Malaysian Journal of Microbiology, Vol 17(6) 2021, pp. 646-660
DOI: http://dx.doi.org/10.21161/mjm.211212

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Partial characterization of bacteriocin-like compound (BLIS) produced by *Burkholderia stagnalis* strain K23/3 against *Burkholderia pseudomallei*

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Received 25 June 2021; Received in revised form 16 August 2021; Accepted 20 October 2021

ABSTRACT

Aims: *Burkholderia pseudomallei*, the human pathogen that causes melioidosis, is intrinsically resistant towards a wide range of antibiotics and there have been reports of acquired resistance towards antibiotics used for melioidosis treatments. Antimicrobial peptides (AMP) such as bacteriocins are gaining the interests of researchers as alternative for treating infections caused by multidrug resistant bacteria. In this study, we aimed to identify *Burkholderia* spp. isolated from soil in Sarawak that possess the potential in inhibiting the growth of *B. pseudomallei* and to further characterize the antagonistic compound produced.
Methodology and results: A total of 50 *Burkholderia* spp. isolates of environmental origin and two isolates of *Ralstonia solanacearum* were screened against five clinical isolates of *B. pseudomallei* using spot-on-lawn assay and flip streak method. *Burkholderia stagnalis* isolate K23/3 showed clear zones of inhibition (ZOI) in both preliminary tests. Cell-free supernatant (CFS) was obtained from *B. stagnalis* K23/3 broth culture and was tested via agar well diffusion assay (AWDA). The antagonistic compound secreted at the early log phase of the bacterial growth was shown to be stable in a wide range of temperatures and pH. Treatment with different enzymes revealed that it was sensitive towards proteinase K, suggesting that it is proteinaceous. The bacteriocin-like-substance (BLIS) was subjected to ammonium sulfate precipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE gel was overlaid with indicator *B. pseudomallei* isolates where the active protein was shown to be less than 7.1 kDa.
Conclusion, significance and impact of study: *Burkholderia stagnalis* isolate K23/3 was able to secrete bacteriocin-like-substance (BLIS) that has the potential in biocontrol of *B. pseudomallei* in the environment or as potential treatment for melioidosis.

Keywords: Antimicrobial peptides, bacteriocin, *Burkholderia pseudomallei*, melioidosis, SDS-PAGE

INTRODUCTION

Melioidosis is a potentially fatal infectious disease that is caused by the bacterium *Burkholderia pseudomallei*. With ineffective antimicrobial treatment, the fatality rate of melioidosis is expected to exceed 70% (Limmathurotsakul *et al*., 2016), making it a life-threatening infectious disease. Melioidosis has been recognized as one of the important human infections in Malaysia (Nathan *et al*., 2018), Singapore and across the north of Australia (White, 2003) and Southeast Asia (Limmathurotsakul *et al*., 2016). Over the past years, this disease has been emerging in tropical developing countries that were not previously reported such as Africa (Birnie *et al*., 2015), Puerto Rico (Doker *et al*., 2015) and Brazil (Rolim *et al*., 2018). A person could be infected by *B. pseudomallei* through inhalation, open wound inoculation or ingestion of contaminated environmental sources (Barnes and Ketheesan, 2005). According to Currie *et al*. (2000), the incubation period for melioidosis is estimated to be 1–21 days and may extend to months and even years. It is influenced by the amount of inoculum, route of infection and underlying host risk factors (Ngauy *et al*., 2005). However, *B. pseudomallei* has the tendency of staying dormant in humans for a prolonged period (Wiersinga *et al*., 2012).

Burkholderia pseudomallei is intrinsically resistant to many classes of commonly used antibiotics such as β-lactams, aminoglycosides, macrolides and fluoroquinolones (Lipsitz *et al*., 2012). Currently, meropenem, trimethoprim/sulfamethoxazole (TMP-SMX) and doxycycline are used for melioidosis treatment (Dance, 2014). However, there were reported possible resistance towards TMP-SMX (Wuthiekanun *et al*., 2005; Yong *et al*., 2016) and meropenem (Yong *et al*., 2016). Although yet to be verified through comprehensive in-depth studies, concerns of possible resistance towards antibiotics is a major concern to the public health. Thus,

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

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there is a need to search for new alternative antimicrobials such as bacteriocin (Cotter *et al*., 2013).

Bacteriocin is an antimicrobial peptide produced by almost every bacterial species (Riley and Chavan, 2007) which acts as the producer's natural defense mechanism against closely related organisms in the same ecological niches (Naz and Rasool, 2013). According to Chikindas *et al*. (2018), bacteriocins are defined as "ribosomally-produced multifunctional substances of a proteinaceous nature, with pronounced antimicrobial activity at certain concentrations". Bacteriocin possesses favorable characteristics that makes it applicable in combating the phenomenon of antibiotic resistance, such as their low toxicity, high potency, availability of both narrow- and broad- spectrum and the possibility of bioengineering the peptides (Cotter *et al*., 2013). Some bacteriocins have been approved and commercialized as bio-preservatives such as nisins, produced by *Lactococcus lactis*, pediocin, produced by *Pediococcus acidilactici*, (Bali *et al*., 2014) and Micocin (combination of carnocyclin, carnobacteriocin and piscicolin, produced by *Carnobacterium maltaromaticum*) (Martin-Visscher *et al*., 2011). Other bacteriocins such as subtilosin (Sutyak *et al*., 2008) are also reported to have potential as biocontrol agent while some are reported to be effective against antibiotic-resistant bacteria *in vitro* (Aunpad and Na-Bangchang, 2007; Odah *et al*., 2019).

A study conducted by Marshall *et al*. (2010) showed that *B. ubonensis* produced bacteriocin-like substance that was effective against *B. pseudomallei*. In another study by Lin *et al*. (2011), they experimented on the effect of *B. multivorans* that was isolated from agricultural crop soil on *B. pseudomallei* growth when co-cultured in sterile soil. In their findings, there were only *B. multivorans* morphotype after 10 days of culturing. After 30 days, the amplicons that were specific to *B. pseudomallei* were no longer detectable which indicates elimination of *B. pseudomallei* from the soil or the concentration of *B. pseudomallei* were too low to be detected. There are more than 80 species in the genus *Burkholderia* that occur naturally in the environment (Depoorter *et al*., 2016) which might have antagonistic activity against *B. pseudomallei*.

From these past studies, it has been shown that there are antagonistic interactions between *Burkholderia pseudomallei* and *Burkholderia* species especially those found within the same environmental niche (Coenye *et al*., 2001; Marshall *et al*., 2010) However, production of an antagonistic compound by *Burkholderia* spp. isolates against Sarawak *B. pseudomallei* which were reportedly unique (Podin *et al*., 2014) has not been previously described. Obtaining compound that is specifically antagonistic against *B. pseudomallei* is important as potential sources of new therapeutic compounds in the pharmacological industry. Thus, the aims of this current study are to identify environmental *Burkholderia* spp. isolates from Sarawak that possess the potential in inhibiting the growth of *B. pseudomallei* and to further characterize the antagonistic compound produced.

