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Plasmid Mediated Salt Tolerance in Root Associated Bacteria from *Erigerone linifolious*

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Abstract: Bacterial strains (which could tolerate 2-3 M NaCl in the growth medium) were isolated from the roots of *Erigerane linifolious.* They were EI-1, EI-2, EI-3 (from histoplane of roots) and REI^{l}-1, REI-2, REI-3, REI-4, REI-5 (from the rhizoplane). All bacterial strains were motile rods (except EI-2 which were cocci), exhibiting either Gram-negative (EI-1, EI-2, EI-3, REI-1) or Gram-variable (REI-2, REI-3, REI-4, REI-5) staining. These strains were affiliated with genus *De/ya* (EI-1), *Vibrio* (EI-3, REI-1), while four Gram-variable strains chaired characters with *Bacillus pumilus.* They have wide temperature and pH ranges with different optima. These strains also exhibit multiple salts/osmolytes/heavy metals/antibiotics resistance. Curing of plasmids from four of these strains revealed that salt tolerance and most of the other resistances were plasmid encoded. Plasmids residing in these halo-tolerant strains were conjugative (except pSH1413I. Hybridization experiments revealed that one of these plasmids (pSH1414) belonged to IncN group of plasmids.

Key words: Halo-tolerant bacteria, moderately halophilic bacteria, conjugative plasmid, cured segregants

Introduction

The ability to adapt to fluctuations in the external osmolarity is pre-requisite for the survival of organisms present in saline environment. The mechanism responsible for the osmotic/salt adaptation have been elucidated in lower and higher organisms. There are remarkable similarities between the cellular responses of bacteria and plants to osmotic stress. They respond to extracellular osmolarity changes by modifying their cytoplasmic osmolarity i.e., by modulating cytoplasmic levels of compatible solutes (Miller and Wood, 1996).

Bacteria, inhabitants of saline environments are either halo-tolerant or halophilic in nature (Kushner and Kamekura, 1988). They protect themselves against these deleterious osmotic effects by the uptake or synthesis of a limited number of solutes. These compatible solutes includes amino acids (glutamate, glutamine, praline), amino acid derivatives (betains, peptides, N-acetylated amino acids), polyoles and sugars etc. (Jebbar *et al*., 1997; Pichereau *et al*., 1997; Mojica *et al*., 1997; Ventosa *et al*., 1998; Engelbrecht *et al*., 1999). Accumulation of these solutes enable them to adapt to a wide range of salt concentrations.

Halotolerant/moderately halophilic bacteria are very important from ecological and industrial point of view. A variety of possible applications of these organisms includes the food processing microbiology, waste water treatment, production of commercially useful compounds like PHB, enzymes, antibiotics, polyols and amino acids and as a source of enzyme protectants have been demonstrated (Galinski and Tindall, 1992). Hence the interest in the isolation and characterization of these bacteria is increasing day by day. We have reported these bacteria from saline soils and a number of plants growing in these soils (Yasmin and Hasnain, 1993a, b, 1997, 1998; Afrasayab and Hasnain, 1999). Our interest is in the mechanisms involved in salt tolerance. In continuation to this, here we are reporting the halo-tolerant/moderately halophilic bacteria associated with the roots of *Erigerone linifolious.*

Materials and Methods

Bacterial Strains, Growth Conditions and Characterization: Following Yasmin and Hasnain (1993a) bacterial isolates were obtained from the histoplane and rhizoplane of *Erigerone linifolius.* In this study only those strains were included which could tolerate more than IM NaCl in the growth medium. Purified strains were characterized morphologically and biochemically following Gerhardt *et al.* (1994). Some biochemical tests were performed on Q.T.S-20 and CO strips (DESTO Laboratories, Karachi, Pakistan). Effects of different concentrations of NaCl, pH, time and temperature on the growth of isolates, both in IM and 0.1 M NaCl supplemented media, were studied (Yasmin and Hasnain, 1997). Heavy metals/antibiotics resistance profile of bacterial strains was worked out by adding different concentrations (50-800 μg ml^{-1}) of filter sterilized solutions of different heavy metal salts (silver nitrate, cadmium chloride, copper sulphate, potassium chromate, cobalt chloride, mercuric chloride, lead nitrate, zinc sulphate) or antibiotics i.e. ampiciline (Ap), kanarnycin (Km), chloramphenicol (Cm), streptomycine (Sm), tetracycline (Tc) in the medium supplemented with 0.1 or 1.0 M NaCl.

