

# Molecular Phylogeny of Arctic Microbes Using Metagenomic Approach

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## Abstract

Metagenomic approach to the characterization of uncultured and mixed bacterial consortium has been used in this study for a few arctic micro-organisms. Arctic soil, collected from one spot at Ny Alesund in Svalbard was used as the source of such organisms and enriched in culture medium. Only psychrotrophic microbes were chosen which could grow from 5°C to 20°C. Total genomic DNA isolated from the consortium was used as template for amplifying the 16S rRNA genes using the conserved forward and reverse primers. The amplified mixture of 1.4 kb DNA was cloned in pGEM-T Easy vector and individual 16S rDNA sequences were determined by automated sequencing. A phylogenetic tree was constructed using MEGA 5 software which enlists 19 isolates in the consortium. Most of these arctic bacteria were *Bacillus* species as these were isolated from a single spot.

## Keywords

Metagenomics, 16S rRNA, Molecular Phylogeny

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## 1. Introduction

Microorganisms constitute the majority of all life forms present in the biosphere [1]. Their total number is estimated to about  $10^{30}$  [2], while the microbial diversity is estimated to be about  $10^6$  to  $10^8$  [3]. Thus classification of microorganisms, establishing their phylogenetic relationship can enrich our understanding of microbial ecology. Normally, characterizing a microbe involves its isolation in pure state, followed by microbiological and biochemical characterization as per Bergey's manual. However, it is estimated that only 1% of the total bacterial biodiversity is culturable in the laboratory using different media. Metagenomics is the study of the genomes of

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whole communities of microbes. Instead of isolating individual pure colonies on nutrient medium plates, one can analyze the whole consortium of microbes by metagenomic approach. Craig Venter (2004) published one of the pioneering studies in sequencing the Sargasso Sea micro-organisms [4]. Using shotgun sequencing of the mixture of genomic DNAs isolated from Sargasso Sea, it was established that the water contained about 1800 varieties of microbes. The study found 150 new bacterial and Archaea species and more than 1.2 million new genes. Recent approach of metagenomics involving direct isolation of 16S rRNA gene and sequencing has proved to be a powerful technique both for studying diversity as well as to look out for functional aspects [5]-[7]. Pace *et al.* [8] first reported direct cloning of environmental DNA using this approach.

Here we describe the extraction of DNA from the enrichment culture of Arctic soil samples, the PCR amplification of 16S RNA genes, the cloning of these genes in pGEM-T Easy vector, the sequencing using universal primers of SP6 and T7 promoters present in the vector and their phylogenetic analysis using bioinformatic tools like MEGA 5 software.

## 2. Materials & Methods

**Soil Samples:** The soil samples were collected from Ny Alesund (79°N, 11°E), Svalbard, Norway under a project sponsored by NCAOR (National Centre for Antarctic and Ocean Research, Goa, India). 1 gm soil sample was incubated in 100 ml Luria broth at 20°C for 48 hours. About 50 µl of the culture was transferred to 5 ml Luria broth for enrichment. Bacterial cells were harvested thereafter.

**DNA Extraction:** Bacterial cells were suspended in 1 ml glucose Tris EDTA buffer (25 mM glucose, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA). 50 µl of Lysozyme of concentration 10 mg/ml was added to it, this mixture was kept at 37°C for 30 minutes. SDS was then added to 1% concentration and kept for 1 hr at 37°C. Phenol-Chloroform mixture (1:1 v/v) was added in equal volume to the mixture. The mixture was shaken for 5 min, and then centrifuged at 10,000 rpm for 10 minutes. The upper layer was taken and the phenol-chloroform extraction was repeated, again the upper layer was taken. 3 M Na-acetate, pH 5.1 was added at 1/10<sup>th</sup> the volume of the above extract, and double volume cold ethanol was added, the mixture was kept overnight at -20°C.

