BIOCHEMICAL AND GENETIC CHARACTERIZATION OF *Halobacterium salinarium* STRAIN ISOLATED FROM TUZ LAKE IN CENTRAL ANATOLIA

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ABSTRACT

BIOCHEMICAL AND GENETIC CHARACTERIZATION OF *Halobacterium salinarium* STRAIN ISOLATED FROM TUZ LAKE IN CENTRAL ANATOLIA

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In this study, a halophilic archaea *Halobacterium salinarium* TG13 which is isolated from Tuz Lake in Central Anatolia was characterized biochemically and genetically. *Halobacterium salinarium* DSM3754 and *Halobacterium salinarium* S9 strains were used as a reference strain through the experiments. In biochemical characterization; total protein profiles of strains was compared by using 1D SDS PAGE. Total protein profile of the isolated strain has shown differences. The SDS-PAGE profile of the purified purple membrane showed only single band by coomassie staining. Molecular weight and p*I* values of the protein

isolated from *Halobacterium salinarium* TG13 and *Halobacterium salinarium* S9 were estimated by 2D SDS-PAGE as 22 kD and 5.4, respectively. Photoactivity of purple membrane of the strains was investigated. pH change of the purple membranes were observed upon illumination. This protein might be corresponded to bacteriorhodopsin. In genetical characterization; polymorphism of genomic DNA of strains was scanned with RAPD-PCR. Plasmid DNA profiles of strains was determined to make use of RFLP technique. RAPD-PCR and RFLP analyses have shown that *Halobacterium salinarium* TG13 is different strain from reference *Halobacterium salinarium* strains (*H.s.* S9 and *H.s.* DSM3754).

Key Words: *Halobacterium salinarium*, bacteriorhodopsin, proton pump, retinal protein, 1D SDS-PAGE, 2D SDS-PAGE, RAPD-PCR, RFLP.

ÖZ

TUZ GÖLÜNDEN İZOLE EDİLMİŞ *Halobacterium salinarium* SUŞUNUN BİYOKİMYASAL VE GENETİK KARAKTERİZASYONU

Çakıcı, Özgür

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Ocak 2004, 94 sayfa

Bu çalışmada İç Anadolu'da bulunan Tuz Gölü'nden izole edilen halofilik bir archaea olan *Halobacterium salinarium* TG13 suşu biyokimyasal ve genetik olarak karakterize edilmiştir. Deneyler sırasında referans olarak *Halobacterium salinarium* DSM3754 ve *Halobacterium salinarium* S9 suşları kullanılmıştır. Biyokimyasal karakterizasyonda, suşların protein profilleri 1D SDS PAGE kullanılarak karşılaştırılmıştır. İzole edilen suşun toplam protein profili değişiklikler göstermektedir. Saflaştırılmış mor zarın SDS PAGE profili coomassie boyaması sonucunda sadece tek bant olarak görülmüştür. *Halobacterium salinarium* TG13 ve *Halobacterium salinarium* S9'dan izole edilen proteinin

molekül ağırlığı ve pI değeri 2D SDS-PAGE kullanılarak sırasıyla 22 kD ve 5.4 olarak bulunmuştur. Suşlardan elde edilen mor zarların fotoaktiviteleri ölçülmüş ve aydınlatma ile mor zarların pH'sının değiştiği gözlenmiştir. İzole edilen bu proteinin bakteriyorodopsin olması muhtemeldir. Genetik karakterizasyon çalışmalarında, suşların genomik DNA'larındaki polimorfizm, RAPD-PCR methodu ile belirlenmiştir. Suşların plasmit DNA profilleri RFLP tekniğiyle belirlenmiştir. Sonuç olarak, protein profilleri ve RAPD-PCR ve RFLP analizleri *Halobacterium salinarium* TG13'ün referans *Halobacterium salinarium* suşlarından (*H.s.* S9 ve *H.s.* DSM3754) farklı bir suş olduğunu göstermektedir.

Anahtar Kelimeler: *Halobacterium salinarium*, bakteriyorodopsin, proton pompası, retinal protein, 1D SDS-PAGE, 2D SDS-PAGE, RAPD-PCR, RFLP.

To My Sister and Mrs. Yücel and Mrs. Eroğlu

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LIST OF SYMBOLS AND ABBREVIATIONS

1D	One Dimensional
2D	Two Dimensional
λ_{max}	Maximal absorption
ADP	Adenosin diphosphate
ATP	Adenosin triphosphate
bR	Bacteriorhodopsin
H.s.	*Halobacterium salinarium*
M.W.	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
ΔpH_{max}	$\Delta pH_{max} = pH_{initial} - pH_{equilibrium}$
pI	Isoelectric point
PM	Purple membrane
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulphate

CHAPTER I

INTRODUCTION

1.1 Introduction

Halobacterium salinarium (formerly *Halobacterium halobium*) is an archaeobacterium which belongs to family of halobactericeae. Halobacteria, members of the domain Archaea, live under extremely halophilic conditions (4M NaCl). They are found in strongly saline lakes (such as Great Salt Lake, the Dead Sea, or the alkaline, saline lakes of East Africa) and also in the worldwide salters where salt is commercially produced from seawater by evaporation. *H. salinarium* is basically aerobic, but may grow anaerobically in the light when containing bacteriorhodopsin or in the dark when supplied with arginine (Krieg et al., 1989).

As a result of adaptation to their environment, many halophilic microorganisms have evolved unique properties of considerable biotechnological and, therefore, commercial significance.

Because of bacteriorhodopsin, *Halobacterium salinarium* has been taken much more interest among the halophilic archaea. Bacteriorhodopsin is

mostly studied protein of *H. salinarium* for application purposes. The biotechnological applications of bacteriorhodopsin will be mentioned in the next section.

There are also other biotechnological applications of *H. salinarium.* Exopolysaccharide of *H. salinarium* is an interesting source for microbially enhanced oil recovery where polymers with appropriate properties (high viscosity even at diluted concentrations and high temperatures, pseudoplasticity, resistance to salt and thermal degradation) act as emulsifiers and mobility controllers. The continuous production of halophilic α-amylase can be performed via whole-cell immobilization of *H. salinarium* in alginate beads and a polyvinyl alcohol film (Bagai and Madamwar 1997). The cells were osmotically stable and showed continuous enzyme production for 45 days. An extracellular serine protease from *H. salinarium* (ATCC 43214) was claimed to be an excellent candidate as a catalyst for peptide synthesis, particularly for glycine-containing peptides (Ryu *et al.* 1994). The enzyme requires 4 M NaCl for optimal catalytic activity and stability in aqueous solutions. Addition of organic solvents, such as dimethylformamide, had a positive effect on enzyme stability in low-salt media. The stabilization of halophilic enzymes by organic cosolvents while lowering the required salt concentration is of practical importance as the corrosive nature of high NaCl concentrations can be avoided. Higher substrate solubilities in the presence of an organic cosolvent can be useful in synthetic reactions catalyzed by enzymes from extreme halophiles (Kim and Dordick 1997). For the harvesting of cells in fermentation processes, buoyancy of cells is desired. DasSarma *et al.* (1999) constructed a recombinant vector capable

of directing the synthesis of gas vesicles in nonfloating cells. The vector contains genes encoding *H. salinarium* proteins that are required for the synthesis of gas vesicles. Transformed cells float in an aqueous medium and can be separated by skimming or decanting.

1.2 Bacteriorhodopsin

Bacteriorhodopsin is a retinal protein present in the purple membrane of *H. salinarium*. In the presence of light, the protein pumps protons out of cells and provides them with the energy to live. The basic function of bacteriorhodopsin as light-driven proton pump was recognized by Oesterhelt (1972). But the discovery of the protein by Stoeckenius was in the 1967. Two further circumstances have driven the huge interest in bacteriorhodopsin. First, it serves as a simple model for certain cell-membrane receptors, known as G-protein-coupled seven-helix receptors, which include most well-known drug targets in humans and which probably operate by a similar switch mechanism (Subramaniam and Henderson, 2000). Second, it is the prototype of membrane transporter. These are biological macromolecules performing the amazing task of transporting ions against and electrochemical potential up to 250 milivolts in the case of bacteriorhodopsin, which translates into a 10,000-fold difference in proton concentration on either side of the membrane. Such transport processes are fundamental to all forms of life (Kühlbrandt, 2000).

1.2.1 Structure and Function of bR

Bacteriorhodopsin (bR) is a 26 kD weighed integral membrane protein which pumps protons across the membrane by using sunlight. It has 248 amino acid residues those are arranged in seven transmembrane helical segments (Figure 1.1). The 3D structure of bR was first determined in 1975 by Richard Henderson and Nigel Unwin using electron micrographs at 7A° resolution of the purple membrane fragments. Clarification studies of the molecular structure of the bR via cryoelectron microscopy at 3.4 A° resolution were carried out by Henderson *et al.* (1990). More detailed information about the structure of bR in 1.55 A° resolution scale was obtained by novel research activities of several laboratories (Figure 1.2) (Luecke *et al.*, 1999).

In tertiary structure bR has retinal chromophore which is enclosed within these helical segments of bR. The chromophore gives the protein its original color that is purple. The retinal chromophore is a derivative of the β-carotene, and it is bound via protonated schiff base to ε-amino group of Lysine-216 near the center of helix G and lies nearly parallel to the plain of membrane (Figure 1.3). In quarternary structure, bacteriorhodopsin forms trimers and these reveal two dimensional hexagonal lattices in purple membrane of the archaea (Figure 1.4). In the proton pumping mechanism of bacteriorhodopsin, this chromophore plays a key role. bR pumps the proton from the inside of the cell to the outside by using the energy it received from sunlight, and as a result, a proton gradient occurs across the membrane.

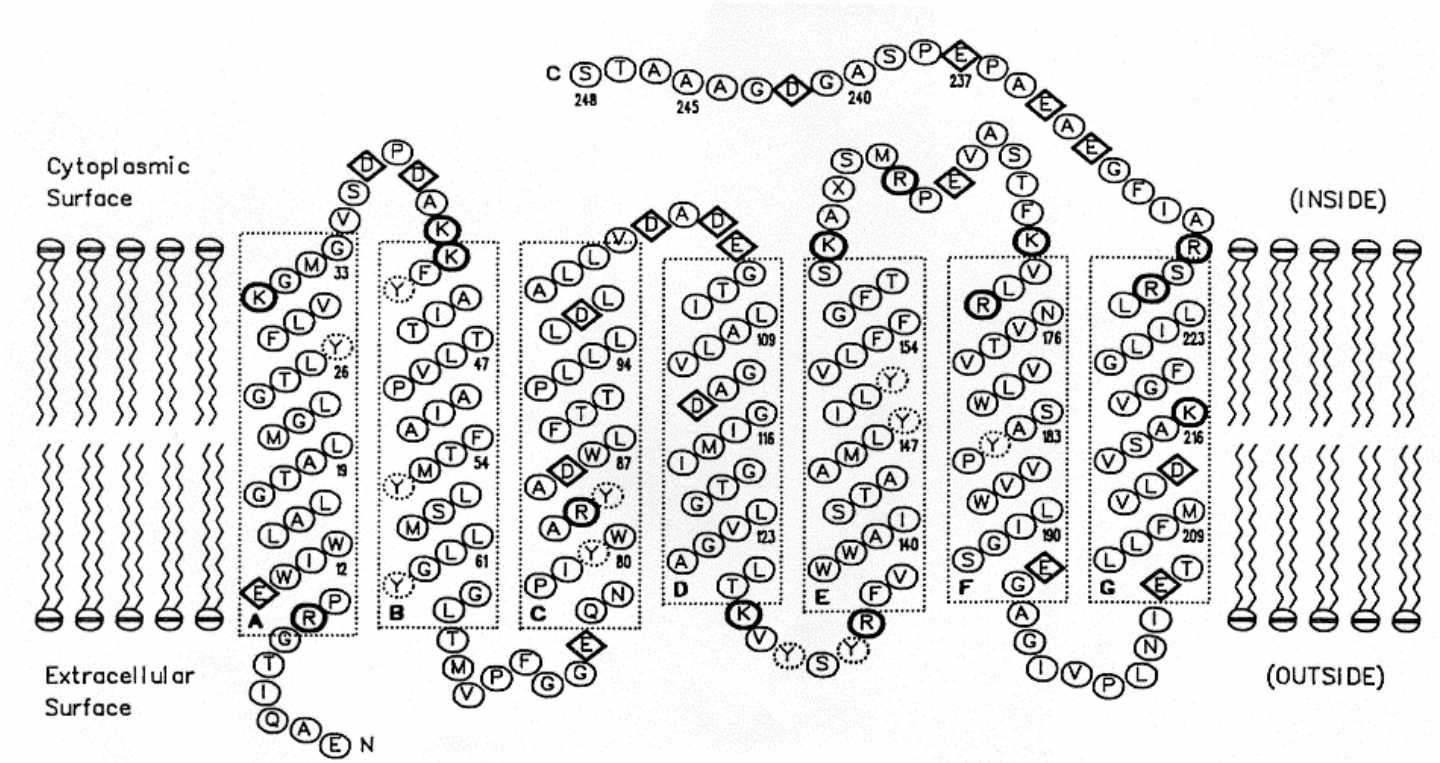

Figure 1.1 Amino acid sequences and putative membrane spanning regions of bacteriorhodopsin. Amino acid abbreviations are as follows: Alanine (Ala, A); Arginine (Arg, R); Asparagine (Asn, N); Aspartic Acid (Asp, D); Cysteine (Cys, C); Glutamine (Gln, Q); Glycine (Gly, G); Histidine (His, H); Isoleucine (Ile, I); Leucine (Leu, L); Lysine (Lys, K); Methionine (Met, M); Phenylalanine (Phe, F); Proline (Pro, P); Serine (Ser, S); Threonine (Thr, T); Tryptophan (Trp, W); Tyrosine (Tyr, Y); Valine (Val, V). Amino acids which under nominal conditions carry a charge are shown in thickened circles (positively charged) or boxes (negatively charged) (Birge 1990).

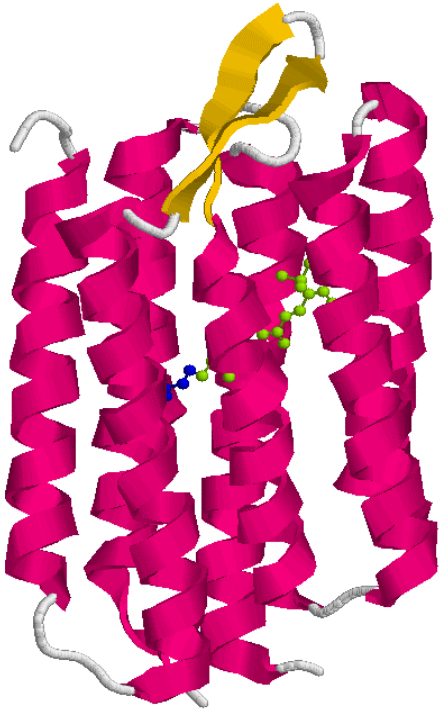

Figure 1.2 Three Dimensional Structure of Bacteriorhodopsin (Luecke *et al.*, 1999).

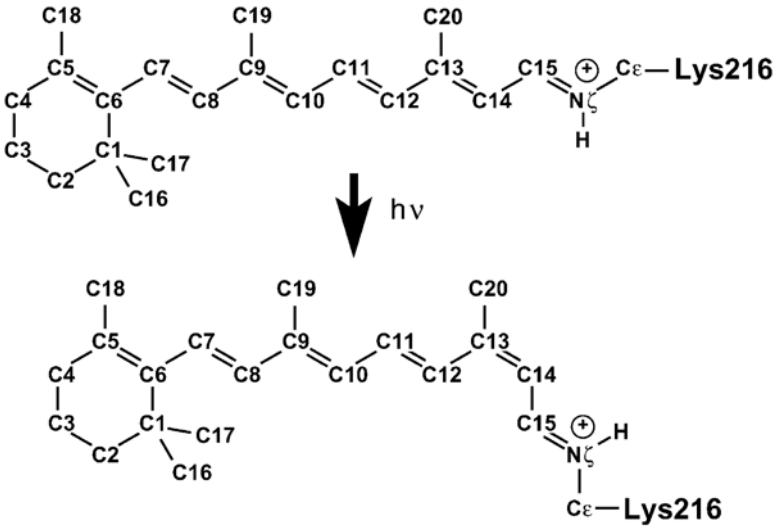

Figure 1.3 Structure of Retinal Chromophore and all-trans - 13-cis conversion by light (Neutze *et al.*, 2002)

With the enforcement of this generated proton gradient, protons are driven to inside of the cell through the ATP-synthase, in order to balance proton concentrations between these two environments. In this step, The ATP-synthase provides the production of the ATP using protons, therefore, the energy that the cell required for would be produced.

