The Malaysian Journal of ANALYTICAL SCIENCES

BACTERIOCIN ISOLATED FROM *HALOMONAS* SP.: A BACTERIAL DING PROTEIN?

(Pemencilan Bakteriosin dari *Halomonas* sp. : Sejenis Bakteria DING protin?)

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Received: 23 November 2014; Accepted: 27 June 2015

Abstract

A marine halophile, *Halomonas* sp. strain M3 was isolated from Straits of Johor, Malaysia and produce bacteriocin CC that acts as bacteriostatic agent. Characterisation of the bacterium showed that optimal growth and bacteriocin production is at ambient temperature, pH of 8-8.5 in nutrient broth medium supplemented with 2.9% w/v NaCI to mimic saltwater conditions. The stability studies indicated that bacteriocin CC is heat-labile (35°C-50°C) and was stable over 2 years when stored in 0.02M Tris-HCI with 30-60% glycerol at 4°C. A loss of activity was detected after proteolytic enzymes treatment, indicating the proteinaceous nature of the antimicrobial compound. The amino acid sequence of bacteriocin CC was obtained by Edman degradation and MALDI-TOF analysis, showed the characteristic sequence of a DING protein (D-I-N-G-G-G-A-T-L-P-Q-A-L-Y-Q) in size 38.9-kDa at pI of 6.8. These proteins constitute a conserved and widely distributed set of proteins found in all kingdoms with ligand-binding activities and hydrolytic enzyme, suggesting a possible role in cell signalling and bio mineralization in DING isolates. Intriguingly, DING proteins also have been involved as an anti-tumour agent in humans. Thus, bacteriocin CC as DING protein family members should be further studied to investigate its potential as a novel antimicrobial agent.

Keywords: antimicrobial activity, bacteriocin, DING protein, *Halomonas* sp. Strain M3

Abstrak

Sejenis halofil marin *Halomonas* sp. strain M3 telah dipencilkan daripada Selat Johor, Malaysia dan menghasilkan bakteriosin CC yang bertindak sebagai ejen bakteriostatik. Pencirian bakteria ini menunjukkan bahawa pertumbuhan yang optima pada suhu bilik, pH 8-8.5 dalam nutrien media yang ditambah dengan 2.9% w/v NaCI untuk meniru keadaan air masin. Kajian kestabilan menunjukkan bahawa bakteriosin CC adalah tahan haba (35°C-50 °C) dan stabil sepanjang 2 tahun apabila disimpan dalam 0.02M Tris-HCI bersama 30-60% gliserol pada 4°C. Kehilangan aktiviti telah dikesan selepas rawatan enzim proteolitik, menunjukkan sifat protin dalam komposisi antimikrob. Urutan asid amino bakteriosin CC telah diperolehi dengan menggunakan degradasi Edman dan analisis MALDI-TOF, menunjukkan ciri protin DING (D-I-N-G-G-G-A-T-L-P-Q-A-L-Y-Q) dalam saiz 38.9-kDa pada pI 6.8. Protin ini membentuk satu set yang terpelihara dan edaran protin ini didapati secara meluas dalam semua hidupan dengan aktiviti pengikatan-ligan dan enzim hidrolitik, mencadangkan peranan yang mungkin ada dalam pengisyaratan sel dan bio mineral dalam pemencilan DING yang lain. Menariknya, DING protin juga terlibat sebagai agen anti-tumor pada manusia. Oleh itu, bakteriosin CC sebagai ahli keluarga protin DING, perlu dikaji lagi untuk menyiasat sekiranya ia berpotensi sebagai ejen antimikrob yang baru.

Kata kunci: aktiviti antimikrob, bakteriosin, DING protin, *Halomonas* sp. Strain M3

Introduction

Since last decades, there are numerous novel compounds have been found from marine organisms with valuable nutraceutical and pharmaceutical potentials [1]. Bacteria like halophilic microorganisms are generally recognized as primary colonizers of this habitat and are able to rapidly secrete bioactive compounds over freshly exposed surface of organisms [2, 3] which thrive in NaCI saturated in marine environments [4]. Halophilic microorganisms can be classified into two groups, the extremely halophilic, survive in high concentration of salt (below 2.5M) and the moderately halophilic which grows in wide salinity: 0.5-2.5M [5]. Moreover, many novel species of halophilic bacteria have been described within the family *Halomonadaceae* during the past few years especially within genus *Halomonas* [6] which have exhibit biological activity, however the exactly activity remains unclear [7-13].

