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Full Length Research Paper

Monitoring of marine *Bacillus* diversity among the bacteria community of sea water

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Bacillus **species are ubiquitous and diverse both in the terrestrial and marine ecosystems. Efforts were made to enrich for marine *Bacillus* using the medium containing manganese that stimulate spore germination an indication of manganese reduction by *Bacillus*. Two strains identified as *Bacillus pumilus* using 16S rRNA gene sequence were isolated from Buzzers Bay seawater at 45 feet dept. The strains spores were centrally located and they were able to tolerate 1.0 M NaCl concentration indicating their marine origin. Although, different enrichment medium tested could not support growth of different species of *Bacillus* in the seawater. The result of the analysis of the 16S rRNA gene sequence of the clone library showed that the bacteria in the seawater are diverse. The diversity of bacteria in the seawater was also indicated by the FISH probe signals showing that marine bacteria at 33-45 dept in Woods Hole sea water are diverse.**

Key words: Bacillus, marine, 16S rRNA, genomic, enrichment.

INTRODUCTION

The genus *Bacillus* comprised a phylogenetically and phenotypically diverse species; they are ubiquitous in terrestrial and freshwater habitat and are also widely distributed in sea water (Ruger, 1989). Members of this genus are heterogeneous in nutritional requirement; *Bacillus* from sea water and marine bottom deposit are moderately halotolerant. They are able to propagate and metabolize under marine conditions. They were previously differentiated form terrestrial strains by their requirement of seawater medium only for growth. Ruger and Hentzschel (1980) described true marine *Bacillus* as strains that are dependent on sodium and potassium ion for growth.

Systematic studies of Bacillus have always focused on terrestrial *Bacillus*, although marine *Bacilli* are noted for their ability to produce different antibiotic, glucanase and cyclic acylpeptides. Also, scientific information is available on the manganese reduction potential of *Bacillus* (Vrind et al., 1986). Study of the small-subunit ribosomal RNA sequences has facilitated the split of the species in the genus *Bacillus* into four distinct clusters (Ash et al., 1991). Efforts have also been made to characterize marine *Bacillus* strains using different phenotypic techniques. However, these techniques are often subject to errors. The few available information on the use of genomic techniques has always focused on the culturing of the organisms before identification. The limitations of these techniques include inability of most of the defined medium to support growth of all the species in an ecosystem and the fact that some marine strains are typically difficult to culture (Ivanova et al., 1999).

However, there is a need for proper understanding of the diversity of marine *Bacillus* using modern molecular techniques. This will be highly relevant to isolation of novel strains with desirable functional characteristics and biotechnological applications. Techniques that involve, extraction of total genome from the samples and subsequent PCR amplification of 16S rRNA gene of the bacteria community using species specific primers target has been used previously to study filamentous bacteria community in a mesotrophic lake (Pernthaler et al., 2004).

Therefore this work is aimed at characterization of *Bac-*

Table 1. The Primer and Probe specificity and sequences used in this study.

Primer/Probe	Sequence Target position(5´ 3´)	(*E. coli* length, nt)
16S-8f	AGA GTT TGA TCM TGC	-
16S-1492 Rev	TAC CTT GTT AYG ACT T	-
Bs16Sr	AAC AGA TTT GTG GGA TTG GC	1275
Bc16Sf	TTC GAA CCA TGC AGT TCA AA	173
5´/5Cy3	AAC AGA TTT GTG GGA TTG GC	1275
EUB	GCT GCCTCCCGTAGGAGT	-

illus strains from Woods Hole deep seawater using the combination of culture and non culture techniques with the following objectives.

Materials and Methods

Sampling

Sew water were obtained from 33 feet and 45 feet dept at Buzzers Bay and Sounds Vineyard at Woods Hole, Massachusetts, USA. Samples were transported into the laboratory and pHs of the samples were determined.

Enrichments

Samples were concentrated by centrifugation and the supernatant discarded. 20 ml of the lower residue was pasteurized at 80°C for 30 min to eliminate non endospore forming bacteria. Samples were diluted serially up to 10^5. The K medium described by Roson and Nealson (1982) containing 80% natural sea water, 2 g peptone, 0.5 g yeast extract, 10 µg EDTA and 100 µg $MnCl_2.4H_2O$ per 100 ml was used as the isolation medium. Plates were incubated at room temperature for 48 h. Colonies with distinct morphology were aseptically subculture onto fresh K medium until pure culture were obtained. Pure culture of the isolates was observed under the phase contrast microscopy (Zeiss Axioplan) at 1000x magnification for cell morphology and presence of endospore.

