1991

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DOI: <https://doi.org/10.31979/etd.49kn-22we>
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A comparative study of nitrate reductase in the Halobacteriaceae

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San Jose State University, 1991

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**A COMPARATIVE STUDY OF NITRATE REDUCTASE
IN THE HALOBACTERIACEAE**

**A Thesis
Presented to
the Faculty of the Department of
Biological Sciences (Microbiology Area)
San Jose State University**

**In Partial Fulfillment
of the Requirements for the Degree
Master of Arts**

**By
Nina Mushell
December, 1991**

Approved for the Department of Biological Sciences
(Microbiology Area)

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Approved for the University

ABSTRACT

A COMPARATIVE STUDY OF NITRATE REDUCTASE IN THE HALOBACTERIACEAE

By Nina Mushell, Major Professor: Dr. Robert D. Haight

Most enzymes from extremely halophilic bacteria exhibit salt-dependent properties. Nitrate reductase, a dissimilatory enzyme, appears to be an exception; when studied in *Haloferax denitrificans*, it was independent of salt concentration for its activity and stability. This investigation was conducted to establish whether the salt-independence of dissimilatory nitrate reductase is an exception for *H. denitrificans* or a general rule in halobacteria. For this purpose, the properties of nitrate reductase in *Haloferax mediterranei* and *Halobacterium larsenni* were studied and compared with *H. denitrificans*. The membrane-bound enzyme was produced under limited oxygen conditions and in the presence of nitrate, the two distinguishable characters of dissimilatory nitrate reductases. Membranes prepared from these cultures catalyzed the production of nitrite, was inhibited by azide and chlorate competitively, and exhibited salt-independent properties. Nitrate protected against the inhibition effect of cyanide, and when cyanide inhibition occurred, it was noncompetitive. Thus it appears that salt-independence of dissimilatory nitrate reductase may be typical in the Halobacteriaceae.

ACKNOWLEDGMENTS

I wish to express my appreciation to Dr. L. I. Hochstein for the suggestion of the project, the opportunity of working in his laboratory, and his advice during the course of my study; to Dr. R. D. Haight for his helpful advice and continued support; to Dr. A. C. Brinegar and Dr. R. G. Fowler for a critical review of the manuscript; to Dr. A. Mandel for being a wonderful teacher and friend; to my family who I love the most, especially my sister, and to the United States of America for being the land of opportunities.

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INTRODUCTION

When exploring life in extreme environments, one can choose bacteria as the key organisms to study. The ability of microorganisms to adapt to extreme environments has long been of interest to microbiologists. A variety of these organisms are found in different type of environmental conditions on Earth, including hot springs, the polar regions, deserts, salt ponds, acid or alkaline waters, and many others. These organisms are important tools for studying many areas of microbial physiology and changes in their structures as a result of adaptation to an external parameter.

Archaeobacteria tend to be found in "extreme" habitats. The archaeobacteria that live in concentrated salt solutions, and are the most highly adapted are the extremely halophilic species of *Halobacterium* . These bacteria are found in waters of high salinity, under conditions of high light intensity and low oxygen tension. Many of their unusual biochemical features are related to either the presence of concentrated salt or the adaptation to light and low oxygen tension. They can be studied to understand the kind of adaptations necessary for the life of bacteria in extreme environment. Halobacteria also carry many interesting implications for their evolution.

The presence of large amounts of salt, especially sodium chloride (NaCl), is known to inhibit the growth of most familiar microorganisms, but the halobacteria have adapted successfully. These organisms require NaCl concentrations above 10% (1 to 2 M) for survival, and optimal growth is often

obtained above 20%. The intracellular cation concentrations of these organisms approximate that of the medium and enable the organisms to overcome the osmotic problems caused by high concentrations of salt (Christian and Walto, 1962; Matheson *et al*, 1976).

The deleterious effects of monovalent salts at high concentrations on biological macromolecules have long been noted (Jencks, 1969; von Hill and Schleich, 1969) and seem to be caused primarily by disruption of ionic linkages. The mechanisms by which cellular components of halobacteria tolerate such high intracellular salt concentration is not understood.

In general, the properties of specific enzymes of extreme halophiles are found to be stable in the presence of at least 1M salt, and they may even require salt at concentrations above this value (Brown, 1964a; Soo-Hoo and Brown, 1967). Thus, rather than being destroyed at high salt concentrations, the macromolecular structures in halophiles appear to be dependent on the presence of salts. The enzymes from halophilic organisms usually require high salt concentrations for both stability and activity. Recently, an unusual and unexpected property of nitrate reductase from an extreme halophile, *Haloferax denitrificans*, was discovered: salt-independence of both activity and stability (Hochstein, unpublished data).

This investigation was conducted to establish whether the salt-independence of this enzyme is an exception for *Haloferax denitrificans* or a general rule in halobacteria. For this purpose the properties of nitrate reductase in *Haloferax*

mediterranei and *Halobacterium larsenni* (Baja 12) were studied and compared with *Haloferax denitrificans*. The induction pattern of nitrate reductase, the activity and stability of the enzyme in the presence and absence of various concentrations of salt, and the inhibition effects of azide, chlorate, and cyanide on the reduction of nitrate were determined.

LITERATURE REVIEW

I. HALOBACTERIA: THEIR ADAPTATION TO HIGH SALT CONCENTRATIONS

A. Taxonomy of the Halobacteriaceae

Nonalkalophilic and alkalophilic halophilic bacteria both belong to the family Halobacteriaceae. The review by Juez (1988) on the taxonomy of the Halobacteriaceae includes the four currently accepted genera of nonalkalophilic halobacteria: *Halobacterium*, *Halococcus*, *Haloarcula*, and *Haloferax*.

In addition to the halophilic archaeobacteria, there are halophilic eubacteria and halophilic eukaryotes. The archaeobacterial halophiles concentrate inorganic ions such as K^+ , Na^+ , and Cl^- intracellularly (Christian and Walto, 1962; Masui and Wada, 1973). The halophilic eubacteria and eukaryotes accumulate, at least to some extent, organic neutral compatible solutes instead of the inorganic salts. Examples of compatible solutes used by various halophilic or halotolerant organisms include glutamate, proline, choline, betaine, polyamines, and glycerol (Rodriguez-Valera *et al*, 1981; Kushner and Kamekura, 1988).

B. General Characteristic of Rod-shaped Halobacteriaceae

The rod-shaped nonalkalophilic Halobacteriaceae include three different genera: *Halobacterium*, *Haloferax*, and *Haloarcula* (Juez, 1988; Torreblanca *et al*, 1986).

The genus *Halobacterium* are extremely halophilic in their requirements for optimal growth and for maintenance of cell stability. They are motile well-defined rods and often gas vacuolated. They form red strongly pigmented colonies which are small and never mucous. They are restricted metabolically, requiring amino acids as growth factors and utilizing a narrow range of substrates. These organisms can be isolated from concentrated salt ponds and solar salterns. Three of the species included in this genus are *H. larsenni*, *H. marismortui*, and *H. salinarium*. *H. salinarium* now includes strains previously described as *H. halobium* and *H. cutirubrum*.

The genus *Haloarcula* forms small, orange or red strongly pigmented colonies. They are able to grow without added amino acids as growth factors, and a variety of different substrates can be used as carbon and energy sources. The salt requirements for growth and for the prevention of lysis are intermediate. The organisms in this genus grow on media with a wide range of salt concentrations and they are found at many geographic sites. They have been isolated from the Dead Sea, Death Valley, and California salterns. Two of the species included in this genus are *H. hispanica*, and *H. vallismortis*.

The genus *Haloferax* includes organisms which are very pleomorphic. The colonies are large, mucous and pigmented (more pigmented at lower salt concentrations). Their response to salt is moderate, with optimal growth taking place in the range of 2.5 to 3 M NaCl. Metabolically they are versatile, not requiring growth factors and utilizing a wide range of substrates. Four of the species included in this genus are *H. denitrificans*, *H. gibbonsii*, *H. volcanii*, and *H. mediterranei*.

C. Internal Concentrations of Cations

Extremely halophilic bacteria require 20-30% (3-4 M) NaCl for optimal growth. In direct measurements of the internal concentrations of salt in *H. salinarium*, grown on a medium containing 3.33 M NaCl and 0.05 M KCl, the internal concentration of chloride ions was similar to that in the medium, while the total concentration of monovalent cations was significantly greater, being 0.8 M in Na⁺ and 5.3 M in K⁺ (Christian and Walto, 1962; Matheson *et al*, 1976). These data show that the ionic concentration inside the cells of this bacterium is similar to that outside, a condition that is thought to prevent osmotically driven cell dehydration. The predominant ion inside is not Na⁺ but K⁺, and the cells concentrate K⁺ in very high amounts from the medium. It has been argued (Brown, 1976), from the observation of the relative preference of many of the *Halobacterium* proteins (particularly ribosomal proteins) for KCl, that the tendency of the cells to accumulate K⁺ and exclude Na⁺ is explained by the need to maintain optimal intracellular conditions.

