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Original Research Article

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Alkane biodegradation under saline conditions

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ABSTRACT

Halophiles have a great potential in value-added compounds production such as ectoine, and use for bioremediation in hypersaline environments. As well as the halophiles, halo-tolerant microbes could be used for the similar application. In addition, screening and maintenance of such microbes could contribute in constructing a namely, 'Bioresource Library' for achieving the sustainable development goals (SDGs). Here, we present the characterization of halophilic-, and halotolerant bacteria, isolated from Okinawa and Hachioji, Japan, and Tunisia.

Using enrichment culture and direct isolation from environmental samples in Tunisia and Okinawa, Japan, totally 100 strains were obtained as moderately halophilic bacteria. For the practical application in marine petroleum remediation, enrichment culture with a mid-chain alkane as the sole carbon source under saline condition were further conducted using soils from Tunisia and Japan (Hachioji, Tokyo). All the isolates exhibited a similar growth rate below 15 % of NaCl suggesting they are halo-tolerant bacteria. Among them, *Sinomicrobium* sp. belonging to CFB group was included as halo-tolerant alkane degrading bacteria from Tunisian soil, suggesting an unique gene resources for alkane degradation. For the genes related for alkane degradation, both of alk B and P450 (CYP153A) genes were detected from the isolates.

KEYWORDS

Halophiles; Halo-tolerant bacteria; Petroleum degradation; Saline conditions.

INTRODUCTION

Halophiles have a great potential in value-added compounds production such as ectoine, and use for bioremediation in hypersaline environments. Particularly, moderate halophiles constitute the most versatile group having a great potential for biological depollution through a wide range of salinity[1].

For the screening of such microbes, Tunisia is one of the interesting area for bioprospecting due to its versatile geographic characters such as the desert (Sahara), Salt lake locally called "sabkhas" (Chott el Jerid), and mountain oasis (Chebika) etc. As well as Tunisia, Okinawa, the only subtropical area in Japan, could be such an area for bioprospecting and also it is interesting to compare their biosphere each other. Such Hypersaline areas are considered as extreme environments polluted so often with hydrocarbons[2]. Thus, bioremediation of these areas could be conducted using the activities of halophilic/halotolerant hydrocarbonoclastic microorganisms[3]. In addition, bioprospecting for various microbes with potential

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hydrocarbon remediation is one of the significant goals not only for environmental preservation but also Sustainable Development Goals[4,5].

Although bacteria from different genera showed their ability to degrade hydrocarbons, the most dominant members belong to genera *Halomonas*, *Halobacterium*, *Marinobacter*, *Alcanivorax*, *Haloferax* and *Haloarcula*[6,7]. To maintain the osmotic balance under saline conditions inside the microbial cells, the majority of moderate halophiles use the compatible solute. Compatible solutes are synthesized and accumulated by halophilic bacteria, including betaine and ectoines[8,9].

We previously examined the isolation of moderate halophilic bacteria from mainly soil samples in Tunisia and Yaeyama Islands of Japan (part of the Okinawa archipelago), followed by the phylogenetic analysis for the isolates as the first step for the comparison of halophilic bacterial library either from Tunisia or Okinawa[10,11]. In addition, genetic tools such as plasmid-vector[12], and integrative-vector system[13], for halophilic bacteria was reported using strain 21a, one of the isolate from this screening project.

In this report, screening program for microbial growth at salinity conditions has been expanded for the use of bioremediation under high salt concentrations. As the results, aliphatic hydrocarbons degrading bacteria with salt tolerant character were obtained from soils in Tunisia and in Japan. Genetic characterization was discussed using the isolates.

RESEARCH METHODS AND MATERIAL

Sampling

Samples for bacterial isolation were collected from various area of Tunisia and Mt foot of Mt. Takao, Tokyo, as shown in TABLE 1. For the sampling in Tunisia, Memorandum of decision on the joint research program have been made by the parties including Tokyo University of Technology, and Laboratory of wastewater and environment/Borj-Cedria Science and Technology EcoPark, Tunisia.

Bacterial strains and growth conditions

Cultivations were carried out in Minimal medium (MM) in the presence of NaCl as described previously[11]. As for the growth kinetics with the isolates, specific growth rates (1(one)/h) were estimated by plotting cell concentration versus time in a log-linear plot, as described by Murarka et al.[14].

