CLONING HALOTOLERANT GENES IN HALOPHILES

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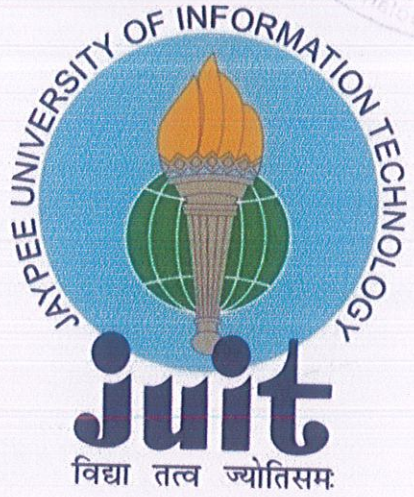

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CERTIFICATE

This is to certify that the work entitled, "**Cloning halotolerant genes in halophiles**" submitted by **Mandvi Singh (081554)** and **Shagoon Preet Kaur(081565)** in partial fulfillment for the award of degree of Bachelors of Technology in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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To all the individuals who have helped us complete our experiments on time, we again express our appreciation. We are obligated to all those who provided reviews and suggestions for improving the results and the topics covered in this project, and extend our apologies to anyone we may have failed to mention.

We would also express our gratitude towards our families and our friends for their support throughout the execution of this study.All copyrights and trademarks that are cited in this document remain the property of their respective owners.

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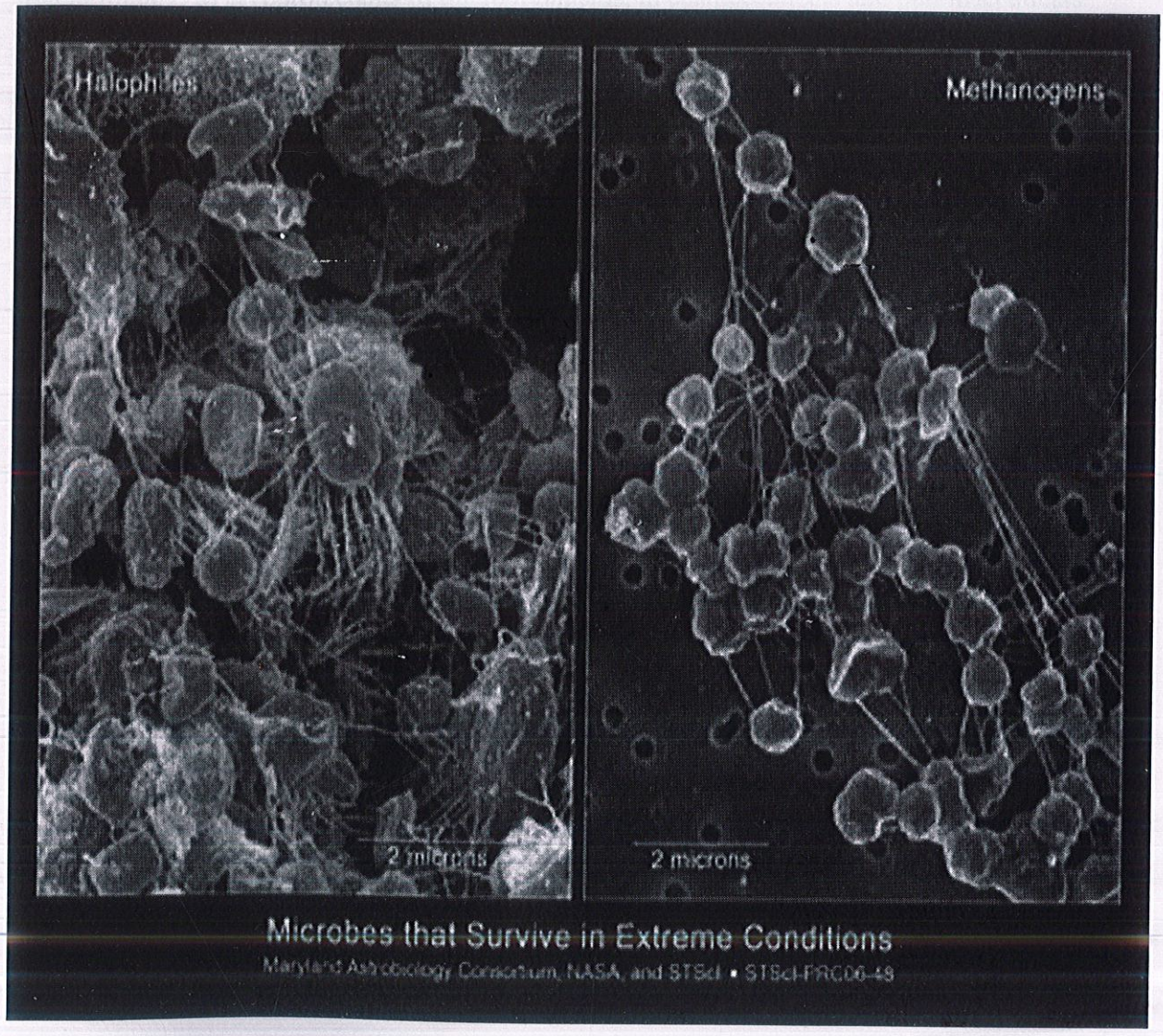
ABSTRACT OF THE DISSERTATION

Halophiles are extremophile organisms that thrive in environments with very high concentrations of salt. The name comes from the Greek for "salt-loving". Halophiles are categorized slight, moderate or extreme, by the extent of their halotolerance. Halophiles can be found anywhere with a concentration of salt five times greater than the salt concentration of the ocean, such as the Great Salt Lake in Utah, Owens Lake in California, the Dead Sea, and in evaporation ponds.
Within the Bacteria halophiles are known in the phyla *Cyanobacteria*, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Spirochaetes*, and *Bacteroidetes*. Within the Archaea the most salt-requiring microorganisms are found in the class *Halobacteria*.



Halophiles are also a good source of genes for a variety of compatible solutes that can function under salty conditions. These solutes protect biomolecules and whole cells against denaturation caused by heating, freezing, desiccation, or chemical agents (Margesin *et al.*, 2001). This property

has attracted commercial attention. Compatible solutes can be used as chemical chaperones for protein folding, cryoprotectants of microorganisms, cosmeceuticals, and pharmaceuticals.

An understanding of halotolerance can be applicable to areas such as arid-zone agriculture, xeriscaping, aquaculture (of fish or algae), bioproduction of desirable compounds (such as phycobiliproteins or carotenoids) using seawater to support growth, or remediation of salt-affected soils,drought resistant crops ,fermentation and food biotechnology.

Halotolerance genes have potential biotechnological importance, as the development of salt tolerant plants can facilitate use of marginal areas for crop production, and allow a wider range of sources of irrigation water. The genes coding for osmotic tolerance can be used as chemical chaperones for protein folding, as cryoprotectants of microorganisms, in cosmeceuticals and pharmaceuticals.

We worked on the halotolerant bacteria Staphylococcus arlettae and our aim was to clone halotolerant genes which have biotechnological importance.we found EIF1A(elongation initiation factor 1A) gene from this bacteria. This gene belongs to the salt toxicity targets along with two other genes i.e. 3'(2'),5'-bisphosphate nucleotidase (*tol1*) and halotolerance protein (*hal2*). The *eif1A* gene that is important for initiation of protein synthesis under saline conditions which is mostly found in extremely halotolerant bacteria.

CHAPTER 1

INTRODUCTION

Halophiles are organisms adapted to thrive in extreme conditions of salinity, distributed all over the world in hypersaline environments, which includes hypersaline brines in arid, coastal, and even deep sea locations, as well as in rock salt mines. The intracellular machinery of these prokaryotes has evolved to function at very high salt concentrations. During recent years, these halophiles have been considered of great interest because of their biotechnological potential, notably for producing genes and enzymes of industrial interest and accumulating a variety of organic compounds, called compatible solutes, useful as enzymes or cell stabilizing agents. Moreover, microorganisms are themselves used in various biotechnological applications (e.g. bioremediation).

