Phylogeny and Physiology of Actinomycetes and Biogeochemical Parameters in Sediments of Eastern Mediterranean Sea

Ilknur Tuncer and Nihayet Bizsel

***Abstract*—In spite of high morphological and phylogenetic diversity, biotechnological and economic importance of actinomycetes, there is a limited number of studies in sediments of Eastern Mediterranean Sea, especially in relation with environmental parameters. The actinomycetes were isolated from deep-basins of Eastern Mediterranean Sea (72–1235 m depths) with regional variability. They were tested physiologically using commercial kits and found that they utilized proteins rather than carbohydrates. According to 16S rRNA gene sequence analysis, highly diverse *Streptomycetes* strains with two of them representing new taxa and also the genera *Nocardiopsis* and *Pseudonocardia* were obtained. Geochemical parameters of the sediments together with enzymatic activity results of the strains underlined the nitrogen limitation in the area.**

***Index Terms*—Actinobacteria, 16S rRNA genes, sediments, Eastern Mediterranean Sea.**

I. Introduction

Actinomycetes are aerobic, nonmotile, and Gram-positive bacteria with high GC content in their DNA. They are very important due to their high diversity and production of secondary metabolites. However, discovery from terrestrial actinomycetes has declined recently and thus marine environment has been opened to research area.

Compared to cultivation-independent methods, cultivation-based studies are generally preferred for further studies such as production of secondary metabolites and determination of physiological characteristics needed in especially identification of new species [1], [2]. Although the *Actinobacteria* accounted minor part in 16S rRNA gene libraries of Mediterranean sediments [3], especially for Eastern Mediterranean Sea sediments as 4-28% of total sequence [4], they were highly isolated from the deep sediments of Eastern Mediterranean Sea in the previous culture-dependent study [5]. Furthermore, highly diverse and antibiotically active actinomycetes were also obtained in coastal zone of Turkish marine environments [6].

Since actinomycetes are biotechnologically and economically important bacteria and there is a limited number of phylogenetic studies in association with environmental parameters in the sediments of Eastern Mediterranean Sea (EMS) which is one of the most oligotrophic regions in the world [4]–[10], in the present study, it was aimed to isolate actinomycetes from sediments of EMS, to physiologically and phyogenetically analyze them and also to analyze biogeochemical parameters of EMS with regional variability.

Manuscript received November 21, 2016; revised March 23, 2017. This work was supported in part by the Scientific and Technological Research Council of Turkey (TUBITAK) and also Scientific Research Project Coordination Unit (BAP), Dokuz Eylul University under project no. COST 110Y193 and 2010.KB.FEN.014, respectively.

Ilknur Tuncer and Nihayet Bizsel are with Institute of Marine Sciences and Technology, Dokuz Eylul University, Turkey (e-mail: tuncerilik@gmail.com, nihayet.bizsel@deu.edu.tr).

II. Materials and Methods

A. Sediment Sampling and Analysis

In the present study, sediment samples were taken from totally 11 stations with 72–1235 m depths of Eastern Mediterranean Sea for both bacterial isolation and sediment analysis (Fig. 1). While the stations C1 and C2 were located at the upper most North Aegean Sea, the stations C6–C8 were at the lower most South Aegean Sea. On the other hand, stations B1–B5 were between Lesvos Island and Karaburun in North Aegean Sea whereas the station C10 was near to Cyclades plateau at the center. The sediment samples were collected into sterile plastic bags, 40 ml glass containers and sterile plastic cores for different processes and kept at –20 ℃ till the analysis.

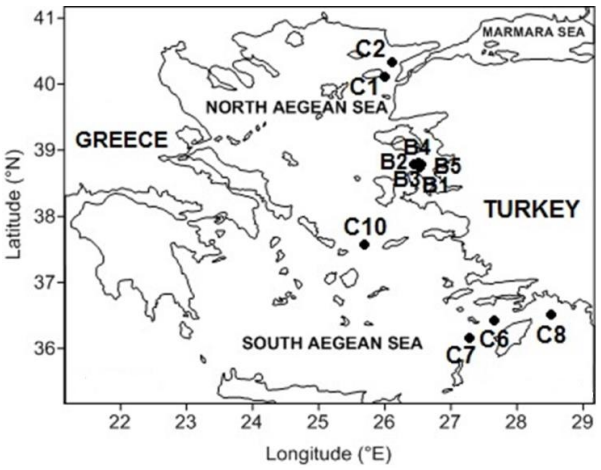

Fig. 1. Stations C (1, 2, 6–8, 10) and B (1–5) in Eastern Mediterranean Sea.

