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New Report on the Occurrence of *Exiguobacterium* sp. AT1b in the Persian Gulf and its Resistance to Mercury Pollution

[1]K. Karami, [1]H. Zolgharnein, [2]M. Mazaheri Assadi, [1]A. Savari and [3]S. Dadollahi
[1]Department of Marine Biology, Khoramshahr University of Marine Science and Technology, P.O. Box 669, Khoramshahr, Iran
[2]Biotechnology Research Center, Iranian Research Organization for Science and Technology, Iran
[3]Department of Environment, Khoramshahr University of Marine Science and Technology, P.O. Box 669, Khoramshahr, Iran

Corresponding Author: K. Karami, Department of Marine Biology, Khoramshahr University of Marine Science and Technology, P.O. Box 669, Khoramshahr, Iran

ABSTRACT

This study was done to investigate of new mercury resistant bacteria in coastal waters of Mahshahr area which the greatest petrochemical chlor-alkali unit of Iran located there. Water and sediment samples were taken and cultured on specific media in laboratory. Traditional biochemical and modern molecular approaches were used for identification. Mercury toxicity was measured via Minimal Inhibitory Concentration method. New observation of a coryneform, the *Exiguobacterium* sp. AT1b was occurred and identified based on 16S rRNA gene homology. Sequence matching was 99% and resistance to mercuric chloride were 75 and 50 ppm in different media. Optimal growth conditions was studied and temperature range plus with optimum pH were determined. Wide range of temperature tolerance and other conditions such as salinity and pH variation tolerance were observed. Agitation was the least affecting parameter on the growth based on optical density measured and analysis of variance results. The *Exiguobacterium* sp. AT1b is one of the best bacteria suggested for environmental studies and simulations.

Key words: Marine bacteria, actinomycetes, pollution, Iran

INTRODUCTION

Persian Gulf is one of the most important marine areas because of around 70% of the world oil exploitation and transportation undergoes across this semi-enclosed sea at the North-West corner of the Western Indian Ocean (Nadim *et al*., 2008). In spite of the great importance, Persian Gulf is still poorly environmentally understood. Political relationships and three extended wars during the past three decades have been covered other aspects of this area (Price *et al*., 1994).

Persian Gulf had been faced with many types of pollution after long time passing from oil trade and development of industrial activities plus with war disadvantages. Furthermore, the genus *Exiguobacterium* is a newly described bacterium which was explained by Collins *et al*. (1983). This genus has 17 described species and more than 294 strains and new members are still adding to that. Members of this genus had formerly been identified as the genera such as *Corynebacterium*, *Brevibacterium* and even *Staphylococcus* (Rodrigues *et al*., 2006) because of some similarities in peptidoglycan structure or fatty acid profile. Modern molecular based techniques revealed it as a new genus separated from coryneforms. This microorganism seems to be a cosmopolitan genus since its wide range of dispersion all over the world. Little of its characteristics

are nowadays well-known, however, resistance to mercury was reported by Wagner *et al.* (2003). Biology of *Exiguobacterium* is still poorly understood and our knowledge limits to relatively small number of reports about sequenced genes and their functions that can be found in international databases (Jakubauskas *et al.*, 2009).

The *Exiguobacterium* sp. AT1b was isolated and described first by Robert F. Ramaley (Nebraska Medical Center, Omaha, NE) and Christine Hendrix (Yellowstone National Park) in 2004. the strain *Exiguobacterium* sp. AT1b was isolated from a slightly alkaline and highly carbonated hot spring water of Angel Terrace, which is part of Mammoth Terrace, Yellowstone National park. Differences in culture methods and identification procedures leads to basically different results hard to compare. Situation which makes the environmental microflora less comparable even in adjacent regions. The aim of the present study was to investigate mercury resistant bacteria in vicinity of an industrial petro-chemical area at North Western of the Persian Gulf.

