J. Viet. Env. **2012**, Vol. 3, No. 2, pp. 71-75
DOI: 10.13141/jve.vol3.no2.pp71-75

JOURNAL of VIETNAMESE ENVIRONMENT

Isolation and identification of marine bacteria from marine mud in Vietnam with antimicrobial activity

Phân lập và nhận dạng các chủng vi sinh vật biển từ mẫu bùn biển ven bờ Việt Nam và hoạt tính kháng khuẩn của chúng

Research article

Thi, Tuyen Do[1]; Dinh, Quyen Le[1]; Dinh, Thi Quyen[1]*; Van, Cuong Pham[2]

[1]*Institute of Biotechnology;* [2]*Institute of Marine Biochemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Caugiay District, Hanoi, Vietnam*

Seventeen bacterial strains were isolated from 9 marine mud samples from the inshore environments of the East Sea. Four bacterial strains showed an inhibition against all tested microorganisms *Staphylococcus aureus* ATCC10832, *Escherichia coli* JM109, and *Fusarium oxysporum*. 16S rRNA sequences of four bacterial strains were obtained by PCR using specific primers. PCR products were cloned into *E. coli* DH5α using pJET1.2 blunt vector. The recombinant plasmids were sequenced and the lengths of these 16S rRNA sequences were ~930bp. The 16S rRNA sequence from the four bacterial DB1.2, DB1.2.3, DB4.2 and DB5.2 strain showed a high identity of 97 to 99% with the 16S rRNA sequence from *Photobacterium* sp., *Oceanisphaera* sp., *Shigella* sp., *Stenotrophomonas* sp, respectively.

Mười bảy chủng vi khuẩn đã được phân lập từ 9 mẫu bùn biển từ các vùng ven bờ biển Việt Nam. Bốn chủng vi khuẩn được ghi nhận có khả năng ức chế mạnh sự sinh trưởng và phát triển của các chủng vi khuẩn Staphylococcus aureus ATCC10832*, Escherichia coli* JM109*, và thậm chí cả nấm Fusarium oxysporum. Trình tự gene 16S rRNA của bốn chủng vi khuẩn này đã được khuếch đại bằng PCR sử dụng cặp mồi đặc hiệu. Sản phẩm PCR được nối ghép vào vector pJET1.2 blunt sử dụng T4 ligase, hình thành plasmid tái tổ hợp và biến nạp vào E. coli DH5α. Khuẩn lạc có plasmid mang phân đoạn DNA chèn được nuôi cấy và tách plasmid. Trình tự 16S rRNA từ 4 chủng DB1.2, DB1.2.3, DB4.2 and DB5.2 chỉ ra có sự tương đồng 97 ÷ 99% so với trình tự 16S rRNA tương ứng của các chủng vi sinh vật biển trên ngân hàng gene thế giới là Photobacterium sp., Oceanisphaera sp., Shigella sp., và Stenotrophomonas sp.*

Keywords: antimicrobial, marine bacteria, 16S rRNA gene

1. Introduction

Heterotrophic bacteria commonly present in marine environments have received little attention, though special groups such as the agar digesters have been extensively investigated. Extensive investigations on marine bacteria in Mandapam were reported (Velankar, 1954; Leifson *et al.*, 1964; Okami *et al.*, 1976; Ramaiah, 2004). Qualitatively, the bacterial flora of marine environments in different parts of the world recorded by different researchers showed some differences. The study of marine bacterial diversity is important in order to understand the community structure and pattern of distribution.

Studies on bacteria from East Sea coastal waters and comparison with marine bacteria recorded in other seas would be interesting, particularly under a consideration of the temperature and depth differences. Competition among microbes for space and nutrient in marine environment is a powerful selection pressure that endows marine microorganisms to produce many natural products possessing medical and industrial values (Armstrong *et al.*, 2001). Furthermore, marine bacteria have potential applications in fish processing and preservation (Okazaki *et al.*, 1975). In the present work, we have isolated and identified marine bacteria from marine mud samples collected in different coastal areas of Vietnam. These bacteria were then tested with antimicrobial activity.

* Corresponding author
E-mail: quyen@ibt.ac.vn

http://dx.doi.org/10.13141/JVE
ISSN: 2193-6471

2. Materials and methods

2.1 Marine mud collection

Nine marine mud samples were collected by specialized equipment in the sea areas of different depths, in August and September, 2010. Captured mud samples were held individually in clear plastic bags to prevent cross-contamination of bacteria between mud samples. The vials containing mud were plugged with cotton wool for ventilation and placed in a cool ice box in the boat to be stored.

