Twenty First Indian Expedition to Antarctica, Scientific Report, 2007
Ministry of Earth Sciences, Technical Publication No. 19, pp 125-133

Isolation and Molecular Characterization of Sea Ice Microbial Communities of East Antarctica

V. Wilsanand

Environmental Biotechnology Division, Rajiv Gandhi Centre for Biotechnology
Jagathy, Thiruvananthapuram-14, Kerala, India

Abstract

During the XXI Indian Antarctic Expedition bacterial samples have been collected from different habitats of Antarctica. A total of 150 pure isolates were obtained by streak plate method. Of these, 64 isolates are marine. Morphological, biochemical, physico-chemical and molecular characterization of the all the strains have been completed. Studies on the salt and temperature tolerance of the marine isolates indicate that all the strains are halotolerant psychrotrophs. Studies on the pH tolerance revealed that they are neutrophiles and tolerated a pH range of 4 to 11.8. PCR based RFLP analysis of various strains indicate that there are 13 genetically different marine species. Partial sequencing of 16 S rRNA of all the RFLP differentiated strains have been completed. Blasting and species identification will be possible only after sequencing the complete 1538 bp gene, which is in progress.

Keywords: Antarctica, Bacteria, Halotolerant, Marine, Microbes, Psychrotrophs, Pack ice, Sea ice.

Introduction

Oceans cover about 70% of the earth's surface and harbour a myriad of organisms. Marine microorganisms are of enormous scientific interest since they have adapted to extreme environments ranging from the cold polar seas at -2°C to the great pressures of the ocean floor. Yet the potential of this domain as the basis for new biotechnologies remain largely unexplored. In fact, the vast majority of the marine microorganisms have yet to be identified. Even for known organisms, there is insufficient knowledge to permit their intelligent management and application. It is supposed that only 1% of the microorganisms living in the marine environment have yet been discovered.

Sea ice is an interesting feature of Polar Regions. The largest expanse of sea ice occurs in the southern ocean. Unlike freshwater ice, sea ice forms a semisolid matrix, permeated by a net work of channels and pores filled

with brine formed from expelled salts as the ice crystal freeze together. Recent studies have demonstrated that the sea ice is a microcosm on its own (Brown & Bowman 2001, Staley &Gosink 1999). Many microorganisms can stand the low temperature and high salinities of the brine channels and pockets within the porous sea ice. Many planktonic organisms including viruses, bacteria, algae, protists, flatworms and small crustaceans stick to ice crystals and become trapped within the brine channels. In recent years, the study of sea ice organism has intensified by the realization that the physiological and biochemical acclimatization that microorganisms thriving in the ice have to undergo may have considerable potential for biotechnological applications. Apart from their ecological and biotechnological importance, the bacteria and algae found in the sea ice have become the focus for new breed of astrobiologists who are enthusiastically scrutinizing the ice covered extraterrestrial bodies for the possibility of existence of life. Bearing these in mind, in the present investigation an attempt has been made to study the biotechnological applications of Antarctic microbes in general and Sea Ice Microbial Communities in particular and the preliminary results obtained are presented here.

Materials and Methods

Sampling and Isolation

During the XXI Indian Antarctic Expedition (summer) conducted during December 2001 - March, 2002, bacterial samples were collected from sea ice and waters of East Antarctica. During the sampling period, the average temperature was -2 °C. The samples were grown in nutrient Broth at 16 °C. They were spread on nutrient agar plates and pure colonies were isolated by streaking. Pure isolates were stored in refrigerator and transported back to the Laboratory in India in refrigerated conditions.

Morphological, biochemical and physiological characterization

The following morphological, biochemical and physiological characterization were examined: Cell morphology; gram staining; motility; oxidase and catalase production; indole production; MR-VP (Methyl Red-Voges Proskauer) test; citrate utilization; nitrate reduction; ammonia and hydrogen sulphide production; oxidation and fermentation; sugar fermentation; salt, pH and temperature tolerance following the IMTECH

manual for identification of microbes. Gram reaction was determined using the Hi Media gram stain kit according to the manufacturer's instructions. Motility of the culture was determined by hanging drop technique. Catalase activity was determined by bubble formation in a 3% hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% tetramethyl -p-phenylenediamine. Oxidation and fermentation was carried out as described by Hugh and Leifson. Tolerance of NaCl was measured in nutrient broth containing various percentages of NaCl. Growth at various temperatures was measured in nutrient agar plates kept at 0, 4, 10, 16, 20, 25, 37 and 45°C. The pH range for growth was determined in nutrient broth with pH values of separate batches of medium adjusted to 2, 4, 5.7, 6.8, 9, and 10.5. The pH was adjusted with 10 M NaOH or HC1.

Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of 16S rRNA

A 1,538 -bp 16S rRNA gene, was amplified from all the strains by using the universal primers

Bacterial 16S forward primer:
5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3';
Bacterial 16S reverse primer
5'-CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3'.

