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INVESTIGATION OF SALT-TOLERANT RHIZOSPHERE BACTERIA FROM SEAWATER-INTRUDING PADDY RICE FIELD IN VIETNAM

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ABSTRACT

Salt-tolerant plant growth-promoting rhizobacteria (ST-PGPR) are known as potential tools to improve rice salinity tolerance. In this study, we aimed to investigate the plant growth-promoting rhizobacteria community richness of the paddy rice fields in Soc Trang and Ben Tre Provinces where were seriously affected by sea level rise. The salinity in the sampling sites ranged from 0.14‰ to 2.17‰ in November 2018, the rainy season. The microbial abundance of samples was evaluated by spreading the samples in tryptic soy agar (TSA) medium supplemented with various concentrations of NaCl. With the increase of salt concentration up to 10% NaCl, a total number of bacteria decreased for all the samples, ranging from 10^6 to 10^4 CFU/g, and bacterial colonies were not observed at 30% NaCl. Among a total of 48 salt-resisting bacteria isolated from the rice paddy field mud surrounding the rice root, 22 isolates were able to produce indole-3-acetic acid (IAA: phytohormone for the plant growth). Seventeen out of 48 isolates were able to grow in the medium without nitrogen or phosphor sources. Six isolates having high IAA producing activity, nitrogen fixation and phosphate solubilization were belonged to *Bacillus* (DT6, LT16, and LHT8), *Halobacillus* (DT8), *Aeromonas* (LHT1), and *Klebsiella* (LHT7) genera. All the sequences of the strains DT6, DT8, LT16, LHT1, LHT7, and LHT8 were registered in the GeneBank with the accession numbers MK335670, MK335671, MK335672, MK335673, MK335674, and MK335675, respectively.

Keywords: PGPR, seawater intrusion, salinity tolerance, Mekong delta, rhizospherebacteria.

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INTRODUCTION

Vietnam is a leading country for rice (*Oryza sativa)* export, a half of rice production and 70% of exported rice comes from the Mekong Delta (Nguyen Thi Minh & Kawaguchi, 2002). Recently, production of rice in this region has been affected by the salt intrusion and draught. In 2013, in Binh Dien District, Ben Tre Province, about a half (500 ha) of 1,158 ha of the rice field were suffered from the draught, lack of water, and high salinity in the soil, resulted in the reduced crop production by 70%. Also, SocTrang Province in the Mekong Delta lost 600 ha of rice field due to salt intrusion. In 2016, 11 out of 13 provinces including Ben Tre and Soc Trang provinces in the Mekong Delta suffered from natural disasters such as draught and salinity. Development of salt-tolerant crops has been a much desired scientific goal but still little success to date (Munns & Tester, 2008). An alternative possible method may be the application of salt-tolerant microbes to rice fields that will enhance crop growth.

Plant Growth Promoting Rhizobacteria (PGPR) play an important role in sustainable agricultural systems. PGPR can promote plant growth because of its ability for non-symbiotic nitrogen fixation, phosphate solubilization, increased iron uptake, suppression of plant pathogenic microorganisms, and regulation of various plant hormone levels, which leads development of resistance to drought and salinity stress. PGPR also can enhance plant growth in a wide range of root-zone salinities, and this strategy can be applied for crops to manage with climate change-induced abiotic stresses (Mapelli et al., 2013).

In this research, we focused on the diversity of salt-tolerant PGPRin the salinity regions of rice paddy fields in the Mekong Delta. Some main groups of PGPR were isolated and identified for future application to improve the rice fieldsofthe currently difficult conditions.

MATERIALS AND METHODS

Sampling

Water samples were collected from six different sites at Dinh Trung, Thanh Phuoc, An Hiep, Dai An 2, Lieu Tu, Lich Hoi Thuong Communes along the coastal areas of the Mekong Delta (Table 1, Fig. 1). Plastic containers used for the collection of samples were pre-washed with 0.05 M HCl and then rinsed with distilled water. After collection, various physicochemical parameters (pH, temperature, salinity, total dissolved solids (TDS), conductivity, dissolved oxygen (DO), oxidation reduction potential (ORP) of the samples were measured using a Horiba U-52 Multiparameter Meter (Horiba, Japan).The rhizosphere rice soils were collected from the paddy fields in the sampling area (Table 1) for isolation and selection of PGPR microbes.

Table 1. Coordinates of the sampling sites in the two target provinces

Sampling sites		Coordinate	
Ben Tre Province	DinhTrung	N: 10°13’18”	E: 106°39’23”
	ThanhPhuoc	N: 10°6’33”	E: 106°41’5”
	An Hiep	N: 10°1’23”	E: 106° 32’27”
Soc Trang Province	Dai An 2	N: 9°34’36”	E: 106°10’12”
	Lieu Tu	N: 9°25’36”	E: 106°7’42”
	Lich Hoi Thuong	N: 9°34’8”	E: 105°36’45”
	Long Phu*	N: 9°34’36”	E: 106°10’12”

Note: *: This site has no water environment but the bare soil.

*Figure 1.*The location of sampling sites in the Ben Tre and Soc Trang provinces

Bacterial Isolation

Salt-tolerant PGPR microbes were characterized by spreading soil samples in the TSA (Tryptic Soy Agar) culture media with variousNaCl concentrations. Briefly, 1 g of rhizosphere soil muds or a root system from each sample was suspended in 9 mL of sterile physiological saline (9 g/L NaCl) and shaken for 15 min at 200 rpm at room temperature. Suspensions were serially diluted in ten-fold and plated in triplicate onto TSA culture media supplemented with various NaCl concentrations (0.5, 1, 1.5, 2, 2.5, 5, 10 and 30%). The number of colonies of each samples were counted and compared.