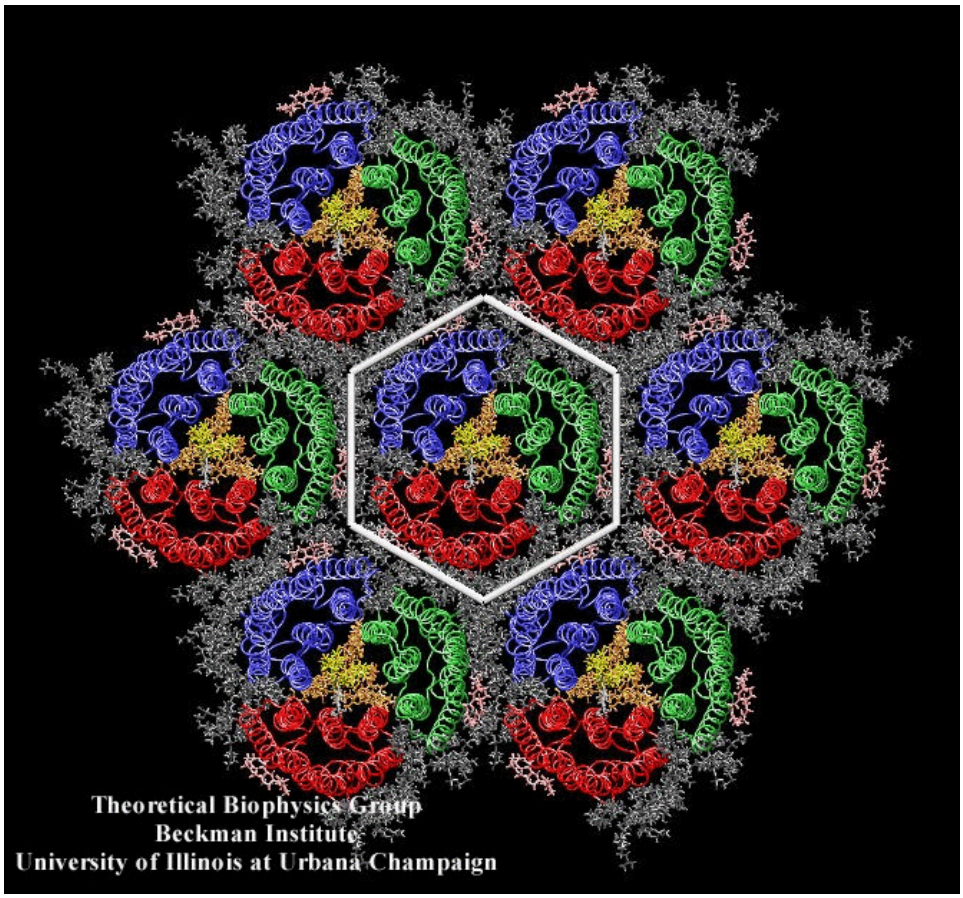

Figure 1.4 Trimer formation and two dimensional hexagonal lattices in purple membrane.

1.2.2 Photocycle of Bacteriorhodopsin

When bR absorb a photon, it follows a cyclic pathway called as photocycle. In the photocyclus, the photocycle contains numerous intermediate states characterized by their absorption maxima in visible range (Neutze, R. *et al.*, 2002). These are bR570 → K590 → L550 → M410 → N560 → O640 (Figure 1.5). The structure of the ground state (bR570) was determined fourteen years ago by electron cryo-microscopy of two-dimensional crystals that form in the cell membrane (Henderson, R. *et al.* 1990). In 1999 and 2000, structures of the K and M intermediates have been determined by X-ray crystallography of microcrystals. This was followed by the structure of the N intermediate determined by electron microscopy of two-dimensional crystals. The M and N intermediates were of bacteriorhodopsin mutants that have slow proton-pumping cycles and are therefore easier to handle. They are now joined by another electron-microscopic structure of a mutant representing the key M intermediate, and by X-ray structures of the wild-type M and L intermediates.

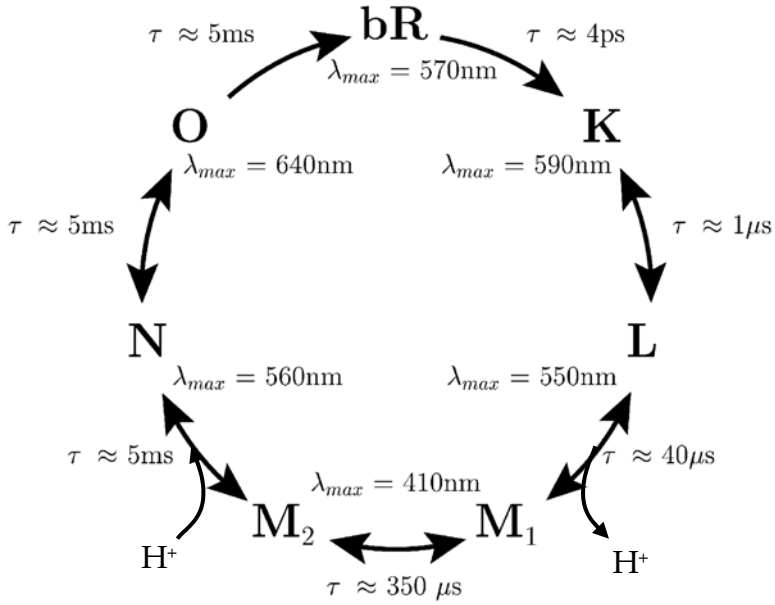

Figure 1.5 Photocycle of Bacteriorhodopsin (Neutze *et al.*, 2002)

The structures of the various intermediates are all slightly different from each other and from the ground state, and it is fascinating to see how they combine to illustrate the proton-pumping cycle. The main events are shown on the Figure 1.6. First, the retinal is hit by a photon and changes its configuration from the all-trans to the 13-cis form (Figure 1.3), which takes about one picosecond (K state). In the 13-cis state, one end of the retinal is twisted around a double bond which causes this part of the pigment to move relative to the protein scaffold. As a result, the proton located on the nitrogen finds itself in an energetically unfavourable environment and within about 50 micro-seconds moves to aspartate 85, by way of tyrosine 89 or aspartate 212 and a tightly bound water molecule, to form the L intermediate. The proton transfer is helped by a small movement of helix C, which brings the side chain of

aspartate 85 closer to the nitrogen atom, as shown by the structure of the L state.

The retinal turns from pink to yellow and straightens as it becomes deprotonated. Because the retinal is tethered at both ends, the nitrogen atom moves upwards towards the cell interior by 0.7 to 1 A° pushing against some bulky residues on helix F. By lever action, the upper part of helix F swings out by about 3.5 A° in late M, while the top of helix G moves partly into its place. In the transition to the N state, the retinal is reprotonated from aspartate 96 and flexes again. The movement of helices F and G opens a narrow channel through which aspartate 96 is reprotonated. In the N to O transition, the proton on aspartate 85 is transferred to an extended network of hydrogen bonds including several water molecules in the lower half of the channel. The end of the positively charged arginine 82 moves downwards slightly during M, facilitating the release of the proton on the outside. Finally, the retinal relaxes to the all-trans form, helices F and G swing back to their original position, and another proton-pumping cycle can begin. The primary motions of the proton-pumping action in bacteriorhodopsin are surprisingly small, involving movements of groups of atoms by 1 A° or less in response to the flexing and unflexing of the retinal. This affects the proton affinity of neighbouring side chains through a change of their local chemical environment. The key event is the straightening of retinal as it sheds its proton in M, as shown by a mutant that has helices F and G stuck in the open state but still pumps protons, albeit inefficiently. The retinal thus acts as a valve in the middle of the membrane, imparting a unique direction to the pumping process.

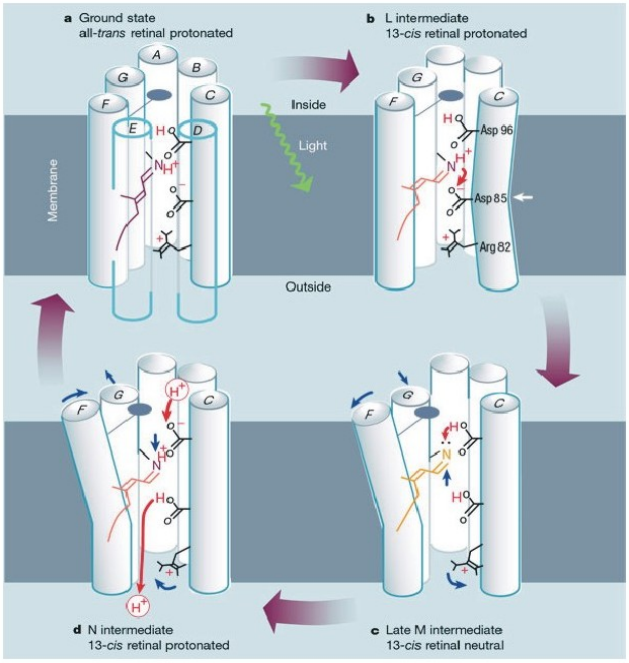

Figure 1.6 Molecular mechanism of proton pumping in bacteriorhodopsin (Kühlbrandt W., 2000).

1.2.3 A Comparison of Bacteriorhodopsin and Rhodopsin

Bacteriorhodopsin and rhodopsin are both retinal based light utilizing integral membrane proteins. The visual rhodopsin is responsible for conversion of light into nerve impulses in the image resolving eyes of mollusks, arthropods, and vertebrates. Despite independent evolutionary development, these systems have converged on protein- and chromophore-binding site designs that are remarkably similar. The bacterial rhodopsins represent a much broader class of proteins that serve both photosynthetic and phototactic functions. The best known of the bacterial rhodopsins is bacteriorhodopsin, which converts light into energy via photon-activated transmembrane proton pumping.

Regardless of function, all rhodopsins appear to share common features, which include a retinyl chromophore in a protein-binding site formed within the inner segments of seven transmembrane helices. Upon the absorption of light, the chromophore isomerizes to generate a batho-chromically shifted photoproduct that stores a significant fraction of the photon energy in both electrostatic and conformational forms (Birge 1990).

Spectroscopic and neutron diffraction studies, amino acid composition, and analogies of rhodopsin with bacteriorhodopsin suggest the secondary structure shown in Figure 1.7 (Hargrave 1982, Adamus 1987). Thus, rhodopsin spans the membranes seven times and it is likely that the 11-cis chromophore linked to Lys-296 spans and intrahelix region similar to that shown in Figure 1.1 for bacteriorhodopsin. The generation of a nerve impulse following excitation of rhodopsin involves a complex series of reactions that ultimately hyperpolarize the plasma membrane of the rod cell in the retina (Stryer 1986, Liebman 1987). The proton pumping of bacteriorhodopsin forms pH gradient across the membrane. The gradient generates a proton-motive force that is used by the bacterium to synthesize ATP from inorganic phosphate and ADP. Isomerization of the retinal chromophore for both of the proteins is different. For bacteriorhodopsin, all-trans retinal is isomerized to 13-cis retinal by the absorption of light. For rodopsin, 11-cis retinal is isomerized all-trans retinal by the absorption of light (Birge 1990). Although there are structural similarities in these proteins, they are quite different from functional point of view.

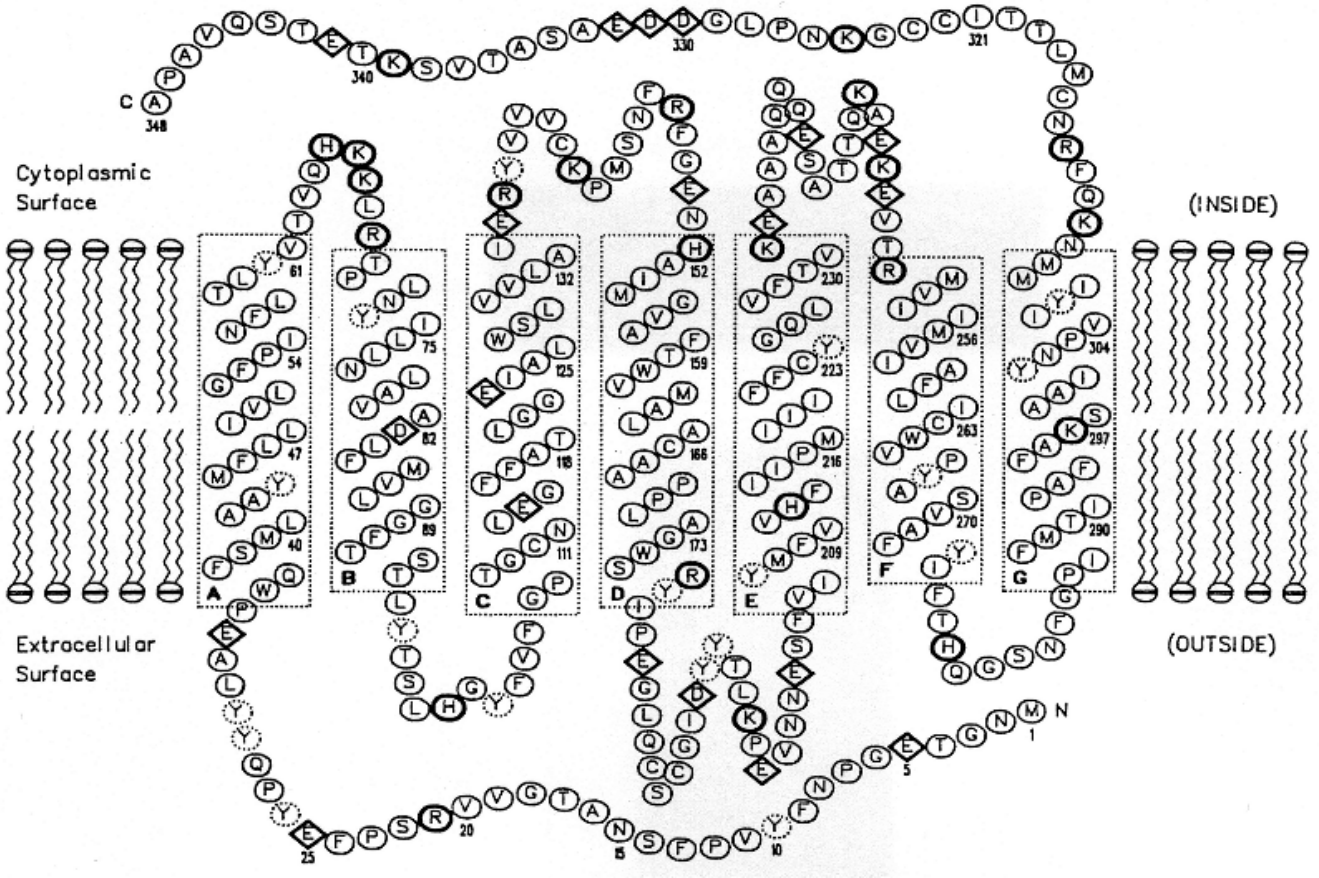

Figure 1.7 Amino acid sequence and putative membrane spanning regions of human rhodopsin. Amino acid codes as in Figure 1.1 (Birge 1990).

1.2.4 Applications of Bacteriorhodopsin

The excellent thermodynamic and photochemical stability of bR has led to many uses in technical applications based on its protonmotive, photoelectric, and photochemical properties. The applications comprise holography, spatial light modulators, artificial retina, neural network optical computing, and volumetric and associative optical memories. A three-dimensional memory is under study. bR is commercially offered in the form of purple membrane patches, isolated from *Halobacterium salinarium* (formerly *H. halobium*) strain S9. It is sold by a Spanish company (COBEL, Barcelona) and a German company (MIB, Munich Innovative Biomaterials) in the lyophilized form with a 75% (by weight) bR content. MIB offers also films and devices made of genetically modified bR for optical information storage and processing. The modified bR (from *H. salinarium* sp. L33) differs from the wild type by a single amino acid exchange and allows adjustment of the membrane lifetime continuously over a range from 10 ms to more than 100 s by changing the extramolecular pH. One of the most interesting applications of bR is its usage as a photosensitive and erasable material for optical information recording and processing, especially in holography. bR films show a high spatial resolution (35,000 lines/mm) and an excellent reversibility (>10^6 write/erase cycles); the spectral range is 400-700 nm.

Highly oriented films composed of purple membranes have been obtained by using two kinds of bispecific antibodies with different

antigen-binding sites, one binding to a specific side of bR and the other to a phospholipid hapten. These films were used in the construction of a light-sensitive photoelectric device (Koyama *et al.* 1994). bR-based color sensors that use biotechnologically modified variants of bR were developed, and their ability to recognize colors has been demonstrated (Frydrych *et al.* 1998). All-optical devices that include bR as photochromic material (e.g., optical switches and modulators, and logic gates, such as optical AND and OR gates), can be used in a wide variety of systems, such as optical signal processes and optical computers (Rao *et al.* 1998). Other patented applications of bR include its use as a bioelement in a motion sensor (Ackley and Shieh 1998), in an image sensor, or in a biocomputer (Kikura *et al.* 1998).

A prototype memory subsystem uses bR molecules to store digital bits. The photocycle of bR makes it an ideal AND data-storage gate. Due to the remarkable stability of bR, the data recorded on a bR storage device should be stable for approximately 5 years; the system should operate nearly as fast as semiconductor RAM (Thompson 1996). The optical properties of bR could be exploited to manufacture electronic ink for laptop displays. Electronic ink obtains its color by reflecting ambient light, not from an internal battery-driven light source. This use would be an important contribution to the problem of battery lifetime in portable computing (American Physical Society 1997).

Closed shells (vesicles) form spontaneously from the purple membrane of *H. salinarium* in the presence of the detergent octylthioglucoside (OTG) at a protein/OTG ratio of 2:1 by weight (Denkov *et al.* 1998). The size distribution of the vesicles was almost independent of the

incubation conditions (mean radius, 17.9-19 nm). The conditions for vesicle formation and the mechanical properties of the vesicles could be of interest with respect to the application of the bR vesicles as light energy converters.

Another application of bR is the renewal of biochemical energy, i.e., the back conversion from ADP to ATP. Such a solar-driven recycling system could be of interest for biotechnological processes that need large amounts of expensive ATP (Groß 1997). A patented ATP-synthesizing device, useful for bioelements, has been obtained by using bR and ATP synthase (Saito *et al.* 1992).

1.3 Molecular Methods for Identification and characterization of bacterial strains

Conventional biological identification methods are available for only limited range of bacterial species. Molecular approach to bacterial identification has broadened the limited range. Molecular identification is concerned nucleic acids, proteins and lipopolysaccharides. They are the only macromolecules which carry enough information in their sequences to allow a simple uniform approach to the study of bacterial diversity. Historically, methods used to isolate and characterize macromolecules have involved complex and time-consuming methods which have prevented their introduction into routine microbiology laboratories. DNA-based methods are emerging as the more reliable, simple and inexpensive ways to identify and classify microorganism. A number of different phenotypic and genotypic methods are presently being employed for microbial identification and classification (Figure

1.8). Some methods which used for identification and characterization of bacterial strains will be summarized.