MATERIALS AND METHODS

Bacterial strains maintenance

Burkholderia pseudomallei (n=5), *Burkholderia* spp. (n=50) isolates and *Ralstonia solanacearum* (n=2) that have been previously characterized using 16S rRNA gene sequencing were obtained from the Institute of Health and Community Medicine (IHCM), Universiti Malaysia Sarawak archival collection that were stored in 20% glycerol in –80 °C (Podin, 2014). *Burkholderia* spp. used in this study are *B. pyrrocinia* (n=9), *B. thailandensis* (n=9), *B. ubonensis* (n=12), *B. cepacia* (n=13), *B. stagnalis* (n=4), *B. diffusa* (n=1) and *Burkholderia* spp. (n=2). Luria-Bertani (LB) agar was prepared by dissolving 10 g tryptone (Oxoid, Hampshire, UK), 5 g NaCl (R&M Chemicals, Essex, UK), 5 g yeast extract (VWR Amresco Life Science, Solon, USA) and 15 g agar (Oxoid, Hampshire, UK) in 1000 mL ultrapure water (ELGA labwater, Germany). Media was sterilized in autoclave (121 °C, 15 min) then poured into Petri dish then stored at 4 °C until further use. Isolates were streaked on LB agar plates and incubated for 24 h. Cultures were kept in 4 °C until further use. Prior to use, subculture was done to obtain a 24-h culture.

Antagonism screening

Two methods were used to test for antagonism between *Burkholderia* spp. and *B. pseudomallei* isolates; namely the spot-on-lawn assay and flip streak method as previously described by Marshall *et al*. (2010) with minor modifications.

Bacterial cell suspension preparation

The "target" and "producer" isolates used for antagonism testing were cultured on LB plates at 39 °C for 24 h. Suspension of bacterial cells was prepared from the overnight culture in 0.85% saline solution. Concentration of the bacterial cell suspension was adjusted to $OD_{600} \approx 0.13$ using a spectrophotometer (BioPhotometer 6131, Eppendorf, Germany).

Spot-on-lawn assay

Bacterial lawn of "target" isolate was prepared by swabbing cell suspension on Mueller-Hinton agar (MHA) plates using sterile cotton swab. Next, 5 µL of each suspension of "producer" isolate was spotted on the prepared bacterial lawn. Sterile 0.85% saline solution was used as control. Plates were left in biosafety cabinet until the spotted "producer" was fully absorbed into the agar and then incubated at 39 °C for 24 h. The plates were observed for the presence of zones of inhibition (ZOI) and results were recorded qualitatively (zones/no zones) daily for 3 days.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(6) 2021, pp. 646-660
DOI: http://dx.doi.org/10.21161/mjm.211212

Flip streak method

Suspension of "producer" isolates were prepared and 5 µL were spot inoculated onto 20 mL MHA (2 per plate) incubated at 39 °C. Sterile 0.85% saline solution was used as control. After 24 h, the agar was aseptically detached from the plate using forceps, inverted and placed back into the Petri dish. Suspensions of "target" isolates were then prepared and 100 µL was inoculated into 10 mL of molten MHA and then poured onto the MHA that was flipped over. Plates were incubated at 39 °C for 24 h and then observed for the presence of ZOI. Results were recorded qualitatively (zone/no zone) daily for 3 days.

Obtaining antagonistic compound in a cell-free state

Cell-free supernatant of potential isolates was obtained as described by Marshall *et al.* (2010) with minor modifications. Potential antagonistic isolates were grown on Luria-Bertani (LB) plates and incubated at 39 °C for 24 h. Cell suspension was prepared and inoculated in fresh LB broth (10% v/v). LB broth was prepared by dissolving 10 g tryptone (Oxoid, Hampshire, UK), 5 g NaCl (R&M Chemicals, Essex, UK) and 5 g yeast extract (VWR Amresco Life Science, Solon, OH) in 1000 mL ultrapure water (ELGA Labwater, Germany). Media was sterilized in autoclave (121 °C, 15 min). Inoculated bacterial culture was shaken at 150 rpm with an orbital shaker (Protech Electronics, Malaysia) and incubated at room temperature and 39 °C, respectively. Culture was then centrifuged at 5,000× *g* for 5 min (Labogene Scanspeed Mini Microcentrifuge, Lynge, Denmark). Supernatant was obtained and filter sterilized using syringe (Becton Dickinson Medical (s), Singapore) attached to Acrodisc® syringe filters with 0.2 µm Supor® membrane (Pall Corporation, Ann Arbor, USA) to remove residual cells. This is referred as cell-free supernatant (CFS).

Agar well diffusion assay (AWDA)

To test for the presence of antagonistic compound in CFS, agar well diffusion assay (AWDA) described by Kindoli *et al*. (2012) with minor modifications was used. On an MHA swabbed with "target" isolate, wells were created using disposable plastic Pasteur pipettes that were cut on the midsection. A total of 80 µL of CFS were then dispensed into each well. Sterile LB broth was used as control. After wells were dry, plates were incubated at 39 °C for 24 h. ZOI were measured and recorded after incubation period. Experiments were done in triplicate.

Bacterial growth study

Potential antagonistic isolate was grown on LB plates and incubated at 39 °C for 24 h. Cell suspension was prepared by inoculated the cells in fresh LB broth (10% v/v) and incubated at room temperature at 150 rpm. Cell growth was monitored (OD at 600 nm) and pH changes recorded using starter 3100 bench pH meter (Ohaus Corporation, Pine Brook, USA) every four hours. Aliquots of CFS were also sampled and kept at –20 °C for detection of antagonism via AWDA.

Characterization of antagonistic compound in cell-free state

Heat stability of antagonistic compound

To determine the heat stability of the compound, the CFS was incubated at 40, 50, 60, 70, 80, 90 and 100 °C, respectively, for 1 h in a digital heat block (Benchmark Scientific Inc, Edison, USA). CFS was also treated at 121 °C for 15 min by autoclaving. Immediately after incubation, treated CFS was cooled to 4 °C prior to testing. CFS without heat treatment was used as control.

Effect of pH on antagonistic compound

Effect of pH on the compound was tested by adjusting the CFS to pH 2, 4, 6, 8, 10 and 12 by adding sterile 5 N sodium hydroxide (NaOH) or 5 N hydrochloric acid (HCl). 5 N NaOH or 5 N HCl with sterile LB broth was used as control.