Bacteriocins defined as proteinaceous antimicrobials from the domain bacteria are coincidently found to be in *Halomonas* sp. strain M3. It constitutes one of the most potent weapons to fight against pathogen infections which mostly discovered in mucous membranes and surfaces in marine organisms [14, 15]. Haloarchaea were the first members of *Archaea* found to produce bacteriocins and recent report describes sulfolobicins from archaebacterium; *Sulfolobus* sp. has a characteristic DING N-terminal sequence [16, 17] and in overview, DING proteins are ubiquitous in living organisms; identified in animals, plants, fungi and several prokaryotes [18-20]. Therefore, this study could be the first reported isolation of a DING protein of bacteriocin isolated from genus *Halomonas*, indicating that DING proteins have now been found in all biological kingdoms. To further assess bacteriocin diversity and determine their biotechnological potential, many other bacteriocins need to be studied. Here, we report optimisation for maximum production of bacteriocin from genus *Halomonas* and their finding of proteins of bacteriocin were characterised as well as will provide wide opportunities as potential antibiotic replacement candidate for treatments in environmental and pharmaceutical activities.

Materials and Methods

Bacteria Strains and Culture Conditions

The marine bacterium; *Halomonas* sp. strain M3 isolated from *Lutjanus erythropterus*, collected in Gelang Patah, Straits of Johor, Malaysia were routinely grown and maintained in marine nutrient broth at 25-30°C. Bacteria strains from human pathogens were maintained in nutrient broth i.e. *Bacillus cereus, Streptococcus faecalis, Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Serratia marcescens, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas diminuta* and *Enterobacter aerogenes* were purchased from Institute for Medical Research (IMR), Kuala Lumpur also fish pathogens were grown in tryptic soy broth i.e. *Streptococcus agalactiae, Stapylococcus scuiri, Micrococcus* sp., *Aeromonas hydrophila, Enterobacter cloacae, Pseudomonas putida, Pseudomonas aeruginosa, Plesiomonas shigelloides* and *Vibrio alginolyticus* were gifted by National Fish Health Research Centre (NAFISH), Penang.

Bacteriocin CC Production

The overnight cultures of *Halomonas* sp. strain M3 at ambient temperature were used for purification via seawater extraction method which was previously described [21] with slightly modified whereby the pellet was collected and supernatant was decanted due to active compounds of antimicrobial activity was found from bacterial cells [22]. After that, the bacterial pellet was extracted by 1.0 ml seawater and 0.1M NaCI in ratio 1:3 and followed by centrifugations at 15,000 rpm for 30 minutes prior to dialyze against 0.1M NaCI using dialysis tubing for 24 hours incubation. Aftermath, the bacteriocin CC was passed through gel filtration; Sephadex G100 (Sigma,USA) and followed by subjected to strongly ion exchange column; Sepharose CL-4B (GE-Healthcare,UK) prior to pooled onto hydrophobic interaction chromatography; Methyl HIC support (Bio-Rad,CA) described [23]. The protein concentration was estimated by Bradford method using bovine serum albumin (Bio-Rad, CA) as standard.

Stability of Salinity, pH, Temperature, Buffer and Stabilizer

Solutions of 0.636 mg of purified bacteriocin per ml in vary salts (NaCI) solution were adjusted to a range of pH values from 6.0 to 8.5 and incubated at ambient temperature for 2 hours. The stability of the bacteriocin CC to heat or freeze was determined by exposing the cell free supernatant to 5-100°C for 10 minutes before exposure to bacteria strains by disk diffusion assay for bacteriocin activity. The seawater, NaCI and Tris-HCI were chosen as buffer for preserver of bacteriocin activity and glycerol as stabilizer solvent was used as mentioned by [24] which is adjusted in range from 10 to 100% and its antimicrobial activity were examined.

Disk Diffusion Assay with Pre-diffusion Step

This method was carried out by sterile disks Whatman filter papers (GE-Healthcare,UK) were placed aseptically over the bacterial culture on nutrient agar plates and incubated at ambient temperature for 24 hours with pre-diffusion step [22]. The unit of measurement of activity is given in mm after the following expression and noted that standard disk diameter is 10 mm.

Proteolytic Digestion and Protein Subunit Structure

To determine whether proteins were responsible for the antimicrobial activity, the protein with enriched bacteriocin was mixed with trypsin (Calbiochem,USA) in ratio 5:1 and incubated at 37°C by 400 rpm agitation within 4 to 6 hours. Subsequently, the treatments were carried out upon samples of purified antimicrobial protein to determine whether it contains subunits. The effects of treatments with β-mercaptoethanol and heat upon the subunit structure of the protein were visualized by SDS-PAGE. The purified protein was dissolved in SDS-PAGE treatment buffer (1.0M Tris-HCl; pH 6.8, 10% SDS, 50% glycerol, 1% bromophenol blue) and divided into four samples. The first sample was exposed to 5% β-mercaptoethanol, the second was heated in a boiling water bath for 95°C in 5 minutes, and the third was exposed to both treatments. The fourth sample was left untreated as a control. The antimicrobial protein was detected within these samples by 12.5% of SDS-PAGE, stained with Coomassie brilliant blue; CBB G-250 (Bio-Rad,CA).