2.2 Bacterium isolation

One gram of mud was dissolved in 9 ml sterile water (10^{-1} dilution) and shaken vigorously for at least 1 full minute. One mL of the dilution 10^{-1} was transferred aseptically to a fresh tube containing 9 mL sterile water (10^{-2} dilution) and mixed thoroughly. This dilution step was repeated to make 10^{-3}, 10^{-4}, and 10^{-5} dilutions. A volume of 100 µL of each diluted solution (but not 10^{-1}) were streaked onto two sterile Petri plates using a glass stick. Plates were inverted, stacked into pipette canisters and incubated at 30°C or room temperature. After three days, three different well isolated colonies were circled on the back of the plate and numbered. Cells of selected colonies were re-streaked onto a nutrient agar plate, incubated at 30°C or room temperature for 24 ÷ 48 h. Each colony was isolated on the basis of morphological appearance and sub-cultured twice to ensure purity.

2.3 Bioassay of antimicrobial activity

All the isolated marine bacteria were screened for antimicrobial activity, using terrestrial microbes including *Staphylococcus aureus* ATCC10832, *Escherichia coli* JM109, *Fusarium oxysporum* (Institute of Biotechnology) as the test microorganisms. Antimicrobial activity was assayed in duplicate using an agar well diffusion assay (De Beer & Sherwood, 1945). The dried crude extracts were dissolved in EtOAc. Twenty mL of LB medium were measured into each Petri dish (90 × 15 mm, inner dimensions). As soon as the agar has gelled, introducing into the dish 100 µL test microorganisms incubated, spreading on surface dish with glass stick, a well was made in the plates with sterile borer (9 mm). The extract compound (50 µL) was introduced into the well and plates were incubated at 37°C for 48 ÷ 72 hrs. All samples were tested in triplicates. Microbial growth was determined by measuring the diameter of zone of inhibition (Maeda & Taga, 1976).

2.4 16S rRNA analysis

Isolates were incubated in 3 mL of HKTS medium for DNA isolation using a modification of the CTAB/phenol-chloroform DNA extraction protocol (Doyle & Doyle, 1987). A DNA fragment of the 16S rRNA gene was amplified from genomic DNA with the forward primer 9F (5′-AGA GTT TGA TCC TGG CTC-3′) and the reverse primer 926R (5′- CCG TCA ATT CCT TTG AGT T-3′) (Sigma-Aldrich, Co., St. Luis, USA) by PCR (Brauman *et al.*, 2001; Wang *et al.*, 2007). The PCR mixture contained 2.5 µL 10x PCR buffer; 2.5 µL of 2 mM dNTP; 2.5 µL of 25 mM $MgCl_2$; 1 µL genomic DNA (50-100 ng); 0.4 µL 5 unit *Taq* polymerase and 1 µL each primer (10 pmol), supplemented with 14.1 µL distilled water to a final volume of 25 µL. The thermocycler conditions were as follows: 95°C/5′; 30 cycles of (95°C/45″, 55°C/1′, 72°C/1′); 72°C/10′. The PCR products amplified from the genomic DNA with both primer 9F and 926R were inserted into the cloning vector pJET1.2/blunt, resulting in pJ16S and then sequenced. DNA sequencing was performed on ABI PRISM 3100 Avant Genetic Analyzer. Sequence alignments were constructed and analyzed using the program MegAlign DNAStar.

3. Results and discussion

3.1 Bacterium isolation

Seventeen bacterial colonies were isolated from 9 samples of marine mud. These were aerobic bacteria and most of them grew at room temperature (26 ÷ 30°C) and others at 37°C. The aim of this study was to isolate as many different types of bacteria as possible, and not the dominants alone. The mud composition broadly reflected the normal distribution of the bacterial types in the environment (Heijs *et al.*, 2006). Dominant bacteria from other marine environments were Gram-negative and motile rod-shaped bacteria (Leifson *et al.*, 1964).

3.2 Antimicrobial activity

Seventeen bacterial isolates from marine mud (seawater, sediment and sea organisms) showed antimicrobial activity against at least one test microbe (Figure 1). They belong mainly to the genera Gamma proteobacteria. Four strains DB1.2, DB1.2.3, DB4.2 and DB5.2 inhibited all tested microorganisms *S. aureus*, *E. coli*, and *F. oxysporum* and identified as *Photobacterium* sp., *Shigella* sp., *Oceanisphaera* sp., and *Stenotrophomonas* sp. Based on their 16S rRNA sequences, respectively. Separation and identification of bioactive compounds with wide antimicrobial spectrum from these marine bacteria are ongoing.

