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THE DIVERSITY OF HALOTOLERANT AND HALOPHILIC BACTERIA IN THE SOIL OF THE NASINUAN SECONDARY FOREST IN MAHA SARAKHAM, THAILAND

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Abstract: In the present study, we investigated the diversity of halotolerant and halophilic bacteria in the soil of the Nasinuan Secondary Forest using the culturable approach. Halotolerant, slightly halophilic, and moderately halophilic bacteria were isolated from soil samples using the halobacteria medium containing 0%, 3%, and 6% w/v sodium chloride, respectively. In total, 55 isolates were obtained. A decreasing trend of bacterial diversity was found with the increase in concentration of sodium chloride in the medium. From a comparison of cell and colony morphologies, 16 isolates were selected for identification based on 16S rDNA sequence analysis. Of the 16 isolates, 14 isolates were halotolerant bacteria and 2 isolates were slightly halophile. Many of them had an ability to grow in a wide range of NaCl (0-8% w/v). The 16 isolates belonged to 5 genera, namely *Bacillus, Enterobacter, Janibacter*, *Rhodococcus,* and *Staphylococcus*. The dominant species belonged to the genus *Bacillus*. The phylogenetic analysis showed genetic diversity covering 5 clusters: Gram-negative rods; Gram-positive rods/cocci, non-endospore-forming; *Bacillus* sensu stricto; *Bacillus* sensu lato; and Gram-positive cocci. This is the first comprehensive study of culturable halotolarant and halophilic bacterial diversity in a non-saline soil of Thailand.

Keywords: Bacterial diversity, bacterial flora, soil bacteria, saline soil**,** salt-affected areas.

Introduction

Soil salinization is one of the most crucial problems of arid and semiarid regions (Sakadevan, & Nguyen, 2010). High salinity results in the reduction of crop productivity and quality (Yamaguchi & Blumwald, 2005; Szabados *et al.,* 2011). In Thailand, the majority of saline soil is in the northeast region, especially in Maha Sarakham Province, where 85% of the area is underlain by the rock salt strata (Wongsomsak, 1986). The moderately and heavily salt-affected areas have been found in almost the entire province (Ghassemi *et al*., 1995), except for the Nasinuan Secondary Forest.

Halotolerant and halophilic bacteria are microorganisms that thrive in saline environments (Javor, 1989; Oren, 2002; Maheshwari, & Saraf, 2015). They have attracted much attention because their physiological properties can facilitate their exploitation for commercial and agricultural purposes (Kushner, 1993; Azua-Bustos & González-Silva, 2014; Singh, 2016; Waditee-Sirisattha *et al.,* 2016). Early and intensive studies of bacterial diversity in saline environments were conducted in salt lakes and salterns (Litchfield & Gillevet, 2002; Hacêne *et al.,* 2004; Hedi *et al.,* 2009; Vahed *et al*., 2011). Most studies of halophilic bacteria in Thailand deal with Thai fish sauce and salted foods, while the studies of saline soils are still limited (Tanasupawat *et al.*, 2006; Chamroensaksri *et al.*, 2010; Sumpavapol *et al.,* 2010). In addition, soil bacteria are the primary organisms that drive the forest ecological processes (Felsmann *et al*., 2015) and their applications in sustainable agriculture are very successful and acceptable (Cavaglieri *et al*., 2005; Yadav & Saxena, 2018). The knowledge regarding halophilic bacterial diversity in the Nasinuan Secondary Forest could provide baseline information for the reclamation of saline soil and the sustainability of the forest ecosystem and agricultural production. It was thus aimed in this work to explore the diversity of halotolerant and halophilic bacteria in the

Nasinuan Secondary Forest and to obtain halotolerant and halophilic bacterial resources for future applications.

Materials and Methods

Site Description and Soil Sample Collection

The Nasinuan Secondary Forest is situated in Kantharawichai District, Maha Sarakham Province, Thailand, with a total area of approximately 19.2 hectares. The weather of the study area is tropical savanna. The sampling sites were divided into 14 plots, according to a previous study (Chookietwattana & Yuwa-amornpitak, 2019). Soil samples were collected seasonally between June 2017 and March 2018. The samples were randomly taken from three subplots for each plot at a depth of 30 cm using a hand auger. Samples from the same plot were mixed thoroughly. Then, one kilogram of the soil samples was taken and analysed within 24 hours.

