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Identification, phylogenetic characterization and preliminary screening of primary and secondary metabolites producing bacteria associated with marine sponge *Axinella donani*

C.E.Rani Juneius*[1] and J.Selvin[2]

[1]*Department of Biotechnology, Hindustan College of Arts and Science, Padur, Chennai -603103,India*
[2] *Department of Bioinformatics, Bharathidasan University,Trichy,India*
Elizabeth.juneius7@gmail.com*[1],selvinj@rediffmail.com[2]

Abstract

Sponges have pharmaceutical and medical relevance being known as a source of bioactive secondary products influencing antimicrobial, antiviral and cytotoxic activities. Study was undertaken on *Axenella donani* available of the coast of Kanyakumari. At Keelamanakudi village, adopting lobster catching technique fisherman netted these sponges yielding nearly 57 morphologically different bacteria. All the 57 isolates when screened showed proteolytic activity (7%), starch hydrolytic activity (12.2%), cellulolytic activity (3.5%). Broad spectrum antimicrobial activity was identified by using 16S rRNA sequencing. SEM examination helped to identify the sponges based on the diameter of the spicules (50 µm), exhibiting a tapered end present in them. Among the 57 isolates studied, two strains identified by 16S rRNA sequencing as *Rhodobacter sphaeroides MSB 57* and *Rhodopseudomonas palustris* MSB 55 showed all the three bacteriolytic activities.

Keywords: SEM, 16SrRNA, cellulolytic, amylolytic, proteolytic, antibacterial, antifungal

Introduction

Sponges have pharmaceutical and medical relevance being known as a source of bioactive secondary products influencing antimicrobial, antiviral and cytotoxic activities. They diet on microorganisms transferring them to the mesohyl, through the choanocyte chambers filtering the sea water and rendering it sterile. Later investigation proved that they are similar in structure to metabolites of sponge associated bacterial activity, suggesting that these metabolites are of microbial origin (Unson *et al.,* 1993). This discovery coupled with ease of cultivating bacteria shifted the focus of research to the cultivation of bacteria from sponges for their bioactive compounds. It is suggested that these metabolites by microbial symbionts serve as a chemical defence against predators (Unson *et al*., 1993).

Materials and methods

Sampling and isolation of bacteria

The study material,*Axenella donani* was *collected* with help of fisherman, off the coast of Kanyakumari. Adopting lobster catching technique fisherman netted these sponges at Keelamanakudi village,. It was transferred to the lab in ice. Collections of sponges were put into sterile plastic bags. The sponges were then

rinsed with sterile seawater and homogenized with blender. The homogenized tissues were serially diluted upto 10-7, spread plate method was carried out on ½ strength ZoBell 2216E marine agar medium and incubated at room temperature for 48 hours. On the basis of morphological features, colonies were pure cultured (Madigan *et al*., 2000).

Morphological examination of sponge by scanning electron microscope

Scanning electron microscope facility from IIT Chennai was used for the morphological study of sponge. A portion of sponge in ethanol was placed onto a specimen holder and optionally coated with gold in a Cressington 108 auto Sputter Coater. The specimens were examined and photographed in a Philips XL30 ESEM scanning electron microscope, high vacuum mode, using BSE detector with accelerating voltage 15 kV.

Screening of primary metabolite production

All the 57 isolates were screened for proteolytic activity, starch hydrolytic activity and cellulolytic activity. Casein milk agar, starch agar and carboxymethyl cellulose agar plates were used and inoculated by a single streak of innoculum in the center of the Petri dish. After 2 days of incubation at 37^{o}C the plates were analyzed for proteolysis, starch hydrolytic activity and cellulolytic properties.

Screening of bioactive compound by cross streak method (lertcanwanichakul & sawangnop., 2008)

Muller Hinton agar plates were prepared and inoculated with various morphologically distinct culture isolated by a single streak of inoculums in the center of the Petri dish. After 2 days of incubation at 37 ºC the plates were inoculated with human pathogens as well as shrimp pathogens such as *Stahylococcus aureus, E.coli and Vibrio paraheamolyticus* by a single streak at a 90° angle to the test isolates. The microbial interactions were analyzed by the observation of the size of the inhibition zone.