The particle size of the sediments was determined by the sieve analysis and the hydrometer method for the larger and the finer particles, respectively according to standard test method for particle size analysis of soils D 422-63 issued by American Society for Testing and Materials [11].

Total and organic carbon and nitrogen contents (TC and TOC, TN and TON, respectively) were obtained using Carlo Erba NC2500 model CHN analyzer, on the other hand, total

doi: 10.18178/ijesd.2017.8.8.1019

and organic phosphorus contents (TP and TOP, respectively) were measured spectrophotometrically [12].

B. Bacterial Isolation and Physiological Analysis

Isolation of bacteria was achieved using seven different sediment processing methods and two isolation media prepared with sterile seawater. The isolation media consisted of the following: M1, 18 g agar, 10 g starch, 4 g yeast extract, 2 g peptone, 1 liter sterile seawater; M2 (Difco™ actinomycete isolation agar, modified), 22 g medium, 5 ml glycerol, 500 ml sterile sea water and 500 ml distilled water. The isolation media M1 and M2 were used with or without six different antibiotics as cycloheximide (100 µg/ml), nystatin (50 µg/ml), polymixin B sulfate (5 µg/ml), rifampin (5 µg/ml), kanamycin sulfate (5 µg/ml), novobiocin (25 µg/ml).

Seven different sediment processing methods were performed. In the first processing method (a) [13], 10 ml wet sediment sample were dried overnight and then 0.5 g dry sediment was aseptically spread in circular fashion onto the agar media. In the dry spot method (b) dry sediment was taken with sterile sponge and put clockwise on the agar media. In the third method (c) [14], 1 ml wet sediment was diluted with sterile seawater (1:4) and then heated for 6 min at 55 ℃. After vortexing for 30 s, 75-100 µl was spread aseptically onto agar-based isolation media. In the fourth method (d) [15], wet sediment was heated for 15 min at 70 ℃ and then spread aseptically on the agar surface in a circular fashion. In the fifth method (e) [16], wet sediment was kept for 30 sec under UV and then spread aseptically in a circular fashion onto the agar media. In the sixth method (f) 1 ml wet sediment sample was diluted with sterile seawater (1:1, 1:10 and 1:100) and then vortexed for 30 s. Then, 75–100 µl was spread aseptically onto agar-based isolation media. In the seventh method (g) without processing, wet sediment sample was aseptically spread onto agar-based isolation media.

All plates were incubated at 26–28 ℃ upto 2 months. The colonies were subcultured on M1 or M2 media and then the isolates were cryopreserved with 50% glycerol at –20 ℃.

All strains were also tested using API ZYM® (BioMérieux, Inc. Durham, USA) and BD BBL™ Crystal™ GP ID (Becton Dickinson and Company, USA) commercial kits according to user's manuals.

C. Nuclear DNA Extraction and 16S rRNA Amplification

Genomic DNA of isolated bacteria was extracted with a commercial kit (Invitrogen, Carlsbad, CA) according to the user's manual for Gram-positive bacterial cell lysate.

The 16S rRNA genes were amplified from genomic DNA by PCR using the universal primer pairs of FC27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RC1492 (5′-TACGGCTACCTTGTTACGACTT-3′) and also the pairs of 63f (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387r (5′-GGGCGGWGTGTACAAGGC-3′). The 50 µl PCR mixture contained 20 to 50 ng of DNA, One Taq Quik-Load 2X Master mix (New England Biolabs, Inc. Beverly, MA), 10 pmol of each primer (Fermentas, Thermo Fisher Scientific, Waltham, MA), and 10 mM deoxynucleoside triphosphate mixture (Fermentas, Thermo Fisher Scientific, Waltham, MA). The PCR program consisted of 30 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 1 min followed by a final extension step at 72 ℃ for 7 min. Amplification products were examined by agarose gel electrophoresis.

D. Sequencing and Phylogenetic Analysis

Sequencing service was taken from Gene Research and Technology (RefGen, Turkey). For the phylogeny, all nucleotide sequences were analyzed using Geneious (version 6.1; Biomatters Ltd., NZ) and compared within the NCBI database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST). Phylogenetic analysis was performed using Mega [17] with 1000 bootstrap neighbor-joining method.

