

# CHARACTERIZATION OF SOME MODERATE AND EXTREME HALOPHILES FROM CULTURE COLLECTION STRAINS AND ENVIRONMENTAL SAMPLES BY COMPARATIVE rRNA SEQUENCE ANALYSIS.

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## INTRODUCTION

rRNA and rDNA analysis is a powerful tool for characterization of microorganisms and the establishment of phylogenetic relationships. Especially interesting is the fact that environmental samples can be used for these purposes (1) as well as cell enrichments and of course pure cultures.

Since the pioneering studies of C.R. Woese in the 1960's, when he applied rRNA to comparative sequencing based evolutionary studies, a lot of work has been done and now rRNAs are accepted as ideal semantides (semantophoric molecules, i.e. genes or transcripts of genes bearing historical records of evolution). As a result of all this work a number of rRNA/rDNA databases are available for getting general information, submitting and retrieving sequences, etc.

In the case of moderate halophiles, defined as those microorganisms that are able to grow optimally in media containing between 3 and 15% NaCl (2), more

molecular analysis are needed in order to clarify the phylogenetic relationship of some of their representatives.

The extreme halophiles, defined as those microorganisms that grow best at 15 to 30% NaCl (2), can be considered a very well defined group, but yet rRNA analysis remains important to define their phylogenetic relationship to other groups and to make easier the identification and characterization of new isolates.

The strains that will be used for this project include four moderate halophiles: *Micrococcus halophilus* DSM 20408, *Marinococcus halobius* ATCC 21727, *Halomonas elongata*, *Hm. meridiana*; and eight extreme halophiles: *Halobacterium salinarium* CCM 2148, *Haloarcula vallismortis* ATCC 29715, *Ha. hispanica* ATCC 33960, *Ha. marimortui* ATCC 43049, *Haloferax volcanii* NCMB 2012, *Halococcus morrhuae* CCM 537, strain no. 617 and strain no. 704.

The environmental samples for this project were collected at different points of a large salt pile located at New Hampshire's port and consist of salt rocks of various types as well as a sludge from the margins of the salt pile. Also sea water (25 and 35 m depth) and sediment taken at Cape Cod Bay will be used.

## MATERIAL AND METHODS

Strains and culture conditions: The four moderately halophilic strains and the eight extreme halophilic strains used in this project are listed in the introduction section. All strains were grown in a medium containing 0.5% (w/v) yeast extract (Difco) in a salt mixture with a final concentration of 10% for the moderate halophiles and 25% for the extreme halophiles (4). The pH was adjusted to 7.5 and the incubation was at 37 C in an orbital shaker at 150 strokes per minute. When necessary, solid media was prepared by adding 2% (w/v) Bacto-Agar (Difco).

For the enrichments and isolations from the environmental samples both kind of media were used.

Isolation of genomic DNA and 16S rRNA sequence analysis: Cells were harvested at approximately late-exponential phase by centrifugation and resuspended in 200 to 400  $\mu$ L TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0). The cell lysis was accomplished by the addition of 200  $\mu$ L lysozyme (50 mg/mL), 20  $\mu$ L pronase (10 mg/mL), 8  $\mu$ L mutanolysine (5000 U/mL), 4  $\mu$ L RNAase (10 mg/mL) and incubation for 1hr at 37 C and gentle revolving motion. DNA extraction and precipitation were performed by the DNA extraction protocol by A. Sghir and J. Dore (unpublished).

Micro polymerase chain reaction (Micro-PCR) amplification of the 16S rRNA gene using the forward primer 008F and the reverse primer 1517R, as well as the forward primer 007F and the reverse primer 1517R, for rDNA-PCR optimization, was carried out with the DNA extracts. Where positive results were obtained the large scale PCR was performed.

The next steps include purification of the amplification products using Costar SpinX microcentrifugation filtration devices, cloning of PCR amplified-environmental and pure cultures rDNA (by either ligation, transformation or preparation of sequence-ready plasmids), screening of clones, sequencing, and phylogenetic positioning of environmental clones. At this point, dendrograms can be generated using a pairwise, weighted, least-squares distance method (3).

## RESULTS AND DISCUSSION

According to the methodology proposed the strains and samples were cultivated in liquid media, then the cells were harvested and their DNA was extracted following the procedures. These DNA extracts were checked by electrophoresis in agarose (1%) gel and no sign of contamination or degradation of the DNA was observed.

The dilutions (1:1000) of these DNA extracts were used for the DNA amplification and different results were obtained depending on the set of primers used. These data are shown in Table 1.

As it was expected PCR products using 008F-15171R primers were obtained only when DNA belonging to eubacteria was used. On the contrary only archaeobacterial DNA gave PCR products if 007F-1517R primers were used, since 007F is an specific primer for archaeobacteria. The sizes of the amplicates, inferred by comparison with  $\Phi$  or 1Kb  $\lambda$  molecular weight markers, were as well the expected ones in all the cases.

Where no band was obtained (*Halococcus morrhuae* NCMB 2012 and strain no. 704), regardless to the set of primers used, was probably due to the fact that in this cases the concentration of DNA was very low but the same dilution was applied (1:1000).

Table 1. PCR products obtained using the primer sets 008F-1517R and 007F-1517R.

Strain	008F-1517R	007F-1517R
<i>Haloferax volcanii</i> NCMB 2012	-	+
<i>Haloarcula marismortui</i> ATCC 43049	-	+
<i>Halococcus morrhuae</i> CCM 537	-	-
<i>Halomonas elongata</i>	+	-
<i>Haloarcula vallismortis</i> ATCC 29715	-	+
<i>Halomonas meridiana</i>	+	-
<i>Haloarcula hispanica</i> ATCC 33960 no. 704	-	+
<i>Micrococcus halobius</i> ATCC 21727	+	-
<i>Marinococcus halophilus</i> DSM 20408 no. 617	+	-
<i>Halobacterium salinarium</i> CCM 2148	-	+
Salt sludge enrichment	+	+
Blue salt enrichment	+	-
White salt enrichment	n.t.	-
Control	n.t.	+
	-	-

+: one single band, -: no band, n.t.: not tested.

In the case of the DNA obtained from *Halobacterium salinarium* CCM 2148 an amplification band was obtained with the 007F-1517R primers but also with the other set suggesting a contamination of the original pure culture with a moderate halophile. This fact was confirmed by microscopic examination and isolation on plates.

Unluckily the rest of the project could not be finished due to a lack of time motivated by an unexpected malfunctioning of the PCR machine.

## CONCLUSIONS

The results so far obtained are good enough to think that this project should be continued in the near future aiming to get all the initial objectives. In any case it has been shown that the environmental samples used (even the marine ones) contain enough halophiles to be detected not only by isolation but also using rRNA molecular analysis.

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