16S rRNA sequencing for the identification of unknown bacteria

Genomic DNA was isolated and PCR was performed using the following primer; Forward primer:5'-AGAGTTTGATCMTGGCTCAG-3' and Reverse primer:5'-AAGGAGGTGWTCCARCC-3'. PCR was performed based on the following programme - Initial Denaturation : 94^{o}C for 3 minutes, Denaturation: 94^{o}C for 1 minute, Annealing: 60^{o}C, for 1minute 30 cycles, Extension: 72^{o}C for 1minute 30sec and Final extension: 72^{o}C for 5 minutes

Polymerized DNA of unknown bacteria was sequenced by gene sequencer. The DNA sequences were then compared for homology with the BLAST database. CLUSTALW, ORF FINDER, BPROM, GENSCAN and FGENESH were also used for the sequence analysis.

Result and discussion

Sponges being continuously exposed to a number of potentially toxic compounds, pollution, and invaders, develop chemical defense systems to protect themselves. Possible contributes to the chemical defences arise from multiple antimicrobial activities against Gram-positive and Gram-negative bacteria and fungi present in seawater (Donia & Hamann, 2003). The digestive strategy of sponges is due to some microbial enzymes involved in nutrition transport in them. This suggestion is supported by the protease, amylase and cellulase activities found in sponge-associated bacteria in this study. For instance, these enzymes can hydrolyze complex compounds such as proteins, lipids, and carbohydrates of algae or microorganisms swallowed or filtrated by sponges into nutritional low-molecular compounds (Anand Prem *et al.,* 2006). Haritha *et al*. (2010) stated that twenty one of the 98 Actinomycetes isolates showed amylolytic activity and seventeen exhibited proteolytic activity while eleven isolates exhibited both amylolytic and proteolytic activities is allied to our findings viz., of the 57 bacterial isolates, 7% were

proteolytic, 12.2% were amylolytic and 3.5% were cellulolytic.

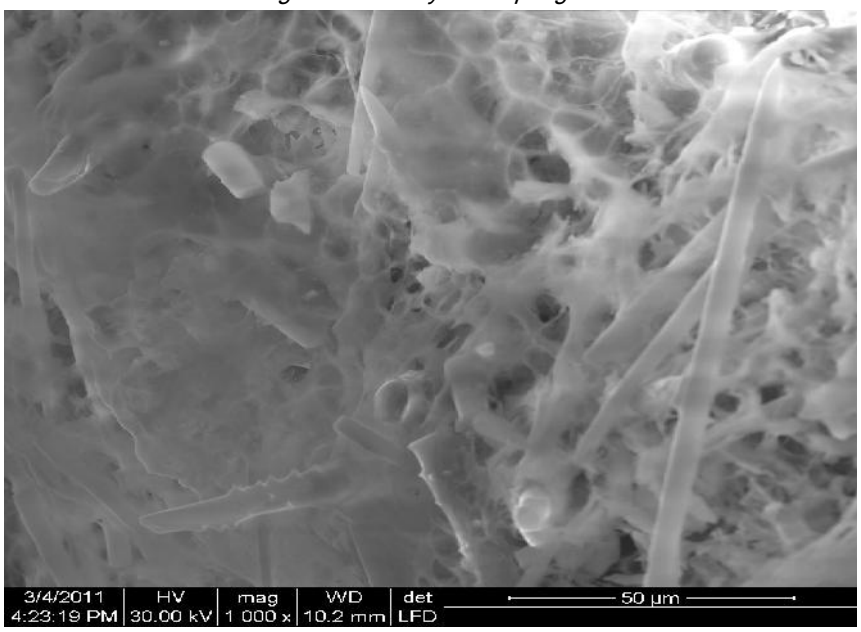
Sponges are a prolific source of biological compounds with diverse bioactivities. However,structural similarities between the metabolites of the sponge and its associated bacteria indicates that these compounds are of bacterial origin. 16S rRNA gene sequence analyses have been widely used to identify and characterize both culturable and unculturable populations of marine bacteria (Althoff *et al.,* 1998, Uzair *et al.,*2008). Three of the seven representative bacterial isolates tested inhibited the growth of *Bacillus subtilis*, a microbe commonly used in bioactivity screening(Luis et al 2006). In this present study also the unknown bacteria were identified using 16SrRNA sequencing. In addition to that, other bioinformatics tools such as ORF FINDER, BPROM, GENSCAN and FGENESH were also used for the sequence analysis helping to assess the gene level difference between the two isolates.