Seeing the importance of halotolerance genes in biotechnology we wanted to find out industrially significant genes in the halotolerant strains. To achieve this, the genes identified in the comparative analysis were used for designing primers from conserved regions. These primers were amplified on genomic DNAs of halotolerant strains through PCR resulting in the identification of fourteen genes of biotechnological importance. These genes included, three transporters: Li^+ Na^+ P-ATPase (*ena1*), halotolerance protein (*hal11*) and glycerol 3-phosphate dehydrogenase (*gpd1*), three genes belonging to regulators of ion transporters: halotolerance proteins (*hal4, hal5)* and auto-inhibited Ca^{2+} ATPase 4 gene (*aca4*), three molecular chaperones: heat shock proteins *dnaK, groEL and groES,* three genes belonging to osmotic tolerance: glycerol-3-phosphate dehydrogenase (*gpd1*) cysteine synthase K (*cysK*) and ectoin biosynthesis (*ectB*) and three genes belonging to salt toxicity targets: elongation initiation factor 1A(*eif-1A*), 3'(2'),5'-bisphosphate nucleotidase (*tol1*) and halotolerance protein (*hal2*). Halotolerance genes (taken from plants, fungi/yeast) identified in the present study in halotolerant bacterial strains are not known in bacteria except for genes *hal2* and *aca4*.

1.1 Habitats of halophiles

Hypersaline habitats are common throughout the world, but extremely hypersaline habitats are rather rare. Most such environments are in hot, dry areas of the world. Salt lakes can vary considerably in ionic composition. Many haypersaline environments have originated by evaporation of sea water e.g. Great Salt Lake. Their salt composition is similar to that of sea water: the dominating ions are sodium and chloride, and the pH is near neutral to slightly alkaline. When

evaporation proceeds, some changes occur in the ionic composition due to the precipitation of gypsum ($CaSO_4.2H_2O$) and other minerals after their solubility has been exceeded. A prime example of such environment is the Dead Sea, a lake in which the concentration of divalent cations exceeds that of monovalent cations and in which the pH is relatively low (around 6.0). Some microorganisms may even simultaneously withstand high salt concentrations (200.0 g/l) and high temperatures upto 68.0°C (Cayol *et al.,* 1994). Even such a hostile environment periodically supports dense microbial blooms (Oren, 1988). Microbial life has adapted to environments that combine high salt concentrations with extremely high pH values. Alkaline soda lakes in Africa, India, China and elsewhere with pH values of 11.0 and higher and salt concentrations exceeding 300.0g/l are teeming with life (Oren, 2002). Microbial life has adapted to environments that combine high salt concentrations with extremely high pH values.

Halotolerant microorganisms play an essential role in food biotechnology for the production of fermented food and food supplements. The degradation or transformation of a range of organic pollutants and the production of alternative energy are other fields of applications of these groups of extremophiles. Halotolerance genes have potential biotechnological importance, as the development of salt tolerant plants can facilitate use of marginal areas for crop production, and allow a wider range of sources of irrigation water. The genes coding for osmotic tolerance can be used as chemical chaperones for protein folding, as cryoprotectants of microorganisms, in cosmeceuticals and pharmaceuticals. Enzymes from halophiles possess a great potential for biotechnological applications, due to their tolerance not only to salt, but also to temperature and extreme pH values.

Halophiles are organisms adapted to thrive in extreme conditions of salinity with distribution in hypersaline environments, such as solar salterns, the Great Salt Lake, the Dead Sea, as well as in rock salt mines (DasSarma *et al.,* 2001). Hypersaline habitats are common throughout the world, but extremely hypersaline habitats are rather rare. Most hypersaline environments are derived from the evaporation of sea water, encouraged by restricted flow, high temperatures, low rainfall, low humidity and high wind speed. Different marine salterns have been studied throughout the world, including salterns in Alicante, Spain; Mexico, San Diego and Newark, California; Bonaire, the Netherlands; Eilat, Israel and Margherita di Savoia, Italy, etc. (Litchfield and Oren, 2001). Hypersaline environments have been reservoirs for the long-term evolution of specifically adapted microorganisms. Halophiles have been mainly isolated from saline lakes, such as the Great Salt Lake in Utah (salinity, >2.6 M) and from evaporitic lagoons and coastal salterns with NaCl concentrations between 1.0 and 2.6 M (Grant *et al.,* 1998). Saline soils are less explored and constitute less stable biotopes than hypersaline waters because they are subjected to periodic significant dilution during rainy periods. Alkaline soda lakes in Africa, India, China and elsewhere

with pH values of 11.0 and higher salt concentrations exceeding 300.0 g/l are teeming with life (Oren, 2002). Some microorganisms may even simultaneously withstand high salt concentrations (200.0 g/l) coupled with high temperatures up to 68.0°C (Cayol *et al.,* 1994).

For many years there has been a considerable interest in a group of microorganisms known as halophiles. Although the early studies on halophiles were based on strains isolated from salted foods because they may contaminate or spoil these products, the majority of recent studies concerning this group of microorganisms have been carried out in hypersaline lakes as well as ponds of marine salterns. The aspects that attracted the interest of researchers were mainly those related to their physiological adaptation to highly saline concentrations and their ecology (Kushner and Kamekura, 1998; Ventosa *et al.,* 1998). Due to the importance in biotechnology, the isolation of new strains from hyper-saline ecosystems is continuing. There are several reports of microbial isolation from highly saline environments. Vreeland and Huval (1991) isolated many strains from brines with a range of salt tolerances, 17.0 - 32.0% (w/v) NaCl. Al-Zabran *et al.* (2002) isolated halotolerant *Nocardiopsis* sp. and a halophilic *Saccharomonospora* sp. from salt marsh soil in Kuwait. Both the microorganisms showed keratinase activities in the culture filtrate in the presence of feather meal as sole source of carbon and nitrogen. During the study of prokaryotic biodiversity in El Golea salt lake, in Algerian Sahara, extreme halophilic microorganisms were isolated from a site containing 290.0 g/1 NaCl (Hacene *et al.,* 2004). Birbiir *et al.* (2004) isolated twelve archaeal strains belonging to the genera *Halobacterium*, *Haloarcula*, *Natrinema* and *Halorubrum,* from salt mine in Central Anatolia, Turkey. The isolated strains produced hydrolytic enzymes like gelatinase, caseinase, amylase, cellulase and lipase. One hundred and sixty-five halophilic archaea were isolated from three different types of hypersaline lakes (Erliannor, Shangmatala and Xilin Soda Lake) in Inner Mongolia (Pan *et al.,* 2006). Joshi *et al.* (2007) isolated 14 bacterial moderate halophiles from alkaline Lonar Lake, Maharashtra, India, which were capable of producing enzymes like amylase and lipase. Isolation of chromium resistant moderately halophilic bacterial strains (9.0% salt tolerant) from Palar river basin, Vellore are also reported (Sundar *et al.,* 2010). These studies included culture-dependent approaches, leading to the isolation and characterization of many novel types of halophiles and new information on the abundance and geographic distribution of the known types, as well as culture-independent studies based on sequencing of DNA recovered from the environment.

Studies on isolation of microorganisms from rock salt mine have been done in the past. For example, Norton *et al.* (1993) isolated seven haloarchaeal strains from rock salt in Winsford salt mine in Cheshire, England belonging to the genus *Halorubrum* and *Haloarcula*. Cojoc *et al.* (2009) have also described the extracellular hydrolytic activities of halophilic bacteria isolated from

subterranean rock salt crystal. Forty-seven bacterial strains were isolated from Winsford salt mine (UK), growing upto 30.0% (w/v) NaCl (Grant *et al.,* 1998).

Two biotechnological processes involving halophiles are highly successful: the production of β-carotene by the green alga *Dunaliella* and the production of ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid), used as a stabilizer for enzymes and now also applied in cosmetic products, from moderately halophilic bacteria.

Rock salt occurrences are limited in India with only two rock salt mines both located in Himachal Pradesh. These sites, to the best of our knowledge, are just the two deposits of rock salts in India that need to be explored for microbial flora and their invaluable genes, enzymes and compounds.

One reason for the interest in halophiles is the need to understand the biochemical mechanisms involved under extreme conditions. Occurrence of novel and stable molecules in halophiles make them valuable for the future biotechnology pursuits. The potential use of bacteriorhodopsin, the retinal protein proton pump of *Halobacterium*, in optoelectronic devices and photochemical processes is being explored, and may well lead to commercial applications in the near future. Another potential promising future application of halophiles could be the enhancement of drought tolerance or salt tolerance of transgenic plants (Yoshida, 2002). The discovery of genes for salinity tolerance plants will provide the basis for effective genetic engineering strategies, leading to greater stress tolerance in economically important crops.

To prevent loss of cellular water under these circumstances, halophiles have developed different basic mechanisms to cope with ionic strength and the considerable water stress. These mechanisms allow halophiles to proliferate in saturated salt solutions and to survive entrapment in salt rock. Two principle mechanisms evolved to lower the chemical potential of cell water, allowing an osmotic adaptation of microorganisms are: "salt-in-cytoplasm mechanism" and "organic-osmolyte mechanism".

