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NAME AND ADDRESS (BLOCK LETTERS PLEASE) SIGNATURE DATE
Physiology of Halophilic Archaea
Isolated from Ancient Salt Deposits

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

By

Rachel Sarah Greedy BSc (Leicester)
Department of Microbiology & Immunology
University of Leicester

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Statement

The work in this thesis was carried out by the author during the period October 1994 to February 1998, under the supervision of Prof. W. D. Grant in the Department of Microbiology and Immunology, University of Leicester, and has not been submitted in full or in part of any other degree.

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Rachel Sarah Greedy
Halophilic archaea (halobacteria) have been isolated from two ancient salt deposits in Britain: namely Winsford Salt Mine in Cheshire and Boulby Potash Mine in Cleveland. These deposits are both over 200 million years old. The origins of these microorganisms are not clear. If they have been actively growing within the salt-mines for over 200 million years, one might expect them to be specifically adapted to the environment within the salt deposit, and thus be metabolically different from other halobacteria, which inhabit surface environments.

Most strains of halobacteria, both from salt-mine and surface environments, could utilise trehalose and glycerol, many can utilise amino acids, but none could utilise glycine betaine. During aerobic growth on glucose, glycerol and trehalose, all halobacteria produce acetic acid together with smaller amounts of others acids such as propionic, butyric, iso-butyric, iso-valeric and pyruvic acids. Acid was not produced during growth on proline. Most strains of halobacteria can grow anaerobically utilising TMAO or DMSO. Some strains can grow using NO$_3^-$ or fumarate. Most strains were able to grow fermentatively in complex growth medium, and a few strains could grow by fermentation of glucose or trehalose.

$\text{CO}_2$ fixation by both salt-mine and surface halobacteria was shown to be highest under aerobic conditions. Incubation under light or dark conditions had no significant effect. $^{13}$C-NMR studies indicted that the major $\text{CO}_2$ fixation was occurring was via pyruvate carboxylase or PEP carboxylase into oxaloacetate, and also into carbamoyl phosphate for synthesis of arginine. Enzyme assays confirmed the presence of pyruvate carboxylase.

Some strains of halobacteria, isolated from salt deposits, have the ability to emulsify and degrade crude oil. Surface strains showed only weak or no emulsification of crude oil. Crude oil may represent a carbon source, which is available to halobacteria within the salt deposits.

The salt-mine strains of halobacteria thus did not differ from the surface strains of halobacteria in the range of carbon sources they could use, the modes of anaerobic growth or the pathways of $\text{CO}_2$ fixation, although no surface strains were shown to degrade crude oil. Therefore it is likely that the salt-mine strains of halobacteria have not grown actively within the salt deposits since their formation, but have either been present in a state of “suspended animation” or are more recent contaminants.
Acknowledgments

Completion of this thesis would not have been possible without the help from the following people, to whom I would like to pass on my gratitude:

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Finally thanks to my parents for all their encouragement and help throughout the years. It is to my parents that I dedicate this thesis.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CHM</td>
<td>classical halophile medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>ECF</td>
<td>ethylchloroformate</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>MM</td>
<td>minimal medium</td>
</tr>
<tr>
<td>MMM</td>
<td>modified minimal medium</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NAD(P)</td>
<td>nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>NAD(P)H₂</td>
<td>reduced nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>orthophosphate</td>
</tr>
<tr>
<td>POR</td>
<td>pyruvate oxidoreductase</td>
</tr>
<tr>
<td>PP&lt;sub&gt;i&lt;/sub&gt;</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RuBisCo</td>
<td>ribulose bisphosphate carboxylase</td>
</tr>
<tr>
<td>RVM</td>
<td>Rodriguez-Valera medium</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
</tbody>
</table>
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1

INTRODUCTION

1.1 General Introduction

Halophilic archaea (halobacteria) have been isolated from two ancient salt deposits in Britain. These ancient salt deposits are Winsford Salt Mine in Cheshire where Triassic (235 – 240 million years old) halite is mined and Boulby Potash Mine where Upper Permian (250 – 260 million years old) halite and potash are mined.

In this introductory chapter the halophilic archaea and the hypersaline environments in which they live are described, paying particular attention to the physiology of halophilic archaea. The development of hypersaline environments, and the ecology of these environments are summarised. The formation of salt deposits, including information on composition and incorporation of organic matter in deposits, is also outlined. In the last section of this introductory chapter the controversies surrounding claims of long-term survival of bacteria and some of the possible mechanisms of survival are discussed.

The isolation of halobacteria from the ancient salt deposits has led to several fundamental questions. Have the halobacteria been present in the salt mine since their formation over 200 million years ago? If so, have they continued to grow under the conditions in the salt mine or have they existed in a state of suspended animation? By studying more about the physiology of the halophilic archaea perhaps we can begin to understand whether they have the metabolic capacity to grow under the conditions in the salt deposits. In this thesis I have looked for metabolic differences between the halobacteria isolated from the salt deposits and those from surface environments. If halobacteria have continued to grow during the
200 million years in the salt deposit they would be expected to be metabolically adapted to the conditions in the salt deposit. In this study I have looked at several aspects of the metabolism of halobacteria which are not completely understood, specifically:

1. An examination has been carried out of the carbon sources utilised by strains isolated from Boulby and Winsford, in addition to strains isolated from surface environments. The ability of these strains to grow anaerobically was also surveyed.

2. Tomlinson and Hochstein (1972b) and Oren & Gurevich (1994a) found that some strains of halobacteria produced acetic acid, pyruvic and lactic acid during growth. In this study further investigations into acid production during growth have been carried out.

3. There is also some controversy surrounding the ability of halobacteria to fix CO$_2$. Various authors have suggested that CO$_2$ fixation is light driven, mediated by bacteriorhodopsin (Danon & Caplan 1977, Oren 1983), that CO$_2$ fixation involves a glycine synthase reaction with CO$_2$, NH$_4^+$ and a methyl carbon derived from the β-cleavage of a propionate molecule (Javor 1988) or that it is carried out using ribulose bisphosphate carboxylase (Altekar & Rajapalan 1990). It has also been suggested by some authors that halobacteria may have the metabolic capacity to grow autotrophically, but autotrophic growth has never been demonstrated (Tindall & Truper 1986, Javor 1988, Oren 1994). In this study CO$_2$ fixation by several strains of halobacteria has been examined to try to elucidate the pathways of CO$_2$ fixation. Attempts have also been made to try to grow halobacteria autotrophically.

4. Bertrand et al. (1990), Kulichevskaya et al. (1991) and Zvygintseva et al. (1995) have all found that some strains of halobacteria can degrade hydrocarbons. As salt deposits are often found in close proximity to oil deposits, the ability of halobacteria isolated from the salt deposits to degrade crude oil was examined.
1.2 Hypersaline Environments and their Ecology

1.2.1 The Development of Hypersaline Environments

Hypersaline environments are those containing salts with concentrations exceeding those of seawater (Edgerton and Brimblecome 1981). The concentration of salts and their nature are variable, and they may be classified into one of two categories: thalassohaline or athalassohaline. Thalassohaline environments are those related by origin to the sea, usually having arisen by evaporation of seawater, for example, solar salterns. The term is also used to refer to any hypersaline environment with the salts in approximately the same proportions as those found in seawater (Williams 1981). In this context, the Great Salt Lake is thalassohaline, although it lies about 1000 km from the Pacific Ocean. Athalassohaline environments are those which are derived from the dissolution of salts of previous depositional origin. The composition of athalassohaline brines depends on the surrounding geology, geography and topography. An example is the Dead Sea. (Grant, Gemmell & McGinity 1998).

The lower limit to which the term hypersaline is applied is not clear. Different authors state values from 3 to 12% salinity (Post et al. 1983, Por 1980). In hypersaline waters the high concentrations of salts is the major reason for the drastic reduction in species diversity (Por 1980). This reduction is seen at around 10-12% salinity. Below this concentration of salts other factors determine the population composition, above this level the salinity is the major determinant. Thus a salt concentration of 10-12% seems to mark the lower limits of a hypersaline environment (Rodriguez-Valera 1992). In thalassohaline environments the upper limits of salinity is around 35%. At this concentration NaCl precipitates and the concentration of salts shifts to a preponderance of magnesium. The point at which NaCl precipitates is also the upper limit of biological activity (Javor 1983a).
### Table 1.1: Major Ion Concentrations of extremely, hypersaline brines (g/l)

<table>
<thead>
<tr>
<th>Ion</th>
<th>Seawater at onset of gypsum saturation</th>
<th>Seawater at onset of NaCl saturation</th>
<th>Seawater at onset of potash saturation</th>
<th>Great Salt Lake, North Arm</th>
<th>Dead Sea</th>
<th>Lake Mushiki</th>
<th>Don Juan Pond</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>10.8</td>
<td>49.5</td>
<td>98.4</td>
<td>61.4</td>
<td>105</td>
<td>39.7</td>
<td>62.2</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.3</td>
<td>6.8</td>
<td>14.5</td>
<td>39.3</td>
<td>11.1</td>
<td>42.4</td>
<td>29.0</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.4</td>
<td>1.7</td>
<td>0.4</td>
<td>0.2</td>
<td>0.3</td>
<td>17.2</td>
<td>low</td>
</tr>
<tr>
<td>K⁺</td>
<td>0.4</td>
<td>2.0</td>
<td>4.9</td>
<td>12.8</td>
<td>6.7</td>
<td>7.6</td>
<td>low</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>19.4</td>
<td>91.5</td>
<td>187</td>
<td>189</td>
<td>181</td>
<td>219</td>
<td>114</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>2.7</td>
<td>12.5</td>
<td>19.3</td>
<td>51.2</td>
<td>27.0</td>
<td>0.4</td>
<td>237</td>
</tr>
<tr>
<td>TDS %</td>
<td>3.5</td>
<td>16.4</td>
<td>32.4</td>
<td>35.4</td>
<td>33.3</td>
<td>32.7</td>
<td>34.2</td>
</tr>
</tbody>
</table>

The origin of most hypersaline environments is seawater. The ionic composition of seawater and some hypersaline brines is shown in Table 1.1. Salinity and ionic composition are extremely constant in the present day oceans, usually about 3.5% (w/v) in most of the Pacific, Atlantic and Indian Oceans, 3.8% in the Sargasso Sea and above 4.0% in the Gulf of Suez (Hatch et al. 1971).

When seawater is separated from the main body of the ocean, evaporation will lead to concentration of the ions present. If the net balance is in favour of evaporation rather than dilution (due to freshwater inflow or rain fall) the result is hypersaline brine. The ion composition depends on the surrounding topography, geology and climate (Grant and Ross 1986). Development usually occurs in tropical or subtropical regions subject to high temperatures and high light intensities. The concentration of Ca\(^{2+}\) and Mg\(^{2+}\), and to a lesser extent SO\(_4^{2-}\), in the surrounding rocks is of paramount importance in the composition of the brine formed. Calcium sulphate is the first substance to precipitate but the quantity is small. When the volume of seawater is reduced to 19% of the starting volume calcium sulphate (gypsum) begins to precipitate. Halite (NaCl) precipitates out when the volume of seawater is at about 9.5% of the original and the salinity has reached 34%. When the volume is reduced to 4%, a complex magnesium and potassium salt (polyhalite K\(_2\)SO\(_4\).MgSO\(_4\).2CaSO\(_4\).2H\(_2\)O) begins to crystallise (Blatt et al 1980, Craig et al 1996). During the reduction in solution volume between 9.5% to 4% more than half the halite will precipitate. The sequence of minerals precipitating from the final 4% of the brine (the bitterns) is complex and variable, depending on temperature and reactions between the final liquids and the earlier formed crystals. Table 1.1 shows concentrations of ions in brines during the concentration process. Two of the precipitates in the final stages are sylvite (KCl) and carnallite (KCl.MgCl\(_2\).6H\(_2\)O). One of the most important physical and chemical characteristics which influences the biology of these systems is the pH. An important effect on the final pH of the brine is that the precipitation of CO\(_3^{2-}\) removes alkalinity and precipitation of Mg\(^{2+}\) minerals such as sepiolite (Mg\(_2\)Si\(_3\)O\(_8\)) contributes H\(^+\). In the presence of high Ca\(^{2+}\), insoluble CaCO\(_3\) forms removing the alkaline CO\(_3^{2-}\), so a high Ca\(^{2+}\) area will produce a neutral to slightly alkaline brine, for example the Great Salt Lake. A high Ca\(^{2+}\)Mg\(^{2+}\) area will produce a neutral to slightly acid brine due to precipitation of
CaCO₃ and Mg²⁺ minerals, e.g. the Dead Sea. A low Mg²⁺Ca²⁺ area will give an extremely alkaline brine because the concentration of Ca²⁺ is always exceeded by CO₃²⁻ e.g. Lake Magadi (Grant and Ross 1986, Grant, Gemmell and McGenity 1998). These processes are illustrated in Fig. 1.1.

**Fig. 1.1 Formation of Hypersaline Brines**

- **CO₃²⁻ SiO₄⁻ HCO₃⁻ Na⁺ Cl⁻ K⁺ SO₄²⁻ Ca²⁺ Mg²⁺**
  - **Low Ca²⁺ [SO₄²⁻]**
    - **Low Mg²⁺**
    - Calcite CaCO₃ precipitation
    - Gypsum precipitation
    - Na⁺ CO₃²⁻ Cl⁻ [SO₄²⁻]
    - SODA LAKE pH 10-11 e.g. Lake Magadi
  - **High Ca²⁺ [SO₄²⁻]**
    - Calcite CaCO₃ precipitation
    - Gypsum precipitation
    - Na⁺ Cl⁻ [SO₄²⁻]
    - SALT LAKE pH 7-8 e.g. Great Salt Lake
  - **High Ca²⁺ [SO₄²⁻]**
    - Sepiolite Mg₂SiO₅·nH₂O precipitation
    - Gypsum CaCO₃ precipitation
    - Na⁺ Mg²⁺ Cl⁻ [SO₄²⁻]
    - SALT LAKE pH 6-7 e.g. Dead Sea
1.2.2 Modern Day Hypersaline Environments

1.2.2.1 Salinas and Sabkas

Salinas develop in arid or semi-arid regions of the world, on the coastline, where a sandy barrier isolates a shallow lagoon or arm of the sea. Salts are replenished by seepage and periodic flooding, and deposited subaqueously by evaporite concentration. They are found around the Mediterranean coast, the Portuguese and Spanish coasts, the Sinai coast, North Africa, Bahamas, Central America and Australia. An example of a salina is Lake Macleod in Western Australia, which developed after isolation from the Indian Ocean 5 000 years ago. Salinas are often exploited by man for salt production (Kendall and Warren 1988).

Sabka is an Ethiopian word referring to a marine lagoon. Sabkas are formed on a high intertidal or supratidal flats where there is periodic flooding by sea water and subsequent evaporation. Capillary brine deposits gypsum and anhydrite subaerially. Examples exist along Baja California, the Gulfs of Suez and Eliat, the Mediterranean coast and the Trucial coast of the Arabian (Persian) Gulf. The Abu Dhabi sabka on the Arabian gulf covers an area of 100 km² with deposits of carbonate, gypsum and anhydrite, and a halite crust in the central region to a total of about 1m in depth (Kendall & Warren 1988). Sabkas may also exist inland around salt lakes.

1.2.2.2 Man-Made Salterns

In many arid or semi-arid regions of the world, man has transformed natural salinas/sabkas into man-made salterns for the production of salt. These salterns are widely distributed throughout the world. Some solar salterns consist of a single pond, but most consist of small ponds connected by canals and ditches. The first ponds are filled with seawater, when the salt concentration increases slightly, the water is allowed to flow or is pumped into the next pond. The salt concentrates due to evaporation, as the water circulates through the system. NaCl precipitates in special ponds called crystallisers (Rodriguez-Valera 1992).
1.2.2.3 Continental Salt Lakes

Salt lakes exist in restricted or closed drainage basins, in arid or semi-arid regions. Examples are the Dead Sea, the Great Salt Lake and salt lakes in California. The ionic composition of these lakes depends on the mineral composition of the surrounding rocks and in-flowing rivers and springs. They are generally athalassohaline. Saline lakes are also found in Antarctica due to low precipitation and low humidity. Freezing of seawater leaves a brine that may reach the point of gypsum formation (Blatt et al. 1980).

1.2.2.4 Cyclic Salt

Salt from sea spray and surface deposition can be carried inland for hundreds of miles by strong winds. In 1839, storms blew salt from the Irish Sea as far as Yorkshire in quantities of tons per acre (Hatch et al. 1971). In Eastern Britain, in 1984 aeolian dust fall, probably of Saharan origin, also contained some cubes of halite (Wheeler 1985).

In the desert of Rajaputana in Northwest India, the vast quantities of salt have been carried by strong winds, bringing salt dust from the Rann of Kutch, a sabka on the Indus River delta. At least 200 000 tons of salt were carried to the desert this way each hot, dry season. During the following monsoon season the salt was washed into local hollows forming temporary salt lakes. The largest of these, Sambhar Salt Lake covered 70 -80 square miles by the end of the rainy season. During the cold, dry season evaporation occurs and these lakes shrink. The annual cycle continues and the salt builds up (Holland 1911). The Triassic salt deposits in Northwest England may be partially explained by aeolian transport.

1.2.2.5 Hypersaline Waste Effluents

The organic chemical industry generates waste brines during the production of pesticides and herbicides, polyhydric chemicals, organic peroxides, pharmaceuticals and other products. Large volumes of waste brines are also generated during oil and gas recovery operations. This waste called produced water is geological formation
water containing high concentrations of salts, crude oil organics and heavy metals. Other sources of hypersaline wastes include landfill leachates, meatpacking and hide curing waste waters, and contaminated ground water. Waste minimisation practices may generate additional waste brines in the future. Reduction in waste volume that concentrates organics and inorganics can occur. In arid parts of the world high salinity industrial wastes are already created. Over 4000m$^3$ per day complex waste with over 3% salt is produced by the Havav Industrial Park in Israel. As additional waste minimisation conservation measures are implemented, the salinity of the waste is expected to increase (Woolard & Irvine 1995). The biological removal of organics from industrial brine wastes could minimise the environmental impact of brine disposal by reducing the overall toxicity of the waste (Woolard & Irvine 1994)

### 1.2.3 Environmental Conditions and Ecology of Hypersaline Environments

#### 1.2.3.1 Environmental Conditions

**Temperature**

Many hypersaline environments occur in regions of the world with arid, tropical climates, but hypersaline lakes are also found in temperate and polar zones. Under conditions of high light intensity, waters with high salinity heat up very rapidly due to a combination of physical and biological factors. Concentrated salt solutions have a low specific heat. The carotenoid pigments of halobacteria are known to trap solar energy and increase the heat absorption. This would be favourable to halobacteria, which are slightly thermophilic (Larsen 1980). As a result high temperatures can be reached with only mild air temperatures. In Mediterranean salterns the temperature may reach 45°C with air temperatures of 30°C (Rodriguez-Valera et al. 1985). Hypersaline waters also cool quickly at night and in the winter. In the Great Salt Lake, air temperatures range from -30°C in the winter to +48°C in the summer (Post 1981). Hypersaline lakes in Antarctica maintain low temperatures year round; for example Organic Lake temperatures range from -14°C to 15°C (Franzmann et al. 1987).
Pressure

Most hypersaline environments are shallow and therefore do not develop a great pressure at the bottom. However the deepest regions of the Dead Sea are about 320m and considerable pressure is therefore exerted on life. Red Sea geothermal brines are another examples of deep hypersaline brines (Rodriguez-Valera 1992).

Nutrient Availability

Post (1977) examined the nutrient levels in the Great Salt Lake. Phosphate was plentiful at the sediment surface. Orthophosphate remained fairly constant but declined during algal blooms. Nitrogen was low or undetectable as ammonia, nitrates or nitrites. Javor (1983a) found the limiting factor in the Dead Sea was phosphate, with ammonia and organic nitrogen abundant (Javor 1983a).

In a saltern in Baja California (Guerrero Negro), the nutrient concentrations were low. This was attributed to the development of extensive benthic microbial mats in the first stages of brine concentration. These mats were presumed to sequester all the nutrients (Javor 1983b). However in a saltern near San Diego nutrient concentrations and plankton productivity were higher and the benthic communities were more restricted (Javor 1983b).

In multi-pond salterns the concentrations of N and P increase as the salt concentration increases, reaching highest values in the crystalliser ponds (Javor 1983a, Rodriguez-Valera et al. 1985).

Light

Hypersaline environments are often located in geographical regions with few cloudy or rainy days per year. As they also tend to be shallow the biota are exposed to strong solar radiation. Dunaliella salina and halobacteria produce carotenoids to serve as protective agents (Rodriguez-Valera 1992). The bacteriorubin carotenoid pigments of halobacteria act primarily as photoprotectants, but are also involved in energy transfer to facilitate photoreactivation of DNA damaged by exposure to UV
Introduction

light (Hescox and Carlberg 1972).

Oxygen

High salt concentrations and high temperatures restrict the solubility of oxygen. The high density of brines also contributes to the easy anaerobiosis by favouring stratification. In lakes with high algal or cyanobacterial populations the oxygen transfer rate to the aerobic microbial population is sufficient to permit a rapid metabolism (Rodriguez-Valera 1992).

Salt Concentration

As discussed earlier in Section 1.2.1., hypersaline environments have a lower salt concentration limit of 10 -12%. Microorganisms in hypersaline environments have to be able to cope with the high ionic strength and the low concentration of freely available water (Trüper and Galinski 1986). Highly concentrated salt solutions are well known for their food-preserving effects. This is due to the resulting low water activity, which causes dehydration of contaminating organisms. Halophilic and halotolerant organisms are defined in Table 1.2. They must possess mechanisms to prevent osmotically driven loss of cytoplasm water since the cytoplasmic membrane is freely permeable to water.

Two different strategies have evolved to survive water stress. In the first mechanism the cytoplasmic membrane allows free entrance of osmotically active solutes and the organism possesses metabolic systems not impaired by high salt concentrations. This is the strategy used by halobacteria (see section 1.1.2). The second mechanism is to allow only slow or limited entrance of outer salts, which then triggers the formation or uptake of organic materials, which are osmotically active. This counter-balances the outer osmotic pressure. These osmoregulants must be compatible with cellular enzymatic activities so are referred to as “compatible solutes” (Brown 1976).

A very large number of halophilic and halotolerant eubacteria have been surveyed using high-performance liquid chromatography (HPLC) and nuclear magnetic
Table 1.2 Salt responses of different microorganisms. From Kushner (1993)

<table>
<thead>
<tr>
<th>Category</th>
<th>Minimal</th>
<th>Optimal</th>
<th>Maximal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nonhalophiles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slight halophiles</td>
<td>0.2</td>
<td>0.2 - 0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Moderate halophiles</td>
<td>0.4</td>
<td>0.5 - 0.2</td>
<td>3.0 - 0.5</td>
</tr>
<tr>
<td>Borderline extreme halophiles</td>
<td>1.0</td>
<td>2.0 - 3.5</td>
<td>4.0</td>
</tr>
<tr>
<td>Extreme halophiles</td>
<td>2.0</td>
<td>3.0 - 4.0</td>
<td>5.2 (saturated)</td>
</tr>
<tr>
<td><strong>Halotolerant</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonhalophiles, which can tolerate high salt concentrations. Extremely halotolerant cells can grow in higher than 2.5 mol l⁻¹ NaCl.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Facultative halophiles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Require about 0.5 mol l⁻¹ NaCl under certain environmental conditions.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Examples:

1. Most terrestrial eubacteria and most freshwater microorganisms.

2. Many marine microorganisms.

3. *Vibrio costicola, Paracoccus halodenitrificans*, many other eubacteria and algae.


5. *Halobacterium, Natronobacterium, Halococcus, Natronococcus spp.* and other “red halophiles”.

6. *Staphylococcus and Halomonas spp.*, some of the latter being extremely “halotolerant”.

7. *Planococcus halophila*
resonance (NMR) used to reveal the compatible solutes for osmoregulation (Truper and Galinski 1986, Wohlfarth et al. 1990, Severin et al. 1992). The compatible solutes fall into the following classes of compounds: sugars and sugar-polyol derivatives, glycine betaine, tetrahydropyrimidines (ectoines), α-amino acids (proline, glutamine), N-acylated diamino acids, N-derivatized carboxamides of glutamine, and methylated sulphur compounds like dimethylsulphoniopropionate. Isolates which have been looked at include anoxygenic phototrophic bacteria and aerobic chemoheterotrophic Proteobacteria of the α and γ-subdivisions, actinomycetes, Gram-positive cocci, bacilli and related species as well as Staphylococcus and Salinicoccus species and moderately halotolerant species of the genera Brevibacterium and Corynebacterium. The various groups of bacteria preferentially utilise particular compatible solutes. Methanogenic archaea characteristically accumulate glycine betaine and the β-amino acids, β-glutamine and Nε-acetyl-β-lysine (Robertson et al. 1990, Lai et al. 1991, Roberts et al. 1992). Polyols, mainly glycerol and arabitol are often found in algae, yeast and fungi, but not in bacteria.

1.2.3.2 Ecology of Hypersaline Environments

Early reports of hypersaline environments described them as inhospitable to life. Grabau (1920) described the shores near the inlet into the Gulf of Kara-Bugaz on the eastern coast of the Caspian Sea. The inflowing current carries thousands of fish into the hypersaline waters of the Gulf, where they die and provide a limitless bounty for sea birds. The accumulation of carcasses is so great that at certain times of the year the birds eat only the eyes of the fish and often only the eye on the upper surface of the fish.

Lotze (1957) stated “Nicht das Leben, sandern der Tod beherrsant die Salzbildungsstatten”, which translates as “Not life, but death rules the locales of salt deposition”. (cf. Evans and Kirkland 1988)

It is now known that primary production can be extremely high in many hypersaline environments. The soda lakes in Africa are recognised as the world’s most productive ecosystems. Measurements of primary production in saline environments
range up to 19 000 mg of carbon assimilated per m$^3$ per day in Lake Aranguadi, Ethiopia. This can be compared to the highest productivity in the oceans, which rarely exceeds 2000 mg of carbon assimilated per m$^3$ per day in coastal upwellings on the Western side of South America and Africa.

Macrophscopic life is limited to the brine shrimp (*Artemia salina*) and two species of brine fly (*Ephydra sp.*). Protozoa have been isolated from enrichments of samples from a hypersaline lagoon in Western Australia- these included several species of flagellate and amoeboid protozoa (Post *et al.*1983). The role of the protozoa in the hypersaline environment does not appear to have been studied.

Primary producers in hypersaline environments are algae mainly of the genus *Dunaliella*, cyanobacteria for example *Aphanothece halophytica*, *Dactylococcopsis salina*, *Spirulina platensis*, *Synechoccus* and *Synchocystis sp.*, and anoxygenic phototrophic bacteria belonging to the genera *Rhodospirillum*, *Chromatium*, *Thiocapsa* and *Ectothiorhodospira*.

A wide range of halophilic and halotolerant eubacteria have been isolated from hypersaline environments including Proteobacteria mainly of the γ-subclass (*Halomonas*, *Vibrio*, *Pseudomonas* species), actinomycetes (*Actinopolyspora*, *Nocardiopsis* species) and Gram positive bacteria (e.g. *Bacillus*, *Micrococcus*, *Marinococcus*, *Salinicoccus* species).

The halophilic archaea or halobacteria are the dominant organisms in hypersaline brines of above 20% (w/v) salt. Densities of halophilic bacteria are often very high for example up to 2.2 x 10$^7$ per ml has been counted in the Dead Sea (Oren 1983a), and counts as high as 4 x 10$^7$ -10$^8$ per ml are not uncommon in the Great Salt Lake (Post 1977). Dense populations of halobacteria impart a red colouration to the environment. Characteristics of the halophilic archaea are described in detail in section 1.3.

Six halophilic anaerobic fermentative genera have been described, containing 9 species. These are *Halobacteroides halobius*, *Halobacteroides acetoethyliticus*,
**Introduction**


The sulphate reducing bacteria (SRB) are a physiological group with the common property to use sulphate as the main electron acceptor in anaerobic metabolism. Biological sulphate reduction has been observed in hypersaline environments (Mathrani et al. 1987, Nissenbaum and Kaplan 1976, Zeikus et al. 1983), but only a few moderately halophilic sulphate reducers have been isolated. SRB have been isolated from hot brines in the Red Sea (Truper 1969), from Solar Lake in Sinai (Caumette et al. 1991), from hypersaline oil field water (Cord-Ruwisch et al. 1985) and from Retba Lake in Senegal (Ollivier et al. 1991). Ollivier et al. (1994) state that many other halophilic sulphate reducers must exist.

Methanogenesis in hypersaline environments has been shown to occur in several locations- a submarine brine pool in Mexico (Brooks et al. 1979), an alkaline lake (Oremland et al. 1982), in the Great Salt Lake (Phelps and Zeikus 1980) and in Lake Retba in Senegal (Mathrani et al. 1987). A halotolerant hydrogenotrophic methanogenic rod growing in up to 5% NaCl was recently isolated and characterised (Ollivier et al. 1994). The highest salt concentration for growth of methanogens using \( \text{H}_2 \) and formate is 8.3%. At present it appears \( \text{H}_2 \) is not an important source of energy for methanogenesis in hypersaline environments. Halophilic methylotrophic methanogens have been reported among three genera and five species: "*Methanohalococcus alcaliphilum*" and "*Halomethanococcus alcaliphilum*" probably belong to the Genus *Methanohalophilus*. These two species and
**Methanolobus oregensis** are halotolerant, however *Methanohalobium evestigatum* is an extreme halophile growing optimally with a salt concentration of 24% (Zhilina and Zavarzin 1987).

Rodriguez-Valera et al. (1985) studied the changes in the populations of aerobic microorganisms in a multi-pond saltern in SE Spain as the salt concentration increased. In ponds with below 15% (w/v) salt many organisms were isolated. Between 15% to 30% salt the populations of *Dunaliella* increased reaching high numbers. Moderately halophilic members of the halophilic eubacteria, including members of the *Pseudomonas-Alteromonas-Alcaligenes* group, *Vibrio* and Gram positive cocci and some halobacteria of the genera *Halofex*, *Haloarcula* and *Halorubrum*. Phototrophic bacteria were also found in this salt range. Among the anoxygenic phototrophs *Chromatium* species and *Rhodospirillum salexigens* were probably predominant. At over 30% salt the diversity greatly decreased, the species found at lower salt concentrations disappeared and large populations of halobacteria developed. Eight species of halobacteria were present from the genera *Halofex*, *Haloarcula*, *Halorubrum* and *Halobacterium*. At over 50% only 4 species of halobacteria were found—*Halofex gibbonsii, Halorubrum saccharovorum, Haloarcula vallismortis* and *Halobacterium halobium (salinarum)*.

Benlloch et al. (1996) carried out a similar study of the same multi-pond saltern, but used methods based on PCR amplification of 16S rRNA genes from DNA extracted directly from the environment, rather than isolation of organisms. They found that bacterial and archaeal primers gave good amplification in all samples except the two ponds of lowest salinity (6.4% and 9.2% w/v salts). These gave no amplification when archaeal primers were used—indicating a low number or absence of archaea at low salinity. Both bacterial and archaeal 16S rRNA genes were amplified from the crystalliser pond (30.8% w/v salt). However, hybridisation of the products from both amplifications to total DNA extracted, blotted on membranes showed a much stronger signal for archaeal rDNA than bacterial rDNA. This indicated that the bacteria had only a small representation in the crystalliser pond. The bacterial DNA in the crystalliser pond belonged to a single group distantly related to α-proteobacteria. In the previous study by Rodriguez-Valera et
al. (1985) no (eu)bacteria had been isolated from this pond. Twelve archaeal 16S rRNA clones were sequenced, and found to be highly similar. The clones were not related closely to any archaea which had previously been isolated from this pond, thus indicating that the diversity of archaea in salterns is wider than previously thought.
1.3 The Extremely Halophilic Archaea
(Halobacteria)

1.3.1 Taxonomy of Halobacteria

The presence of halobacteria in brines as indicated by their red colouration was first noted by the ancient Chinese over 4000 years ago (Baas-Becking 1931). The works of Pliny also mention highly, pigmented brines (Jones 1963). However, halobacteria were not isolated in pure culture until the beginning of this century, and it was not until much more recently that the full diversity of the halobacteria has been realised.

Kleban (1919) was probably the first person to isolate what we would now describe as halobacteria. While investigating the problem of the reddening of salted fish, he described red, extremely halophilic isolates, which lysed when placed in distilled water. He referred to rod shaped isolates as "Bacillus halobius ruber" and sarcinae as "Sarcina morrhuae".

Harrison and Kennedy in 1922 looked again at the taxonomy of isolates causing the reddening of salted fish and suggested isolates should be placed in the species "Pseudomonas salinaria". These were later transferred to "Serratia salinaria" (Tindall 1992).

In the early 1930's Petter and Schoop became interested in the taxonomy of this group of organisms. Petter (1932) worked with rod-shaped strains isolated from salted herrings and salted cod, which she assigned, to "Bacterium halobium" or "Bacterium trapanicum". Schoop (1935a, 1935b) was the first to suggest the generic designations Halococcus and Halobacterium. Also in this period Lochhead (1934) isolated a number of organisms from salted hides.

The first record of isolation of halophilic bacteria from a naturally occurring halophilic environment- the Dead Sea was by Elazari-Volcani (1940). These organisms were first assigned to the genus Flavobacterium, but Elazari-Volcani
(1957) later transferred these organisms and those of Petter and Lochhead to the new genus *Halobacterium*.

In the early 1950's N.E. Gibbons began a collection of extremely halophilic organisms, including those from Petter and Lochead and newly isolated strains. Also in the 1950's Anderson described organisms isolated from salted hides and salt in Australia (Anderson 1954).

Until the 1980s only the two genera of halobacteria, first described by Schoop (1935a, 1935b), were recognised; the genus *Halobacterium* consisting of rod-shaped or pleomorphic isolates and the genus *Halococcus* consisting of coccoid isolates. The discovery of the haloalkaliphilic strains in particular indicated that the diversity of halobacteria was much wider. The haloalkaliphilic strains were placed in two genera named *Natronobacterium* and *Natronococcus* (Soliman and Truper 1982, Tindall *et al.* 1984, Grant and Ross 1986). Since then several other generic groups have been described and in 1989 Grant and Larsen proposed the new taxon *Halobacterales*, to include the single family *Halobacteriaceae*, to accommodate all the then described genera.

Polar lipid analysis has been used extensively in the grouping of halobacteria. Chemotaxonomic studies, including DNA-16S rRNA hybridisation and DNA-DNA homologies (Ross and Grant 1985) and 16S rRNA analysis (McGenity, Gemmell and Grant 1998) indicate that there are at least 11 distinctive groups among the halobacteria.

In 1986, Torreblanca *et al.* had described two new genera; *Haloarcula* to include *H. vallismortis, H. trapanicum, H. hispanica, H. californiae*; and *Haloferax* to include *H. volcanii, H. mediterranei* and *H. gibbonsii* (the genus *Haloferax* had previously been proposed by Javor *et al.* in 1982).

In addition within the genus *Halobacterium*, the species *Hb. saccharovorum, Hb. sodomenase, and Hb. lacuprofundi* were clearly distinct from *Hb. salinarum*. Results from 16S rRNA/DNA hybridisation (Grant and Ross 1986, Ross and Grant
1985), 16S rRNA sequencing (Franzmann *et al.* 1988), immunological studies (Conway de Marcario *et al.* 1986), and polar lipid analysis (Tindall 1990a, Tindall 1990b) showed that the *Hb. saccharovorum* group deserved a separate taxonomic status within the *Halobacteriaceae*. McGenity and Grant (1995) described the Genus *Halorubrum* to include these species.

In 1995, Oren *et al.* proposed the Genus *Halobaculum*, to include the newly isolated organism responsible for the Dead Sea blooms and Kamekura & Dyall-Smith proposed the genus *Natrialba* to include non-pigmented strains of halobacteria.

Kamekura *et al.* (1997) showed using 16S rRNA that the haloalkaliphiles were much more diverse than previously thought and did not all fit into two distinct groups *Natronobacterium* and *Natronococcus*. They proposed a new genus *Natronomonas*, renaming *Natronobacterium pharaonis* as *Natronomonas pharaonis*. *Natronobacterium vaculatum* was transferred to *Halorubrum* as *Halorubrum vaculatum*. This strain differs from other members of the genus *Halorubrum* as it is the only obligately alkaliphilic member and also has several other biochemical differences. *Natronobacterium magadii* was transferred to the genus *Natrialba* as *Natrialba magadii*.

In 1985 Ross & Grant had shown clearly that species such as *Hb. salinarum* (NCIMB 786), *Halobacterium trapanicum* (NCIMB 784) and *Halobacterium halobium* (NCIMB 777) fell into a separate taxonomic group away from other *Halobacterium* species or any of the other named genera. McGenity, Gemmell and Grant (1998) have carried out an extensive phylogenetic analysis of halobacterial 16S rRNA gene sequences (Fig. 1.2) and on the basis of these and other taxonomic data proposed a new genus *Natrinema* gen. nov. to include these three species. *Hb. salinarum* NCIMB 786 was renamed as *Natrinema pellerubra*, and *Hb halobium* NCIMB 777 and *Hb. trapanicum* NCIMB 784 were renamed as a single species, *Natrinema pallida*.

The taxonomy of the halobacteria is still not completely clarified. One of the
problems has been the incomplete history of strains deposited in culture collections, as discussed by Tindall (1992). At present there are 11 described genera but these still do not adequately describe the whole diversity of halobacteria. 16S rRNA clones from a crystalliser pond described by Benlloch et al. (1995) form a separate distinct phylogenetic group, as does a clone 2MT16 isolated from a salt marsh by Munson et al. 1997 (see Fig. 1.2, McGenity, Gemmell and Grant 1998). Walsby (1980) discovered a square halophilic bacterium, although it has not yet been isolated in pure culture, polar lipid analysis suggests it must belong to a previously undescribed genus of halobacteria (Oren et al. 1996).

The current position of the taxonomy of the halophilic archaea or halobacteria is as follows:

Domain: Archaea
Kingdom: Euryarchaeota
Division: Mendosicutes
Class: Archaeobacteria
Order: Halobacteriales
Family: Halobacteriaceae
Genus: Halobacterium
     Halorubrum
     Haloarcula
     Haloferax
     Halococcus
     Halobaculum
     Natrialba
     Natronobacterium
     Natronococcus
     Natronomonas
     Natrinema

(Fig.1.2)
Fig. 1.2: Phylogenetic tree showing the relationship between species of halobacteria based on 16S rDNA sequences. (From McGenity et al. 1998)
1.3.2 General Characteristics of Halobacteria

The extremely halophilic archaea or halobacteria have an obligate requirement of at least 1.5 M NaCl for growth and to maintain the structural integrity of the cell. Many grow optimally at concentrations of 4M NaCl (Grant & Ross 1986, Grant, Gemmell & McGenity 1998). Colonies of halobacteria are often red, pink or orange coloured due to the possession of C₅₀ carotenoids (bacteriorubins). Dense populations of halobacteria impart a red colouration to the environment. Recently, colourless strains lacking this pigmentation have been identified (McGenity 1994, Kamekura & Dyall-Smith 1995). Strains that produce gas vesicles form opaque, white or pink colonies. A purple hue is often seen in strains containing bacteriorhodopsin (Grant et al. 1998).

Halobacteria are found world-wide in hypersaline environments. They are the most halophilic organisms known, and form the dominant microbial population when hypersaline waters approach saturation (Rodriguez-Valera et al. 1981).

Halobacteria may be a variety of shapes: cocci, rods, ribbons, cups, discs, trapezoids, squares and irregular shaped morphologies. Single species may also be very pleomorphic, often depending on growth conditions. *Halobacterium (Haloarcula) vallismortis* can be pear shaped with a filamentous tail (Gonzalez et al. 1978) and *Halobacterium (Haloferax) volcanii* which is generally disc shaped, can form hypomicrobium-like structures. The different morphologies may help cells to more efficiently exchange nutrients and gases (Norton 1992).

Osmoregulation by halobacteria is achieved by accumulation of K⁺ inside the cell. The internal concentration of *Halobacterium cutirubrum* (now *Hb. salinarum*) grown in 4M NaCl and 0.01M KCL is about 3.5M KCL, 1M NaCl and 0.1 M MgCl₂. Some halobacteria possess glycine betaine. This is often accumulated by eubacteria and by methanogenic archaea as a compatible solute for osmoregulation. In the halobacteria it is complexed with phosphatidyl glycerophosphate in the cell membrane, rather than free in the cytoplasm. Its role is therefore, probably not as a compatible solute, but it may play a role in stabilising the cell membrane (Nicholaus
As a consequence of the high intracellular solute concentration, the biochemical machinery is specifically adapted (Eisenberg et al. 1992). The ribosomes of halobacteria require around 3M KCl for stability. Some halobacterial enzymes such as aspartate transcarbamylase need high concentrations of salt for maximal activity and are rapidly inactivated below 2M salt. Other enzymes function well both in the absence of salt and in up to 4M NaCl or KCl, i.e. isocitrate dehydrogenase (Kushner 1986).

Halobacterial proteins contain an excess of negatively charged (acidic) groups with the excess of aspartate and glutamate being as much as 20%. Studies on halobacterial proteins also identified acid clusters in the N-terminal regions (Kogut & Russel 1987). Lanyi (1974) found that halophilic proteins had a lower concentration of hydrophobic amino acids when compared to non-halophilic counterparts suggested that borderline nonpolar residues participated in hydrophobic interactions and that this was encouraged under high salt concentrations. Many halobacterial proteins have now been sequenced and the structures of malate dehydrogenase and dihydrofolate reductase have now been determined. These have confirmed the earlier observations by Lanyi. Malate dehydrogenase, for example, has an excess of charged amino acids on its surface where they number twice as many as the positive amino acids. A number of the acidic amino acid residues are also involved in the formation of salt bridges leading to stabilisation of the folded protein (Danson & Hough 1997).

The cell wall of 	extit{Hc. morrhuae} is an extensively cross-linked sulphated heteropolysaccharide (Schleifer et al. 1982). In 	extit{Hb. salinarum} and probably all the non-coccoid halobacteria however, the external cell wall is a S-layer consisting mainly of glycoprotein (Mescher and Strominger 1976). This incorporates a regular array of morphological units arranged in a hexagonal pattern. The quartenary structure of the cell wall is dependent on the presence of Mg$^{2+}$ and Na$^+$ ions. The cell membrane of halobacteria exhibits typical archaeal properties, in containing isopranyl glycerol diether linked polar lipids (White 1995).
Introduction

Under conditions of light and low oxygen levels some species of halobacteria produce photopigments (rhodopsins). Five types of photopigments have been described- bacteriorhodopsin, halorhodopsin, two sensory rhodopsins (SRI and SRII) and slow-rhodopsin. Bacteriorhodopsin functions as a proton pump, which is energised directly by light energy (unlike photosynthesis, which is an example of indirect transformation of light energy via redox reactions producing the electrochemical potential). Bacteriorhodopsin is present in purple patches in the cell membrane, in amongst the usual red cell membrane, so it is often referred to as the "purple membrane". Bacteriorhodopsin consists of a large polypeptide (248 amino acids, 26 486 d) folded in to seven α-helices forming a transmembrane channel. Located in the middle of the channel and attached to the bacteriorhodopsin is a retinal pigment (a C20-carotenoid)) attached via a Schiff base to a lysine residue on the protein. When the retinal absorbs light, the bacteriorhodopsin translocates protons out of the cell and a proton potential is created (White 1994). The proton potential can then drive ATP synthesis via a membrane ATP synthase, and also drives a Na⁺/K⁺ antiport. Thus, under conditions of low oxygen concentrations and illumination halobacteria can obtain energy and also export Na⁺ and accumulate K⁺ to maintain the osmotic balance. It has been shown in continuous culture experiments that under low oxygen and nutrient levels, illumination allows populations to reach higher growth rates and higher population densities (Rodriguez-Valera et al. 1983).

Halorhodopsin is structurally similar to bacteriorhodopsin. This is a light driven inward Cl⁻ pump. In the dark halobacteria actively accumulate Cl⁻, probably using ATP (Lanyi 1990, Duschl and Wagner 1986).

A further role of the photopigments is that they make phototaxis possible (Bickel-Sandkötter et al. 1996). Hb. salinarum possesses two photosensory pigments SRI and SRII (λ_max: 565 nm and 490 nm respectively). They are present in relatively low abundance compared to halorhodopsin and bacteriorhodopsin (about 100 fold less than halorhodopsin). These photoreceptors control photoattraction (towards green light) and photophobic (towards blue/UV irradiation) responses.
1.3.3. Metabolism of Halobacteria

Halobacteria are chemoheterotrophs. The classical view of the members of the family *Halobacteriaceae* was that they had complex nutritional requirements and would only grow in defined media with the addition of a large number of amino acids (Tindall 1989). This idea arose because early nutritional studies focused particularly on a limited group; namely, those halobacteria isolated from proteinaceous material such as salted meat or fish. However sugar-utilising strains of halobacteria were reported by Elazari-Volcani in 1940 and by Breed in 1957 and it is now known that many halobacteria can utilise a wide variety of carbon and energy sources including sugars, amino acids, carboxylic acids and alcohols. (Rodriguez-Valera *et al.* 1980, Tomlinson and Hochstein 1972a, Gonzalez *et al.* 1978, Javor 1984). Halobacteria can also grow in the absence of oxygen.

1.3.3.1 Glycolysis in halobacteria

The halophilic archaea which have the ability to utilise glucose, do so via a modification of the Entner-Doudoroff pathway, in which oxidation precedes phosphorylation (see Fig 1.3). Unusually, the glucose dehydrogenase has dual cofactor specificity; NADP was found to be the most efficient electron acceptor, but cell extracts also reduced NAD (Tomlinson *et al.* 1974). This feature is not unique to the halobacteria among the archaea, but nicotinamide-dependant dehydrogenases of eubacteria and eukarya are characteristically specific for either NAD or NADP.

The Embden-Meyerhoff glycolytic pathway (Fig. 1.4) is probably not operative in halophilic archaea growing on glucose as 6-phosphofructokinase is absent. $^{13}$C-NMR experiments with *Hb. salinarum* (*halobium*) indicate the presence of a glycolytic pathway in addition to the modified Entner-Doudoroff pathway (Sonawat *et al* 1990). A modified Embden-Meyerhoff pathway was shown to operate in *Ha. vallismortis* during growth on fructose. In this case fructose was phosphorylated to fructose 1-phosphate in an ATP-dependent reaction, followed by conversion to fructose 1,6-bisphosphate by ketohexokinase, rather than entry by the usual phosphoenolpyruvate-dependent fructose phosphotransferase system (Altekar &
Fig. 1.3 The Classical and Modified Entner-Doudoroff Pathways

**Classical Entner-Doudoroff pathway**

1. **Glucose** + ATP → **Glucose-6-phosphate**
2. **Glucose-6-phosphate** + NAD(P) → **NAD(P)H + H^+**
3. **6-Phosphogluconate**
4. **6-Phosphogluconate** + H²O → **2-keto-3-deoxy-6-phosphogluconate**
5. **2-keto-3-deoxy-6-phosphogluconate** → **2-keto-3-deoxygluconate**
6. **2-keto-3-deoxygluconate** + H²O → **Pyruvate**
7. **Pyruvate** → **Glyceraldehyde 3-Phosphate**
8. **Glyceraldehyde 3-Phosphate** + Pi + NAD⁺ → **1,3-Bisphosphoglycerate**
9. **1,3-Bisphosphoglycerate** → **3-phosphoglycerate**
10. **3-phosphoglycerate** → **2-phosphoglycerate**
11. **2-phosphoglycerate** + H₂O → **phosphoenolpyruvate**
12. **phosphoenolpyruvate** + ADP + ATP → (**pyruvate** + ADP + ATP)

**Modified Entner-Doudoroff pathway in halobacteria.**

1. **Glucose** + ATP → **Glucose-6-phosphate**
2. **Glucose-6-phosphate** + NAD(P) → **NAD(P)H + H^+**
3. **6-Phosphogluconate**
4. **6-Phosphogluconate** + H²O → **2-keto-3-deoxy-6-phosphogluconate**
5. **2-keto-3-deoxy-6-phosphogluconate** → **2-keto-3-deoxygluconate**
6. **2-keto-3-deoxygluconate** + H²O → **Pyruvate**
7. **Pyruvate** + NAD⁺ → **NADH**
8. **NADH** + H⁺ → **2-phosphoglycerate**
9. **2-phosphoglycerate** + H₂O → **phosphoenolpyruvate**
10. **phosphoenolpyruvate** + ADP + ATP → (**pyruvate** + ADP + ATP)
Fig. 1.4: Pathways of glucose catabolism: the Embden-Meyerhoff glycolytic pathway and the Entner-Doudoroff pathway.

Glucose

ATP

ADP

NADP^+

NADPH + H^+

Glucose-6-phosphate

Fructose-6-phosphate

ATP

ADP

Fructose 1,6-bisphosphate

Dihydroxyacetone phosphate

Glyceraldehyde 3-phosphate

Pi + NAD

NADH +

1,3-bisphosphoglycerate

3-phosphoglycerate

2-phosphoglycerate

H₂O

phosphoenolpyruvate

ADP

ATP

pyruvate

6-phosphogluconate

2-keto-3-deoxy-6-phosphogluconate

H₂O

Emdben Meyerhoff

Entner-Doudoroff

Introduction
Introduction


All the enzymes required for gluconeogenesis have been detected in *Hb. salinarum* (*halobium*). The enzyme fructose 1,6 bisphosphate aldolase acts in gluconeogenesis, but has also been described as acting in the opposite direction during the growth of *Ha. vallismortis* on fructose.