Plasmid characterization: Presence of plasmid was detected by gel-electrophoresis of total cell lysate (Thomas, 1984). While plasmid DNA was isolated by a modified version of Birnboim and Doly (1979). Before extraction bacterial pellet was washed with STE (Sambrook *et al*., 1989) and isolated DNA was cleaned with WIZARD PCR clean up (Promega, England) system. To characterize plasmid, conjugation experiments were performed. For conjugation both plate mating and broth mating techniques were used (Willetts, 1988). *E. coli* K12 strains CSR603 (Se), SK1592 (Cm^{R}). *P. putida* strain UVCI (Rif^{R}) and *B. subtilis* strains IA311 (Sm^{R}), IA754 (Cm^{R}) and IA746 (Ere) were used as recipients. Transconjugants were selected on agar plates supplemented with 1-2 M NaCl and respective antibiotic. In transformation experiments, plasmid DNA was transformed into different hosts *(E. coli* K12 strains MV10, HB101, NM259, TG1). *E. coli* cells were made competent by the method of Thomas (1981). Selection of transformants was on NaCl supplemented agar plates. Bacterial strain used other than isolates were obtained from M. Rosenberg, S.K and F. Laboratories USA. (*E. coli* strains), Mark Baily, UK. (*P. putids* strain) and *Bacillus* stock center, USA (*Bacillus* strains).

Hybridization experiments: Hybridization experiments were performed using steroid hapron digoxigenin (DIG-dUPT) kit.

1.5 µl of denatured plasmid DNA was dot blotted on positivety charged nylon membranes (Boehringer Mannheim Biochemical, Germany). DNA fragments of plasmids belonging to different incompatibility groups N, P, CI and X) (Couturier *et al*., 1988) were used as probes. The hybrids were detected by enzyme-linked immuno assay using an antibody conjugate antidigoxigenin-alkaline phosphate conjugate) and subsequent enzyme-catalyzed color reaction with 5-bromo, 4-chloro, 3- indolyle phosphate and nitroblue tetrazolium salt.

Curing experiments: For curing of plasmid/s residing in salt tolerant strains different curing agents were used. Curing experiments [using high temperature, ethidium bromide (EtBr), sodium dodecyle sulphate (SOS) and trimethoprim (TMP)], were performed as described by Yasmin and Hasnain (1996) While for lysozyme treatment cells from the fresh cultures were suspended in the TE buffer (Sambrook *et al*., 1989) having 10 µg ml^{-1} of lysozyme. After 5,10,15 and 20 minutes treatment at room temperature, cells were harvested, washed and resuspended in lysozyme free TE buffer and plated on L-agar plates. Growth obtained was checked for sensitivity to NaCl by replica plating. Plasmid loss in NaCl sensitive colonies was confirmed by gel-electrophoresis of total cell lysate.

Results

Isolation of bacteria: Initially many bacterial strains were isolated from the roots of *Erigerone linifolious* (at 1 M NaCl concentration), they were taken to higher. level of NaCl and only those which could bear more than 1 M NaCl in the growth medium were selected. Total eight strains Were selected. Three of them were obtained from the histoplane (EI-1, EI-2, EI-3), while five were from the rhizoplane (REM, REI-2, REI-3, REI-4, REI-5) of *Erigerone*.

