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Isolation of Genes Encoded for Salinity Tolerance from Halophytic *Streptomyces* Species using Polymerase Chain Reaction

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Seven unrepeated halotolerant *Streptomyces* strains were isolated from the Red Sea in Jeddah at Saudi Arabia. These halotolerants were screened against different concentrations of the salt stress as an attempt to isolate some salt tolerance genes by using polymerase chain reaction (PCR). These halotolerants were identified using morphological, physiological, and biochemical characteristics as well as by sequence profiling for 16S rRNA gene using universal primer. These halotolerants were found have a varied range of salt tolerance particularly with increasing NaCl concentration in the growth medium up to 150g.L^{-1}. It was also noted that all these strains tolerated NaCl concentrations up to 100g.L^{-1}. However, when NaCl concentration was raised to 130g.L^{-1}, all isolates gave moderate growth except one which gave good growth named *Streptomyces albus* R-5. As per an increasing of NaCl concentration up to 150g.L^{-1}, the growth of six isolates was inhibited completely, but *Streptomyces albus* R-5 gave moderate growth. On the molecular level, PCR was successfully used for isolating the *mtlD* gene (1150 bp) from three isolates (*S. indigoferus* R-1, *S. purpeofuscus* R-3, and *S. albus* R-5) and *P5CR* gene (831 bp) from four isolates (*S. herbaricolor* R-7, *S. aburaviensis* R-2, *S. albolongus* R-4 and *S. chrysomallus* R-6). In addition, the fructan-accumulating (*sacB*) gene was detected in *S. albus* R-5 by amplification of a fragment of a size of about 1665 bp. These results confirmed the ability to use of PCR for isolation or detection of any gene based on its nucleotide sequencing in any microorganism.

Key words: Salt stress, Gene amplification, Halotolerants, Streptomyces, Osmoregulants.

Streptomycetes are widely distributed in terrestrial and aquatic habitats[1]. Alvarez-Mico[2], Bhave[3] and Su[4] reported that halotolerant *Streptomyces* isolated from soil and/or marine were considered as sources of new compounds from their metabolites. Shori[5] identified seven streptomycete isolates able to grow in the presence of 7 % NaCl in the starch nitrate agar medium from soil samples of Western region (Taif, Makkah and Jeddah). Streptomycetes are bacteria able to grow in soil, sea water and aquatic habitats due to their ability to tolerate salt. Moreover, some streptomycetes were recorded as halophilic streptomycetes. The scientists succeeded in the isolation of some salt tolerant genes from a few different kinds of bacteria[6] including actinomycetes[7]. Halophilic or salt tolerant actinomycetes are being developed as model organisms to disclose the mechanism and microbial physiology under extreme environments[8]. Most microorganisms subjected to water stress accumulate organic solutes to control their internal water activity, maintain the appropriate cell volume and turgor pressure, and protect intracellular macromolecules[9]. Prokaryotes have developed two strategies to cope with increasing salinities. One is to accumulate and adjust the internal concentration of inorganic ions such as K^+ and Cl^- to values that counteract the external osmolarity[10]. A different strategy is the accumulation of

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osmolytes or compatible solutes including sugars (trehalose), free amino acids (e.g., glutamate and proline), and quaternary ammonium compounds (e.g., glycine betaine, proline betaine, butyrobetaine, and carnitine) that bacteria accumulate from de novo synthesis or from externally provided osmoprotectants such as choline, the precursor of glycine betaine[11,12,13,14]. Approaches to the study of genetic processes have recently been developed for several moderate halophiles, opening the way toward an understanding of haloadaptation at the molecular level[15,16,17,18,19,20,21]. Tarczysnki[22] showed that a bacterial gene encoding mannitol-1-phosphate dehydrogenase, *mtlD*, was engineered for expression in higher plants. Vander-Meer[23] investigated the significance of the metabolism of fructans (polyfructosylsucroses) in plants by studying the advantages of fructan metabolizing plants over those unable to synthesize and degrade these nonstructural storage carbohydrates using two constructs containing the fructosyltransferase genes of either *Bacillus subtilis* (*sac*B which encodes levansucrase) or *Streptococcus mutans* (ftf). Qiu-DongLiang[24] described several mechanisms related to salt tolerance in plants, i.e., the accumulation of micro-molecular osmotic substances (such as proline, betaine, polyol, polyamine and fructan), the synthesis of macromolecular proteins (such as late embryogenesis abundant protein, osmotin, water channel protein, K^+ channel protein), the activity of ATPase and related gene expression. Saito[25] reported that the gene encoding a 2,6-beta-Dfructan 6-levanbiohydrolase (LF2ase) (EC 3.2.1.64) that converts levan into levanbiose was cloned from the genomic DNA of *Streptomyces exfoliatus F3-2*. The gene encoded a signal peptide of 37 amino acids and a mature protein of 482 amino acids with a total length of 1560 bp. This study is aimed to use the polymerase chain reaction (PCR) to isolate some salt tolerance genes from some *Streptomyces* species isolated from salt water in Saudi Arabia.