1.3.3.2 Conversion of Pyruvate to Acetyl-CoA and Citric Acid Cycle

The conversion of pyruvate to acetyl-CoA is carried out by pyruvate oxidoreductase (POR) in halobacteria. Pyruvate oxidoreductase is also used by the other members of the archaea including the thermophiles and the methanogenic archaea (Schäfer & Schönheit 1991, Kerscher & Oesterhelt 1981a, 1981b, 1982, Kerscher et al. 1982, Zeikus et al. 1977). Halobacteria (and thermophiles) use ferredoxin as the electron acceptor. Methanogens use the deazaflavin derivative F420. In eukaryotes and many bacteria the pyruvate dehydrogenase multienzyme complex catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA.

Halophilic archaea also possess a complete oxidative citric acid cycle to generate energy and key intermediates for biosynthesis (Danson 1988a, Danson et al.1985, Danson & Hough 1992, Ghosh & Sonawat 1998). Others members of the archaea such as *Thermoplasma acidophilum* and *Thermoplasma volcanium* are facultative organotrophs also operate a full citric acid cycle (Segerer, et al. 1988). Many of the sulphur-dependent thermophilic archaea use a reductive citric acid cycle to fix CO2 (Kandler & Stetter 1981, Schäfer et al. 1986, Schäfer et al. 1989). Partial citric acid cycles are observed in methanogenic archaea (Ekiel et al. 1985, Weimer & Zeikus 1979).

Some members of the archaea catabolise acetyl-CoA by routes other than the citric acid cycle. *Tp. thermoplasma* and *Pyrococcus furiosus* generate ATP by the conversion of acetyl CoA into acetate. In *Archeoglobus fulgidus* acetyl CoA oxidation proceeds via a carbon monoxide dehydrogenase pathway (Thauer et al. 1989, Moller-Zinkmann & Thauer 1990)
1.3.3.3 Carbon utilisation

From a taxonomic point of view the carbon utilisation of the 11 genera of the halobacteria is as follows.

**Halobacterium**: Most strains have a complex nutrition and preferentially utilise amino acids as a source of carbon and energy. Many strains are proteolytic. Ducharme et al. (1972) found that arginine was rapidly removed from the media by *Hb. cutirubrum* (*salinarum*). Arginine is degraded via the arginine desimidiasde pathway, and the resulting citrulline converted to ornithine with the production of carbamoyl-phosphate in a reaction catalysed by ornithine carbamoyl transferase (Larsen 1967). The utilisation of aromatic amino acids by *Halobacterium salinarum* was studied by Lobyreva et al. (1987). This species could grow on L-phenylalanine, L-tyrosine and L-tryptophan. However an excess of tyrosine or tryptophan depressed the growth. A general overview of metabolic pathways in *Halobacterium* does not appear to have been published (Larsen & Grant 1989).

**Halorubrum**: Members of this genera are described by McGenity & Grant (1995) as able to utilise several sugars - glucose, mannose, ribose, maltose, and sucrose, and acid is sometimes produced. The type species of this genus *Halorubrum saccharovorum* (*saccharovorum* = sugar devouring) and also *Halorubrum trapanicum* are both described as requiring amino acids for growth. Both produce acids during growth on sugars. The third species in this genus *Halorubrum lacusprofundi* utilises many carbon sources including glucose, galactose, mannose, ribose, lactose, succinate, formate, acetate and ethanol without production of acid. It is not described as requiring amino acids.

**Natrinema**: Members of this genus are able to grow on casamino acids and certain sugars (McGenity, Gemmell and Grant 1998).

**Haloarcula**: Species in the genus *Haloarcula* do not require amino acids for growth and can metabolise a diverse range of substrates (Javor 1984). All produce acid from sugars.
**Introduction**

*Haloferax*: The species *Haloferax mediterranei* does not require amino acids for growth. It can grow on a wide range of substrates including lactate, acetate, amino acids and sugars. Acid is produced after growth on sugars (Torreblanca et al. 1986, Grant & Larsen 1986).

*Halococcus*: Javor (1984) found that *Halococcus* strains grew on a wide range of substrates. However, elsewhere it is reported that *Halococcus* species have a complex nutrition, requiring a number of amino acids, purines and pyrimidines for growth (Grant and Larsen 1989). *Halococcus morrhuae* can not utilise glucose as a main source of carbon and energy and does not produce acid or gas after growth on glucose (Grant and Larsen 1989).

*Natronobacterium*: A detailed study of the nutrition of the genus *Natronobacterium* does not appear to have been carried out. Growth of *Natronobacterium gregoryi* is stimulated by ribose, fructose, glucose, mannitol, and sucrose.

*Natronomonas*: *Natronomonas pharaonis* (formally *Natronobacterium pharaonis*) can not utilise sugars (Grant & Lasen 1987).

*Natronococcus*: Growth of the species *Natronococcus occultus* is stimulated by glucose, ribose, sucrose, and xylose (Grant 1989). Acetate is also metabolised by *Natronococcus occultus* (Kevbrina & Plakinov 1992).

*Natrialba*: *Natrialba asiatica* will grow on carbon sources including glucose, arabinose, xylose, maltose and starch (Kamekura & Dyall-Smith 1995). However *Natrialba magadi* (formally *Natronobacterium magadii*) can not grow on sugars but its growth is stimulated by acetate (Grant & Larsen 1989).

1.3.3.4. Production of Acids During Growth

Several strains of halobacteria have been reported as producing acid during growth on sugars. Tomlinson & Hochstein (1972b) looked at acid production of a strain called M6, after growth on glucose, galactose, lactose, fructose, and sucrose. They identified the acids produced as acetic acid and pyruvic acid. The highest amounts
of acids produced were after growth on glucose, but minimal amounts of acid were produced after growth on galactose, sucrose or lactose. It was suggested that when M6 was grown on glucose it lacked effective routes for the metabolism of acetate and pyruvate.

Oren & Gurevich (1994a) have also studied production of acids during growth on glucose and glycerol. During growth on glycerol, *Haloferax* species produced lactate and acetate, *Haloarcula* species and *Halobacterium saccharovorum* (now *Halorubrum saccharovorum*) produced acetate and pyruvate. During growth on glucose *Halobacterium saccharovorum* produced pyruvate only. *Haloferax mediterranei* and *Haloferax denitrificans* produced acetate only. *Haloferax gibbonsii* produced lactate and acetate. *Haloferax volcanii*, *Haloarcula marismortui*, and *Haloarcula vallismortui* produced acetate and pyruvate. In general quantities of acids were highest when strains were grown on glycerol. *Halobacterium cutirubrum (salinarum)* produced no measurable amounts of acid during growth on glycerol or glucose. The concentrations of lactate produced were between 1.0 – 5.0 mM, between 0.1 – 3.3 mM acetate was measured and between 0.1 and 1.6 mM of pyruvate was detected.

1.3.3.5 Glycerol metabolism

Probably all halobacteria can use glycerol (Oren 1994). Glycerol is probably one of the most available carbon sources for halobacteria in the natural environment (discussed in section 1.3.3.11) Many halobacteria produce acid when glycerol is added to the growth media. Oren & Gurevich (1994a) showed that *Haloferax* cultures grown in the presence of glycerol converted part of the glycerol to D-lactate and acetate, while cultures of the two *Haloarcula* cultures tested produced pyruvate and acetate from glycerol.

There are two pathways of glycerol metabolism among prokaryotes. In the first glycerol is phosphorylated to glycerol 3-phosphate by glycerol kinase. Glycerol 3-phosphate is then dehydrogenated to dihydroxyacetone phosphate. In the second pathway, glycerol is oxidised to dihydroxyacetone by glycerol dehydrogenase
followed by phosphorylation. Prokaryotes, which utilise glycerol, may possess one or both pathways (Oren 1994).

Baxter & Gibbons (1954) showed that *Hb. salinarum* had a NAD$^+$ dependent glycerol dehydrogenase activity. More recent studies have shown that glycerol dehydrogenase activity is restricted to the *Hb. salinarum* group (Oren & Gurevich 1994b). Glycerol kinase activity was demonstrated in all strains tested including *Hb. salinarum* strains with glycerol dehydrogenase activity (Oren & Gurevich 1994b, Rawal *et al.* 1988a).

13.3.6 Acetate metabolism

Javor (1983) has shown that several members of the halophilic archaea can grow using acetate as the sole carbon and energy source, these include *Ha. marismortui* and *Hf. volcanii*, but not *Hr. saccharovorum*, *Ha. vallismortis*, or *Hb. salinarum*. Klevbrina & Plakinova (1992) have shown that *Nc. occultus* can also use acetate as the sole source of carbon and energy.

In most organisms the growth using acetate involves the operation of the glycylxylate cycle, involving the enzymes isocitrate lyase and malate synthase. Aitken & Brown (1969) detected the presence of both enzymes in *Hb. salinarum* if acetate was present in the growth medium. More recently, Oren & Gurevich (1995b) have investigated the presence of isocitrate lyase amongst the halobacteria. They found high levels of isocitrate lyase in *Haloferax* *spp.*, low levels of activity in *Hb. salinarum* (halobium), *Ha. marismortui* and *Ha. vallismortis* and no activity in *Hb. salinarum*, *Hr. sodomense* and *Halobaculum gomorrae*. The enzyme was induced only when acetate was present and other nutrients were limited. Kevbrina & Plakunov (1992) also found isocitrate lyase activity in *Nc. occultus*.

13.3.7 Lactate metabolism

The presence of at least three different lactate dehydrogenases has been shown in the halophilic archaea. The presence or absence is correlated with the taxonomic position of the particular species.
An NAD⁺-linked D-lactate dehydrogenase from *Ha. marismortui* has been isolated and characterised (Hecht et al. 1990). A study by Rawal *et al.* (1988a) confirmed the presence of the NAD⁺ linked lactate dehydrogenase in *Ha. vallismortis* and also in *Hf. mediterranei*. A low activity was also detected in *Hb. salinarum*.

The presence of NAD⁺-independent lactate dehydrogenases is widespread in the halophilic archaea. Baxter & Gibbons (1954) first reported the presence of a NAD⁺-independent lactate dehydrogenase in *Hb. salinarum* over 40 years ago. Oren & Gurevich showed that both NAD⁺-independent L-lactate dehydrogenase and D-lactate dehydrogenase activities were present in all strains tested (Oren & Gurevich 1995). *Hb. salinarum* isolates had very low L-lactate dehydrogenase activity. *Haloarcula* species, which exhibit very high NAD⁺-linked D-lactate dehydrogenase showed very low activities of both NAD⁺-independent L and D lactate dehydrogenases.

Little is known of the function of the lactate dehydrogenases in the halophilic archaea. The equilibrium of the NAD⁺-linked reaction greatly favours formation of lactate from pyruvate and NADH. However, *Ha. marismortui* and *Ha. vallismortis*, which display a high activity of the NAD⁺-linked enzyme, produced pyruvate rather than lactate when grown in the presence of glycerol or sugars (Oren and Gurevich 1994a). Also although most stains show NAD⁺-independent L-lactate dehydrogenase activity, L-lactate added to the media was not used unless lactate racemase was also present (Oren 1994). *Hf. volcanii* and two *Haloarcula* species tested showed lactate racemase activity. No activity was demonstrated in *Hb. salinarum, Hr. saccharovorum* or *Hf. mediterranei* (Oren & Gurevich 1995).

A further pathway, which may contribute to the formation of D-lactate in the halophilic archaea, is the methylglyoxal bypass. In this pathway, methylglyoxal is formed from dihydroxyacetone phosphate by methylglyoxal synthase, wherefore glyoxalase I produces the D-lactoyl derivative of glutathione, or in the case of the halophilic archaea, γ-glutamylcysteine. Glyoxalase I activity was found in all halophilic archaea tested; however distribution of methylglyoxal synthase was species dependent. No activity was found in *Hb. salinarum* strains, while other
strains including *Hr. saccharovorum* contained the enzyme (Oren & Gurevich 1995). Unusually, the activity of *Hf. mediterranei* methylglyoxal synthase was highest in the absence of salt, the presence of 3M NaCl or KCl caused about 50% inhibition.

1.3.3.8 Utilisation of hydrocarbons and crude oil

Crude oil is a very complex mixture of mainly hydrocarbons, but with significant representation of other elements such as oxygen and sulphur, and smaller amounts of nickel and vanadium. Many evaporite deposits occur in close proximity to oil deposits. Petroleum has been mined from, and tar seeps occur, in the Great Salt Lake (Ward & Brock 1978). Bacteria have been shown to colonise tar balls formed from oil seeps, leaking drill holes and faults in the lake floor of the Great Salt Lake (Post 1977).

Bertrand *et al.* (1990) isolated several strains of halobacteria from a salt marsh in the South of France able to degrade eicosane (saturated C20 hydrocarbon). One of the isolates EH4 (*Halobacterium* or related) was able to degrade both odd and even saturated hydrocarbons (tetradecane, hexadecane, eicosane, heneicicosane), saturated isoprenoid alkanes (pristane) and aromatic hydrocarbons (acenaptene, phenanthrene, anthracene and 9-methyl anthracene). Kulichevskaya *et al.* (1991) showed strains of *Hb. salinarum*, *Hf. volcanii*, and *Hb. distributum* emulsified and grew on petroleum hydrocarbons, but petroleum degradation was not detected in *Hb. halobium (salinarum)*, *Hr. saccharovorum*, *Hf. mediterranei* and *Halococcus turkmenicus*. Zvyagintseva *et al.* (1995) isolated two species of *Haloferax spp.* from the Kalamkass oil field in Russia, which degraded n-alkanes and iso-prenoids. Also Emerson *et al.* (1994) isolated a member of the genus *Haloferax* which was able to utilise a number of aromatic substrates (e.g. benzoate, cinnamate and phenylpropionate) as the sole carbon and energy source.

A field study on the degradation of hydrocarbons in the Great Salt Lake was carried out by Ward and Brock (1978). Enrichment cultures with [1-\(^{14}\)C] hexadecane with salinities of 20.4% and higher showed signs of degradation although no \(^{14}\)CO\(_2\) was evolved during the first five days of incubation. However there is no indication of type of bacteria involved.
The pathways and enzymic basis of hydrocarbon degradation have not been elucidated in halobacteria (Oren 1994).

The diphosphodiphytanyl diglycerol membrane lipids of the halobacteria have been shown to have an emulsifying effect producing a water in oil emulsion (Post & Collins 1982). Halobacterial cells have been shown to enhance oil release from Utah tar sands, thus are potentially useful for enhancing oil recovery. (Post & Al-Harjan 1988).

1.3.3.9 Anaerobic Metabolism

At least four alternative electron acceptors have been shown to replace oxygen in the respiration of different representatives of the halophilic archaea; nitrate, dimethylsulphoxide (DMSO), trimethylamine-N-oxide (TMAO) and fumarate. Oxygen is often in short supply in hypersaline environments as a result of both the frequently high microbial densities and the limited solubility of oxygen at high salt concentrations.

Anaerobic growth with nitrate as the electron acceptor with the formation of the gaseous products dinitrogen and/or nitrous oxide (denitrification),

\[ \text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2 \]

has been demonstrated in all Haloarcula species tested; Ha. vallismortis, Ha. marismortui, Ha. hispanica, and Ha. japonica (Tindall 1992) and in two Haloferax species; Hf. denitrificans and Hf. mediterranei (Mancinelli & Hochstein 1986, Tindall 1992). Other species such as Hb. sodomense, Hb. distributum, Hf. volcanii and Natronobacterium pharonis slowly reduce nitrate to nitrite without the formation of gas (Tindall 1992). Some species do not reduce nitrate for example Hf. gibbonsei. Several members of the Halobacterium, Haloarcula and Haloferax can use either dimethylsulphoxide as an electron acceptor reducing it to
dimethylsulphide, trimethylamine N-oxide (TMAO) to trimethylamine (TMA) or fumarate to succinate (Oren and Truper 1990, Oren 1991). Oren (1994) suggests that only TMAO is present in high concentrations in nature. Marine animals (teleosts and elasmobranchs) contain TMAO in amounts of up to 7% of their dry weight. As halobacteria often live on salted fish it is possible that they may use TMAO as a potent and natural electron acceptor.

*Hb. salinarum* strains are able to grow anaerobically by fermentation of arginine to citrulline. The consumption of arginine is accompanied by the equimolar appearance of ornithine in the media (Hartman 1980). This suggests that the degradation occurs by the arginine deiminase pathway; this pathway requires the enzymes arginine deiminase (ADI), carbamate kinase (CK) and catabolic ornithine carbamylase (COTLase). Ornithine carbamoyl transferase has been isolated from *Hb. salinarum* (Dundas 1970). Oren and Gurevich (1994) confirmed the ability of the above strains to utilise arginine, they also found that *Na. pharaonis* could grow anaerobically with arginine added to the media, but they found no anaerobic growth with arginine in any other species tested.

1.3.3.10 CO₂ Fixation by Halobacteria

Among prokaryotes there are three main pathways of CO₂ fixation. The Calvin cycle is found in, most photosynthetic bacteria, cyanobacteria and chemoautolithotrophs, as well as photosynthetic eukaryotes. The reductive tricarboxylic acid pathway occurs in the green photosynthetic bacteria, *Hydrogenobacter, Desulphobacter, Sulpholobus* and *Thermoproteus*. The acetyl-CoA pathway is found in methanogenic archaea, some sulphate-reducing bacteria and in acetogens.

There is some controversy surrounding the ability of halobacteria to fix carbon dioxide. Oren (1983) speculated that halobacteria may contribute significantly to light-dependent CO₂ assimilation in the Dead Sea. Danon and Caplan (1977) and Oren (1983) suggested that CO₂ fixation is linked to the possession of bacteriorhodopsin, which might mediate a light driven fixation of CO₂.
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Danon and Caplan (1977) demonstrated the ability of *Hb. halobium (salinarum)* to fix carbon dioxide when grown anaerobically. The reaction required light, ATP and the integrity of the electron transfer chain. Propionate had a stimulatory effect on the CO₂ fixation. This suggested that the reaction occurring could be of the type:

\[ Z_{\text{red}} + \text{CO}_2 + \text{propionyl CoA} \rightarrow Z_{\text{ox}} + \alpha\text{-ketobutyrate + CoA} \]

Javor (1988) carried out further studies on CO₂ fixation in halobacteria. Cells were grown heterotrophically and then incubated semi-anaerobically for several days to induce bacteriorhodopsin in strains which produce it. Prior to assaying for CO₂ fixation cells were subjected to starvation overnight. *Halobacterium* spp., *Halococcus* spp., and *Haloarcula* spp. fixed CO₂ under light and dark conditions. Light enhanced CO₂ fixation in *Hb. halobium (salinarum)* but inhibited it in *Hf. volcanii* and *Haloarcula* strain GN-1. Propionate enhanced CO₂ fixation in some but not all strains. NH₄⁺ enhanced CO₂ fixation, with glycine being the major amino acid synthesised. Little or no α-ketobutyrate was detected among the early products of CO₂ fixation. Javor accordingly proposed a novel pathway for non-reductive CO₂ fixation involving a glycine synthase reaction with CO₂, NH₄⁺ and a methyl carbon derived from the β-carbon cleavage of propionate.

Rajagopalan & Altekar (1991) analysed the products of CO₂ fixation in heterotrophically grown *Hf. mediterranei*. The dominant product of CO₂ fixation following incubation with NaH¹⁴CO₃ and pyruvate or propionate was pyruvate. The major amino acids, which became labelled, were glutamic acid, aspartic acid and alanine, but no incorporation into glycine was observed (Rajapalan and Altekar 1991). Rajagopalan & Altekar (1991) therefore concluded that a glycine synthesis reaction was not responsible for the CO₂ fixation.

Ribulose bisphosphate carboxylase (RuBisCo) activity has been detected in *Hf. mediterranei*, *Hf. volcanii* and *Ha. marismortui*. (Rawal et al. 1988, Altekar and Rajagopalan 1990) and the enzyme from *Hf. mediterranei* has now been purified and characterised (Rajagopalan & Altekar 1994). *Hf. mediterranei* also possesses all the other enzymes necessary for the reductive pentose phosphate cycle (Rawal et al. 1988). No RuBisCo activity was detected in *Ha. vallismortis*, several *Hb. salinarum*
strains and *Hr. saccharovorum* (Altekar and Rajagopalan 1990). The CO₂ fixation was stimulated by the addition of ATP and NADH and it was suggested that hydrogen could be a possible reductant (Altekar & Rajagopalan 1990). The levels of RuBisCo activity are at best only a few percent of those typically found in autotrophic bacteria (Oren 1994). The strains with RuBisCo activity all accumulate poly-β-hydroxybutyrate (PHB) and Altekar and Rajagopalan (1990) suggest that the CO₂ fixation could be directed towards the production of PHB.

Halobacteria have, however, never been shown to be capable of autotrophic growth (Tindall and Truper 1986, Javor 1988, Oren 1994).

1.3.3.11 Nutrition of Halobacteria in the Natural Environment

Although there have been numerous studies of the metabolism of pure cultures of halobacteria "in vitro" very little is known about the modes of nutrition in the natural environment. Studies of nutrition of halobacteria in the Great Salt Lake (Fendrich and Schink 1988) and in the Dead Sea (Oren 1995a, Oren 1995b, Oren and Gurevich 1986) have been carried out.

Fendrich & Schink (1988) found that the levels of glucose and glycerol in the Great Salt Lake were below detectable limits (5.5 μmol L⁻¹ and 10.0 μmol L⁻¹), with the acetate level between 6.1 - 8.6 μmol L⁻¹. Transformation rates of acetate, glucose and glycerol in water samples taken from the North (22% salt) and South (8.5% salt) arms of the Great Salt Lake were measured. Production of ¹⁴CO₂ from [U-¹⁴C] labelled acetate, glucose and glycerol was examined over 8 days. In the water samples from the South Arm CO₂ production from all substrates started in the first twelve hours. In the sample from the North Arm only acetate was degraded within 12 hours, degradation of glucose and glycerol began 12 - 24 hours after substrate addition. After 8 days the substrate was completely transformed; about 70% being recovered as ¹⁴CO₂. Water samples contained bacteria and no eukaryotes, but no indication is given of numbers of bacteria or types.
In the Dead Sea the main primary producers are species of the unicellular green algae *Dunaliella*. *Dunaliella* spp. accumulates glycerol intracellularly in molar concentrations as a compatible solute. In the Dead Sea blooms of *Dunaliella* spp. are often accompanied by mass developments of halophilic archaea (Oren 1994, Oren 1988; Oren 1993). Glycerol leaking out of healthy or decaying *Dunaliella* cells is probably the main carbon and energy source enabling the dense communities of halophilic archaea to built up (Oren and Gurevich 1994b).

Oren (1994) incubated samples from the Dead Sea and saltern crystalliser ponds with [U-\(^{14}\)C] glycerol. They found that the natural communities of halophilic archaea depleted all added glycerol. Around 10% of the label was found in acetate and lactate and in the case of the Dead Sea sample label was also found in pyruvate. Upon further incubation of Dead Sea samples, after depletion of the glycerol, pyruvate disappeared rapidly, while acetate and lactate disappeared only slowly. In the saltern brines the lactate formed was degraded after depletion of the glycerol, but the concentration of labelled acetate decreased only slowly. In a further study Oren (1995b) showed that respiratory electron transport activity in the Dead Sea was stimulated more than 2-fold by the addition of glycerol, although in samples from the saltern crystalliser pond the effect of adding glycerol was less pronounced. This effect was not stimulated by addition of sugars, organic acids, amino acids or inorganic nutrients.

Oren (1995a) determined acetate uptake and turnover rates for the community in the Dead Sea and in saltern crystalliser ponds. Acetate is formed from glycerol. Values of \([Kt + Sn]\) (the sum of the substrate affinity and the substrate concentration present in situ) for acetate measured in saltern crystalliser ponds were around 4.5 - 11.5 \(\mu\)M, while in the Dead Sea during a *Dunaliella* bloom values of up to 12.8 \(\mu\)M were found. Maximal theoretical values (\(V_{max}\)) of acetate uptake in saltern crystalliser ponds were 12 -56 nmol l\(^{-1}\) h\(^{-1}\) with estimated turnover times for acetate (\(T_t\)) between 127 - 730 hr at 35°C. \(V_{max}\) values measured in the Dead Sea were between 0.8 to 12.8 nmol l\(^{-1}\) h\(^{-1}\) with turnover times in the range of 320 -290 hr. \(V_{max}\) values for acetate were much lower than those for glycerol. Comparisons with pure cultures of halophilic archaea grown under different conditions showed that the
natural communities were not adapted for preferential use of acetate. Both in natural brines and in pure cultures of halophilic archaea, acetate incorporation rates rapidly decreased above the optimum pH, probably since acetate enters the cell only in its unionised form. The low affinity for acetate together with the low potential utilisation rates result in long acetate turnover times, which explains the accumulation of acetate observed when low concentrations of glycerol are supplied to the natural communities of halophilic archaea.

Glycerol is of course, not the only compatible solute used for osmoregulation in hypersaline environments. Mateeva et al. (1993) looked at accumulation of amino acids and glycine betaine by bacteria isolated from high salinity stratal waters of oil fields. They found that the major osmoprotectant was glycine betaine, accumulated up to 5M. The amino acids, which played the most significant role, were glutamic acid (348 mM) and serine (38.8 mM). It is therefore a possibility that in some hypersaline environments compatible solutes such as glycine betaine and amino acids may be present in significant quantities and could be potential carbon and energy sources for halophilic archaea.

Mopper and Taylor (1986), Diaz et al. (1992) and Shieh (1965) have reported catabolism of glycine betaine by aerobic bacteria. Diaz and Taylor (1996) isolated bacteria from Mono Lake, a moderately hypersaline (90 ppt) and alkaline lake (pH 7.7) in California, which grew on glycine betaine and dimethylsufonopropionate (DMSP).

1.4 Ancient salt deposits

1.4.1 Location and composition of ancient salt deposits

Evaporite rocks are those which were originally precipitated from a saturated surface or near surface brine in hydrological systems driven by solar evaporation (Warren 1996). Evaporite deposits are widespread in the geological record both in time and location. Evaporite rocks underlie approximately 25% of continental areas (Blatt et al.1980). Evaporites have been recorded from the Precambrian through
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Phanerozoic time. Some of the largest evaporite deposits are associated with the Permian period. Permian evaporite beds probably covered more than 4.5 million km$^2$ of the earth’s surface and produced nearly 1.3 million km$^3$ of rock salt. Beds of gypsum and halite from several hundreds to thousands of meters thick are known in several locations in the world. Many of these contain fossil and textural evidence of having formed in shallow water. The complete evaporation of a body of seawater as deep as the Mediterranean Sea, which averages about 1370 m would only produce 24 m of halite and 1.4 m of gypsum. Thus, these thick evaporite sequences did not form as a result of a single evaporitic sequence in a deep basin, but through the continual evaporation of water from a partially isolated basin that was episodically fed with seawater for thousands of years (Craig et al. 1996).

Many regions of the world have salt domes, including Europe, South America, the Middle East and the former Soviet Union. They are particularly abundant in the area on the north of the Gulf of Mexico. These occur where bedded salt rises upwards through weaker sediments due to the salt having a lower density and lower rigidity than the overlying rocks and slowly becoming buoyed towards the surface. The deformation may be due to unequal loading or tectonism. Salt domes range from 100 m to more than 2 km across and have risen through the sediments by as much as 12km. Salt domes are major sources of sulphur and petroleum (Craig et al 1996, Schreiber 1988).

1.4.1.2 Winsford Salt Mine, Cheshire

The Cheshire basin in England is filled with Triassic sedimentary rocks. The Mercia mudstone (formally known as the Keuper Marls), which forms part of the succession, contains two halite horizons. The upper horizon- the Wilksley Halite is up to 400m thick and the lower horizon- Northwich Halite is up to 290m thick (Fig.1.5) The Northwich halite sub-outcrops against glacial drift and marl of Pleistocene age at Winsford. The halite is made up of individual units of bedded rock salt separated by marl bands. There are 9 zones present at Winsford, but only zones D and F have been mined (Fig. 1.6). The glacial drift is porous and allows percolation of groundwater down to the bedrock dissolving salt from the sub-outcrop of the saliferous beds giving rise to a long-standing horizon of brine (Fig.
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1.6). Most of the brine seeps out to sea, but some wells to the surface forming brine springs (wyches). These were common in Cheshire and Shropshire, and were exploited for hundreds of years. The pumping of this "wild brine" resulted in many springs drying up and also in widespread subsidence. The marls are impermeable and prevent penetration of brine to any greater depth. The halite horizons are not only separated by clastic material but also contain thin layers of clastic material. The halite horizons also contain polygonal features, which can be seen on the mine roof. The mine has corresponding V-shaped fissures on the walls. These fissures contain bands of secondary salt and clastic materials. These features may have been caused by thermal contraction (Tucker and Tucker 1987).

Winsford Salt Mine in Cheshire, England has been mined since 1844. Rock salt is mined using the "room and pillar" method, where salt pillars are left uncut to support the roof. This has created a series of chambers and tunnels 7.6m high and 6 to 20m wide (Longley-Cook 1989). The salt is cut and blasted. It is then crushed, treated with potassium ferrocyanide as an anti-caking reagent, transported to the surface and stored in the open. The rock salt is used mainly for de-icing roads and a small amount is used as sugar beet fertiliser (Longley-Cook 1989). Solution mining is also carried out, at Holford, east of Northwich (Longley-Cook 1989).
Fig 1.5 Generalised sequence of Triassic sediments above the Keuper Sandstone in the central part of the Cheshire-Shopshire basin (Arthurton 1980)
Fig. 1.6 Schematic representative of a typical section of strata of the Northwich Halite Formation at Winsford, Cheshire (Longley-Cook 1989)
The mines are ventilated by fans, which drive air through the tunnels at a rate of 10,000 m$^3$ min$^{-1}$. To remove moisture from the air it is first circulated through old workings known as dewatering tunnels. The water forms condensation on the cool walls. The dried air is then circulated to the mine faces. The condensation on the walls forms brine pools on the mine floor and salt efflorescences of recrystallised salt are formed on the walls (Longley-Cook 1989).

1.4.1.3 Boulby Potash Mine, Cleveland

The halite and potash beds mined at the Boulby, Cleveland in England are part of the Upper Permian Zechstein deposit. The Zechstein Sea probably covered an area of 250,000 km$^2$ ranging from Northern England to Germany and Poland. There were several cycles of salt deposition. The evaporite beds in the Boulby area were laid down in the third and fourth cycles (EZ3 and EZ4). The Boulby Halite and Potash which are mined are of cycle EZ3.

In contrast to the very thick beds of halite in the Cheshire basin, the Zechstein deposit has a sequence of beds in each cycle. In EZ3 the order of deposition was anhydrite (CaSO$_4$), halite (NaCl) and sylvite (NaCl + KCl). Within Boulby Potash there are also regions rich in camallite (KCl$\cdot$MgCl$_2$$\cdot$6H$_2$O). It is believed that the deposits of the EZ3 and EZ4 Zechstein cycles were formed in a narrow arm of the Zechstein Sea, and further evidence indicates that they were formed on extensive salt flats on the edges of the diminishing Zechstein sea (Smith and Crosby 1979). The strata overlying the Zechstein salts are shown in Fig. 1.7 The Bunter sandstone is a Triassic aquifer with 4% w/v NaCl at the top and 18% w/v NaCl at the base. There is a small inflow of Bunter brine in to the mine workings at Boulby Mine. The Boulby halite bed has undergone very little mineralogical change. It has variable content of clay, sylvite and anhydrite. However, Boulby potash has undergone extensive chemical and physical change. It has been subjected to 2 stages of diagenesis, firstly upward brine migration, causing almost complete recrystallisation, probably of camallite to produce sylvite. Secondly plastic flow of sylvite occurred when the evaporites were at their maximum depth to undulate the top of the bed (Woods 1979). Fig. 1.8 shows a schematic representation of the strata at Boulby.
Fig. 1.7: Section of the strata overlying the Permian evaporites at Boulby

- Boulder clay
- Ironside
- Liassic Shales
- Rhaetic
- Keuper Marl
- Bunter Sandstone
- Bunter Mudstone
- Permian Mudstone
- Series of Permian Evaporites

Drift

Jurassic

Triassic

Permian
Fig. 1.8 Schematic representation of the strata of the Permian Evaporites at Boulby, Cleveland

Introduction

- Upper Anhydrite
- Upgang Formation
- Carnalite
- Transition zone
- Boulby Potash
- Boulby Halite
- Billingham Main Anhydrite

The biogenic-in-hypersaline environments are extremely high. Algae and plants growing nearby may also export organic materials to the system. A hypersaline lake in Xilitla has been documented where the presence of the water level in salinity changes led to submersion and desiccation of the organisms growing on the banks of the lake. Organic carbonates (carbonate seston) may also contribute to the carbon cycling in hypersaline environments (Oliver et al., 1999).

Organic matter generated in an aquatic environment has several possible fates. It may be preserved intact in the circumstances, but more commonly it is partially or completely degraded, with the potential to release nutrients and recirculate into the ecosystem.
Boulby Potash Mine has been mined since 1974. It is exploited mainly for potash, but also for smaller amounts of halite. The Boulby Potash deposit lies at about 1050m depth and as a result the temperature of the mine is 37 - 40°C. The principle component of Boulby Potash is sylvinite (sylvite and halite). It is mined by cutting and blasting roadways with pillars left for support. The Boulby Potash is underlain with Boulby Halite. This is also cut where the potash is thin and to make roadways for transportation and ventilation. The potash is crushed on the surface and the sylvite concentrated by flotation in brine. The sylvite is sold for use as fertiliser and the halite for de-icing roads. Processing waste (salt and insoluble minerals) is discharged into the sea (Gemmell 1996).

Brine is common in Boulby mine. Some is from external origins, such as the Bunter sandstone, however other brines are residual formed from diagenetic changes to minerals. Drilling fluid and surface waters are other sources of brines in the mine. Pockets of gas- N\textsubscript{2} and CH\textsubscript{4} also occur in the mine, the origin of these is unknown, but they may occur in situ (Sonnenfeld 1984).

1.4.2 Organic Matter and Fluid Inclusions in Ancient Evaporites

In this section the processes by which organic matter becomes incorporated in evaporite deposits are discussed and the amount and type of organic matter found in ancient salt deposits is described.

The biomass in hypersaline environments is extremely high. Algae and plants growing nearby may also input organic matter in to the system. A hypersaline lake in Africa has been described where the increase in the water level in the rainy season leads to submersion and death of the vegetation growing on the banks of the lake. Organic osmolytes (compatible solutes) may also contribute to the carbon cycling in hypersaline environments. (Ollivier et al. 1994)

Organic matter generated in an aquatic environment has several possible fates. It may be preserved intact in rare circumstances, but more commonly it is partially or
completely destroyed. In the open ocean, the Black Sea and in lakes the primary zone of destruction is in the oxygenated water column. This is probably also true in evaporitic environments. In evaporitic environments this part of the water column is thin—usually only tens of meters. The O$_2$ concentration is less than in marine water and therefore the destructive capacity is less. _Artemia salina_ are abundant in many hypersaline environments. The faecal pellets sink rapidly through the oxygenated region, so the organic matter in the faecal pellets is removed quickly from contact with aerobic bacteria.

Below the oxic zone anaerobic bacteria will feed on sinking algal and bacterial debris. The sulphate reducing bacteria metabolise simple organic compounds forming CO$_2$ and hydrogen sulphide. At the boundary between the oxic and anoxic conditions the hydrogen sulphide and O$_2$ are utilised by sulphur-oxidising bacteria or photosynthetic sulphur bacteria. These bacteria are likely to be the principal source of organic matter that becomes entombed within the sediments (Evans and Kirkland 1988).

During the first few cm or tens of cm of sediment organic matter is degraded by the sulphate reducing bacteria. The sediments are usually fine-grained carbonates or marls with restricted permeability preventing movement of brine in to the sediment. Burrowing metazoans are absent in the sediments of hypersaline lakes. Consumed sulphate can therefore only be replaced by very slow downward diffusion and slow replacement of sulphate will limit the oxidation of organic matter by the sulphate-reducers. Under favourable conditions, amino acids, fatty acids and molecular hydrogen are generated by fermentation and hydrolysis by bacteria, allowing sulphate reduction to occur, but the high salinity of the brine may mean that their activity is decreased (Klug _et al._ 1985). Under these circumstances oxidation of organic matter by sulphate reducers would be diminished. Enrichments from sediments from a hypersaline lake in Senegal, produced acetate from cellulose degradation, which then accumulated. Acetate, propionate or butyrate were not metabolised 2 months after addition to the enrichment cultures (Ollivier _et al._ 1994).

Below the region where sulphate is present, the methanogens are found.
Degradation of organic matter is likely to proceed slowly in this zone (Evans and Kirkland 1988). Ollivier et al. (1994) suggest that above salinity of 15% sulphate reduction and methanogenesis are often low or even absent.

Therefore, sediments rich in organic matter are formed due to the incomplete oxidation of organic matter and slow accumulation of mineral matter. In the Dead Sea, Neev and Emery (1976) found up to 2% total organic content (TOC) in sediments, although in a previous study by Nissenbaum et al. (1972) the TOC was found to be in the range 0.23% to 0.4%. The sediment below the anoxic water column contained higher concentrations of hydrocarbons, fatty acids, amino acids, humic acids and fulvic acids and chlorophyll derivatives than sediments from below the oxygenated water. In strongly anoxic areas fulvic and humic acid made up 40 - 50% of TOC but only 1.5 - 3.2 % in oxygenated margins. The isoprenoid hydrocarbons phytane and pristane were also present. Nissenbaum et al. (1972) found that 80% of hydrocarbons from deep basin sediments were n-alkane C15 and C16, however these were only 4 -16% in shallow water sediments. Fatty acids found were both free and hydrolysable (bound) acids, most of the free acids were C16 to C18. The organic matter was rapidly converted into an insoluble non-extractable complex in oxic regions, but was preserved in the anoxic environment (Evans and Kirkland 1988).

Organic matter accumulating in the sediments has been measured at the Alviso Salterns in San Francisco Bay. Measurements of Dissolved Organic Carbon (DOC) of brines from three different salinities showed that the DOC increased as salinity increased- Table 1.4 (Hite et al.1984). Most of the DOC is was probably the result of death and autolysation of algae and bacterial cells, and the breakdown of the mucilage produced by cyanobacteria.

In cores penetrating up to 50cm of pond sediments Klug et al. (1985) measured TOC content and volatile fatty acids (VFA) from five different salinity sites. They found that the TOC increased in up to 9% salinity. A maximum of 23.1 % TOC was found in sediments of the pond with 9% salinity. TOC values decreased in
Introduction

Table 1.4 Dissolved organic carbon (DOC) from three brines from the Alviso salt ponds (From Hite et al. 1984).

<table>
<thead>
<tr>
<th>Brine Density</th>
<th>Saturation Field</th>
<th>TOC (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.16</td>
<td>Gypsum</td>
<td>223</td>
</tr>
<tr>
<td>1.21</td>
<td>Halite</td>
<td>249</td>
</tr>
<tr>
<td>1.30</td>
<td>Halite-potash</td>
<td>2419</td>
</tr>
</tbody>
</table>

sediments in ponds with 15 and 30% salts. This may be due to dilution by rapidly precipitating gypsum and halite. The amount and spectrum of VFA was found to increase with increasing salinity. Acetic acid, the principle VFA, increased with increasing salinity in all pond sediments reaching a value of 802.33 μmolL-1 in the upper 1cm of sediment of the 30% salinity pond. Propionic, isobutyric, butyric and isovaleric acids were also found.

Ultimately, most of the organic matter becomes incorporated into kerogen. Kerogen consists of organic polymers of high molecular weight and complex structure. Kerogen forms from degradation products of algal cell and microbial by-products. In a deep-water evaporitic environment the kerogen is rich in hydrogen. After sufficient burial and chemical decomposition it may yield oil. Szatmari (1980) suggests that most of the worlds largest oil deposits could have been derived from evaporite source rocks, especially halite. Examples where oil reservoirs are likely to have originated from evaporite deposits include the Todilto Limestone Formation in SW Colorado and NW New Mexico, the Paradox basin in Utah and Colorado, the Michigan Basin, the Devonian Basin of Western Canada, the Jurassic Smackover formation oil in the Gulf Coast Region of the USA, and the Permian Zechstein Basin (Evans and Kirkland 1988).
There is substantial organic geochemical data on the Paradox Member halite of the Hermosa formation in SE Utah. 147 samples were analysed, of these 70 had a TOC content of below the detectable level (0.001%). In the other samples the content ranged from 0.001% to 0.28%. Similar low values were found for halite from the A-1 salt in the Michigan Basin and halite from Cretaceous evaporites in Thailand (Hite and Anders 1991). However Treesh and Friedman (1974) found higher TOC values of 0.44 - 2.14% for Silurian halite in the Michigan Basin.

Inclusions of droplets and veinlets of oil and bitumen occur frequently in the evaporite deposits in the Northern Caspian region (Sonnenfeld 1984). Preserved spores and pollen often occur in most rock salt and potash deposits. Evaporite beds frequently contain streaks of organic matter and black bituminous stringers (Sonnenfeld 1984). Hydrogen is a common gas in halite and potash deposits as a result of reduced consumption by sulphate reducers and methanogens. Combustible mixtures of gases causing explosions and fires have been encountered in salt and potash mines in Europe (Sonnenfeld 1984). These gases most commonly occur in micro-inclusions and consist mainly of nitrogen, and smaller amounts of methane, higher alkanes, H2, helium, neon and argon.

Fluid inclusions may comprise up to 10% of the volume of halite deposits. They persist in halite throughout its burial history. The fluid inclusions are formed as a result of the porosity of halite rapidly decreasing within a few metres of burial. At depths of 30 - 40m porosity and permeability is lost and only isolated intracrystalline voids filled with fluid inclusions remain. (Schr€iber 1988). Hite and Anders (1991) analysed inclusions of hydrocarbons from some Paradox halites finding predominantly light (C1 -C5) hydrocarbons. It has been shown that halobacteria can become entrapped in fluid inclusions during their formation and that they can survive entrapment for at least six months (Norton & Grant 1988).
1.4.3. Microbial Ecology of Salt Mines

Both halobacteria and eubacterial halophiles have been isolated from Boulby and Winsford salt mines. The main difference between the salt mines and surface hypersaline environments is that due to the lack of light there is no primary production. Carbon must be obtained as secondary carbon from within the deposit. Winsford Mine is much cooler than many hypersaline environments with an average temperature of 12°C all year round. The temperature in Boulby is 37 - 40°C. Within the mines there is a large variety of different environments.

In Winsford mine, surface air is drawn through a network of disused tunnels in which water vapour condenses in shallow brine pools (pH 5.8 -7.0). These are saturated and cold (13°C) throughout the year. The halite of the tunnel walls is moist and covered in efflorescences of recrystallised salt. The brine horizon which overlays the Northwich halite wells to the surface at a few locations in Cheshire and Shropshire forming surface springs and pools. In the brine pools within the mine eubacterial halophiles were found to be dominant. The brine is almost pure, saturated NaCl brine. Dominance of eubacterial halophiles over halobacteria in a saturated brine has not been described elsewhere in nature. McGenity (1994) suggested that the low temperature of the Winsford salt mine may allow the eubacterial halophiles to out compete the halobacteria. In efflorescences of recrystallised salt halobacteria formed about 50 % of the population (Norton et al. 1993, McGenity 1994). Solution mining, in which boiling water is injected in to the deposit and pumped out at Lostock, about 5 miles from Winsford, is also carried out. The solution mining brine yielded only halobacteria, when tested immediately on receipt. However two years after sampling it was found to contain equal numbers of eubacteria and halobacteria. Soil in regions fed by the brine horizon yielded more than 1000 colonies per gram of soil. All colonies were eubacterial. Brine in Lower Wych pond gave very low counts on CHM, but a salt crust from the edge of the pond produced 5 x 103 colonies per gram of which 1% per red and presumed halobacteria. This was the only surface site to yield halobacteria.

The chemistry and microbiology of Boulby salt mine were found to vary widely
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among samples. Boulby halite and potash are overlaid by Permian Bunter Mudstone, followed by Triassic Bunter Sandstone, forming an aquifer with 18% NaCl at its base. Bunter brines enter the salt deposits along mine shafts and through fissures in the Mudstone rocks (McGenity 1994). Counts of $1.6 \times 10^3$ to $6.4 \times 10^3$ cfu ml$^{-1}$ were obtained from brines, which are believed to have originated from Bunter brine which has prolonged contact with halite and potash seams. These brines contained halobacteria only. In contrast the brine, which entered along mine shafts, with no contact with Permian strata, yielded 45% eubacteria. Some of the brines in the Boulby mine are believed to be residual and of Permian origin. It is possible the halobacteria have been associated with the brines or rock salt giving rise to them for 230 Ma (McGenity 1994). It is also note worthy that two brine samples and an efflorescence collected as drips from the ceiling proved to be sterile (McGenity 1994) The source of these brines was unknown, but it illustrates that the microbiology of the brines very much depends on the origin of the brine.

Halobacteria have also been isolated from rock salt placed in enrichment media. Norton and Grant (1988) isolated halobacteria from 4 out of 17 rock salt samples from Winsford, however McGenity obtained only 2 out of 456 positive enrichments from rock salt (McGenity 1994).

Both eubacterial halophiles and halobacteria have also been isolated from Wieliczka Salt Mine in Poland; the Green River Valley, USA; the Khorat basin, Thailand; Solno and Gora Solution Mines, Poland, Mariager, Denmark; Delfzijl, Netherlands; Hengelo, Netherlands; Stade, Germany and a salt mine near Bad Ischl, Austria (Table 1.5). No bacteria could be isolated from brines from the Kali and Salz Mines in Germany (Gemmell 1996).
### Table 1.5: Isolation of Bacteria from Salt Deposits

<table>
<thead>
<tr>
<th>Salt Deposit</th>
<th>Geological Period and Age (Ma BP)</th>
<th>Type of Microorganisms % of halobacteria isolated</th>
<th>Numbers of Microorganisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wieliczka Salt Mine, Poland</td>
<td>Lower Miocene (c. 20)</td>
<td>Halobacteria (30 - 80%), Eubacteria</td>
<td>varied from 0 to 6.9 x 10^4 ml^-1</td>
<td>Gemmell 1996</td>
</tr>
<tr>
<td>Green River Valley, Wyoming, USA</td>
<td>Lower / Middle Miocene (C.50)</td>
<td>Halobacteria (3-20%), Eubacteria</td>
<td>5.27 x 10^7 ml^-1 at 30°C incubation, 8.7 x 10^3 ml^-1 at 37°C</td>
<td>Gemmell 1996</td>
</tr>
<tr>
<td>Khorat Basin, Thailand</td>
<td>Upper Cretaceous / Lower Tertiary (c. 65)</td>
<td>Halobacteria (10 - 70%), Eubacteria</td>
<td>4.4 x 10^5 ml^-1</td>
<td>Gemmell 1996</td>
</tr>
<tr>
<td>Winsford Salt Mine, England</td>
<td>Middle Triassic (Anisian Stage, 235 - 240)</td>
<td>Halobacteria, Eubacteria (proportion very variable)</td>
<td>varied from 480 ml^-1 to 2.5 x 10^5 ml^-1 (Gemmell 1996), 1.1 x 10^4 to 5 x 10^3 ml^-1 (McGenity 1994)</td>
<td>McGenity 1994, Gemmell 1996</td>
</tr>
<tr>
<td>Solno and Gora Solution Mines, Inowroclaw, Poland</td>
<td>Upper Permian (PZ3, 250-260)</td>
<td>Halobacteria, Eubacteria (Solno few halobacteria, significant halobacteria in Gora)</td>
<td>10^5 ml^-1</td>
<td>Gemmell 1996</td>
</tr>
<tr>
<td>Hengelo, Netherlands</td>
<td>Upper Permian (250-260)</td>
<td>Halobacteria (48 - 66%), Eubacteria</td>
<td>approx. 800 ml^-1</td>
<td>Gemmell 1996</td>
</tr>
<tr>
<td>Delfzijl, Netherlands</td>
<td>Upper Permian (250-260)</td>
<td>Halobacteria (1 - 100%), Eubacteria</td>
<td>3.9 - 4.0 x 10^5 ml^-1</td>
<td>Gemmell 1996</td>
</tr>
<tr>
<td>Mariager, Denmark</td>
<td>Upper Permian (250-260)</td>
<td>Halobacteria (40-50%), Eubacteria</td>
<td>9.7 x 10^4 - 1.0 x 10^5 ml^-1</td>
<td>Gemmell 1996</td>
</tr>
<tr>
<td>Stade, Germany</td>
<td>Upper Permian (250-260)</td>
<td>Not Known</td>
<td>5.93 - 5.3 x 10^3 ml^-1</td>
<td>Gemmell 1996</td>
</tr>
<tr>
<td>Werra and Hannover, Germany</td>
<td>Upper Permian (Z1 and Z2, 250-260)</td>
<td>No bacteria</td>
<td>0</td>
<td>Gemmell 1996</td>
</tr>
<tr>
<td>Salt Mine, Bad Ischl, Austria</td>
<td>Permian</td>
<td></td>
<td></td>
<td>Stan-Lotter et al. 1993</td>
</tr>
</tbody>
</table>
Isolation of bacteria from the salt deposits at Boulby and Winsford led to several fundamental questions. Have these bacteria been present in the salt deposits since their formation millions of years ago? Or have they been introduced at a more recent date? Is it possible for bacteria to survive for millions of years and then be revived? Or could populations of bacteria continue to slowly grow for this period of time under the conditions of the salt mine?