Characterization of salt-tolerant bacteria: Bacterial colonies of the isolates were circular, entire with either convex (EI-1, EI-2, EI-3, REI-1, REI-4) or flat (REI-2, REI-3, REI-5) Elevation. Color of colonies was orange (EI-1), yellow (EI-3, REI-1), lemon yellow (EI-2) or off-white (REI-2, REI-3, REI-4, REI-5). All isolates were motile, rod shaped (except EI-2, which was cocci) and Gram-negative. Cells of REI-2, REI-3 , REI-4, REI-5 were mainly Gram-negative with very little proportion of Gram-positive or Gram-variable cells. All strains were non-capsulated and non-spore former, Only one of these strains (EI-1) was strictly aerobic in nature, while rest of them were facultative anaerobic. They had cytochrome-oxidase, catalase, nitrate reductase enzymes but lack urease enzyme. None of them could denitrify. All of them could hydrolyze gelatin and esculine and were able to produce acid from arabinose. Positive results for methyl red (all excluding EI-1), vogesproskaure (EI-2, REI-2, REI-3, REI-4), arginine dihydrolase (EI-2), acid production from glucose (EI-1) and casein hydrolysis (REI-1) were observed with different strains. EI-1 and EI-2 showed negative results for phenylalanine deaminase, on the other hand only these two (EI-1, EI-2) could hydrolyze starch. For majority of the other characters these strains showed high level of similarity i.e. all of these bacterial strains gave negative results for ONPG, citrate and malonate utilization, lysine decarboxylase, ornithine decarboxylase, H_2S production, tryptophan deaminase, indole production, acid production from maltose, sucrose, manitol, rhamnose, sorbitol, inositol and pigment production.

Effect of NaCl, time, temperature and pH on the growth of bacteria: Bacterial strains were grown in L-broth supplemented with different concentrations of NaCl for 24 hours. After that optical density was measured at 600 nm. These bacterial strains could tolerate NaCl upto 2 M (EI-2, REI-4), 2.5 M (EI-3, REI-1, REI-2, REI-3, REI-5) and 3 M (EI-1) in rich medium (Fig. 1a). Growth of EI-1 and REI-5 was better manifested in medium supplemented with NaCl. They could grow optimally in medium supplemented with 1.5 M NaCl. These bacterial strains could also grow in the medium containing $NaHCO_3$ (0.1-0.5 M), Na_2SO_4 (0.1-1 M), $MgSO_4$, KNO_3 , KCl (0.1-2 M) both in the presence and absence of 1 M NaCl (data not shown). Growth behavior of isolates over time in the presence or absence of NaCl revealed that in REI-5 growth in 1 M NaCl supplemented medium was better till 24 hours but later it excelled in low osrnolarity medium. In EI-2 initially growth was better expressed in salt supplemented medium but after 16 hours it superseeded in medium of low salt concentration (Fig. 1b).

All strains had a wide temperature range. They could grow from 24-42°C. Growth of EI-3 and REI-3 was very poor at 24°C. Optimal temperature for their growth was 28-32°C (EI-2), 37°C (EI-1, EI-3, REM) and 42°C (REI-2, REI-3, REI-4, REI-5) (Fig. 2a). Temperature range and temperature optima were same in both with and without 1 M NaCl supplemented media. For the determination of pH range, bacterial strains were grown in L. broth adjusted to different pHs (both in the presence of IM and 0.1 M NaCl) and growth was monitored after 24 hours at 600 nm. These results revealed that EI-2, REI-2, REI-3, REI-4, REI-5 have more broader pH range in 0.1 M supplemented medium as compared to 1 M medium, while EI-1 have wide pH range (6-9) in the presence of 1 M NaCl while in 0.1 M medium, it can grow at pH 7 only. pH optima were different for different strains as well as in the two media (Fig. 2b).

Antibiotics/heavy metal resistance profile: For antibiotic resistance profile Ap, Sm, Tc, Cm and Km were considered. All these strains were sensitive to Ap (except EI-2, which could resist 100 µg ml^{-1}) Cm, Sm and Tc, whereas all of them conferred resistance to various levels of Km. They could tolerate 10 µg ml^{-1} (EI-1, REI-4), 20 µg ml^{-1} (REI-2, REI-5), 36 µg ml^{-1} (REI-1), 40 µg ml^{-1} (EI-3) and 50 µg ml^{-1} (EI-2) of kanamycin. EI-2 and EI-3 could tolerate Km both in the presence and absence of 1 M NaCl. Resistance pattern of these strains to different heavy metals showed that majority of them were sensitive to the salt of mercury, silver, cadmium and copper. None of them could grow in the presence of Hg or Ag (only EI-3 could tolerate 50 pg ml^{-1} of $AgNO_3$ in the absence of IM NaCl). Whereas EI-2 (both in 0.1 M and 1 M NaCl media) and EI-3 (only in the absence of 1M NaCl) could tolerate salt of copper upto 150 µg ml^{-1}. EI-2, REI-2, REI-3, REI-4, REI-5 also conferred resistance to cadmium (Table 1). All strains exhibited resistance behavior to different concentrations of cobalt, chromium, nickel, lead and zinc (EI-1 was sensitive to Zn) (Table 1). EI-1 showed sensitive behavior to these metals in the absence of NaCl but manifested resistance against Co, Cr, Ni and Pb in the presence of 1 M NaCl. Generally cadmium toxicity was reduced with the increase in salt concentration while zinc toxicity was increased with the increase in the NaCl concentration for most of the strains.

