# Chapter One

# INTRODUCTION

## 1.1 Extreme Environments

In general, moderate environmental conditions act to support a wide range of living organisms and usually have pH around neutrality, temperature between 20 and 40°C, air pressure not exceeding 1 atmosphere, adequate amounts of available water, and a source of nutrients (Satyanarayana *et al*., 2005). In contrast, extreme environmental conditions can be described as having a drastically reduced biodiversity with most organisms present being microorganisms (Gomes and Steiner, 2004). The range of extreme environments include high temperature conditions between 55 and 121°C or low temperature environments between – 2 and 10°C, high alkalinity environments that have pH values above 9 or high acidity environments that have pH values lower than 4 and high salinity environments containing 2 – 5 M NaCl (Gomes and Steiner, 2004, Hough and Danson, 1999, Van Den Burg, 2003). There are also high pressure environments that have hydrostatic pressures reaching up to 1400 atmospheres (Satyanarayana *et al*., 2005) and environments with high levels of ionizing radiation or the presence of heavy metals (Irwin, 2010). In addition, there are manmade extreme environments like cool houses, steam heated buildings and acid mine waters (Satyanarayana *et al*., 2005).

The anthropocentric definition of an extreme environment is an environment that deviates significantly from conditions suitable for human life in terms of temperature, pH or osmotic balance. A more scientific definition is that any environment where species diversity is very low and almost all are microorganisms is an extreme environment (Gilmour, 2010). Extreme conditions are usually not transient but remain constant in their physicochemical properties i.e. they are an inherent part of the ecosystem. The microorganisms which show optimal growth and not just tolerate extreme conditions are called extremophiles (Madigan and Orent, 1999).

## 1.2 Extremophilic Microoganisms

Extremophile comes from the Latin word "extremus" which means extreme and the Greek word "philia" which means love. Extremophiles can be defined in various ways according to the point of approach. The term extremophile, first used by MacElroy in 1974, refers to an organism that can grow and thrive in extreme environmental

conditions, as reported by Hendry (2006). Some researchers defined extremophiles as those microbes that thrive in abnormal environments where higher organisms such as mammals would be killed (Madigan and Orent, 1999, Irwin, 2010). The most wide ranging definition states that any organism which can live in extreme conditions should be considered to be extremophile (Rothschild and Mancinelli, 2002). However, Wainwright suggested the term "extremodure" to describe microorganisms which can survive, but cannot grow in extreme conditions (Wainwright, 2003). Essentially, one or more stress factors is necessary for the viability of extremophilic microorganisms (Satyanarayana *et al*., 2005). Most of the extremophiles that have been recently identified belong to the domain Archaea, however many bacteria as well as some eukaryotes can tolerate extreme environmental conditions (Van Den Burg, 2003).

Studies on extreme environments focus on the diversity of microorganisms and the molecular and regulatory mechanisms involved in growing in extreme environments. Extremophiles are structurally prepared to adapt to extreme environmental conditions. Extremophiles may adapt to one stress (e.g. high temperature or alkalinity) as either thermophiles or alkaliphiles. Furthermore, some extremophiles can adapt to more than one stress, for example, those which can adapt to both high temperature and alkalinity and hence called thermoalkaliphiles (Gomes and Steiner, 2004). Thus, extremophiles can be grouped depending on the stress conditions they can tolerate and subsequently thrive in (e.g. alkaliphiles, acidophiles, halophiles, thermophiles, psychrophiles and osmophiles) (Gilmour, 1990, Horikoshi, 1991, Gomes and Steiner, 2004, Hendry, 2006, Oarga, 2009, Irwin, 2010). Moreover, it is interesting to note that many extremophilic bacteria are amongst the most primitive of bacteria, as suggested by their location close to the root of the universal phylogenetic tree (Figure 1.1 and Table 1.1).

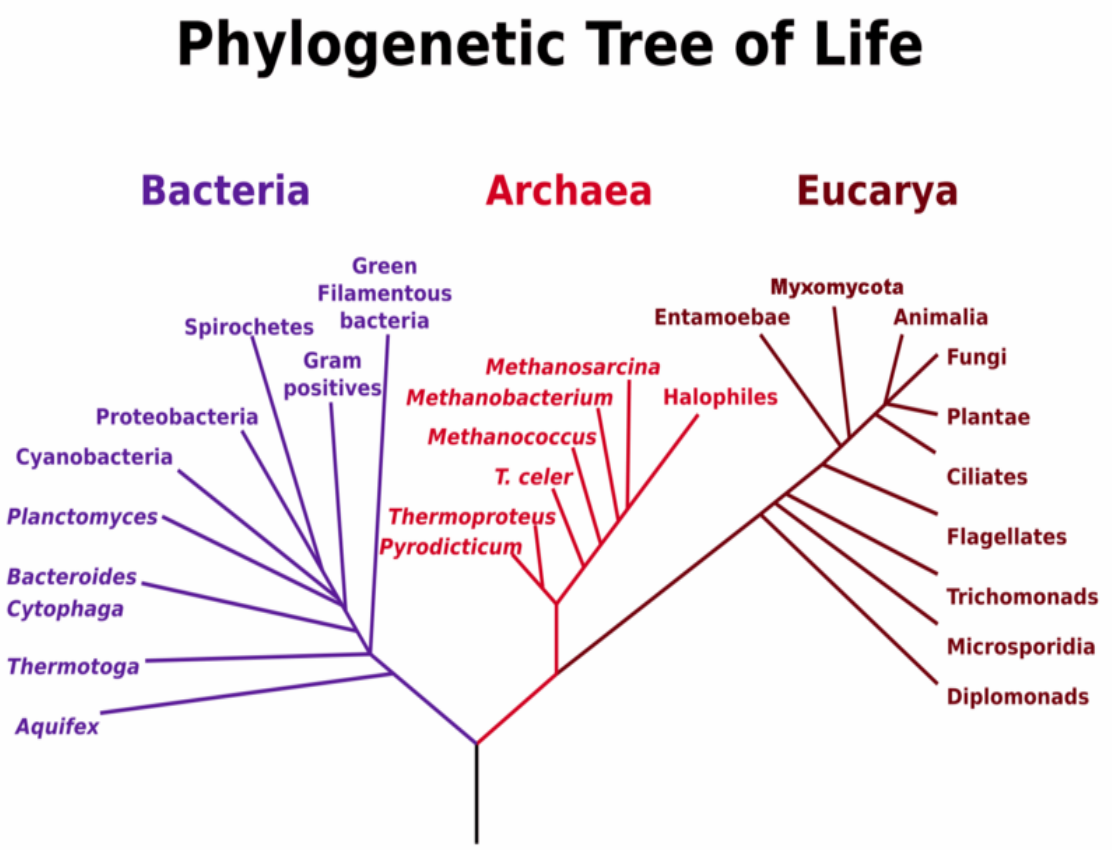



**Figure 1.1.** Universal phylogenetic tree as determined from comparative small subunit rRNA gene sequence analysis (16S and 18S rRNA genes), based on information in (Madigan, 2012). The bacterial phyla (e.g. *Thermotoga* and *Aquifex*) near the base of the tree are all thermophilic.

| Phenotype | Environment | Typical genus ( Bacteria and Archaea) |
|---|---|---|
| Thermophilic | 55 – 80°C | *Methanobacterium*, *Thermoplasma*, *Thermus**, some *Bacillus** species |
| Hyperthermophilic | 80 –113°C | *Aquifex**, *Archaeoglobus*, *Hydrogenobacter**, *Methanothermus*, *Pyrococcus*, *Pyrodictium*, *Pyrolobus*, *Sulfolobus*, *Thermococcus*, *Thermoproteus*, *Thermotoga** |
| Psychrophilic | – 2 to 10°C | *Alteromonas**, *Psychrobacter** |
| Halophilic | 2–5 M NaCl | *Haloarcula*, *Halobacterium*, *Haloferax*, *Halorubrum* |
| Acidophilic | pH<4 | *Acidianus*, *Desulfurolobus*, *Sulfolobus*, *Acidithiobacillus** |
| Alkaliphilic | pH>9 | *Natronobacterium*, *Natronococcus*, some *Bacillus** species |

**Table 1.1.** Extremophiles and their environments, genera marked * belong to the domain Bacteria; all others are Archaea. Modified from (Hough and Danson, 1999, Van Den Burg, 2003).

### 1.2.1 Extremes of Temperature and Life

#### 1.2.1.1 Thermophiles

Studying extreme environments has led to the discovery of a variety of microorganisms that can thrive in extreme conditions. Among these groups is the thermophile group where microorganisms can grow at high temperature levels. Due to the unusual properties of thermophilic microorganisms, they have become a key target of exploitation for biotechnology (Irwin, 2010). The term thermophile is used for those microbes which can grow at temperatures between 45 – 80ºC, while if they grow in environments with temperatures between 80ºC and 113°C then they can be called hyperthermophiles (Vieille and Zeikus, 2001, Stetter, 2006). Proteins and nucleic acids are normally denatured at temperatures reaching 100$^{o}$C and denaturation of lipids leads to an increase in fluidity of membranes to a lethal degree, while 100°C is optimal for growth of some hyperthermophiles (Madigan and Orent, 1999). Both thermophiles and hyperthermophiles have been assumed to be a rich source of many extremozymes (thermozymes or thermoenzymes) which are thermostable and can resist intracellular damage and disintegration when exposed to high temperatures (Vieille and Zeikus, 2001, Hendry, 2006).

Many prokaryotic microorganisms can grow at 50$^{o}$C (Satyanarayana *et al*., 2005), thus thermophiles are much more common than hyperthermophiles (Rothschild and Mancinelli, 2002). Thermophiles and hyperthermophiles can be found in different parts of the same areas where the temperature is high (e.g. hot springs and hydrothermal vents). Meanwhile thermophiles can be isolated from ordinary environments such as sun-heated soils, composts, oil wells and geothermally heated oil reservoirs (Madigan and Orent, 1999, Rothschild and Mancinelli, 2002, Irwin, 2010).

A variety of species of Bacteria and Archaea have been classified as thermophiles. These include *Thermus aquaticus, T. thermophilus, Thermodesulfobacterium commune, Sulfolobus acidocaldarius, Thermomicrobium roseum, Dictyoglomus thermophilum, Methanococcus vulcanicus, Sulfurococcus mirabilis* and *Thermotoga maritima*. Hyperthermophilic microorganisms on the other hand include, *Methanococcus jannaschii, Acidianus infernos, Archaeoglobus profundus, Methanopyrus kandleri, Pyrobaculum islandicum, Pyrococcus furiosus, Pyrodictium occultum, Pyrolobus fumarii, Thermococcus littoralis, Ignicoccus islandicum* and *Nannoarchaeum equitans* (Ghosh *et al*., 2003, Satyanarayana *et al*., 2005).

1.2.1.2. Psychrophiles

Psychrophiles are defined as those cold loving extremophiles that are capable of growth at temperatures of 15$^{o}$C or lower with maximum growth at temperatures of approximately 20$^{o}$C and a minimal growth temperature at 0$^{o}$C or lower (Morita, 1975). Low temperature environments can act as a stress factor and induce intracellular changes. These changes include the dangerous formation of ice crystals which rip cell membranes and cease solution chemistry due to the absence of liquid water. Intracellular freezing of water is almost always lethal (Feller and Gerday, 1997, Rothschild and Mancinelli, 2002, Madigan, 2012). However cold adapted organisms can protect themselves by producing cryoprotectants or antifreeze in which these molecules are responsible for preventing the formation of ice crystals that destroy cell membranes (Irwin, 2010). Psychrophiles usually exist in cold environments such as oceans where the average temperature is around 5$^{o}$C, and deep oceans have a constant temperature of approximately 1-4$^{o}$C. Psychrophilic microorganisms can exist in refrigerated environments and hence are potential contaminants of chilled food, so that psychrophiles are significant as food spoilage agents (Morita, 1975). Psychrophiles are widely used in industrial applications, at least partly because of ongoing efforts to decrease energy consumption, as a result of their low temperature catalytic activity and low thermal stability. Psychrophiles tend to have molecules with increased flexibility (Bentahir *et al*., 2000, Van Den Burg, 2003).

A number of genera have been reported to contain psychrophilic microorganisms including, *Alcaligenes*, *Alteromonas*, *Aquaspirillum*, *Arthobacter*, *Bacillus*, *Bacteroides*, *Brevibacterium*, *Gelidibacter*, *Methanococcoides*, *Methanogenium*, *Methanosarcina*, *Microbacterium*, *Micrococcus*, *Moritella*, *Octandecabacter*, *Phormidium*, *Photobacterium*, *Polaribacter*, *Polaromonas*, *Psychroserpens*, *Shewanella* and *Vibrio* (Satyanarayana *et al*., 2005).

### 1.2.2. Extremes of pH and Life

#### 1.2.2.1. Alkaliphiles (High pH)

Very high or very low concentrations of hydrogen ions (extremes of pH) will have a big effect on microbial activity and the ability of microorganisms to live in an environment. Alkaliphilic microorganisms require high pH (≥ 9) to thrive in addition to certain amounts of sodium ions normally in the form of NaCl and in the presence of glucose as a carbon source for their life style (Horikoshi, 1999, Le Romancer *et al.*, 2007). A minimum of 0.5 mM $Na^+$ is essential to maintain pH homeostasis (Sugiyama *et al*., 1985). Alkaliphiles can be isolated from a wide range of environments including pH neutral ones. The interest in alkaliphiles is due to their significance in industrial applications and particularly the utilization of their enzymes (Horikoshi, 1991, Peddie *et al*., 1999). Further details of alkaliphiles will be discussed in section 1.3.

#### 1.2.2.2 Acidophiles (Low pH)

Acidophiles are defined as those organisms which are largely prokaryotes and able to grow down to pH 1 with an optimal growth at pH of less than 4 (Magan, 2007). Presence of hydrogen ions in specific amounts is of significant importance to microorganisms in order to enhance metabolism and hence affect their viability, particularly in relation to energy (ATP) generation (Rothschild and Mancinelli, 2002). To grow at low pH, acidophiles must maintain their internal cytoplasmic pH close to neutral (maintain a pH gradient of several pH units) across the cellular membrane while producing ATP by the influx of protons through the $F_0/F_1$ ATPase (Baker-Austin and Dopson, 2007). There are more than one theory of pH homeostasis for adaptation in acidic environments which involve restricting proton entry by the cytoplasmic membrane and purging of protons from the cytoplasm. Thus movement of protons into the cell is decreased by intracellular net positive charge, and hence the cells have a positive inside membrane potential (Satyanarayana *et al*., 2005). Acidophiles contribute to numerous biogeochemical cycles which include the iron and sulphur cycles. The biodiversity of extreme acidophiles has recently attracted considerable attention with regard to their physiology and phylogenetic affiliations. The ability to use inorganic chemicals as electron donors is common among extreme acidophiles, as ferrous iron and elemental sulphur represent two major energy sources in many extremely acidic environments (Druschel *et al.*, 2004, Johnson and Hallberg, 2008). Johnson and

Hallberg stated that acidophiles display considerable diversity in how they assimilate carbon; some are obligate autotrophs, others obligate heterotrophs, while a large number use either organic or inorganic carbon, depending on the availability of the former (Barrie Johnson and Hallberg, 2008, Johnson and Hallberg, 2008).

### 1.2.3. High Salinity and Life (Halophiles)

Halophiles are those microbes which love salt and brine environments, where these microorganisms have the capacity to balance the osmotic pressure of the environment and resist the denaturing effect of salt. Halophilic microorganisms could be found in all three domains of life: Archaea, Bacteria, and Eukarya (Ma *et al*., 2010, Prakash, 2012). Halophiles prevent loss of water and adjust turgor in a highly saline environment by accumulation of osmotically active compounds. These compounds are normally small, neutral, highly soluble organic molecules that do not interfere with cellular metabolism, and are accumulated by either salt-induced *de novo* synthesis or salt-induced uptake from the medium, they are called compatible solutes (Oren, 2002, Frings *et al*., 1993). Halophiles are discussed in detail in section 1.4.

### 1.2.4. Other Environmental Extremes and Life

(Madigan, 2012) stated that studies of barotolerant and barophilic (piezophilic) microorganisms in deep sea show the existence of both types. Barotolerant and barophilic microorganisms can live at enormous hydrostatic pressures which are associated with depths of several kilometres (Le Romancer *et al*., 2007).

Surface microorganisms have developed various mechanisms to protect themselves from destructive radiations, (e.g. UV radiation). Microorganisms found in the atmosphere are exposed (and presumed to be resistant) to ambient radiation. UV light is damaging to DNA leading to the formation of dimers which may lead to lethal effects to the organisms. Two of the most UV resistance bacteria are *Deinococcus radiodurans* and the cyanobacterium *Synechococcus* sp. (Yang *et al.*, 2008, Snider *et al.*, 2012).

## 1.3. Alkaliphilic and Alkalitolerant Microorganisms

Alkaliphilic bacteria are considered as one of the extremophile groups and are characterized by the ability of thriving in alkaline (pH 9 or more) environments, where certain molecules, notably RNA, breakdown (Madigan, 1997). The microorganisms that can inhabit and grow in extremely alkaline environments can be divided into two main groups; alkaliphilic microorganisms and alkalitolerant microorganisms (Krulwich and Guffanti, 1989, Yumoto, 2002). Microorganisms that grow very well at pH values of 9 or 10 - 12, but cannot grow or grow slowly at pH values below 9 are considered to be alkaliphilic (Atanasova *et al*., 2008). Haloalkaliphilic microorganisms grow in environments which contain alkaline pH of 9 and high salinity up to 5 M NaCl, which is 10 times the saline/salt content of normal ocean water (Horikoshi, 1999). An example of an alkaliphilic microorganism is *Bacillus alcalophilus*, which has an intracellular pH between 8.4 and 9.0 (Kroll, 1990, Horikoshi, 1999). Alkalitolerant microorganisms, are those microorganisms that grow at pH 10 or above, and at the same time show good growth at or near neutral pH. These microorganisms are also known as facultative alkaliphiles (Krulwich and Guffanti, 1989).

Cultivation of alkaliphiles in the laboratory requires specially formulated media containing sodium salts for growth and buffering to the optimum pH of 9 to 11 (Kurono and Horikoshi, 1973). The presence of sodium ions in the surrounding environment has proved to be essential for effective solute transport through the membranes of alkaliphiles (Horikoshi, 1999, Horikoshi, 2008). Proton and or sodium electrochemical gradients are critical to energy generation at the plasma membrane of all living cells and in turn control pH homeostasis at the same time (Krulwich and Masahiro Ito, 2001 ).

### 1.3.1 Diversity of Alkaliphilic Microorganisms

The scientific study of alkaliphiles started fairly recently when Horikoshi began his experiments on alkaliphilic bacteria in 1968. However, the use of alkaliphilic microorganisms has a long lasting history in Japan (Horikoshi, 1999). Alkaliphilic microorganisms can be found in a wide range of environments including neutral and alkaline habitats and in some instances can even be isolated from acidic environments (Horikoshi, 1996, Horikoshi, 1999). Other environments include alkaline springs, and unexpected environments like garden soil, which is sometimes not truly alkaline.

However, it is found that soil samples and faeces are good sources of alkaliphilic microorganisms (Grant *et al*., 1990, Jones *et al*., 1994). Alkaliphilc microorganisms have been isolated from a wide range of environments for industrial applications (Yumoto *et al*., 2000).

Haloalkaliphilic microorganisms have been found in extremely alkaline saline environments including soda lakes and soda deserts (e.g. the Wadi Natrun in Egypt, the Rift Valley lakes of East Africa such as Lake Magadi, and the western soda lakes of the United States) (Horikoshi, 1999, Rees *et al*., 2004, Satyanarayana *et al.*, 2005).

### 1.3.2 Alkaline Environments (Habitats)

Alkaliphiles can be found where stable naturally occurring alkaline conditions are maintained. A combination of geological, geographical and climatic conditions plays a great role in this regard. Soda lakes and soda deserts represent the major type of naturally occurring highly alkaline environment, and may exhibit pH values of >11.5 (Grant, 2006). Alkaline environments may also include those of industrial environments at food processing plants (Grant *et al*., 1990, Jones *et al*., 1994) and industrially derived waste waters (ve Habitatlar, 2002).

Alkalinity of environments may also be chiefly attributed to biological activity, such as sulphate reduction or ammonification (Horikoshi, 1991). (Grant, 2006) observed that two types of alkaline environments exist on Earth. The first type is that of high calcium ($Ca^{2+}$) environments which are chiefly represented by groundwater, bearing high levels of CaOH. However the second type is low $Ca^{2+}$ environments and is primed by the presence of sodium carbonate ($Na_2CO_3$) and with low levels of magnesium ($Mg^{2+}$) ions, such as soda lakes and deserts. These environments are the most stable, naturally occurring highly alkaline environments found worldwide, where pH values of 10 and above are common (Ma *et al*., 2004, Tiago *et al*., 2004).

### 1.3.3 Mechanism of Alkaliphilic Cytoplasmic pH Regulation

(Mitchell, 1961) described the chemiosmotic theory. This theory illustrates the key role that membranes play in the formation of metabolic energy in the form of ATP (Albers *et al*., 2001). Electron transport in the cytoplasmic membrane of bacteria induces a difference in electrochemical potential of $H^+$ across the membrane (proton motive force, PMF, Δp), which is the sum of both membrane potential (transmembrane electrical potential) and transmembrane pH gradient, and is required for production of ATP by $H^+$-ATPase.

The respiratory chain pumps out $H^+$ from the inside to the outside of the membrane associated with electrochemical gradients due to the flow of electrons from NADH to $O_2$. ATPase produces ATP by translocating $H^+$ from the outside to the inside of the cell membrane of bacteria. Moreover, the cell membrane of bacteria possesses a number of transporter systems for obtaining substrates from the outside of the membrane, which work using the electrochemical potential of $H^+$ across the cell membrane (Δp) as their driving force.

Protons are involved in almost every physiological and biochemical reaction. Accordingly, it is necessary for every living cell to regulate its cytoplasmic pH around an optimal and compatible pH for cellular activities and stability of most proteins that support life and growth (Padan *et al*., 2001). Alkaliphilic bacteria, should maintain their intracellular pH near normality (i.e. within a fairly narrow range around pH 7) to grow in extreme environments with > pH 9 (Booth, 1985, Yumoto, 2002). Alkaliphiles require sodium ($Na^+$) for optimal growth and viability (Kurono and Horikoshi, 1973). The surrounding environment should contain $Na^+$ ions because their presence is essential for driving solute transport through the cell membrane in alkaliphiles. A $Na^+$ / $H^+$ antiporter system produces a sodium motive force that drives substrates accompanied by $Na^+$ into the cell (Kitada and Horikoshi, 1977, Horikoshi, 1999) (Figure 1.2).

