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Full Length Research Paper

Isolation, classification, phylogenetic analysis and scanning electron microscopy of halophilic, halotolerant and alkaliphilic actinomycetes isolated from hypersaline soil

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Actinomycetes were isolated by plating of serially diluted samples onto humic acid-vitamin agar prepared with or without NaCl and characterized by physiological and phylogenetical studies. A total of 16 strains were isolated from hypersaline soil in Mongolia. All strains showed alkaliphilic characteristics and were able to abundantly grow in media with pH 9.0. Six strains were halotolerant actinomycetes (0 to 12.0% NaCl) and 10 strains were moderately halophiles (3.0 to 12.0% NaCl). Most strains required moderately high salt (5.0 to 10.0%) for optimal growth but were able to grow at lower NaCl concentrations. Among the 16 strains, 5 were able to grow at 45°C. Among the group of moderate halophilic actinomycetes, 9 strains were phylogenetically detected under the genus *Nocardiopsis* in the family *Nocardiopsaceae*. Results of scanning electron microscopic study demonstrated that most of the moderately halophilic strains under the genus *Nocardiopsis*, produce synnemata structure. Overall phylogenetic analysis based on 16S rRNA gene sequences analysis revealed that all the 16 strains fell into 4 different genera: *Nocardiopsis* (9 strains), *Isoptericola*, (2 strains), *Nesterenkonia* (2 strains), *Streptomyces* (3 strains). This preliminary study demonstrated that the genus *Nocardiopsis* is abundant and a recoverable actinomycetes group in Mongolian saline soil. Based on the position in phylogenetic tree of 16 isolates, there were new clusters which supposed to be novel species under the genera *Nocardiopsis* (possible new species 1) and *Isoptericola* (possible new species 1) in the family *Nocardiopsaceae* and *Promicromonosporaceae*, respectively.

Key words: Classification, moderate halophilic and halotolerant actinomycetes, hypersaline soil, phylogenetic analysis, scanning electron microscope.

INTRODUCTION

The aerobic halophilic bacteria have been scarcely studied as compared to the extensive literature on the physiology, biochemistry and ecology of the aerobic red halophilic archaea (family *Halobacteriaceae*). Research on the halophilic and halotolerant bacteria often seems to be less fascinating than the study of the archaea, with their unique adaptations, including a highly saline cytoplasm, specialized salt-requiring proteins, and the

unique high driven proton and chloride pumps bacteriorhodopsin and halorhodopsin (Kushner, 1989). Moderately, halophilic bacteria constitute a heterogenous physiological group of microorganisms which belong to different genera. During the last decade, the extensive studies on hypersaline environments that have been carried out in many geographical areas have permitted the isolation and taxonomic characterization of a large number of moderately halophilic species.

Hypersaline environments are ubiquitous and halophiles and can survive in environments that limit the growth of most organisms. A common phenomenon in hypersaline environments is the occurrence of gradients in salinity as a result of the evaporation of sea water. Halophiles are characterized based on their requirement of salt for growth in hypersaline conditions. In contrast, halotolerant bacteria do not require NaCl for growth, although they grow in high salinity and in environments devoid of high concentration of salt. To describe microorganisms according to their behavior toward salt, different classification schemes have been devised. Although several classifications or categories have been proposed (Ollivier et al., 1994; Ramos-Cormenzana, 1989; Truper and Galinski, 1986; Vreeland, 1987), the most widely used is that of Kushner (1978), who defined moderate halophiles as organisms growing optimally between 0.5 and 2.5 M salt. Bacteria that are able to grow in the absence of salt as well as in the presence of relatively high salt concentrations (e.g., 8% in the case of *Staphylococcus aureus*) are designated halotolerant (or extremely halotolerant if growth extends above 2.5 M). A rare case of a bacterium that requires at least 2 M salt (optimal growth at 3.4 M), such as is exemplified by the actinomycete *Actinoployspora halophyla* (Gochnauer et al., 1975), is considered a borderline extreme halophile (Johnson et al., 1986; Kushner, 1978). According to Ollivier et al. (1994), halophiles can be classified into three groups on the basis of their response to NaCl: (a) slight halophiles which grow optimally at 2.0 to 5.0% NaCl (0.2 to 0.85 M); (b) moderate halophiles that show rapid growth at 5.0 to 20.0% NaCl (0.85 to 3.4 M) and the extreme halophiles which show optimally grow at 20.0 to 30.0% NaCl (3.4 to 5.1 M). According to DasSarma and Arora (2001), the non-halophiles grow optimally at less than 2.0% NaCl (0.2 M). Halophilic microorganisms are defined as organisms that have optimal growth in concentrations above 0.2 M NaCl (2.0%) and some are known to thrive above 5 M NaCl (30.0%). Many halophiles and halotolerant microorganisms can grow over a wide range of salt requirement or salt tolerance at times depending on environmental and nutritional factors. These extremophiles have been found in a variety of microenvironments including salt lakes and brines, saline soils, cold saline environments, alkaline saline habitats, and salted fish, meat and other foods (Ventosa et al., 1998 a, b). Originally, it was thought that Archaea dominated the hypersaline environments but it is now known that even in extreme saline conditions near the saturation point of NaCl, there are examples of halophiles from a wide variety of taxa including eukaryotes (Das Sarma and Arora, 2001), protozoa (Das Sarma and Arora, 2001; Rothschild and Mancinelli, 2001), fungi (Rothschild and Mancinelli, 2001), cyanobacteria (Rothschild and Mancinelli, 2001), Archaea (Das Sarma and Arora, 2001), Bacteria (Rothschild and Mancinelli, 2001; Dyall-Smith and Danson, 2001), diatoms (Rothschild and Mancinelli, 2001) and green algae (Rothschild and Mancinelli, 2001). Identification and classification of halophilic microorganisms is generally achieved through comparison of the 16S rDNA sequence (Anton et al., 2000) or based on chemotaxonomic criteria (Kamekura, 1998).

