrations: 100 µg of ampicillin/ml, 5, 20, 34 µg of chloramphenicol/ml for *E. coli*.

### DNA isolation

The *L. acidophilus* was incubated in MRS broth at 30°C in 48 to 72 h. The white colonies with the milky smell were picked up and transferred onto MRS agar. The incubation condition was at 37°C in 24 to 48 h aerobically. The genomic DNA was isolated according to Sambrook et al. (1989).

### Sequencing and analysis of *minC*

Based on the similarity of *minC* of *L. rhamnosus* strains sequenced and deposited in DNA Data Bank of Japan (DDBJ), the *minC* of *L. acidophilus* was thought to have a high similarity at the 5 and 3 ends of *minC* of *L. rhamnosus*. In order to clone, the primers used to isolate *minC* were designated from the *minC* of *L. rhamnosus*. The primer set was as sense 5'-GTGGACAGTGTGGTCTTAAAAGGTC-3' and an antisense 5'-CTACATTGCCTCCATTTGCTTAAATAATC-3'. The genomic DNA from *L. acidophilus* VTCC-B-871 strain was used as template to perform Polymerase chain reaction (PCR). The PCR fragment was amplified and sequencing was performed with the ABI PRISM 310 genetic analyzer using the BigDye terminator cycle sequencing ready reaction kit according to the manufacturer's protocols. The *Lactobacillus minC* gene was confirmed and analyzed using FastA. The protein molecular mass, pI, were calculated on an ExPASy Proteomics Server. The sequence data obtained in this study has been submitted to the DDBJ.

### Overexpression of *minC* and microscopy studies

The *L. acidophilus minC* gene was amplified by PCR with a sense primer CHE1 (5'-CATATGGACAGTGTGGTCTTAAAAGGTC-3') (the *NdeI* site is underlined) and an antisense CHE2 (5'-CTCGAGCATTGCCTCCATTTGCTTAAATAATC-3') (the *XhoI* site is underlined). The amplified *minC* was subcloned into pGEM-T vector and then checked again by DNA sequencing. The *minC* fragment was cut out from pGEM-T vector by *NdeI* and *XhoI* double-digestion and inserted into the same sites of pET-21(a+) to produce pET-21(a+)/*minC*. *E. coli* BL21(DE3)pLysS transformed with pET-21(a+)/*minC* was grown at 37°C in Luria-Bertani (LB) medium supplemented with appropriate antibiotics but chloramphenicol at 5, 20, and 34 µg/µl to OD<sub>600</sub> = 0.4 to 0.5, after which 2 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to culture to induce at 37°C for 5 h. Light microscopy was used to observe the morphological changes in *E. coli*.

### Antimicrobial activity test

Antimicrobial effects were tested on *S. aureus* ATCC 25923, *E. coli* ATCC 9637, *Salmonella typhi* ATCC 19430, *C. albicans* ATCC 14053, *P. aeruginosa* ATCC 27853 by the agar diffusion method. The tested microorganisms were propagated twice and then grown for 18 to 24 h in 10 ml of appropriate growth media. Turbidity of the culture broth was compared with McFarland tubes to give an estimate of bacterial population (1 × 10<sup>6</sup> CFU/ml). Supernatant of the cell after expression were collected after centrifugation at 12,000 × g for 15 min, and the clear supernatant was sterilized by filtration (0.45 µm), thus yielding cell-free filtrates.

The wells (ø 6 mm) were then prepared and filled using 100 µl of cell-free filtrate. The inoculated plates were incubated for 18 to 24 h at appropriate temperatures, and the diameter of the inhibition zone was measured in millimeters with calipers. The measurements recorded were from the edge of the zone to the edge of the wall. The study was also optimized for the antimicrobial activities in cultures containing 1% glucose, 1% saccharose and 1% manitol.

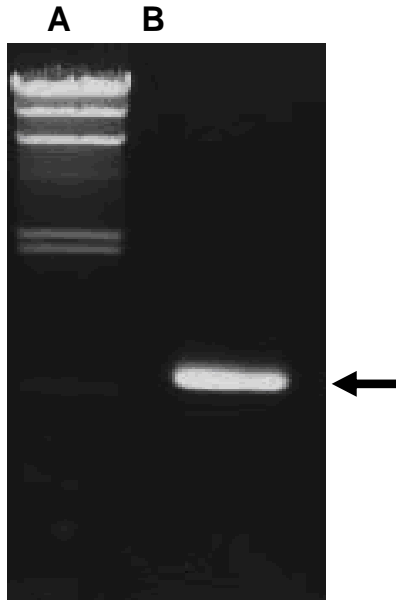
### Partial characterization of the antimicrobial agent

To test the thermostability and sensitivity to protease, both tests were performed according to Lewus et al. (1991). 100 µl of the supernatant were incubated 15 min at 60, 70, 80, 90 and 100°C within 15 min, followed by the bacteriocin test. To test the sensitivity to protease, the supernatant was treated with proteinase K (10 mg/ml) and the mixture was incubated 30 min at 37°C, followed by the bacteriocin tests.