It has been argued (Skulachev, 1978) that the gradients of K^+ and Na^+ achieved across the cell membrane represent energy reservoirs of large capacity; indeed, the gradient of Na^+ (out > in) which forms in *H. halobium* has been shown to drive active transport systems for all nutritionally important amino acids. It has been suggested that the large amounts of energy expended by the extremely halophilic bacteria to establish cation gradients is part of the salt-tolerance mechanism in these organisms.

D. Cell Envelope and Cell Membrane

The cytoplasm of *Halobacterium halobium* and closely related halobacteria is surrounded by two layers, a cytoplasmic membrane and an external layer (sometimes called the "cell wall") that is about 60-150 Å thick, composed of a hexagonal array of glycoprotein particles, and contains no muramic acid, diaminopimelic acid, D-amino acids, or teichoic acids (Kushner, 1978; Stoeckenius and Rowen, 1967).

The cell envelope and outer membrane of halobacteria are exposed to high salt levels at all times. When the NaCl concentration of the medium is reduced, the halophilic rod-shaped cells become spherical. If the concentration is lowered further the cells lyse. This is not a direct osmotic effect but is due to the fact that the cell wall of *Halobacterium* is stabilized by Na^+ ions. When there is insufficient Na^+ the wall breaks and the cell lyses (Kushner, 1964). This requirement for high levels of monovalent salts (or somewhat lower levels of

divalent salts) appears to be due mainly to the acidic nature of the envelope proteins. Furthermore, the surface charge density is increased by acidic groups on the membrane lipids. It would seem that these excess negative charges are shielded by the presence of high concentrations of cations (Brown, 1964b).

When the membranes of halobacteria grown aerobically were dialyzed against distilled water, small red membrane fragments were recovered. In cells grown under conditions of high light intensity and low oxygen tension, patches of purple membrane develop within the red membrane (Stoeckenius, 1976). These purple patches are bacteriorhodopsin, a carotenoid analogous to rhodopsin, the pigment of the animal eye, which functions in vision (Oesterhelt and Stoeckenius, 1971; Kushwaha *et al*, 1975). Bacteriorhodopsin is able to absorb light and generate a proton gradient across the membrane, leading to ATP synthesis. At the high salt concentration at which halobacteria grow, oxygen solubility is very low, so that oxygen deficiencies can frequently develop. Rhodopsin-mediated ATP synthesis enables the organism to synthesize ATP under anoxic conditions which permits the organism to survive in nature when oxygen is not available (Oesterhelt, 1973; Danon and Stoeckenius, 1974). In addition, in the intense sunlight of their environment, halobacteria are probably protected to some extent against UV damage and photodynamic oxidation by these carotenoid pigments.

E. Proteins

Because of the high internal salt concentration, not only the cell envelope but also the internal macromolecules of the archaeobacterial halobacteria must have adapted to an environment of concentrated salt. One major class of macromolecules that shows such adaptation is the proteins. Enzymes of halobacteria tolerate high salt concentrations. Moreover, for many enzymes as well as for protein-containing structures like ribosomes in *Halobacterium*, high concentrations of monovalent cations are in fact essential for both structural integrity and biological activity (Lanyi, 1974). However, the optimal levels of cations for structural integrity and biological activity might be different for a particular protein. Although different kinds of salt dependent behavior are exhibited by halobacterial enzymes, they always show considerable enzyme activity even at very high concentrations of salt. The salt response of enzymes from halophilic bacteria are highly variable. Little or no activity at NaCl concentrations below 0.1 M have been reported for some halophilic enzymes. These enzymes are aspartate transcarbamylase (Lieberman and Lanyi, 1969), nicotinamide adenine dinucleotide (NADH) menadione reductase (Lanyi, 1969a; Lanyi, 1969b), cytochrome oxidase (Lieberman and Lanyi, 1972), polynucleotide phosphorylase (Peterkin and Fitt, 1971), citrate synthase (Cazzulo, 1973), and malic enzyme (Cazzulo and Vidal, 1972) from *H. cutirubrum*. These enzymes exhibited maximal activity between 2 to 4 M NaCl or KCl, but several were inhibited at the highest salt concentration tested.

Another class of enzymes, including malic dehydrogenase (Holmes and Halvorson, 1965), ornithine transcarbamylase and arginine desmitase (Dundas and Halvorson, 1966) of *H. salinarium*, and threonine deaminase from *H. cutirubrum* (Lieberman and Lanyi, 1972) showed maximal activity between 0.5 and 1.5 M NaCl, both being inhibited at higher salt concentrations.

Finally, some enzymes from halophilic bacteria do not require salt or are inhibited by low concentrations, such as fatty acid synthetase (Pugh *et al*, 1971), and RNA-dependent RNA polymerase (Louis and Fitt, 1971) of *H. cutirubrum* and amylase (Good and Hartman, 1970) of *H. halobium*.

Other enzyme properties are also salt dependent. Salt concentration and the allosteric regulation of threonine deaminase from *H. cutirubrum* by ADP, for example, are related (Lieberman and Lanyi, 1972). In addition, it has been found that the subunit association of some halophilic enzymes is dependent on salt (Brown, 1976). There are no satisfactory explanations for observed effects of salt or for the role of cations in enzyme stability and activity. Over all, the proteins of halobacteria are more acidic (acidic amino acids are negatively charged) than those of nonhalophilic bacteria. This is true of the total proteins, the ribosomal proteins, and of several isolated enzymes. It has been suggested (Brown, 1964a) that salts stabilize the enzymes by acting as a screen that shields negatively charged carboxyl groups on the outer surface of the enzymes from each other.

It was suggested that very high concentrations of NaCl or certain other salts might be needed to maintain hydrophobic interactions in proteins of extreme halophiles. Such interactions, normally very important in maintaining protein structure, are weaker than usual in halophilic proteins (Lanyi, 1974).

F. Ribosomes and *In Vitro* Protein Synthesis

The ribosomes of *H. cutirubrum* require high salt concentrations for stability. In addition to the salt requirement for their stability, the ribosomes of *H. cutirubrum* (and probably of the other halobacteria) are unique in their possession of large numbers of acidic proteins (Bayley and Kushner, 1964). To maintain stable 70S, 50S, and 30S forms, about 3M KCl and 0.1M Mg^{2+} are needed (Visentin *et al*, 1972). At low concentration of salt, e.g. 1mM of $MgCl_2$, which maintains *Escherichia coli* ribosome structure, most of the proteins are lost from the subunits of the halobacterial ribosomes (Strøm and Visentin, 1973).

Optimal *in vitro* protein synthesis in halobacteria takes place in the presence of 3.8M KCl, 1.0M NaCl, 0.4M NH_4Cl , and 0.04M Mg^{2+} . A number of halobacterial amino acyl-tRNA synthetases function best in 3.8M KCl. Once it was suggested that the adaptation may have been a direct consequence of the effect of salt in altering the reading of codons during translation. Studies with artificial messenger RNAs showed that codon assignments for the *H. cutirubrum* system were the same as in nonhalophiles (Bayley, 1976).

Synthesis of proteins in halobacteria is initiated by methionine, not N-formylmethionine. The initiating tRNA^{Met} and other tRNAs have a pseudouridine or modified pseudouridine instead of ribothymidine in the "common arm." Some of these characteristics resemble the protein synthesizing system in eukaryotic rather than prokaryotic cells (Bayley and Morton, 1978).

G. Genetics of Halobacteria

The halobacteria present as many surprises in their genetic properties as they have presented in their physiological and biochemical ones. It has long been known that these organisms contain large amounts of extra chromosomal ("satellite") DNA. For many species the chromosomal DNA has a G+C content of 66-68%, whereas that of the satellite DNA is 57-60%. The satellite DNA can account for 11-36% of the total DNA in some species (Moore and McCarthy, 1969). Recent studies have suggested that the extrachromosomal DNA may consist of plasmids (Pfeifer, 1988). Simon (1978) found that the presence of a plasmid was correlated with that of gas vacuoles in a *Halobacterium* species. In the absence of the plasmid, defective gas vesicles with different protein structure and organization were formed. Weidinger *et al* (1979) found a large plasmid, pHH1 of a size of 150kb, in *H. halobium* that contains genes coding for gas vacuoles and probably bacteriorhodopsin and bacterioruberin.

The spontaneous mutation frequency in *H. halobium* can be extraordinarily high, based on three phenotypic markers (purple membrane, bacterioruberin,

and gas vacuole synthesis) observable by changes in colony pigmentation on agar plates. Colorless mutants of *H. halobium* were found to arise with a frequency of about 10^{-3} . One of these mutants formed all transphytoene, which appears to be a common intermediate in bacterioruberin and retinal synthesis. Mutants deficient in gas-vacuole formation arose at a frequency of 10^{-2} ; those deficient in bacterioruberin, bacteriorhodopsin, and retinal, at a frequency of about 10^{-4} . Reversions were also observed but at somewhat lower frequencies. It was suggested that spontaneous mutations arose by insertions in chromosomal and plasmid DNA, and the possibility was considered that there might be "hot spots" in the plasmids such that one insertion could trigger other insertions. Indeed, restriction endonuclease digestion has shown that most genetic changes are accompanied by alterations in plasmid pHH1 (Weidinger *et al*, 1979; Pfeifer *et al*, 1981).