MATERIALS AND METHODS

Study area: Mahshahr is an industrial city located in 30° 33' N and 49° 13' E in Khuzestan province, Iran (Tabatabaie *et al.*, 2009). Great port and full transportation system and frequent factories have been maden it as a special industrial zone; therefore, sampling area selected near sewage outfalls of the petro-chemistry at area around 30° 24' to 30° 27' N and 49° 06' to 49° 08' E. Seven stations sampled for surface waters and sediments. Environmental parameters such as temperature, pH, dissolved oxygen were measured *in situ*. Water and sediment samples held in sterilized plastic bottles at 4°C ice bags until transferred to the laboratory.

Sediment samples were serially diluted with Phosphate Buffer Saline (PBS) to 10^{-4} level, then spreaded onto Sea Water Nutrient Agar (SWNA) amended with 25 μg mL^{-1} Hg^{2+} as $HgCl_2$. This threshold concentration of Hg was suggested by Ramaiah and De (2003) and bacteria could tolerate that, were known as Bacteria Highly Resistant to Mercury (BHRM). Grown colonies were picked up into new media to ensure purity of cultures. Mercury toxicity test was done in terms of Minimal Inhibitory Concentration (MIC) method. Mercury concentrations in SWNA plates were in range from 0 to 100 ppm with 25 ppm intervals.

The MIC defined as the concentration of toxicant which causes no visible growth. Plates were incubated at 30°C for 3 days. Biochemical tests such as catalase, nuclease and oxidase were performed on pure cultures. Temperature and pH optimal range were studied in the range of 6°C in refrigerator to 55°C and 3 to 11, respectively. Agitation effect were also studied in range of 120 to 200 round per minute (rpm) on a rotary shaker.

Growth was measured in terms of Optical Density (OD) in 600 nm on a spectrophotometer. DNA extraction was made by means of a DNA extraction kit DNG plus (Cinnagen, Iran) and 16S rRNA gene was amplified with Polymerase Chain Reaction(PCR) using primers 27f (5`-AGAGTTTGATCC TGGCTCAG-3`) and 1492r (5`-GGTTACCTTGTTACGACTT-3`) with following program: 94°C for 5 min followed by 35 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 2 min. A final extension was for 5 min at 72°C. PCR products were purified using DNA purification kit (Geni, Pin, USA) according to manufacturer's instruction. PCR products were sequenced with an ABI Prism 3100 capillary DNA sequencer. Sequencing results were analyzed with BLAST program comparing GenBank database which is available at: http://ncbi.nlm.nih.gov/.

RESULTS

New finding for the Persian Gulf microflora was the *Exiguobacterium* sp. strain AT1b. Both forward and reverse strands of 16S rDNA matched 99% which reflects the precise and reliable

Table 1: Phenotypic characteristics that differentiate strain AT1b. and related type strains

Characteristic	1	2	3	4	5	6	7
Habitat	Glacial water	Creamery waste	Drainage from	Microbial	Garden pond	Permafrost	Chlor-alkali
	fish-processing	mat from					waste
	plant	lake water					
Colony morphology							
Size (mm)	2-4	2-5	1-5	2-3	2-4	3.5-4	3-5
Shape	Round	Irregular	Round	Round	Round	Round	Irregular
Colour	Yellowish	Yellowish	Orange	Orange	Orange	Bright orange	Bright yellowish
Growth temperature (°C)							
2.5	W	-	W	-	-	+	-
5	+	-	+	-	-	+	+
37	-	W	+	+	+	+	+
Maximum growth	30	37	40	41	41	40	50
temperature (°C)							
Gelatinase	-	+	+	+	+	+	+
Lipase	-	-	-	-	-	+	+
Phosphatase	+	+	+	+	-	+	+
Starch hydrolysis	-	+	+	+	+	ND	+
Citrate utilization	+	+	+	-	-	ND	+
Lysine decarboxylase	+	-	-	-	+	ND	W
Arginine decarboxylase	+	-	-	+	+	ND	W
DNase, caseinase	-	+	+	+	+	ND	+
Tryptophan deamination	-	+	+	+	-	ND	+
Methyl red test	-	+	+	+	+	ND	+
Malonate utilization	+	-	-	-	-	ND	-
DNA G+C content (mol%)	48	47	47	47	48	47.7	48.6
Peptidoglycan	Lys-Gly	Lys-Gly	Lys-Gly	ND	Lys-Asp	Lys-Gly	Lys-Gly