The present investigation aimed at the isolation and characterization of halophilic actinomycetes from hypersaline soils from different parts of Mongolia, which is a new investigation in Mongolia. Mongolia is one of the biggest Asian countries and recently, this attracted the attention of naturalists and other researchers to search for actinomycetes (Norovsuren et al., 2007) as a potential source of bioactive secondary metabolites. Mongolia also has preserved ecosystem with rich biodiversity and for this reason we conducted this study.

MATERIALS AND METHODS

Isolation of actinomycetes

Hypersaline soil samples were collected from Uvs and Dornod Provinces, Mongolia (Figures 1 and 2). Soil samples were collected by removing the surface loose litter layer, underlying 5 to 10 cm depth and before drying at room temperature for 7 days, pH values were determined. Humic acid-vitamin agar prepared with 0, 5 and 10% NaCl were used for isolation of actinomycetes. About 0.5 g soil samples were diluted with 5 ml sterilized distilled water and aliquot of 0.1 ml was spread on HV agar media. After incubation for 2 weeks at 28°C, actinomycetes colonies that developed on the plates were counted and numbers were expressed in colony forming units (CFU) (Figure 3). Actinomycetes colonies were picked and purified on yeast extract-starch agar (YS) (composed of 1.0% starch and 0.2% yeast extract in distilled water, pH 7.2) and yeast extract-glucose agar (YG) (composed of 1.0% starch and 1.0% glucose in distilled water, pH 7.2) with or without 5% NaCl.

Effect of NaCl concentration on growth of actinomycetes strains

Range of NaCl% in growth was observed on YS and YG agar without and with 3, 5, 8, 10, 12, 15 and 18% NaCl. Optimum NaCl concentration for growth was estimated in YG broth with 0, 3, 5, 8, 10 and 12% NaCl at 28°C for 7 days on a mini shaker at 342 rpm. After growth, the cultures were harvested by centrifugation and measured the dry biomass. Growth at pH 5, 7, 9 were observed in YG broth and on YS agar supplemented with 5% NaCl adjusted to the respective pH. Range of growth temperature was observed on

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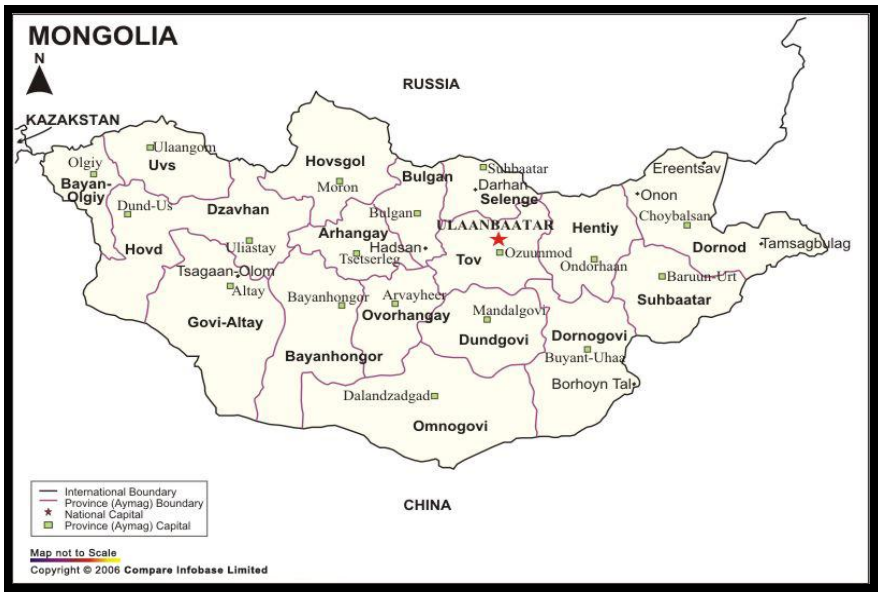

Figure 1. Map of Mongolia showing the study sites, Uvs and Dornod Province.