The frequency of genetic alterations suggested to Sapienza and Doolittle (1982a and b) that the *Halobacterium* genome may contain many transposable elements of regions of sequence homology promoting recombination in and between chromosomal and plasmid DNAs. These workers showed that the genomes of *H. halobium* and *H. volcanii* contain many repeated sequences. Repeated sequences are common in plant and animal cells and the halobacteria, thus far, seem to have the most complex repeated sequences of any prokaryote.

II. NITRATE REDUCTION

A. Denitrification and Nitrate Respiration

Denitrification is defined as the reduction of nitrate (NO_3^-) to gaseous products (Payne, 1973). The physiological significance of denitrification is the generation of ATP through anaerobic respiration: nitrate serves as the terminal electron acceptor in an electron transport chain. The branch point in the electron transport chain occurs at cytochrome *c*. Electrons are transferred to the terminal cytochrome oxidase a_3 and then to oxygen. In the absence of oxygen, electrons are transferred directly to nitrate from cytochrome *c*, a process which involves the participation of an iron-molybdenum complex (Fewson and Nicholas, 1961). Upon reduction of nitrate, other nitrogenous oxides are generated that also serve as terminal electron acceptors in the same or different electron transport chains. Therefore, denitrification is a series of anaerobic respiration processes through which nitrate is reduced to N_2 :

Each reductive step by itself is coupled to the generation of ATP and should be able to support growth (Koike and Hottori, 1975).

Some organisms can not carry out different steps of the pathway: (1) organisms capable of reducing nitrate only to nitrite; (2) organisms capable of

reducing nitrate to nitrous oxide; (3) organisms capable of reducing nitrate to nitrite and nitric oxide to nitrous oxide. The organisms in subcategory 1 do not produce a gaseous product; hence, they are not denitrifiers. This limited dissimilatory reduction of nitrate to nitrite is called nitrate respiration.

Nitrate reductase has been studied in several different higher plants and bacteria, including some strains of *Escherichia coli* (Nasoon, 1962; Taniguchi and Itagaki, 1960). The functional properties and physical characteristics of the enzymes found in different organisms vary widely. Nitrate reductase in *Escherichia coli* is principally membrane-bound, and it has been shown to catalyze the translocation of protons across the cytoplasmic membrane which is coupled to the oxidation of ubiquinol by nitrate (Garland *et al*, 1975).

This enzyme, which contains molybdenum, iron-sulfur centers, and cytochrome *b*, possesses three types of polypeptide subunits, α , β , and γ (approximate M_r 155000, 60000, 20000, respectively) (Vincent and Bray, 1978). The latter, smallest subunit is the apoprotein of cytochrome *b*, cytochrome *b*-556 NO_3^- . The enzyme, consistent with its proton-translocating ability, occupies a transmembranous location within the cytoplasmic membrane. The γ -subunit (a *b* type cytochrome) whose structural gene is part of the nitrate reductase operon, has been located at the periplasmic face of the membrane whereas the α and β -subunits are exposed at its cytoplasmic face. The γ -subunit is necessary for the oxidation of quinol by nitrate but not for the oxidation of reduced benzyl viologen. The α -subunit has been reported to be the catalytic

subunit on the basis that, when the α -subunit is cleaved by proteolysis, the enzyme activity (benzyl viologen to nitrate) is lost. However, the β -subunit can undergo proteolysis, to become substoichiometric, without loss of activity with benzyl viologen. The role of the β -subunit in the oxidation-reduction process is not known, but it is involved in membrane attachment (Ingledew and Poole, 1984; Boxer and Clegg, 1975).

The mutants lacking nitrate reductase activity have been isolated based on their ability to grow anaerobically in the presence of chlorate (Ruiz-Herrera *et al*, 1969). This resistance of these mutants is due to the lack of a functional nitrate reductase which is necessary to reduce the chlorate to the toxic species, chlorite. These mutants are usually designated *ChL*. The structural genes for nitrate reductase (α , β , and γ subunits) are organized in an operon at 27 minutes on the *E. coli* map (Bonney *et al*, 1981). The operon appears to be under the control of a regulatory gene, designated *nir* R and mapping at 29 minutes, which controls the expression of various anaerobic respiratory chain components.

B. Enzyme Kinetics and Inhibition Characteristics

The nitrate reductases associated with denitrification and respiration (i.e. dissimilation) are with one exception, membrane-bound enzymes (Burke *et al*, 1981). The dissimilatory enzymes from different organisms share many properties; they contain molybdenum, heme and nonheme iron, and acid-labile sulfur (Tiedge *et al*, 1985). The molybdenum cofactor has been identified in the

nitrate reductases from *Escherichia coli*, *Proteus mirabilis*, *Bacillus licheniformis*, *Rhodobacterium capsulatus*, and *Pseudomonas denitrificans* (Hochstein, 1988).

The apparent K_M for nitrate is affected by the reductant. When reduced viologen dyes are used, the values range from about 100 μM to 1.5 mM (Stouthamer, 1976).

Dissimilatory nitrate reductases are inhibited by azide, and the inhibition is competitive with respect to nitrate (Carlson, 1982). Nitrate reductase activity is also inhibited by cyanide. In *Escherichia coli*, the dithionite-reduced form of the enzyme is the most cyanide sensitive. Nitrate protects the dithionite-reduced enzyme against cyanide inhibition, even though the inhibition is presumably noncompetitive with respect to nitrate (Adams and Mortenson, 1982).

Apparently there are three enzymes in *Escherichia coli* that act on nitrate and chlorate. They differ in substrate specificity and in their sensitivity towards inhibitors. They are nitrate reductase A and B and chlorate reductase C. Nitrate reductase A always has a role in nitrate respiration. It is inhibited by azide and is a membrane bound enzyme. Chlorate is also a substrate for this enzyme. Nitrate reductase B is a soluble enzyme found in the cytoplasmic fraction. It is inhibited by chlorate and azide. Chlorate reductase C does not reduce nitrate. Chlorate is the substrate for this enzyme and azide the inhibitor. It is localized in both membrane and cytoplasmic fractions. Nitrate reductase A is induced

under anaerobic conditions in the presence of nitrate (Stouthamer, 1976). The properties differentiating these enzymes are given in Table 1.

The nitrate reductase from *Escherichia coli*, a type A nitrate reductase, reduces chlorate and bromate in addition to nitrate. The turnover numbers follow the order chlorate>nitrate>bromate in the presence of reduced methyl viologen (Stouthamer, 1976).

C. Evolution and Nitrate Respiration

Little is known about the role of nitrate in the evolution of bacterial energy-generating mechanisms. Denitrification occurs among a diverse number of eubacteria (Jeter and Ingraham, 1981). Denitrifying bacteria are commonly regarded to have evolved from nitrate-respiring bacteria. Whether denitrification is the precursor of aerobic respiration is still a question (Betlach, 1982).

The occurrence of nitrate respiration and denitrification in several halobacteria, archaebacteria isolated from salterns, has been shown (Mancinelli and Hochstein, 1986; Hochstein *et al*, 1984; Hochstein and Tomlinson, 1985). Some of their properties are suggestive of a kind of bacteria one might expect to have dominated the Earth some 3-4 billion years ago.

Although archaebacteria have undergone considerable changes since they originated, they can be studied to understand the evolutionary relationships between the nitrate respirers and denitrifiers.

Property	Enzyme		
	<u>Nitrate reductase</u>		<u>Chlorate reductase</u>
	A	B	C
Reduction of nitrate	+	+	—
Interaction with chlorate	Substrate	Inhibitor	Substrate
Inhibition by azide	+	+	—
Localization in cell-free extract	Cytoplasmic membrane	Cytoplasm	Cytoplasmic membrane

Stouthamer, 1976

TABLE 1

Differentiating properties of various enzymes that reduce nitrate and chlorate.

MATERIALS AND METHODS

I. BACTERIAL STRAINS AND CULTURE CONDITIONS

The organisms used in this study were *Haloferax denitrificans*, *Halobacterium larsenni* (Baja 12), and *Haloferax mediterranei* (strain R4). Baja 12 is an extremely halophilic denitrifying bacterium isolated from a Baja California saltern and deposited with the American Type Culture Collection. *H. mediterranei* was obtained from Dr. Brian J. Tindall (Institut Fur Mikrobiologie, Rheinische Friedrich-Wilhelms Universitat, Bonn, Germany). The membrane fraction of *H. denitrificans* was provided by Frank Lang from Dr. Hochstein's laboratory (NASA/Ames Research Center, Moffett Field, Mountain View, CA). *H. larsenni* and *H. denitrificans* were grown at 37°C in a complex medium (HYH-2) of the following composition per liter of deionized water: NaCl (176 g), MgCl₂·6H₂O (20 g), KCl (2 g), CaCl₂·2H₂O (0.1 g), HEPES(N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (13 g) (Aldrich Chemical Co., Milwaukee, WI), yeast extract (5 g) (Difco, Detroit, MI) and Hy-Case SF (2 g) (Humko-Sheffield Chemical Co., Norwich, NY). *H. mediterranei* was grown at 37°C in a complex medium (M-2) of the following composition per liter of deionized water: NaCl (200 g), MgCl₂·6H₂O (20 g), KCl (2 g), CaCl₂·2H₂O (0.2 g), Tris (12.1 g), yeast extract (5 g), Hy-Case SF (5 g), and glycerol (10 ml) (Hochstein and Dalton, 1968). The pH of HYH-2 was adjusted to 6.7 and M-2 to 7.4 before autoclaving for 15 minutes at 20 psi.