During the last ten years several groups have isolated microorganisms from the deep subsurface (hundreds to thousands of metres below the surface) and it now seems that subsurface bacteria are ubiquitous. In 1987 the Department of Energy (DOE) drilled several deep bore holes near the Savannah River nuclear processing facility in South Carolina. A diverse range of microorganisms were isolated from cores from depths of up to 500m. More recently the DOE’s Subsurface Science Program has isolated microorganisms from depths extending to 2.8 km below the surface and from formations with temperatures of up to 75°C (Fredrickson & Onstott 1996). Parkes and co-workers have found high numbers of microorganisms from sediments deep below the ocean floor (Parkes et al. 1995. Parkes et al. 1996, Barnes et al. 1998). Stevens and McKinley (1995) have found bacteria living within igneous formations composed of layers of basalt, and Pederson has detected bacteria in water flowing through deep fractures in granite (Pederson et al. 1997, Kotelnikova and Pederson 1997). Igneous rock forms from molten magma, therefore microbes within igneous rock such as basalt or granite must have been carried there by the flow of groundwater. Infiltration of groundwater into rock can be exceedingly slow. In the Savannah River facility for example it has been calculated that ground water at the deepest sites has been isolated from the surface for millions for years. Therefore the subsurface bacterial community must be at least several million years old.

There have been many reports of preservation of microorganisms for long periods of
time. Kennedy et al. (1994) drew up a database of microbial preservations and revivals after periods of 50 years. Table 1.6 shows survival of microorganisms for over 1 million years. Is it really possible that microorganisms could survive for periods of millions of years? This section discusses some of the arguments for and against survival of microorganisms for long periods of time.

Table 1.6: Table showing survival of microorganisms for periods of over 1 million years.

<table>
<thead>
<tr>
<th>Estimated period of survival (Million years)</th>
<th>Micro-organisms</th>
<th>Origin of specimen /Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>650</td>
<td>Not identified</td>
<td>Precambrian rocks from the Algonkian</td>
<td>Lipman (1928, 1931), Farrell (1933)</td>
</tr>
<tr>
<td>650</td>
<td><em>Bacillus circulans</em></td>
<td>Precambrian salts from Irkutsk</td>
<td>Dombrowski (1960, 1961a, b, 1963)</td>
</tr>
<tr>
<td>600</td>
<td><em>Kakabekia umbellata</em></td>
<td>Ammonia-rich soils from Harlech, Wales</td>
<td>Siegel &amp; Guimarro (1966)</td>
</tr>
<tr>
<td>430</td>
<td>Encapsulated diplococcus-like bacteria</td>
<td>Silurian salts from New York State</td>
<td>Reiser &amp; Tasch (1960)</td>
</tr>
<tr>
<td>430</td>
<td>Bacteria</td>
<td>Middle-Devonian salts</td>
<td>Dombrowski (1960, 1961a,b, 1963), Sussman &amp; Halvorson 1966, Muller &amp; Schwartz (1953), Rippel (1935), De Ley et al. (1966)</td>
</tr>
<tr>
<td>300</td>
<td>Diplococci</td>
<td>Permian mine salt</td>
<td>Reiser &amp; Tasch (1960)</td>
</tr>
<tr>
<td>300</td>
<td>Bacteria</td>
<td>Permian Zechstein and Kali salts. Halophilic pseudomonads were also found from salt springs at Bad Nauheim</td>
<td>Dombrowski (1960, 1961a, b,1963), Sussman &amp; Halvorson (1966), Rippel (1935), Muller &amp; Schartz (1953), De Ley et al. (1966)</td>
</tr>
</tbody>
</table>
### Table 1.6: Table showing survival of microorganisms for periods of over 1 million years (Cont.)

<table>
<thead>
<tr>
<th>Period</th>
<th>Microorganisms</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>225 – 270</td>
<td><em>Halorubrum saccharovorum</em>, <em>Halobacterium salinarum</em>, <em>Haloarcula sp.</em></td>
<td>1200m depth in Permian potash mine at Boulby, Cleveland, England</td>
<td>Rippel (1935), Muller &amp; Schartz (1953), De Ley et al. (1966), Norton et al. (1993)</td>
</tr>
<tr>
<td>200 MY and various ages</td>
<td>Thermophilic anaerobes, chemoheterotrophic bacteria and fungi</td>
<td>From five sites sampled as part of the DOE Deep Microbiology Subprogram</td>
<td>Colwell et al. (1992), Fredrickson (1992), Russell et al. (1992), Balkwill (1993)</td>
</tr>
<tr>
<td>190</td>
<td>Bacteria</td>
<td>Jurassic salt from a Humble Oil salt Dome</td>
<td>Reiser &amp; Tasch (1960)</td>
</tr>
<tr>
<td>24 – 40</td>
<td>Bacillus</td>
<td>Viable <em>Bacillus sphaericus</em> from isolated from the gut of a bee entombed in amber.</td>
<td>Cano et al. (1995)</td>
</tr>
<tr>
<td>15 – 200</td>
<td>Bacilli and cocci</td>
<td>Pennsylvania anthracite coal</td>
<td>Lipman (1931) Farrell &amp; Turner (1932), Burke &amp; Wiley (1937), Turner (1932)</td>
</tr>
</tbody>
</table>
Claims of survival and revival for long periods of time have been met with some scepticism from the scientific community. It is easier to believe reports of survival of bacteria for periods of tens to hundreds of years. Examples include *E. coli* frozen in pony faecal matter from Scotts Antarctic expedition of 1911, *B. anthracis* spores prepared by Louis Pasteur in 1888 which were revived in 1956 or *Clostridium* spp. from the abdomen of mummies in catacombs in Bohemia which were up to 250 years old (Kennedy et al. 1994). The main difficulty is proving that these organisms are truly ancient or if there is another explanation for the apparent revivals.

It is possible that bacteria, which have survived over along period of time, may have gone through periods of growth when conditions are favourable, followed by periods of non-growth or dormancy. Thus the bacteria isolated would not be truly ancient. Most rock formations are very low in nutrients and may not be able to support microbial growth. The term dormant or anabiotic is used to describe a reversible state of low metabolic activity in which cells can persist for long periods of time without division (Kaprelyants et al. 1993).

Some types of bacteria produce morphologically specialised structures for survival. Members of the genera *Bacillus*, *Clostridium*, *Sporolactobacillus* and *Desulfotomaculum* produce endospores. These are formed under conditions of carbon, nitrogen or phosphorous limitation. Endospores are highly resistant to heat,
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desiccation, pesticides, antibiotics and dyes. The water content of the spore is very low. Viable spores of *Bacillus subtilis* and *B. licheniformis* were found in dry soil attached to plants which had been stored at Kew Gardens Herbarium for 200–300 years. *Methylosinus trichosporium* forms exospores. Bacteria belonging to the genera *Azotobacter* and *Methlocystis* form thick-walled spherical cells known as cysts. Members of the genera *Myxococcus* and *Sporocytophaga* form myxospores (Schlêgel 1995). However endospores are by far the most resistant to heat.

Kaprelyants *et al.* (1993) state that non-sporulating bacteria also seem to be able to exist in a dormant form. As early as the 1950’s Bisset claimed that nearly all bacteria possess resting stages, which he called microcysts (Bisset 1952). Small, ovoid forms of bacteria are normally observed in natural systems when observed by light microscopy (Stevenson 1978). Stevenson maintained that these represented dormant forms of bacteria responding to unfavourable conditions. Roszak and Colwell (1987) expanded this idea to suggest that they represent a spore-like form of non-spore forming bacteria.

It has been shown in the laboratory that when bacteria are subjected to conditions of starvation they round up and decrease in size from 15 to 300 fold (Roszak and Colwell 1987). These cells have been called ultramicrobacteria, ultramicrocells, dwarf cells, mini-, pico- and nanobacteria by various authors (Morita 1988). These cells are usually less than 0.3μm, show slow growth and do not increase significantly in size when inoculated onto nutrient rich media (Torella & Morita 1981). Bacteria, which have been shown to form ultramicrobacteria under conditions of starvation, include *Spirillum, Cellovibrio, Aerobacter, Leucothrix, Flavobacterium, Cytophaga,* and *Vibrio* (Roszak & Colwell 1987). In addition to a decrease in size the following biochemical observations have been made: an increase in surface hydrophobicity, a decrease in lipid and RNA content of the cell, changes in fatty acid content, utilisation of poly-β-hydroxybutyrate and protein degradation. Some time after the onset of starvation the rate of endogenous respiration sharply decreases and the ATP content of the starved bacteria steadily decreases. Other changes that are seen are condensed cytoplasm, accumulation of specific proteins and inability to multiply using traditional plating techniques.
(Kapreyants et al. 1993). However respiration continues and can be measured using iodonitrotetrazolium (INT)-violet (Amy et al. 1993).

Some strains of halobacteria have also been reported to form cyst-like structures (Kostrikina et al. 1991). It was observed that *Halobacterium distributum* formed three types of cyst-like structure; clusters of tightly packed cells of an irregular shape surrounded by a capsule, single rods with a thickened cell wall and rounded cells with a three-layer capsule. They also observed cyst formation in *Halococcus turkenicus* where capsule would surround two or more cells.

Halobacteria have also been shown to survive entrapment in fluid inclusions within salt crystals for at least 6 months. When sodium chloride crystallises in the natural environment living halobacteria are often entrapped within the fluid inclusions formed. Salt crystals were grown under controlled conditions in the laboratory and representatives of all the major groups of halobacteria, except *Halococcus*, were shown to survive entrapment for at least 6 months (Norton & Grant 1988).

Many investigators have gone through very scrupulous procedures to avoid contamination by surface contaminants during sampling. Cano et al. (1995) revived a *Bacillus* sp. from the gut of a bee entombed in amber 25 – 40 million years ago. They carried out very fastidious sampling procedures and controls to ensure there was no contamination. Sampling was carried out in a Class II laminar flow cabinet. The amber pieces were surface sterilised chemically, then cracked and the tissue within removed. Trypticase Soy Broth (TSB) was inoculated with samples of the solutions used in the sterilisation process and with pieces from the exterior and interior of the amber to check for any contamination. To test for environmental contaminants plates of tpytic soy agar were open in the hood for the period of the sampling. These plates were then incubated. The effectiveness of the surface sterilisation procedure was tested, by soaking pieces of amber in a culture of *B. subtilus*, before the surface sterilisation procedure was carried out. The sterilised amber pieces were then transferred in to TSB and the tubes incubated to look for any bacterial growth.
Radiocarbon dating does not appear to have been attempted to verify the age of revived microorganisms. This technique makes use of the fact that all living things absorb $^{14}$C from the nutrients they use but once an organism dies the $^{14}$C begins to decay. A drawback is that the half-life of $^{14}$C is 5700 years. The technique can only be used to date material less than 50 000 years old; however it could be used to confirm/ disprove whether organisms were older than 50 000 years. A further drawback is that as soon as an organism is revived it will start to absorb nutrients containing $^{14}$C. Also at least 1 mg of material is needed to carry out this technique which could be problematic is only small numbers of preserved bacteria are available (Kennedy et al. 1994).

There are several theoretical considerations that limit the length of survival by a microorganism. The decay of biological molecules is a very important consideration. Paabo et al. (1989) state a peptide bond can last for up to 108 years in fossil shells and bones. Ancient DNA, however, isolated from tissue is heavily modified. Estimates put less than 1% of DNA from museum and archaeological finds as undamaged. So how could it survive for millions of years? As DNA is a phosphate based it many survive better when contained in phosphate minerals rather than dead tissues (Anon 1994). Also if the metabolism of a bacterium were to continue at a very, very slow rate this may prevent the DNA from decay (Kennedy et al. 1994).

Microorganisms are also susceptible to radiation. Sneath (1962) stated that 90% of bacterial spores would be killed by an accumulated radiation dose of $10^5$ R. Rocks contain an inherent radioactivity. Sneath estimated that depending on the rock type microorganisms could be expected to live between $10^4$ to $10^5$ years based on the radiation model of death. All living things also contain a radiation source- $^{40}\text{K}$. If this was the only radiation a microorganism was exposed to then Sneath estimated that they might survive for $10^9$ years. It must be noted that the radioactivity of rock types and the resistance of microorganisms to radiation vary widely. Micrococcus radiodurans is resistant to up to $6.5 \times 10^6$ R of radiation (Matthews 1993).

The scientific implications of preserved microorganisms are far reaching. If the
organisms are truly ancient they could be very important for studying evolutionary theories. The whole genome of a pre-evolved microorganism would be available for study. They may also be important for biotechnology. It is possible that these organisms may have the ability to make novel products that have been lost to modern day organisms (Kennedy et al. 1994).
1.6 Aims of this Thesis

The aim of this thesis is to try to ascertain whether halobacteria isolated from ancient salt deposits are metabolically adapted to live deep within the deposits. Specifically:

1. To compare growth using various carbon sources and the ability to grow anaerobically between salt mine halobacteria and halobacteria from surface environments.

2. To compare the production of volatile fatty acids by surface and subsurface isolates of halobacteria.

3. To investigate whether halobacteria from the salt mines can degrade crude oil, a possible subterranean carbon source.

4. To elucidate any pathways of CO$_2$ fixation in halobacteria.
All chemicals and enzymes used in this study were obtained from Sigma Chemical Co Ltd. (Poole, Dorset), unless otherwise stated.

2.1 Archaeal and Bacterial Strains

Archaeal and bacterial strains, which were isolated from Boulby potash mine, Winsford salt mine and Lostock solution mine and used in this study are shown in Table 2.1. Other halobacterial strains, which were used in this study, are shown in Table 2.2.

Table 2.1: Strains isolated from Boulby and Winsford salt deposits and Lostock solution mine.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolated from</th>
<th>Genus</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mine, Boulby potash mine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>014.1</td>
<td>Brine pool, Boulby potash</td>
<td><em>Halobacterium</em></td>
<td>McGenity 1994, Norton et al. 1993</td>
</tr>
<tr>
<td></td>
<td>mine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br1</td>
<td>Solution mining brine, Lostock.</td>
<td><em>Haloarcula</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>Br2</td>
<td>Solution mining brine, Lostock.</td>
<td><em>Haloarcula</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>Br4</td>
<td>Solution mining brine, Lostock.</td>
<td><em>Haloarcula</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>Br5</td>
<td>Solution mining brine, Lostock.</td>
<td><em>Haloarcula</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>Br7</td>
<td>Solution mining brine, Lostock.</td>
<td><em>Haloarcula</em></td>
<td>McGenity 1994,</td>
</tr>
</tbody>
</table>
**Materials and Methods**

**Table 2.1: Strains isolated from Boulby and Winsford salt deposits and Lostock solution mine (Cont.).**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolated from</th>
<th>Genus</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br8</td>
<td>Solution mining brine, Lostock</td>
<td><em>Haloarcula</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>54P</td>
<td>Rock salt, Winsford salt mine</td>
<td><em>Halorubrum</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>011.1</td>
<td>Recrystallised salt, Winsford salt mine</td>
<td>Bacteria (Genus not known)</td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>G002.1</td>
<td>Brine pool, Winsford salt mine</td>
<td><em>Halorubrum</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>G005.1</td>
<td>Brine pool, Winsford salt mine</td>
<td><em>Halorubrum</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>G014.2</td>
<td>Halite crystals, brine and marl, Winsford salt mine</td>
<td><em>Halorubrum</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>G015.2</td>
<td>Recrystallised salt, Winsford salt mine</td>
<td><em>Halorubrum</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>Malk 006.3</td>
<td>Brine pool, Winsford salt mine</td>
<td><em>Halorubrum</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>GBr1.1</td>
<td>Solution mining brine, Lostock</td>
<td><em>Halorubrum</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>2Wnr5.1</td>
<td>Rock Salt, Winsford salt mine</td>
<td><em>Halorubrum</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>0011.2</td>
<td>Recrystallised salt, Winsford salt mine</td>
<td>Bacteria (Genus not known)</td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>Glu 008.1</td>
<td>Brine pool, Winsford salt mine</td>
<td><em>Halorubrum</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>Glu 006.2</td>
<td>Brine pool, Winsford salt mine</td>
<td><em>Halorubrum</em></td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>001.2</td>
<td>Brine, pool, Winsford salt mine</td>
<td>Bacteria (Genus not known)</td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>001.3</td>
<td>Brine, pool, Winsford salt mine</td>
<td>Bacteria (Genus not known)</td>
<td>McGenity 1994,</td>
</tr>
<tr>
<td>AnBr1.3.1</td>
<td>Solution mining brine, Lostock</td>
<td><em>Haloarcula</em></td>
<td>McGenity 1994,</td>
</tr>
</tbody>
</table>

*a: Complete 16S rRNA sequence was obtained for this strain (McGenity 1994).*

*b: Partial sequencing of the 16S rRNA was carried out for this strain (McGenity 1994).*

*c: Genus determined using polar lipid analysis (McGenity 1994).*
Table 1.2: Other halobacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Culture Collection and Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haloferax mediterranei</em></td>
<td>CCM 3361&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Halococcus morrhuae</em></td>
<td>NCIMB 787&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Haloarcula vallismortis</em></td>
<td>ATCC 21975&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Haloarcula marismortui</em></td>
<td>ATCC 43049&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Halarubrum saccharovorum</em></td>
<td>NCIMB 2081&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Halobacterium salinarium</em></td>
<td>NCIMB 764&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Halobacterium salinarum (cutirubrum)</em></td>
<td>CCM 2088&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Halobacterium salinarum (halobium)</em></td>
<td>NCIMB 2090&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Halorubrum sodomense</em></td>
<td>ATCC 33755</td>
</tr>
<tr>
<td><em>Halobacterium halobium (Natrinema pallida)</em></td>
<td>NCIMB 777&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>: CCM: Czechoslovak Collection of Microorganisms, J E Purkyne University, Brno, Czechoslovakia  
<sup>b</sup>: NCIMB: National Collection of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland  
<sup>c</sup>: ATCC: American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA.
2.2 Media used for growth and maintenance of Halophilic Archaea and Bacteria

2.2.1: Classical Halophile Medium (CHM)

This growth medium was used for the growth and maintenance of all strains used in this study (unless otherwise stated). This medium is based on that of Payne et al. (1960).

\[
\begin{align*}
\text{Yeast Extract} & \quad 10.0 \\
\text{Casamino acids} & \quad 7.5 \\
\text{KCl} & \quad 2.0 \\
\text{Trisodium citrate} & \quad 3.0 \\
\text{Starch} & \quad 5.0 \\
\text{MnCl}_2.4\text{H}_2\text{O} (50 \text{ g l}^{-1}) & \quad 1.0 \text{ ml} \\
\text{FeSO}_4.7\text{H}_2\text{O} (0.36 \text{ g l}^{-1}) & \quad 1.0 \text{ ml} \\
\text{Agar (if required)} & \quad 15.0 \\
\text{NaCl} & \quad 200.0 \\
\text{MgSO}_4.7\text{H}_2\text{O} & \quad 20.0
\end{align*}
\]

The first eight ingredients are made up in 300 ml of dH₂O and the pH corrected to 7.6 using NaOH. The remaining two ingredients are made up in 700 ml of dH₂O. These two components are autoclaved separately and mixed after cooling to about 60°C.
2.2.2 Minimal Medium (MM) for Halobacteria

A minimal media was developed to allow the study of growth on single carbon sources. Tris-HCl (0.2M) was included to maintain the pH at 7.6. Without the buffer the pH fell to around pH 6- too low for growth of many strains of halobacteria.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>2.0 gl⁻¹</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1.0 gl⁻¹</td>
</tr>
<tr>
<td>NaCl</td>
<td>200.0 gl⁻¹</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>20.0 gl⁻¹</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>2.0 gl⁻¹</td>
</tr>
<tr>
<td>MnCl₂·4H₂O (50 gl⁻¹)</td>
<td>1 ml</td>
</tr>
<tr>
<td>FeSO₄·7H₂O (0.36 gl⁻¹)</td>
<td>1 ml</td>
</tr>
<tr>
<td>Carbon Source (see below)</td>
<td>10</td>
</tr>
<tr>
<td>Agar (if required)</td>
<td>15</td>
</tr>
</tbody>
</table>

Buffered with Tris-HCl (0.2 M) to pH 7.6

Carbon Sources- glucose, glycerol, trehalose, alanine, proline, glycine betaine and glutamic acid were autoclaved separately and added after cooling.
2.2.3 Modified Rodriguez-Valera Medium (RVM)

A defined media was developed to study acid production by halobacteria. This media was adapted from that of Rodriguez-Valera et al. (1980)

**Organics**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_4$Cl (Fisher)</td>
<td>2.0 g l$^{-1}$</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.5 g l$^{-1}$</td>
</tr>
<tr>
<td>CaCl$_2$.6H$_2$O</td>
<td>1.0 g l$^{-1}$</td>
</tr>
<tr>
<td>KCl (Fisher)</td>
<td>4.0 g l$^{-1}$</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.2 g l$^{-1}$</td>
</tr>
<tr>
<td>MES (0.1M)</td>
<td>19.52 g l$^{-1}$</td>
</tr>
</tbody>
</table>

**Salt**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (Fisher)</td>
<td>156.0 g l$^{-1}$</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>13.0 g l$^{-1}$</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O (Fisher)</td>
<td>20.0 g l$^{-1}$</td>
</tr>
</tbody>
</table>

**Carbon source**

(e.g. glucose, glycerol, trehalose, glycine betaine, alanine, proline, glutamic acid)

**Vitamin solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-aminobenzoic acid</td>
<td>4 mg</td>
</tr>
<tr>
<td>D-(+) biotin</td>
<td>1 mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>10 mg</td>
</tr>
<tr>
<td>Calcium D (+) pantothenate</td>
<td>5 mg</td>
</tr>
<tr>
<td>Pyridoxine dihydrochloride</td>
<td>15 mg</td>
</tr>
</tbody>
</table>

Made up in 100ml of sodium phosphate buffer (10mM, pH 7.1), filter sterilised and stored at 4°C.

**Thiamine Solution**

10 mg in 100 ml

(Filter sterilised and prepared fresh)
Materials and Methods

Vitamin B\textsubscript{12}  
(Filtered sterilised and stored at 4°C)

5mg in 100ml

Trace element solution

<table>
<thead>
<tr>
<th>Element</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO\textsubscript{4}.7H\textsubscript{2}O</td>
<td>(7.5 mM)</td>
</tr>
<tr>
<td>H\textsubscript{2}O\textsubscript{3}</td>
<td>(0.5mM)</td>
</tr>
<tr>
<td>MnCl\textsubscript{2}.4H\textsubscript{2}O</td>
<td>(0.5 mM)</td>
</tr>
<tr>
<td>CoCl\textsubscript{2}.6H\textsubscript{2}O</td>
<td>(0.8 mM)</td>
</tr>
<tr>
<td>NiCl\textsubscript{2}.6H\textsubscript{2}O</td>
<td>(0.1 mM)</td>
</tr>
<tr>
<td>CuCl\textsubscript{2}.2H\textsubscript{2}O</td>
<td>(0.01 mM)</td>
</tr>
<tr>
<td>ZnSO\textsubscript{4}.7H\textsubscript{2}O</td>
<td>(0.5 mM)</td>
</tr>
<tr>
<td>NaMo\textsubscript{4}.2H\textsubscript{2}O</td>
<td>(0.15mM)</td>
</tr>
<tr>
<td>HCl (25% / 7.7M)</td>
<td>(100mM)</td>
</tr>
</tbody>
</table>

Made up in 987 ml of dH\textsubscript{2}O

The organics part of the medium is made up in 250ml dH\textsubscript{2}O and the pH corrected to 7.0 with KOH. The salt part is made up in 700 ml dH\textsubscript{2}O. The carbon source is made up in 50 ml of dH\textsubscript{2}O. These three components are autoclaved separately and mixed after cooling to about 60°C. 1 ml of trace element solution, 1 ml of vitamin solution, 1 ml of Vitamin B\textsubscript{12} solution and 1 ml of thiamine solution are then added aseptically.

2.3 Growth of Halobacteria in Minimal Media

The ability to grow in minimal media with the addition of a single carbon source was investigated. The carbon sources chosen are all utilised as compatible solutes by halophilic and halotolerant eubacteria, and thus may become available to halobacteria in the natural environment. Carbon sources investigated were glycerol, glycine betaine, alanine, glutamic acid and proline. Growth in minimal medium with the addition of glucose was also studied. Initially growth on minimal media was investigated by looking at growth of each strain on agar plates. Once it was established that the strains could grow on the minimal medium with a single carbon source growth was investigated in broth. Starter cultures were grown in minimal media + 10gL\textsuperscript{-1} carbon source to an OD (660 nm) of 0.5. 0.5 ml of the starter culture
Materials and Methods

was then used to inoculate 10 ml of minimal media with carbon source in a universal tube in duplicate. The initial O.D. was about 0.05. Growth was followed by measuring O.D. at 660 nm.

2.4 Growth of halobacteria in defined media

In order to study the production of acids by halobacteria a defined medium was developed. The ability of strains to grow in Modified Rodriguez-Valera with glucose as a carbon source following a number of transfers was examined. It was important to ascertain that strains were utilising the glucose in the growth medium and were not growing using the carbon sources contained in the initial inoculant.

Starter cultures were grown in CHM to an OD (660 nm) of 0.5. 0.5 ml of the starter culture was used to inoculate 10ml of RVM + glucose in a universal tube in duplicate and the growth followed by measuring the OD (660 nm). When the OD reached 0.5 this was used to inoculate a second tube of RVM + glucose. When the OD in this tube reached 0.5 this was used to inoculate a third tube. In this way the growth of halobacteria in this media following a number of transfers was followed.

The growth of halobacteria in minimal media with the addition of glucose as a carbon source was also studied following a number of transfers using the method described above.

2.5 Analysis of acids produced during aerobic growth of Halobacteria

2.5.1 Growth and preparation of cells.

The production of acid during growth under aerobic conditions of the following strains was investigated: 54R, E4, O14.1, BbpA1, Br3, Hr. saccharovorum, Hb. salinarum, Ha. marismortui and Hc. morrhuae.
Materials and Methods

Strains were grown to late exponential phase in 250ml of RVM with the addition of 10 gL⁻¹ of one of the following carbon sources: glucose, trehalose, glycerol or proline. *Hb. salinarum* will not grow in RVM with glucose, trehalose or proline so was grown with glycerol only. Cells were spun down at 10 000 rpm for 30 minutes. The supernatant was then filtered using a 0.2 μm filter. Filtered supernatant was then stored at 20°C until use.

### 2.5.2 Extraction of free acids and analysis using GC

10μl or 100μl of valeric acid internal standard solution was added to a 10ml sample of filtered supernatant. This gave a final concentration of 0.25 or 2.5mM valeric acid. Samples were then basified by adding 5% NaOH drop by drop. This converts the free acids to non-volatile salts. The sample was then evaporated using a rotary evaporator with 2 acetone/ dry ice cold finger condensers and a conventional water trap immersed in an acetone/ dry ice mix. Samples were evaporated to less than 0.5 ml. Alternatively some samples were freeze-dried. The residue was then washed out with 2 x 1 ml alkaline distilled water (20ml dH₂O + 1 drop 5% NaOH). The samples were then washed by shaking with 2 x 1ml ether to remove any neutral compounds (alcohols, esters etc.) not removed by evaporation. The samples were then acidified with 2 to3 drops of 50% H₂SO₄. Acids were then extracted with 2 x 0.25 ml ether. The extracts were pooled and then used for analysis using Gas Chromatography (GC). It was sometimes necessary to pass the samples through non-absorbent cotton wool to remove any precipitate. Analysis by GC was carried out on the same day as extraction as acids can be lost during storage. Samples were stored at 20°C.

**Gas Chromatography of Free Acids**

**GC Conditions 1**

Gas Chromatogram: Pye Unicam GCD

Column Type: SGE BP21 bonded phase megabore column

Length: 12 m

Film: 0.5 μm

Column: ID:0.53 mm

Detector: Flame Ionisation (FID)

Oven Temp. 54°C (held for 1 minute) ramping at 16°C min⁻¹ to 160°C.
Materials and Methods

N₂ flow: 20 ml min⁻¹
Sample injection volume: 1.0 μl

GC Conditions

GC chromatogram: Pye Unicam 4500
Detector: flame ionisation (FID)
Column Type: Hewlett Packard FFAP capillary column
  Length: 25 m
  Film thickness: 0.25μm
  Column ID: 0.25 mm
Oven temperature: 150°C (isothermal)
Helium pressure: 15 lb/in²
Sample injection volume: 1.0 μl

A solution of 2.5 mM acetic, propionic, butyric, isobutyric, valeric and isovaleric acids was injected prior to any samples, to check the performance of the chromatogram. Between injection of each sample the syringe was rinsed 12 times with ether to prevent any contamination. Peaks were identified by comparison of retention times with authentic samples and also by spiking samples with authentic samples and looking for co-elution.

Quantification of free acids using an internal standard

Valeric acid (0.25 or 2.5 mM final concentration) was used as an internal standard. This was added to the sample prior to the extraction procedure. By adding the internal standard prior to the extraction procedure variations in the extraction efficiency are corrected for. In addition using an internal standard corrects for variable losses from the syringe prior to injection and variations in injection size. A 2.5 mM solution of the following acids was prepared: acetic, propionic, isobutyric acid, butyric, isovaleric and valeric acid. The sample was divided into three portions and prepared for GC as described above. Each sample was then run three times using GC under the conditions shown above. A mean peak area was calculated for each acid, including valeric acid. A correction factor was calculated to allow the concentration of the other acids to be calculated from the area of the valeric acid peak. Due to variable detector response to molecules of different sizes and the different extraction efficiency of the different
acids, although the starting concentration of acids is the same the peak size is not equal for all the acids.

Concentration of acids in samples is calculated from the size of the valeric acid internal standard peak as follows:

\[
\frac{\text{Area of sample peak} \times \text{correction factor}}{\text{Area of valeric acid peak}} \times \text{Conc. of valeric acid}
\]

### 2.5.3 Derivatisation of acids to methyl esters

Samples were basified with 5% NaOH (as for free acids). Samples are then dried to complete dryness by rotary evaporation. The residue was washed out with 2 x 2ml of methanol and the sample was dried again using the rotary evaporator. The residue was washed out with 2 x 0.5 ml of methanol and transferred to a reaction vial. Two drops of concentrated H\textsubscript{2}SO\textsubscript{4} were added and the reaction vial was heated in a heating block at 100 – 110°C for one hour. The reaction vial was allowed to cool. The sample was then neutralised with saturated Na\textsubscript{2}CO\textsubscript{3}. Neutralisation must be done to completion to remove all residual acid. Na\textsubscript{2}CO\textsubscript{3} was added drop by drop until there is no further effervescence. The pH was checked with pH paper. Any precipitate was removed by passing the sample through a Pasteur pipette containing a plug of non-absorbent cotton wool (these must be washed with toluene first). The neutralised sample was then extracted with 2 x 0.25 ml of toluene (HPLC grade) in a separation tub. Extracts were pooled and dried by passing through dry MgSO\textsubscript{4} in a pasteur pipette. The dried extracts were then examined by GC.

**GC Conditions for Methyl Esters**

- **GC chromatogram**: Pye Unicam 4500
- **Detector**: flame ionisation (FID)
- **Column Type**: Hewlett Packard Innowax column
  - **Length**: 30 m
  - **Film thickness**: 0.25μm
  - **Column ID**: 0.25 mm
- **Oven temperature**: 60°C (2 min.) increasing by 16°C to 150°C (20 min.)
- **Injection volume**: 1.0 μl
Material and Methods

Helium pressure: 15 lb/in²

2.5.4 Derivatisation of acids to ethyl esters

Method 1: Derivatisation using Ethanol and an Acid Catalyst

The same method as for methyl esters was used (section 2.5.3), except ethanol was used in place of methanol.

Method 2: Derivatisation using ethylchloroformate (ECF) (Husek 1995)

0.2 ml of filtered culture supernatant was added to 0.4 ml of acetonitrile and 0.2 ml of ethanol. The sample was then shaken for about one minute to precipitate any protein in the sample and then centrifuged for 10 minutes at 2000 g. The supernatant was then transferred to a fresh tube. The pH of the sample was adjusted to >pH 8 by adding 2M NaOH (2-3 μl). The sample was then extracted 2 times with 0.5 ml of hexane. 20 μl of ECF and 40 μl of pyridine were added. The sample was shaken briefly and effervescence occurred. 0.25 ml of dichloromethane and 0.5 ml of sodium bicarbonate solution (1M) were added. The sample was left to stand for 2-3 minutes. The upper aqueous layer was then removed and discarded. The organic phase was dried by passing through MgSO₄ in a Pasteur pipette. The sample was then be analysed using GC.

GC Conditions for Ethyl Esters

The same GC conditions were used as for methyl esters (section 2.6.3).

2.5.5 Pyruvate Assay using Lactate Dehydrogenase

Lactate dehydrogenase catalyses the following reaction:

\[
\text{Pyruvate} + \text{NADH} \quad \rightleftharpoons \quad \text{lactate} + \text{NAD} \\
\text{(High A340)} \quad \text{ (Low A340)}
\]

In the presence of excess NADH all the pyruvate is converted to lactate. The
reduction of absorbance at 340 nm due to oxidation of NADH to NAD is proportional to the amount of pyruvate originally present. The change in $A_{340}$ was proportional for pyruvate concentrations from 0.01 - 0.1 mM. A standard curve was prepared using a range of pyruvate concentrations from 0.01 mM to 0.1 mM.

**Assay Mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH (1.0 mg in 2.2 ml 1.5 M Tris-HCl buffer pH 7.5)</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>1.9 ml</td>
</tr>
<tr>
<td>culture supernatant</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

The assay mixture was made up in a 3 ml cuvette and inverted several times. The initial $A_{340}$ nm was measured and then 50μl of lactate dehydrogenase (bovine heart, approx. 1000 Uml$^{-1}$) was added immediately. The cuvette was inverted several times and the $A_{340}$ nm was read again after 5 minutes.

### 2.6 Anaerobic growth by halobacteria.

Nitrate, dimethylsulphoxide (DMSO), trimethyl-N-oxide (TMAO) and fumarate have been shown to replace oxygen in the respiration of some members of the halobacteria (Tindall 1992, Mancinelli & Hochstein 1986, Oren & Truper 1990, Oren 1991). Additionally some strains have been shown to have the ability to grow anaerobically by fermentation of arginine to citrulline (Hartmann 1980). The ability of strains to grow anaerobically in CHM alone and with the addition of nitrate, DMSO, TMAO, fumarate or arginine was examined.

CHM was prepared with the addition of 25 mM PIPES and buffered to pH 7.6. Sterile solutions of NaNO$_3$, TMAO, DMSO, fumarate and arginine were added to the CHM to give a final concentration of 40mM. 10ml aliquots of each media were put into universal tubes. Starter cultures were grown in CHM aerobically to an OD of 0.5. Starter cultures and universal tubes containing the growth media were then placed in an anaerobic cabinet (Don Whitley, atmosphere of 10% hydrogen in nitrogen) for 24 hours to deplete any oxygen prior to their use. Each 10ml of media was inoculated with 0.5 ml of the starter culture inside the anaerobic cabinet. Tubes were examined...
Materials and Methods

for growth after 7, 14, 21 and 49 days. The ability to grow anaerobically using one of the alternative electron acceptors was scored positively if growth was observed in media with addition of the electron acceptors before growth occurred in the negative control of CHM only.

The ability of strains to grow in MM with the addition of glucose, glycerol, trehalose and glycine betaine with and without the addition NaNO₃ or TMAO was examined. Starter cultures were grown aerobically in MM + glucose, glycerol, trehalose or glycine betaine. Anaerobic growth was monitored by removing 1 ml portions and measuring the OD (660 nm) after 7, 14 and 21 days. The ability of strains to grow after several anaerobic transfers was also examined.

The production of acid during anaerobic growth was measured for some cultures. The method described in section 2.5.2 was used.

2.7 Biodegradation of crude oil by halobacteria

2.7.1 Initial Studies


Strains were grown in MM for 7 days. 2 ml of this culture was then used to inoculate 50 ml of MM with 2% (v/v) crude oil in a 200 ml bottle. Negative controls contained MM + oil but were uninoculated. Bottles were incubated at 37°C in a shaking incubator. Bottles were examined by eye and under the microscope at weekly intervals for growth and changes in the appearance of oil.

2.7.2 Studies of Crude Oil Degradation Using Gas Chromatography and Gas Chromatography-Mass Spectrometry

Strains used: Br2, Br3, Br4, Br5, Br6, E4, 54R, 54P, Br1, Br7, O14.1. (These strains were selected as they showed the greatest amounts of emulsification and biodegradation in the initial studies.)
Materials and Methods

Strains were grown for 7 days in MM and inoculated into 50 ml MM + 2% (v/v) crude oil or 50 ml RVM + 2% (v/v) crude oil. Bottles were examined at weekly intervals for growth and changes in the appearance of oil. After 8 weeks the degradation of the oil was analysed using Gas Chromatography.

Preparation of samples for GC and GC-MS

200µl of an internal standard mix containing 14mM nonane and 12mM squalane was added to each 50ml sample growth medium. Crude oil was extracted from the growth medium by using 25 ml of chloroform. 1.0 ml of the chloroform extract was passed through dried MgSO₄ in a Pasteur pipette to remove any remaining water from the sample. The sample could then be used for GC or GC-MS as below.

Gas-Chromatography

GC Chromatogram: Pye Unicam 4500
Detector: FID
Integrator: SP4270
Column: Scientific Glass Engineering BP5 (25m, 0.22 ID)
Oven Temp: 55°C (5 min.) ramping up at 6°C min⁻¹ to 250°C (15 min)
Injector Temp: 250°C
Detector temp: 300°C
He carrier gas: 15 lb/in2
Injection Size: 1.0 µl

Gas Chromatography-Mass Spectometry

GC-MS System: Hewlett Packard
Column: Alltech RSII (25m, 0.25 mm ID)
Oven Temp: 60°C (5 min.) increasing at 5°C min⁻¹ to 150°C (5 min)
increasing to 250°C (5 min)
Injector Temp: 250°C
Injection Size: 1.0 µl
Identification of peaks was carried out by co-chromatography with authentic alkanes or by comparison of retention times and mass spectra with published data (Jones et al. 1986, Wang et al. 1994, Han & Calvin 1969, Gallegos 1971, McLafferty & Stauffer 1989)

Calculations of Percentage Biodegradation

Biodegradation of hydrocarbons was calculated as follows. In the control samples the mean peak area for each hydrocarbon was calculated. This included mean peak areas for the internal standards nonane and squalane. The area of the nonane and squalane peaks in the biodegraded samples was compared to the mean nonane and squalane areas in the control and a normalisation factor calculated. The peak areas in the biodegraded samples were then multiplied by this normalisation factor. This factor allows for any variations in the efficiency of the extraction procedure and the size of the sample injected onto the GC column. Changes in the area of the peaks should be due to biodegradation only. The normalised peak area for each hydrocarbon was then compared to the area of that hydrocarbon in the control and the percentage hydrocarbon remaining after biodegradation was be calculated.

2.7.3 Growth of halobacteria with hydrocarbons as the main source of carbon and energy.


Growth on the following hydrocarbons was investigated: alkanes (dodecane -C12, undecane -C11, eicosane- C20), cyclic alkanes (cyclohexane), isoprenoid branched alkane (pristane -C19), polyaromatic hydrocarbons (phenanthracene, anthracene), aromatic compounds (sodium benzoate), alkenes (hexadecene).

10 ml of MM was aliqotted into universal tubes. The hydrocarbons were added to give 50mM final concentration. Starter cultures were grown in MM for 6 days and 0.5 ml of starter culture was used to inoculate the MM + hydrocarbon. OD (660 nm) was measured after 7 and 14 days.
2.8 Autotrophic Growth and CO$_2$ Fixation by Halobacteria.

2.8.1 Autotrophic Growth

The following halobacterial strains were used in this experiment: 54R, E4, BbpA1, 014.1, Br3, *Hr. saccharovorum*, *Hr. sodomense*, *Ha. marismortui*, *Hc. morrhuae*, *Hb. salinarum*, *Hb. salinarum (halobium)* (bacteriorhodopsin producing strain).

100ml cultures of halobacteria were grown to mid-log phase in CHM. Half of this culture was spun down at 10 000 g for 20 minutes, washed in 10ml of salt solution (SS). SS consisted of NaCl 200 gl$^{-1}$ and MgSO$_4$ 20 gl$^{-1}$. Cell were then spun again and the pellet re-suspended in 10ml of SS. The flasks containing the remaining 50ml of cells were covered in a double layer of parafilm and incubated at 37°C under illumination for 4 days. These conditions should induce the production of bacteriorhodopsin (Javor 1988). After the 4 days incubation period cells were then spun and re-suspended as above.

0.5 ml of re-suspended cells were used to inoculate eight universal tubes containing 15ml of modified minimal media (MMM) pH 7.6. The media was modified by the omission of the yeast extract and the addition of the following: 5mM NaHCO$_3$, vitamin mix (as used in RVM) 1mlL$^{-1}$, trace elements (as used in RVM) 1 mlL$^{-1}$, thiamine (10 mgml$^{-1}$) 1 mlL$^{-1}$, Vitamin B$_{12}$ (5mg in 100ml) 1 mlL$^{-1}$, NH$_4$Cl 2.0glL$^{-1}$. Half of the tubes contained 0.5% agar to make a sloppy agar in which oxygen would be limited. Each strain was incubated under the following conditions: light and shaken, light and static, dark and shaken and dark and static in both liquid media and sloppy agar. Wrapping tubes in aluminium foil to prevent any light reaching the cells provided dark conditions. Tubes were incubated at 37°C in illuminated incubators. The OD (660 nm) of cells was measured immediately after inoculation, and after 7, 14 and 21 days in the liquid media. The tubes containing the sloppy agar were examined by eye for any growth.
2.8.2 Whole Cell Assays of CO₂ Fixation in Halobacteria

2.8.2.1 Semi-anaerobic incubation and illumined conditions.

Initial experiments carried out to investigate CO₂ fixation by halobacteria used a method similar to Javor (1988). Javor grew cells to late exponential phase and then incubated cells for several days semi-anaerobically (flasks covered with two layers of parafilm) and illuminated to induce bacteriorhodopsin synthesis. In this experiment the effect of the period of semi-anaerobic incubation was investigated. Cells were grown in 200ml of CHM in a 500ml Duran bottle to late exponential phase (7-10 days). The cells were then divided into three separate flasks. One flask was incubated semi-anaerobically illuminated for 6 days, another flask was incubated semi-anaerobically in the dark for 6 days and the remaining cells were used immediately to assay CO₂ fixation as follows.

Cells were centrifuged and suspended in BSS (4.28M NaCl, 81 mM MgSO₄·7H₂O, 27mM KCl, 20mM MOPS, pH 7.4) to give an OD (660nm) of 0.5. Javor (1988) added 1% glycerol to the BSS and incubated cells overnight to deplete them of nutrients, then centrifuged again and re-suspended in BSS. In these experiments there was no overnight incubation period. Formalin-killed cells were used as a control, where 10ml of diluted cells were treated with 1ml of formalin. Cells were sparged with N₂ for 30 minutes to remove CO₂. 2ml of cells were then placed in Wheaton bottles, which were sealed and crimped, and then preincubated at 37°C for 1 hour. 10µl of NaH¹⁴CO₃ (containing 0.186 MBq) was then added to each bottle and they were incubated statically for two hours at 37°C under illumination. After 2 hours incubation was terminated by placing the bottles on ice. From each bottle two 0.5 ml aliquots were removed and added to 10ml of 10% trichloroacetic acid (TCA). Aliquots were then filtered using GF/C (Whatman) filters and washed with 10ml of 10% TCA. Filters were dried by heating in an oven at 80°C for about two hours. Filters were then added to 3ml of Optiphase Safe Scintillation fluid and counted three times in a scintillation counter (United Technologies Packard Minaxi Tri-Carb 400 Series).

The protein content of the cells was determined using Peterson’s modification of the micro-Lowry method (Peterson 1977, Lowry et al. 1951).
2.8.2.2 Aerobic and anaerobic conditions.

Cells were grown in 200ml of CHM in a 500ml Duran bottle to late exponential phase (7-10 days). Cells were then diluted using CHM to give an OD (660 nm) of 0.5 This eliminated the need to centrifuge cells, which may cause some damage. In addition, experiments had shown CO$_2$ fixation occurred at a similar rate when cells were diluted in CHM as compared to centrifuging and resuspending cells in SS. Cells were then placed in an anaerobic cabinet (Don Whitley, atmosphere of 10% hydrogen in nitrogen) overnight (15 hours) to deplete CO$_2$ from the media. 2 ml of cells were then placed in a Wheaton bottle which was sealed and crimped. Transfer of cells and crimping was carried out in the anaerobic cabinet for cells, which were to be incubated anaerobically and on the bench for cells to be incubated aerobically. For each strain and set of conditions duplicates were set up. Cells were then incubated for 1 hour at 37°C either anaerobically in the anaerobic cabinet or aerobically shaking at approx. 200 rpm. Cells were incubated illuminated with two 20W strip lights. After the preincubation for 1 hour 10μl of NaH$^{14}$CO$_3$ (containing 0.186 MBq) was then added to each bottle and they were incubated for two hours at 37°C illuminated under aerobic or anaerobic conditions. After the incubation period the radioactivity incorporated in the cells was assayed as described above.

2.8.2.3 Light and dark conditions

The effect of light and dark incubation was carried out as above, except all bottles were incubated aerobically. Dark conditions were obtained by covering the Wheaton bottles with foil.

2.8.2.4 Addition of substrates

The experiments examining the effect of addition of carbon sources on CO$_2$ fixation were carried out as above, except that after cells had reached late exponential phase they were spun down at 10,000 rpm for 30 minutes. They were then re-suspended in BSS to give an OD of 0.5 at 660 nm and incubated over night in the anaerobic cabinet before using for the assay. The substrates were added to the cells prior to the preincubation period. 20μl of 1M solutions were added to give a final concentration of 10mM. In one set of experiments the effect of propionate and NH$_4^+$ was studied. In the second set the effect of adding glucose, acetate, pyruvate and NH$_4^+$ was studied.
2.8.3 Enzyme Activity in Cell Extracts of Halobacteria

2.8.3.1 Preparation of cell extract

Unless stated otherwise the cell extract was prepared as below. Cells were grown in CHM for 5 to 6 days. Cells were then stored for no longer than 7 days at 4°C until use. 100 ml of cells were spun at 1 400 g for 20 minutes, the supernatant removed, cells were re-suspended in SS and spun again as before. The pellet was then re-suspended in 1ml of Tris-HCl buffer (0.5M) pH 7.8 containing 3M KCl. Cells were sonicated (Sanyo Soniprep 150) for 8 -10 times for 15 seconds with 15 second intervals. Cells were then centrifuged at 10 000 g for 20 minutes. The supernatant constituted the cell free extract. The cell extract was used immediately or stored on ice for not longer than 2 hours. The protein content of the cell extract was determined using Peterson's modification of the micro-Lowry method (Peterson 1977, Lowry et al. 1951).

2.8.3.1 Ribulose Bisphosphate Carboxylase Assay (EC 4.1.1.39)

Activity was determined using the method of Rajagopalan & Altekar 1994.

Assay Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume of 250µl</td>
<td></td>
</tr>
<tr>
<td>MgCl₂ (50mM)</td>
<td>50</td>
</tr>
<tr>
<td>NaH¹⁴CO₃ (18.5 MBqml⁻¹)</td>
<td>5</td>
</tr>
<tr>
<td>Tris-HCl buffer (0.6M) pH 7.8 4M NaCl</td>
<td>62.5</td>
</tr>
<tr>
<td>Cell extract</td>
<td>82.5</td>
</tr>
<tr>
<td>Ribulose bisphosphate (30mM)</td>
<td>16</td>
</tr>
</tbody>
</table>

Cells were grown in CHM or minimal media containing 0.5% sodium butyrate and 0.05% NaHCO₃ until late exponential phase was reached. The cell extract was then prepared as previously described. All the components of the assay mix, except RuBP were added in an eppendorf tube and incubated for 10 minutes at 30°C. Ribulose-1,5-bisphosphate carboxylase from spinach (0.04 units) was used as a positive control. The RuBP was then added and the tubes were incubated for 30 minutes at 30°C in a water bath. 25 µl of concentrated HCl was then added to each tube to terminate the
reaction. To remove unincorporated CO\textsubscript{2} it was necessary to evaporate samples to dryness in a heating block or a boiling water bath. Each sample was then redissolved in 250\(\mu\)l of dH\textsubscript{2}O. Two 100\(\mu\)l aliquots were then removed from each tube and each added to 3ml of Optiphase Safe scintillation fluid. Radioactivity in each tube was counted for one minute three times using a scintillation counter.

2.8.3.3 Pyruvate Carboxylase (EC 6.4.1.1) Assays

Three methods were used to assay for pyruvate carboxylase.

Method A: Spectrophotometric Assay based on Milrad de Forchetti & Cazzulo 1976 and Rawal et al. 1988a

\begin{align*}
\text{Pyruvate} + \text{ATP} + \text{HCO}_3^- &\rightarrow \text{oxaloacetate} + \text{ADP} + \text{Pi} \quad (1) \\
\text{Oxaloacetate} + \text{NADH} &\rightarrow \text{malate} + \text{NAD}^+ \quad (2)
\end{align*}

Pyruvate carboxylase catalyses reaction 1. The assay is based on the reaction catalysed by malate dehydrogenase (reaction 2). The reduction of NADH can be followed at 340 nm.