Figure 2. Hypersaline and salty sampling sites of Uvs and Dornod Province in Mongolia.

YS agar supplemented with 5% NaCl.

Scanning electron microscopy

Cell morphology was observed under a light microscope and by scanning electron microscopy (SEM). For SEM, the agar blocks containing the organisms (cultivated on YS agar medium supplemented with 5% NaCl for 14 days at 28°C) were fixed with the vapor of 1% osmium tetroxide and dried samples were sputter coated and viewed with scanning electron microscope (Tamura et al., 1994).

Survival at 50°C

For the survival at 50°C, two slopes of yeast glucose agar were inoculated from a 14 to 30 day culture in glucose broth. One of the

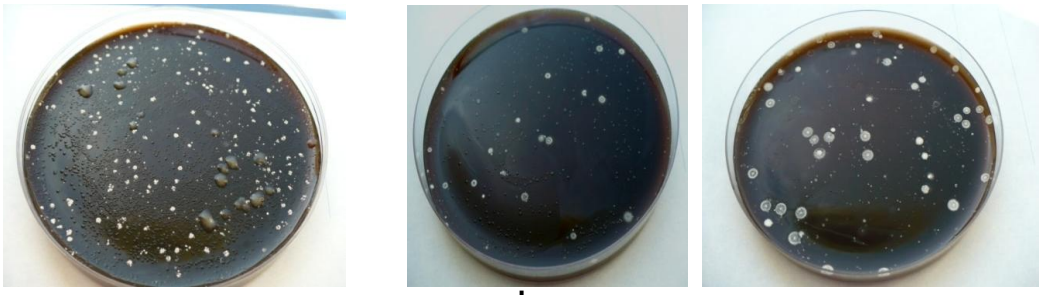

Figure 3. Isolation of halophilic and halotolerant actinomycetes from hyper saline soil of Mongolia. a: Humic acid vitamin agar (HVA) with 0% NaCl (soil no. 44); b: HVA with 5% NaCl (soil no. 44) and c: HVA with 5% NaCl (soil no. 44).

Table 1. Effect of salt concentrations on colony forming unit (cfu) of actinomycetes isolated from Mongolian saline soil.

Sample number	Location	0% NaCl with HV agar (cfu x 10^2/g of soil)	5.0% NaCl with HV agar (cfu x 10^2/g of soil)	10.0% NaCl with HV agar (cfu x 10^2/g of soil)	Strains isolated
S-26	Uvs province	> 800	226	31	MN07-A0385, MN07-A0386, MN07-A0387, MN07-A0388, MN07-A0389
S-30	Dornod province	> 800	67	69	MN07-A0390, MN07-A0391, MN07-A0392, MN07-A0393, MN07-A0394, MN07-A0395
S-44	Dornod province	264	96	37	MN07-A0396, MN07-A0397, MN07-A0398, MN07-A0399, MN07-A0400

two slopes was quickly heated to 50°C in a water bath and then held in another water bath at 50°C inside a constant temperature incubator for 8 h. After heating, the slope was quickly cooled, incubated at 28°C for 7 days and observed for growth. The unheated culture was also incubated at 28°C for 7 days and inspected for growth.

16S rDNA sequencing and phylogenetic analysis

Genomic DNA was extracted and purified as described by Marmur (1961) and Saito and Miura (1963), but with slight modifications: after lysis, we used 20% SDS and protease K to denature proteins and phenol/chloroform/isoamyl alcohol (25:24:1, by volume) to remove denatured proteins. 16S rRNA gene sequences were analysed as described by Tamura and Hatano (2001). Sequence analysis was performed with an ABI Prism BigDye Terminator cycle sequencing kit (PE Applied Biosystems) and an automatic DNA sequencer (model 3130 Genetic Analyzer: PE Applied Biosystems). Blast analysis (Altschul et al., 1990) was used to compare the 16S rRNA gene sequences of all the strains with sequences from the NCBI nucleotide database and sequence alignments were generated using CLUSTAL_X program (Thompson et al., 1997) with corresponding sequences (available in the DDBJ/EMBL/GenBank databases). The evolutionary distance matrices (Kimura two-parameter model) were calculated and phylogenetic trees were inferred using the neighbor-joining (NJ) method in CLUSTAL_X package. Minimum evolution (ME) (Rzhetsky and Nei, 1992), maximum parsimony (MP) (Eck and Dayhoff, 1966) and neighbor-joining (Saitou and Nei, 1987) trees were constructed using MEGA4 (Molecular Evolutionary Genetics Analysis version 4; Tamura et al., 2007). The evolutionary distances were computed using the maximum composite likelihood method. Stability of the resultant tree topologies was evaluated by bootstrap analysis (Felsenstein, 1985) based on the neighbor-joining dataset of 1000 resamplings using CLUSTAL_X and MEGA 4 packages. The 16S rRNA gene sequences determined in this study were deposited in the GenBank/EMBL/DDBJ database.