An inoculum was prepared by first growing the cells aerobically in a 250 ml Erlenmeyer flask containing 25 ml of medium while rotating the flask at 200 rpm

on a New Brunswick Gyrotory model G10-21 shaker at 37°C until the culture entered its stationary phase. The cells were then adapted to "anaerobic growth" (oxygen limiting condition) by next inoculating 70 ml of medium contained in a 500 ml Erlenmeyer flask using 7 ml of the aerobically grown cells and rotating the flask at 105 rpm at 37°C. At this speed the surface of the medium never broke but rather appeared to roll back and forth. These conditions were sufficient to initiate but not sustain aerobic growth as evidenced by the initial production of carotenoid pigments and the eventual accumulation of nitrite (Mancinelli and Hochstein, 1986; Hochstein and Tomlinson, 1985). Actively denitrifying cultures were thus obtained by growing cells under this "anaerobic" or oxygen limiting condition at 37°C in the complex medium to which potassium nitrate was added to a final concentration of 0.25%(w/v). As the culture entered the stationary phase, 20 ml was used to inoculate 1000 ml of medium with 0.25% nitrate added contained in a 2000 ml Erlenmeyer flask. The flask was incubated at 37°C under "anaerobic" conditions. The growth of cultures was determined turbidimetrically with a Klett-Summerson colorimeter fitted with a red filter (No. 66).

II. PREPARATION OF MEMBRANE FRACTIONS

Cell-free extracts were prepared from cells grown in 1 liter amounts of the complex medium. The cells were harvested when in stationary phase by centrifugation at 5,900 x g in a Sorvall SS-34 rotor for 30 minutes at 4°C, suspended in 20 mM HEPES buffer (20 mM HEPES, 3 M NaCl, 10 mM MgCl₂, 100 µM dithiothreitol, pH 7.4) so that the ratio of cells to buffer was 0.1 (wet

wt/vol), and then disrupted by passage through a French pressure cell operated at 5,000 psi. When the disrupted cell suspension became maximally viscous, a small quantity (1-2 mg) of powdered DNase I (Sigma, St. Louis, MO) was added and the mixture was stirred at room temperature (approx. 22°C) until the viscosity was negligible (about 30 minutes). Whole cells and debris were removed by centrifugation in a Sorvall SS-34 rotor at 5,900 x g for 30 minutes at 4°C. The supernatant (crude extract) was decanted and centrifuged in a Beckman 60 Ti rotor at 250,000 x g for 75 minutes at 4°C. The supernatant was decanted and designated as the cytoplasmic fraction and stored at 4°C. The sediment was made up in the 20 mM buffer described above and centrifuged in a Beckman 60 Ti rotor at 250,000 x g for 60 minutes at 4°C. The supernatant was discarded and the sediment was suspended in the 20 mM HEPES buffer to a protein concentration of approximately 20 mg/ml. This fraction, designated as the membrane fraction, was stored at 4°C.

III. ANALYTICAL METHODS

A. Enzyme Assays and Determinations

Nitrate reductase activity was assayed at 37°C and pH 7.0 in a reaction mixture that contained the following additions in a total volume of 2.5 ml: 50 mM TES buffer (2-Tris methyl amino-1-ethanesulfonic acid), pH 7.2 (2 ml); 1.0 M potassium nitrate (20 µl); 25 mM methyl viologen (20 µl); 230 mM sodium dithionite, dissolved in 10 mM NaOH (100 µl); water (360 µl); and enzyme. Sodium dithionite (pH 7.2) was prepared immediately before use by dissolving

80 mg of dithionite in 1.9 ml of NaOH (0.01 N) and 100 μ l NaOH (2N). A solution of 1M KNO₃ was prepared and 20 μ l was added to the reaction mixture to supply the 20 μ mole substrate requirement; similarly 20 μ l of 0.5 mM NaNO₂ solution was used as Standard (STD) (50 nmols nitrite). Formaldehyde (HCHO) at 0.75% was prepared by adding 2 ml of 37% HCHO to 98 ml of distilled water. The 4% (w/v) sulfanilamide was prepared in 9% HCl. To prepare the NED-Sulfa solution for daily use 0.08% NED (N-1-Napthylethylene diamine) and 4% sulfanilamide were mixed (1:1). In the enzyme assay, 2ml of 50 mM TES (pH 7.2), 20 μ l of 25 mM methyl viologen (Sigma Chemical Co., St. Louis, MO), and 100 μ l of 230 mM sodium dithionite were used. The total volume of 2.5 ml was achieved by addition of distilled water as required.

The reaction was initiated at 37°C by the addition of nitrate (following preincubation of the other components for 2 minutes without enzyme and for 1 minute with enzyme at room temperature) and terminated after 10 minutes by forcefully agitating the reaction mixture with a vortex mixer. This mixing resulted in the rapid oxidation of dithionite, as evidenced by disappearance of blue color of the reduced methyl viologen (within 15 sec), indicating that no reducing power was left in the solution. A typical enzyme assay was performed as follows:

After 10 minutes at room temperature, the absorbance was determined at 540nm with the Gilford StaStar Spectrophotometer (Showe and DeMoss, 1968).

For each set of assays, blanks and standards were set up. For the blank the membrane fraction was excluded from the reaction mixture, and for the standard the membrane fraction was replaced by nitrite (50 nmols).

B. Enzyme Unit and Specific Activity

A unit of nitrate reductase activity is that amount of enzyme which produces 1 μ mol of nitrite/min per milliliter of the membrane or cytoplasmic fraction after correcting for any nitrite produced in the absence of either enzyme or nitrate. The specific activity for nitrate reductase is defined as the units of enzyme per mg of protein.

C. Protein Determination

The total protein content of the extracts was measured at 37°C in the presence of sodium dodecylsulfate (at a final concentration of 1%, wt/vol) by the bicinchoninic acid (BCA) procedure (Smith *et al*, 1985) using bovine serum albumin (Fraction V) as the protein standard. Reagents for the BCA assay were obtained from Pierce (Rockford, IL). Bovine serum albumin was obtained from the Sigma Chemical Company (St. Louis, MO).

D. Requirements of Nitrate Reduction.

The enzyme activity of various volumes of membrane fractions was studied. The membrane fraction of *H. larsenni* was diluted 1:20 and that of *H. mediterranei* 1:50. For protein concentration of the membrane fractions refer to Table 3.

Enzyme activity of each membrane fraction was examined over a different range of preincubation periods (5 to 45 minutes) to identify the time within which changes in enzyme activity was linear and to determine possible time-dependent inactivation of the enzyme.

The requirement for the components of the enzyme activity assay was determined by performing the assay and measuring the enzyme activity in the absence of each component in the reaction solution.

E. Enzyme Kinetic Studies

To determine the value of K_m and V_{max} for nitrate, the normal nitrate reductase assay was performed while the amounts of nitrate were varied in the range of 1-16 mM. The K_m and V_{max} were determined using the Lineweaver-Burke plot.

F. Inhibition Studies

Solutions of KCN were made by adding potassium cyanide to the required volume of 50 mM Tris HCl and adjusting the pH to 7.2 with 1 N HCl under the

hood. The cyanide inhibition assay was optimized using different concentrations of cyanide and different preincubation times. For each preincubation time, two series of tubes were set up: one with and one without cyanide. Each test was conducted by incubation of a mixture of methyl viologen, dithionite, and membrane fraction with or without cyanide for a preincubation time of 5 to 35 minutes. The rest of the assay was performed the same as for the typical enzyme assay. For each test, the enzyme activity in the absence of cyanide was considered as 100% activity. When studying the inhibition effect of cyanide, the membrane fraction, potassium cyanide, sodium dithionite and methyl viologen were incubated for 15 minutes at room temperature before the 10 minutes incubation period at 37°C. The rest of the assay was typical. The inhibition effect of several concentrations of KCN was determined at nitrate concentrations of 1-16 mM.

The inhibition effect of chlorate was also determined since chlorate is known to be a substrate for nitrate reductase. Prior to inhibition assays, a set of assays were performed to study the effect of reagent addition order. These assays were performed as follows:

Variation in the order of addition of chlorate, nitrate, and membrane fraction caused no detectable change in inhibition rate. Therefore, chlorate and membrane fraction were added to the reaction mixture at the same time and the

reaction was started a minute later by the addition of nitrate. The inhibition effect of cyanide on this system in different concentrations was determined as described earlier.

Sodium azide inhibition was also studied. To first determine the amount of azide needed for inhibition, various amounts of azide, from 0-100 nmol, were added to the nitrate reductase activity assay and the mixture was preincubated for 2 minutes. Enzyme was then added and the mixture was again incubated for an additional minute. Nitrate was then added to start the reaction.

