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New Bacterial Species Isolated from Malaysian Sea Cucumbers with Optimized Secreted Antibacterial Activity

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Abstract: In this study, several Malaysian sea cucumber species that have traditional medicine value were selected and from them, the native bacterial population was isolated. Optimization of growth was designed and all bacterial secretions were tested for antibacterial properties. 30 bacterial types were isolated and 7 types recorded moderate antibacterial activity against *K. pneumoniae*, *S. marscens*, *P. aeruginosa* and *E. feacalis*. Antibacterial plate screening was done, with various testing parameters. Turbidometry revealed a single dose of the 10x concentrated crude antibacterial extracts were effective in preventing pathogenic growth for up to 4 hrs. PCR and subsequent sequencing of the 16S rDNA showed that the bacterial species were from the halopholic *Bacillus* and *Klebsiella* genera.

Key words: Sea cucumber bacteria, antibacterial peptide, antibacterial screening, turbidometry and spectrophotometry, 16S rDNA

INTRODUCTION

Sea cucumbers have long been used as a source of traditional medicines in Malaysia[1]. According to legend, the healing properties of the sea cucumber became evident when fishermen who hurt themselves applied the coelomic fluid from sea cucumbers on their wounds and discovered that the wounds healed faster[1]. The medicinal preparation is called "gamat" and there are several species of sea cucumber that are used, mainly *Holothuria atra*, *Stichopus hermanii* and *Stichopus horrens*[2]. Modern research has shown that besides wound healing, sea cucumber extracts have anticoagulant and antithrombosis compounds[3], cholesterol and lipid reducing compounds[4], anticancer and antitumor compounds[5-9] and antibacterial compounds[10-14]. Russian researchers showed that cucumarioside derived from the species *Cucumaria japonica* have potent immunomodulatory properties, exhibiting high efficacy against *E. coli*, *Proteus mirabilis*, *Neisseria meningitidis* BT-2, *Salmonella Minnesota*, *Pertussis meningoencephalitis* and *Salmonella typhimurium*[13,14].

To date, all isolated antibacterial agents belong to the triterpene glycoside structural configuration. Several subtypes have been determined, namely synallactosides (non-sulfated triterpene glycosides) from *Synallactes nozawai*[15], hemoiedemosides (trisulfated triterpene glycosides) from *Hemoiedema spectabilis*[16], liouvillosides (trisulfated triterpene glycosides) from *Staurocucumis liouvillei*[17], calcigerosides from *Pentamera calcigera*[18], eximisoside from *Psolus eximius*[19], koreoside from *Cucumaria koraiensis*[20] and DS-penaustrosides from *Pentacta australis*[21].

Some work was previously done on obtaining antibacterial agents from Malaysian sea cucumber species[22]. However, the researchers were unsuccessful in isolating an antibacterial agent.

MATERIALS AND METHODS

Raw materials: Several sea cucumbers were obtained from the coastal waters of Pangkor Island, Perak, Malaysia. The samples were kept alive and subsequently stored at 4°C after testing for further use. Approximately 20 liters of seawater was also taken from the collection site, to be used in media preparation.

Microbial isolation: The sea cucumbers were dissected and 6 sampling zones were chosen; coelomic fluid, stomach, intestines, inner body tissue, brown gastrointestinal tissue and outer skin layer. Spread plates were prepared using the supernatant resulting from vortexing the various samples with sterile distilled water. LB agar made with artificial seawater or LB-SW media (Merck, Darmstadt, Germany) was used in plating steps. Incubation was overnight at 37°C.

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Subculturing was carried out using LB-SW plates but a relatively poor yield was obtained. So, culturing media was changed to Brain Heart Agar with seawater or BHA-SW (Merck, Darmstadt, Germany). Single colonies were selected from the subcultured plates and transferred to universal bottles containing sterile Brain Heart Broth with seawater or BHB-SW (Merck, Darmstadt, Germany). The bottles were incubated in a rotary shaker running at 150 rev min^{-1} at 37°C.

Two-milliliter samples were taken at 24h intervals. The samples were placed in Eppendorf tubes and centrifuged at 13,000rpm for 10 min. The resulting supernatant was then used for subsequent antibacterial screening tests.

Antibacterial screening test: Overnight cultures of 10 selected human pathogenic bacteria were prepared, using LB broth and universal bottles. To prepare the test plates, LB agar plates were first prepared, with 25mL LBA $plate^{-1}$. 50µL of overnight culture was added to 5mL of an LBA-LBB mix (ratio 2:1) and the mixture was poured onto an LB plate. Eppendorf pipette tips (diameter mm) were used to punch holes into the agar according to a predetermined pattern. 50 µL of the bacterial supernatants was pipetted into each well. The plates were incubated overnight at 37°C.