RESULTS

Colony forming units of actinomycetes isolated from Mongolian saline soils collected from three different places of Uvs and Dornod province are shown in Figure 3 and Table 1. Total actinomycetes populations were observed to decrease at 10% NaCl (61.5 to 86.3%) when compared with the number of actinomycetes at 5% NaCl (Table 1).

Actinomycetes were isolated from salty soil of Mongolia and characterized by physiological and phylogenetical studies. A total of 16 strains were isolated from salty soil. All strains showed alkaliphilic characteristics on YS and YG agar media with pH 7.0 to 9.0 and were able to grow optimally in YG broth media with pH 7.0 to 9.0 (Table 3). Based on the growth intensity on YS agar with or without NaCl, 6 strains were classified as halotolerant actinomycetes (0 to 12.0% NaCl) and 9 strains were moderately halophiles (3.0 to 12.0% NaCl). Among the group of moderately halophilic actinomycetes, 1 strain

Table 2. Classification of halotolerant and halophilic actinomycetes isolated from Mongolian saline soil.

Classification[a]	Isolates
Non-halophilic/halotolerent[b] (0-0.2 M) (0-1.2%)	MN07-A0386, MN07-A0387, MN07-A0388, MN07-A0389, MN07-A0391, MN07-A0399
Slight halophilic (0.2-0.5 M) (1.2-2.3%)	Not detected
Moderate halophilic (0.5-2.5 M) (2.3-14.5%)	MN07-A0385, MN07-A0390, MN07-A0392, MN07-A0393, MN07-A0394, MN07-A0395, MN07-A0396, MN07-A0397, MN07-A0398, MN07-A0400
Extreme halophilic (2.5-5.2 M) (14.5-30.0%)	Not detected

[a]According to the Kushner (1978); [b]Bacteria that are able to grow in the absence of salt as well as in the presence of relatively high salt concentrations (e.g., 8.0% NaCl in the case of *Staphylococcus aureus*) are designated halotolerent (or extremely halotolerant if growth extends above 14.5% NaCl) (Kushner, 1978).

designated as MN07-A0385 was able to grow in upto 15.0% NaCl (3.0 to 15.0% NaCl) (Table 2). Most of the strains required moderately high salt (5.0 to 10.0%) for optimal growth; however, they were able to grow at lower NaCl concentrations (Table 3).

Based on the growth intensity in different temperature on YS agar, 5 strains were able to grow at 10.0 to 45.0°C, 8 strains at 10.0-37.0°C, 2 strains at 15.0 to 37.0°C and 1 strain at 5.0 to 37.0°C (Table 3). Based on the growth intensity at 0.5 M of different chlorides, all the 16 strains grew well with 0.5 M KCl and NaCl. But no growth was observed with 0.5 M $MgCl_2$ and $CaCl_2$ except for the strain MN07-A0388 which showed growth with 0.5 M $MgCl_2$ (Table 3).

Results of scanning electron microscopic study demonstrated that most of the strains under the genera *Nocardiopsis* and *Streptomyces* abundantly produced synnemata structure (Figure 4).

Phylogenetic analysis based on 16S rRNA gene sequence analysis revealed that all the 16 strains fell into 4 different genera: *Nocardiopsis* (10 strains), *Isoptericola*, (2 strains), *Nesterenkonia* (1 strain) and *Streptomyces* (3 strains) (Figure 5).

This study demonstrated that strains under the genus *Nocardiopsis* are abundant in Mongolian hypersaline soil and recoverable (Table 3). Based on the position in phylogenetic tree of 16 isolates with other validly published species in the corresponding genera, there were new clusters which are supposed to be possible novel species under the genera *Nocardiopsis* and *Isoptericola* in the family *Nocardiopsaceae* and *Promicromonosporaceae* (Figure 5).

DISCUSSION

Distribution of actinomycetes in hypersaline soils

The oceans are the largest bodies of saline water with average salinities ranging from 32 to 35 psu. Salinities, generally originate as a result of evaporation of seawater. Such environments are inhabited by halophiles, the salt loving organisms. Halophiles are distributed in hypersaline environments all over the world, mainly in natural hypersaline brines in arid, coastal, and deep sea locations as well as in artificial salterns. Halophiles include prokaryotes and eukaryotes which are adapted to these hypersaline environments at the highest salt concentrations or close to the solubility limit of NaCl.