MATERIALS AND METHODS

Isolation of Streptomyces Bacteria

Samples were collected on the 3rd of February 2013 from the waters of Red Sea in Jeddah at Saudi Arabia. The samples were collected at a distance of 2 meters from the sea surface. The samples were mixed vigorously in sterile glass bottles. Samples were plated on selective agar plates (2 % w/v), within 24 hours after collection, and then incubated at 20ºC. Three different media were used; ½ ISP2; Malt extract (5 gm), yeast extract (2 gm), glucose (2 gm), natural sea water (0.5 L) and distilled water (0.5 L), Kusters streptomycete isolation medium (modified); Glycerol (10 gm), Casein (0.3 gm), KNO_3 (2 gm), $FeSO_4.7H_2O$ (0.25 mg), H_2SO_4 (0.5 mg), natural sea water (0.5 L) and distilled water (0.5 L), and actinomycete isolation medium without $MgSO_4$[26]. The pH of the isolation media was adjusted at 8.2. All media contained 50 % sea water and was supplied with Cycloheximide (50 µl.ml^{-1}) and Nalidixic acid (30 µl.ml^{-1}). Selected isolates were transferred to ½ ISP2 agar medium to ensure pure colonies, and incubated for 16 days before storing as glycerol stock in micro well plates at -80°C.

Identification of Streptomyces Isolates

Abdulkhair[27] reported that, the morphological and cultural characteristics of *Streptomyces* isolates were detected according to Shirling and Gottlieb[28]. For electron micrograph, ISP4 agar medium was inoculated and incubated for seven days at 28°C. A plug of the culture was removed and fixed in glutaraldehyde (2.5 % v/v), washed with water and post-fixed in osmium tetraoxide (1 % w/v) for 1 h. The sample was washed twice with water and dehydrated in ascending ethanol before drying in a critical point drying apparatus (Polaron E3000) and finally coated in gold and examined in a JEOLISM 541OLV scanning electron microscope at 15 Kv.

16S rRNA Identification by PCR

Identification was carried out by 16S rRNA sequencing. 16S rRNA was amplified in a thermocycler (Gene Amp PCR System 2400) by using universal primers of 27f (5'-AGA GTT TGA TCC TGG CTC AG -3') and 1525r (5'-AAG GAG GTG ATC CAG CC-3') under the following condition: 94°C for 5 min, 35 cycles of 94°C for 60 s, 55°C for 60 s, 72°C for 90 s and final extension at 72°C for 5 min. The product was directly sequenced by a BigDye terminator cycle sequencing kit (PE Applied Biosystems USA) on an ABI 310 automated DNA sequencer (Applied Biosystems, USA). Homology of the 16S rRNA sequence of isolate was analyzed by using BLAST program

from GenBank database[29]. ***Detection of Salt-Tolerance Range***

To detect the ability of the *Streptomyces* isolates to grow on increasing salt concentrations, the method given by Saleh[30] and Mohamed[31] were followed. In the experiment, different concentrations of NaCl [(0.05 is the normal concentration of the medium) (10 and 15 %)] were separately added to starch nitrate agar medium[32]. The inoculated plates were incubated at 28°C ± 2 up to 15 days to ensure the growth of the tested isolates. The growth of *Streptomyces* isolates was determined and recorded as recommended by Mahfouz and Mohamed[33]. Plates containing salt-free medium were used as control.