A Lineweaver-Burk plot showing the effect of inhibition was obtained for each inhibitor. Once the type of inhibition was established, the inhibition constant K_i was determined. To allow errors in individual determinations to be averaged out the K_m' value for a competitive inhibition (determined from the intercept on the $1/[S]$ axis of the Lineweaver-Burk plot) and the slope or interception for the noncompetitive inhibition were plotted against the inhibitor concentration $[I]$. The graphs were linear and the intercept on the $[I]$ gave the $-K_i$ values.

The concentrations of each inhibitor used for the determination of K_i were as follows:

ORGANISM	[KCN] mM	[NaClO ₃] mM	[NaN ₃] μM
<i>H. larsenni</i>	0, 0.25, 0.5	0, 1.0, 2.0	0, 1.25, 2.5
<i>H. mediterranei</i>	0, 0.2, 0.5	0, 1.6, 3.2	0, 1.25, 2.5

To study the effect of different concentrations of inhibitors on nitrate reduction a set of typical enzyme assays were performed using the following concentrations:

MEMBRANE FRACTION	[KCN] mM	[NaClO ₃] mM	[NaN ₃] μM
<i>H. larsenni</i>	0 to 1.5	0 to 120	0 to 100
<i>H. mediterranei</i>	0 to 1.5	0 to 30	0 to 100

G. Nitrate Protection Assay

The membrane fraction was preincubated in the presence and/ or absence of cyanide, dithionite, and nitrate:

1. M.F.	<u>15 min</u> ►	M.V. & S.D.	<u>2 min.</u> ► NO ₃ ⁻ <u>10 min.</u> ► Stop
2. M.F. & KCN	<u>15 min</u> ►	M.V. & S.D.	<u>2 min.</u> ► NO ₃ ⁻ <u>10 min.</u> ► Stop
3. M.F. & KCN & S.D.	<u>15 min</u> ►	M.V.	<u>2 min.</u> ► NO ₃ ⁻ <u>10 min.</u> ► Stop
4. M.F. & KCN & S.D.	<u>15 min</u> ►		<u>2 min.</u> ► M.V. <u>10 min.</u> ► Stop
5. M.F. & KCN & S.D. & NO ₃ ⁻	<u>15 min</u> ►		<u>2 min.</u> ► M.V. <u>10 min.</u> ► Stop

After 15 minutes incubation at room temperature the required components and/ or methyl viologen were added and incubated for 2 minutes. The reaction was then initiated by adding either the nitrate or viologen as needed, and the reaction was stopped after 10 minutes.

IV. THE SALT-DEPENDENCE STUDIES

A series of experiments were performed to determine the effect of salt concentration on the activity and the stability of nitrate reductase. To study the stability of the enzyme in different concentrations of salt, a series of 20 mM HEPES buffers (H buffers) were prepared containing salt concentrations ranging from 0 M to 4 M. The membrane fractions were originally prepared in H buffer with 3 M NaCl. These fractions were diluted (1:20 for *H. larsenni* and *H. denitrificans*, 1:50 for *H. mediterranei*) in H buffers with different concentrations of salt. After determining the enzyme activity of the preparations, the preparations were stored at 4°C. The activities of the stored fractions were determined for the next seven days and again after 45 days. When diluted in the H buffers, the membrane fractions carried some salt which increased the final salt concentration of each fraction as follows:

Original Salt Concentrations of H buffer	Final Salt Concentrations of Membrane Fractions of	
	<i>H. larsenni</i> & <i>H. denitrificans</i>	<i>H. mediterranei</i>
0 M	0.060 M	0.020 M
1 M	1.060 M	1.020 M
2 M	2.060 M	2.020 M
3 M	3.060 M	3.020 M
4M	4.060 M	4.020 M

To study the activity of the enzyme in the presence of various concentrations of salt, enzyme activity was determined in the presence of salt in the assay buffer.

For this purpose, TES buffer was prepared containing various concentrations of salt ranging from 0 to 4 M. The rest of the assay was typical.

RESULTS AND DISCUSSION

I. GROWTH OF BACTERIA AND INDUCTION OF NITRATE REDUCTASE

To study the nitrate reductase of halobacteria, *H. larsenni* (Baja 12) and *H. mediterranei* were grown in HYH-2 and M-2 media respectively. Growth of *H. larsenni* was initiated after a lag of about 3-5 hours when incubated under "anaerobic conditions" with or without nitrate, and the culture reached stationary phase after 22 hours (Fig. 1). A doubling time of approximately 4.5 hours was observed. Nitrite production was initiated during exponential growth (Fig. 2). As the culture entered stationary phase, the concentration of nitrite in the media rapidly increased and reached maximum after approximately 45 hours. After 45 hours little if any change was observed in the amount of nitrite in the media. When *H. larsenni* was grown in the absence of added nitrate, some nitrite was detected (less than 10% of that produced in the presence of 0.25% nitrate).

H. mediterranei grew with a doubling time of approximately 2.5 hours (Fig. 3) in the presence of nitrate under "anaerobic conditions", but considerable variation in nitrite production was observed. Exponential growth was accompanied by the production of nitrite when nitrate was added (Fig. 4) and no nitrite was found in the cultures without added nitrate. The nitrite yield reached a maximum at about 25 hours, the time at which the culture entered the stationary phase.

FIGURE 1

Growth of *H. larsenni*.

The bacteria were grown under three different conditions: (a) fast shake, 200 rpm, for aerobic growth, (b) slow shake, 105 rpm, for anaerobic growth in presence of 0.25% nitrate (denitrifying growth), (c) slow shake, 105 rpm, for anaerobic growth in the absence of nitrate as control for denitrifying growth. Growth was measured turbidimetrically with a Klett-Summerson Colorimeter using a red (No. 66) filter.

FIGURE 2

Production pattern of nitrite in culture of *H. larsenni*.

The organism was grown in complex medium in the absence or presence of 0.25% nitrate. The concentration of NO₂⁻ in the growth medium was determined by a diazotization procedure described in the methods section.

FIGURE 3

Growth of *H. mediterranei*.

The bacteria were grown under three different conditions: (a) fast shake, 200 rpm, for aerobic growth, (b) slow shake, 105 rpm, for anaerobic growth in presence of 0.25% nitrate (denitrifying growth), (c) slow shake, 105 rpm, for anaerobic growth in the absence of nitrate as control for denitrifying growth. Growth was measured turbidimetrically with a Klett-Summerson Colorimeter using a red (No. 66) filter.

FIGURE 4

Production pattern of nitrite in culture of *H. mediterranei*.

The organism was grown in complex medium in the absence or presence of 0.25% nitrate. The concentration of NO₂⁻ in the growth medium was determined by a diazotization procedure described in the methods section.

The nitrite level remained at maximum level for some 25 hours after which it rapidly disappeared. The disappearance of nitrite was accompanied by an observation of gas production which was not observed in the culture without added nitrate. Although the gas was not analyzed, it was assumed to be dinitrogen. This interpretation was consistent with earlier observations of nitrate reduction and gas production in *H. mediterranei* and a number of other halobacteria when grown anaerobically in media containing nitrate (Mancinelli and Hochstein, 1986; Javor, 1984; Gonzalez *et al*, 1978; Elzar-Volcani, 1957; Tomlinson and Hochstein, 1972; Tomlinson and Hochstein, 1976; Rodriguez-Valera *et al*, 1983).

The variation in denitrification pattern and product formation of *H. larsenni* and *H. mediterranei* could be an intrinsic property of these organisms or a reflection of the required nitrate and oxygen concentrations. The pattern of product formation during denitrification could depend upon the nitrate concentration. When *H. denitrificans* was grown in the presence of 0.1% nitrate, the only product detected was dinitrogen (Tomlinson *et al*, 1986).

When grown in the presence of 0.5% nitrate, *H. denitrificans* produced nitrite, nitrous oxide, and dinitrogen. An extreme halophile, designated strain D4, produced nitrite, nitrous oxide, and dinitrogen during exponential growth when grown in the presence of 0.1 and 0.5% nitrate (Hochstein and Tomlinson, 1985).

The induction pattern of the enzymes leading to gas production (nitrate and nitrite reductases) as well as the various factors regulating their synthesis has been studied in several organisms. The synthesis of the nitrate reductase of *Escherichia coli* K-12 is controlled by two principal growth conditions: the presence of nitrate, which acts to induce, and aeration of the medium, which serves to repress. The control is similar to that found in *Aerobacter aerogenes* (*Enterobacter aerogenes*) (Pichinoty and D'Ornano, 1961) in that vigorous aeration will repress enzyme synthesis even in the presence of nitrate. The basal level of enzyme in highly aerated cultures is 2 to 4% of that observed in cultures that are derepressed in enzyme production by anaerobiosis. The fully induced levels, obtained in anaerobic cultures in the presence of nitrate, were 20-fold higher (Show, 1968). In the case of *H. larsenni* a 10-fold increase in the level of nitrite production was observed in cultures grown "anaerobically." The anaerobic condition utilized in this study, however, might have not been sufficient to fully derepress the enzyme synthesis. The increase in the detected amount of nitrite in the cultures was observed when the culture entered its stationary phase. This can be correlated with the increase in the enzyme synthesis as a result of the decrease in oxygen concentration which occurs when culture density increases. *H. mediterranei* grown "anaerobically" in the presence of nitrate, however, had a slightly higher initial rate of nitrite production during the exponential phase than did *H. larsenni* (Figs. 2 & 4). These results may indicate the difference in the requirements for the enzyme to appear in each of these organisms. Perhaps, the derepression of the enzyme in *H. larsenni* requires somewhat longer time and is more sensitive to oxygen than that of *H. mediterranei*.