Optimization: Based on initial test results, optimization was done to increase overall production and expression of antibacterial agents. 2mL 50% glucose was added to the BHB-SW + sea cucumber bacterial mix. The supernatants were heated at 90°C for 5 min to test for thermostability. Concentration was also done using a vacuum concentrator (Eppendorf, Hamburg, Germany). Initially, 8x concentrated samples were used, followed by 10x concentrated samples.

Turbidometry and spectrophotometry: Three bottles were prepared per bacterial supernatant to be tested. Each contained 10mL LBB and 5 µL of overnight culture. 2mL 10x concentrated supernatant was added to bottle 1, 2mL ampicillin (50 µg mL^{-1}) was added to bottle 2 and 2mL sterile distilled water was added to bottle 3. The bottles were incubated in a rotary shaker (150 rev min^{-1}, 37°C). Absorbance readings (600 nm wavelength) were taken at 60 min intervals for the 1st 3 hrs and at 30 min intervals for the subsequent 3 hrs. A growth curve was plotted with the obtained readings.

DNA isolation and PCR: Total DNA isolation was done on 7 bacterial strains whose supernatant secretions yielded the highest and most consistent antibacterial activity. A Qiagen DNA isolation kit (Qiagen, Hilden, Germany) was used. Primers specific to 16S rDNA were used in subsequent PCR steps (Farouk, unpulished). Electrophoresis using a 1% agarose gel was done to obtain the approximate size of the DNA fragments, using a 1kb DNA ladder marker as a comparison.

Data analysis: Measurements of the clear zone around the wells from the antibacterial screening test stage gave a rough estimate of the efficacy of the antibacterial agent. This in turn provided the selection criteria for selecting the best 7 bacterial strains for turbidometry and PCR work. Absorbance readings obtained from turbidometric analysis helped determine the efficacy of the antibacterial agents over time.

RESULTS AND DISCUSSION

A total of 30 bacterial strains were isolated from two *H. atra* juveniles. After subculturing, the bacteria were grown in LB broth (Merck Darmstadt, Germany) and Brain Heart Broth (Merck Darmstadt, Germany). All media contained seawater. Bacterial secretions from all 30 isolates were tested using the well diffusion screening method[23]. 7 strains were selected for further optimization steps, spectophotometric analysis and phylogenetic identification via PCR done on the 16s rDNA sequence. Selection criterion was based on average inhibition zone diameter after 7 days of testing.

The results show that the test samples record moderate activity against the selected human pathogens, as compared to ampicillin (positive control, 50µg mL^{-1} concentration). The high loading volume (50µL, 10x concentrated) of the test samples suggests that although antibacterial agents are present, further purification is necessary.

Well diffusion was chosen over disc diffusion for the antibacterial screening as a higher loading volume could be used (50µL as opposed to approximately 5 µL) and also because all test solutions were not hydrophobic, which would normally cause problems when using the well diffusion method[23].

Turbidometric analysis showed that the antibacterial agents were effective in the first 2.5 hrs of a single dose administration. At 5 hrs, test sample absorbance readings were lower than the negative control, suggesting that bacterial cell count at the lag phase was also lower.

Data analysis from both antibacterial screening and turbidometry showed that J2:7 and J2:10 were most effective against *S. typhimurium*. From Fig. 4, we see that even after 5 hrs, J2:10 had a low absorbance (0.144), whereas J2:7 recorded a lower reading (0.027) than ampcillin (0.040). When loading concentration was reduced from 2mL test solution per 10mL broth, to 1mL solution per 10mL broth, antibacterial activity was reduced, evident by the quicker increase in absorbance readings versus time. Lag phase absorbance was also higher compared to the higher loading concentration of the test solution.

Previous research showed that antibacterial agents isolated from the coelomic fluid of *Holothuria scabra* was very effective against Gram negative bacteria but not Gram positive species, especially against *K. pneumoniae* and *P. vulgaris*[24]. The samples J1:2 and J2:2 were quite effective against *P. vulgaris* and *K. pneumoniae* but faired better against *S. typhimurium* (Table 1-3) which is a Gram negative bacterium.