Isolation of Halophilic Bacteria from Soil Samples

The halobacteria medium (Atlas, 1997) containing 0%, 3%, and 6% w/v NaCl were used for the isolation of halotolerant bacteria, slightly halophilic bacteria, and moderately halophilic bacteria according to the classification of microorganisms in response to salt (NaCl) in which they grow best (Kushner, 1993). The pH of the medium was 7.2, unless otherwise stated. Ten-fold serial dilutions of the soil samples were made using a sterile normal saline solution, and 0.1 mL of each was spread out over the isolation medium. The plates were incubated for 2 to 3 days at 37°C. Each different colony type (according to size, color, edge, and texture) in each plate was picked and streaked on the halobacteria medium containing NaCl at the same concentration as they were first isolated to obtain single colonies. This procedure was repeated to purify the isolates. All bacterial isolates were maintained on halobacteria agar slants for short-term applications and preserved in 50% glycerol stocks at –70°C for long-term applications.

Phenotypic and Genotypic Characterization of Bacterial Isolates

The phenotypic characteristics of halophilic bacterial isolates, namely colony morphology (evaluated from first picked colonies from the original soil dilution plate), Gram-staining reaction, and cell morphology were examined. All bacterial isolates were classified on the basis of their response to salt (NaCl) in which they grow best (Kushner, 1993). Bacterial growth at various NaCl concentrations (0, 3, 8, 15, 20, 25, and 32% w/v), pH (4, 5, 7, 9, and 11), and temperatures (10, 25, 35, 45, and 50°C) was determined by transferring 0.1 mL of pre-culture (approximate 10^8 CFU/mL) to 4.0 mL of halobacteria broth. Growth was determined by measuring an optical density at wavelengths of 600 nm after 48 hours of incubation. An optical density of below 0.05 meant that there were no growth and the highest optical density meant that the culture medium had an optimum condition for growth. For testing growth at various NaCl concentrations, the pH of the culture medium was fixed at 7.2 and the incubation temperature was kept at 37°C. For testing growth at various pH, the medium containing NaCl at an optimum concentration of each isolate was used and the incubation temperature was kept at 37°C. For testing growth at various temperatures, the medium was kept at a pH level of 7.2 and NaCl at the optimum concentration of each isolate was used.

For genotypic characterization, genomic DNA was extracted using an InstaGene Matrix kit (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. The 16S rRNA genes of bacterial isolates were amplified by PCR using primers 27F 5'(AGAGTTTGATCMTGGCTCAG)3' and 1492R 5'(TACGGYTACCTTGTTACGACTT)3'. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30 µL reaction mixture by using a *EF-Taq* (SolGent, Korea) as follows: initial denaturation at 95°C for 2 minutes, followed by 35 cycles of 95°C for 1 minutes, 55°C, and 72°C for 1 minutes, and extension at 72°C for 10 minutes. The

amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The sequencing primers were 785F 5' (GGATTAGATACCCTGGTA)3' and 907R 5'(CCGTCAATTCMTTTR AGTTT)3'. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5 minutes, followed by 5 minutes on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The 16S rRNA gene sequences were aligned to the nucleotides database provided by the National Center for Biotechnology Information (NCBI, U.S.A.) using the BLASTN (Basic Local Alignment Search Tools) (Altschul *et al*., 1990). The alignment scores and the percent sequence identity were determined for the closest identity. A multisequence alignment was performed using Clustal W. A phylogenetic tree was constructed by the neighbor-joining method using MEGA 6.0 (Tamura *et al*., 2013) with 1,000 bootstrap replications.

Results and Discussion

Isolation of Bacteria from Soil Samples and Phenotypic Characterization

The present study is the first report on halotolerant and halophilic bacterial diversity in the Nasinuan Secondary Forest, a non-salt affected area of Maha Sarakham. Forty-two soil samples were collected seasonally throughout the year and subjected for isolation of halotolerant and halophilic bacteria. It is worth mentioning that halotolerant bacteria grow best at <1% w/v salt and can tolerate high salt, whereas halophile bacteria require high salt for growth (Kushner, 1993). Halophilic bacteria are divided into several groups according to salt concentrations in which they grow best. The slightly and moderately halophilic bacteria are defined as bacteria that grow best at 1-3%, and 3-15% w/v NaCl, respectively. As such, the halobacteria media with the final NaCl concentration at 0%, 3%, and 6% w/v NaCl were used for the isolation of halotolerant, slightly halophilic, and moderately halophilic bacteria. Of the 55 bacterial isolates obtained, the 31, 17, and 7 isolates were isolated from the halobacteria media containing 0%, 3%, and 6% w/v NaCl, respectively. These results are in agreement with the findings of Del Moral *et al*. (1987) and Ventosa *et al*. (1998), who found that bacterial diversity decreased as the salt concentration of the media increased.