Prior to amplification, bacteria were grown for 24 h in nutrient broth on a rotary shaker at 200 rpm at 20°C. Cells were then harvested by centrifugation and suspended in ultra pure RNAse DNAse free distilled water. The cells were then heated at 95°C for 4 min and centrifuged at 12,000 rpm for 4 min. The supernatant was used for PCR reactions. Amplification was conducted in a final volume of 50 uL with 0.5 uL of bacterial suspension (optical density (OD600) of 0.5), 0.2 uM of each primer, 1.0 mM MgC12, 200 uM dNTPs, and 2.5 U of *TaqTM* DNA polymerase. Amplifications were carried out in a thermo-cycler (eppendorf Mastercycler gradient) with the following program: initial lysis of bacterial cells and denaturation for 5 min at 95°C, 30 cycles of denaturation (30 s at 95°C), annealing (30 s at 45°C), extension (2 min at 72°C), final extension (10 min at 72°C). Amplification of DNA was confirmed by electrophoresis in 1% (w/v) agarose gels. Gels were stained in an aqueous solution of 10 mg/ml ethidium bromide and photographed using Biorad gel doc system. PCR products were digested with the restriction endonucleases *Hae II, Hae III, Hind III, EcoRI, Bam HI.* Ten microlitres of PCR products were digested with 2 U of the respective restriction enzyme for specified hours and temperatures by the manufacturers. The

digestion products were separated by electrophoresis on 3 % agarose gels/ and or % acrylamide gels. Gels were stained with ethidium bromide and photographed using Biorad gel doc system. Pattern analysis of DNA fragments was used to differentiate organisms.

16S rRNA Sequence Determination

The RFLP differentiated PCR amplified 16S rRNA product was separated by electrophoresis on a 1% agarose gel dissolved in IX TAE buffer containing ethidium bromide. The DNA was visualized under UV light and the 1538 bp fragment was excised from the gel. The DNA fragment was then purified using GFX™ PCR DNA and Gel Band purification kit supplied by Amersham Biosciences. The purified products was subjected to PCR reactions using the same forward and reverse primers and sequenced using automated DNA sequencer, Applied Biosystems. Based on the sequencing data obtained with the above primers further internal oligonucleotide primers were designed and used for sequencing the complete 1538 bp. The BLAST database of the National Center for Biotechnology Information (NCBI)/ribosomal database project, Michigan state university was used to compare resolved sequences of the isolates.

Results and Discussion

During the XXI Indian Antarctic Expedition bacterial samples have been collected from different habitats of Antarctica. A total of 150 pure isolates were obtained by streak plate method. Of these, 64 isolates are marine. Data on nature and location of sample collection is given in Table 1. Agarose gel showing PCR amplification of 1538 bp gene and RFLP analysis of selected strains are presented in Fig. 1 and Fig. 2 respectively. Distribution of various genetically differentiated strains is given in Table 2. Results on the morphological and biochemical characterization

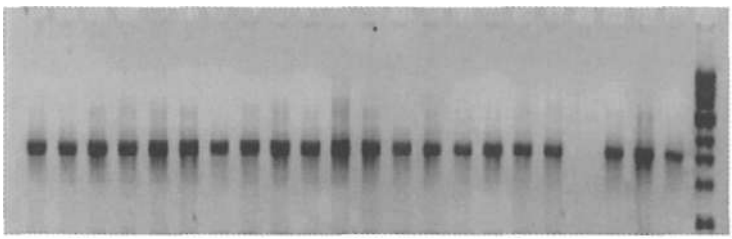

Fig. I: Gel showing amplification of16S rRNA gene of various strains (extreme right lane is lkb ladder Marker)

Table 1: Nature and location of sample collection

Site/location	Sampling site	Location
SL1	Antarctic waters	67° 06″ 86″ S; 12° 35′ 61″ E
SL2	Antarctic waters	69° 51′ 57 ″ S; 12° 43′ 60″ E
SL3	Indian Bay seawater	69° 56′ 47″ S; 11° 55′ 23″ E
SL4	Russian bay seawater	70° 02′ 23″ S; 11° 37′ 15″ E
SL5	Indian ice shelf	69° 59′ 18.93″ S; 11° 59.06′ 8″ E
SL6	Lazarev bay ice shelf	70° 03′ 37″ S; 12° 33′ 50″ E
SL7	Russian bay ice shelf	70° 03′ 23″ S; 019° 37′ 15″ E
SL8	Sea ice	69° 51′ 57″ S; 12° 43′ 60″ E
SL9	Sea ice	69° 56′ 47″ S; 11° 55′ 23″ E.

