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ISOLATION, SCREENING AND CHARACTERIZATION OF MARINE BACTERIOCIN-PRODUCING BACTERIA FOR THE DEVELOPMENT OF POTENTIAL DRUGS IN AQUACULTURE

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Abstract: Marine bacteria are a wealthy source of useful antimicrobial compounds including bacteriocins as ribosomally synthesized antibiotic peptides or proteins. Only few reports have been published worldwide on marine producers of bacteriocin or bacteriocin-like substance (BLIS), especially on marine animal-associated bacteria. Up to date, related publications have absolutely been empty in Vietnam. The present research aims to screen and characterize marine bacteriocinogenic bacteria as well as assess the properties of bacteriocins for using them as multi-functional drugs of anti- and pro- biotics in aquaculture. The results have indicated ten bacteria strains isolated from several reared animals expressed their bacteriocin activity against selected target pathogens. Bacteriocinogenic bacteria were then be identified and phylogenetically analyzed based on 16S rRNA gene sequences. The findings contribute new knowledge on the biodiversity, evolution, physiology, ecology of marine microorganisms and their potential applications as multi-functional drugs in aquaculture in Vietnam.

Keywords: *Bacteriocin, Bacteriocinogenic bacteria, Marine aquaculture, Probiotics.*

PHÂN LẬP, TUYỂN CHỌN VÀ NGHIÊN CỨU ĐẶC ĐIỂM SINH HỌC CỦA VI KHUẨN BIỂN SINH BACTERIOCIN NHẰM ĐỊNH HƯỚNG ỨNG DỤNG DƯỢC PHẨM TRONG NUÔI TRỒNG THỦY SẢN

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Tóm tắt: Vi khuẩn biển sản sinh nhiều chất kháng khuẩn khác nhau bao gồm bacteriocin có bản chất protein. Cho đến nay nghiên cứu về vi khuẩn biển sinh bacteriocin còn hạn chế, nhất là về vi khuẩn dính bám trên động vật biển. Những nghiên cứu như vậy còn bỏ ngỏ ở Việt Nam. Nghiên cứu này nhằm tuyển chọn và xác định đặc điểm sinh học của các vi khuẩn biển sinh bacteriocin nhằm định hướng ứng dụng làm thuốc đa năng (kháng sinh thế hệ mới, probiotic) trong nuôi trồng thủy sản. Kết quả đã phân lập được 10 chủng vi khuẩn sinh bacteriocin từ một số động vật nuôi biển. Chúng đã được định danh và phân tích phát sinh chủng

loại dựa trên trình tự đoạn gen 16S rDNA. Nghiên cứu này cung cấp những dữ liệu mới về đa dạng sinh học và tiến hóa của vi sinh vật biển cũng như khả năng ứng dụng của chúng dùng làm thuốc đa năng phục vụ nuôi trồng hải sản ở Việt Nam.

Từ khóa: *Bacteriocin, Nuôi trồng hải sản, Probiotic, Vi khuẩn sinh bacteriocin*

I. INTRODUCTION

The development of sustainable aquaculture for food security requires of the discovery of novel drugs to replace of the use of traditional antibiotics. Scientific communities have proposed friendly alternatives such as vaccines, antibiotic substitutes or probiotics (Corripio-Myar *et al.*, 2007; Sahu, 2008). Bacteriocin-producing bacteria could be an excellent candidate with dual role because bacteriocin would be used as an environmental-friendly antibiotic substitute, whereas bacteria would be a potential probiotic (Gillor *et al*., 2008).

Marine bacteria are a wealthy source of useful antimicrobial compounds in which bacteriocins are ribosomally synthesized antibiotic peptides or proteins (Wilson *et al*., 2010). However, only few reports were worldwide published on marine producers of bacteriocin or bacteriocin-like substance (BLIS), especially on marine animal-associated bacteria (Desriac *et al*., 2010). Currently, some bacteriocinogenic bacteria were found in marine animals such as oysters, barnacles, sponges, tunicates, sea urchins (Wilson *et al.,* 2010), seahorses (Balcazar *et al.,* 2010), and olive flounders (Heo *et al.*, 2011). Up to date, related publications have absolutely been empty in Vietnam.

Assessing the biodiversity of bacteriocin-producing bacteria could provide the deeper understanding on physiology, ecology and evolution of marine microoganisms in the interaction with their host animals and target bacteria. Moreover, novel bacteriocins or BLISs could potentially be used as food additives or as marine drugs to human, livestock and aquaculture animals (Rodriguez et al., 2002; Chen, Hoover, 2003; Cotter et al., 2005). In our knowledge, this is the first report on bacteriocin-producing bacteria isolated from marine animals in Vietnam.