All those partial 16S rRNA gene sequences have been deposited into GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under the accession numbers KC815756–KC815772, KF366670, KF366671, KF366674, KF366679 and KF366682.

III. Results

A. Sediment Parameters

Grain size analysis showed that the deep-basins had finest particles as expected. While the stations C1, B1, B3, B5 were sand and B2, B4 were sand-silt-clay, the stations C2, C6–C8, C10 were clayey silt (Table I). For the chemical contents of the sediments, the station C1 had the lowest carbon and phosphorus values whereas stations C6–C8 had the highest carbon values (Table I). On the other hand, the carbon ranges of the stations B1–B5 were 5.3–9.6% and 1.0–3.3 % for TC and TOC, respectively as in between compared to the rest. While phosphorus contents were not changed among stations, except C1, the variation in nitrogen values was obvious (Table I) as indicating the nitrogen playing much more dynamic role in sediments.

TABLE I: The Sediment ParameterS for Stations in Eastern Mediterranean Sea

Stations	Depth (m)	Sediment type	TC (%)	TOC (%)	TN (%)	TON (%)	TP (%)	TOP (%)
B1	104	sand	9.6	1.1	0.02	BDL	0.048	0.015
B2	198	sand-silt-clay	5.3	1.7	0.24	BDL	0.032	0.017
B3	159	sand	6.4	2.8	0.06	BDL	0.053	0.008
B4	190	sand-silt-clay	5.4	1.0	0.02	BDL	0.038	0.010
B5	106	sand	5.7	3.3	0.20	0.15	0.037	0.007
C1	72	sand	2.9	0.6	0.94	BDL	0.011	0.004
C2	522	clayey silt	5.0	2.8	0.02	BDL	0.033	0.016
C6	703	clayey silt	15.3	9.0	0.57	0.21	0.026	0.011
C7	1035	clayey silt	9.6	5.3	0.11	BDL	0.030	0.013
C8	1235	clayey silt	17.9	1.6	0.38	0.01	0.028	0.011
C10	661	clayey silt	4.4	1.9	0.30	0.12	0.030	0.016

TC: total carbon; TOC: total organic carbon; TN: total nitrogen; TON: total organic nitrogen; TP: total phosphorus; TOP: total organic phosphorus; BDL: below detection limit.

B. Phylogeny and Physiology of Actinomycetes

Totally 22 actinomycete strains were successfully isolated from mostly M1 and then M2 media and among seven sediment processing methods, the methods (a) and (f) which were overnight drying wet sediment and diluting wet sediment with sterile seawater were highly efficient.

The isolates generally showed high phosphatase (alkaline or acid), lipase or esterase, aminopeptidase activities (90%, 81% and 68% of all isolates, respectively), in contrast to low enzymatic activities for carbon utilization such as glucosidase or galactosidase activities with 44% of isolates and also 15% averaged activity against related substrates for glucosaminidase, maltosidase, urase, trehalase, lactase, methyl glucosidase, sucrase, mannitol dehydrogenase, amylase, arabinase, fructokinase, glycerol kinase, glucuronidase, mannosidase, fucosidase and dehydrogenase. According to the average activity values for each station, the strains isolated from the station C10 showed the lowest activities whereas the highest glucosidase or galactosidase activities were obtained from stations C6–C8 (Table II). On the other hand, while the isolates from the station C2 gave the second lowest phosphates activity, the station C1 had the lowest glucosidase or galactosidase activities (Table II). Like this separation between closer stations C1 and C2, the isolates from deeper B stations (B2 and B4) showed lower activities compared to shallower B stations (B1, B3 and B5) (Table II). In fact, overall B stations had the highest aminopeptidase activity among all stations (Table II).

TABLE II: FOR EACH STATION, THE AVERAGE VALUES OF ENZYMATIC ACTIVITIES AGAINST THE RELATED SUBSTRATES

Stations	Phosphatase activity (%)	Aminopeptidase activity (%)	Lipase or esterase activity (%)	Glucosidase or galactosidase activity (%)
B1	100	67	83	55
B2	100	74	92	33
B3	100	75	100	40
B4	80	71	67	45
B5	100	83	100	45
C1	100	58	100	20
C2	60	67	100	50
C6	90	79	50	40
C7	100	58	100	60
C8	100	50	100	80
C10	40	25	33	50

The isolates were found highly affiliated with three families as *Nocardiopsaceae*, *Pseudonocardiaceae* and *Streptomycetaceae* (Table III). When the phylogenetic tree was constructed using nearly full 16S rRNA gene sequence of representative isolate for each nearest type strain, it was seen that the tree clearly supported those three families of the order *Actinomycetales* (Fig. 2).