For the isolation of bacteria, 1 g of rhizospherical soil from each sample was suspended in 9 mL of sterile physiological solution (9 g/L NaCl) and shaken for 15 min at 200 rpm at room temperature. Suspensions were serially diluted ten-fold and plated in triplicate onto TSA culture medium. Then, colonies were randomly selected from the TSA medium or NaCl-TSA medium agar plates and spread onto the original medium for three times to avoid contamination risks. Pure isolates were frozen in 25% glycerol at (-)80 ^{o}C (Mapelli et al., 2013; Ferjani et al., 2015; Soussi et al., 2016).

In vitro Screening of Bacterial Isolates for their Plant Growth Promoting (PGP) Activities

All isolates were first screened on Pikovskaya's agar plates for phosphate solubilization as described by Jiang et al. (2020). The production of indole-3-acetic acid (IAA) was detected by the method described by Patten & Glick (2002). The ability of nitrogen fixation was estimated according to Singh (2013) and Cappuccino and Welsh (2019).

Molecular Identification of Isolates

The isolated bacteria were identified based on 16S rDNA sequences. The total DNA of the isolated bacteria were used for PCR amplification of 16S rDNA using the 16S rDNA universal primer set (27F:AGAGTTTGATCMTGGCTCAG; and 1492R:CGGYTACCTTGTTACGACTT). The PCR products were sequenced by Macrogen (Seoul, Korea). The partial sequence of 16S rDNA of each isolate was blasted in NCBI for the identification of the isolate. Then, the DNA sequences were aligned with highly identical sequences from NCBI database using ClustalW tool in BioEdit software v7.0.5.3 for sequence identity comparison (Hall, 1999). The phylogenetic trees were constructed from aligned sequences using Mega software (Tamura et al., 2013). Minimum Evolution method with the best nucleic acid substitution model and Bootstrap method with 1000 replications were applied for phylogenetic tree reconstruction.

RESULTS AND DISCUSSION

Environmental factors in the sampling sites

The salinity, temperature, pH, TDS, conductivity, dissolved oxygen, reduction potential of the water samples from each site were summarized in table 2. The salinity, pH, turbidity, DO and conductivity of the water were the highest at Thanh Phuoc sampling site, 2.2‰, 32.6 ^{o}C, 8.1, 2.7 g/L, 12.2 mg/L and 4,700 µS/m, respectively. Meanwhile, the water sample from Dai An 2 showed the

lowest value of salinity, turbidity, DO and conductivity. The temperature of the sampling sites ranged from 29 ºC to 34 ºC, and the pH values ranged from 7.4 to 8.1 (Table 2). The data confirmed that there was salt intrusion in the several water environments of rice paddy fields examined.

Environmental parameters of the sampling sites showed that the rice paddy field of target provinces are suffering from the seawater intrusion. The rice cultivation was heavily affected by salinity, particularly at the Thanh Phuoc site where the maturation of rice plant require longer time than normal growth. In our sampling, the rice at the study site of Dai An 2 where the soil is low salinity was matured and could be harvested completely, whereas in other study sites, some of the rice remained at immature stage. Such retarded growth of the rice was supposed to be caused by salt stress that results in panicle sterility, especially at pollination and fertilization stages due to some genetic mechanisms and nutrient deficiencies (Hussain et al., 2017).

Table 2. Physico-chemical featuresof water samplesfromthe rice fields studied

Sampling Sites	Salinity (‰)	Temperature (ºC)	pH	TDS (g/L)	Conductivity (µS/m)	DO (%)	DO (mg/L)	ORP (mV)
DinhTrung	0.68	34.15	7.4	0.90	1630.5	134.1	7.52	-38.15
ThanhPhuoc	2.17	32.64	8.15	2.68	4735.0	171	12.22	-31.8
An Hiep	1.29	29.12	7.89	1.64	2722.5	114.6	8.74	-27.7
Dai An 2	0.14	30.19	7.85	0.19	335.0	27.8	2.09	-56.2
Lieu Tu	0.68	30.59	7.61	0.89	1505.0	78.65	5.74	-41.55
Lich Hoi Thuong	1.07	32.66	7.4	1.38	2425.0	118.3	8.49	-13.15

Microbial abundance of the rhizosphere bacteria in the soil mud of rice roots

The total numbers of bacterial colonies appeared on the TSA medium containing various NaCl concentrations were described in Fig. 2. With the increase of NaCl concentration, the colony count decreased for all the samples, ranging from 10^4 to 10^6 CFU/gr. The number of colonies was the lowest at NaCl concentration of 5% and 10%, and colonies were not observed at 30% of NaCl. The density of bacteria varied at different sites. At 0.5% NaCl concentration, the number of bacterial colonies was the highest at Dai An 2 and An Hiep sites, and the lowest at Dinh Trung and Lieu Tu. However, increasing the NaCl in the TSA, the number of colonies was reduced significantly for the samples of all the study sites (for example, 36% reduction of Dinh Trung sample, 48% for Lieu Tu and Dai An samples and about 40% for An Hiep sample) except for the Thanh Phuoc sample, which gave rather consistant number of colonies at various NaCl concentrations up to 2.0% NaCl and then reduced at 5% and 10% NaCl.