The reaction mix was made up in a 1ml cuvette. The reaction was started by the addition of ATP. The blank contained no NADH. An ATP dependent fall in NADH indicates the presence of pyruvate carboxylase. All assays were carried out in duplicate or triplicate. Three different assay mixtures were used containing 0.68M NaCl, 1M KCl and 0.68M KCl and 1M KCl.
Materials and Methods

**Assay Mixture 1: 0.68M NaCl**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Volume of 1ml</td>
<td>μl</td>
</tr>
<tr>
<td>Tris-HCl buffer (0.5M) pH 8.5</td>
<td>500</td>
</tr>
<tr>
<td>Sodium pyruvate (1M)</td>
<td>5</td>
</tr>
<tr>
<td>ATP (10mM)</td>
<td>22.2</td>
</tr>
<tr>
<td>MgCl₂ (50mM)</td>
<td>100</td>
</tr>
<tr>
<td>NaHCO₃ (0.1M)</td>
<td>150</td>
</tr>
<tr>
<td>NADH (10mM)</td>
<td>15</td>
</tr>
<tr>
<td>Malate dehydrogenase (Sigma: bovine heart)</td>
<td>0.5 IU</td>
</tr>
<tr>
<td>Cell extract (0.9 mg ml⁻¹ protein)</td>
<td>200</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to make 1 ml</td>
</tr>
</tbody>
</table>

Cell extract was prepared in buffered saline solution (BBS): 4.28M NaCl, 81mM MgSO₄.7H₂O, 27 mM KCl, 20 mM MOPS, pH 7.4

**Assay Mixture 2: 1M KCl, 0.68M NaCl**

As above except 500μl of Tris-HCl buffer was replaced by 250μl of Tris-HCl (0.5M) pH 8.5 buffer with 250μl of 4M KCl.

**Assay Mix 3: 1M KCl**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume of 1ml</td>
<td>μl</td>
</tr>
<tr>
<td>KCl (4M)</td>
<td>100</td>
</tr>
<tr>
<td>Tris-HCl (50mM) pH 7.5</td>
<td>200</td>
</tr>
<tr>
<td>Na pyruvate (1M)</td>
<td>5</td>
</tr>
<tr>
<td>ATP (10mM)</td>
<td>22.2</td>
</tr>
<tr>
<td>Acetyl-CoA (10mM) (if included)</td>
<td>50</td>
</tr>
<tr>
<td>Malate dehydrogenase (Sigma: bovine heart)</td>
<td>0.5 IU</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>100</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>150</td>
</tr>
<tr>
<td>Cell extract</td>
<td>200</td>
</tr>
<tr>
<td>H₂O</td>
<td>to make 1 ml</td>
</tr>
</tbody>
</table>

Cell extract was prepared in Tris-HCl buffer (50mM) pH 7.5 with 3M KCl.
Method B: Modified Spectrophotometric Assay

High levels of NADH oxidation were observed independently of ATP using method A. Ultracentrifugation at 100 000 x g reduced the NADH oxidation activity but did not eliminate it. This activity made it very difficult to observe any ATP dependent activity and so the modification described was developed. The reaction was allowed to proceed for 5 min without the addition of NADH and malate dehydrogenase. 1ml of 10% TCA was then added to precipitate the protein, the sample was neutralised and the NADH and malate dehydrogenase were then added. This procedure eliminated the NADH oxidase activity. Positive controls in which 5 mM oxaloacetate was included showed this assay method was successful.

Method C: Radioisotopic Assay

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl (4M)</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Tris-HCl buffer (0.5M) pH 7.5</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Na pyruvate (0.5M)</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>ATP (10mM)</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>MgCl₂ (50mM)</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Acetyl-CoA (10mM)</td>
<td></td>
<td>12.5</td>
</tr>
<tr>
<td>NaH¹⁴CO₃ (18.5 MBq ml⁻¹)</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>cell extract (40 -50 mg ml⁻¹ protein)</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>to make 250μl</td>
</tr>
</tbody>
</table>

The cell extract was prepared in 50mM Tris-HCl buffer (pH7.5) with 3M KCl. The cell extract was pre-incubated with all the components of the assay, except the NaH¹⁴CO₃, at 37°C for 10 minutes. After addition of the isotope tubes were incubated at 37°C for 30 minutes. 25μl of concentrated HCl was then added to each tube to terminate the reaction. Tubes were then heated at 90°C for 3 hours to remove unincorporated CO₂. The dried samples were then dissolved in 250μl of dH₂O. Two 100μl aliquots from each tube were added to 3ml of Optiphase Safe scintillation fluid and counted three times in a scintillation counter. Each set of conditions was carried out in duplicate.
2.8.3.4 PEP Carboxylase (EC 4.1.1.31) Assays

Both spectrophotometric and radioisotopic assays were carried out.

A: Spectrophotometric assays (Based on the method of Canovas & Korberg 1969)

\[ \text{PEP} + \text{CO}_2 \rightarrow \text{oxaloacetate} + \text{Pi} \ (1) \]
\[ \text{Oxaloacetate} + \text{NADH} \rightarrow \text{malate} + \text{NAD} \ (2) \]

PEP carboxylase catalyses reaction 1. Malate dehydrogenase catalyses reaction 2. By adding malate dehydrogenase to the assay mix the reaction can be followed by measuring the reduction of NADH at 340 nm.

**Assay Mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Volume of 1 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris-HCl buffer (0.5M) pH 8.5</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>MgCl(_2) (50mM)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>NADH (10mM)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>KHCO(_3) (0.1M) freshly prepared</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA (10mM)</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>malate dehydrogenase (Sigma: bovine heart)</td>
<td>2 IU</td>
<td></td>
</tr>
<tr>
<td>PEP (50mM)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>cell extract</td>
<td>50,100, 200 or 400</td>
<td></td>
</tr>
<tr>
<td>dH(_2)O</td>
<td>to make 1ml</td>
<td></td>
</tr>
</tbody>
</table>

The reaction mix was made up in a 1 ml cuvette. The reaction was started by adding PEP. The blank contained no NADH. Positive controls containing 0.5 IU of PEP carboxylase from *E. coli* (Sigma) or 5 mM of oxaloacetate were also used. All assays were carried out in duplicate or triplicate. NADH oxidase activity was seen in high levels without the addition of PEP. This could be partially removed by ultracentrifugation at 100 000 x g for 30 min. Allowing the reaction to proceed without addition of NADH and malate dehydrogenase, precipitating the protein with TCA and then neutralising the sample as described above for pyruvate carboxylase eliminated the NADH oxidase activity. Assays were carried out with 0.68 M NaCl, 1M KCl and 0.68M NaCl, 1M NaCl and 2M NaCl.
B: Radioisotopic assay

**Assay Mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume of 250μl</td>
<td>μl</td>
</tr>
<tr>
<td>KCl (4M)</td>
<td>25</td>
</tr>
<tr>
<td>Tris-HCl buffer (0.5 M) pH 7.5</td>
<td>50</td>
</tr>
<tr>
<td>MgCl$_2$ (50 mM)</td>
<td>25</td>
</tr>
<tr>
<td>Acetyl-CoA (10mM)</td>
<td>12.5</td>
</tr>
<tr>
<td>PEP (50mM)</td>
<td>25</td>
</tr>
<tr>
<td>NaH$^{14}$CO$_3$ (18.5 MBqml$^{-1}$)</td>
<td>5</td>
</tr>
<tr>
<td>cell extract (20mg ml$^{-1}$ protein)</td>
<td>50</td>
</tr>
</tbody>
</table>

The radioisotopic PEP assay was carried out as for radioisotopic pyruvate carboxylase assay.

**2.8.3.5 Effect of ATP on pyruvate and PEP dependent CO$_2$ fixation**

In order to try to distinguish between CO$_2$ fixation catalysed by PEP carboxylase and pyruvate carboxylase the effect of ATP on CO$_2$ fixation was examined. Pyruvate carboxylase requires ATP, whereas PEP carboxylase does not.

This assay was carried out using same radioisotopic method as for the pyruvate carboxylase and PEP carboxylase assay. Tubes contained 5mM pyruvate, 5mM PEP, or both 5mM PEP and 5mM pyruvate. The effect of omitting ATP (10mM) on pyruvate and PEP dependent CO$_2$ fixation was studied.

**2.8.3.6 Effect of avidin on PEP and pyruvate dependent CO$_2$ fixation**

Pyruvate carboxylase is a biotin containing enzyme. Avidin from egg white binds to biotin-containing proteins and therefore should inhibit the activity of pyruvate carboxylase. 1 unit of avidin will bind to 1μg of biotin.

These assays were carried out as for the pyruvate and PEP carboxylase assays. A control containing pyruvate carboxylase (0.2 units) from bovine liver was also carried out. Cell extract was incubated for 30 minutes at 37°C with avidin (2.5 units).
Materials and Methods

tubes containing biotin and avidin, excess biotin (25µg) was incubated at 37°C for 10 minutes prior to incubation with cell extract.

2.8.3.7 NADP⁺-linked Malate Enzyme (EC 1.1.1.40) Assays

The NADP⁺-linked Malate enzyme (also called malic enzyme) is responsible for the following reaction:

\[ \text{L-malate} + \text{NADP}^+ \quad \leftrightarrow \quad \text{pyruvate} + \text{CO}_2 + \text{NADPH} \]

This enzyme requires Mn²⁺ or Mg²⁺ ions for activity. Two assay methods were used to look for malate enzyme activity: a spectrophotometric assay and a radioisotopic assay.

A: Spectrophotometric Assay (Bhaumik & Sonawat 1994)

Cell extract was prepared using the usual method of sonication modified using a buffer containing Tris-HCl (0.1M) pH 7.2 containing 1.2M NaCl.

**Assay Mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume of 1ml</td>
<td>100</td>
</tr>
<tr>
<td>Malate (15mM)</td>
<td>100</td>
</tr>
<tr>
<td>NADP (4mM)</td>
<td>100</td>
</tr>
<tr>
<td>MnCl₂ (50mM)</td>
<td>120</td>
</tr>
<tr>
<td>Tris-HCl buffer (0.1M) pH9 containing 4M NaCl</td>
<td>250</td>
</tr>
<tr>
<td>Cell extract</td>
<td>250</td>
</tr>
<tr>
<td>dH₂O</td>
<td>180</td>
</tr>
</tbody>
</table>

The assay mix was made up in a 1ml cuvette. The reaction was started by the addition of the cell extract. The cell extract contained 25 mgml⁻¹ protein. A blank was used containing no NADP and the reaction was monitored by following the reduction of NADP at 340 nm.
Materials and Methods

B: Radioisotopic Assay (Adapted from the method of Hughes et al. 1995)

Assay Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Volume of 250µl</td>
<td></td>
</tr>
<tr>
<td>KCl (4M)</td>
<td>25</td>
</tr>
<tr>
<td>Tris-HCl buffer (0.1M) pH 7.5</td>
<td>50</td>
</tr>
<tr>
<td>MgCl₂ (50mM)</td>
<td>50</td>
</tr>
<tr>
<td>Malate (50mM)</td>
<td>50</td>
</tr>
<tr>
<td>NADP (1mM)</td>
<td>50</td>
</tr>
<tr>
<td>cell extract</td>
<td>50</td>
</tr>
<tr>
<td>NaH¹⁴CO₃ (18.5 MBqml⁻¹)</td>
<td>5</td>
</tr>
</tbody>
</table>

Assay performed as for pyruvate carboxylase radioisotopic assay. A positive control contained 0.3 units of malic enzyme (chicken liver).

2.5.3.8 PEP Carboxykinase (EC 4.1.1.49) Assays

\[ \text{PEP} + \text{CO}_2 + \text{GDP/ADP} \overset{\text{PEP carboxykinase}}{\rightarrow} \text{Oxaloacetate} + \text{GTP/ATP} \]

PEP carboxykinase catalyses the above reaction. PEP carboxykinase requires the presence of GDP or ADP for activity. Higher organisms are usually GDP specific and bacterial PEP carboxykinases are ADP specific. However some bacteria including *Arthrobacter globiformis, Alcaligenes eutrophus* and *Corynebacterium glutamicum* have GDP specific enzymes. The enzyme also requires Mn²⁺ ions. The assay mix used was based on that used by Pickett et al. 1994 except that 1M KCl was included in the assay mix and only 10mM PEP was used.

Assay Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume of 250µl</td>
<td></td>
</tr>
<tr>
<td>KCl (4M)</td>
<td>25</td>
</tr>
<tr>
<td>Tris-HCl buffer (0.5M)</td>
<td>50</td>
</tr>
<tr>
<td>MgCl₂·6H₂O (50mM)</td>
<td>25</td>
</tr>
<tr>
<td>MnCl₂·6H₂O (50mM)</td>
<td>25</td>
</tr>
<tr>
<td>CoASH (10mM)</td>
<td>1.25</td>
</tr>
<tr>
<td>ADP (2mM) or GDP (2mM)</td>
<td>10</td>
</tr>
</tbody>
</table>

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Materials and Methods

Cell extract 50
PEP (50mM) 50
NaH$^{14}$CO$_3$ (18.5 MBq ml$^{-1}$) 5
H$_2$O 8.75

The assay was carried as for previous radioisotopic assays.

2.8.3.9 PEP carboxytransphosphorylase (EC 4.1.1.38) Assay

This enzyme catalyses the following reactions:

$$\text{PEP + CO}_2 + \text{Pi} \leftrightarrow \text{oxaloacetate} + \text{Pp}$$

$$\text{PEP + Pi} \rightarrow \text{pyruvate} + \text{PPi}$$

The activity of this enzyme is dependent on the presence of Pi and also Mg$^{2+}$, Mn$^{2+}$ or Co$^{2+}$ ions. The presence of a thiol stimulates the rate of oxaloacetate formation. Assay used was adapted from the method of Wood et al. (1969).

**Assay Mix**

- Total volume of 250μl
- PEP (20mM) 25
- K$_2$HPO$_4$ (0.1 M) pH 6.8 25
- or Tris-HCl (0.5M) pH 6.8 25
- MgCl$_2$ (50mM) 60
- CoCl$_2$ (3mM) 8.3
- KCl (4M) 25
- cell extract 50
- NaH$^{14}$CO$_3$ 5
- dH$_2$O 51.7

A standard mix was made up containing all the assay mix components except the CoCl$_2$, cell extract and NaH$^{14}$CO$_3$. This was saturated with CO$_2$ for 5 minutes prior to use. The cell extract was prepared in either 0.2 M phosphate buffer pH 6.8 with 3 M KCl and 1 mM mercaptoethanol or Tris-HCl buffer (0.5M) pH 6.8 with 3 M KCl and 1 mM mercaptoethanol using the method described above. The cell extract in Tris-HCl was used for a Pi free control. The cell extract contained 20 mg ml$^{-1}$. The assay was carried out as for pyruvate carboxylase.
2.8.3.10 Acetyl-CoA Carboxylase (EC 6.4.1.1.) Assay

This assay was adapted from the method of Hughes et al. 1995. Acetyl-CoA carboxylase carries out the following reaction:

\[
\text{Acetyl-CoA} + \text{ATP} + \text{HCO}_3^- \rightarrow \text{Malonyl CoA} + \text{ADP} + \text{Pi} + \text{H}^+ 
\]

**Assay mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume of 250μl</td>
<td></td>
</tr>
<tr>
<td>KCl (4M)</td>
<td>25</td>
</tr>
<tr>
<td>Tris-HCl buffer (0.5M) pH 7.5</td>
<td>50</td>
</tr>
<tr>
<td>MgCl(_2) (50mM)</td>
<td>25</td>
</tr>
<tr>
<td>Acetyl-CoA (10mM)</td>
<td>5</td>
</tr>
<tr>
<td>ATP (10mM)</td>
<td>25</td>
</tr>
<tr>
<td>NaH(^{14})CO(_3) (18.5 MBq ml(^{-1}))</td>
<td>5</td>
</tr>
<tr>
<td>cell extract (20 mg ml(^{-1}) protein)</td>
<td>50</td>
</tr>
<tr>
<td>dH(_2)O</td>
<td>65</td>
</tr>
</tbody>
</table>

Assay carried out using the same method as for pyruvate carboxylase and PEP carboxylase.

**2.8.4: \(^{13}\)C-labelling experiments using NMR, TLC and ion exchange chromatography**

To investigate the pathways of CO\(_2\) fixation further, the incorporation of \(^{13}\)CO\(_3\)\(^{-}\) into amino acids was investigated using \(^{13}\)C-NMR. This technique allows the labelling in individual carbon atoms in amino acids to be observed. The pathways can then be elucidated. Thin-layer chromatography was also used to confirm the identities of the amino acids seen using NMR. Quantitative amino acid analysis was carried out at Cambridge University.

**2.8.4.1 Preparation of Whole Cells for NMR**

Cells were grown in 100ml of CHM containing 20mM NaH\(^{13}\)CO\(_3\) or MM + glucose containing 20mM NaH\(^{13}\)CO\(_3\) (min. 98 atom % \(^{13}\)C; Isotec Inc). In order to obtain the natural abundance \(^{13}\)C-NMR spectra of amino acids of 54R, cells were grown in 1L of
Materials and Methods

CHM. Cells were grown to late exponential phase, then fractionated using the method of Pickett et al. (1994). The cells were harvested by centrifugation at 9 000 x g for 25 minutes. Cells were re-suspended in SS and spun again for 25 minutes. The pellet was then re-suspended in 2ml of buffer solution containing 20mM Tris-HCl pH 8, 10mM magnesium acetate, 30mM NH₄Cl and 6mM mercaptoethanol. Cells were sonicated (Sanyo Soniprep 150) eight times for 15 seconds with 15 second intervals. 1 mg of DNase was added and cells were kept on ice for 15 minutes. Samples were then centrifuged at 12 000 x g for 30 minutes, the supernatant removed and spun in an ultracentrifuge at 150 000 x g for 90 minutes. The cytoplasmic proteins in the supernatant were precipitated with ethanol (60% v/v) at 4°C for 1 hour and collected by centrifugation at 12 000 x g for 15 minutes. The membrane proteins were also collected from the pellet produced after ultra-centrifugation. The pellet was re-suspended in 500μl of chloroform: methanol: water (1:2:0.3 v/v), left at room temperature for 30 minutes and then centrifuged at 12 000 x g for 30 minutes. The supernatant was removed and the protein precipitated with ethanol (60% v/v) and collected as before. The cytoplasmic and membrane protein pellets were re-suspended in 500- 1000μl of dH₂O (depending on the amount of pellet) and pooled.

The sample was then placed in an acid washed pyrex tube, which was placed in a hydrolysis vessel containing 0.5 ml of 6M HCl. The vessel was evacuated and filled with nitrogen several times, then finally evacuated. The protein was then hydrolysed for 18 hours at 110°C. The resulting protein hydrolysate was freeze-dried prior to use for NMR spectroscopy.

2.8.4.2 Preparation of Cell Extract for NMR

In these experiments, cell extract was incubated with NaH¹⁴CO₃, NaH¹³CO₃ or unlabelled NaHCO₃ to compare the behaviour of cell-free extract, which was used in numerous enzyme assays, to the behaviour of whole cells. Cell extract was prepared in Tris-HCl (0.5M) buffer pH 7.6 containing 3M KCl using the same method as for the enzyme assays.
**Materials and Methods**

**Assay Mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume of 5ml</td>
<td>µl</td>
</tr>
<tr>
<td>Cell extract</td>
<td>(Expt 1: 40 mg·ml⁻¹ protein, Expt 2: 7.5 mg·ml⁻¹ protein)</td>
</tr>
<tr>
<td>KCl (4M)</td>
<td>500</td>
</tr>
<tr>
<td>Tris-HCl buffer (0.5M) pH 7.5</td>
<td>1000</td>
</tr>
<tr>
<td>MgCl₂ (50mM)</td>
<td>500</td>
</tr>
<tr>
<td>Acetyl-CoA (10mM)</td>
<td>250</td>
</tr>
<tr>
<td>PEP (50mM)</td>
<td>500</td>
</tr>
<tr>
<td>Na pyruvate (0.5M)</td>
<td>50</td>
</tr>
<tr>
<td>ATP (5 mg·ml⁻¹)</td>
<td>110</td>
</tr>
<tr>
<td>dH₂O</td>
<td>99</td>
</tr>
<tr>
<td>NaH¹⁴CO₃ (18.5 MBq·ml⁻¹)</td>
<td>100</td>
</tr>
<tr>
<td>OR NaH¹³CO₃</td>
<td>0.17g</td>
</tr>
<tr>
<td>OR unlabelled NaHCO₃</td>
<td>0.17g</td>
</tr>
</tbody>
</table>

The assay mixture was incubated for 24 hours at 37°C. The samples containing NaH¹³CO₃ and unlabelled NaHCO₃ were then prepared for NMR spectroscopy using the same method as for whole cells. The reaction containing NaH¹⁴CO₃ was terminated by adding 500µl of HCl. A 250µl aliquot was removed and evaporated to dryness for approximately three hours in a heating block. The dried sample was redissolved in 250µl of dH₂O. Two 100µl aliquots were added to 3ml of Optiphase Safe scintillation fluid. Radioactivity was counted three times in a scintillation counter. This was used as a positive control.

**2.8.4.3 ¹³C-NMR Spectroscopy**

Principles of NMR and the use of NMR in studies of biosynthesis are described in Appendix 1. ¹³C-NMR spectra were obtained on a Bruker DRX400 spectrometer operating at 100.61272 MHz for the ¹³C nucleus. Standard composite pulse decoupling of ¹H was used. Spectra were accumulated with 64K data points and processed with 3 to 6 Hz line broadening. Samples were dissolved in approximately 0.5 ml D₂O (Goss Scientific Instruments; 99.9% min.) and the pH was adjusted to pH 1. Between 27000 to 40000 scans were obtained. Signals in spectra were assigned by
Materials and Methods

comparison to those in the literature (Ekiel et al. 1983, Pickett et al. 1994, Pretsch et al. 1989) and by comparison to $^{13}$C- NMR spectra recorded for authentic samples.

A semi-quantitative approach was used to calculate relative enrichment with $^{13}$C of carbon atoms in amino acids. The ratio of the intensity of the signal for each carbon atom in authentic amino acids was calculated. The ratio of the intensity of the signal for each carbon in each amino acid observed in the spectrum for the protein hydrolysate from cells grown with the $^{13}$C label was also calculated. Identical instrument settings were used to obtain spectra for both authentic amino acids and protein hydrolysate from halobacteria. A comparison was made, for each amino acid, of the ratio seen in the protein hydrolysate with the ratio in the authentic amino acid. This method allowed the enrichment of individual carbon atoms in amino acids to be assessed. If the ratio of the intensities of the signals for a particular amino acid was close to that seen in an authentic sample of that amino acid, it was considered not to be enriched. However, if one peak for one carbon atom was much larger than predicted compared to the other signals for that amino acid, then that carbon atom was considered to be enriched. Using this method to look for enrichment, a carbon atom was considered to be significantly enriched if the intensity of its signal was greater than twice the intensity predicted from the authentic sample. If the intensity was greater than 5 times the predicted intensity the enrichment was recorded as “high” (see Table 5.18). For example, in the spectra for 54R (grown on CHM and H$^{13}$CO$_3$), the intensity of signals representing glutamic acid C1, C2, C3 and C4 had a ratio of 4: 1: 1: 6. However the intensity of signals for C1 – C4 in an authentic sample of glutamic acid had a ratio of 0.4: 1: 1: 0.5. Therefore both C1 and C4 must be enriched, C1 by approximately 10 times and C4 by approximately 12 times.

2.8.4.4 Thin layer chromatography (TLC) of amino acids

The protein hydrolysate used for NMR was also analysed using TLC to confirm the identification of amino acids. Samples (0.2 - 0.4 µl) were spotted on to cellulose coated aluminium TLC plates. The plates were the run in two dimensions. The 1st phase consisted of n-butanol: pyridine: H$_2$O (1: 1:1) and the 2nd phase consisted of butanol : acetic acid: H$_2$O (12: 3: 5). After drying, plates were sprayed with ninhydrin (0.5% in butan-1-ol) and developed by heating at 95°C for a few minutes. Spots were
identified by comparing with chromatograms of authentic amino acids.

2.8.4.5 Quantitative Amino Acid Analysis by Gas Phase Hydrolysis and Ion Exchange Chromatography

Amino acid analysis was carried out by Peter Sharratt, Protein and Nucleic Acid Chemistry Facility, Dept. Of Biochemistry, University of Cambridge. The following method was used. Protein was prepared from 50ml of cells grown in CHM with 20mM NaH$^{13}$CO$_3$, as described above for NMR experiments. The pellet (about 4 mg) was dissolved in 1 ml of water. An aliquot (100μl) was placed in a pyrolysed tube and a solution of norleucine was added (typically 200 nmoles, 2.5 mM). The solution was concentrated to dryness in a centrifugal evaporator and then placed in a hydrolysis vessel containing 500μl of 6M hydrochloric acid and 68 μl of 1-dodecanethiol. The vial was evacuated, then flushed with argon. This process was repeated several times prior to evacuation. The vial was then placed in an oven at 115.5°C for 22 hours. The tube was removed from the vial and dried under vacuum at room temperature. 100μl of sodium citrate buffer (pH 2.2) was then added. The resulting solution was filtered under centrifugation through a 0.2μm filter. An aliquot of the filtrate (usually 2μl) was injected into a sample-loading capsule followed by 20μl of loading buffer. The loading capsule was placed in an amino acid analyser (Pharmacia Alpha Plus Series II) and chromatography was performed on an ion exchange resin (Ultropac 8 Polysulphonate) eluting with a series of buffers over the pH range 3.2 to 6.45. Peak detection was performed by mixing the eluate with ninhydrin at 135°C and measuring the absorbance at 570 and 440 nm.
3

RESULTS AND DISCUSSION:
GROWTH OF HALOBACTERIA

3.1: Growth of Halobacteria in Minimal Medium (MM) with a Single Carbon Source

The ability of salt-mine strains to grow on plates of minimal medium (MM) with the addition of glucose, trehalose, glycerol and glycine betaine was investigated. Growth was scored as positive if growth was better than on plates containing MM only. Often it was difficult to decide if there was a significant difference between the growth on MM only and the growth on MM with an additional substrate. For this reason the growth of several strains was investigated in broth, in which growth could be compared more easily by measuring the OD. Growth in MM broth with the additional of glycerol, trehalose, glycine betaine, alanine, proline or glutamic acid was tested. These compounds were chosen as they are produced by bacteria as compatible solutes for osmoregulation. They therefore represent compounds, which are likely to be present in relatively high concentrations in the hypersaline environment. Growth on glucose was also tested. Growth of some strains on pyruvate, acetate, iso-butyrate, butyrate and iso-valerate was tested.

Table 3.1 shows the growth of a range of salt-mine and surface strains on plates containing MM with the addition of glucose, glycerol, trehalose, and glycine betaine. Fig. 3.1 – 3.24 show the growth of 5 salt mine strains and four surface strains in MM broth with the addition of glucose, glycerol, trehalose, glycine betaine, alanine, proline and glutamic acid. The growth observed on plates and in broth generally agreed well.
**Table 3.1:** Table showing the ability of strains isolated from salt mines to grow on plates of minimal media with the addition of glucose, glycerol, trehalose and glycine betaine.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose</th>
<th>Trehalose</th>
<th>Glycerol</th>
<th>Glycine betaine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br1</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Br2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Br3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Br4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Br5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Br6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Br7</td>
<td>+</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Br8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>011.1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>014.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>G002.1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>G005.1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>G014.2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G015.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mali 006.3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GBr1.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glu006.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2Wnr5.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0011.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>001.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>54R</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>54P</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glu 008.1</td>
<td>+</td>
<td>+</td>
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Results and Discussion: Growth of Halobacteria

**Fig. 3.1:** Growth of 54R in minimal media (MM) containing glucose, glycerol, trehalose and glycine betaine.

**Fig. 3.2:** Growth of 54R in minimal media (MM) containing proline, alanine and glutamate.

**Fig. 3.3:** Growth of 54R in minimal media (MM) containing acetate, pyruvate, isobutyrate, butyrate, isovalerate and propionate.
Results and Discussion: Growth of Halobacteria

Fig. 3.4: Growth of *Hr. saccharovorum* in minimal media (MM) containing glucose, glycerol, trehalose and glycine betaine.

Fig. 3.5: Growth of *Hr. saccharovorum* in minimal media (MM) containing proline, alanine and glutamate.

Fig. 3.6: Growth of *Hr. saccharovorum* in minimal media (MM) containing acetate, pyruvate, isobutyrate, butyrate, isovalerate and propionate.
Results and Discussion: Growth of Halobacteria

Fig. 3.7 Growth of E4 in minimal media (MM) containing glucose, glycerol, trehalose and glycine betaine.

Fig. 3.8: Growth of E4 in minimal media (MM) containing proline, alanine and glutamate.

Fig. 3.9: Growth of E4 in minimal media (MM) containing acetate, pyruvate, isobutyrate, butyrate, isovalerate and propionate.
Results and Discussion: Growth of Halobacteria

Fig. 3.10 Growth of Ha. marismortui in minimal media (MM) containing glucose, glycerol, trehalose and glycine betaine.

Fig. 3.11: Growth of Ha. marismortui in minimal media (MM) containing proline, alanine and glutamate.

Fig. 3.12: Growth of Ha. marismortui in minimal media (MM) containing acetate, pyruvate, isobutyrate, butyrate, isoalrate and propionate.
Results and Discussion: Growth of Halobacteria

Fig. 3.13 Growth of Br3 in minimal media (MM) containing glucose, glycerol, trehalose and glycine betaine.

Fig. 3.14 Growth of Br3 in minimal media (MM) containing proline and alanine.

Fig. 3.15 Growth of Hc. morrhuae in minimal media (MM) containing glucose, glycerol, trehalose and glycine betaine.
Results and Discussion: Growth of Halobacteria

Fig. 3.16: Growth of Hc. morrhuae in minimal media (MM) containing proline, alanine and glutamate.

Fig. 3.17: Growth of O14.1 in minimal media (MM) containing glucose, glycerol, trehalose and glycine betaine.

Fig. 3.18: Growth of O14.1 in minimal media (MM) containing proline, alanine and glutamate.
Results and Discussion: Growth of Halobacteria

Fig. 3.19: Growth of BbpA1 in minimal media (MM) containing glucose, glycerol, trehalose and glycine betaine.

Fig. 3.20: Growth of BbpA1 in minimal media (MM) containing alanine, proline and glutamate.

Fig. 3.21: Growth of Hb. salinarum in minimal media (MM) containing glucose, glycerol, trehalose and glycine betaine.
Results and Discussion: Growth of Halobacteria

Fig. 3.22: Growth of Hb. salinarum in minimal media (MM) containing proline, alanine and glutamate.

Fig. 3.23: Growth of Hb. salinarum (halobium) in minimal media (MM) containing glucose, glycerol, trehalose and glycine betaine.

Fig. 3.24: Growth of Hb. salinarum (Hb.halobium) in minimal media (MM) containing proline, alanine and glutamate.
None of the strains tested on plates could grow using glycine betaine (Table 3.1), although the growth of *Hb. salinarum* was slightly enhanced by the addition of glycine betaine to MM broth (Fig. 3.21). Many of the salt mine strains could grow on both the sugars tested (glucose and trehalose) and glycerol (Table 3.1, Figs. 3.1, 3.5, 3.9, 3.13 & 3.15). These strains belonged mainly to the genera *Haloarcula* and *Halorubrum*, but also included O14.1 and BbpA1 (which have been shown to be related to *Hb. salinarum*), and Br3 (which is a member of the genus *Halococcus*). Of the surface strains tested *Ha. vallismortis*, *Ha. marismortui*, *Hr. saccharovorum* and *Hc. morrhuae* could grow on glucose, glycerol and trehalose (Table 3.1, Figs. 3.4, 3.10 & 3.15).

Some of the salt mine strains were more fastidious. Br4 and Br5 were shown to grow on glycerol only and not glucose or trehalose. Br2 could grow on glucose only and not glycerol or trehalose (Table 3.1). Br2, Br4 and Br5 have been shown by polar lipid analysis to belong to the genus *Haloarcula*. They therefore represent salt-mine strains, which are more fastidious than the surface strains *Ha. vallismortis* and *Ha. marismortui*. Strains G002.1 and G005.1 grew on trehalose and glycerol, but not glucose. Strains 011.1 and G014.2 grew on trehalose and glucose, but not glycerol. Malk 006.3 grew on glucose and glycerol, but not trehalose (Table 3.1). Strains G002.1, G005.1, 011.1, G014.2 and Malk 006.3 have been shown to belong to the genus *Halorubrum*, however they are more limited by the carbon sources they can utilise than the surface strain *Hr. saccharovorum*.

*Hb. salinarum* could grow well on glycerol only, although glycine betaine gave slight enhancement of growth in broth (Table 3.1, Fig. 3.17). *Hb. salinarum* (*Hb. halobium*) could not utilise any of the added substrates as sole carbon and energy source (Table 3.1, Fig. 3.19). However BbpA1 and O14.1, which have been shown to be related to *Hb. salinarum*, could both utilise glucose, glycerol, and trehalose (Figs. 3.17 & 3.19).

Amongst the bacterial halophiles tested growth of 0011.2 and 001.3 was not enhanced by addition of any of the substrates tested, but 001.2 grew with the addition of glucose and trehalose, but not glycerol or glycine betaine (Table 3.1).
The ability to utilise amino acids and carboxylic acids was tested using less strains than the ability to grow on glucose, glycerol, trehalose and glycine betaine, but the ability to utilise amino acids and carboxylic acids varied much more amongst those strains which were tested. Growth with an amino acid as the single carbon source was in many cases only slightly better than with the MM only. 54R and \textit{Hc. morrhuae} were enhanced by the addition of alanine, proline and glutamic acid (Figs. 3.2 & 3.16). Growth of Br3 on glutamic acid was not tested, but it was enhanced by alanine and proline (Fig. 3.14). Enhancement of the growth of 54R, \textit{Hc. morrhuae} and Br3 by alanine was less than the enhancement of growth by proline or glutamic acid. \textit{Hr. saccharovorum} and \textit{Ha. marismortui} were enhanced by proline and glutamate, but not by alanine (Figs. 3.5 & 3.11). E4 was enhanced only slightly by proline (Fig. 3.8) and O14.1 was enhanced only slightly by alanine (Fig. 3.10). Growth of BbpA1, \textit{Hb. salinarum} and \textit{Hb. salinarum} (\textit{Hb. halobium}) was not enhanced by addition of any of the amino acids (Figs. 3.20, 3.22 & 3.24).

54R and \textit{Hr. saccharovorum} could both grow on pyruvate, \textit{Hr. saccharovorum} could grow on acetate but 54R could not, neither strain could grow on any of the other carboxylic acids tested (Fig. 3.3 & 3.6). \textit{Ha. marismortui} could grow on pyruvate and butyrate and none of the other carboxylic acids tested, including acetate (Fig. 3.12). E4 was found to grow on all the carboxylic acids tested, although growth on isovalerate was not as good as growth on the other carboxylic acids (Fig. 3.9). In many organisms the growth on acetate involves the operation of the glyoxylate cycle, involving the enzymes isocitrate lyase and malate synthase. Isocitrate lyase activity has been shown to be present at low levels in members of the genus \textit{Haloarcula}, but no activity has been shown in members of the genus \textit{Halorubrum} (Oren & Gurevich 1995b).

Most of the salt mine strains tested fell into the genera \textit{Halorubrum} or \textit{Haloarcula} as these were isolated in the largest numbers from Boulby and Winsford mines. Among the salt mine isolates of \textit{Halorubrum} half of the strains grew on glucose, glycerol and trehalose, as did \textit{Hr. saccharovorum}. The other half of the strains were more fastidious than \textit{Hr. saccharovorum}. The growth of the strain 54R was tested on a wide range of substrates and was found to behave in a similar way to \textit{Hr. saccharovorum}, except that except 54R can utilise alanine and \textit{Hr. saccharovorum} can not. \textit{Hr.
Results and Discussion: Growth of Halobacteria

Saccharovum can utilise acetate and 54R can not. McGenity & Grant (1995) describe members of the genus Halorubrum as able to utilise several sugars. This study has shown that some members can also utilise pyruvate and amino acids as the main carbon and energy source.

Among the salt mine strains of Haloarcula, 4 out of the nine strains tested were more fastidious than both Ha. marismortui and Ha. vallismortis, with the remaining five strains behaving like Ha. marismortui and Ha. vallismortis. Growth of E4 was tested on a wider selection of carbon sources. This was the only strain shown to grow on iso-butyrate, propionate, and iso-valerate. The growth of E4 on glucose and glycerol was only slightly better than on MM, with the OD reaching a maximum of 0.6 during growth on glucose and a maximum OD of 0.55 during growth on glycerol. During growth of Ha. marismortui on these substrates the highest OD measured was over 1.5. Therefore there were some metabolic differences between the salt mine strain E4 and the surface strain Ha. marismortui. Members of the genus Haloarcula have previously been described as metabolising a wide range of substrates (Javor 1984).

The growth of only two strains of Halococcus was tested. Br3 and Hc. morrhuae behaved in a very similar way on all the substrates tested. This study shows that members of the genus Halococcus can utilise sugars including glucose, as well as glycerol and amino acids. This is in contrast to Grant & Larsen (1989) who reported that glucose could not be used as the main source of carbon and energy by Hc. morrhuae.

Members of the genus Halobacterium are known to have a complex nutrition and require amino acids for growth (Grant & Larsen 1989), however Halobacterium salinarum was able to grow well in MM with the addition of glycerol. MM contains a small amount of yeast extract and the amino acids required may be supplied in this. Neither Hb. salinarum nor Hb. salinarum (halobium) could grow in MM with the addition of alanine, proline or glutamic acid. The salt mine strains BbpA1 and O14.1 have been shown by sequencing of 16S rRNA to be most closely related to members of the genus Halobacterium. However they can both grow well using glucose, glycerol and trehalose as the sole carbon source supplementing MM, O14.1 can also utilise alanine. They both therefore behave differently from Hb. salinarum and Hb.
**Results and Discussion: Growth of Halobacteria**

*salinarum (halobium)*. BbpA1 and O14.1 both have a different polar lipid pattern to *Hb. salinarum* R1 (McGenity 1994). The genus *Halobacterium* is characterised by the presence of two sulphated glycolipids- S-TGD-1 and S-TeGD (Grant & Larsen 1989). O14.1 and BbpA1 do not possess these glycolipids and the cells are more round and pleomorphic than those of *Hb. salinarum* R1 (McGenity 1994). McGenity discussed whether these strains should be placed in the genus *Halobacterium* or whether they should be placed into a new genus. The different metabolic capacities of BbpA1 and O14.1 provide further evidence that they should be placed in a new genus.

This study has shown that the majority of halobacteria tested can grow on glycerol and trehalose. The unicellular green algae *Dunaliella* accumulates glycerol intracellularly in molar concentrations. *Dunaliella* spp. are the most numerous primary producers in hypersaline environments (Rodriguez-Valera et al. 1985). Glycerol leaking out of healthy or decaying *Dunaliella* is probably one of the most available carbon sources in the Dead Sea (Oren & Gurevich 1994b). Trehalose is accumulated by a number of organisms which have been isolated from hypersaline environments for example *Actinopolyspora* spp., *Micrococcus* spp. and *Ectothiorhodospira* spp., although many of these strains also accumulate glycine betaine, often to higher concentrations than trehalose (Severin et al. 1992).

Alanine, glutamate and proline can also be utilised as a carbon and energy source by some halobacteria. Alanine has been shown to be accumulated as a compatible solute by *Streptomyces griseolus* and *Paracoccus denitrificans*. Glutamate is accumulated by a number of halophilic bacteria including *Micrococcus* spp., *Halomonas* spp., *Paracoccus halodentificans* and *Vibrio* spp. Both alanine and glutamate are usually accumulated as minor components of a "cocktail" of osmolytes. Strains that accumulate alanine or glutamate, usually also accumulate ectoine and/or hydroxyectoine to much higher concentrations then alanine or glutamate (Severin et al. 1992). Proline is accumulated by some species of algae (mainly *Bacillorphyceae*), halotolerant *Bacillus* spp., some *Salinicoccus* spp. and some *Planococcus* spp. (Galinski 1993). Thus amino acids such as alanine, proline and glutamate may be available as carbon sources to halobacteria in the hypersaline environment, but they are probably not as plentiful as glycerol or trehalose.
Glycine betaine and ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) represent two of the most predominant compatible solutes (Severin et al. 1992). Ectoines are accumulated by a wide range of chemoheterotrophic halophilic bacteria and glycine betaine by anoxygenic phototrophic bacteria. Many chemoheterotrophic halophilic bacteria also accumulate hydroxy-ectoine (Severin et al. 1992). The ability of halobacteria to grow on ectoine and hydroxy-ectoine has not been tested in this study, as these compounds are not commercially available. Glycine betaine is typically accumulated at concentrations above 0.5 M by species such as Chromatium and Ectothiorhodospira and also by halophilic methanogens (Severin et al. 1992, Galinski 1993). Ectothiorhodospira excretes glycine betaine under dilution stress and therefore glycine betaine occurs regularly in hypersaline brines such as Solar Lake, Sinai and Wadi Natrun, Egypt (Truper & Galinski 1990). Mateeva et al. (1993) found that, bacteria in the high salinity stratal waters of oil fields, accumulated glycine betaine in concentrations of up to 5M. Despite the evidence that glycine betaine is likely to be present at high concentrations in hypersaline environments, halobacteria do not appear to have the ability to utilise glycine betaine as a carbon and energy source. Oren (1990) has also previously reported that halobacteria can not grow using glycine betaine.

The compatible solutes, which are produced by halophilic bacteria, are likely to represent an important source of carbon for halobacteria. However not all of the compounds likely to be available can be utilised by halobacteria.

Are compatible solutes likely to be available as carbon sources in the salt deposits? Is it possible that they would become laid down with the salt during formation of the deposits? Sediments of hypersaline lakes are often have high TOC contents due to the high productivity of these environments (Neev & Emery 1976, Hite et al. 1984, Klug et al. 1985) however, ultimately, most of the organic matter, which is deposited becomes incorporated into kerogen. So it seems unlikely that the compatible solutes present in the hypersaline brines would remain in an unchanged form deep within the salt deposit. Salt deposits often contain streaks of organic matter (Sonnenfeld 1984), but the nature of this matter has not been analysed.
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In both Boulby and Winsford mines, populations of chemoheterotrophic bacteria were isolated in addition to the halophilic archaea (McGenity 1994). The compatible solutes accumulated by these bacteria have not been established, but if these bacteria are similar to chemoheterotrophic halophilic bacteria from surface environments, they are likely to accumulate mainly ectoines, hydroxy-ectoine or trehalose with smaller amounts of glutamate, alanine or proline (Severin et al. 1992). Glycine betaine and glycerol are synthesised and used as compatible solutes by phototrophic bacteria and green algae, respectively, and not chemoheterotrophic bacteria. Glycine betaine and glycerol are likely to be present only in relatively small concentrations or perhaps absent from the salt deposits.

Most of the salt deposit strains could utilise both trehalose and glycerol well. Some salt-mine strains could utilise the amino acids alanine, proline and glutamate, but no strains could use glycine betaine. The salt-mine strains show the same pattern of utilisation of compatible solutes as the surface strains of halobacteria, even though the range of compatible solutes available to strain mine strains is probably more limited than those available to surface strains of halobacteria. The ability of salt-mine strains to utilise glycerol indicates that perhaps, contrary to our expectations, glycerol is present within the salt-mine. If phototrophic bacteria and green algae were the dominant members of the hypersaline brine prior to its deposition, perhaps some glycerol became incorporated into the deposit. Or perhaps glycerol is used as a compatible solute by members of the present salt-mine community. If the compatible solutes accumulated by the salt-mine halophilic bacteria were investigated, this would contribute to the knowledge of the carbon sources available to halobacteria in the salt-mine. Chemical analysis of the range of carbon compounds present in Boulby and Winsford mines would help to discover whether the metabolism of salt-mine halobacteria is well adapted to life in the salt deposits.
3.2: Growth of halobacteria in defined medium.

The growth of 54R, E4, BbpA1, O14.1 and Br3 in a defined medium: modified Rodriguez-Valera medium (RVM) with glucose as the carbon source was investigated. It was important to ascertain that cells were growing on the carbon source provided - in this case glucose, and not on carbon sources carried over in the inoculant. Growth following a number of transfers was therefore studied. Starter cultures were grown in CHM to an OD of 0.5. 0.5 ml of the starter culture was used to inoculate 10 ml of RVM + glucose in a universal tube (transfer I). When growth reached an OD of 0.5 this was then used to inoculate a fresh tube of RVM + glucose (transfer II) and when growth in this tube reached 0.5 then this was used to inoculate a further tube of RVM + glucose (transfer III). The effect of the addition of vitamins and minerals on growth in RVM + glucose was investigated. The concentrations of vitamins and minerals added are given in section 2.2.3. The growth in MM + glucose following a number of transfers was also investigated.

Fig. 3.25 to 3.29 show that growth by 54R, E4, O14.1, BbpA1 and Br3 in RVM + glucose without the vitamins and minerals, reached a high OD (about 2.5) after the first transfer whereas, after the second transfer the OD reached was reduced, and after the third transfer the OD was fell even further (to about 0.5 or less). This indicates that cells were using components of CHM for growth.
Results and Discussion: Growth of Halobacteria

Fig. 3.25: Growth of 54R in RVM + glucose following three transfers.

I: indicates growth after 1st transfer from CHM to RVM
II: indicates growth after 2nd transfer from RVM to RVM
III: indicates growth after 3rd transfer from RVM to RVM

Fig. 3.26: Growth of E4 in RVM + glucose following three transfers.

I: indicates growth after 1st transfer from CHM to RVM
II: indicates growth after 2nd transfer from RVM to RVM
III: indicates growth after 3rd transfer from RVM to RVM
Fig. 3.27: Growth of BbpA1 in RVM + glucose following three transfers.

I: indicates growth after 1<sup>st</sup> transfer from CHM to RVM
II: indicates growth after 2<sup>nd</sup> transfer from RVM to RVM
III: indicates growth after 3<sup>rd</sup> transfer from RVM to RVM

Fig. 3.28: Growth of O14.1 in RVM + glucose following three transfers.

I: indicates growth after 1<sup>st</sup> transfer from CHM to RVM
II: indicates growth after 2<sup>nd</sup> transfer from RVM to RVM
III: indicates growth after 3<sup>rd</sup> transfer from RVM to RVM
Results and Discussion: Growth of Halobacteria

**Fig 3.29: Growth of Br3 in RVM + glucose following three transfers.**

I: indicates growth after 1st transfer from CHM to MM  
II: indicates growth after 2nd transfer from RVM to MM  
III: indicates growth after 3rd transfer from RVM to MM

For strains 54R and E4 (Fig. 3.25 & 3.26) when the vitamins and minerals were added the growth was maintained at high levels (OD of 2.5) even after the third transfer of cells into RVM + glucose. Growth of 54R and E4, in RVM + glucose with the vitamins and minerals, reached much higher levels than growth in MM + glucose, where the maximum OD reached was only about 0.6 for both 54R and E4. Growth of O14.1 reached a high OD (2.5) even after three transfers into RVM + glucose supplemented with vitamins and minerals, although the growth rate slowed down after each transfer (Fig. 3.28). Growth of BbpA1 in RVM + glucose with vitamins and minerals reached an OD of 2.5 after the first transfer, but this was reduced to an OD of about 1.7 after the third transfer (Fig. 3.27). This was still higher than RVM + glucose with no vitamins and minerals, where after three transfers the OD reached 0.3, or growth in MM + glucose where the OD reached 0.5. When vitamins and minerals were added to RVM + glucose the growth of Br3 after the first transfer reached an OD of 2.5, after the second transfer reached about 1.7 but after the third time reached an OD of about 2. The growth rate slowed down with each transfer (Fig. 3.29). These results indicate that the reduction of the maximum OD reached after each transfer from RVM + glucose to RVM + glucose (no vitamins and minerals) is due to
the lack of one or more essential vitamins or minerals, which are required for the growth of these strains of halobacteria.

Fig. 3.30, 3.31 & 3.33 show that with strains 54R, E4 and O14.1 after the transfer from CHM to MM + glucose (Transfer I) a high OD of around is reached (2.0 for E4 and O14.1 and 1.3 for 54R). After the second transfer the maximum growth reached is lower (OD of 0.6 for 54R and around 1.0 for E4 and O14.1). After the third transfer the maximum OD reached is the same as after the second transfer. This is a similar pattern to RVM + glucose (without the addition of the vitamins and minerals) and indicates that after the first transfer components of the CHM are utilised. When the vitamins and minerals are added to RVM + glucose the reduction in the level of growth following the transfers does not occur.

*Fig 3.30: Growth of 54R in MM+ glucose following three transfers.*
Results and Discussion: Growth of Halobacteria

Fig 3.31: Growth of E4 in MM+ glucose following three transfers.

![Graph showing growth of E4](image)

I: indicates growth after 1st transfer from CHM to MM
II: indicates growth after 2nd transfer from MM to MM
III: indicates growth after 3rd transfer from MM to MM

Fig 3.32: Growth of O14.1 in MM+ glucose following three transfers.

![Graph showing growth of O14.1](image)

I: indicates growth after 1st transfer from CHM to MM
II: indicates growth after 2nd transfer from MM to MM
III: indicates growth after 3rd transfer from MM to MM
Results and Discussion: Growth of Halobacteria

Growth in MM + glucose by 54R, E4 and O14.1 must therefore be limited by the availability of certain vitamins or minerals. In one strain, BbpA1, after the first transfer the OD reached a maximum of 0.6 after 100 hours, after the second transfer it took 170 hours to reach the same maximum OD of 0.6, however after the third transfer it reached a maximum OD of 1.0 after 300 hours. This seems to demonstrate that BbpA1 was very slowly adapting to grow in MM + glucose after growing in the nutritionally richer CHM, perhaps this strain has the ability to synthesise a vitamin, which limits growth in the other strains.