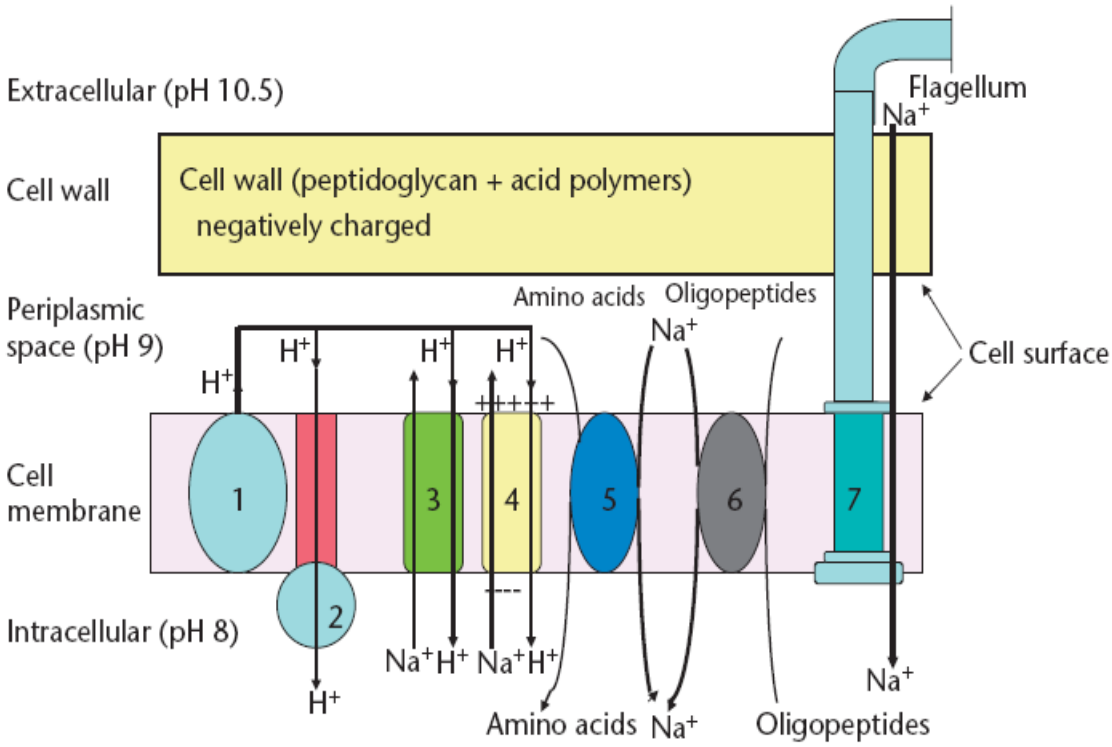



**Figure 1.2.** The movement of ions across the cell membrane of a Gram-negative alkaliphilic bacterium. 1. Respiratory chain pumping $H^+$ into the periplasmic space, 2. $F_1F_0$ – ATPase, 3. $Na^+/H^+$ antiporter driven by ΔpH 4. $Na^+/H^+$ antiporter driven by ΔΨ, 5. Amino acids/$Na^+$ symporter, 6. Oligopeptides/$Na^+$ symporter, 7. Flagellar motor. (adapted from Horikoshi, 2008).

## 1.4 Halophiles

Halophiles are salt-loving organisms that flourish in saline environments; this reflects a physiological need for salt above common values and is characterized by their demand for high concentration of salts, especially NaCl. Halophilic microorganisms have the capacity to balance the osmotic pressure of the environment and resist the denaturing effect of salt. Halophilic microorganisms could be found in all three domains of life: Archaea, Bacteria, and Eukarya (Ma *et al*., 2010, Prakash, 2012, DasSarma and DasSarma, 2012, Arahal *et al.*, 2008). Microorganisms show a wide range of salt tolerance and some demonstrate a requirement for salt. Halophiles can be classified according to their requirement for sodium chloride into slightly, moderately or extremely halophilic (Table 1.2). In addition halotolerant bacteria which are microorganisms that can grow over a large variety of salt concentrations where they can adapt to salinities as high as 4 or 5 M NaCl, however they can show optimum growth rate in the absence of salt (Gilmour, 1990, Oren, 2002). Halophiles have been isolated from various saline environments, for example salt lakes such as the Dead Sea or the Great Salt Lake; salterns, subsurface salts formation (brines, ponds) and solar salts and soils which are a source of halophilic microorganisms in which the soil adds to the water stress caused by salt concentrations (Kunte *et al*., 2002).

| | NaCl % Requirements | Molarity Requirements | Survival without NaCl | Remarks |
|---|---|---|---|---|
| Slight Halophilic | 1- 6 % | 0.2-1M | -ve | Wide range |
| Moderate Halophilic | 6 – 15 % | 0.5 - 2.5 M | -ve | Marine |
| Extreme Halophilic | 15 – 30 % | 2 - 5.2 M | -ve | True Halophiles |

**Table 1.2.** The main categories of halophilic microorganisms. Halotolerant organisms can survive in the absence of salt.

### 1.4.1 Adaptation of Halophilic Microorganisms

Two mechanisms are used to allow growth of microorganisms in high salinities, the high salt-in mechanism that uses inorganic compounds and the low salt-in mechanism that uses organic compounds (Gilmour, 1990). The organic osmolyte mechanism depends on special solutes called compatible solutes where their main function is to maintain osmotic equilibrium across the cell membrane. In addition the low salt-in-cytoplasm strategy is more flexible than the high salt in strategy that uses inorganic compounds (Kunte *et al*., 2002). Microorganisms that adapt to moderate and high salt environments use a variety of solutes, organic and inorganic solutes, to counter external osmotic pressure. Accordingly compatible solutes are divided into zwitterionic solutes, uncharged solutes, anionic solutes (carboxylates) and cationic solutes (phosphate and sulphate). Organic compatible solutes are synthesised by many microorganisms. In addition, organic compatible solutes can be accumulated from the environment.

#### 1.4.1.1 Inorganic Ions

Inorganic ions, mainly $K^+$ and $Cl^-$, are accumulated in the cytoplasm to a level that resembles the external salt concentration in the high salt-in mechanism. When the external NaCl concentration is increased in the surrounding environment, the cells accumulate KCl in the cytoplasm to counteract the external osmotic pressure, $Na^+$ is never accumulated in the cells above around 500 mM (Oren, 2002, Roberts, 2005). Salt-in mechanism of high salt adaptation was first discovered in extremely halophilic Archaea. The Archaea is the main group which uses the salt-in cytoplasm mechanism. Despite the fact that NaCl is the predominant salt in the medium, in the haloarchaeal cytoplasm, it was found that $K^+$ ions accumulated in the cell as proven in *Haloferax volcanii*. This potassium surge will be followed by an intracellular sodium increase when the bacteria enter the stationary phase of growth (Kunte *et al*., 2002).

#### 1.4.1.2 Organic Solutes

Organic solutes are osmolytes with low molecular weight called compatible solutes which do not interfere with the main function of the cell or with its metabolism and normal enzymatic activity. Halophilic microorganisms produce and/or accumulate organic compatible solutes such as ectoine, glycine betaine and proline. Due to *de novo* synthesis of compatible solutes or their presence in the environment, halophilic cells can accumulate compatible solutes and preserve the same enzymatic machinery as non

halophiles needing only minor adjustment of their ribosomal proteins (Kunte *et al*., 2002). In addition to their function of maintaining an osmotic equilibrium across the cell membrane, compatible solutes are effective stabilizers of proteins, membranes and even the whole cell from denaturation, inactivation and inhibition by hyperosmotic stress. This mechanism is widely spread among bacteria and eukaryotes and is found in some methanogenic archaea (Schubert *et al*., 2007, Oren, 2008, Konrad and Bar-Zvi, 2008, Fallet *et al*., 2010, Kunte *et al*., 2002).

| Organism | Main cytoplasmic solutes | Minimum water activity($a_w$) |
|---|---|---|
| Non phototropic Bacteria/ Freshwater cyanobacteria | Amino acids (mainly glutamate or proline)/sucrose, trehalose | 0.98 - 0.90 |
| Marine cyanobacteria | Sucrose | 0.92 |
| Marine algae | Mannitol, various glycosides, dimethylsulfoniopropinate | 0.92 |
| Halophilic cyanobacteria | Glycine betaine | 0.92 - 0.75 |
| Halophilic anoxygenic phototrophic purple Bacteria | Glycine betaine,ectoine, trehalose | 0.90 - 0.75 |
| Extremely halophilic Archaea and some Bacteria | KCl | 0.75 |
| *Dunaliella* (halophilic green algae) | Glycerol | 0.75 |
| Xerophilic and osmophilic yeasts | Glycerol | 0.83 - 0.62 |
| Xerophilic filamentous fungi | Glycerol | 0.72 - 0.61 |

**Table 1.3.** Compatible solutes of microorganisms (*Reproduced and updated from Madigan et al., 2012*).

## 1.5 Aquatic Microbial Ecology

Water is the solvent of life. It covers 70% of the earth's surface and it is an essential component of all cells. Water is a vital factor affecting the growth of microorganisms and required for life of cells. Water cycle on earth gives the opportunity for the exchange of components of water and subsequently microbes harboured in it. According to the type of water (salt and fresh water), organisms differ from one type to another based on osmotic properties. Many microbes inhabit ocean waters, they are very often small cells and this can be categorized as a typical characteristic of organisms that adapt to the harsh life of nutrient-poor environments (Lim, 1989, Madigan, 2012).

### 1.5.1 Ecological Factors in Aquatic Environments

Aquatic environments are highly variable in the factors that control the life and growth of microorganisms. In aquatic environments, oxygen-producing as well as oxygen-consuming organisms are present and the balance between photosynthesis and respiration controls the natural life cycles of oxygen, carbon and other elements such as phosphorus, nitrogen and metals. The wide range of aquatic bacterial diversity communities are found to be in harmony with each other in the same environment in terms of all being closely related and not competing for resources by a combination of habitat, metabolic and behavioural differentiation (Hunt, 2008). A more favorable environment for bacterial growth was found to be at interfaces due to nutrient accumulation from the bulk aqueous phase at such sites (Kjelleberg *et al*., 1982).

#### 1.5.1.1 UV Light in Environmental Bacteriology

Life on earth is solely based on solar energy either directly or indirectly, and one of these radiations is the ultraviolet light (UV). UV radiation strength in the environment estimated by standard measurement must be taken into consideration and put as an index to evaluate the conditions where humans and other living organisms exist. The UV index varies from zero at night to extremely high at midday. UV index is between 0-8 in the UK (8 is rare; 7 may occur on exceptional days, mostly in the two weeks around the summer solstice (*weatheronline.co.uk*) while it is between 5-16 in Saudi Arabia (*uk.weather.com*). UV irradiation can be classified as UVA, UVB and UVC

radiation. UVA light predominates at the earth's surface followed by small amount of UVB compared to UVA. UVC can be absorbed by the ozone layer where only 90% of UVB radiation can be absorbed by this layer in addition to the aid of water vapour, oxygen and carbon dioxide in the atmosphere. UVC is harmful to living beings (WHO, 2002). Surface microorganisms have developed a number of mechanisms to overcome and protect themselves from the destructive effect of UV radiation. The most important role of UV radiation is the damaging effect to DNA and thus the lethality to organisms (Snider *et al*., 2012). UV radiation has cytotoxic and genotoxic effects. As a result of UV radiation, a variety of DNA photolesions result including cyclobutane pyrimidine dimers (CPDs), 6-4 photoproducts and pyrimidine photohydrates. These lesions lead to mutagenic effects which will result in indogenic repair. These repair mechanisms include nucleotide excision repair (NER) and base excision repair (BER) (Reed *et al.*, 1996).

### 1.5.2 Survival of Extremophile Microorganisms in Non-Extremophilic Freshwater Habitats

It is not uncommon to find extremophilic microorganisms in non-extremophilic environments and this phenomenon is often associated with using molecular methods to identify microorganisms without culturing of the microorganisms (Purdy *et al*., 2004). Many microbial strains have been found in environments where they are not expected to grow. Alkaliphilic bacteria have been found in acidic soil samples with pH 4.0 as well as from neutral and alkaline soils (Horikoshi, 1999).

## 1.6 Molecular Approaches and Microbial Biodiversity

Traditionally the detection of microorganisms in water samples and other environmental samples is restricted to the possibility of cultivation of these microorganisms. Recently due to the use of molecular techniques it is possible to find out the content of any environmental sample's biodiversity without cultivating the organisms and this leads to knowledge of biodiversity of microbes and their interaction in complex environments (Gilbride *et al*., 2006). It has been thought that some organisms were limited to environmental extremes. However the discovery of Archaeal 16S rRNA gene sequences in sea water has changed many perceptions (Purdy *et al.*, 2004). The use of molecular techniques has revealed tremendous bacterial diversity due to its wide spectrum compared to classical method of survey (Hunt, 2008). The main goal for molecular approaches is to detect a broad range of prokaryotes in the environment and routinely rely on finding and classifying 16S rRNA genes which are then amplified using polymerase chain reaction (PCR) and primers with broad specificity (DeSantis *et al*., 2007).

## 1.7 Aims of the Project

Water samples were obtained from Bradford and Lathkill rivers, Derbyshire UK; as well as Alasfar Lake and Irrigation Canals, Hassa, Saudi Arabia. The aims of the project are listed below.

- To isolate extremophilic microorganisms from non extremophilic environments.
- To examine and compare the biodiversity of water samples from the Lathkill and Bradford rivers in the UK and the Alasfar Lake, Saudi Arabia.
- To identify and fully characterize selected microorganisms isolated from the water samples.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Sampling Sites

### 2.1.1 Al-Asfar Lake

Al-Asfar Lake is situated 13 kilometres (8.07 miles) to the east of the centre of the Al-Hassa region, and is one of the most important shallow wetland lakes in the Eastern Province of Saudi Arabia as well as the Gulf area. Al-Hassa is the largest oasis in the world, being approximately 20,000 hectares in size, as well as being one of the largest and oldest agricultural centres in the Arabian Peninsula. An irrigation system was put in place in 1971 and delivers 328,000,000 cubic metres of spring water to about 22,000 farms, with additional water supplied by treated wastewater from Al-Hofuf sewage station. The excess drainage water is collected by a drainage network and discharged into two evaporation lakes which are called Al-Asfar and Al-Uyoun. The lake has good wetlands, sabkhas and sand dunes as well as large expanses of open water. Salt tolerant vegetation is present in some of the sabkha areas and huge stands of *Phragmites* reeds occur around much of the lake. The habitat is very important for wildlife and birds in particular and is not something you would expect to find in a large desert.


**Figure 2.1.** Aerial image of the location of Al-Asfar lake in the Eastern Province of Saudi Arabia.

**Figure 2.2.** Al Asfar lake, Eastern Province, Saudi Arabia.

### 2.1.2 Bradford and Lathkill Rivers

2.1.2.1 Lathkill River

The River Lathkill is in the Peak District National Park in Derbyshire, England. First recorded in 1280, the name "Lathkill" possibly has Scandinavian roots, the old Norse *hlada-kill* translating as "narrow valley with a barn. In times of high rainfall the source of the river is Lathkill Head Cave, but in normal conditions the river rises from springs slightly further down the valley, close to the village of Monyash, west of Bakewell, and flows generally eastward past the village of Over Haddon and through the village of Alport (where it is joined by the River Bradford) until it meets the River Wye near Rowsley. Roughly six and a half miles from source to outfall, it is the only river in the district that flows over limestone for its entire length.

The river valley, known as Lathkill Dale, is popular with tourists who visit to enjoy its natural beauty and wildlife. The dale has a history of lead mining, and among the trees on the north side of the valley can be seen the remains of the 19th century Mandale Mine, including an old aqueduct and the ruined pump house, used as one of the last attempts to keep the mines drained and workable (Wikipedia web site).

2.1.2.2 Bradford River

The River Bradford is in the Peak District National Park in England. Its source is near Middleton-by-Youlgrave and after passing below Youlgreave it joins the River Lathkill at Alport. Less than 2 miles in length, its waters are very clear due to the limestone rock over which it flows, and its width has been enhanced by a number of weirs which also encourage white-throated dippers to breed in the pounds created. The valley is known as Bradford Dale, and the Limestone Way passes through it (Wikipedia web site).

## 2.2 Collection of Water Samples

### 2.2.1 Bradford and Lathkill Rivers

Various samples were collected from around Bradord and Lathkill Rivers into sterile 500 ml Duran bottles or sterile 50 ml Falcon tubes. Each sample was labelled at the time and a photograph taken at each sampling location. The pH of samples was measured using a Mettler Toledo MP225 pH meter. Samples were stored at 4ºC until required. The first set of samples were collected in March 2009 and the second set in June 2010.

### 2.2.2 Al Asfar Lake

Eight water samples were aseptically collected from the Al Asfar lake region as follows:

- Sample no.1: collected from treated water used for agriculture (Fudhol Station point)
- Sample no.2: collected from the pathway of treated water next to treatment station (Alluwaimi point)
- Sample no.3: taken from the canal of treated water for agriculture purposes (Bani Ma'an adjacent point)
- Sample no.4: Al Asfar drainage pathway 3-4 km prior to reaching the main lake near Alomran Reservoir
- Sample no.5: Al Asfar drainage pathway 2 km prior to reaching the main lake
- Sample no.6: Al Asfar lake (western point)
- Sample no.7: Al Asfar lake (northern point)
- Sample no.8: Al Asfar lake (eastern point)

All samples were collected aseptically into sterile medical tubes.

## 2.3. Growth Media

All media were prepared using distilled water and unless stated otherwise sterility was achieved by autoclaving for 20 min at 121°C (15 lbs $in^{-1}$).

Horikoshi medium was prepared using a slightly modified method as described by Horikoshi (1999) and is shown in Table 2.1 below:

| Ingredient | g $litre^{-1}$ |
|---|---|
| Yeast extract | 5 |
| Tryptone | 5 |
| Glucose | 10 |
| $KH_2PO_4$ | 1 |
| $Mg_2SO_4.7H_2O$ | 0.2 |
| $Na_2CO_3$ | 10 |

**Table 2.1.** Composition of normal Horikoshi medium for alkaliphilic microorganisms.

Two types of Horikoshi media, categorized as A and B media, were used where type A uses sodium carbonate to generate high pH while type B contains the organic buffer CAPS.

### 2.3.1. Horikoshi Medium Type A

This medium was prepared by initially dissolving 5 g tryptone, 5 g yeast extract and 0.2 g Mg $SO_4$. $7H_2O$ in 600 ml of distilled water in a 1000 ml Duran bottle. The volume was then made up to 650 ml with distilled water. Three separate solutions were prepared by adding 10.6 g $Na_2CO_3$ to 180 ml of distilled water and then adjusted to pH 10 using either 1 M $H_2SO_4$ or 1 M NaOH. The solution then was made up to 200 ml with distilled water. 10 g glucose was added to 100 ml of distilled water. 1 g $KH_2PO_4$ was added to 50 ml of distilled water.

$Na_2CO_3$ and the first three solutions (tryptone, yeast extract and $MgSO_4.7H_2O$) were mixed together and adjusted to the pH 10 again. This solution and glucose and $KH_2PO_4$ were autoclaved at 120°C for 20 minutes. Glucose and $KH_2PO_4$ were then aseptically added to the main solution to avoid glucose caramelisation and phosphate precipitation.

A small amount of medium was then removed aseptically and checked to ensure that the pH was still 10.

For solid medium, 15 g bacteriological agar No.1 (Oxoid) was added to the main solution before making it up to 650 ml. After autoclaving, solutions were put in a water bath at 50°C. Once cooled to 50°C, $Na_2CO_3$, glucose and $KH_2PO_4$ solutions were added to the main solution, mixed and immediately poured in plates.

### 2.3.2. Horikoshi Medium Type B

This medium was prepared by dissolving 5 g tryptone, 5 g yeast extract and 0.2 g Mg $SO_4$. $7H_2O$ in 700 ml of distilled water in a 1000 ml Duran bottle. 50 ml of 1M CAPS buffer was then added and the pH was adjusted to 10 using 1M $H_2SO_4$ and 1M NaOH. The volume was then made up to 850 ml with distilled water. Two separate solutions were prepared by adding 10 g glucose to 100 ml of distilled water and 1 g $KH_2PO_4$ was added to 50 ml of distilled water.

All three solutions were then autoclaved and once cooled the three solutions were combined aseptically and gently mixed to give 1 litre. A small sample of medium was then removed aseptically and the pH checked to ensure that it is pH 10.

For solid medium, 15 g bacteriological agar No.1 (Oxoid) were added to the main solution before making it up to 850 ml. After autoclaving, solutions were put in a water bath at 50°C. Once cooled to 50°C, glucose and $KH_2PO_4$ solutions were added to the main solution, mixed and immediately poured into plates.

### 2.3.3. LuriaBertani (LB) Medium

LB medium consisted of 5 g yeast extract, 10 g tryptone and 10 g NaCl in 1 litre of distilled water to produce liquid medium. Solid medium consisted of 5 g yeast extract, 10 g tryptone 10 g NaCl and and 15 g bacteriological agar No.1 (Oxoid) to form 1 litre. Various NaCl concentrations were used to form different molarities of LB medium.

### 2.3.4. Minimal Medium M9

M9 minimal medium was prepared by dissolving 11.3 g M9 minimal salts (Sigma M-6030) in 980 ml distilled water and adjusting the salt concentration as required. The following four solutions were autoclaved separately and added as follows; 1 ml $MgSO_4$ (1 M), 9 ml $NH_4Cl$ (5 g in 45 ml distilled water), 9 ml glucose (15 g in 45 ml distilled water) and 1 ml $CaCl_2$ (100 mM). Solid M9 minimal medium was prepared by adding 15 g bacteriological agar No.1 (Oxoid).

## 2.4. Morphology of Bacterial Stains

### 2.4.1. Gram Stain

A Gram stain was used to determine the shape of bacterial cells, the stain was carried out on overnight bacterial cultures (Kirkpatrick *et al*., 1993). A droplet of water was placed onto a slide, and an inoculating loop was used to transfer some bacterial cells into the water droplet. The slide was air-dried and then heat fixed by passing through a Bunsen flame. The cells were then washed in an excess of crystal violet solution and

then treated with iodine solution as a mordant for approximately one minute. Ethanol was adopted to decolourise the stain (for about 30 seconds). The cells were then counterstained with safranin solution for 1 minute and the excess stain was removed by washing and blotting dry. The slides were examined under a light microscope at 1000 times magnification (oil immersion). Gram-positive cells appear purple, while Gram negative cells appear pink in colour.

### 2.4.2. Motility of Bacteria

The motility of the bacteria was carried out using soft agar stabbing (tube method). LB medium was prepared where it was modified by adding 5 g bacteriological agar no.1 (Oxoid) to make the medium soft to ease clarification of bacterial movement.

## 2.5. Purity and Maintenance of the Strains

The purity of strains was monitored by streaking a loopful of the culture on agar plates. After incubation the plates were first examined for colonial morphology and then single colonies were picked off and observed as a wet mount using a phase contrast microscope.

All isolated strains were maintained by adding 2 ml of overnight culture (active inocula) to 2 ml of sterile glycerol (50% v/v) and kept in a freezer at – 20ºC or – 80ºC. When required, the frozen cells were thawed at room temperature and inoculated into 250 ml conical flasks plugged with a piece of sponge containing 50 ml of the appropriate medium and incubated overnight at a suitable temperature on a rotary shaker at 250 rpm. When pure colonies have developed on the plates a piece of parafilm was stretched around each plate's edge and then one set of plates was stored at room temperature and the other set in the fridge until required. Sub-culturing of the strains was also performed routinely once every two weeks to minimize contamination over time by adding 1 ml of overnight culture into 250 ml conical flasks containing 50 ml of the medium incubated at the appropriate temperature according to the type of bacteria and placed on a rotary shaker at 250 rpm and then kept at room temperature until required.

## 2.6. Effect of Medium pH on Growth of Bacteria and Growth Curve Determination

The effect of pH on the growth of bacteria was determined using overnight cultures in Horikoshi medium. 1 ml of active inoculum from each strain was inoculated into Horikoshi medium pH 8, pH 9 and pH 10 and the optical density (OD) at 600 nm was measured using the Unicam Helisα spectrophotometer against a distilled water blank in 1 ml plastic cuvettes. To produce a growth curve, 4x 250 ml conical flasks containing 50 ml of each pH value Horikoshi medium were each inoculated with 1 ml of the same pH adapted cells from an overnight culture. The $OD_{600}$ was measured against a water blank immediately after inoculation then every one hour over an incubation period at 25ºC on a rotary shaker at 250 rpm. The growth curves were plotted against time of incubation.

## 2.7 Effect of Salt Stress on Growth of Bacteria and Growth Curve Determination

The effect of salt stress was measured by adding NaCl to LB medium to give 0.5 M, 1 M, 1.5 M, 2 M, 2.5 M, 3 M, 3.5 M and 4 M. In contrast for M9 minimal medium, different molarities were used to find out their effect on growth of halophilic as well as haloalkaliphilic strains. 1 ml of active inoculum from each strain was inoculated into LB medium and the optical density (OD) of 600 nm was measured using the Unicam Helisα spectrophotometer against a distilled water blank in 1 ml plastic cuvettes. To produce a growth curve, 4x 250 ml conical flasks containing 50 ml of each molarity medium were inoculated with 1 ml of the same molarity adapted cells from an overnight culture. The $OD_{600}$ was measured against a water blank initially after inoculation then every hour over the incubation period at 37ºC on a rotary shaker at 250 rpm. The growth curves were plotted against time of incubation.