1.2 Salt-in-cytoplasm strategy

The "salt-in-cytoplasm mechanism", first discovered in *Halobacteria,* is considered the typical strategy of osmoadaptation in extreme halophiles. Despite the abundance of NaCl in the typical haloarchaeal environment, halophiles keep the cytoplasm relatively free of sodium. The effect of the accumulation of potassium and/or sodium in the cytoplasm is that the cytoplasm is exposed to an increased ionic strength. To adapt the enzymatic machinery to an ionic cytoplasm, proteins of halophilic anaerobic bacteria and halophilic archaea contain an excess of acidic amino

acids over basic residues (Eisenberg, 1987). Instead, potassium accumulates in the cell and together with its counter ion Cl^-, K^+ can be found in molar concentrations in the cytoplasm. Because the K^+ concentration inside the cell is 100 times higher than in the surrounding environment, a part of the proton motive force must be used to maintain the ion gradient. This leads to a predominance of charged amino acids on the surface of enzymes and ribosomes which is thought to stabilize the hydration shell of the molecule when in high ionic surroundings. In low salinity environments, the excess of negatively charged ions will destabilize the molecule's structure, due to repulsion when the shielding cations are removed (Eisenberg, 1992). This mechanism explains the fact that organisms employing the salt in cytoplasm strategy display a relatively narrow adaptation and their growth is restricted to highly saline environments (Oren, 2002). However, in habitats with saturated salt concentrations, halophilic archaea outcompete organic-osmolyte producers, proving members of the "salt-in-cytoplasm mechanism" as *extreme* halophiles.

1.3 Organic-osmolyte strategy

In response to an osmotic stress, halophilic/halotolerant organisms mainly accumulate organic compounds like sugars, polyols, amino acids and/or amino acid derivatives either by *de novo* synthesis or by uptake from the surrounding environment. These non-ionic, highly water-soluble compounds do not disturb the metabolism, even at high cytoplasmic concentrations and are thus aptly named *compatible solutes* (Brown, 1976). Halophilic cells using compatible solutes can basically preserve the same enzymatic machinery as non-halophiles, needing only minor adjustments in their interior proteins (i.e. ribosomal proteins), which are slightly more acidic than the cytoplasmic proteins in *Escherichia coli* (Falkenberg, 1976). Halophiles employing the organic-osmolyte mechanism are more flexible than organisms employing the "salt-incytoplasm strategy" because even though they display wide salt tolerance, they can also grow in low salt environments. The organic osmolyte mechanism is widespread among bacteria and Eukarya and also present in some methanogenic archaea (Robertson, 1992; Lai, 1992).

1.4 Identification, Characterization and Significance of Halophiles

The extensive studies on hypersaline environments carried out in many geographical areas have permitted the isolation and taxonomic characterization of a large number of halophilic species. In an early study of halophilic bacteria, Hof (1935) inoculated salt mud from a solar salt facility on Java onto a variety of media of different salinities. In addition to red archaeal types, different types of white colonies were isolated, including endospore containing *Bacillus* species able to grow at 24.0% NaCl. *Pseudomonas beijerinckii* was isolated from salted beans, it grew from 3 to 18% salt

but not at 0.5%, showing its obligate halophilic character.

Amoozegar *et al.* (2003) have isolated a moderately halophilic, Gram-positive, spore-forming bacterium from saline soil surface of the Karaj region, Iran. The strain was strictly aerobic with rod-shaped cells that occurred singly, in pairs or short chains. It was non-motile and had an ellipsoidal endospore located centrally or subterminally. Growth occurred at 10.0–49.0°C and in the pH range 6.0–9.6. Strain MA-2T grew at salinities of 1.0–24.0% (w/v) NaCl, showing optimal growth at 10.0% (w/v) NaCl. The DNA G+C content was 41.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain was showing the closest phylogenetic relationship to *Halobacillus litoralis* and *Halobacillus trueperi.* On the basis of phenotypic and chemotaxonomic characteristics, 16S rRNA gene sequence analysis and DNA–DNA similarity data, it was proposed that the strain should be placed in the genus *Halobacillus* as the type strain of a novel species, *Halobacillus karajensis* sp. nov.

Table 1.1 Potential of halophilic microorganisms in biotechnology

Organism	Application	Reference
Halomonas elongate KS3	Compatible solutes like ectoine and hydroxyectoine used in cosmetics	Sauer and Galinski, 1998
Halobacteirum salinarum S9	Bacteriorhodopsin is used for optical data processing (optical switches, holography, information storage)	Margesin and Schinner, 2001
Halobacterium cutirubrum	Liposomes are used in cosmetic and medicine for the transport of compounds to specific target sites in the body	Margesin and Schinner, 2001
Natrialba sp.	Exopolymer poly (γ-D-glutamic acid) (PGA) used as abiodegradable thickner, gelling agents and emulsifiers, sustained release material (drug carrier in the food or	Kunioka, 1997; Hezayen *et al.*, 2000

	pharmaceutical industry)	
Haloferax mediterranei	Polyhydroxyalkanoates (PHA) and poly β- hydroxybutyric acid (PHB) are used in bioplastic production of pharmaceutical and clinical importance, also used in delayed drug release, bonereplacement and surgical sutures	Rodriguez-Valera and Lillo, 1992; Lafferty *et al.*, 1988
Halobacterium halobium	Extracellular serine proteases are used as catalyst for synthesis of glycine containing peptides inpresence of organic solvents	Ryu *et al.*, 1994
Lactobacillus plantarum	Fermented cucumbers and sauerkraut (pickled cabbage)	Margesin and Schinner, 2001
Halococcus sp., *Bacillus* sp., *Vibriobacillus* sp. and *Stephylococcus* sp.	Thai fish sauce preparation	Thongthai and Suntinanalert, 1991; Yongsawatdigul *et al.*,, 2007
Shewanella sp. and *Colwellia* sp. (Antarctic marine bacteria)	Productin of polyunsaturated fatty acid (PUFA) used as dietary supplement	Nichols *et al.*, 1993; Nichols and Russell, 1999
Halobacterium sp.	Bioremediation of oil spills in saline habitats	Kulichevskaya *et al.*, 1992

1.5 OBJECTIVES

1) Isolation of genomic DNA from staphylococcus arlettae.

2) Identification of halotolerant genes. 3) Cloning of halotolerant genes.

CHAPTER 2

REVIEW OF LITERATURE

2.1 CLASSIFICATION

Staphylococcus arlettae

KINGDOM: BACTERIA

PHYLUM:FIRMICUTES

CLASS:BACILLI

ORDER:BACILLALES

FAMILY:STAPHYLOCOCCACEAE

GENUS:STAPHYLOCOCCUS

SPECIES:S.arlettae

MORPHOLOGY:Gram positive cocci,0.5-1.5µm.non-motile,non spore-forming

ECOLOGY:Isolated from human and animal(mammals and birds)skin,mucous membranes,also from milk,chicken litter.

It is an aerobic bacteria with salt tolerance of 19%.so it is an extreme halophile.

SIGNIFICANCE: Staphylococcus Arlettae, the bacteria that promises to revolutionize the treatment of effluents from the textile industry.

2.2 Halotolerance genes

Halophiles are a good source of genes encoding proteins for a variety of compatible solutes that can function under salty conditions. These solutes protect biomolecules and whole cells against denaturation caused by heating, freezing, desiccation, or chemical agents. One such gene Na^+/H^+ antiporter have a role in salt tolerance in both fungi and bacteria (Jia *et al.*, 1992; Nozaki *et al.*, 1998). Mulet *et al.* (1999) have characterized two novel paralogous genes, *hal4* and *hal5*, which

encode partially redundant protein kinases required for ion homeostasis. It was observed that the overexpression of either *hal4* or *hal5* confers enhanced tolerance to sodium and lithium.