The abundance of salt resistant rhizophere bacteria in the rice paddy soil from the sampling provinces were characterized by the conventional method based on the number of colonies on the TSA medium containing various concentration of NaCl. Due to the limitation of the methods, the present results did not cover the whole picture of microbiome of the samples. Instead, as the first step, morphological description of the colonies of the isolated rhizobacteria are summarized in the supplementary figure 1. Apparently, the samples from the different sites has different dominant colonies on the medium supplemented with various concentrations of NaCl. The abundance of the cultivable bacteria varied from site to site and it did not correlate with the salinity of the sampling site. The high salinity was supposed to support the stable community in the case of Thanh Phuoc sample, in that the number of bacterial colony was not changed significantly at different concentrations of NaCl up to 2%, while the abundance of the samples from other sites dramatically decreased with the increase of NaCl concentration from 0.5% to 1%. We assume that the high salinity of the soil of

Thanh Phuoc favored the salt-resistant bacteria, thereby the total number of bacteria did not change significantly when the NaCl concentration increased from 0.5% to 2%.

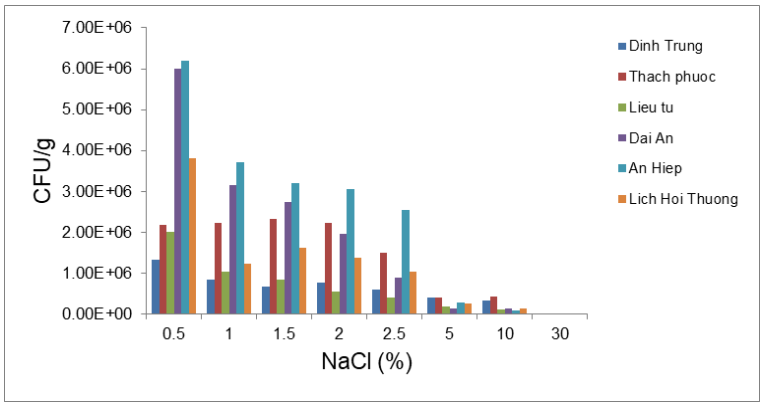

Figure 2. The abundance of the bacteria in the samples from sites cultured in TSA supplemented with various concentrations of NaCl

IAA production, phosphate solubilization and nitrogen fixation of the isolates

The rhizosphere bacteria isolated from the TSA plates with NaCl concentration of 2.5% or higher were used for this study. A total of 48 isolates were obtained from the TSA with high NaCl concentration. Their IAA production, phosphate solubilization and nitrogen fixation capacity were tested and the results were shown in table 3.

Table 3. IAA production, phosphate solubilization and nitrogen fixation properties of the isolates

No	Isolates	IAA	Phosphate solubilization	Nitrogen fixation	No	Isolates	IAA	Phosphate solubilization	Nitrogen Fixation
1	DT.MR1_1	-	-	-	25	LT. MR1_7	-	-	-
2	DT.MR1_2	+	-	G+	26	LT.MR1_16	+	G+	G+
3	DT.MR1_3	-	-	G+	27	ĐA2. MR_1	-	G++	G+
4	DT.MR1_4	+	G+	-	28	ĐA2. MR_3	-	G+	G+N+
5	DT.MR1_5	+	-	-	29	ĐA2. MR_4	+	-	-
6	DT.MR1_6	++	G++	G+	30	ĐA2. MR_5	+	-	G+
7	TP. MR1_1	+	-	G+	31	ĐA2. MR_6	+	G+	-
8	TP. MR1_2	-	-	G+	32	ĐA2. MR_7	+	-	-
9	TP. MR1_5	-	-	G+	33	AH. MR1_1	++	G++	-
10	TP. MR1_6	+	G+	G+	34	AH. MR1_2	-	-	G+
11	TP. MR1_7	-	-	G+	35	AH. MR1_3	+	G+	-
12	TP. MR1_8	+	-	-	36	AH. MR1_4	-	-	-
13	TP.MR1_10	+	G++	G++	37	AH. MR1_5	-	-	-
14	LT. MR_1	-	-	G+	38	AH. MR1_6	-	-	-
15	LT. MR_2	-	-	G+	39	LHT. MR1_1	+	G+	G+
16	LT. MR_3	++	G+	G+	40	LHT. MR1_2	+	G++	G+
17	LT. MR_4	-	-	-	41	LHT. MR1_3	+	-	-
18	LT. MR_5	-	G++	G+	42	LHT. MR1_4	-	G+	-
19	LT. MR1_1	+	-	-	43	LHT. MR1_5	-	-	-
20	LT. MR1_2	-	-	-	44	LHT. MR1_6	-	G++	G+
21	LT. MR1_3	-	G+	G+	45	LHT. MR1_7	-	G+P+	G+N+
22	LT. MR1_4	-	G++	G+	46	LHT. MR1_8	+	G+	G+
23	LT. MR1_5	-	-	G+	47	LHT. MR1_16	+	G+	G+
24	LT. MR1_6	-	-	-	48	DT.MR1_8	+	G+	G+
Total					48	23	22	28	

Notes: G++: Strong growth; G+: Weak growth; P+ or N+: Positive for P solubilization or NH_3 production.