The culture of *H. mediterranei* showed no detectable amount of nitrite when it was well into the stationary phase. At the same time, production of some gaseous products was observed in the media. The apparently concomitant disappearance of nitrite and the appearance of gas suggests that nitrite was reduced further to gaseous products in the denitrification pathway, due in part, perhaps, to a thorough scavenging of oxygen at this stage of growth. This interpretation is consistent with the previous reports indicating that the later reductases in the denitrification sequence are somewhat more O₂ sensitive than are the earlier reductases (Bettach, 1981; Payne, 1973; Sacks and Barker, 1949). It has been reported that nitrite reductase in *Pseudomonas aeruginosa* requires somewhat longer for derepression than does the nitrate reductase. The concentration of O₂ which controls the repression-depression threshold of this enzyme is unknown (Van Hartingsvett and Stouthamer, 1973).

Overall it seems that the induction of the enzymes associated with denitrification occurs when conditions become anaerobic. Although, for a variety of organisms, denitrification can occur in the presence of oxygen (Boody and Davis, 1987; Hochstein *et al*, 1984; Meiburg *et al*, 1980; Robertson and Kuenen, 1984a and b), or may even require low concentrations of oxygen for the enzyme induction to occur (Aida *et al*, 1986; Payne *et al*, 1971). A significant levels of enzyme may be present as a result of anaerobiosis in the absence of nitrate or other nitrogenous oxides (Hochstein and Tomlinson, 1988).

II. MEMBRANE FRACTIONS AND ASSOCIATED NITRATE REDUCTASE ACTIVITY

Nitrate reductase activity in *H. larsenni* and *H. mediterranei* was found in the membrane and cytoplasmic fractions of both organisms when grown in the presence of nitrate (Table 2). When cells were disrupted by passage through a French press and separated into "soluble" and "particulate" fractions by centrifugation, approximately 81 ± 3.5 percent of the enzyme activity was localized in the particulate fraction of the cells which sedimented along with the membrane fractions. The soluble enzyme, found in the cytoplasmic fraction, was most likely due to fragments of a larger particulate structure than to a truly soluble protein.

The properties of nitrate reductase found in different organisms may vary widely. Nitrate reductase in *Escherichia coli* (Yamaguchi) is principally membrane-bound and is linked functionally to cytochrome b_1 (Lida and Taniguchi, 1959; Itagaki *et al*, 1961; Itagaki *et al*, 1962). This enzyme was solubilized and purified by Taniguchi and Itagaki (1960) who reported its sedimentation coefficient as 25S and calculated its molecular weight to be 1 million. A soluble nitrate reductase not linked to cytochrome has been obtained by Nicholas and Nasan (1955).

In a study of nitrate reductase of *Escherichia coli* K12, it was reported that the nitrate reductase of this organism was principally particulate in nature and that a small amount of activity found in soluble form seemed to be associated with

Bacterial Strain	Membrane Fraction			
	Activity %	Enzyme Activity	Specific Activity	Protein mg/ml
<i>H. larsenni</i>	82	3.1	0.19	16.2
<i>H. larsenni</i>	76	2.4	0.13	18.5
<i>H. mediterranei</i>	84	16.2	0.6	27.2
<i>H. mediterranei</i>	82	14.5	0.5	27.8

TABLE 2

The enzyme activity of the *H. larsenni* and *H. mediterranei* membrane fractions.

The nitrate reductase activity of the membrane fractions extracted from cells "anaerobically" grown in the presence of the 0.25% nitrate was determined as described in the Methods section. The table presents the data collected from two preparations. The enzyme activity is expressed as $\mu\text{mol NO}_2^-$ produced per minute per milliliter of the fraction. The specific activity is the enzyme activity per milligram of protein.

small membrane fragments rather than to a homogeneous soluble enzyme. The partition of nitrate reductase activity between the two fractions depended on the method of cell disruption used (Showe and DeMoss, 1968).

The nitrate reductase activities of membrane fractions from two preparations of *H. larsenni* (Baja 12) were 3.08 and 2.4 $\mu\text{mol (ml.min)}^{-1}$. In the case of *H. mediterranei* the enzyme activities were 16.2 and 14.5 $\mu\text{mol (ml.min)}^{-1}$.

III. REQUIREMENTS FOR NITRATE REDUCTASE ACTIVITY

The presence of nitrate, methyl viologen and dithionite was necessary for the reduction of nitrate to nitrite. Nitrite was not produced when only one of these or the membrane fraction was omitted, except that in the absence of methyl viologen a small amount of nitrite was detected (Table 3).

Enzyme activity (production of nitrite) showed dependency on the membrane fraction concentration in the presence of a constant amount of substrate (nitrate) (Figs. 5 and 6) and on the time of incubation (Figs. 7 and 8). Catalytic activity increased in a linear fashion as the concentration of membrane fraction was elevated. The duration of incubation and concentration of the membrane fraction used in enzyme assays were determined from the linear portion of the plotted graphs.

Components of the Assay	<i>H. larsenni</i> Enzyme Activity	<i>H. mediterranei</i> Enzyme Activity
Complete	2.5	15.2
– Methyl Viologen	0.035	0.028
– Sodium Dithionite	<0.01	<0.01
– Nitrate	<0.01	<0.01
– Membrane Fraction	<0.01	<0.01

TABLE 3

Requirements of nitrate reductase activity.

The assay was carried out as described in the Methods section using membranes prepared from cells grown "anaerobically" in the presence of nitrate. The complete reaction was carried out in the presence of methyl viologen, dithionite, membrane fraction (enzyme), and nitrate. The effect of each component on the enzyme activity was determined by excluding it from the complete reaction mixture.

FIGURE 5

Enzyme activity of different concentrations of membrane fraction (*H. larsenni*).

Different volumes of *H. larsenni* membrane fraction diluted 1:20 were incubated in the presence of methyl viologen ($0.5 \mu\text{mol}$), and dithionite ($23 \mu\text{mol}$) for one minute at room temperature. The reaction was started by adding nitrate ($20 \mu\text{mol}$) and incubating at 37°C . The reaction was terminated after 10 minutes. The enzyme activity was calculated to be $2.4 \mu\text{mol/ml. min.}$

FIGURE 6

Enzyme activity of different concentrations of membrane fraction (*H. mediterranei*).

Different volumes of *H. mediterranei* membrane fraction diluted 1:50 were incubated in the presence of methyl viologen ($0.5 \mu\text{mol}$), and dithionite ($23 \mu\text{mol}$) for one minute at room temperature. The reaction was started by adding nitrate ($20 \mu\text{mol}$) and incubating at 37°C . The reaction was terminated after 10 minutes. The enzyme activity was calculated to be $15 \mu\text{mol/ml. min.}$

FIGURE 7

Effect of incubation time on enzyme activity (*H. larsenni*).

The membrane fraction of the *H. larsenni* was incubated at 37°C for different periods of time in the presence of methyl viologen, dithionite, and nitrate. Twenty μ l of the membrane fraction diluted 1:20 were assayed. The enzyme activity was calculated to be 2.5 μ mol/ml. min.

FIGURE 8

Effect of incubation time on enzyme activity (*H. mediterranei*).

The membrane fraction of the *H. mediterranei* was incubated at 37°C for different periods of time in the presence of methyl viologen, dithionite, and nitrate. Ten μ l of the membrane fraction diluted 1:50 were assayed. The enzyme activity was calculated to be 15 μ mol/ml. min.

IV. HALOPHILIC NITRATE REDUCTASE AND ITS SALT-DEPENDENT PROPERTIES

The primary purpose of this investigation was to study the effect of various salt concentrations on the activity and the stability of the extremely halophilic nitrate reductase. This enzyme from *H. denitrificans* has been studied by Hochstein (unpublished data) and it was found to be active and stable in the absence as well as the presence of various concentrations of salt.

The nitrate reductases from *H. larsenni*, and *H. mediterranei* along with *H. denitrificans* were studied in this work and displayed independence from salt concentration for stability and activity. These enzymes retained more than 80-90% (*H. larsenni*, and *H. mediterranei*) and 70% (*H. denitrificans*) of their activities during a period of seven days of incubation at 4°C in the presence of different concentrations of salt ranging from 0.060 M to 4M. The enzymes remained active (approx. 70%) even after 45 days of incubation (Tables 4 and 5). The results suggested that nitrate reductase is an exception among halophilic enzymes; it did not exhibit a time-dependent inactivation and was stable in the presence of different concentrations of salt as well as the absence of salt.