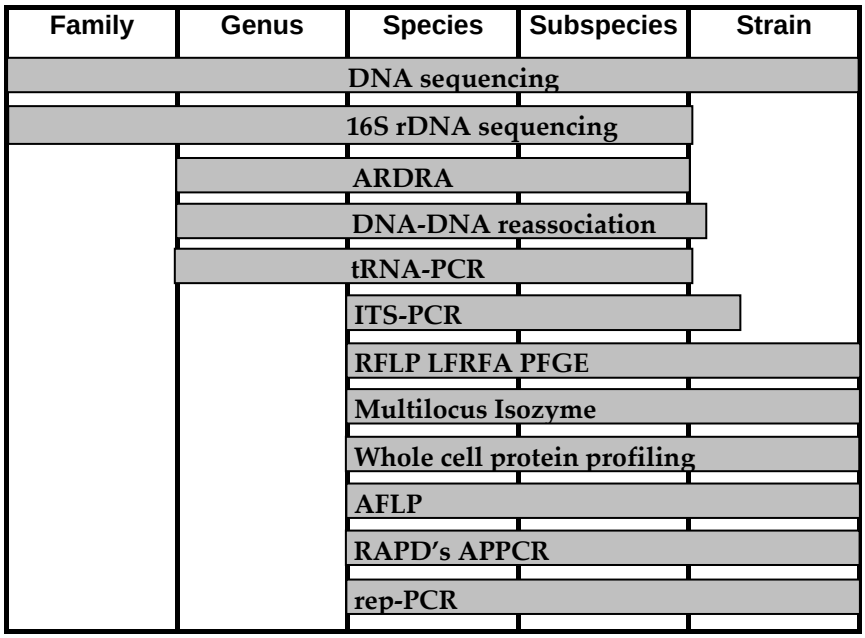

Figure 1.8 Relative resolution of various fingerprinting and DNA techniques.

1.3.1 Genetic Characterization

1.3.1.1 RFLP Analysis

The analysis indigenous plasmid sequence variation appears to be useful to complement taxonomic and ecological studies (King 1989; Stahl 1991). Plasmid profiles can also be used to distinguish between isolates presumed to come from a common source, although due to plasmid instability, they can not always be used to prove or refute the recent

common ancestry of a group of strains (Earnshaw and Gidley 1992). Problems of interpretation of plasmids may arise if different molecular forms (covalently closed circular, open circular or linear) of a single plasmid are visualized on the same gel, thereby giving three bands where only one plasmid is present. In addition, problems may arise when using plasmid analysis for typing purposes, particularly if preparations contain large plasmids, because agarose gel electrophoresis is not a sensitive method of detecting small differences between plasmids of similar size. Similarly, simple analysis of plasmid profiles will not detect the difference between two dissimilar plasmids of identical size. Such difficulties can be resolved by using enzymes termed restriction endonucleases to generate 'plasmid fingerprints' (Earnshaw and Gidley 1992).

Restriction endonucleases are enzymes that recognize a specific base sequence of DNA, typically 4-6 base pairs (bp) long, and then cleave the DNA at a defined position in relation to the specific recognition sequence. Over 400 restriction endonucleases have been isolated from bacteria and characterized (Roberts and Macelis, 1991). Some of the restriction endonucleases used most commonly for plasmid fingerprinting are listed in Table 1, together with their respective recognition sequences. Restriction endonucleases isolated from different species of bacteria may have the same recognition sequence; these are termed isoschizomers. The natural function of restriction endonucleases is thought to be the protection of bacteria from foreign DNA. Methylation of DNA at the recognition sequence prevents cleavage.

Foreign DNA lacks this methylation and is therefore cleaved (Old and Primrose, 1985).

The specificity of DNA cleavage by restriction endonucleases means that complete digestion of a particular sequence of DNA by a specified enzyme or combination of enzymes will result in the production of a reproducible set of linear fragments, generated according to the frequency and location of the specific enzyme recognition sequence(s).

Table 1.1 Common restriction endonucleases used for the generation of DNA fingerprints

Enzyme	Recognition sequence*
*Apa*I	GGGCC/C
*Bam*HI	G/GATCC
*Bgl*I	GCCNNNN/NGGC
*Bgl*II	A/GATCT
*Cla*I	AT/CGAT
*EcoR*I	G/AATTC
*EcoR*V	GAT/ATC
*Hind*III	A/AGCTT
*Hinf*I	G/ANTC
*Hpa*II	C/CGG
*Kpn*I	GGTAC/C
*Mlu*I	A/CGCGT
*Pst*I	CTGCA/G
*Pvu*II	CAG/CTG
*Sac*I	GAGCT/C
*Sca*I	AGT/ACT
*Sma*I	CCC/GGG
*Stu*I	AGG/CCT
*Xho*I	C/TCGAG

** N indicates a random nucleotide base; / indicates cutting site in relation to the recognition sequence.*

Plasmid DNA molecules can, therefore, be compared by examining the number and size of fragments generated by digestion of the DNA with restriction endonucleases. The pattern of fragments produced is termed the plasmid fingerprint, while variations observed between related molecules are termed restriction fragment length polymorphisms (RFLPs). Restriction fragment length polymorphism (RFLP) of plasmids has been used in epidemiological studies to subtype many bacteria [e.g. *Salmonella typhimurium* (Platt *et al.* 1988), *Vibrio anguilarum* (Olsen and Larsen 1990)] and to determine relationship within and between pathovars of *Pseudomonas syringae* (King 1989) and *Xantomonas campestris* (Lazo and Gabriel 1987).

1.3.1.2 RAPD-PCR Analysis

A common strategy underlies three PCR-based methods for DNA fingerprinting developed in the early 1990s. Arbitrarily primed PCR (AP-PCR) (Welsh and McClelland 1990), random amplified polymorphic DNA (RAPD) assay (Williams *et al.* 1990) and DNA amplification fingerprinting (DAF) (Caetano-Anollés *et al.* 1991) are all based on the use of arbitrary primers to perform the PCR amplification of random genomic DNA fragments. Each primer (or combination of primers) generates a characteristic pattern of amplification products which is visualised by either radionuclide incorporation or ethidium bromide staining or silver staining. Polymorphisms between individuals (or strains) are detected as differences between the patterns of DNA fragments amplified from the different DNAs using a given primer(s).

This PCR-based strategy provides a number of advantages over classic DNA fingerprinting through restriction fragment length polymorphism (RFLP) analysis since it allows easy and rapid generation of polymorphic markers by using very small amounts of starting DNA. Furthermore, unlike other PCR-based fingerprinting methods, it does not require any knowledge of target DNA sequence.

The above cited methods, as originally developed, differ from one another in the length of primers used, amplification conditions, separation and, as already mentioned, visualization of amplified DNA fragments. Fingerprint patterns produced are also markedly different, varying from quite simple (RAPD) to highly complex (DAF).

RAPD assay involves PCR amplification of genomic DNA using short primers (9 or 10 bases), separation of the amplification products on agarose gels and their detection by ethidium bromide staining. Compared with AP-PCR and DAF, this method is easier, faster and less expensive (agarose gels vs polyacrylamide gels and ethidium bromide staining versus radionuclide incorporation or silver staining). However, the fingerprint patterns it generates contain relatively few bands which could, in some cases, be disadvantageous.

The RAPD technique allows detection of polymorphisms in closely related organisms (e.g., different populations of single species or individuals within a population) and, therefore, provides a powerful tool for such tasks as gene mapping, marker-assisted selection in breeding programs, population and pedigree analysis, phylogenetic studies and individual and strain identification. This method has been used to estimate the diversity of *Lactobacillus* species and strains (Tailliez *et al.*

1996, Nigatu et al. 2001), to type strains of *Lactobacillus plantarum* (Johansson *et al*. 1995) and to study non-starter lactic acid bacteria in cheese (Fitzsimons *et al.* 1999), characterization of *Bacillus thuringiensis* (Brousseau et al., 1993), identification of *Rhizobium* spp. (Harrison et al., 1992).

1.3.1.2.1 Principles

The RAPD assay is a PCR amplification performed on genomic DNA templates using a short, arbitrary oligonucleotide primer and a low annealing temperature, conditions that ensure the generation of several discrete DNA products. Each of these anonymous but reproducible fragments is derived from a region of the genome that contains, on opposite DNA strands, two primer binding sites located within an amplifiable distance of each other (e.g., within a few thousand nucleotides). Polymorphisms between individuals (or strains) result from sequence differences which inhibit primer binding or otherwise interfere with amplification; therefore, they can be simply detected as DNA fragments that are amplified from one individual (or strain) but not from another.

Although in AP-PCR and RAPD protocols, as first described (Welsh and McClelland 1990; Williams *et al.* 1990), a single arbitrary primer is involved, it has been shown that performing amplifications using two arbitrary primers generates reproducible patterns that are different from those obtained with each single primer (Welsh and McClelland 1991; Micheli *et al.* 1993). It has also been shown that more than 50 % of the

products generated by pairwise combination of primers are different from those generated by either primer alone (Welsh and McClelland 1991). This modification increases the analytical power of the RAPD technique since a higher number of polymorphisms can be detected when the number of primers used are equal; for instance, 15 different patterns are obtained with only 5 available primers (used either individually or in pairwise combinations), thus increasing the chance to detect polymorphic bands. The use of pairwise combinations of primers provides a further advantage: sequencing of RAPD products generated by two different primers can be directly performed by cycle sequencing.

Although the sequence of RAPD primers is arbitrarily chosen, two basic criteria, indicated by Williams *et al.* (1990, 1993), must be met: a minimum of 40 % G+C content (50 %-80 % G+C content is generally used) and the absence of palindromic sequences. Furthermore, when using pairwise combinations of primers, sequences must be designed so as to avoid complementarity between primers to be used together (Micheli *et al.* 1993).

1.3.2 Biochemical Analysis

1.3.2.1 Protein Analysis

A microbial cell expresses some 2000 different proteins which form a rich source of information for the characterization, classification and identification of microorganisms. Polyacrylamide gel electrophoresis (PAGE) of cellular proteins yields complex banding patterns which can be considered as highly specific fingerprints of the strains investigated.

These protein electrophoregrams are highly reproducible provided strains are cultivated under reproducible conditions and standardized techniques are used. In one-dimensional (1D) gels each protein band usually consists of a number of structurally different proteins having identical electrophoretic mobility.

The value of protein electrophoresis in microbial systematics has been established for over 20 years (for reviews see Jackman, 1985, 1987; Kersters & De Ley, 1980; Kersters, 1985; Vauterin et al, 1993). The rationale for the application of electrophoresis of cellular proteins in microbial systematics is that bacterial strains with 90 to 100% DNA relatedness display only minor differences in their protein fingerprints; strains with at least 70% DNA homology tend to have similarities in their protein electrophoregrams (Owen & Jackman, 1982; Kersters, 1985). Hence, the electrophoretic separation of cellular proteins is a sensitive technique that mainly provides information on the similarity of strains within the same species or subspecies. Depending on the protein electrophoretic variation within a given taxon individual strains can often be recognized by small, but specific and reproducible differences in part of their protein patterns.

A number of different protein-electrophoretic techniques are currently used in microbial systematics: A mixture of soluble cytoplasmic proteins of a bacterial strain, or proteins solubilized by treatment with a denaturing agent, e.g. sodium dodecyl sulphate (SDS), is submitted to PAGE and stained with a general staining compound for polypeptides. The resultant banding patterns are compared without any attempt to characterize individual protein bands. Native proteins of bacterial strains

are submitted to electrophoresis in a suitable support matrix (e.g. starch gel, polyacrylamide gel, or polyacrylamide-agarose mixtures) and the gels stained for specific enzymes. These zymograms are used to compare electrophoretic mobility variants of enzymes between different strains.

Several types of electrophoretic separations in polyacrylamide gels are applied in bacterial systematics: continuous PAGE, discontinuous or disc PAGE, isoelectric focusing, two-dimensional PAGE and gradient PAGE. The following types of bacterial proteins can be compared by PAGE: soluble, native proteins; proteins solubilized by e.g. SDS; cell-envelope and ribosomal proteins; radiolabelled proteins.

1.4 AIM

The aim of this study is to isolate a new *Halobacterium salinarium* strain from Tuz Lake and to characterize and identify it. For this purposes, the isolated strain will be compared with selected *Halobacterium salinarium* strains by use of biochemical and genetic methods.

The following steps will be carried out for biochemical characterization of the starins; (i) comparison of total protein patterns of the strains, (ii) determination of pI and MW values of bR which isolated from the strains, (iii) photoactivity measurement of bRs. The following experiments will be conducted, for genetic characterization of the starins; (i) comparison of restriction fragment length polymorphisms (RFLP) of plasmids of the strains, (ii) comparison of random amplified polymorphic DNA (RAPD) of genomic DNA of the strains. After

identification and characterization of the new strain, it can be used for biotechnological applications.

CHAPTER II

MATERIAL AND METHODS

2.1 Materials

The chemicals used in this study were obtained from SIGMA Co., MERCK, FLUKA, DUCHEFA, BIRPA, Riedel-de Haën, SCP, Appli Chem, and Prona. The enzymes, dNTP and marker DNA for molecular studies were obtained from MBI Fermentas. DNA cleaving enzyme DNAase was from ROCHE. For the calibration of pH meter, standard buffers of 4, 7, and 9 were obtained from INGOLD. The bacteriological peptone (L-37) and agar were from OXOID. Dialysis sack was from SIGMA Company. Crystal violet, iodine, potassium iodide, and safranine for Gram staining were obtained from BTR (Bio-Test Lab.). Marker proteins for SDS-PAGE were from SIGMA Company.

In this study, three strains of *H. salinarium* were used. One of these strains was a gift of Prof. Dr. Oesterhelt from Max Plant Ins. (Germany) which is known as strain S9. Second one was from DSMZ – (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - German

Collection of Microorganisms and Cell Cultures). The last one was isolated from Tuz Lake in Central Anatolia in Turkey during this study.

2.2 Methods

2.2.1 Growth of *Halobacterium salinarium* strains

Halobacterium salinarium strains were grown as described by Oesterhelt and Stoeckenius (1974) at 39 °C in a sterilized medium under illumination. The medium contains 250 g of NaCl, 20 g of $MgSO_4 \cdot 7H_2O$, 3 g of trisodium citrate · $2H_2O$, 2 g of KCl and 10 g of Oxoid bacteriological peptone L-37 in 1 Lt. The pH of the medium was adjusted around 6.5-7.4 by 10 N NaOH. The medium was sterilized at 121 °C for 20 minute. The strains are maintained on slants of the same medium containing 1.8 % agar and transferred every 3 months. After incubation at 39 °C for seven days, they have been kept at 4 °C. Before the each growth, a single colony was grown in 2 ml growth medium in a test tube overnight to activate the strain.

2.2.2 Isolation of a *Halobacterium salinarium* strain from Tuz Lake

Water samples which were collected from Tuz Lake in sterile bottle were used to isolate *Halobacterium salinarium*. Some aliquot of the sample was made dense by centrifugation and used to spread on sterilized solid medium containing 1.8% agar in petri dishes. The petri dishes were incubated for seven days at 39 °C under illumination. After seven days, pink-purple colored colonies were picked up and each was streaked on agar medium in separate petri dishes. The petri dishes were incubated

under the same conditions for seven days. Last step was repeated to be sure that the colonies were same. Finally, the better-grown, well isolated pink-purple colonies were transferred to slants as stock cultures.

2.2.3 Microbial Characterization of the *H. salinarium* strains

2.2.3.1 Gram staining

The morphological characterization was observed before and after Gram staining by using the light microscope connected to the computer through a camera. The bacterial smear was covered with crystal violet reagent on a slide for 1 minute. Then, after rinsing the slide by water, the smear was rinsed with iodine reagent and allowed to remain for 1 minute followed by rinsing with water. After that, the ethanol (96%) was applied until dye no longer runs off from the smear (15-20 seconds) followed by rinsing with water again. Then, the smear was covered with safranin for 1 minute and rinsed with water (Lansihg et al., 1990). At the end, the dried slides were observed under the light microscope and photograph was taken through the camera which connected with a computer.

2.2.3.2 Gelatin Hydrolysis Test

The gelatine test was carried out by inoculating the new strain to a 10 ml growth medium containing 1.2 g gelatine in a test tube. This tube together with the control one (without bacteria) was incubated for one week at 39°C and shaken at 120 rpm under illumination of light intensity of 100 W/m^2. After one week the tubes were kept at 4°C in the

refrigerator for one hour. After one hour the tubes were observed. The growth medium in the control tube became solid whereas in the bacteria containing tube it stayed liquid.

2.2.3.3 Growth Curve

Growth was followed by measuring the optical density in UV-visible spectrophotometer (Shimadzu UV Mini 1240) at 660 nm. The standard curve was constructed by drawing the optical densities against the incubation time.

2.2.4 Biochemical Characterization of the *H. salinarium* strains

2.2.4.1 Total Protein Isolation

Total protein exraction of strains was made according to B. Pot et al. (1994). Overnight activated strains were transferred into 25 ml growth medium in 100 ml flask and incubated at 39 °C for seven days as explain before. Wet weight of cells per ml was estimated by centrifugation of 1 ml sample at 12,000 g for 5 minutes. Cells of 50 mg wet weight were used for the isolation. The cells were harvested by centrifugation at 13,000 g for 15 minutes and the resulting pellet washed two or three times with basal salt solution (growth medium without peptone). Washed cells were suspended in 0.9 ml sample treatment buffer (0.062 M Tris-HCl buffer containing 5% (v/v) mercaptoethanol and 10% (v/v) glycerol; final pH 6.8). The suspension was mixed thoroughly. 0.1 ml 20% SDS was added into the suspension and mixed again. The cell suspension was heated at 95-100 °C for 10 minutes and centrifuged at

10,000 g for 10 minutes. The supernatant was stored at -20 °C for further use.