## 2.8. Measurement of Respiration Rate of Bacterial Cells (Oxygen Uptake)

Respiration rate was measured using a modified Clarke oxygen electrode (Hansatech Scientific Instruments, Kings Lynn, UK) with a Servoscribe 1S potentiometric chart recorder as described by Delieu and Walker (Delieu and Walker, 1972). The electrode consists of platinum wire sealed in plastic as the cathode and an anode of circular silver

wire bathed in a saturated potassium chloride (KCl) solution which forms a bridge between the electrodes. The electrodes were separated from the reaction mixture (chamber) by an oxygen- permeable Teflon membrane. The reaction mixture in the Perspex container was stirred constantly with a small magnetic stirring rod.

When a voltage was applied across the two electrodes using the polarising meter the platinum electrode became negative with respect to the reference electrode and the oxygen in the solution is thought to undergo electrolytic reduction at the cathode.

$$4H^{+} + O_2 + 4e^{-} \longleftrightarrow 2H_2O$$

The flow of current in the circuit when the polarising volts were set between 0.5 and 0.8 V varied in linear relationship to the partial pressure of oxygen in solution. The current flowing was measured by connecting the electrode to a sensitive potentiometric chart recorder. The reaction chamber of oxygen electrode was maintained at a constant temperature similar to the culture temperature (25°C), by circulating water from a temperature controlled water bath.

The calibration of the oxygen electrode was achieved by placing 2 ml of distilled water in the reaction chamber for 15 minutes and setting the chart recorder to 100% oxygen saturation point and then a small amount of sodium dithionite was added to remove all oxygen from the chamber to give the 0% oxygen concentration point. After washing, air saturated water was added and this gave the 100% value. The difference between 0% and 100% is known as the range. The sodium dithionite reacts with dissolved oxygen and removes it from the solution as shown below:

$$Na_2S_2O_2 + O_2 + H_2O \longleftrightarrow NaHSO_4 + NaHSO_3$$

To calculate the respiration rate we need to know the concentration of $O_2$ in air saturated water at 25°C and the protein content of the bacterial suspension. Then the following equation is used:

**$O_2$ uptake or Respiration Rate (µmoles $O_2$ mg protein$^{-1}$ h$^{-1}$) =**

$$\frac{\text{standard}}{\text{range}} \times \frac{\text{Number of units change}}{\text{Time}} \times \frac{60}{\text{mg protein present in sample}}$$

- **Standard:** Amount of oxygen solubility in 2 ml medium (sample) = 0.660 µmoles ml$^{-1}$ at 30°C or 0.722 µmoles ml$^{-1}$ at 25°C
- **Range:** Units taken from calibration (0 – 100%)
- **Number of units:** Number of units are read directly from the chart recorder, it is normal to draw best fit straight line over 5 min
- **Time:** The length of time in minutes for which the sample was measured
- **60:** This converts the time from minutes to hours
- **Protein present in sample (mg):** this relates to the amount of protein in a sample of 2 ml of cells from the Bradford assay.

To prepare cells for measurements in the oxygen electrode, the $OD_{600}$ of all cultures was measured and 20 ml of cells from each sample (grown overnight at pH 8, pH 9, and pH 10 in Horikoshi medium and 0.5 M, 1 M, 1.5 M, 2 M, 2.5 M, 3 M, 3.5 M and 4 M LB medium) were transferred to a 50 ml Falcon tube and harvested by centrifugation at 3000 *g* for 10 minutes and then each bacterial pellet was resuspended in 10 ml of fresh Horikoshi medium of the same pH and or the same molarity LB medium. The cells were also normally concentrated two fold and the protein content was determined. The viability of the cultures grown overnight was measured by adding 0.5 ml of concentrated cells to 1.5 ml of fresh medium of the same pH or salinity in the chamber of oxygen electrode (keeping the overall volume at 2 ml to allow standardisation of the results). The plunger was placed on top and left for 5 minutes without illumination to induce oxygen uptake. The plunger was removed and the sample unloaded using a Pasteur pipette. Distilled water was used to clean both the chamber and the plunger.

## 2.9. Determination of Protein Content

### 2.9.1. Determination of Standard Curve

A standard curve was produced using Bovine serum albumin (BSA) in the range of 0 – 100 µg by dissolving 250 mg of BSA in 50 ml of distilled water. The final concentration of protein in the stock solution was 5 mg $ml^{-1}$ (5 µg $µl^{-1}$). The stock solution was used to make a range of protein concentrations by a series of dilutions as shown below in Table 2.2.

| Tube number | Volume of BSA Stock solution (µl) | Volume of distilled water (µl) | Total volume in each tube (µl) | Amount of Protein (µg) |
|---|---|---|---|---|
| 1 | 0 | 100 | 100 | 0 (Blank) |
| 2 | 2.5 | 97.5 | 100 | 12.5 |
| 3 | 5 | 95 | 100 | 25 |
| 4 | 7.5 | 92.5 | 100 | 37.5 |
| 5 | 10 | 90 | 100 | 50 |
| 6 | 15 | 85 | 100 | 75 |
| 7 | 20 | 80 | 100 | 100 |

**Table 2.2.** Components in test tubes which were needed to make a standard protein curve.

Three replicates were made for each test tube. To each test tube, 3 ml of Bradford Reagent (Sigma) were added then thoroughly mixed and left on the bench for 5 min. Optical density was measured for each sample at 595 nm using a 3 ml glass cuvette. The blank was used to zero the Unicam Helisα spectrophotometer. The protein standard curve was plotted from which protein concentration for each sample can be determined. Standard error for each sample were calculated and shown on the curve.

### 2.9.2. Determination of Sample Protein

Soluble protein in bacterial cells was measured using the method described by Bradford (Bradford, 1976). Bovine serum albumin (BSA) was used as the standard as described previously.

Samples were prepared for protein determination as follows:

- 0.1 ml from the same concentrated cells which were prepared for respiration rate measurements was placed into a clean test tube and then 0.9 ml of 1 M NaOH was added. The sample was then thoroughly mixed and heated at 90°C for 10 minutes.
- The test tube was cooled on ice for 5 minutes and dried outside and then centrifuged for 10 minutes in bench centrifuge at full speed (3000 *g*).
- 0.3 ml of the resulting supernatant was added to 3 ml of Bradford's reagent in a fresh test tube and the contents were thoroughly mixed. After 5 minutes incubation at room temperature the optical density was measured in the Unicam Helisα spectrophotometer against 0.1 ml water plus 3 ml of Bradford's reagent (blank) at 595 nm.

The protein content of the samples was determined by reading (μg protein) from the standard curve, divided by 0.3 to get μg protein $ml^{-1}$ and then multiplied by 10 to take into account the dilution by NaOH.

## 2.10. Determination of Intracellular Enzyme Activities of Bacterial Cell Free Extracts

50 ml culture of bacteria grown in normal medium were transferred into 50 ml Falcon tubes and harvested by centrifugation at full speed (3000 g) in a bench top centrifuge for

15 minutes. The bacterial pellet was resuspended in 4 ml of fresh growth medium of the same pH or salinity. Four x 1 ml aliquots of this 4 ml resuspension were placed into Eppendorf tubes and then were placed immediately on ice until required. Bacterial cells were disrupted using an ultrasonic disintegrator (MSE Soniprep 150). The sonication vessel was surrounded by an ice-water mixture and the cells disrupted by sonication for 2 x 20 seconds with 10 seconds breaks between each 20 seconds of sonication to allow cooling. The probe (1 cm diameter) was used at a power setting of 7 microns and then cells debris were removed by centrifugation at 13000 *g* for 60 seconds at 4°C. The supernatant fluid obtained was referred to as the crude cell free extract (CFE). It was kept on ice until used for measurements. A Bradford assay was performed, as described previously, on CFE to determine the protein concentration for use in calculating the specific activity of the enzymes.

### 2.10.1. General Assay Conditions

Continuous assay of enzyme activity was carried out using a Unicam Helisα spectrophotometer, the temperature of the cuvette was maintained at room temperature (20 – 25 °C). Both glass and Quartz 4 ml cuvettes with 1 cm light path were used in all cases. For all assays the reaction rate was initially linear and proportional to the amount of extract present. Any activity measured before addition of the substrate was subtracted from the reaction rate.

2.10.2. Determination of Malate Dehydrogenase Enzyme Activity

Malate dehydrogenase is an enzyme found in the TCA cycle (citric acid cycle) and it catalyses the following reaction:

$$\text{L-Malate} + NAD^{+} \longleftrightarrow \text{Oxaloacetate} + NADH + H^{+}$$

The assay was based on the method described by (Reeves *et al.*, 1971).

The assay mixture contained:

| | |
|---|---|
| 2.0 ml | Defined growth medium ( 50 mM Tris- HCl buffer pH 7, 8.5 and 10) |
| 0.05 ml | 1.5 mM NADH |
| 0.2 ml | 7.5 mM Oxaloacetate pH 7.5 |
| 100 µl | Cell-free extract |

Distilled water to a final volume of 3.0 ml

All reagents, with the exception of oxaloacetate, were added to the 3 ml cuvette and then background rate of reaction measured at 340 nm for 1 minute. The reaction was started by addition of oxaloacetate to the same 3 ml cuvette and represents the decrease in absorbance at 340 nm $min^{-1}$. Enzyme activity was expressed as µmoles NADH oxidised $min^{-1}$. mg $protein^{-1}$. The extinction coefficient of NADH at 340 nm is 6.22 x $10^3$ litre $mole^{-1}$ $cm^{-1}$.

**2.10.3. Determination of Fumarase Enzyme Activity**

Fumarate + $H_2O$ ⟷ L -malate

The method of (Hill and Bradshaw, 1969) was used to detect the formation of fumarate from malate.

The reaction mixture contained:

| | |
|---|---|
| 1.5 ml | Defined growth medium (50 mM Tris- HCl buffer pH 7, 8.5, 9, 9.5 and 10) |
| 0.1 ml | 1 M L – malate (sodium salt) |
| 0.025 ml | Cell- free extract |

Distilled water to a final volume of 3.0 ml

The reaction was started by addition of 0.1 ml L – malate and the increase in absorbance at 240 nm (with deuterium lamp and quartz cuvette) was followed against water blank. Enzyme activity is expressed as µmole fumarate produced $min^{-1}$. mg $protein^{-1}$. The extinction coefficient of fumarate at 240 nm is 2.44 L $\mu mol^{-1}$ $min^{-1}$.

## 2.11 Sensitivity to Antibiotics

Estimation of the sensitivity of strains to antibiotics was carried out using a standard disk dispenser (Oxoid) method (Bauer *et al.*, 1966). A set of antimicrobial susceptibility test discs were obtained from Oxoid: Penicillin G (P) 10 units, Tetracycline (TE) 50 µg, Erythromycin (E) 15 µg, Amoxycillin (AML) 25 µg, Ampicillin (AMP) 25 µg, Sulphoacethoxazole Trimethoprim (SXT) 25 µg, Vancomycin (VA) 30 µg, Cefoxitin (FOX) 30 µg, Chloramphenicol 30 µg, Streptomycin (S) 10 µg, Imipenem (IPM) 10 µg, Gentamycin (CN) 30 µg and Lincomycine (MY) 15 µg. 0.1 ml of the fresh culture was spread on pH 8, 9 &10 Horikoshi medium plates and LB medium of 0.17, 1, 2, 2.5, 3 & 3.5 M LB plates were spread using sterile glass spreader and then left for 10 minutes to dry. The antimicrobial disks were put on the bacterial film. Three plate replicates were used to perform the test and a plate without any antibiotics was used as a control. The plates were incubated (uninverted) at 25°C and 37°C overnight depending on the strain. Inhibition zones were measured by ruler in cm and then the mean and standard deviation of the inhibition zone for each antimicrobial was calculated.

## 2.12 Anaerobic Growth

Anaerobic growth determination was carried out using fresh overnight cultures in normal Horikoshi medium pH 10 using Petri dishes containing solid medium. The plates were inoculated and placed in an anaerobic jar and incubated overnight at 25ºC.

## 2.13 Utilisation of Carbon Sources Measured in Biolog 96 Well Plates

Bacteria were grown in appropriate media at an appropriate temperature in 250 ml flasks overnight. The bacteria were then washed with M9 minimal medium (without any carbon source) three times to avoid any interference by organic chemical sources. A diluted preparation ($OD_{600}$ = 0.2) for all strains was made by addition of M9 minimal

medium. The dilute bacterial suspension was used to inoculate 96 well plates (Biolog, Hayward, USA) using a multi channel pipette. Plates were then read (1 second per well) to evaluate the amount of reaction between bacteria and chemical and or carbon sources. Initial reading was taken directly after inoculation and the second reading was taken after overnight incubation.

## 2.14 Molecular Biology Techniques

### 2.14.1 Genomic DNA Extraction

QIAgen Genomic-Tip kit was used as first attempt to extract genomic DNA from all four strains. The second method used was CTAB method as described by Chen *et al.* (Chen *et al.*, 2001). PowerSoil DNA kit (MoBio Laboratories Inc, California) was used as the third method of extraction. ANACHEM Key Prep kit was the fourth method used in this study to extract genomic DNA from all strains. For all kits, the manufacturer's instructions were followed.

### 2.14.2 Chemicals, Enzymes and General Reagents

All general molecular biology and biochemistry chemicals used in this study were obtained from Sigma Aldrich and Roche. All chemicals used were of molecular biology grade. Standard molecular biology enzymes were supplied from New England Biolabs (NEB), Bioline and Promega. $dH_2O$ was produced by a Millipore MilliQ system. $ddH_2O$ was produced by autoclaving MilliQ $dH_2O$ for a period of 20 minutes at 121$^{o}$C in acid-cleaned glass containers.

### 2.14.3 Oligonucleotides

All oligonucleotides were synthesized by Eurofins MWG GmbH. Oligonucleotides were stored as 0.1mM stock solutions made up in $ddH_2O$ for use. Oligonucleotides were stored at 4$^{o}$C for short-term use and -20$^{o}$C for long-term storage.

### 2.14.4 Polymerase Chain Reaction (PCR) Amplification of 16S rRNA

Following extraction of genomic DNA, polymerase chain reaction (PCR) was carried out in order to amplify the 16S rRNA gene, the primers used to amplify the 16S rRNA gene were two universal bacterial primers: Forward primer (F: 5' CCGAATTCG TCG ACA ACA GAG GAT CCT GG 3') and Reverse primer (R: 5' CCC GGG ATC CAA GCT TAC GGC TAC CTT GT 3') designed to target the conserved regions of the 16S rRNA gene (Weisburg *et al.*, 1991). PCR mix was used either as a master mix (Fermentas) or the reaction mixture contained the following reagents in a 0.2 ml thin walled PCR tube: 39 µl Distilled Water, 5 µl 10x Buffer, 2.5 µl 50 mM $MgCl_2$, 0.5 µl Forward Primer, 0.5 µl Reverse Primer, 1 µl 25 mM dNTPs, 1 µl genomic DNA and 0.5 µl Taq polymerase (Bioline).

Amplifications were carried out in a MyCycler thermocycler (BioRad) and began with an initial denaturation step consisting of 94°C for 3 min followed by 30 cycles consisting of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C followed by a final extension at 72°C for 5 minutes.

### 2.14.5 Purification of PCR Products

PCR reactions were cleaned up using QIAgen PCR purification kit and ANACHEM Key Prep Purification kit as per the manufacturer's protocols. This stage was used to remove any remaining primers and dNTPs. After the clean-up, the PCR product was sent for sequencing.

### 2.14.6 Agarose Gel Electrophoresis

Following PCR and purification processes, gel electrophoresis was used to check and confirm the correct gene had been amplified (16S rRNA is 1.5 kbp). The gel was made by adding 2 ml of 50X TAE into a conical flask, add distilled water up to 100 ml and add 1 g of agarose (ICN Biomedicals Inc.) to produce a 1% gel, This mixture was then heated in a microwave until the agarose had melted, after which it was allowed to cool whilst being stirred, and 5 µl of ethidium bromide (Biorad #161-0433) was added prior to pouring into a Biorad Subcell GT electrophoretic tank with a 30 well comb. Once the gel had set, it was covered with 1X TAE buffer and run at 90-100 V using a Biorad PowerPack 300. PCR products were loaded on the gel as follows, 2 µl of the PCR reaction was added to 2 µl of loading dye and analysed on a 1% agarose gel against 1 µl

of 1 kb GeneRuler ladder (Fermentas). Gels were visualised using the Uvitec "Uvidoc" mounted camera system.

### 2.14.7 The Use of SYBR Green 1 Nucleic Acid Gel Stain

This stain was used due to its high staining sensitivity and safety compared to ethidium bromide. It is usually used in case of documenting damaged DNA to visualise the streak or stream of DNA on the gel. SYBR Green stain was diluted in 1 X TAE buffer, 50 µl stain in 500 ml of 1 X TAE buffer. The agarose gel electrophoresis was carried out as usual and the dye put in a plastic container covered with aluminium foil for further protection from light, then put on slow rotary shaker for 20 – 40 minutes.

### 2.14.8 *Staphylococcus warneri* Cell Wall Disruption Prior to gDNA Extraction

A fast Prep machine was used for breakage of cell wall of *Staphylococcus warneri*. Two sizes of tubes A & B were used, B size was the suitable one due to its genomic band on gel. This band was confirmed by PCR amplification. In the absence of the Fast Prep machine, lysostaphin (Sigma – Aldrich) was used for cell wall lyses.

### 2.14.9 TOPO Cloning Kit

#### 2.14.9.1 Ligation

The ligation stage is used to ligate 16S rRNA gene into a vector or plasmid which is a naturally occurring circular piece of DNA which most bacteria possess. Bacteria use them to spread antibiotic resistance genes between each other for example, which is useful to use as a selective marker.

#### 2.14.9.2 Transformation & Miniprep Procedure

After ligation of 16S rRNA gene into the vector, competent *E. coli* cells were transformed with the vector containing the 16S rRNA insert. Basically, transformation is the process of forcing the *E. coli* cells to take up the plasmid DNA. Competent *E. coli*

cells are made especially containing sticky ends so that they can take up plasmid. Ligation reaction and transformation were carried out by using TOPO 10 cloning reaction protocol.

2.14.9.3 Digestion

The digestion step is usually used to confirm if the plasmid has the correct insert or not. This involves cutting the DNA in a specific place using a restriction enzyme. Restriction digests usually contain 1 µl of enzyme, 1 µl 10X buffer (appropriate to the enzyme), 2 µl plasmid DNA and 6 µl MilliQ water, and then the mixture was incubated at 37°C for 2 hours. The results are checked using gel electrophoresis and samples containing the correct insert are sent to Medical School for sequencing. The sequences were then compared to other sequences using the NCBI Blast web site. For high quality plasmid DNA, the PureLinkTM HiPure Plasmid DNA MiniprepKit was used as described in the manufacturer's protocol.

**2.14.10 Competent Cell Preparation**

Cells were streaked onto agar and incubated overnight at 37°C. A single colony was inoculated into 25 ml of LB in a 250 ml flask and incubated at 37°C. Flasks were monitored frequently using the spectrophotometer till cells reached the density of $OD_{600}$ 0.45 – 0.5. The cell culture was then transferred to 20 ml Falcon tubes and left on ice for 10-15 minutes. At 4°C cells were pelleted by centrifugation at 3000 g for 10 minutes. Complete removal of supernatant was carefully performed; in addition the tube was inverted to remove remaining liquid.

The cells were then resuspended in 1/3 of the initial volume (8 ml) of RF1 solution, and left on ice for 15 minutes according to Hanahan (1985). The cells were pelleted again, and the supernatant decanted. The cell pellet was then resuspended in 1/12.5 of the original volume (2 ml) of RF2 solution, and incubated on ice for 15 minutes. Resuspended cells were prepared as aliquots using pre-chilled 1.5 ml Eppendorf tubes in ice on 100µl aliquots, and stored at – 80°C.

A plasmid (pUC19) was transformed into a competent cell (*DH5α*) in order to have plasmid miniprep ready for the process of cloning.

### 2.14.11 Plasmid Preparation

Small scale preparation was carried out using the QIAprep Spin Plasmid Miniprep system. This system utilizes the principles of the alkaline lysis method of (Bimboim and Doly, 1979) followed by plasmid purification from a silica resin. The kit was purchased from Qiagen and used as follows: 1.5-3ml of overnight *E. coli* cultures containing the required plasmid were pelleted by centrifugation at 13000 g for 5 minutes. Each supernatant was discarded and the cells resuspended in 250 µl of buffer P1 (50mM Tris-HCl, pH 8.0, 10mM EDTA and 100µg/ml of RNase A), 250µl of buffer P2 (200mM NaOH and 1%SDS) was then added to lyse the cells, and the contents of the tube mixed via inversion. 350 µl of buffer N3 (3M potassium acetate pH 5.5) was then added to neutralize the lysis reaction and the tube inverted several times to mix the contents.

The complete reaction was then centrifuged at 13000 *g* for minutes to form a compact pellet consisting of genomic DNA and lysed cells.

The supernatant was recovered and applied to a QIAprep spin column, seated in a 2ml microcentrifuge tube. The spin column was then centrifuged at 13000 *g* for 1 minute and the flow through discarded. 0.5 ml of buffer PB (a proprietary buffer containing guanidine thiocyanate and propan-2-ol) was then added to remove any residual endonucleases or contaminating proteins and the spin column was centrifuged at 13000 *g* for 1 minute.

The column was then washed by adding 0.75 ml of buffer PE (a proprietary buffer containing 70% ethanol) and centrifuged at 13000 *g* for 1 minute. The flow through was discarded and a second centrifugation at 13000 *g* was used to remove any residual buffer PE. The QIAprep column was then transferred to a fresh microcentrifuge tube and 50µl of buffer EB (10mM Tris-HCl pH 8.5) was added to the centre of the column, before centrifugation at 13000 *g* for 1 minute. Using this system up to 15 µg of DNA can be purified from 1 ml of culture depending on the copy number of the plasmid. Plasmid DNA obtained from this method can then be used directly for DNA manipulation or sequencing.

### 2.14.12 Plasmid Transformation

A 1-5 µl of pUC19 was added to 50 – 200 µl of competent cells (*DH5α*), and kept on ice for 30 minutes. Heat shock followed by putting the mixture at 42°C for 90 seconds (sharp). The mixture was then transferred immediately onto ice for 2 minutes.

400 µl LB medium were added to each 100 µl competent cells and incubated at 37°C for 1 hour. In order to find very fine colonial growth, Eppendorf tubes were centrifuged for one minute at 10000 *g*, decanted partially and half amount of the supernatant was left. Pipetting of the pellet with the remaining supernatant up and down was adopted and spread (50 – 100 µl) on LB agar (containing Ampicillin, IPTG, X – gal). Plates were incubated overnight at 37°C. On the second day the expected outcome were blue colonies on the plate.

### 2.14.13 Miniprep Preparation of pUC19

One blue colony from plasmid transformation was picked and put into 5 ml LB + 5µl ampicillin (selective media) and was incubated overnight at 37°C.

The whole tube was centrifuged, supernatant decanted and then, Qiaprep (Qiagen) protocol was followed.

### 2.14.14 Ligation of Plasmid and Insert

DNA fragments were ligated to plasmid vector (containing sticky ends) using the enzyme T4 DNA ligase (Promega). Ligation reactions were carried out in 20 µl volumes using an approximate molar ratio between 1:1 and 3:1 (insert DNA: plasmid DNA). A 10 x buffer T4 ligase was chosen according to manufacturer. The reaction was incubated at room temperature for 2 hours or at 16°C overnight.

Plasmid (pUC19) was used as a control (without ligation with insert) to find out if there any growth in the process of blue white screening. The pUC19 should not give any growth or very few white colonies in this case.