Strains: 1, strain HHS 31T; 2, *Exiguobacterium acetylicum* DSM 20416T; 3, *E. oxidotolerans* JCM 12280T; 4, *E. antarcticum* DSM 14480T; 5, *E. undae* DSM14481T; 6, *E. sibiricum* DSM 17290; 7, *E.* sp. AT1b was isolated in present study. Data for all strains except *E.* sp. AT1b are not from this study and hereby indicated. All of the strains shown are motile, Gram-positive, rod-shaped, do not form endospores, grow at temperatures between 10 and 30. +: Positive; -: Negative; ND: Not done/no data available; +/-: Variable reaction; W: Weakly positive

results for species identification.; morphological characteristics consisting of Gram positive non-spore forming bacilli with rounded ends. Catalase and Nuclease positive which appears singly, in pairs and less frequently in chains. Colonies appear white on SWNA medium pH 8 and the pigment does not diffuse into the medium. This bacterium is facultative anaerobe but growth was more enhanced aerobically as the others reported. However, no significant difference showed between shaking speeds in ANOVA test, a slight increase in growth was observed at 180 rpm agitation (data not shown). The MICs were 75 and 50 ppm in solid and liquid media, respectively.

Broad range of living temperature was observed foe *Exiguobacterium* sp. AT1b isolated from Persian Gulf waters which was from 6 to 50°C with optimum around 37°C and the best growth was observed in slightly alkaline media with pH range from 7 to 8. Colony morphology had similarities with *Bacillus* colonies but resembled to the typical Actinomycetes from initial days of growth. Oxidase reaction was positive. Use of multiple sources of carbon was observed except for malonate. The isolated strain was also able to growth on Nutrient Agar and Nutrient Broth and thus determined that it has advanced osmotic tolerance capabilities. Common features of seven species *Exiguobacterium* are compared in Table 1 for more details and exactly comparisons.

DISCUSSION

Present results were mainly in concordance with general characteristics of this bacterium obtained by other researchers (Ramaley *et al*., 2004). This high level of resistance to mercury had never been recorded in literature for this species nor did for growth temperature range, therefore this strain is more eurythermal than isolated by Yumoto *et al*. (2004) that was able to growth from 15 to 50°C. Growth pH that was in accordance with its original conditions and similar to above mentioned researchers that reported in pH 8.8 on TSA medium. Colony shape and color similarities with the genus *Bacillus* is because of phylogenetically relationship among these two taxa (Farrow *et al*., 1994). Oxidase reaction was positive similar to the most of the *Exiguobacterium* strains (Funke *et al*., 1997), whereas Collins *et al*. (1983) reported the genus as oxidase negative. Low level of growth temperature is similar to *E. aurantiacum* reported by Collins *et al*. (1983) which was 7°C and *E. sibiricum* and *E. oxidotolerans* that were 5°C (Chaturvedi and Shivaji, 2006). This species had common characteristics with *E. marinum* described by Kim *et al*. (2005) isolated from tidal flat of the Yellow Sea in Korea. The *E. marinum* was grown on marine agar 2216 (MA; Difco) at 30°C in two days. The *E.* sp. strain AT1b. showed excellent survival after exposure to a long-term freeze, rapid growth at low temperatures, osmotic tolerance and age of the permafrost sediment from which it was cultured, characteristic which is similar to *E. sibiricum*. Through analysis of the transcriptome, experiments can be designed to study microbial survival under a variety of harsh conditions. The issue of long-term survival is of interest in the field of astrobiology. Organisms that survive such hostile environments may be used as models for understanding cellular responses on astral bodies. This strain can be considered as an extremophil species due to harsh conditions of habitat and tolerance potentials for stress factors such as temperature, pH and osmotic variations. Furthermore, it is suitable for educational and research purposes as well as applied aspects such as bioremediation.

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