Table 1: Measurement of inhibition zones during antibacterial activity screening (29/06/06)

Sample name	Test pathogen and measurement of inhibition zone (+/- 0.5mm)										
	S. m	S. t	S. e	K. p	P. v	E. c	S. a	B. s	S. d	P. a	E. f
FASCJ1CFUIA 2	3.0	3.0	4.0	-	-	-	-	-	-	3.0	-
FASCJ2CFUIA2	-	-	4.5	-	-	-	4.5	3.5	-	3.0	-
FASCJ2BUIA3	-	-	4.0	-	-	-	5.0	3.0	-	3.5	-
FASCJ2BTUIA6	-	-	4.5	-	-	-	3.5	-	-	-	-
FASCJ2BTUIA7	-	-	5.5	-	-	-	3.5	-	-	-	-
FASCJ2IUIA10	-	-	7.0	-	-	-	5.0	-	-	3.5	-
FASCJ1I^{80}UIA12	4.5	-	-	-	-	-	3.5	-	-	5.5	-

Table 2: Measurement of inhibition zones during antibacterial activity screening (30/06/06)

Sample name	Test pathogen and measurement of inhibition zone (+/- 0.5mm)										
	S. m	S. t	S. e	K. p	P. v	E. c	S. a	B. s	S. d	P. a	E. f
FASCJ1CFUIA 2	-	-	-	8.0	-	3.5	5.5	6.5	-	-	8.5
FASCJ2CFUIA2	3.0	-	-	-	-	-	5.5	3.5	7.5	5.5	8.0
FASCJ2BUIA3	3.5	-	-	-	-	-	3.5	4.5	5.0	4.5	6.5
FASCJ2BTUIA6	3.0	-	-	-	-	-	4.5	-	3.5	5.5	3.5
FASCJ2BTUIA7	3.0	-	-	-	-	-	3.5	-	3.5	4.0	3.0
FASCJ2IUIA10	3.5	-	-	-	-	-	3.0	-	4.5	-	4.5
FASCJ1I^{80}UIA12	-	-	-	8.0	-	7.5	6.5	7.5	-	-	9.5

Table 3: Measurement of inhibition zones during antibacterial activity screening (03/07/06)

Sample name	Test pathogen and measurement of inhibition zone (+/- 0.5mm)										
	S. m	S. t	S. e	K. p	P. v	E. c	S. a	B. s	S. d	P. a	E. f
FASCJ1CFUIA 2	3.0	-	-	-	4.5	-	5.5	3.5	7.5	5.5	8.0
FASCJ2CFUIA2	-	-	-	3.5	-	-	3.0	3.0	-	4.5	-
FASCJ2BUIA3	3.0	-	-	3.5	-	-	-	4.5	-	4.5	-
FASCJ2BTUIA6	-	3.5	-	4.0	-	-	-	3.5	-	4.5	-
FASCJ2BTUIA7	3.5	4.5	-	3.5	-	-	-	6.5	-	4.0	-
FASCJ2IUIA10	-	-	-	4.5	-	-	3.5	3.5	-	4.5	-
FASCJ1I^{80}UIA12	3.0	-	-	5.0	5.5	-	4.5	3.0	-	-	4.5

Key:

S. m = *S. marscens* ATCC 274
S. t = *S. typhimurium* ATCC 13311
S. e = *S. epidermitis* ATCC 14990
K. p= *K. penumoniae* ATCC 12658
P. v = *P. vulgaris* ATCC 6380
E. c = *E. coli* ATCC 10536
S. a = *S. aureus* ATCC 6538
B. s = *B. subtilis* ATCC 10783
S. d = *S. dysenteriae* ATCC 11835
P. a= *P. aeruginosa* ATCC 10197
E. f= *E. feacalis* ATCC 19433

Although both these samples were obtained from the coelomic fluid of *H. atra* Jaeger, it was not the fluid itself that had the antibacterial agent but rather the bacteria in it. This was confirmed by filtering some coelomic fluid using a 45 micron syringe filter (Sartorius, Goetinggen, Germany) and then comparing its efficacy against the bacterial secretions J1:2 and J2:2. The coelomic fluid showed no activity, which proves the antibacterial activity from J1:2 and J2:2 are from bacterial cell secretions and not from the sea cucumber itself.

Cucumarioside, from *C. frondosa* was effective against both Gram positive and negative bacteria[3,14]. Other isolates also had varied efficacy[15-21]. Comparatively, the test samples in this research were more effective against the Gram negative test pathogens (*S. typhimurium* and *K. pneumoniae*). The assumption here is the test samples may have a terpenoid structure as well, which would explain nominal efficacy against Gram positive bacteria. Most of the triterpene antibacterial agents have large polysaccharide groups and also some sulfates[15-21], thus hindering transport across the thicker cell membranes of Gram positive bacteria. High efficacy against Gram negative bacteria could be due to the effectiveness of the terpenoids against the lipopolysaccharide layer of the Gram negative pathogens[25].