Screening and characterization of plasmids: Gel-Electrophoresis of the total cell lysate revealed the presence of single plasmid in all these strains. These plasmids

Table 1: Heavy metal resistance profile of bacteria isolated from *Erigerone*

STRAINS/NaCl conc.		Salts of Heavy Metals (µg ml^{-1})								
		Ag	Cd	Co	Cr	Cu	Hg	Ni	Pb	Zn
EI-1	0.1 M	0	0	0	0	0	0	0	0	0
	1.0 M	0	0	100	200	0	0	400	600	0
EI-2	0.1 M	0	100	200	400	150	0	400	600	100
	1.0 M	0	200	200	400	150	0	400	600	200
EI-3	0.1 M	50	0	400	400	150	0	400	600	100
	1.0 M	0	0	400	400	0	0	400	600	0
REI-1	0.1 M	0	0	400	500	0	0	400	600	100
	1.0 M	0	0	400	500	0	0	400	600	100
REI-2	0.1 M	0	0	400	400	0	0	400	600	200
	1.0 M	0	100	400	400	0	0	400	600	0
REI-3	0.1 M	0	0	400	400	0	0	400	600	200
	1.0 M	0	100	400	400	0	0	400	600	100
REI-4	0.1 M	0	0	400	300	0	0	400	600	200
	1.0 M	0	100	400	500	0	0	400	600	0
REI-5	0.1 M	0	0	400	300	0	0	300	600	200
	1.0 M	0	200	400	500	0	0	300	600	100

Table 2: Efficiency of lysozyme/TMP to cure plasmid conferring resistance to salt in bacterial strains

A)

Strains	Plasmids	Conc. of LYSOZYME (µg ml^{-1})	Time (min)	Colonies		%Age of	
				Screened	NaCl sensitive	Deleted plasmid	Cured plasmid
EI-1	pSH1412	10	5	228	0		
			10	228	106 (46.49%)	73.34	26.66
			15	228	182 (79.83%)	46.67	53.33
			20	228		-	-
REI-4	pSH1418	10	5	228	0	-	-
			10	228	52 (22.80%)	100	0
			15	228	200 (87.71%)	66.67	33.33
			20	228	228 (100%)	50	50
REI.5	pSH1419	10	5	228	0	-	-
			10	228	0	-	-
			15	228	13 (5.7%)	100	0
			20	228	210 (92.10%)	71.43	28.57B

B)

Strains	Plasmids	MIC of TMP (µg ml^{-1})	Conc. of TMP for curing (µg ml^{-1})	Colonies		%Age of	
				Screened	NaCl sensitive	Deleted plasmid	Cured plasmid
EI-3	pSH1412	> 100	80	228	188 (82.45%)	84.62	15.38

Table 3: Resistance/sensitivity of parental and cured strains against different salts

	Salts									
	$NaHCO_3$ (0.5 M)		Na_2SO_4 (1 M)		$MgSO_4$ (2 M)		KNO_3 (2 M}		KCl (2 M)	
Strains	P	C	P	C	P	C	P	C	P	C
EI-1	-	-	+	-	+	-	-	-	-	-
EI-3	+	-	+	+*	+	-	+	-	+	-
REI-4	+	-	+	-	+	-	+	-	+	-
REI-5	+	-	+	-	+	+*	+	+*	+	+*

+*Resistance level dropped to 0.5 M (for Na_2SO_4, $MgSO_4$, KNO_3 (or 1 M (for KCl). P = Parental strain, C = Cured strain

Table 4: Resistance/sensitivity of parental and cured strains against different osmolytes and heavy metal salts