These growth experiments indicate that growth in MM + glucose and in RVM + glucose is limited by the availability of certain vitamins and minerals. If vitamins and minerals are added to RVM + glucose a high OD can be reached after several transfers from RVM + glucose to RVM + glucose by 54R, E4, BbpA1, O14.1 and Br3. These strains can utilise glucose as the sole carbon and energy source when supplied with vitamins and minerals. Some members of the halophilic archaea are described as requiring amino acids for growth, these include members of the genera Halorubrum, Halococcus and Halobacterium. None of the strains used in this study require amino acids for growth, including 54R (a member of the genus Halorubrum),
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Br3 (a member of the genus *Halococcus*) and strains BbpA1 and O14.1 (related to *Halobacterium*). It has been reported that members of the genus *Halococcus* require a number of purines and pyrimidines for growth (Grant & Larsen 1989), however this study shows that these are not required by strain Br3.

Defined growth media have been designed for *Halobacterium* spp. Onishi *et al.* (1965) developed a media containing 15 amino acids, two nucleotides and glycerol. Grey & Fitt (1976) later showed that the nucleotides served as sources of phosphate. Mevarech & Werczberger (1985) used a defined media containing glycerol and succinate for growth of *Haloferax volcanii*, but several groups have reported that this medium did not support good growth (Kushner 1993). Kushner (1993) found that the medium of Mevarech & Werczberger supported better growth of *Hf. mediterranei* if vitamins were added to the media. Kushner observed a similar pattern of growth to that seen in the experiments carried out in this study, in that when cells were transferred from complex media to defined media, without vitamins, they would grow very well for the first two transfers, but not on subsequent transfers. When the medium of Mevarech & Werczberger is supplemented with thiamin and biotin it has been found to support the growth of *Hr. saccharovorum*, *Hb. trapanicum*, and *Ha. vallismortis* (Kauri *et al.* 1990).

In this study it has been demonstrated that RVM, supplemented with vitamins and trace elements, supports growth of several strains of halobacteria and is therefore suitable for use in studies where a defined medium is needed. The one disadvantage of RVM is the large amount of precipitate it contains.
3.3 Production of acids during growth of halobacteria.

A number of different approaches were investigated to analyse and quantify the acid products produced during growth by halobacteria. Previous work on analysis of organic acids, mainly as metabolic products in human body fluids, suggested that gas chromatography (GC) would be the most effective tool for separating and identifying individual analytes (Morrison et al. 1992, Mardens et al. 1992, Eder 1995, Whitehead, Kim & Prizont 1976, Lehotay & Clarke 1995, Carlsson 1972). A further advantage of GC is that it can be used in combination with mass spectrometry to aid identification of analytes. Oren & Gurevich (1994a) had previously found that lactic acid, pyruvic acid and acetic acid were produced by halobacteria during growth on glycerol and glucose. The method chosen required that these three acids could be separated and quantified. Three main approaches were investigated—firstly analysis of free acids, secondly derivatisation of acids to methyl esters and thirdly derivatisation of acids to ethyl esters.

In initial studies extraction of free acids into ether was carried out (as in section 2.6.2) prior to analysis using a BP21 megabore column (details of this column are given in section 2.6.2). Extraction of acids into ether was suitable for carboxylic acids including aryl acids such as benzoic acid, diacids such as malonic acid, and hydroxyacids such as lactic acid. However pyruvic acid has a low solubility in ether compared to water\(^1\), and may also be lost during the drying stage (Chalmers & Watts 1977), so this method was not suitable for the extraction of pyruvic acid. The BP21 megabore column could be used to separate fatty acids from formic acid (C1) up to much longer fatty acids. It could also separate benzoic acid, malonic acid, lactic acid, and pyruvic acid. However there were problems associated with this column. The baseline was very uneven making it difficult to distinguish small peaks and probably resulting in inaccurate integration of peaks. Additionally the column was very fragile. The FFAP column was much more sensitive than the megabore column; the baseline was straight resulting in better resolution and more accurate integration of peaks. The

\(^1\) The partition coefficient between ether and water for pyruvic acid is 0.15 (ether /\(H_2O\)). For propionic acid it is 1.7 (ether /\(H_2O\)).
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FFAP column could be used at lower attenuation settings so lower concentrations of acids could be detected. Additionally an isothermal oven temperature could be used to analyse acids on the FFAP column. A temperature program had to be used with the megabore column. When a temperature program is used a period of time is required to cool the oven down to the starting temperature after each run, which increases the time required for each run. One disadvantage was that lactic acid and pyruvic acid could not be detected using the FFAP column.

Methyl esters are less polar and more thermally stable than free acids; as a consequence they are easier to analyse than free acids. Free acids were derivatized to methyl acids by heating with methanol and an acid catalyst at 100°C for one hour (see section 2.6.3). This method was successful for short chain fatty acids, lactic acid and pyruvic acid. However only 5% of pyruvic acid was converted to methyl pyruvate compared to 50 – 70% for other acids, including lactic acid. The Innowax capillary column (see section 2.6.3) gave good separation of methyl pyruvate, methyl lactate and methyl esters of fatty acids greater than C6. Small methyl esters eluted close to the solvent peak due to their high volatility, making analysis of these very difficult.

To overcome the problem of the high volatility of methyl esters derivatisation of acids to ethyl esters was investigated (see section 2.6.4). The higher molecular weight of ethyl esters compared to methyl esters meant that the ethyl esters of acids smaller than C6 eluted further away from the solvent peak. Derivatisation to ethyl esters using ethanol and an acid catalyst was carried out successfully for fatty acids and lactic acid but again ethyl pyruvate was produced only at very low efficiency. Production of ethyl esters using ethyl chloroformate (ECF) was also investigated (section 2.6.4). Husek (1995) described a procedure in which ethyl esters could be produced instantaneously by reaction of acids with ethylchloroformate. It was not necessary to dry samples so loss of acids during drying were avoided and no long period of heating was required. Using this protocol however, produced a large number of side reactions and large amounts of un-reacted acids were also present. Ethyl lactate could not be produced using this method. The presence of a large number of peaks in addition to the ethyl esters made analysis very difficult.
Due to the problems associated with analysis of pyruvic acid by GC, an enzymatic approach to quantifying pyruvate using lactate dehydrogenase was investigated (section 2.6.5). This was found to be a much more sensitive method than GC and concentrations as low as 0.01 mM could be detected. Other acids were analysed by extraction into ether and analysis using both the FFAP capillary column and the BP21 megabore column. Extraction into ether was a faster method of analysis than derivatisation to methyl or ethyl esters as no heating period is required. The FFAP column was used to accurately quantify short chain acids. The minimum concentration of acetic acid detectable was 0.02 mM, that of propionic acid 0.005 mM, that of isobutyric and butyric acids 0.001 mM and that of iso-valeric acid 0.0005 mM. The BP21 column was used to detect any lactic acid in samples. The minimum concentration of lactic acid detectable using the BP21 megabore column was 0.1 mM.

The major acid produced by halobacteria during aerobic growth on glucose, glycerol and trehalose was acetic acid (see tables 3.2, 3.3, 3.4 & 3.5). The concentration of acetic acid was found to range from 0.3 mM to 12.7 mM. Strains 54R, Ha. marismortui, O14.1 and Br3 produced more acetic acid during growth on glycerol than on the other substrates tested. Hr. saccharovorum and Hc. morrhuae produced more acetic acid during growth of glucose than on the other substrates tested. BbpA1 produced the most acetic acid during growth on trehalose. E4 produced equal amounts of acetic acid during growth on glucose and glycerol and this was more than the amount produced during growth on trehalose. Only one strain produced acetic acid during growth on proline (54R), which produced 0.2 mM acetic acid. This is much lower than the amount produced on glycerol or glucose by 54R.

Small amounts of propionic acid (0.01 – 0.3 mM), iso-butyric acid (0.001 – 0.04 mM), butyric acid (0.002 – 0.1 mM), and iso-valeric (0.002 – 0.07 mM) acid were also produced during growth on glucose, glycerol and trehalose. 54R, Hr. saccharovorum, E4, Ha. marismortui, Hb. salinarum and BbpA1 were found to produce propionic, iso-butyric, butyric and iso-valeric acids in addition to acetic acid on either glucose, glycerol or trehalose. Hc. morrhuae produced propionic, iso-butyric and butyric acids in addition to acetic acid, but did not produce iso-valeric acid after growth on any of the substrates tested. O14.1 produced only acetic acid and butyric acid on the substrates tested. Several strains (Ha. marismortui, O14.1 and Hc. morrhuae) produced propionic and butyric acids in addition to acetic acid.
### Table 3.2: Production of acid during aerobic growth in RVM + glucose.

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD (660 nm) of cells</th>
<th>Mean concentration of acid(^a, b) (mM)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Std Dev shown in brackets</td>
<td>Acetic</td>
<td>Propionic</td>
<td>Iso-butyric</td>
<td>Butyric</td>
<td>Iso-valeric</td>
<td>Pyruvic</td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S4R</td>
<td>2.1</td>
<td></td>
<td>0.3 (0.2)</td>
<td>0.01 (0.004)</td>
<td>0.02 (0.09)</td>
<td>0</td>
<td>0.02 (0.004)</td>
<td>0</td>
<td>0.331</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hr. saccharovorans</em></td>
<td>1.8</td>
<td></td>
<td>8.8 (0.5)</td>
<td>0.07 (0.003)</td>
<td>0.03 (0.06)</td>
<td>0.1 (0)</td>
<td>0.01 (0)</td>
<td>0</td>
<td>9.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>2.0</td>
<td></td>
<td>1.5 (0.5)</td>
<td>0.07 (0.0006)</td>
<td>0.03 (0.008)</td>
<td>0.008 (0.005)</td>
<td>0.009 (0.007)</td>
<td>0</td>
<td>1.617</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hsa. marismortui</em></td>
<td>2.3</td>
<td></td>
<td>1.2 (0.03)</td>
<td>0.03 (0.002)</td>
<td>0.007 (0.001)</td>
<td>0.04 (0)</td>
<td>0.01 (0)</td>
<td>0</td>
<td>1.287</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BbpA1</td>
<td>1.1</td>
<td></td>
<td>4.5 (0.001)</td>
<td>0</td>
<td>0.02 (0.03)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.523</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br3</td>
<td>2.3</td>
<td></td>
<td>1.8 (0.5)</td>
<td>0.02 (0.02)</td>
<td>0.005 (0.002)</td>
<td>0.05 (0.003)</td>
<td>0.02 (0.002)</td>
<td>0</td>
<td>1.895</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hc. morrhuae</em></td>
<td>1.8</td>
<td></td>
<td>15.6 (0.2)</td>
<td>0.1 (0.01)</td>
<td>0.03 (0.003)</td>
<td>0.1 (0.005)</td>
<td>0</td>
<td>0</td>
<td>15.83</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>N/A</td>
<td></td>
<td>0.06 (0.08)</td>
<td>0</td>
<td>0.001 (0.001)</td>
<td>0.002 (0.002)</td>
<td>0.002 (0.002)</td>
<td>0</td>
<td>0.011</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\): Acetic, propionic, iso-butyric, butyric and iso-valeric acids were measured using GC (See Section 2.6). The minimum detectable concentration of acetic acid was 0.02 mM, that of propionic acid was 0.005 mM, that of iso-butyric and butyric acid 0.001 mM and that of iso-valeric was 0.0005 mM.

\(^b\): Pyruvic acid was measured using lactate dehydrogenase and measuring the change in A\(_{340}\) nm (see Section 2.6). The minimum concentration detected by this method was 0.01 mM.
### Results and Discussion: Growth of Halobacteria

#### Table 3.3: Production of acid during aerobic growth on RVM + glycerol

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD (660 nm) of cells</th>
<th>Mean concentration of acid(^{\pm}) (mM)</th>
<th>Std Dev shown in brackets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acetic</td>
<td>Propionic</td>
</tr>
<tr>
<td><strong>Strain OD</strong></td>
<td><strong>2.0</strong></td>
<td><strong>2.5</strong></td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td><em>Hr. saccharovorum</em></td>
<td><strong>2.2</strong></td>
<td><strong>1.4</strong></td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td><em>E4</em></td>
<td><strong>2.2</strong></td>
<td><strong>1.5</strong></td>
<td><strong>0.02</strong></td>
</tr>
<tr>
<td><em>Ha. marinocoral</em></td>
<td><strong>2.1</strong></td>
<td><strong>12.7</strong></td>
<td><strong>0.08</strong></td>
</tr>
<tr>
<td><em>O14.1</em></td>
<td><strong>2.2</strong></td>
<td><strong>2.5</strong></td>
<td><strong>0</strong></td>
</tr>
<tr>
<td><em>BbpA1</em></td>
<td><strong>1.1</strong></td>
<td><strong>0.3</strong></td>
<td><strong>0</strong></td>
</tr>
<tr>
<td><em>Hb. salinarum</em></td>
<td><strong>2.2</strong></td>
<td><strong>2.2</strong></td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td><em>Br3</em></td>
<td><strong>1.9</strong></td>
<td><strong>11.2</strong></td>
<td><strong>0.1</strong></td>
</tr>
<tr>
<td><em>Hc. morrhuae</em></td>
<td><strong>2.2</strong></td>
<td><strong>2.3</strong></td>
<td><strong>0</strong></td>
</tr>
<tr>
<td>Control</td>
<td>N/A</td>
<td><strong>0.4</strong></td>
<td><strong>0</strong></td>
</tr>
</tbody>
</table>

*a: Acetic, propionic, iso-butyric, butyric and iso-valeric acids were measured using GC (See Section 2.6). The minimum detectable concentration of acetic acid was 0.02 mM, that of propionic acid was 0.005 mM, that of iso-butyric and butyric acid 0.001 mM and that of iso-valeric was 0.0005 mM.*

*b: Pyruvic acid was measured using lactate dehydrogenase and measuring the change in A\(_{340}\) nm (See section 2.6). The minimum concentration detected by this method was 0.01 mM.*
**Results and Discussion: Growth of Halobacteria**

**Table 3.4: Production of acid during aerobic growth on RVM + trehalose**

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD (660 nm) of cells</th>
<th>Concentration of acid(^a,b) (mM)</th>
<th>Std Dev shown in brackets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acetic acid</td>
<td>Propionic</td>
</tr>
<tr>
<td>54R</td>
<td>1.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Hr. saccharovorum</em></td>
<td>2.3</td>
<td>1.6 ((0.04))</td>
<td>0</td>
</tr>
<tr>
<td>L4</td>
<td>2.3</td>
<td>0.8 ((0.04))</td>
<td>0.03 ((0.02))</td>
</tr>
<tr>
<td><em>Hs. marismortui</em></td>
<td>2.2</td>
<td>1.9 ((0.6))</td>
<td>0</td>
</tr>
<tr>
<td>O14.1</td>
<td>1.7</td>
<td>0.9 ((0.02))</td>
<td>0</td>
</tr>
<tr>
<td>BbpA1</td>
<td>1.987</td>
<td>7.8 ((0.2))</td>
<td>0.3 ((0.004))</td>
</tr>
<tr>
<td>Br3</td>
<td>2.2</td>
<td>1.4 ((0.04))</td>
<td>0</td>
</tr>
<tr>
<td><em>Hs. marismortui</em></td>
<td>2.3</td>
<td>1.3 ((0.2))</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>N/A</td>
<td>0.3 ((0.02))</td>
<td>0.01 ((0.02))</td>
</tr>
</tbody>
</table>

\(a\): Acetic, propionic, iso-butyric, butyric and iso-valeric acids were measured using GC (See Section 2.6). The minimum detectable concentration of acetic acid was 0.02 mM, that of propionic acid was 0.005 mM, that of iso-butyric and butyric acid 0.001 mM and that of iso-valeric was 0.0005 mM.

\(b\): Pyruvic acid was measured using lactate dehydrogenase and measuring the change in \(A_{340}\) nm (see section 2.6). The minimum concentration detected by this method was 0.01 mM.
### Table 3.5: Production of acid during aerobic growth on RVM + proline

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD (nm) of cells</th>
<th>Concentration of acid a,b (mM)</th>
<th>Std Dev shown in brackets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acetic acid</td>
<td>Propionic</td>
</tr>
<tr>
<td>S4R</td>
<td>1.9</td>
<td>0.2 (0.06)</td>
<td>0</td>
</tr>
<tr>
<td>H. saccharovorum</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E4</td>
<td>1.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H. marismortui</td>
<td>1.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O14.1</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BbpA1</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Br3</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hc. morrhuae</td>
<td>1.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>N/A</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a: Acetic, propionic, iso-butyric, butyric and iso-valeric acids were measured using GC (see section 2.6). The minimum detectable concentration of acetic acid was 0.02mM, that of propionic acid was 0.005 mM, that of iso-butyric and butyric acid 0.001 mM and that of iso-valeric was 0.0005 mM.

b: Pyruvic acid was measured using lactate dehydrogenase and measuring the change in A340 nm (see section 2.6). The minimum concentration detected by this method was 0.01 mM.
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*morrhuae*) produced only acetic acid and no other acids during growth on trehalose. Pyruvic acid was produced by strain BbpA1, during growth on glycerol and trehalose. The concentration of pyruvic acid measured during growth on both glycerol and glucose was 0.02 mM. No lactic acid was produced by any of the strains tested.

For all strains, except BbpA1, the total amount of acid produced during growth on trehalose was lower than on both glucose and glycerol. Interestingly 54R did not produce any acid during growth on trehalose. The extraction was repeated several times but no acid was detected. Only one strain, 54R, produced any acid during growth on proline.

It should be noted that small amounts of acids were measured in the un-inoculated controls (see tables 3.2, 3.3, 3.4, and 3.5). Acetic acid (up to 0.4 mM) was mainly found, but very small amounts of propionic, iso-butyric, butyric and iso-valeric acids were also found. In the majority of cases the total amount of acid found in the control was much less than that found in the samples. Powers *et al.* (1995) reported that acetate was ubiquitously found in distilled, de-ionised water, in many polar solvents (including ether), in reagents and in the environment.

Tomlinson & Hochstein (1972b) also found that acetate was the major acid produced during growth of halobacteria (strains M4, M6, M7, Gt-1 and U-18) on glucose. They found acetate was produced in smaller quantities during growth on sucrose, fructose, lactose and galactose than during growth on glucose. It was found that pyruvate was produced during growth on these substrates, but the amount of pyruvate produced was less than the amount of acetate produced. Oren & Gurevich (1994a) found that *Hr. saccharovorum, Ha. marismortui,* and *Ha. vallismortis* produced acetate and pyruvate during growth on glucose and glycerol. However, *Hb. salinarum* (*cutirubrum*) produced no acids during growth on glucose or glycerol. Additionally it was found that *Halofex* strains produced lactate and acetate during growth on glucose and glycerol. It should be noted however that both Tomlinson & Hochstein (1972b) and Oren & Gurevich (1994a) used growth media containing complex organic carbon
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sources (yeast extract or casamino acids), whereas in this study a completely defined medium has been used.

In this study only one strain, BbpA1, was found to produce pyruvate during growth on glycerol and trehalose. Pyruvate is not commonly produced as an end-product of fermentation by bacteria. Production of pyruvate has usually been explained by a limited capacity of the enzymatic systems of the metabolic pathways down stream from pyruvate (Le Bloas et al. 1993, Cocaign-Bousquet & Linley 1995). In this study (section 3.1) growth of strains 54R, E4, *Hr. saccharovorum* and *Ha. marismortui* in minimal media was shown to be strongly enhanced by the addition of pyruvate. In their study, Oren & Gurevich (1994a) found that after glycerol was depleted, the pyruvate, which had been excreted by cells, was then rapidly depleted. In this study pyruvate may also have been used up subsequently and so not detected during analysis. No strains were found to produce lactic acid. However no *Haloferax* strains were included in this study and the production of lactic acid may be limited to the genus *Haloferax*. Oren & Gurevich (1994a) found that only members of this genus produced lactic acid. The production of acids such as propionic, iso-butyric, butyric, and iso-valeric by halobacteria has not been reported before. Some species of hyperthermophilic Archaea have been shown to ferment sugars and peptides to acetate and other volatile fatty acids such as propionate, isobutyrate, and isovalerate. These include species in the orders *Thermococcales, Desulphurococcales, Pyrodictiales* and *Thermoproteales*.

Oren & Gurevich (1994a) explain the production of acid during growth as an overflow effect, resulting from repression and induction of certain enzymatic activities in the presence of excess sugar or glycerol. However, there is other evidence which suggests that glucose is being metabolised, at least partially by fermentation, producing acetate as the major fermentation product. Tomlinson & Hochstein (1972b) measured the oxygen uptake during growth of strain M6 (a *Hr. saccharovorum* strain) on glucose, and found that only 18% of the theoretical amount of oxygen required for the complete oxidation of glucose was consumed. The addition of 2, 4-dinitrophenol resulted in only a small increase in the amount of oxygen consumed. 2, 4-dinitrophenol is a specific uncoupler of oxidative phosphorylation. This suggests that oxidative assimilation of glucose is negligible and fermentation of glucose is
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occurring. The enzyme acetyl-CoA synthetase (ADP-forming) has been found in *Hr. saccharovorum* (Schafer et al. 1993). This enzyme is responsible for acetate formation during fermentation in *Pyrococcus furiosus*, *Pyroc. woesei*, *Thermococcus celer*, *Desulphurococcus amylyticus* and *Hyperthermus butylicus*. Acetyl-CoA synthase (ADP forming) couples acetate formation from acetyl-CoA with the phosphorylation of ADP via substrate level phosphorylation:

\[
\text{Acetyl-CoA + ADP + P} \rightarrow \text{acetate} + \text{ATP} + \text{CoA}
\]

Although acetate was the major acid produced by halobacteria the small amounts of propionic, iso-butyric, butyric and iso-valeric acids produced indicate that other fermentation pathways must also be operating in halobacteria.

The halobacteria do possess a full citric acid cycle (Aitken & Brown 1969, Danson 1988, Danson et al. 1985, Ghosh & Sonawat 1998), although Hochstein (1978) found that glucose represses the activity of citrate synthase and malate synthase. In contrast Bhaumik & Sonawat (1994) found that the presence of glucose enhanced the condensing activity of citrate synthase. Bhaumik & Sonawat found that although the citric acid cycle was operative as far as \(\alpha\)-ketoglutarate in the presence of glucose, it did not operate after this point, although the studies by Ghosh & Sonawat (1998) indicate that the TCA cycle is fully operative in *Hb. salinarum* during growth in complete media with glucose as an additional carbon source. If glucose does inhibit citrate synthase then this would explains why glucose is not respired oxidatively, instead acetyl-CoA is converted to acetate the main fermentation product observed in this study. Energetically fermentation is much less productive than the citric acid cycle and oxidative phosphorylation. The acetate synthetase reaction produces one ATP per acetyl-CoA, whereas if acetyl-CoA was to enter the citric acid cycle 10 ATP are produced per molecule.

Trehalose (\(\alpha\)-D-glucopyranosyl-\(\alpha\)-D-glucopyranoside) consists of two linked glucose molecules; therefore it may be surprising that the amounts of acids produced by growth on trehalose were consistently lower than those produced by growth on glucose, suggesting that oxidative phosphorylation was occurring to a greater extend
during growth on disaccharides than during growth on glucose. Tomlinson & Hochstein (1972b) suggested that perhaps disaccharides are oxidised prior to cleavage, thus explaining why a different pattern of metabolism is observed with disaccharides from that with glucose. It is more likely perhaps that an excess of glucose in the growth media inhibits citrate synthase, but the presence of trehalose does not, thus less acid is often produced during growth on disaccharides.

Significant amounts of acids were produced by growth on glycerol indicating that glycerol may also have an inhibitory effect on citrate synthase. Glycerol is converted by the enzymes glycerol kinase or glycerol dehydrogenase into dihydroxyacetone, whereby it enters glycolysis. Both these enzymes have been demonstrated in halobacteria (Rawal et al. 1988a, Oren & Gurevich 1994b). Glycerol dehydrogenase activity is limited to the genus Halobacterium, whereas glycerol kinase activity was found in all other members of halobacteria tested including those members of Halobacterium which contain glycerol dehydrogenase (Oren 1994). The addition of glycerol to samples of water from the Dead Sea has been shown to increase the activity of the respiratory electron chain by more than 2-fold as estimated by reduction of INT to INT-formazan (Oren 1995b). The addition of glycerol to samples from saltern crystalliser ponds also significantly increased the respiratory activity (Oren 1995b). Despite the evidence that glycerol is metabolised oxidatively significant amounts of acid were produced during growth on glycerol. This may be due to the limited enzyme capacity of enzymes such as citrate synthase, resulting in the production of acetate in addition to oxidative metabolism of glycerol.

Most strains of halobacteria did not produce acid during growth on proline. The pathways of proline metabolism in halobacteria are not known although it is likely to be converted by a series of reactions to α-ketoglutarate, via glutamate, whereby it enters the citric acid cycle. Halobacteria have been shown to grow by fermentation of amino acids. Arginine is fermented via citrulline to ornithine and carbamoylphosphate, which is subsequently degraded to carbon dioxide and ammonia (Hartman et al. 1980). Other members of the Archaea such as Thermococcales, Desulphurococcales, Thermogales, Pyrodictiales and Thermoproteales have been reported to ferment peptides to acetate and other products, however the metabolic pathways have not been established (Schönheit & Schäfer 1995).
Results and Discussion: Growth of Halobacteria

The stoichiometry of production of acids during growth by halobacteria is not clear since the amount of substrate utilised by halobacteria in these studies was not quantified. The concentration of glucose (for example) added to the media was approximately 50mM. \textit{Hc. morrhuae} (for example) produced 15.6 mM acetate. This indicates that only approximately a sixth of the glucose is converted to acid products. A proportion of the glucose must be incorporated into cell material and the rest of the glucose may be oxidised to CO\textsubscript{2}. In order to estimate the percentage of glucose used in fermentation, the amount of substrate utilised and the molar growth yield need to be measured, in addition the oxygen uptake and CO\textsubscript{2} production.

\textit{Ma et al.} (1997) have recently shown that pyruvate: ferredoxin oxidoreductase (POR) from the hyperthermophilic archaeon, \textit{Pyrococcus furiosus}, catalyses the formation of acetaldehyde from pyruvate, in addition to the formation of acetyl-CoA (Fig. 3.34). \textit{Ma et al.} (1997) suggest that this may be a general property of all 2-ketoacid oxidoreductases. Since halobacteria also contain POR it is interesting to speculate whether the POR of halobacteria also has this activity. \textit{P. furiosus} also contains two aldehyde-utilising enzymes- aldehyde Fd oxidoreductase (AOR) and NADP(H)-dependent alcohol dehydrogenase (ADH). AOR is proposed to be the primary enzyme responsible for oxidising the acetaldehyde forming acetate (Fig. 3.35). It would be interesting to look for the presence of AOR in halobacteria, since this represents a possible pathway of acetic acid formation. This may explain why acetate is produced from glycerol and glucose in addition to oxidative respiration of these substrates, even through full oxidation to CO\textsubscript{2} of substrates is energetically much more favourable.

The production of acetate by halobacteria has interesting implications for the microbial environment. Acetate is an important carbon source for methanogens such as \textit{Methanosarcina} and \textit{Methanotherrix}, and for sulphate reducers such as \textit{Desulphomaculum}, \textit{Desulphobacter}, \textit{Desulphonema} and others. Additionally, some strains of halobacteria can utilise acetate. In this study (section 3.1) it has been shown that strains E4 and \textit{Hr. saccharovorum} have the ability to utilise acetate. Javor (1984) showed that several strains of halobacteria have the ability to utilise acetate. In a community of several different species of halobacteria the production of acetate by one strain may provide another strain with a substrate on which it can grow.
Results and Discussion: Growth of Halobacteria

**Fig. 3.34: Reactions catalysed by POR in Pyrococcus furiosus (Ma et al. 1997).**

A: \[
\text{COO-} + \text{CoA} \xrightarrow{\text{oxidative decarboxylation}} \text{CoA} + \text{CO}_2
\]

B: \[
\text{COO-} \xrightarrow{\text{decarboxylation}} \text{H} + \text{CO}_2
\]

**Fig. 3.35: Proposed pathway for the metabolism of aldehydes produced during glucose and amino acid fermentation in heterotrophic hyperthermophilic archaea (Ma et al. 1997).**

Glucose Amino acids

\[
\begin{align*}
\text{R—CO—COOH} & \quad \text{POR} \\
& \quad \text{CoASH} \\
& \quad \text{Fd(ox) + CoASH} \\
& \quad \text{Fd (red)} \\
& \downarrow \\
\text{R—CO—SCoA + CO}_2 & \quad \text{Fd (red)}
\end{align*}
\]

\[
\begin{align*}
\text{R—CHO} & \quad \text{NADPH} \quad \text{NADP}^+ \\
& \quad \text{ADH} \\
& \quad \text{Fd(ox)} \\
& \downarrow \\
\text{R—CH}_2\text{OH} & \quad \text{AOR} \\
& \quad \text{Fd(ox)} \\
& \quad \text{Fd (red)}
\end{align*}
\]

\[
\begin{align*}
\text{R—COOH} & \quad \text{ACS} \\
& \quad \text{ADP + Pi} \\
& \quad \text{ATP} \\
& \downarrow \\
\text{R—COOH}
\end{align*}
\]

POR, AOR, IOR, VOR and KGOR represent oxidoreductases that use pyruvate, aldehydes, indolepyruvate, 2-keto isovalerate, and 2-ketoglutarate, respectively.

ACS: acetyl CoA synthetase

ADH: alcohol dehydrogenase
3.4: Anaerobic growth by halobacteria

The ability of strains of halobacteria to grow in CHM with the addition of the alternative electron acceptors DMSO, TMAO, NO₃⁻ and fumarate or with the addition of arginine is shown in Table 3.6. Most strains could grow by utilising TMAO and DMSO. The exceptions were BbpA1, which could not use TMAO and *Hc. morrhuae*, which could also use DMSO. Oren and Trüper (1990) showed that anaerobic growth of strains belonging to the genera *Halobacterium*, *Haloarcula* and *Halofex* was enhanced by the addition of TMAO or DMSO, whereas *Halorubrum sodomense* could not use DMSO or TMAO. In this study it has been shown that some members of the genus *Halorubrum-54R* and *Hr. saccharovorum* have the ability to use DMSO and TMAO for anaerobic growth. This study also showed that some members of the genus *Halococcus*—*Hc. morrhuae* and Br3 can use TMAO and DMSO, which had not previously been reported.

The following strains could grow anaerobically using NO₃⁻: E4, *Ha. marismortui*, O14.1 and *Hb. salinarum*. (Table 3.6). Mancinelli and Hochstein (1986) showed that *Ha. vallismortis*, *Ha. marismortui* and *Hf. mediterranei* could grow anaerobically using NO₃⁻ as the terminal electron acceptor. Oren and Truper (1990) showed that members of the genera *Halofex*, *Haloarcula* and *Halorubrum* could reduce NO₃⁻ to NO₂⁻, but did not investigate whether this reduction was accompanied by anaerobic growth. In this study there is no evidence for the anaerobic growth by reduction of NO₃⁻ by members of the Genus *Halorubrum*, although Oren and Truper (1990) showed that *Hr. sodomense* could reduce nitrate. In this study nitrate has also been shown to enhance the anaerobic growth of some but not all members of the genera *Halobacterium*, which has not previously been demonstrated. This study shows that members of the genus *Halococcus* can not grow by reduction of NO₃⁻, although *Hc. morrhuae* has been reported as having the ability to reduce nitrate to nitrite (Grant & Larsen 1989). It therefore seems that among the halobacteria the ability to reduce nitrate is not necessary accompanied by the ability to grow anaerobically.
Table 3.6: Growth of halobacteria under anaerobic conditions in CHM with the addition of DMSO, TMAO, NaNO3, fumarate, and arginine.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DMSO</th>
<th>TMAO</th>
<th>NaNO3</th>
<th>Fumarate</th>
<th>Arginine</th>
<th>CHM only</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4</td>
<td>++</td>
<td>++</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>54R</td>
<td>++</td>
<td>+</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BbpA1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Br3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O14.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Hr. saccharovorum</em> NCM B664</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Ha. marismortui</em> ATCC 43047</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Hb. salinarium</em> NCMB 764</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Hb. salinarium</em> (Hb. halobium) NCMB 2090</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Hc. morrhuae</em> NCMB 787</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

++: Positive growth occurred within 7-14 days.
+: Positive growth occurred after 30-49 days.
-: no growth occurred or growth occurred after the same number of days as in un-supplemented CHM.

O14.1, BbpA1 and *Hb. salinarum* (halobium) were the only strains found to be capable of using fumarate for anaerobic growth (Table 3.6). Oren (1991) found that strains from the genera *Halobacterium* and *Haloferax* could grow anaerobically using fumarate, but members the genus *Haloarcula* could not. The ability to use fumarate seems to be limited to members of *Halobacterium* and *Haloferax*, indeed in this study only *Hb. salinarum* (Hb. halobium) and O14.1 and BbpA1 (two strains which are related to *Hb. salinarum*) were shown to use fumarate for anaerobic growth.

The ability to grow anaerobically by fermentation of arginine was first demonstrated in *Halobacterium halobium* R1M1 (Hartmann et al. 1980). Members of the genera *Halobacterium* and *Natronobacterium* have subsequently been shown to have this ability, but members of *Halorubrum, Haloferax* and *Haloarcula* could not grow by fermentation of arginine (Oren 1994a). In this study no strains were shown to grow by
Results and Discussion: Growth of Halobacteria

fermentation of arginine, despite testing members of the genus *Halobacterium* and related strains such as BbpA1 and O14.1 (Table 3.6).

It should be noted that in these studies many strains grew anaerobically in un-supplemented CHM, indicating that they must be growing by some kind of fermentation (Table 3.6). The growth in un-supplemented CHM was slower than with the addition of an electron acceptor, with growth not visible by eye until after 30 days incubation. Strains, which could not grow in un-supplemented CHM, were members of the genus *Halococcus* (Br3 and *Hc. marrhuae*) and *Hb. salinarum*. Javor (1984) has reported that many strains of halobacteria could grow anaerobically in complex medium.

The ability of several strains of halobacteria to grow anaerobically in MM with glucose, glycerol, trehalose or glycine betaine as the main carbon source and NO\(_3^-\), TMAO or no electron acceptor was investigated. The results are shown in Tables 3.7 and 3.8. Many strains grew well anaerobically in minimal media with glucose, glycerol, trehalose or glycine betaine as the carbon source and NO\(_3^-\) or TMAO as the electron acceptor. Many strains could also grow anaerobically in MM with NO\(_3^-\) or TMAO, but no additional carbon source indicating they were utilising carbon sources provided in the yeast extract. E4, O14.1 and *Ha. marismortui* grew well in minimal media + glycine betaine with either NO\(_3^-\) or TMAO, however these strains also grew well in MM without supplementation and growth was no better with the addition of betaine than in un-supplemented MM indicating that these strains can not utilise glycine betaine as a carbon source.
### Results and Discussion: Growth of Halobacteria

Table 3.7: Anaerobic Growth in Minimal Media with the addition of glucose and trehalose as carbon sources and NaNO₃ and TMAO as electron acceptors.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MM only</th>
<th>MM + glucose</th>
<th>MM + trehalose</th>
</tr>
</thead>
<tbody>
<tr>
<td>e⁺ acceptor</td>
<td>None</td>
<td>NaNO₃</td>
<td>TMAO</td>
</tr>
<tr>
<td>54R</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>E4</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>BbpA1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O14.1</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Br3</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>*H. saccharovorum</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NCMB 2081</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>*H. marismortui</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ATCC 43047</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>*Hb. salinarium</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NCMB 764</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>*Hc. morrhuae</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NCMB 787</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

++: good growth: OD (660 nm) reached at least 0.1 after 21 days incubation.
+: some growth: OD (660 nm) reached between 0.05 - 0.1 after 21 days incubation
-: no growth.
*: growth occurred after three transfers.

Table 3.8: Anaerobic Growth in Minimal Media with the addition of glycerol and glycine betaine as carbon sources and NaNO₃ and TMAO as electron acceptors.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MM + glycerol</th>
<th>MM + glycine betaine</th>
</tr>
</thead>
<tbody>
<tr>
<td>e⁺ acceptor</td>
<td>None</td>
<td>NaNO₃</td>
</tr>
<tr>
<td>54R</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E4</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>BbpA1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O14.1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Br3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>*H. saccharovorum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NCMB 2081</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>*H. marismortui</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>ATCC 43047</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>*Hb. salinarium</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NCMB 764</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>*Hc. morrhuae</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

++: good growth: OD (660 nm) reached at least 0.1 after 21 days incubation.
+: some growth: OD (660 nm) reached between 0.05 - 0.1 after 21 days incubation
-: no growth.
*: growth occurred after three transfers.
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Some strains could grow in MM only or MM + glucose, trehalose or glycine betaine without the addition of NO$_3^-$ or TMAO (Table 3.7 & 3.8). These strains must be growing by fermentation. O14.1 could use glucose or trehalose as a substrate for fermentation. This strain could also grow in MM or CHM anaerobically. *Ha. marismortui* could also use glucose and trehalose for fermentative anaerobic growth. E4 and Br3 could only use trehalose for fermentative anaerobic growth. No strains could grow anaerobically by fermentation with glycerol as the carbon source. Javor (1984) found that many strains of halobacteria could grow by fermentation of carbohydrates under anaerobic conditions and a few could grow by fermentation of glycerol.

In section 3.3 the production of acid during aerobic growth with glucose, glycerol and trehalose as carbon sources was studied. Acid was produced after growth on glucose, glycerol and trehalose. Growth on glycerol often produced substantial amounts of acid, this indicated energy production via substrate level phosphorylation was occurring, yet it has been shown in this section that glycerol can not support anaerobic growth by fermentation. Thus there does not however seem to be a correlation between the production of acid during aerobic growth and the ability to grow anaerobically by fermentation. In the study by Javor (1984) it was noted that many strains which had somewhat better growth on glycerol than glucose aerobically grew better on glucose than glycerol anaerobically.

Production of acid during anaerobic growth on selected growth media by *Ha. marismortui*, E4, and O14.1 was measured (Table 3.9). These growth media were chosen as they supported the highest levels of anaerobic growth by halobacteria. Acetic acid was found to be the major acid produced during anaerobic growth at concentrations ranging from 0.5 mM to 13.2 mM. Propionic acid, iso-butyric acid, butyric acid and iso-valeric acid were also detected but at much lower levels than acetic acid. The production of acid indicates energy is produced by substrate level phosphorylation (fermentation) in addition to or as an alternative to the use of NO$_3^-$ or TMAO as an electron acceptor. It may be the case that fermentation occurs after the depletion of NO$_3^-$ or TMAO. It would be informative to measure the depletion of the carbon source and the electron acceptor, the production of acid and the reduced
### Table 3.9: Production of acid during anaerobic growth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth Media</th>
<th>OD (660 nm) of cells</th>
<th>Mean Concentration of acids a,b (mM)</th>
<th>Std Dev shown in brackets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acetic</td>
<td>Propionic</td>
</tr>
<tr>
<td><em>Hal. marismortui</em></td>
<td>MM + NaN03</td>
<td>0.170</td>
<td>0.5 (0.03)</td>
<td>0.3 (0.003)</td>
</tr>
<tr>
<td><em>Hal. marismortui</em></td>
<td>MM + TMAO</td>
<td>0.123</td>
<td>2.6 (0.3)</td>
<td>0.4 (0.03)</td>
</tr>
<tr>
<td><em>Hal. marismortui</em></td>
<td>MM + glycine betaine + NaN03</td>
<td>0.116</td>
<td>0.5 (0.03)</td>
<td>0.7 (0.03)</td>
</tr>
<tr>
<td><em>Hal. marismortui</em></td>
<td>MM + trehalose + NaN03</td>
<td>0.281</td>
<td>10.8 (0.06)</td>
<td>0.4 (0.001)</td>
</tr>
<tr>
<td><em>Hal. marismortui</em></td>
<td>MM + glycerol + NaN03</td>
<td>0.468</td>
<td>8.2 (0.7)</td>
<td>0.1 (0.008)</td>
</tr>
<tr>
<td>E4</td>
<td>MM + glycine betaine + TMAO</td>
<td>0.128</td>
<td>2.5 (0.4)</td>
<td>0.4 (0.02)</td>
</tr>
<tr>
<td>E4</td>
<td>MM + trehalose + NaN03</td>
<td>0.375</td>
<td>5.3 (0.1)</td>
<td>0.1 (0.004)</td>
</tr>
<tr>
<td>E4</td>
<td>MM + glycerol + TMAO</td>
<td>0.328</td>
<td>13.2 (0.9)</td>
<td>0.7 (0.05)</td>
</tr>
<tr>
<td>O14.1</td>
<td>MM + NaN03</td>
<td>0.157</td>
<td>1.7 (0.01)</td>
<td>0.3 (0.002)</td>
</tr>
<tr>
<td>Control</td>
<td>MM + glycerol + TMAO</td>
<td>N/A</td>
<td>0.2 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>MM + glycine betaine + TMAO</td>
<td>N/A</td>
<td>0.4 (0.2)</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>MM + glycerol + NaN03</td>
<td>N/A</td>
<td>0.3 (0.6)</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>MM + trehalose + NaN03</td>
<td>N/A</td>
<td>0.3 (0)</td>
<td>0</td>
</tr>
</tbody>
</table>

a: Acetic, propionic, iso-butyric, butyric and iso-valeric acids were measured using GC (See Section 2.6). The minimum detectable concentration of acetic acid was 0.02mM, that of propionic acid was 0.005 mM, that of iso-butyric and butyric acid 0.001 mM and that of iso-valeric was 0.0005 mM.

b: Pyruvic acid was measured using lactate dehydrogenase and measuring the change in A₃₄₀ nm (see section 2.6). The minimum concentration detected by this method was 0.01 mM.
electron acceptor, and the molar growth yield to produce balanced equations for the anaerobic growth with different substrates and electron acceptors. It is significant that, although the amount of growth (as indicated by the OD) was much less under anaerobic conditions than under aerobic conditions, the amount of acid produced is similar. Thus a larger proportion of the carbon source is being converted to acids under anaerobic conditions than under aerobic conditions.

The availability of molecular oxygen is greatly reduced at high salt concentrations. Additionally, the high microbial densities often observed in surface hypersaline environments might further deplete the oxygen levels (Oren & Trüper 1990), although photosynthesis by *Dunaliella*, for example, will produce oxygen. The ability of halobacteria to grow anaerobically is thus not surprising, however the availability of electron acceptors such as NO$_3^-$, TMAO and DMSO in the natural environment could be limited. Nitrate concentrations in hypersaline environments have generally been found to be low, with ammonia being the most predominant inorganic nitrogen compound. Formation of nitrate via nitrite from ammonia (nitrification) has not been demonstrated in halophilic environments and no halophilic nitrifying bacteria are known (Oren 1994b). Concentrations of DMSO, TMAO or fumarate in hypersaline environments do not appear to have been studied.

DMSO has been shown to be present in surface sea water, where it is probably generated from the oxidation of dimethyl sulphide (DMS), which is formed by the enzymatic cleavage of dimethylsulphoniopropionate (DMSP) (Oren & Trüper 1990). DMSP is used as an osmolyte and synthesised by macroalgae, phytoplankton and higher plants (Diaz & Taylor 1996). Bacteria have, however, been isolated from the moderately hypersaline, alkaliphilic Mono Lake, which can grow on DMSP yielding DMS (Diaz & Taylor 1996) and therefore, DMSO may be formed in hypersaline environments.

TMAO is often present in high concentrations in marine animals such as teleost and elasmobranch fish. Halobacteria have often been found on salted or dried fish. Another possible source of TMAO is by oxidation of trimethylamine (TMA). TMA can be oxidised to TMAO by methylotrophic bacteria, but to my knowledge this process has not been demonstrated in the hypersaline environment. Two strains of
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anaerobic halophilic bacteria *Acetohalobium arabaticum* (Zhilina & Zavarzin 1990a, Zhilina & Zavarzin 1990b) and *Sporohalobacter lortetii* (Oren 1990) can degrade glycine betaine to TMA. In Mono Lake the estimated TMA pool in the upper sediments was 1μM and in Westend pond, Virgin Islands (9 - 13% salt) the TMA concentration at the top of the sediment was 6μM (Oren 1990).

It has been reported that thiosulphate enhances the aerobic growth of halobacteria (Newton & Javor 1985) and also that elemental sulphur can be reduced by halobacteria under aerobic or microaerophilic conditions (Tindall & Truper 1986). It has not been investigated whether halobacteria can utilise thiosulphate or elemental sulphur for anaerobic growth, however many members of the Archaea utilise sulphur compounds as terminal electron acceptors during growth (Schonheit & Schafer 1995, Segerer *et al.* 1985, Stetter & Caag 1983, Zillig *et al.* 1985). Elemental sulphur is produced extracellularly by *Ectothiorhodospira spp.* and therefore theoretically might be available for use by halobacteria in the surface hypersaline environment.

Since there is no photosynthesis within the salt mines oxygen is likely to be even more limited than in surface hypersaline environments. Halobacteria have been shown to survive within fluid inclusions, but any oxygen present in fluid inclusions would be quickly depleted. The ability of salt-mine strains to grow anaerobically is probably an important property. There is no information about the availability of NO₃⁻, TMAO, DMSO or fumarate within the salt deposits. However since the main origin of DMSO is likely to be DMSP, which is accumulated as an osmoprotectant by macroalgae and higher plants, then DMSO is unlikely to be present within the salt-mines. The degradation of glycine betaine produces TMA, which can be oxidised to TMAO. Glycine betaine is used as a compatible solute by phototrophic bacteria but these are not present in the salt deposits. If high concentrations of phototrophs were present in the hypersaline environment, prior to deposition, some glycine betaine may have been incorporated into the deposit, but it is also unlikely that TMAO would be present in high concentrations in the salt mines.

This study has shown that both salt-mine and surface strains of halobacteria have the ability to grow anaerobically. The ability to grow using DMSO or TMAO was widespread among members of halobacteria, including strains both
from the salt-mines and surface environments. The ability to grow using NO$_3^-$ was less widespread and observed in both salt-mine and surface strains of *Haloarcula* and *Halobacterium* and related strains. The ability to grow anaerobically using fumarate was found to be limited to members of *Halobacterium* and related strains, including strains from both surface and salt-mine environments. Most strains, except members of the genus *Halococcus*, could grow by fermentation in CHM and some strains could also grow fermentation of glucose and/ or trehalose.
Results and Discussion: Degradation of Crude Oil By Halobacteria

4

RESULTS AND DISCUSSION:
DEGRADATION OF CRUDE OIL BY HALOBACTERIA

The initial studies of biodegradation of crude oil by halobacteria showed that they had a significant effect on the structure of the oil (Table 4.1). The oil in the un-inoculated controls remained as a thin layer on the surface of the growth medium. When shaken up the un-inoculated oil formed small, rounded particles. Some strains of halobacteria caused the oil to stick together in large globules e.g. Br5 (Fig. 4.1) and Br7. Several strains emulsified the oil- oil droplets were very small and dispersed through out the growth medium e.g. Br3, E4, O14.1 (Fig. 4.2) In some samples there was a mixture of larger droplets and very small droplets e.g. 54R, 54P (Fig. 4.3). In several samples, the shape of the droplets of oil changed from even-shaped round droplets to uneven fragments, which often had tails or a bubble-like appearance. The change in the shape of the oil particles was often accompanied in a change in the colour from a dark brown to a much lighter brown e.g. Br3, E4. Emulsification or a change in the structure of the oil does not necessarily indicate that the oil is being degraded. The ether-linked glycerol membrane lipid of halobacteria has been shown to have surfactant properties and an ability to emulsify oil (Post & Collins 1982, Post & Al-Harjan 1988). The formation of emulsions is an important process in the uptake of hydrocarbons by bacteria, and bacteria that effectively degrade crude oil also exhibit strong emulsifying activity (Leahy & Colwell 1990). It was clear in some samples that the volume of the oil had decreased compared to the control e.g. O14.1 and Br7. It is also important to note that that the surface strains of halobacteria tested showed much poorer emulsification of crude oil than the salt mine strains.
Results and Discussion: Degradation of Crude Oil by Halobacteria

Fig. 4.1: Appearance of MM + crude oil after inoculation with Br5 and appearance of control after two weeks incubation.

