

## Plasmid Coded Halotolerance in Mangrove Soil Bacteria

Nuzhat Ahmed, Fehmida Fasim, Zafar Nawaz and Fouad M. Qureshi  
Centre for Molecular Genetics, University of Karachi, Karachi, Pakistan

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**Abstract:** Increasing salinity in the soil and irrigation water is a global concern of paramount importance especially to Pakistan. Isolation was carried out from soil samples of Mangrove area near 'REHRI', two isolates CMG350 (*Staphylococcus saprophyticus*) and CMG351 (*Pseudomonas paucimobilis*) were selected for further studies. Character halotolerance in both the isolates was plasmid born, as cured derivatives lost the character. Maximum accumulation of Na<sup>+</sup> i.e., 12.5% was observed by CMG351 whereas 9.8% was observed by CMG350. Halotolerance was found associated with proteins encoded by plasmids.

**Key words:** Mangrove, soil bacteria, halotolerance

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### Introduction

The most serious and urgent problem of cultivable land is salinity. The elevation of ground water or evaporation of irrigation water leaves salt as a residue in the upper part of the soil profile which disturbs the agricultural productivity this condition is referred to as "Salinity" (Siddiqui, 1990). Like other parts of the world, Pakistan is equally affected by salinity an economic survey estimated that about 9.5 million hectares of land in the country had been affected by salinity.

The sea and other aquatic environments harbour a variety of microorganism. Marine and mangrove soil bacteria have been reported to tolerate high salt concentrations, as they can grow in elevated levels of osmotic stress by their ability to adapt to the changes in their surroundings. These adaptive changes may include the synthesis or accumulation of compounds known as osmoprotectant (proline, glycine, betaine, choline, trehalose, etc.) or cations (K<sup>+</sup>, Ca<sup>++</sup>, etc.) from surroundings. By these means they balance their osmotic strength with their surroundings (Le Rudulier *et al.*, 1984) and hence prevent dehydration. Proline and glycine betaine are the most preferred osmoprotectant used by the microorganisms. When betaine and proline are present in the growth medium, large amount of the osmoprotectant are transported into the cytoplasm by the osmotically regulated proP and proU gene products (Cairney and Booth, 1985; Cairney *et al.*, 1985).

Plasmid mediated salt tolerance had been reported by many workers (Qureshi *et al.*, 1994). Takeyama *et al.* (1991) detected a small plasmid pSY 10 (2.7 kb) in a marine bacterium *Cynechococcus sp.* NKBG 042902, the copy number of the plasmid was depended on the salinity. A five fold increase in the copy number of plasmid was observed when salinity of the growth medium was increased from 0% to 3% NaCl.

*Staphylococcus aureus* is among the most halotolerant of the non-halophilic eubacteria, growing up to 3.5 M NaCl (Scott, 1953). Plasmid mediated salt tolerance have been reported by many workers. Castillo *et al.*, 1992 were the first to identify, isolate and restriction map of a plasmid from moderately halophilic eubacteria. High intracellular concentration of sodium thought to interact with proteins and lipids. Eisenburg and Watchel (1987) have proposed that competition for water between negatively charged protein residues and external salt may be involved in stabilizing proteins in high salt concentration. The present study was carried out to isolate salt to tolerant bacteria and to study the mechanism of salt tolerance.

## **Materials and Methods**

### **Collection of samples**

Samples were collected in sterilized 250 ml broad mouth glass bottles from different locations of mangrove areas of "REHRI" along the coast of the Arabian Sea. Temperature, pH and salinity of the soil were checked and recorded.

### **Isolation and maintenance of pure cultures**

One gram of wet soil from each samples was suspended in 10 ml of sterilized distilled water. Salt tolerant strains isolated by standard procedure. Isolated cultures were maintained on nutrient agar slabs supplemented with salt at 4°C and preserved in glycerol.

### **MIC of NaCl**

Minimal inhibitory concentration of the NaCl was determined by the standard procedure. Briefly both strains were grown on nutrient agar plates containing different concentrations of NaCl (0.7 M to 4.0 M). Plates were observed after 24 h for the growth.

