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การสร้างฐานข้อมูลพันธุกรรมของ *Bacillus amyloliquefaciens* KKU14 ที่สร้างสารต้าน *Burkholderia pseudomallei*

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Construction of Genomic DNA Library of *Bacillus amyloliquefaciens* KKU14 Harboring Antimicrobial Activities Against *Burkholderia pseudomallei*

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หลักการและวัตถุประสงค์: โรคเมลิออยด์เป็นโรคติดเชื้อเกิดจากแบคทีเรีย *Burkholderia pseudomallei* ซึ่งเป็นเชื้อที่มีอัตราการดื้อยาปฏิชีวนะหลักสูงมาก จึงจำเป็นต้องค้นหายาชนิดใหม่ที่มีประสิทธิภาพสูงขึ้น การศึกษาก่อนหน้านี้พบว่าแบคทีเรีย *B. amyloliquefaciens* KKU14 สามารถสร้างสารเปปไทด์ต้านจุลชีพซึ่งยับยั้งเชื้อ *B. pseudomallei* ได้หลายสายพันธุ์ การศึกษานี้มีวัตถุประสงค์เพื่อหายีนที่สร้างสารเปปไทด์ต้านจุลชีพยับยั้งเชื้อ *B. pseudomallei* โดยการสร้างฐานข้อมูลพันธุกรรมของ *B. amyloliquefaciens* KKU14 ซึ่งจะเป็นประโยชน์ในการศึกษาคุณสมบัติของสารในการยับยั้งเชื้อต่อไป

วิธีการศึกษา: สร้างฐานข้อมูลพันธุกรรมของ *B. amyloliquefaciens* KKU14 โดยใช้เวกเตอร์ pCC2FOS ทำการคัดเลือกหาโคลนที่มีฤทธิ์ต้าน *B. pseudomallei* ด้วยวิธี solf agar overlay และ agar well diffusion

ผลการศึกษา: ได้สร้างฐานข้อมูลพันธุกรรมของ *B. amyloliquefaciens* KKU14 สำเร็จ โดยชิ้นดีเอ็นเอที่แทรกมีขนาดประมาณ 30-40 กิโลเบส จากการคัดเลือกเบื้องต้นด้วยวิธี solf agar overlay พบว่ามีโคลนที่สามารถยับยั้ง

Background and Objective: Melioidosis is a serious community-acquired infectious disease caused by a Gram-negative bacterium *Burkholderia pseudomallei*. Several strains are highly resistant to commonly used antibiotic drugs, so the discovery of novel potential drugs becomes strictly necessary. Previous study indicated that antimicrobial peptides (AMPs) produced by *Bacillus amyloliquefaciens* KKU14 showed a broad spectrum of activity to strongly inhibit *B. pseudomallei*. In this study, genes encoding AMP against *B. pseudomallei* from the genome of *B. amyloliquefaciens* KKU14 were investigated through genomic DNA library. The identified AMP genes can be further characterized.

Materials and Methods: The genomic DNA library of *B. amyloliquefaciens* KKU14 was constructed by using pCC2FOS vector. The library clones were overlaid by soft agar containing *B. pseudomallei* strain P37. The clones with clear zones were confirmed by agar well diffusion method.

Results: Genomic libraries of *B. amyloliquefaciens* KKU14 were successfully constructed. Five recombinant clones

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B. pseudomallei สายพันธุ์ P37 ได้ 5 โคลน แต่เมื่อทดสอบด้วย agar well diffusion พบว่ามี 1 โคลนที่สามารถยับยั้ง *B. pseudomallei* ได้

สรุป: การสร้างฐานข้อมูลพันธุกรรมของ *B. amyloliquefaciens* KKU14 และแสดงออกใน *E. coli* พบโคลนที่ออกฤทธิ์ต้าน *B. pseudomallei* สายพันธุ์ P37 ซึ่งจะมีการวิเคราะห์หาลำดับเบสของดีเอ็นเอเพื่อบ่งชี้ยีนที่เกี่ยวข้องต่อไป

Conclusion: A recombinant clone from *B. amyloliquefaciens* KKU14 genome showed antimicrobial activities inhibiting *B.pseudomallei* strain P37. DNA sequencing and data analysis will be proceeded to identify the involving genes.