The possibility of the presence of toxic bacteria was supported by the finding that organic extracts prepared from sponge samples displayed toxicity, when analyzed in vitro using leukemia cells. The present study also revealed that the antagonistic bacteria MSB 57 isolated from the sponge *Axinella donani* is *Rhodobacter sp*, results similar to study by Chandrasekaran *et al*.,(2010) who stated that among the purple non-sulfur bacterial isolates, *Rhodobacter sphaeroides*, showed maximum antagonistic activity. The findings suggest that the antagonistic extracts from *Rhodobacter sphaeroides* could be used as an effective antibiotic in controlling *Vibrio* spp., in aquaculture systems.

Anand *et al*. (2005) reported that Seventy-five marine bacterial strains associated with four species of sponges (*Echinodictyum* sp., *Spongia* sp., *Sigmadocia fibulatus* and *Mycale mannarensis*) were isolated off the Tuticorin coast and Gulf of Mannar region. The agar overlay method was used to screen for antibiotic production by these strains against four bacteria, viz., *Bacillus subtilis*, *Escherichia coli*, *Vibrio parahaemolyticus*, and *Vibrio harveyi* and one fungal pathogen, viz., *Candida albicans*. The selected isolates of *Axinella donani* also showed antagonistic activity against *Escherichia coli* and *Vibrio parahaemolyticus* (Isnansetyo & Kamei, 2006).

Lertcanawanichakul & Sawangnop (2008), adopted cross streak method to screen bioactive compound producing bacteria from the sponge. The present study adopted the same methodology to find out antagonistic bacteria associated with *Axinella donani. Staphylococcus aureus, Escherichia coli* and *Vibrio parahaemolyticus* were used as a test strains. Among the 57 isolates MSB 55 and MSB 57 showed broad spectrum activity. The unknown bacteria were identified by Phynotypic and Genotypic method (16srRNA sequencing), which revealed the broad spectrum activity were *Rhodobacter sphaeroides* MSB 57 and *Rhodopseudomonas palustris* MSB 55.

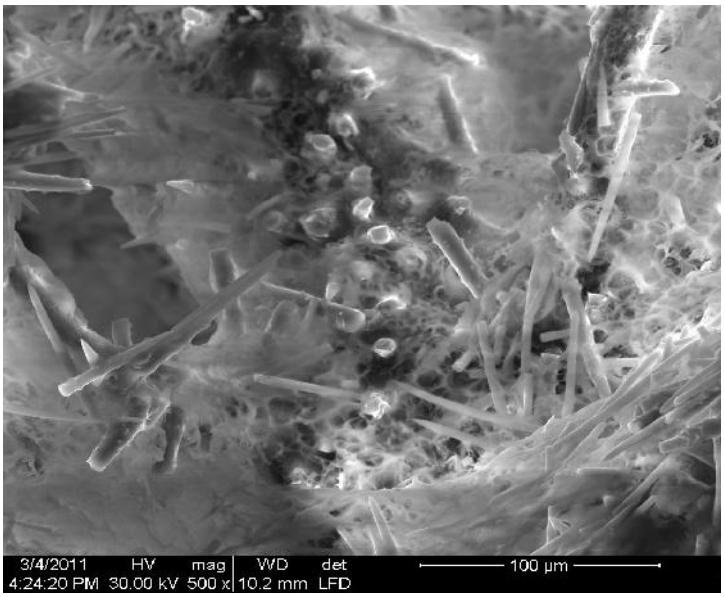
Fig.1. SEM analysis of sponge



Identification of sponge by scanning electron microscope (SEM)

SEM examination helped to identify the sponge based on the shape of the spicules present inside the sponge. The diameter of the spicules was 50 µm. They were tapered at one end. Fig. 1 & 2. shows the SEM analysis of sponge.

Fig.2.SEM analysis of sponge



Primary metabolite production

The sponge *Axinella donani* was used in this study and 57 morphologically different bacteria were isolated from the sponge. All the 57 isolates were screened for their primary metabolite production ability in order to know whether these sponges associated bacteria had any industrial value. The result revealed that some of these members are proteolytic, some are starch hydrolytic and some are cellulolytic. Though the main aim of this study is about the therapeutic values of sponge associated bacterial secondary metabolites, the primary metabolite production was studied because some literature says the relationship between the proteolytic activity and the ability to produce therapeutic compounds like Diketopyperazins. Fig.3 shows percentage distribution of primary metabolite production by the sponge associated bacteria.