This equation was used for insert vector calculations:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of insert : vector} = \text{ng of insert}$$

Transformation should follow ligation process using DH5α competent cells. The new clones (constructs) were recovered using Miniprep kit (Qiaprep spin Miniprep, Qiagen). Confirmation of the ligation of plasmid and insert by restriction digestion again after miniprep obtained with *Hind*III *and Bam*H1to make sure that the white colonies found in blue white screening were really cloned. In addition the identification was confirmed by restriction map. The expected sized fragments were 1.5 and 2.68 kbp.

### 2.14.15 Removal of 5' Terminal Phosphate Groups from DNA

In order to decrease vector religation during DNA ligation reactions, 5' terminal phosphates were removed using calf intestinal phosphatase (CIP). De-phosphorylation reactions were carried out in 50 µl volumes containing up to 1µg DNA, 1/10 volume of 10XCIP buffer (supplied with the enzyme) and 1 unit of CIP per pmole of DNA ends (e.g. 1 µg of a 3000 bp fragment of DNA contains 1 pmole DNA ends). The reaction mixture was incubated at 37°C for 60 minutes. The reaction was stopped by adding EDTA to final concentration of 5 mM and incubation at 75°C for 10 minutes. The de-phosphorylated DNA samples were then purified by phenol-chloroform extraction and ethanol precipitation or the buffer was removed using Sephacryl columns (MoBiTec).

### 2.14.16. Purification of DNA Fragments from Agarose Gels

For the purification of DNA fragments from agarose gel slices, a MinElute Gel Extraction kit was used. The DNA was excised from 1% agarose gels using a clean scalpel blade and transferred to a microfuge tube. The tube was then weighed and 3 volumes of buffer QG (a proprietary buffer containing guanidine thiocyanate) added to 1 volume of agarose (i.e. 300 µl to 100 mg agarose). The tube was then transferred to a heat block at 55°C for 10 minutes, with vortexing every two minutes. Once the agarose had dissolved one gel volume of isopropanol was added to the microfuge tube and

mixed before the contents were poured into a MinElute spin column and centrifuged at 13000 *g* for 1 minute. After centrifugation the flow-through was discarded and 500 µl of buffer QG added before centrifuging at 13000 *g* for 1 minute. Again the flow-through was discarded before the addition of 750µl of buffer PE (a proprietary buffer containing ethanol) and centrifugation to remove salt and carbohydrates, the flow-through was again disposed of, and the spin column was centrifuged at 13000 *g* for 1 minute to remove residual ethanol. The spin column was then transferred to a fresh microfuge tube and 10-50µl of EB (10 mM Tris-HCl, pH 8.0) was added to the centre of the silica matrix and incubated at room temperature for 1 minute before elution of the purified DNA by centrifugation at 13000 *g* for 1 minute. DNA purified in this manner can then be used immediately in any downstream applications.

### 2.14.17 Restriction Digestion Analysis

The PCR product (insert) as well as the plasmid (pUC 19) were digested with *Bam* H1 and *Hin* III accompanied with proper buffer selected according to the manufacturer (Promega).

The Composition of a typical reaction mixture:

| Component | Quantity |
|---|---|
| DNA | 10 µl |
| 2x10 Buffer (E) | 2µl |
| BSA | 1µl |
| Enzyme *Bam H*1 | 0.5µl |
| Enzyme *Hind* III | 0.5µl |
| dd$H_2O$ | To 20µl total |

The mixture was incubated for 2-4 hours. Then it was heat treated for inactivation at 75°C for 20 minutes. After this step both digests were gel loaded. The result was 1.5 kbp for the insert and 2.68 for the vector.

The DNA fragment bands for the plasmid and inserts were excised from the agarose gel with clean sharp scalpel under UV transluminator. These DNA fragments were subjected to gel extraction technique using Mini Elute Gel protocol (Qiagen).

### 2.14.18 Primer Design and Cloning

Primer design was carried out in order to facilitate the process of ligation (cloning). Universal primers (section 2.14.4) were modified by adding unique restriction sites. *Hind*III *and Bam*H1enzymes were chosen according to the gene map and their proper location at the start and end of the gene on vector (pUC19) (Figure 2.3).

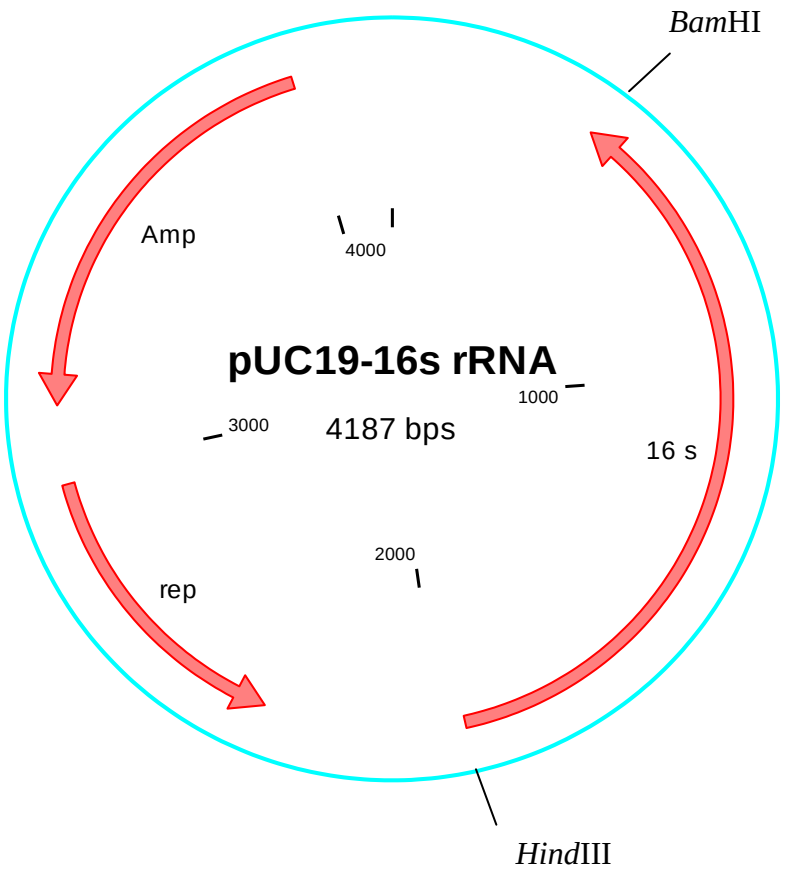



**Figure 2.3.** The location of the two sites for the restriction enzymes (*Hind*III and *Bam*H1) at the start and the end of the gene on pUC19 vector.

Designed primers with forward (*Hind*III) and reverse (*Bam*H1) were synthesized by Eurofins MWG synthesis GmbH.

***Hind*III**

**For.:** 5'CGCGAAGCTTCCGAATTCGTCGACAACAGAGGATCCTGG -3'

***Bam*H1**

**Rev.:** 5' CGCGGGATCCCCCGGGATCCAAGCTTACGGCTACCTTGT -3'

### 2.14.19 Phylogenetic (DNA Sequence) Analysis

For the phylogenetic placement of strains, 16S rRNA gene sequences were processed by The National Collection of Industrial, Marine and Food Bacteria (NCIMB) using the MicroSeq database and the EMBL public database.

### 2.14.20 DNA Quantification

Where required, DNA quantitation was carried out to ensure that the same amount of DNA was loaded into gel wells.

Sample (2 µl) was added to 98 µl elution buffer (EB) added to 2 µl of the sample, mixed and put in spectrophotometer using special UV cuvettes (UVette, eppendorf) and OD was measured.

100 ÷ 2 = 50 (dilution factor).

$OD_{260}$ + dilution factor = amount of DNA µg/ml.

## 2.15 Ultraviolet Light Effect on Bacteria

A serial dilution was set up using tubes filled with sterile distilled water (9 ml) inoculated with 1 ml sample to form the first dilution $10^{-1}$, the second dilution formed by addition of 1 ml of the first one to the second tube $10^{-2}$ and so on.

### 2.15.1 Control Samples

A 100 µl from each dilution was taken and streaked on normal LB agar and incubated overnight. On the following day plates were counted to find out the number of colonies in each dilution.

### 2.15.2 UV Light Treated Samples

The same procedure was followed for control samples, but with the addition of ultra violet light (UV) exposure of plates at various times and at a wavelength of 254 nm (power = 40 Joules). Barriers around the UV chamber must be in place to avoid infiltration of light that would weaken the intensity of UV light and for safety. Glass Petri dishes (pyrex) were autoclaved and filled with a $10^{-1}$ sample to a height of about 0.3 cm or 3 to 4 ml of liquid sample. Petri dishes were put on a shaker moved right and left with vibratory movement to make sure all sample were exposed to UV light to the same amount. The height of the lamp was between 25 and 30 cm.

A series of time exposures was tested until a total killing time was reached at various dilutions. Exposure time started with more than one minute where it was found that short time exposures had no significant effect. The strain specific time exposures to UV light were as follows. *Exiguobacterium* was exposed to UV for 5, 10, 20, 30, 45 and 60 minutes while *Halobacillus blutaparonensis* was exposed for 110, 115, 120, 125 and 130 seconds. *Staphylococcus warneri* was prone to UV radiation at 60, 90, 105, 110, 120, 135 and 150 seconds. Pellets were harvested after exposure to avoid autogenic repair followed by gDNA extraction.

To look for the presence of DNA regeneration a period of recovery time after UV exposure was used either in the light or the dark before gDNA extraction.

## 2.16 Electron Microscopy

Electron microscopy was carried out using scanning electron microscope (SEM) and transmission electron microscope (TEM). Both types were used to examine *Exiguobacterium, Halobacillus blutaparonensis* and *Staphylococcus warneri* cells to find out cellular changes which might occur due to UV light radiation (Section 2.15). The analyses were performed in the Biomedical Sciences Department.

### 2.16.1 Scanning Electron Microscope (SEM)

To produce very high-resolution images of a sample surface, SEM was used. The first step was to prepare the sample for examination by pelleting 1.5 ml of overnight culture for 10 minutes at 3000 *g* and discarding the supernatant. The cells were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer for 4 hours at $40^0$C, cells were washed then in 0.1 M phosphate buffer, twice with 15 minute intervals at 40°C.

Secondary fixation was carried out in 2% aqueous osmium tetroxide for 1 hour at room temperature. The wash step was repeated and dehydration was achieved through a graded series of ethanol solutions as follows:

75% ethanol for 15 minutes
95% ethanol for 15 minutes
100% ethanol for 15 minutes
100% ethanol for 15 minutes
100% ethanol dried over anhydrous copper sulphate for 15 minutes.

All the above steps were carried out at room temperature.

The cells were then air dried from hexamethyldisilazane. Initially they were placed in a 50/50 mixture of 100% ethanol and hexamethyldisiazane for 30 minutes followed by 30 minutes in 100% hexamethyldisilazane. The cells were then allowed to air dry overnight before mounting.

Upon completion of drying, the cells were mounted on 12.5 mm diameter stubs, attached with Sticky Tabs and then coated in an Edwards S150 B sputter coater with approximately 25 nm of gold. The cells were examined in a Philips XL-20 Scanning Electron Microscope (SEM) at an accelerating voltage of 20 Kv.

### 2.16.2 Transmission Electron Microscope (TEM)

TEM has been used to obtain thin, minimally deformed cells that allow for the observation of the internal structures of cells. After preparing cells as described in section 2.16.1 in an Eppendorf tube, fresh 3% glutaradehyde in 0.1 M phosphate buffer was added to the pellet (re-suspended to ensure optimal fixation) and left overnight at 40°C. The cells were then washed twice in 0.1 M phosphate buffer, at 40°C with a 30 minute interval.

Secondary fixation was carried out in 2% aqueous osmium tetroxide for 2 hours at room temperature, washed in buffer as above. This was followed by dehydration through a graded series of ethanol solutions at room temperature as described in section 2.16.1, except the last dehydration step which was repeated twice. The cells were then placed in an intermediate solvent, propylene oxide, for two changes of 15 minutes duration. Infiltration was accomplished by placing the cells in a 50/50 mixture of propylene oxide/Araldite resin. The cells were left in this 50/50 mixture overnight at room temperature.

The cells were then left in full strength Araldite resin for 6-8 hours at room temperature (resin was changed after 3-4 hours) after which they were embedded in fresh Araldite resin for 48-72 hours at 60°C.

Araldite resin contained:
CY212 resin 10 ml
DDSA hardener 10 ml
BDMA accelerator 1 drop per 1 ml of resin mixture

Semi-thin sections approximately 0.5 μm thick were cut on a Leica ultramicrotome and stained with 1% Toluidine blue in 1% Borax.

Ultrathin sections, approximately 70-90 nm thick, were cut on a Leica ultramicrotome and stained for 25 minutes with saturated aqueous uranyl acetate followed by staining with Reynold's lead citrate for 5 minutes. The sections were examined using a FEI

Tecnai TEM at an accelerating voltage of 80 Kv. Electron micrographs were taken using a Gatan digital camera.

## 2.17 Determination of Compatible Solutes Using Nuclear Magnetic Resonance Spectroscopy (NMR)

Samples (5ml) of *Exiguobacterium* strains were put into 15 ml tubes, centrifuged at 3000 *g* for 10 minutes then the supernatant was discarded. The pellets were kept in a -80°C freezer until the analysis was due.

The pellets were thawed, resuspended in 1 ml of distilled water and vortexed for 1 minute at room temperature. Then the samples were sonicated (2 x 20 seconds) and centrifuged at full speed in the microfuge for 10 minutes. The supernatants were transferred into two 1.5 ml Eppendorf tubes, transferred to -80°C freezer for 2 hours and then freeze dried for two days. Freeze dried samples were prepared for Nuclear Magnetic Resonance (NMR) analysis by dissolving them in 500 µl of D2O in Eppendorf tube and then 5 µl of trimethyl syle propionate (TSP) were added. Next, the dissolved sample was transferred into an NMR tube and run in the NMR as described by (Frings *et al*., 1993).

## 2.18 Statistics

Most of the experiments in this study were carried out in triplicate and error bars represent standard errors of the means. If no error bars are shown, they were smaller than the symbol used to represent the mean. For experiments carried out in duplicate, both values plus the average are shown.

# CHAPTER 3

# MOLECULAR IDENTIFICATION OF BACTERIA ISOLATED FROM UK AND HASSA SAMPLES

## 3.1 Introduction

Since the discovery of bacteria by Antoni Van Leeuwenhoek in 1684 many attempts at isolating and identifying bacteria have taken place (Madigan, 2012). In recent years, the rapid development of molecular techniques has highlighted this field of microbiology. Bacteria used to be identified to the genus level using classical microbiology tools of identification with special reference to biochemical tests which may lead to species identification. Such phenotypic microbial identification is of value in initial description of unknown organisms. Therefore classical microbiology identification techniques depend on characteristic morphological features as well as physiological ones and are now supplemented by genotypic analysis (Scow, 2001)

Genotypic analysis depends on the polymerase chain reaction (PCR) (Chen *et al*., 2001) which is used to amplify specific genes from whole genome DNA extracts. Microbial biodiversity studies can be carried out using PCR directly on environmental water or soil samples prior to cultivation and detection. Metagenomic studies of microorganisms whether cultured or non cultured ones can be achieved using direct amplification by PCR. Uncultured species genomes are believed to form the major part of the genetic data of the natural world and constitute a rich source of information (e.g. novel products) (Singleton, 2010).

Ribosomal (r) RNA is the main molecular marker widely used for the purpose of detection and identification due to its presence in all organisms. rRNA has the same structural skeleton in all organisms though it differs in length and this makes it easy to identify by size alone (Olsen *et al*., 1986). The 16S rRNA gene is used for prokaryotic species identification (Archaea and Bacteria) and the homologous 18S rRNA gene is used for eukaryotic organisms. Molecular methods are much more reliable for relatedness and classification of microorganisms than phenotypic information because they are informative with regard to evolutionary relationships (Woese, 1987).

Water samples were collected from the Bradford and Lathkill rivers in the Derbyshire Peak District as well as from Alasfar Lake and Irrigation Canals in the Hassa Region, Saudi Arabia as described in section 2.1. Two strains (ABr1 and AL2) were isolated from Bradford and Lathkill rivers by selecting for cells that will grow at high pH. Four strains (6aFLTR, 6aFLSK, 4CFLTR and 4M6) were isolated from Alasfar Lake and Irrigation canals, Saudi Arabia by subjecting them to high salinity conditions. All six strains were identified to the genus level using 16S rRNA gene sequencing. This 16S

rRNA gene was amplified using bacterial primers by the PCR technique (Chen *et al*., 2001). PCR products were ligated into plasmids in two ways, the classical one using the pUC19 cloning vector or by using the pCR2.1 cloning vector kit. Cloning vectors were transferred into *E. coli* (DH5α) competent cells. Plasmid was then isolated and 16S rRNA gene sequenced as described in sections 2.14 and 2.15, respectively. The sequences from strains of UK and Saudi Arabia were compared to known 16S rRNA gene sequences using NCBI Blast function. Phylogenetic information was retrieved using the same programme allowing the construction of phylogenetic trees to show the genetic relatedness to known strains in the data base.

## 3.2 Results and Discussion

### 3.2.1 Extraction of Genomic DNA

The genomic DNA with high molecular weight and high quality was extracted from unknown bacterial cultures as described in section 2.12.1. ABr1 and AL2 organisms were grown on Horikoshi medium while 4cFLTR, 6aFLTR, 6aFLSK and 4M6 cultures were grown on LB medium to produce biomass required for DNA extraction.

Genomic DNA extraction process normally involves breaking cells by digesting the cell wall, followed by removal of the cell fragments and debris using centrifugation and then nucleic acid pellet precipitation and the final step is the purification. Fig. 3.1 shows the standard Hyperladder I, which was used in all agarose gels to allow the size of bands to be estimated. Fig. 3.2 shows the successful genomic DNA extraction of ABr1 and AL2 organisms (lanes 1 and 4) and from 4cFLTR, 6aFLTR, 6aFLSK and 4M6 (lanes 5, 6, 8 and 9) using Anachem Key Prep genomic DNA Extraction Kit. This image shows the proof of the purity of DNA and efficiency of Anachem Key Prep genomic DNA Kit in removing polysaccharides, proteins and other contaminating molecules. Due to using RNAase enzyme, the genomic DNA is of high molecular weight and free from RNA contamination which is in turn ready for 16S rRNA gene amplification using PCR technique.

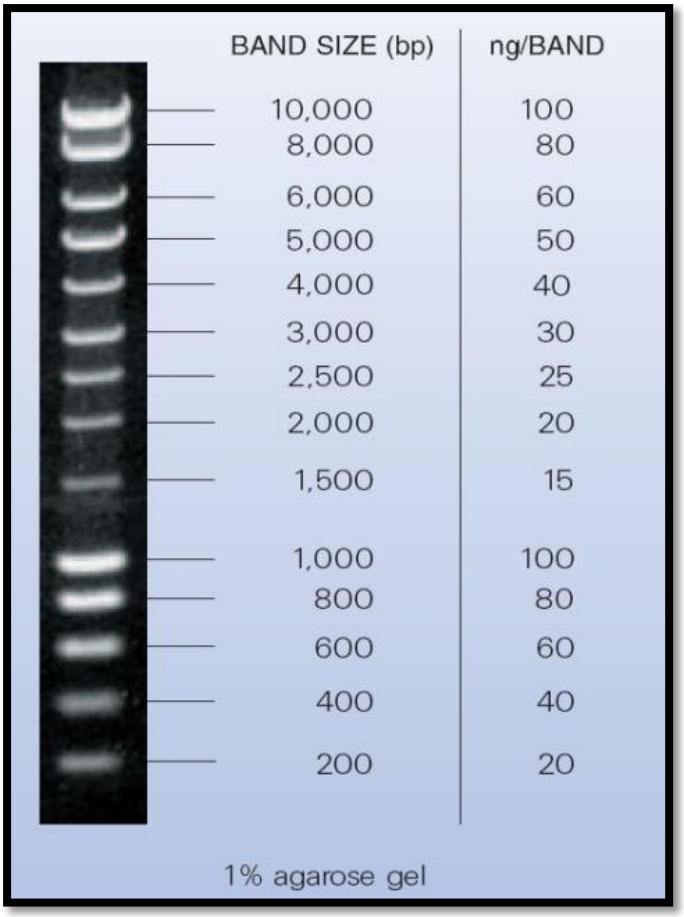



**Figure 3.1 Standard Hyperladder I produces a pattern of 14 regularly spaced bands, 10,000-1000bp and each lane (5 µl) provides 720 ng of DNA.**

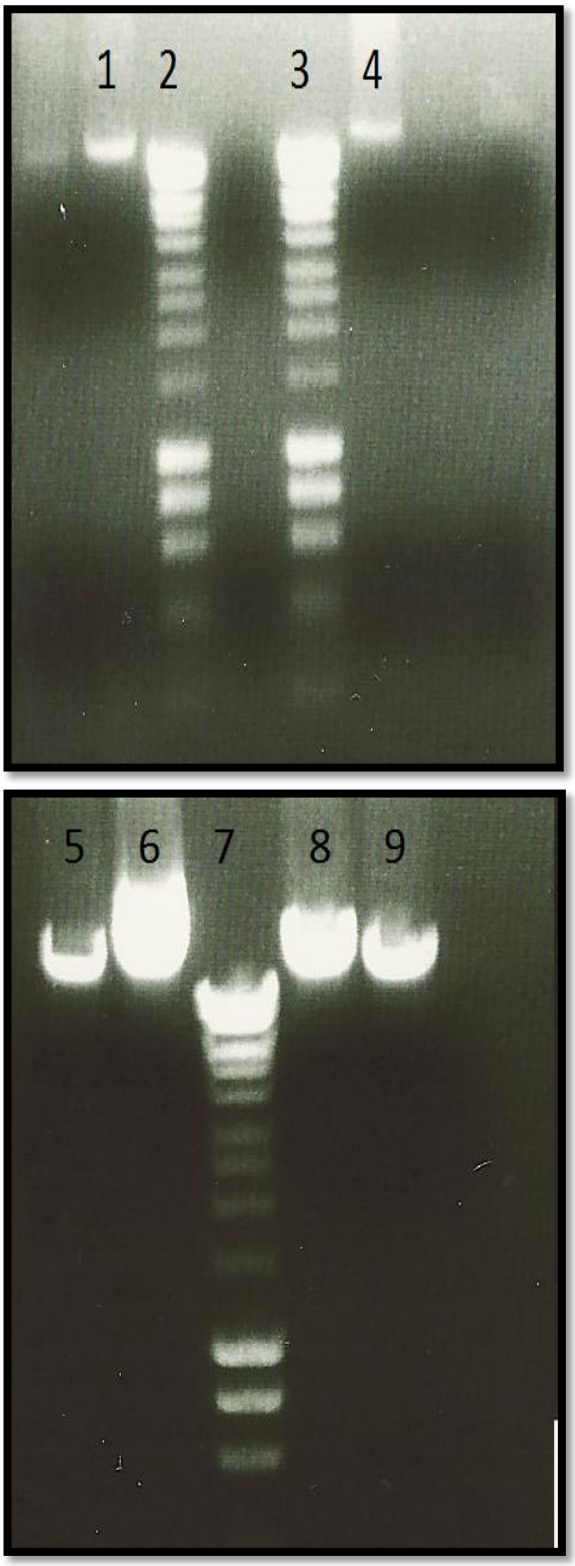



**Figure 3.2 Agarose gel (1%) electrophoresis with ethidium bromide showing 1kb DNA ladder lanes (2, 3 & 7). Genomic DNA extraction with a size over 10000 base pairs from strains ABr1 and AL2 (Lanes 1 and 4) and from strains 4cFLTR, 6aFLTR, 6aFLSK and 4M6 (Lanes 5, 6, 8 and 9). Genomic DNA extracted using Anachem Key Prep Genomic DNA Kit.**

### 3.2.2 PCR Amplification of 16S rRNA gene of Bacterial Strains

PCR amplification process was carried out to copy and amplify the 16S rRNA gene of bacterial genomic DNA extracted from strains ABr1, AL2, 6aFLTR, 6aFLSK, 4cFLTR and 4M6 as shown in section 2.12.4.