As shown in table 3, 23 out of 48 isolates produced the plant hormone, IAA; 22 isolates could grow on the phosphate medium with/without clear zone of phosphate solubilization; and 25 isolates could grow on the medium without nitrogen supplementation and some of them produced ammonium. While 2 isolates, DT.MR1_6 and LT.MR1_16, showed high IAA production activity, 4 isolates, LHT.MR1_2, LHT_MR1_7, LHT.MR1_8, DT.MR1_8, showed high phosphate solubilization and nitrogen fixation activities. Hereafter, those biologically active isolates were relabeled as DT6, LT16, LHT1, LHT7, LHT8, and DT8, respectively. Three (LT16, LHT8 and DT8) out of those 6 isolates were obtained from TSA culture with 10% of NaCl.

In high salinity condition, the growth of plants in general, particularly of rice, is affected via the reduction of auxin (IAA), phosphorus and nitrogen uptake. Previous study showed significant reduction of IAA level of rice after exposure to salinity stress over for 5 days (Nilsen and Orcutt, 1996). In addition, seed priming of salt-intolerant wheat cultivars with different sources of auxins (IAA, IBA and tryptophane) was diminished by salt stress (Iqbal and Ashraf, 2013). It was also reported that the high salinity reduced the phosphorus uptake of plant roots by sorption processes (Rojas-Tapias et al., 2012). The saline stress inhibits N uptake process of rice due to an antagonistic effect of salt ions with NO_3^- and NH_4^+ (Teh et al., 2016). The high salinity condition resulted in the reduction of the rice height and nitrate content in the rice shoot and root due to Cl^- antagonism. Therefore, identification/isolation of the salt-resistant isolates with high activities of IAA production, phosphorous solubilization and/or nitrogen fixation is necessary to improve the salinity fields for better crop of rice.

Species identification of the selected isolates using partial sequences of 16S rDNA

The six isolates with the high activities of IAA production, phosphorous solubilization and nitrogen fixation under salinity condition were selected and identified using molecular taxonomy methods. The isolates were cultured to produce pure biomass, and their total DNA was extracted, and 16S rDNA was amplified using PCR reaction with the universal primer set. The PCR products were sent to Macrogen (Korea) for sequencing, and the six samples were sequenced completely and blasted in the NCBI GeneBank. The results showed that they belong to *Bacillus* (DT6, LT16, and LHT8), *Halobacillus* (DT8), *Aeromonas* (LHT1), and *Klebsiella* (LHT7) genera. All the sequence data of DT6, DT8, LT16, LHT1, LHT7, and LHT8 isolates were registered tothe Genebank with the accession number of MK335670, MK335671, MK335672, MK335673, MK335674 and MK335675, respectively.

As shown in the phylogenetic tree (Supplementary figure 2), the DT6 isolate is highly similar (98.9%) to *Bacillus aerophilus* strain *BC13-3* (KJ616371.1) and *B. altitudinis* strain HICAS60 (JX254660.1). The partial sequence (860 bp) of the DT6 16S rDNA gene is clustered to *B. altitudinis*, although this gene has nine nucleotides different from both strains (*B. aerophilus and B. altitudinis*) (supplementary data). The LT16 isolate was similar (99.7%) to *B. aquimari*s strain GSP18 (AY505499.1) and *B. aquimaris* strain PPL-S5 (KM226904.1). The LHT8 was similar (99.8%) to *B. marisflavi* strain R3 (KY928104.1). The DT8 was similar (99.8%) to *Halobacillus* sp. GSP34 (AY505519.1) và *Halobacillus* sp. GSP15(AY505518.1). The LHT1 isolate was highly similar to *Aeromonas caviae* GSH8M-1 (99.9%, AP019195.1:86381-87921). Lastly, the LHT7 isolate was highly similar (99.9%) to *Klebsiella pneumonia* subsp. strain JNM8C2 (CP030857.1:249514-251063).

Recently, salt-tolerant microbes were of great interest because their properties will allow potential application in the salt intruding agricultural areas. Nguyen et al. (2002) screened the microbes in the rice fields in Long An and Tien Giang provinces to isolate the salt-tolerant microbes. His group found that the isolates mostly belonged to *Bacillus* and *Azotobacter* genera

with the saline tolerance upto 10‰ NaCl (Minh, 2018), which was far lower tolerance level than those of our isolates reported here. All of identified isolates were able to grow normally in the condition of 50‰ NaCl and expressed the plant promoting activities. It was noticeable that the rice in Long An and Tien Giang provinces are tolerant to lower saline stress than the rice in Ben Tre and Soc Trang provinces.

Among the identified isolates, LHT7 and LHT1 belonged to the species that were reported to be ubiquitous pathogens in the environment, while the other 4 isolates belonged to the moderate halophilic bacteria. The LHT7 strain was identified as *Klebsiella pneumoniae*, which is found in all types of waters (fresh, brackish, and salt) and capable of expressing putative virulence factors (Podschun et al., 2001). The strain LHT1 was identified as *Aeromonas caviae* that are recognized as emerging pathogen causing diarrhea in children and found in estuarine environments with various salinity levels (Shivaji et al., 2006). Since the isolates LHT1 and LHT7 were identified as *Aeromonas caviae* and *Klebsiella pneumoniae*, repectively, the water sources used for farming in the study area were assumed to be contaminated with human feces. The LT16 and LHT8 strains were identified as *Bacillus aquimaris* and *Bacillus marisflavi*, respectively, which were reported to have optimal growth at 2−5% NaCl (Yoon et al., 2003b). It is interesting that genetically the DT6 strain is equally similar to two airborne bacteria, i.e. *Bacillus aerophilus* and *B. altitudinis* (Shivaji et al., 2006). The DT6 strain was isolated in the medium with 5% NaCl. As for the salt tolerance property of two airborne *Bacillus* species, *B. aerophilus* can grow in high salt concentration upto 16%, whereas the salt tolerance of *B. altitudinis* was only 2%. Thus, in terms of salt tolerance, the DT6 strain is more similar to *B. aerophilus* than to *B. altitudinis.* The strain DT8 was identified as a member of genus *Halobacillus*, which comprises of species having different physiological characteristics including salt tolerance. The strain DT8 can grow in the presence of NaCl at 10% but not at 30%. In contrast, *H. trueperi*, a representative of this genus, can grow at 30% NaCl concentration (Spring et al., 1996; Yoon et al., 2003a). Thus, DT8 might not be *H. trueperi* but a new strain of *Halobacillus.*