It is important to distinguish between salt effects on enzyme activity and stability. Sometimes stability is much more dependent than activity on high salt concentrations. For one example, the aspartate transcarbamylase (ATCase) of *H. cutirubrum* is about four times as active in 3.5 M NaCl as in 2 M NaCl but

% of Original Enzyme Activity (7 Days of Storage)			
[NaCl]	<i>H. larsenni</i>	<i>H. mediterranei</i>	<i>H. denitrificans</i>
0 M	89±3	95±4	100
1 M	89±2	100	72±5
2 M	90±3	91±2	71±5
3 M	83±3	100	74±2
4 M	94±6	98±1	71±1

% of Original Enzyme Activity (45 Days of Storage)			
[NaCl]	<i>H. larsenni</i>	<i>H. mediterranei</i>	<i>H. denitrificans</i>
0 M	72±2	85±2	56±3
3 M	83±3	94±1	75±2

TABLES 4 and 5

Effect of different concentrations of salt on the stability of nitrate reductase.

Nitrate reductase activity of the membrane fractions of *H. larsenni*, *H. mediterranei*, and *H. denitrificans* were determined daily during the first 7 days and once after 45 days*. The membrane fractions were diluted in H buffers containing different concentrations of salt ranging from 0 M to 4 M and stored at 4°C. The enzyme assays were performed as described in the Materials and Methods section. The data of four assays are expressed as the mean ± standard deviation.

* Data shown here indicate only the results after 7 and 45 days of storage.

almost 500 times as stable, judged by the half-life (8 hours and 1 minute, respectively) of the enzyme at the two salt concentrations (Norberg *et al*, 1973).

When investigated the effect of salt concentration on nitrate reductase activity, the membranes of *H. denitrificans*, *H. larsenni*, and *H. mediterranei* showed slightly higher activity at 0 M salt than that at 4 M (Table 6). The fact that the enzyme activity did not decline by decreasing the salt concentration shows that nitrate reductase activity of these enzymes does not require high concentrations of salt. The slightly higher activity in lower salt concentration could be explained by the fact that the proteins of halobacterial membranes solubilize at low salt concentration, or could be due to a change in the enzyme conformation resulting in higher enzyme activity or affinity for substrate.

Nitrate reductase, an enzyme from a halophilic organism exhibiting nonhalophilic properties, might be a good model to study the possible mechanism of action of a protein in the presence and absence of salt. A better understanding of the way proteins function in concentrated salt would be obtained by detailed structural comparisons of halobacterial nitrate reductase with analogous enzyme from nonhalophilic organisms.

It has been suggested that the destabilization of halophilic enzymes at salt concentrations where nonhalophilic enzymes are stable might be due to weaker hydrophobic interactions at low salt concentrations in halophiles than in

[NaCl]	% of Enzyme Activity		
	<i>H. larsenni</i>	<i>H. mediterranei</i>	<i>H. denitrificans</i>
0 M	100	100	100
1 M	83±4	83±3	73±6
2 M	82±7	83±4	77±3
3 M	79±1	84±4	74±2
4 M	66±7	79±9	66±4

TABLE 6

Effect of different concentrations of salt on the activity of nitrate reductase.

Nitrate reductase activity of the membrane fractions of *H. larsenni*, *H. mediterranei*, and *H. denitrificans* were determined. Enzyme assays were performed using H buffer containing salt concentrations ranging from 0 M to 4 M as described in the Materials and Methods section. The data of four assays are expressed as the mean $\pm$ standard deviation.

nonhalophiles (Lanyi, 1974; Bayley and Morton, 1978). Therefore, more salt is required for enhancing and stabilizing hydrophobic interactions in the halophilic proteins with a lower hydrophobicities. The proteins from halophilic sources, which are stable at low ionic strength, have relatively high hydrophobicities (Bayley and Morton, 1978; Werber and Mevarech, 1978a and b).

The enzymes in *Halobacterium* are abnormally high in acidic amino acid residues. It has been suggested that the charge shielding at high salt concentrations might be required for stability and, in some cases, results in a conformational changes that increase enzyme activity (Lanyi, 1974). The gas vacuoles, found in some strains of *H. halobium*, are morphologically unchanged in distilled water. As expected, the protein components of these structures are much less acidic than the bulk protein of the organism (Krants and Ballou, 1973).

When a group of ribosomal proteins from *H. cutirubrum* were compared with the same proteins from *Escherichia coli*, it was shown that the halophilic proteins contain significantly more borderline hydrophobic residues (e. g., alanine and threonine) than the nonhalophilic proteins (Louis and Fitt, 1971). It was suggested, therefore, that the new hydrophobic bonding created at high salt concentration involves the marginally hydrophobic groups, which are normally not well suited for nonpolar interaction (Lanyi, 1974).

The molecular weights of halophilic enzymes correspond very well to their nonhalophilic counterparts in many cases. However, molecular size differences have been reported for the DNA-dependent RNA polymerase of *H. cutirubrum*, which functions similarly to the *Escherichia coli* enzyme, but its molecular weight is only 36,000 (including both subunits), rather than a half-million (Louis and Fitt, 1971).

A better understanding of structure, amino acid sequence and composition, and size of halophilic nitrate reductase is required to explain the response of this protein to salt.

V. ENZYME KINETICS AND INHIBITION CHARACTERISTICS

In studying the nitrate reductase from *H. larsenni* and *H. mediterranei*, the inhibitory effects of azide, chlorate, and cyanide were examined and the Michaelis constant value for nitrate was determined. The K_M for nitrate reductase of *H. larsenni* was 0.17 ± 0.03 mM and for *H. mediterranei* was 0.047 ± 0.006 mM. The K_M value for *H. larsenni* compares very well with the K_M value of 0.2 mM reported for the nitrate reductase of *H. marismortui* (Werber and Mevarach, 1978a and b) and the K_M value of 0.27 mM for *H. denitrificans*, but the K_M value for *H. mediterranei* is very different and smaller indicating a higher affinity for nitrate (Table 7).

The nitrate reductase from both organisms was extremely sensitive to azide and the inhibition was competitive with respect to nitrate (Figs. 9 and 10).

<u>Michaelis constant for nitrate (mM)</u>		<u>Reference</u>
<i>Escherichia coli</i>	1.5	Stouthamer, 1976
<i>Micrococcus halodenitrificans</i>	1.3	Stouthamer, 1976
<i>Klebsiella aerogenes</i>	0.1	Stouthamer, 1976
<i>Micrococcus denitrificans</i>	0.25	Stouthamer, 1976
<i>H. marismortui</i>	0.2	Werber, 1978
<i>H. denitrificans</i>	0.27	
<i>H. larsenni</i>	0.17	
<i>H. mediterranei</i>	0.047	

TABLE 7

The K_M (Michaelis constant value) of nitrate reductase from different organisms for nitrate.

FIGURE 9

Azide inhibition of nitrate reductase activity (*H. larsenni*).

Twenty μ l of 1:20 diluted membrane fraction of *H. larsenni* were preincubated for one minute in the presence of 1.25 μ M or 2.5 μ M NaN₃ at room temperature before the reaction was initiated by the addition of different concentrations of nitrate.

FIGURE 10

Azide inhibition of nitrate reductase activity (*H. mediterranei*).

Twenty μ l of 1:50 diluted membrane fraction of *H. mediterranei* were preincubated for one minute in the presence of 1.25 μ M or 2.5 μ M NaN₃ at room temperature before the reaction was initiated by the addition of different concentrations of nitrate.

Competitive inhibition is recognized from plots of $1/v_0$ (initial velocity) vs. $1/[S]$ (different concentration of the substrate) in the presence of different fixed concentrations of inhibitor. These plots characteristically give a family of straight lines intersecting at a common intercept on the $1/v_0$ axis. The presence of a competitive inhibitor thus increases the apparent K_M of the enzyme for substrate, i. e., causes it to require a higher substrate concentration to achieve its maximum velocity. The apparent K_M for the substrate will be greater than the true K_M by the increase in the intercept on the $1/[S]$ axis. The nitrate reductase activities of *H. larsenni* and *H. mediterranei* were strongly inhibited, approximately 70% at 80 μM (50% in the presence of 35-40 μM) (Figs. 11 and 12). The inhibitor constant K_i value of azide for *H. larsenni* was 0.65 μM and that of *H. mediterranei* was 0.25 μM . The enzyme from *H. denitrificans* was inhibited by μmolar concentrations of azide ($K_i = 1.2 \mu\text{M}$), and that inhibition was also shown to be competitive (Hochstein, unpublished data).

In the case of cyanide, the inhibition was noncompetitive and the K_i value was 0.15 mM (Figs. 13 and 14). Noncompetitive inhibition is also recognized from plots of $1/v_0$ vs. $1/[S]$ in the presence of different fixed concentrations of inhibitor. The plots differ in slope but do not share a common intercept on the $1/v_0$ axis. The intercept on the $1/v_0$ axis is greater for the inhibited than the uninhibited enzyme, indicating that V_{max} is decreased by the inhibitor and cannot be restored regardless of how high the substrate concentration may be. A noncompetitive inhibitor can combine with either the free enzyme or the enzyme-substrate complex, interfering with the action of both.

