Antimicrobial and Antiadhesive Activities of the Crude Biosurfactant from Bacillus sp. Against Clinical Isolates of Acinetobacter baumannii from Srinararind Hospital

Pontapan Polyiam, Chotima Photisap, Patcharaporn Boottanun, Surasakdi Wongratanaicheewin, Rasana Wongratanaicheewin, Umaporn Yordpratum

1Department of Microbiology, 2Biochemistry, 3Melioidosis Research Center, Faculty of Medicine, Khon Kaen University, Thailand

**Background and Objectives:** 
Acinetobacter baumannii is a cause of hospital-acquired infection. It can form biofilm on medical devices which is related to antibiotic resistance and the chronic infections. Biosurfactants (BS) were produced from microorganisms that have antibacterial, antifungal, and antiviral activities. Previous study showed BS from Bacillus sp. prevent biofilm formation of pathogens in human. The aims of this study were to determine the activities of crude BS from Bacillus sp. to inhibit the growth and adhesion of clinical A. baumannii isolates from Srinararind hospital.

**Methods:** Two samples of crude BS were extracted from 2 isolates of B. amyloliquefaciens KKU3 and KKU14. Crude biosurfactants were determined antimicrobial activity on 96-well plates and antiadhesive activity on intravenous (IV) tubes.

**Results:** Crude BS sample from B. amyloliquefaciens KKU14 strain can inhibit the growth of five clinical isolates A. baumannii which were inhibited in range of 2-3%, 32-33%, 35-37%, 78-82% and 81-84% for 50, 80, 100, 150, and 200 mg/ml, respectively, and inhibited adhesion of A. baumannii on IV tube when compare

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*Corresponding author: Umaporn Yordpratum, Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand E-mail : umapornyo@kku.ac.th*
Introduction

The biofilm formation of A. baumannii on medical devices especially implanted catheters which related to urinary tract infection. This bacteria is a causative agents of resistance among hospital-acquired infection and ventilator-associated pneumonia patients in Srinagarind hospital, Thailand. The bacterial biofilm increase antibiotic resistance and contributes to the chronicity of infections that is an important problem of worldwide public health.

Biosurfactants (BS) are new compound using in the medical field for protection and removal of bacterial biofilm have increased during the past decade. The wide range of BS chemical structure are synthesized and secreted from microorganisms vary from low to high-molecular-mass. These compounds have amphipathic property of hydrophilic and hydrophobic moieties within the same molecule that show the effect to bacterial membrane. The various Bacillus subtilis strains produced the potent extracellular lipopeptide-surfactin BS. In 2008, the first evidence reported that the uncharged and negatively charged of BS-lipopeptides from B. subtilis can alter the morphology of gram-negative bacteria. Bacillus amyloliquefaciens produced lipoprotein-bamylolcin biosurfactant which strongly inhibited the growth of plant-pathogenic fungi. The cell-free supernatant of B. amyloliquefaciens inhibit the growth of Candida albicans. BS-pseudofactin II can be used as a disinfectant or surface coating agent against colonization and biofilm formation of uropathogens on different surfaces e.g. implants or urethral catheters. The crude BS produced by lactobacilli exhibit strong antimicrobial, antiadhesive and antibiofilms properties against multi-drug-resistant pathogens. In this study focus on BS of Bacillus sp. which are isolated from soil in the absence Burkholderia pseudomallei area, because we expected they may secreted the BS against B. pseudomallei and may have antimicrobial, antiadhesive and antibiofilms inhibit other pathogens which can inhibit A. baumannii.

Therefore, the BS is an alternative therapeutic approach in medical application for the prevention and/or treatment of hospital-acquired infections. Here is the first study about the activity of crude BS from Bacillus spp. can inhibit adhesion of A. baumannii on intravenous tube.

Materials and methods

Strains and growth conditions

Two isolates of B. amyloliquefaciens strains KKU3 and KKU14 were isolated from the soil samples by Miss Chotima Photsap and Miss Patcharaporn Boottanan, meliodosis research center, Faculty of Medicine, Khon Kaen University, Thailand. Five clinical isolates of A. baumannii were provided from Srinagarind hospital laboratory, Faculty of Medicine, Khon Kaen University, Thailand. All bacteria were stored at -80 °C in Luria-Bertani
(LB) medium (Difco, Sparks, MD, USA) containing 20\% (v/v) glycerol.