## RESULTS

### Analysis of *minC* gene of *L. acidophilus* VTCC-B-871

After PCR performance, a PCR product was amplified (Figure 1). After cloning, a *minC* gene of 657 bp was sequenced. This sequence showed 99.1% similarity to *L. rhamnosus* GG and was deposited in the DDBJ database under accession no. AB755424. The *minC* protein of *L. acidophilus* VTCC-B-871 consisted of 218 amino acids with a calculated pI of 5.31 and Mw of 23509 Da. The protein exhibits 99% identity to *minC* from *L. rhamnosus* GG (ID: 8421052). Interestingly, there were similarities of 60 to 70 nucleotides at both 5' end and 3' end of *L.*



**Figure 1.** The PCR product of *minC* gene from isolated *Lactobacillus acidophilus*. A:  $\lambda$ Hind III marker, B: PCR product. The arrow showed the PCR product.

*rhamnosus* GG and *L. acidophilus* VTCC-B-871. Figures 2 and 3 showed the similarity of the nucleotides in the sequence at both ends and the difference of the nucleotides of A to C at the position of 70 nt and G at the 590 nt of the sequence, respectively. Based on the similarity, the designated primer set would be used to identify these *Lactobacillus* strains.

#### Expression of *minC* gene in *E. coli* and its effect on cell division

To study the function of *L. acidophilus minC* protein, this work tried to test whether the *L. acidophilus minC* protein was functional in *E. coli* cells, the *E. coli* BL21(DE3)plyS was introduced with plasmid pET-21(a+)/*minC*. The recombinant *E. coli* cells were cultured in media containing without or with chloramphenicol at 5, 20 and 34  $\mu\text{g}/\mu\text{l}$ .

The over-expression was done under Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) with or without combination of glucose, saccharose, manitol. Under the condition of 2 mM IPTG for expression, the cells transformed with the pET-21(a+) exhibited normal rod-shaped morphology (Figure 4) and pET-21(a+)/*minC* exhibited the filament formation after 5 h of over-expression. This result indicated that *Lactobacillus minC* was functional across species. The morphology of *E. coli* harboring *minC* in different sugars also formed filamentation after 5 h-overexpression.

#### Antimicrobial activities

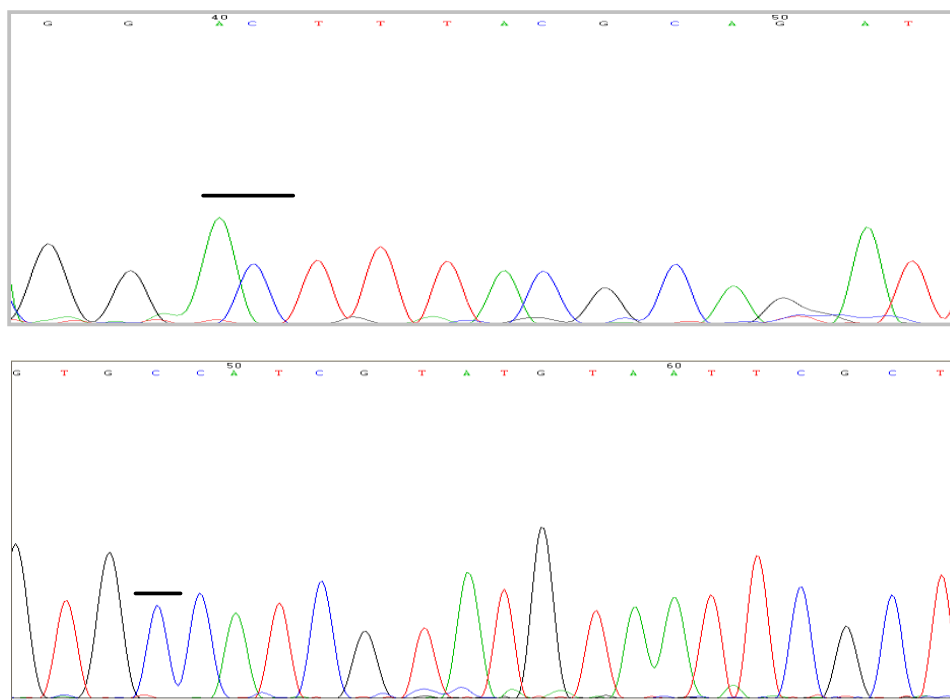
The supernatants of the overexpressed cell cultures with and without glucose, saccharose, manitol were centrifuged and used to test the activities on the above mentioned pathogens. The results showed the activities on *P. aeruginosa* (Figure 5). The activities were also obtained in the cultures without ampicillin and cloramphenicol surprisingly (Figure 5). The antimicrobial agent was tested on the other strains (data not shown). The antimicrobial agent might be the colicin secreted from *E. coli*. This result revealed that *minC* might be related to bacteriocin production. The tests also used the samples from the *E. coli* without harboring *Lactobacillus minC*. Consequently, there was no inhibition zone formed by this strain (Figure 5). Presented in Figure 5 was the inhibition of the growth of *P. aeruginosa* ATCC 27853 by the action of the supernatants. The inhibition zone diameters were 17 to 19 mm and were not significant between conditions ( $P < 0.05$ ). To characterize the agent, the supernatant was treated with high temperature and protease K which showed that the antimicrobial agent in the supernatant was heat-stable and damage in protease K (Figure 6). However, there was not significant in five levels of tested temperature ( $P < 0.05$ ). The results presented the inhibition zone in Figure 6.