Fig. 4.2: Appearance of MM + crude oil inoculated with O14.1 after two weeks incubation.
Fig. 4.3: Appearance of MM + crude oil inoculated with 54R and 54P after 2 weeks incubation.
### Table 4.1: Table showing the change in the appearance of crude oil after incubation with halobacteria.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Appearance of Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 weeks</td>
</tr>
<tr>
<td>Control</td>
<td>Layer across the surface, small round droplets when shaken</td>
</tr>
<tr>
<td>Br1</td>
<td>large droplets</td>
</tr>
<tr>
<td>Br2</td>
<td>small &amp; large droplets</td>
</tr>
<tr>
<td>Br3</td>
<td>As control</td>
</tr>
<tr>
<td>Br4</td>
<td>As control</td>
</tr>
<tr>
<td>Br5</td>
<td>very large clumps</td>
</tr>
<tr>
<td>Br6</td>
<td>As control</td>
</tr>
<tr>
<td>Br7</td>
<td>Large clumps</td>
</tr>
<tr>
<td>Br8</td>
<td>As control</td>
</tr>
<tr>
<td>54P</td>
<td>Large droplets and very small particles</td>
</tr>
<tr>
<td>54R</td>
<td>Large lumps, very tiny droplets dispersed through medium</td>
</tr>
<tr>
<td>E4</td>
<td>As control</td>
</tr>
<tr>
<td>O14.1</td>
<td>Some large particles and some smaller ones.</td>
</tr>
<tr>
<td>O01.3</td>
<td>As control</td>
</tr>
</tbody>
</table>
Results and Discussion: Degradation of Crude Oil By Halobacteria

Table 4.1 (Cont.): Appearance of oil after incubation with strains of halobacteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>Appearance of Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 weeks</td>
</tr>
<tr>
<td>(Hb.) salinarium</td>
<td>Large droplets</td>
</tr>
<tr>
<td>(Hr.) saccharovorum</td>
<td>Large droplets</td>
</tr>
<tr>
<td>(Hc.) morrhuae</td>
<td>As control</td>
</tr>
<tr>
<td>(Ha.) vallismortis</td>
<td>As control</td>
</tr>
</tbody>
</table>

Strains Br3, Br5, Br7, 54R, 54P, E4 and O14.1 were chosen to investigate further. These strains exhibited the greatest degree of emulsification or loss of oil in the initial experiment. Gas chromatography and gas chromatography-mass spectrometry were used to analyse the alkanes present in the crude oil and look for loss of alkanes compared to the control. It was important that losses of hydrocarbons in the samples inoculated with halobacteria were compared to losses from un-inoculated controls containing oil. This is because abiotic losses of the most volatile hydrocarbons occur due to evaporation. It is important to ascertain that losses from inoculated samples are due to biological activity (Sepic et al. 1995). Fig. 4.4 shows a gas-chromatogram of the un-inoculated oil after 8 weeks incubation. Peaks 4, 6, 8, 9, 11, 13, 15, 16, 18, 20, 23, and 24 were identified as C24, C22 and C20 –C11 straight chain alkanes on the basis of their co-elution with authentic alkanes. Peak 12 was identified as pristane (C19 isoprenoid alkane) on the basis of its co-elution with authentic pristane. The straight chain alkanes C21, C23, C25 and C26 and isoprenoid alkanes C13, C14, C15, C16, C18 and C20 were identified by comparison of their retention times with retention times on similar GC columns in the literature (Jones et al.1986, Jones personal communication 1997). In addition, mass spectra were obtained for the all the peaks observed. The mass spectra of straight chain alkanes have a characteristic appearance. Groups of peaks spaced 14 units apart (corresponding to CH₂) and each having the \(C_nH_{2n+1}\) as the most abundant unit occurs with increasing abundance (Budzikiewicz, Djerassi and Williams 1967). It is therefore easy to identify straight chain alkanes. The mass spectra produced for peaks 2, 3, 5 and 7 fitted with their identification as C26, C25, C23 and C21 straight chain alkanes. The mass spectrum obtained for peak 7 (C21 alkane) is shown in Fig.4.5
Fig. 4.4: Gas-Chromatogram of Crude Oil (control) after 8 weeks incubation

Results and Discussion: Degradation of Crude Oil By Halobacteria
Fig. 4.5: Mass Spectrum of peak 7- (Identified as C21 n-alkane).
The mass spectra obtained for peaks 10, 14, 17, 19 and 21 appeared very similar to those obtained for the straight chain hydrocarbons, indicating that they are also aliphatic hydrocarbons. Ions with relatively large m/z 113 and 183 ions are characteristic of isoprenoid alkanes (Dr. Martin Jones personal communication). The mass spectra produced for peaks 10, 14, 17, 19 and 21 were consistent with their identification as C20, C18, C16, C15, C14 and C13 isoprenoid alkanes. The mass spectrum of peak 14 (identified as C18 isoprenoid) is shown in fig. 4.6 and shows the characteristic m/z 113 and 183 ions.

Fig. 4.7 shows the gas chromatogram of oil incubated with Br3 for 8 weeks, this illustrates a typical chromatogram after degradation of oil by halobacteria. If this chromatogram is compared to that for the control oil in Fig. 4.4 it is clear that the profile of the oil has changed and degradation of some components of the oil has occurred. The extent of the biodegradation of oil after 8 weeks incubation for each strain tested are shown in Fig. 4.8 - 4.24. Strain E4 did not degrade any of the alkanes present in the crude oil (Fig. 4.22 & 4.23). The other strains all carried out some degradation. O14.1 degraded only about 40% of C11 and C12 alkanes and no significant degradation of branched alkanes (Fig. 4.20 & 4.21). S4P degraded C11 - C14 alkanes together with C13 - C16 isoprenoid alkanes (Fig. 4.8 & 4.9). The ability of S4R to degrade oil added to both MM and RVM was examined. When inoculated into MM + crude oil S4R degraded C11 and C12 alkanes and C13 and C14 alkanes (Fig. 4.10 & 4.11), but when S4R was inoculated into RVM more extensive degradation was observed with degradation of C11 - C14 alkanes and C13 - C16 isoprenoid alkanes (Fig. 4.12 & 4.13). Br7 degraded C11 - C15 alkanes and C13 - C16 isoprenoid alkanes (Fig. 4.16 & 4.17). The most significant biodegradation was carried out by strains Br3 and Br5. Br5 degraded significant amounts of C11 - C20 straight chain alkanes and C13 - C20 isoprenoid alkanes (Fig. 4.14 and Fig. 4.15). Br3 degraded significant amounts of C11 to C14 straight chain alkanes and C13 - C16 isoprenoid alkanes (Fig. 4.18 and Fig. 4.19). These experiments indicated that some strains of halobacteria can degrade both odd and even numbered straight chain alkanes and branched hydrocarbons, which are present in crude oil.
Results and Discussion: Degradation of Crude Oil By Halobacteria

Fig. 4.6: Mass Spectrum of peak 14 (identified as C18 isoprenoid alkane)
Results and Discussion: Degradation of Crude Oil by Halobacteria

Fig. 4.7: Gas-Chromatogram of crude oil biodegraded by Br3 after 8 weeks incubation.
Results and Discussion: Degradation of Crude Oil By Halobacteria

**Fig. 4.8** Biodegradation of straight chain alkanes in crude oil by 54P after 8 weeks.

Error bars indicate +/- one std. dev.

**Fig. 4.9** Biodegradation of branched chain alkanes in crude oil by 54P after 8 weeks.

Error bars indicate +/- one std. dev.
Results and Discussion: Degradation of Crude Oil By Halobacteria

**Fig. 4.10:** Biodegradation of straight chain alkanes in crude oil by 54R (MM) after 8 weeks.

Error bars indicate +/- one std. dev.

**Fig. 4.11:** Biodegradation of branched chain alkanes in crude oil by 54R (MM) after 8 weeks.

Error bars indicate +/- one std. dev.
Results and Discussion: Degradation of Crude Oil By Halobacteria

**Fig. 4.12:** Biodegradation of straight chain alkanes in crude oil by 54R (RVM) after 8 weeks.

![Bar graph showing biodegradation of straight chain alkanes in crude oil by 54R (RVM) after 8 weeks.](image)

Error bars indicate +/- one std. dev.

**Fig. 4.13:** Biodegradation of branched chain alkanes in crude oil by 54R (RVM) after 8 weeks.

![Bar graph showing biodegradation of branched chain alkanes in crude oil by 54R (RVM) after 8 weeks.](image)

Error bars indicate +/- one std. dev.
Results and Discussion: Degradation of Crude Oil by Halobacteria

**Fig. 4.14:** Biodegradation of straight chain alkanes in crude oil by Br5 after 8 weeks.

Error bars indicate +/- one std. dev.

**Fig. 4.15:** Biodegradation of branched chain alkanes in crude oil by Br5 after 8 weeks.

Error bars indicate +/- one std. dev.
Results and Discussion: Degradation of Crude Oil by Halobacteria

**Fig. 4.16:** Biodegradation of straight chain alkanes in crude oil by Br7 after 8 weeks.

[Graph showing biodegradation of straight chain alkanes]

Error bars indicate +/- one std. dev.

**Fig. 4.17:** Biodegradation of branched chain alkanes in crude oil by Br7 after 8 weeks

[Graph showing biodegradation of branched chain alkanes]

Error bars indicate +/- one std. dev.
**Fig. 4.18: Biodegradation of straight chain alkanes in crude oil by Br3 after 8 weeks**

Error bars indicate +/- one std. dev.

**Fig. 4.19: Biodegradation of branched chain alkanes in crude oil by Br3 after 8 weeks**

Error bars indicate +/- one std. dev.
Results and Discussion: Degradation of Crude Oil By Halobacteria

**Fig. 4.20: Biodegradation of straight chain alkanes in crude oil by O14.1 after 8 weeks**

Error bars indicate +/- one std. dev.

**Fig. 4.21: Biodegradation of branched chain alkanes in crude oil by O14.1 after 8 weeks**

Error bars indicate +/- one std. dev.
Results and Discussion: Degradation of Crude Oil by Halobacteria

**Fig. 4.22: Biodegradation of straight chain alkanes in crude oil by E4 after 8 weeks**

Error bars indicate +/- one std. dev.

**Fig. 4.23: Biodegradation of branched chain alkanes in crude oil by E4 after 8 weeks**

Error bars indicate +/- one std. dev.
Experiments were also carried out to investigate the ability of halobacteria to grow with hydrocarbons as the main source of carbon. The ability of strains to grow in MM with the addition of undecane (C11 alkane), dodecane (C12 alkane), eicosane (C20 alkane), pristane (C19 isoprenoid alkane), phenanthracene and anthracene (polyaromatic hydrocarbons), benzoate, cyclohexane and hexadecene (Table 4.2). Growth of Br3 was shown to be enhanced by eicosane, pristane, benzoate, cyclohexane, and hexadecene, but not by undecane or dodecane. In many cases the addition of the alkane to the media enhanced growth, as determined by measuring the OD (660 nm), only by about 15%, for example addition of undecane, dodecane and eicosane on the growth of 54R and 54P. Growth of Br5 was enhanced slightly by eicosane, but not by any of the other hydrocarbons tested. This was surprising as Br5 had shown good biodegradation of crude oil. Benzoate enhanced the growth of 54R, 54P, Br3 and Br5 indicating the halobacteria have the ability to grow using aromatic compounds as a carbon source. Emerson et al. (1994) reported that Haloferax strain D1227 could use benzoate, cinnamate and phenylpropanoate as the sole carbon and energy source. This study indicates that members of the genera Haloarcula (Br5), Halorubrum (54R, 54P) and Halococcus (Br3) can also utilise aromatic compounds. It is interesting that the strains which can utilise benzoate all originate from either rock salt or solution mining brine.

It was surprising that the addition of undecane and dodecane to MM did not cause more enhancement of growth in the strains tested, as these were commonly completely degraded in crude oil. The concentration added to the MM was 50 mM, this is much higher than the concentration added as part of the crude oil. Therefore, further experiments were carried out adding 5 mM only of each alkane but this did increase enhancement of growth of any strains. Experiments were carried out where 50mM undecane, dodecane, pristane and eicosane were added to 200 ml of media in a 500 ml conical flask, inoculated with 54R and incubated shaken. Cells should have been better aerated than in the previous experiments, which were carried out in shaken universal tubes. The initial steps in the catabolism of aliphatic hydrocarbons involve oxidation of the substrates by oxygenases, for which molecular oxygen is needed. Fig. 4.24 shows that some enhancement of growth was observed with dodecane, eicosane and pristane, but not undecane (which was degraded by 54R in crude oil).
Table 4.2: Growth of halobacteria in MM with the addition of 50mM hydrocarbon after 14 days incubation.

<table>
<thead>
<tr>
<th></th>
<th>54R</th>
<th>54P</th>
<th>Br7</th>
<th>Br3</th>
<th>BbpA1</th>
<th>O14.1</th>
<th>Br5</th>
<th>E4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undecane</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dodecane</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>Eicosane</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Pristane</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anthracene</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Benzoate</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hexadecene</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

+: indicates enhancement of growth by at least 100%  
+-: indicates enhancement of growth by at least 15%  
-: indicates there was no enhancement of growth compared to MM only.

Fig. 4.24: Growth of 54R in MM + 50 mM undecane, dodecane, eicosane and pristane.
Results and Discussion: Degradation of Crude Oil By Halobacteria

High concentrations of hydrocarbons may cause inhibition of biodegradation either by oxygen limitation or by toxic affects to the cells. It is possible that at 5 or 50mM these alkanes are toxic to the cells. The physical structure of the hydrocarbon is a very important factor in influencing the degradation of hydrocarbons and it is possible that the physical structure of the pure alkanes made them difficult to be degraded by halobacteria, whereas when they are present as a mixture in crude oil they are more physically accessible to the halobacteria. Undecane, dodecane and pristane are liquid at room temperature, whereas eicosane is a waxy solid at room temperature. The pure hydrocarbons tended to form a layer over the surface of the growth media, whereas the crude oil was broken up into particles. It was certainly true that emulsification of crude oil occurred to a much greater extent than emulsification of pure alkanes.

Bertrand et al. (1990) isolated several strains of halobacteria from a salt marsh, which were able to degrade eicosane (C20 alkane). One of these strains- EM4, was able to degrade C14, C16, C20 and C21 alkanes, pristane, and aromatic hydrocarbons such as acenaphthene, phenanthrene, anthracene and 9-methyl anthracene. C20 and C21 alkanes and pristane were present in the water of the salt marsh. Kulichevskaya et al. (1991) isolated a strain of halobacteria-H-352 from the stratum fluid of an oil deposit which was able to degrade almost completely the C10- C30 alkanes in crude oil, but had a more limited ability to degrade isoprenoid alkanes. This strain was identified as Halobacterium distributum. They found that many strains of halobacteria could decompose petroleum and a few could decompose hexadecane, at least as determined by visual and microscopic determinations. Zvyagintseva et al. (1995) isolated two strains of halobacteria from enrichments in media containing crude oil which had been inoculated with brine from the Kalamkass oil field. Both strains belonged to the genus Haloferax. Both n-alkanes and isoprenoid alkanes were degraded in the oil added to the enrichment, however Zvyagintseva et al. (1995) do not state whether these two strains in pure culture could also degrade alkanes and isoprenoid alkanes.

The strains, which were shown to have the ability to degrade alkanes present in crude oil, fall into four taxonomic groups- Halorubrum (54R, 54P), Haloarcula (Br5, Br7), Halococcus (Br3) and Halobacterium (O14.1). E4 is Haloarcula sp. and this strain was not shown to degrade the alkanes present in crude oil. It is very interesting that
those strains which showed the most significant degradation of oil: 54R, 54P, Br5, Br7 and Br3 are all either isolated from rock salt or solution mining brine. 54R, 54P, Br5 and Br3 could also utilise benzoate as a source of carbon and energy. For these strains there is strong circumstantial evidence that they are autochthonous residents of the salt deposit. In previous studies of the ability to degrade hydrocarbons by halobacteria, strains of halobacteria with this ability have always been isolated from environments in which hydrocarbons are present. Several studies have shown that the hydrocarbon-oxidising ability of a community is greater if it has previously been exposed to hydrocarbons (Leahy & Colwell 1990). Perhaps these salt-mine strains have a greater ability to degrade crude oil because they are exposed to hydrocarbons in the salt deposit. Inclusions and veinlets of oil occur frequently in the evaporite deposits of the Northern Caspian region (Sonnenfeld 1984).

To summarise, several strains of halobacteria were shown to have a strong emulsifying effect on crude oil. Gas-chromatography has shown that there is significant biodegradation of alkanes in crude oil by 54P, 54R, Br5, Br7 and Br3. None of the surface strains of halobacteria were shown to have a strong emulsifying effect on crude oil.
5

RESULTS AND DISCUSSION:

AUTOTROPHIC GROWTH AND CO₂ FIXATION BY HALOBACTERIA

5.1 Autotrophic growth

To investigate whether halobacteria could grow autotrophically, cells were inoculated into modified minimal media (MMM) containing 5mM NaHCO₃ together with vitamins and minerals and incubated under light and dark conditions, both shaken and static. Tubes containing agar were observed by eye for growth, but no growth was observed in any tubes. The OD in liquid media was measured on days 0, 7, 14 and 21 and is shown in Figs. 5.1- 5.11. For most strains the OD fell between 0 to 7 days and then continued to fall more slowly between 7 to 21 days, indicating that autotrophic growth was not occurring. The only example where a fall in OD was not observed was Hc. morrhuae, where when incubated under dark and shaken conditions the OD remained constant between 0 to 7 days and then increased from 0.039 to 0.053 between 7 to 14 days. After 21 days this fell to 0.018 (Fig. 5.7a). This could indicate that some growth was occurring. Additionally when Hc. morrhuae was preincubated illuminated and semi-anaerobic for 4 days and then inoculated into MMM, the OD initially dropped after 7 days but then was seen to rise again after 14 days, although not as high as the initial OD. Hc. morrhuae grows in clumps making it difficult to measure the OD accurately as the cells tend to settle out in the bottom of the cuvette and the amount of variation between duplicate cuvettes was greater for this strain than many of the others. Therefore these growth experiments need to be repeated using some other indication of growth, for example protein concentration, before it can be confirmed that Hc. morrhuae can grow autotrophically.
Results and Discussion: Autotrophic Growth and CO₂ Fixation by Halobacteria

**Fig. 5.1a:** Graph showing the change in OD (660 nm) following inoculation of 54R into MMM with 5 mM NaHCO₃.

![Graph showing the change in OD (660 nm) following inoculation of 54R into MMM with 5 mM NaHCO₃.](image)

Error bars indicate +/- one std. dev.

**Fig. 5.1b:** Changes in OD (660 nm) following inoculation of 54R in to MMM with 5 mM NaHCO₃. 54R was pre-incubated semi-anaerobically and illuminated for 4 days.

![Changes in OD (660 nm) following inoculation of 54R in to MMM with 5 mM NaHCO₃. 54R was pre-incubated semi-anaerobically and illuminated for 4 days.](image)

Error bars indicate +/- one std. dev.
Results and Discussion: Autotrophic Growth and CO₂ Fixation by Halobacteria

Fig. 5.2a: Changes in OD (660 nm) following inoculation of Hr. saccharovorum in to MMM with 5 mM NaHCO₃.

Error bars indicate +/- one std. dev.

Fig. 5.2b: Changes in OD (660 nm) following inoculation of Hr. saccharovorum into MMM with 5 mM NaHCO₃. Hr. saccharovorum was pre-incubated semi-anaerobically and illuminated for 4 days.

Error bars indicate +/- one std. dev.
Results and Discussion: Autotrophic Growth and CO$_2$ Fixation by Halobacteria

Fig. 5.3a: Changes in OD (660 nm) following inoculation of Hr. sodomense into MMM with 5 mM NaHCO$_3$.

Error bars indicate +/- one std. dev.

Fig. 5.3b: Changes in OD (660 nm) following inoculation of Hr. sodomense into MMM with 5 mM NaHCO$_3$. Hr. sodomense was pre-incubated semi-anaerobically and illuminated for 4 days.

Error bars indicate +/- one std. dev.
Results and Discussion: Autotrophic Growth and CO₂ Fixation by Halobacteria

**Fig. 5.4a:** Changes in OD (660 nm) following inoculation of E4 into MMM with 5 mM NaHCO₃.

![Graph showing changes in OD (660 nm) with light and dark conditions for different days.]

Error bars indicate +/- one std. dev.

**Fig. 5.4b:** Changes in OD (660 nm) following inoculation of E4 into MMM with 5 mM NaHCO₃. E4 was pre-incubated semi-anaerobically and illuminated for 4 days.

![Graph showing changes in OD (660 nm) with light and dark conditions for different days.]

Error bars indicate +/- one std. dev.
Results and Discussion: Autotrophic Growth and CO₂ Fixation by Halobacteria

Fig. 5.5a: Changes in OD (660 nm) following inoculation of Ha. marismortui into MMM with 5 mM NaHCO₃.

Error bars indicate +/- one std. dev.

Fig. 5.5b: Changes in OD (660 nm) following inoculation of Ha. marismortui into MMM with 5 mM NaHCO₃. Ha. marismortui was pre-incubated semi-anaerobically and illuminated for 4 days.

Error bars indicate +/- one std. dev.
Results and Discussion: Autotrophic Growth and CO₂ Fixation by Halobacteria

Fig. 5.6a: Changes in OD following inoculation of Br3 into MMM with 5 mM NaHCO₃.

![Graph showing changes in OD following inoculation](image)

Error bars indicate +/- one std. dev

Fig. 5.6b: Changes in OD following inoculation of Br3 into MMM with 5 mM NaHCO₃. Br3 was pre-incubated semi-anaerobically and illuminated for 4 days.

![Graph showing changes in OD following inoculation](image)

Error bars indicate +/- one std. dev
**Results and Discussion: Autotrophic Growth and CO$_2$ Fixation by Halobacteria**

**Fig. 5.7a:** Changes in OD following inoculation of *Hc. morrhuae* into MMM with 5 mM NaHCO$_3$.

![Graph showing changes in OD over time with different conditions](image)

Error bars indicate +/- one std. dev.

**Fig. 5.7b:** Changes in OD following inoculation of *Hc. morrhuae* into MMM with 5 mM NaHCO$_3$. *Hc. morrhuae* was pre-incubated semi-anaerobically and illuminated for 4 days.

![Graph showing changes in OD over time with different conditions](image)

Error bars indicate +/- one std. dev.
Results and Discussion: Autotrophic Growth and CO₂ Fixation by Halobacteria

**Fig. 5.8a:** Changes in OD following inoculation of BbpA1 into MMM with 5 mM NaHCO₃.

![Graph showing changes in OD following inoculation of BbpA1 into MMM with 5 mM NaHCO₃.](image)

Error bars indicate +/- one std. dev

**Fig. 5.8b:** Changes in OD following inoculation of BbpA1 into MMM with 5 mM NaHCO₃. BbpA1 was pre-incubated semi-anaerobically and illuminated for 4 days.

![Graph showing changes in OD following inoculation of BbpA1 into MMM with 5 mM NaHCO₃.](image)

Error bars indicate +/- one std. dev
Results and Discussion: Autotrophic Growth and CO₂ Fixation by Halobacteria

**Fig. 5.9a:** Changes in OD following inoculation of O14.1 into MMM with 5 mM NaHCO₃.

![Graph showing OD changes over time for different conditions.](image)

Error bars indicate +/- one std. dev

**Fig. 5.9b:** Changes in OD following inoculation of O14.1 into MMM with 5 mM NaHCO₃. O14.1 was pre-incubated semi-anaerobically and illuminated for 4 days.

![Graph showing OD changes over time for different conditions.](image)

Error bars indicate +/- one std. dev
**Fig. 5.10a:** Changes in OD following inoculation of *Hb. salinarum* into MMM with 5 mM NaHCO$_3$.

Error bars indicate +/- one std. dev

**Fig. 5.10b:** Changes in OD following inoculation of *Hb. salinarum* into MMM with 5 mM NaHCO$_3$. *Hb. salinarum* was pre-incubated semi-anaerobically and illuminated for 4 days.

Error bars indicate +/- one std. dev
Fig. 5.11a: Changes in OD following inoculation of *Hb. salinarum* (halobium) into MMM with 5 mM NaHCO₃.

Fig. 5.11b: Changes in OD following inoculation of *Hb. salinarum* (halobium) into MMM with 5 mM NaHCO₃. *Hb. salinarum* (halobium) was pre-incubated semi-anaerobically and illuminated for 4 days.
Results and Discussion: Autotrophic Growth and CO₂ Fixation by Halobacteria

Although no growth was observed in any of the strains tested, the effect of the different incubation conditions on the survival of the cells is interesting. For many strains, e.g. 54R, E4, Br3, O14.1, *Hb. salinarum, Hb. salinarum (halobium)*, and *Hr. sodomense*, the OD fell much more quickly and to a lower level under the light static conditions than any of the other conditions (Fig. 5.1, 5.3, 5.4, 5.6, 5.9, 5.10). In many of the strains the OD of cells incubated in the dark remained much higher than those incubated in the light. This is particularly evident in BbpA1 (Fig. 5.7b), *Hb. salinarum* (Fig 5.9 a + b) and *Hb. salinarum (halobium)* (Fig. 5.10 a + b).

Incubation for several days semi-anaerobically and illuminated should induce bacteriorhodopsin in those strains that produce it. *Hb. salinarum, Hb. salinarum (halobium)* and *Hb. sodomense* have been shown to produce bacteriorhodopsin (Oren 1994a). However, a period of illuminated, semi-anaerobic incubation prior to inoculation into MMM did not induce autotrophic growth in any of the strains tested. For most strains a fall in the OD of the cells occurred in a very similar pattern to cells which had not been preincubated e.g. 54R, E4, *Ha. marismortui*, Br3, *Hr. sodomense*, and *Hb. salinarum* (Figs. 5.1, 5.3, 5.4, 5.5, 5.10). However, preincubation semi-anaerobically changed the way some strains behaved. For example, the OD of *Hr. saccharovorum* did not fall as rapidly or as low after the pre-incubation period (Fig. 5.2). In *Hb. salinarum (halobium)* with no preincubation, the OD of cells incubated under light conditions fell to a low level but under dark conditions only a small reduction in OD was observed. In this strain, after the period of pre-incubation, the OD of the light incubated cells and dark and shaken cells fell to a low level but the dark and static cells behaved as before (Fig. 5.11). BbpA1 is a further example of a strain where its behaviour after the preincubation period changes. With no preincubation period the light static incubated cells fell to a low OD, but the cells incubated under other conditions remained at levels at least 50% of the OD at day 0. After the pre-incubation period, the cells incubated under light, shaking conditions also fell to a low level while the dark incubated cells remained at a higher OD (Fig. 5.8).

Activity of the enzyme ribulose 1,6-bisphosphate carboxylase/oxygenase (RuBisCo) has been demonstrated in several strains of halobacteria (Rawal et al. 1988, Altekar &
**Results and Discussion: Autotrophic Growth and CO₂ Fixation by Halobacteria**

Rajagopalan 1990, Rajagopalan & Altekar 1994). Strains, which have this activity, include *Hf. mediterranei*, *Hf. volcanii* and *Ha. marismortui*. No RuBisCo activity could be demonstrated in *Hb. salinarum*, *Hb. cutirubrum*, *Hr. saccharovorum*, or *Ha. vallismortis* (Altekar & Rajagopalan 1990). *Hf. mediterranei* also possesses all the other enzymes necessary for the Calvin cycle (Oren 1994a). Danon and Caplan (1977) showed that CO₂ fixation in a bacteriorhodopsin-producing strain required light. Oren (1983) and Danon and Caplan (1977) suggested that CO₂ fixation is linked to the possession of bacteriorhodopsin. Additionally, Rodriguez-Valera *et al.* (1983) showed that light could act as an energy source for *Hb. salinarum* (*halobium*) cells when grown under conditions of low nutrients and low oxygen. It is therefore theoretically possible that halobacteria may be capable of photoautotrophic growth using the enzymes of the Calvin cycle together with bacteriorhodopsin (Danson & Hough 1992).

In the experiments described in this section (Figs. 5.1-5.11), no autotrophic growth has been demonstrated under the conditions tested, however no external electron source was provided which may have a bearing on the results obtained. Incubation semi-anaerobically, illuminated for 4 days prior to inoculation into MMM did not induce autotrophic growth, although it should induce bacteriorhodopsin in *Hb. salinarum* strains and *Hr. sodomense*.

In the experiments described in this section, tubes were observed only for 21 days. It is possible that if tubes were observed for a longer period, evidence of growth may have been seen. Additionally, strictly anaerobic conditions were not tested. It was interesting that survival of strains in MMM was generally the lowest in light static conditions, although these are the conditions in which bacteriorhodopsin should be synthesised. High levels of bright light can induce formation of oxygen radicals, which are toxic to cells and this could be a possible explanation for the lower levels of survival under light conditions. However halobacteria are exposed to high levels of light in surface hypersaline environments and they possess enzymes such as catalase, peroxidase, catalase-peroxidase and superoxide dismutase to deal with oxygen radicals (Salin & Brown-Peterson 1993). McGenity (1994) found that all salt mine strains of halobacteria were catalase positive and most were also oxidase positive. Survival in most strains was greatest under dark conditions. This may be significant...
when considering the salt deposit strains which may have been present in the dark for millions of years. It would be interesting to look at protein expression under these different conditions; perhaps low levels of light induce expression of proteins, which aid survival.
5.2. Whole Cell Assays of CO₂ fixation

Experiments were carried out to investigate fixation of ¹⁴CO₂ by halobacteria under different incubation conditions and with the addition of various substrates. In 1988 Javor carried out CO₂ fixation experiments in halobacteria. In these experiments Javor grew cells to late exponential phase and then incubated cells semi-anaerobically for several days to induce bacteriorhodopsin synthesis. To investigate whether a period of semi-anaerobic incubation is necessary for CO₂ fixation in halobacteria, CO₂ fixation in cells before and after a period of semi-anaerobic incubation was compared. Bacteriorhodopsin is induced, in those strains that produce it, under semi-anaerobic and illuminated conditions. Rates of CO₂ fixation after semi-anaerobic incubation in the light and in the dark were compared. If CO₂ fixation is related to possession of bacteriorhodopsin then there should be a difference in levels of CO₂ fixation between the light and dark incubated cells.

For most strains the period of semi-anaerobic incubation had no effect on or reduced the amount of CO₂ fixation compared to levels prior to the incubation period (Table 5.1). However, in *Ha. marismortui* semi-anaerobic incubation in both the light and dark increased levels of CO₂ fixation by about 25%. In *Hb. salinarum (halobium)*, which is known to produce bacteriorhodopsin, semi-anaerobic incubation in the light reduced CO₂ fixation, but incubation semi-anaerobically in the dark increased rates of CO₂ fixation compared to rates prior to the incubation period. The amount of CO₂ fixation of strain BbpA1 increased after pre-incubation in the dark, but not after pre-incubation in the light when compared to the level of CO₂ fixation, without a pre-incubation period.

When levels of CO₂ fixation after semi-anaerobic incubation in the light are compared to those in the dark, for several strains there was no difference in the amount of CO₂ fixed. 54R, *Hb. salinarum (halobium)*, BbpA1 and *Hb. salinarum* had higher levels of CO₂ fixation after incubation in the dark. O14.1, Br3 and *Hf. mediterranei* had higher levels after incubation in the light compared to that in the dark, but this level was lower than the CO₂ fixation level with no pre-incubation. Neither O14.1, Br3 or *Hf. mediterranei* have been shown to produce bacteriorhodopsin.
Table 5.1: Effect of pre-incubating cells under semi-anaerobic and illuminated conditions prior to assaying CO₂ fixation in halobacteria.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean CO₂ fixed (nanomol mg protein⁻¹ in 1 hour) (Std. Dev. shown in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-incubation: None</td>
</tr>
<tr>
<td>Hr. saccharovorun</td>
<td>0.38 (0.14)</td>
</tr>
<tr>
<td>Ha. marismortui</td>
<td>4.6 (0.23)</td>
</tr>
<tr>
<td>54R</td>
<td>0.98 (0.065)</td>
</tr>
<tr>
<td>E4</td>
<td>4.39 (2.23)</td>
</tr>
<tr>
<td>O14.1</td>
<td>1.76 (0.21)</td>
</tr>
<tr>
<td>Hb. salinarum (halobium)</td>
<td>7.46 (0.29)</td>
</tr>
<tr>
<td>Hc. morrhuae</td>
<td>1.81 (0.39)</td>
</tr>
<tr>
<td>Br3</td>
<td>7.48 (1.1)</td>
</tr>
<tr>
<td>BbpA1</td>
<td>4.09 (0.08)</td>
</tr>
<tr>
<td>Hb. salinarium</td>
<td>0.64 (0.85)</td>
</tr>
<tr>
<td>Hf. mediterranei</td>
<td>3.19 (0.3)</td>
</tr>
</tbody>
</table>

CO₂ fixation was assayed in all cells under aerobic illuminated conditions at 37°C. Background counts of scintillation counts only had a mean of 15. Scintillation fluid plus a GC/F filter had a mean count of 14. Formalin killed cells had a mean count of 24. This figure was subtracted from the count for each sample prior to calculating the CO₂ fixation.
These results (Table 5.1) show that a period of semi-anaerobic incubation is not necessary to demonstrate CO₂ fixation in halobacteria. Semi-anaerobic incubation in the light, which is known to induce bacteriorhodopsin production, does not enhance CO₂ fixation. Therefore in the strains of halobacteria tested in this study, CO₂ fixation is unlikely to be linked to the possession of bacteriorhodopsin.

The differences in levels of CO₂ fixation before and after the period of semi-anaerobic CO₂ fixation are presumably due to changes in the metabolism which occur during the semi-anaerobic incubation, for example a switch to anaerobic respiration or fermentation.

Table 5.2 compares ¹⁴CO₂ fixation during incubation in CHM under aerobic and strictly anaerobic conditions. All tubes were illuminated. In all strains, except E4, CO₂ fixation under aerobic conditions was higher than under anaerobic conditions. Levels of CO₂ fixation under aerobic conditions varied from 0.027 – 4.59 nanomoles CO₂ mg protein⁻¹ hour⁻¹. Anaerobic CO₂ fixation of many strains was close to the limit of detection with counts only slightly higher than for formalin killed cells (negative control). The level of CO₂ fixation under aerobic conditions was 4 times higher than that under anaerobic conditions for Hb. salinarum (cutirubrum). For Ha. marismortui, CO₂ fixation was over 9 000 times higher under aerobic conditions compared to anaerobic conditions. For the other strains, except E4, the level of CO₂ fixation was between 8 and 400 times higher in aerobic conditions than in anaerobic conditions. No CO₂ fixation was recorded for E4 under aerobic conditions (Table 5.2), that is counts were no higher than for the formalin killed cells which were used as a negative control. This result is surprising as CO₂ fixation was recorded under aerobic conditions for E4 in the previous experiment (Table 5.1). E4 demonstrated CO₂ fixation under anaerobic conditions at a level, which was comparable to the CO₂ fixation of the other strains under anaerobic conditions.
Results and Discussion: Autotrophic Growth and CO₂ Fixation

Table 5.2: CO₂ Fixation by halobacteria under Aerobic and Anaerobic Conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean CO₂ fixed (nanomoles mg protein⁻¹ in 1 hour) (Std Dev. shown in brackets)</th>
<th>AEROBIC</th>
<th>ANAEROBIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>&lt; 0.00015</td>
<td>0.014</td>
<td>(0.011)</td>
</tr>
<tr>
<td>Hr. saccharovorum</td>
<td>0.12 (0.015)</td>
<td>0.0018</td>
<td>(0.0026)</td>
</tr>
<tr>
<td>Br3</td>
<td>0.19 (0.075)</td>
<td>0.025</td>
<td>(0.0024)</td>
</tr>
<tr>
<td>Hc. morrhuae</td>
<td>0.17 (0.029)</td>
<td>0.011</td>
<td>(0.0047)</td>
</tr>
<tr>
<td>O14.1</td>
<td>0.49 (0.027)</td>
<td>0.019</td>
<td>(0.029)</td>
</tr>
<tr>
<td>Hf. mediterranei</td>
<td>0.089 (0.019)</td>
<td>0.0063</td>
<td>(0.0065)</td>
</tr>
<tr>
<td>BbpAl</td>
<td>0.085 (0.021)</td>
<td>0.0063</td>
<td>(0.0087)</td>
</tr>
<tr>
<td>Hb. salinarum halobium</td>
<td>4.59 (0.19)</td>
<td>0.012</td>
<td>(0.011)</td>
</tr>
<tr>
<td>54R</td>
<td>2.57 (0.36)</td>
<td>0.029</td>
<td>(0.026)</td>
</tr>
<tr>
<td>Ha. marismortui</td>
<td>4.12 (0.15)</td>
<td>0.00045</td>
<td>(0.00096)</td>
</tr>
<tr>
<td>Hb. salinarum (cutirubrum)</td>
<td>0.027 (0.01)</td>
<td>0.0032</td>
<td>(0.0047)</td>
</tr>
</tbody>
</table>

CO₂ fixation was assayed at 37°C under illumination by two 20W strip lights. Formalin killed cells were used as a control. These had scintillation counts of 20-28 per minute. This figure was subtracted from the count for the samples to calculate CO₂ fixed. Background counts in scintillation fluid were 18 per minute. The scintillation fluid plus glass fibre filters also had mean counts of 18 per minute.
Table 5.3 shows the effect of illumination on CO₂ fixation by halobacteria under aerobic conditions. For several strains there was no significant difference between cells which were illuminated and those which were dark. Strains E4 and *Ha. marismortui* showed higher levels of CO₂ fixation in the dark. *Hb. salinarum (cutirubrum)* showed a higher level of CO₂ fixation in the light than the dark. This is a bacteriorhodopsin producing strain.

The effect of light and dark incubation on CO₂ fixation during anaerobic incubation was also investigated in some strains (Table 5.4). The level of anaerobic CO₂ fixation was low in all strains tested and illumination had no significant effect on any of the strains tested.

It should be noted that the salt-mine isolates of halobacteria did not show particularly high or low levels of CO₂ fixation under any of the conditions tested compared to the surface strains of halobacteria. 54R and *Ha. marismortui* had the most consistently high rates of CO₂ fixation. These strains were therefore used to investigate the effects on CO₂ fixation of the addition of various substrates.

Javor (1988) and Danon & Caplan (1977) reported that propionate enhanced CO₂ fixation in halobacteria. Table 5.5 shows the effect of propionate on CO₂ fixation by 54R and *Ha. marismortui*. These experiments showed that propionate, either with or without the addition of NH₄⁺, had no significant effect on CO₂ fixation of 54R or *Ha. marismortui*. 
Results and Discussion: Autotrophic Growth and CO₂ Fixation

Table 5.3: CO₂ fixation by halobacteria under light and dark conditions (aerobic).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean CO₂ fixed (nanomol mg protein⁻¹ in 1 hour)</th>
<th>(Std. Dev. shown in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LIGHT</td>
<td>DARK</td>
</tr>
<tr>
<td>E4</td>
<td>0.00029</td>
<td>0.0021</td>
</tr>
<tr>
<td></td>
<td>(0.00078)</td>
<td>(0.0030)</td>
</tr>
<tr>
<td><em>Hr. saccharovorum</em></td>
<td>0.069</td>
<td>0.078</td>
</tr>
<tr>
<td></td>
<td>(0.0060)</td>
<td>(0.013)</td>
</tr>
<tr>
<td>Br3</td>
<td>0.51</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>(0.053)</td>
<td>(0.039)</td>
</tr>
<tr>
<td><em>Hc. morrhuae</em></td>
<td>0.17</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>(0.042)</td>
<td>(0.047)</td>
</tr>
<tr>
<td>O14.1</td>
<td>0.54</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>(0.053)</td>
<td>(0.031)</td>
</tr>
<tr>
<td>BbpAl</td>
<td>0.54</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>(0.053)</td>
<td>(0.12)</td>
</tr>
<tr>
<td>54R</td>
<td>3.05</td>
<td>3.20</td>
</tr>
<tr>
<td></td>
<td>(0.081)</td>
<td>(0.15)</td>
</tr>
<tr>
<td><em>Ha. marismortui</em></td>
<td>2.19</td>
<td>3.04</td>
</tr>
<tr>
<td></td>
<td>(0.40)</td>
<td>(0.12)</td>
</tr>
<tr>
<td><em>Hb. salinarum</em> (cutirubrum)</td>
<td>0.027</td>
<td>0.0071</td>
</tr>
<tr>
<td></td>
<td>(0.01)</td>
<td>(0.0079)</td>
</tr>
</tbody>
</table>

CO₂ fixation was assayed at 37°C. Light conditions were produced using two 20W strip lights. Covering bottles with foil produced dark conditions. Formalin killed cells were used as a control. These had scintillation counts of 29–34 per minute. This figure was subtracted from the count for the samples to calculate CO₂ fixed. Background counts in scintillation fluid were 13 per minute. The scintillation fluid plus glass fibre filters also had mean counts of 13 per minute.
Table 5.4: CO₂ fixation by halobacteria under light and dark conditions (anaerobic).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean CO₂ fixed (nanomol mg protein⁻¹ in 1 hour)</th>
<th>(Std. Dev. shown in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LIGHT</td>
<td>DARK</td>
</tr>
<tr>
<td><em>Hf. mediterranei</em></td>
<td>0.011 (0.017)</td>
<td>0.014 (0.0096)</td>
</tr>
<tr>
<td><em>BbpAl</em></td>
<td>0.0047 (0.0072)</td>
<td>0.0085 (0.015)</td>
</tr>
<tr>
<td><em>Hb. salinarum (halobium)</em></td>
<td>0.0060 (0.0054)</td>
<td>0.0035 (0.0048)</td>
</tr>
<tr>
<td>54R</td>
<td>0.0019 (0.0044)</td>
<td>0.0067 (0.011)</td>
</tr>
<tr>
<td><em>Ha. marismortui</em></td>
<td>0.0013 (0.0024)</td>
<td>0.0011 (0.0015)</td>
</tr>
</tbody>
</table>

CO₂ fixation was assayed at 37°C in an anaerobic cabinet. Light conditions were produced using two 20W strip lights. Covering bottles with foil produced dark conditions. Formalin killed cells were used as a control. These had scintillation counts of 19–22 per minute. This figure was subtracted from the count for the samples to calculate CO₂ fixed. Background counts in scintillation fluid were 24 per minute. The scintillation fluid plus glass fibre filters also had mean counts of 16 per minute.

Table 5.5: Effect of addition of propionate on CO₂ fixation by halobacteria.

<table>
<thead>
<tr>
<th>Substrates Added</th>
<th>Mean CO₂ fixed (nanomol mg protein⁻¹ in 1 hour)</th>
<th>(Std. dev. shown in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>54R</td>
<td><em>Ha. marismortui</em></td>
</tr>
<tr>
<td>None</td>
<td>0.51 (0.15)</td>
<td>1.24 (0.028)</td>
</tr>
<tr>
<td>Propionate</td>
<td>0.34 (0.062)</td>
<td>0.88 (0.12)</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.42 (0.15)</td>
<td>1.42 (0.13)</td>
</tr>
<tr>
<td>Propionate + NH₄⁺</td>
<td>0.37 (0.12)</td>
<td>1.73 (1.00)</td>
</tr>
</tbody>
</table>

CO₂ fixation was assayed at 37°C under aerobic conditions. Formalin killed cells were used as a control. These had a mean cpm of 20. This figure was subtracted from the count for the samples to calculate CO₂ fixed. Scintillation fluid only had a mean count of 19 and scintillation fluid with the addition of a glass fibre filter had a count of 14.
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Table 5.6 shows the effect on CO₂ fixation of adding NH₄⁺, acetate, glucose and pyruvate in 54R and *Ha. marismortui*. Whereas addition of acetate increased the CO₂ fixation in 54R only slightly, the addition of glucose or pyruvate increased CO₂ fixation by about 4 times. Addition of NH₄⁺ also slightly increased the CO₂ fixation in 54R. In *Ha. marismortui*, acetate increased CO₂ fixation slightly, pyruvate increased it by about 50%, but glucose increased it by over 12 times. Addition of NH₄⁺ only increased CO₂ fixation slightly in *Ha. marismortui*. When NH₄⁺ was added together with glucose or pyruvate an increase in the amount of CO₂ fixation, compared to the amount with glucose or pyruvate only, was observed. This may indicate that the CO₂ incorporated into the cell is being used in amino acid biosynthesis. It is interesting that glucose had a greater stimulatory effect on CO₂ fixation than pyruvate in *Ha. marismortui*, although glucose is metabolised via pyruvate.

**Table 5.6: Effect of addition of carbon and nitrogen sources on CO₂ fixation**

<table>
<thead>
<tr>
<th>Substrate Added</th>
<th>Mean CO₂ fixed (nanomol mg protein⁻¹ in 1 hour) (Std. Dev. shown in brackets)</th>
<th>54R</th>
<th><em>Ha. marismortui</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.56 (0.26)</td>
<td>0.75 (0.28)</td>
<td></td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.83 (0.086)</td>
<td>0.92 (0.055)</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>0.84 (0.11)</td>
<td>0.89 (0.059)</td>
<td></td>
</tr>
<tr>
<td>Acetate + NH₄⁺</td>
<td>0.69 (0.046)</td>
<td>0.83 (0.10)</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>2.25 (0.18)</td>
<td>9.59 (1.91)</td>
<td></td>
</tr>
<tr>
<td>Glucose + NH₄⁺</td>
<td>2.18 (0.41)</td>
<td>11.07 (1.64)</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>2.38 (0.096)</td>
<td>1.10 (0.078)</td>
<td></td>
</tr>
<tr>
<td>Pyruvate + NH₄⁺</td>
<td>1.86 (0.12)</td>
<td>1.59 (0.17)</td>
<td></td>
</tr>
</tbody>
</table>

CO₂ fixation was assayed at 37°C under aerobic conditions. Formalin killed cells were used as a control. These had a mean cpm of 35. This figure was subtracted from the count for the samples to calculate CO₂ fixed. Scintillation fluid only had a mean count of 27 and scintillation fluid plus a glass fibre filters had a mean count of 16.
Danon & Caplan (1977) investigated CO₂ fixation in *Halobacterium salinarum* R1 (formally *Hb. halobium*) under anaerobic conditions. This is a bacteriorhodopsin producing strain. They found that CO₂ fixation occurred only under illumination and not in the dark. In this study CO₂ fixation of *Hb. salinarum* (*halobium*) was approximately 400 times higher under aerobic conditions than anaerobic conditions (Table 5.2). Illumination had no significant effect on levels of CO₂ fixation under anaerobic conditions (Table 5.4).

Danon & Caplan (1977) investigated the effect of adding succinate, malate, citrate, pyruvate and propionate on CO₂ fixation, but only propionate had a noticeable stimulatory effect. Javor (1988) found that propionate stimulated CO₂ fixation in *Hb. salinarum* R1, and *Haloarcula* sp. GN-1, but not in *Hf. volcanii*. Javor also found that pyruvate enhanced CO₂ fixation in all strains tested, but acetate had no effect or actually decreased CO₂ fixation. Altekar & Rajagopalan (1990) found that in *Hf. mediterranei* the CO₂ fixation was enhanced by propionate, pyruvate and glucose.

In this study CO₂ fixation by 54R and *Ha. marismortui* was enhanced by pyruvate and glucose, but not propionate or acetate. It therefore appears that enhancement of CO₂ by addition of different substrates is strain specific. It should be noted that 54R and *Ha. marismortui* can utilise glucose and pyruvate as carbon and energy sources for growth but not acetate or propionate. It should also be noted that in the experiments carried out by Javor (1988) and Rajagopalan & Altekar (1990) the cells were “semi-starved” overnight in basal medium with 1% (v/v) glycerol prior to the CO₂ fixation assays. Cells in this study were grown to mid-exponential phase and then diluted in CHM. Cells in this study were therefore in a different physiological state to those used by Javor (1988) and Rajagopalan & Altekar (1990).

In this study much higher rates of CO₂ fixation were observed under aerobic conditions than under anaerobic conditions. In strains 54R and *Ha. marismortui* CO₂ fixation was enhanced by glucose and pyruvate. This suggests that the major CO₂ fixation in these two strains is anaplerotic fixation replenishing the citric acid cycle. Accordingly, assays were carried out to look for enzymes involved in anaplerotic reactions (section 5.3).
5.3. Enzyme Activity in Cell Extracts

In this section assays were carried to investigate the enzymes responsible for CO$_2$ fixation in strains 54R and *Ha. marismortui*. These strains were chosen as they had consistently high levels of CO$_2$ fixation in whole cell assays.

RuBisCo activity has been demonstrated in *Hf. mediterranei* and *Hf. volcanii* (Rawal *et al.* 1988b, Altekar & Rajagopalan 1990), and also at a lower activity in *Ha. marismortui* (Altekar & Rajagopalan 1990). No RuBisCo activity could be demonstrated by 54R or *Ha. marismortui* in this study (Table 5.7), despite using the method of Altekar & Rajagopalan (1990) and growing cells in a medium containing 0.5% Na butyrate and 0.05% NaHCO$_3$, which Rajagopalan & Altekar (1994) used to stimulate RuBisCo activity.

Three different assay methods were used to look for pyruvate carboxylase activity. Results from the enzymatic and radioisotopic assays are shown in Tables 5.8 and 5.9. Both showed that 54R and *Ha. marismortui* exhibit pyruvate-dependent CO$_2$ fixation. The radioisotopic assay (Table 5.9) showed that the CO$_2$ fixation was reduced by omission of ATP. CO$_2$ fixation by 54R was reduced by the absence of acetyl-CoA from the assay mix. Pyruvate carboxylase requires ATP for activity and it is positively regulated by acetyl-CoA in many organisms. Therefore these results provide evidence for pyruvate carboxylase activity. Pyruvate carboxylase activity has previously been demonstrated in *Hf. mediterranei* and *Ha. vallismortis* (Rawal *et al.* 1988a). $^{13}$C-NMR studies using labelled pyruvate have also indicated that pyruvate carboxylase is present in *Hb. salinarum* (Bhaumik & Sonawat 1994, Ghosh & Sonawat 1998). Pyruvate: ferredoxin oxidoreductase (POR) has been shown to catalyse exchange between H$^{14}$CO$_3^-$ and pyruvate (Raeburn & Rabinowiz 1971). This is an ATP independent reaction. This enzyme is responsible for the conversion of pyruvate to acetyl-CoA in halobacteria and may also be responsible for some of the CO$_2$ fixation, which is observed in this experiment.
### Table 5.7: Rubisco Assay

<table>
<thead>
<tr>
<th>Assay Mixture</th>
<th>Mean Count per Minute (Std Dev shown in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ha. marismortui</td>
</tr>
<tr>
<td>CHM</td>
<td>MM + butyrate + NaHCO₃</td>
</tr>
<tr>
<td>Complete assay mix</td>
<td>145 (37)</td>
</tr>
<tr>
<td>minus RuBP</td>
<td>151 (26)</td>
</tr>
<tr>
<td>minus NaH¹⁴CO₃</td>
<td>18 (4)</td>
</tr>
<tr>
<td>RuBisCo (0.04 units)</td>
<td>154137 (39783)</td>
</tr>
</tbody>
</table>

Background counts of scintillation fluid only had a mean count of 19. Each assay contained 72µg of protein.
Results and Discussion: Autotrophic Growth and CO₂ Fixation

Table 5.8: Enzymatic Assay for pyruvate carboxylase.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Assay Conditions</th>
<th>NADH oxidised (μmol in 5 minutes per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Complete Assay Mix</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>54R</td>
<td>0.68M NaCl</td>
<td>1.1 x 10⁻³</td>
</tr>
<tr>
<td>54R</td>
<td>1MKCl, 0.68M NaCl</td>
<td>6.4 x 10⁻⁴</td>
</tr>
<tr>
<td><em>Ha. marismortui</em></td>
<td>1M KCL</td>
<td>1.2 x 10⁻³</td>
</tr>
</tbody>
</table>

Table 5.9: Radiolabeled assay for pyruvate carboxylase.

