Isolation and Characterization of Salt Tolerant Strains of *Sinorhizobium meliloti* ♦

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الخلاصة

تم عزل أربعة وعشرون عزلة من بكتريا *Sinorhizobium meliloti* من العقد الجذرية لنباتات الجت. جمعت النباتات المضيفة للبكتريا من مناطق زراعية مختلفة من محافظة نينوى العراق. وجد أن خمسة عزلات وهي FA7, FA8, FA10, FA11 و FA12 متحملة لـ 6 % NaCl. كانت للعزلات الخمسة القابلية على النمو في وسط الرايزوبيا الادنى. لقد أظهرت العزلات المدروسة مقاومة متعددة للمضادات الحيوية. وجد ان نباتات الجت الاكثر كفاءة في أنتاج المجموعة الخضرية عندما لقحت بالعزلتين FA8 و FA7. أظهرت دراسة تغيير الدالة الحامضية في وسط MSY الصلب أنها كانت تتغير باتجاه الحامضية. لقد تم ملاحظة أختزال وحدات تكوين المستعمرات (CFU) تحت ظروف الشد الملحي بالمقارنة مع الظروف القياسية. تم دراسة جزيئات سطح الخلية مثل كلوكونات β-(1 ← 3) و ليبفات السليولوز والسكر المتعدد الخارجي. دراسة تحييد بلازميدات التكافل باستخدام درجة الحرارة أظهرت أنه على الأقل أحد جينات تحمل الملوحة يقع على البلازميدات التعايشية.

ABSTRACT

Twenty four strains of *Sinorhizobium meliloti* were isolated from root nodules of alfalfa plants. Host plants collected from different agroclimatic regions of Ninavah province-Iraq. Five strains, *vis*. FA7, FA8, FA10, FA11 and FA12 were found tolerant to 6 % NaCl. All the five strains were able to grow on rhizobial minimal medium. The five studied strains showed multiple antibiotic resistance. The more sufficient production in shoot group in alfalfa plants found when the plants inoculated with FA8 and FA7 strains. pH changes in MSY solid medium study revealed that changes in pH towards acidity. Reduction in colony forming units (CFU) was observed under salt conditions in comparison with normal conditions. Production of cell surface molecules in these five strains , such as, β-(1 ⟶ 3) glucans, cellulose fibrils and exopolysaccharides were also studied. Heat curing of Sym plasmids study revealed that at least one of the salt tolerant genes lays on Sym plasmids.

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Introduction

Atmospheric N_2 fixed symbiotically by the association between *Rhizobium* species and legumes represents a renewable source of N for agriculture (1). Soil salinity is a major limiting nitrogen fixation factor because of its adverse effects on the growth of the host plant, on its root nodule bacteria and on symbiotic development (2). Rhizobia vary in efficacy of osmoregulation-response to salt stress (3,4,5). This capacity of adaptation of the microbial population was exhibited in a study of the survival of *Bradyrhizobium japonicum* in sludge-amended soils (6). Some salinity tolerant rhizobia were reported which showed better nodulation and nitrogen fixation than salinity sensitive rhizobia under saline conditions (7). Though these salt tolerant strains seem to accumulate osmoprotectants such as glycine betaine, proline betaine glutamic acid etc., the biochemical mechanisms of salt tolerance is not fully understood (5).

Several cell surface molecules like exopolysaccharides and β-(1→2) cyclic glucans, which play a role in early stages of infection process also play a role in stress tolerance. β-(1 →2) glucan has been found to play a major role in osmotic adaptation (8). Nogales *et al.* (9) identified eight gene loci required for adaptation to high external NaCl. Seven different genes involved in salt tolerance were isolated and characterized from *S. meliloti* mutants (10). Miller-Williams *et al.* (11) studied the determinants necessary for adaptation to high NaCl concentrations and they isolated salt-sensitive mutants of *S. meliloti* strain Rm1021. Payakapong *et al.* (12) identified two clusters of genes involved in salt tolerance in *Sinorhizobium* sp. strain BL3. Bacem *et al.* (13) isolated salt tolerant rhizobia from a Tunisian oasis that are highly effective for symbiotic N_2 fixation.

Selection of salt tolerant and efficient strains is very useful as the selected strains can be introduced into soils of respective stress where competition from naturally occurring rhizobia is lacking. Keeping these observations in mind, the present work was taken for collection of a number of strains of rhizobia from different agroclimatic regions of Ninavah State/ Iraq, selection of stress tolerant strains in the laboratory as well as to characterize these strains for their symbiotic performance and other features.