2-DE and gel documentation

About 5.88 mg/ml of protein was loaded onto the pH 4-7, 7cm IPG strips (Bio-Rad,CA), followed by process of passive rehydration for 16 hours. First dimension IEF (Isoelectric focusing) was performed on the IEF protean cell (Bio-Rad,CA). Strips were equilibrated with 6.0M urea, 2% SDS, 0.375M Tris-HCI (pH 8.8), 20% glycerol, 130mM DTT and distilled water for 10 minutes followed by a second equilibration with 6.0M urea, 2% SDS, 0.375M Tris-HCI (pH 8.8), 20% glycerol, 135mM Iodoacetamide and distilled water for 10 minutes prior to 2-DE. Gels loaded with proteins were stained by CBB G-250 and gel documentation was carried out as mentioned [25].

Determination of Mode of Action by MIC and Scanning Electron Microscope (SEM)

The mode of action of bacteriocin either cytocidal or cytostatic was determined and *Enterobacter aerogenes* and *Pseudomonas putida* were chosen as representatives of human and fish sources respectively. Both bacteria treat with proteinaecous were grown on dialysis strip which is placed on agar prior to incubation at ambient temperature for overnight. The fixation of bacteria with 5% glutaraldehyde in 0.1M sodium cacodylate was incubated for another 24 hours. Aftermath, bacteria treated were washed out by graded ethanol concentration; 20 to 80% in 15 minutes and followed 100% within 30 minutes and leaves at room temperature for several minutes prior to observed under SEM microscope. The Minimal Inhibitory Concentration (MIC) of the antimicrobial compounds was determined by a micro broth dilution assay in 96-well microplate whereas the plate was covered by lid and mixed at room temperature for 2 hours prior to pre-diffusion step and placed at room temperature on next day for 24 hours [26]. Then, data was read by Benchmark Microplate Reader (Bio-Rad,CA) at 570 nm. The bacterial growth and MIC of proteinaceous antimicrobials that reduces at MIC_{50} (required to inhibit the growth of 50% the strain tested) or MIC_{90} (required to inhibit the growth of 90% the strain tested) were calculated as described by [23].

Protein Sequencing by Edman Degradation and Mass Spectrometry

The N-terminal amino acid sequence of antimicrobial protein was determined by Automated Edman Degradation which using a 494 Procise Protein Sequencing System instrument (Applied Biosystems,USA). The PVDF membrane was rinsed three times, alternating water and 50% methanol/water. Blot was excised into smaller pieces, loaded onto sequencer cartridge and was run under standard conditions as per manufacturer instructions. Pulsed Liquid PVDF was used as sequencing method. Meantime, the solubilised peptides were trypsin digested and extracted according to standards protocol [27]. Peptides were used for MALDI-TOF MS analysis, using a 4800 Proteomics Analyzer instrument (Applied Biosystems,USA). Spectra were analysed to identify protein of interest using Mascot sequence matching software (Matrix Science): http://www.matrixscience.com with Ludwig NR Database and taxonomy set to Bacteria.

Results and Discussion

Bacteriocin Purification and Stability of Activity

Apparently, the antimicrobial activity had eliminated by distilled water instead of seawater (supplementary appendix) during extraction bacteriocin using modified method as reported [21] whereby active crude pellets inhibit most species of gram negative and three of gram positive bacteria after modified, rather than only two species of gram negative bacteria from human and fish pathogens. The antibacterial protein was purified from concentrated bacterial pellet CC by gel filtration, ion-exchange and hydrophobic interaction chromatography as described in Methods. Table 1 summarizes the purification. About 2.09 g of purified protein was obtained from 100 ml of concentrated pellet. The specific activity of the purified material was increased about 16-fold over that of the crude extract, and on SDS-PAGE, it gave a single band of 40-kDa.

Table 1. Yield of the purification of bacteriocin activity from *Halomonas* sp.

Step	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	512	42.16	12.14	1	100
Gel filtration	500	6.325	79.1	6.5	97.7
Ion exchange	256	2.167	118.1	9.7	50
Hydrophobic Interaction	128	0.636	201.3	16.6	25

For the purposes of this purification, one unit of antibacterial activity was defined as the minimal amount necessary to produce inhibit in log-phase of bacteria strains growing in broth medium by the micro broth dilution assay. The stability of the *Halomonas* sp. strain M3 antibacterial protein as well as its resistance to changes in salinity, pH, temperature, buffer and stabilizer for storage as well as volume extractor was assessed. The bacteriocin CC production maximized when incubated at 35-50°C (data not shown) at pH of 8.0 upon supplementation with 2.9% w/v NaCI (0.49M), 0.02M Tris-HCI and 30-60% glycerol after extract using volume 1.0 ml during purification. In purified form, the protein could be stored in solution at 4°C for at least 2 years without loss of activity, whereas the unfractionated crude extract lost activity within 4 months when stored under the same conditions, probably because of the presence of proteolytic enzymes. In addition, the greater size of inhibition zones was detected after 2 hours of exposure to pH values within the range of 6.0 to 8.5 and Tris-HCI used as buffer for maintaining the bacteriocin activity. These data (supplementary appendix) indicate that the protein is sensitive to denaturation. It could be noted that the bacteriocin activity increased with an increase of glycerol up to 60% and subsequently decreased with further rise in glycerol.