II. MATERIALS AND METHODS

1. Animal sampling

Three species of marine animals were collected in Nha Trang Bay and Cam Ranh Bay including cobia (*Rachycentron canadum)*, snubnose pompano (*Trachinotus blochii*), and ornate spiny lobster (*Panulirus ornatus*). Total 15 animal samples were collected at 5 areas with 5 samples per species. At the sampling sites, animals were aseptically beheaded, peeled and gut region separated using sterile sharp knife and forceps. The gut was then dispensed in pre-weighed sterile 1% peptone water taken in a screw cap test tube. Samples were then brought to the laboratory in insulated containers for further analysis.

2. Bacteria isolation

Marine bacteria were isolated by the spread plate method. Gut samples were homogenized with an appropriate volume of sterile 1% peptone water at 30^0C for 24h and serial dilutions plated onto trypticase soy broth (TSB) supplemented with 2% agar and 1% NaCl. The plates will then be incubated at 30^0C for 24-48 h. The representative colonies developed on the plates will be picked up and purified (Nithyanand, Pandia, 2009).

3. Assay for bacteriocin activity

Bacteriocin activity was determined by agar-well diffusion method. Isolates were grown on trypticase soy agar (TSA) media and incubated at 37°C. Next day cell-free neutralized supernatants were be obtained by centrifugation (6000 rpm, for 30 min, 4°C) and adjusted to pH 7.0 with 1 N NaOH. Plates were overlaid with 3 ml soft agar containing $1x10^6$ cells of selected target bacteria (Todorov, Dicks, 2009). These target bacteria include a Gram-posivtive represensative *Bacillus cereus* B1.1 and a Gram-negative shrimp/fish pathogen *Vibrio cholerae* V1.1 obtained from the collection of local microorganisms at Nha Trang University. Wells (5 mm diameter) were cut and 100 µL of supernatant fluid of the test organism was poured into each well. Next day zone of inhibition of the target strain around the well was measured (Todorov, Dicks, 2009).

4. Genomic DNA extraction and Polymerase Chain Reaction

The DNA of bacterial strains was extracted by alkaline lysis method using the kit Wizard®SV Genomic DNA Purification System (Promega, USA). Purified DNA samples were used as templates for amplification of 16S rDNA gene segments using eubacterial universal primers (Integrated DNA Technologies, USA), namely forward primer 16S-27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer 16S-1492R (5'-ACG GCT ACC TTG TTA CGA CT-3') (Lane *et al*., 1991). The PCRs were performed in 50 µl reactions containing 2 µl (10 ng) of template DNA, 0.5 µM each primer, 1.5 mM of $MgCl_2$, 50 µM each dNTP, and 1 U Taq DNA polymerase along with 1X Taq buffer as recommended (Promega, USA). Amplification was performed in a DNA thermal cycler (Biorad) as follows: 40 cycles of 1 min per cycle at 95 °C, and 1 min at 55 °C, followed by an increase to 72 °C over 2 min. Extension of the amplified product was at 72 °C for 5 min. The amplified products were be visualized in a 1% agarose gel stained with ethidium bromide.

5. Gene sequencing and phylogenetic analyses

The PCR product of the 16S rRNA genes of bacterial isolates was purified using the PCR Clean Up System Kit (Promega, USA) and used as template for sequencing using dye-labelled dideoxy terminator (Big Dye Terminator v. 3.1, Applied Biosystems) on an ABI Prism 3700 DNA Analyser (Applied Biosystems). The 16S rRNA gene sequences of bacterial isolates and reference sequences available in GenBank were used for sequence analysis at the National Center for Biotechnology Information (NCBI) using BLAST (http://www.ncbi.-nlm.nih.gov/BLAST). All the 16S rRNA gene sequences were aligned using

ClustalW (Larkin et al., 2007), and regions with gaps were removed using BioEdit (Hall, 1999). Model selection was used to determine the best fit model with the lowest Bayesian Information Criterion score for Neighbor Joining (NJ) (Saitou, Nei, 1987; Tamura *et al*., 2004) and Maximum Likelihood (ML) analysis, which was then used to construct a NJ or ML tree using the MEGA5 program (Tamura *et al*., 2011). The robustness of the tree topology was tested by bootstrap analysis with 1,000 resamplings (Felsenstein, 1985).