The results of Gram-staining reaction of the 55 isolates revealed that most of them were Gram-positive bacteria (Figure 1). These results agreed with the study by Schimel *et al*. (2007). The soil texture as the loamy sand - sandy loam of the soil samples (Chookietwattana & Yuwa-amornpitak, 2019) caused inconsistent change in soil moisture content, soil pH, soil salinity, and soil temperature by permitting rapid movements of materials moving through it, including air, water, and microorganisms (Rodriguez-Valera *et al*., 1981). The Gram-positive bacteria can better tolerate such a fluctuating environment compared with the Gram-negative bacteria as they have the stronger cell walls (Schimel *et al*., 2007). The teichoic acids found in the cell walls of Gram-positive bacteria provide the negative charges of the cell surface as a whole, thus increasing the mobility and transport of these bacteria in the environment. These findings may also be due to an extremely low amount of soil organic matter and total nitrogen in the soil samples (Chookietwattana & Yuwa-amornpitak, 2019) since Gram-positive bacteria are more prevalent in soil of low quality, while Gram-negative bacteria dominates in soils of higher quality (Zhou *et al*., 2017). A total of 50 out of the 55 isolates were Gram-positive rods and endospore-forming. These results could be due to the fact that spore-forming bacteria are likely to be widely dispersed by wind and migratory birds (Jones *et al*., 1998). The ability to produce spores helps these bacteria endure and survive in arid environments with nutrients for low growth (Reynolds & Pepper, 2000).

From the preliminary phenotypic observation (data not shown), 16 out of the 55

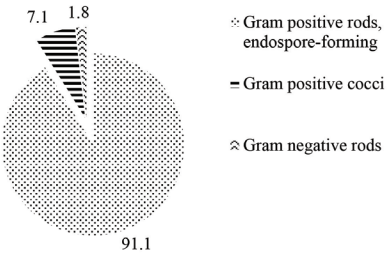

Figure 1: The results of microscopic observation of the Gram test

isolates were selected for further characterization. Although we specified the salt concentrations of the media to provide the optimal saline conditions for each halophilic bacterial group, we found that many isolates, especially the ones that were isolated from the medium containing either 3% or 6% w/v NaCl, could not grow in the media containing the same NaCl content. This is due to the fact that the halotolerants occupy the more diverse groups than those that tolerate only a small concentration (1% w/v) to a high concentration (up to saturation) of salt (Larsen, 1986). Therefore, to eliminate the taxonomic problems with halophiles, growth in response to salt was determined. The bacterial growth at various pH and temperature levels was also determined. The responses of bacterial isolates to salt (NaCl), pH, and temperature were summarized in Table 1. Quesada *et al*. (1982), Rodriguez-Valera *et al*. (1981) and Zahran *et al*.

Table 1: The characteristics of 16 isolates in response to salt, pH, and temperature levels

Isolate code	Degree of halophilia*	NaCl range	Optimal NaCl	pH range	Optimal pH	Temperature range	Optimal temperature
1. RSPG 10at	Halotolerant	0-3	0	5-9	7	25-50	50
2. RSPG 1A	Halotolerant	0-8	0	5-9	7	25-35	25
3. RSPG 2d	Halotolerant	0-3	0	7-9	9	35-50	50
4. RSPG 8aad	Halotolerant	0-3	0	7-9	9	25-35	35
5. RSPG 1N	Halotolerant	0-8	0	4-9	7	25-50	50
6. RSPG 3h	Halotolerant	0-3	0	4-9	7	25-35	35
7. RSPG 1T	Halotolerant	0-3	0	5-7	7	35-50	50
8. RSPG 10t	Halotolerant	0-3	0	5-9	9	25-50	50
9. RSPG 8k	Slightly halophile	0-8	3	5-9	7	25-50	35
10. RSPG 10am	Halotolerant	0-8	0	5-9	5	25-50	45
11. RSPG 3m	Slightly halophile	0-8	3	7-9	7	25-50	35
12. RSPG 15ac	Halotolerant	0-8	0	5-9	7	10-35	35
13. RSPG 5ag	Halotolerant	0-3	0	7	7	25-50	35
14. RSPG 12f	Halotolerant	0-3	0	4-9	5	25-45	45
15. RSPG 15aa	Halotolerant	0-3	0	5-9	9	25-35	35
16. RSPG 7aac	Halotolerant	0-3	0	5-9	7	35-50	35

*The degree of halophilic was interpreted by comparison with the classification of microorganisms' response to salt (NaCl) as described by Kushner (1993).