Screening of bioactive compounds by cross streak method

Bioactive compound producing bacteria were screened by cross streak method done by using three pathogens. Among the 57 isolates studied, two strains showed wide activity against all the three pathogens, which showed their broad spectrum activity; while around 75.4% of the strains were showing no inhibitory activity against any of the three pathogens. Antagonistic activity was noticed only with 24.5% of the isolates. Fig. 4. predicts the percentage distribution of antagonistic activity by cross streak method. Fig.5 is representing the sequencing of 16SrRNA.

Fig.3. Percentage distribution of primary metabolite production by the sponge associated bacteria.

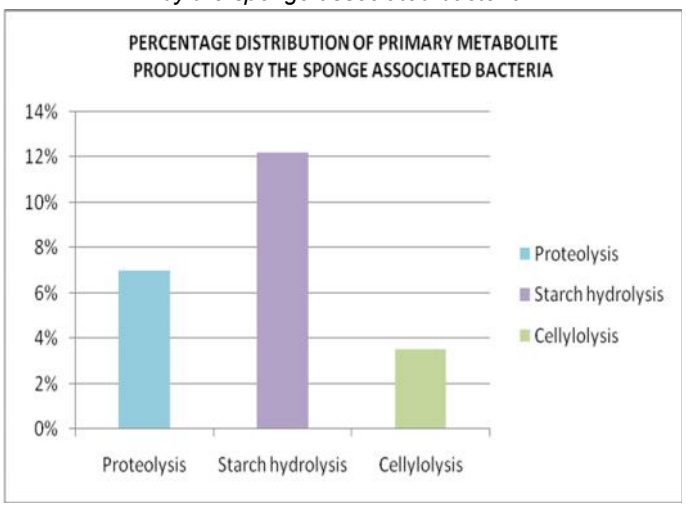


Fig.4.Percentage distribution of antagonistic activity by cross streak method

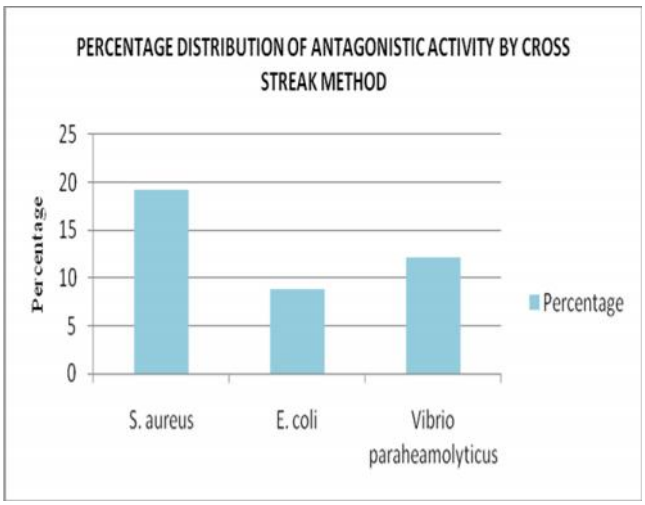
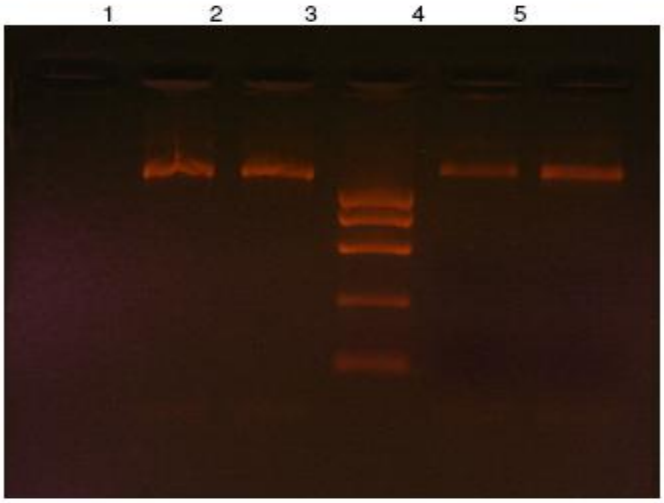


Fig.5.16SrRNA sequencing



Lane 1: PCR product of Sample 1
Lane 2: PCR product of Sample 2
Lane 3: HELINI 250bp DNA Ladder
[100bp, 250bp, 500bp, 750bp and 1000bp]
Lane 4: Purified PCR product of Sample 1
Lane 5: Purified PCR product of Sample 2

Screening of primary and secondary metabolites from sponge associated bacteria revealed that the sponge *Axinella donani* acts as a reservoir of intracellular halophylic bacteria with multi potentials to produce both primary metabolites such as α-amylase and protease enzymes and secondary metabolites like antibacterial and antifungal agents. 16S rRNA sequencing served as a better tool for the identification of the unknown bacteria which was used to identify the bacteria as *Rhodobacter sphaeroides* MSB 57 and *Rhodopseudomonas palustris* MSB 55. In addition to that, other bioinformatics tools such as ORF FINDER, BPROM, GENSCAN and FGENESH were used for the sequence analysis, helping to assess the gene level difference between the two isolates (Fig.6,7,8,9,10,11 & Table 1).