Growth at different NaCl concentration

Isolates were grown in 0.5, 1.0 and 2.0 M NaCl concentrations. Optical density of the culture were determine after 18 h of incubation at 37 °C.

DNA extraction

20 ml of water sample was filter using the 0.2 µm type GTTP Millipore filter (Millipore Ireland), The Ultraclean soil DNA isolation kit (Mo Bio Laboratories USA) was used to extract bacteria genomic DNA directly from the 0.2 µm type GTTP Millipore filter following the manufacturer's instructions.

Polymerase chain reaction and cloning

Amplification of 16Sr RNA gene was by using bacteria primer and *Bacillus* species specific primer (Table 1). The extracted 2.0 µl genomic DNA from the sample and 0.5 µl of the 18 h *Bacillus* culture were used as PCR templates. Master mix for reaction was made such that each reaction contain 25µl total reaction volume of PCR buffer (10x); 200 µM dNTPs (deoxynucleoside triphosphate) Promega USA; 15 pMol forward primer 16S_8f (5´- AGA GTT TGA TCM TGC- 3´) and 15 pMol reverse primer 16SrBs (5´-AAC AGA TTT GTG GGA TTG GC- 3´) (Integrated DNA Technology Inc. USA); 1 U Taq polymerase (Invitrogen USA) and 6.5 µl milliQ water. The PCR reaction was carried out in a thermal cycler (Eppendof Master Cycler) under the following conditions: 95°C for 5 min; 25 cycle of 95°C for 30 s, 46°C for 30 s and extension at 72 °C for 1.5 min. This was followed by a final extension at 72 °C for 5 min.

The amplified rDNAs were cloned into TOPO vector (TOPO TA cloning kit cloning kit; Invitrogen, USA) and transformed into competent cells of *Escherichia coli* as described by the manufacture. The transformed cells were plated on Luria-Bertani agar plates containing 50 µg of kanamycin and incubated overnight at 37°C. The colonies were grown in super broth medium for 18 h, and spin down at 2800 rpm for 8 min. Plasmid were exacted by alkaline lyses and sequenced in Applied Biosystems Sequencer.

Phylogenetic analysis

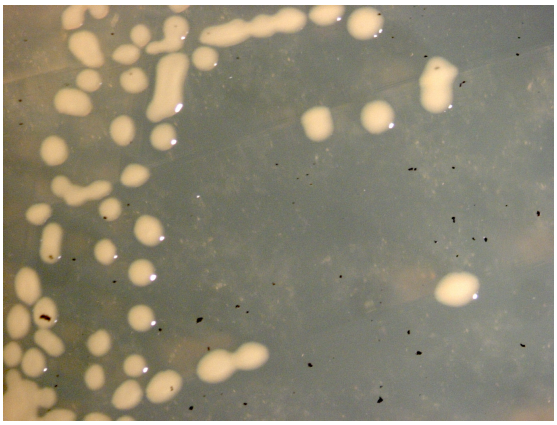
The partial sequence were tested for chimeras through the Ribosomal Database Project CHIMERA_CHECK program. All sequences were again analyzed via Blast to identify their closest relatives. Phylogenetic analyses were performed with the ARB software package (www. Arb-home.de). The ARB database was complemented with sequence from the GeneBank database that was related to the seawater lineages and to marine *Bacillus* cluster. For the reconstruction of a phylogenetic tree, only 16Sr DNA sequences affiliated with this subphylum were considered. 50% base frequency filter was applied to these sequences to perform maximum-parsimony, neighbor-joining and maximum-likelihood analyses. Downloaded partial sequences of the closely related sequence types from marine samples were subsequently added to the consensus tee in accordance with maximum-parsimony criteria, without introducing changes in the topology based on the complete sequences.