### **Growth curve**

Growth rate was measured by determining optical density (O.D) at 600nm. (Photic-100 spectrophotometer) at hourly interval till stationary phase (Miller, 1972).

### **Isolation of plasmid DNA**

Plasmid DNA was isolated by the modified method of Kado and Liu (1981). The isolated plasmid was loaded on horizontal agarose gel using bromophenol blue as tracking dye and ethidium bromide for staining. Gel was visualized on transilluminator with 366 nm UV light source (Model HB-1) and photographed with Polaroid Camera (DS-34).

### **Conjugation and curing of plasmid DNA**

The Conjugation experiments were performed by patch mating method (Bopp *et al.*, 1983), modified method of filter mating (Anna, 1990) were used. *E. coli* HB101 (100ug ml<sup>-1</sup> Sm<sup>+</sup>, NaCl<sup>-</sup>) was used as a recipient strain, Plasmid DNA was cured by serial dilution (Bopps *et al.*, 1983) and by treatment with ethidium bromide and acridine orange (Miller, 1972).

**Accumulation of sodium ions**

Accumulation of sodium ions was studied as described by Goddard and Bull (1989). Standard curve was also prepared and calculated the percentage of internal sodium content (Macaskie and Dean, 1987)

**Protein analysis**

Quantitative analysis of protein was carried out by Lowry Method

**Results**

Fourteen salt tolerant strains were isolated from the collected samples of Mangrove area near REHRI. These strains showed tolerance to different concentrations of NaCl. Among those, two strains, *Staphylococcus saprophyticus* (CMG350) and *Pseudomonas paucimobilis* (CMG351) tolerant to 3.5 M and 1.5 M NaCl respectively, were selected for further studies.

Significant difference was observed in growth pattern of CMG350, in presence of additional salt a very short log phase was observed as compared to the control samples (grown in absence of additional NaCl) whereas no significant difference was observed in growth pattern of CMG351 when subjected to media with additional NaCl, showed somewhat similar pattern in presence and absence of NaCl (Fig. 1 and 2)

Three plasmids (pNA1, pNA2, pNA3) were isolated from CMG351 while in case of CMG350 failed to isolate plasmid DNA. Conjugation between CMG351 and *E. coli* HB101 was unsuccessful, whereas between CMG350 and HB101 was successful and transconjugants grew on selective plates containing 2.5 M NaCl and 100 µg/ml of streptomycin (Table 1).

The plasmid DNA of CMG351 was cured at 70 µg/ml of acridine orange and plasmid DNA CMG350 was cured at 90 µg/ml of ethidium bromide. Upon curing the tested strains lost their ability to grow in presence of NaCl.

Table 1: Characteristics of donor, recipient and transconjugants

Donor characters	Recipient's characters	Cross	Transconjugants characters
CMG351	HB101	HB101 ×	Unsuccessful
NaCl <sup>r</sup> , Sm <sup>r</sup>	NaCl <sup>r</sup> , Sm <sup>r</sup>	CMG351	
CMG351	HB101	HB101 ×	HB101
NaCl <sup>r</sup> , Sm <sup>r</sup>	NaCl <sup>r</sup> , Sm <sup>r</sup>	CMG351	NaCl <sup>r</sup> , Sm <sup>r</sup>

Table 2: Accumulation of sodium ions

Strain code	Time					
	24	48	72	96	120	144
CMG350	9.8%	8.7%	6.8%	4.7%	1.5%	0%
CMG351	-	12.5%	0%	0%	0%	0%

Table 3: Intracellular protein content

Strain	In presence of additional NaCl (0.7M)	In absence of additional NaCl
CMG351	479.88µg	333.25µg
CMG350	306.59 µg	573.19µg
CMG350T	346.59µg	480.82µg