Keywords: Antimicrobial peptide, Fosmid library, *Bacillus amyloliquefaciens*, *Burkholderia pseudomallei*

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Introduction

Burholderia pseudomallei is a Gram-negative, soil saprophyte and pathogenic bacterium which is the cause of melioidosis, a serious community-acquired infectious disease in the endemic areas. Unlike other soil bacteria, *B. pseudomallei* is difficult to be destroyed. Furthermore, the lack of protective vaccine against *B. pseudomallei* increases biosecurity concerns regarding the use of *B. pseudomallei* as biological weapons. Currently, the best way of treating melioidosis is the use of antibiotic drugs. However, many clinical strains are now intrinsically resistant to almost all available antibiotic drugs, which limit their therapeutic use during patient treatment[1], so the discovery of novel potential drugs is immediately necessary. Prior to the development of conventional antibiotics, antimicrobial peptides (AMPs) have been used as an efficient source of antibiotics[2]. The development of new antibacterial alternatives is the most apparent approach to attack those antibiotic resistant strains. Amino acid composition of AMPs including amphipathicity, cationic charge, and small size allow them to attach to and insert into membrane bilayers which are heavily negatively charged of phospholipid headgroups to form pores[3]. As AMPs kill microbes primarily through generation of membrane pores, it is inherently more difficult for the organisms to develop resistance that differs from conventional antibiotics, generally targeting metabolic enzymes that may selectively develop resistance[4].

Recently, AMPs produced by members of the genus *Bacillus* were shown to have a broad spectrum of activity against pathogenic microorganisms. Therefore, AMPs of the genus *Bacillus* may be promising alternatives to conventional antibiotics for the effective treatment of single drug- and multi drug-resistant infectious pathogens[2]. These compounds can be synthesized both ribosomally and nonribosomally as secondary metabolites. *Bacillus amyloliquefaciens* is a greatly interesting species, and many strains in this species can produce multiple antimicrobial compounds to suppress fungal and bacterial growth *in vitro*[5,6]. They have a broad antibacterial spectrum against both Gram positive and negative bacteria such as Listeria monocytogenes, *Bacillus cereus*, *Serratia marcescens,* and *Pasteurella haemolytic*[7]. Based on previous study, the bacterial strain KKU14 isolated from soil was first identified as *B. amyloliquefaciens*, and showed high antimicrobial activity against several strains of *B. pseudomallei*, especially the drug resistant strains[8]. The AMPs against *B. pseudomallei* from strain KKU14 consist of both ribosomal and nonribosomal peptide synthesized as secondary metabolites[8]. These unknown AMPs isolated from *B. amyloliquefaciens* KKU14 might be potential candidates for a melioidosis treatment, and may be used as a single drug or as a combination with other conventional antibiotics to increase the effectiveness of melioidosis treatment and to control the pathogen in the environment.

In 2001, a small antimicrobial peptide, namely indirubin, was obtained from metagenomic libraries[9]. Another antimicrobial compound was successfully screened from cyanobacterial metagenomic libraries in fosmid system (pCC2FOS vector)[10]. Metagenomic

library is thus a potential technology to provide valuable natural compounds including antimicrobial drugs. Here, we report the first use of genomic library construction of *B. amyloliquefaciens* KKU14 in a fosmid system to screen for genes with antimicrobial activities against *B. pseudomallei*. The identified AMP genes can be further characterized. This may lead to the discovery of a new potential antibiotic.

Materials and methods

Bacterial strains and vectors

B. amyloliquefaciens KKU14 was previously isolated from soil sample that contained no *B. pseudomallei*[8]. *B. pseudomallei* strain P37, a clinical strain without a lysogenic phage, was received from Melioidosis Research Center, faculty of Medicine, Khon Kaen University, Thailand. The fosmid, pCC2FOS (Epicentre Biotechnologies) was used for construction of the genomic DNA library. *Escherichia coli* EPI300 (Epicentre Biotechnologies) was used as the host strain for transfection. The host strain was grown in Luria-Bertani (LB) (Difco Laboratories; Sparks, MD) broth media at 37°C with shaking at 200 rpm or on LB agar plates containing 12.5µg/ml chloramphenicol for selection of the fosmid clones.