Isolation of Salt Stress Genes

Fifty ml in 250 ml conical flask of starch nitrate broth medium[32] supplemented with 10 % NaCl were inoculated separately with each of the *Streptomyces* isolates. After incubation at 28°C ± 2 for 6 days on a rotary shaker (160 rpm), the mycelium was collected and pulverized in liquid nitrogen[34]. To extract, purify and adjust the DNA concentration to 100ng.µl^{-1}, the method given by Mahfouz and Mohamed[33] was followed. Six oligonucleotide primers specific to three salt tolerance genes (Table 1 here) were used. PCR was conduced in a volume of 50¼l[31]. Amplification was performed in a Perkin-Elmer (Gene Amp PCR System 2400) Thermocycler for 35 cycles after initial denaturation for 4 min at 95°C. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min. The primer extension was extended to 7 min at 72°C in the final cycle. The PCR amplified products were detected by electrophoresis on 1.5 % agarose gel in 1X TAE buffer at 80 volts for 1 hour[35]. PCR fragments were visualized by staining gels with ethidium bromide (0.5µg.ml^{-1}) and photographed under UV light using a Polaroid camera.

RESULTS AND DISCUSSION

There seven unrepeated halotolerant *Streptomyces* strains were isolated from the Red Sea in Jeddah at Saudi Arabia. Data in Table 2 (here) show the salt tolerance range of these isolates which used for isolation of some salt stress genes via polymerase chain reaction (PCR). Results showed that these isolates varied in their salt tolerance range, in particular, with increasing NaCl concentration in the growth medium up to 150g.L^{-1}. It was also noted that all the *Streptomyces* isolates had a good growth at NaCl concentrations (0.05, 5, and 10 %). However, when NaCl concentration was raised to 13 %, all isolates were found have moderate growth except *S. albus* R-5 gave good growth. The results also showed that all *Streptomyces* isolates gave weak growth at 15 % of NaCl concentration except the same isolate *S. albus* R-5 gave moderate growth. These results are approximately similar to which reported by Mohamed[36], who stated that, some highly halotolerant *Streptomyces* isolates have the ability to grow at 15-18 % of NaCl concentrations. These isolates named *Streptomyces violans* Da-3, *S. alboflavus* Is-10, *S. bobili* PS-12 and *S. hawaiiensis* Si-8. On the molecular level, a trial was done to detect the three salt tolerance genes (*P5CR*, *mtlD* and *sacB*) in the DNA extracted from the isolated halotolerant *Streptomyces* bacteria using PCR. Results in Table 3 (here) showed that the *P5CR*

Table 1. The sequences of six oligonucleotide primers used for isolation of three salt tolerance genes

Primers	Sequences (5'——————————————————3')	Sizes (nt)	EPP (bp)
	P5CR gene		
P1	GGA GAT CTA ACA ATG GAG ATT CTT CCG ATT CGG GCG G	34	831
P2	GGG ATA TCT TAG CTC TGT GAG AGC TCGCGG C	31	
	mtlD gene		
P3	CGA GAT CTA ACA ATG AAA GCA TTA CATTTG GCG C	34	1150
P4	GGG ATA TCT TAT TGC ATT GCT TTA TAAGCG G	31	
	sacB gene		
P5	CCA GAT CTA AAG AAA CGA ACC AAA AGCC	28	1665
P6	CCG ATA TCT TAT TTG TTA ACT GTT ATT GTC C	31	

EPP: Excepted PCR products.