PCR amplification and phylogenetic analyses

Genomic DNA preparation, PCR amplification of 16SrRNA and 16S rRNA gene sequence-based phylogenetic analyses were carried out as described previously[15]. In brief, The phylogenetic tree based on the 16S rRNA gene sequence was constructed by using the neighbor-joining method[16] and the Kimura two-parameter model for distance correction[17]; the tree was constructed with the CLUSTALW program, version 1.4[18]. PCR amplification of CYP153A-P450 genes (producing an approximately 800 bp product) and alkB (an approximately 550bp) were performed using primer pairs of P450F/P450R, and alkBwf/ alkBwr, respectively, as described previously[19]. General DNA manipulations were performed as described by Sambrook et al.[20].

Analyses

The growth of bacterial cells was spectrophotometrically estimated by measuring the optical density of the culture at 660 nm. Alkanes degradation was analyzed by GC (Shimadzu GC-1700; Shimadzu Co., Kyoto, Japan) after extracting the reaction solution with ethyl acetate at pH < 1 by using a J&W DB-5 capillary column (length 30 m). The run conditions were as follows: start temperature, 80 °C; ramp rate, 10 °C min–1; and final temperature, 250 °C for 4 min. The nucleotide sequences of the PCR-amplified genes were determined by using the ABI model 3500 and BigDye terminator kit, version 1.1 (Applied

TABLE 1: Sampling area for the isolation in this study

Country	Region	Description
Tunisia	El Djem	Soil
Tunisia	Mahares	Soil
Tunisia	Matmata	Dry soil
Tunisia	Sahara	Desert sand
Tunisia	Chott El Jerid	Salt lake
Tunisia	Chebika	Forest soil
Tunisia	Grand canyon	Forest soil
Japan	Okinawa	Sub tropical area
Japan	Hachioji (Tokyo)	Field soil
Japan	Takao (Tokyo)	Mountain

Biosystems Inc., CA), according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Identification of moderately halophilic bacteria from Tunisia and Japan

In our previous study, we have isolated ca 40 strains of halophilic bacteria from Tunisian environmental samples.Phylogenetic analysis of the isolates revealed that the isolates belong to phylums, γ-proteobacteria represented by the genus: *Halomonas, Chromohalobacter, Salinicola, Marino*bacter; firmicutes represented by the genus: *Bacillus* and *Hallobacillus* and Actinobacteria represented by *Brevibacterium*[9]. In addition, 45 strains were obtained from environmental samples in Okinawa.with a good diversity as shown in TABLE 2. The identification of isolate strains highlighted the predominance of Halomonas genus well knowon as moderate halophilic bacteria. The interest in halomonas species was previously confirmed due to their ability to produce exoploysaccharides, exoenzymes, osmolytes and bioplastics[21,22].

Halo-tolerant bacteria degrading n-alkanes isolated from Tunisia

Enrichment culture using the Tunisian environment samples with n-tetradecane under high NaCl concentration resulted in the positive growth only from two samples from Chebika, Tunisia. Only 2 strains out of 12 isolates showed stable assimilation of n-tetradecane under 10 % of NaCl. Figure 1 showed the phylogenetic positions of the isolates based on the 16SrRNA sequences.

TABLE 2: Isolates able to grow on medium with 10 % NaCl from Tunisia and Japan

Phylum	Genus	Tunisia	Japan
Proteobacteria	*Chromohalobacter*	0	1
Proteobacteria	*Halomonas*	3	36
Proteobacteria	*Kangiella*	1	0
Proteobacteria	*Marinobacter*	1	1
Proteobacteria	*Salinicola*	0	1
Proteobacteria	*Vibrio*	3	0
Firmicutes	*Bacillus*	13	2
Firmicutes	*Halobacillus*	6	1
Firmicutes	*Marinococcus*	3	0
Firmicutes	*Oceanobacillus*	1	1
Firmicutes	*Salinicoccus*	2	1
Firmicutes	*Staphylococcus*	2	0
Firmicutes	*Paenibacillus*	1	0
Firmicutes	*Virgibacillus*	0	1
Actinobacteria	*Brevibacterium*	0	1

