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EVIDENCE FOR A PLASMID CONFERRING SALT-TOLERANCE IN THE PLANT-ROOT ASSOCIATED, DIAZOTROPH KLEBSIELLA SP. NIAB-I.

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SUMMARY

Using analytical and preparative methods,we demonstrated the presence of an indigenous plasmid (pNIAB-I) in a diazotroph, *Klebsiella* sp. NIAB-I isolated, from the roots of Kallar grass, growing on saline lands in Pakistan. The plasmid is approximately 50 kilobase (kb) in size . Transformation experiments indicated that non-halophilic bacteria such as *E. coli* K12 strain (MV10) and *K. pneumoniae* M5AI on acquiring this plasmid become tolerant to high salt (NaCl) and alkaline pH.

INTRODUCTION

In adaptations of cells to high salt environment, basic osmotic and ion regulations are important. The mechanism of adjustment of the bacterial cell to NaCl stress is not fully understood. L-proline and glycine betaine are accumulated inside the cell under salt stressed conditions in enteric bacteria (Measures 1975, Galsinki & Truper 1983).Their role in osmoregulation has been studied in detail (Cairney et al 1985, Andersen et al 1988).

Christian (1955a,b) demonstrated that L-proline had an osmoprotective property. Csonka (1980) isolated salt-tolerant mutants of *Salmonella typhimurium* which over-produce L-proline. Later, the construction of a conjugative plasmid (F' pro) carrying a mutation leading to proline-over-production was described (Csonka 1981).This plasmid, when present in *Salmonella* (Csonka 1981) and *K. pneumoniae* M5AI (Le-Rudulier et al 1982), conferred increased osmotic tolerance imposed by NaCl. Jakowec et al (1985) also constructed two stable recombinant plasmids pMJ101 and pMJ102 which conferred proline over-production and osmotic tolerance.

This paper describes the isolation and characterization of a natural plasmid (pNIAB-I) from a new nitrogen-fixing *Klebsiella* sp. NIAB-I (Qureshi et al. 1988). This plasmid, when present in *E. coli* K12 (MV10) and *K. pneumoniae* M5AI confers increased tolerance to salt (NaCl) and alkalinity.

MATERIALS AND METHODS

Bacterial strains and growth conditions:

Bacterial strains listed in Table 1 were grown aerobically in a shaking incubator at 37°C in Luria broth (LB) medium (10g/l Bacto-tryptone, 5g/l Bacto yeast extract and 5g/l NaCl). The osmolarity of the LB medium was increased by the addition of NaCl as indicated. Solid medium with elevated osmolarity was

Table 1: List of Bacterial Strains Used

STRAIN	DESCRIPTION	SOURCE
Klebsiella sp. NIAB 1	Wild type, N_2-fixer, Indole -ve, isolated from the roots of kallar grass growing on saline sodic soils in the Shahkot area near Faisalabad, Pakistan.	Zafar et. al. (1987), Qureshi et. al. (1988)
K. pneumoniae M5a1	Wild type, N_2-fixer, Indole +ve, originally isolated by P. W. Wilson, also known as *K. oxytoca*. Now held at NCIB No. 1224	Dr. David Lowe, AFRC, Lab for Nitrogen Fixation, Sussex, England
Escherichia coli (MV10)	Derived from *E. coli* K12, plasmid less strain	Dr. Helinski, University of California, San Diego, USA.

prepared by adding 1Molar NaCl to the nutrient agar . In both cases, the pH of the medium was adjusted to 8.0 with NaOH.

Detection and Plasmid DNA Isolation:

To detect plasmid, vertical (Echhdart 1978) and horizontal (Simon 1984) agarose gels of varying concentrations were used. Plasmid DNA (pNIAB-I) from Klebsiella sp. NIAB-I was isolated and purified by alkaline lysis (Brinboim and Dolly 1979) as described in Boehringer manual (1985). The purified DNA was resuspended in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) and used in subsequent analysis.

Restriction endonuclease digestion:

Cleavage of plasmid DNA with restriction endonucleases was carried out in buffers according to the manufacturer's instructions (BRL USA). About 1 ug of plasmid DNA was digested with 30-35 units of the appropriate enzyme in a volume of 40 to 50 ul at 37°C for one hour. The reaction was terminated by adding 8-10 uls of stop solution (10 mM EDTA, 15% Ficoll, 400,000 (Sigma), 1.5% SDS and 0.05% bromophenol blue in distilled water). Fragments were separated by electrophoresis on 0.8% horizontal agarose slab gels for 3 hours with constant 50 volts. Staining of gels and photographic conditions were as described in Maniatis et al (1989).

Molecular weight estimation:

The molecular weight of the plasmid (pNIAB-I) was estimated by plotting the relative mobility of the restricted fragments of the plasmid versus the logarithm of the molecular weights of Lamda Hind III fragments (Maniatis et al 1989).

Transformation:

Bacterial cells of *K. pneumoniae* M5AI and *E. coli* K12 (MV1O) strains were made competent by calcium chloride shock at 42 C and were transformed with pNIAB-I(0.1to 0.5 ug) as described by Maniatis et al (1989). For the selection, $3x10^7$ transformed cells of M5AI and MV10 were spread onto nutrient agar plates containing 1Molar NaCl, pH 8.0.