2.2.4.2 Purple Membrane Isolation

The isolation of purple membrane of the strains was carried out according to Oesterhelt (1995).

2.2.4.2.1 Preparation of packed cells

Activated strain was transferred into 38 ml growth medium in 100 ml flask and incubated at 135 rpm on rotary shaker (Thermoshake, Gerhardt) for 3 days at 39 °C under illumination. Each of four 240 ml growth medium in 500 ml flasks were inoculated with 10 ml of 40 ml grown culture. The last four flasks were incubated for 4 days at the same conditions. *H. salinarium* cells from 1 liters of growth culture were harvested by centrifugation for 30 minutes at 7000 rpm (Sorvall RC-5B, GS3 rotor) and 4 °C. The supernatant was decanted and the cell pellets were resuspended in 25 ml of basal salt solution (growth medium without peptone). The suspension was stored at -20 °C until use.

2.2.4.2.2 Preparation of purple membrane fragments

All operations were carried out at room temperature. The dialyses of packed cells and centrifugations were done at 4 °C. Twenty five milliliters of packed cell suspension was mixed with 1000 unit of DNase I and dialyzed for 24 hours at 4 °C against 2 liters of distilled water. DNase I solution was prepared by dissolving 5 mg of DNase I (2000

U/mg) in 1 ml of 0,15 M NaCl and adequate volume (~100µl of the stock) of this solution was added in the suspension, in order to prevent the development of excessive viscosity from liberated DNA during dialysis. The red lysate was centrifuged at 50,000 g for 40 minutes (Sigma 3K30, 12158 rotor) and the red supernatant was decanted. The pellet was resuspended in 30 ml of distilled water by vortexing. The suspension was centrifuged at 50,000 g for 40 minutes again and the pellet was resuspended in 30 ml of distilled water by vortexing. The last step was repeated until the supernatant is nearly colorless. The last pellet was resuspended in 1-2 ml of distilled water.

2.2.4.2.3 Sucrose Density Gradient

The Purple Membrane suspension which gained in the previous step was layered over a linear 30-50% (w/w) sucrose density gradient on 1 ml of 60% sucrose cushion at the bottom of the tubes of a swing-out rotor with 10 ml capacity. The gradient was prepared by use of hand-made glass gradient mixer and peristaltic pump (DYNAMAX Peristaltic pump Model RP-I, RAININ). 4 ml of 30% (w/w) sucrose solution was loaded in first vessel and 4 ml of 50% (w/w) sucrose solution was loaded in second vessel. To make gradient, peristaltic pump at 10 rpm and stirrer which is under second vessel were started simultaneously. The prepared tubes were centrifuged at 100,000 g for 17 hours at 15 °C (Ultracentrifuge Sorvall Combi Plus Rotor TH-641). Upper part of the gradient was removed with a vacuum aspirator and then the purple band was collected with a pasteur pipette in a minimal volume. Sucrose was

removed by dilution to 25 - 30 ml distilled water and repeated centrifugation at 50,000 g for 40 minutes.

2.2.4.3 Protein Determination

The protein concentrations of cell extracts were determined according to Bradford method (Bradford, 1976). Bradford reagent was prepared by mixing 500 mg of Coomassie Brilliant Blue G-250 in 250 ml 95% ethanol. 100 ml 85% (w/v) phosphoric acid was added to this solution. The resultant solution was diluted to 1 liter with distilled water and filtered. Before use the concentrated Bradford reagent (5x) was diluted to 1x with distilled water.

5 - 20 µl of sample was diluted to 5 ml with distilled water in a test tube. 5 ml Bradford reagent was added. The tube was vortexed, and left at room temperature for at least 10 minutes. The absorbance at 595 nm was measured with a UV-Visible spectrometer (SHIMADZU UVMINI 1240) against blank solution containing 500 µl distilled water and 5 ml Bradford reagent. Bovine Serum Albumin (BSA) was used as a standard at following concentrations: 5, 10, 20, 30, 40, 50, 60 µg/ml.

2.2.4.4 Characterization of total proteins by 1D SDS-PAGE

Total protein profiles of the strains and the molecular weight of bR were determined by SDS-PAGE which was performed with 4% stacking and 12% separating gels according to Laemmli (1970). Marker proteins in range of 14.2 - 66 kD were used to determine the molecular weight of the

protein bands. Bromophenol blue was added to the samples as tracking dye and electrophoresis was achieved under constant current (10 mA in stacking and 20 mA in separating gel) in a Bio Rad Protean-II xi vertical electrophoresis chamber at 4 ^{o}C. When tracking dye reached near the lower end, the run was stopped.

2.2.4.5 Coomassie Blue Staining

The staining was carried out according to Pot et al. (1994). The gel was fixed by shaking gently for 30 minutes in fresh 3% trichloroacetic acid solution in gel container. The fixation solution was poured off and the staining solution made up of 0.25% Coomassie Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid was added. The gel was shaken gently for at least 1 hour. After staining, the gel was destained by shaking for three times 30 minutes in destaining solution containing 25% (v/v) methanol and 10% (v/v) acetic acid.

2.2.4.6 Characterization of membrane proteins by 2D SDS-PAGE

2D SDS-PAGE was done according to Naqvi et al. (1994). IEFGS was contained urea, ampholines 5-8, ampholines 3-10, Nonidet P-40 and CHAPS (Appendix F). Gels were casted in 1.5 mm inner diameter 150 mm long glass tubes. Samples were directly extracted in IEFGS. Then IEFGS containing desired amount of protein was made up to total gel volume (250 µl) with addition of IEFGS and mixed with 0.4 µl TEMED and 1 µl of freshly prepared 2.5% ammonium persulfate. Following a brief vortex shake, the contents were collected in a micropipette tip. Gel tubes

were marked at 140 mm from lower end and were filled slowly from the bottom while being held horizontal. When the gel solution reached the mark, tubes were blocked with thumb at their distal end and closed at the bottom end by stretching a piece of parafilm using the other hand now free from pipetting operation. Still keeping the upper end blocked by the thumb, parafilm was first pressed against the bottom face and then against the sides of the gel tube by rolling in the fingers. Filled tubes were then held vertically, tapped gently for 4-5 times to dislodge any bubble staying on the inner surface of the gel tube, layered 5 µl of overlay solution on the upper end and left vertically in tube stand. It takes about 20 minutes for polymerization to complete.

IEF was generally started one hour after filling the last tube. Cathodic solution was 20 mM NaOH while anodic solution contained 10 mM phosphoric acid. Run was performed with constant voltage of 400 for the first two hours followed by 800 volts for 14 hours (12,000 volt-hours total). For removing gels from tubes a 250 µl pipette tip was cut at rear end to fit a syringe outlet. Water was pressurized through the syringe by inserting the lower end of the micropipette tip directly into the upper end of the gel tube. Gels were extruded on a piece of parafilm and dripped off any remaining liquid with a piece of filter paper.

The second dimension SDS-PAGE on 12% acrylamide gels was performed essentially according to Laemmli (1970) except that no stacking gel was used. IEF gels were loaded to second dimension gels (160x160x1.5 mm) without any equilibration and no overlay gel was

employed. Separation was performed at 10°C using 10 mA constant current for the first 30 minutes and then 25 mA for rest of the run.
For determination of pH gradient, blank gel that was run simultaneously in IEF were sliced into 1 cm long pieces while discarding 5 mm from either ends. These pieces were transferred to 1.4 ml of 1 M KC1 in eppendorf tubes and kept overnight at 4 °C, before measuring their pH at 25 °C.

2.2.4.7 Silver Staining

After electrophoresis gel was stained by silver staining. The gel was fixed in a fixative solution of 12% acetic acid, 50% methanol for at least 1 hour.The fixed gels was silver stained according to Blum (1987). The gel was washed 3 times with 50% ethanol to remove acetic acid. After pretreatment with thiosulphate , gel was rinsed with distilled water 3 times and incubated in silver nitrate solution with formaldehyde for 20 minutes. Rinsed gel was developed for appearance of bands. The detailed procedure is given in Appendix G.

2.2.4.8 Measurement of Photoactivity Purple Membrane Fragments

The photoactivities of *Halobacterium salinarium* purple membrane fragments were measured as pH change in the suspension of purple membrane fragments under illumination and in dark with respect to time. The experimental setup is shown in Figure 2.1. *Halobacterium salinarium* purple membrane fragments were diluted with 4 M NaC1 solution to have a 3 ml suspension of 5 μM bacteriorhodopsin

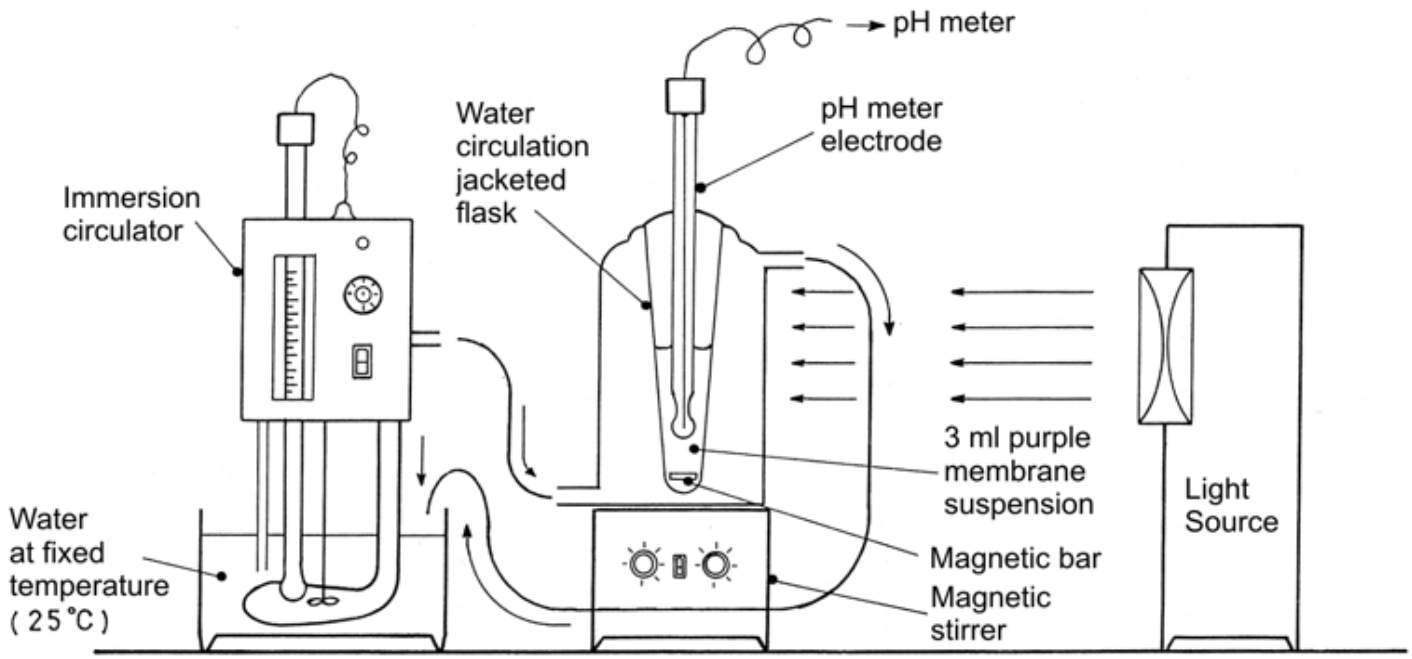

Figure 2.1 Scheme of the measuring system.

concentration. The suspension was loaded in a water jacketed vessel. The temperature of the suspension was kept constant at 25°C with water circulation and it was continuously mixed by means of a magnetic stirrer. The pH of the suspension was continuously measured by means of a combined pH electrode connected to a pH meter. Initially, the system was incubated in dark until constant pH level was settled. Then, the system was illuminated with a 1000 Watt-lamp from a 30 cm distance. The pH values were recorded with respect to time. After steady pH values were attained, the lamp was turned off and the changes in pH were recorded until the system established a stable pH level.

2.2.5 Genetic Characterization

2.2.5.1 Genomic DNA Isolation (mini preparation)

The isolation of genomic DNA was carried out according to Bazzicalupo and Fancelli (1997). Overnight activated strains from single colony were transferred into 10 ml growth medium (defined in section 2.2.1) in 50 ml flask and incubated at 39 °C for three or four days (mid-log phase). The cells were collected by centrifugation of 1.5 ml of the culture for 2 minutes at 8000 rpm in microcentrifuge (Micromax RF Thermo IEC). The cell pellet was suspended in 500 µl of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and added 30 µl SDS together with 5 µl proteinase K (20 mg/ml). The tube was mixed by inversion and incubated at 37 °C for 1 hour to allow cell lysis. 100 µl of 5M NaCl was added and vortexed for few seconds. 80 µl 10% CTAB (cetyl-trimethyl ammonium bromide) was added. After mixing, the tube was heated 10 minutes at 65 °C.

An equal volume (about 800 µl) of chloroform:isoamyl alcohol (24:1) was added and vortexed for a few seconds and centrifuged for 5 minutes at 11000 rpm. The aqueous upper phase was collected in a new tube. Equal volume of phenol:chloroform:isoamyl alcohol was added and mixed by vortex and centrifuged for 5 minutes at 11000 rpm. The upper aqueous phase was recovered in a fresh tube and added 2 µl RNase (10 mg/ml) and incubated for 30 minutes at 37 °C. Approximately an equal volume of isopropanol was added and the DNA was precipitated for 5 minutes at room temperature and centrifuged for 5 minutes at 11000 rpm. The supernatant was discarded and the pellet washed with 70% ethanol and centrifuged again for 5 minutes at 11000 rpm. The pellet was dried under vacuum and solubilized in 10-20 µl of sterile TE.

2.2.5.2 Plasmid DNA Isolation (mini preparation)

Plasmid DNA isolation was carried out according to Sambrook et al. (1989). Single colony were transferred into 15 ml growth medium in 50 ml falcon tube and incubated at 39 °C for three or four days (mid-log phase). 1.5 ml of the culture was poured in to microfuge tube and centrifuged at 12,000g for 2-3 min at 4 °C in the microfuge (Micromax RF Thermo IEC). The remainder of the culture was stored at 4 °C. The medium was removed by aspiration. The bacterial pellet was left as dry as possible. Plasmid DNA was extracted by the alkaline lysis method. The bacterial pellet was resuspend in 100 µl of ice-cold Solution I (50 mM glucose, 25mM Tris·Cl (pH 8.0), 10mM EDTA (pH 8.0)) by vigorous vortexing. 200 µl of freshly prepared Solution II (0.2 N NaOH and 0.1%

SDS) was added. The tube was closed tightly and the contents were mixed by inverting the tube rapidly five times. The tube stored on ice. 150 µl of ice-cold Solution III (60% (v/v) of 5M potassium acetate, 11.5% (v/v) glacial acetic acid, 28.5% (v/v) H_2O) was added. The tube was closed and mixed gently by inverting to disperse Solution III through the viscous bacterial lysate. The tube was stored on ice for 3-5 minutes. The tube was centrifuged at 12000g for 5 minutes at 4 °C in a microfuge. The supernatant was transferred into a fresh tube and added an equal volume of phenol:chloroform:isoamylalcohol (25:24:1). The tube was mixed by inverting and centrifuged at 12000g for 4-5 minutes at 4 °C in a microfuge. The supernatant was transferred into fresh tube. The double stranded DNA was precipitated with two volumes of ethanol at room temperature. The tube was mixed by inverting and allowed to stand for at least 20 minutes at -20 °C. After the incubation, the mixture was centrifuged at 12000g for 5 minutes at 4 °C in a microfuge. The supernatant was removed by gentle aspiration. The tube was stand in an inverted position on a paper towel to allow all of the fluid to drain away. Any drops of fluid adhering to the walls of the tube were removed. The pellet of double stranded DNA was rinsed with 1 ml 70% ethanol at 4 °C. The supernatant was removed by gentle aspiration and the pellet of nucleic acid was allowed to dry in the air for 10 minutes. The nucleic acids were redissolved in 20 µl of TE (10 mM Tris·Cl (pH 8.0), 1mM EDTA (pH 8.0)) containing DNase free RNase A (20µg/ml) and stored at -20 °C. Plasmid DNA amount was estimated spectrophotomerically at 260nm and 280nm. To visualize the plasmid DNA and verify the

method, 5 µl aliquots taken from the samples were run by agarose electrophoresis in 0.6 % (w/v) agarose at 70 V for 1-2 h.

2.2.5.3 Agarose Gel Electrophoresis

All DNA products were checked with agarose gel electrophoresis. Depending on the size of the nucleic acid, different agarose concentrations ranging from 0.6% to 1.5% (w/v) were used. Appropriate amount of agarose was added to 50 ml of 0.5 X TBE solutions and heated to melt the agarose. While cooling to room temperature 3 µl of ethidium bromide solution was added. The gel was solidified in the tray. The electrophoresis was carried out in 0.5 X TBE buffer at constant voltage 60-80 V. After visualization of DNA bands under UV illumination photos of the gels were taken.

2.2.5.4 RAPD-PCR Assay

RAPD-PCR Assay was performed according to Micheli et al. (1997). Amplification reactions were performed in volumes of 25 µl containing $MgCl_2$, *Taq* polymerase buffer, dNTPs, ten base primers, genomic DNA and *Taq* DNA polymerase. A master mix was prepared with following composition; 2 µl of 25 mM $MgCl_2$, 2.5 µl of 10x *Taq* polymerase buffer, 2.5 µl of 1mM dNTPs and 2.5 µl of 2 µM primer (60% G+C). Distelled water was added to a final volume of 24 µl per sample and mixed well. 24 µl of master mix was dispensed to each reaction tube. 1µl of genomic DNA of strains (25 ng/µl) was added into each reaction tube. The reaction mixtures were mixed gently by tipping the tubes and spun

briefly to remove air bubbles. The mixtures were overlaid with 15 µl mineral oil. The tubes were placed in the thermocycler and initial denaturation step was performed at 96 °C for 5 minutes. After denaturation step, the tubes were chilled on ice and 1U of *Taq* DNA polymerase was added. The tubes were placed in thermocycler programmed for 45 cycles of denaturation at 94 °C for 1 minute, annealing at 36 °C for 1 minute and extension at 72 °C for 2 minutes. Samples were stored at 4 °C. PCR products were analyzed by electrophoresis in 1.5 % (w/v) agarose gels and detected by staining with ethidium bromide.