Partial 16SrRNA sequence analysis (ca 750 bp) with the strain 21-3 and 22-1 showed significant homology to *Sinomicrobium oceani* SCSIO 03483 (99.86 %, accession no. JQ352762), and *Mycobacteirum aubagnense* (98.93 %, AY859683), respectively. In addition, gene analysis responsible for alkane degradation was examined with degenerate PCR for alkB and P450, those were responsible genes for the initial hydroxylation of alkanes under aerobic cultivation (TABLE 3). As a result, clear PCR DNA fragment was amplified with TN22-1 genome template when using degenarete primer-set for P450. Sequence analysis for the fragment (658 bp) revealed 82.67 % identity with nucleotide sequence of *Mycobacterium chubuense* strain NBB4 (accession number GU174754) and 82.38 % identity with primary sequence of *Mycolicibacteirum* sp. (WP 044524727). In contrast, no positive DNA fragment was amplified from genomic DNA of strain TN21-3 neither with P450 or alkB primer-set, suggesting that the genes responsible for the alkane hydroxylase could be a novel one since alkanes degradation of *Sinomicrobium* sp. has little been reported.

Halo-tolerant bacteria degrading n-alkanes isolated from Japan

Screening program for the alkanes degrading bacteria under high salinity condition was further examined using environmental samples in Japan. Overall, 72 strains were isolated from the enrichment culture with n-tetradecane (nC14) as the sole carbon source, followed by the degradation test for nC14 at 10 % NaCl. As a result, 3 strains (namely MHA3a, MHA4a, and MHA5a) showed a stable growth with nC14 as the sole carbon source under high salt concentration. As shown in Figure 2, they showed a significant growth at high NaCl concentration as 10 %, although no growth observed with strain *Rhodococcus* sp. 11B[23], a typical n-alkane degrading bacteria under the same

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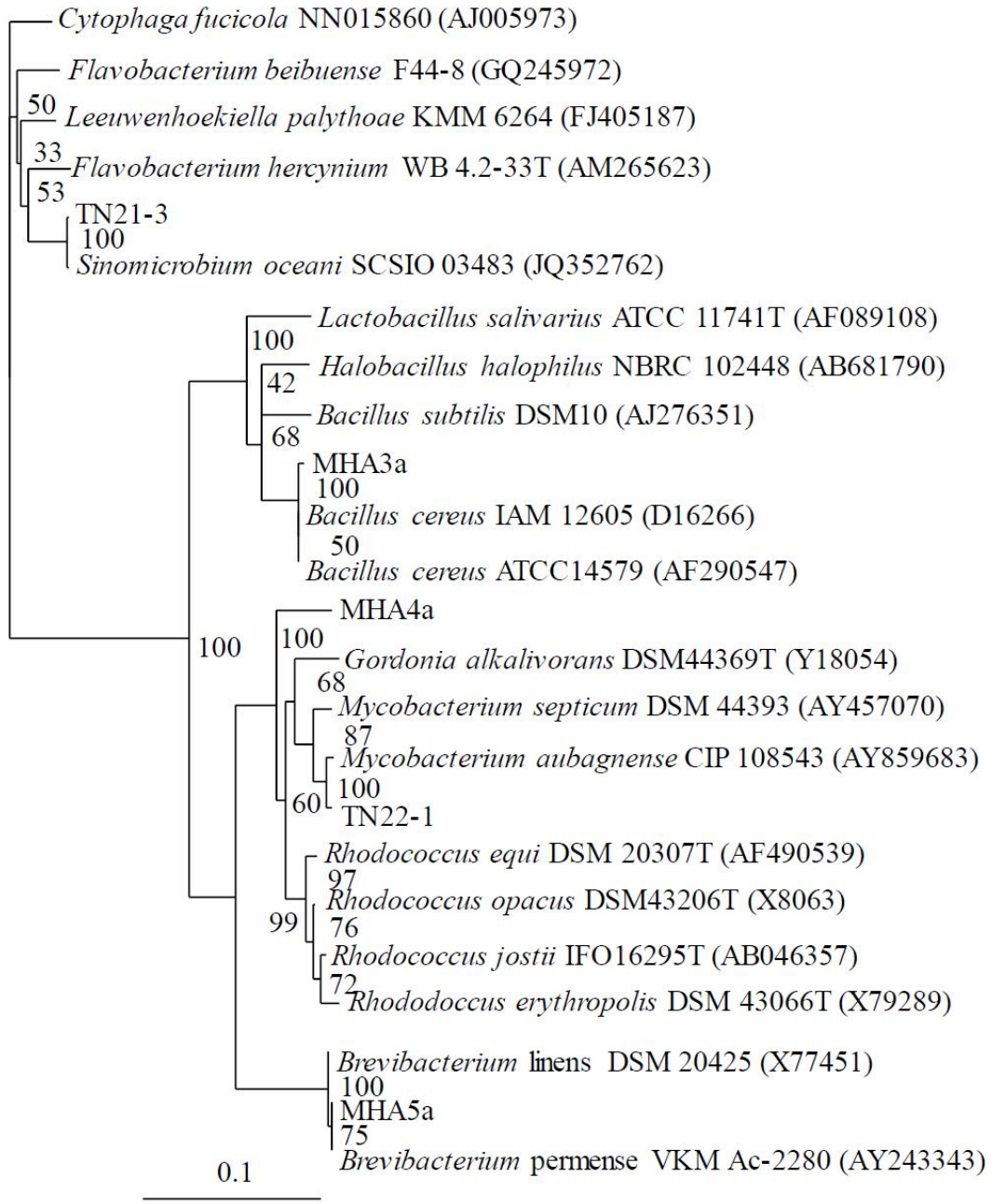