gene was detected in the DNA extracts of four species, namely, *S. herbaricolor* R-7, *S. aburaviensis* R-2**,** *S. albolongus* R-4 and *S. chrysomallus* R-6 (Figure 1) (here), while, *mtlD* gene was found in the DNA extracts of *S. indigoferus* R-1, *S. purpeofuscus* R-3, and *S. albus* R-5 (Figure 2) (here). In addition, the *sacB* gene as PCR fragments with sizes of about 831, 1150 and 1665 bp was amplified for the three genes under investigation respectively. These results indicate the differences in the genetic make up of different species under study. They also indicate that different mechanisms are involved in conferring salt tolerance in these *Streptomyces* species. One can recommend that, the isolated salt tolerance genes require more confirmation via some molecular studies, i.e., nucleotide sequencing aligning with those previously isolated genes. Furthermore, these genes can be constructed and used in the production of transgenic plants conferring tolerance to salt stress in the future. Ronde[37] showed that the L-DELTA1-pyrroline-5-carboxylate reductase (*P5CR*) gene controls the common step in the both pathways governing the biosynthesis of proline from ornithine and glutamic acid. Abebe[38] showed that ectopic expression of the *mtlD* gene of *Escherichia coli* for the biosynthesis of mannitol in wheat (*Triticum aestivum* L. cv Bobwhite) improved tolerance to water stress and salinity. They concluded that the improved growth performance of mannitol-accumulating calluses and mature leaves was due to other stress-protective functions of mannitol. Also, Wang-HuiZhong[39] integrated *mtlD* gene into the rice genome mediated by *Agrobacterium tumefaciens* LBA4404 (pBIM).

Seven halotolerant *Streptomyces* isolates were identified using morphological, physiological, and biochemical characteristics as shown in Table 4 (here). Spore mass color of the five isolates (R-1, R-2, R-4, R-5, and R-7) is gray while the spore mass color of the isolates R-3 and R-6 is white and red respectively. The isolates R-1, R-2, R-5, R-6, and R-7 were found have smooth spore surface while R-3 and R-4 isolates have hairy one. The electron microscopy analysis illustrated that all isolates have spiral spore chain. On starch nitrate agar medium, light yellowish brown color of substrate mycelia was detected at four plates of R-1, R-3, R-4, and R-5 isolates while light gray color was detected at plates of R-2, R-6, and R-7 isolates. All these isolates do not have the ability to produce diffusible pigments. The cell wall biochemical analysis proved that all isolates have LL-DAP and not detected sugar pattern. These results emphasized that the actinomycete isolates are related to genus *Streptomyces*[40,41].

The physiological characteristics are

Table 2. Salt tolerance range of seven *Streptomyces* isolates

No.	*Streptomyces* isolates	NaCl Concentrations (%)				
		0.05	5.0	10.0	13.0	15.0
1	*S. indigoferus* R-1	+++	+++	+++	++	+
2	*S. aburaviensis* R-2	+++	+++	+++	++	+
3	*S. purpeofuscus* R-3	+++	+++	+++	++	+
4	*S. albolongus* R-4	+++	+++	+++	++	+
5	*S. albus* R-5	+++	+++	+++	+++	++
6	*S. chrysomallus* R-6	+++	+++	+++	++	+
7	*S. herbaricolor* R-7	+++	+++	+++	++	+

+: Weak growth. ++: Moderate growth. +++: Good growth.

Table 3. PCR detection of salt tolerance genes from the DNA extracts of seven *Streptomyces* isolates

No.	*Streptomyces* isolates	PCR detected genes		
		P5CR	*mtlD*	*sacB*
1	*S. indigoferus* R-1	-	+	-
2	*S. aburaviensis* R-2	+	-	-
3	*S. purpeofuscus* R-3	-	+	-
4	*S. albolongus* R-4	+	-	-
5	*S. albus* R-5	-	+	+
6	*S. chrysomallus* R-6	+	-	-
7	*S. herbaricolor* R-7	+	-	-

+: Detected, -: Not detected.

varied widely among seven isolates. Amylase enzyme can be produced by all isolates; protease and lecithinase are produced only by R-1, R-2, R-4, and R-7 isolates; lipase is produced by R-2 and R-5 isolates; catalase is produced by R-1, R-3, R-6, and R-7 isolates; pectinase is produced by R-1, R-3, R-4, and R-5 isolates; urease is produced only by R-3 and R-4 isolates. Two isolates only (R-5 and R-7) which were found have the ability to produce the melanoid pigment on peptone yeast-extract iron agar, tyrosine agar, and tryptone yeast-extract broth media. H_2S gas is produced by R-1, R-2, and R-7 isolates. Nitrate was reduced completely by four isolates R-1, R-2, R-5, and R-6. Coagulase enzyme is produced by R-1, R-2, R-4, and R-7 isolates. R-1, R-5, and R-6 isolates were found utilized citrate as carbon source. All isolates do not have the ability to degrade esculin and xanthine, which are supplemented in liquid media as carbon and nitrogen sources respectively. All