Hypersaline environments are those with salt concentrations above that of sea water (3.3% total dissolved salts), sodium and chloride are the dominating ions and the pH is near neutral to slightly alkaline as these environments result from evaporation of sea water. Halophiles employ a wide variety of mechanisms to survive in the hypersaline environment. The most common methods of avoiding desiccation involve using organic osmotic solutes such as glycerol, glycine betaine, or amino acids to prevent water loss (Ventosa et al., 1998).

Table 1 shows the total colony actinomycetes population at each hypersaline sample collected from Uvs and Dornod Province, Mongolia. The number of actinomycetes colonies is much higher in medium without NaCl from all sampling sites. The highest actinomycetes count was observed in sample no. S-26 from Uvs Province with 5.0% NaCl in HV agar, but lowest counts with 10.0% NaCl in HV agar as compared to sample no. S-30 and S-44, which were collected from Dornod Province (Table 1). Based on the 16S rDNA gene sequence analysis and morphological characteristics using light microscope and scanning electron microscope, among the total actinomycete population, 62.5% strains were under the genus *Nocardiopsis*, 18.7% in the genus *Streptomyces*, 12.5% in the genus *Isoptericola* and 6.2% under the genus *Nesterenkonia* (Table 1). The microbial diversity in hypersaline environments have been studied using the analyses of 16S ribosomal RNA genes amplified PCR from DNA extracted from samples taken from the environment. Molecular phylogenetic studies indicate a great phylogenetic and physiological diversity of Archaea.

Table 3. Classification, physiological characterization and generic identity of halotolerant and moderately halophilic actinomycetes isolated from Mongolian saline soil.

Isolates	Classification[e]	NaCl % for growth on YS agar	Optimum NaCl % for growth in YG broth	Growth pH	Growth temperature (°C)	Survival at 50°C	Generic identification
MN07-A0386	Halotolerant (0-0.2 M) (0 - 1.2 %)[e,f]	0-10.0	No growth	7.0-9.0	15.0-37.0	-	*Streptomyces*
MN07-A0387	Halotolerant	0-12.0	12.0	5.0-9.0	10.0-45.0	+	*Streptomyces*
MN07-A0388	Halotolerant	0-12.0	8.0	5.0-9.0	10.0-45.0	+	*Nocardiopsis*
MN07-A0389	Halotolerant	0-10.0	No growth	7.0-9.0	15.0-37.0	-	*Streptomyces*
MN07-A0391	Halotolerant	0-12.0	5.0	7.0-9.0	5.0-37.0	d[b]	*Isoptericola*
MN07-A0399	Halotolerant	0-10.0	8.0	7.0-9.0	10.0-37.0	d	*Isoptericola*
MN07-A0385	Moderate halophilic (0.5 - 2.5 M) (2.3 - 14.5 %)[e]	3.0-15.0	10.0	7.0-9.0	10.0-37.0	+	*Nocardiopsis*
MN07-A0390	Moderate halophilic	3.0-12.0	8.0	7.0-9.0	10.0-37.0	+	*Nocardiopsis*
MN07-A0392	Moderate halophilic	3.0-10.0	10.0	7.0-9.0	10.0-45.0	-	*Nocardiopsis*
MN07-A0393	Moderate halophilic	3.0-10.0	No growth	7.0-9.0	10.0-37.0	-	*Nesterenkonia*
MN07-A0394	Moderate halophilic	3.0-12.0	8.0	7.0-9.0	10.0-37.0	+	*Nocardiopsis*
MN07-A0395	Moderate halophilic	3.0-12.0	12.0	7.0-9.0	10.0-45.0	+	*Nocardiopsis*
MN07-A0396	Moderate halophilic	3.0-10.0	8.0	7.0-9.0	10.0-37.0	-	*Nocardiopsis*
MN07-A0397	Moderate halophilic	3.0-10.0	12.0	7.0-9.0	10.0-45.0	-	*Nocardiopsis*
MN07-A0398	Moderate halophilic	3.0-10.0	10.0	7.0-9.0	10.0-37.0	+	*Nocardiopsis*
MN07-A0400	Moderate halophilic	3.0-10.0	10.0	7.0-9.0	10.0-37.0	-	*Nocardiopsis*

Growth of all the 16 strains was positive at 0.5 M (2.3%) of NaCl and KCl and negative for 0.5 M of $MgCl_2$ and $CaCl_2$. b: d, doubtful; NG, no growth; e, according to the description of Kushner (1978); f, bacteria able to grow in the absence of salt as well as in the presence of relatively high salt concentrations (e.g., 8.0% NaCl in the case of *S. aureus*) are designated halotolerant (or extremely halotoletrant if growth extends above 14.5% NaCl) (Kushner, 1978).