III. RESULTS AND DISCUSSION

1. Isolation and screening of bacteriocin-producing bacteria from marine animals

Total 93 bacterial strains were isolated from guts of marine animals including 20 strains from cobia (*R. canadum)*, 30 from spiny lobster (*P. ornatus*) and 43 from snubnose pompano (*T. blochii*). Among them, 27 strains (5 strains from cobia (25%), 12 from spiny lobster (40%) and 10 from snubnose pompano (23%)) were found to inhibit the growth of at least one of two target microorganisms *Bacillus cereus* B1.1 or *Vibrio cholerae* V1.1 on plates. Finally, total 10 strains were expressed to produce bacteriocins as a weapon to kill other bacteria as revealed by the completed deactivation of antibacterial activity of their culture extracts after the treatment with Proteinase K, or trypsin, or both (Table 1). Similar results have shown that the cell-free culture supernatants from 13 of 250 bacterial strains isolated from seahorses exhibited antibacterial activity against at least one of the indicator strains (Balcazar *et al*., 2010). However, only three of these 13 strains were found to produce bacteriocins or BLISs

Table 1. Antibacterial activities against *Bacillus cereus* B1.1 of the bacteria isolated from guts of marine animals (D-d, mm)

Animal hosts	Bacterial strains	Cell-free culture supernatants after pH adjustment to neutral	Cell-free culture supernatants after pH adjustment and protease treatment	
		Control	Proteinase K	Trypsin
Cobia (*R. canadum)*	CT1.1	14	0	0
Snubnose pompano (*T. blochii*)	D9	17	0	0
	D10	14	0	10
	D15	22	0	15
	D16	16	0	0
	D18	8	0	0
Ornate spiny lobster (*P. ornatus*)	G1	14	0	0
	L5	16	0	0
	M2	16	0	0
	N14	15	0	0

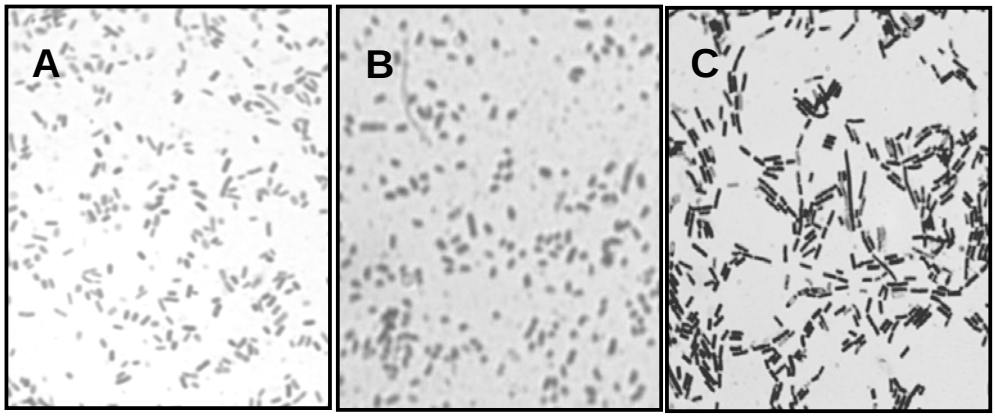

Figure 1. Gram staining cells of the strains CT1.1 (A), D10 (B) and M2 (C) isolated from cobia, snubnose pompano and spiny lobster, respectively.

2. Identification and phylogenetic analysis of bacteriocin-producing bacteria

The 16S rRNA genes of all 10 bacteriocin-producing isolates were amplified, sequenced and published in Genbank under accession numbers (Table 2). Following the sequence alignment using BLAST, these strains are identified, which belong to four different genera (Table 3). Of which, 9 (90%) strains were Gram-negative bacteria and only one (10%) was Gram-positive. Out of the 10 bacteriocinogenic bacteria strains identified, 5 (50%) belonged to the genus *Proteus*, 2 (20%) to *Klebsiella*, 2 (20%) to *Alcaligenes*, and 1 (10%) to *Bacillus*. *Proteus* strains were found in all three marine animal species.

