Monitory of Marine *Bacillus* among the Bacteria community of the Buzzers bay and Sounds Vineyard Woods Hole Seawater

by

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Abstract

*Bacillus* species are ubiquitous and diverse both in the terrestrial and marine ecosystem. Efforts were made to enrich for marine *Bacillus* using the medium containing manganese that stimulate spore germination and indicated manganese reduction of *Bacillus*. Two strains identified as *Bacillus pumilus* using 16Sr RNA gene sequence were isolated from Buzzers Bay seawater at 45 feet dept. The strains spores were centrally located and they were able to tolerate 1.0M NaCl concentration an indication of their marine origin. Although different enrichment medium could not support growth of many species of *Bacillus* in the seawater. The result of the analysis of the 16Sr RNA gene sequence of the clone library showed that the bacteria in the seawater are diverse, but the primers sets were not able to target marine *Bacillus* specifically. The diversity of bacteria in the seawater was also indicated by the FISH probe signal, this showed that marine bacteria at 33-45 dept in Woods Hole sea water are diverse.
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 the 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 are previously differentiated form the terrestrial strains by their ability to requirement for growth only in seawater medium. Ruger and Hentzschel (1980) described true marine *Bacillus* as strains that are dependent on sodium and potassium ion for growth.

Systematic of *Bacillus* as always focused on terrestrial *Bacillus*, although marine *Bacilli* are noted for their ability to produce different antibiotic, glucanase and cyclic acylpeptides and scientific information is available on the manganese reduction potential (Vrind *et al*., 1986). Study of the small-subunit ribosomal RNA sequences as aided the split of the species in the genus *Bacillus* into four distinct clusters Ash *et al*., (1991). Efforts has also been made to characterize marine *Bacillus* strains using different phenotypic techniques, this techniques are often subject to errors, the few available information on the use of genomic techniques as 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 strains are typically difficult to culture.

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 with biotechnological applications. Techniques that involve, extraction of total genome from the samples and subsequent PCR amplification of 16Sr RNA gene of the bacteria community using species specific primers targeted 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 *Bacillus* strains from Woods Hole deep seawater using the combination of culture and non culture techniques with the following objectives.

To enrich for different marine *Bacillus* strains from seawater samples.

To use combination of cultural and non cultural techniques to study the diversity of *Bacillus* species.

To determine some functional characteristic of selected strains of the marine *Bacillus*. 
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, pH of the samples were determined.

Enrichments

Samples were concentrated by centrifugation and the supernatant discarded, 20ml of lower residue was pasteurized at $80^\circ C$ for 30 mins 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, 10µg EDTA, 100µg MnCl$_2$.4H$_2$O 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 M, 1.0 M and 2.0 M NaCl concentrations, Optical density of the culture were determine after 18 hr of incubation at 37 °C.

DNA Extraction

Twenty milliter of water sample was filter using the 0.2µm 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 0.2µm type GTTP Millipore filter following the manufacturer’s instructions.

Polymerase Chain Reaction
Amplification of 16Sr RNA gene 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 2.5µl PCR buffer (10x); 0.5µl dNTPs (deoxynucleoside triphosphate) Promega USA; 2.0µl forward primer 16S_8f (5´- AGA GTT TGA TCM TGC- 3´) and 2.0µl reverse primer 16SrBs (5´-AAC AGA TTT GTG GGA TTG GC- 3´) (Integrated DNA Technology Inc. USA); 0.2µl Taq polymerase (Invitrogen USA) and 6.5µl milliQ water. The PCR reaction was carried out in a thermal cycler (Eppendof Master Cycler) under the following conditions: in 25 cycle of 95°C for 5min; 95°C for 30 sec, 46 °C for 30 sec. Extension for 72 °C for 1.5 min and 72 °C for 5 min.

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

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

Cloning of the 16S rDNA PCR Product and sequencing

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

Phylogenetic analysis
The partial sequence 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 tree in accordance with maximum-parsimony criteria, without introducing changes in the topology based on the complete sequences.

Florescence in situ hybridization (FISH)

Sample preparation

Thirty milliter of water sample was filter using the 0.2µm type GTTP Millipore filter (Millipore Ireland), Cells were fixed by embedding the filters in 50% ethanol. The filters were added to 300ul hybridization buffer (5M NaCl, 1M Tris-HCl pH 8.0, 10% Formamide, 10% SDS) containing 5ng/ul oligonucleotide probe µl⁻¹ (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)

Fluorescence microscopy

The hybridized cells on the filter section were floured with 20ul 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 epiflorescence microscope equipped with DAPI and Rhodamine filter and Axion Cam MRC 5 camera. DAPI and Rhodamine image were captured and analyzed.
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 1. The organisms are rod shaped Figure 2 and they produced endospore that is centrally located and resemble that of *Bacillus* spp. sensu stricto Figure 3. The organisms tolerate 1.0M NaCl with an average OD of 0.9 at 600 nm wavelength. No growth was detected in the 2.0 M NaCl concentration.

*Phylogenetic analysis of the total community*

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 16Sr RNA clone library of the community genome. The group of bacteria detected from the clone library is shown Figure 3. However, the result of the 16Sr RNA sequencing reveal the identity of the isolate as closest relative of *Bacillus pumilus* and marine bacterium, result of the ARB analysis of the isolate is shown in Figure 4. The bacteria probe used for the FISH analysis also supported the presence of diverse bacteria in the seawater samples Figure 6a,b.
Figure 1. Colonies of *Bacillus pumilus* grown on K medium.

Figure 2. Cellular morphology of the isolate under the microscope

X 1000 magnification

Figure 3. Phase contrast micrography of *Bacillus* cells and the centrally located endospore indicated by the arrow

X1000 M
Figure 3 The dendrogram of clone library of the bacteria community of the seawater
Figure 4 The dendrogram of ARB Bacillus species isolated from seawater.
Conclusion

Marine Bacillus are not in relative abundance in the sea water tested in this work, different enrichment techniques are needed to be develop to aid isolation of the bacteria from the seawater. Species specific primer and probe will be highly useful for the detection of both the culturable and culturable Bacillus community in the seawater. Further investigation will be required to understand the physiology of the Bacillus pumilus isolated in this study.

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References