Table 1.2 Halotolerance genes reported in different organisms

Organism Category	Source Organism	Gene	Organism	Source Organism	Gene
Archaea	*Halobacterium salinarum*	*bop*	Fungi	*Saccharomyces cerevisiae*	*hal4*
Bacteria	*Halomonas elongata*	*ectA*		*Saccharomyces cerevisiae*	*hal5*
	Halomonas elongata	*ectB*		*Saccharomyces cerevisiae*	*hal11*
	Halomonas elongata	*ectC*		*Saccharomyces cerevisiae*	*ena1*
	Lactococcus lactis	*dnaK*		*Debaryomyces hansenii*	*ena2*
	Tetragenococcus halophila	*groEL*		*Saccharomyces cerevisiae*	*ppz1p*
	Lactococcus lactis	*groES*		*Saccharomyces cerevisiae*	*ppz2p*
	Listeria monocytogenes	*cysK*		*Saccharomyces cerevisiae*	*nhx1*
	Sinorhizobium meliloti	*rtsE*		*Saccharomyces cerevisiae*	*ura5*
	Listeria monocytogenes	*ccpA*		*Arabidopsis thaliana*	malic enzyme
	Listeria monocytogenes	*gap*	Plants	*Beta vulgaris*	*ura4*
	Listeria monocytogenes	*gbuA*		*Sesuvium portulacastrum*	*stp*
	Halomonas elongata	*nhaD*		*Arabidopsis thaliana*	*gsk1*
	Sinorhizobium meliloti	*relA*		*Lophopyrum elongatum*	*esi47*
	Halobacterium salinarum	*ndk*		*Beta vulgaris*	*stp4*
Fungi	*Debaryomyces hansenii*	*gpd1*		*Arabidopsis thaliana*	*rcy1*
	Saccharomyces cerevisiae	*tol1*		*Capsicum annuum*	*czf1*

Saccharomyces cerevisiae	*trk1*	*Beta vulgaris*	*eif-1A*
Candida albicans	*cnh1*	*Chlamydomonas sp. W80*	*bbc1*
Lodderomyces elongisporus	*hal2*	*Arabidopsis thaliana*	*aca4*
Candida tropicalis	*hal3*	*Beta vulgaris*	*ck2*

The gene *cnh1* encodes a protein of 840 amino acids that exhibits high levels of similarity in sequence, size, and structural and functional domains to a group of known Na^+/H^+ antiporters of fungi. Gene deletion and heterologous-expression studies demonstrated that these proteins are involved in pH regulation of the cell and in salt tolerance. When it was heterologously expressed in a *S. cerevisiae* strain severely defective in Na^+ extrusion, *cnh1* conferred resistance to 800 mM NaCl, indicating that *cnh1* may play a similar role in *Candida albicans* cells. Deletion of *cnh1* lead to slower growth rates in a gene.

Empadinhas *et al.* (2004) reported that compatible solutes can be produced in recombinant hosts, e.g., *E. coli* or yeast, which are more suitable in bioprocesses. A prerequisite for this approach is the availability of the synthetic pathway genes for cloning and the construction of a corresponding recombinant organism. This approach was chosen for mannosylglycerate where the bifunctional mannosylglycerate synthase from *Dehalococcoides ethenogenes* was overproduced in *E. coli* and *Saccharomyces cerevisiae*. Compatible solutes from halophiles can be used as chemical chaperones for protein folding, cryoprotectants of microorganisms, cosmeceuticals, and pharmaceuticals (Lentzen and Schwarz, 2006).

Kurz *et al.* (2006) reported a Na^+/H^+ antiporter in *H. elongata,* sequence comparison results showed that these antiporters belong to the *nhaD* family which is so far found exclusively in cell membranes of marine/haloalkaliphilic γ-proteobacteria. The gene was cloned and expressed in *E. coli*. Antiporter deficient NaCl and LiCl sensitive *E. coli* mutants EP432 and KNabc were partially complemented by a plasmid carrying the *H. elongata nhaD* gene. Surprisingly the LiCl sensitivity of *E. coli* strain DH5α having a complete homeostasis system was increased when *nhaD* was co-expressed.

Vaupotic *et al.* (2007) reported that the 3′-phosphoadenosine-5′-phosphatase encoded by *hal2* gene, is required for the removal of the cytotoxic 3′-phosphoadenosine-5′-phosphate produced during sulfur assimilation in eukaryotes. Two *HAL2*-like genes, *HwHAL2A* and *HwHAL2B,* were

cloned from *Hortaea werneckii* isolated from saltern-inhabited extremely halotolerant black yeast. When the *HwHAL2* genes were transferred to the salt sensitive *Saccharomyces cerevisiae,* the resulting organism tolerated 1.8 M NaCl or 0.8 M LiCl, the highest reported salt concentrations at which *S. cerevisiae* can survive.

Aphanothece gene encoding 3-phosphoglycerate dehydrogenase (*PGDH*) was isolated and functionally characterized from *A. halophytica* which was isolated from the Dead Sea and is known to accumulate significant amounts of betaine at high salinity. The *ApPGDH* gene was transferred into *E. coli*, which has a betaine synthetic pathway via choline oxidation; it was also transferred into *Arabidopsis* plants, in which the betaine synthetic pathway was introduced via glycine methylation. The results manifested that the heterogeneous expression of *ApPGDH* significantly enhanced betaine levels in both betaine accumulating and non-accumulating organisms. Overexpression of *ApPGDH* in *E. coli* resulted in a large increase of serine and glycine levels (Waditee *et al.,* 2003 and 2007).

Ectoine ((4S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid), a compatible solute that acts against halostress in microorganisms was originally discovered by Galinski *et al.* (1985) in *Halorhodospira halochloris*. It is an extremely halophilic phototrophic eubacterium, isolated from Wadi Natrun, Egypt, produce an organic solute (ectoine) in response to sudden changes in salinity of the growth medium. To synthesize ectoine continuously ectoine genes *ectABC* of the halophilic bacterium *Chromohalobacter salexigens* were expressed into an *Escherichia coli* strain using the expression vector pASK-IBA7. The transgenic *E. coli* synthesized 6 g liter^{-1} of ectoine (Galinski *et al.,* 1985 and Schubert *et al.,* 2007).

Overexpression of a chaperone protein, *dnaK,* from a halotolerant cyanobacterium *Aphanothece halophytica* (*ApDnaK*) which was isolated from the Dead Sea, conferred the tolerances for salt in transgenic tobacco plants. In another study enhanced tolerance to abiotic stresses such as high salinity, drought and low temperature in tobacco plants was seen when transferred from a halotolerant cyanobacterium *Aphanothece halophytica* to tobacco plants (Sugino *et al.,* 1999 and Hibino *et al.,* 1999 and Takabe *et al.,* 2008).

Table 1.3 Classification and biological function of genes detected in halotolerant strains

Gene	Protein Name	Domain	Function
ectB	Diaminobutyrate-2 Oxoglutarate Transaminase	Aminotransferases class-III	Osmotic Tolerance
hal4	Halotolerance Protein 4	Protein kinase domain	Regulators of ion transporters
hal5	Halotolerance Protein 5	Protein kinase domain	Regulators of ion transporters
groEL	60 Kda Chaperonin	60 Kda Chaperonin	Chaperones
groES	10 Kda Chaperonin	10 Kda Chaperonins	Chaperones
eif-1A	Translation Initiation Factor	S1 domain	Salt toxicity targets
tol1	3'(2'),5-Bisphosphonucleoside 3'(2')-Phosphohydrolase	Inositol monophosphatase domain	Salt toxicity targets

2.3 APPLICATIONS OF HALOTOLERANT GENES IN BIOTECHNOLOGY:

Halotolerant or halophilic microorganisms, able to live in saline environments, offer a multitude of actual or potential applications in various fields of biotechnology.The technical applications of bacteriorhodopsin comprise holography, spatial light modulators, optical computing,and optical memories. Compatible solutes are useful as stabilizers of biomolecules and whole cells, salt

antagonists, or stress-protective agents. Biopolymers, such as biosurfactants and exopolysaccharides, are of interest for microbially enhanced oil recovery. Other useful biosubstances are enzymes, such as new isomerases and hydrolases, that are active and stable at high salt contents. Halotolerant microorganisms play an essential role in food biotechnology for the production of fermented food and food supplements.The degradation or transformation of a range of organic pollutants and the production of alternative energy are other fields of applications of these groups of extremophiles.

One possible strategy to recover saline land for agricultural use is the transfer of halotolerance from halophilic organisms to crops of agronomic value. Transgenic tobacco plants acquired resistance to salt stress after introduction of the *dnaK1* gene from the halotolerant cyanobacterium *Aphanothece halophytica,* which can grow in saline conditions up to 3 M NaCl. DnaK1 was constitutively expressed.After 3 days of treatment with 0.6 M NaCl, the sodium content in leaves of the transgenic plant remained at levels similar to those in nonstressed plants. Dnak/Hsp70 proteins are known to play a role in the recovery of cells from stress.Herewith, the positive role of heat-shock proteins for enhanced salt tolerance has been demonstrated for the first time (Sugino et al. 1999).