(1992) reported that the majority of microflora in saline soil was halotolerant, while Echigo *et al*. (2005) found numerous halotolerant bacteria from non-saline soil. Thus, it is not surprising that soil samples that were non-saline (electrical conductivity ranging from 0.03-0.08 dS/m, data not shown) showed mostly halotolerant bacteria, instead of halophilic bacteria. Only two slightly halophile bacteria were found and optimally grew at 3% w/v NaCl. The majority of isolates showed optimal growth at a pH level of 7 and temperature of 35°C, which is the most favorable level for common bacteria. The upper limit of NaCl concentration permitting growth for many isolates obtained in this study was up to 8% w/v NaCl.

Genotypic Characterization of Bacterial Isolates

To provide a more reliable classification, the genotypic characterization was performed with the help of phenotypic data. Sequences of PCR-amplified 16S rRNA genes of the 16 isolates were analysed (about 1,400 nucleotides). All 16S rRNA gene sequences of the isolates were compared with the nucleotide sequence database and deposited in the GenBank. The results of the search are summarized in Table 2. Sixteen isolates exhibited similarities higher than 99% with *Bacillus* (11 isolates), *Staphylococcus* (2 isolates), *Enterobacter* (1 isolate)*, Janibacter* (1 isolate), and *Rhodococcus* (1 isolate). These results are similar to the findings of Echigo *et al*. (2005), who found that the *Bacillus* was a predominant genus in non-saline soil.

From the phylogenetic analysis, the isolates were divided into five clusters, as shown in Figure 2. The Gram-negative rods group was assigned to Cluster I, which comprised only one isolates related to *Enterobacter hormaechei*. Cluster II consisted of *Janibacter anophelis* and *Rhodococcus rhodochrous,* which exhibiting Gram-stain-positive to Gram-stain-variable

Table 2: Summary of information of the 16S rDNA sequence comparison

Isolate code	Closest relative	Information of organisms used for sequences comparison	
		Accession number	Reference
1. RSPG 10at	*Bacillus australimaris*	JX680098	Liu *et al.* (2013)
2. RSPG 1A	*Bacillus cereus*	NR074540	Ivanova *et al.* (2003)
3. RSPG 2d	*Bacillus glycinifermentans*	KT005408	Kim *et al*. (2015)
4. RSPG 8aad	*Bacillus indicus*	NR029022	Suresh *et al.* (2004)
5. RSPG 1N	*Bacillus licheniformis*	NR118996	Ludwig *et al.* (1992)
6. RSPG 3h	*Bacillus megaterium*	CP009920	Johnson *et al.* (2015)
7. RSPG 1T	*Bacillus subtilis*	CP002905	Earl *et al.* (2012)
8. RSPG 10t	*Bacillus paralicheniformis*	KY694465	Dunlap *et al.* (2016)
9. RSPG 8k	*Bacillus safensis*	NR041794	Venkateswaran *et al.* (2001)
10. RSPG 10am	*Bacillus siamensis*	KY643639	Gondil *et al.* (Unpublished)
11. RSPG 3m	*Bacillus wiedmannii*	KU198626	Miller *et al.* (2016)
12. RSPG 15ac	*Enterobacter hormaechei*	CP017179	Chavda *et al.* (2016)
13. RSPG 5ag	*Janibacter anophelis*	NR043218	Kämpfer *et al.* (2006)
14. RSPG 12f	*Rhodococcus rhodochrous*	NR037023	Rainey *et al.* (1995)
15. RSPG 15aa	*Staphylococcus gallinarum*	NR028667	Lambert *et al.* (1998)
16. RSPG 7aac	*Staphylococcus saprophyticus*	NR074999	Kuroda *et al.* (2005)

