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Studies of the Paclitaxel Delivery and Antimicrobial Activities Generated by MinD from *Lactobacillus acidophilus* VTCC-B-871

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In this study, the *minD* gene from *Lactobacillus acidophilus* VTCC-B-871 was introduced into *Escherichia coli* BL21(DE3)plysS using pET 21(a+) vector. The overexpression under 0.5mM Isopropyl β-D-1-thiogalactopyranoside within 5 hours caused filamentation and 24 hours caused minicells while in the glucose condition, the cells were filaments, interestingly. The minicells was packaged with paclitaxel at 10 mg/ml in 10 to 18 hours. After extraction of the packaged cells and high performance liquid chromatography performance, there was paclitaxel exisiting in the minicells. The study also tried to test antimicrobial activities and consequently, *Pseudomonas aeruginosa* ATCC 27853 was detected. The antimicrobial agent still showed the activities after treated with temperature at 100°C, 90 °C, 80°C, 70 °C, 60 °C in 15 minutes, but loss activity after protease K treatment at 10 mg/ml for 30 minutes. The characterization pointed the antimicrobial agent seemed a bacteriocin or colicin in *Escherichia coli*. As a result, MinD might affect on bacteriocin production in the host. The present work was the first report in characterizing the *Lactobacillus* MinD homolog that might be useful in antibiotic fields as well as drug delivery for paclitaxel.

Key words: Morphology change, *Lactobacillus acidophilus* VTCC-B-871, MinD, Minicell, Paclitaxel, Antimicrobial activities.

MinD protein acts as the inhibitors of cell division by blocking septum formation at all potential division sites (polar and mid sites) (Szeto J *et al*., 2001). In *E. coli*, there was MinC, MinD and MinE (De Boer PAJ *et al*., 1988). MinD showed the ATP hydrolysis (De Boer PAJ *et al*., 1991). In the other hand, *Bacillus subtilis* contained MinCD homologues and DivIVA acts topologically, but not MinE (Stahlber H *et al*., 2004). In human, there was only MinD according to the complete human sequence. It was also noticed that the entire nucleotide sequences of the *Streptomyces* genomes of *Streptomyces coelicolor* and *Streptomyces avermitilis* have reported (Bently SD *et al*., 2002; Ikeda H *et al*., 2003) and these strains carried the MinD homolog, but not MinC or MinE. The MinD homolog harbored by *Streptomyces lavendulae* ATCC 25233 has also been characterized and might have a role other than cell division (Nguyen HKT *et al*., 2008). Recently, the deletion of *minD* in enterohemorrhagic *Escherichia coli* EHEC caused reduced adherence to human epithelioid cervical carcinoma (HeLa) and human colonic adenocarcinoma (Caco-2) cells as compared to wild-type those were studied (Parti RP *et al*., 2011). The *minD* mutant formed minicells and filaments owing to aberrant cytokinesis. Inactivation of *minC*, *minD* or *oxyR* in *Neisseria gonorrhoeae* attenuated its ability to bind and invade urethral epithelial cells without altering its potential to induce IL6 and IL8 release (Parti RP *et al*., 2011).

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Resistance of drug was one of our biggest problems in therapy, especially cancer and infectious disease therapy. Therefore, the requirements for disease treatment were essential and developed rapidly with the involvement in numerous physical and chemical methodologies. Remarkably, bacterial minicells were anucleate nanoparticles produced as a result of inactivating the genes controlling the normal bacterial cell division, due to depressing the polar sites of cell division, may provide the better way for cytotoxic drug delivery. The minicells were also prepared from genetically defined *minCED*(-) chromosomal deletion bacteria and then the subsequent minicells were purified. The deletion of *minCED*(-) out of the bacteria cell may affect on their growth under their control so far (De Boer PAJ *et al*., 1989). MinD might show other function. MinD oscillation depend on temperature (Ahmed T *et al*., 2006). The study suggest the way of minicell production that may be the better way for cancer treatment. Paclitaxel was used as an anti-proliferative agent for the prevention of restenosis of coronary stents; locally delivered to the wall of the coronary artery, a paclitaxel coating limits the growth of neointima (scar tissue) within stents (Heldman *et al*., 2001). Until now, the pathogens as *Candida albicans*, *Salmonella typhi*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* were the causes of infectious diseases. Therefore, obtaining the new antibiotic or the mechanism of antibiotic producing was essential.