Table 4. Morphological, physiological, and biochemical characteristics of seven halotolerant *Streptomyces* isolates

Characters	Items	Results *Streptomyces* isolates						
		R-1	R-2	R-3	R-4	R-5	R-6	R-7
Morphology	Spore mass	G	G	W	G	G	R	G
	Spore surface	S	S	H	H	S	S	S
	Spore chain	Sp	Sp	Sp	Sp	Sp	Sp	Sp
	Color of substrate mycelia	LyB	Lgy	LyB	LyB	LyB	Lgy	Lgy
	Color of diffusible pigment	-	-	-	-	-	-	-
Biochemistry of cell wall	Diaminopimelic acid (DAP)	LL	LL	LL	LL	LL	LL	LL
	Sugar pattern	ND	ND	ND	ND	ND	ND	ND
	Amylase production	+	+	+	+	+	+	+
	Protease production	+	+	-	+	-	-	+
	Lipase production	-	+	-	-	+	-	-
	Lecithinase production	+	+	-	+	-	-	+
	Catalase production	+	-	+	-	-	+	+
	Pectinase production	+	-	+	+	+	-	-
	Urease production	-	-	+	+	-	-	-
	Melanoid pigment production	-	-	-	-	+	-	+
Physiology	H_2S production	+	+	-	-	-	-	+
	Nitrate reduction	+	+	-	-	+	+	-
	Coagulation of skim milk	+	+	-	+	-	-	+
	Citrate utilization	+	-	-	-	+	+	-
	Degradation of esculin	-	-	-	-	-	-	-
	Degradation of xanthine	-	-	-	-	-	-	-
	Growth with sodium azide	-	-	-	-	-	-	-
	Growth with thallus acetate	-	-	-	-	-	-	-
	Maximum growth temperature (°C)	35	45	40	40	45	45	45
	Growth at 7 % NaCl	+	+	+	+	+	+	+
	Resistance to ampicillin (10 µg)	-	-	+	+	+	-	-
	Resistance to erythromycin (15 µg)	-	-	-	-	-	+	-
Antimicrobial activity	*Staph. aureus* ATCC-33591	+	+	-	-	+	+	-
	P. aeruginosa ATCC-19429	-	+	+	-	+	-	-
	Escherichia coli ATCC-13706	-	+	+	-	+	-	-
	Bacillus sabtilis ATCC-11774	+	+	-	-	+	+	-
	Candida albicans IMRU-3669	+	-	-	+	+	+	+
	Aspergillus niger IMI-31276	+	-	-	+	+	+	+

G: gray, W: white, R: red, S: smooth, H: hairy, Sp: spiral, LyB: light yellowish brown, Lgy: light gray, -: not produced, ND: not detected, +: produced or growth.

isolated can not grow on the medium containing thallus acetate and/or sodium azide, which are considered toxic substances for them. The maximum growth temperature for R-2, R-5, R-6, and R-7 isolates at 45°C, R-3 and R-4 at 40°C, and R-1 at 35°C. As per isolation of these isolates from highly saline waters of Red Sea, all of them can grow well on the medium containing 7 % NaCl. Three isolates (R-3, R-4, and R-5) are resistant to ²-lactam antibiotics represented by ampicillin (10µg), while all of them are sensitive to aminoglycoside antibiotics represented by erythromycin (15µg) except one is R-6 isolate. R-1 and R-6 isolates were found have the ability to produce antibacterial activity towards Gram-positive bacteria and antifungal activity. R-2 isolate has antibacterial activity towards Gram-positive and Gram-negative bacteria but does not have any effect against fungi. R-3 isolate has only anti Gram-negative effect. R-4 and R-7 isolates were found have only antifungal activity. R-5 isolate can produce broad spectrum antimicrobial effect because it has inhibitor effect against Gram-positive and Gram-negative bacteria and fungi.