<table>
<thead>
<tr>
<th>Assay Content</th>
<th>54R Mean Count per Minute (Std Dev shown in brackets)</th>
<th><em>Ha. marismortui</em> Mean Count per Minute (Std Dev shown in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete assay mix</td>
<td>5398 (216)</td>
<td>5194 (1409)</td>
</tr>
<tr>
<td>Minus Acetyl-CoA</td>
<td>3828 (77)</td>
<td>7457 (606)</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>3979 (1149)</td>
<td>2081 (579)</td>
</tr>
<tr>
<td>Minus pyruvate</td>
<td>224 (72.4)</td>
<td>290 (30)</td>
</tr>
<tr>
<td>Minus NaH¹⁴CO₃</td>
<td>24 (7)</td>
<td>22 (4)</td>
</tr>
<tr>
<td>Minus cell extract</td>
<td>216 (40)</td>
<td></td>
</tr>
</tbody>
</table>

54R cell extract contained 2 mg protein per assay. *Ha. marismortui* cell extract contained 2.5 mg protein per assay. Scintillation fluid only had a mean cpm of 19.
Two different methods were also used for assaying PEP carboxylase. However no activity could be demonstrated by the spectrophotometric assay. The radioisotopic assay, which is more sensitive than the spectrophotometric assay, showed that PEP-dependent CO$_2$ fixation was occurring (Table 5.10). Omission of acetyl-CoA reduced CO$_2$ fixation. PEP carboxylase is positively regulated by acetyl-CoA. This indicates that PEP carboxylase may be present in 54R and *Ha. marismortui*.

**Table 5.10: Radioisotopic Assay for PEP Carboxylase**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Counts per minute (Std Dev shown in brackets)</th>
<th>54R</th>
<th>Ha. marismortui</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete assay mix</td>
<td></td>
<td>816</td>
<td>251</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(197)</td>
<td>(70)</td>
</tr>
<tr>
<td>Minus acetyl-CoA</td>
<td></td>
<td>279</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(19)</td>
<td>(11)</td>
</tr>
<tr>
<td>Minus PEP</td>
<td></td>
<td>116</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10)</td>
<td>(18)</td>
</tr>
<tr>
<td>Minus NaH$^{14}$CO$_3$</td>
<td></td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(17)</td>
<td>(5)</td>
</tr>
<tr>
<td>PEP Carboxylase</td>
<td></td>
<td>6186</td>
<td></td>
</tr>
<tr>
<td>(0.125 units)</td>
<td></td>
<td></td>
<td>(1208)</td>
</tr>
<tr>
<td>Minus cell extract</td>
<td></td>
<td>120</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(21)</td>
</tr>
</tbody>
</table>

*Each assay contained 1 mg of protein. Scintillation fluid only had a count of 17.*
However, PEP is converted into pyruvate by pyruvate kinase as part of glycolysis and the activity of this enzyme has been shown in halobacteria (Rawal et al. 1988a). Pyruvate can be converted into PEP by the enzymes PEP synthetase or pyruvate orthophosphate dikinase (Fig. 5.12). Both PEP synthetase and pyruvate orthophosphate dikinase require ATP. The conversion of pyruvate to PEP or PEP to pyruvate must be considered when interpreting the results of these experiments. Additionally, the enzymes PEP carboxykinase and PEP carboxytransphosphorylase also convert PEP and CO$_2$ into oxaloacetate (Fig. 5.13). Like, PEP carboxylase they do not require ATP.

**Fig. 5.12: Interconversions of PEP and pyruvate**

**Pyruvate Kinase**

(1) \( \text{PEP} + \text{ADP} + H^+ \rightarrow \text{Pyruvate} + \text{ATP} \)

**PEP Synthetase**

(2) \( \text{Pyruvate} + \text{ATP} \rightarrow \text{PEP} + \text{AMP} + \text{Pi} \)

**Pyruvate Orthophosphate Dikinase**

(3) \( \text{Pyruvate} + \text{ATP} + \text{Pi} \rightarrow \text{PEP} + \text{AMP} + \text{PPi} \)

**Fig. 5.13: Anaplerotic reactions of PEP**

**PEP Carboxylase**

(1) \( \text{PEP} + \text{HCO}_3^- \rightarrow \text{Oxaloacetate} + \text{Pi} \)

**PEP Carboxykinase**

(2) \( \text{PEP} + \text{CO}_2 + \text{GDP} \rightarrow \text{Oxaloacetate} + \text{GTP} \)

**PEP Carboxytransphosphorylase**

(3) \( \text{PEP} + \text{CO}_2 + \text{P}_i \rightarrow \text{Oxaloacetate} + \text{ADP} + \text{PP}_i \)
An experiment was carried out to compare the relative rates of CO\textsubscript{2} fixation with PEP and pyruvate with and without the presence of ATP. The results are shown in Table 5.11. In both 54R and \textit{Ha. marismortui} CO\textsubscript{2} fixation was higher with the addition of 5mM pyruvate than 5 mM PEP. Interestingly, in 54R, when both 5mM pyruvate and 5mM PEP were added, the CO\textsubscript{2} fixation was 3 times greater than the sum of the CO\textsubscript{2} fixation with 5mM pyruvate only and the CO\textsubscript{2} fixation with 5mM PEP only. In 54R both PEP and pyruvate-dependent CO\textsubscript{2} fixation are inhibited by omission of ATP (Table 5.11). This suggests that pyruvate carboxylase is present as this enzyme requires ATP but PEP carboxylase does not. It also suggests that at least some of the PEP is being converted to pyruvate. If PEP is being converted to pyruvate and pyruvate carboxylase is responsible for CO\textsubscript{2} fixation, then PEP-dependent CO\textsubscript{2} fixation is inhibited if ATP is omitted. However the CO\textsubscript{2} fixation, when pyruvate or PEP are present, is not completely inhibited by omission of ATP. This may suggest that ATP is present in the cell extract preparation or it could suggest that ATP-independent CO\textsubscript{2} fixation is occurring e.g. via PEP carboxylase, PEP carboxykinase, PEP carboxytransphosphorylase or catalysed by POR. These results therefore indicate that pyruvate carboxylase and at least one other enzyme are responsible for CO\textsubscript{2} fixation in 54R. Table 5.11 suggests that pyruvate dependent CO\textsubscript{2} in \textit{Ha. marismortui} is not inhibited by omission of ATP from the assay. However in a previous experiment (Table 5.9) pyruvate-dependent CO\textsubscript{2} fixation in \textit{Ha. marismortui} was shown to be inhibited by omission of ATP. If pyruvate-dependent CO\textsubscript{2} fixation is unaffected by ATP, then pyruvate carboxylase can not be responsible for the CO\textsubscript{2} fixation. The CO\textsubscript{2} fixation observed may be due to the exchange catalysed by pyruvate: ferredoxin oxidoreductase or pyruvate may be converted to PEP and CO\textsubscript{2} fixation carried out by PEP carboxylase or other enzyme. PEP-dependent CO\textsubscript{2} fixation was however, inhibited by the omission of ATP (Table 5.11). An explanation for PEP-dependent CO\textsubscript{2} fixation being reduced by absence of ATP is that some of the PEP is being converted to pyruvate and a subsequent pyruvate carboxylase reaction is being inhibited by lack of ATP. However if this were the case, then the pyruvate dependent CO\textsubscript{2} fixation would also be reduced by the omission of ATP. Thus these results do not unequivocally indicate pyruvate carboxylase is responsible for the CO\textsubscript{2} fixation in \textit{Ha. marismortui}.
Table 5.11 The effect of ATP on PEP and pyruvate dependent CO₂ fixation.

<table>
<thead>
<tr>
<th>Assay Mixture</th>
<th>Mean Count per minute (Std Dev shown in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>54R</td>
</tr>
<tr>
<td>PEP</td>
<td>4996 (1653)</td>
</tr>
<tr>
<td>+ ATP</td>
<td></td>
</tr>
<tr>
<td>PEP</td>
<td>2456 (248)</td>
</tr>
<tr>
<td>- ATP</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>7136 (123)</td>
</tr>
<tr>
<td>+ ATP</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>5868 (927)</td>
</tr>
<tr>
<td>- ATP</td>
<td></td>
</tr>
<tr>
<td>Pyruvate + PEP</td>
<td>39510 (1570)</td>
</tr>
<tr>
<td>+ ATP</td>
<td></td>
</tr>
<tr>
<td>Pyruvate + PEP</td>
<td>20203 (1745)</td>
</tr>
<tr>
<td>- ATP</td>
<td></td>
</tr>
<tr>
<td>-Pyruvate -PEP</td>
<td>665 (58)</td>
</tr>
<tr>
<td>+ ATP</td>
<td></td>
</tr>
<tr>
<td>PEP + ATP</td>
<td></td>
</tr>
<tr>
<td>- cell extract</td>
<td></td>
</tr>
<tr>
<td>Pyruvate+ ATP</td>
<td></td>
</tr>
<tr>
<td>-cell extract</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td></td>
</tr>
<tr>
<td>Carboxylase (0.125 units)</td>
<td></td>
</tr>
</tbody>
</table>

Each assay contained 1.5 mg of protein. Background counts of scintillation fluid only had a mean of 18.
A further difference between the enzymes pyruvate carboxylase and PEP carboxylase is that pyruvate carboxylase is a biotin-containing enzyme. Therefore adding avidin, which binds to biotin, to the assay mixture should inhibit the activity of pyruvate carboxylase. If the added avidin is preincubated with excess biotin then the inhibitory effect should be lost. Experiments were carried out to determine the effect of avidin on pyruvate and PEP dependent CO$_2$ fixation in 54R and *Ha. marismortui*. The results are shown in Tables 5.12 and 5.13.

Avidin inhibited pyruvate-dependent CO$_2$ fixation in 54R by about 40% (Table 5.12). When the avidin was preincubated with biotin the loss of activity was reduced to about 30% (Table 5.12). In 54R avidin did not inhibit PEP-dependent CO$_2$ fixation; however if the avidin was preincubated with biotin and then added to the assay mix a reduction in CO$_2$ fixation of about 70% was observed. In *Ha. marismortui* avidin reduced pyruvate-dependent CO$_2$ fixation by about 20% and PEP-dependent CO$_2$ fixation by 80% (Table 5.12). When the avidin was preincubated with biotin, the pyruvate-dependent CO$_2$ fixation by *Ha. marismortui* was slightly higher than when incubated with avidin alone, but PEP-dependent CO$_2$ fixation in *Ha. marismortui* was even lower than when incubated with avidin only. The inhibition of pyruvate-dependent CO$_2$ fixation by avidin indicates that a biotin-containing enzyme is responsible for CO$_2$ fixation. This provides evidence for the activity of pyruvate carboxylase in 54R and *Ha. marismortui*. In 54R there was no inhibition of PEP-dependent CO$_2$ fixation by avidin, thus indicating that another non-biotin enzyme is responsible for CO$_2$ fixation e.g. PEP carboxylase, PEP carboxykinase or PEP carboxytransphosphorylase. In *Ha. marismortui* the PEP-dependent CO$_2$ fixation was inhibited by avidin, which could suggest pyruvate carboxylase is active, however when the avidin was preincubated with biotin there was an even larger reduction in the level of CO$_2$ fixation (in a similar way in 54R the PEP-dependent CO$_2$ fixation was not inhibited by avidin alone but it was inhibited by avidin when it had been preincubated with biotin). This does not fit with the activity of a biotin-containing enzyme such as pyruvate carboxylase. Thus it seems that in both *Ha. marismortui* and 54R there is also a second enzyme present, in addition to pyruvate carboxylase, which is responsible for CO$_2$ fixation.
Results and Discussion: Autotrophic Growth and CO₂ Fixation

Table 5.12 Effect of avidin on pyruvate and PEP dependent CO₂ fixation

<table>
<thead>
<tr>
<th>Assay Mix</th>
<th>Mean count per minute (Std Dev shown in brackets)</th>
<th>54R</th>
<th>Ha. marismortui</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td></td>
<td>2718 (238)</td>
<td>2089 (403)</td>
</tr>
<tr>
<td>Minus pyruvate</td>
<td></td>
<td>215 (13)</td>
<td>246 (25)</td>
</tr>
<tr>
<td>Pyruvate + avidin</td>
<td></td>
<td>1721 (60)</td>
<td>1725 (50)</td>
</tr>
<tr>
<td>Pyruvate + Avidin</td>
<td></td>
<td>1888 (124)</td>
<td>1798 (92)</td>
</tr>
<tr>
<td>+ Biotin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP</td>
<td></td>
<td>3909 (994)</td>
<td>5042 (2388)</td>
</tr>
<tr>
<td>- PEP</td>
<td></td>
<td>218 (15)</td>
<td>170 (14)</td>
</tr>
<tr>
<td>PEP + avidin</td>
<td></td>
<td>4867 (1876)</td>
<td>823 (50)</td>
</tr>
<tr>
<td>PEP + avidin + biotin</td>
<td></td>
<td>1306 (136)</td>
<td>730 (86)</td>
</tr>
</tbody>
</table>

The protein content of 54R cell extract in each assay was 1 mg and the protein content of Ha. marismortui was 0.6 mg. 2.5 units of avidin and 25 μg of biotin were used in each assay. Negative controls containing no cell extract had a count of 186 and background scintillation counts had a mean of 15.
### Results and Discussion: Autotrophic Growth and CO₂ Fixation

**Table 5.13: Assay to determine the effect of avidin on Pyruvate Carboxylase**

<table>
<thead>
<tr>
<th>Assay Mixture</th>
<th>Mean Count per minute (Std Dev shown in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1M KCl</td>
</tr>
<tr>
<td>Pyruvate Carboxylase + Pyruvate</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>(48)</td>
</tr>
<tr>
<td>Pyruvate Carboxylase + Pyruvate + avidin</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>(38)</td>
</tr>
<tr>
<td>Pyruvate Carboxylase + Pyruvate + avidin + biotin</td>
<td>279</td>
</tr>
<tr>
<td></td>
<td>(99)</td>
</tr>
</tbody>
</table>

Avidin used per assay was 1 unit. 0.2 units of pyruvate carboxylase (bovine liver) were used. 10µg of biotin were used per assay. Background scintillation counts had a mean of 19.
The assay mixture used in these experiments contains 1M KCl and it is possible that this affects the binding of avidin to biotin and may have influenced the results shown in Table 5.12. A control experiment was carried out with bovine pyruvate carboxylase (Table 5.13). When no KCl was present in the assay mixture, the presence of avidin reduced the activity by about 90%, and when the avidin was preincubated with biotin the activity was reduced by only 40%. Therefore the response of the bovine pyruvate carboxylase to avidin was as expected. When the assay was carried out in the presence of 1M KCl the activity of the bovine pyruvate carboxylase was inhibited by about 90% and the addition of avidin or avidin and biotin had no significant further inhibitory effect. Thus the effect of 1M KCl on the binding of avidin and biotin could not be assessed.

The results suggest that 54R and *Ha. marismortui* possess pyruvate carboxylase and that another carboxylation enzyme is also present. Assays to try to detect activity of PEP carboxykinase and PEP carboxytransphosphorylase were carried out. PEP carboxykinase is dependent on ADP or GDP for activity. Table 5.14 shows that PEP-dependent CO$_2$ fixation was not significantly inhibited by the omission of ADP or GDP from the assay mix in 54R, and only slightly inhibited in *Ha. marismortui*. It is therefore unlikely that 54R or *Ha. marismortui* possess any PEP carboxykinase. It is possible that ADP or GDP could be present in the cell extract preparation, thus not clearly showing the effect of adding ADP and GDP. The activity was strongly inhibited by omission of MgCl$_2$ and MnCl$_2$ from the assay mix. PEP carboxykinase has an absolute requirement for a divalent ion such as Mg$^{2+}$ or Mn$^{2+}$, but PEP carboxylase and PEP carboxytransphosphorylase also require divalent ions for activity. PEP carboxykinase plays an important role in reverse direction (formation of PEP from oxaloacetate) during gluconeogenesis, although in the presence of high concentrations of CO$_2$ the reaction goes predominantly in the direction of oxaloacetate formation. Halobacteria have been shown to have most of the enzymes responsible for gluconeogenesis (D’Souza & Altekar 1983), and strains *Ha. vallismortis* and *Hf. mediterranei* have been shown to have PEP carboxykinase activity (Rawal *et al.* 1988a).
The enzyme PEP carboxytransphosphorylase requires Pi for activity. In this assay (Table 5.14), PEP-dependent CO$_2$ activity is observed but there is no significant reduction of activity when Pi is absent in either 54R or *Ha. marismortui*. In both strains there was a reduction of activity when MgCl$_2$ and CoCl$_2$ were omitted. **There is therefore no evidence for PEP carboxytransphosphorylase activity in 54R or *Ha. marismortui***.

In both the PEP carboxykinase assay (Table 5.14) and the PEP carboxytransphosphorylase assays (Table 5.15), the PEP dependent CO$_2$ fixation was strongly inhibited by the absence of divalent ions. In both assays high levels of PEP dependent CO$_2$ fixation was observed, but could not be attributed fully to PEP carboxykinase or to PEP carboxytransphosphorylase. PEP carboxylase requires the presence of a divalent ion such as Mg$^{2+}$, Mn$^{2+}$, or Co$^{2+}$. Pyruvate carboxylase requires the presence of Mg$^{2+}$ ions. Thus the PEP dependent activity observed in the PEP carboxykinase and PEP carboxytransphosphorylase could be explained by the activity of PEP carboxylase or pyruvate carboxylase together with pyruvate kinase. Utter & Kolenbrander (1972) state that the identification of PEP-dependent fixation of CO$_2$ by crude extracts is not always a simple matter. The presence of biotin containing proteins such as pyruvate carboxylase, acetyl Co-A carboxylase or propionyl-CoA carboxylase could be confirmed by running cellular proteins on an SDS-PAGE gel, Western blotting and probing with avidin-horseradish peroxidase. The isolation of the genes for pyruvate carboxylase and PEP carboxylase and construction and analysis of defined mutants would help to increase understanding of the role of PEP carboxylase and pyruvate carboxylase in anaplerotic carboxylation in halobacteria.

No malate enzyme activity could be demonstrated in 54R or *Ha. marismortui* using the spectrophotometric method, although the radioisotopic assay showed that both 54R and *Ha. marismortui* exhibit malate dependent CO$_2$ fixation activity (Table 5.16). The activity observed is dependent on NADP and MgCl$_2$. This provides evidence that malate is being converted to pyruvate by NADP-linked malate enzyme followed by pyruvate dependent CO$_2$ fixation.
Table 5.14 PEP Carboxykinase Assay

<table>
<thead>
<tr>
<th>Assay Mix</th>
<th>Mean Count per Minute (Std Dev shown in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>54R</td>
</tr>
<tr>
<td>Complete (ADP)</td>
<td>37477 (6825)</td>
</tr>
<tr>
<td>Complete (GDP)</td>
<td>34458 (2910)</td>
</tr>
<tr>
<td>Minus ADP/GDP</td>
<td>32976 (928)</td>
</tr>
<tr>
<td>Minus MgCl₂/MnCl₂</td>
<td>1225 (395)</td>
</tr>
<tr>
<td>Minus PEP</td>
<td>216 (17)</td>
</tr>
<tr>
<td>Minus cell extract</td>
<td>149 (10)</td>
</tr>
</tbody>
</table>

54R cell extract contained 1 mg protein per assay. Ha. marismortui cell extract contained 0.6 mg protein per assay. Background scintillation counts were 21.
Table 5.15: PEP Carboxytransphosphorylase Assay

<table>
<thead>
<tr>
<th>Strain</th>
<th>Count per minute (Std Dev shown in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>54R</td>
</tr>
<tr>
<td>Complete</td>
<td>2971 (643)</td>
</tr>
<tr>
<td>Minus phosphate (Tris-HCl buffer)</td>
<td>2338 (394)</td>
</tr>
<tr>
<td>Minus MgCl₂ and CoCl₂</td>
<td>506 (40)</td>
</tr>
<tr>
<td>Minus PEP</td>
<td>573 (83)</td>
</tr>
<tr>
<td>Minus NaH₁⁴CO₃</td>
<td>20 (6)</td>
</tr>
<tr>
<td>Minus cell extract (phosphate buffer)</td>
<td>243 (20)</td>
</tr>
<tr>
<td>Minus cell extract (Tris-HCl buffer)</td>
<td>217 (13)</td>
</tr>
</tbody>
</table>

All assays contained 1 mg of protein. Background scintillation counts had a mean of 21.
Table 5.16: Malic Enzyme Assay

<table>
<thead>
<tr>
<th>Assay Mixture</th>
<th>Mean Counts per Minute (Std Dev in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>54R</td>
</tr>
<tr>
<td>Complete</td>
<td>21791 (1576)</td>
</tr>
<tr>
<td>Minus malate</td>
<td>914 (28)</td>
</tr>
<tr>
<td>Minus NADP</td>
<td>4114 (392)</td>
</tr>
<tr>
<td>Minus MgCl₂</td>
<td>23893 (984)</td>
</tr>
<tr>
<td>No cell extract</td>
<td></td>
</tr>
<tr>
<td>Complete assay mix</td>
<td></td>
</tr>
<tr>
<td>Malic Enzyme (0.3 unit)</td>
<td>118762 (10097)</td>
</tr>
<tr>
<td>+ Cell extract</td>
<td></td>
</tr>
</tbody>
</table>

Background scintillation counts had a mean of 18. 54R cell extract contained 1.0 mg protein per assay. Ha. marismortui cell extract contained 0.5 mg protein per assay.
Malate enzyme catalyses the following reaction:

\[
\text{L- malate} + \text{NADP}^+ \rightarrow \text{pyruvate} + \text{CO}_2 + \text{NADPH}
\]

Although this reaction is reversible in most organisms malate enzyme usually catalyses the oxidative decarboxylation of malate rather than the reductive carboxylation of pyruvate (Kornberg 1966). Bhaumik & Sonawat have previously demonstrated malate enzyme activity in \textit{Hb. salinarum} (1994).

Assays were also carried out to look for the presence of acetyl-CoA carboxylase. The presence of acetyl-CoA in assay mixtures has been shown to enhance CO$_2$ fixation when pyruvate or PEP are present (Tables 5.9 and 5.10). Results from the acetyl-CoA carboxylase assay are shown in table 5.17. \textbf{These results show that there is no acetyl-CoA carboxylase activity in 54R or \textit{Ha. marismortui}.} This not surprising since this enzyme catalyses the first reaction in the synthesis of fatty acids in bacteria whereas archaea do not possess fatty acids in their lipids.

Acetyl-CoA is known to positively regulate PEP carboxylase and pyruvate carboxylase and this presumably is the explanation for the positive effect on CO$_2$ fixation observed in previous assays (Table 5.9 & 5.10).

The results shown in Tables 5.7 – 5.17 indicate that 54R and \textit{Ha. marismortui} possess the enzymes pyruvate carboxylase and NADP-linked malate enzyme. PEP-dependent CO$_2$ fixation was observed, possibly indicating that these strains also possess PEP carboxylase, but the activity of PEP carboxylase and the activity of pyruvate carboxylase together with pyruvate kinase can not be distinguished in these assays. Additionally, pyruvate: ferredoxin oxidoreductase (POR) activity probably amounts for some of the CO$_2$ fixation observed. There is no evidence for any RuBisCo, PEP carboxykinase, PEP carboxytransphosphorylase, or acetyl-CoA carboxylase activity in strains 54R or \textit{Ha. marismortui}. Thus, pyruvate carboxylase and pyruvate: ferredoxin oxidoreductase are probably responsible for the $^{14}$CO$_2$ fixation observed by whole cells in section 5.2.
Table 5.17: Acetyl-CoA Carboxylase Assay

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>Counts per minute</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>54R</td>
</tr>
<tr>
<td>Complete</td>
<td>158 (19)</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>171 (15)</td>
</tr>
<tr>
<td>Minus Acetyl-CoA</td>
<td>237 (25)</td>
</tr>
<tr>
<td>Minus MgCl₂</td>
<td>202 (14)</td>
</tr>
<tr>
<td>Minus NaH₁₄CO₃</td>
<td>18 (4)</td>
</tr>
<tr>
<td>Minus cell extract</td>
<td>173 (13)</td>
</tr>
</tbody>
</table>

Background scintillation counts had a mean of 14. 54R cell extract contained 0.6 mg protein per assay. Ha. marismortui contained 1.2 mg of protein per assay.
Pyruvate carboxylase and PEP carboxylase play important roles in anaplerotic replenishment of the TCA cycle. Anaplerotic reactions were defined by Kornberg (1966) as those reactions which enable the provision of energy to be maintained under conditions in which removal of biosynthetic precursors would otherwise interrupt the pathways of energy supply. PEP carboxylase is the most widely distributed enzyme for the replenishment of oxaloacetate among prokaryotes. In most eukaryotes, pyruvate carboxylase is the only enzyme present for this function. Pyruvate carboxylase is found in some *Bacillus* and *Pseudomonas* species and PEP carboxykinase is found in some anaerobic bacteria. The gyoxylate cycle is used as an anaplerotic sequence in some *Arthrobacter* strains (Krulwich & Pelliccione 1979). Several microorganisms contain more than one enzyme for the replenishment of oxaloacetate. These include *Pseudomonas citronellolis*, *Pseudomonas flourescens*, and *Azotobacter vinelandii*, (Park et al. 1997), which all contain both PEP carboxylase and pyruvate carboxylase. *Corynebacterium glutamicum* has been shown to possess pyruvate carboxylase, PEP carboxylase and PEP carboxykinase (Peters-Wendisch et al. 1997).
5.4: $^{13}$C labelling experiments using NMR, TLC and ion exchange chromatography.

$^{13}$C-NMR was used to study the labelling of amino acids after growth of halobacteria in CHM containing 20 mM $^{13}$CO$_3^-$ and to deduce the pathways of CO$_2$ fixation. Principles of NMR and its use in studies of biosynthesis are described in Appendix 1. The chemical structures of amino acids are given in Appendix 2. The protein hydrolysate of halobacteria was also analysed using TLC. Quantitative amino acid analysis was carried out on protein prepared from strains 54R and Br3.

The chemical shifts of authentic amino acids at pH 1 are shown in Table 5.18. It was important to carefully adjust the pH of samples to minimise variations in chemical shifts. Chemical shifts of authentic amino acids varied between runs by a maximum of +/- 0.05 ppm. Some amino acids had chemical shifts, which were close to each other and which made assignment of signals difficult. For example the C1 of glutamic acid and C1 of isoleucine both had chemical shifts of 171.9. However, any peaks with this chemical shift were assigned to glutamic acid as no other isoleucine signals were detected in the NMR spectra. In addition isoleucine was not detected in any samples by TLC (see Table 5.19). Amino acid analysis (Table 5.20) showed that glutamic acid made up 12- 15% of total amino acids whereas isoleucine made up only 4%. Other examples where chemical shifts were close to each other included leucine C4 and arginine C4 both with a chemical shift of 24.2 and valine C2 and threonine C2 (with chemical shifts of 58.5 and 58.6 respectively) (Table 5.18). Tris, which was used as a buffer, was shown to have chemical shifts of 59.7 and 61.9. Serine C3 also has a chemical shift of 59.7.

Bi-dimensional Thin Layer Chromatography (TLC) using n-butanol: pyridine: H$_2$O (1:1:1) as the first phase and butanol: acetic acid: H$_2$O (12:3:5) as the second phase was used to analyse the amino acid content of the protein hydrolysate (Fig 5.14, Fig. 5.15). The protein hydrolysate from all strains was shown by TLC to contain aspartic acid, threonine, serine, glutamic acid, glycine, valine and leucine (Table 5.19).
Table 5.18: $^{13}$C chemical shifts of amino acids. Relative signal intensity is shown in brackets. Spectra were recorded at pH 1.0 in D$_2$O using the conditions described in Section 2.8.4.3.

<table>
<thead>
<tr>
<th>Amino Acid$^b$</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>C7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>171.9</td>
<td>52.4</td>
<td>25.2</td>
<td>29.8</td>
<td>176.7</td>
<td>(0.6)</td>
<td>(0.7)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>171.2</td>
<td>49.6</td>
<td>34.0</td>
<td>173.5</td>
<td>(1)</td>
<td>(1)</td>
<td>(0.5)</td>
</tr>
<tr>
<td>Arginine</td>
<td>172.0</td>
<td>52.8</td>
<td>27.3</td>
<td>24.2</td>
<td>40.7</td>
<td>157.2</td>
<td>(1.2)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>171.7</td>
<td>54.4</td>
<td>35.9</td>
<td>134.4</td>
<td>129.8$^c$</td>
<td>129.6$^c$</td>
<td>128.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>171.6</td>
<td>54.4</td>
<td>35.0</td>
<td>125.8</td>
<td>131.2$^d$</td>
<td>116.3$^d$</td>
<td>155.4</td>
</tr>
<tr>
<td>Serine</td>
<td>170.5</td>
<td>54.9</td>
<td>59.7</td>
<td>(0.3)</td>
<td>(1.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>170.7</td>
<td>58.6</td>
<td>65.5</td>
<td>19.2</td>
<td>(1.2)</td>
<td>(1.1)</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>171.8</td>
<td>58.5</td>
<td>29.3</td>
<td>17.1</td>
<td>17.6</td>
<td>(1)</td>
<td>(1)</td>
</tr>
<tr>
<td>Alanine</td>
<td>174.8</td>
<td>35.5</td>
<td>31.3</td>
<td>(0.9)</td>
<td>(1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>173.0</td>
<td>51.6</td>
<td>39.1</td>
<td>24.2</td>
<td>21.9</td>
<td>21.1</td>
<td>(0.7)</td>
</tr>
<tr>
<td>Lysine</td>
<td>170.5</td>
<td>52.8</td>
<td>26.6</td>
<td>21.7</td>
<td>29.5</td>
<td>39.3</td>
<td>(1.0)</td>
</tr>
<tr>
<td>Histidine</td>
<td>172.1</td>
<td>52.1</td>
<td>25.3</td>
<td>118.6</td>
<td>134.6</td>
<td>126.6</td>
<td>(1.1)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>171.9</td>
<td>57.5</td>
<td>36.1</td>
<td>25.0</td>
<td>11.2</td>
<td>14.4</td>
<td>(1.2)</td>
</tr>
<tr>
<td>Glycine</td>
<td>170.0</td>
<td>40.4</td>
<td>40.4</td>
<td>(0.3)</td>
<td>(1.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a: Chemical shifts varied +/- 0.05 ppm.
b: 5mg of each amino acid was dissolved in approx. 0.5 ml D$_2$O.
c: C5 and C9 of phenylalanine are chemically equivalent (see structures in Appendix 2), thus both have the same chemical shift. The signal intensity represents both C5 and C9. Likewise C6 and C8 of phenylalanine are chemically equivalent.
d: C5 and C9 of tyrosine are chemically equivalent (see structures in Appendix 2), thus both have the same chemical shift. The signal intensity represents both C5 and C9. Likewise C6 and C8 of tyrosine are chemically equivalent.
Amino acids were separated on cellulose coated plates. The first phase consisted of n-butanol : pyridine: H₂O (1:1:1) and the second phase consisted of n-butanol: acetic acid: H₂O (12:3:5) as described in section 2.8.4.4.
Results and Discussion: Autotrophic Growth and CO$_2$ Fixation

Fig. 5.15: Thin layer chromatogram of 54R protein hydrolysate

The protein hydrolysate (0.4 µl) was separated on cellulose coated plates. The first phase consisted of n-butanol: pyridine: H$_2$O (1:1:1) and the second phase consisted of n-butanol: acetic acid: H$_2$O (12:3:5) as described in section 2.8.4.4.
Table 5.19 Amino acids present in protein hydrolysate of halobacteria as shown by Thin Layer Chromatography.

<table>
<thead>
<tr>
<th>Strain</th>
<th>AMINO ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
</tr>
<tr>
<td>54R</td>
<td>+</td>
</tr>
<tr>
<td>Hr. saccharovorum</td>
<td>+</td>
</tr>
<tr>
<td>Ha. marismortui</td>
<td>+</td>
</tr>
<tr>
<td>E4</td>
<td>+</td>
</tr>
<tr>
<td>Hf. mediterranei</td>
<td>+</td>
</tr>
<tr>
<td>Hb. salinarium</td>
<td>+</td>
</tr>
<tr>
<td>O14.1</td>
<td>+</td>
</tr>
<tr>
<td>Hc. morrhuae</td>
<td>+</td>
</tr>
<tr>
<td>Br3</td>
<td>+</td>
</tr>
<tr>
<td>Hb. salinarum</td>
<td>+</td>
</tr>
<tr>
<td>(Hb. cutirubrum)</td>
<td></td>
</tr>
</tbody>
</table>

Amino acids were separated by bi-dimensional thin layer chromatography (TLC) on cellulose coated plates. The first phase consisted of n-butanol: pyridine: H₂O (1:1:1) and the second phase consisted of butanol: acetic acid: H₂O (12:3:5) as described in section 2.8.4.4.
Table 5.20: Amino Acid Analysis by Ion Exchange Chromatography of Total Cell Protein of 54R and Br3

<table>
<thead>
<tr>
<th>Amino Acid Residue</th>
<th>54R</th>
<th></th>
<th>Br3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nanomoles amino acid per 50 ml cells (mean of two samples)</td>
<td>% of total amino acids</td>
<td>Nanomoles amino acid per 50ml cells (mean of two samples)</td>
<td>% of total amino acids</td>
</tr>
<tr>
<td>D + N</td>
<td>3702</td>
<td>13.5</td>
<td>525</td>
<td>12.5</td>
</tr>
<tr>
<td>T</td>
<td>1527</td>
<td>5.5</td>
<td>264</td>
<td>6.3</td>
</tr>
<tr>
<td>S</td>
<td>1272</td>
<td>4.6</td>
<td>224</td>
<td>5.3</td>
</tr>
<tr>
<td>E + Q</td>
<td>4203</td>
<td>15.3</td>
<td>514</td>
<td>12.2</td>
</tr>
<tr>
<td>G</td>
<td>2816</td>
<td>10.2</td>
<td>466</td>
<td>11.0</td>
</tr>
<tr>
<td>A</td>
<td>2869</td>
<td>10.4</td>
<td>432</td>
<td>10.2</td>
</tr>
<tr>
<td>V</td>
<td>2174</td>
<td>7.9</td>
<td>334</td>
<td>7.9</td>
</tr>
<tr>
<td>M</td>
<td>456</td>
<td>1.7</td>
<td>76</td>
<td>1.8</td>
</tr>
<tr>
<td>I</td>
<td>1153</td>
<td>4.2</td>
<td>159</td>
<td>3.8</td>
</tr>
<tr>
<td>L</td>
<td>1994</td>
<td>7.2</td>
<td>322</td>
<td>7.6</td>
</tr>
<tr>
<td>Y</td>
<td>718</td>
<td>2.6</td>
<td>125</td>
<td>3.0</td>
</tr>
<tr>
<td>F</td>
<td>856</td>
<td>3.1</td>
<td>135</td>
<td>3.2</td>
</tr>
<tr>
<td>H</td>
<td>606</td>
<td>2.2</td>
<td>111</td>
<td>2.6</td>
</tr>
<tr>
<td>K</td>
<td>725</td>
<td>2.6</td>
<td>117</td>
<td>2.8</td>
</tr>
<tr>
<td>R</td>
<td>1421</td>
<td>5.2</td>
<td>219</td>
<td>5.2</td>
</tr>
<tr>
<td>P</td>
<td>1038</td>
<td>3.8</td>
<td>194</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Amino acid analysis was performed using a Pharmacia Alpha Plus Series II amino acid analyser and chromatography was performed on Ultropac 8 Polysulphonate ion exchange resin as described in 2.8.4.5.
Results and Discussion: Autotrophic Growth and CO₂ Fixation

In most strains, alanine, tyrosine, arginine and phenylalanine were also observed using TLC (Table 5.19). These amino acids were also the most commonly observed amino acids using NMR (Tables 5.21 & 5.22). Lysine and proline were also observed using TLC in most strains but these were not commonly observed using NMR. Histidine was only observed in 54R by TLC, but this was not observed using NMR in any strains. Methionine, and isoleucine were not observed in samples using TLC or NMR. Amino acid analysis was carried out using ion exchange chromatography at Cambridge University on protein prepared from 54R and Br3 (Table 5.20). This revealed that aspartic acid, glutamic acid, glycine and alanine were present in the highest quantities (over 10% of total amino acid concentration). Methionine, histidine, lysine, isoleucine, tyrosine, proline and phenylalanine were present as less than 5% of the total amino acid concentration. Methionine, histidine, lysine, isoleucine, and proline were not observed by TLC or NMR. Phenylalanine and tyrosine which, although were present at concentrations of around only 3%, were detected by both NMR and TLC. Acid hydrolysis (used to prepare amino acids for NMR, TLC and ion exchange chromatography) is known to result in complete destruction of tryptophan and partial destruction of serine and threonine. Protein prepared from strains 54R and Br3 had very similar % amino acid compositions, although the overall concentration of amino acids from Br3 was much lower than for 54R. This probably reflects the difficulty in lysis of Br3 cells prior to the preparation of the protein.

Attempts were made to obtain natural abundance (unlabelled) spectra for the amino acids of 54R grown in CHM with no 13C label. This would have enabled us to compare labelled and unlabelled spectra to deduce where the label was becoming incorporated. When cells from 100ml of growth medium were used no signals were detected. However when cells from 1L of growth medium were used, the resulting product was hard to dissolve in D₂O, but peaks corresponding to aspartic acid (C1 – C4) were observed (Fig 5.16). Aspartic acid was shown to be present in high concentration compared to other amino acids present by amino acid analysis (Table 5.20). A peak with a chemical shift of 59.7 was also observed; this chemical shift corresponds to Tris buffer.
Results and Discussion: Autotrophic Growth and CO₂ Fixation

Fig. 5.18: $^{13}$C-NMR Spectrum of the protein hydrolysate of 54R after growth in CHM (no $^{13}$C label).

![Diagram of the $^{13}$C-NMR Spectrum]

- Asp C4
- Aap C1
- Tris
- Asp C2
- Aap C3

- 177.242
- 173.364
- 179.082
- 157.563
- 139.118
- 137.613
- 134.492
- 33.570
- 28.130
- 24.706
- 22.309
- 18.897
- 17.147
.In addition, peaks were observed with chemical shifts of 137.6, 30.3, and 24.7 (Fig. 5.16). These peaks could not be assigned to any amino acid. The low solubility of this sample may mean that it is not truly representative of the amino acids present.

Although aspartic acid was the only amino acid observed in the natural abundance spectra of 54R, the assumption that all the other signals in spectra for samples grown with NaH\(^{13}\)CO\(_3\) represent carbons which are enriched with \(^{13}\)C, was not made. Some of the signals observed in the spectra may be natural abundance signals (see Fig. 5.17). The ratio of the intensity of the signal for each carbon atom in authentic amino acids was calculated. The ratio of the intensity of the peaks representing each carbon atom for authentic amino acids was found to be close to those in spectra shown in Pouchert & Bennke (1993). The ratio was compared to the ratio in amino acids in the protein hydrolysate of the cells grown with \(^{13}\)C. This allowed relative enrichment to be calculated. Signal to noise ratios were relatively poor despite the large number of scans carried out. This was due to the small sample size and probably also due to low incorporation of the \(^{13}\)C label. The accuracy of integration of peaks is reduced when signal to noise ratio is poor. Calculations of precise percentages of enrichment were not calculated, as they may have been inaccurate and misleading.

Due to the poor noise to signal ratio in some spectra, signals for some carbons in amino acids, which were present only in low concentrations, were lost under the noise. This meant it was not always possible to assess whether an amino acid was enriched. This occurred particularly for phenylalanine and tyrosine. In the spectrum for the protein hydrolysate of 54R (Fig. 5.17), for example, a peak was observed with the chemical shift of 116.3. This was assigned to tyrosine C\(_6\)/C\(_8\). No other amino acids have chemical shifts, which are near to this. However the six other signals expected for tyrosine were not visible and were assumed to be lost under the background noise. In an authentic sample of tyrosine the ratio of the intensity of the peaks C1: C2: C3: C4: C5/C9: C6/C8: C7 was 0.2: 0.3: 0.3: 0.2: 0.9: 1.0: 0.3. (C5 and C9 and C6 and C8 are chemically equivalent therefore only one signal is seen for each pair- see Appendix 2 for structures of amino acids). Therefore it is not surprising the peaks for C1 – C4 and C7 are not observed, as they would be small in comparison to the peak for C6/C8, and must be lost under the noise.
**Fig. 5.17:** $^{13}$C-NMR Spectrum of the protein hydrolysate of 54R after growth in CHM with 20 mM NaH$^{13}$CO$_3$. 

Results and Discussion: Autotrophic Growth and CO$_2$ Fixation
Results and Discussion: Autotrophic Growth and CO₂ Fixation

However we would expect to also see a peak for C5/C9, but do not, therefore signal for the C6/C8 peak must be enriched with ¹³C, but it is not possible to calculate the amount of enrichment without any signals to compare it to. Also in the spectrum for the protein hydrolysate of *Ha. marismortui* (Fig. 5.25), very small peaks are observed for phenylalanine C5/C9, C6/C8 and C7, however these were not accompanied by the remaining four signals for phenylalanine, as these must be lost under the noise, but without the presence of the remaining signals it is impossible to assess if these signals are enriched.

The ¹³C-NMR spectrum for 54R grown in CHM with 20 mM Na¹³HCO₃ is shown in Fig. 5.17. The high levels of labelling of amino acids in the protein hydrolysate from 54R were observed in glutamic acid C1, aspartic acid C1 and C4 and arginine C6 (Tables 5.21 & 5.22). Labelling was also observed in threonine C4, leucine C1, glycine C1, phenylalanine C6/C8 and tyrosine C6/C8 (Table 5.21 & 5.22). Many strains showed a similar labelling pattern to 54R with high levels of labelling seen in glutamic acid C1, aspartic acid C1 and C4, arginine C6 and leucine C1 (Tables 5.21 & 5.22). Labelling was also observed in glutamic acid C5 in most strains but at a lower level than labelling in glutamic acid C1. Labelling was also observed in serine C1, threonine C4, alanine C1, glycine C1, phenylalanine C6/C8 and C7 and tyrosine C6 in one or more strains (Table 5.21 & 5.22).

Aspartic acid is formed directly from oxaloacetate (Fig 5.19). As such the labelling in aspartic acid directly reflects that in oxaloacetate. If, as predicted from the enzyme assays in section 5.2, pyruvate carboxylase and/or PEP carboxylase are catalysing CO₂ fixation forming oxaloacetate we would expect to see labelling at C4 of aspartate (Fig. 5.18 and Fig. 5.19). It should be noted that ¹³C-NMR can not be used to distinguish between fixation by PEP carboxylase and that by pyruvate carboxylase, as they both result in labelling at the same position of oxaloacetate. High levels of labelling are also observed in C1 of aspartate in some samples. This can be explained by a backward flow of the TCA cycle to the symmetrical succinate molecule before it cycles back round to oxaloacetate (Fig 5.18). This produces labelling in oxaloacetate in both carboxyl groups and thus produces aspartate with both C1 and C4 labelled (Fig. 5.19).
Table 5.21: $^{13}$C labelling in amino acids of halobacteria when grown with $^{13}$CO$_2$

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glutamic Acid</th>
<th>Aspartic Acid</th>
<th>Arginine</th>
<th>Serine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{13}$C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$^{13}$C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(x 28)</td>
<td></td>
<td>(x 8)</td>
<td>ND</td>
</tr>
<tr>
<td>54R</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>(x 6)</td>
<td></td>
<td>(x 60)</td>
<td>ND</td>
</tr>
<tr>
<td>Hr. saccharovorum</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>(x 8)</td>
<td></td>
<td>(x 9)</td>
<td>ND</td>
</tr>
<tr>
<td>H4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(x 7)</td>
<td></td>
<td>(x 7)</td>
<td>ND</td>
</tr>
<tr>
<td>Ha. marismortui</td>
<td>++</td>
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<td>++</td>
<td>+</td>
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<td>(x 9)</td>
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<tr>
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<td></td>
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<tr>
<td>Hf. mediterranei</td>
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<td>+</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td></td>
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<td>(x 7)</td>
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</tr>
<tr>
<td>O14.1</td>
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<td>+</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(x 6)</td>
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<td>(x 6)</td>
<td>ND</td>
</tr>
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<tr>
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<td>(x 10)</td>
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</tr>
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<td>-</td>
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<td>ND</td>
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<td></td>
<td>(x 6)</td>
<td></td>
<td>(x 6)</td>
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</tr>
<tr>
<td>Hc. morrhuae</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

+ : Indicates positive enrichment with $^{13}$C  
++: Indicates a high level of enrichment with $^{13}$C, figures in brackets indicate approximate enrichment  
-: Indicates no enrichment occurred  
ND: indicates that enrichment could not be determined
Results and Discussion: Autotrophic Growth and CO₂ Fixation

Table 5.22: $^{13}$C labelling in amino acids of halobacteria when grown with $H^{13}CO_3$(Cont.)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Threonine</th>
<th>Leucine</th>
<th>Alanine</th>
<th>Glycine</th>
<th>Phenylalanine</th>
<th>Tyrosine</th>
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<tr>
<td></td>
<td>C4</td>
<td>C1</td>
<td>C1</td>
<td>C1</td>
<td>C6/C8</td>
<td>C7</td>
</tr>
<tr>
<td>54R</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td><em>Hr. saccharovorum</em></td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>E4</td>
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<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td><em>Ha. marismortui</em></td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Hb. salinarum</em></td>
<td>-</td>
<td>++</td>
<td>(x 9)</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(Hb. cutirubrum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hf. mediterranei</em></td>
<td>+</td>
<td>++</td>
<td>(x 8)</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>O14.1</td>
<td>+</td>
<td>++</td>
<td>(x 6)</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td><em>Hb. salinarium</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Br3</td>
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<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td><em>Hc. morrhuae</em></td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

+: Indicates positive enrichment with $^{13}$C

++: Indicates a high level of enrichment with $^{13}$C, figures in brackets indicate approximate enrichment

-: Indicates no enrichment occurred

ND: indicates that enrichment could not be determined
Results and Discussion: Autotrophic Growth and CO₂ Fixation

Fig. 5.18: Incorporation of \(^{13}\)CO₂ into TCA cycle.

PEPC = PEP carboxylase
PC = pyruvate carboxylase
OOR: 2-oxoglutarate: ferredoxin oxidoreductase
\* = labelling from PEP carboxylase activity
\# = labelling from pyruvate carboxylase activity
\^ = labelling resulting from scrambling at succinate
\o = labelling resulting from OOR
Labelling of the carboxyl group of pyruvate followed by the action of pyruvate carboxylase producing oxaloacetate labelled at C1 would also produce labelling at C1 of aspartate. Possible mechanisms which would label the carboxyl group of pyruvate are shown in Fig. 5.21. The action of malate enzyme would produce pyruvate labelled at the carboxyl group if the malate had become labelled at both carboxyl groups as a result of the backflow of the TCA cycle (Fig. 5.18 & 5.21). Malate enzyme activity has been demonstrated in a 54R and *Ha. marismortui* in this study (See section 5.3 and Table 5.16). Bhaumik & Sonawat (1994) studied pyruvate metabolism using $^{13}$C-NMR and [2-$^{13}$C] pyruvate. The labelling patterns they observed in glutamic acid indicated that backflow between oxaloacetate and succinate was occurring and also indicated that malate was being converted to pyruvate by way of malate enzyme. Ghosh & Sonawat (1998) used both [2-$^{13}$C] and [3-$^{13}$C] pyruvate to study the TCA cycle in *Hb. salinarum*. In this study further evidence was given for the activity of malate enzyme, but the labelling patterns indicted that back reactions from oxaloacetate to succinate were not occurring. POR has been shown to catalyse isotope exchange between pyruvate and H$^{13}$CO$_3^-$ (Raeburn & Rabinowitz 1971).
Results and Discussion: Autotrophic Growth and CO$_2$ Fixation

Fig. 5.20: Formation of glutamate from $\alpha$-ketoglutarate.

\[
\begin{align*}
\text{alpha-ketoglutarate} & \quad \text{glutamic acid} \\
\text{labelling from pyruvate carboxylase} & \quad \text{labelling from PEP carboxylase} \\
\text{labelling due to reverse TCA cycle} & \\
\end{align*}
\]
Fig. 5.21: Possible pathways by which $^{13}$CO$_2$ becomes incorporated into pyruvate

A:

\[
\begin{align*}
\text{malate enzyme} & \quad \overset{\text{malate}}{\longrightarrow} & \overset{\text{pyruvate}}{\longrightarrow}
\end{align*}
\]

(labelling due to symmetrical nature of succinate)

B:

\[
\begin{align*}
\text{Acetyl-CoA carboxylase} & \quad \overset{\text{Acetyl-CoA}}{\longrightarrow} & \overset{\text{Pyruvate}}{\longrightarrow}
\end{align*}
\]

C:

\[
\begin{align*}
\text{isotope exchange} & \quad \overset{\text{Pyruvate: ferredoxin}}{\longrightarrow} & \overset{\text{Oxidoreductase}}{\longrightarrow}
\end{align*}
\]

i: labelling due to isotope exchange with pyruvate
\(^\wedge\): labelling due to scrambling at succinate
*: labelling from pyruvate carboxylase
#: labelling from PEP carboxylase
#: labelling from Acetyl-CoA activity
This produces a pyruvate molecule with the carboxyl group labelled. After the reaction with pyruvate carboxylase to form oxaloacetate this would then result in labelling in C1 of aspartate (Fig. 5.21). A further mechanism by which the carboxyl group of pyruvate would become labelled is from acetyl-CoA carboxylase activity (Fig. 5.21). However no acetyl-CoA carboxylase activity was detected in assays carried out in this study (section 5.2) so this explanation is unlikely. In several samples, labelling in C4 was much higher than labelling in C1, however in other samples the labelling in C1 was all most as high as in C4 suggesting that either a large proportion of the label must flow through succinate or that the level of isotope exchange catalysed by POR is high. Threonine is synthesised directly from aspartate (Fig. 5.19). In several samples we see labelling in C4 of threonine as expected if C4 of aspartate is labelled. However no labelling was observed in C1 of threonine which would result from C1 labelling of aspartate.