The study selected the cell division inhibitor MinD of *Lactobacillus acidophilus* VTCC-B-871 as research object because of the use of *Lactobacillus acidophilus* in medicinal products (Kaur IP *et al*.,2001).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

Escherichia coli BL21(DE3)pLysS was purchased by Promega. The pET21(a+) used for overexpression was purchased by Novagen. *Lactobacillus acidophilus* VTCC-B-871 purchased by Vietnam type culture collection (VTCC). *Escherichia coli* BL21(DE3)pLysS was used as an expression strain. *Lactobacillus* strain were grown on MRS (de Man JD *et al*., 1960) for 72-96 hours at 30°C. *Escherichia coli* strain was grown in Luria-Bertani (LB) for 18-24 hours at 37°C with shaking at 200 rpm. When required, antibiotics as ampicillin and chloramphenicol were added to media. Isopropyl â-D-1-thiogalactopyranoside (IPTG) was used at 0.5 mM.

Introduction of MinD into E. coli and light microscopy

The *Lactobacillus acidophilus minD* was amplified by PCR with a sense primer BHE1 (5'-CATATGGGGACAGCGTTAGTAGTGACTTC-3') (the *NdeI* site is underlined) and an antisense BHE2 (5'-CTCGAGGATGGCGATGGAACAATTTTGAC-3') (the *XhoI* site is underlined). The amplified *minD* was cut by *NdeI* and *XhoI* double-digestion and inserted into the same sites of pET-21(a+) to produce pET-21(a+)/*minD*. The transformation was performed according to Sambrook *et al*., 1989. The *E. coli* BL21(DE3)pLysS transformed with pET-21(a+)/*minD* was grown in LB medium supplemented with appropiate antibiotics at 37°C to OD600 = 0.3, after which 0.5 mM IPTG was added to culture to induce at 28°C – 37 °C from 5 to 24 hours. Light microscopy was used to observe the morphological changes in *E. coli*. The study also used 1% glucose as inducer instead of IPTG.

Minicell generation and paclitaxel packaging

The cells were overexpressed in the medium contaning chloramphenicol at 34 µg/ml for 24 hours, the cells were taken out and observed under light microscope to make sure that the minicells were generated. The collected cells were suspended in phosphate buffer solution (PBS), pH7.0 and then incubated in paclitaxel at 10 µg/ml from 10 hours to 18 hours.

High performance liquid chromatography (HPLC) analysis

The cell suspensions incubated with paclitaxel at 10 µg/ml from 10 hours to 18 hours were centrifuged at 12.000 g x 15minutes. The supernatants were used to analyze for the paclitaxel remaining . In the other hand, the cells was washed five times with phosphate buffer solution (PBS). Then, the cells were ground with glass beads for 30minutes in isopropanol and PBS (1: 5). The extract and the above supernatants were analyzed by HPLC. The HPLC system (Shimadzu, Kyoto, Japan) consisted of LC-8A solvent delivery module and UV-Visible spectrophotometric detector and phenomenax C-18 column (250x4.60 mm-5microns) was used for the analysis. Sensitivity was set at

0.001 a.u.f.s. Mobile phase consisting of water, acetonitrile and methanol (40:30:30: v/v) was used at a flow rate of 1.0ml/min. The elute was monitored using a UV/VIS detector set at 228nm (Rajender G *et al*., 2009).

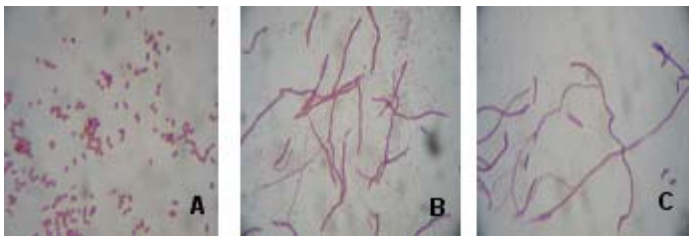
Antimicrobial activity tests

Antimicrobial effects were tested on *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 9637, *Salmonella typhi* ATCC 19430, *Candida albicans* ATCC 14053, *Pseudomonas aeruginosa* ATCC 27853 were determined by the agar diffusion method. The tested microorganisms were propagated twice and then grown for 18-24 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 (1×10^6 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 18-24 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. 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 at 60, 70, 80, 90 and 100°C within 15 minutes (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 in after incubated 30 min at 37°C, followed by the bacteriocin tests.

RESULTS

Overexpression of MinD from Lactobacillus acidophilus

The *minD* gene introduced into *Escherichia coli* by transformation of pET21(a+)/*minD*. After induction of IPTG or glucose for 5 hours at 37 °C, the *E. coli* cells became filaments (Fig. 1). The *E. coli* harbored pET21(a+) without



A: *E. coli* harboring pET21(a+), B: *E. coli* harboring pET21(a+)/*minD* and induced by IPTG, C: *E. coli* harboring pET21(a+)/*minD* and induced by glucose