2.3.1 Fermentation products:

Halotolerant microorganisms play an essential role in various fermentation processes that occur in the presence of salt. These organisms catalyze the fermentation, thereby producing various compounds that give the characteristic taste, flavor, and aroma to the resulting products.In the production of pickles (fermented cucumbers),brine strength is increased gradually from 5% to 15.9%(w/v) NaCl. Fermentation of Sauerkraut(pickled cabbage)occurs in the presence of 2.25%–2.5% salt.*Lactobacillus plantarum* is the most essential species in both cases.*Halobacterium salinarum, Halococcus* sp.,*Bacillus* sp.,pseudomonads, and coryneform bacteria are used in the production of an Asian (Thai) fish sauce (nam pla) in which fish is fermented in concentrated brine (Thongthai and Suntinanalert 1991).

2.3.2 Biological waste treatment:

Hydrocarbons

The ability of halophiles/halotolerants to oxidize hydrocarbons in the presence of salt is useful for the biological treatment of saline ecosystems contaminated with petroleum products.Successful

bioremediation of oil spills has been observed in marine, Arctic, and Antarctic environments(Delille et al. 1998; Margesin and Schinner 1999). In contrast to the assumption of an inverse relationship between oil biodegradation and high salinity, i.e., above 20% (w/v) (Ward and Brock 1978), a new halo- and thermotolerant *Streptomyces albaxialis* was found that was able to degrade crude oil and petroleum products even in the presence of 30% (w/v) NaCl (Kuznetsov et al. 1992).

2.3.3 Detoxification of chemical warfare agents:

Several halophilic isolates produce organophosphorus acid anhydrases (OPAA) with hydrolytic activity against several organophosphorus chemicals and related compounds, such as sarin and soman. These enzymes have considerable potential for the decontamination and demilitarization of chemical warfare agents. G-type nerve agent-degrading enzyme activity was found in halophilic *Alteromonas* JD6.5 (DeFrank et al. 1993). A cloned gene, encoding OPAA from this strain, is used for the production of a recombinant enzyme that is involved in the recently patented enzymatic detoxification of organophosphorus compounds.The composition may be prepared as a dry powder and reconstituted with water when needed, and can be applied to contaminated surfaces or substances (Cheng and DeFrank 2000).

2.3.4 Agriculture:

Ice-nucleation activity (INA), i.e., the ability to freeze water at subzero temperatures higher than –7° or –5°C, is of advantage in biotechnology for the energy-saving production of artificial snow and ice. In food processing, it is used for ice cream production and efficient freeze concentration without loss of flavor (Lundheim and Zachariassen 1999).The transfer and expression of the responsible gene in nonpathogenic bacteria could be of considerable economic interest. The ice nucleation gene *inaZ* from phytopathogenic *Pseudomonas syringae* has been successfully expressed in various moderately halophilic bacteria(Arvanitis et al. 1995).

Peptidyl prolyl *cis-trans* isomerase (PPIase) is useful for the regeneration of denatured protein, for the stabilization of proteins, for the production of recombinant protein, and for the development of novel immunosuppressant and physiologically active substances. Iida et al. (1997) patented a novel cyclophilin type PPIase gene by amplifying the genome DNA of *Halobacterium cutirubrum* DSM669.

2.4 CLONING:

Cloning in biotechnology refers to processes used to create copies of DNA fragments (molecular cloning), cells (cell cloning), or organisms. The term also refers to the production of multiple copies of a product.

Cloning of any DNA fragment essentially involves four steps.

1. Fragmentation - breaking apart a strand of DNA
2. Ligation - gluing together pieces of DNA in a desired sequence
3. Transfection - inserting the newly formed pieces of DNA into cells
4. Screening/selection - selecting out the cells that were successfully transfected with the new DNA.

A ligation procedure is used where the amplified fragment is inserted into a vector (piece of DNA). The vector (which is frequently circular) is linearised using restriction enzymes, and incubated with the fragment of interest under appropriate conditions with an enzyme called DNA ligase. Following ligation the vector with the insert of interest is transfected into cells. A number of alternative techniques are available, such as chemical sensitivation of cells, electroporation, optical injection and biolistics. Finally, the transfected cells are cultured. As the aforementioned procedures are of particularly low efficiency, there is a need to identify the cells that have been successfully transfected with the vector construct containing the desired insertion sequence in the required orientation. Modern cloning vectors include selectable antibiotic resistance markers, which allow only cells in which the vector has been transfected, to grow. Additionally, the cloning vectors may contain colour selection markers, which provide blue/white screening (alpha-factor complementation) on X-gal medium. Nevertheless, these selection steps do not absolutely guarantee that the DNA insert is present

in the cells obtained. Further investigation of the resulting colonies must be required to confirm that cloning was successful.

pGEM®-T Easy Vector Systems

The pGEM®-T Easy Vector Systems are convenient systems for the cloning of PCR products. They offer all of the advantages of the pGEM®-T Vector Systems with the added convenience of recognition sites for EcoRI and NotI flanking the insertion site. Thus several options for removal of the desired insert DNA with a single restriction digestion are provided. The pGEM®-T Easy Vector System II contains JM109 Competent Cells in addition to all of the pGEM®-T Easy

Vector System I components.

TA cloning is a subcloning technique that doesn't use restriction enzymes and is easier and quicker than traditional subcloning. The technique relies on the ability of adenine (A) and thymine (T) (complementary basepairs) on different DNA fragments to hybridize and, in presence of ligase, become ligated together. PCR products are usually amplified using Taq DNA polymerase which preferentially adds an adenine to the 3' end of the product. Such PCR amplified inserts are cloned into linearized vectors that have complementary 3' thymine overhangs. Commercialized kits with pre-prepared vectors and PCR reagents are currently sold, greatly speeding up the process.

2.5 TRANSFORMATION:

In molecular biology transformation is the genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane(s). Transformation occurs naturally in some species of bacteria, but it can also be effected by artificial means in other cells. Bacteria that are capable of being transformed, whether naturally or artificially, are called competent. Transformation is one of three processes by which exogenous genetic material may be introduced into a bacterial cell, the other two being conjugation (transfer of genetic material between two bacterial cells in direct contact) and transduction (injection of foreign DNA by a bacteriophage virus into the host bacterium). "Transformation" may also be used to describe the insertion of new genetic material into nonbacterial cells, including animal and plant cells; however, because "transformation" has a special meaning in relation to animal cells, indicating progression to a cancerous state, the term should be avoided for animal cells when describing introduction of exogenous genetic material.

Selection and screening in plasmid transformation:

Because transformation usually produces a mixture of relatively few transformed cells and an abundance of non-transformed cells, a method is necessary to select for the cells that have acquired the plasmid. The plasmid therefore requires a selectable marker such that those cells without the plasmid may be killed or have their growth arrested. Antibiotic resistance is the most commonly used marker for prokaryotes. The transforming plasmid contains a gene that confers resistance to an antibiotic that the bacteria are otherwise sensitive to. The mixture of treated cells is cultured on media that contain the antibiotic so that only transformed cells are able to grow.

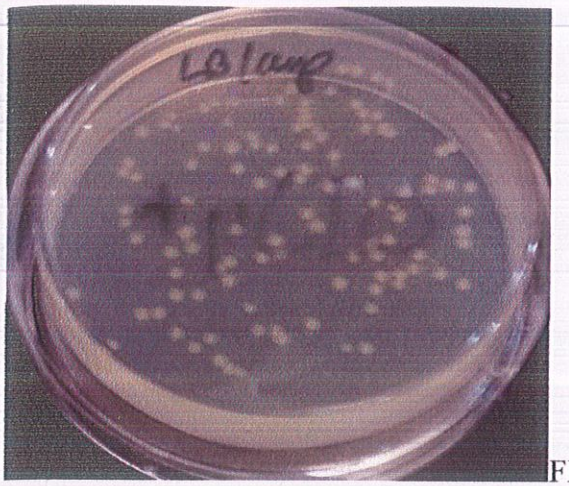


FIGURE1.1Genetically transformed ampicillin resistant

FIGURE 1.2 Resistance of antibiotic ampicillin

The plate labeled "Amp"/"B2" should not have bacterial growth because the bacteria are killed because they did not have resistance to the antibiotic ampicillin. Bacterial growth on the "Amp"/"B1" plate is from cells that took up plasmids added in step 2 and that became resistant to ampicillin. There is extensive bacterial growth on both of the "No Amp" plates because the antibiotic was not present and both resistant and nonresistant bacteria could grow.