2.2.5.5 RFLP Analysis

RFLP analyses were done on the plasmids isolated from strains. The plasmids were digested with *Pst*I and *Hind*III restriction endonucleases. Digestion reactions were performed in volumes of 25 µl containing 30 µg plasmid DNA of the strains, 1x Enzyme Buffer, 30 unit enzyme and distilled water at 37 °C for overnight. The fragments of the digested plasmids were separated by horizontal electrophoresis in 0.6% (w/v) agarose gel at 35V for 5-6 hours. The size of restriction fragments was estimated using computer program (BioProfil Bio-1D V99.04 Vilber-Lourmat).

CHAPTER III

RESULTS AND DISCUSSIONS

3.1 Identification of the strain of *Halobacterium salinarium* from Tuz Lake

Identification of the *Halobacterium salinarium* strain was achieved by comparative analysis with reference strains *Halobacterium salinarium* DSM3754, *Halobacterium salinarium* S9.

3.1.1 Isolation of the strain by selective media

Halobacterium genus can be differentiated from *Haloferax* genus as *Halobacterium* strains do not use carbohydrates as carbon and energy sources; and, it is differentiated from *Natronobacterium* and *Natronococcus* genera as *Halobacteria* strains are neutrophilic, growing in pH range of 5.0-8.0 (Bergey, 1994).

As detailed in Sections 2.2.1, the selective media used for isolation was at 4 M concentration of NaCl, lacking carbohydrates and its pH was adjusted to be around 6.5-7.4. The water samples spread on selective media developed colonies under aerobic conditions and the colonies

were in pink-purple color. Hence, it is deduced that the isolated strain does not necessitate carbohydrates for growth, it is aerobic, and the pH range for its growth covers the values in between 6.5-7.4. It can be concluded that the results given above support the claim that the isolated strain is a member of Halobacterium genus. The colonies formed on streaked petri plates are shown in Figure 3.1. The color differences of the strains which were used for comparisons were shown in Figure 3.2 and 3.3.

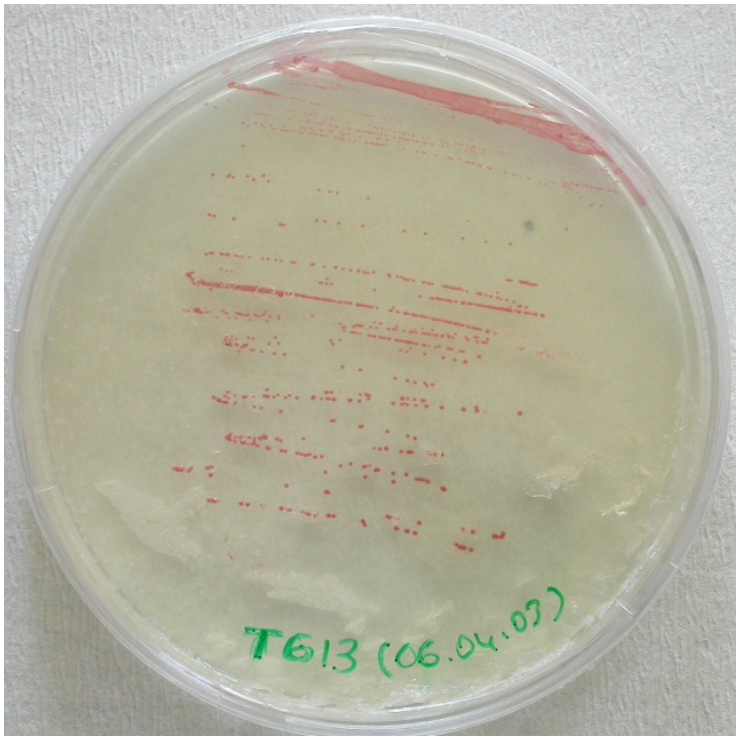

Figure 3.1 Petri plate streaked with isolated strain from Tuz Lake, Central Anatolia, Turkey.

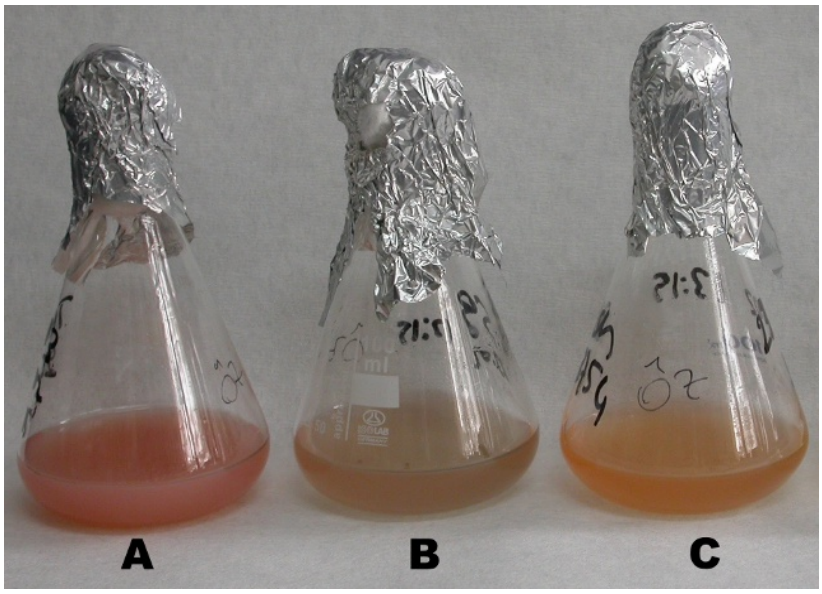

Figure 3.2 Color differences of the strains in the medium on 7th day of growth at 39 °C in shaking incubator; A: *Halobacterium salinarium* TG13, B: *Halobacterium salinarium* S9, C: *Halobacterium salinarium* DSM3754.

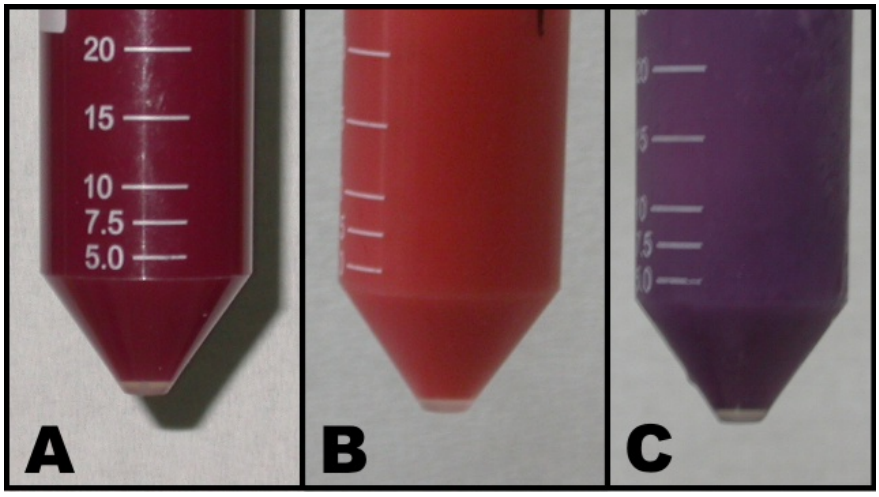

Figure 3.3 Color differences of packed cells of the strains; A: *Halobacterium salinarium* TG13, B: *Halobacterium salinarium* DSM3754, C: *Halobacterium salinarium* S9.

3.1.2 Morphological Characterization of the strain

For morphological characterization of the strain, samples from liquid cultures at early stationary phase were examined via light microscope before and after Gram staining was performed.

The members of Halobacterium genus have irregular rod-shaped cells staining Gram negative; and, most of the species of this genus, including *Halobacterium salinarium,* are motile (Bergey, 1994). *Halobacterium salinarium* cells are propelled by a monopolarly or bipolarly inserted flagellar bundle, which are too thin to be visible in the light microscope under standard conditions (Kandler et al., 1993).

In this study, the living cells of the isolated strain were apparently obtained as motile and rod-shaped using light microscope (Figure 3.4). The rod-shape of the cells was emphasized after staining and the cells stained pink, i.e. gram negative (Figure 3.5). As a conclusion, the results obtained are in accordance with the features of *Halobacterium salinarium* species.

3.1.3 Growth curves of *Halobacterium salinarium*

The growth of *Halobacterium salinarium* strains in 100 ml growth culture were followed by measuring absorbance at 660 nm. The growth curve of the strains was shown in the Figure 3.6. The strains were reached the end of the logarithmic phase after 5 days. Similarly, Lorber and DeLucas (1990) have demonstrated that *H. salinarium* S9 was reached stationary phase after 5-6 days in shaken flask.

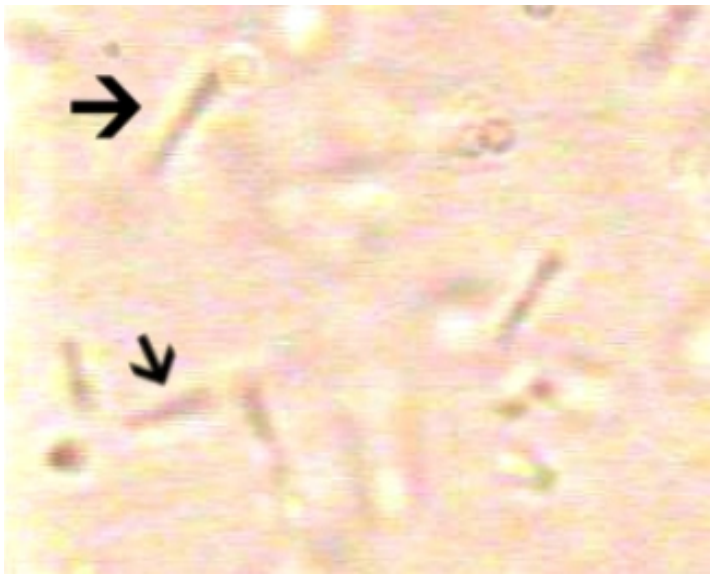

Figure 3.4 *Halobacterium salinarium* cells of the isolated strain were motile and rod-shaped (Prior light microscope, Pro-Series High Performance CCD camera and Image-Pro Plus software, x1000)

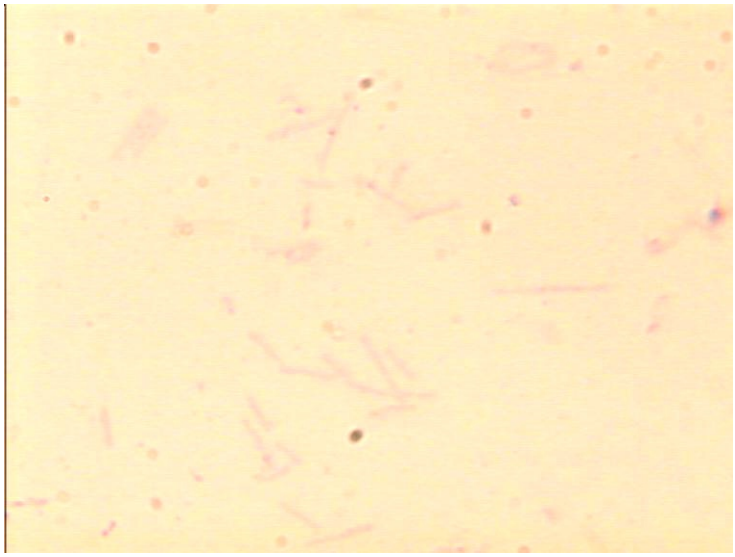

Figure 3.5 Gram staining result of the isolated strain. The cells were found to be gram-negative (Prior light microscope, Pro-Series High Performance CCD camera and Image-Pro Plus software, x1000)

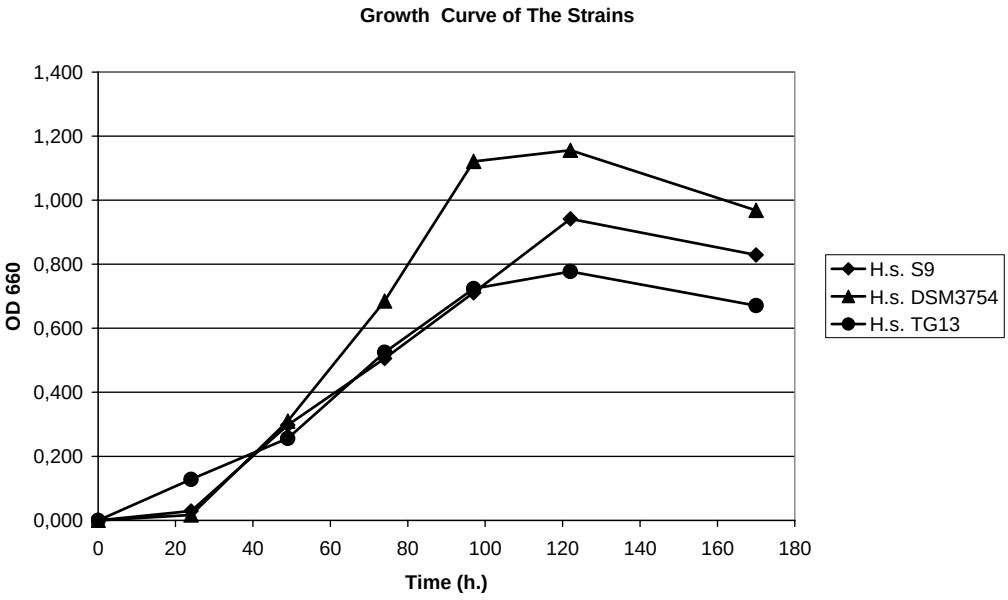

Figure 3.6 Growth profiles of different *Halobacterium salinarium* strains at 39 °C under illumination and shaking at 120 rpm.

3.1.4 Gelatin hydrolysis test

The isolated strain *H. salinarium* TG13 was compared with reference strains *H. salinarium* DSM3754, *H. salinarium* S9 in terms of hydrolyzing ability of gelatin. Each medium containing gelatin in tubes was inoculated with strains. After seven days of growth, each medium inoculated became liquefied. Whereas the medium without inoculation as control stayed solid after keeping them at 4 °C for a while (Figure 3.7). This result shows that the isolated strain and reference strains have the extracellular enzymatic activity for hydrolyzing gelatin.

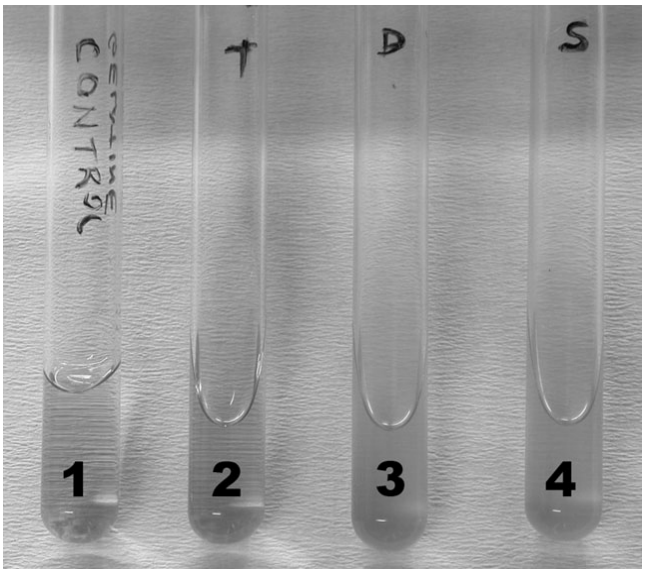

Figure 3.7 Gelatine hydrolysis test results. The inoculated tubes were liquefied, whereas the control tube was still rigid after a week of incubation at 39 °C. (Tube 1: Control, Tube 2: *H. salinarium* TG13, Tube 3: *H. salinarium* DSM3754, Tube 4: *H. salinarium* S9)

In *Halobacterium* genus, *Halobacterium salinarium* is only the bacteria which can utilize gelatin. The others (*H.volcanii, H.saccharovarum, H.vallismortis, H.pharaonis, H.lacusprofundi, H.sodomense* and *H.trapanicum*) have shown negative result for gelatin utilization (Krieg Bergey, 1994), so the isolated strain has supposed to be a member of *Halobacterium salinarium* species.

3.2 Biochemical Characterization of the strain

3.2.1 Comparison of Total Protein Profiles of the Strains

Total protein extraction was carried out from the strains after one week of growth. The protein concentration was determined by Bradford method (Bradford, MM. 1976). Protein profiles of the strains were

characterized by use of one dimensional gel electrophoresis (1D SDS PAGE). Two SDS-polyacrylamide gels were run concurrently. One of the gels was stained by coomassie blue and the other one was stained by silver staining. The protein concentrations which applied to the gels were 30 μg and 10 μg, respectively. To determine the molecular weight of the protein bands, molecular weight markers were used in range of 14.2 kD to 66 kD. Also, isolated purple membrane was used to find out the place of the bacteriorhodopsin bands in the protein profiles of the strains. The bR concentrations applied to the gels were 0.87 μg for coomassie staining and 0.218 μg for silver staining, respectively. The differences between protein profiles of the strains were apparent in Figure 3.8. Comparison of the protein profiles of different *H. salinarium* strains according to their molecular weights were summarized in APPENDIX E. The corresponded bR band which had apparent M.W. of 22 kD was observed intense band in three of the strains (*H.s.* TG13, S9, L33). Molecular weight of bR was discussed in section 3.2.2.3.