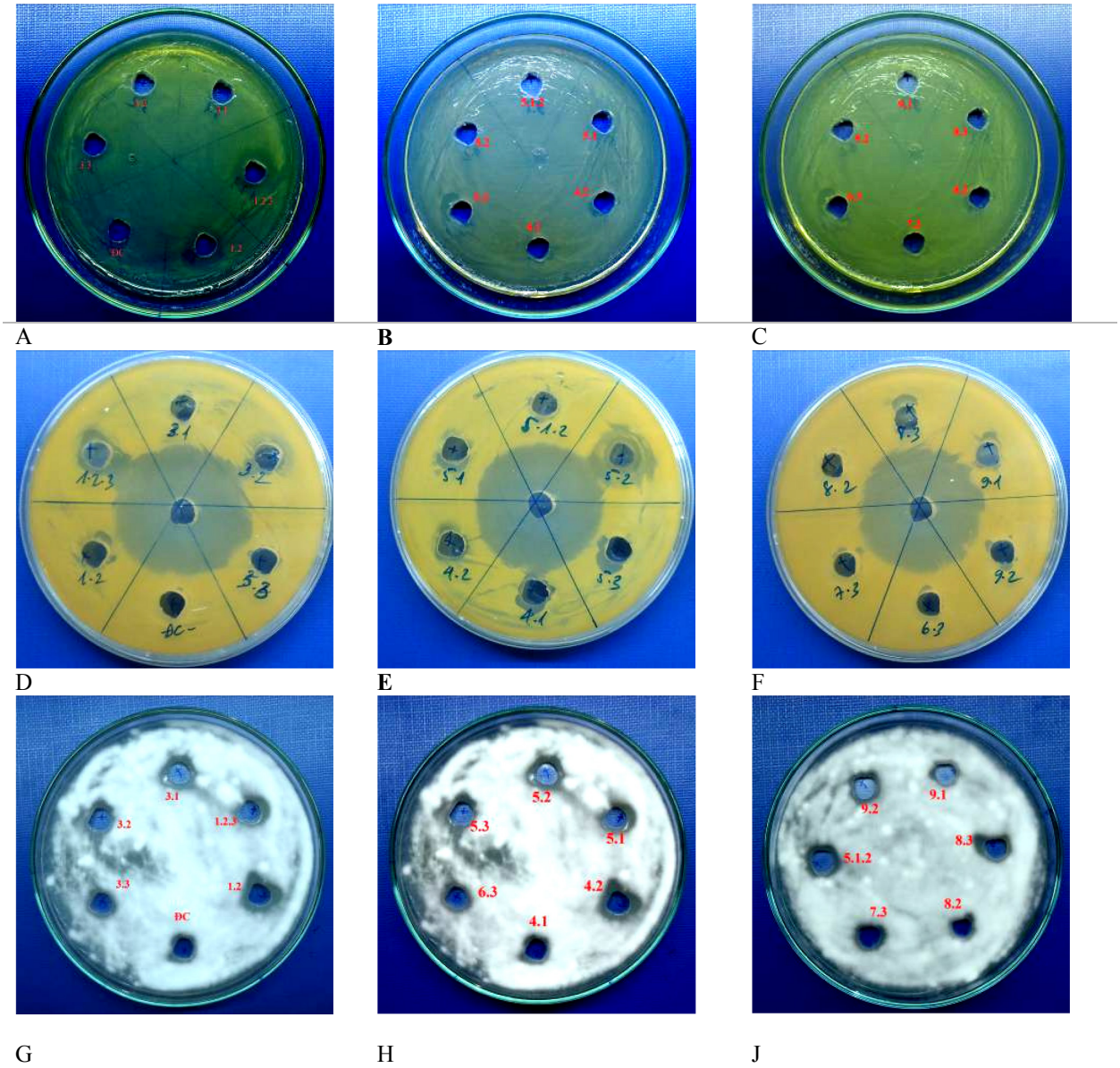

Figure 1. Antimicrobial activity of marine bacteria using agar diffusion assay against tested microorganism *E. coli* JM109 (A-C), *S. aureus* ATCC10832 (D-E), *F. oxysporum* (G-J)

3.3 Clonning 16S rRNA gene

DNA from these bacteria are purified, running on a 0.8% agarose gel electrophoresis and used as a template to amplify the 16S rRNA gene with a pair of specific primers for 16S rRNA of bacteria segments. PCR products with the size of ~ 930 bp (Figure 2) and cloned in *E. coli* DH5α. PCR products were cloned into *E. coli* DH5α using pJET1.2 blunt vector. The recombinant plasmids were sequenced and the lengths of these 16S rRNA sequences were ~ 930 bp (Figure 3A).