Turbidometric analysis is a relatively good way to determine bacterial growth versus time and also to decipher the MIC of the antibacterial agents. In this experiment, the antibacterial agent was introduced at t = 0 and spectophotometric readings were taken at preset time intervals. This type of procedure shows antibacterial efficacy versus time, based on antibacterial agent concentration. Another method used was to introduce the antibacterial agent after the broth was inoculated with one of the human pathogens and incubated for 5 hrs. Incubation time was based on initial turbidometric analysis, whereby the lag phase of the negative control was noted at approximately 4 hrs. The second method yielded no significant drop in initial absorbance readings, showing that although the antibacterial agents are bactericidal, they are not bacteriolytic.

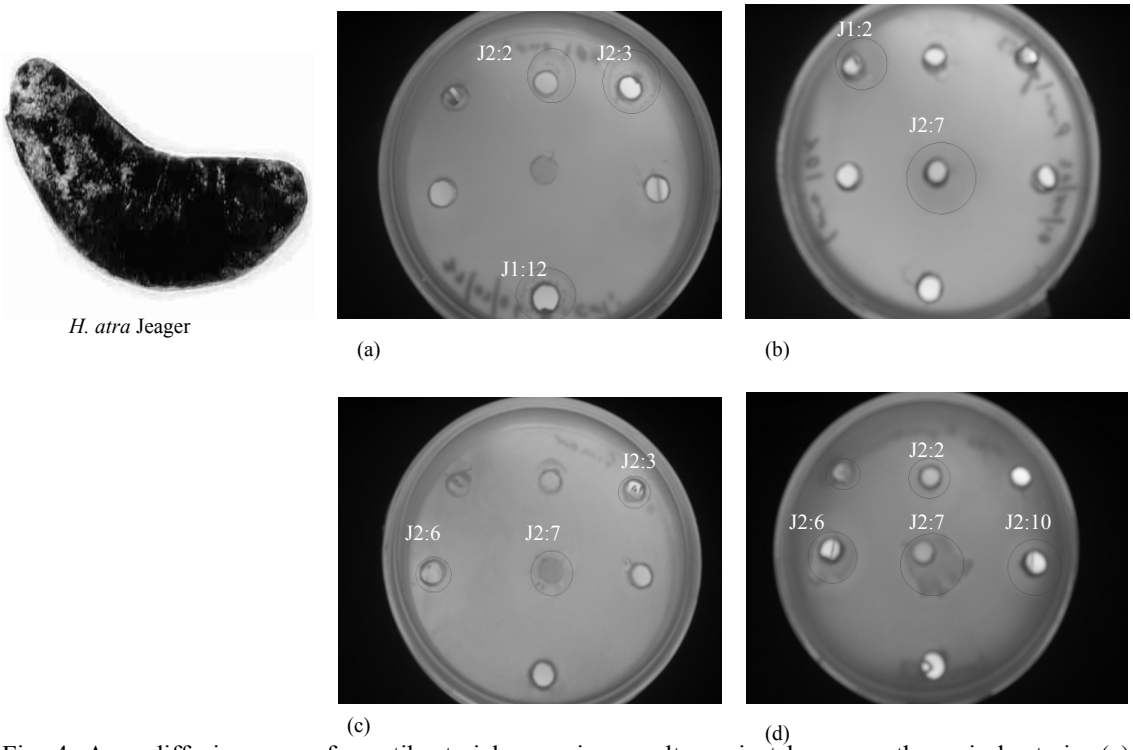

Fig. 4: Agar diffusion assay for antibacterial-screening results against human pathogenic bacteria; (a) against *P. vulgaris*, (b) against *E. coli*, (c) against *S. typhimurium*, (d) against *K. pneumoniae*