Table 2. Sequencing results of 16S rRNA genes of bacteriocin-producing bacteria

No.	Strains	Sequence size (bp)	Genbank accession numbers
1	*Klebsiella* sp. L5	1408	KC213802
2	*Klebsiella* sp. M2	1382	KC213803
3	*Bacillus* sp. D9	851	KC213798
4	*Alcaligenes* sp. D16	1342	KC213795
5	*Alcaligenes* sp. D18	1270	KC213796
6	*Proteus* sp. CT1.1	1409	KC213808
7	*Proteus* sp. G1	1388	KC213811
8	*Proteus* sp. N1.4	835	KC213812
9	*Proteus* sp. D10	1280	KC213809
10	*Proteus* sp. D15	1290	KC213810

It is not surprised that almost all strains belong to *Klebsiella* and *Proteus* genera within the family Enterobacteriaceae, a large family of Gram-negative bacteria of many harmless symbionts and even more familiar pathogens (O'Hara

et al., 2000; Martínez *et al*., 2004). Members of this family are a normal part of the gut flora found in the intestines of humans and other animals. Besides, two strains D16 and D18 belong to the *Alcaligenes* genus within the family Alcaligenaceae, which often occur in the soil and water, and some live in the intestinal tract of vertebrates (Van Trappen *et al*., 2005). *Alcaligenes* species have been used for the industrial production of non-standard amino acids. Finally, the strain D9 was identified as *Bacillus* sp. The genus *Bacillus* includes Gram-positive, rod-shaped bacteria, which has been found ubiquitously in nature. They often secrete many kinds of biologically active compounds including enzymes and bacteriocins (Abriouel *et al*., 2011). With two exceptions of *B. anthracis* as a pathogen of anthrax and *B. cereus* causing a foodborne illness similar to that of *Staphylococcus, Bacillus* species are considered as safe to human and popularly used as probiotics to human and animals.

Table 3. The percent identity of 16S rRNA gene sequences from bacteriocin-producing bacteria with the nearest related sequences in Genbank

Accession	Bacterial strains in Genbank	Our strains	
A. *Klebsiella* spp. L5 and M2		**L5**	**M2**
HQ288920.1	*Klebsiella pneumoniae* A18	99.9%	99.9%
HM352365.1	*Klebsiella* sp. HaNA22	99.9%	99.9%
AP006725.1	*Klebsiella pneumoniae* NTUH-K2044	99.9%	99.9%
GQ259887.2	*Klebsiella pneumoniae* strain 211	99.8%	99.8%
JQ305691.1	*Klebsiella variicola* ISB-6	99.7%	99.7%
B. *Bacillus* sp. D9			**D9**
JX544748.1	*Bacillus cereus* CP1		100%
JX442945.1	*Bacillus thuringiensis* NA2		100%
JX423633.1	*Bacillus anthracis* KS-1		100%
JX188065.1	*Bacillus subtilis* AB30		100%
JN411469.1	*Bacillus cereus* IARI-CW-47		99%
C. *Alcaligenes* spp. D16 and D18		**D16**	**D18**
AY346138.1	*Alcaligenes* sp. IS-17	100%	100%
AY994311.1	*Alcaligenes* sp. PAOAC171	99.9%	99.9%
DQ001156.1	*Rhodobacter sphaeroides* strain B	99.9%	99.9%
HQ386706.1	*Alcaligenes* sp. x-1	99.9%	99.9%
JX293288.1	*Alcaligenes faecalis* G4	99.8%	99.8%
D. *Proteus* spp. D10 and D15		**D10**	**D15**

Accession	Bacterial strains in Genbank	Our strains	
JQ975907.1	*Proteus mirabilis* XJ134-12-3NF1	100%	100%
AB680401.1	*Proteus mirabilis* NBRC 13300	100%	100%
JF775423.1	*Proteus penneri* YCY34	100%	100%
HQ259933.1	*Proteus penneri* Z2	100%	100%
GQ856254.1	*Proteus vulgaris*	99.5%	99.5%
E. *Proteus* spp. CT1.1 and G1		**CT1.1**	**G1**
JN566137.1	*Proteus* sp. LS9(2011)	99.9%	99.9%
JN644538.1	*Proteus vulgaris* BD2_1A	99.9%	99.9%
JN092605.1	*Proteus vulgaris* FFL20	99.9%	99.9%
JN092599.1	*Proteus hauseri* FFL13	99.9%	99.9%
JN092596.1	*Proteus hauseri* FFL9	99.9%	99.9%
F. *Proteus* sp. N1.4			**N1.4**
JX203251.1	*Proteus penneri* YAK6		99.8%
JN092595.1	*Proteus penneri* FFL8		99.8%
HQ116441.1	*Proteus vulgaris* Dahp1		99.8%
GU361619.1	*Proteus vulgaris* IVTMP1		99.8%
DQ499636.1	*Proteus vulgaris*		99.8%