Parental Strains		Resistant to	Cured Strains	Resistant to	Resistance Lost with Curing
A-	**Osmolytes**				
EI-1		-	EI-1.1	-	-
EI-3		glu, suc	EI-3.1	glu	suc
RE!-4		glu, suc	REI-4.1	glu	suc
REI-5		glu, suc	REI-5.1	glu	suc
B-	**Heavy metals**				
EI-1		-	EI-1.1		
EI-3		Ag, Ca, Ni, Cr, Pb, Zn, Cu	EI-3.1	Co, Ni, Cr, Pb	Ag, Zn. Cu
REI-4		Co, Ni, Cr, Pb, Zn,	REI-4.1	Co, Ni, Zn	Cr, Pb
REI-5		Co, Ni, Cr, Ph, Zn	REI-5.1	Ni, Zn	Co, Cr. Pb

were designated as pSH1412 (EI-1), pSH1413 (EI-2), pSH1414 (EI-3), PSH (REI-1), pSH1416 (REI-2), pSH1417 (REI-3), pSH1418 (REI-4), pSH1419 (REI-5). Plasmids p51-11412, pSH1413, p51-11414, p51-11415 were larger plasmid (>50 kb), while pSH1416-pSH1419 were relatively smaller plasmids (9 kb). *HinadII* and *EcoRI* (separate and in combination with one another) restriction pattern (Fig. 3) revealed that Psh1416, pSH1417, pSH1418 and pSH1419 have similar restriction fragments. Plasmids 01-11414 and pSH1415 yielded many faint fragments of varying sizes with these restriction endonucleases, hence their exact size can not be obtained. Results of hybridization experiments revealed that plasmid pSH1414 had some sequence similarity with IncN (plasmid pUL2432) group of plasmids. Whereas others did not showed any homology with IncN, P, Q or X group of plasmids.

To probe whether salt tolerant property was plasmid encoded, transformation and conjugation experiments were performed. Plasmid DNA (0.1 μg) was transformed to *E. coli* strains and selection of transformants was on L-agar plates supplemented with 1 M NaCl. Transformation experiments were totally unsuccessful as none of the plasmids were able to transform any of the four recipients (MV10, TG1, HB101, NM259) strains.

Strains EI-3, REI-1, REI-2, REI-5 yielded transconjugants when *E. coli* strain HB101 was used as recipient, whereas in case of EI-1 and EI-1, EI-3, REI-2, 8EI-4, transconjugants were obtained when *E. coli* strain CSR603 and SKi 592 were used as recipients, respectively. Plasmids residing in EI-1 and REI-1 were also able to promote their transfer to *B. subtilis* strain IA311 whereas none of the donor strains were able to conjugate with *P. putida* strain UVC1 or *B. subtllis* strains 1A754 or 1A746.

To amplify the relationship of plasmid and salt tolerance property, curing experiment were performed. For that purpose different curing agents (high temperature, EtBr, SDS, TMP and lysozyme) were used. These experiments revealed that high temperature, ethidium bromide and SDS were not suitable curing agents for the removal of plasmids from these strains. Although with SDS treatment, some NaCl sensitive colonies were obtained in case of REI-4 and REI-5 but complete removal of plasmid was not exhibited. With the trimethoprim treatment, all strains yielded NaCl sensitive colonies at different subinhibitory concentrations of TMP, but plasmid loss was observed only in EI-3 (80 μg ml^{-1}). Whereas in the salt sensitive colonies of other strains plasmids were present. In REI-4 salt sensitive-colonies plasmids of larger size, relative to that of parental strain, were observed. Lysozyme appeared to be a successful curing agent for removing plasmid from three of these strains i.e., from EI-1, REI-4 and REI-5 (Table 2). However variation was observed in the duration of treatment for plasmid removal. In EI-1, 10 and 15 minutes treatment, in 8EI-4, 15 and 20 minute treatment and in REI-5, twenty minutes treatment with lysozyme was requisite for plasmid removal. For each parental strain one cured derivative was selected which was named after the name of original strain i.e., EI-1.1 (EI-1), EI-3.1 (EI-3), REI-4.1 (REI-4) and REI-5.1 (REI-5). The identity of cured derivatives was confirmed by morphological, biochemical and physiological characteristics of parental strains. With the loss of plasmid change in colony color of EI-1 (orange to off white) and (EI-3) (yellow to off white) was observed. Cured derivatives of EI-3, REI-4 had smaller and somewhat swollen cells in contrast with the rod shaped cells in the parental strains, while in remaining strains the cell size was slightly smaller. Otherwise no change in morphological and in all biochemical characters were observed.