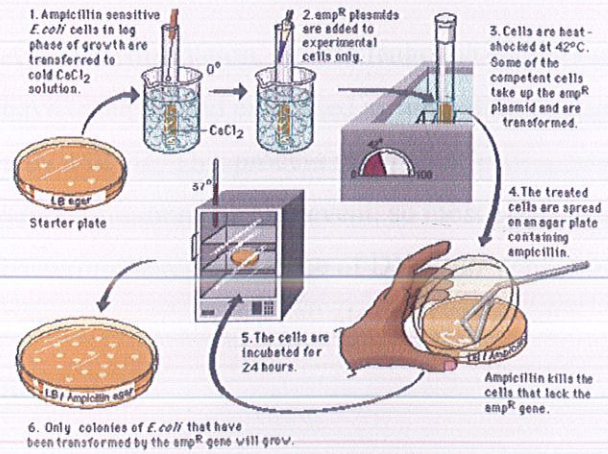


FIGURE 1.3: TRANSFORMATION

How do we isolate a plasmid we want?

- We introduce the reclosed (ligated) products into E. coli, a process called "transformation", and select for bacteria resistant to a drug (such as amipicillin, for example).
- We screen the individual bacterial colonies to find one that contains a plasmid of the correct structure.

Bacteria naturally take up DNA from their environment, and we call that process transformation. When we are transforming DNA in the laboratory (i.e. for experimental purposes), we have several ways of making the uptake of DNA by E. coli cells more efficient.

- One method is to starve the cells in ice-cold calcium chloride solution, add a sample of ligated DNA, and "heat-shock" the cells at 42 degrees C for a short period of time - about 45 seconds. The fluidity of the membrane increases to the point where DNA is taken up by the cell.
- A second method is to deliver an electric shock to the cell, releasing a charged capacitor with a field strength in the sample of approximately 1200 volts per millimeter. The DNA is swept into the cells as the membranes are temporarily breached. This process is called electroporation.

Selection

After transformation, we challenge the bacteria with an antibiotic (such as ampicillin). If the E. coli have taken up and expressed an ampicillin resistance gene on a plasmid, they will live - otherwise they will die. This process is called selection, because we are selecting which bacteria may survive. Transformation is a rare event, so most bacteria in an experiment are killed by the antibiotic. If a bacterium takes up a piece of DNA that cannot be maintained in a cell (e.g. if it lacks an origin of DNA replication) that cell also will not survive.

Screening

At this stage we have a bacteriological plate (agar medium containing ampicillin) with bacterial colonies on it. Each colony contains a different plasmid type, because each was grown up from a single transformed cell. What we do now is to isolate DNA from each colony (or a small growth of

cells propagated from the colony), and analyze the structure of the plasmid with restriction enzymes or by DNA sequencing.

We can use gel electrophoresis to identify the sizes of restriction fragments that are released from the plasmid and to check the purity of the preparation.

CHAPTER 3

MATERIALS AND METHODS

3.1 CULTURING STAPHYLOCOCCUS ARLETTAE

Four luria bertini agar plates were made and they were incubated at 37° C to check for contamination .

Next day plates were streaked with glycerol stock sample of staphylococcus arlettae.

Luria bertini broth was inoculated with colony picked from agar plate .the culture were grown in LB medium overnight at 37°C with shaking at 175 rpm.Growth was observed after 24 hours .

3.2 TO ISOLATE GENOMIC DNA FROM STAPHYLOCOCCUS ARLETTAE

Labwares:

Falcon tube,Conical flask,Eppendorf tubes,Measuring cylinder,Falcon tube stand,inoculation loop,ice bucket,,gloves,cotton.

Equipments:

Laminar hood,ph meter,spirit lamp,shaker incubator,autoclave,weighing balance,4°c refrigerator,mini centrifuge,water bath,-20°c refrigerator.

Chemicals:

LB broth,TE buffer,10%SDS,10N NAOH,phenol(water saturated),chloroform,3M sodium acetate,isopropanol.70%ethanol,milliQ water,6 N HCl,glacial actetic acid.

PROCEDURE:

A. CULTURE OF STAPHLOCOCCUS ARLETTAE

One colony from luria bertini agar was picked up from inoculating loop and was transferred into LB broth(20ml).Then it was incubated overnight @37°C,175 rpm.

Isolation of genomic dna:

Total genomic DNA of *S.arlettae* was isolated by the method of Wilson (1997).

1. 1.5 mL of culture broth in three sterile eppendorf tubes were centrifuged at 10000 rpm for 2 mins at 4°C.
2. Supernatant was discarded by inverting the tube.these two steps were repaeated for 4 times to increase the cell mass in the same tube.
3. The pellet obtained was resuspended in 467µL TE buffer and 30 µL of 10%SDS.
4. Incubated at 37°C for 1 hour.
5. 1:1 volume of phenol and chloroform was added(500µL).
6. Mixed by inverting 2-3 times.
7. Centrifuged at 10,000g for 10 mins at 4°C.
8. The upper aqueous phase was transferred to a fresh eppendorf tube.
9. 50µL of 3M sodium acetate was added.
10. 500µL of isopropanol was added.

11. Mixed well by inverting 2-3 times.

12. Kept in -20°C for 30 mins.

13. Centrifuged @10,000g for 10 mins at 4°C.

14. Supernatant was discarded.

15. 70%ethanol(1ml) was added to the pellet(ethanol washing).

16. Repeat ethanol washing if pellet is not whitish.

17. The pellet was air dried by keeping the mouth of the tube open. Do not overdry.

18. 50µL of TE buffer was added to the pellet.

19. Stored at 4°C.

3.3 Gel electrophoresis

Gel electrophoresis was done after DNA extraction, to check the presence of DNA.

Materials required:

- Agarose
- TAE 50x buffer
- Distilled water
- Ethidium bromide
- DNA sample
- Lambda DNA
- Gel electrophoresis set up.

Procedure:

- Dilute 50X Tris-Acetate buffer for a final concentration of 1X.

- Prepare the gel such that it is 1.0% agarose. Weigh out approximately 0.5g of the agarose powder.
- Add agarose in 50 mL of 1X TAE buffer.
- Heat the agarose-TAE buffer mixture in intervals to ensure the agarose is completely dissolved.
- While the mixture is cool enough, add 2.5 µL ethidium bromide.
- Pour the mixture in the casting tray and is kept for around 30 minutes to solidify, with comb in it.
- After the gel has cooled completely and solidified the combs is removed and the tray inserted properly into the gel chamber.
- Pour enough 1X TAE buffer into the chamber to cover the gel and fill the wells.
- Load 1µL DNA with loading dye.
- Load 1 µL lambda DNA (200ng per µL)
- The gel is then run at 100V for around 30 minutes.
- The gel is then studied under UV light.

3.4 ***PCR set up***

The 16S rRNA gene was amplified by PCR universal forward (5'-CCAGCAGCCGCGGTAATACG-3') and reverse (5'-ATCGG(C/T)TACCTTGTTACGACTTC-3') primers for Bacteria (Lu *et al.,* 2000). The PCR was performed in a final reaction mixture (25.0 µl) containing 1µL of genomic DNA, 0.5µL of each primer, 0.5 µL of dNTPs (Intron Technologies), 0.25µLTaq polymerase (Intron Technologies) and 2.5µL 10x reaction buffer (Intron Technologies).

Amplification reactions were performed with the following cycling conditions: initial denaturation for 4.0 min at 95.0°C followed by 30.0 cycles of 30.0 s at 94.0°C, 1 min at 52.0°C and 1min at 72.0°C with a final extension for 7.0 min at 72.0°C and cooling to 4.0°C.

Primers designed to amplify halotolerance genes(TABLE 1.4)

S.NO.	GENE	PRIMER SEQUENCE(FORWARD)	PRIMER SEQUENCE(REVERSE)	ANNEALING TEMPERATURES USED
1.	groEL	accgcgtcaccatcgaccagga	tccgagccttcgccgccaacaa	63°C
2.	*eif1A*	tgcaaaagcgaatctggatccgtg	cccatggttcaacaagcaccacg	61°C
3.	tol1	gcgaggtcgtggccatctac	gggctggcatcggccttttc	61°C

3.5 PREPARATION OF COMPETENT CELLS:

A loopful of glycerol stock of E.Coli DH5α was inoculated into 5 mL LB broth.it was incubated at 37°C at 300 rpm for overnight.next day OD reading of the inoculated broth at

600nm was taken.1.5 mL of 5mL LB broth culture was inoculated to 180mL LB broth(fresh) under aseptic conditions.it was incubated at 37°C at 300 rpm.OD was checked at 600 nm.the bacterial cells were transferred to sterile ice cold tubes.then the culture was placed on ice bucket for 10 mins to bring down the culture to 0°C.the cells were recovered by centrifugation at 2700g for 10 mins at 4°C.the media was decanted from the cell pellets.the tubes were stood inverted on a pad of paper towels for 1 min to allow the last traces of media to drain away.the pellet was resuspended by swirling or gentle vortexing in 30mL of ice cold Mgcl2-Cacl2 solution(80mM Mgcl2,20mM Cacl2).the cells were recovered by centrifugation at 2700g for 10 mins at 4°C.the medium was decanted from the cell pellets.the tubes were placed in an inverted position on a pad of paper towels for 1 min to allow the last traces of media to drain away.the pellet was resuspended by swirling or gentle vortexing in 2mL of ice cold 0.1M Cacl2.70µL of DMSO was added to the final volume and mixed by inverting the tube for 2 mins.incubated on ice for 15 mins.another 70µL of DMSO was added and mixed as done above.200µL of the sample was aliquoted into microfuge tubes.