Analysis of the 16S rRNA gene sequences of our 10 isolates showed significant differences with their closest phylogenetic relatives (Fig. 2). Two strains L5 (Genbank accession number KC213802) and M2 (KC213803) belonging to the genus *Klebsiella,* which were isolated from spiny lobster, were phylogenetically identical, both sharing 99.9% and 99.7% similarity with *Klebsiella pneumonia* (HQ288920) and *K. variicola* (JQ305691) as the nearest relatives, respectively. The species *K. pneumonia* and *K. variicola* form distinct lines within the phylogenetic tree and thus the strains L5 and M2 could represent the species *K. pneumonia.* Therefore, using phylogenetic analysis these two strains could be classified into a defined species although the 16S rRNA gene sequences of *K. pneumonia* and *K. variicola* are more than 99% identical.

The strain D9 (KC213798) isolated from snubnose pompano (*T. blochii*) was found to be closely related (100% similarity) to four *Bacillus* species, *B. cereus* (JX544748.1), *B. thuringiensis* (JX442945.1), *B. anthracis* (JX423633.1), and *B. subtilis* (JX188065.1) (Table 3), showing the limited resolution of the 16S rRNA gene sequences for species-level identification in the *Bacillus* genus.

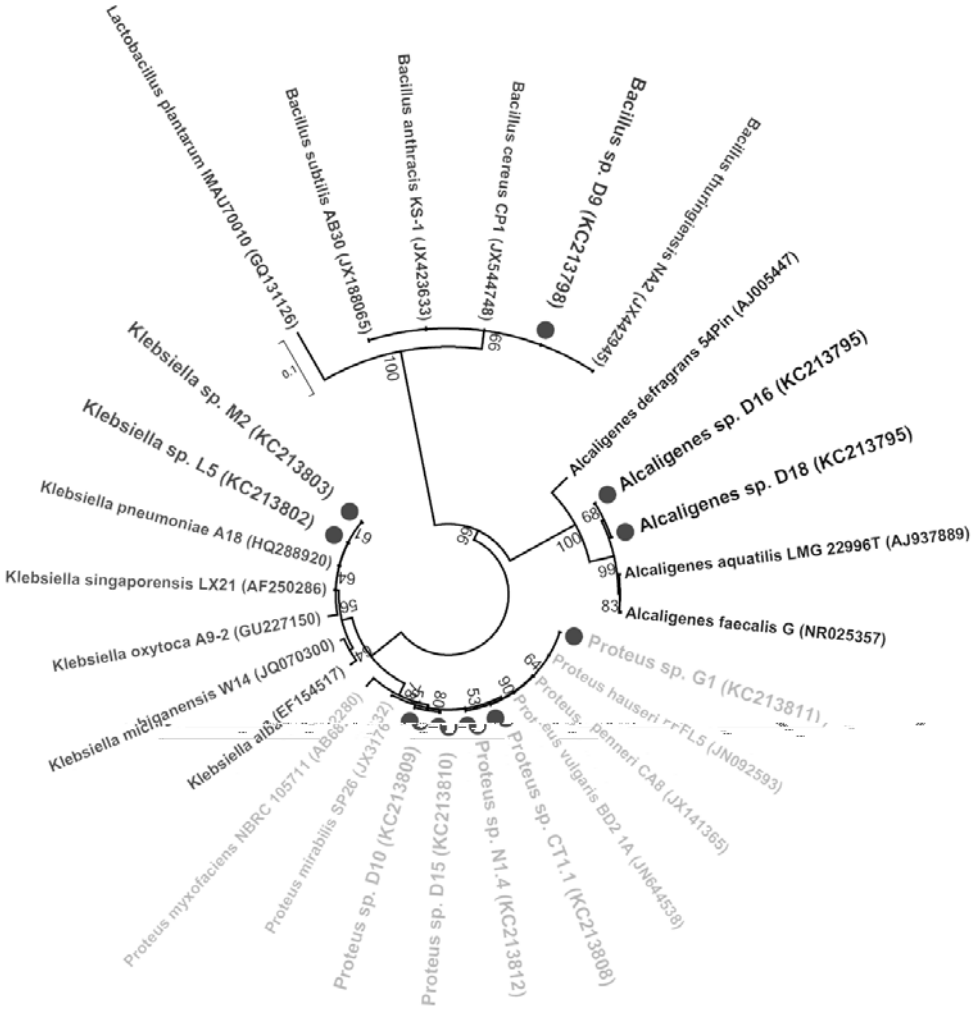

Fig. 3. Maximum-Likelihood phylogenetic tree based on the comparative analysis of 16S rRNA gene sequences showing the relationships between the bacteriocin-producing bacteria and related species.