Enzyme susceptibility of antagonistic compound

For determination of enzymes susceptibility, proteinase K (EC 3.4.21.64) (Qiagen, Hilden, Germany), trypsin (EC 3.4.21.64), (Amresco, Solon, USA), pepsin (EC 3.4.23.1), α-amylase (EC 3.2.1.1) (HiMedia, Mumbai, India) and lipase (EC 3.1.1.3) (Sigma-Aldrich, St. Louis, USA) solutions were added to aliquots of CFS to a final concentration of 1mg/mL. Samples were incubated at 37 °C for 2 h and then heated at 80 °C for 10 min to inactivate the enzymes (Yi *et al*., 2016). Untreated CFS and enzyme with sterile LB broth was used as control.

All treated samples were tested using AWDA. Percentage residual inhibitory activity of the compound was calculated using the formula below.

Residual activity (%) = (ZOI of treated samples/ZOI of control) × 100%

Partial purification of antagonistic compound

CFS was precipitated with ammonium sulfate (BDH, Poole, England) to a concentration of 75%. Solid ammonium sulfate was added slowly to supernatant with constant stirring. After all salt had dissolved completely, CFS was incubated at 4 °C with stirring and left overnight. Pellet was collected after centrifugation at 13,000× *g* for 20 min at 4 °C in an Avanti JXN-26 centrifuge (Beckman Coulter, California, USA). Precipitated protein was resuspended in PBS (pH 7.2) in 1% of initial volume. It was then dialyzed overnight against 1000 mL of similar buffer using 6000–8000 Da molecular weight cutoff regenerated cellulose membranes (Fisher Scientific, Nepean, Canada). Dialyzed product was stored at –20 °C until further use.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(6) 2021, pp. 646-660
DOI: http://dx.doi.org/10.21161/mjm.211212

SDS-PAGE and bioassay

The molecular weight of the antagonistic compound was estimated through Tris-Glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) described by Laemmli (1970). Mini Protean 3 electrophoresis unit (BioRad Laboratories, Hercules, USA) was used to conduct SDS-PAGE. Discontinuous gel system which consists of stacking and resolving gel was used. LB broth precipitated with 75% ammonium sulfate was used as negative control. Electrophoresis was conducted at 80 V for 120 min. Direct detection of protein of interest from SDS-PAGE gel was then conducted as described previously by Bhunia *et al*. (1987), with minor modifications. After electrophoresis, gel was cut into half. The gel containing the protein molecular weight markers (SDS-PAGE molecular weight standards, broad range, BioRad Laboratories, Hercules, USA), sample and negative control was stained with Coomassie Brilliant Blue (CBB) (CBB Stain One Super, Nacalai Tesque, Kyoto, Japan) for visualization. For bioassay detection, the other half of gel was immediately fixed in a solution containing 20% isopropanol (Merck, Darmstadt, Germany) and 10% acetic acid (R&M Chemicals, Essex, UK) for 2 h, then equilibrated in sterile deionized water for 4 h. The strips were then placed in sterile Petri dish and overlaid with soft MHA (0.7% agar) containing 1% (v/v) overnight indicator culture. Plates were incubated at 39 °C for 24 h. Presence of ZOI was observed and position of band containing the antagonistic compound was compared to the stained gel.

Statistical analysis

Results were expressed in mean and standard deviation of triplicate. To determine if there were significant difference between treated samples and control, one-way analysis of variance (ANOVA) was applied, followed by Tukey's post-hoc test using IBM SPSS Statistics version 22 software (IBM, New York, USA). Evaluation was done separately against five 'target' isolates. In all analyses, $p<0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Antagonistic screening

Spot-on-lawn assay

Most techniques for bacteriocin detection involve the diffusion of the antimicrobial peptide through an agar medium (Somkuti and Steinberg, 2002). In the initial preliminary screening, spot on lawn assay was used in selecting potential isolates that can inhibit the target *B. pseudomallei* isolates, MSHR5079, MSHR5084, MSHR5095, MSHR5105 and MSHR6793. This method enables cell-to-cell interaction between the producer and target isolates. Results were recorded qualitatively, as shown in Supplementary Table S1.

From 52 isolates tested, 28 isolates showed antagonism against at least one *B. pseudomallei* tested (Table 1). However, only one isolate, *B. stagnalis* K23/3, that showed clear ZOI against all five *B. pseudomallei* tested, as shown in Figure 1. Other isolates with weak inhibition presented cloudy or unclear ZOI around the spot inoculated culture. Results were in contrast with previous studies which reported that *B. ubonensis* showed antagonism against *B. pseudomallei* (Marshall *et al*., 2010). This is because production of bacteriocin is strain-specific instead of species-specific (Nakatsuji *et al*., 2017; Newstead *et al*., 2020). Furthermore, bacteria isolated from different geographical regions may have different susceptibility to antimicrobials as well. For instance, *B. pseudomallei* isolated from central Sarawak presented different gentamicin susceptibility compared to previously reported study (Podin *et al*., 2014).

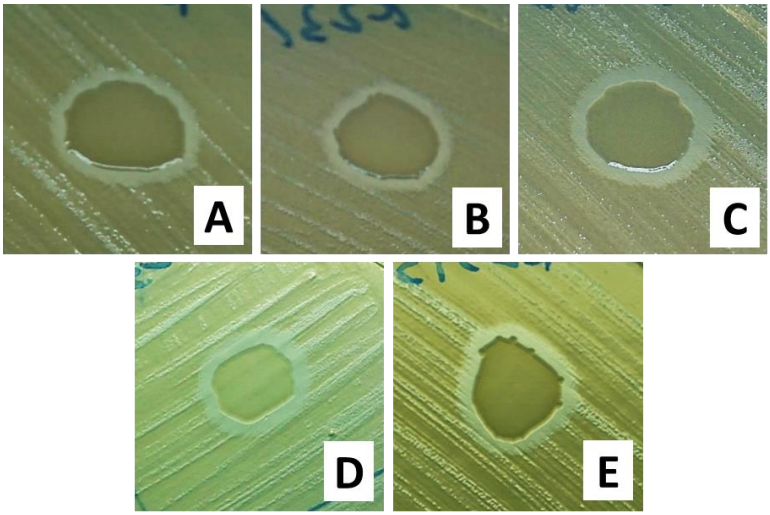

Figure 1: Presence of clear ZOI around *Burkholderia stagnalis* K23/3 spotted on lawn of (A) MSHR5079, (B) MSHR5084, (C) MSHR5095, (D) MSHR5105 and (E) MSHR6793.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(6) 2021, pp. 646-660
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Table 1: Antagonism between producer and target isolates.