The mixture was centrifuged at 10,000 rpm for 10 min; the pellet was treated with 70% ethanol and was vortexed. Centrifugation was done at 10,000 rpm for 5 minutes. The pellet so obtained was dried at 37°C. It was dissolved in 0.25 ml TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 10 µl of the above was tested on 0.8% agarose gel electrophoresis (0.2 gm agarose in 25 ml 1X TAE buffer (40 mM Tris-Acetate, pH 8.3 and 1 mM EDTA) and 1 µg/ml Ethidium bromide solution).

**PCR for Amplification of 16S rDNA:** A master mixture for 12 tubes containing 25 µl final reaction volume was prepared according to the following composition, 30 µl 10X buffer with MgCl<sub>2</sub> (1.5 mM), 6 µl of 10 mM dNTP, 24 µl universal forward primer (27F: 5'AGA.GTT.TGA.TCC.TGG.CTC.AG3'), 24 µl of reverse primer (1492R:5' TAC.GGY.TAC.CTT.GTT.ACG.ACT.T3') of 20 pmoles/µl concentration, 6 µl Taq Polymerase of 5 units/µl and 186 µl water. The mixture was divided in 12 tubes with 23 µl each, 2 µl of genomic DNA of the mixed consortium as template was added to get the final 25 µl reaction volume. The thermo-cycler was programmed according to the following, 5 minutes of denaturation at 94°C followed by 30 cycles at 94°C for 1 minute, 45 seconds at 52°C annealing and 72°C for 3 minutes of extension time. The product was tested on 1.5% agarose gel electrophoresis.

### 2.1. pGEM-T Vectors

The pGEM-T Easy vector (Promega Corp) was used for the construction of recombinant plasmids. pGEM-T vectors are linearized vectors with a single 3' thymidine at both the ends. The overhanging T ends prevents recirculization of vectors and is complementary to the extra A residue at the 3'-ends of the products of PCR [9] [10]. The pGEM-T vectors have T7 and SP6 phage promoters flanking multiple cloning sites within  $\alpha$ -peptide coding region of the enzyme  $\beta$ -galactosidase. Insertional inactivation of  $\alpha$ -peptide region allows selection by blue/white method, using Isopropyl thiogalactoside (IPTG) as the inducer and 5-Bromo, 4-chloro, 3-Indolyl  $\beta$ -galactoside as the chromogenic substrate.

**Ligation of PCR Amplicons of 16S rDNA:** 20 µl of amplicons were taken and 2.5 µl of 10X buffer with ATP was added to it. 1 µl of pGEM-T Easy vector was added to the mixture followed by 1.5 µl of T4 DNA ligase. The mixture was kept overnight at 6°C [9]. An aliquot was tested on 1% agarose gel electrophoresis.

## 2.2. Transformation of *E. coli*

XLI Blue (Tet<sup>r</sup>) strain was taken and inoculated in Luria broth and shaken overnight. 1 ml of the culture was inoculated in 50 ml Luria broth and was kept in shaker incubator at 37°C till the mid log phase ( $A_{600} \approx 0.5$ ). The culture was aseptically centrifuged at 8000 rpm for 10 minutes. Cell pellets so obtained was suspended in 10 ml of 0.1 M CaCl<sub>2</sub> followed by centrifugation at 8000 rpm for 10 minutes. The cell pellet was resuspended in 1 ml of 0.1 M CaCl<sub>2</sub>. 0.1 ml aliquot was taken in sterile Eppendorf tube, 5 µl of PCR product ligated to pGEM-T Easy vector (Promega) was added for transformation.

The above mixture was kept at 4°C for 30 minutes. Heat shock at 42°C was given for 45 seconds. 0.5 ml of Luria broth was added to the tube and was incubated at 37°C for 45 minutes in an incubator shaker. 0.1 ml aliquot was placed on LB-Ampicillin plates and was incubated overnight at 37°C. Blue and White colonies were observed. Plasmid preparations from individual white colonies were done using the alkaline lysis method [9] [11] followed by phenol extraction and alcohol precipitation [10].