RESULTS

Detection of Plasmid DNA:

The electrophoretic separation of cell lysate of *Klebsiella* sp. NIAB-1 is shown in Fig. 1. Two plasmids bands were seen when the samples were run on horizontal Eckhardt gels (Fig.1A).However, in other gelsthe electrophoretic analysis of plasmid DNA from Klebsiella sp. NIAB-I exhibited only one band (Fig.1B). It may be suggested that the plasmid from NIAB-I is in multimeric forms and was designated as pNIAB-I. CsCl-ethidium bromide density gradient centrifugation of SDS alkaline lysis DNA from NIAB-I provided additional evidence for the existence of a plasmid DNA (data not shown).

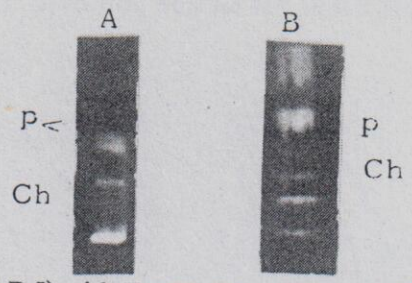

Fig. 1 Horizontal (A) and Vertical (B) agarose gel electorphoresis of cell lysate from *Klebsiella* sp.NIAB-1. Position (Ch) Chromosomal (p) Plasmid

Restriction Endonucleases analysis:

The restriction pattern of plasmid DNA (pNIAB-I) with EcoRI, Bam HI and Hind III is presented in Fig. 2. Lamda DNA digested with Hind III was used as a standard marker. The restriction enzymes used cleaved the plasmid DNA (pNIAB-I) into more than eight fragments each. Based on the comparison of electrophoretic mobility of fragmented DNA with that of Lamda Hind III fragments, a molecualr weight of approximately 50 kilobase (kb) was determined for plasmid pNIAB-I.

Transformation:

To examine, whether, the salt-tolerance in *Klebsiella* sp. NIAB-I is plasmid mediated, the plasmid was transformed to other organisms. No growth was observed with *K. pneumoniae* M5AI and *E. coli* K12 (MV10) strains on selective plates containing 1M NaCl whereas 55 to 60 colonies were observed for NIAB-I. 15 and 10 colonies of transformed M5AI and K12 (MV10) cells, respectively, were found to

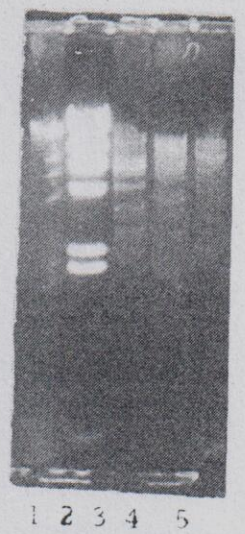

Fig.2. Restriction pattern of plasmid pNIAB-1 .Lanes (1) Uncut pNIAB-1, (2) Lamda Hind 111 marker, (3, 4 & 5) restriction digest of pNIAB-1 with BamH1 ,EcoR1 and Hind III respectively.

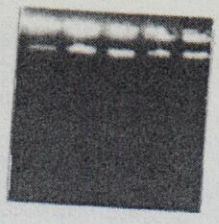

Fig. 3. Quick colony lysis screening of the transformants. The cell lysates werre loaded on 0.8% horizontal agarose gel.
Position (Ch) Chromosomal, (p) Plasmid

grow on the selective medium. The transformation efficiency was less in *E. coli* K12 (MV10) strain than that of *K. pneumoniae* M5AI. Salt resistant transformants were tested for the presence of plasmid. Fig.3 shows that all the transformants tested contained the plasmid.

Salt resistant transformants of M5AI and MV10 containing the plasmid and the parent bacterial strains namely NIAB-I, M5AI and K12 (MV10) strain were grown in 100 ml Luria broth containing 0.6, 0.8 and 1M NaCl at pH 8.0 in 500 ml side arm flask. The results of these experiments are presented in Fig. 4.