Construction of genomic DNA library

The Genomic DNA of *B. amyloliquefaciens* KKU14 was extracted by the phenol-chloroform extraction method modified from Anderson and Mckay, 1983, and Wilson 1990[11, 12]. Ten micrograms of genomic DNA were used for a library construction. DNA at a concentration of 500ng/µl was randomly sheared using 200 µl small-bore pipette tip aspirated and expelled for 100-300 times. The sheared DNA fragments were end-repaired to generate blunt (5' phosphorylated) ends by the End-Repair Enzyme Mix included in the kit. The size of approximately 40 kb of end-repaired DNAwere extracted from the gel using phenol-chloroform method modified from Sambrook, 1989[13]. The blunt-ended DNA was ligated into the pCC2FOS vector. Then, the ligated DNA was packed into phage particles by MaxPlax Lambda Packaging Extracts (Epicentre Biotechnologies) and added into *E. coli* EPI300 cells. The infected *E. coli* EPI300 cells were spread on an LB agar supplemented with chloramphenicol and incubated at 37 °C overnight.

Analysis of inserted DNA

Ten library clones were randomly selected for restriction analysis to confirm the presence of the inserted DNA. The recombinant fosmid DNA extraction using alkaline lysis with SDS (miniprep) was done as previously described by Green and Sambrook[14]. The extracted DNA was subsequently digested by FastDigest *Bam*HI (Thermo Fisher Scientific) and analyzed by agarose gel electrophoresis.

Screening for active clones against *B. pseudomallei* by soft agar overlay method

The antimicrobial activity of the library clones was preliminary screened through the soft agar overlay method[10] using *B. pseudomallei* strain P37 as an indicator strain. *B. pseudomallei* strain P37 was grown in 3 ml LB medium at 37 °C with shaking at 200 rpm for 16 h. The overnight culture of *B. pseudomallei* strain P37 was diluted in 2.5 ml 0.7% LB agar (45 °C) to a final concentration of 2×10^5 cfu/ml and overlaid on pretreated library clones. The pretreatment of library clone was done as followed: after incubation overnight at 37°C and another 3 days at room temperature, the library clones were induced by overlay with 1xFosmid Library AutoInduction Solution (Epicentre Biotechnologies). The induced clones were vaporized with chloroform in a closed container for 15 min in order to lyze their cell walls. After soft agar overlay, the library plates was incubated at 37 °C for overnight and further incubated at room temperature for 3-7 days. The active clones against *B. pseudomallei* strain P37 can be investigated by observing the inhibition zone, which showed a clear halo around the clones.

Screening for active clones against *B. pseudomallei* by agar well diffusion method

Preparation of the samples

The positive library clones obtained from the soft agar overlay method were grown in 5 ml LB medium containing chloramphenicol and Fosmid Library AutoInduction Solution with shaking at 200 rpm, 37 °C

overnight. The overnight culture was centrifuged at 13,000 rpm for 15 min. The supernatant was filtered while the cell pellets were used for extraction of intracellular proteins by sonication. The pellets were resuspended in 1 ml of 0.01 M Tris-HCl buffer, pH 7.0. The mixtures was sonicated using 20% of maximal amplitude and pulsed for 6 min before the lysate was filtered[10]. Three samples including the cell culture, the cell extracts, and media supernatant were further used for antimicrobial activity tests using *B. pseudomallei* strain P37 as an indicator strain.