Plasmids are cut check BglII restriction enzymes for 2 bands, the size corresponds to the 16S rRNA (~ 1 kb) and pJET1.2 Blunt (~ 3 kb) (Figures 2A, 2B). Also products with mold amplification plasmid size 1 kb corresponds to the insert 16S rRNA (Figure 2B).

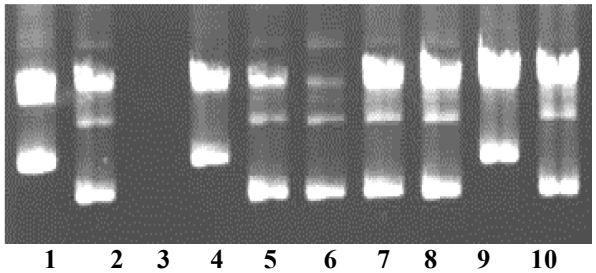

Figure 2. Plasmid products - 1, 4, 9: plasmid with the insert; 2, 5, 6, 7, 8: plasmid without the insert; 10: pJET1.2 control

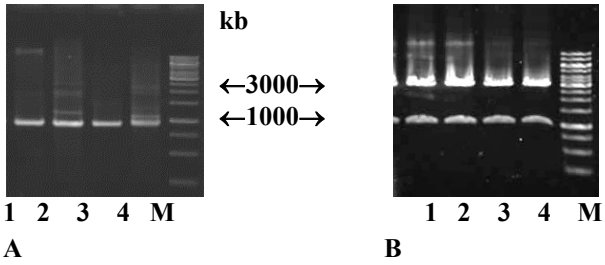

Figure 3. (A) PCR products and (B) Plasmid products cutting by restriction enzymes of four bacterial DB1.2, DB1.2.3, DB4.2 and DB5.2 strain, were isolated from 9 marine mud samples

3.4 16S rRNA analysis of strains DB1.2, DB1.2.3, DB4.2 and DB5.2

16S rRNA sequences obtained from four strains inhibiting all tested microorganisms were compared directly with sequences in the NCBI database using Basic Local Alignment Search Tool (BLAST). In the phylogenetic tree (Figure 4), sequences aggregated into four clusters in conformity with the bacterial classes Gamma proteobacteria. Four bacterial strains DB1.2, DB1.2.3, DB4.2 and DB5.2 showed an identity of 97 ÷ 99% with *Photobacterium* sp., *Oceanisphaera* sp., *Shigella* sp., *Stenotrophomonas* sp, respectively.

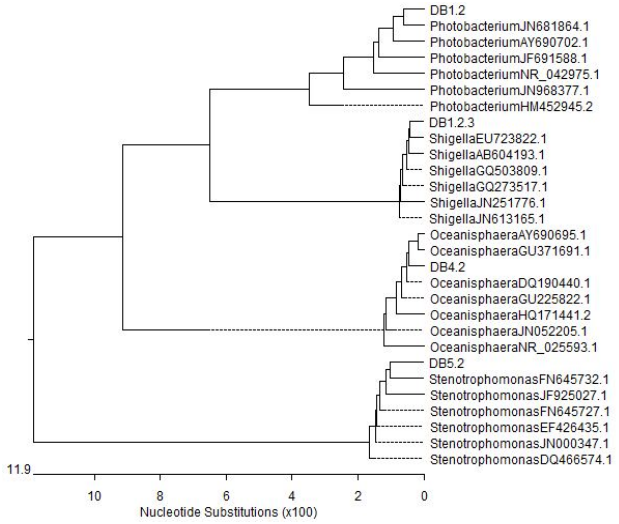

Figure 4. Phylogenetic tree based on 16S rRNA gene sequences from marine strains DB1.2, DB1.2.3, DB4.2, and DB5.2. Other codes referred to the strains deposited in GenBank

4. Discussion

From 9 marine mud samples we have isolated 17 bacteria colonies belonging to a single family, the Gamma proteobacteria, which are common microflora in marine mud (Das *et al.*, 2006; Anil Kumar *et al.*, 2008; Bhatnagar, Kim, 2010; Lucena *et al.*, 2010). In the marine environment, 90% of bacteria are Gram-negative with different characteristics and the Gram-negative cell wall is better adapted for survival in the marine environment (Velankar, 1954; Das *et al.*, 2006). These bacteria were tested with antimicrobial activity. Four strains showed inhibition against all tested microorganisms. The assay implied that the antimicrobial metabolites produced by four strains with wide antimicrobial spectrum were different. Due to a competitive role for space and nutrient, the marine bacteria associated with marine invertebrates and seaweeds could produce more antibiotic substances. These marine bacteria were expected to be potential resources of natural antibiotic products.

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