Materials and Methods

1. *Bacterial strains*

Twenty four strains of *Sinorhizobium meliloti* were isolated from root nodules of alfalfa plants. The plants were collected from different agroclimatic locations of Ninavah State/ Iraq.

2. *Host plant cultivar*

The seeds of alfalfa were obtained from local market.

3. *Media*

3.1. *Mannitol Salt Yeast Extract* (*MSY*) *Medium* (14), (g/l) : mannitol, 10; yeast extract, 0.2; K_2 HPO_4, 0.2; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.1 and $CaCl_2.2H_2O$, 0.05, pH was adjusted to 6.8. This medium was used for growing and maintenance of rhizobial strains.

3.2. *Yeast Extract Mnnitol* (*YEM*) *Medium* (15), (g/li) : mannitol, 10; yeast yxtract, 10; K_2 HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2 and NaCl, 0.1, pH was adjusted to 6.8. This medium was used for testing succinylated exopolysaccharides, cyclic β-(1→3) glucans, cellulose fibrils production as well as for symbiotic plasmids heat curing study.

3.3. *Tryptone Yeast Extract* (*TY*) *Medium* (16), (g/li) : tryptone, 5.0; yeast extract, 3.0 and $CaCl_2.2H_2O$, 0.12, pH was adjusted to 7.0. This medium was used for testing motility. TY swarm plates contained 0.3 % (w/v) agar (17). This medium was also used for symbiotic plasmids heat curing study.

3.4 *Rhizobial Minimal Medium* (*RMM*) (18) :

Solution A, (g/l) : $Na_2HPO_4.12H_2O$, 0.45; $(NH_4)_2SO_4$, 2.0; $FeCl_3$, 2.0; $MgSO_4.7H_2O$, 0.1 and $CaCl_2.2H_2O$, 0.04. This solution, after adjusting its pH to 7.0, was autoclaved.

Solution B: This solution, contains glucose (20 %) in distilled water, was filter sterilized.

To prepare 1.0 l of RMM, 10 ml of solution B was added to 990 ml solution A. This medium was used for testing auxotrophs of rhizobial salt tolerant strains.

3.5. *Nitrogen free* (*NF*) *Plant Medium* (19), (g/l) : $CaCl_2$, 132; $MgSO_4.7H_2O$, 120; KH_2PO_4, 100; $Na_2HPO_4.2H_2O$, 150; Fe-citrate, 0.05; $MnSO_4.$ $2H_2O$, 0.11; $CuSO_4.5H_2O$, 0.025; $ZnSO_4.7H_2O$, 0.28; $CoCl_2.6H_2O$, 0.024; H_3BO_3, 0.062 and $NaMoO_4.2H_2O$, 0.024. pH was adjusted to 6.0. This medium was used for authentication and symbiotic response of salt tolerant strains.

4. *Maintenance of rhizobial strains*

Purified isolated strains were streaked on slants of MSY solid medium. After a growth period of 24-48 hours at 28±2 °C, slants were stored at 4 °C in a refrigerator. Reculturing of rhizobial strains was done each two months.

5. *Supplements to media*

5.1. *Antibiotics*

Five antibiotics with four concentrations, viz., 100, 200, 300 and 400 µg/ml were used in this study. Stock solutions of tetracycline hydrochloride (Tc), chloramphenicol (Cm) and ampicillin (Am) were prepared in ethanol, while streptomycin sulphate (Sm) solution was prepared in distilled water. Nalidixic acid (Nal) was dissolved in o.o5 N sodium hydroxide. Antibiotic solutions were sterilized by passing them through 0.45µm membrane filters and stored at 4 °C. Different concentrations of antibiotic were added to the autoclaved medium after cooling it to 50 °C, just before plating (20).

5.2. *Salt*

Sodium chloride was added to the medium before autoclaving. Percentage concentration method (%, w/v) was followed for supplementing this salt into the medium.

5.3. *Dyes*

Aniline blue for cyclic β-(1→3) glucans production or calcoflour white for testing succinylated exopolysaccharides was added to the YEM medium at the rate 0.02 % (w/v), while congo red for testing cellulose fibrils production was added to the same medium at 0.1mg/ml final concentration. Each of these reagents was added to the medium before autoclaving (17).