Evaluation of Mode of Action

The minimum concentration of antibacterial protein necessary to inhibit indicator strains in suspension was calculated as $6.5x10^{-2}$ – 5.37µg/ml at MIC_{50} and 7.0 - 48 µg/ml at MIC_{90} values. *S. faecalis, P.aeruginosa, S. marcescens* from human and all of fish pathogens were not recoverable at MIC_{90}. In order to compare the concentration of protein necessary to cause suppression of cells with that necessary for growth inhibition, two strains previously shown to be susceptible to the bacteriostatic effect of the protein were selected and viewed under SEM microscopy. A comparison between the protein concentration necessary to cause cell death and that necessary to cause growth inhibition is shown in supplementary appendix.

Identification of Antimicrobial Protein Band

The antimicrobial proteins were rehydrated in IPG strips pH 4-7 by 2-D gels and spot of antimicrobial proteins were depicted in Figure 1(a) shown at about pI of 6.8 which concluded that these proteins are negative charges and it would bind to anion exchange column and that is why increasing of yield bacteriocin upon ion exchange chromatography. Samples of purified protein which had undergone treatment with 5% β-mercaptoethanol, heat

only, or heat and β-mercaptoethanol and the untreated control were fractionated by SDS-PAGE to observe whether any of the treatments would split the protein into subunits. Band protein which treated with 5% β-mercaptoethanol only and untreated control sample were disappeared.

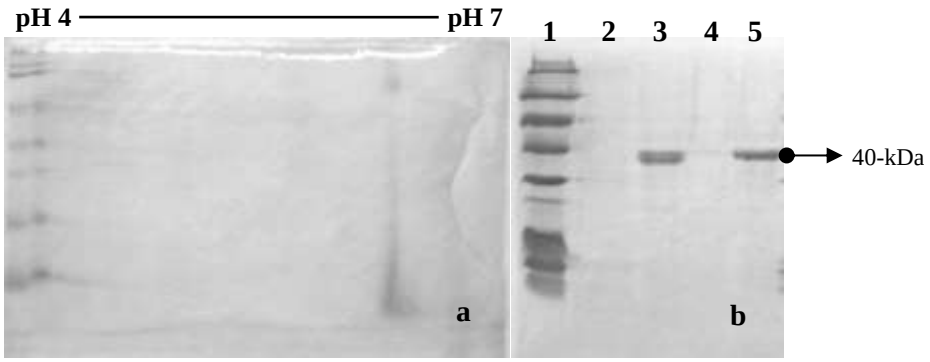

Figure 1. **(a)** the antimicrobial proteins showed negative charge and estimated at pI of 6.8, in 2-D PAGE gel and **(b)** display purified antimicrobial protein after treatments aimed at no breaking intersubunit bonds. Lane 1, molecular weight standard. Lane 2, no treatment (control). Lane 3, heat only. Lane 4, 5% β-mercaptoethanol only. Lane 5, 10% β-mercaptoethanol and heat.

This is indicating that disulphide linkages between proteins were not reduced, since β-mercaptoethanol is a reducing agent which functioned to break down and eliminate any S-S linkages between proteins. Upon treatment with β-mercaptoethanol and heat, or heat only, the protein ran as a single band of approximately 40-kDa in size compared with molecular weight standards. This would suggest that the antibacterial protein does not contain subunits joined together by covalent bonds alone in Figure 1(b). Clearly, this protein has single subunits structure in isolation; however, more information would be necessary to determine the exact size and structure of the subunits which make up the antimicrobial protein. The antimicrobial activity was abolished by trypsin digestion, demonstrating that the bacteriocin activity is entirely due to proteinaceous factors (data not shown).

N-terminal Sequence and MALDI-TOF Analysis

A sample of purified protein was prepared for N-terminal sequencing as follows and band proteins were blotted onto PVDF membrane as described in Materials and Methods and sequenced for the first 15 amino acids in the N-terminal region. As results, amino acid sequence of this region was as follows: **M**(Met)- **D**(Asp)- **I**(Ile)- **N**(Asn)- **G**(Gly)- **G**(Gly)- **G**(Gly)- **A**(Ala)- **T**(Thr)- **L**(Leu)- **P**(Pro)- **Q**(Gln)- **A**(Ala)- **L**(Leu)- **Y**(Tyr)- **Q**(Gln)-. This N-terminal sequence was compared with previously identified protein sequences present within the Swiss Prot database, Protein Databank (PDB), NCBI protein reference sequences and no proteins were found to have similar sequences in either their N-terminal regions or any other region (supplementary appendix). In addition, we also had attempted to find the antimicrobial peptides in Anti-microbial Peptides Database and Analysis System (APD) which can be accessed at (http://aps.unmc.edu/AP/main.php).