FIGURE 11

Inhibition effect of different concentrations of azide on nitrate reductase activity (*H. larsenni*).

Twenty μ l of 1:20 diluted membrane fraction of *H. larsenni* were preincubated for one minute in the presence of different concentrations of NaN₃ at room temperature before the reaction was initiated by the addition of 20 μ mol of nitrate.

FIGURE 12

Inhibition effect of different concentrations of azide on nitrate reductase activity (*H. mediterranei*).

Twenty μ l of 1:50 diluted membrane fraction of *H. mediterranei* were preincubated for one minute in the presence of different concentrations of NaN₃ at room temperature before the reaction was initiated by the addition of 20 μ mol of nitrate.

FIGURE 13

Cyanide inhibition of nitrate reductase activity (*H. larsenni*).

Twenty μl of 1:20 diluted membrane fraction of *H. larsenni* were preincubated for 15 minutes in the presence of 0.25 mM or 0.5 mM KCN at room temperature before the reaction was initiated by the addition of different concentrations of nitrate.

FIGURE 14

Cyanide inhibition of nitrate reductase activity (*H. mediterranei*).

Twenty μl of 1:50 diluted membrane fraction of *H. mediterranei* were preincubated for 15 minutes in the presence of 0.20 mM or 0.40 mM KCN at room temperature before the reaction was initiated by the addition of different concentrations of nitrate.

It was found that the preincubation of cyanide and membrane fraction of *H. larsenni* and *H. mediterranei* was necessary for effective inhibition. The enzyme was more sensitive to cyanide inhibition in the presence of dithionite suggesting that cyanide reacted with the reduced form of the enzyme. The enzyme showed no loss of activity when incubated with dithionite alone. When the enzyme was incubated in the presence of nitrate prior to cyanide, the inhibition was only 30-34 percent as compared to an inhibition of 84-91% by cyanide in the presence dithionite (Table 8). These results indicated that nitrate protected the enzyme against cyanide inhibition. Hochstein (unpublished data) has studied the protection effect of nitrate and proposed that upon combining with nitrate, the cyanide binding site is no longer accessible to the inhibitor. However, once nitrate reduction took place, the enzyme became progressively inhibited. Cyanide is a potent inhibitor of nitrate reductase activity; in the presence of 1mM cyanide 90% of enzyme activity was inhibited (50% in the presence of 0.25 mM) (Figs. 15, 16, 17 and 18).

The inhibition by chlorate was also competitive ($K_i = 750 \mu\text{M}$ and $250 \mu\text{M}$ for *H. larsenni* and *H. mediterranei*, respectively) (Figs. 19 and 20). Chlorate also competitively inhibited the enzyme from *H. denitrificans* ($K_i = 360 \mu\text{M}$) (Hochstein, unpublished data). Since chlorate is known to be a substrate for nitrate reductase, the inhibition was expected to be competitive with respect to nitrate. Thirty mM of chlorate resulted in an inhibition of 50-60% of nitrate reductase activity (50% in presence of 20-25 mM) (Figs. 21 and 22).

These results indicate that the properties of nitrate reductases of the two halobacteria studied in this work are similar to the dissimilatory enzyme of other bacteria. The salt-independency of this halophilic enzyme is also suggestive of a nonhalophilic and possible eubacterial origin.

Components Present During Preincubation	% of Enzyme Activity	
	<i>H. larsenni</i>	<i>H. mediterranei</i>
Enzyme	100	100
+ KCN	55	43
+ Dithionite	100	100
+ Dithionite, KCN	16	9
+ Dithionite, Nitrate	100	100
+ Dithionite, Nitrate, KCN	70	76

TABLE 8

Inhibition of nitrate reductase activity by cyanide and the protective effect of nitrate.

The membrane fractions were preincubated at room temperature in Tris HCl, pH 7.2. After 15 minutes of preincubation, dithionite and nitrate were added in those cases where they were not present during preincubation, and the reaction was started by the addition of methyl viologen. The concentration of cyanide used in this test was 0.5 mM.

FIGURE 15

Effect of different concentrations of cyanide on nitrate reductase activity (*H. larsenni*).

Twenty μ l of 1:20 diluted membrane fraction of *H. larsenni* were preincubated for 15 minutes in the presence of different concentrations of KCN at room temperature before the reaction was initiated by the addition of 20 μ mol of nitrate.

FIGURE 16

Inhibition effect of different concentrations of cyanide on nitrate reductase activity (*H. mediterranei*).

Twenty μ l of 1:50 diluted membrane fraction of *H. mediterranei* were preincubated for 15 minutes in the presence of different concentrations of KCN at room temperature before the reaction was initiated by the addition of 20 μ mol of nitrate.

FIGURE 17

Effect of preincubation time on cyanide inhibition of nitrate reductase activity (*H. larsenni*).

Twenty μ l of 1:20 diluted membrane fraction of *H. larsenni* were preincubated for different periods of time in the presence of 0.5 mM KCN at room temperature before the reaction was initiated by the addition of 20 μ mol of nitrate.

FIGURE 18

The effect of preincubation time on cyanide inhibition of nitrate reductase activity (*H. mediterranei*).

Twenty μ l of 1:50 diluted membrane fraction of *H. mediterranei* were preincubated for different periods of time in the presence of 0.5 mM KCN at room temperature before the reaction was initiated by the addition of 20 μ mol of nitrate.

FIGURE 19

Chlorate inhibition of nitrate reductase activity (*H. larsenni*).

Twenty μ l of 1:20 diluted membrane fraction of *H. larsenni* were preincubated for one minute in the presence of 0.10 mM or 0.20 mM ClO_3^- at room temperature before the reaction was initiated by the addition of different concentrations of nitrate.

FIGURE 20

Chlorate inhibition of nitrate reductase activity (*H. mediterranei*).

Twenty μ l of 1:50 diluted membrane fraction of *H. mediterranei* were preincubated for one minute in the presence of 1.6 mM or 3.2 mM ClO_3^- at room temperature before the reaction was initiated by the addition of different concentrations of nitrate.

FIGURE 21

Inhibition effect of different concentrations of chlorate on nitrate reductase activity (*H. larsenni*).

Twenty μ l of 1:20 diluted membrane fraction of *H. larsenni* were preincubated for one minute in the presence of different concentrations of ClO_3^- at room temperature before the reaction was initiated by the addition of 20 μ mol of nitrate.

FIGURE 22

Inhibition effect of different concentrations of chlorate on nitrate reductase activity (*H. mediterranei*).

Twenty μ l of 1:50 diluted membrane fraction of *H. mediterranei* were preincubated for one minute in the presence of different concentrations of ClO_3^- at room temperature before the reaction was initiated by the addition of 20 μ mol of nitrate.

SUMMARY

Most enzymes from extremely halophilic bacteria exhibit salt-dependent properties. Nitrate reductase appears to be an exception; when it was studied in *H. Larsenni*, *H. mediterranei* (R4), and *H. denitrificans*, it was independent of salt concentration for its activity and stability. These nitrate reductases were not unusual when compared to other dissimilatory nitrate reductases. The enzyme was membrane-bound and was inhibited by azide and chlorate competitively. Nitrate protected the enzyme against the inhibition effect of cyanide and when cyanide inhibition occurred, it was noncompetitive. The K_M value of 170 μM nitrate for the enzyme from *H. larsenni* compares very well with the apparent K_M of 200 μM of *H. marismortui* and 270 μM of *H. denitrificans*. The apparent K_M of the enzyme from *H. mediterranei* was 47 μM , an unusually low K_M for nitrate. These values, however, are within the range of apparent K_M values reported for nitrate reductases from other bacteria. Finally, the enzyme was produced only under limited oxygen conditions and in the presence of nitrate, the two distinguishable characters of dissimilatory nitrate reductases.

The presence of a salt-independent enzyme in halobacteria suggests a nonhalophilic origin for this enzyme. The presence of a nonhalophilic gene could suggest gene transfer from a nonhalophilic bacterium; however, the presence of nitrate reductase with salt-independent properties in three different species of halobacteria isolated from widely separated parts of the world would seem to argue against the idea of gene transfer from nonhalophilic bacteria to halophilic bacteria. For such a transfer to occur a denitrifying nonhalophilic organism carrying the enzyme should have been present at each one of these

places with the conditions such that the gene could be transferred. It is also difficult to explain an efficient mechanism with which the information required for synthesis of enzyme and its associated cytochromes were transferred from nonhalophilic bacteria to halophilic ones. The satellite DNA in *Halobacterium* could represent transferred genes. If DNA had been transferred from a progenitor of eukaryotes into a prokaryotic ancestor at about the time these two lines were diverging, it could explain some of the eukaryotic features found in *Halobacterium*. Even if the halophilic nitrate reductases might have eukaryotic origin, they have undergone considerable adaptation in order to be able to function in their extreme environment.

Additional studies to determine the functional and structural properties of nitrate reductase of halobacteria would help to understand the nonhalophilic properties and origin of this enzyme.

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