5.4 *pH indicator dye*

Bromothymol blue dye was added to the MSY solid medium for the pH changes study at the rate of 2.5 mg/ 100 ml medium before autoclaving (20).

6. *Isolation of rhizobial strains from their host plant*

Vincent (15) procedure was followed for isolating rhizobial strains from the root nodules. Three to four pinkish nodules were washed in distilled water and exposed to 95 % (v/v) ethanol for 2-4 minutes. These nodules were then washed in sterile distilled water and immersed in 0.1 % (w/v) acidified $HgCl_2$ ($HgCl_2$, 1.0 gm; conc. HCl 5 ml and water 1.0 liter) for 3-6 minutes. The surface sterilized nodules were washed thoroughly 5-7 times with sterile water to remove traces of mercuric chloride and alcohol. The nodules were then crushed aseptically in 1.0 ml

sterile saline (0.85 % w/v NaCl) with a sterilized glass rod. Suspension (0.1 ml) was spread on MSY solid medium and the plate was incubated at 28±2 °C for 2-4 days. Transparent mucoid or gummy colonies were picked for further purification.

7. *Screening for salt tolerance*

The growth of all isolated strains of *S. meliloti* was tested by streaking these strains on MSY medium containing (0, 2, 4, 6 and 8 %) of NaCl. Incubation was done at 28±2 °C for 4-6 days (21).

8. *Plant inoculation studies*

Alfalfa seeds were sterilized as described by Vincent (15) and transferred onto nitrogen free agar slants in 20 x 2.5 cm tubes. Two 2-days old seedlings in each tube were inoculated with 10^8 cells (suspended in sterile distilled water) of a particular rhizobial strain. The growth conditions for the plants were 2000 lux light, a photoperiod of 16 hr, a dark period of 8 hr and 25 °C temperature. The morphological features of plants were recorded six weeks after inoculation. For determining the dry plant shoot weight, the plant tops were collected and dried in an oven at 65 °C for 72 hr and then weighted. Reisolation of bacteria from nodules was done to confirm the nodule occupancy by a particular strain.

9. *Symbiotic plasmids heat curing study*

To emphasize the position of salt tolerance genes whether it lay on chromosome or on symbiotic plasmids, heat curing of Sym plasmids procedure was done as follows: Approximately 10^{10} cells from a log phase culture of salt tolerant strain grown on TY medium were evenly spread on the surface of solid YEM medium. The cultures were incubated at 37 °C for 7 days, during which no growth occurred. The YEM plates were placed at room temperature, and after 5 days, about 100 single colonies arose per plate (22). To confirm symbiotic plasmids curing; plant nodulation test, antibiotic sensitivity and 6 % NaCl tolerance were done as reported by Vincent (15), Hussein (20) and Sadowsky *et al.* (21).

Results and Discussion

1. *Isolation of salt stress tolerating rhizobial strains*

Among studied strains five strains *vis.* FA7, FA8, FA10, FA11 and FA12 could grow on MSY medium supported with 6 % NaCl, 8 % K_2SO_4 and pH 4.5 (data not shown) choosed for further studies. Kumar (23) and Hussein (20) have isolated rhizobial strains from Indian soils which tolerated 4 and 5 % NaCl, respectively. Screening of rhizobial strains for survival and growth in salt stress laboratory media has resulted in some

success in improving the nodulation of some legumes in stressed soils (24). Tolerance of *S. meliloti* strains to salt stress due to ability of rhizobial cells to accumulate potassium ions inside the cells (25).

2. *Antibiotic resistance patterns of salt tolerant stress*

Results of this study showed that the five *S. meliloti* strains were sensitive to 100 µg/ml of Tc whereas two strains (FA11 and FA12) were able to grow on MSY medium supplemented with 200 µg/ml of (Cm), increasing the concentrations of this antibiotic up to 400 µg/ml resulted in inhibition of growth of all the studied strains. These strains also were resistant to Am up to 400 µg/ml. FA10 strain was able to grow with 300 µg/ml Sm whereas there is no growth with 300 and 400 µg/ml for all strains. All strains, except FA12 grew on MSY medium supplemented with Nal up to 400 µg/ml. Multiple antibiotic resistances to antibiotics explain existences of these strains in soil and then enter symbiotic relation with their host plant (6). The antibiotic resistance may be helpful in further genetic analysis of salt tolerance in these strains (20).