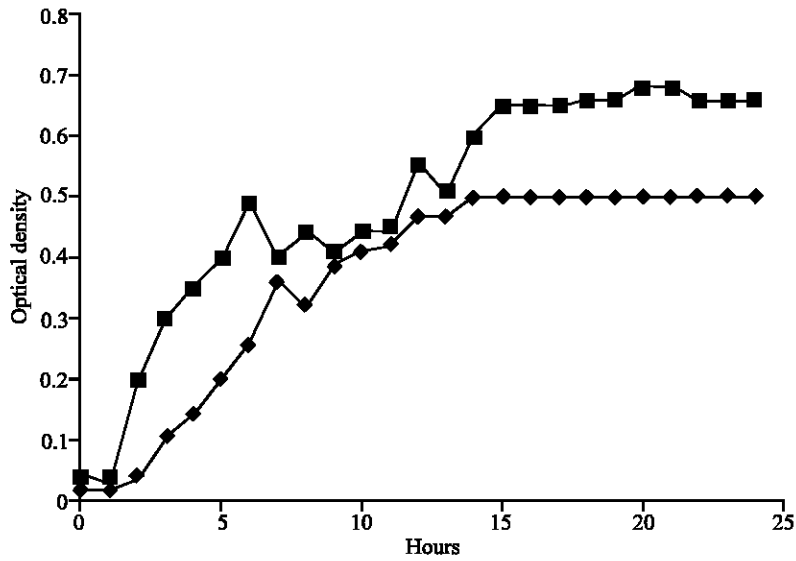


Fig. 1: Growth pattern of CMG350 (■) in absence and (◆) in presence of additional NaCl

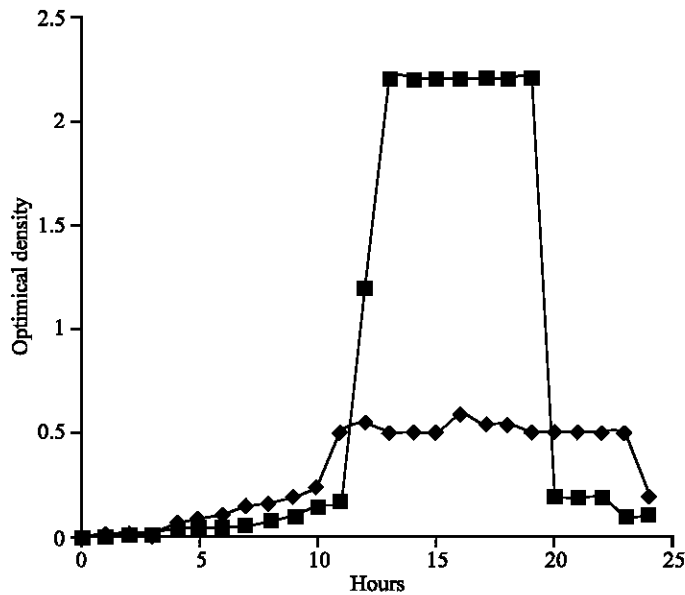


Fig. 2: Growth pattern of CMG351 (■) in absence and (◆) in presence of additional NaCl

Both the strains showed accumulation of sodium. In CMG350 maximum accumulation i.e., 9.8% was observed after 24 h and 12.5% was noticed in CMG351 after 48 h (Table 2).

In CMG351 an increase in amount of protein was estimated in presence of additional NaCl as compared to control while in CMG350 less protein content in presence of additional NaCl same situation was observed in transconjugant as well (Table 3).

## Discussion

The sea and other aquatic environments harbour a variety of microorganisms. Bacteria isolated from sea can tolerate high salt concentrations as compared to bacteria isolated from fresh water and other sources because salinity of the sea is very high, especially in case of back water (Amir *et al.*, 1992).