CONCLUSION

In conclusion, moderate halophilic bacteria were isolated from rice paddy fields.In total, 48 isolates of salt-resistant bacteria were obtained from the rice root mud using TSA medium supplemented with high concentrations of NaCl. Among these isolates, 22 isolates were able to produce IAA (phytohormone for the plant growth). Several isolates were found to possess the capability of nitrogen fixation and phosphate solubilization. Six of them that possess high activity of IAA, nitrogen fixation and phosphate solubilization, were identified to be *Bacillus* (DT6, LT16, and LHT8), *Halobacillus* (DT8), *Aeromonas* (LHT1) and *Klebsiella* (LHT7) genera. Four out of six isolates were potential PGPR bacteria for promoting rice growth in the saline condition. For future application for promoting the rice growth in the high saline condition, further investigation including co-fermentation of the isolates and their antagonistic properties is essential.

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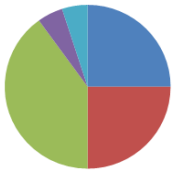
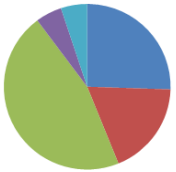
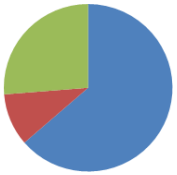
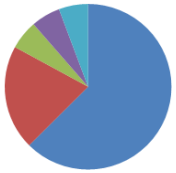
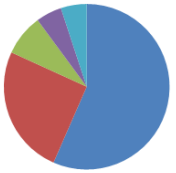
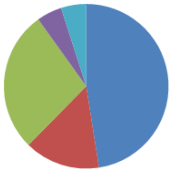
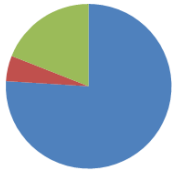
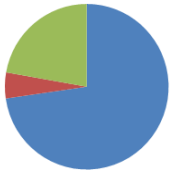
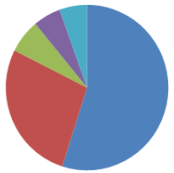
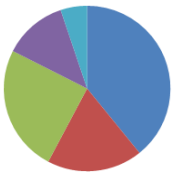
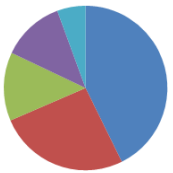
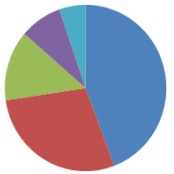
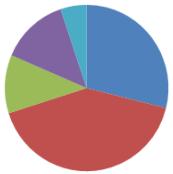
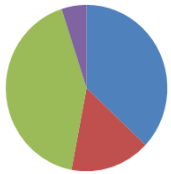
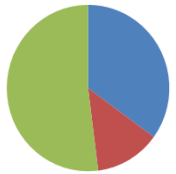
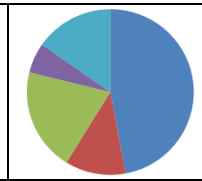
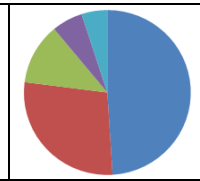
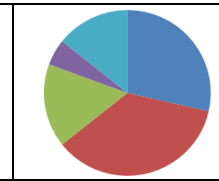
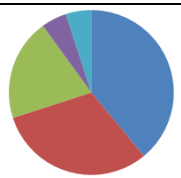
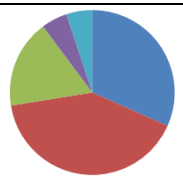
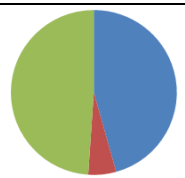
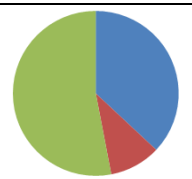
APPENDIX

Supplementary Figure 1. Composition structure of the different color and shape colonies in the samples cultured in the different concentration of NaCl, only colonies that possessed more than 1% were counted and calculated

NaCl% / Sites	0.5%	1.0%	1.5%	2.0%	2.5%	5.0%	10.0%
Thanh Phuoc							
Lieu Tu							
Dai An							
An Hiep							