Unfortunately, they (APD) also displayed no close homology to any protein sequences found in APD database. Masses produced from MALDI-TOF were submitted for protein blast and identification using MASCOT database search mentioned in Methods. As consequence, the sequences matched in protein hits; DOVWY2 described D-I-N-G- from *Pseudomonas fluorescence* with unknown species in taxonomy identified. According to data in appendix, the theoretical mass and pI of bacteriocin CC were calculated to be 38.7-kDa and 6.8 respectively, which was in small different agreement with the mass of 38.9-kDa and pI of 7.71 determined by MALDI-TOF. Thus, we considered that actual pI value of bacteriocin CC is based on MALDI – TOF's results since it showed precise and accurate result.

Bacterial DING Proteins

DING proteins, named according to their four conserved N-terminal amino acid residues, encompass a recently discovered protein family. DING proteins are highly conserved in animal and plant isolates, but more variable in a range of microbial proteins (supplementary appendix). Similarly, the result obtained from both method i.e. N-terminal Edman degradation and MALDI-TOF revealed that sequence coverage of bacteriocin CC from *Halomonas* sp. strain M3 is 54% from MALDI-TOF and 100% from N-terminal protein sequencing with the total score of coverage is 49.4 only.

Table 2. The summary of phosphate-binding protein superfamily [19].

Class	% identity with eukaryotic DING proteins	Bacterial strain	Database Reference	Origin
PstS	25-30%	*P.fluorescens* SBW25	**GL020393**	2
Soluble component of ABC transporter in *pst* operon		*P.aeruginosa* PA01	**PA5369**	3
		P.putida KT2440	**PP2656**	3
AP	40-50%	*P. aeruginosa* Pa01/1 – Lap A[1]	**PA0688**	3
Associated with type II secretion systems; in *hxc* operon		*P. aeruginosa* Pa01/2 – Lap B[1]	**PA0689**	3
		P. fluorescens Pf5/2	**CP000076.1** **Protein AAY92033.1**	4
		P. fluorescens Pf01	**CP000094.2** **Protein ABA74910.1**	4
DING	70-75%	*P.fluorescens* SBW25	**GL019191**	2
DING N-terminus; in *hxc* operon		*P. fluorescens* Pf5/1	**CP000076.1** **Protein AAY92032.1**	4
		P.aeruginosa PA14	**RefSeq ZP_00138283.2**	5

[1]**Lap**: **L**ow MW **A**lkaline **P**hosphatase, [2]PseudoDB database reference number in uncompleted genome, [3]PseudoDB database ORF number in completed genome, [4]Gen Bank reference number, [5]The PA14 genome is being sequenced by the ParaBioSys consortium (Harvard, MA)

Indeed, both results are adequate to prove that bacteriocin have authentical of DING proteins. However despite much effort, no complete gene sequences are known for these proteins and only a single complete amino acid sequence i.e. *Pseudomonas* genus [28-30] and most of these proteins have molecular weights of 38 to 40-kDa and thus, this is corresponding to *Halomonas* sp. strain M3 which is 38.9-kDa. Previous study discovered that DING protein is a novel member in a prokaryotic phosphate-binding protein superfamily which extends into the eukaryotic kingdom as well as PstS and AP proteins (Figure 1 and Table 2). [19]. The PstS proteins are periplasmic in gram-negative organisms, have 20–25% identical in the amino acid sequence with fragments of eukaryotic DING proteins, and as a phosphate-binding protein (PBP) for bacterial ABC cassette phosphate-uptake systems, which are ubiquitous in bacteria but do not have the DINGGG- N-terminal sequence.

A second family is much less well-characterised, but comprises alkaline phosphatases, which are 40–50% identical with eukaryotic DING proteins. These ‘‘AP family’’ genes are not found in pst operons, but are also induced by low phosphate concentrations, and the resulting proteins are secreted into the bacterial environment [31, 32]. The bacterial DING proteins as a third family are identical in 70–80% with eukaryotic DING proteins and the gene for one such protein, PfluDING from *P. fluorescens* SBW25, has been cloned and expressed in *Escherichia coli*. It binds a single phosphate ion, but has no detectable phosphatase activity [29, 30].