These results agree with the results for the population of actinomycetes colonies in different salty, saline, alkaline and hypersaline soils. The genus *Nocardiopsis* is comprised of 29 species with validly published names, including several recently described species (Hozzein et al., 2004; Li et al., 2004; Sabry et al., 2004). Members of the genus *Nocardiopsis* have been reported to predominate in saline, alkaline soils (Tang et al., 2003), hypersaline soils (Li et al., 2006) and several recognized species have been isolated from such sources (Al-Tai and Ruan, 1994; Chun et al., 2000; Al-Zarban et al., 2002; Li et al., 2003; Hozzein et al., 2004; Li et al., 2004).

The genus *Nesterenkonia* was proposed through the reclassification of *Micrococcus halobius* (Onishi and Kamekura, 1972) as *Nesterenkonia halobia* (Stackebrandt et al., 1995). Phylogenetic analysis based on 16S rRNA gene sequences showed that the genus *Nesterenkonia* belongs to the family *Micrococcaceae* of the order *Actinomycetales* (Stackebrandt et al., 1995, 1997). Currently, the genus comprises eleven recognized species and most of these species were isolated from hypersaline or alkaline habitats such as saline soils (Li et al., 2004, 2005), hypersaline lake in eastern Antarctica (Collins et al., 2002), soil in the eastern desert of Egypt solar salt (Onishi and Kamekura, 1972), seafood (Yoon et al., 2006), soda-lake or alkaline waste water from Ethiopia (Delgado et al., 2006) and black liquor treatment system of a cotton pulp mill (Luo et al., 2009). All these moderate halophilic species belong to the low G+C group of the gram-positive phylum, only *Nesterenkonia* (formerly *Micrococcus halobius*) is within the high G+C

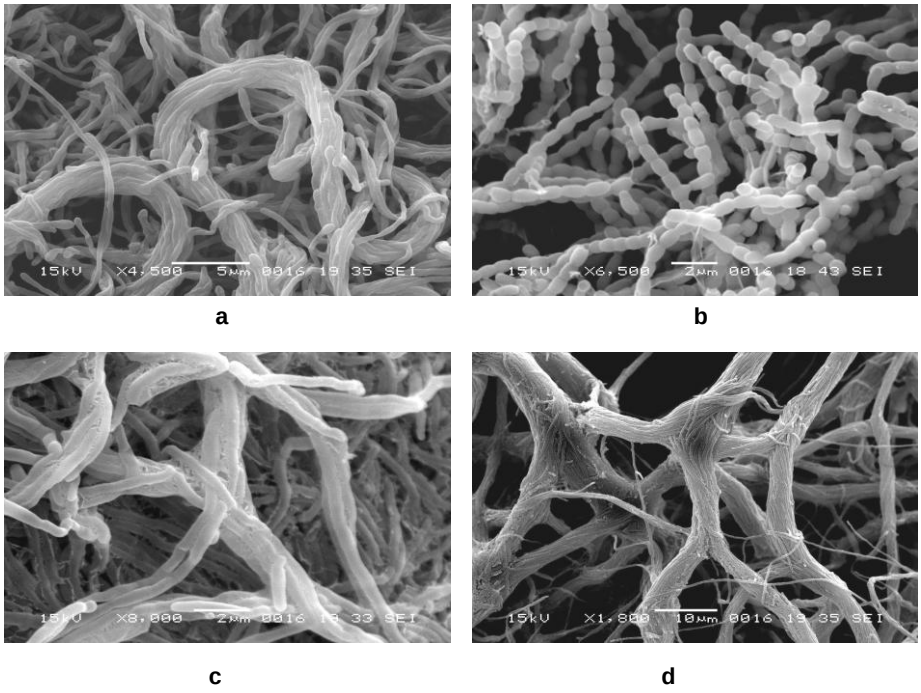

Figure 4. SEM photographs of a. MN07-389, b. MN07-387, c. MN07-400, d. MN07-388, grown on YS agar at 28°C for 3 weeks (arrows indicate synnemata formation).