The majority of the strains formed a highly diverse clade with members belonging to the genus *Streptomyces* highly isolated from stations B compared to stations C and there were also the genera *Nocardiopsis* and *Pseudonocardia* (Table III). When a limit of 98.5 to 99% was considered, as revised and proposed by [18], the *Streptomyces* strains 317CA6Y12 and 41BA4Y12 isolated from stations B4 and C6 had a probability of representing new taxa (Table III).

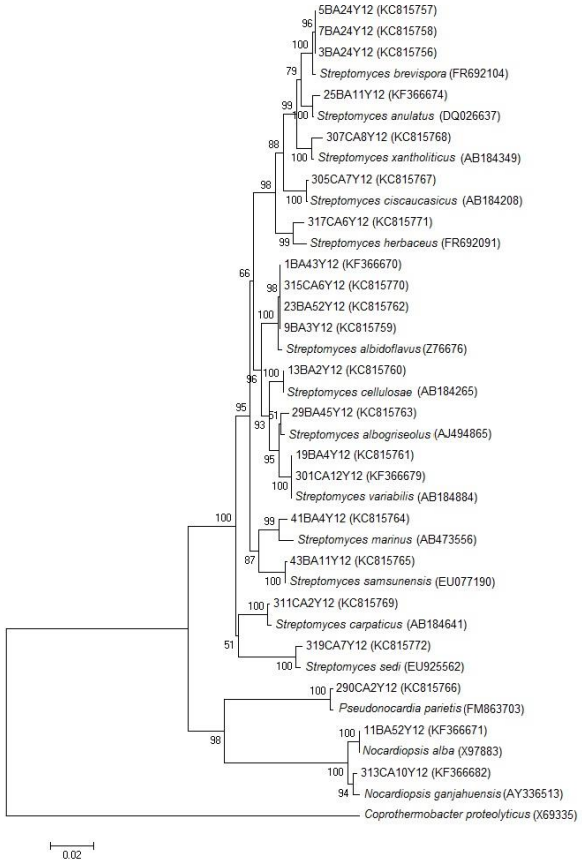

Fig. 2. For the order *Actinomycetales*, neighbor-joining distance tree constructed in Mega using partial 16S rRNA gene sequences. GenBank accession numbers were given in parentheses. Bootstrap values calculated from 1000 re-samplings were in percentage. *Coprothermobacter proteolyticus* was used to position the root.

TABLE III: THE LIST OF ISOLATES REPRESENTING THE CLASS *ACTINOBACTERIA* WITH THE NEAREST TYPE STRAINS ACCORDING TO THEIR 16S RRNA SEQUENCES

Stations	Isolates	Nearest type strains	Pairwise similarity (%)
B1	25BA11Y12	*Streptomyces anulatus*	99.64
	43BA11Y12	*Streptomyces samsunensis*	99.93
B2	3BA24Y12	*Streptomyces brevispora*	99.78
	5BA24Y12	*Streptomyces brevispora*	99.78
	7BA24Y12	*Streptomyces brevispora*	99.78
	13BA2Y12	*Streptomyces cellulosae*	100
B3	9BA3Y12	*Streptomyces albidoflavus*	99.71
B4	1BA43Y12	*Streptomyces albidoflavus*	99.71
	29BA45Y12	*Streptomyces albogriseolus*	99.35
	41BA4Y12	*Streptomyces haliclonae*	98.60
	19BA4Y12	*Streptomyces variabilis*	100
B5	11BA52Y12	*Nocardiopsis alba*	100
	23BA52Y12	*Streptomyces albidoflavus*	99.71
C1	301CA12Y12	*Streptomyces variabilis*	100
C2	290CA2Y12	*Pseudonocardia parietis*	99.78
	311CA2Y12	*Streptomyces carpaticus*	99.71
C6	315CA6Y12	*Streptomyces albidoflavus*	99.71
	317CA6Y12	*Streptomyces herbaceus*	98.93
C7	305CA7Y12	*Streptomyces ciscaucasicus*	99.93
	319CA7Y12	*Streptomyces sedi*	99.57
C8	307CA8Y12	*Streptomyces xantholiticus*	99.34
C10	313CA10Y12	*Nocardiopsis ganjiahuensis*	99.57