Two universal primers were chosen to amplify the 16S rRNA gene according to (Chen *et al*., 2001). These primers are designed to target the conserved regions of the 16S rRNA gene (Weisburg *et al*., 1991) (Table 3.1).

**Table 3.1.** Oligonucleotide primers obtained from Eurofins (mwg/operone) Germany

| Primer | Sequence(5`-3`) | Reference |
|---|---|---|
| **16S UN1.FOR** | **CCGAATTCG TCG ACA ACA GAG GAT CCT GG** | **(Chen *et al*., 2001)** |
| **16S UN1. REV** | **CCC GGG ATC CAA GCT TAC GGC TAC CTT GT** | **(Chen *et al*., 2001)** |

Figure 3.3 shows the amplification product of 16S rRNA gene for ABr1 and AL2 (lanes 1 and 2) and 6aFLTR, 6aFLSK, 4cFLTR and 4M6 (lanes 4 - 7). The results revealed the success of the amplification process of 16S rRNA gene from genomic DNA of strains with correct fragment band size for 16S rRNA gene of approximately 1.5 kb. The PCR product (16S rRNA gene) was cleaned up using Anachem Key Prep PCR Purification Kit protocol. This step removes any possibility of the presence of compounds that could affect the purity of the 16S rRNA gene (e.g. proteins, extra primers, salts and dNTPs). (see section 2.12.5).

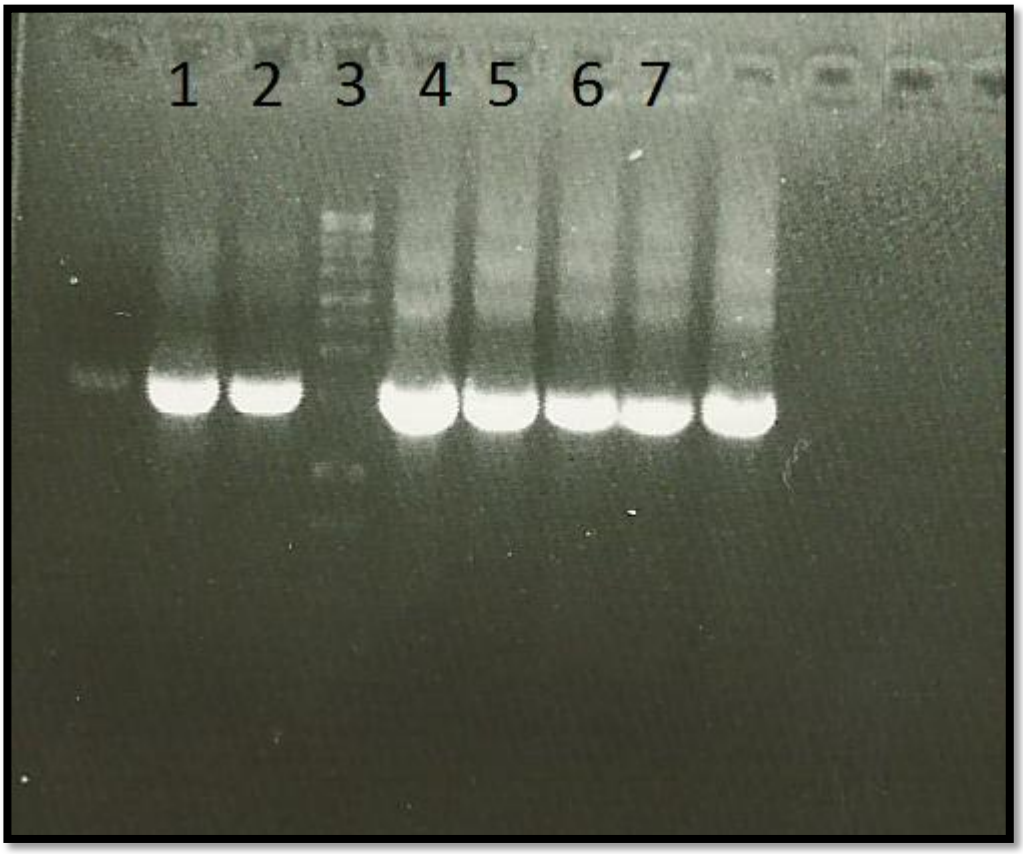



**Figure 3.3.** Agarose gel electrophoresis (1%) with ethidium bromide showing the amplification product of 16S rRNA gene with a size of 1.5 kb from the PCR involving the use of universal bacterial primers for the genomic DNA of ABr1 and AL2 organisms (lanes 1 & 2) and 6aFLTR, 6aFLSK, 4cFLTR and 4M6 organisms (lanes 4-7). Ladder is lane 3.

### 3.2.3 Cloning of PCR Products and Transformation of *E.coli*

3.2.3.1 Cloning using vector pUC19

Primer design was carried out in order to facilitate the process of ligation (cloning). Universal primers were modified by adding unique restriction sites. *Hind*III *and Bam*H1 enzymes were chosen according to the gene map and their location at the start and end of the gene as revealed on the vector pUC19 (Fig. 3.4).

The 16S rRNA gene sequences can be obtained directly from the purified PCR product. However, the sequence returned from direct sequencing of PCR products is often only 800 - 1000 bp, which is significantly shorter than the full 1500 bp. Therefore, in this study the amplified 16S rRNA genes of both strains ABr1 and AL2 were ligated into pUC19 vector (2.68 kb) in order to obtain a longer read of the insert sequences (1.5 kb) for better comparison with other sequences in the genomic DNA databases. Transformation (the process of the *E. coli* DH5α competent cells taking up the plasmid by heat shock) with the vector pUC19 containing the 16S rRNA gene insert was performed using ampicillin as the selectable marker with blue-white screening using X-gal and IPTG as described in section 2.15. Successful ligation of 16S rRNA gene into the plasmid (pUC19) is confirmed by the production of white colonies on the plates (Fig 3.5). Blue colonies confirm that there was no insertion, the lacZ gene was transcribed and the X-gal substrate was used by the *E. coli* cells. DNA fragments were ligated to plasmid vector (containing sticky ends). Transformation should follow the ligation process using DH5α competent cells. The new clones (constructs) were recovered using a miniprep kit. Confirmation of the ligation of plasmid and insert was confirmed by restriction digestion again after miniprep using *Hind*III *and Bam*H1to make sure that the white colonies found in blue white screening were really cloned. In addition the identification was confirmed by restriction map (Fig 3.6). The expected sized fragments were 1.5 and 2.68 kbp for the insert and vector, respectively.

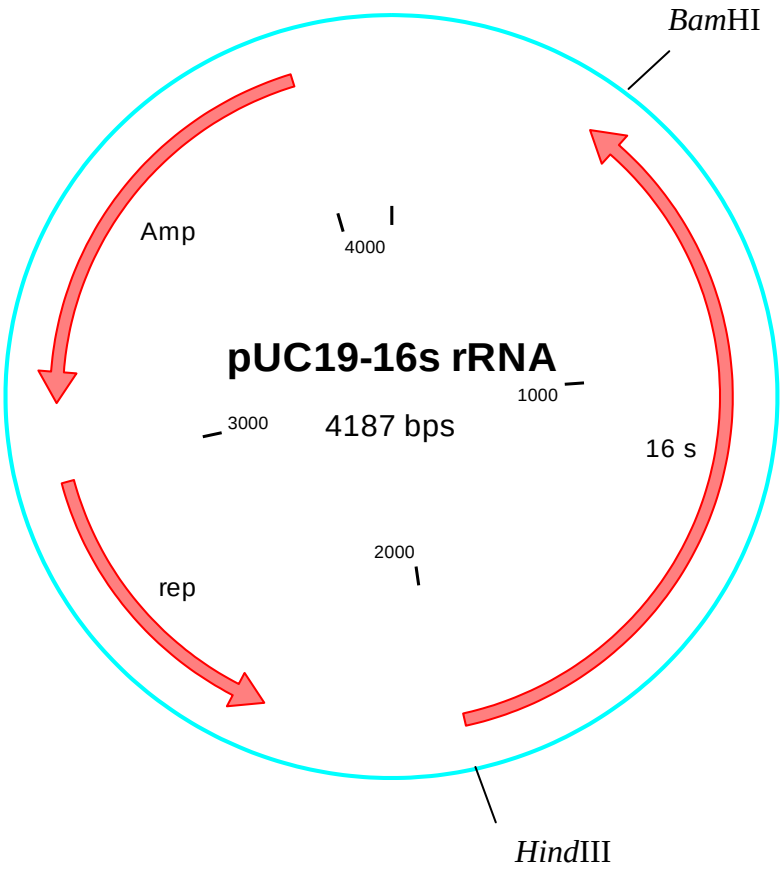



**Figure 3.4** pUC19 vector showing 16S rRNA gene and the location of *Hind*III and *Bam*H1 restriction sites at the start and end of the gene.

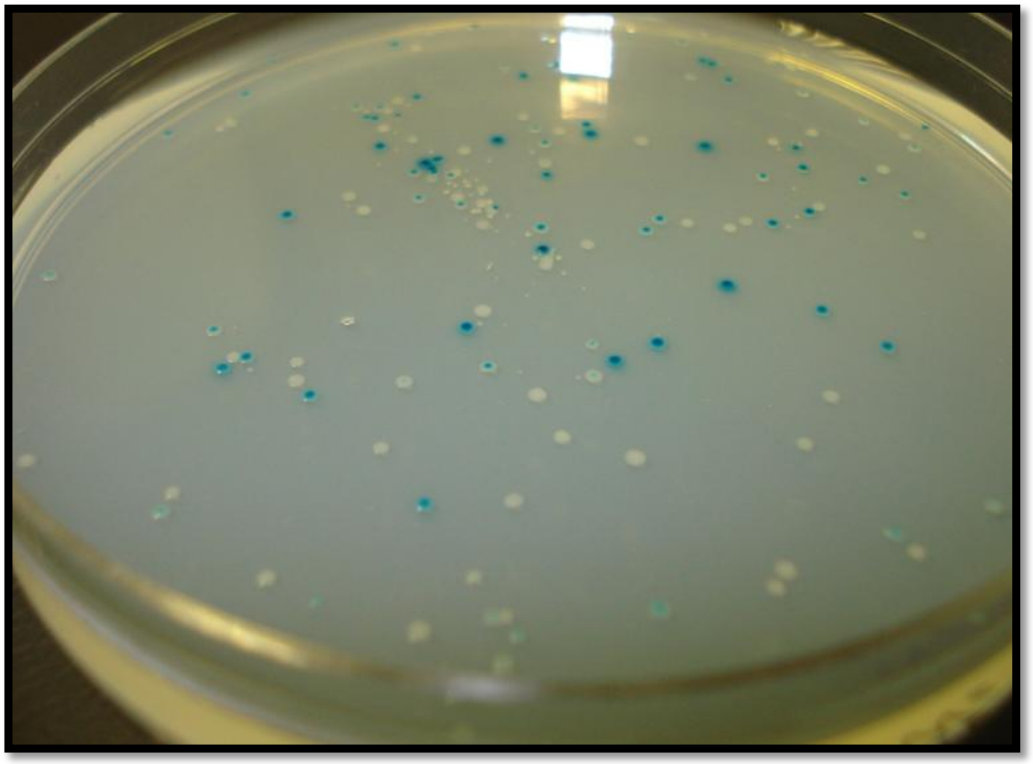


**Figure 3.5.** LB agar plate containing 50 µl $ml^{-1}$ ampicillin and 40 µl 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) showing the results of blue-white screening for the detection of successful ligation. β-glactosidase hydrolyses X-gal and releases a relatively insoluble blue dye. Competent DH5α colonies on this plate may give blue colour, indicating the presence of vector without cloned DNA. However, many of the colonies are colourless, indicating that amplified 16S rRNA gene has been inserted into the vector and the *lacZ* gene has been disrupted.

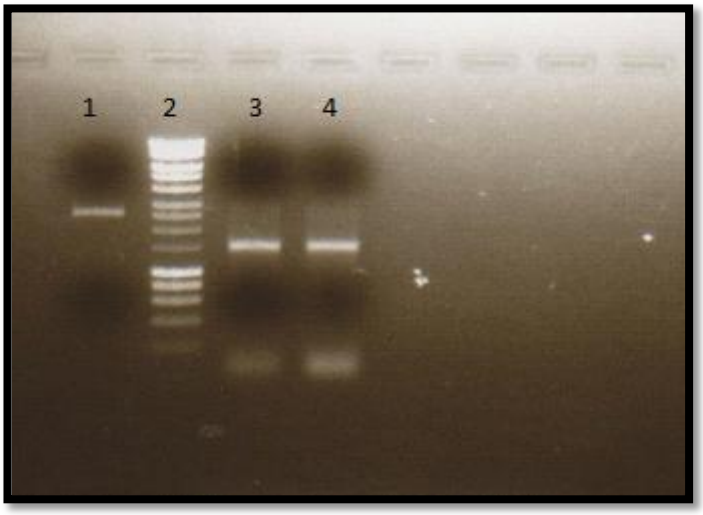



**Figure 3.6.** Agarose gel (1%) electrophoresis with ethidium bromide showing the restriction endonuclease analysis of vector pUC19 (lane 1) digested with *Hind*III and BamH1 for both strains ABr1 (lane 3) and AL2 (lane 4). Lane 2 shows the 10 kb DNA ladder. The upper band indicates the pUC19 vector, with a size of 2.68 kb and the lower band indicates the 16S rRNA gene with 1.5 kb size.

3.2.3.2 Cloning of PCR Products using Topo TA Cloning kit

Cloning can be carried out using Topo TA Cloning kit (Invitrogen) in which amplified 16S rRNA gene of strains 6aFLTR, 4M6 and 4cFLTR were ligated into pCR2.1 vector (3.9 kb) in order to obtain a longer read of the sequences (1.5 kb) for better comparison with other sequences in the genomic DNA databases. Transformation (the process of the *E. coli* DH5α competent cells taking up the plasmid by heat shock) with the vector containing the 16S rRNA gene insert was performed using ampicillin as the selectable marker with blue-white screening using X-gal as described above in section 3.2.3.1.

Plasmid DNA was cut using *Eco*R1 to confirm that the plasmid has the correct insert. pCR2.1 is 3.9 kbp in length and has *Eco*R1 sites where the 16S rRNA sequence (1.5 kbp) should have inserted. Each digest produced two products (vector pCR 2.1 and 16S rRNA gene) at 3.9 kb and 1.5 kb bands, respectively (Fig 3.7).

The full cloning process is summarized in Fig 3.8.

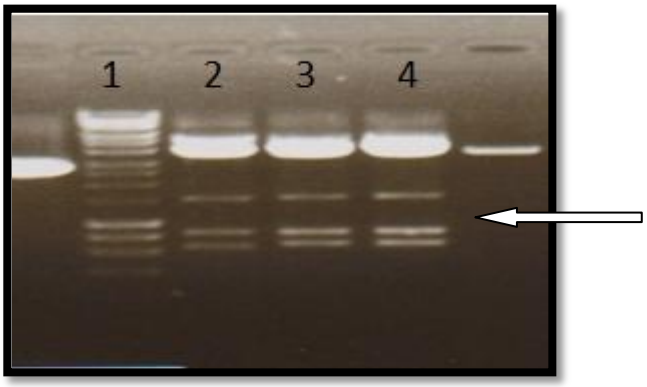



**Figure 3.7.** Agarose gel (1%) electrophoresis containing ethidium bromide showing 10 kb DNA ladder (lane 1). Restriction endonuclease analysis of vector pCR2.1 digested with *Eco*R1 for strains 6aFLTR, 4M6 and 4cFLTR (lanes 2, 3 and 4) respectively. The upper band indicates the pCR2.1 vector at 3.9 kb while the lower band indicates the 16S rRNA gene with size of 1.5 kb.

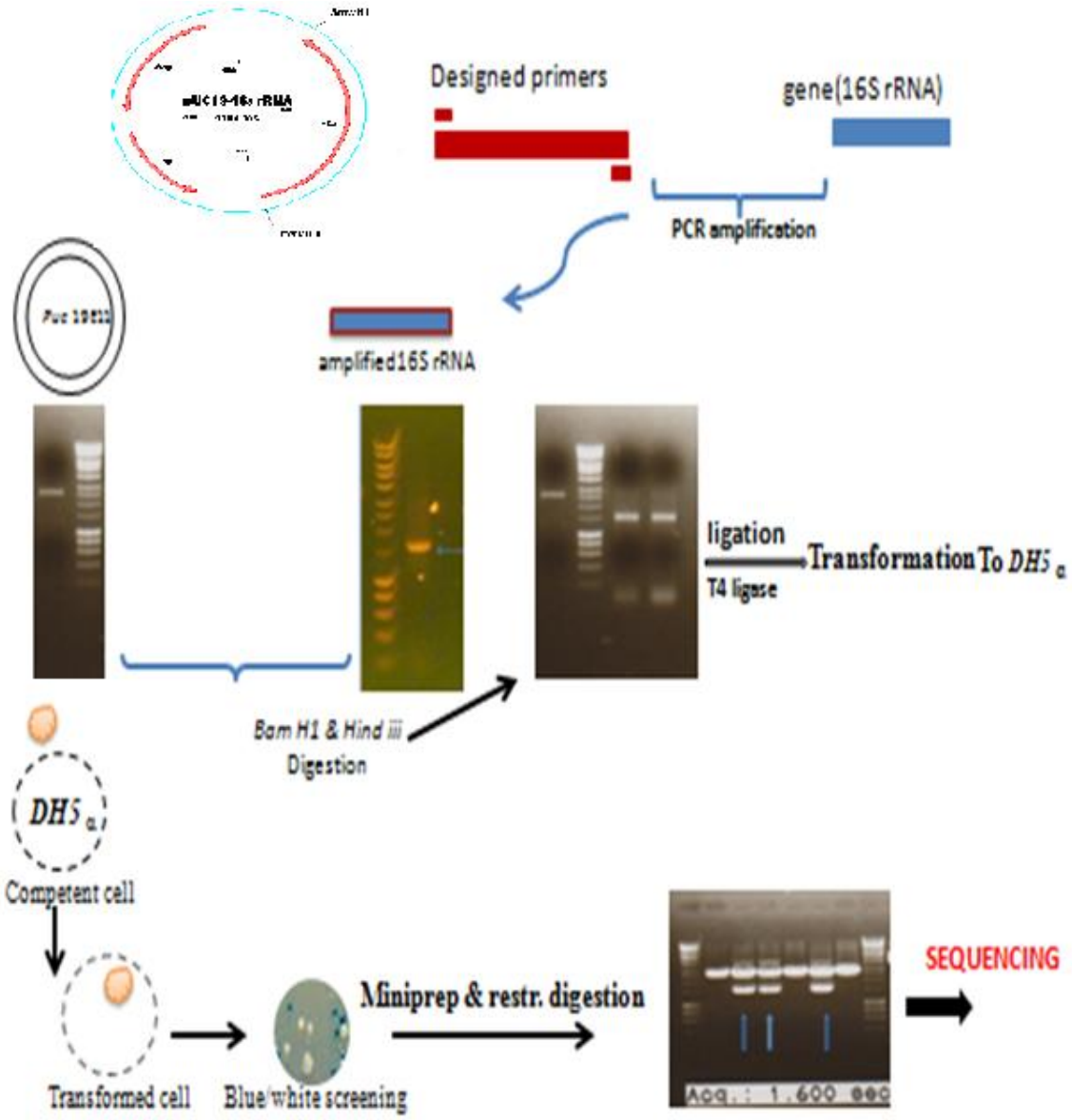



**Figure 3.8.** Panoramic image for the process of cloning starting from localization of endoclease enzymes, primer designing, ligation, transformation to DH5α and restriction digestion till being ready for sequencing.

### 3.2.4 Sequencing of 16S rRNA Gene of Bacterial Isolates

The vector containing the correct sized insert was sent to the Medical School for strains 6aFLTR, 4M6, 4cFLTR, ABr1 and AL2, while 6aFLSK was sent to NCIMB at Aberdeen for sequencing. The sequences obtained are shown in Figures 3.9 to 3.14 and vary in length from 368 to 760 bp. In the next section, the 16S rRNA gene sequences obtained were compared to other sequences using a number of computer software sequencing analysis programme packages which are available to assist in sequence searches.

CACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACA
AGACCCGGGAACGTATTCACCGCAGTATGCTGACCTGCGATTACTAGCGAT
TCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGANAACGGCT
TTCTGGGATTGGCTCCACCTCGCGGCTTCGCTGCCCTTTGTACCGTCCATTGT
AGCACGTGTGTAGCCCAACTCATAAGGGGCATGATGATTTGACGTCATCCC
CACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAACTGAATG
GTGGCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATC
TCACGACACGA

**Figure 3.9** 16S rRNA gene sequence for the Gram-positive facultative anaerobic strain AL2, length of sequence is 368 bp.

AAGNNCCTCTAGAGGTACCGGGCCCCCCCTCGAGGTCTATGGCAGCTAACT
CTTCTTTATCCAATCCCACTGACTGACATCGTTGCCTCCAGCCTTCTCGATTG
CCTCCGCTACGCTCAAGCTCTGACCAGGCTGAATACCATAGCCGATGCGCA
GGCCATGCGGATTCGAGGGCGTCTTGCAGTCCATGGTGCACTCCTTGCGCAG
CACAGTGTCTACCTCCACGGACGAGAAGAAGGCAACCGACATGGGCAGATC
CTGCAGTCCGATGCGGGACACACAAAAGGAGCGCGTCATTAGGTTTGTGAT
GTGCATGGCCAGCGCCGCTTTGGGCGATAGCTCTTCCTTGACCAAGTAGCGC
AATTCATCATGGAAGCTCAAGCAGAATCTAACATGGGATCCCATCAGCCAT
CGCATGCTCACCAGCATCAGATGAAGGAAGTCCACTGCACCGCTCTGTACC
ACCCAATTGATGCGCGTTGGCAGGAAACGTTGCTCCTGCTCCGGTCCAGTAT
CCGCCTCCAATGCTCGACTAAGACGTCCGCCCAGGAACGGCGTTCGTGGCT
GCGATCCCGTGGCAATCTCTTCCAGGCGATTGAACATGGCGCTTTCCGTGCC
TCNNTGCCAGTTGGGACGATGAAATACCTCTGCNNGGGTGCGGTTGCGTTG
AGTGGCCAGTCGGGAAGCCTCGTANCTGCTGTANGNCCTGTCCTCCNNTTCG
TCATGAAACTCCTCTCGCAATCGATACACTCGCTTGCCCTTTGTGA

**Figure 3.10** 16S rRNA gene sequence of Gram-positive facultative anaerobic strain ABr1 nucleotide sequence, length of sequence is 760 bp.

ACACGTGGGCAACCTGCCTGTAAGATCGGGATAACTCCGGGAAACCGGGGC
TAATACCGGGTAATACTTTCTTTCGCATGAAGGAAAGTTGAAAGATGGCTTC
TCGCTATCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTA
ACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGA
ATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAACGATGA
AGGTCTTCGGATCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCGTGCG
AATAGAGCGGTACCTTGACGGTACCTAACGAGGAAGCCCCGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTG
GGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCACGG
CTCNACCGTGGAGGGTCATTGGAAACTGGGGAACTTGA

**Figure 3.11** 16S rRNA gene sequence of Gram-positive, spore forming bacterium strain 4M6, length of sequence is 548 bp.

CCGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAA
CAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACAC
GTGGAAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATA
CCGGATAACATATTGAACCGCATGGTTCAATAGTGAAAGGCGGCTTTGCTG
TCACTTATAGATGGATCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTT
ACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGA
ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG
CAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTC
GGATCGTAAAACTCTGTTATCAGGGAAGAACAAATGTGTAAGTAACTGTGC
ACATCTTGACGGTACCTGATCAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAG
CGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTG
GAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAAT
TCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCCAGTGGCG
AAGGCGACTTTCTGGTCTGTAACTACCGCTATT

**Figure 3.12** 16S rRNA gene sequence of Gram-positive coccus strain 4cFLTR, length of sequence is 747 bp.

ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGGG
TACTTGCTACCCGCTGACGAGCGGCGRACGGGTGAGTAATGCATAGGAATC
TGCCCGATAGTGGGGGATAACCTGGGGAAACCCAGGCTAATACCGCATACG
TCCTACGGGAGAAAGGGGGCTCCGGCTCCCGCTATTGGATGAGCCTATGTC
GGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAACGATCCGTAGCT
GGTCTGAGAGGATGATCAGCCACATCGGGACTGAGACACGGCCCGAACTCC
TACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCC
AGCCATGCCGCGTGTGTGAAGAAGGCCCTCGGGTTGTAAAGCACTTTCAGC
GAGGAAGAACGCCTAGTGGTTAATACCCATTAGGAAAGACATCACTCGCAG
AAGAAGCACCGGCTAACTCC

**Figure 3.13** 16S rRNA gene sequence of non-fermentative Gram-negative rod strain 6aFLTR, length of sequence = 479 bp.

GATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGG
GTAGCTTGCTACCCGCTGACGAGCGGCGRACGGGTGAGTAATGCATAGGAA
TCTGCCCGATAGTGGGGGATAACCTGGGGAAACCCAGGCTAATACCGCATA
CGTCCTACGGGAGAAAGGGGGCTCCGGCTCCCGCTATTGGATGAGCCTATG
TCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAACGATCCGTAG
CTGGTCTGAGAGGATGATCAGCCACATCGGGACTGAGACACGGCCCGAACT
CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATC
CAGCCATGCCGCGTGTGTGAAGAAGGCCCTCGGGTTGTAAAGCACTTTCAG
CGAGGAAGAACGCCTAGTGGTTAATACCCATTAGGAAAGACATCACTCGCA
GAAGAAGCACCGGCTAACTCC

**Figure 3.14** 16S rRNA gene sequence of non-fermentative Gram-negative rod strain 6aFLSK, length of sequence = 480 bp.

### 3.2.5 Phylogenetic Analysis

The 16S rRNA gene sequences of both bacterial isolates from the Lathkill and Bradford rivers were compared with the highly similar sequences available from NCBI GenBank library using the BLAST programme. The other programme used was the EMBL nucleotide sequence database which forms part of the European Nucleotide Archive in addition to the use of MicroSeq database programme. Sequence comparison results using MicroSeq show that ABr1 is most similar to *Exiguobacterium acetylicum*, but only at 91% identity (Table 3.2 and Figure 3.15). This identity score is not sufficient to confirm ABr1 as a member of the *Exiguobacterium* genus. However, using the NCBI BLAST database, ABr1 16S rRNA gene sequence was shown to be 99.9% identical to an *Exiguobacterium* species (Figure 3.16). Strain AL2 went through a similar process, closest match was to *E. acetylicum* on Microseq, but the definitive match on BLAST was to *Exiguobacterium* species with an identity of 98.7% (Table 3.3 and Figures 3.17 and 3.18).

The 16S rRNA gene of the 4M6 strain showed the closest match on the Microseq database to *Virgibacillus halodenitrificans at* 94.02% (Table 3.4 and Figure 3.19). However, after searching the EMBL public database the closest match was to *Halobacillus blutaparonensis* at 100% identity (Fig 3.20).

Sequence analysis of the 6aFLTR strain revealed the closest match to this isolate on the Microseq database was to *Halomonas venusta* at 99.5% (Table 3.5 and Fig 3.21). A sequence comparison between 6aFLTR and 6aFLSK showed that they were very closely related (99.8% identity), so 6aFLSK was also identified as *H. venusta* (Fig 3.22).

EMBL database sequence search for 4cFLTR strain reveals the closest match to be *Staphylococcus warneri* at 99% identity (Table 3.6). On the NCBI database, 4cFLTR had a 100% identity match to *S. warneri* (Fig 3.23).

Table 3.2 Top 10 hits for similarity between 16S rRNA gene sequence of the facultative anaerobic strain ABr1 and other related species/strains based on MicroSeq.

| Representative sequence | Closest matches | Matches Identity |
|---|---|---|
| ABr1 | *Exiguobacterium acetylicum* | 91.04 % |
| | *Bacillus agaradhaerens* | 84.4 % |
| | *Bacillus selenitireducens* | 83.76 % |
| | *Kurthia zopfii* | 84.11 % |
| | *Gracilibacillus dipsosauri* | 83.75 % |
| | *Bacillus pumilus* | 83.45 % |
| | *Bacillus clarkii* | 83.4 % |
| | *Bacillus azotoformans* | 83.15 % |
| | *Bacillus pycnus* | 83.7 % |
| | *Kurthia gibsonii* | 82.89 % |

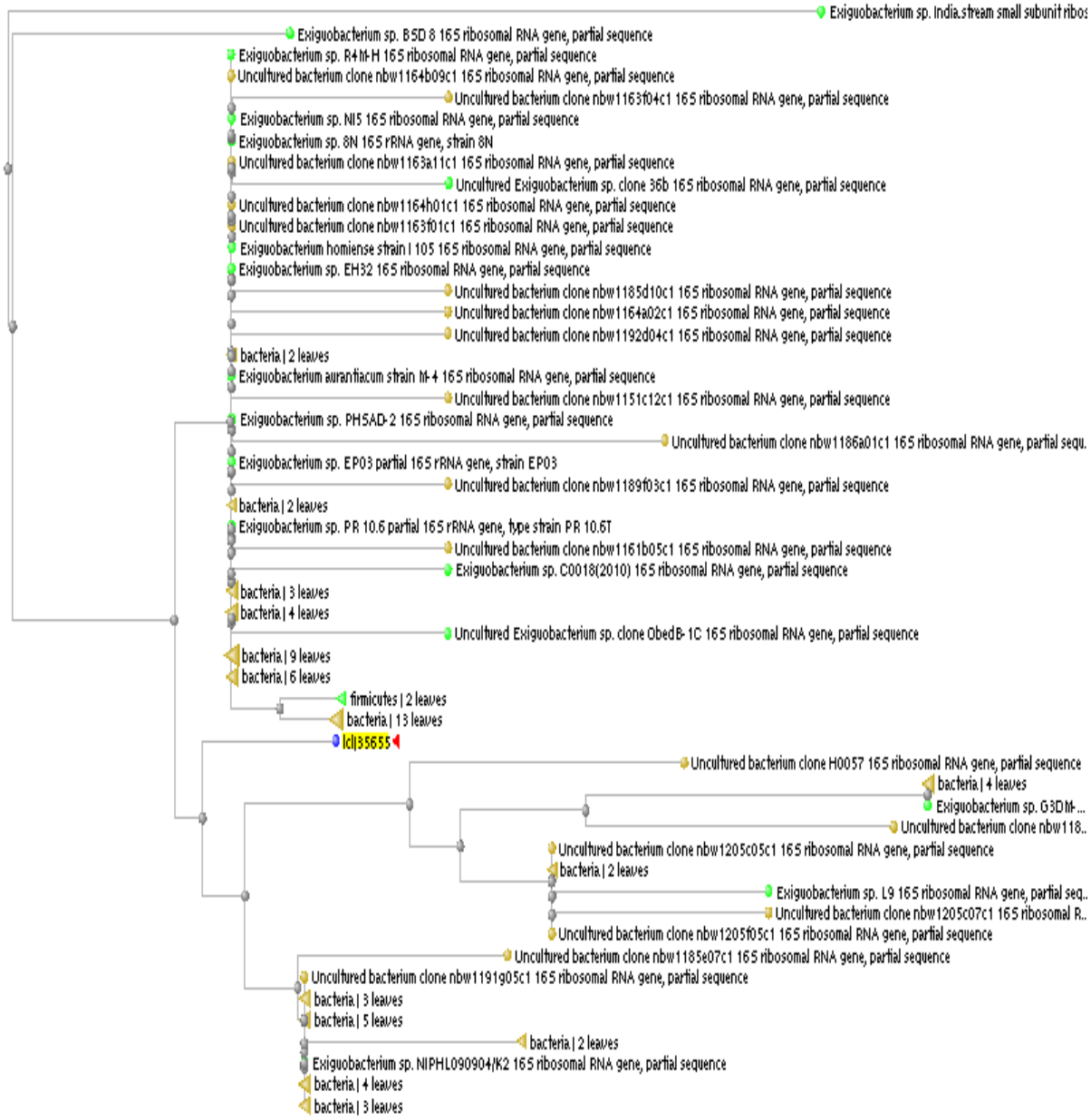



**Figure 3.15** Neighbour joining phylogenetic tree for ABr1 strain associated with other members of the Firmicutes based on 16S rRNA gene sequences. ABR1 strain is marked in yellow.

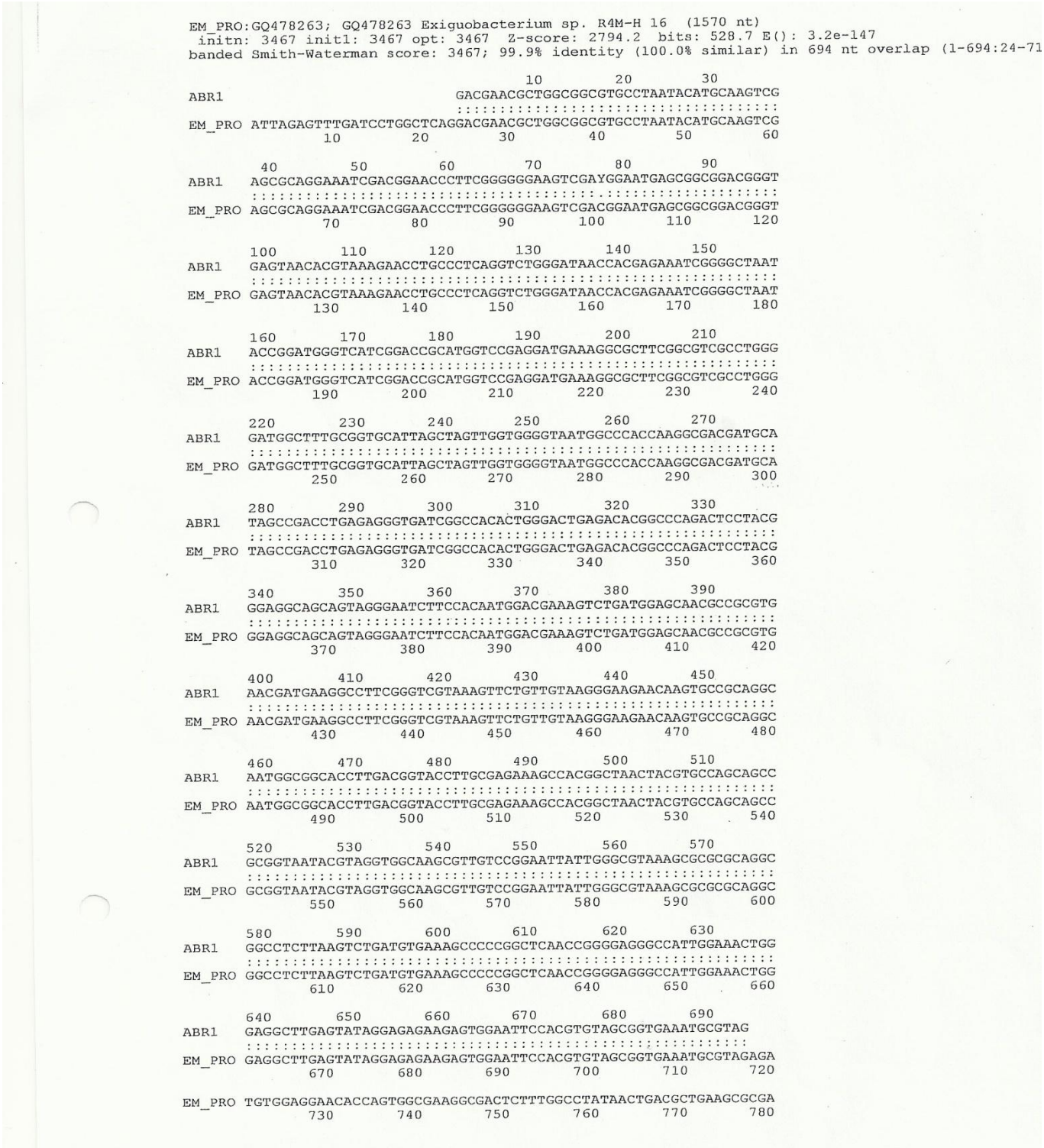



**Figure 3.16** The highest percentage identity match following BLASTN comparison of ABr1 16S rRNA gene sequence was to *Exiguobacterium spp.* 16S rRNA. The identity match was 99.9%.

**Table 3.3** Top 10 hits similarity between 16S rRNA gene sequence of the facultative anaerobic strain AL2 and other related species/strains based on MicroSeq.

| Representative sequence | Closest matches | Matches Identity |
|---|---|---|
| AL2 | *Exiguobacterium acetylicum* | 90.98 % |
| | *Bacillus agaradhaerens* | 84.4 % |
| | *Brevibacillus chshinensis* | 83.76 % |
| | *Kurthia zopfii* | 83.73 % |
| | *Gracilibacillus dipsosauri* | 83.52 % |
| | *Bacillus pumilus* | 83.36 % |
| | *Bacillus clarkii* | 83.26 % |
| | *Bacillus azotoformans* | 82.92 % |
| | *Bacillus pycnus* | 82.92 % |
| | *Kurthia gibsonii* | 82.86 % |

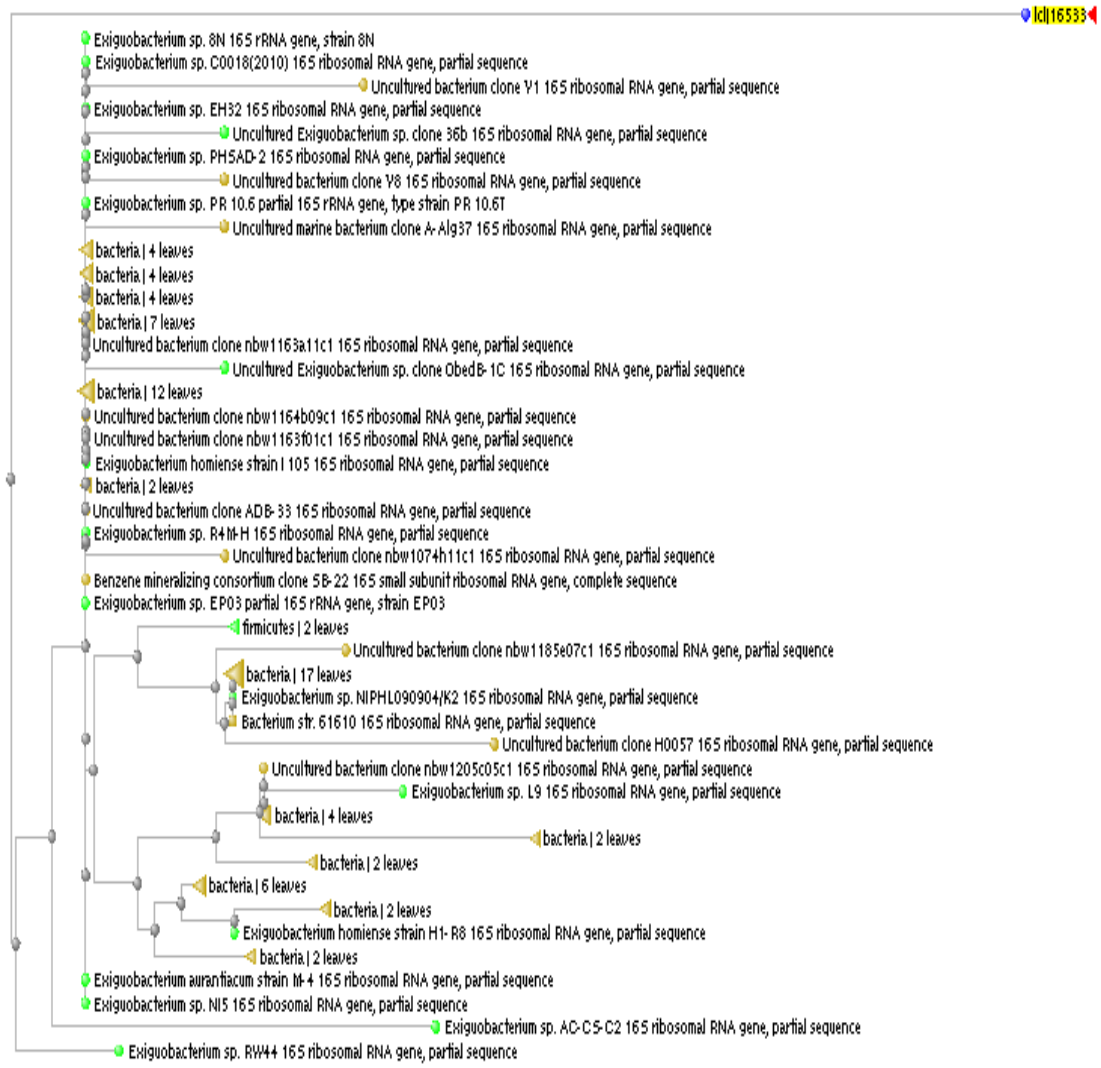



**Figure 3.17** Neighbour joining phylogenetic tree for AL2 strain associated with other members of the Firmicutes based on 16S rRNA gene sequences. AL2 strain is marked in yellow.

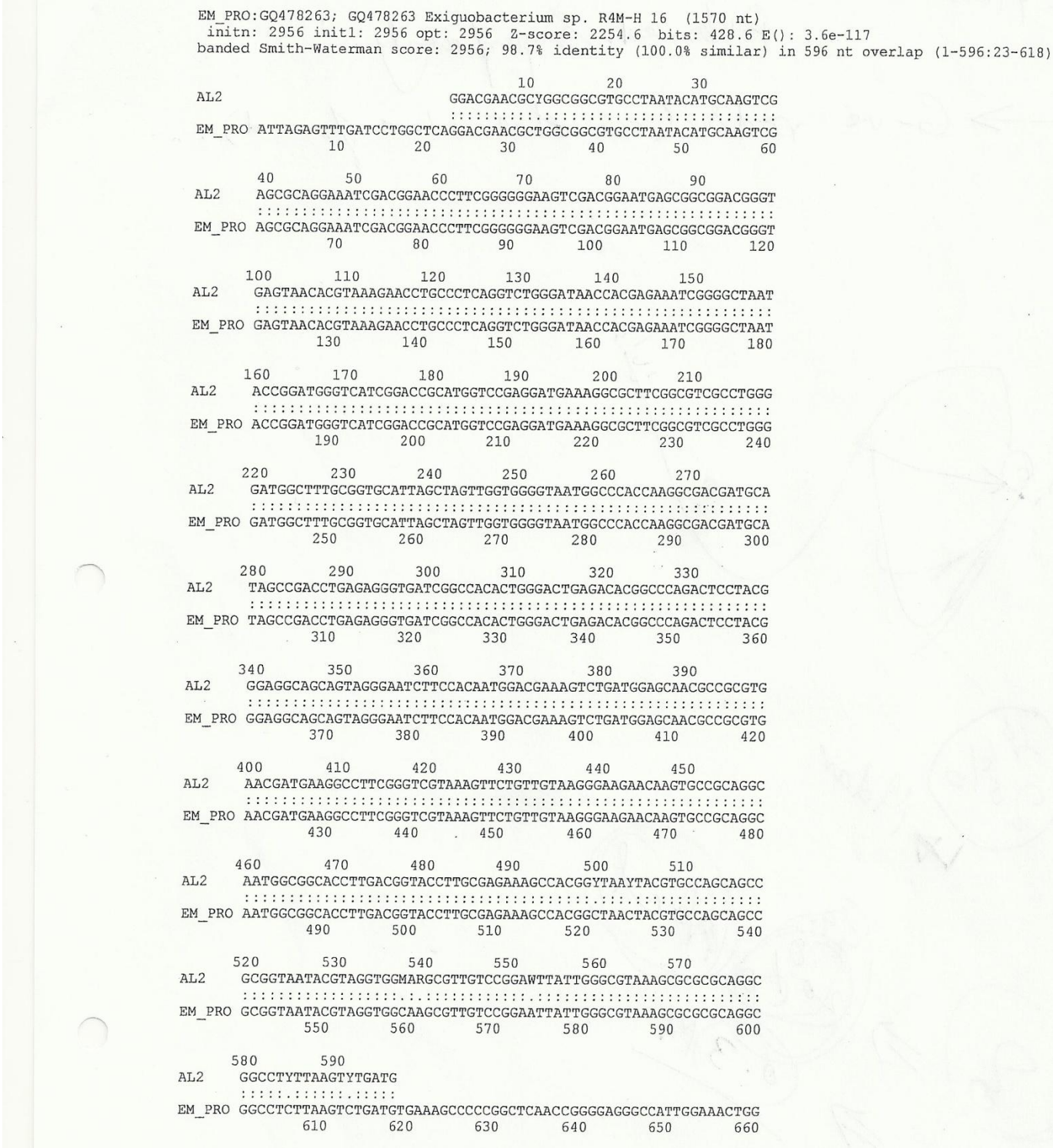



Figure 3.18 The highest percentage identity match following BLASTN comparison of AL2 16S rRNA gene sequence was to *Exiguobacterium spp.* 16S rRNA. The identity match was 98.7%.