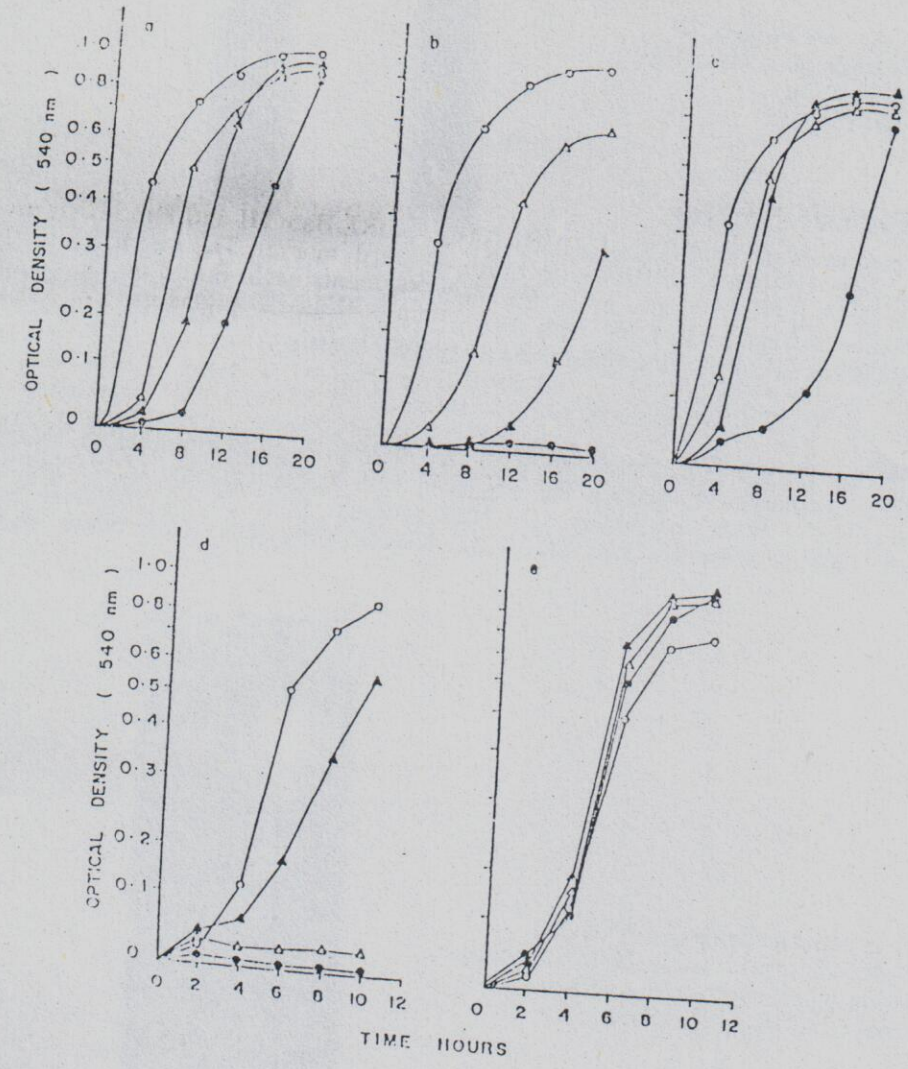

Fig.4.Effect of NaCl concentration adjusted at pH 8.0 on the growth of (a) NIAB-1 (b) M5a1 (c) M5a1 harbouring pNIAB-1 (d) MV10 and (e) MV10 harbouring pNIAB-1. The optical density of the cultures is plotted as the function of incubation time. Open circle represents control, close circle 1M NaCl, close triangle 0.8M NaCl and open triangle 0.6M NaCl.

It was observed that in general, the lag phase of the organisms increased as the osmotic strength of the medium imposed by NaCl concentration was increased. Sodium chloride concentrations in the medium reduced the growth of parent strains M5AI and K12 (MV10) strain (Fig. 4 b,d). However, the growth pattern of transformant M5AI + pNIAB-I was similar to that of NIAB-I strain at different salt concentrations (Fig. 4 a,c). Similarly, the growth behaviour of K12 (MV10) strain harbouring pNIAB-I (Fig. 4 c) showed even better salt-tolerance than the parent strains (Fig. 4 a,d) indicating that plasmid (pNIAB-I) is well expressed in (K12) MV10 strain. At the end of the experiment, pH dropped from 8.0 to 7.2-7.0. Efforts to cure plasmid (pNIAB-I) from the wild type NIAB-I by conventional methods (heating, detergent, acridine orange) were not successful. However, transformants from M5AI and K12 (MV10) strain were cured with 100 ug/ml acridine orange.

DISCUSSION

The main finding presented in this communication is that diazotroph *Klebsiella* sp. NIAB-I carries a plasmid (pNIAB-I) which confers salt-tolerance in this organism. This observation was confirmed by genetic transformation of pNIAB-I to other organisms of the family Enterbacteriaceae such as *K. pneumoniae* M5AI and *E. coli*K12 (MV10) strain. This plasmid (pNIAB-I), when present in the above mentioned strains is expressed and confers salt-tolerance to the level of the parent strain i.e. *Klebsiella* sp. NIAB-I (see Fig.4). The availability of salt-tolerance genes carried by the naturally occurring plasmid is the first step towards the enhancement of salt- tolerance phenotype in prokaryotes .

In the previous reports, Csonka (1981) obtained a conjugative plasmid (F'pro) carrying a mutation leading to proline over-production and also conferred osmotolerance. Jakowec et al (1985) also constructed osmotolerant recombinant plasmids by cloning a 10.6 kb DNA segment and the pro 74 mutation. However, Morishita (1978) had suggested that salt-tolerance in halophilic bacteria *Spirillum lunatum* SR^{+} was controlled by a plasmid and its expression occurred only in the salt environment at the level of RNA polymerase.

The work presented in this paper suggests that salt tolerance genes are present on the plasmid. Studies on the localization of plasmid encoded proteins presumed to be involved in salt tolerance is in progress.

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