Fig. 1. *Escherichia coli* after 5 hour overexpression

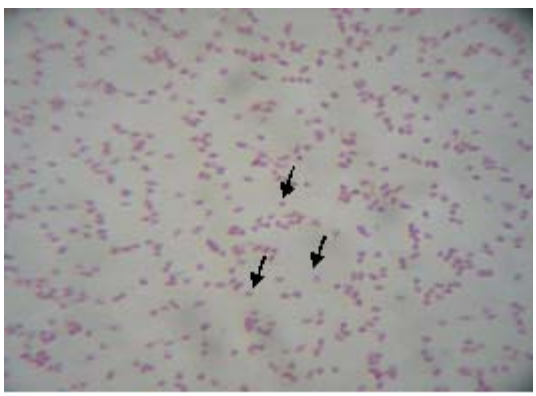


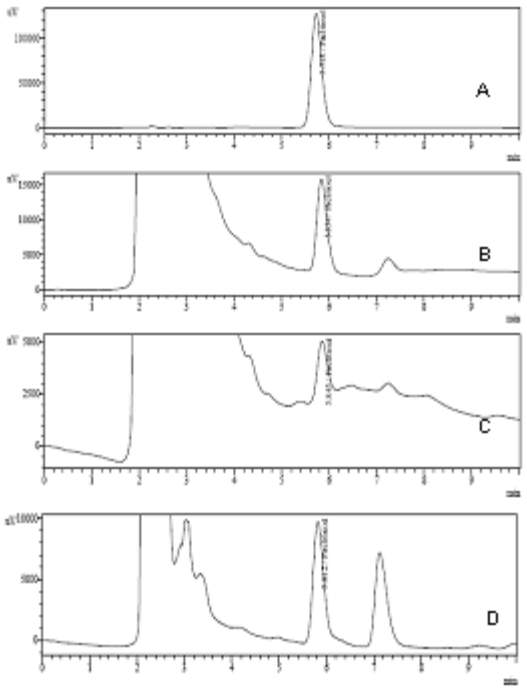
Fig. 2. *Escherichia coli* harboring pET21(a+)/*minD* after 24 hour overexpression induced by IPTG. The black arrows showed minicells

Fig. 3. *Escherichia coli* harboring pET21(a+)/*minD* after 24 hour overexpression induced by glucose

minD showed the normal rod (Fig. 1). As a result, *minD* of *Lactobacillus acidophilus* was functional across species.

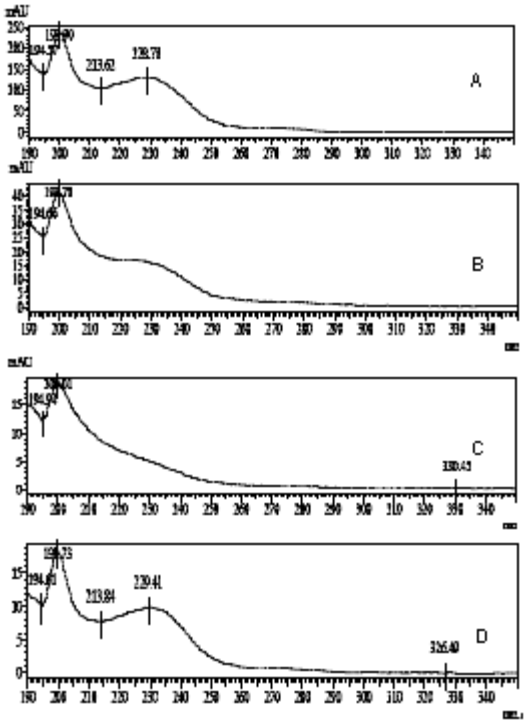
Minicell generation and paclitaxel packaging analysis

The *Escherichia coli* by transformation of pET21(a+)/*minD* for 24 hours, the overexpression could form minicell under IPTG (Fig. 2) and filaments under glucose (Fig. 3). Therefore, the cells were collected in the IPTG condition and incubated with paclitaxel. After extraction paclitaxel from the cells, the extract was checked by HPLC. The retention time was 5.812 minutes (min) that pointed the existance of paclitaxel packaged with



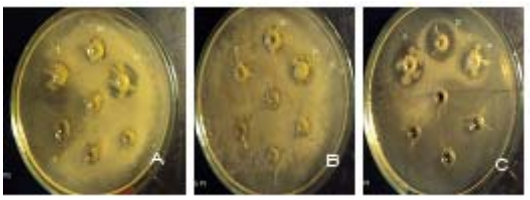
A: Standard paclitaxel, B: The supernatant fraction after incubated with paclitaxel infor 10 hours, C: The supernatant fraction after incubated with paclitaxel for 18 hours, D:The extract of the cells incubated with paclitaxel for 10 hours. The retention time of these sample was 5.718, 5.834, 5.845, 5.812, respectively

Fig. 4. Chromatogram analysis of paclitaxel



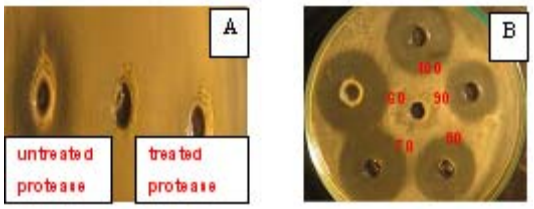
A: Standard paclitaxel, B: The supernatant fraction after paclitaxel incubation for 10 hours, C: The supernatant fraction after paclitaxel incubation for 18 hours, D: The extract of the cells incubated with paclitaxel for 10 hours.