The value of protein electrophoresis in microbial systematics has been established for over 30 years (Pot, 1994). 1D SDS PAGE was used to rapidly screen large numbers of unknown strains to group them into related clusters (Hesselberg and Vreeland, 1995). A total of 102 strains received as Corynebacterium 'group JK' were characterized by SDS-PAGE of their whole-cell proteins (Jackman and Pelczynska, 1986).

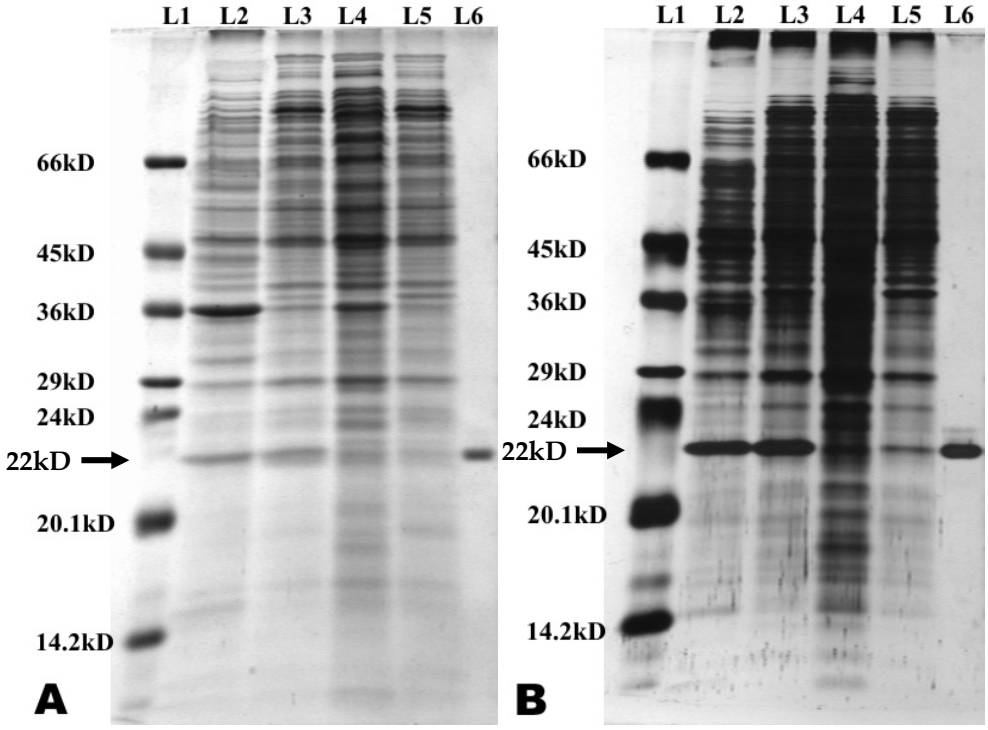

Figure 3.8 SDS-PAGE profiles of total proteins of the strains; Gel contained 12% acrylamide. A: Coomassie blue stain. B: Silver stain. Lane 1: Marker proteins, Lane 2: *H.salinarium* TG13, Lane 3: *H.salinarium* S9, Lane 4: *H.salinarium* DSM3754, Lane 5: *H.salinarium* L33, Lane 6: Pure purple membrane isolated from *H.salinarium* TG13. In each well, equal amounts of protein were loaded (0.87 µg for coomassie staining (A) and 0.218 µg for silver staining (B).)

3.2.2 Characterization and comparisons of Purple Membrane of *Halobacterium salinarium* strains

3.2.2.1 Sucrose density profiles

Further purification of purple membrane fragments was achieved by using of a linear 30 to 50% sucrose density gradient with 60% sucrose bottom cushion. Centrifugation was carried out in swingout rotor. The profile of the sucrose density gradient of *H. salinarium* TG13 was same as

the profile of *H. salinarium* R1 and NRL defined by Oesterhelt (1974). Red band and Purple band were observed clearly in the tube of *H. salinarium* TG13 (Figure 3.9). Only purple band was observed in the tube of *H. salinarium* S9. Neither red band nor purple band was clearly observed in the tube of *H. salinarium* DSM3754. Therefore, purple membrane fragments were not able to be isolated from *H. salinarium* DSM3754. The purple band was collected an explained in section 2.2.4.2.3 and used for further characterization studies.

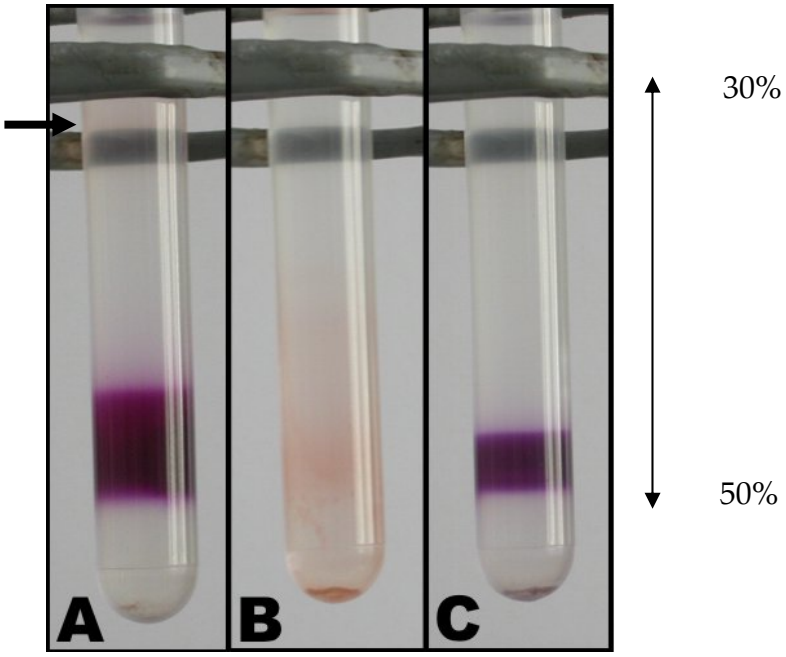

Figure 3.9 Sucrose density profile of the strains; A: *Halobacterium salinarium* TG13 (The arrow shows red band), B: *Halobacterium salinarium* DSM3754, C: *Halobacterium salinarium* S9. The sucrose density gradient was formed linearly 30-50% sucrose on 60% sucrose bottom cushion.

3.2.2.2 Absorption spectra of the purple membrane fragments

The spectroscopic analysis of the purple membrane fragments was performed. In order to find maximal absorption, the purple membrane fragments were scanned between 260 nm and 800 nm wavelengths in UV-visible spectrophotometer. For *H. salinarium* TG13, maximal absorptions were observed at 280 nm and 563 nm wavelength and For *H. salinarium* S9, maximal absorptions were found at 280 nm and 567 nm (Figure 3.10 - 3.11). The maximal absorption at 280 nm was corresponded to protein content of the sample and the maximal absorptions at 563 nm and 567 nm were corresponded to retinal chromophore group of bacteriorhodopsin. In the native membrane bacteriorhodopsin isolated from *H. salinarium* R1 and NRL has an absorption maximum at 560 nm (Oesterhelt and Stoeckenius, 1974).

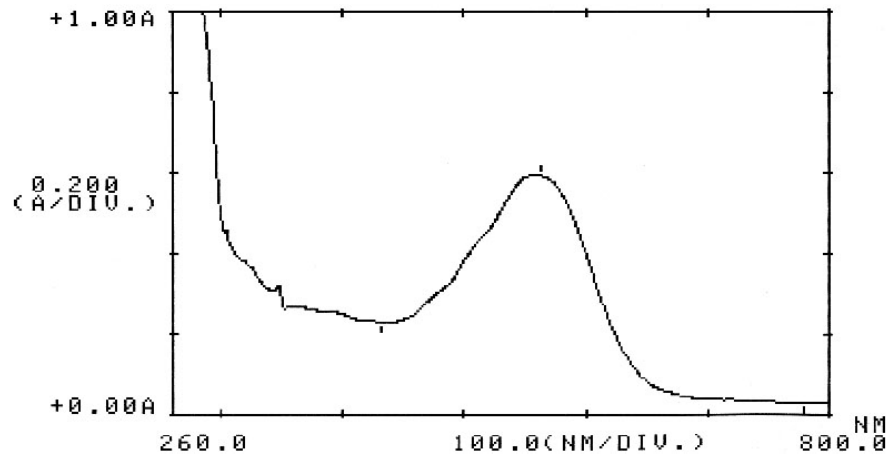

Figure 3.10 Spectrum of purple membrane of *H. salinarium* TG13

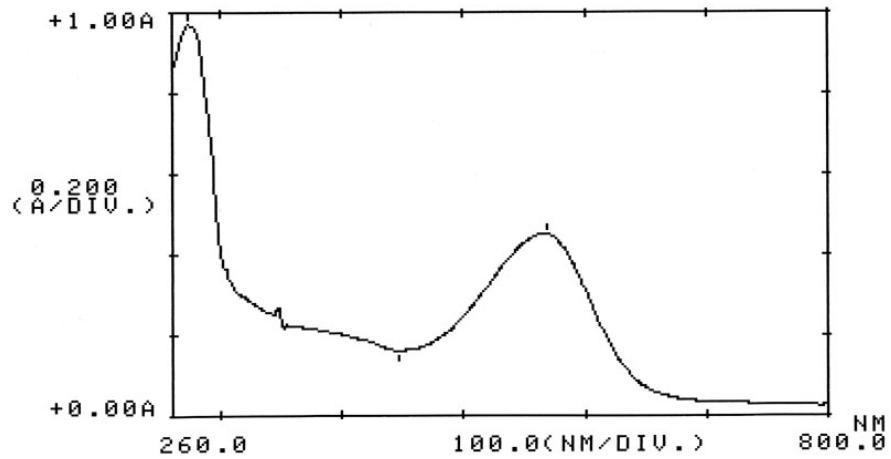

Figure 3.11 Spectrum of purple membrane of *H. salinarium* S9

3.2.2.3 Molecular weight of the isolated bacteriorhodopsin from the strains

Molecular weight determination of bacteriorhodopsin in the purple membrane fragments was estimated by use of 1D SDS-PAGE. Molecular weight of bacteriorhodopsin of *H. salinarium* TG13 and S9 strains were compared. Because of insufficient bacteriorhodopsin content, *H. salinarium* DSM3754 strain was not compared with other strains. The molecular weight of the bacteriorhodopsin of *H. salinarium* TG13 and S9 strains were found to be same around 22 kD (Figure 3.12). In the literature, the molecular weight of the bacteriorhodopsin isolated from *H. salinarium* R1 was defined as 26 kD (Oesterhelt 1971). Later, the homogenous bacteriorhodopsin isolated from *H. salinarium* S9 had an apparent M.W. of 22 kD in electrophoresis in the presence of SDS (Lorber and DeLucas, 1990).

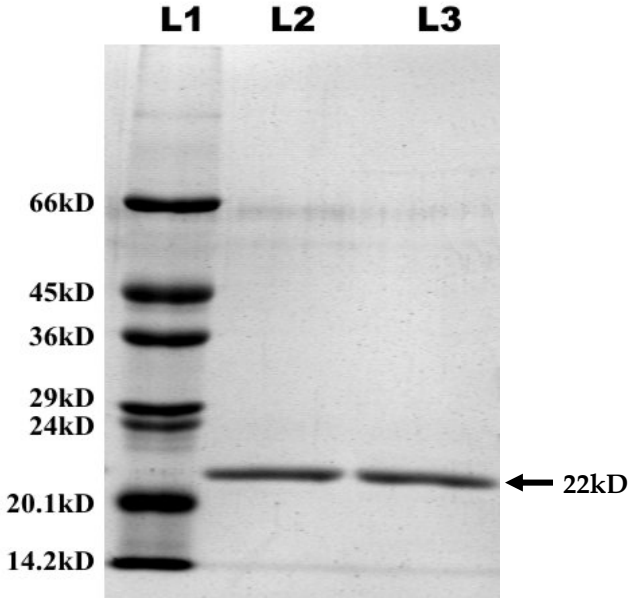

Figure 3.12 Molecular weight of bacteriorhodopsin isolated from *H. salinarium* TG13 and S9 strains. The gel was visualized by coomassie blue stain. Lane1: Marker proteins, Lane2: 1 μg PM of *H. salinarium* S9, Lane 3: 1 μg PM of *H. salinarium* TG13). The gel contained 12% acrylamide and run at the room temperature.

3.2.2.4 p*I* values of bR in PM fragments isolated from the strains

Since bR is the only protein present in PM, We can determine the p*I* values of bR directly using PM without further purification. The p*I* values of bacteriorhodopsin isolated from *H. salinarium* TG13 and *H. salinarium* S9 was determined by use of 2D SDS-PAGE. The p*I* value of bacteriorhodopsin was determined according to standard curve data gained by measuring pH of 1 cm fragments of blank IEF gels (Figure 3.13 and 3.14). As a result, the p*I* values of each bacteriorhodopsin gained from the strains was observed around 5.4 (Figure 3.15 and 3.16). The p*I* value of bacteriorhodopsin isolated from *H. salinarium* JW-3, R1 and S9

strain was found to be 5.2 by Miercke et al. (1989) and Lorber and DeLucas (1990). When we compare these two results, they are very close and latter result was gained by use of IEF in slab gel. The differences might be come from experimental differences. Purity of purple membrane isolated from *H. salinarium* TG13 was compared with total protein profiles of *H. salinarium* TG13 lysate (Figure 3.17). The bacteriorhodopsin spot of *H. s.* TG13 was almost pure when compared with total protein profile of the strain (Figure 3.15). In the result of total protein 2D SDS-PAGE of *H. salinarium* DSM3754, the bacteriorhodopsin spot was not observed (Figure 3.18).

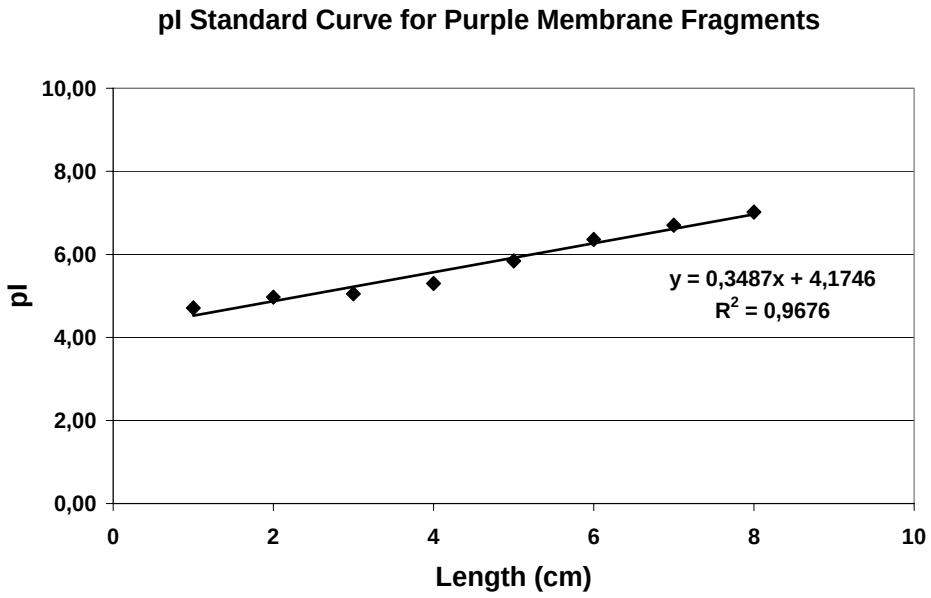

Figure 3.13 p*I* Standard curve for IEF of purple membrane fragments isolated from *H. salinarium* TG13 and *H. salinarium* S9.

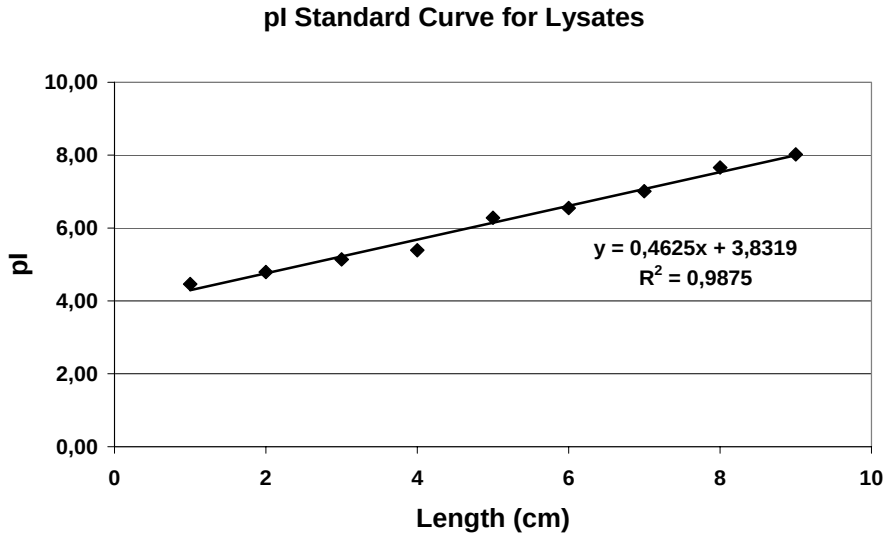

Figure 3.14 p*I* Standard curve for IEF of lysates of *H. salinarium* TG13 and *H. salinarium* DSM3754.

3.2.2.5 Photoactivity of Purple Membrane Fragments of the strains

The photoactivity of pure purple membrane fragments of *H. salinarium* TG13 and S9 were measured. pH changes upon illumination with respect to time (explained in section 2.2.4.8) Figure 3.19 shows the results of photoactivity measurement of *H.salinarium* TG13 and S9 pure purple membrane fragments. The bR concentration was 5 μM and initial pH values of the solutions were 5.76 for *H. salinarium* TG13 and 5.77 for *H. salinarium* S9. As soon as the light was on, there was a proton dissociation and decrease in pH values of the solution within 22 minutes for the strain TG13 and 25 minutes for the strain S9. When the light was turned off, due to uptake of proton, an increase in pH was observed.