As shown in Table 5 (here) all isolates were found have good growth with starch as a sole carbon source, while they have moderate growth with D-glucose, D-galactose and D-fructose. R-1 and R-7 isolates could be grown weakly with D-xylose but the other isolates could not grow completely. All isolates have weak growth with D-mannose and raffinose except R-2 isolate does not have any growth. On contrary, all isolates could not grow with rhamnose and mannitol except R-1 isolate which demonstrated weak growth. The results showed that L-arabinose is a non-fermentable sugar for all isolates which could not grow with it completely. The results revealed that all isolates gave weak growth with *meso*-Inositol, lactose, maltose, trehalose and sucrose sugars. On

Table 5. Utilization of different carbon and nitrogen sources by seven *Streptomyces* isolates

Carbon/ Nitrogen sources	Results *Streptomyces* isolates						
	R-1	R-2	R-3	R-4	R-5	R-6	R-7
D-Xylose	+	-	-	-	-	-	+
D-Mannose	+	-	+	+	+	+	+
D-Glucose	++	++	++	++	++	++	++
D-Galactose	++	++	++	++	++	++	++
Rhamnose	+	-	-	-	-	-	-
Raffinose	+	-	+	+	+	+	+
Mannitol	+	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-
meso-Inositol	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+
D-fructose	++	++	++	++	++	++	++
Sucrose	+	+	+	+	+	+	+
Starch	+++	+++	+++	+++	+++	+++	+++
L-Cysteine	-	-	-	-	-	-	-
L-Valine	+	+	+	+	+	+	+
L-Histidine	+	+	+	+	+	+	+
L-Phenylalanine	+	+	+	+	+	+	+
L-Hydroxproline	+	+	+	+	+	+	+
L-Lysine	+	+	+	+	+	+	+
L-Arginine	-	-	+	+	+	+	+
L-Serine	+	+	+	-	-	+	+
L-Tyrosine	-	-	-	-	+	+	+

+: Weak growth, -: No growth, ++: moderate growth, +++: good growth

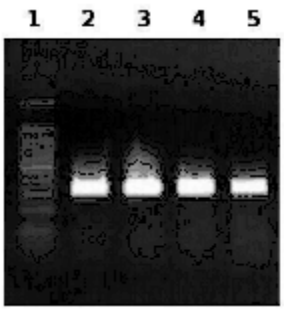

Fig. 1. Agarose gel electrophoresis of amplified *P5CR* gene from the DNA of four *Streptomyces* isolates; *S. aburaviensis* R-2 (lane 2), *S. albolongus* R-4 (lane 3), *S. chrysomallus* R-6 (lane 4), and *S. herbaricolor* R-7 (lane 5)

Fig. 2. Agarose gel electrophoresis of amplified *mtlD* gene from the DNA of *S. indigoferus* R-1 (lane 2), *S. purpeofuscus* R-3 (lane 3) and *S. albus* R-5 (lane 4)

the other hand, L-Cysteine was found is an unavailable nitrogen source for all isolates because they could not grow with it completely. Moreover, all isolates were found have weak growth with L-Valine, L-Histidine, L-Phenylalanine, L-Hydroxproline, and L-Lysine. R-1 and R-2 isolates did not have any growth with L-Arginine but the other isolates gave weak growth. R-4 and R-5 isolates did not have any growth with L-Serine but the other isolates gave weak growth. R-1, R-2, R-3, and R-4 isolates did not have any growth with L-Tyrosine but the other isolates gave weak growth. Identification process has been carried out according to Williams[40] and Hensyl[41] and numerical taxonomy of *Streptomyces* species program. All isolates were identified by determining 16S rRNA gene sequence using PCR. The resulted sequences were aligned with available almost compete sequence of type strains of family streptomycetaceae. In view of all the previously recorded data, the identification of actinomycete isolates (R-1, R-2, R-3, R-4, R-5, R-6, and R-7) was suggestive of being similar to *Streptomyces indigoferus*, *Streptomyces aburaviensis*, *Streptomyces purpeofuscus*, *Streptomyces albolongus*, *Streptomyces albus*, *Streptomyces chrysomallus*, and *Streptomyces herbaricolor* respectively.

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