Agar well diffusion procedure

An overnight culture of *B. pseudomallei* strain P37 was inoculated in fresh LB medium (1%v/v) and incubated at 37°C, 200 rpm for 4 h until log phase. Approximately 10^5-10^6 CFU/ml cells were swabbed on MHA plate. The plate was punched by sterile pipette tip to obtain 6.6 mm wells. Then, 100 µl of each prepared samples was added into agar wells. Ceftazidime (60 µg/ml; Sigma-Aldrich, St. Loius, MO) was used as a positive control. LB medium containing 12.5 µg/ml chloramphenicol and Fosmid Library AutoInduction Solution was used as a negative control. Thereafter, the plate was incubated overnight at 37°C and inhibition zone against *B. pseudomallei* strain P37 was monitored.

DNA pattern analysis of inserted DNA

The recombinant fosmids were extracted by QIAprep®SpinMiniprep Kit (QIAGEN), according to the manufacturer's instructions. One microgram of the extracted DNA was digested by 2 µl FastDigest *Nco*I (Thermo Scientific) for 15 min at 37 °C and then analyzed by agarose gel electrophoresis.

Calculation of number of library clones for a complete library

The approximate number of library clones for a complete fosmid library was determined by Clark-Carbon equation (see below) using a 99% confidence level[15].

$$N = \ln(1 - P)/\ln(1 - f)$$

N is the required number of fosmid clones.

P is the desired probability (expressed as a fraction).

f is the proportion of the genome contained in a single clone.

Results

Construction of genomic DNA library

Genomic DNA of *B. amyloliquefaciens* KKU14 at 1,500 ng/µl concentration has been successfully extracted for DNA library construction (Figure 1a). It was shown that the genomic DNA could be sheared using a 200 µl pipette tip. By pipetting up and down for at least 200 times, an average DNA fragment size of 40 kb could be obtained (Figure 1b). After end-repairing, more DNA was gained by phenol-chloroform extraction method when compared to GELase Enzyme (according to the manufacturer's instructions), obaining DNA concentrations of 25 ng/µl and 10 ng/µl, respectively (Figure 1c).

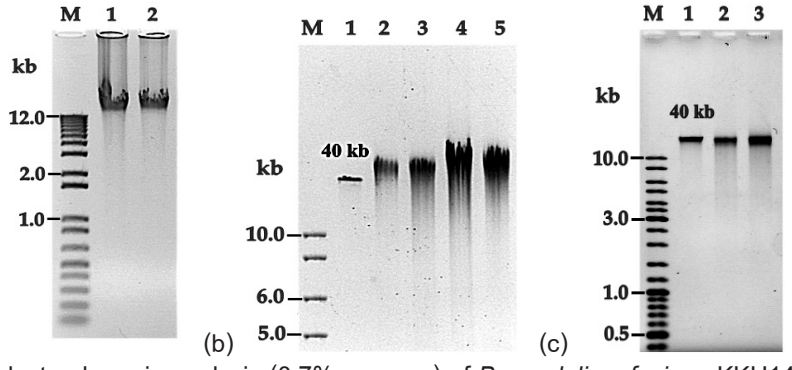


Figure 1 Agarose gel electrophoresis analysis (0.7% agarose) of *B. amyloliquefaciens* KKU14 genomic DNA (a) Lane M: DNA molecular weight marker, lane 1 and lane 2: extracted genomic DNA of *B. amyloliquefaciens* KKU14. (b) Shearing of genomic DNA, lane M: DNA molecular weight marker, lane 1: 40 kb DNA marker, lane 2: genomic DNA before the shearing procedure, lane 3-5: genomic DNA after pipeting up and down with 200 µl pipette tip for 100 times (lane 3), 200 times (lane 4), 300 times (lane 5). (c) Lane M: DNA molecular weight marker, lane 1: 40 kb DNA marker, lane 2: End-repaired DNA after gelase enzyme extraction., lane 3: end-repaired DNA after phenol-chloroform extraction.

When end-repaired DNA was sufficiently done, two genomic DNA libraries of *B. amyloliquefaciens* KKU14 had been constructed using pCC2FOS vector. In the first library, 4,700 clones were gained, while 500 clones were obtained in the second library, as shown in Table 1. The restriction enzyme *Bam*HI could be used for analysis of inserted DNA and showed that all of the random library clones contained the linearized fosmid of approximately 8 kb and the average inserted DNA size of 30-40 kb (Figure 2).