3. *Test for growth on Rhizobial Minimal Medium (RMM)*

Results of this study revealed that the five studied strains were able to grow on RMM solid medium after 4-5 days incubation period. This result revealed that non of these strains suffered from auxotrophy (26). Such these strains are useful for genetic studies (12).

4. *Symbiotic characteristics of alfalfa plants inoculated with NaCl tolerant strains*

Results of this study showed that the nodules induced by all the studied strains were pinkish in color and were located on both primary and lateral roots. The nodules were cylindrical in shape except in the strain FA11, the induced nodules were branched (Table 1). The plants inoculated with FA12 strain showed maximum mean shoot lengths (20.5 Cm). Mean number of days for the appearance of first nodule varied from 7.2 in strain FA12 to 10.1 in strain FA11. The mean number of nodules per plant ranged from 3.5 in strain FA10 to 7.2 in strain FA12. The minimum mean nodule dry weight per plant (0.4 mg) was observed in strain FA10, whereas strain FA12 produced the maximum value (2.1 mg) for this character. The mean shoot dry weights per plant ranged from 18.9 in strain FA10 to 44.1 mg in strain FA8. The more sufficient production in shoot group in alfalfa plants was found when the plants were inoculated with FA8 and FA7 strains, respectively. However, the performance of these strains under salt stress conditions in the field remains to be studied (20).

Table (1) Symbiotic characteristics of alfalfa plants inoculated with NaCl tolerant strains of *Sinorhiobium meliloti*

Strains	Mean shoot length (cm)	Mean no. of days to first nodule	Nodule characteristics			Mean shoot dry weight (mg)
			Mean no./plant	Shape	Mean dry wt. (mg) /plant	
FA7	17.5*±1.2	8.4±1.0	4.7±2.0	Cylindrical	1.3±0.4	40.5±2.9
FA8	17.0±0.5	8.0±0.5	5.0±1.4	-do-	1.6±0.2	44.1±4.7
FA10	09.9♦±1.7	9.3±1.2	3.5±1.9	-do-	0.4±0.2	18.9♦±1.3
FA11	14.5±0.9	10.1±0.9	6.0±1.2	Branched	1.9±0.1	22.3±5.1
FA12	20.5±0.7	7.2±0.8	7.2±1.7	Cylindrical	2.1±0.6	36.0±0.8
Control	5.5±1.1	------	------	------	------	09.0±0.2

* Each value is mean of ten plants, Cont. = control (uninoculated plants), ♦ Difference significantly from the control ($P < 0.05$),
± = Standard deviation (S.D.).

5. *pH changes during growth of the NaCl tolerant strains*

On solid MSY medium containing 6 % NaCl change to acid pH, as shown by the change of bromothymol blue dye to yellow color, occurred after 48 hours in all *S. meliloti* strains. Only one salt tolerant strain (FA7) changed the pH to acidic on solid medium under normal condition. Payakapong *et al.* also obtained colonies from salt tolerance strains of *Sinorhizobium* sp. showed acidic reactions on YEM agar containing bromothymol blue (12). Howieson (27) reported that during the growth of *S. meliloti* in yeast extract/ sugar preparation, incomplete oxidation of sugars takes place which yields acid end products. But other workers Hernández and Focht (28); Cadahia *et al.* (29) found no correlation between growth and pH changes of the medium in case of cowpea and chickpea rhizobia. The pH changes during growth seem to be strain specific(20).

6. *Colony forming units (CFUs) of the NaCl tolerant strains*

When strains were grown in medium without and with 6 % NaCl, CFU decreased in 6 % NaCl medium in all strains as compared to that under normal condition (Table 2). Maximum CFU value was 3.3 x 10^9/ ml after 72 hr of incubation under salt stress condition for FA8 strain. Minimum CFU value was 1.4 x 10^8/ ml after 24 hr of incubation under salt stress condition for FA10 strain. Under normal condition (without salt stress), maximum CFU value was 4.4 x 10^9/ ml after 72 hr incubation for FA8 strain. Minimum CFU under same condition was 2.9 x 10^9/ ml after 24 hr incubation for FA10 strain. Other researchers also showed inhibition in growth under salt condition (12, 20).