group (Mota et al., 1997; Stackebrandt et al., 1995). Saline soils appear to yield mostly halotolerant rather than halophilic microorganisms, presumably reflecting adaptation to periodic edisodes of relatively high dilution (Quesada et al., 1982, 1983). Saline soils have been somewhat neglected as compared to hypersaline aquatic environments. The isolation of novel halophilic *Actinopolyspora* and *Nocardiopsis* species from salty soils in Death Valley (Calif.), Alcante and Iraq (Al-Tai and Ruan, 1994; Rosenberg, 1983; Yamada et al., 1954; Yorkovsky and Silver, 1997) suggests that a wealth of interesting unknown halophilic microorganisms may be present in these soils. Studies have unequivocally confirmed the abundance of halophilic bacteria in saline soils. It was also reported that the strains under the three genera of mesophilic actinomycetes such as *Actinopolyspora*, *Microbispora* and *Amycolatopsis* were isolated from the saline soil samples of Kuwait (Abbas, 2006). The species composition in soils differs greatly from that of the aquatic environments discussed previously (Ventosa et al., 1998a, b), while the dominant types of actinobacteria encountered in saline soils belongs to members of the genus *Micrococcus* (possibly *Nesterenkonia*) (Quesada et al., 1982; Rodriguez-Valera, 1988).

The genus *Isoptericola* was proposed by reclassification of *Cellulosimicrobium variabile* (Bakalidou et al., 2002) as *Isoptericola variabilis* (Stackebrandt et al., 2004). Currently, the genus *Isoptericola* comprises 4 species with validly published names and most of these species were isolated from saline soils in China (Zhang et al., 2005) and soil from Korea (Yoon et al., 2006) and Domitilla (Groth et al., 2005).

Grouping of halophilic and halotolerant actinomycetes

Halophilic and halotolerant microorganisms can be found in each of the 3 domains of life: Eukarya, Bacteria and Archae. Halophilic microorganisms can be conventionally grouped according to their requirements for NaCl for growth. Slightly halophilic organisms in marine environments can grow in the presence of 2 to 3% NaCl. The moderate halophilic grow over a much wider NaCl concentration range (5 to 20%, w/v). The extreme halophiles, including the well-known halobacteria and halococci, are able to grow in saturated NaCl and unable to grow in the presence of NaCl concentrations lower than 12%. The occurrence of actinomycetes in high saline environments and the tolerance of these organisms to high concentrations of salts have been described by Gottlieb (1973). There is however a preference to lower optimal and maximal salt concentrations for growth and thus microorganisms are classified according to their requirements and tolerance to salt.

The common denominator for all moderately halophilic

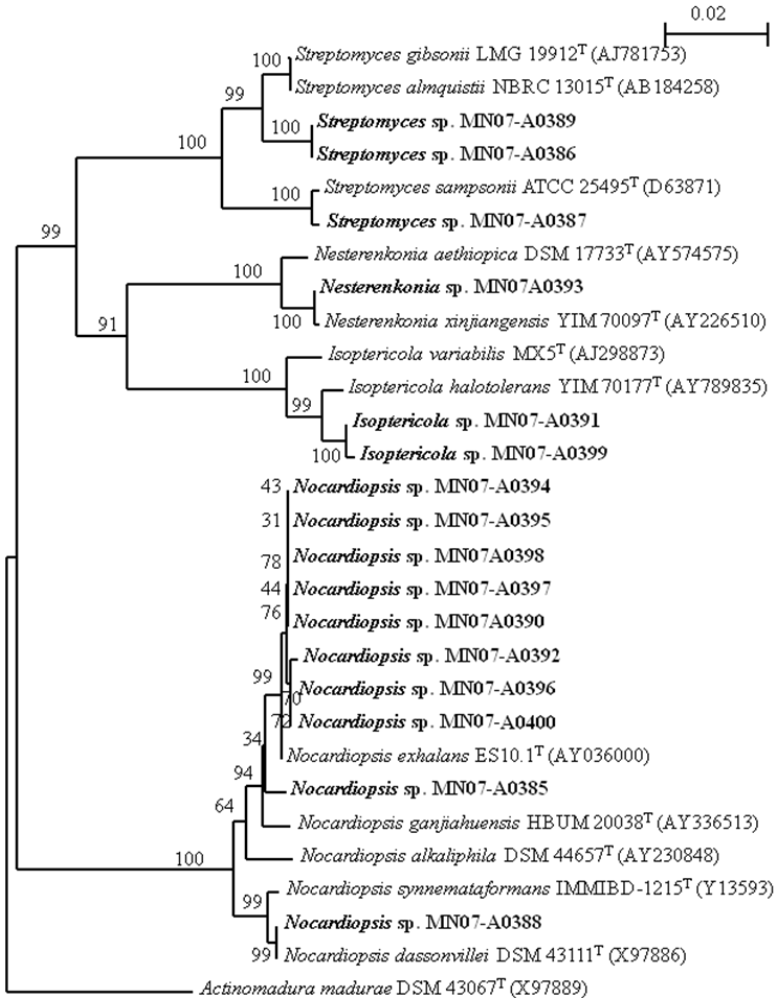

Figure 5. NJ phylogenetic tree based on the almost complete 16S rRNA gene sequence analysis and showing the relationships between 10 strains of moderately halophilic and 6 strains of halotolerant actinomycetes in 4 different genera.

bacteria is their requirement for salt and their ability to tolerate high salt concentrations. Salt requirement and tolerance are highly variable among the different species. Moreover, these parameters are by no means constant, since they may vary according to the growth temperature and the nature of the nutrients available (Kushner, 1993).