3.6 TRANSFORMATION OF COMPETENT CELLS:

1-To transform the cacl2 treated cells, directly transfer 200µl of each suspension of competent cells to a chilled sterile eppendorf tube.

2-DNA was added to each tube.The content were mixede by swirling gently.

3-Tubes were kept on ice for 30 mins.

4-Tubes were then transferred to water bath (42°c).

5-Tubes were kept at RT for 90 secs.

6- Tubes were transferred rapidly to ice box.the cells were allowed to chill for 2 mins.

7-800 µl of LB medium was added to each tube.

8- cultures were incubated at 37°c for 45 mins.Transfer 200µl of transformed competent cells into LB agar medium in petriplates .The culture was spread using bent rod.

9-plates were inverted and incubated at 37°C.

10-Transformed colonies appeared after 18 hours.

3.7 PLASMID ISOLATION:

1-8µl of E.coli FH5α strain with PUC18 was inoculated into 20ml LB broth containing 16µl ampicilin.

2- Incubated overnight at 37°c at 150 rpm.

3-Second day optical density was measured at 600nm.

4-1.5 ml of culture was transferred to a microfuge tube.

5- Centrifuged at 15000 rcf for 30 secs.

6- Supernatent was discarded.

7- 100µl of ALS I was added to pellet.

8- Pellet was resuspending by vortexing.

9- 200µl ALS II was added.

10- Mixed by gently inverting 3 times.

11- 150 µl of ALS III was added.

12- Tube was incubated in ice for 5 mins.

13- Centrifuged at 15000 rcf at 4°c for 5 mins.

14- Supernatent transferred to fresh appendorf tube.

15- 2 volumes of absolute ethanol was added.

16- Gently mixing by inverting 10 times.

17- Centrifuged at 15000 rcf at 4°c for 5 mins.

18-Supernatent was discarded.

19- 1ml of 70% ethanol was added to pellet.

20- Dislodge the pellet.

21- Again centrifuge.

22- Discard the supernatant.

23- 70% ethanol washing was repeated for 3 times.

24- Air dried the pellet.

25- Pellet was dissolved in 50µl of mili-Q water.

26- Analyzed on gel.

CHAPTER 4

RESULTS AND ANALYSIS

4.1 streaking of sample(staphylococcus arlettae) on LB agar plates

FIGURE 1.4 STREAKING

The purified DNA is then resuspended and stored in 50 µL TE buffer .To check the quality of the extracted DNA, a sample is run on an agarose gel, stained with ethidium bromide, and visualised under UV light.

4.2 ISOLATED GENOMIC DNA

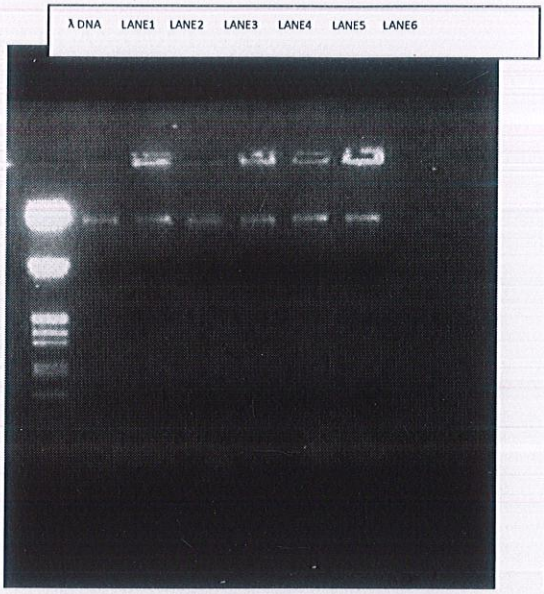


FIGURE 1.5: Representative gel picture of isolated genomic DNA.

DNA Ladder(Bangalore Gene I): double digest,digestion with ECORI and HINDIII

We observed DNA bands and quantified the DNA using nanodrop.

4.3 PCR RESULT:

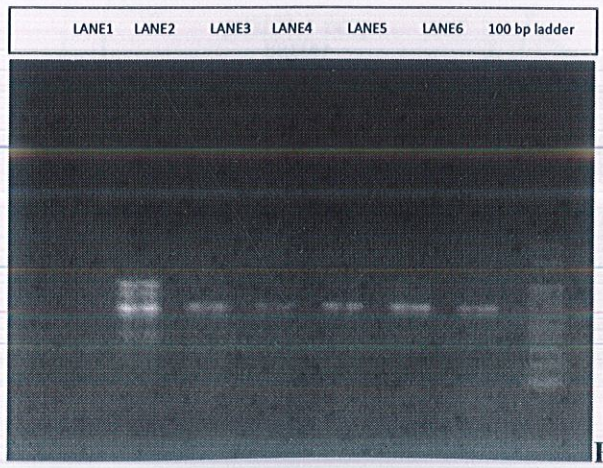


FIGURE1.6 PCR OF ISOLATED DNA

We used gradient PCR with temperature ranging from 59-63.5. We observed multiple bands with size of 650-1000bp.

4.4 PLASMID DNA:

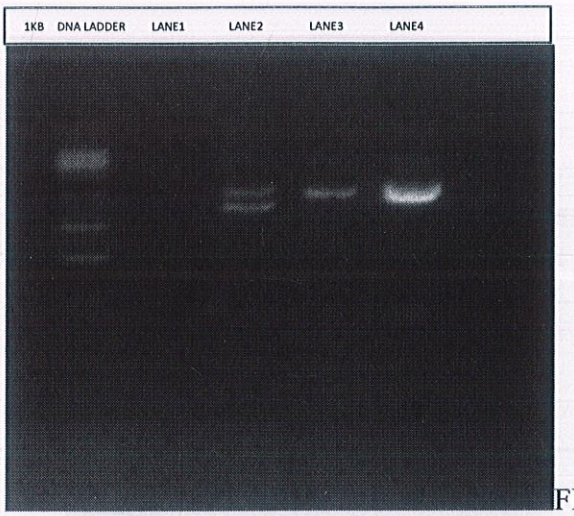
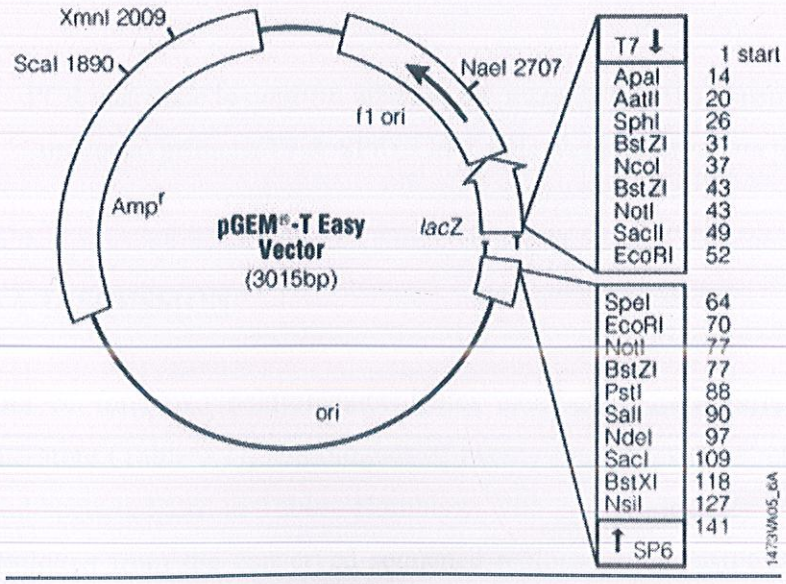


FIGURE 1.7: PLASMID DNA ON GEL

We observed bands in lane2,lane3 and lane4.

4.5 PCR OF PLASMID DNA:



The high-copy-number pGEM®-T and pGEM®-T Easy Vectors contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the coding region for the α-peptide of β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening on indicator plates containing X-Gal and IPTG. Both the pGEM®-T and pGEM®-T Easy Vectors contain numerous restriction sites within the multiple cloning region. The pGEM®-T Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes EcoRI, BstZI and NotI, thus providing three single-enzyme digestions for release of the insert.