Producer isolates		Target isolates				
		MSHR 5079	MSHR 5084	MSHR 5095	MSHR 5105	MSHR 6793
B. pyrrocinia	Bp5	-	-	+	-	-
B. thailandensis	Bt6	-	-	+	+	-
B. thailandensis	Bt7	-	-	+	+	-
B. thailandensis	Bt8	-	-	+	+	-
B. thailandensis	Bt9	-	-	+	+	-
B. ubonensis	Bu4	-	+	+	-	-
B. ubonensis	Bu5	-	+	+	-	-
B. ubonensis	Bu7	-	+	-	-	-
B. ubonensis	Bu8	-	-	-	-	-
B. ubonensis	Bu9	-	+	-	-	-
B. ubonensis	Bu10	-	+	-	-	-
B. ubonensis	Bu11	-	+	-	-	-
B. ubonensis	K26/1	-	++	+	-	-
B. cepacia	Bc1	-	-	+	-	-
B. cepacia	Bc2	-	-	+	-	-
B. cepacia	Bc6	-	+	-	+	+
B. cepacia	Bc8	-	+	+	+	+
B. cepacia	Bc9	-	+	+	-	-
B. cepacia	Bc10	-	+	-	-	-
B. cepacia	K25/2	-	+	-	-	-
B. cepacia	S5/2	-	+	-	-	-
B. stagnalis	S4/15	-	-	+	-	-
B. stagnalis	S4/16	-	-	+	-	-
B. stagnalis	S4/18	-	-	+	-	-
B. stagnalis	K23/3	++	++	++	++	++
B. diffusa	Bd1	-	+	-	-	-
Ralstonia solanacearum	Rs1	-	+	-	-	+
Ralstonia solanacearum	Rs2	-	+	-	-	+
Burkholderia spp.	Burk1	-	+	-	-	-

++, strong inhibition (ZOI ≥ 2 mm); +, weak inhibition (ZOI < 2 mm); -, no inhibition.

Table 2: Presence of ZOI in flip streak assay.

Producer isolates		Target isolate				
		MSHR 5079	MSHR 5084	MSHR 5095	MSHR 5105	MSHR 6793
B. cepacia	Bc10	+	+	+	+	+
B. cepacia	S5/2	+	+	+	+	+
B. stagnalis	K23/3	++	++	++	++	++

++, strong inhibition (ZOI ≥ 2 mm); +, weak inhibition (ZOI < 2 mm); -, no inhibition.

Flip streak assay

Isolates that showed antagonism in the spot-on-lawn assay were further studied in flip streak assay. This assay is also known as the indirect or deferred assay as there is no direct contact between the producing and target isolates (Kékessy and Piguet, 1970; Somkuti and Steinberg, 2002). Antagonistic activities present in the spot-on-lawn assay might be caused by the presence of bacteriophage (Alam *et al*., 2011). Hence, flip streak assay was used to distinguish the inhibition is caused by bacteriocin-like inhibitory substance (BLIS) or bacteriophage (De Vuyst and Vandamme, 1994). The ZOI produced on the opposite side of the agar will indicate that the antagonistic activity was caused by proteins instead of bacteriophage. Unlike bacteriocin, bacteriophage does not have the ability to diffuse through agar medium (Alam *et al*., 2011). Results of this assay were recorded qualitatively (Supplementary Table S2). Three isolates that showed antagonistic activity against *B. pseudomallei* tested are presented in Table 2.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(6) 2021, pp. 646-660
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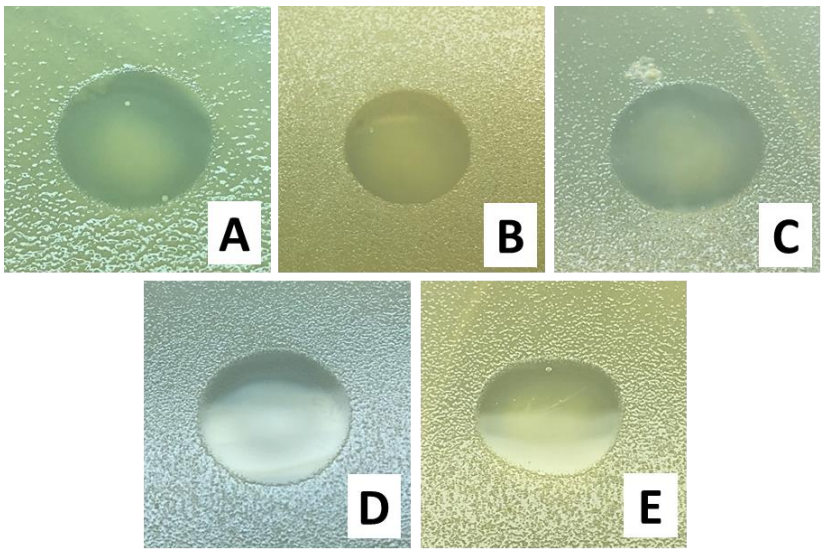

Figure 2: Presence of clear ZOI in flip streak assay of *Burkholderia stagnalis* isolate K23/3 against (A) MSHR5079, (B) MSHR5084, (C) MSHR5095, (D) MSHR5105 and (E) MSHR6793.

Two isolates of *B. cepacia* showed slight inhibition on the surface of the inoculated agar but *B. stagnalis* K23/3 showed clear inhibition against all five *B. pseudomallei* tested, as shown in Figure 2. The clear ZOI on the agar surface may be caused by a non-bacteriophage compound that is released by *B. stagnalis* K23/3.

Antagonism activity test on cell-free supernatant (CFS)

Isolate *B. stagnalis* K23/3 was selected for further investigation because of its high antagonism towards *B. pseudomallei* isolates tested. According to van der Wal *et al*. (1995), bacteriocins are proteins that are released into the extracellular environment. To investigate if the antagonistic compound was secreted constitutively, isolate *B. stagnalis* K23/3 was cultured in LB broth for 24 h. Cell-free supernatant (CFS) was then obtained and tested via AWDA. AWDA is widely used to study antimicrobial activity of plants or microbial extracts (Magaldi *et al*., 2004; Valgas *et al*., 2007). According to Magaldi *et al*. (2004), this method is simple, reproducible, inexpensive and easy to evaluate.