## 2.3. Sequencing

The insert sequences of 16S rDNA in different cloned plasmids were sequenced. The sequencing was outsourced from the companies, Biobharti at Salt Lake, Kolkata and Xcelris at Ahmedabad, India. The primers used for the sequencing reactions were either SP6 promoter or T7 promoter sequences, which flank the inserts in opposite orientation.

## 2.4. Methodology for Bioinformatic Analysis

Nineteen different sequences of 16S rDNA were deposited at NCBI. The sequences were published with accession number KF711832-KF711850. To determine the closest resembling species of the new isolates, n-BLAST was performed with the NCBI database and the sequences with maximum e-value with 100% query coverage were used for outgrouping [12] [13]. The sequences were analyzed by MEGA 5 software, where the sequences in Fasta format were aligned by ClustalW and the alignments were refined manually using the TrimaAl tool. Gaps and ambiguously aligned positions were excluded. Phylogenetic tree was constructed using the MEGA 5 software by the statistical maximum-likelihood method. The bootstrap analysis was carried out on 1000 replicates. Phylogenetic tree was constructed using the Tamura Nei substitution model with gamma distributed (G) using eight rate categories. The detailed phylogenetic tree is appended in the supplement.

## 3. Results & Discussion

### 3.1. Amplification of 16S rRNA Genes from the Arctic Consortium

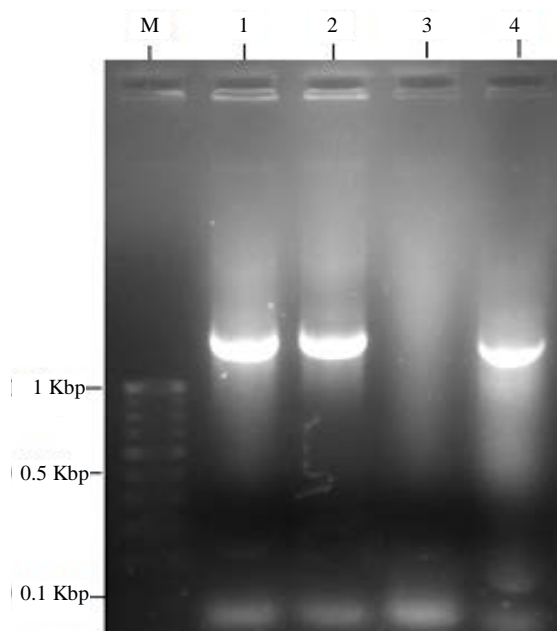
Using the universal forward and reverse primers for bacterial 16S rDNA sequence, the amplification product with the genomic DNA of the consortium as template was observed in 1.5% agarose gel electrophoresis. As expected this showed a single DNA band of 1.4 kb size (Figure 1).

### 3.2. Cloning of the Mixed 16S rDNA Sequences

The different sequences present in the amplified 16S rDNA product were separated by cloning in A:T vector in *E. coli* XLI-Blue doing the blue-white selection on Ampicillin-X-gal-IPTG plates. The blue colonies arise from cells carrying just the vector and white colonies contain vector with inserts. Randomly picked white colonies were processed for plasmid isolation and sequencing in automated sequencer.