Salt resistance profile of cured segregants in all the four cases revealed that none of them were able to grow even in the presence of 0.5 M NaCl. Resistance patterns of cured strains for other salts/heavy metals/antibiotics etc. were also disturbed. Derivative strains (EI-1.1, EI-3.1, REI-4.1, REI-5.1) were either sensitive to these salts/metals or their resistance level was dropped (Table 3 and 4). Cured segregants of EI-3 were sensitive to Ag, Zn, Cu, whereas derivatives of REI-4 and REI-5 showed sensitivity to Cr, Pb and Co, Cr, Pb salts, respectively (Table 4).

Discussion

In saline environment two types of micro-organisms exist i.e., halophilic and halotolerant. NaCl is indispensable for the growth of halophilic bacteria. They are further classified into different groups depending upon the requirement of NaCl concentration for optimal growth, while halo-tolerant do not require NaCl for their growth but could tolerate higher levels of it (Kushner, 1978). Bacterial strains EI-2, EI-3 and REI-2 were halotolerant, REI-1, REI-3, REI-4 were slightly halophilic, while EI-1 and REI-5 were moderately halophilic. At higher concentrations (2 M and above) bacterial cell density was drastically reduced. Many workers (Gouesbet *et al*., 1992; Bernard *et al*., 1993; Cummings and Gilmour, 1995; Welsh *et al*., 1996) reported decrease in the growth rate of bacteria at higher concentrations of salt. They related this inhibition both with the increase in the lag phase and reduction in the specific growth rate.

All these isolates shared many morphological and biochemical characters. Histoplane bacteria showed slightly more variation in the biochemical attributes as compared to rhizoplane bacteria. Only one strain (EI-2) exhibit cocci cell shape, remaining were rod shaped. On the basis of their biochemical behavior one of them i.e., EI-1 was grouped with genus *Delya,* while EI-3 and REI-1 shared characters with genus *Vibrio* (Holt *et al*., 1994). The four Gram-variable strains (REI-2, REI-3, REI 4, REI-5) shared characters with *Bacillus pumilus* (Hasnain and Thomas, 1996) while affinities of EI-2 remained uncertain. All bacterial isolates could grow in a wide range of temperature and pH. Although they have different optimal temperatures for growth, but all of them were mesophilic in nature. Temperature optima were same both in 1 M NaCl supplemented and without NaCl media (though the bacterial growth was very poor in the absence of 1 M NaCl). Strain REI-3 was alkaliphilic, while remaining were facultative alkaliphilic in nature. Many other workers have described facultative alkaliphilic bacteria (Steele *et al*., 1992; Yasmin and Hasnain, 1993a, b, 1997, 1998) from saline habitats. Moderately halophilic and alkaliphilic methanogens which could grow over a wide pH range from 6.8-9 were also reported by Ni *et al*. (1994).

The strains described here were sensitive to Sm, Tc, Ap and Cm and were resistant to Km. Excluding EI-2 and EI-3, which showed resistance to Km, both in the presence and absence of IM NaCl, remaining could tolerate it only in the presence of IM NaCl. They also exhibit multiple heavy metal resistance. Bacterial strains showed low level resistance to cadmium, zinc, copper and comparatively high level resistance to cobalt, chromium, nickel and lead. They were sensitive to mercury and silver (except EI-3, which could tolerate 50 μg ml^{-1} of Ag. Heavy metal resistance profile of these bacteria both in with and without 1 M NaCl media showed that Cd toxicity decreased, while Zn toxicity increased (except for EI-2) with the increase in NaCl concentrations. Strain EI-1 showed sensitivity against all metals when grown in 0.1 M NaCl