FASTA sequence

Sample-1

ATTAAGGCCTCCTTGTTTCGACTTCACCCCAGTCATGAATCCCACCGTGGGAAGCGCCCTCCTTACGGTTAGGCTAC
CTACTTCTGGTGAAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGACAT
TCTGATCCGCGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTACGATCGGGTTTC
TGAGATTGGCTCCCCCTCGCGGGTTGGCGACCCTCTGTCCCGACCATTGTATGACGTGTGAAGCCCTACCCATAAG
GGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCATTAGAGTGCTCTTGCGTAGC
AACTAATGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAG
CACCTGTGTTCCGGTTCTCTTGCGAGCACGGCCAAATCTCTTCGGCTTTCCAGACATGTCAAGGGTAGGTAAGGTTT
TTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTTAATCTTGC
GACCGTACTCCCCAGGCGGTCAACTTCACGCGTTAGCTGCGCTACTAAGGCCTAACGGACCCAACAGCTAGTTGAC
ATCGTTTAGGGCGTGGACTACCAGGGTATGTAATCCTGTTTGCTCCCCACGCTTTCGTGTCTGAGCGTCAGTATTATC
CCAGGGGGCTGCCTTCGCCATCGGTATTCCTCCACATATCTACGCATTTCACTGCTACACGTGGAATTCTACCCCCC
TCTGACATACTCTAGCTCGGCAGTTAAAAATGCAGTTCCAAGGTTGAGCCCTGGGATTTCACATCTTTCTTTCCGAAC
CAACTACACACGCTTTACGCCCAGTCATTCCGATTAGCAGCATTGCATCCTACGTATAACTGCGGCTGCTGGCACGT
ACGTAGCTAGTGCTGATCTGCAGATACGGTACAGCAGTATCCCGTATTATGGATACTTTTCTTCTCTGCAAAAGAACT
TACACCCGACGCATACATCATACACGCAGGATGGCTGGATCAGGCTACT

Sample-2

ATGGGGCTCCTTGTTACGACTTCACCCCAGTCATGAATCCCACCTTGGGAAGCGGGCTCCTTACGGTTAGGCTACCT
ACTTCTGGTGAAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGACATTC
TGATCCGCGATTACTAGCGATTCCGACTTCACGCAGGCGAGTTGCAGACTGCGATCCGGACTACGATCGGGTTTCTG
AGATTGGCTCCCCCTCGCGGGTTGGCGACCCTCTGTCCCGACCATTGTATGACGTGTGAAGCCCTACCCATAAGGG
CCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCATTAAAGTGCTCTTGCGTAGCAA
CTAATGACAAGGGTTGGGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCA
CCTGTGTTCCGGTTCTCTTGCGAGGACGGCCAAATCTCTTCGGCTTTCCAGACATGTCAAGGGTAAGTAAGGGTTTT
CGCCTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGA
CCGTACTCCCCAGGCGGTCAACTTCACTCTTTAGCTGCGCTACTAAGGCCTAACGGCACCAACAGCTAGTTGACATC
GTCTAGGGCGTGGACTACCAGAGTATCTAATCATGTTTGCTCCTCACGCTTTCGTGTCTGAGCGTCAGTATTATCACA
GGAGACAGCCTTCTCCATCGGTATTGCTCTACATATCTACTCATTTCACTGCTACACGTGGAATTCTACCCCCCTCTG
ACATACTCCTACCTCAGCAGTTAAAAATGAAGTTCCACGGTTGAGCCCTGGGATGTCACATCTTTCTTTCCGAACAGC
CTACACACGCTTTACTCCCAGTAATTAATATTGACACTTGCACCCTACGTAATTACCGCGGCTGCTGGCACGTAGTAA
GTCGGTGCTTATCTGCAGATACCGTCAGCATTATCCCGCATTAAGGGAAGACATTTACATTCTCTGCCACAAGAACTT
TACAATCCCGAAATGCCTACATCATGCATGCCGGCATGCCTGATCATGGCTTACCCCTATTGCCCAAAAATACCCCCT
GCCTGC