Results for this study showed no antagonistic activity was observed in the AWDA when broth culture of *B. stagnalis* K23/3 was incubated at 39 °C, while the opposite was observed when broth culture was incubated at 26 °C (room temperature). This showed that temperature of bacterial growth plays a significant role in the production of the antimicrobial compound. There were no reported optimum temperature of *B. stagnalis* yet, but De Smet *et al*. (2015) described that this species grew at 37 °C and most isolates in their study grew at 42 °C. The optimum temperature for *B. pseudomallei* growth was reported to be 37 to 42 °C (Dance, 2000; Chen *et al*., 2003). Similar results were observed by Todorov and colleagues (2004), whereby antagonistic compound of *Lactobacillus plantarum* was produced at 30 °C, but no production at 36 °C. Several other studies also showed that the optimal temperature for bacteriocin production was lower than the optimum temperature for the growth of the producer strain such as nisin Z by *Lactococcus lactis* (Matsusaki *et al*., 1996) and amylovorin by *Lactobacillus amylovorus* (De Vuyst *et al*., 1996). According to De Vuyst *et al*. (1996), growth at lower temperature causes lower growth rates which results in more energy available for bacteriocin production.

Bacterial growth curve

Figure 3 shows the compound chart of growth curve, pH of culture and measurement of ZOI produced. CFS was only obtained on 16th hour of culture onwards because results from preliminary investigation that involved hourly sampling showed that the compound was only released after 16 h (data not shown), which is the early exponential phase of the growth (Figure 3). Antagonistic activity reached its maximum at the stationary phase with slight reduction after the stationary phase. This is similar to most of the bacteriocin production kinetics reported by previous work. In a study by Barboza-Corona *et al*. (2007), *Bacillus thuringiensis* produced bacteriocin-like inhibitor substances at the middle of the logarithmic phase and reached its maximum production at the start of stationary phase, while bacteriocin LaKS400 produced by *Lactobacillus acidophilus* KS 400 increased during the exponential phase and reached the maximum production at the stationary growth phase with reduction was also seen after the stationary phase (Gaspar *et al.*, 2018). Other bacteriocins such as thuricin (Favret and Yousten, 1989), tochicin (Paik *et al.*, 1997) and cerein (Naclerio *et al.*, 1993) were also detected at early exponential phase.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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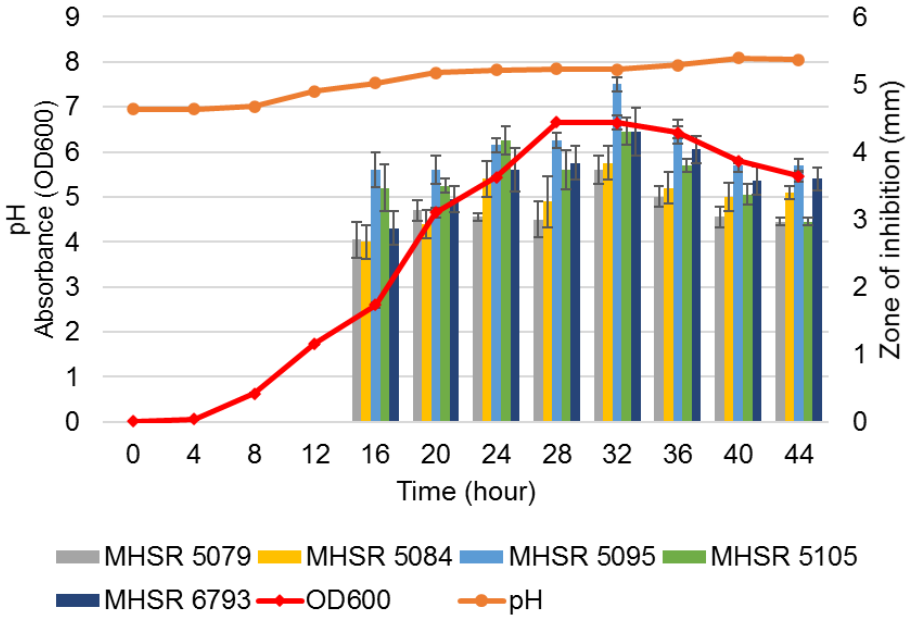

Figure 3: Growth curve of K23/3, pH changes and production of antagonistic compound tested against MSHR5079, MSHR5084, MSHR5095, MSHR5105 and MSHR6793.

Gray *et al.* (2006) reported that thuricin 17 produced by *Bacillus thuringiensis* NEB17 may be classified as product of secondary metabolism as the production begins at the mid-exponential phase and continued in the stationary phase. Metabolites from *Bacillus amyloliquefaciens* that could inhibit the growth of *B. pseudomallei* in the study of Boottanun *et al*. (2017) were produced from the mid-exponential to mid-stationary phase (12-72 h) and activity decreased after 78 h of culturing. They concluded that this fits the characteristic of secondary metabolites as well. The results obtained from this current study seem to concur with these studies which suggest that the biosynthesis of the compound produced by *B. stagnalis* K23/3 follows the pattern of secondary metabolite synthesis.

Secondary metabolites can be induced by various factors, such as stress, starvation or environmental factors and quorum sensing that uses small peptides as inducer (Kleerebezem and Quadri, 2001). Primary metabolites, on the other hand, are growth associated and the product concentration increases with cell concentration (Sakthiselvan *et al*., 2019). Therefore, the production of primary metabolites follows closely to the growth curve of the producer cell. In this study, the production of the antagonistic compound was influenced by temperature of the culture and in Figure 3, it is revealed that the compound is produced from the mid-exponential to the mid-stationary phase of the culture. These characteristics suggest that this compound is a product of secondary metabolism, instead of primary metabolism.

It was previously reported that secondary metabolites are not essential in the growth, development or reproduction of the producing bacteria but they play important ecological roles in interacting with other organisms with different purposes especially for soil bacteria (Tyc *et al*., 2017). Some of the functions of secondary metabolites include natural defense against other organisms, transportation of metal, symbiotic compound between microbes and other organisms, as sexual hormones and also differentiation effectors (Demain and Fang, 2000). Examples of microbial secondary metabolites are antibiotics, growth hormones, antitumor agents (Ruiz *et al*., 2010) and bacteriocins (Gray *et al*., 2006).

In this study, pH of the culture slightly increased from 6.9 to 7.5 at the 16th hour of incubation. Ratzke and Gore (2018) mentioned that microbes are able to increase or decrease the pH of their environment that maybe beneficial or harmful for their own growth. Since the slight increment of pH is at the lag phase of the bacterial growth, the bacteria could be altering the environment for better growth.

Characterization of CFS obtained from *B. stagnalis* K23/3

Bacteriocins vary in molecular weight, biochemical properties, spectrum of antimicrobial activity and mechanism of action (Salazar *et al.*, 2017). Thus, in exploratory work of antimicrobial substance, characterization is usually done to determine the nature and the possible compound produced. CFS of *B. stagnalis* K23/3 broth culture was treated with different temperatures, pH and enzymes to identify the nature and characteristics of the compound that affect the inhibition. Table 3 shows the results of all the treatment parameters.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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DOI: http://dx.doi.org/10.21161/mjm.211212

Table 3: Antagonistic activity of CFS in percentage after different treatments.