Table 1 Genomic libraries of *B. amyloliquefaciens* KKU14 generated and screened in this study

Library	Number of library clones	Insert size	Screening procedure	Number of positive clones
First	~4,700	~40 kb	Soft agar overlay	0
Second	~500	~30-40 kb	Copy number induction and chloroform vaporization prior to soft agar overlay	5

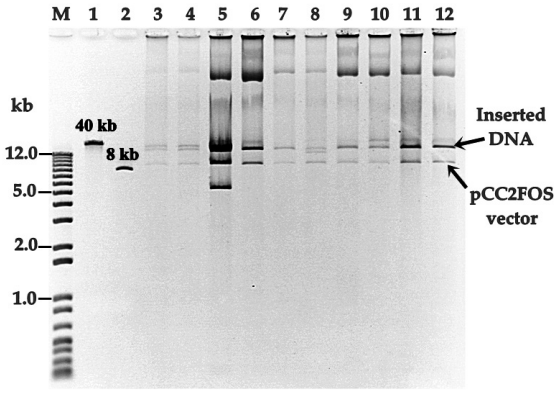
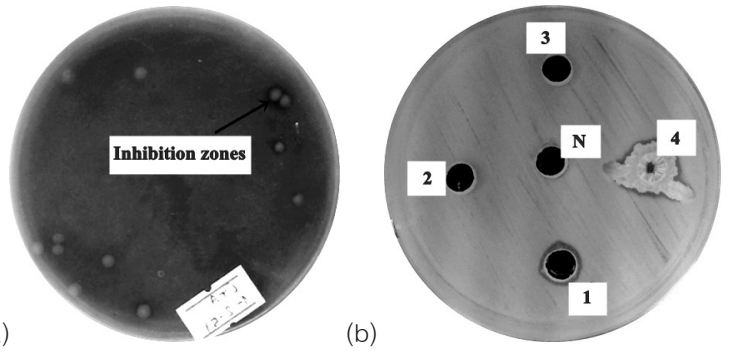


Figure 2 Agarose gel electrophoresis (0.7% agarose) of the inserted DNA analysis. Lane M: DNA molecular weight marker, lane 1: 40 kb DNA marker, lane 2: linearized pCC2FOS vector, lane 3-12: Recombinant fosmids from random selected library clones digested with *BamH*I.

Antimicrobial activity against *B. pseudomallei*

Five recombinant clones from the second library showed antimicrobial activities against *B. pseudomallei* strain P37 by exhibiting inhibition zones (Figure 3a). After confirmation by agar well diffusion, only one clone exhibited antimicrobial activity in the culture supernatant fraction (Figure 3b). The clone No. 5 showed the same result as the clone No. 2 and 3 (no inhibition zone) (data not shown).



(a) (b)

Figure 3 Antimicrobial assay (a) Soft agar overlay assay showing inhibition zones of the clones potentially baring antimicrobail activities against *B. pseudomalei* stain P37. (b) Agar well difusion assay using culture supernatant of 4 positive clones. Clone No.1 showed inhibition zone while clones 2, 3 showed no activity and 4 with *Bacillus* spp. contamination. N : LB containing chloramphinicol as a negative control.

DNA pattern analysis of inserted DNA

The DNA patterns of recombinant fosmids extracted from selected positive clones was shown in Figure 4. Different DNA patterns from these clones indicated that the library clones contained dissimilar DNA inserted fragments. Therefore, these positive clones might include different genes involving in antimicrobial activities.