Fig.6. Phylogenetic analysis of msb 55: showed 97% similarity with Rhodopseudomonas palustris

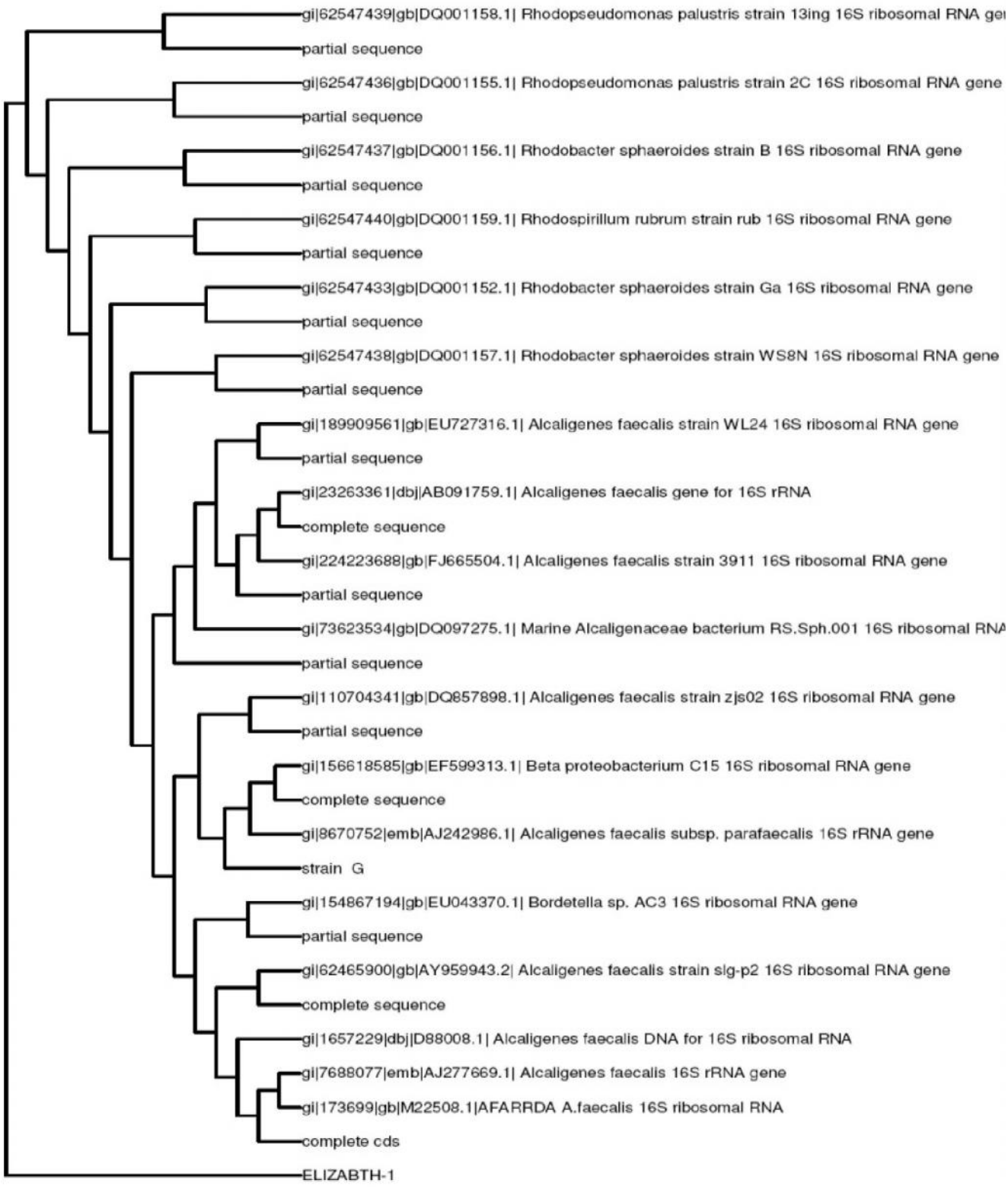


Fig.7. Phylogenetic analysis of MSB 57: showed 94% similarity with Rhodobacter sphaeroides