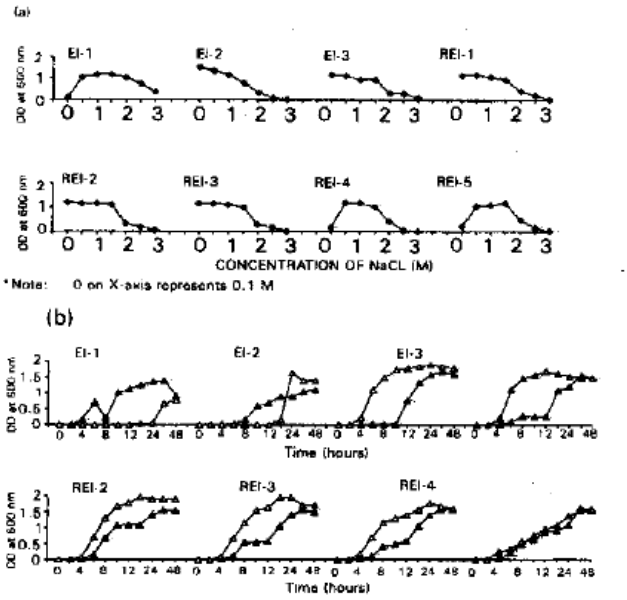

Fig. 1: a) Growth behaviour of bacterial strains in the presence of different concentrations of NaCl.
b) Growth curves of bacterial strains in the presence and absence of 1 M NaCl

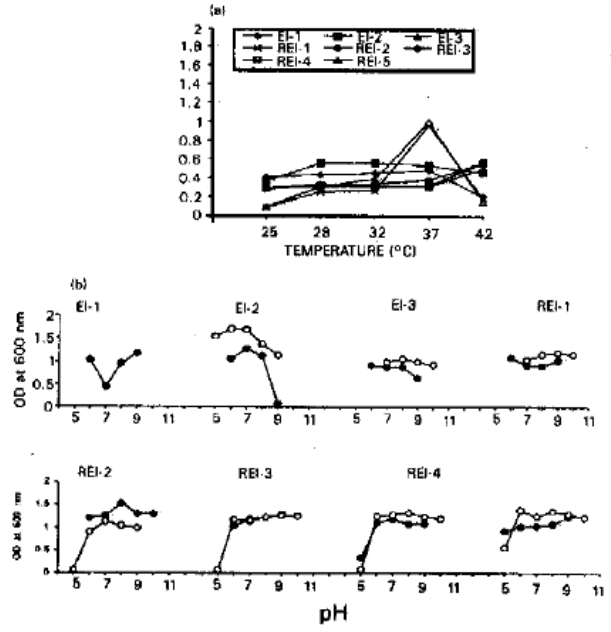

Fig. 2: a) Temperature range of bacterial strains. b) pH range of bacterial strains in the presence (I) and absence (0) of 1 M NaCl

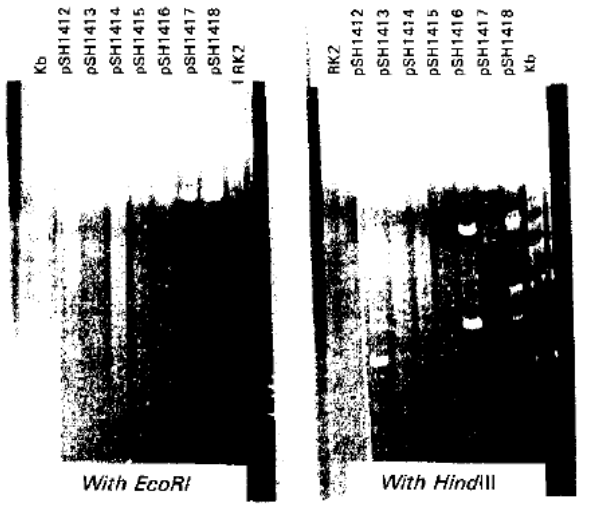

Fig. 3: Restriction pattern of plasmids (pSH1412-pSH1418) after digestion with *EcoRI* and *HindIII*

medium and became resistant to Co, Cr, Ni and Pb, when they were present along with 1 M NaCl in the medium. Many workers reported that halophilic bacteria generally tolerate high concentrations of most antimicrobial agents at their optimal salinity (Quevedo-Sarmiento *et al*., 1989; Nieto *et al*., 1993), however a decrease in the salinity resulted in an enhanced sensitivity of *Halomonas, Chromobacter* and *Salinivibrio* strains to many antimicrobial agents (Vreeland, 1992; Kunte and Galinski, 1995; Coronado *et al*., 1995).