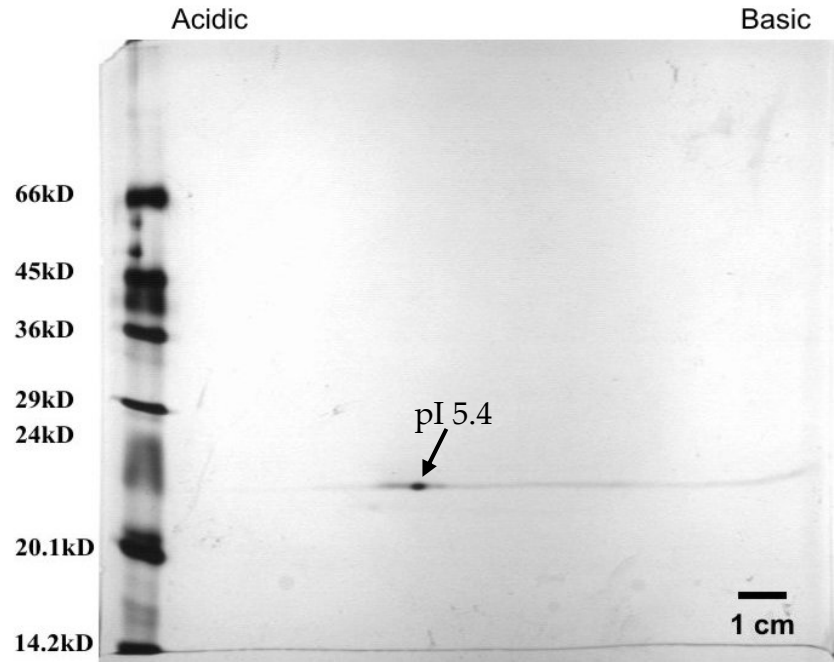

Figure 3.15 2D-SDS PAGE result of PM fragment of *H. salinarium* TG13. Gel was stain silver stain, p*I*: 5.4, M.W.: 22 kD.

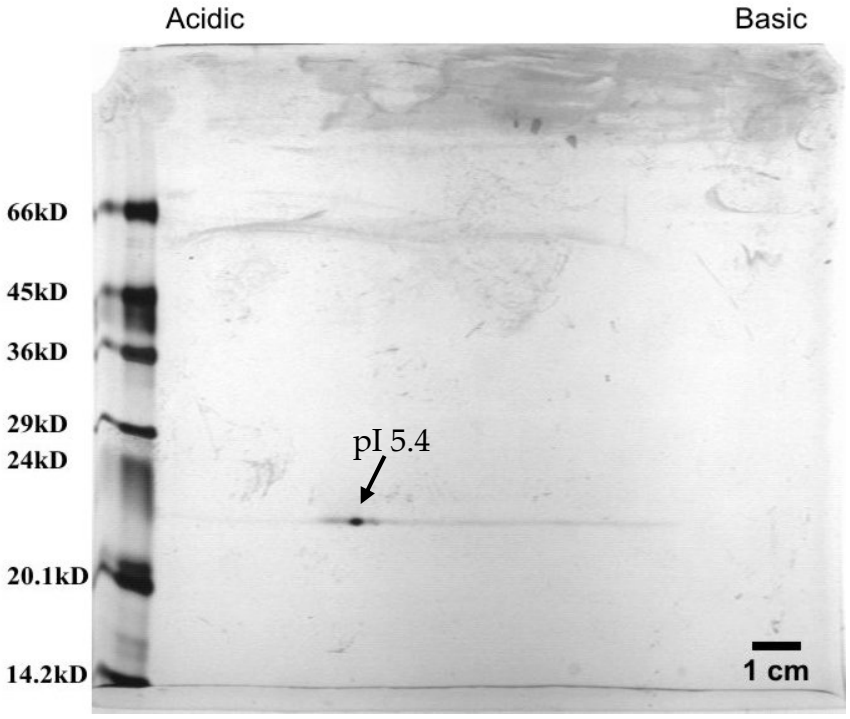

Figure 3.16 2D-SDS PAGE result of purple membrane fragment of *H. salinarium* S9. Gel was stain silver stain, p*I*: 5.4, M.W.: 22 kD.

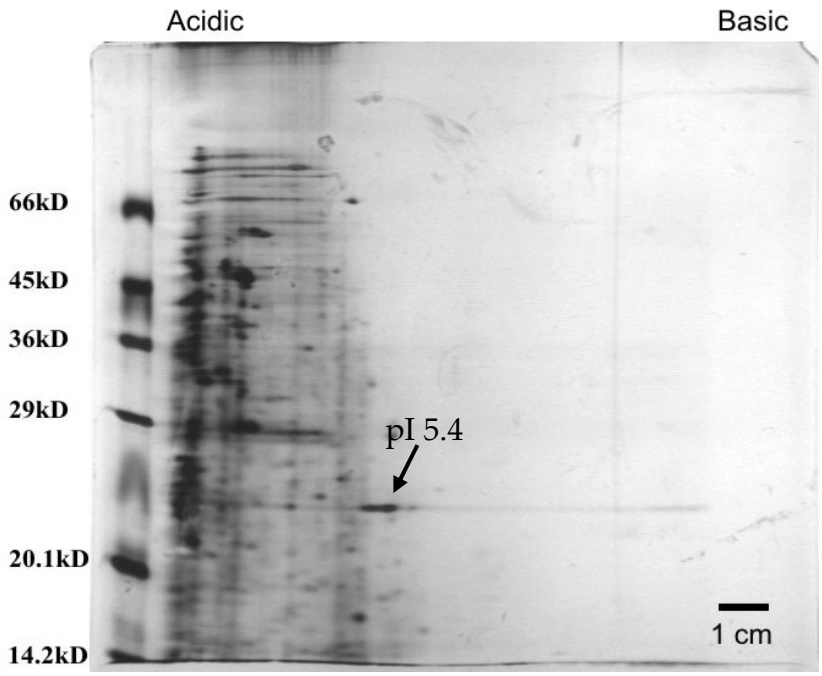

Figure 3.17 2D-SDS PAGE result of lysate (total protein) of *H. salinarium* TG13. Gel was stain silver stain.

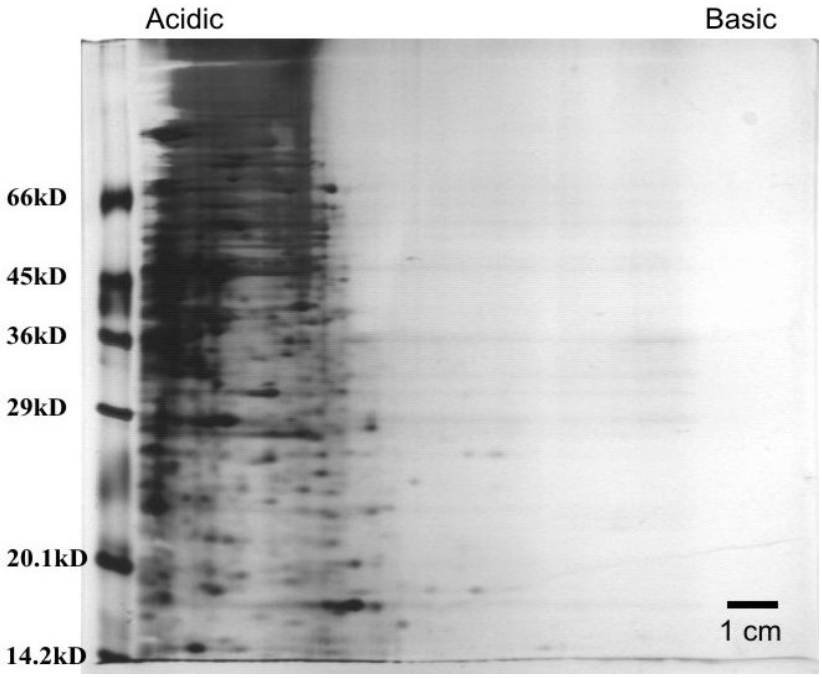

Figure 3.18 2D-SDS PAGE result of lysate (total protein) of *H. salinarium* DSM3754. Gel was stained silver stain.

It was observed that the reaction system was reached equilibrium within 22-25 minutes in the strain TG13 and the strain S9, respectively. The maximum ΔpH defined as the difference between pH initial and pH equilibrium values ($\Delta pH = pH_{initial} - pH_{equilibrium}$) for both light and dark phases. The maximum ΔpH values of PM of *H. salinarium* TG13 and *H. salinarium* S9 were found to be about 0.09 and 0.04, respectively. Upon comparison of proton dissociation and association capacities in light and dark phases, PM of *H. salinarium* TG13 was found to have higher proton dissociation and association capacity than PM of *H. salinarium* S9. The profile of photoactivity curves are in agreement with the previous studies on PM fragments (Yücel et al., 1995; Zabut, 1987, 1990).

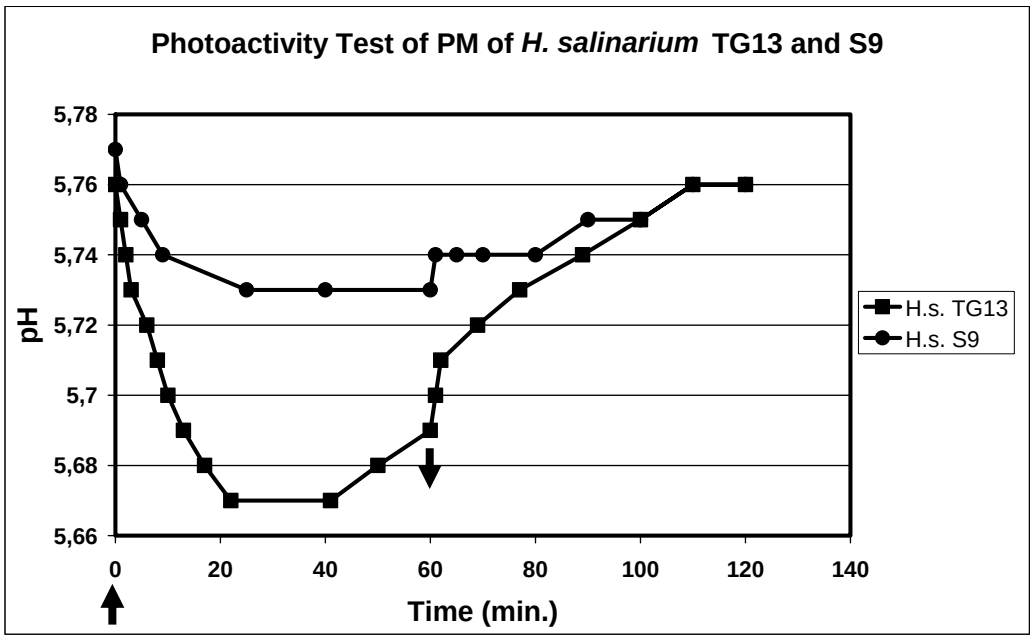

Figure 3.19 Photoactivity result of pure purple membrane fragments isolated from *H. salinarium* TG13 and *H. salinarium* S9. 25 °C Temp., 4 M NaCl, 5 μM bR (↑ Light On - ↓: Light Off).

3.3 Genetic Characterization of the strains

3.3.1 Results of RAPD-PCR Assay

The genomic DNAs of three different strains (*H. salinarium* TG13, *H. salinarium* S9 and *H. salinarium* DSM3754) were compared by RAPD-PCR analysis. The quantity of genomic DNA of the strains for each reaction of the PCR was 25 ng. As random primer, three different primers were designed. The G+C % contents of the primers were 50%, 60%, and 80%. The 60% G+C content primer was used for the analysis, since, the comparable pattern was observed at 60% G+C content. Polymorphism between strains was apparent (Figure 3.21). While conventional PCR assays can be used to amplify DNA sequences that are characteristic of a particular species or even strain, the method has the drawback that specific oligonucleotide primers are required, thereby making knowledge of the DNA sequence of the organism being studied an essential prerequisite. Analysis of randomly amplified polymorphic DNA fingerprints, also known as arbitrarily primed PCR, removes this requirement by using a primer(s) chosen without regard to the sequence of the genome to be fingerprinted (Welsh and McClelland, 1990; Williams et al., 1990). Thus, this method requires no previous knowledge of molecular biology of the organism to be investigated, and can therefore be applied to any species from which DNA can be prepared. The genome size of *H. salinarium* is 4.3 Mbp (Joshi *et al*., 1963). Genomic DNA of *E. coli* strains (strain 037, 641, 642) were assayed by Williams et al. (1990) to determine whether short primers could be used to amplify DNA segments from small genomes. The results shown indicate that

genomes as small as *E. coli* (4.7 Mbp) will support amplification, and that bacteria can be distinguished according to the banding patterns of their DNA on an agarose gel.

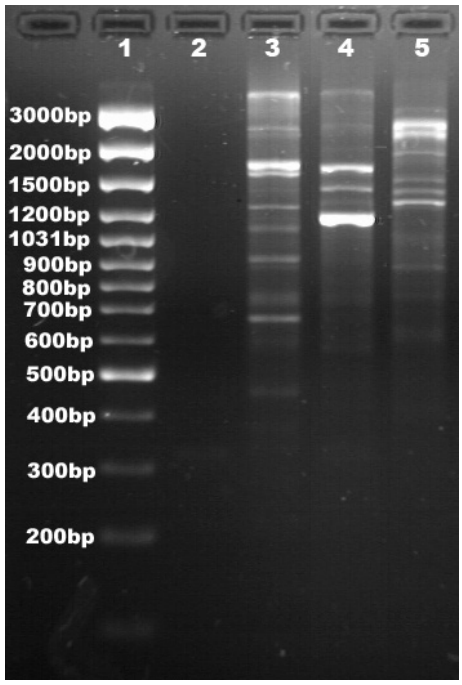

Figure 3.20 RAPD-PCR result of *H. salinarium* strains. Lane 1: Markers, Lane 2: Negatif control, Lane 3: *H.salinarium* TG13, Lane 4: *H.salinarium* S9, Lane 5: *H.salinarium* DSM3754.

3.3.2 Results of RFLP Analysis

RFLP of plasmids has been used in epidemiological studies to subtype many bacteria line *Salmonella typhimurium* (Platt et al., 1988) *Vibrio anguilarum* (Olsen and Larsen, 1990) and *Thermus* (Moreira et al., 1995). As a result of the plasmid DNA isolation by alkaline lysis method, a

single band was observed for each of the strain (*H.salinarium* TG13, *H.salinarium* S9, *H.salinarium* DSM3754) in the gel electrophoresis (Figure 3.22). The single band was thought covalently closed circular DNA (CCC). The calculation of the plasmid size for plasmids >20kb is not accurate, when traditional electrophoresis is used (Moreira et al., 1995). Since most of the plasmid had target sequences for *Hind*III and *Pst*I, these restriction endonucleases were chosen for enzyme digestion reaction of the plasmids of the strains. To compare differentiation of plasmids among the strains, each of plasmid of the strains were digested with *Hind*III and *Pst*I. Electrophoretic separation of *Hind*III and *Pst*I fragments of plasmids of the strains has showed difference among the strains (Figure 3.23). Although, low amount of DNA was used in the literature for RFLP analysis (Pfeifer et al. 1981), in our experiment, 30 μg of sample of DNA was needed for comparable enzyme digestion pattern. The similarity of restriction fragments size may reflect homology of DNA sequences of plasmids. The *Hind*III and *Pst*I restriction fragment profiles were analyzed by computer software (Bio-Profil, Bio-1D V99.04 Vilber Lourmat) (APPENDIX H). The *Hind*III and *Pst*I restriction profiles of plasmid DNA isolated from strain TG13, S9, DSM3754 had an extensive restriction fragment length polymorphism, although some of the fragments appear to have similar molecular weights.

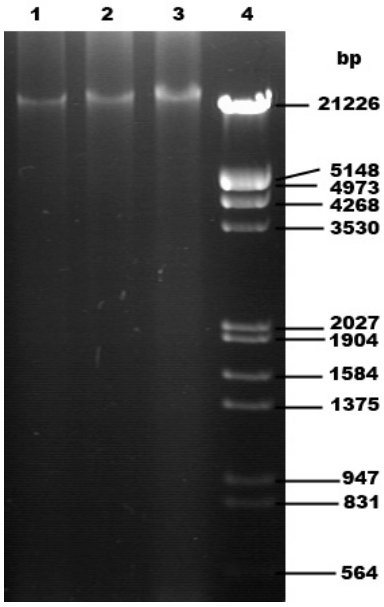

Figure 3.21 Undigested plasmid DNA profile of the strains. Lane 1 *H.salinarium* TG13, Lane 2 *H.salinarium* S9, Lane 3 *H.salinarium* DSM3754, Lane 4 Marker.