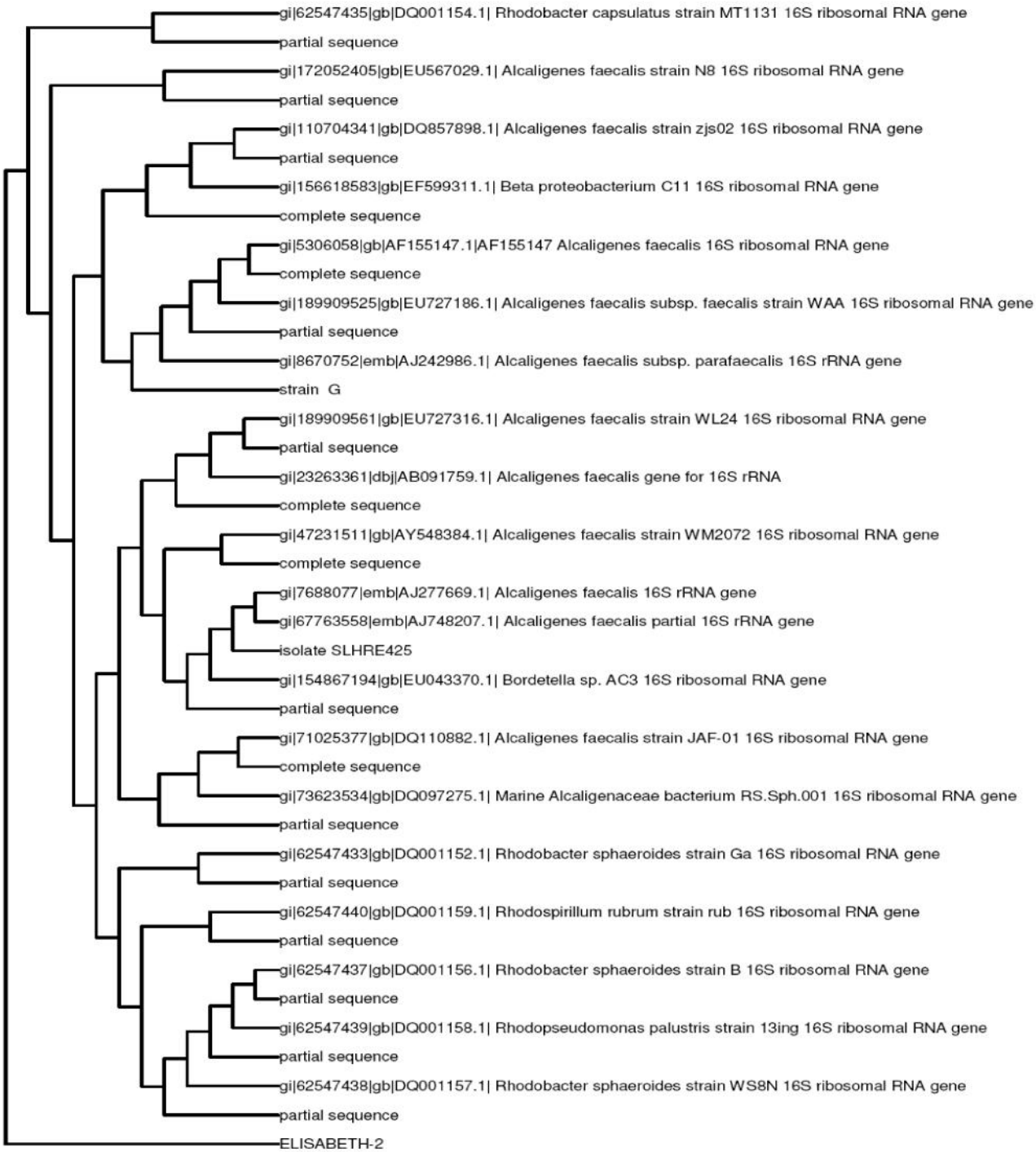


Fig. 8. ORF finder

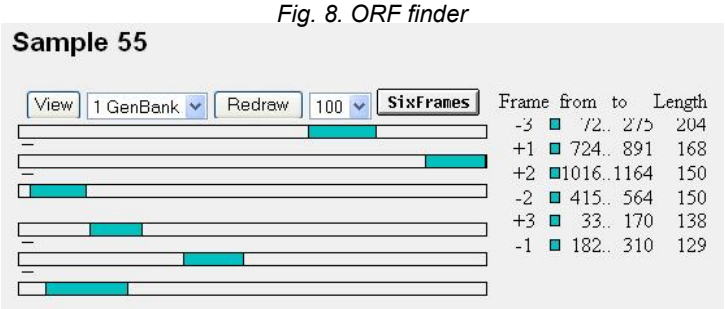


Fig. 9.BPROM result

```
>Sample 55
 Length of sequence-      1165
 Threshold for promoters -  0.20
 Number of predicted promoters -      3
 Promoter Pos:   1094 LDF-  4.96
 -10 box at pos.   1079 CTTTACAAT Score    66
 -35 box at pos.   1057 TTTACA    Score    47
 Promoter Pos:    227 LDF-  2.56
 -10 box at pos.    212 GACTACGAT Score    39
 -35 box at pos.    195 TTGCAG    Score    49
 Promoter Pos:    772 LDF-  2.54
 -10 box at pos.    757 CAGTATTAT Score    62
 -35 box at pos.    734 CTCACG    Score     5

 Oligonucleotides from known TF binding sites:

 For promoter at   1094:
       ompR:  TTTACATT at position    1057 Score -  11
       rpoE:  ACTTTACA at position    1078 Score -  10
       tyrR:  TTTACAAT at position    1080 Score -   8
 For promoter at    227:
      rpoH3:  CTCCCCCT at position     238 Score -   9
 For promoter at    772:
       arcA:  TCATGTTT at position     722 Score -   6
       soxS:  TTGCTCCT at position     728 Score -   9
```

Interpretation: Three Promoter regions and transcription factor binding sites were found at the 1094,227,772 position.

Fig.10. GENSCAN result

```
Sequence Sample : 1146 bp : 51.62%
C+G : Isochore 3 (5100 - 57.00 C+G%)
Parameter matrix: HumanIso.smat
```

Fig. 11.ORF FINDER

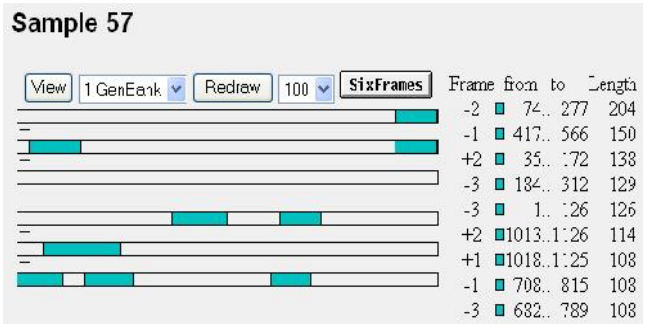


Fig.12.BPROM

```
>Sample 57
 Length of sequence-      1127
 Threshold for promoters -  0.20
 Number of predicted promoters -      3
 Promoter Pos:    541 LDF-  3.49
 -10 box at pos.    526 AGGTAAGGT Score    42