CMG350 was identified as *Staphylococcus saprophyticus* and CMG351 as *Pseudomonas paucimobilis* by their cellular morphology, cultural characters and API-system. CMG 350 was able to tolerate 3.5M NaCl while CMG351 of (*Pseudomonas*) was tolerating 1.5 M NaCl. It has been reported that when microorganisms are subjected to high concentrations of salts they synthesis permeases which enable them to take up prolines and betaines from the media (Cairney *et al.*, 1985) while in some other conditions under high salt concentrations they produced enzymes for synthesis of trehalose or glutamates, or proline (Dinnebier, 1988 and Omori, 1992). Presence of salt tolerance genes on plasmids has been reported earlier by Qureshi and Malik (1990) in *Klebsiella sp.* Loss of salt tolerance in the cured derivatives of both the isolates indicated that salt tolerant genes are plasmid-borne. This observation was further supported by plasmid isolation of *P. paucimobilis* and its cured derivatives. Three plasmid bands of molecular weight 1.60 kbp, 1.73 kbp and 23.44 kbp were observed in CMG351 whereas no plasmid band was observed in its cured derivative. In case of CMG350 although plasmid isolation was unsuccessful but salt tolerant character was transferred by conjugation, this confirmed the earlier observations that salt tolerant genes are plasmid coded and further suggested that the plasmids are conjugative and perhaps have a broad host range. Conjugation was not successful in case of CMG351 there could be three possibilities either the plasmid was non-conjugative and may require a mobilizing plasmid or it may be because of strong restriction system of host, that transconjugants were not obtained (Old and Primrose, 1989).

Surprisingly, the transconjugants of CMG350 MIC of NaCl was low i.e. 2.5 M, it could be due to several reasons:

- Reduced efficiency of the host RNA polymerase to bind to plasmid DNA (Kolenc *et al.*, 1988).
- Impaired translation because of inability of the plasmid encoded gene product into host biochemical pathways (Jacoby *et al.*, 1978).
- Reduced copy number of plasmid (1) due to (a) restrictive host may reduce the plasmid size and restricted gene may include the gene responsible for high copy number of plasmid (b) because of small volume of cell, copy number of plasmid may reduce.

Results obtained by estimation of total protein content of cells of *Pseudomonas paucimobilis* synthesized an increased amount of proteins in presence of additional Na<sup>+</sup> concentration these proteins may be stress proteins. Hecker *et al.* (1988) reported that when *Bacillus subtilis* was grown in 4% NaCl there was a rapid increase in the synthesis rate of a group of protein. Our result corresponds with the findings of Hecker *et al.*, 1988.

However analysis of total protein content of *Staphylococcus saprophyticus* CMG350 and transconjugants revealed that low levels of proteins were present in presence of additional NaCl.

It may be due to the utilization of cell energy for the efflux of Na<sup>+</sup> it may be possible that *S. saprophyticus* synthesized trehalose (disaccharide) to overcome plasmolysis in high osmotic conditions or accumulates K<sup>+</sup> to balance the intracellular and extracellular concentrations.

*Pseudomonas paucimobilitis* CMG350 showed maximum accumulation after 48 h than it decreased,, which indicated lysis of cells (Amir *et al.*, 1993). It may be due to accumulation of Na<sup>+</sup> to a high level, the cell lost its ability to resist such

In case of *S. saprophytic* maximum accumulation was observed after 24 h of growth after that there was a concomitant decrease in intracellular Na<sup>+</sup> content. This suggested that the cells accumulated Na<sup>+</sup> and there was other mechanism, which enabled cell to survive in high osmotic pressure and cell excluded Na<sup>+</sup> as it was toxic to cell.

Our findings suggests that in both the strains plasmid encoded protein is evolved in salt tolerance which may have some role in uptake of osmolytes from media or synthesis of osmoprotectant like trehalose and glutamates