IV. Discussion

The geochemical composition of the study area was generally in the range of Aegean Sea. In addition to the decrease in grain sizes with depth, the chemical contents of the sediments reflected the regional variability. The stations B closer to the coastal area as Lesvos Island, Izmir and Candarli Bays in North Aegean Sea, were distinguished from other stations. On the other hand, the geochemical variations among stations C were also seen. Although both stations C1 and C2 were located in the upper most North Aegean Sea having complex bottom topography and highly dynamic hydrographic conditions influenced by Aegean Sea and nutrient-rich Black Sea, the station C1 was near to the mouth of Dardanelle Strait carrying Black Sea water passing through Marmara Sea to Aegean Sea. While the stations C6–C8 were located in the lower most South Aegean Sea influenced by both Aegean Sea and oxygen-depleted and oligotrophic Levantine Sea, the station C10 was near to Cyclades plateau at the center. Furthermore, the stations C6–C8 had higher carbon contents than the previous results as 0.33–15.63% and 1.30–13.10% for TC, 0.22–6.76% and 0.42–0.99% for TOC in North and South Aegean Sea, respectively [4], [10], [19]–[23]. However, for the nitrogen contents, the station C2 had lower TN values than the previous 0.02–0.07% in North Aegean Sea [21] and the station C6 had higher TON than the previous 0.04–0.10 % in South Aegean Sea [4].

In the study area, actinomycete diversity was found lower compared to previous studies in Eastern Mediterranean Sea. In the study of sediments in Cretan Sea, isolated actinomycetes were phylogenetically affiliated with the genera *Arthrobacter*, *Blastococcus*, *Corynebacterium*, *Micromonospora*, *Pseudonocardia*, *Streptomyces* [5]. However, in spite of high phylogenetic diversity in higher taxa in [5], *Streptomyces* strains were found more diverse in the present study. On the other hand, in addition to the isolation of alkaliphilic actinomycetes MA1-1 from sediments of Izmir Bay, Aegean Sea [8], much more diverse *Streptomyces* strains and one Nocardia *alba* strain were seen among 66 antimicrobially active strains of 261 actinomycetes isolated from the sediments in Turkish coasts of Black Sea, Aegean Sea and Mediterranean Sea [6].

In the present study, the isolation and diversity of actinomycetes were much higher from stations closer to coastal areas in North Aegean Sea compared to deeper stations in Eastern Mediterranean Sea. Moreover, the relation between enzymatic activities of actinomycetes and the chemical contents of the sediments in addition to the geographical variability was clearly seen in this study. Actinomycete strains isolated from the study area showed high aminopeptidase, phosphatase, lipase, esterase activities, in contrast to glucosidase, galactosidase, and even much lower trehalase, lactase, sucrase, amylase, arabinase, urease, etc. activities. In addition to a positive correlation between aminopeptidase activity and N limitation as an indication of the utilization of organic nitrogen for bacterial growth in the study of [24], it was also shown that bacterial biomass in the sediments of Aegean Sea displayed a positive correlation with the sedimentary protein concentration [7]. It was also given that carbohydrates were a reservoir of non-utilised organic carbon in oligotrophic deep-sea sediments [7]. Thus, in the present study, enzymatic activity results and organic nitrogen depletions in the sediments as underlying N limitation supported this protein utilization rather than carbohydrates and also reflected the geochemical effects on bacterial characteristics.

V. Conclusion

As a result, in this study, it was indicated that phenotypic and phylogenetic diversity of actinomycetes changed in accordance with geographical differences and environmental factors. For further studies i.e. identification of new species and production of secondary metabolites, the present study provided morphologically different actinomycete strains especially with probability of representing new taxa.

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Ilknur Tuncer was born in Turkey, 1980. She received the BSc degrees in chemistry and genetics from Bogazici University, Turkey, the MSc degree in marine sciences from University of Wales, UK and the PhD degree in marine sciences from Dokuz Eylul University, Turkey in 2015. Her current reseach interests are marine environment, microbiology, phylogeny, and species-area relationship.

Nihayet Bizsel was born in Turkey. She received her PhD degree in marine sciences from Dokuz Eylul University, Turkey in 1996. Her current reseach interests are marine environment, and phytoplankton.