 -35 box at pos.    506 TTTCCA    Score    42
 Promoter Pos:    229 LDF-  2.56
 -10 box at pos.    214 GACTACGAT Score    39
 -35 box at pos.    197 TTGCAG    Score    49
 Promoter Pos:    882 LDF-  1.99
 -10 box at pos.    867 AGTTAAAAA Score    43
 -35 box at pos.    847 CTGACA    Score    31

 Oligonucleotides from known TF binding sites:

 For promoter at    541:
       glpR:  TCGAATTA at position     548 Score -   9
 For promoter at    229:
      rpoH3:  CTCCCCCT at position     240 Score -   9
 For promoter at    882:
      rpoH2:  ATTCTACC at position     834 Score -   8
```

Interpretation: Three Promoter regions and transcription factor binding sites were found at the 541,229,882 position.

Table 1. Difference between MSB 55 & MSB 57

S.NO	Analysis	MSB 55	MSB 57
1.	ORF FINDER	FRAME 3 from reverse strand is the best ORF which potentially encodes a protein	FRAME 2 from reverse strand is selected as the best ORF which potentially encodes a protein
2.	BPROM RESULT	Three Promoter regions and transcription factor binding sites were found at the 1094,227,772 position.	Three Promoter regions and transcription factor binding sites were found at the 541,229,882 position.
3.	GENSCAN	51.62% C+G Two exonic region was found which is an initial exonic region at the positions 519,931	No peptides predicted
4.	FGENESH	One gene was found at the position from 3 to 170. GAPCYDFTPVMNPTLGSGLLTVRLPTSGETH SHGVTGGVYKTRERIHRDILIRDY	One gene was found at the position from 35 to 172 MNPTVGSALLTVRLPTSGETHSHGVTGG VYKTRERIHRDILIRDY

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