Characteristics of hyper saline environments

During evaporation, some changes may occur in the ionic composition out of precipitation of gypsum ($CaSO_4.2H_2O$) or other minerals once their solubility has been exceeded.

Adaptations to high and changing salt concentrations

To be able to live at high salt concentrations, halophilic and halotolerant microorganisms must maintain cytoplasm which is osmotically isotonic with the outside medium. The soil habitat is inherently inhomogenous and it can be expected that a wide range of salinities might be present in any of the saline soil (Grant, 1991).

Diversity of hypersaline microorganisms

The domain bacteria contains many types of halophilic and halotolerant microorganisms, spread over a large number of phylogenetic groups (Ventosa et al., 1998a, b), and reported that halophiles are also found among the actinomycetes, within the lineages of Gram-positive bacteria (Firmicutes). In general, it may be stated that most halophiles within the domain bacteria are moderate rather than extreme halophiles. Gram-positive bacteria, mainly include moderate halophilic species in the genera *Halobacillus*, *Bacillus*, *Marinococcus*, *Salinococcus*,

Nesterenkonia and *Tetragenococcus*. Actinomycetes from saline soils include *Actinopolyspora halophila* which grows best at moderate NaCl concentrations and is one of the few heterotrophic bacteria that can synthesize the compatible solute glycine-betaine and *Nocardiopsis halophila*, which uses a hyperoxydative of ectoine and beta-glutamate as compatible solutes (Ventosa et al., 1998a, b). Adaptation and adaptability of halophilic bacteria depend on the regulation of the synthesis of organic osmolytes as glycine-betaine, ectoine, glucosylglycerol and others.

In our study, it was found that salt requirement and tolerance of many species vary according to growth conditions such as temperature and medium composition (data not shown). The growth temperature should be specified, especially for the determination of the lower salt range enabling growth. It was reported that *Marinococcus halophilus* grows at NaCl concentrations as low as 0.01 M at 20°C but at least 0.5 M is required at 25°C (Novitsky and Kushner, 1976). Similarly, *S. costicola* can grow between 0.5 and 4 M NaCl at 30°C but can grow to 0.2 M at 20°C (Kushner, 1978).

From our data, it may be concluded that 75% of the total isolated actinomycetes are able to grow on agar media supplemented with 10.0% NaCl; 37% with 12.0% NaCl and 6.0% isolates with upto 15.0% NaCl (Table 3).

Conclusion

The enormous diversity of halophilic microorganisms is disbursed in the three domains of life, each with its own interesting and unique properties. There is hardly a hypersaline niche in nature that is not occupied by some halophiles (Oren, 1999). The existing types of Archaea, Bacteria and Eukarya that are able to withstand the stress exerted by salt concentrations upto halite saturation, exhibit a large metabolic diversity that empowers hypersaline ecosystems to function. Hypersaline environments, especially salt marshes are wild and beautiful components of coastal lands. They and their inhabitants make these unique ecosystems fascinating to study. Comparatively little is known about the processes occurring in the salt marshes and their importance to adjacent ecosystems. When examined superficially, they appear rather simple, but a closer investigation reveals tremendous diversity of form and process, which make it unwise to extrapolate the knowledge gained at one site to other marshes.

Halophiles produce a large variety of stable and unique biomolecules that may be useful for practical applications. Although, the current commercial uses of the halophiles are quite significant and many novel and unique properties of many of these organisms, suggest that they have greater potential for biotechnology (Rodriguez-Valera, 1992). Finally, halophiles are an interesting class of extremophilic organisms that have adapted to harsh hypersaline conditions. The diversity of microorganisms in these environments is also of growing interest. The recent findings of bacterial and archaeal metabolic activity suggest that these environments may harbor diverse consortia of microbes that are not easily cultured. Occurrence of novel and stable molecules in halophiles make them valuable for the future biotechnology pursuits.

Systematic and phylogenetic studies have defined a large number of species to be included within the moderate halophilic bacteria, distributed over at least half of the major phylogenetic branches of the bacteria. Molecular ecology techniques available nowadays should be used to determine in more detail the ecological distribution of these halophilic microorganisms and the roles they play in hypersaline environments, as well as their contribution to microbial transformation process. The use of such techniques would enable the elucidation of the biodiversity of moderate halophilic bacteria and the identification of species that constitute the predominant populations in these extreme habitats.

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