Florescence *in situ* hybridization (FISH)

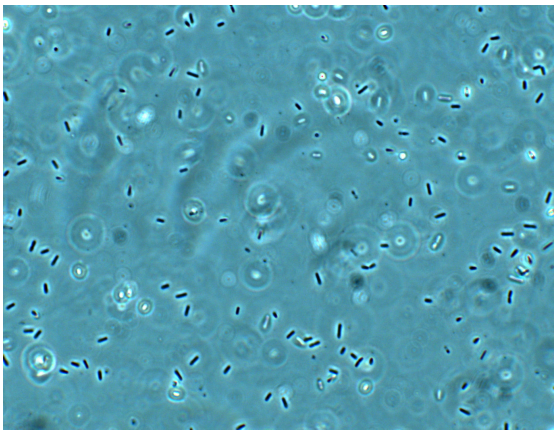
30 ml of water sample was filter using the 0.2 µm type GTTP Millipore filter (Millipore Ireland). Cells were fixed by embedding the filters in 50% ethanol. The filters were added to 300 µl hybridization buffer (5 M NaCl, 1 M Tris-HCl pH 8.0, 10% formamide, 10% SDS) containing 5 ng/µl oligonucleotide probe (Oligonucleotide probe were CY3 labeled, Integrated DNA Technology Inc. USA). The bacteria/hybridization buffer/probe mixture was incubated for 90 min at 46°C to allow hybridization. The mixture was then washed

with washing buffer (5 M NaCl, 1 M Tris-HCl, 0.5 M EDTA, 10% SDS)

The hybridized cells on the filter section were floured with 20 µl of DAPI (4', 6-diamidino-2-phenylindo (DAPI; 1µg/ml) for 3 min and wash in DAPI wash solution, dried and mounted onto a slide using Citifluor glycerol/PBS solution AF (Citiflour Ltd, London) mounting medium. Slides were viewed under oil immersion using the using the Zeiss imager M1 epiflouresence microscope equipped with DAPI and Rhodamine filter and Axion Cam MRC 5 camera. DAPI and Rhodamine image were captured and analyzed.



a



b

Figure 1. (a) Colonies of *Bacillus pumilus* on K medium. (b) Cellular morphology of the isolate under the microscope.

RESULTS AND DISCUSSION

The culture technique used in this study did not bring about isolation of many marine *Bacillus*, although two strains were isolated and identified as *Bacillus* due to their endospore formation and colonial morphology as shown in Figure 1a. The organisms are rod shaped (Figure 1b) and they produced endospore that is centrally located and resemble that of *Bacillus* spp. sensu stricto (Figure 1c). The organisms tolerate 1.0 M NaCl with an average OD of 0.9 at 600 nm wavelength. No growth was detected in the 2.0 M NaCl concentration.

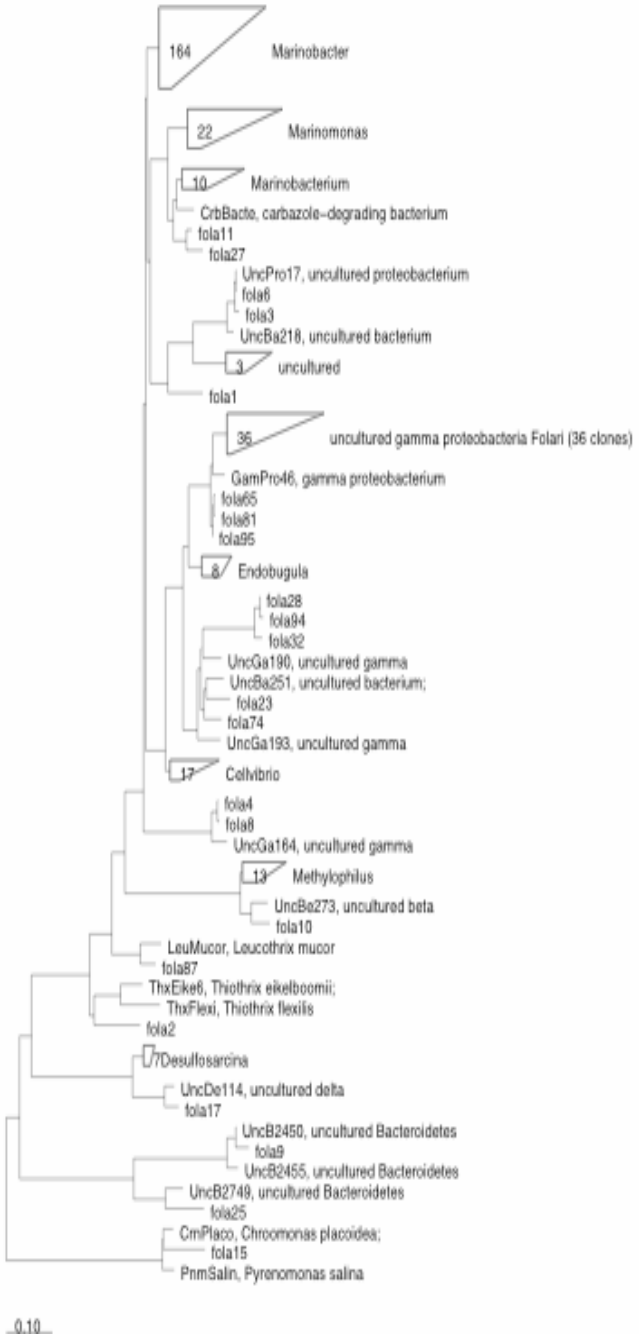


Figure 2. The dendrogram of ARB of the clone library for the bacteria community in seawater