Table 3.4 Top 10 hits for similarity between 16S rRNA gene sequence of the spore forming strain 4M6 and other related species/strains based on MicroSeq.

| Representative Sequence | Closest matches | Matches Identity |
|---|---|---|
| 4M6 | *Vigibacillus halodenitrificans* | 94.02 % |
| | *Bacillus halophilus* | 92.87 % |
| | *Gracilibacillus dipsosauri* | 92.69 % |
| | *Vigibacillus pantothenticus* | 91.87 % |
| | *Bacillus firmus* | 89.83 % |
| | *Alkalibacillus haloalkaliphilus* | 89.8 % |
| | *Bacillus oleronius* | 83.4 % |
| | *Bacillus sporothermodurans* | 89.64 % |
| | *Bacillus decolorationis* | 89.6 % |
| | *Bacillus smithii* | 88.98 % |

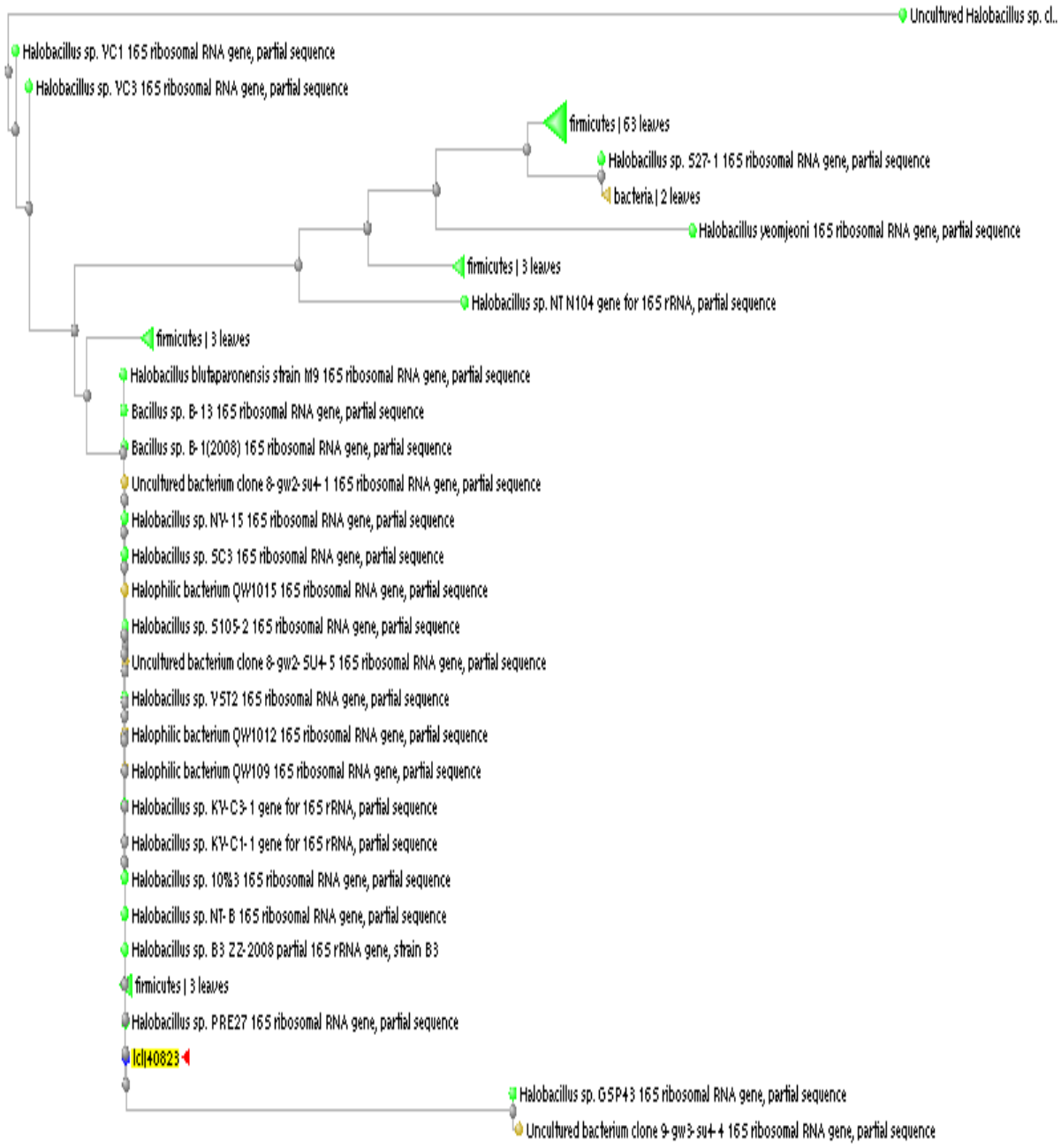
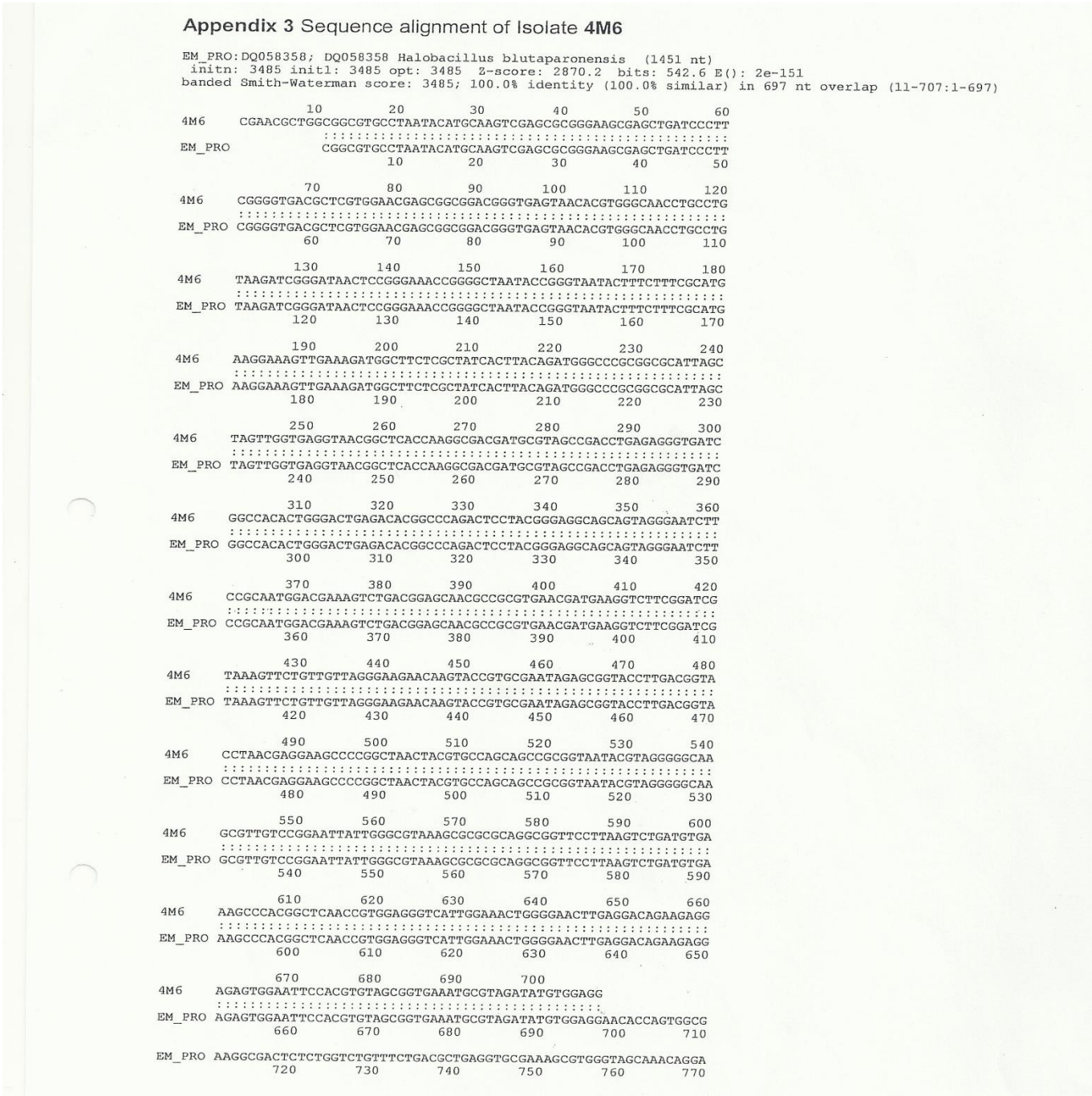



**Figure 3.19** Neighbour joining phylogenetic tree for 4M6 strain associated with other members of the Protobacteria based on 16S rRNA gene sequences. 4M6 strain is shown in yellow.



**Appendix 3** Sequence alignment of Isolate **4M6**

```
EM_PRO:DQ058358; DQ058358 Halobacillus blutaparonensis  (1451 nt)
 initn: 3485 init1: 3485 opt: 3485  Z-score: 2870.2  bits: 542.6 E(): 2e-151
banded Smith-Waterman score: 3485; 100.0% identity (100.0% similar) in 697 nt overlap (11-707:1-697)

                10        20        30        40        50        60
4M6     CGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCGGGAAGCGAGCTGATCCCTT
                ::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO          CGGCGTGCCTAATACATGCAAGTCGAGCGCGGGAAGCGAGCTGATCCCTT
                        10        20        30        40        50

              70        80        90       100       110       120
4M6     CGGGGTGACGCTCGTGGAACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CGGGGTGACGCTCGTGGAACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTG
              60        70        80        90       100       110

             130       140       150       160       170       180
4M6     TAAGATCGGGATAACTCCGGGAAACCGGGGCTAATACCGGGTAATACTTTCTTTCGCATG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TAAGATCGGGATAACTCCGGGAAACCGGGGCTAATACCGGGTAATACTTTCTTTCGCATG
             120       130       140       150       160       170

             190       200       210       220       230       240
4M6     AAGGAAAGTTGAAAGATGGCTTCTCGCTATCACTTACAGATGGGCCCGCGGCGCATTAGC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AAGGAAAGTTGAAAGATGGCTTCTCGCTATCACTTACAGATGGGCCCGCGGCGCATTAGC
             180       190       200       210       220       230

             250       260       270       280       290       300
4M6     TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATC
             240       250       260       270       280       290

             310       320       330       340       350       360
4M6     GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
             300       310       320       330       340       350

             370       380       390       400       410       420
4M6     CCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAACGATGAAGGTCTTCGGATCG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAACGATGAAGGTCTTCGGATCG
             360       370       380       390       400       410

             430       440       450       460       470       480
4M6     TAAAGTTCTGTTGTTAGGGAAGAACAAGTACCGTGCGAATAGAGCGGTACCTTGACGGTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TAAAGTTCTGTTGTTAGGGAAGAACAAGTACCGTGCGAATAGAGCGGTACCTTGACGGTA
             420       430       440       450       460       470

             490       500       510       520       530       540
4M6     CCTAACGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CCTAACGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAA
             480       490       500       510       520       530

             550       560       570       580       590       600
4M6     GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGA
             540       550       560       570       580       590

             610       620       630       640       650       660
4M6     AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGGACAGAAGAGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGGACAGAAGAGG
             600       610       620       630       640       650

             670       680       690       700
4M6     AGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGG
        :::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCG
             660       670       680       690       700       710

EM_PRO AAGGCGACTCTCTGGTCTGTTTCTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAACAGGA
             720       730       740       750       760       770
```

**Figure 3.20** The highest percentage identity match following BLASTN comparison of 4M6 16S rRNA gene sequence was to *Halobacillus blutaporensis* 16S rRNA. The identity match was 100%.

**Table 3.5** Top 10 hits similarity between 16S rRNA gene sequence of the non-fermentative strain 6aFLTR and other related species/strains based on MicroSeq.

| Representative sequence | Closest matches | Matches Identity |
|---|---|---|
| 6aFLTR | *Halomonas venusta* | 99.45 % |
| | *Halomonas aquamarina* | 96.77 % |
| | *Halomonas pacifica* | 92.07 % |
| | *Halomonas cupida* | 91.29 % |
| | *Cobetia marina* | 90.96 % |
| | *Halomonas halophila* | 90.96 % |
| | *Halomonas salina* | 90.96 % |
| | *Chromohalobacter marismortui* | 90.95 % |
| | *Chromohalobacter salexigens* | 90.61 % |
| | *Chromohalobacter canadensis* | 90.48 % |

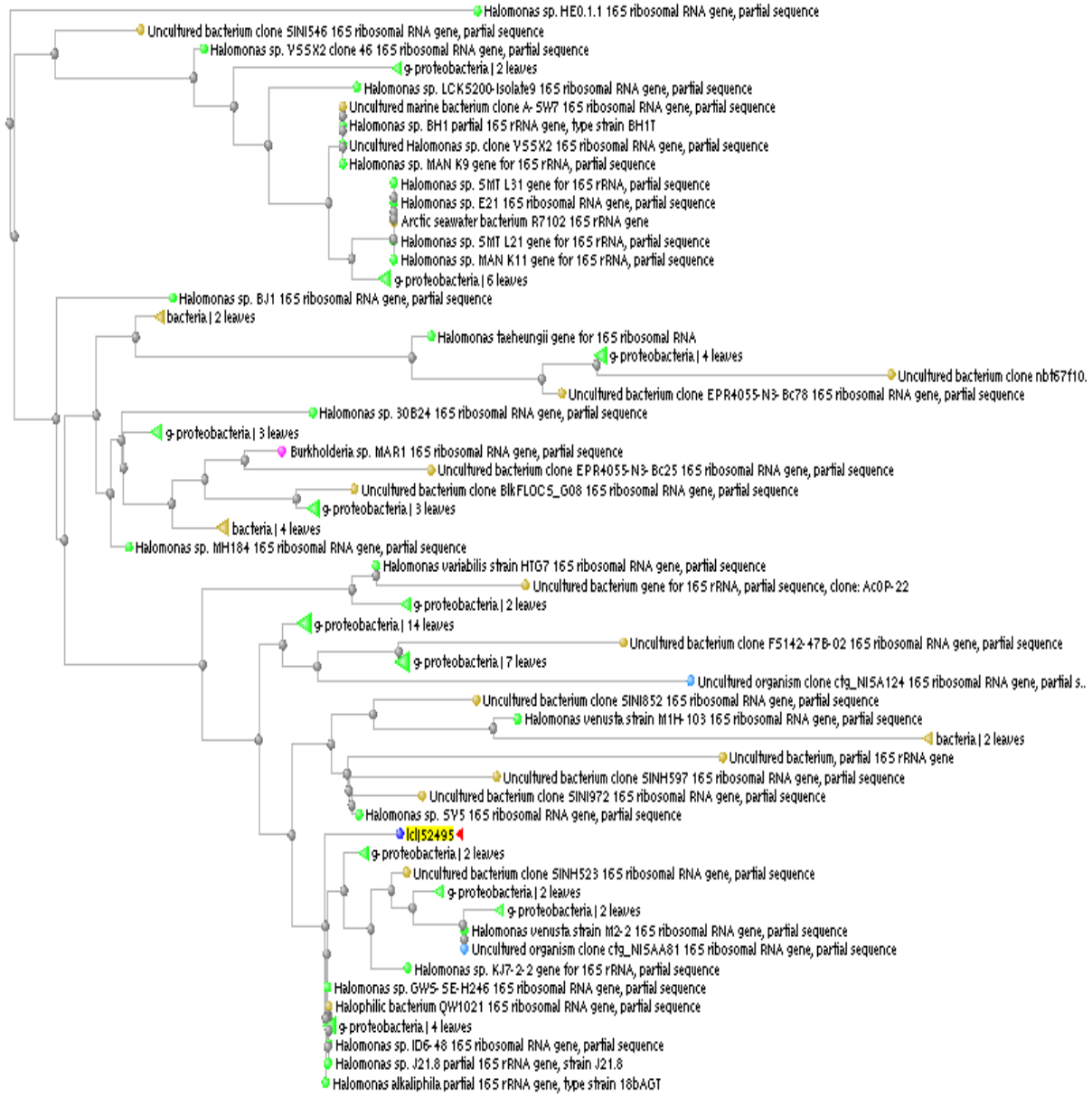



**Figure 3.21** Neighbour joining phylogenetic tree for 6aFLTR strain associated with other members of the Protobacteria based on 16S rRNA gene sequences. Strain 6aFLTR is highlighted in yellow.

```
>_ 6.a FLTR                                                479 nt vs.
>_ 6.a FLSK                                                480 nt
scoring matrix: , gap penalties: -12/-2
99.8% identity;        Global alignment score: 1902

                10        20        30        40        50
261315 -ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGGGTAGCTTGC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGGGTAGCTTGC
_               10        20        30        40        50        60

      60        70        80        90       100       110
261315 TACCCGCTGACGAGCGGCGRACGGGTGAGTAATGCATAGGAATCTGCCCGATAGTGGGGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      TACCCGCTGACGAGCGGCGRACGGGTGAGTAATGCATAGGAATCTGCCCGATAGTGGGGG
_               70        80        90       100       110       120

     120       130       140       150       160       170
261315 ATAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGGGGGCTCCGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      ATAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGGGGGCTCCGG
_              130       140       150       160       170       180

     180       190       200       210       220       230
261315 CTCCCGCTATTGGATGAGCCTATGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      CTCCCGCTATTGGATGAGCCTATGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAA
_              190       200       210       220       230       240

     240       250       260       270       280       290
261315 GGCAACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACATCGGGACTGAGACACGGCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      GGCAACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACATCGGGACTGAGACACGGCC
_              250       260       270       280       290       300

     300       310       320       330       340       350
261315 CGAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      CGAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAG
_              310       320       330       340       350       360

     360       370       380       390       400       410
261315 CCATGCCGCGTGTGTGAAGAAGGCCCTCGGGTTGTAAAGCACTTTCAGCGAGGAAGAACG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      CCATGCCGCGTGTGTGAAGAAGGCCCTCGGGTTGTAAAGCACTTTCAGCGAGGAAGAACG
_              370       380       390       400       410       420

     420       430       440       450       460       470
261315 CCTAGTGGTTAATACCCATTAGGAAAGACATCACTCGCAGAAGAAGCACCGGCTAACTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
_      CCTAGTGGTTAATACCCATTAGGAAAGACATCACTCGCAGAAGAAGCACCGGCTAACTCC
_              430       440       450       460       470       480
```

**Figure 3.22.** Sequence comparison of 6aFLTR and 6aFLSK showing that they are virtually identical (99.8%).

Table 3.6 Top 8 hits similarity between 16S rRNA gene sequence of the facultative anaerobic strain 4.c FLTR and other related species/strains based on the EMBL database.

| Representative sequence | Closest matches | Matches Identity |
|---|---|---|
| 4.c FLTR | *Staphylococcus warneri L37603* | 99.00 % |
| | *Staphylococcus warneri VCU121* | 99.00 % |
| | *Prevotella dentalis DSM 3688* | 99.00 % |
| | *Staphylococcus sp. es1* | 99.00 % |
| | *Staphylococcus epidermidis ATCC 12228* | 97.00 % |
| | *Staphylococcus epidermidis RP62A* | 97.00 % |
| | *Staphylococcus sp. PB1* | 98.00 % |
| | *Staphylococcus epidermidis M23864:W2(gre)* | 97.00 % |

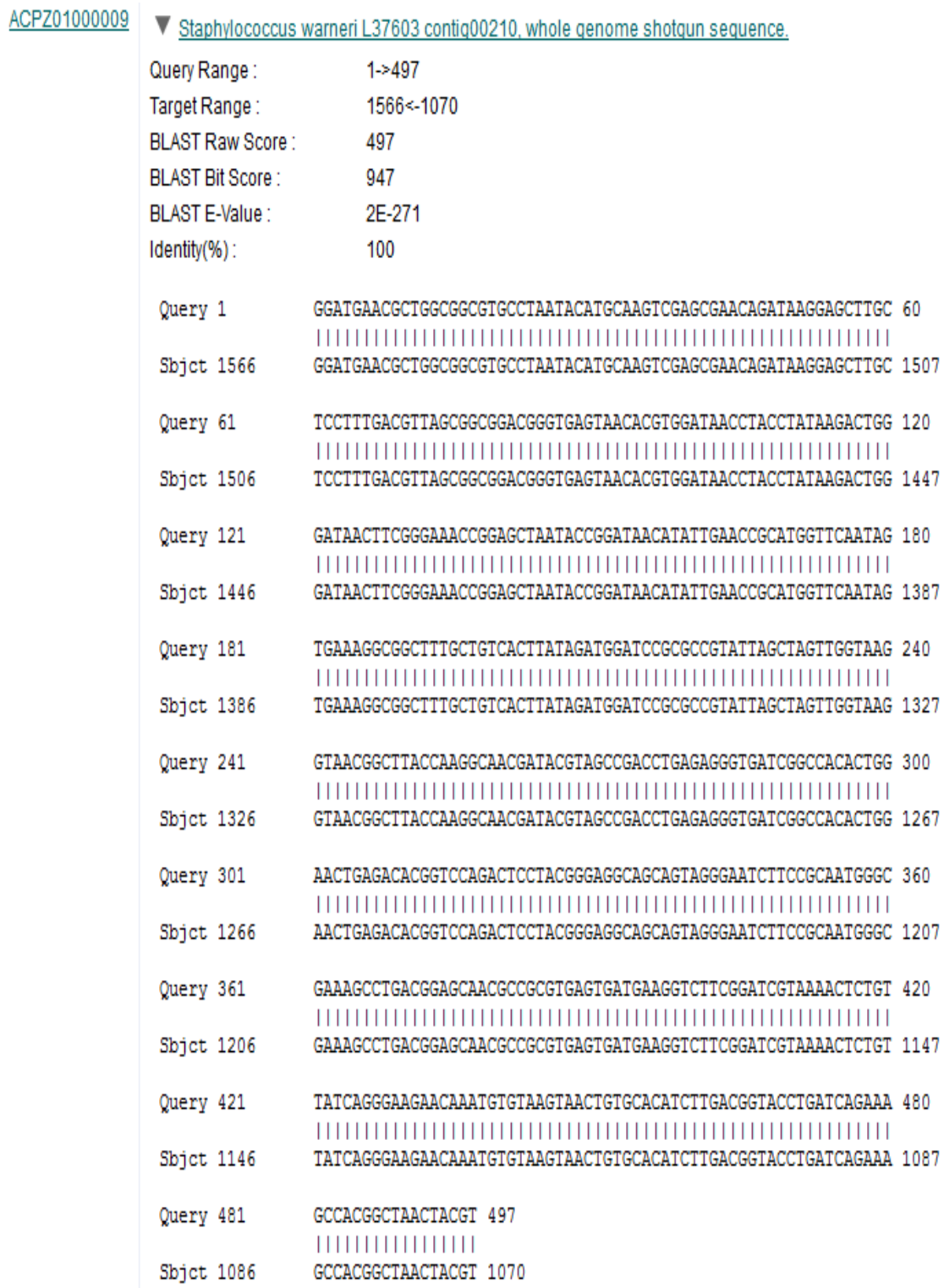



**Figure 3.23.** The highest percentage identity match for 4cFLTR on the NCBI (BLAST) database was *Staphylococcus warneri* (100% identity).

## 3.3 Conclusions

Six bacterial strains have been isolated from Bradford and Lathkill Rivers, UK and Al-Asfar Lake, Al-Hassa region Saudi Arabia. Two strains (ABr1 and AL2) were isolated from the UK water samples and four strains (6aFLTR, 6aFLSK, 4cFLTR and 4M6) were isolated from the Saudi Arabian water samples. In addition to the usual classical identification methods, bacterial strains have been identified in the laboratory according to molecular identification techniques, starting with the extraction of genomic DNA, followed by PCR amplification and purification of 16S rRNA gene, and the transformation of competent *E.coli* (*DH5α*) cells. The sequences have been subjected to computer software analyses in which they were compared with other sequences using the NCBI GenBank library using BLAST programme, the MicroSeq database and the EMBL public database.

Strains ABr1 and AL2 were shown to be similar to unidentified strains of the genus *Exiguobacterium* with sequence identity of 100%. 4cFLTR strain was shown to match *Staphylococcus warneri* at 99-100% identity. Strain 4M6 was identified as *Halobacillus blutaparonensis* with 100% identity. The sequence results for 6aFLTR and 6aFLSK strains showed them both to be *Halomonas venusta* with the highest identity match of 99.5%.

*Exiguobacterium* genus was first described by (Collins *et al*., 1983) who stated that this genus belongs to the expanding group of coryneform bacteria and is closely related to the *Bacillus* genus within the order Bacillales. *Exiguobacterium* strains are Gram – positive rod shaped, alkaliphilic, halotolerant, facultative anaerobic non- spore forming bacteria (Suzuki *et al*., 2005). Many strains of *Exiguobacterium* can grow well in a pH range of 7-10. They can be isolated from industrial wastewater as well as rivers and lakes, and are capable of lowering the pH of highly alkaline wastewater (Kulshreshtha *et al*., 2010). *Exiguobacterium* species have also been implicated in human infections, where it was obtained from patients with bacteraemia (Pitt *et al.*, 2007). Some strains of *Exiguobacterium* can be used in bioremediation (e.g. in the biological removal of toxicants in the environment) of hexavalent chromium (Cr VI), a harmful agent which leads to morphological changes and growth reduction in plants and carcinogenic effects in man and animals (Okeke, 2008).

*Halomonas* species are halotolerant and (sometimes) alkaliphilic Gram-negative rod-shaped bacteria that grow aerobically at the optimum temperature of 37°C, and at pH

7.5-10. Due to its halophilic nature, *Halomonas* are usually found in water sources with high salinity levels (Romano *et al*., 2006). *Halomonas* species can be involved in human health issues and some strains are assumed to be pathogenic bacteria causing infections and contaminations (Stevens *et al*., 2009).

*H. venusta* is a moderately halophilic, non fermentative Gram-negative rod-shaped bacterium and it has been reported as a human pathogen in a wound of a female patient. The bacterium source originated from a fish bite (Von Graevenitz *et al.*, 2000).

*Staphylococcus warneri* is Gram-positive coccus-shaped bacterium, it is coagulase-negative and is a common commensal organism found as part of the skin flora on humans and animals and commonly found in the flora of human epithelia and mucosal membranes (Campoccia *et al*., 2010). Like other coagulase-negative staphylococci, *S. warneri* rarely causes disease, but may occasionally cause infection in patients with compromised immunity. However, *S. warneri* has also been categorized as a pathogenic bacteria that can cause serious diseases like endocarditis (Dan *et al.*, 1984), and ventricular and orthopaedic infections (Campoccia *et al*., 2010). This bacterium is of significant value in veterinary medicine due to its pathological effect in cattle in which it causes abortion and it is also associated with meningoencephalitis in dogs (Espino *et al*., 2006).