Glutamic acid is synthesised from α-ketoglutaric acid (Fig. 5.20). Thus labelling of oxaloacetate due to the activity of pyruvate carboxylase and conversion of this to α-ketoglutaric acid via the TCA cycle will lead to labelling in C1 of glutamic acid (Fig. 5.18 and 5.20). Some labelling of glutamic acid was also observed in C5. One possible explanation for this is that reversal of the TCA cycle is occurring (Fig. 5.18). Halobacteria contain the enzyme 2-oxoglutarate: ferredoxin oxidoreductase. This enzyme is responsible for the conversion of α-ketoglutarate to succinyl CoA but this step is reversible. In many organisms this step is catalysed by the 2-oxoglutarate dehydrogenase complex and is irreversible. Conversion of succinyl-CoA to α-ketoglutarate would result in labelling at C1, C2 and C5 of α-ketoglutarate, giving labelling at C1, C2 and C5 of glutamic acid (Fig. 5.20). Labelling at C2 and C5 of glutamic acid originate from labelling in succinyl-CoA and the labelling at C1 is a result of incorporation of CO₂ by 2-oxoglutarate: ferredoxin oxidoreductase. Although labelling was observed in both C1 and C5 of glutamic acid no labelling was observed in C2 of glutamic acid. ¹³C-NMR studies of the TCA in Hb. salinarum by Bhaumik & Sonawat (1994) and Ghosh & Sonawat (1998) indicated that the TCA cycle was active only in the oxidative direction and provided no evidence for reversal of the TCA cycle. A second explanation for is that 2-oxoglutarate: ferredoxin oxidoreductase, catalyses an isotope exchange between α-ketoglutarate and ¹³CO₂ in a similar manner to the isotope exchange catalysed by POR. If ¹³C label became
incorporated into the C5 position of α-ketoglutarate this would in turn lead to labelling in C5 of glutamic acid, however 2-oxoglutarate: ferredoxin oxidoreductase has never been shown to catalyse this activity. Both pyruvate and 2-oxoglutarate: ferredoxin oxidoreductases have been purified from \textit{Hb. halobium} (Kerscher \& Oesterhelt 1981, Plaga \textit{et al.} 1992). The molecular masses were found to be 256 kDa and 248 kDa respectively by SDS/PAGE. Both enzymes have a $\alpha_2\beta_2$ subunit structure and contain two iron-sulphur clusters and two molecules of thiamin diphosphate. Both enzymes are highly similar in properties, including the catalytic mechanism, and therefore the catalysis of isotope between α-ketoglutarate and $^{13}$CO$_2$ is a feasible explanation for the labelling observed at the C5 position of glutamate.

Arginine is synthesised from glutamic acid via ornithine and citrulline. Labelling was observed in many samples in C6 of arginine. The origin of C6 in arginine is carbamoyl phosphate, which is synthesised by the enzyme carbamoyl phosphate synthetase from NH$_4^+$, ATP, H$_2$O and CO$_2$ (Fig. 5.22). Thus this represents another way in which $^{13}$CO$_2$ becomes incorporated into cells. Labelling of glutamic acid at C1 and C5 would produce labelling in arginine C1 and C5, but this was not observed.

Labelling was also observed at C1 of leucine and alanine. These amino acids are formed from pyruvate (Fig.5.23). Possible mechanisms by which pyruvate could become labelled have been discussed earlier and are shown in Fig. 5.21. Labelling of the carboxyl group of pyruvate would result in the labelling of C1 of leucine and alanine (Fig. 5.23).

Some labelling was also observed in C6/C8 and C7 of phenylalanine and in C6/C8 of tyrosine. In tyrosine and phenylalanine the C6 and C8 carbon atoms can not be distinguished by $^{13}$C-NMR as they are chemically equivalent (the chemical structures are shown in Appendix 2). Phenylalanine and tyrosine are synthesised initially from PEP and erythrose 4-phosphate via shikimate and chorismate (Fig.5.24). Labelling in C6 and C7 of the aromatic ring is consistent with labelling of the carboxyl group in pyruvate, resulting in labelling in erythrose 4-phosphate. Labelling would also be expected at the C1 position of phenylalanine and tyrosine, which originates from the carboxyl group of PEP. However no labelling was observed at C1 position.
Fig. 5.22: Formation of arginine via ornithine and citrulline and incorporation of $^{13}$C label.

\[
\Delta CO_2 + NH_4^+ + ATP + H_2O \rightarrow H_2N-\overset{\text{carbamoyl phosphate}}{\text{O}}-P-O^-\text{carbamoyl phosphate}
\]

\[
\overset{\text{COO-}}{\text{H-CH-NH_2}} \quad \overset{\text{NH_2}}{\text{CH}} \quad \overset{\text{CH_2}}{\text{CH}} \quad \overset{\text{CH_2}}{\text{CH}} \quad \overset{\text{NH_3+}}{\text{H}} \quad \overset{\text{NH_3+}}{\text{NH_3+}} \quad \overset{\text{COO-}}{\text{COO-}} \quad \overset{\text{COO-}}{\text{COO-}}
\]

\[
\overset{\text{COO-}}{\text{H-C-NH_2}} \quad \overset{\text{NH_2}}{\text{H}} \quad \overset{\text{H}}{\text{H}} \quad \overset{\text{NH_3+}}{\text{NH_3+}} \quad \overset{\text{COO-}}{\text{COO-}} \quad \overset{\text{COO-}}{\text{COO-}} \quad \overset{\text{COO-}}{\text{COO-}}
\]

\[
\overset{\text{COO-}}{\text{H-C-NH_2}} \quad \overset{\text{NH_2}}{\text{H}} \quad \overset{\text{H}}{\text{H}} \quad \overset{\text{NH_3+}}{\text{NH_3+}} \quad \overset{\text{COO-}}{\text{COO-}} \quad \overset{\text{COO-}}{\text{COO-}} \quad \overset{\text{COO-}}{\text{COO-}}
\]

\[
\overset{\text{COO-}}{\text{H-C-NH_2}} \quad \overset{\text{NH_2}}{\text{H}} \quad \overset{\text{H}}{\text{H}} \quad \overset{\text{NH_3+}}{\text{NH_3+}} \quad \overset{\text{COO-}}{\text{COO-}} \quad \overset{\text{COO-}}{\text{COO-}} \quad \overset{\text{COO-}}{\text{COO-}}
\]

*: labelling from PEP carboxylase
#: labelling from pyruvate carboxylase
\(\Delta\): labelling from carbamoyl phosphate
Results and Discussion: Autotrophic Growth and CO₂ Fixation

Fig. 5.23: Formation of leucine and alanine, and incorporation of $^{13}$C label.

\[
\begin{align*}
\overset{i}{\text{COO}}^- & \overset{\text{CH}_3}{\text{pyruvate}} \quad \overset{\text{i}^\wedge\text{COO}^-}{\overset{\text{H}}{\text{-NH}_3^+}} \\
\overset{i}{\text{COO}^-} & \overset{\text{H}}{\text{-NH}_3^+} \\
\overset{\text{leucine}}{\text{CH}_2} & \overset{\text{H}_3\text{C-C-CH}_3}{\text{alanine}}
\end{align*}
\]

i: labelling due to isotope exchange with pyruvate
\^: labelling due to scrambling at succinate
Results and Discussion: Autotrophic Growth and CO₂ Fixation

Fig. 5.24: Formation of phenylalanine, tyrosine, glycine via 3-phosphoglycerate.

\[ \begin{align*}
\text{pyruvate} & \quad \rightarrow \quad \text{oxaloacetate} \\
\text{ribulose-4-phosphate} & \quad \rightarrow \quad \text{3-phosphoglycerate} \\
\text{erythrose-4-phosphate} & \quad \rightarrow \quad \text{shikimate} \\
\text{phenylalanine} & \quad \rightarrow \quad \text{chorismate} \\
\text{tyrosine} & \quad \rightarrow \quad \text{glycine}
\end{align*} \]

\( ^{13} \text{C} \): labelling with 13-C
Phenylalanine and tyrosine were both shown to be present at low concentrations by amino acid analysis (Table 5.20). The carboxyl groups have a much lower signal intensity than the C6/C8 and C7 carbons, therefore even if they were enriched with $^{13}$C, the signals may still have been lost under noise. Labelling was also observed in serine C1 and glycine C1. These amino acids are formed from 3-phosphoglycerate. If pyruvate is labelled at the carboxyl group then this would result in labelling at the C1 of serine and glycine (Fig. 5.24).

Among the ten strains of halobacteria which were studied, most showed a similar pattern of labelling in glutamic acid, aspartic acid and arginine (Tables 5.21 & 5.22). Labelling in leucine, alanine, glycine, phenylalanine and tyrosine could not be determined, in several strains, due to the lack of signals for these amino acids as a result of the lower concentrations of these amino acids. The strains Br3 and *Hc. morrhuae* seem to show a different labelling pattern from the other strains (Table 5.21 & 5.22). The overall level of labelling was lower than for many of the other strains. They showed no labelling in aspartate C4 or glutamic acid C1, which was shown by all the other strains, and is indicative of CO$_2$ incorporation as a result of the activity of pyruvate carboxylase or PEP carboxylase. Additionally, there was no labelling in threonine C4, which was shown by several other strains and also indicates the activity of pyruvate or PEP carboxylase. No labelling was seen in arginine C6, in contrast to the majority of the other strains. This indicates that labelled CO$_2$ was not being incorporated into carbamoyl phosphate. Labelling was observed in leucine C1 and phenylalanine C7 in the protein hydrolysate of Br3. This indicates that the carboxyl group of pyruvate was becoming labelled, probably as a result of POR activity. Br3 and *Hc. morrhuae*, however, both show labelling in glutamic acid C5, indicating that reversal of the TCA cycle may be occurring. These observations indicate that CO$_2$ fixation by these two strains of halococci is not occurring by the route of pyruvate carboxylase or PEP carboxylase. CO$_2$ fixation by whole cells of these strains was observed to occur at similar levels as other strains of halobacteria (section 5.2), but these results indicate that CO$_2$ fixation in these strains may be occurring by a different mechanism than for the rest of the halobacteria tested. It should also be noted that there was some evidence of autotrophic growth in *Hc. morrhuae* (section 4.1). The CO$_2$ fixation pathways of *Hc. morrhuae* and Br3 deserve further investigation.
Halobacterium salinarum and other members of the genus Halobacterium have been shown to be metabolically different from many of the other members of halobacteria. For example they can not utilise glucose or trehalose as the sole source of carbon and energy (see section 3.1) and some require the addition of amino acids for growth (Larsen & Grant 1989). Members of this genus also have the ability to produce bacteriorhodopsin. Some authors have suggested that CO$_2$ fixation in halobacteria is linked to the possession of bacteriorhodopsin (Oren 1983, Danon & Caplan 1977). The $^{13}$C-NMR labelling patterns of the amino acids in Hb. salinarum and Hb. salinarum (cutirubrum) were similar to many of the other members of the halobacteria (Tables 5.21 & 5.22), indicating that CO$_2$ fixation of these strains is occurring primarily by fixation into oxaloacetate by pyruvate carboxylase or PEP carboxylase and also into carbamoyl phosphate for the synthesis of arginine. Labelling was observed in glutamic acid, aspartic acid, arginine and alanine in both strains. The observation of $^{13}$C label in these amino acids indicates that they can be synthesised by Halobacterium spp., although members of these genera often require amino acids for growth. Labelling was also observed in leucine in Hb. salinarum (cutirubrum) and in glycine in Hb. salinarum.

Hf. mediterranei has been shown to possess RuBisCo and also all the other enzymes necessary for the pentose phosphate cycle. The $^{13}$C labelling patterns of this strain did not differ from the other strains of halobacteria tested (Table 5.21& 5.22), indicating that fixation by pyruvate carboxylase/PEP carboxylase and into carbamoyl phosphate were the main pathways of incorporation of CO$_2$. RuBisCo discriminates strongly between $^{12}$CO$_2$ and $^{13}$CO$_2$. Cell carbon fixed by RuBisCo is enriched in $^{12}$CO$_2$ compared to the CO$_2$ of the growth media (Park & Epstein 1960). Thus even if RuBisCo was actively fixing CO$_2$ in this strain, only low levels of $^{13}$C label would be incorporated into the cell.

The strain Ha. marismotui was grown in both CHM + 20mM NaH$^{13}$CO$_3$ and MM + glucose + 20mM NaH$^{13}$CO$_3$ and a $^{13}$C-NMR spectra obtained for the protein hydrolysate produced (Fig. 5.25 & 5.26). The spectra produced after growth in MM + glucose was simpler than that produced after growth in CHM, but the labelling patterns were similar (Table 5.23). Labelling was observed in glutamic acid C1, aspartic acid C4 and threonine C4 in both samples indicating CO$_2$ was being
incorporated by the pyruvate carboxylase (or PEP carboxylase) pathway. Labelling was also observed in both samples in aspartate C1, indicating that either reversal of the TCA cycle to succinate and scrambling of label was occurring, or that pyruvate was becoming labelled as a result of the activity of POR. Both samples also showed labelling in arginine C6 indicating CO\textsubscript{2} was incorporated into carbamoyl phosphate. Both samples also showed labelling in glycine C1, which indicates pyruvate is becoming labelled. Labelled pyruvate would also produce the labelling in tyrosine C6/C8 and C7, which was observed after growth on MM + glucose, but not after growth on CHM. Labelling in tyrosine C6/C8 was, however, observed in strains 54R, \textit{Hr. saccharovorum} and E4 after growth on CHM. After growth on glucose no labelling in glutamic acid C5 was observed; this was seen after growth on CHM. Labelling in glutamic acid C5 is could be explained by reversal of the TCA cycle. This result indicates that the reversal of the TCA cycle does not occur when glucose is present. Glucose has been shown to inhibit citrate synthase activity in \textit{Hr. saccharovorum} (Hochstein 1978), however the labelling observed in glutamic acid C1 indicates that the TCA cycle is still operating in the presence of glucose.

The amino acid-labelling pattern was also investigated in a cell free extract prepared from 54R, which was incubated with pyruvate, PEP and NaH\textsuperscript{13}CO\textsubscript{3}. Much more extensive labelling was observed in the first experiment than in the second experiment (Table 5.24). The reduction in labelling in the second experiment is probably a result of the lower protein content in the cell extract preparation. In the first experiment, labelling was observed in glutamic acid C1 and C5, aspartic acid C4, threonine C4, leucine C1 and alanine C1. The pattern of labelling was, therefore, similar to that obtained for 54R after growth in CHM. Labelling in glutamic acid C1, aspartic acid C4 and threonine C4 indicate that labelling of oxaloacetate is occurring due to carboxylation by pyruvate carboxylase or PEP carboxylase (See Fig.5.18, 5.19 & 5.20). Labelling in glutamic acid C5 indicates that a reversal of the TCA cycle may be occurring, but the lack of labelling in aspartate C1 indicates that there is no flow from succinate back to oxaloacetate.
Fig. 5.25: $^{13}$C-NMR Spectrum of the protein hydrolysate of Ha. marismortui after growth in CHM with 20mM NaH$^{13}$CO$_3$. 

Results and Discussion: Autotrophic Growth and CO$_2$ Fixation
Results and Discussion: Autotrophic Growth and CO$_2$ Fixation

Fig. 5.26: $^{13}$C-NMR Spectrum of the protein hydrolysate of Ha. marismortui after growth in MM + glucose with 20mM NaH$^{13}$CO$_3$. 

[Diagram showing peaks and assignments]
Results and Discussion: Autotrophic Growth and CO₂ Fixation

Table 5.23: $^{13}$C enrichment of amino acids in Ha. marismortui when grown with $H^{13}CO_3^−$.

A comparison between growth in CHM and growth in MM + glucose.

<table>
<thead>
<tr>
<th>Growth Conditions</th>
<th>Glutamic acid</th>
<th>Aspartic Acid</th>
<th>Arginine</th>
<th>Tyrosine</th>
<th>Threonine</th>
<th>Glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1</td>
<td>C5</td>
<td>C1</td>
<td>C4</td>
<td>C6</td>
<td>C6</td>
</tr>
<tr>
<td>CHM</td>
<td>++ (8)</td>
<td>+</td>
<td>++ (9)</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MM + glucose</td>
<td>++ (10)</td>
<td>-</td>
<td>+</td>
<td>++ (12)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+: Indicates positive enrichment with $^{13}$C

++: Indicates a high level of enrichment with $^{13}$C

-: Indicates no enrichment occurred

ND: indicates that enrichment could not be determined

Table 5.24: $^{13}$C enrichment of amino acids when cell extract prepared from 54R was incubated with PEP, pyruvate, and $H^{13}CO_3^−$.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Glutamic acid</th>
<th>Aspartic Acid</th>
<th>Threonine</th>
<th>Leucine</th>
<th>Alanine</th>
</tr>
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<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C1</td>
<td>C5</td>
<td>C4</td>
<td>C4</td>
<td>C1</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+: Indicates positive enrichment with $^{13}$C

-: Indicates no enrichment occurred

Counts for the positive control containing $^{14}CO_3^−$ in expt. 1 were 105817, in expt. 2 counts were 92509.
Results and Discussion: Autotrophic Growth and CO\textsubscript{2} Fixation

The labelling of C\textsubscript{1} of alanine and leucine indicates that pyruvate C\textsubscript{1} is becoming labelled (see Fig. 5.23). The absence of glucose would mean that a higher proportion of oxaloacetate is probably being used for gluconeogenesis rather than entering the TCA cycle. This may explain why C\textsubscript{1} of alanine and leucine become labelled but C\textsubscript{1} of aspartate does not.

To summarise the information gained from the \textsuperscript{13}C-NMR experiments the labelling shown in aspartate C\textsubscript{4}, threonine C\textsubscript{4} and glutamic acid C\textsubscript{1} agrees with the fixation of CO\textsubscript{2} via pyruvate carboxylase or PEP carboxylase. Labelling of arginine C\textsubscript{6} indicates that CO\textsubscript{2} is incorporated into carbamoyl phosphate and this is then used in the synthesis of arginine. Labelling of leucine C\textsubscript{1}, alanine C\textsubscript{1}, serine C\textsubscript{1}, glycine C\textsubscript{1}, phenylalanine C\textsubscript{6} and C\textsubscript{7} and tyrosine C\textsubscript{6} indicate that the carboxyl group of pyruvate must become labelled. Labelling in the pyruvate carboxyl group could also be a result of malate enzyme activity, which has been shown in this study (section 5.3) and by other authors (Bhaumik & Sonawat 1994, Ghosh & Sonawat 1998). Some of the labelling observed is likely to be a result of isotope exchange between pyruvate and H\textsuperscript{13}CO\textsubscript{3}, which is known to be catalysed by pyruvate: ferredoxin oxidoreductase. Glutamic acid C\textsubscript{5} was shown to be labelled in some samples. This suggests that reversal of the TCA cycle may be occurring in halobacteria.

Propionate-stimulated CO\textsubscript{2} fixation in halobacteria has been observed by several authors (Danon & Caplan 1977, Oren 1983, Javor 1988, Altekar & Rajagopalan 1990, Rajagopalan & Altekar 1991), but was not shown in this study (section 5.2). Danon & Caplan (1977) and Oren (1983) suggested that propionate-stimulated CO\textsubscript{2} fixation led to the synthesis of \(\alpha\)-ketobutyrate. In the study by Javor (1988), analysis of keto-acids after \textsuperscript{14}CO\textsubscript{2} incorporation in the presence of propionate showed that \(\alpha\)-ketobutyrate was absent or a very minor product. Javor (1988) found that pyruvate was the major product which became labelled after both pyruvate and propionate stimulated CO\textsubscript{2} fixation. A similar result was also observed by Rajagopalan & Altekar (1991). These results could be explained by isotope exchange catalysed by POR. The amino acid labelling patterns observed in this study indicate that pyruvate becomes labelled after incubation with \textsuperscript{13}CO\textsubscript{2}.
Javor (1988) suggested a novel pathway of CO₂ fixation involving a glycine synthase reaction with CO₂, NH₄⁺, and a methyl carbon derived from the β-cleavage of propionate. Both Javor (1988) and Rajagopalan & Altekar (1991) found that propionate-stimulated CO₂ fixation is sensitive to trimethoprim, which is an inhibitor of dihydrofolate acid reductase. This enzyme is responsible for the regeneration of tetrahydrofolate from dihydrofolate. Tetrahydrofolate derivatives serve as donors of Cl₁ units in a variety of biosyntheses including the conversion of serine to glycine. Rajagopalan & Altekar (1991) observed no incorporation of ¹⁴CO₂ into glycine and therefore it seems unlikely that CO₂ fixation occurs via a glycine synthesis pathway as suggested by Javor.

The major pathway of propionate utilisation in many prokaryotes, including Propionibacterium, Ochromonas, Rhizobium and E. coli, is via the propionyl-CoA carboxylase pathway. Propionate is first activated to propionyl-CoA, then carboxylated to methyl-malonyl-CoA and finally isomerised to succinyl CoA. E. coli can also oxidise propionate to propionyl-CoA to pyruvate, whereby it is converted to acetyl-CoA and enters the TCA cycle. If halobacteria were utilising propionate by the propionyl-CoA pathway, then this would explain the enhancement of CO₂ fixation by the addition of propionate. Javor (1988) suggested that propionate is partly metabolised by this route in halobacteria. In this study, no propionate enhanced CO₂ fixation was observed in strains 54R or Ha. marismortui. It has also been shown that these two strains cannot utilise propionate as the main carbon and energy source (section 3.1), thus must lack the necessary enzymes for the propionyl-CoA pathway. Javor (1988) found that propionate-stimulated CO₂ fixation was not universal amongst the halobacteria. Growth of E. coli on propionate involves an adaptive process, the presence of Vitamin B₁₂ and the induction of B₁₂ containing enzymes. Thus, growth on propionate and propionate stimulated CO₂ fixation in halobacteria might be dependent on the presence of Vitamin B₁₂. The pathways of propionate utilisation in halobacteria could be investigated using ¹³C labelling propionate and ¹³C-NMR. A thorough investigation of the ability to utilise propionate as a carbon source, the presence of the enzymes necessary for the propionyl-CoA pathway and propionate stimulated CO₂ fixation should be carried out to confirm whether propionate-stimulated CO₂ is due to the induction of the propionate-CoA pathway.
Both Danon & Caplan (1977) and Oren (1983) observed light-dependent CO$_2$ fixation in halobacteria which contained bacteriorhodopsin. Oren (1983) showed that the presence of the bacteriorhodopsin containing purple membrane was essential for light-dependent CO$_2$ fixation. The purple membrane enables light energy to be transformed into a proton gradient which can be utilised for ATP generation (Danon & Stoeckenius 1974). Danon & Caplan (1977) and Oren (1983) suggest that absorption of light by bacteriorhodopsin leads to CO$_2$ fixation. There is no strong evidence for light-dependent CO$_2$ fixation in this study. CO$_2$ fixation of *Hb. salinarium (cutirubrum)* under aerobic conditions (Table 5.3) was shown to be enhanced slightly by illumination, but CO$_2$ fixation by other bacteriorhodopsin containing strains such as *Hb. salinarum* and *Hb. salinarum (halobium)* was not enhanced by light. Pre-incubation semi-anaerobically under illumination, so to induce bacteriorhodopsin, did not enhance CO$_2$ fixation. (Table 5.1). It is possible that only low levels of bacteriorhodopsin were produced under the conditions used and no attempt to measure the amount of bacteriorhodopsin was attempted. It would be informative to use $^{13}$C NMR to study the pathways of $^{13}$CO$_2$ fixation by bacteriorhodopsin containing strains under illuminated conditions. It is possible that the generation of ATP resulting from the absorption of light by bacteriorhodopsin results in increased CO$_2$ fixation by pyruvate carboxylase or that CO$_2$ fixation may occur by a completely different pathway. The relationship between the functioning of bacteriorhodopsin and the mode of metabolism have not been investigated. Additionally, there is very little knowledge about the distribution and activity of bacteriorhodopsin in the natural environment. *In vivo* absorption spectra of biomass from crystalliser salt ponds at Eliat, Israel did not show the broad shoulder of absorption at 570 nm, which is characteristic for bacteriorhodopsin (Oren *et al.* 1992) although bacteriorhodopsin was detected in the saltern ponds at Exportadora del Sal, Baja California (Javor 1983) and in the Dead Sea (Oren 1983). In the salt deposits there is however no light and so photoassimilation of CO$_2$ is not possible.
5.5: Summary of CO$_2$ fixation in halobacteria

No autotrophic growth was demonstrated by any strains of halobacteria. It has been demonstrated that incorporation rates of CO$_2$ are highest under aerobic conditions in all strains of halobacteria tested (except E4). $^{13}$CO$_2$ incorporation into amino acids was examined using $^{13}$C NMR. This indicated that the major routes of incorporation of CO$_2$ were by PEP carboxylase or pyruvate carboxylase into oxaloacetate, and also into carbamoyl phosphate for the synthesis of arginine. Enzyme assays in cell extracts indicated that pyruvate carboxylase is present in strains 54R and Ha. marismortui. PEP-dependent CO$_2$ fixation was also observed in cell extracts of 54R and Ha. marismortui, but the activity of pyruvate carboxylase together with pyruvate kinase and PEP carboxylase could not be distinguished in these assays. Fig. 5.27 summarises the CO$_2$ fixation pathways in halobacteria that are indicated by these experiments. The role of pyruvate carboxylase and PEP carboxylase is anaplerotic i.e.: to replenish TCA cycle intermediates, which are used in biosynthesis. It therefore seems that the major fixation of CO$_2$ in halobacteria is anaplerotic replenishment of the TCA cycle.
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Fig. 5.27: Summary of CO₂ fixation pathways in halobacteria deduced from $^{13}$C-NMR studies and enzymatic studies.

Amino acids labelled with $^{13}$C
Enzymes indicted by $^{13}$C labelling
Enzymes shown by enzymatic assays
There are several possible explanations explaining the origin of the halobacteria within the salt-deposits. They may have become embedded in the evaporites around 200 Ma ago when the salts were deposited. Within the salt deposits they may have grown very slowly or even at a normal rate using carbon available in the deposit. Alternatively they could have survived in a state of "suspended animation" with no active metabolism or reproduction. It is also possible that they may have gone through cycles of metabolic activity followed by periods of metabolic inactivity. It has been shown in laboratory experiments that halobacteria can survive within fluid inclusions for periods of at least several years (Norton & Grant 1988). If the halobacteria within the salt-deposits have been actively growing we would expect them to have evolved over the period of 200 million years to be metabolically adapted to life within the salt deposit. The selective pressures on halobacteria within the salt deposits would be different from those on halobacteria in surface environments, so we would expect metabolic differences between salt-mine halobacteria and halobacteria isolated from surface hypersaline environments to have evolved. However, if the halobacteria have remained in a state of "suspended animation" for around 200 million years then they will have maintained the metabolism of the population from the surface hypersaline environment and we would expect to see few differences between the salt-mine halobacteria and those isolated from modern surface hypersaline environments. However, an alternative hypothesis about the origin of the salt-mine halobacteria is that they are modern contaminants from surface hypersaline environments. In this case the metabolism of the salt-mine bacteria would also be no different from that of isolates from surface hypersaline environments.

The presence of a wide variety of strains of both halobacteria and halophilic bacteria within the salt mines suggests that the communities are well established. Isolation of halobacteria from residual brines, solution mining brine and rock salt also suggests that the salt-mine halobacteria are autochthonous residents of the salt-deposits. However halobacteria isolated from Winsford salt mine have a high optimal temperature for growth, compared to the low ambient temperature of the mine (around
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12°C (McGenity 1994). If halobacteria have been actively growing one would expect them to have adapted, growing optimally at the temperature of the mine. This suggests that halobacteria have either been present within the mine for a long period of “suspended animation” or that they have been introduced into the mine at a much more recent date.

54R was isolated from inside crystals of rock salt from Winsford Salt Mine. The 16S rRNA of 54R has been completely sequenced (McGenity 1994) and it has been shown to be a member of the genus *Halorubrum*. This strain shows many similarities in its metabolism to *Halorubrum saccharovorum*. Both 54R and *Hr. saccharovorum* could grow using glucose, glycerol, trehalose, pyruvate and some amino acids. Both strains produced acetic acid and other organic acids during growth on glucose and glycerol, although *Hr. saccharovorum* produced acid during growth on trehalose whereas 54R did not. Both strains could utilise DMSO and TMAO for anaerobic growth, but not fumarate, arginine or NO$_3^-$.

The $^{13}$C labelling patterns in amino acids after growth with $^{13}$CO$_2$ were similar, although 54R showed higher levels of CO$_2$ fixation than *Hr. saccharovorum*. 54R showed stronger emulsification of crude oil than *Hr. saccharovorum*. 54R could degrade both straight and branched-chain alkanes.

E4 was isolated from a brine pool in Winsford salt mine. Partial sequencing of 16S rRNA indicated that it is a member of the genus *Haloarcula*. E4 was shown to be phylogenetically close to *Ha. marismortui* and *Ha. sinaiiensis* (McGenity 1994). The metabolism of E4 was similar to that of *Ha. marismortui* in many aspects, although E4 grew well in MM with the addition of organic acids including acetate, iso-butyrate, propionate and iso-valerate, on which *Ha. marismortui* did not grow on. E4 showed poorer growth on substrates such as glycerol and glucose, which *Ha. marismortui* grew well on. Members of the genus *Haloarcula* that have been described are able to use a variety of carbon sources (Javor 1984), although some members of this genus isolated from the salt mine were more fastidious, growing only on glycerol or only on glucose. E4 and *Ha. marismortui* both produced acid during growth on glucose, glycerol and trehalose. Both strains grew anaerobically using DMSO, TMAO and NO$_3^-$. Both strains also grew well anaerobically in minimal media supplemented with TMAO or NO$_3^-$ with a single carbon source and produced acid during anaerobic
growth. Levels of CO$_2$ fixation were higher in *Ha. marismortui* than in E4, however the labelling in amino acids after growth with $^{13}$CO$_2$ showed was similar.

Br3 was isolated from solution mining brine at Lostock. Its origin was, therefore, rock salt, which was dissolved away by the water pumped into the mine. Complete sequencing of the 16S rRNA revealed it was a member of the genus *Halococcus* (McGenity *et al.* 1994). Its closest relative was found to be *Hc. salifodinae*, which was isolated from Permian rock salt in Austria (Denner *et al.* 1994). The sequence analysis of Br3 revealed it diverged from *Hc. morrhuae* about 200 Ma ago (McGenity 1994). These factors strongly suggest that Br3 was an autochthonous resident of the Triassic Cheshire salt deposit. However in this study Br3 and *Hc. morrhuae* have been shown to be metabolically very similar, utilising the same range of carbon sources and producing acid during growth on glucose, glycerol and trehalose. One difference was that *Hc. morrhuae* could utilise only TMAO, whereas Br3 could utilise both DMSO and TMAO for anaerobic growth. The $^{13}$C labelling in amino acids after growth with $^{13}$CO$_2$ was similar and differed from the labelling pattern seen in other members of the halobacteria. Br3 was shown to emulsify and degrade crude oil, whereas *Hc. morrhuae* had no effect on crude oil.

O14.1 was shown by partial sequencing to be phylogenetically related to *Hb. salinarum* (McGenity 1994). The complete sequence of the 16S rRNA was obtained for BbpA1 and this was also shown to be related to *Hb. salinarum* (McGenity 1994). However, both strains had different polar lipid patterns from *Hb. salinarum*. The genus *Halobacterium* is characterised by the presence of two sulphated glycolipids, S-TGD-1 and S-TeGD; in addition it possesses $C_{20}C_{20}$ diethyl derivatives of three phospholipids PG, PGP and PGS (Grant & Larsen 1989). Neither BbpA1 or O14.1 contained any detectable amounts of glycolipids. O14.1 had $C_{20}C_{20}$ diether derivatives of PG, PGP and PGS. BbpA1 had both $C_{20}C_{20}$ and $C_{20}C_{25}$ diether derivatives of PG, PGP and PGS (McGenity 1994). The sulphated glycolipid S-TGD1 is associated exclusively with the purple membrane (Kates 1992). If strains have been present in the salt deposits for 200 Ma in the dark then they may have lost the ability to make the purple membrane. The nutrition of strains BbpA1 and O14.1 also differs from that of *Hb. salinarum*. Both BbpA1 and O14.1 could grow using glucose, glycerol and trehalose as the sole carbon source supplementing minimal medium (MM). O14.1
could also utilise alanine. *Hb. salinarum*, however could only use glycerol and the
growth of *Hb. salinarum (halobium)* in MM was not enhanced by addition of any of
the carbon sources tested. O14.1, BbpA1 and *Hb. salinarum* all produced acid during
growth on glycerol. O14.1, BbpA1 and *Hb. salinarum (halobium)* were the only
strains tested in this study, which could use fumarate as an electron acceptor in
anaerobic growth. O14.1 and *Hb. salinarum* could also grow anaerobically using
DMSO, TMAO, and NO₃⁻. *Hb. salinarum (halobium)* also used DMSO and TMAO,
but not NO₃⁻. BbpA1 could only use TMAO, in addition to fumarate. The ¹³C
labelling in amino acids after growth with ¹³CO₂ were similar for O14.1, *Hb.
salinarum* and *Hb. salinarum (halobium)*. The labelling pattern of BbpA1 was not
determined. O14.1 was shown to emulsify crude oil and demonstrated a limited ability
to degrade alkanes in crude oil whereas *Hb. salinarum* had very little effect on crude
oil. There were, therefore, both similarities and some significant differences between
*Hb. salinarum* and BbpA1 and O14.1. Several other strains of halobacteria, which
were identical to BbpA1 in partial 16S rRNA sequence were also isolated from
Boulby and Winsford mines (McGenity 1994). It has been reported that a strain of
halobacteria with a polar lipid patterns like BbpA1 has been isolated from a Spanish
saltern (McGenity 1994). So although this group is not unique to the salt mines it may
be that it is more predominant in the salt-mine environment than the surface
environment.

Emulsification of crude oil by several strains of salt-mine halobacteria was observed
in this study. The surface isolates of halobacteria tested did not show good
emulsification of crude oil. Salt-mine strains 54R, 54P, Br3, Br5 and Br7 carried out
significant biodegradation of crude oil. It is interesting that 54R and 54P were isolated
from rock salt from Winsford mine and Br3, Br5 and Br7 were isolated from solution
mining brine from Lostock. Several studies have shown that the hydrocarbon-
oxidising ability of a bacterial community is greater if it has previously been exposed
to hydrocarbons (Leahy & Colwell 1990). Perhaps these salt-mine strains have the
ability to degrade crude oil, because they have been exposed to hydrocarbons within
the salt deposit. Inclusions and veinlets of oil occur frequently in the evaporite
deposits of the Northern Caspian region (Sonnenfeld 1984).
Compatible solutes are accumulated to high intracellular concentrations by bacteria and algae for osmoregulation. They, therefore, represent a large potential pool of carbon for the growth of halobacteria. The type of compatible solutes accumulated is specific for different groups of bacteria. Algae accumulate mainly glycerol and glycerol is therefore likely to be present at high concentrations in surface hypersaline environments due to the high population density of the algae Dunaliella. Within the salt-mines, phototrophic bacteria and algae are absent. Phototrophic bacteria accumulate mainly glycine betaine as a compatible solute. Thus glycerol and glycine betaine are not likely to be found within the salt-mines at high concentrations. If glycerol and glycine betaine were present at high concentrations within the hypersaline environment prior to deposition, some may be present within the salt-deposits. Chemoheterotrophic bacteria, which are present within the salt-mines, accumulate mainly ectoines and trehalose and also smaller amounts of glutamate, alanine and proline. The ability of halobacteria to grow on compatible solutes has been studied in this thesis. If salt-mine bacteria are well adapted to the salt-mine environment we might expect them to grow best on the compatible solutes which are likely to be present in the salt mines at the highest concentrations. These are likely to be trehalose and amino acids. However both salt-mine and surface isolates of halobacteria showed similar patterns of utilisation of compatible solutes. No strains could grow on glycine betaine, many strains could grow on trehalose and glycerol and some strains could grow using alanine, proline and glutamic acid. There is not therefore a good correlation between the ability to grow on different compatible solutes and the carbon sources, which are most likely to be present at highest concentrations in the salt deposit.

To conclude, the salt-mine strains did not differ greatly in their metabolic capacities from the surface strains. This would suggest that rather than actively growing within the salt-mines, they have either been present within the salt mines in a state of "suspended animation" or that they have been introduced into the salt-mines at a more recent date. One significant difference between the surface isolates and the salt-mine isolates was the ability of some salt-mine strains to degrade crude oil. The strains with this ability were all isolated from either rock salt from Winsford or solution mining brine from Lostock. Crude oil represents a potential carbon source for halobacteria.
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within inclusions in the salt-deposits. It would be interesting to investigate whether hydrocarbons or crude oil are present within inclusions in the Cheshire salt deposit.

In this study only five strains of salt-mine halobacteria have been studied in detail. It may be that these five strains are not typical members of the salt-mine community. Additionally, only particular aspects of the physiology were studied. A better approach to comparing the physiology of the two groups may be to screen much larger numbers of bacteria from the salt mines and surface environments to look for differences between the groups in a much wider number of physiological traits. However, in this study the aim was not merely to compare the two groups of organisms, but also to investigate aspects of the physiology in more detail. This would not have been possible with a much larger number of strains.

The population of halobacteria in both Boulby and Winsford mines consisted mainly of *Haloarcula* and *Halorubrum* species (McGenity 1994, Gemmell 1996). These are also the major groups isolated from surface environments such as solar salterns. Does this similarity reflect the similarity of the environments? Is the range of carbon sources available similar in both environments? In which case we would not expect to find many physiological differences between salt-mine halobacteria and halobacteria from surface environments. Or could this similarity reflect the similarity of the enrichment procedures used to isolate halobacteria from both environments? The development of gene probes would allow us to assess whether the strains which are isolated in the lab are truly representative of the main types present in the salt deposits. Other strains may exist which are metabolically very different from surface salt mine strains. In the study of salterns carried out by Benlloch *et al.* (1996), where DNA was isolated directly from the salterns and amplified by PCR, the clones were found to be not closely related to any archaea which had previously been isolated from the salterns. This indicated that the diversity was wider than had previously been indicated by the isolation of bacteria. The same could hold true for the salt-mine environment.

There is no information available regarding the nutrients available in the salt deposits at Boulby and Winsford. Information regarding the types of carbon compounds present, which might be used by as carbon and energy sources or as electron acceptors by halobacteria would be very useful. It would be particularly interesting to
investigate the nutrients available in residual brines and within fluid inclusions in the salt deposits. As some strains of halobacteria isolated from rock salt and solution mining brine have been shown to degrade alkanes it would be interesting to look for the presence of these in the salt deposits. This information would advance the understanding of the ecology of the salt mines greatly and would allow an assessment of whether halobacteria are metabolically well adapted to the environment.

Very little information is known about the types of (eu)bacteria which are present within the salt deposits. Characterisation of many strains of halophilic bacteria from salt deposits is currently being carried out. These, obviously, have a huge influence on the dynamics of the community in the salt deposits. The types of compatible solutes accumulated by these bacteria are particularly important, as this represents a large pool of potential carbon sources for halobacteria within the salt mines.

In addition to comparing halobacteria from the salt-mines with those from surface environments several new observations about halobacteria have been made regarding acid production during growth, anaerobic growth, and particularly CO₂ fixation by halobacteria. In this study, it has been observed that all strains of halobacteria tested produced acid products during growth on glycerol and glucose, with most also producing acid during growth on trehalose. Only one strain produced acid during growth on proline. Acetic acid was found to be the main acidic product, but smaller amounts of propionic, isobutyric, butyric, iso-valeric and pyruvic acids were also found. Tomlinson & Hochstein (1972b) and Oren & Gurevich (1994a) have both found that acetic acid was produced by halobacteria during growth on certain substrates. Oren & Gurevich (1994a) found that *Hr. saccharovorum, Ha. marismortui* and *Ha. vallismortis* produced pyruvate in addition to acetate, however pyruvate production by these strains was not observed in this study. BbpA1 was the only strain found to produce pyruvate during growth. The production of propionic, iso-butyric, butyric and iso-valeric acids by halobacteria has not been reported before. The production of acid during growth on sugars and glycerol is not fully understood. The enzyme acetyl-CoA synthetase is present in *Hr. saccharovorum* (Schafer et al. 1993). This enzyme couples acetate formation from acetyl-CoA with the phosphorylation of ADP via substrate level phosphorylation. The halobacteria do possess a full citric acid cycle, so it is not clear why some acetyl CoA is converted to acetate rather than...
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entering the TCA and being fully oxidised. The enzyme pyruvate: ferredoxin oxidoreductase (POR) has recently been shown to produce acetaldehyde, in addition to acetyl-CoA, in *Pyrococcus furiosus*. *P. furiosus* also possesses an enzyme aldehyde oxidoreductase (AOR), which converts acetaldehyde to acetate. If POR in halobacteria also produces acetaldehyde, this could explain why acetate is produced as a product during oxidative metabolism.

An investigation into the modes of anaerobic growth by halobacteria was carried out. The anaerobic growth of the genus *Halococcus* had not previously been investigated and members of this genus were shown to be able to utilise TMAO and DMSO. This study also showed that members of the genus *Halorubrum* can use TMAO and DMSO for anaerobic growth, also not previously shown. The study confirmed the findings of Oren & Truper (1990) in showing that members of the genera *Halobacterium* and *Haloarcula* could use DMSO and TMAO for anaerobic growth and also confirmed the findings of Mancinelli & Hochstein (1986) in showing that members of the genus *Haloarcula* can use NO$_3^-$ for anaerobic growth. In addition, it was shown that some members of the genus *Halobacterium* and closely related strains can grow anaerobically using NO$_3^-$ as the terminal electron acceptor. This study also confirmed that anaerobic growth utilising fumarate is limited to members of the genera *Halobacterium* and *Haloferax* (Oren 1991). In this study no strains were shown to grow by fermentation of arginine, although other authors have shown that *Halobacterium* spp. can grow by this mechanism (Hartman *et al.* 1980, Oren 1994a). Strains were also shown to grow anaerobically by fermentation in complex media (CHM) or with glucose or trehalose, confirming the results of Javor (1983).

There has been much controversy surrounding the ability of halobacteria to fix CO$_2$ or even grow autotrophically. No autotrophic growth has been demonstrated by halobacteria in this study. CO$_2$ fixation experiments carried out in this study indicate that CO$_2$ fixation is highest under aerobic conditions. Light or dark conditions or induction of bacteriorhodopsin did not significantly affect the levels of CO$_2$ fixation. $^{13}$C labelling in amino acids after growth with $^{13}$CO$_3$ indicated that the major CO$_2$ incorporation was into oxaloacetate via pyruvate carboxylase or PEP carboxylase. CO$_2$ was also incorporated into carbamoyl phosphate for the synthesis of arginine. $^{13}$C labelling patterns indicated that CO$_2$ fixation by reversal of the TCA cycle could be
occurring, but further work is needed to confirm this. Enzyme assays confirmed that halobacteria possess pyruvate carboxylase. The experimental evidence presented in this study indicates that the major CO$_2$ fixation in halobacteria, like many other heterotrophic microorganisms, is anaplerotic, for replenishment of the TCA cycle.


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Appendix 1

$^{13}$C-Nuclear Magnetic Resonance (NMR)

Principles of NMR (Based on Kemp 1991)

In this section the basic principles underlying NMR are described. Nuclei with a nuclear spin can be detected by nuclear magnetic resonance (NMR). Nuclei having a spin quantum number of $\frac{1}{2}$ include $^1$H, $^{13}$C, $^{19}$F, and $^{31}$P. Under the influence of an external magnetic field the nuclei can take up one of two orientations, either aligned with the field (low energy) or opposed to the field (high energy). In addition to aligning itself with or opposing an external magnetic field the nucleus also has precessional motion (Fig. 8.1). This is a movement of the nuclei around the axis of the applied magnetic field.

*Fig. 8.1: Representation of precessing nuclei, and the transition between aligned and opposed conditions. $B_0$ is the external magnetic field.*

If nuclei are irradiated with a beam of radiofrequency energy of the correct frequency the low energy nuclei can absorb this energy and move to the higher energy state.
level. The precessing nuclei will only absorb energy from the radiofrequency source if the precessing frequency is of the same frequency as the frequency of the radiofrequency beam. When this occurs the nucleus and the radiofrequency beam are said to be in resonance.

When a NMR spectrum is recorded nuclei are exposed to a powerful external magnetic field and will precess. They do not all precess at the same frequency. By irradiating the precessing nuclei with radiofrequency of the appropriate frequency, nuclei are promoted from the lower energy level to the higher level. The absorption of energy is recorded in the form of a NMR spectrum. The high-energy nuclei can undergo energy loss (or relaxation) by transferring energy to the surroundings. Even with a large external magnetic field the energy difference between the two levels is very small. When nuclei in the lower energy level undergo transitions to the higher energy level the populations of the two energy levels may approach equality. If this occurs no further absorption of energy can occur and the resonance signal will fade out. This situation is described as saturation of the signal.

The precise frequency, at which a nucleus precesses, depends on a number of factors including the chemical environment of the nuclei. The position of signals observed in a NMR spectrum is known as the chemical shift. The chemical shift position is expressed in δ units, which are proportional differences in parts per million (ppm) from an appropriate reference standard, usually tetramethyilsilane (TMS). In proton NMR the chemical shift range is ca. 10 ppm, whereas in the $^{13}$C NMR it is ca. 200 ppm. In proton NMR the area under each signal is proportional to the numbers of protons in that environment. The area under each signal is routinely integrated. It should be noted that in a spectrum with a noisy baseline the accuracy of the integration is reduced (McMurry 1996, Kemp 1991). $^{13}$C-NMR has a number of advantages and disadvantages over $^1$H-NMR. Firstly the natural abundance of $^{13}$C is only 1% compared to 99.98% for $^1$H. The magnetic moment of $^{13}$C is only about $\frac{1}{4}$ that of $^1$H. Thus $^{13}$C-NMR is relatively insensitive compared to proton NMR. However $^{13}$C-NMR has a large chemical shift range (200 ppm) compared to proton NMR (10ppm). This means that individual carbon atoms within the same molecule can easily be detected. Measurement of the number of protons in any environment (measured by integration of peak areas) is routine and accurate in proton NMR.
Signals produced by $^{13}$C-NMR can not be easily be accurately integrated to give an indication of the number of carbon atoms in that environment. In the pulsed FT mode, used for normal $^{13}$C-NMR, pulses of radiofrequency energy are applied with only short delays in between each pulse. Carbon nuclei with the longest relaxation times do not fully relax between pulses. The signals are therefore slightly saturated and of lower intensity. Quaternary carbons tend to have long relaxation times and therefore lower signal intensities. The nuclear Overhauser effect (nOe) causes enhancement of certain $^{13}$C peaks. The nOe effect is greatest on carbons attached to protons and has no effect on quaternary carbons. CH3 groups normally show a more intense signal than CH2 groups, which in turn have a more intense signal than CH groups. Quaternary carbons have the lowest intensities due to their long relaxation times and lack of enhancement by nOe. The nOe can be eliminated using special pulse sequences Additionally the saturation effect can be overcome by using longer delays between pulses, but this results in a substantial increase in the time required to produce the NMR spectrum (Kemp 1991).

Use of $^{13}$C-NMR in Metabolic Studies

$^{14}$C Tracer studies have been used extensively in the last 50 years for elucidation and characterisation of metabolic pathways. The feasibility of using $^{13}$C-enriched substrates for studying metabolism was indicated as early as 1972 (Eakin et al. 1972). However it is only since the development of high field instruments, within the last fifteen years, which has made metabolic studies using $^{13}$C-NMR possible. The major advantage of using $^{13}$C-NMR is that it allows labelling in individual carbon atoms in molecules to be detected. The entry of a $^{13}$C label into several metabolites can be investigated without the need for chemical isolation or purification. When $^{14}$C labels are used, the analysis of labelling patterns in individual compounds is a very tedious process. The low level of natural abundance of $^{13}$C is useful, as is means that $^{13}$C labelled precursors can be used in metabolic labelling experiments with little background interference. Using $^{13}$C label avoids the hazards associated with using radioisotopes. A further advantage of using NMR is that it is a non-invasive technique and can be used to study intact cells (Ekiel, Smith & Sprott 1983, Scott & Baxter 1981, Badar-Goffer & Bachelard 1991) $^{13}$C-NMR is a relatively insensitive technique, and the use of $^{14}$C labelled precursors remains a
Appendices

much more sensitive method. $^{13}$C label can only be accurately observed in metabolites which are extensively labelled and present at relatively high concentrations (Scott & Baxter 1981, Badar-Goffer & Bachelard 1991).
Appendix 2

Chemical structure of amino acids commonly observed by NMR in this study (Section 5.4)

- Glycine
- Alanine
- Leucine
- Serine
- Threonine
- Aspartate
- Glutamate
- Phenylalanine
- Tyrosine
- Arginine