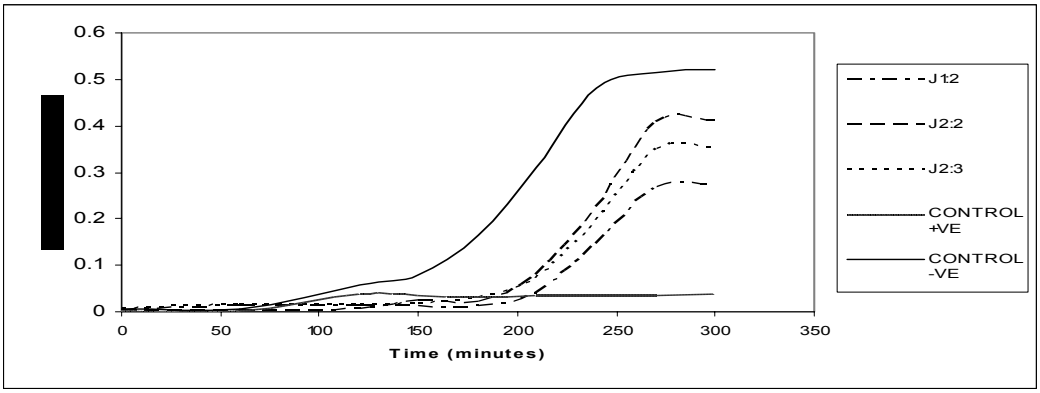

Fig. 5: Turbidometric analysis of J1:2, J2:2 and J2:3 secretion for antibacterial activity against *S. typhimurium*

16S rRNA phylogenecity testing showed that the bacteria were halophilic *Klebsiella* and *Bacillus* species. The strains were strictly halophillic, which was confirmed by using 2 versions of BHA; 1 with sea water and 1 without. No growth was recorded when non-salt media were used. The bacteria also required high nutritional content (10% glucose w/v) before antibacterial agent production was obtained. The postulation here is a synergistic relationship exists between the sea cucumber and the bacteria, whereby in return for nutrition, protective antibacterial agents are produced for the sea cucumber. Care was taken to exclude all possible bacteria that were not part of the sea cucumber i.e. bacteria from the seawater. This ensures accuracy in testing and also validates phylogenecity testing in that only bacteria indigenous to the sea cucumber are of interest and are thus tested.

The antibacterial peptides are also thermostable. This was established when a duplicate set of the crude extracts were heated (90^{o}C, 5 min) before antibacterial screening was carried out. Concentration of samples gave a better antibacterial resistance, suggesting that

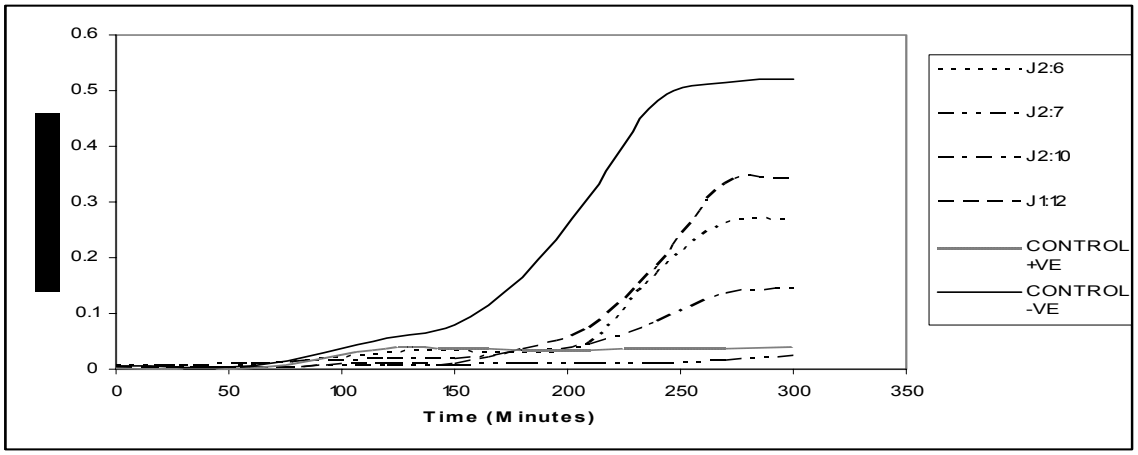

Fig. 6: Turbidometric analysis of J2:6, J2:7, J2:10 and J1:12 secretion for antibacterial activity against *S. typhimurium*

although antibacterial peptides exist in the crude extracts, they are at a low concentration. There was no significant change in antibacterial efficacy when both heating and concentration were carried out, as compared to just using the concentrated samples. This is possibly due to the presence of polysaccharides and sulfated groups, if the antibacterial agents are terpenoid in nature, or a stable quaternary structure, if the antibacterial agent is a polypeptide. Structural analysis is yet to be done.

CONCLUSION

Several strains of bacteria were isolated from various parts of the sea cucumber, *H. atra*. From this, bacterial secretions and supernatants from growth in broth were tested for antibacterial activity. Out of the 30 isolated strains, 7 showed moderate to high antibacterial activity. Optimized media was used to increase antibacterial peptide production. Turbidometry and antibacterial screening showed that the extracts were most effective against *S. typhimurium*, *K. pneumoniae*, *E. coli* and *P. vulgaris*. The extracts from 2 strains, J2:7 and J2:10 gave the highest antibacterial activity. Cloning and expression will be done using these 2 strains.

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