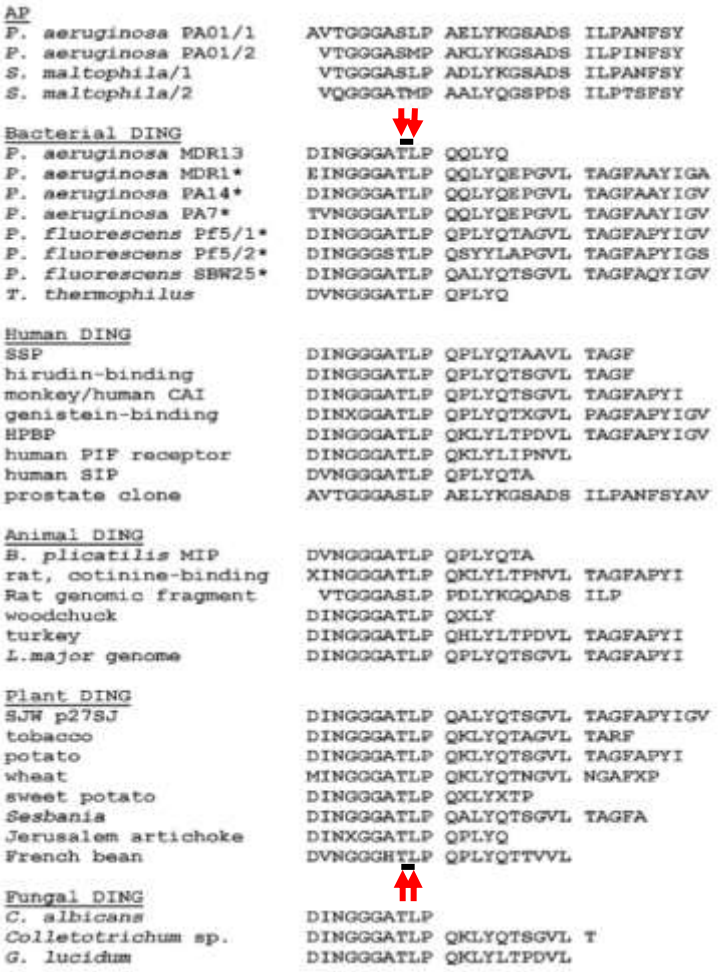

Figure 2. DING N-terminal sequences. The sequences were aligned in taxonomic groups, approximately as ordered in the text. Asterisks (*) indicate the database accession numbers [33]. The vicinity of residues; T L conserved in phosphate binding sites, indicated by red arrow and these residues also possessed in DING protein of *Halomonas* sp. strain M3 (DINGGGA**TL**PQALYQ).

Many DING proteins have been isolated in eukaryotes and are associated with both normal and pathological function in mammals and supposed to be having phosphate-binding protein. Most of DING proteins with enzyme activity have demonstrated a hydrolytic activity such as phosphatase, phosphodiesterase and nucleotidase activity as being discovered in bacterial DING isolates [18], also their phosphate-binding function suggests a role in bio mineralization and coincidently, it is similarly with *Halomonas* sp. strain M3 which had bio mineralization compounds and it related to carbonate precipitation process [22].

In addition, it was mentioned that DING derived peptides had potent to against glioblastomas and possibly other tumour types [34]. Moreover, a moderate halophilic alkaline phosphatase from *Halomonas* sp. 593 had acidic protein which size about 40-kDa, indicated their protein had a high content of acidic amino acid residues accompanied by an increase of negative charge on the protein surface and showed alkaline phosphatase activity [4], however they does not possessed the DINGGG- N-terminal sequence [5]. Meanwhile, phosphate is an essential but often limiting nutrient and multiple Pi transport systems have evolved in bacteria to facilitate efficient uptake of Pi

from the environment which under conditions of Pi limitation, a high-affinity transport system (Pst) is induced and operates for Pi scavenging. Therefore, they inhibit the pathogens that had treaded on surface of host.

When the amount of phosphate (Pi) in their cell is lacking then, they had sequester the Pi from environment include pathogens who adhere on host for bacterial competitive growth which live in phosphate limiting medium for survival [33, 35]. This circumstance (suppress and inhibit the cell) was coincide to mode of action in DING protein of *Halomonas* sp. strain M3 which bacteriocin CC act as suppress the growth of pathogens and then it multiplied the foreign cells.

Conclusion

The plausible relevance in antimicrobial proteins purified from *Halomonas* sp. strain M3 in the present study is supported by their high potency. With minimal inhibitory concentrations in the submicromolar range these proteins might well act alone or in synergism with other antibacterial factors to protect marine organism against exploitation by several pathogens in marine environment. Although we cannot yet demonstrate an experimental link between cytostatic effect and the phosphate-binding site of the *Halomonas* sp. strain M3 DING protein, one obvious speculation is that the DING protein acts as a carrier for an organic phosphate metabolite with mitogenic activity [36, 37].

This hypothesis could be extended to suggest that the activities of bioactive phytochemicals could depend upon their effects on the binding of this endogenous DING ligand as well as the investigation of DING- associated disease states should consider possible bacterial involvement. This first report of isolation bacterial DING of halophilic microorganism in genus *Halomonas* would give advancement to other species in studying genetic engineering of bacteriocins and its possible development of drugs with this antimicrobial peptide.

Acknowledgement

We thank our staff and members at the Universiti Teknologi Malaysia for their support. This research was supported by an FRGS grant from the Ministry of Higher Education Vot 78317 with cooperation and collaboration from Mr. Azmi Rani, Brackishwater Aquaculture Research Centre, Department of Fisheries, 81550 Gelang Patah, Johor Bahru, Johor Darul Ta'zim as well as to Dr. Siti Zahrah Abdullah and Dr. Kua Beng Chu from National Fish Health Research Centre (NaFISH), Fisheries Research Institute, 11960 Batu Maung, Pulau Pinang, Malaysia for supply the indicator of strains isolated from fishes origin.