Lich Hoi Thuong							
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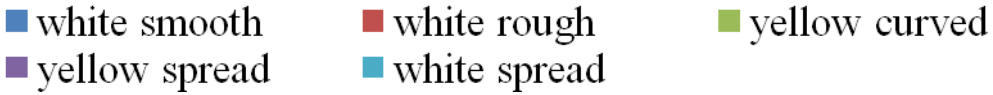
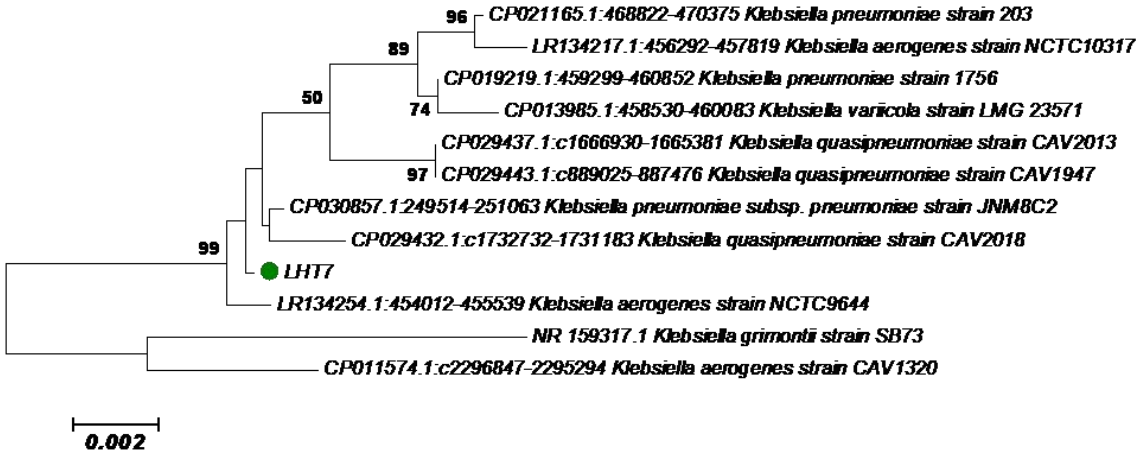
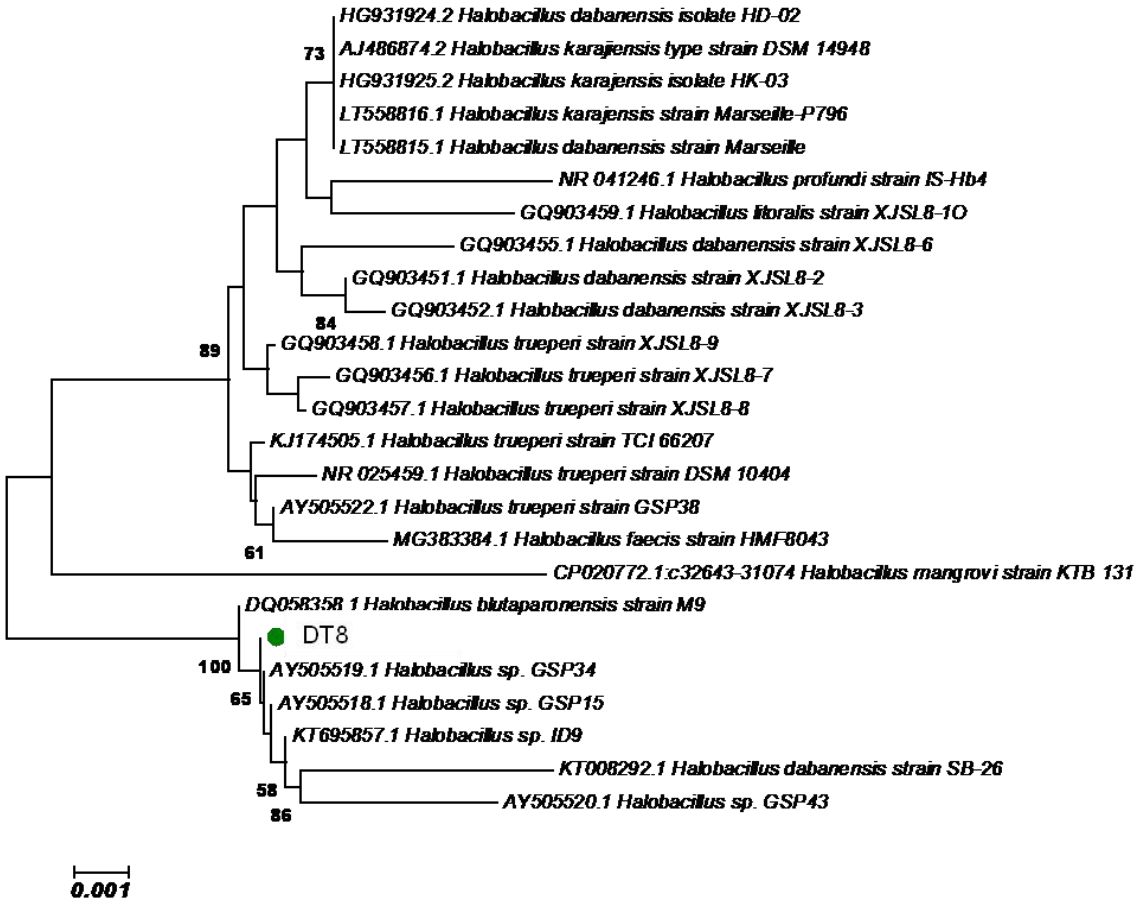
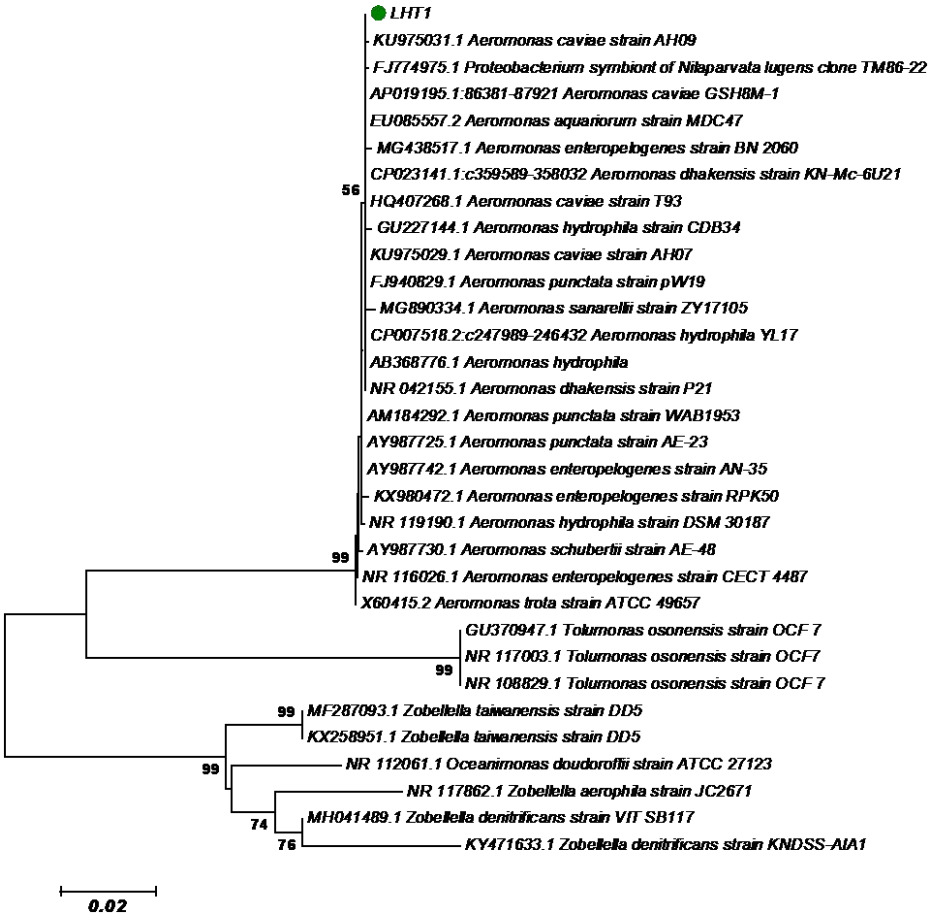