Parameters	*Burkholderia pseudomallei* isolates				
	MSHR 5079	MSHR 5084	MSHR 5095	MSHR 5105	MSHR 6793
Temperature (°C)					
40	98.3 ± 2.9	107.2 ± 5.0	144.1 ± 7.8*	114.1 ± 16.5	108.2 ± 8.5
50	89.9 ± 10.6	111.6 ± 5.0	123.7 ± 12.8	117.2 ± 12.4	111.5 ± 5.7
60	98.3 ± 12.8	102.9 ± 6.6	135.6 ± 7.8*	128.1 ± 10.8*	114.8 ± 5.7
70	82.0 ± 9.2	106.9 ± 6.0	93.5 ± 7.8	96.1 ± 8.1	92.2 ± 2.2
80	106.0 ± 9.2	113.7 ± 7.9	89.6 ± 10.3	92.2 ± 6.0	80.5 ± 11.9
90	84.0 ± 10.4	84.5 ± 15.8	83.1 ± 12.5	84.4 ± 9.0	81.8 ± 15.6
100	28.2 ± 9.7*	39.0 ± 7.6*	56.0 ± 3.3*	73.8 ± 2.2*	41.7 ± 7.4*
Autoclaved	0*	0*	0*	0*	0*
pH					
2	43.7 ± 6.5*	53.4 ± 10.9*	50.6 ± 7.3*	59.5 ± 16.4*	54.9 ± 11.2*
4	86.4 ± 4.5	94.4 ± 8.8	84.0 ± 5.7	81.4 ± 11.3	80.8 ± 2.4
6	88.4 ± 10.0	89.2 ± 8.1	92.5 ± 2.2	95.8 ± 8.3	89.6 ± 9.0
8	86.6 ± 2.6	100.0 ± 8.8	94.9 ± 4.4	102.8 ± 4.8	89.0 ± 10.3
10	87.7 ± 4.7	82.2 ± 14.8	89.7 ± 11.1	69.1 ± 11.3*	89.2 ± 7.1
12	52.7 ± 14.6*	82.4 ± 4.7	74.0 ± 12.3*	59.7 ± 4.5*	71.1 ± 10.4*
Enzyme (1 mg/mL)					
Amylase	91.5 ± 6.6	96.5 ± 2.6	97.2 ± 4.4	106.7 ± 7.2	106.1 ± 1.5
Trypsin	90.0 ± 13.2	93.1 ± 9.0	88.7 ± 4.2	90.4 ± 8.2	95.4 ± 10.7
Lipase	92.7 ± 4.8	90.9 ± 3.0	86.5 ± 8.1	86.9 ± 5.6	98.0 ± 1.7
Pepsin	68.7 ± 3.6*	61.7 ± 4.9*	66.0 ± 4.7*	63.7 ± 4.5*	49.0 ± 2.9*
Proteinase K	0*	0*	0*	0*	0*

* indicates significant difference with untreated CFS (p<0.05).
Data are the mean of triplicates ± S. D.
Residual activity (%) = (ZOI of treated samples/ZOI of control) × 100%

Heat treatment

There was substantial difference of activity between heated samples and the control at 40 and 60 °C when tested against MSHR5095. Notable increment was also seen when sample heated at 60 °C was tested against MSHR5105. Similar observation was made by Marshall and colleagues, whereby antimicrobial activity was enhanced after CFS was treated from 50-70 °C. In their study, ZOI produced after 50 °C and 70 °C treatment for an hour were increased 2.4-fold, compared to untreated CFS. Presence of heat could have caused conformational changes towards the antimicrobial compound, thus enhancing more intermolecular interaction that increases the activity (Marshall *et al*., 2010). However, at higher temperature of 100 °C and 121 °C, activity of CFS showed significant reduction (p<0.05) when tested on all target isolates. Only 28.2% of residual activity was seen when CFS heated at 100 °C tested on MSHR5079. When CFS was autoclaved at 121 °C for 15 min, the compound was deactivated completely. Bacteriocin produced by *Enterococcus faecalis* 478 studied by Phumisantiphong *et al.* (2017) was also deactivated after autoclaved and heated at 100 °C for an hour. Temperature higher than 90 °C would have caused denaturation of protein which decreased or inactivate the reactivity of the antagonistic compound released by *B. stagnalis* K23/3 against target bacteria. Characterizing CFS according to heat stability helps to exclude inhibition caused by the presence of extracellular proteases and hydrogen peroxide (H_2O_2). In preparing CFS from *Lactobacillus curvatus* P99, Funck *et al*. (2019) stated that heating the CFS at 80 °C for 10 min can destroy extracellular proteases and H_2O_2. In this study, activity remained after heat treatment of 80 °C for an hour thus inhibition caused by proteases or H_2O_2 can be excluded.

pH stability

Based on Table 3, it shows the CFS compound was relatively stable in a wide range of pH but was optimum at the range of pH 4 to 8. Decreased activity was observed when pH of CFS was adjusted to extreme acidic condition, whereby 43.7 to 59.5% of activity remained after pH was adjusted to pH 2. In extreme alkali condition, activity of the compound decreased when tested against MSHR5079 and MSHR5105, with remaining activity of 52.7% and 59.7%, respectively. Different results were seen in other bacteriocins studied, whereby the bacteriocins favour acidic conditions compared to alkali conditions. For instance, *Lactobacillus crustor* produced bacteriocin that was more active at pH 2 to pH 6. Partial loss of activity occurred at pH 10 and pH 11, where antimicrobial activity retained were 79.25% and 70.89%,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(6) 2021, pp. 646-660
DOI: http://dx.doi.org/10.21161/mjm.211212

respectively (Yi *et al*., 2016). Besides determining the stability of compound in different pH, adjusting the pH of the CFS containing the antagonistic compound also eliminates the possibilities of inhibition caused by other metabolites such as organic acids that could be produced by the bacterium. In the study by Phumisantiphong *et al*. (2017), CFS was adjusted to pH 7 to eliminate the possibility of inhibition caused by presence of organic acids. Based on the obtained results from this current study, the compound is stable in the range of pH 4-8, thus showing that inhibition was not due to the presence of organic acids.