Note: The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.1112)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 21.5043% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 570 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Bacteriocin-producing strains in this research with Genbank accession numbers are highlighted.

Also isolated from snubnose pompano, two strains D16 (KC213795) and D18 (KC213796) belong to the genus *Alcaligenes*. They were phylogenetically identical, both sharing 100% and 99.8% similarity with an unidentified *Alcaligenes* strain (AY346138) and *A. faecalis* (JX293288), respectively. The phylogenetic tree (Fig. 2) very well separates different species within the genus *Alcaligenes*, thus indicating that the strains D16 and D18 most probably represent a novel species within the genus.

Five *Proteus* strains CT1.1 (KC213808), D10 (KC213809), D15 (KC213810), G1 (KC213811) and N1.4 (KC213812) were found in three different marine animal species. The phylogenetic tree (Fig. 2) shows their different clusterings within the genus *Proteus*. The strains D15 and D10 isolated from the snubnose pompano *T. blochii* has *P. mirabilis* (JQ975907) and *P. penneri* (JF775423) as the nearest relative (100% similarity) whereas the strain N14 isolated from lobster shares 99.8% similarity with both *P. penneri* (JX203251) and *P. vulgaris* (HQ116441). The last strain from the lobster, G1, was also identical in 16S rRNA gene sequence to the strain CT1.1, which was isolated from the cobia *R. canadum*. Both these strains share 99.9% similarity with *P. vulgaris* (JN644538) and *P. hauseri* (JN092599) as the nearest relatives.

The mixed clustering of *Proteus* species in the phylogenetic tree could be explained by the fact that *P. vulgaris* was shown to be a heterogeneous group with at least three biogroups (Brenner *et al*., 1978) or six genomospecies (O'Hara *et al*., 2000) based on DNA-DNA hybridization. *P. vulgaris* biogroup 1 (genomospecies 1) was negative for indole production, salicin fermentation and aesculin hydrolysis, whereas the remaining two biogroups were both positive for indole production. Biogroup 2 (genomospecies 2) was positive for salicin and aesculin but biogroup 3 was negative for both. In 1982, biogroup 1 was named *Proteus penneri* (Hickman *et al*., 1982).

In 2000, strains in biogroup 3 were separated into four distinct groups, designated as *Proteus* genomospecies 3, 4, 5 and 6 using DNA hybridization (O'Hara *et al*., 2000), in which *Proteus* genomospecies 3 was renamed *P. hauseri* due to its negative reactions for salicin fermentation, aesculin hydrolysis and deoxyribonuclease. *Proteus* genomospecies 4, 5 and 6 remain unnamed because of unclear phenotypic differentiation.

Clearly, taxonomy of the genus *Proteus* is on the way to be discovered. For example, our unpublished results on API 50CHE analysis of sugar fermentation reactions in the strain CT1.1 showed this bacterium identical to unnamed *Proteus* genomospecies 4, which was differentiated from known *Proteus* species by its ability to ferment L-rhamnose (O'Hara et al., 2000). Therefore, naming all strains in our *Proteus* culture collection is necessary. In addition, the genus *Proteus* is considered as a frequent cause of urinary tract infections in human although it is not usually a nosocomial pathogen. Thus assessing their pathogenicity for humans and animals are required, followed by studies on bacteriocin production and their application as probiotics in aquaculture.

V. CONCLUSION

Total ten bacteriocin-producing bacteria strains were isolated from three species of marine reared animals in Vietnam, which expressed their bacteriocin activity against at least one of selected target bacteria, *Bacillus cereus* B1.1 or *Vibrio cholerae* V1.1. Bacteriocinogenic bacteria were then identified based on 16S rRNA gene sequences, which indicated that they have belonged to four different genera including *Proteus*, *Klebsiella*, *Alcaligenes*, and *Bacillus*. *Proteus* strains were found in all three marine animal species. Finally, phylogenetic diversity of 16S rRNA genes of our 10 isolated strains was analyzed, which showed significant differences with their closest phylogenetic relatives. In some cases, they could represent novel species within their corresponding genus.

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