Figure 1: Phylogenetic analysis of the isolates based on the 16SrRNA sequence. Bootstrap probabilities are indicated at the branch points. The accession numbers are shown in parentheses. Cytophaga fucicola NN015860 (AJ005973) was used as the outgroup.

TABLE 3: Summary of the characterization of the isolates

Strain	Origin*	Top match (acc. No.)	Degradation**	PCR	
				alkB	P450
TN21-3	Tun	*Sinomicrobium oceani* SCSIO 03483 (JQ352762)	C12-30	X	X
TN22-1	Tun	*Mycobacterium aubagnense* CIP 108543 (AY859683)	C12-30	X	O
MHA3a	Jpn	*Bacillus cereus* IAM12605 (D16266)	C10-16	X	X
MHA4a	Jpn	*Corynebacterium variabilis* NCDO 2097T (X53185)	C12-20	O	X
MHA5a	Jpn	*Brevibacterium permense* VKM Ac-2280 (AY243343)	C10-20	O	X

*** Origin of the isolate: Tun;Tunisia, Jpn; Japan; **Carbon number of the n-alkanes**

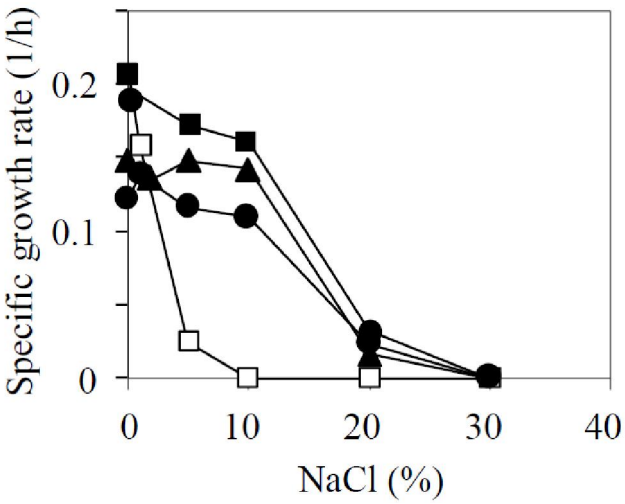

Figure 2: Effect of NaCl concentration on the cellular growth of the isolates. Closed circles; strain MHA3a, closed squares; MHA4a, and triangles; MHA5a. Data for non-salt tolerant alkane degrading bacteria, *Rhodococcus* sp. strain 11B (open squares) were also shown.

condition. According to the profiles in the relationship between NaCl concentration and specific growth rate, all of them were suggested to be salt-tolerant, but not halophilic bacteria.

They assimilated broad range of alkanes from C10 to C20, fatty acid, and trigriceride (data not shown). Genetic analysis responsible for the alkanes degradation revealed the presence of alkB gene but not P450, which was found in the halo-tolerant bacteria from Tunisia as described above.

CONCLUSION

In this report, we have isolated and characterized alkanes degrading bacteria under saline conditions from environmental samples in Tunisia and Japan. These microbial library might be useful for petroleum remediation of the contaminated sea or wastewater treatment with high salt concentration.

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