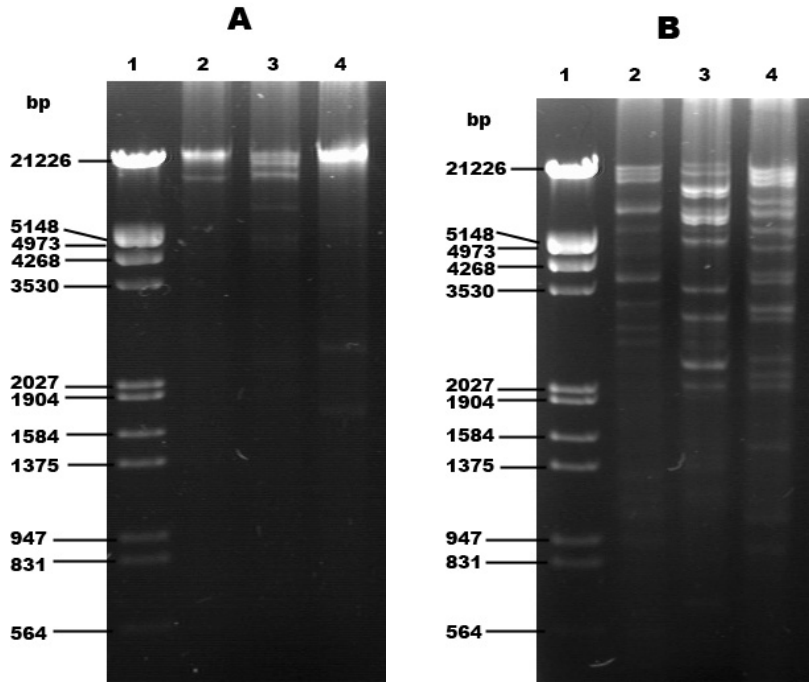

Figure 3.22 RFLP Results. A: Agarose gel electrophoretic separation of HindIII fragments of plasmid DNA from the strains. Lane 1 Marker, Lane 2 *H.salinarium* TG13, Lane 3 *H.salinarium* S9, Lane 4 *H.salinarium* DSM3754. B: Agarose gel electrophoretic separation of PstI fragments of plasmid DNA from the strains. Lane 1 Marker, Lane 2 *H.salinarium* TG13, Lane 3 *H.salinarium* S9, Lane 4 *H.salinarium* DSM3754.

3.3.3 Comparative Analysis of the Results

The results of both biochemical and genetic analysis of *H. salinarium* strains were compared and summarized in Table 3.1

Table 3.1 Summary of biochemical and genetic analysis results.

Analysis	New Strain (*H.s.* TG13)	*H.s.* S9	*H.s.* DSM3754
Growth	5 days	5 days	5 days
Color of cells	reddish purple	pink	purple
Shape of cells	rod	rod	rod
Gram Staining	gram negative	gram negative	gram negative
Gelatin	+	+	+
Total Protein Profile	Show difference	Show difference	Show difference
Photoactivity of bR (ΔpH_{max})	0.09	0.04	N/A
λ_{max} of pure PM	563 nm	567 nm	N/A
M.W. of bR	22 kD	22 kD	N/A
pI value of bR	5.4	5.4	N/A
RFLP[a]	Show difference	Show difference	Show difference
RAPD-PCR	Show difference	Show difference	Show difference

[a] HindIII and PstI were used as restriction endonucleases.

CHAPTER IV

CONCLUSION

Isolated strain TG13 from Tuz Lake in central Anatolia has characterized by biochemical and genetic techniques and compared with reference *Halobacterium salinarium* strain. The strain was grown successfully both on solid and in liquid medium of *Halobacterium salinarium* at 4 M NaCl. The color of the strain was observed reddish-purple. The strain has rod shape and it is motile. Gram staining has shown that the strain is of gram negative type. It has also gelatin hydrolyzing ability. The only protein band found in 1D SDS-PAGE of purple membrane fragment of the strain was corresponded to 22 kD. The spectroscopic analysis of purple membrane has resulted in maximum absorption at 280 nm and 563 nm. The p*I* value of the protein was found as 5.4. The protein has also shown proton pump activity upon illumination. Findings on the protein isolated from TG13 strain demonstrate that it is bacteriorhodopsin. Total protein, RAPD-PCR and RFLP analyses have shown that the *Halobacterium salinarium* TG13 is different strain from reference strains (*H.s.* S9 and *H.s.* DSM3754). As a result of genetic and biochemical analyses, it has been concluded that the isolated strain TG13 from Tuz Lake might be a different *Halobacterium* strain.

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APPENDIX A

Growth of Halobacterium salinarium strains

The growth of *Halobacterium salinarium* in a flask was carried out in a shaking incubator at 39 °C with 120 rpm under overhead illumination of 150-Watt lamb (section 2.2.1). The growth was followed spectrometrically (section 2.2.3.3). The experimental data are given in Table A.1 to A.3.

Table A.1 Growth of *Halobacterium salinarium* TG13

Time (h.)	OD660
0	0,000
26	0,128
36	0,256
57	0,525
82	0,724
130	0,777
169	0,671

Table A.2 Growth of *Halobacterium salinarium* S9

Time (h.)	OD660
0	0,000
24	0,029
49	0,298
74	0,506
97	0,711
122	0,942
170	0,829

Table A.3 Growth of *Halobacterium salinarium* DSM3754

Time (h.)	OD660
0	0,000
25	0,017
49	0,310
74	0,685
97	1,121
122	1,156
170	0,968

APPENDIX B

Photoactivity Measurements

The photoactivity measurements of PM fragments of *Halobacterium salinarium* strains were carried out as described in section 2.2.4.8. The experimental data are given in Table B.1 to Table B.2.

Table B.1 Photoactivity result of pure PM fragments isolated from *H. salinarium* TG13 (5 μM bR, 4 M NaCl, 25 °C, 1000 Watt-lamb at 30 cm distance).

Time (min.)	pH	Light
0	5,76	ON
1	5,75	
2	5,74	
3	5,73	
6	5,72	
8	5,71	
10	5,70	
13	5,69	
17	5,68	
22	5,67	
41	5,67	
50	5,68	
60	5,69	
61	5,70	OFF
62	5,71	
69	5,72	

Table B.1 (Continued)

Time (min.)	pH	Light
77	5,73	OFF
89	5,74	
100	5,75	
110	5,76	
120	5,76	

Table B.2 Photoactivity result of pure PM fragments isolated from *H. salinarium* S9 (5 μM bR, 4 M NaCl, 25 °C, 1000 Watt-lamb at 30 cm distance).

Time (min.)	pH	Light
0	5,77	ON
1	5,76	
5	5,75	
9	5,74	
25	5,73	
40	5,73	
60	5,73	
61	5,74	OFF
65	5,74	
70	5,74	
80	5,74	
90	5,75	
100	5,75	
110	5,76	
120	5,76	

APPENDIX C

SDS-PAGE Stock Solutions, Sample Buffer and Gel Formation

10% SDS SOLUTION

Dissolve 5 g SDS in 50 ml dH_2O with gentle stirring.

SAMPLE BUFFER

3.8 ml dH_2O
1 ml 0.5 M Tris-HCl, pH 6.8
1.6 ml glycerol
1.6 ml 10%SDS
0.4 ml ß-mercaptoethanol
0.4 ml 0.05% (w/v) bromophenol blue

30% ACRYLAMIDE/BIS (200 ml)

During preparation of this solution precautions should be made by wearing gloves and mask (acrylamide is a nerve toxin)

Weigh 58.4 g acrylamide in 500 ml beaker
Add 1.6 g N'N'-bis-methylene-acrylamide
Pour some 150 ml dH_2O into the beaker, cover the beaker with Aluminium foil and mix on a magnetic stirrer.

When complete dissolution is achieved, complete the volume to 200 ml by adding dH_2O.
Store at 4°C in the dark. (Maximum shelf life of this solution is 30 days.)

1.5 M Tris-HCl BUFFER, pH 8.8 (300 ml):

Weigh 54.45 g Tris base and dissolve in 150 ml dH_2O.
Stir by magnetic stirrer and adjust pH to 8.8 with 10 N HCl.
Complete the volume to 300 ml with dH_2O and store at 4°C.

0.5 M Tris-HCl BUFFER, pH 6.8 (100 ml):

Weigh 6 g Tris base and dissolve in 60 ml dH_2O.
Stir by magnetic stirrer and adjust pH to 6.8 with 10N HCl.
Complete the volume to 100 ml with dH_2O and store at 4°C.

5x STOCK RUNNING BUFFER (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3)

Weigh 15 g Tris base and 72 g glycine and dissolve in 1 Lt dH_2O.
Do not adjust the pH. Store at 4°C.

1x WORKING RUNNING BUFFER

Pour 600 ml of 5X stock buffer solution into the electrophoresis tank and dilute it with dH_2O to total 3 Lt.
Stir with magnetic stirrer and gently add 3 g SDS. Place the tank in refrigerator or cold room and provide a gentle stirring on magnetic stirrer.

10% APS (should be prepared freshly)

Weigh 0.06 g APS in an Eppendorf tube and dissolve in 600 µl dH_2O by vortex mixing.

PREPARATION OF SDS-PAGE GEL SOLUTION (12%)

Separating Gel

40 ml of 30% acrylamide/bis
33.5 ml dH2O
25 ml 1.5 M Tris-HCl, pH 8.8
1 ml of 10% SDS solution
500 µl of 10% APS
50 µl TEMED

Stacking Gel

1.3 ml of 30% acrylamide/bis
6.1 ml dH2O
2.5 ml 0.5 M Tris-HCl, pH 6.8
100 µl of 10% SDS solution
50 µl of 10% APS
10 µl TEMED

APPENDIX D

Molecular Weight Marker for SDS-PAGE

STANDARD M.W. MARKER SOLUTION

Prepare separate marker solutions in Eppendorf tubes according to the following table

MW marker	MW	mg of marker	Sample buffer (ml)
Bovine albumin	66,000	5	1.4
Egg albumin	45,000	5	1
Glyceraldehyde-3-P DH	36,000	1	0.300
Carbonic anhydrase	29,000	1	0.400
Trypsinogen	24,000	1	0.200
Trypsin inhibitor	20,100	1	0.200
α-Lactalbumin	14,200	1	0.400

WORKING STANDARD M.W. MARKER SOLUTION

Add 5 µl of each marker into an Eppendorf tube and mix by vortex. Boil for 60 seconds and dilute 1:1 with sample buffer. Keep at -20°C for further use. (Several such tubes can be prepared at the same time and stored at -20°C)

APPENDIX E

Comparison of the protein profiles of *H. salinarium* strains

Total protein profile of *Halobacterium salinarium* strains was analyzed by by using computer software (Gel-Pro Analyzer V3.1). The results are given in Table E.1

Table E.1 Comparison of molecular weight of the protein bands showed in the Figure 3.8. The bands was matched with ± 2% differences. (Lane 1: Marker proteins, Lane 2: *H.salinarium* TG13, Lane 3: *H.salinarium* S9, Lane 4: *H.salinarium* DSM3754, Lane 5: *H.salinarium* L33, Lane 6: Pure purple membrane isolated from *H.salinarium* TG13.)

	Lanes					
Rows	**1**	**2**	**3**	**4**	**5**	**6**
r1			91.315	91.603	91.315	
r2		89.877	90.164	90.74	90.164	
r3			88.438		88.726	
r4			86.712	87	87	
r5		85.562	82.973			
r6		81.247	81.822	82.685	82.11	
r7		78.658	78.658	79.808	78.945	
r8		76.644	76.932	77.795	77.219	
r9		75.781	74.055		74.342	
r10		73.767	71.178	71.753	71.753	
r11		70.315	69.452	69.452	69.74	
r12			67.151	67.151	67.438	

Table E.1 (Continued)

	Lanes					
Rows	**1**	**2**	**3**	**4**	**5**	**6**
r13	66	66.288	66		66.288	
r14		63.411	61.973		62.26	
r15		60.534	60.822	60.822		
r16		57.37				
r17		55.356	55.356	55.356	55.644	
r18			53.055		53.342	
r19			51.329	51.616	51.904	
r20		48.164	48.164	48.452	48.452	
r21	45	44.063	43.875	43.125	44.25	
r22			42.188	41.813	42.375	
r23		41.438	40.125	40.125	40.313	
r24		39.75	38.25		38.438	
r25	36	35.881	36.75	36.563	36.75	
r26		33.153	34.102	33.864		
r27			32.085	32.203	32.203	
r28		31.254		31.373		
r29	29	28.8	29.237	29.356	29.356	
r30	24		24.6	24.6	24.2	
r31			23.776	23.686	23.866	
r32				22.969		
r33		22.341	22.61	22.521	22.61	22.521
r34				20.459		
r35	20.1				19.613	
r36				18.762		
r37				17.059	16.937	
r38		15.66		16.146		
r39	14.2					
r40				11.524	11.524	

APPENDIX F

Isoelectric Focusing Solution

30% ACRYLAMIDE/BIS (200 ml)

During preparation of this solution precautions should be made by wearing gloves and mask (acrylamide is a nerve toxin)

Weigh 58.4 g acrylamide in 500 ml beaker
Add 1.6 g N'N'-bis-methylene-acrylamide
Pour some 150 ml dH_2O into the beaker, cover the beaker with Aluminum foil and mix on a magnetic stirrer.
When complete dissolution is achieved, complete the volume to 200 ml by adding dH_2O.
Store at 4°C in the dark. (Maximum shelf life of this solution is 30 days.)

ISOELECTRIC FOCUSING GEL SOLUTION (IEFGS)

During preparation of this solution precautions should be made by wearing gloves since urea and acrylamide/bis solution are both toxic.

Weigh 10 g urea in a small (100 ml) beaker.
Add 0.3 g CHAPS.
Pour 7.4 ml dH_2O.
Add 3 ml 30% Acrylamide/bis solution.

Mix the solution on a magnetic stirrer.
Add 200 µl of ampholine (pH 5-8) and 800 µl of ampholine (pH 3-10)
Add 100 µl Nonidet P-40 (cut approx. 3 mm of the tip of a plastic tip so that the viscous detergent can be poured easily).
Transfer the solution into a vacuum flask and degas it for a few minutes until the bubbles are completely disappeared.
Transfer the solution into a 25 ml beaker.
Place a filter in the metal tip of the Millipore assembly.
Collect approximately 5 ml of this solution into an empty plastic syringe and connect the metal tip to the syringe outlet.
Filter the IEFGS into Eppendorf tubes.
From time to time, change the filter inside the metal tip.
The IEFGS can be stored at -20°C for further use.

2.5% APS (should be prepared freshly):

Weigh 1.3 mg APS in an Eppendorf tube and dissolve in 52 µl dH_2O by vortex mixing.

CATHOLYTE, 20 mM NaOH SOLUTION (UPPER SOLUTION)

Weigh 0.4 g NaOH and dissolve in 500 ml dH_2O.
Transfer the solution to a vacuum flask and degas it for a few minutes.
Keep at refrigerator.

ANOLYTE (LOWER SOLUTION)

Pour 3 Lt dH_2O into electrophoresis tank.
Add 3 ml of 85% phosphoric acid.
Keep at refrigerator or cold room and provide stirring on a magnetic stirrer (the lower solution should be constantly stirred throughout the isoelectric focusing).

APPENDIX G

Silver Staining of Proteins in Polyacrilamide Gels

The preparation of stock solution of silver staining is given in Table G.1. The staining procedure is given in Table G.2.

Table G.1 Stock solution of silver staining.

SOLUTION	PREPARATION STEPS
Fixer (this soln. can be used several times)	150ml MeOH 36ml Acetic acid 150µl 37%Formaldehyde → complete to 300ml with dH_2O
50% EtOH	600ml pure EtOH 600ml dH_2O
Pretreatment soln.	0.08 g $Na_2S_2O_3.5H_2O$ 400ml dH_2O → mix with a glass rod → take 8 ml and set aside
Silver nitrate soln.	0.8 g silver nitrate 400ml dH_2O 300µl 37%Formaldehyde
Developing soln.	400ml dH_2O 9g potassium carbonate 8ml from pretreatment soln. 300µl 37%Formaldehyde
Stop soln.	200ml MeOH 48ml Acetic acid → complete to 400ml with dH_2O

Table G.2 Staining of Polyacrilamide gels by silver stain.

	STEP	SOLUTION	TIME OF TREATMENT	COMMENTS
1	Fixing	Fixer	**> 1 hr**	Overnight incubation is all right
2	Washing	50% EtOH	**3 X 20 min.**	2^{nd} & 3^{rd} soln. Should always be fresh
3	Pre-treatment	Pretreat soln.	**1 min.**	**Time should be exact**
4	Rinse	dH_2O	**3 X 20 Sec.**	**Time should be exact**
5	Impregnate	Silver Nitrate soln.	**20 min.**	
6	Rinse	dH_2O	**2 X 20 Sec.**	**Time should be exact**
7	Developing	Developing soln.	**~ 10 min.**	**After a few minutes, add some dH_2O to proceed the reaction slowly. Time should be determined by observation of color development**
8	Wash	dH_2O	**2 X 2 min.**	
9	Stop	Stop soln.	**> 10 min.**	**The gels can be kept in this soln. Overnight.**

APPENDIX H

*Hind*III and *Pst*I fragments of plasmid DNA of the strains.

The size of of *Hind*III and *Pst*I fragments of plasmid DNA isolated from the strains (*H. salinarium* TG13, *H. salinarium* S9, *H. salinarium* DSM3754) was estimated by using computer software (BioProfil Bio-1D V99.04 Vilber-Lourmat). The results are given in Table H.1 and H.2.

Table H.1 Agarose gel electrophoretic separation of HindIII fragments of plasmid DNA from the strains. Lane 1 Marker, Lane 2 H.salinarium TG13, Lane 3 H.salinarium S9, Lane 4 H.salinarium DSM3754.

	MW-RF			
	L1	L2	L3	L4
1	21.226	22.558	22.558	22.025
2	4.973	16.659	20.871	2.567
3	4.268		17.696	1.766
4	3.530		9.813	
5	2.027		5.168	
6	1.904		4.777	
7	1.584			
8	1.375			
9	0.947			
10	0.831			
11	0.564			

Table H.2 Agarose gel electrophoretic separation of PstI fragments of plasmid DNA from the strains. Lane 1 Marker, Lane 2 *H.salinarium* TG13, Lane 3 *H.salinarium* S9, Lane 4 *H.salinarium* DSM3754.

	MW-RF			
	L1	L2	L3	L4
1	21.226	22.067	22.627	21.226
2	4.973	20.479	20.852	19.733
3	4.268	18.619	15.360	17.882
4	3.530	11.065	9.068	12.640
5	2.027	6.941	5.700	10.468
6	1.904	3.905	3.565	7.588
7	1.584	3.322	3.071	6.740
8	1.375	2.871	2.324	4.973
9	0.947	2.678	2.057	4.065
10	0.831			3.827
11	0.564			3.230
12				3.093
13				2.399
14				2.184
15				2.057
16				1.523