Enzyme susceptibility

When treated with proteinase K, the activity of the antagonistic compound was totally deactivated. Antimicrobial activity was partially reduced with pepsin treatment. This suggests that the antagonistic compound has protein moiety, which greatly contributed to the inhibition of all five target isolates tested. Antimicrobial activities were retained after trypsin, lipase and α-amylase solution treatments. This finding indicates that the antagonistic compound is proteinaceous, without lipid or carbohydrate moieties that play significant roles for antagonism against target isolates. Different proteinase cleaves different peptide bonds in a protein thus, it is possible that trypsin and pepsin do not disrupt the functional sites of the antagonistic compound. Montville and Kaiser (1993) suggested two requirements are needed for an antagonistic bacterial compound to be classified as bacteriocin. The requirements are the inability to kill the producer cells and has proteinaceous moieties. In proving the compound to be a protein, it is sufficient to be deactivated by one or more proteases (Montville and Kaiser, 1993). Cherif *et al*. (2001) also reported that the inhibitory compound produced by *B. thuringiensis* may be classified as a bacteriocin after confirming the proteinaceous nature of the compound by proteinase K treatment. In this present study, the antagonistic compound in CFS was deactivated totally by proteinase K and its inhibitory activity was partially reduced by pepsin. The secreted compound did not cause any inhibition against the producer cells as well. Thus, we can suggest that this compound is a bacteriocin or bacteriocin-like inhibitory substance (BLIS).

Partial purification of antagonistic compound

In detecting antimicrobial protein in SDS-PAGE, secreted protein in CFS was first concentrated. Ammonium sulfate precipitation method was used to extract all proteins in the CFS was reported by previous work on bacteriocins (Boottanun *et al*., 2017; Le *et al*., 2019). In this present study, ammonium sulfate precipitation concentrated the compound by 1.86-fold. Since the antagonistic compound can be precipitated by ammonium sulfate, this indicates that the compound could be peptides or proteins.

SDS-PAGE and bioassay

SDS-PAGE was performed to estimate the molecular mass of the antagonistic compound. This method also enables the direct detection of antagonistic protein by overlaying soft agar seeded with indicator isolate onto the gel strips. It may not be the most accurate method in determining the molecular mass of the compound, but it was able to show the presence of one or more antimicrobial protein. Godič Torkar and Bogović Matijašić (2003) reported that, it is possible for the production of more than one bacteriocin substance by the same producer strain. For example, Le *et al*. (2019) observed ZOI produced by two bands with approximately 27 and 10 kDa, respectively after overlaying the SDS-PAGE gel strip with indicator isolates. In this study, several bands were seen from the stained SDS-PAGE gel, as shown in Figure 4A. Lane 2 shows negative control of the experiment, which is sterile LB broth precipitated with 75% ammonium sulfate. A blob was observed in the negative control lane, which could be the proteins in LB broth. Half of the gel which contains protein marker was stained with Coomassie Brilliant Blue (CBB). CBB binds non-specifically to almost all proteins and it is widely used because of its rapid and simple staining, and it is inexpensive (Beer and Speicher, 2018). In determining the band of interest, the other half was fixed in fixing solution containing 20% 2-propanol and 10% acetic acid. The fixing solution ensured the proteins to remain in the gel so it can be detected in the overlay assay and it has been shown to removed SDS from the gel (Crupper *et al*., 1997). After fixing, soft agar inoculated with indicator isolate was overlaid onto the fixed gel. Figure 4B shows the presence of ZOI after the gel overlay assay.

The SDS-PAGE analyses showed that the protein of interest is smaller than 7.1 kDa. Interestingly, the expected size of the protein was larger than 30 kDa, as the protein of interest was in the retentate after concentrating the CFS using a 30 kDa molecular weight cutoff (MWCO) protein concentrator (Thermofisher Scientific, Boston, MA, USA) (results not shown). Similar situation was observed by Kato *et al*. (1994) regarding their work with plantaricin, bacteriocin produced by *Lactobacillus plantarum*. From their ultrafiltration analysis, the bacteriocin appeared to be larger than 200 kDa in culture broth. However, the protein size was estimated to be approximately 2.2 kDa from SDS-PAGE overlay assay (Kato *et al*., 1994). This could be due to the exposure of SDS detergent and reducing agents which broke down the protein which remained functional. When working on leuconocin produced by *Leuconostoc paramesenteroides*, Lewus *et al*. (1992) reported that the leuconocin S might have formed an aggregate that was dispersed by the presence of SDS in gel or might be cleaved thus producing a lower molecular weight band. Due to cationic and hydrophilic interactions, bacteriocin could be present with other macromolecules in crude extracts but eventually disintegrated during purification processes

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(6) 2021, pp. 646-660
DOI: http://dx.doi.org/10.21161/mjm.211212

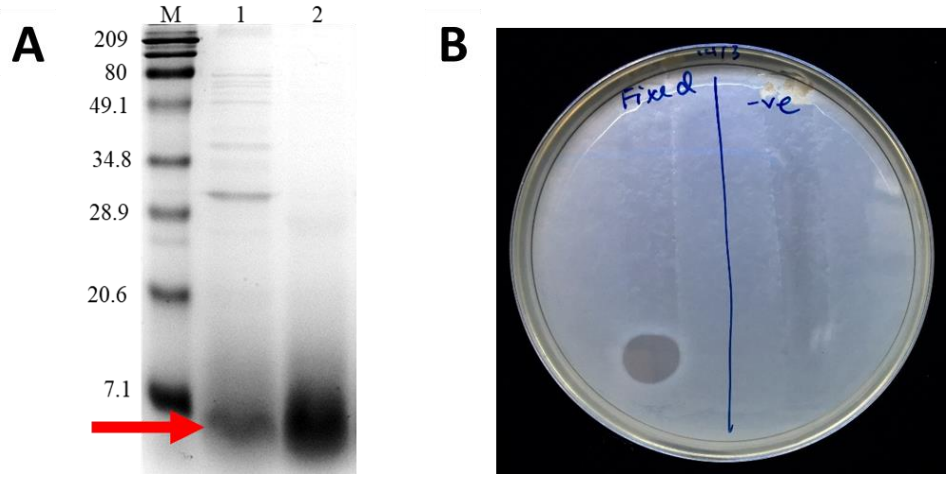

Figure 4: (A) Stained SDS-PAGE imaged with the Bio-Rad Gel Doc XR+ Imaging System (Bio-Rad, Hercules, USA). M: Broadrange marker, Lane 1: Concentrated CFS, Lane 2: Negative control. Red arrow indicates band corresponding to the position of ZOI produced. (B) Fixed gel strips overlaid with "target" isolate producing a single ZOI.

while maintaining the activity of the compound (Godič Torkar and Bogović Matijašić, 2003). In another work done using *L. plantarum*, Jiménez-Díaz *et al*. (1993) showed that disintegration of these high-molecular-weight aggregates, although incomplete, seemed to occur upon ammonium sulfate precipitation step. Therefore in this work, ammonium sulfate and the reagents in SDS-PAGE might have disintegrated the crude compound produced by isolate *B. stagnalis* K23/3 in the broth culture. Since there was still biological activity after SDS-PAGE, the protein could be refractory to detergent action or it is able to renature during the assay (Héchard *et al*., 1992).