Supplement Figure 2. Phylogenetic trees of the isolates: the numbers at the node of clades are bootstrap percentage (%). The number at scale bar is the genetic distance. The reference sequence labels include NCBI accession number, species name, and strain's voucher



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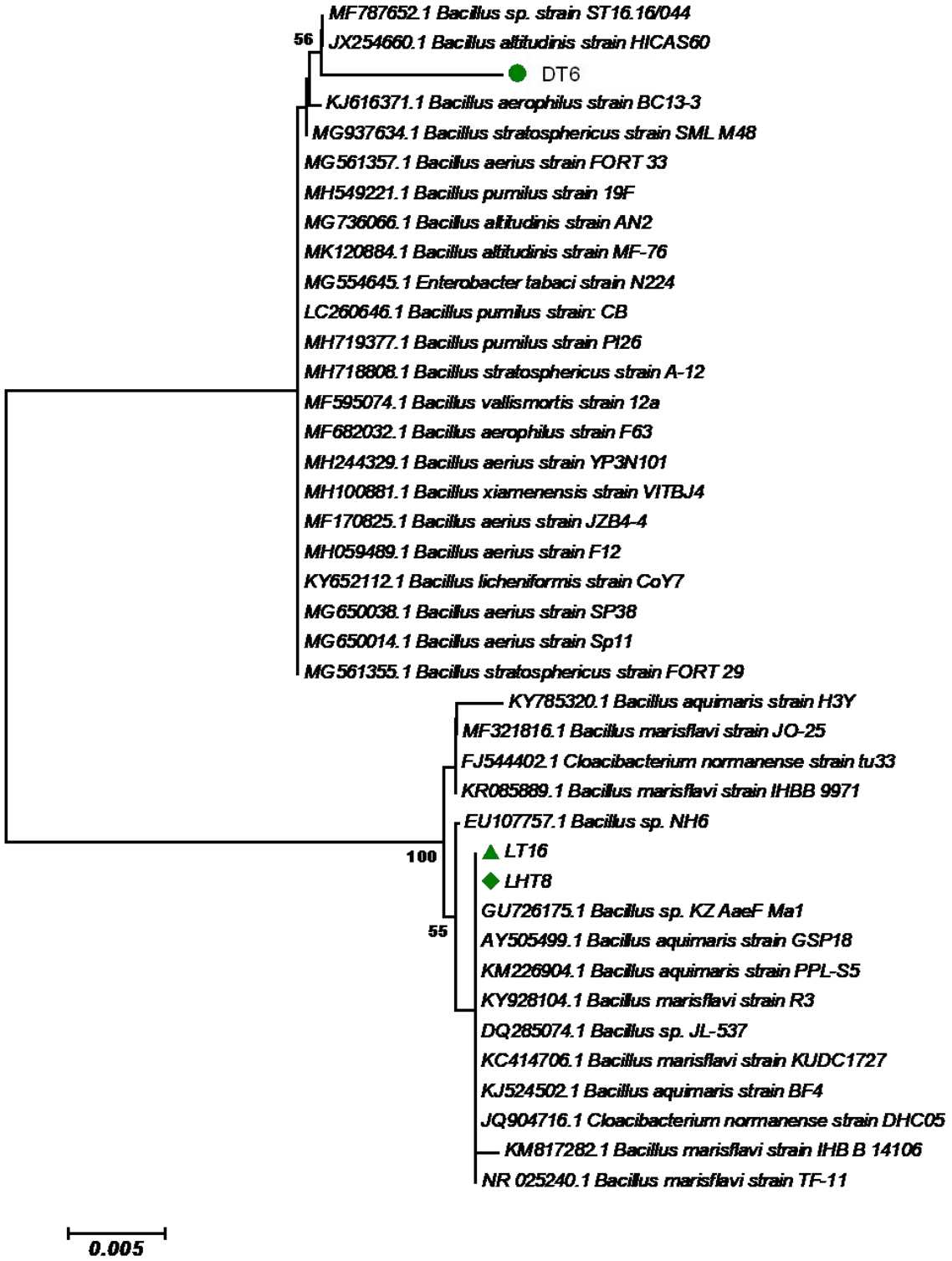
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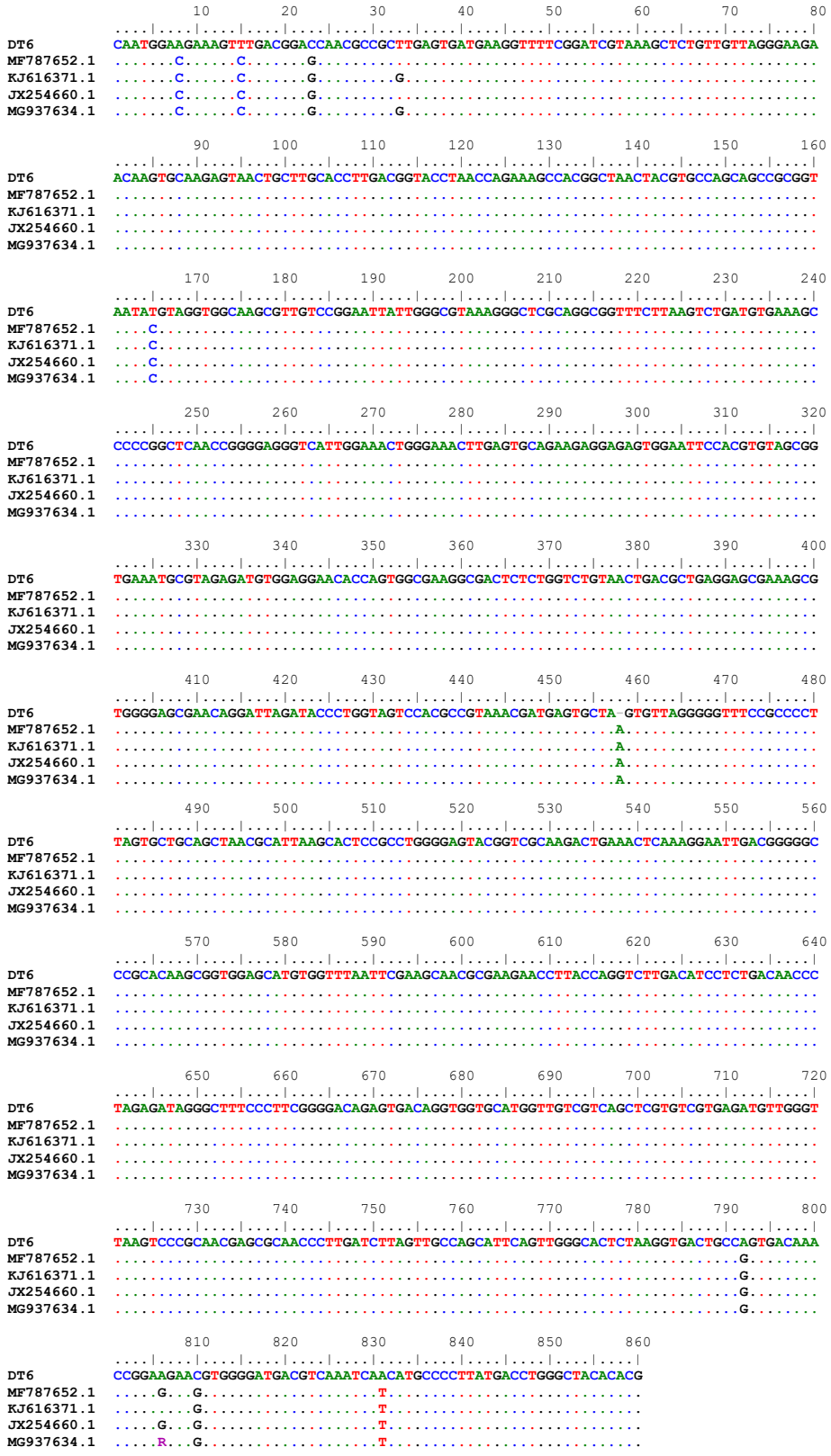
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JX254660.1 .....C.....C.....G.....
MG937634.1 .....C.....C.....G.....G.....
90 100 110 120 130 140 150 160
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KJ616371.1 .....
JX254660.1 .....
MG937634.1 .....
170 180 190 200 210 220 230 240
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KJ616371.1 ....C.....
JX254660.1 ....C.....
MG937634.1 ....C.....
250 260 270 280 290 300 310 320
DT6 CCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGG
MF787652.1 .....
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JX254660.1 .....
MG937634.1 .....
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KJ616371.1 .....
JX254660.1 .....
MG937634.1 .....
410 420 430 440 450 460 470 480
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JX254660.1 .....A.....
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490 500 510 520 530 540 550 560
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