and rod-coccus lifecycle (Rainey *et al.,* 1995; Kämpfer *et al.,* 2006). The evolutionary relationship of Clusters I and II was distant from any other cluster. The phylogenetic tree confirmed the genetic diversity of the *Bacillus*. In this study, we divided *Bacillus* into two clusters, namely Clusters III and IV based on the branching patterns. Cluster III contained six species, namely *Bacillus australimaris, Bacillus safensis, Bacillus subtilis, Bacillus paralicheniformis, Bacillus licheniformis,* and *Bacillus glycinifermentans*, which *Bacillus subtilis* and *Bacillus licheniformis* are representative species of the *Bacillus* sensu stricto**,** hence the cluster name. Cluster IV consisted of five species, namely *Bacillus siamensis*, *Bacillus indicus, Bacillus megaterium*, *Bacillus cereus*, and *Bacillus wiedmannii*, which *Bacillus cereus* was the well-known species in the *Bacillus* sensu lato group (Zeigler, & Perkins, 2009). Cluster V consisted of the genus *Staphylococcus,* which is a sister group among the Gram-positive bacilli (Götz, *et al*. 2006). Since the *Bacillus* spp. are the most dominant group involved in \biogeochemical cycling mainly mediated by their diverse physiological characteristics, a more comprehensive analysis of the structure of the *Bacillus* community is needed to understand their roles and dynamics in the natural soil system. Many *Bacillus* strains have a wide range of biotechnological applications in enzyme production, antibiotic production, biofertilizer, biocontrol, and bioremediation (Zeigler & Perkins, 2009). Thus, several salt tolerant and thermotolerant *Bacillus* strains obtained from this study have potential future applications.

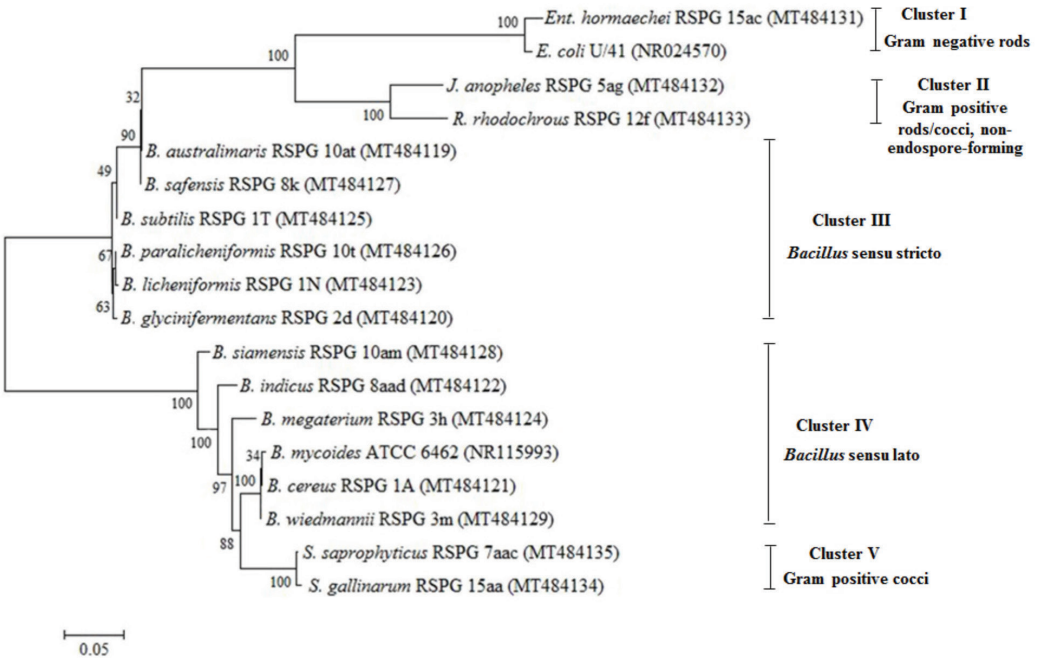

Figure 2: Neighbor-joining phylogenetic tree showing the relationships of the sixteen bacterial isolates obtained from the Nasinuan Secondary Forest. The GenBank accession numbers for all taxa are indicated in the parenthesis. *E. coli* strain U 5/41 (Cilia *et al*., 1996) and *Bacillus mycoides* strain ATCC 6462 (Soufiane, & Cote, unpublished) were used as reference strains for the Gram-negative and Gram-positive bacteria, respectively

Conclusion

In the present study, we have demonstrated that the diverse taxa of halotolerant and halophilic bacteria thrive in non-saline soil. We could isolate 55 bacterial strains. Most of them were Gram-positive rods and endospore-forming, which belonged to the genus *Bacillus*. This study offers a promising start for understanding the bacterial communities inhabiting the secondary forest soil and also provides valuable microbial resources for biotechnological applications.

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