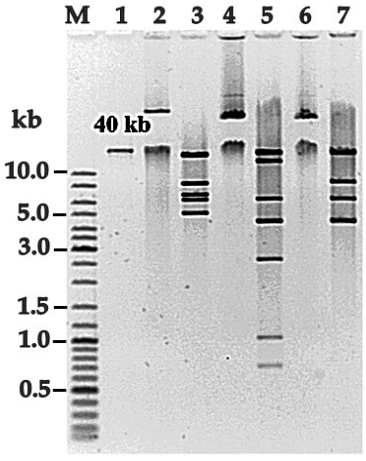


Figure 4 DNA pattern analysis (1% agarose) of the positive clones. Lane M: DNA molecular weight marker, lane 1: 40 kb DNA marker, lane 2: undigested recombinant fosmid of 40 kb marker cloned into pCC2FOS, lane 3: digested recombinant fosmid of 40 kb marker cloned into pCC2FOS by *Nco*I as positive control, lane 4: undigested recombinant fosmid of positive clone No.1. lane 5 digested recombinant fosmid of positive clone No.1 by *Nco*I, lane 6: undigested recombinant fosmid of positive clone No.2, lane 7 digested recombinant fosmid of positive clone No.2 by *Nco*I.

Discussion

Fosmid vectors are suitable for cloning of toxic compounds since they are low copy number vectors, which can prevent hosts from toxicity. The pCC2FOS vector chosen for our library construction is suitable for cloning genomic inserts of approximately 40 kb in size. Large DNA inserts may cover large gene cluster in *Bacillus* genome, which is an advantage for functional-based screening method. Since the genome of *B. amyloliquefaciens* contains an average size of approximately 3.9-4.0 Mb[16-18], theoretically, obtaining of the fosmid library about 460 clones should cover an entire genome of *B. amyloliquefaciens* KKU14 according to Clark-Carbon equation. In the first library construct, 4,700 clones were obtained which was sufficient to cover of the genome. However, no positive clones were found from the first library screening. In the second library construct, 500 clones were gained. These low numbers of clones might be caused by a low amount of DNA inserts which led to lower ligation products. Unlike the first library screening with untreated clones, in the second library screening, the clones were pretreated by vaporizing with chloroform, which would help partially lyse the cell walls and thereby releasing intracellular proteins. Therefore, despite small number of clones, antimicrobial activities were detected in the second library screening. Detection of library clones exhibiting antimicrobial activities might be obstructed due to many factors, for examples, low-copy-number plasmid might prevent monitoring clones with weakly expressed foreign genes. Moreover, expression of foreign genes in the fosmid library containing the large insert size might be limited by vector promoter[19]. Besides, some expressed antimicrobial compounds were not easily secreted from the host. This hypothesis was supported by the observation that the culture supernatant of some positive clones was not able to inhibit *B. pseudomallei* strain P37. The use of *E. coli* as a heterologous expression host may limit the ability to express DNA from other organisms. It was reported that expression of toxic protein in *E. coli* is often confronted with two serious problems as toxicity to the host and sensitivity to proteases[20]. Despite these limitations, there have been reports finding small peptide exposing antimicrobial activity against *B. subtilis* from soil-DNA in BAC vector system[9], or antimicrobial compounds from cyanobacterial metagenomic libraries in the fosmid system[10]. These examples showed that gene involving in natural product synthesis can be cloned and expressed in a heterologous host. Although a genomic library technology is a potentially important new tool for drug discovery, the

discovery rate of novel structural classes of antimicrobial molecules has declined[9]. Functional metagenomics approach is dependent on successful heterologous expression of the gene or genes responsible for their function, as well as detection of gene product function[21]. The functional screenings are not always successfully done by expressing in the heterologous host. Sophie and coworkers reported that a new antibacterial activity against *B. cereus* was found upon screening in *B. subtilis*, but it was not active when the corresponding DNA fragment was expressed in *E. coli* [22]. The fosmid library bearing antimicrobial peptide genes were therefore difficult to be detected through functional screening. Other option for searching antimicrobial genes which might be obstructed in functional screening is using genome sequence analysis to predict the potential genes.

Conclusion

Cloning of large genomic DNA fragments of *B. amyloliquefaciens* KKU14 into fosmid vector and expressing in *E. coli* was shown to be able to screen and discover clones containing antimicrobial activities against *B. pseudomallei* strain P37. Identification of the involving genes remains to be done. The positive clones will be subcloned into plasmid vector and screened for active clones with antimicrobial activities against *B. pseudomallei*. The antimicrobial genes will be indentified by DNA sequencing, gene prediction, and conserved region analysis.

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