Table 2. Colony forming units (CFUs) of NaCl tolerant strains in MSY medium in presence and absence of 6 % NaCl

Strain	Type of condition	CFUs x 10^9/ml at incubation period (hr)		
		24	48	72
FA7	S	2.5*±1.3	3.0±0.4	3.2±1.1
	N	3.8±0.9	4.1±0.7	4.3±0.5
FA8	S	2.4±0.7	3.2±2.1	3.3±0.9
	N	3.6±1.1	4.2±0.6	4.4±1.2
FA10	S	1.4±1.2	1.8±0.9	1.9±0.3
	N	2.9±0.9	3.5±0.5	3.3±0.7
FA11	S	2.3±0.5	2.8±1.3	2.7±1.1
	N	3.1±1.3	4.0±0.8	4.1±1.3
FA12	S	1.7±0.8	2.5±0.5	2.3±0.8
	N	3.3±0.6	3.8±0.9	3.7±0.6

S = Stress condition, N = Normal condition,* Average of three replicates, ± = Standard deviation (S.D.).

7. *Production of cell surface molecules under normal and salt stress condition*

Under normal condition the ability to produce cyclic β-(1→3) glucans was present in the all studied strains. This ability increased under salt stress condition (6 %) for the strain FA7 and FA12. Also all the studied strains were able to produce cellulose fibrils and the salt stress conditions have no effect on the production of cell surface molecule in any of the strains. The ability of production of succinylated exopolysaccharide (SEPS) was unaffected by salt stress in studied strains except FA10, where the production increased under salt stress conditions in comparison with normal condition. Swamynathan and Singh (12) reported that the purine auxotrophy has effect on β-(1→3) production.

In this study all the strains were prototroph so the production of these cell surface molecules was normal. Hussein (20) also reported that the salt stress condition has no effect on cellulose fibrils production. Many researchers revealed the role of exopolysaccharide in rhizobium-legume symbiosis especially in initiation and elongation of infection thread when rhizobium invades root hairs of host plants and induce N_2 fixing root nodule (30, 31, 32). Howieson *et al.* (24) reported that there is no relation between production of exopolysaccharides and stress condition in *S. meliloti*, while in *Rhizobium* spp. the production of exopolysaccharide increased under stress condition (33).

8. *Motility of S. meliloti salt tolerant strains under normal and salt stress conditions*

Higher motility of a rhizobial strains is helpful to it in its competitive ability (17). Results showed that the values of motility of *S. meliloti* salt tolerant strains after 18 hr of inoculation decreased about one

fourth in FA8 and FA11 strains under salt stress conditions in comparison with normal conditions (without salt stress) (Table 3).

Salt stress condition resulted in decreasing the motility values in FA10 and FA12 up to one third. Less effect of salt stress was on FA7 strain in comparison with normal conditions, therefore this strain may be used as inoculants under salt stress conditions. Hussein (20) revealed that salt stress has different effect on motility of *S. meliloti* salt tolerant strains.

Table 3. Motility of *S. meliloti* salt tolerant strains growing in MSY medium with and without 6 % NaCl

Strains	Motility after 18 hrs of incubation (mm)	
	Under normal conditions	Under stress conditions
FA7	19.4*±4.3	18.6±3.6
FA8	08.6±2.5	6.5±1.0
FA10	10.3±2.1	6.1±1.9
FA11	20.5±3.3	15.4±2.4
FA12	12.0±2.9	8.9±1.7

* Each value is an average of three replicates,
± = Standard deviation (S.D.)

9. *Symbiotic plasmids heat curing study*

Results revealed that heat curing of *S. meliloti* salt tolerant strains resulted in Nod$^-$ phenotype when alfalfa plants inoculated with these strains. Obtained colonies after heat curing of symbiotic plasmids of salt tolerance strains showed sensitivity against Cm, Am, Sm and Nal antibiotics when the MSY solid medium supplemented with minimum concentration (100 µg/ml) of the mentioned antibiotics. These results mean successful of heat curing of symbiotic plasmids. The loss of *Rhizobium* nodulation properties strains has been reported from many laboratories (34). Zurkowski (35) isolated many non-nodulating mutants of *Rhizobium leguminosarum bv. Trifolii* after incubation at an elevated temperature with a high frequency of loss of nodulation properties.

After heat curing of *S. meliloti* salt tolerant strains, growing colonies were recultured on MSY medium supplemented with 6 % NaCl. No visible growth was noticed for the five studied strains. This result revealed that at least one of the salt tolerant genes lay on Symbiotic plasmids. Payakapong *et al.* (12) identified two gene clusters for salt tolerance in both fast and slow growing rhizobia.

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