### 3.3. Submission of 16S rDNA Sequences and Phylogenetic Analysis

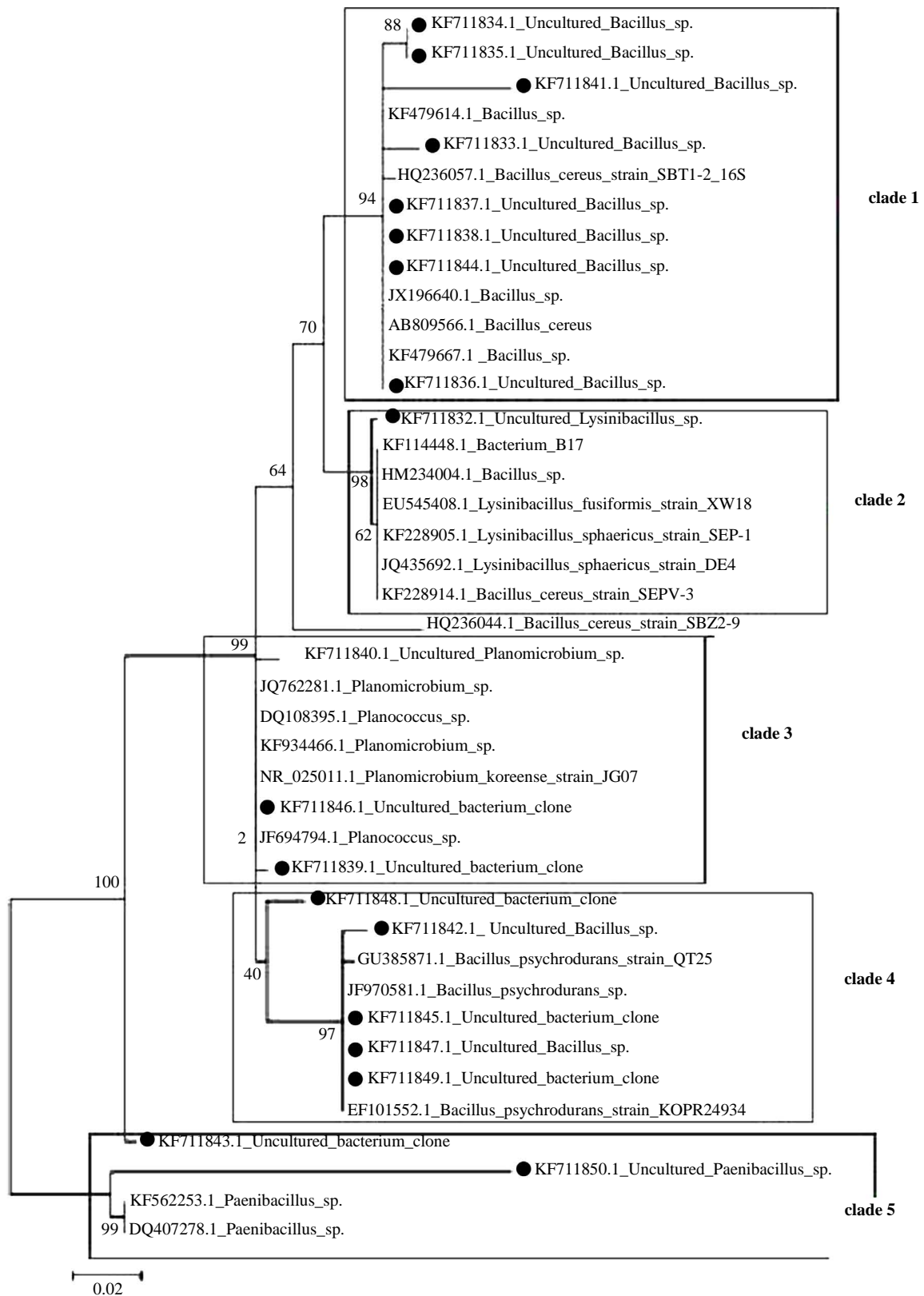
All the DNA sequences generated from the inserts of the recombinant pGEM-T vector were submitted to the NCBI database and the accession numbers were given to 19 such independent sequences (Table 1). A phylogenetic analysis was conducted using maximum likelihood for the 19 dataset of 16S rDNA sequences belong to uncultured and mixed bacterial consortium. A general substitution model (GTR) was applied for analyses. The analyzed 19 dataset of metagenomics dataset of mixed bacterial consortium revealed the occurrence of the major genus belong to the order *Bacillales* and least number of genus belong to order *Hemiptera*. The dominant group



**Figure 1.** PCR amplified 16S rDNA products from three random mixed genomic DNA samples from Arctic region (Lanes 1, 2, 4). The lane marked M is 100 bp ladder and lane 3 is controlled without any template DNA.