**Inoculum preparation**
Each strain of Bacillus sp. was cultivated in LB broth, incubation in shaker incubator at 37 °C, 200 rpm for 18 h.

**Biosurfactant production**
BS was produced from each strain of Bacillus sp. according to Phithnaree et al.\(^\text{14}\) Production medium was McKeen medium which consisted of (w/v) 0.5-2.5\% glucose, 1.0\% monosodium glutamate, 0.3\% yeast extract, 0.1\% MgSO\(_4\).7H\(_2\)O, 0.1\% K\(_2\)HPO\(_4\), 0.05\% KCl and 0.1\% (v/v) of trace element (0.64 g of MgSO\(_4\).7H\(_2\)O, 0.16 g of CuSO\(_4\).5H\(_2\)O in 100 mL of distilled water).\(^\text{14}\)
The inoculum size was 5\% (v/v). Then, incubated conditions at 30 °C, 200 rpm for 36 h. The cultured medium was centrifuged at 13,000×g at 4 °C for 15 min. The supernatant was filtered through 0.2 \mu m membrane filter. The BS was isolated from filtrated supernatant by precipitation with 6 N HCl solution and left at 4 °C overnight. The precipitate was dissolved in acid water, neutralized to pH 8 using 2 N NaOH and finally, the crude BS was lyophilized.\(^\text{15}\) The crude BS powder was resuspended in phosphate-buffered saline (PBS) before testing.

**Antimicrobial assay**
Antimicrobial activity of BS was determined according to Janek et al.\(^\text{16}\) Briefly, 50 \mu L volumes of LB medium were dispensed into each wells of a 96-well microplate. Then, 50 \mu L volumes of crude BS at various concentrations solution in PBS were added into the microplate wells and mixed with the medium. Then, 2 \mu L of each overnight clinical isolate A. baumannii culture (OD\(_{600}\) = 0.1) was inoculated to each well. Negative control was sterile medium with BS and growth control was medium with bacterial suspension (without BS) and positive control was 0.1% TritonX-100 with bacterial suspension. The microplates were incubated for 24 h at 37 °C. Measuring the growth using microplate reader at wavelength 600 nm. The percentages of growth inhibition for each clinical isolate at different crude BS concentrations was calculated as follow.

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\% \text{growth inhibition} = \left(1 - \frac{\text{OD}_{1}}{\text{OD}_{c}}\right) \times 100.
\]

Where OD\(_{1}\) represents the optical density of the well with a given crude BS concentration and OD\(_{c}\) is the optical density of the control well (growth without crude BS).

**Antiadhesive assay on intravenous tubes (IV tubes)**
Antiadhesive ability of BS to A. baumannii on intravenous tube was determined according to Janek et al.\(^\text{10}\) Briefly, a sterile tube containing 4 cm of sterile IV tube\(^\text{10}\) (Unomedical A/S ConvaTec Company, Denmark) was pretreated with 2 mL of 20 mg/mL of crude BS by adding in a tube (while untreated tube was treated with PBS), incubated for 2 h at 37 °C and subsequently washed twice with PBS. Overnight culture of A. baumannii (OD\(_{600}\) = 1.0) were added in IV tubes and incubated overnight at 37 °C. The A. baumannii culture was removed and IV tubes were washed with distilled water for 3 times. After washing, IV tubes were fixed with 3 mL of absolute ethanol for 10 min and stained with 3 mL of 0.1% crystal violet for 20 min. The stained biofilm was rinsed gently 3 times with distilled water, allowed to dry at room temperature for 15 min. The results were observed the purple of crystal violet compared between treated and untreated tubes which the purple indicated the concentration of A. baumannii on IV tubes. Assay was carried out three independent experiments.

**Statistical analysis**
A paired t test was used to compare mean-values of two population (treated and untreated with crude BS) by the statistical significance in p-value, significant different at p<0.05.