Plasmids were discerned in all bacterial isolates. These plasmids were small (except pSH1412) and routine methods were not suitable as the interference of nucleases was higher and yield was poor. Majority of strains harbor transferable plasmids and salt tolerance mechanism was controlled by plasmids. Although there are some reports on the transfer of plasmid from moderately halophilic eubacteria to *E. coli,* but those plasmids, carried the genetic determinants that mediate resistance to antibiotics (Fernandez-Castillo *et al*., 1992; Vargas *et al*., 1995). On the basis of conjugation results, plasmid pSH1412 and pSH1415 were identified as broad host range plasmids. Host strains of these plasmids were Gram-negative. Many of the broad host range plasmids do exist among Gram-negative bacteria (Old and Primrose, 1994) and these plasmids usually belong to IncN or IncP group. But in present case none of the two broad host range plasmids (pSH1412, pSH1415) showed sequence similarity with IncN, P, 0 or X group of plasmids. One plasmid pSH1414 was identified as IncN plasmid but that was not broad host ranged. Plasmid pSH1413 could not conjugate with any of the recipient strains used. This might be due to the non-conjugative nature of the plasmid or due to some other factors (host restriction mechanism, physico-chemical conditions, host physiology etc.) which are responsible for successful conjugation (Rochelle *et al*., 1989; Fernandez-Castillo *et al*., 1992). Transformation experiments with these salt-tolerant plasmids were not successful although four different *E. coli* recipient strains (TGI, C600, MV10, HB101) were used. It might be due to the failure of the plasmid to replicate in the *E. coli* cells or plasmid borne markers might not be expressed in the new environment (Old and Primrose, 1994).

As far as the hybridization experiments are concerned the plasmid which did not showed homology with Inc N, P, Q or X groups, might belong to some other incompatibility group or they might not be able to hybridize with any of the replicon probes of plasmids from enteric bacteria. As attempts to

classify pSH1451 (resident of another salt tolerant bacteria) by hybridization with existing replicon probes of plasmids from enteric bacteria proved negative (Hasnain and Thomas, 1996). Lack of homology in large number of plasmids might demonstrate the limitations of the present classification system and the apparent diversity of the environmental plasmids. Bidinost *et al*. (1994) reported that plasmid pMJ 101 (from *Vibrio ordalii*) belong to a new incompatibility group as it was not related to any of the plasmid incompatibility group contained in the bank of rep probes described by Couturier *et al*. (1988).

Association between salt-tolerance property and plasmid was also confirmed by curing experiments. Four of these salttolerant/moderately halophilic strains were successfully cured with TMP (EI-3) and lysozyme (EI-1, REI-4, REI-5) treatment. Although high temperature, EtBr and SDS were also used but they were not effective. Failure in getting the cured segregants with these agents might either be due to the unsuitability of these chemicals for these strains or due to the culturing conditions. Nevertheless with some curing agents (SOS, TMP and lysozyme), sizes of the plasmids in salt sensitive derivatives of these strains were relatively smaller (except for REI-4 with TMP), which reflect that deletions were taking place in the plasmids but the complete loss was not accomplished. TMP treated cells of REI-4 (sensitive to NaCl) had larger plasmids than the original strains. Similar results were obtained by Dufour *et al.* (1991), where curing not only Eliminated the plasmid but also modified the size of plasmid pOSS which was larger than the original plasmid. Anyhow curing of plasmids from EI-1, EI-3, REI-3, REI-4, clearly revealed that NaCl resistance was plasmid determined. For these strains curing efficiency was 15.38% (EI-3), 26.6-53.3% (EI-1), 33-50% (REI-4) and 28.57% (REI-5). Efficiency of curing may vary from <0.1-100% and this variation depends on the plasmid and the particular bacterial host, which carry the plasmid (Stanisich, 1988), Comparison of cured segregants and parental strains showed that certain morphological characters were affected by the presence or absence of plasmid but not the biochemical characters, while physiological behavior may also be altered if all these characters are plasmid mediated. In majority of these cured strains, resistance against different salts/metals/antibiotics was found to be plasmid encoded, as with the loss of plasmid (cured derivatives) these resistances were either lost or their resistance level was dropped. The ongoing discussion revealed that salt tolerance in these strains was plasmid mediated. Many of these plasmids were conjugative, carry markers for salt/ osmolytes/heavy metals/antibiotics resistance and these plasmids were different in nature from one another.

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