#### DISCUSSION

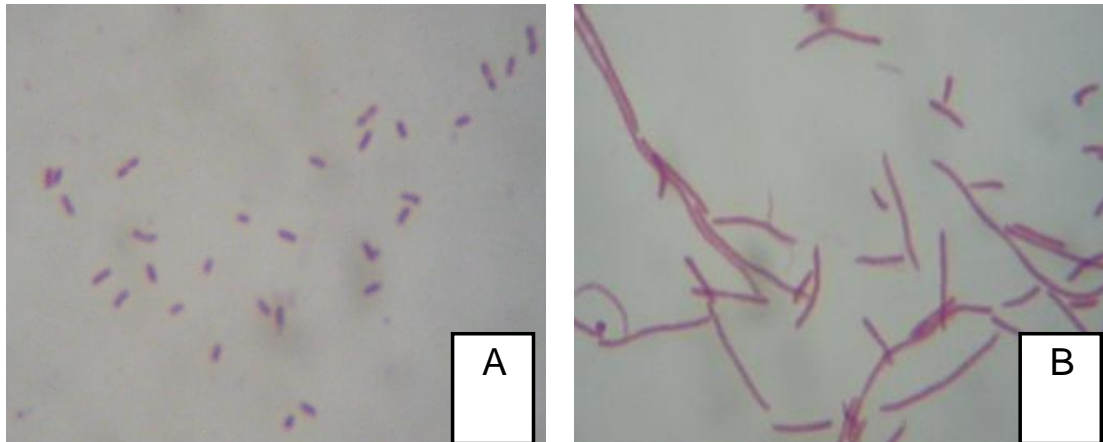
The PCR product was amplified successfully in Figure 1 and the *minC* sequence was confirmed and analysed in Figure 2. It meant that *L. acidophilus* and *rhamnosus* had the similarity in cell division. Especially, the sequences had the similarity at both ends that might be convenient to identify these strain or that they were closely related. The *minC* gene was overexpressed in cultures with and without sugars, but the filamentation formation still developed. The sugars supplied the energy for cell growth as Adenosine-5'-triphosphate (ATP) or Guanosine-5'-triphosphate (GTP). *MinC* activated with *minD* and FtsZ (De Boer et al., 1991). However, *minD* required ATP while FtsZ required GTP for their functions (Walker et al., 1982). The inhibition on *Pseudomonas* might reveal a colicin in the host *E. coli*. The study tried to test the activities in cultures without any antibiotic as well as in heat treatment and consequently, there were also the inhibition. In the Figure 5, the supernatants from *E. coli* without harboring *minC* did not show the antimicrobial activities. Therefore, *minC* of *Lactobacillus* introduced into *E. coli* had oscillation and interaction with *minCDE* of *E. coli* and change the conformation of the cell membrane and caused colicin to be secreted. In the lower concentration of chloramphenicol as 5  $\mu\text{g}/\text{ml}$ , the activities was weaker than the concentration of chloramphenicol at 20 and 34  $\mu\text{g}/\text{ml}$  (Figure 5). With the result without chloramphenol in the combination of the