References

1. Ngo, D-H., Vo, T-S., Ngo, D-N., Wijesekara, I. and Kim, S-K. (2012). Biological activities and potential health benefits of bioactive peptides derived from marine organisms. *Biological Macromolecules*, 51: 378-383.
2. Faulkner, D.J. (2000). Marine natural products. *Natural Product Reports,* 17: 7-55.
3. Chen, L., Wang, G., Bu, T., Zhang, Y., Wang, Y., Liu, M. and Lin, X. (2010). Phylogenetic analysis and screening of antimicrobial and cytotoxic activities of moderately halophilic bacteria isolated from the Weihai Solar Saltern (China). *World Microbiology Biotechnology,* 26: 879-888.
4. Pašić, L., Velikonja, B.. H. and Ulrih, N. P. (2008). Optimization of the culture conditions for the production of a bacteriocin from halophilic archaeon Sech7a. *Preparative Biochemistry Biotechnology,* 38: 229-245.
5. Ishibashi, M., Yamashita, S. and Tokunaga, M. (2005). Characterization of halophilic alkaline phosphatase from *Halomonas* sp. 593, a moderately halophilic bacterium. *Biosciences Biotechnology Biochemistry,* 69 (6): 1213-1216.
6. Arahal, D., R., Russell, H., Carol, V., Litchfield, D., Melanie, R., Mormile, B., Tindall, J., Aharon, O., Victoria, B., Quesada, E. and Ventosa, A. (2007). Recommended minimal standards for describing new taxa of the family *Halomonadaceae*. *Systematic Evolutionary Microbiology,* 57: 2436–2446.
7. Isnansetyo, A. and Kamei, Y. (2003). MC21-A, a bactericidal antibiotic produced by a new marine bacterium, *Pseudoalteromonas phenolic* sp. Nov. O-BC30T, against methicillin-resistant *Staphylococcus aureus*. *Antimicrobial and Chemotheraphy,* 47 (2): 480-488.
8. Bitzer, J., Große, T., Wang, L., Lang, S., Beil, W. and Zeeck, A. (2006). New aminophenoxazinones from a marine *Halomonas* sp.: fermentation, structure elucidation and biological activity. *Antibiotics,* 59 (2): 86-92.