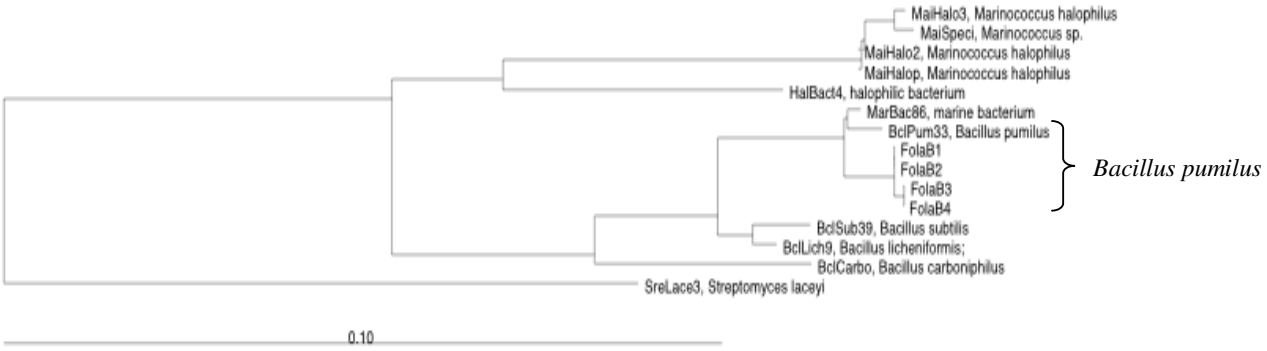
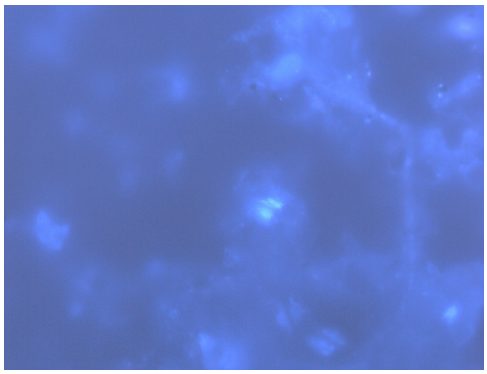
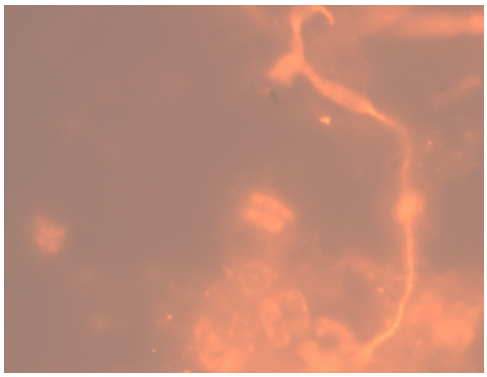


Figure 3. The dendrogram of ARB *Bacillus* species isolated from seawater.



a



b

Figure 4. Photomicrograph of marine bacteria of FISH analysis of the sample (a) DAPI stain and (b) Rhodamine signal of Eub hybridization probe.

The results of the ARB and the blast profile showed that the primers used in this study was not species specific and could not detect *Bacillus* from the 16S rRNA clone library of the community genome (Figure 3). The group of bacteria detected from the clone library is shown Figure 2. However, the 16S rRNA sequencing reveals the identity of the isolate as closest relative of *Bacillus pumilus* and marine bacterium. The bacteria probe used for the FISH analysis also supported the presence of diverse bacteria in the seawater samples (Figure 4a, b).

The results from this work indicated clearly the presence of marine bacilli in the sea water and the limitations of culturing the organism. Although two strains of *Bacillus pumilus* was isolated by the enrichment method used in this study, there is a need to develop novel enrichment techniques to aid isolation of diverse strains of marine bacilli. Species specific primers and probes will be highly useful for the detection of the *Bacillus* community in the seawater. Further investigation will be required to understand the physiology of the *B. pumilus* isolated in this study.

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