CONCLUSION

Preliminary screening of *Burkholderia* spp. against *B. pseudomallei* using the spot-on-lawn and flip streak method have successfully identified potential antagonistic isolate against this pathogen. Results obtained showed that antagonism between tested isolates are strain-specific instead of species-specific. By characterizing the compound according to heat and pH stability and enzymes susceptibility, it is suggested that the compound produced is a protein and could be a bacteriocin or a bacteriocin-like inhibitory substance (BLIS). Direct detection of bioactive compound through SDS-PAGE gel overlaid with target isolates further confirmed that the compound of interest is a protein. Further purification and identification of protein using LC-MS/MS may be carried out in the future to further understand the antagonistic protein. This preliminary finding maybe useful for application in the biocontrol of *B. pseudomallei*.

ACKNOWLEDGMENTS

The authors would like to thank Universiti Malaysia Sarawak for providing the basic facilities and resources for the research work. The authors would also like to express their appreciation to the Melioidosis Research Team of Menzies School of Health Research, Darwin, Northern Territory, Australia for allowing the use of the code of the isolates used in this study. The first author, Sherene Tan Hoon-Li, was a postgraduate scholar under the sponsorship of the Zamalah Siswazah UNIMAS (ZSU) Scholarship scheme.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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DOI: http://dx.doi.org/10.21161/mjm.211212

SUPPLEMENTARY INFORMATION

Table S1: Antagonism between producer and target isolates.

Producer isolates		*B. pseudomallei* target isolates				
		MSHR 5079	MSHR 5084	MSHR 5095	MSHR 5105	MSHR 6793
B. pyrrocinia	Bp1	-	-	-	-	-
B. pyrrocinia	Bp2	-	-	-	-	-
B. pyrrocinia	Bp3	-	-	-	-	-
B. pyrrocinia	Bp4	-	-	-	-	-
B. pyrrocinia	Bp5	-	-	+	-	-
B. pyrrocinia	Bp6	-	-	-	-	-
B. pyrrocinia	Bp7	-	-	-	-	-
B. pyrrocinia	Bp8	-	-	-	-	-
B. pyrrocinia	Bp9	-	-	-	-	-
B. thailandensis	Bt1	-	-	-	-	-
B. thailandensis	Bt2	-	-	-	-	-
B. thailandensis	Bt3	-	-	-	-	-
B. thailandensis	Bt4	-	-	-	-	-
B. thailandensis	Bt5	-	-	-	-	-
B. thailandensis	Bt6	-	-	+	+	-
B. thailandensis	Bt7	-	-	+	+	-
B. thailandensis	Bt8	-	-	+	+	-
B. thailandensis	Bt9	-	-	+	+	-
B. ubonensis	Bu1	-	-	-	-	-
B. ubonensis	Bu2	-	-	-	-	-
B. ubonensis	Bu3	-	-	-	-	-
B. ubonensis	Bu4	-	+	+	-	-
B. ubonensis	Bu5	-	+	+	-	-
B. ubonensis	Bu6	-	-	-	-	-
B. ubonensis	Bu7	-	+	-	-	-
B. ubonensis	Bu8	-	-	-	-	-
B. ubonensis	Bu9	-	+	-	-	-
B. ubonensis	Bu10	-	+	-	-	-
B. ubonensis	Bu11	-	+	-	-	-
B. ubonensis	K26/1	-	++	+	-	-
B. cepacia	Bc1	-	-	+	-	-
B. cepacia	Bc2	-	-	+	-	-
B. cepacia	Bc3	-	-	-	-	-
B. cepacia	Bc4	-	-	-	-	-
B. cepacia	Bc5	-	-	-	-	-
B. cepacia	Bc6	-	+	-	+	+
B. cepacia	Bc7	-	-	-	-	-
B. cepacia	Bc8	-	+	+	+	+
B. cepacia	Bc9	-	+	+	-	-
B. cepacia	Bc10	-	+	-	-	-
B. cepacia	Bc11	-	-	-	-	-
B. cepacia	K25/2	-	+	-	-	-
B. cepacia	S5/2	-	+	-	-	-

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(6) 2021, pp. 646-660
DOI: http://dx.doi.org/10.21161/mjm.211212

B. stagnalis	S4/15	-	-	+	-	-
B. stagnalis	S4/16	-	-	+	-	-
B. stagnalis	S4/18	-	-	+	-	-
B. stagnalis	K23/3	++	++	++	++	++
B. diffusa	Bd1	-	+	-	-	-
Ralstonia solanacearum	Rs1	-	+	-	-	+
Ralstonia solanacearum	Rs2	-	+	-	-	+
Burkholderia spp.	Burk1	-	+	-	-	-
Burkholderia spp.	S6/2	-	-	-	-	-

++, strong inhibition (ZOI ≥ 2 mm); +, weak inhibition (ZOI < 2 mm); -, no inhibition.

Table S2: Presence of ZOI in flip streak assay.

Producer isolates		Target isolate				
		MSHR 5079	MSHR 5084	MSHR 5095	MSHR 5105	MSHR 6793
B. pyrrocinia	Bp5	-	-	-	-	-
B. thailandensis	Bt6	-	-	-	-	-
B. thailandensis	Bt7	-	-	-	-	-
B. thailandensis	Bt8	-	-	-	-	-
B. thailandensis	Bt9	-	-	-	-	-
B. ubonensis	Bu4	-	-	-	-	-
B. ubonensis	Bu5	-	-	-	-	-
B. ubonensis	Bu7	-	-	-	-	-
B. ubonensis	Bu9	-	-	-	-	-
B. ubonensis	Bu10	-	-	-	-	-
B. ubonensis	Bu11	-	-	-	-	-
B. ubonensis	K26/1	-	-	-	-	-
B. cepacia	Bc1	-	-	-	-	-
B. cepacia	Bc2	-	-	-	-	-
B. cepacia	Bc6	-	-	-	-	-
B. cepacia	Bc8	-	-	-	-	-
B. cepacia	Bc9	-	-	-	-	-
B. cepacia	Bc10	+	+	+	+	+
B. cepacia	K25/2	-	-	-	-	-
B. cepacia	S5/2	+	+	+	+	+
B. stagnalis	S4/15	-	-	-	-	-
B. stagnalis	S4/16	-	-	-	-	-
B. stagnalis	S4/18	-	-	-	-	-
B. stagnalis	K23/3	++	++	++	++	++
B. diffusa	Bd1	-	-	-	-	-
Ralstonia solanacearum	Rs1	-	-	-	-	-
Ralstonia solanacearum	Rs2	-	-	-	-	-
Burkholderia spp.	Burk1	-	-	-	-	-

++, strong inhibition (ZOI ≥ 2mm); +, weak inhibition (ZOI < 2mm); -, no inhibition.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538