**Results**
Five clinical isolates A. baumannii (A1, A2, A3, A4, and A5) were treated with crude BS KKU14 from B. amyloliquefaciens at 50, 80, 100, 150 and 200 mg/mL. The antimicrobial activity of crude BS KKU14 against 5 clinical A. baumannii in dose-dependent manner as shown
in figure 1. The results showed that 5 clinical isolates of A. baumannii were inhibited with crude BS KKV14 in the ranges of 2-3%, 32-33%, 35-37%, 78-82% and 81-84% at 50, 80, 100, 150 and 200 mg/ml, respectively. Therefore, crude BS KKV14 may be used as an antimicrobial agents for treatment in the future. Two crude BS samples from B. subtilis couldn't kill A. baumannii (data not shown). However, this crude BS should be purified and characterized structure of effective compound. Characterization of secondary metabolites and other secreted compounds from B. subtilis and also B. amyloliquefaciens may have advantages for medical application.

The sterile IV tubes were treated with crude BS KKV14 at 20 mg/ml then inoculated with A. baumannii 18 h culture. The results revealed that crude BS KKV14 had antiadhesive activity against A. baumannii on IV tube by observed the intensity of blue color when compared with untreated tube as shown in figure 2. Crude BS KKV14 at 20 mg/ml couldn't kill A. baumannii in vitro from antimicrobial assay (not show data). However, low

**Figure 2** Crude BS KKV14 inhibits biofilm formation on intravenous tubes. Pretreatment of crude BS KKV14 on IV tubes before adding with overnight cultures of A. baumannii. The sterile tubes were compared between treated (+) and untreated (-) tube with crude BS at 20 mg/ml and control tube (C). Assay was carried out three independent experiments.

**Figure 1** Antimicrobial activity of crude BS KKV14 against 5 clinical isolates of A. baumannii. Crude BS KKV14 was used at 50, 80, 100, 150 and 200 mg/ml. Used a commercial surfactant 0.1% TritonX-100 as a positive control (PC) and untreated by BS as a growth (G) control. The results represent the mean values. Paired t test was used for determine the statistical significance of the treated versus untreated conditions (*, p<0.05).
concentration (20 mg/ml) of crude BS may be used as a cleaning agents or antibiofilm forming agents on equipments or medical devices.

Discussions

Biosurfactants are the one group of secondary metabolites which are secreted from Bacillus sp., have a wide range of chemical structure and several biological activity such as antiadhesive, antimicrobial, antiviral, antifungal, antitumor, and immunosuppressive activities that allow the bacterium to survive in its natural environments. These compounds are amphiphilic property that show the effect to bacterial membrane. The extracted-BS from lactobacilli showed strong inhibition the growth of multi-drug-resistant pathogens include A. baumannii, E. coli, and S. aureus which showed non-toxic to mammalian cell line at 25-200 mg/ml. The 50 mg/ml of extracted-BS could inhibit adhesion of A. baumannii and E. coli while S. aureus was used at 25 mg/ml. Therefore, the ability of BS to inhibit pathogens was related with strain of biosurfactant-producing bacteria and dose-dependent manner. In this study, crude BS of B. amyloliquefaciens KKV14 strain at various concentrations were used to determine antiadhesive activity, we found that 20 mg/ml of crude BS KKV14 could prevent adhesion of A. baumannii.

The purified-surfactin-BS from B. subtilis inhibit biofilm formation of Salmonella enterica on urethral catheter. The crude BS KKV14 showed antiadhesive activity on IV tube (as the pretreatment) as it inhibit initial step of biofilm forming that may use as the antimicrobial-coating agent. However, posttreatment of crude BS KKV14 on IV tube was needed to confirm antibiofilm-forming activity. Moreover, crude BS should be tested cytotoxicity. Although, high concentration of crude BS show non-toxic may have not to purify or classify BS. Finally, characterization of effective compound in crude BS is very important for applications in the future.

Conclusions

Crude BS KKV14 sample from B. amyloliquefaciens can inhibit the growth of 5 clinical isolates of A. baumannii which are inhibited in the ranges of 2-3%, 32-33%, 35-37%, 78-82% and 81-84% at 50, 80, 100, 150 and 200 mg/ml, respectively. Crude BS KKV14 also has antiadhesive activity against A. baumannii on intravenous (IV) tube when compare with untreated tube. Thus, BS may be used as a new cleaning agent for washing the equipments in laboratory or a coating agent on medical devices for prevention the pathogens contamination particular implanted medical for long term and may be an alternative therapeutic agent in the future.

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