## References

- Amir, R., N. Ahmed and M. Talat, 1993. Salt-tolerance in mangrove soil bacteria. Pak. J. Marine Sci., 2: 129-135.
- Anna, H., Seepfeldous and Alfred, 1990. Conjugation experiments, J. Bacteriol., 172: 1663-1666.
- Bopp, L.H., A.M. Chakrabarty and H.L. Ehrlich, 1983. Chromate resistance plasmid in *Pseudomonas flourances*. J. Bacteriol., 155: 1105-1109.
- Cairney, J. and I.R. Booth, 1985. *S. typhimurium* prop gene encodes a transport system for osmoprotectant betaine, J. Bacteriol., 164: 1218-1223.
- Cairney, J., I.R. Booth and C.F. Higgins, 1985. Osmoregulation of gene expression in *Salmonella typhirium*: proU encodes an osmotically induced betaine transport system, J. Bacteriol., 164: 1224-1232.
- Castillo, F., R.C. Vargas, J.J. Nieto, A. Ventosa and F. Ruiz-Berraquero, 1992. Characterization of a plasmid from moderately halophilic eubacteria. J. Gen. Microbiol., 138: 113-1137.
- Dinnbier, U., E. Limpinsel, R. Schmid and P. Bakker, 1988. Transient accumulation of potassium glutamate and its replacement by *trehalose* during adaptation of growing cells of *Escherichia coli* k-12 to elevated sodium chloride concentrations. Archives of Microbiol., 150: 348-357.
- Eisenberg, H. and E.J. Wachtel, 1987. Structural studies of halophilic proteins, ribosomes and organelles of bacteria adapted to extreme salt concentrations. Annual Reveiws of Biophysics, Biophysiology and Chem., 16: 69-92.
- Goddard, P.A. and A.T. Bull, 1989. The isolation and characterization of bacteria capable of accumulating silver. Appl. Microbiol. Biotechnol., 31: 308-313.
- Hecker, M., C. Heim, U. Volker and L. Wolfel, 1988. Induction of stress proteins by sodium chloride treatment in *Bacillus subtilis*. Archive of Microbiol., 150: 564-566.
- Jacoby, G.A., J.E. Rogers, A.E. Jacob and R.W. Hedges, 1978. Transposition of *Pseudomonas* toluene degrading genes and expression in *Escheria Ecoli*. Nature, 284: 179-180.
- Kado, C.I. and S.T. Liu, 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol., 145:1365-1373.

- Kolenc, R.J., W.E. Inniss, R.C. Bernard and C.W. Robinson, 1988. Transfer and application of mesophilic plasmid mediated degradative capacity in a psychotrophic bacterium. *Applied Environmen. Microbiol.*, 54: 638-641.
- Le Rudulier, D., A.R. Strøm, A.M. Dandekar, L.T. Smith and R.C. Valenatine, 1984. Molecular biology of osmoregulation. *Science*, 214: 1064-1068.
- Macaskie, L.E. and A.C.R. Dean, 1987. Cd accumulation by a *citrobacter species* immobilized on gel and solid supports. *Enzyme Microbiology Technol.*, 9: 2-4
- Miller, H.J., 1972. 'Experiments in Molecular Genetics'. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.
- Old, R.W. and S.B. Primrose, 1989. 'Principles of gene manipulation, An introduction to genetic engineering'. Blackwell Scientific Publications, Oxford, London.
- Omori, K.S., S. Suzuki, Y. Imai and S. Komatsubara, 1992. Analysis of the proBA operon from a proline-producing strain *Serratia marcescens*. *J. Gen. Microbiol.*, 138: 693-699.
- Qureshi, F.M., Z. Nawaz, P. Naim and N. Ahmed, 1994. Recent advances in biochemical research in Pakistan. (Eds Q.R.S.N. Halim, M. Ishaq and A.A. Azhar) Karachi University Press, Karachi Pakistan, pp: 301-310.
- Qureshi, J.A. and K.A. Malik, 1990. Evidence for a plasmid conferring salt tolerance in the plant root associated, Diazotroph *Klebsiella* species. *Biotechnology Letters*, 12: 783-788.
- Scott, W.J., 1953. Water relation of *staphylococcus aureus* at 30°C. *Aust. J. Biol. Sci.*, 6: 549-564.
- Siddiqui, S. K., 1990. Biochemical and Genetic studies of salt tolerance in indigenous bacterial isolates, M.Sc. Thesis, University of Karachi, Karachi, Pakistan.
- Takeyama, H., J.G. Burgess, H. Sudo, K. Sode and T. Matsunaga, 1991. Salinity- dependant copy number increase of a marine *cyanobacterial* endogenous plasmid. *FEMS Microbiology Letters*, 90: 95-98.