Table 2: Distribution of bacterial species identified in Eastern Antarctica

	Site/location								
Species	SL1	SL2	SL3	SL4	SL5	SL6	SL7	SL8	SL9
M1	+	+	+	+	+	+	+	+	+
M12	-	-	+	+	-	-	-	-	-
M14	-	-	-	+	-	-	-	-	-
M34	+	-	-	-	-	-	-	-	-
M35	+	-	-	-	-	-	-	-	-
M36	+	-	-	-	-	-	-	+	-
M41	-	-	-	-	-	-	-	+	-
M42	-	-	-	-	-	-	-	+	-
M43	-	-	-	-	-	-	-	+	-
M45	-	-	-	-	-	-	-	+	-
M47	-	-	-	-	-	-	-	+	-
M50	-	-	-	-	-	-	-	-	+
M74	-	-	-	-	+	-	-	-	-
M75	-	-	-	-	+	-	-	-	-
M80	-	-	-	-	+	-	-	-	-

Table 3: Morphology and biochemical characteristics of genetically differentiated marine bacterial isolates of Antarctica

Code	M1	M12	M14	M34	M35	M41	M42	M43	M45	M47	M75	M80	M90
Gm stain	+ ve	– ve	variable	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Morphology	rods	short rods	short rods	rods	short rods	rods	rods	rods	rods	rods	short rods	short rods	short rods
Motility	motile	motile	motile	motile	motile	motile	motile	motile	motile	motile	motile	motile	motile
Oxidase	+	_	+	-	-	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole	_	_	-	-	-	-	-	-	-	-	-	-	-
Methyl Red	_	+	-	-	-	-	-	-	-	-	-	-	-
Voges Proskauer	_	+	+	+	+	+	+	+	+	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+
NO_3Red	+	+	+	+	+	+	+	+	+	+	-	-	-
H_2S Prod	_	-	-	+	+	-	-	-	-	-	_	-	-
NH_3 prod	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidation	_	+	+	+	+	+	+	+	+	+	-	-	-
Fermentation	_	+	+	-	+	+	+	+	+	-	-	-	-
Glucose	_	+	+	+	+	+	+	+	+	-	-	-	-
Sucrose	_	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	_	+	+	-	-	_	_	-	-	-	-	-	-
Mannitol	_	_	-	+	-	_	_	-	-	-	-	-	-

Table 4: Tolerance limit of genetically differentiated Antarctic marine bacteria to various physico-chemical parameters

Code	Salt Tolerance (%)				pH tolerance								Temperature Tolerance (^{O}C)							
	0	7	10	25	2	4	5.7	6.8	9	10.5	11.8	14	0	5	10	14	20	25	37	45
M1	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-
M12	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
M14	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
M34	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
M35	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
M41	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
M42	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
M43	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
M45	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
M47	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
M75	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
M80	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
M90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

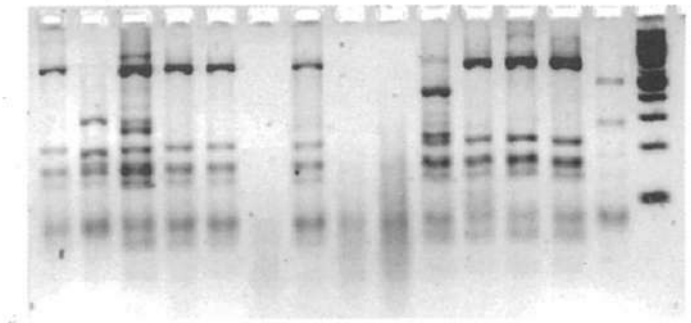

Fig. 2: Gel showing PCR based RFLP analysis of some marine strains (extreme right lane is Marker)

of the genetically differentiated strains are presented in Table 3. Tolerance limits of these genetically differentiated strains to various physico-chemical parameters are given in Table 4.

Interestingly all the marine isolates tolerated a pH range of 4 to 11.8. Few strains (M35, M42, M45, M47, M80 and M90) tolerated upto a pH of 2. Notably strain M90 tolerated a pH range of 2 to 14. All the marine strains tolerated 25% salt concentration. Surprisingly, all of them have grown in zero salt concentration also. This indicates that all the marine strains are halotolerants. Results on temperature tolerance indicate that all the strains are psychrotrophs. All of them have grown at 0°C and near room temperature. Expect seven strains all other strains studied got amplified by the universal primers used. The 7 strains could not amplify even with two other primers studied. Preliminary RFLP results indicate the presence 16 genetically different marine bacterial species. Partial sequencing of all the 16 species were carried out. Since the total 1538 bp was not sequenced, identification of the species is incomplete. Blasting and species identification will be possible only after sequencing the complete 1538 bp gene, which is in progress.

Tolerance of high salt concentrations, low temperature and extreme pH require unique adaptation strategies leading to new natural products, which are different from known structures of terrestrial organisms. These new marine products may have enormous potential for various biotechnological applications and studies in these directions are in progress.

Acknowledgement

I am extremely grateful to the Department of Ocean Development, Govt, of India and Director, National Centre for Antarctic and Ocean

Research for giving an opportunity to participate in the XXI Indian Antarctic Expedition. I am also thankful to the Director, Rajiv Gandhi Center for Biotechnology, Trivandrum, India, for the facilities provided to carry out the work.

References

Brown M.V., Bowman J.P., (2001). A molecular phylogenetic survey of sea-ice microbial communities (SIMCO). FEMS Microbiol. Ecol. Vol. 35, pp 267-275.

IMTECH (1995). Basic Microbiology Laboratory Mannual. MTCC, IMTECH, pp 74.

Staley J. T. , Gosink J. J., (1999). Poles apart: biodiversity and biogeography of sea ice bacteria. Annu. Rev. Microbiol., Vol. 53, pp 189-215.