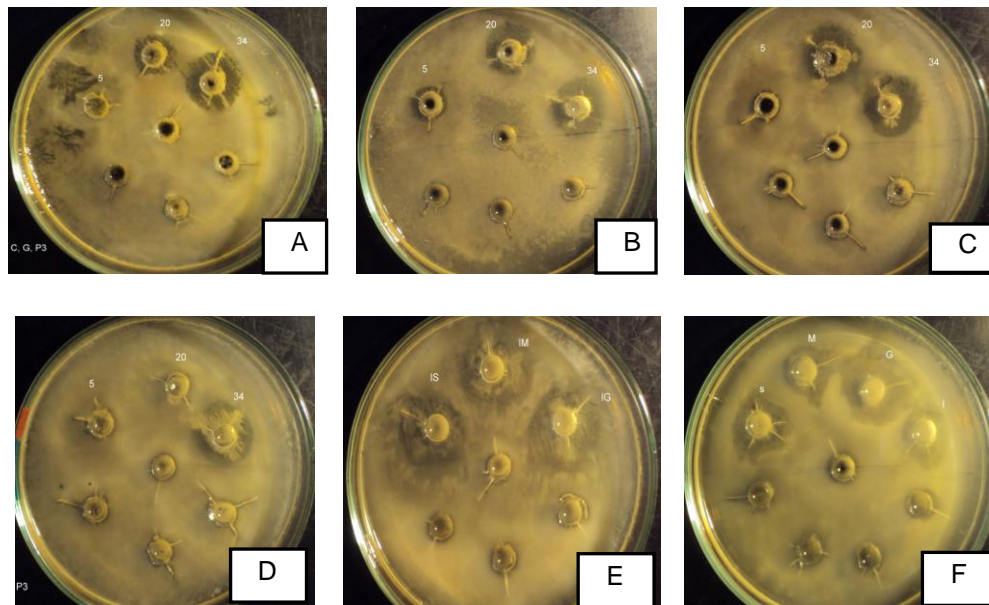
**Figure 2.** Comparison of the nucleotide segment at both 5 and 3 ends of *L. acidophilus* and *L. rhamnosus* using Blast search. The red letters indicate the similarity at 5 and 3 ends. The green letters indicate that the different nucleotides appeared first at both ends. The stars show the sequence remaining portion.



**Figure 3.** The chromatogram portion of sequence showed the first different site and the last different sites at the both ends of nucleotide sequence. The upper: the black short line on the peaks of A and C indicate that the different nucleotides appeared first at 5 end. The lower: the black short line on the peaks of C indicate that the complementary nucleotide G appeared at 3 end.



**Figure 4.** Morphology of *E. coli* cells harboring *minC* gene from *Lactobacillus acidophilus*. A: *E. coli* BL21 (DE3)plysS cells harboring pET 21a(+), B: pET-21a(+)/*minC*. The morphology was analyzed by light microscopy. The scale bar is 5  $\mu$ .



**Figure 5.** Antimicrobial activity test on *Pseudomonas aeruginosa*. The activities are shown in cultures containing A: glucose, B: IPTG, C: saccharose, D: manitol, E: IPTG combined sugars, F: without antibiotics. The white letters indicate the supernatants from *E. coli* BL21 (DE3)plysS cells harboring pET-21a(+)/*minC*.

control, the used antibiotics were lysed during culture (Benjamin et al., 2009). Clearly, *minC* play a role in antimicrobial agent or bacteriocin production.

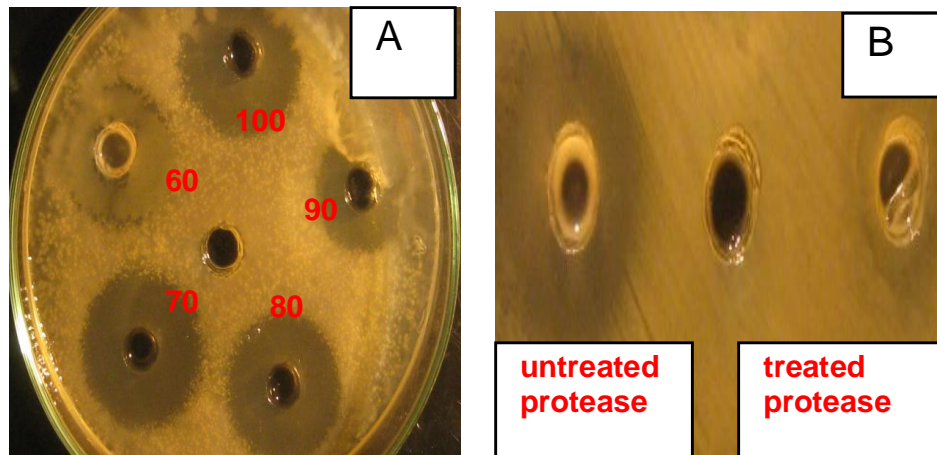
### CONCLUSION

The study was the first report that *minC* play a role in antimicrobial agent or suspected colicin. The study also detected *minC* in *L. acidophilus* that was benefit in identi-

fication of *L. rhamnosus* and *L. acidophilus*. The *minC* homologue was also over-expressed in *E. coli* successfully.

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**Figure 6.** Antimicrobial activity tests on *Pseudomonas aeruginosa*. A: After heat treatment at 100, 90, 80, 70, and 60°C, B: protease K treatment.

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