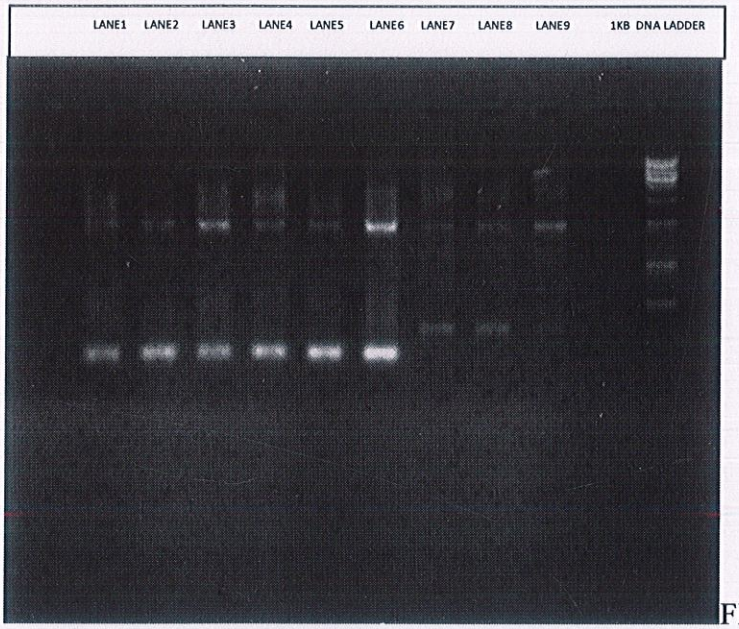


FIGURE 1.8: PCR OF PLASMID DNA

PCR was done to confirm whether the insert is there in plasmid or not with specific primers and universal primers(T7 & SP6).2 kb bands were observed on the gel. Also some faint bands with the size of 3 kb were observed.

4.6 DISCUSSION:

Out of forty-two halotolerance genes catalogued from different organisms through published literature (Table 3.1), homologues of twenty-one genes were retrieved in the halotolerance archaea and bacteria listed in Table . Homologs with ≥70.0% identity were selected and PCR primers were designed from the conserved sequence regions using FastPCR (Kalendar *et. al.*, 2009).Expected

amplicon size of EIf1A gene is 1 kb and we observed bands of 2 kb on the gel.The result can be validated further using sequencing.The gene is being sequenced.

CHAPTER5

CONCLUSION

Halotolerance is the adaptation of living organisms to conditions of high salinity. Halophiles are a group of bacteria that live in highly saline environments, and indeed in many cases require the salinity to survive. Halophytes are salt-tolerant higher plants.Fields of scientific research relevant to halotolerance include biochemistry, molecular biology, cell biology, physiology, ecology, and genetics.

An understanding of halotolerance can be applicable to areas such as arid-zone agriculture, bioproduction of desirable compounds using seawater to support growth, or remediation of salt-affected soils. In addition, many environmental stressors involve or induce osmotic changes, so knowledge gained about halotolerance can also be relevant to understanding tolerance to extremes in moisture or temperature. Conventional agricultural species could be made more halotolerant by gene transfer from naturally halotolerant species (by conventional breeding or genetic engineering) or by applying treatments developed from an understanding of the mechanisms of halotolerance. In addition, naturally halotolerant plants or microorganisms could be developed into useful agricultural crops or fermentation organisms.

The tremendous diversity of halophilic microorganisms found in nature is still far from being fully exploited.

The mining of halotolerant strains for halotolerance genes revealed that most of the strains are having useful genes, which can be cloned to full length and used in the development of transgenics for various agricultural and industrial applications. Halophiles are also a good source of genes for a variety of compatible solutes that can function under salty conditions. These solutes protect biomolecules and whole cells against denaturation caused by heating, freezing, desiccation, or chemical agents (Margesin *et al.,* 2001). This property has attracted commercial attention. Compatible solutes can be used as chemical chaperones for protein folding, cryoprotectants of microorganisms, cosmeceuticals, and pharmaceuticals (Lentzen and Schwarz, 2006).

REFERENCES

1. Alqueres SMC, Almeida RV, Clementino MM, Vieira RP, Almeida WI, Cardoso AM, Martins OB (2007) Exploring the biotechnological applications in the archaeal domain. Braz J Microbiol. 38: 398–405

2. Baxter BK, Litchfield CD, Sowers K, Griffith JD, DasSarma PA and Das–Sarma S (2005) Microbial diversity of Great Salt Lake. In Adaptation to life at high salt concentrations in Archaea, Bacteria, and Eukarya Edited by: Cimerman GN, Oren A, Plemenitas A. Dordrecht, Springer, Netherlands, pp. 9–26

3. Bayley ST, Morton RA (1978) Recent developments in the molecular biology of extremely halophilic bacteria. CRC Crit Rev Microbiol. 6: 151–205

4. DasSarma S and Arora P (2001) A general review on Halophiles. In Encyclopedia of life sciences. Nature publishing group/ www.els.net.

5. de Champdore M, Staiano M, Rossi M and D'Auria S (2007) Proteins from extremophiles as stable tools for advanced biotechnological applications of high social interest. J R Soc Interface. 4: 183–191.

6. Eisenberg H, Mevarech M and Zaccai G (1992) Biochemical, structural, and molecular genetic aspects of halophilism. Adv Protein Chem. 43: 1–62

7. Eisenberg H, Wachtel EJ (1987) Structural studies of halophilic proteins, ribosomes, and organelles of bacteria adapted to extreme salt concentrations. Annu Rev Biophys Biophys Chem. 16: 69–92

8. Grant WD (1991) General view of halophiles. In Superbugs – Microorganisms in extreme environments. Horikoshi K and Grant WD (eds) Tokyo, Japan: Springer-Verlag pp. 15–37

9. Grant WD, Gemmell RT and Mcgenity TJ (1998) Halobacteria – the evidence for longevity. Extremophiles 2: 279–288

10. Horikoshi K (1999) Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol Rev. 63: 735–750

11. Kushner DJ (1978) Life in high salt and solute concentrations: halophilic bacteria, p. 317–368. In D. J. Kushner (ed.) Microbial life in extreme environments. Academic Press, Ltd. London, United Kingdom

12. Margesin R and Schinner F (2001) Potential of halotolerant and halophilic microorganisms for biotechnology. Extremophiles 5: 73–83

13. Oren A (2002) Halophilic microorganisms and their environments. Kluwer Academic Publisher London.

14.Oren A (2008) Microbial life at high salt concentrations: phylogenetic and metabolic diversity. Saline Systems 4: 2

15. Lederberg, Joshua (1994). The Transformation of Genetics by DNA: An Anniversary Celebration of AVERY, MACLEOD and MCCARTY(1944) in Anecdotal, Historical and Critical Commentaries on Genetics. The Rockfeller University, New York

16. Mandel, Morton; Higa, Akiko (1970). "Calcium-dependent bacteriophage DNA infection". Journal of Molecular Biology 53

17. Cohen, Stanley; Chang, Annie and Hsu, Leslie (1972). "Nonchromosomal Antibiotic Resistance in Bacteria: Genetic Transformation of Escherichia coli by R-Factor DNA".

18. Santos, H., and da Costa, M.S. (2002) Compatible solutes of organisms that live in hot saline environments. Environmental Microbiology

19. Oren, A. (2002) Molecular ecology of extremely halophilic Archaea and Bacteria. FEMS Microbiology Ecology

20. Gutierrez, M.C., Kamekura, M., Holmes, M.L., Dyall-Smith, M.L., and Ventosa, A. (2002) Taxonomic characterisation of Haloferax sp. ("H. alicantei") strain

BRIEF BIO-DATA OF STUDENTS

Mandvi Singh:

I am currently pursuing B.Tech in Biotechnology and will be completing the degree in June, 2012 from Jaypee University of Information Technology. My current CGPA is 6.9 and my interests lie in Genetic Engineering, Microbiology and Animal tissue culture. I am looking forward to pursue M.B.A in Biotechnology . My objective is to contribute to the scientific community by using my skills and knowledge in the field of Biotechnology.

Shagoon preet kaur:

I am currently pursuing B.Tech in biotechnology and will be completing the degree in June, 2012 from Jaypee University of Information Technology. My current CGPA is 8.1 and my interests lie in Genetic Engineering, Molecular Biology and Reproductive Biology. I am preparing to pursue MS and my objective is to use all the knowledge that I have gained and the skills that I have learnt during my educational period, in all my future pursuits in scientific research and in turn not only add value to myself but also make some contribution for the betterment of the mankind.