Fig. 5. The UV spectrum of paclitaxel



A: The activities showed in cultures containing glucose, B: IPTG, C: IPTG combined with glucose. The white letters indicated the supernatants from cultures of *E. coli* BL21 (DE3)plysS cells harboring pET-21a(+)/*minD*

Fig. 6. Antimicrobial activity tests on *Pseudomonas aeruginosa*



A: After protease K treatment, B: After heat treatment at 100°C, 90 °C, 80 °C, 70 °C, 60 °C

Fig. 7. Antimicrobial activity tests on *Pseudomonas aeruginosa*

the cells when compared with the retention time at 5.718 min of the (Fig. 4). The paclitaxel still remained in the supernatants after 10 hours to 18 hours with the retention time at 5.834 mins and 5.845 min, respectively (Fig. 4). The drug packaged after 18 hours was higher than 10 hours. Indeed, the peak area of sample after 18 hours was lower than in 10 hours (Fig. 4). The drug detected from the cell extraction was 70-80% of the paclitaxel used in the 10 hour-incubation (Fig. 4). By the UV spectrum analysis, the transmittance reduced in the 10 hour-incubation (Fig. 5).

Antimicrobial activities

The supernatants of the overexpressed cell cultures in IPTG with and without glucose were centrifuged, then used to test the actvities on the above mentioned pathogens. The results showed the activities on *Pseudomonas aeruginosa* (Fig. 6). The antimicrobial agent might be the bacteriocin secreted from *E. coli* called colicin. This results revealed that MinD might be related to bacteriocin production. In the other hand, the *E. coli* without harboring *Lactobacillus* MinD did not show the inhibition (Fig. 6). 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 (Fig. 7). The results also pointed that the antimicrobial activties were not be affected by used chloramphenicol or ampicillin.

DISCUSSION

The study tried to overexpress the *minD* gene by inducing with IPTG and then the generated minicell was packaged with the paclitaxel. The MinCDE prevented minicell formation under the condition of chemicals, temperature and the length of expression, the minicells could be produced. During of the overexpression study, glucose was added to the culture without IPTG, the morphology differentation was similar to IPTG phenomenon. The results showed that glucose could give the phenomenon of cell differentiation. The study will bring out the explanation of the glucose consuming in human because MinD homolog also exist in human. The morphology alteration occured when using glucose for MinD. It meant that glucose might be play a role in cell division in human hopefully. ATP and glucose are similar because they are both chemical sources of energy used by cells. They are very different in terms of composition and structure. Glucose is gradually broken apart during cellular respiration, and the energized electrons from glucose are carried by the carrier molecules NAD and FAD to the electron transport chain. When the H+ build up enough, they rush back into the inner mitochondrial space through special "ATP-maker enzymes" that spin and build the ATP. In the study, glucose also caused the division as in IPTG condition that might be glucose and switch I or II domain of MinD play a role in cell division (Walker JE *et al*.,1982). The study tried to test the activities in cultures without any antibiotic and consequently, there were also the inhibition. The results confirmed that the activities were not artifact. In the Fig. 5, the supernatants from the *E. coli* without harboring *minD* did not show the antimicrobial acivities. Therefore, MinD 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 5µg/ml, the activities was weaker than the concentration of chloramphenicol at 20µg/ml and 34µg/ml (Fig. 5). With the result without chloramphenol in the combination of the control, the used antibiotics were lysed during culture (Benjamin PBD *et al*., 2009). In the palitaxel packaging, there were the palitaxel peaks in HPLC of the tested samples that meant the minicells generated could be used as drug delivery for palitaxel. The MinD had caused the structure cell wall alteration that could bind to palitaxel. With HPLC to check the palitaxel after extracted from the cells, the peak of palitaxel existed. Clearly, palitaxel could not be converted to other derivatives. More studies should be performed to understand well so far.

CONCLUSIONS

The study has successfully overexpressed the *minD* gene by inducing with IPTG and glucose. The results showed that glucose could give the phenomenon of cell differentiation. The mechanism of only glucose used in the induction of cell division *minD* gene caused filaments was not understood and . The study will bring out the explanation of the glucose consuming

in human because MinD homolog also exist in human. The study could support the paclitaxel by minicells and MinD could be benefit in bacteriocin production. That was the first report of MinD from *Lactobacillus acidophilus* VTCC-B-871.

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