9. Mabinya, L., V., Cosa, S., Mkwetshana, N. and Okoh, A., I. (2011). *Halomonas* sp. OKOH – a marine bacterium isolated from the bottom sediment of algoa bay – produces a polysaccharide bioflocculant: partial characterization and biochemical analysis of its properties. *Molecules,* 16: 4358-4370.
10. Ojima, T., Saburi, W., Yamamoto, T. and Kudo, T. (2012). Characterization of *Halomonas* sp. strain H11 α-glucosidase activated by monovalent cations and its application for efficient synthesis of α-D-glucosylglycerol. *Applied and Environmental Microbiology,* 78 (6): 1836-1845.
11. Chen, W. and Liu, Y. (2013). Isolation and identification of *Halomonas* sp. ZSCW-10: a moderately halophilic bacteria strain with cellulose activity. *Advances Materials Research,* 749: 236-241.
12. Donio, M., B., S., Ronica, F., A., Viji, V., T., Velmurugan, S., Jenifer, J., S., C., A., MichaelBabu, M., Dhar, P. and Citarasu, T. (2013). *Halomonas* sp. BS4, a biosurfactant producing halophilic bacterium isolated from solar salt works in India and their biomedical importance. *SpringerPlus,* 2 (149): 1-10.
13. Juan, L., Fuchao, L., Ling, L., Peng, J. and Zhaopu, L. (2013). Inhibitory activity of an extract from a marine bacterium *Halomonas* sp. HSB07 against the red-tide microalga *Gymnodinium* sp. (Pyrropphyta). *Chinese Journal of Oceanology and Limnology,* 31 (6): 1241-1247.
14. Peschel, A. and Sahl, H.S. (2006). The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nature Reviews Microbiology,* 4: 529-536.
15. Kanagasabhapathy, M. and Nagata, S. (2008). Cross-species induction of antibacterial activity produced by epibiotic bacteria isolated from Indian marine sponge *Pseudoceratina purpurea. World Microbiology Biotechnology,* 24: 687–691.
16. Di Maro ,A., De Maio, A., Castellano, S., Parente, A., Farina, B., and Faraone-Mennella, M.R. (2009). The ADP-ribosylating thermozyme from *Sulfolobus solfataricus* is a DING protein. *Biology Chemistry,* S1431–S6730.
17. Ellen, A., F., Rohulya, O., V.,Fusetti, F., Wagner, M., Albers, S-V. and Arnold J. M. Driessen. (2011). The sulfolobicin genes of *Sulfolobus acidocaldarius* encode novel antimicrobial proteins. *Bacteriology,* 193 (17): 4380-4387.
18. Pantazaki, A., A., Tsolkas, G., P. and Kyriakidis, D.A. (2008). A DING phosphatase in *Thermus thermophilus*. *Amino Acids,* 34: 437 – 448.
19. Berna, A., Bernier, F., Chabriere, E., Perera, T. and Scott, K. (2008). DING proteins; novel members of a prokaryotic phosphate-binding protein superfamily which extends into the eukaryotic kingdom. *Biochemistry Cell Biology,* 40: 170–175.
20. Collombet, J.,M., Elias, M., Gotthard, G., Four, E., Renault, F., Joffre, A., Baubichon, D., Rochu, D. and Chabrière, E. (2010). Eukaryotic DING proteins are endogenous: an immunohistological study in mouse tissues. *PLoS ONE e9099,* 5 (2): 1-7.
21. Longeon, A., Peduzzi, J., Barthe´, M. L., Corre, S., Nicolas, J-L. and Guyot, M. (2004). Purification and partial identification of novel antimicrobial protein from marine bacterium *Pseudoalteromonas* species strain X153. *Marine Biotechnology,* 6: 633–641.
22. Atirah., W.S.N. (2011). Identification and characterization of antimicrobial peptides in *Halomonas* sp. from *Lutjanus erythropterus* epidermis. Msc. Thesis. Universiti Teknologi Malaysia, Skudai.
23. Azemin, A. and Omar, M., S., S. (2014). Detection and activity of bacteriocin produced by *Halomonas* sp. strain M3. *Proceeding of International Postgraduate Conference on Science and Mathematics,* 2: 47-58.
24. Giek Far, C. and Rashid, N. A. (2005). Flavin reductase from *Citrobacter freundii* A1. Ph.D. Thesis. Universiti Teknologi Malaysia, Skudai.
25. Chong, K., Joshi, S., Jin, L., T. and Shu-chien, A., C. (2005). Proteomics profiling of epidermal mucus secretion of a cichlid (*Symphysodon aequifasciata*) demonstrating parental care behaviour. *Proteomics,* 5: 2251-2258.
26. Mousavi, S. M., Wilson, G., Raftos, D., Mirzargar, S. S. and Omidbaigi, R. (2011). Antibacterial activities of a new combination of essential oils against marine bacteria. *Aquaculture International,* 19 (1): 205-214.
27. Bringans, S., Eriksen, S., Kendrick, T., Gopalakrishnakone, P., Livk, A., Lock, R. and Lipscombe, R. (2008). Proteomic analysis of the venom of *Heterometrus longimanus* (Asian black scorpion). *Animal Proteomic,* 8 (5): 1081-1096.
28. Lewis, A.,P. and Crowther, D. (2005). DING proteins are from *Pseudomonas*. *FEMS Microbiology Letters*, 252: 215–222.

29. Scott, K. and Wu, L. (2005). Functional properties of a recombinant bacterial DING protein: comparison with a homologous human protein. *Biochim Biophys Acta*, 1744: 234–244.
30. Ahn, S., Moniot, S., Elias, M., Chabriere, E. and Kim, D. (2007). Structure-function relationships in a bacterial DING protein. *FEBS Letters*, 581: 3455–3460.
31. Tan, A.,S. and Worobec, E.,A. (1993). Isolation and characterization of two immunochemically distinct alkaline phosphatases from *Pseudomonas aeruginosa*. *FEMS Microbiology Letters*, 106: 281–286.
32. Ball, G., Durand, E., Lazdunski, A. and Filloux, A. (2002). A novel type II secretion system in *Pseudomonas aeruginosa*. *Molecular Microbiology*, 43: 475–485.
33. Berna, A., Scott, K., Chabriere, E. and Bernier, F. (2009). The DING family of proteins: ubiquitous in eukaryotes, but where are the genes?. *Bioessays*, 31: 570–580.
34. Perera, T., Berna, A., Scott, K., Lemaitre-Guillier, C. and Bernier, F. (2008). Proteins related to St. John's Wort p27SJ, a suppressor of HIV-1 expression, are ubiquitous in plants. *Phytochemistry*, 69: 865–872.
35. Zhang, X.,X., Scott, K., Meffin, R. and Rainey, P.,B. (2007). Genetic characterization of psp encoding the DING protein in *Pseudomonas fluorescens* SBW25. *BMC Microbiology*, 7: 114.
36. Belenky, M., Prasain, J., Kim, H. and Barnes, S. (2003). DING, a genistein target in human breast cancer: a protein without a gene. *Nutrition*, 133: 2497S–2501.
37. Riah, O., Dousset, J.,C., Bofill-Cardona, E. and Courriere, P. (2000). Isolation and microsequencing of a novel cotinine receptor. *Cellular Molecule Neurobiology*, 20: 653–664.