**Table 1.** Accession numbers provided by NCBI for the isolates from Arctic sample.

Accession number	Specie identified
Clade 1	
KF711834.1	Uncultured Bacillus sp
KF711835.1	do
KF711841.1	do
KF711833.1	do
KF711837.1	do
KF711838.1	do
KF711844.1	do
KF711836.1	do
Clade 2	
KF711832.1	Uncultured Lysinibacillus sp
Clade 3	
KF711840.1	Uncultured Planomicrobium sp
KF711846.1	Uncultured Bacterium clone
KF711839.1	do
Clade 4	
KF711848.1	do
KF711842.1	Uncultured Bacillus sp
KF711845.1	Uncultured Bacterium clone
KF711847.1	Uncultured Bacillus sp
KF711849.1	Uncultured Bacterium clone
Clade 5	
KF711843.1	Uncultured Bacterium clone
KF711850.1	Uncultured Paenibacillus sp



**Figure 2.** Phylogenetic tree of the arctic microbes.

in clone library is belonging to order *Bacillales* which was profound revealed the supremacy of the genus *Bacillales* phylotypes in soil samples from Ny Alesund (collected location). The 16S rDNA analyses revealed the 99% affiliation of the gram positive bacteria of order *Bacillales* to *Pseudococcidae*. The genera represented in the study are the members of *Bacillales* i.e., *Bacillus*, *Lysinibacillus*, *Placomicrobium*, *Paenibacillus* were found to cluster significantly with the possibly similar outgroups (Figure 2).

All these genera belonging to the gram positive bacteria, namely KF711833, 34, 35, 36, 37, 38, 41, 44 were clustered in clade 1 which showed 88% and 94% similarity. Clade 4 showed the clustering of the sequences KF711842, 45, 47, 48, 49 which share the phenotype similar to the *Bacillus* with 97% similarity. These organisms are aerobic or facultative anaerobic and thrive well in the adverse environmental conditions, as in lack of elements such as phosphorus, nitrogen, or oxygen combined with an excessive supply of carbon sources which substantiates its dominant existence in the regions around Ny Alesund.

KF711832 branched deeply in the clade 2 along with the Genus *Lysinibacillus* and *Bacillus*, however, it shares 98% similarity with *Lysinibacillus* when compared to *Bacillus* genus. *Lysinibacillus* is a common environmental organism which is typically a facultative anaerobe that is resistant to heat, chemical and ultraviolet lights. The members of *Planomicrobium*, apparently omnipresent, grouped in clade 3, its presence is seemingly second to *Bacillus*. This organism currently accommodates the aerobic, gram-positive bacteria. In clade 5 the sequences KF711843 and KF711850 were clustered with Genus *Paenibacillus* with 99% similarity. *Paenibacillus* is a genus of facultative anaerobic which is capable of fixing nitrogen, pattern formation and self-organization. It reflects social behaviors of bacteria act as a scratch for the evolutionary development of the collective action of cells in higher organisms. Possibly all isolates identified are facultative anaerobic bacteria which reflects the characteristic of sampling site. The anaerobic conditions developed temporarily may due to the presence of melting ice.

The present study provides the valuable baseline data on the microbial communities in the arctic region around Ny Alesund (79°N, 11.8°E), Svalbard, Norway. The phylogenetic analyses would enable us to understand the bacterial diversity in the selected area. The organisms identified in the area are non-affiliated genera, which substantiates that the microenvironment can be a “hot-spot”. Analyzing the bacterial communities through phylogenetic analyses may lead to the identification of new organisms with cold adaptation mechanism, which may act as a source for the novel genes and products.

## 4. Conclusion

The present study is aimed at identifying uncultured bacteria from a virgin region of the earth, i.e. North polar soil collected from the northernmost habitable place on earth at 79°N and 11.8°E. Peculiarly, most of the isolates turned out to be *Bacillus* or *Lysinibacillus* species. Metagenomic approach provides a powerful tool to isolate and characterize such unculturable species from environmental samples.

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## Declaration

There is no conflict of interest among the authors regarding the contents of this paper.

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