# Chapter Four

# PHYSIOLOGICAL CHARACTERISTICS OF *Exiguobacterium*

## 4.1 Introduction

In Chapter 3, the two isolates from the Bradford and Lathkill rivers, ABr1 and AL2, were identified as belonging to the genus *Exiguobacterium.* The genus *Exiguobacterium* was proposed in 1983 by Collins *et al*. based on a chemotaxonomic study of five strains of Gram-positive bacteria isolated from potato-processing effluent (Collins *et al*., 1983). Members of the new genus *Exiguobacterium* can be identified based on their cell wall composition, which is based on Lys-Gly peptidoglycan type. The type species is *E. aurantiacum* and the pH range for growth is 6.5 – 11.5, with optimal growth between pH 8.5 – 9.5.

Over the following 20 years, only two other isolates, one from a garden pond in Germany (*E. undae*) and the other from a lake in Antarctica (*E. antarcticum*) have been added to the *Exiguobacterium* genus (Fruhling *et al*., 2002). However, between 2004 and 2008, a further nine species of *Exiguobacterium* have been identified: *E. oxidotolerans* - an alkaliphile showing high levels of catalase activity isolated from a Japanese fish processing plant (Yumoto *et al.*, 2004); *E. aestuarii* and *E. marinum* – slightly halophilic species from a tidal flat in Korea (Kim *et al.*, 2005); *E. mexicanum* and *E. artemiae* – slightly halophilic species isolated from brine shrimp (*Artemia franciscana*) cysts (López-Cortés *et al*., 2006); *E. sibiricum* – psychrotolerant strain isolated from permafrost (Rodrigues *et al*., 2006); *E. indicum* – slightly halophilic, psychrotolerant and alkalitolerant strain isolated from glacier melt water in the Himalayas (Chaturvedi and Shivaji, 2006); *E. profundum* – moderately thermophilic strain isolated from a deep sea hydrothermal vent in the Pacific Ocean (Crapart *et al*., 2007) and *E. soli* – a psychrophilic strain isolated from the McMurdo dry valleys in Antarctica (Chaturvedi *et al*., 2008).

Recently, two further strains of *Exiguobacterium* have been isolated, *E. aquaticum* – from a lake in India (Raichand *et al.*, 2012) and *E. himgiriensis* – slightly alkaliphilic and slightly halophilic strain again isolated from the Himalayan region (Singh *et al*., 2013). The *Exiguobacterium* genus comprises psychrophilic, mesophilic and thermophilic strains, some are alkaliphilic and all seem to show some tolerance to increased salinity. The genus *Exiguobacterium* is closely related to the *Bacillus* genus and is placed in the order Bacillales, within the phylum Firmicutes (Vishnivetskaya *et al*., 2009).

Several strains of *Exiguobacterium* show properties that are of interest to industry and agriculture. (Kulshreshtha *et al*., 2012) demonstrated the usefulness of an *Exiguobacterium* strain DSM21148 in reducing the pH of alkaline wastewater from pH 12.0 to pH 7.5. The pH reduction was due to excretion of an organic acid (probably formic acid) into the medium. Another *Exiguobacterium* strain (2Sz) was an efficient remover of pesticide (Vishnivetskaya *et al.*, 2009). There are a few reports in the literature (Pitt *et al*., 2007) which suggest that *Exiguobacterium* species can be isolated from humans suffering from a range of diseases such as periodontis and cellulitis.

In 2012, it was announced that the genome sequence of *E. antarcticum* was available and can be accessed in the GenBank database under CP003063.1 (Carneiro *et al.*, 2012). This psychrophilic strain of *Exiguobacterium* was chosen for sequencing because of its potential roles in bioremediation and as a source of cold adapted enzymes. Therefore, the genus *Exiguobacterium* has environmental as well as public health hazard significance in addition to its industrial use (Kulshreshtha *et al*., 2012).

In this chapter, the two strains isolated from the River Bradford and the River Lathkill (ABr1 and AL2), which were identified as *Exiguobacterium* strains in Chapter 3, will be further characterised in terms of their physiology and ability to grow well at various pHs.

## 4.2 Results and Discussion

### 4.2.1 Isolation and Selection of Microorganisms

In order to isolate and grow alkaliphilic bacteria, a highly alkaline (pH >9) medium must be used. For this purpose, Horikoshi medium (Table 2.1) was selected and prepared for the initial isolation of strains (Horikoshi, 1999). Ten environmental samples of water with a variety of original pH values were collected from different places in both the Bradford and Lathkill rivers in the Derbyshire Peak District (Alsull, 2010) as shown in Table 4.1.

| | Bradford River | | | | | Lathkill River | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Original pH | 7.8 | 8.3 | 8.3 | 8.2 | 8.3 | 8.0 | 8.2 | 7.5 | 7.9 | 8.1 |

**Table 4.1.** Original pH readings of river water samples. Samples 1 to 5 were collected from the edge of the River Bradford between the villages of Youlgreave and Alport. Samples 6 to 10 were collected from the edge of the River Lathkill in the area between the village of Over Haddon and the Conksbury Bridge. All pH readings of samples were carried out using a Mettler Toledo MP225 pH meter.

To isolate alkaliphilic bacteria (i.e. bacteria with optimum growth at pH 9 or above), from the river samples, serial sub-culture and streak plating techniques were used repeatedly, with alkaline Horikoshi medium (liquid and solid plate media). Single colonies were selected according to the findings of best growth at pH 10. Cells were

checked in terms of morphology and motility and were examined microscopically using Gram stain reaction and motility test respectively to find out more information about the bacterial isolates.

Using these techniques, two strains of bacteria were selected and designated as ABr1 (from River Bradford) and AL2 (from River Lathkill). Cells of these strains showed most resistance to alkaline pH and subsequently were successfully grown at pH 10 in Horikoshi medium at an early stage in the adaptation test processes. As described in Chapter 3, both strains have been shown to belong to the *Exiguobacterium* genus and will henceforth be called *Exiguobacterium* ABr1 and *Exiguobacterium* AL2.

### 4.2.2 Characteristic Features of *Exiguobacterium* ABr1 and *Exiguobacterium* AL2

Table 4.2 presents the basic characteristic features of *Exiguobacterium* ABr1and AL2 strains grown in pH 10 liquid Horikoshi medium at 25ºC overnight. It was found that the two strains had Gram-positive cell wall morphology and the cell shape for both strains is rod-shaped.

On solid Horikoshi medium the colony morphology of the two strains was observed to be semi-translucent, circular, regular and entire. ABr1 has a dry texture appearance while AL2 was mucoid in texture. Furthermore, ABr1 strain had deep orange coloured colonies, whereas AL2 strain had faint orange colonies on plates.

Examination of growth temperature range of the two strains showed no changes in morphology or colony characteristics when plates or flasks were incubated at 37ºC.

| Name of Bacterium | Gram Stain Reaction | Motility | Cell Shape | Colony Colour | Type of Growth |
|---|---|---|---|---|---|
| *Exiguobacterium* ABr1 | positive | Positive | rod shape | deep orange | aerobic & non-aerobic |
| *Exiguobacterium* AL2 | positive | Positive | rod shape | faint orange | aerobic & non-aerobic |

**Table 4.2.** The basic characteristic features of *Exiguobacterium* ABr1and AL2 strains. Cells were grown in pH 10 liquid Horikoshi medium and were incubated in 25ºC constant temperature with continuous shaking at 250 rpm overnight.

### 4.2.3 Growth Characteristics of *Exiguobacterium* Strains ABr1and AL2 at Different pH Values

Growth curves were carried out for *Exiguobacterium* ABr1and AL2 to observe the effect of external pH on growth rates at pH 8, pH 9 and pH 10 in Horikoshi medium. Bacterial growth was quantified and monitored using direct optical density (OD) at 600 nm measured with Unicam Helisα spectrophotometer (Fig. 4.1). It appears that both strains grow well over the pH range 8 to 10, but there was a long lag phase before growth commenced at pH 8 for strain AL2. This suggests that *Exiguobacterium* AL2 is an alkaliphilic strain.

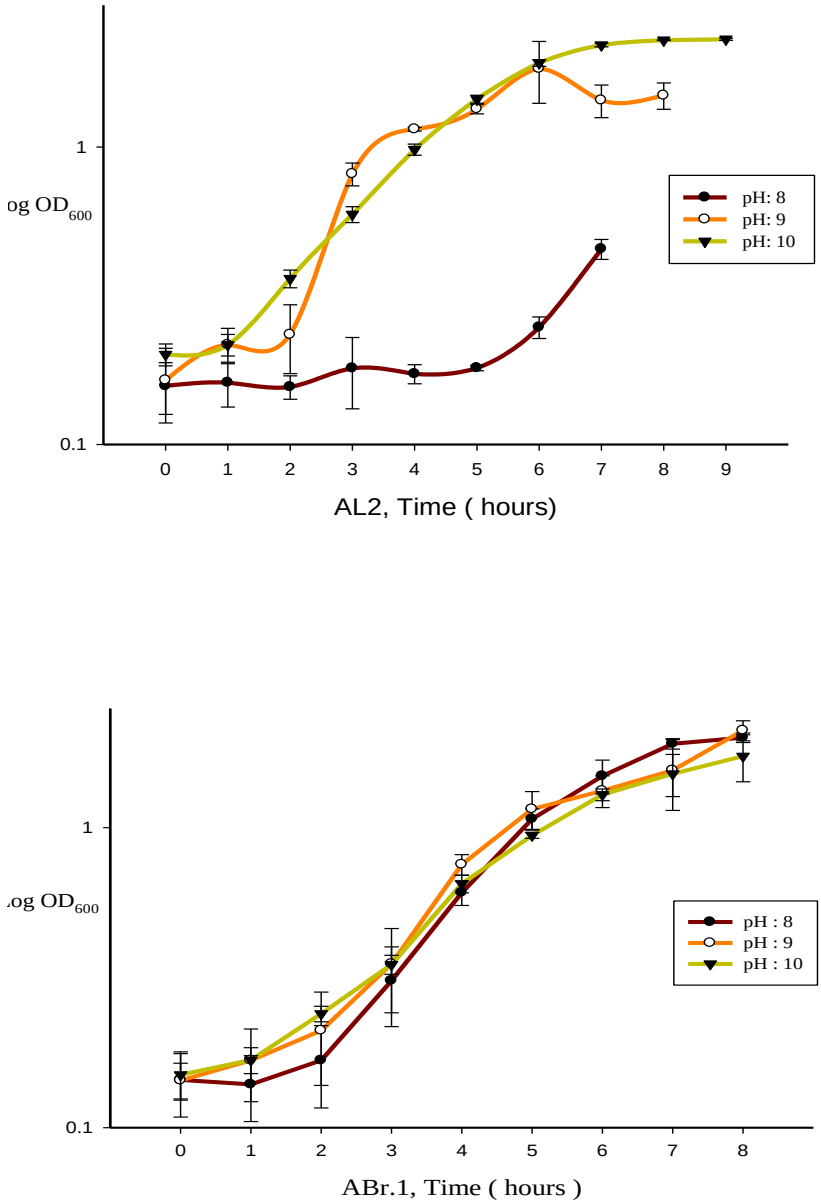



**Figure 4.1.** Growth curves for *Exiguobacterium* ABr1and AL2 strains after repeated sub-culture period (fully adapted period). Cells were grown in Horikoshi medium at pH 8, 9 and 10, incubated in a 25ºC constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm each hour. Data points are the means of three replicates plus or minus standard error.

### 4.2.4 Effect of External pH on the Respiration Rate

Respiration rate of bacteria, as a function of oxygen concentration, was measured using a Clarke-type oxygen electrode. Both *Exiguobacterium* strains (ABr1and AL2) were tested for the effect of external pH on the rate of uptake of oxygen over pH range of pH 8 to 10 in Horikoshi medium. Cells were harvested by centrifugation then the pellets re-suspended in Horikoshi medium with different pH values. Then oxygen uptake of bacterial strains was determined by the oxygen electrode and the results are shown in Table 4.3.

**Respiration Rate (Mean ± S.D)**
(μmoles $O_2$ mg protein$^{-1}$ h$^{-1}$)

| Growth pH | pH 8 | pH 9 | pH10 |
|---|---|---|---|
| ABr1 | 59.4 ± 23.34 | 50.5 ± 20.07 | 92.2 ± 25.47 |
| AL2 | 52.7 ± 16.23 | 38.5 ± 10.82 | 46.5 ± 14.11 |

**Table 4.3.** Respiration rate for *Exiguobacterium* ABr1and AL2 at pH 8, 9 and 10. 20 ml of cells from each strain grown at pH 8, pH 9, and pH 10 in Horikoshi medium overnight at 25°C on an orbital shaker at 250 rpm were harvested by centrifugation and resuspended in 10 ml of fresh Horikoshi medium of the same pH. The $O_2$ uptake was measured. Data points are the means of three replicates plus or minus standard error.

The highest rate of respiration for strain ABr1was at pH 10, while for AL2, the highest rate of respiration was at pH 8. However, it should be noted that the error bars associated with the respiration rates are large and there is probably little evidence to support statistically significant trends in the results shown in Table 4.3. It is best to conclude that good rates of respiration were found for both strains at all three pH values tested.

### 4.2.5 Antimicrobial susceptibility test

Antibiotic resistance tests were carried out using the standard disk method as described in Section 2.9. Bacterial cells were spread on agar plates of Horikoshi solid plate media with pH 8, 9 and 10. Antibiotic discs were placed on plates and inhibition zones were calculated after overnight incubation. Figure 4.2 shows examples of plates used in the antibiotic sensitivity tests.

In *Exiguobacterium* ABr1, it is found that the sensitivity to antibiotics may be affected by the pH. The inhibition zone may differ either when pH was increased or decreased. Most antibiotic inhibition zones were slightly affected or showed no changes when exposed to pH 10 or lower. Ampicillin (AMP) and Gentamycin (CN) inhibition zones were lower at pH 10 than the rest of the group. Some antibiotics were not affected by increasing pH increasing such as cefoxitin (FOX) which showed increased zones of inhibition from 2.5 ± 0.22 at pH 8 to 3 ± 0.11at pH 10 (Table 4.4).

| Antibiotic | pH8 | pH9 | pH10 |
|---|---|---|---|
| AMP | 4.0± 0.07 | 3.3± 0.15 | 2.9± 0.05 |
| AML | 3.3± 0.14 | 3.1± 0.05 | 2.8± 0.16 |
| VA | 1.4± 0.04 | 1.4± 0.05 | 1.5± 0.05 |
| TE | 2.3± 0.11 | 2.1± 0.05 | 2.4± 0.10 |
| E | 2.2± 0.0 | 2.2± 0.11 | 2.4± 0.17 |
| P | 3.2± 0.11 | 3.2± 0.05 | 2.8± 0.20 |
| FOX | 2.5± 0.22 | 2.8± 0.25 | 3± 0.11 |
| MY | 2.1± 0.09 | 1.9± 0.51 | 2.3± 0.20 |
| S | 1.5± 0.11 | 1.5± 0.11 | 1.7± 0.10 |
| IPM | 1.8± 0.09 | 1.5± 0.00 | -ve |
| C | 1.9± 0.07 | 2.1± 0.10 | 2.1± 0.10 |
| CN | 2.1± 0.04 | 2.1± 0.05 | 1.5± 1.3 |
| SXT | 2.5± 0.10 | 2.7± 0.05 | 2.6± 0.05 |

**Table 4.4.** Antibiotic sensitivity tests for *Exiguobacterium* ABr1. Data points are the means of three replicates plus or minus standard deviations for zones of inhibition.

| Antibiotic | pH8 | pH9 | pH10 |
|---|---|---|---|
| AMP | 4.1± 0.10 | 3.8± 0.05 | 3.9± 0.25 |
| AML | 3.7± 0.26 | 3.2± 0.20 | 3.4± 0.15 |
| VA | 1.3± 0.11 | 1.2± 0.05 | 1.3± 0.10 |
| TE | 1.4± 0.00 | 1.3± 0.10 | 1.1± 0.20 |
| E | 1.3± 0.05 | 1.3± 0.05 | 1.4± 0.10 |
| P | 3.06± 0.23 | 3.1± 0.11 | 3.03± 0.28 |
| FOX | 3.2± 0.20 | 3.3± 0.10 | 3.3± 0.10 |
| MY | 2.1± 0.05 | 2.2± 0.15 | 2.2± 0.10 |
| S | 1.7± 0.10 | 1.7± 0.05 | 1.8± 0.11 |
| IPM | 3.2± 0.20 | 2.6± 0.30 | 1.6± 0.05 |
| C | 2.5± 0.20 | 2.4± 0.05 | 2.5± 0.17 |
| CN | 2.1± 0.05 | 2.2± 0.00 | 2.1± 0.05 |
| SXT | 2.3± 0.32 | 2.06± 0.05 | 2.1± 0.15 |

**Table 4.5.** Antibiotic sensitivity tests for *Exiguobacterium* AL2. Data points are the means of three replicates plus or minus standard deviations for zones of inhibition.

Table 4.5 shows similar results were obtained for *Exiguobacterium* AL2. Most antibiotic inhibition zones were slightly affected or showed no changes when exposed pH 10 or lower. It is noteworthy that all the antibiotics tested had an inhibitory effect on the growth of *Exiguobacterium* ABr1 or AL2.

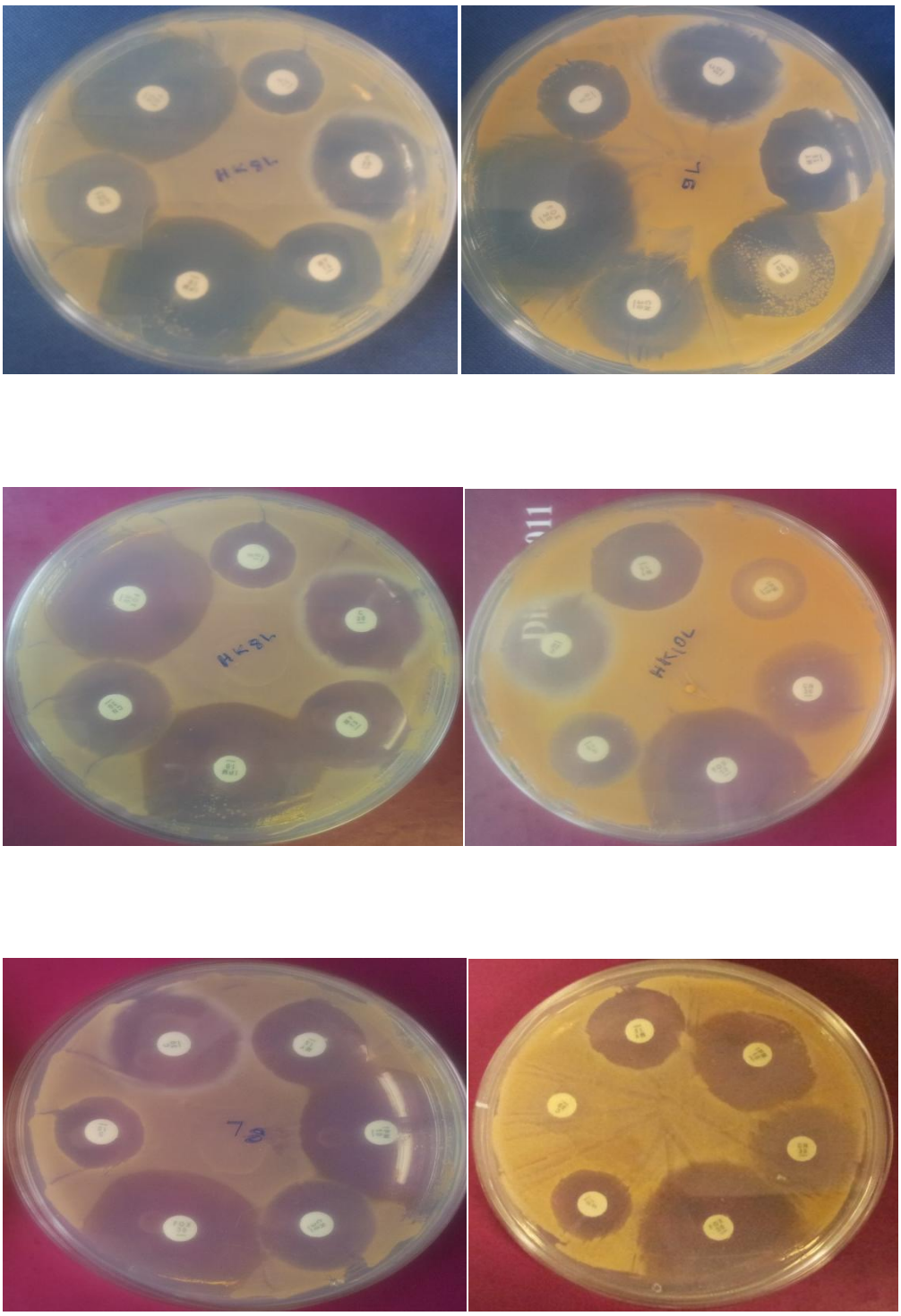


**Figure 4.2.** Examples of antibiotic inhibition zones for *Exiguobacterium* ABr1 and AL2 when exposed to Antimicrobial Succeptibility Test Discs (Oxoid).

### 4.2.6 Biolog Plate Results for *Exiguobacterium* ABr1 and AL2

To allow a wide range of carbon sources to be tested for their ability to support growth of *Exiguobacterium* ABr1 and AL2, Biolog GP2 (Gram-positive) plates were purchased. In the absence of the full Biolog identification system the GP2 plates were measured in the lab plate reader. This technique relies on the irreversible reduction of a tetrazolium redox-dye to the purple formazan as an indicator for organic substrate oxidations. ABr1 and AL2 were grown in normal Horikoshi medium (pH 10) in 250 ml flasks overnight. The bacteria were then washed with M9 minimal medium (without any carbon source) three times to avoid any carryover of carbon sources from the Horikoshi medium. Biolog GP2 plates were inoculated with washed bacterial cells to give an $OD_{600}$ of 0.2 and incubated overnight at 25°C. The results are shown in Table 4.6. Two points should be kept in mind, firstly a number of unusual carbon sources are included in the Biolog GP2 plate to help to identify a wide range of organisms and secondly the tetrazolium reaction measures oxidation of the substrate, but not actual growth. Therefore, it is not surprising that many of the carbon sources could not be used by the *Exiguobacterium* strains. It is clear from Table 4.6 that the two strains differ in their carbon source utilization. The following compounds were utilized well by both strains: β-hydroxybutyric acid, α-ketovaleric acid, propionic acid, pyruvic acid, L-serine, 2-3-butanediol, glycerol and several phosphorylated sugars.

| GP2 BIOLOG | AL2 *Exiguobacterium* | ABr1 *Exiguobacterium* |
|---|---|---|
| Water | 0.317 | 0.358 |
| α- cyclodextrin | - | - |
| β- cyclodextrin | ± | - |
| Dextrin | ± | - |
| glycogen | - | - |
| inulin | ± | - |
| mannan | - | - |
| tween 40 | + | ± |
| tween 80 | + | ± |
| N-acetyl-D-Glucosamine | - | - |
| N-acetyl- β-D-Mannosamine | - | - |
| Amygdalin | - | - |
| L-Arabinose | - | - |
| D-Arabitol | - | - |
| Arbutin | - | - |
| D-cellobiose | ± | - |
| D-fructose | - | - |
| L-fucose | - | - |

| | | |
|---|---|---|
| D-galactose | - | - |
| D-galacturonic acid | - | - |
| Gentiobiose | - | - |
| D-Gluconic acid | - | - |
| α-D-Glucose | - | - |
| m-Inositol | - | - |
| α-D-Lactose | + | ± |
| Lactulose | - | - |
| Maltose | - | - |
| Maltotriose | - | - |
| D-Mannitol | - | - |
| D-Mannose | ± | - |
| D-Melezitose | ± | - |
| D-Melibiose | - | - |
| α-Methyl-D-Galactoside | - | + |
| β-Methyl-D-Galactoside | ± | - |
| 3-Methyl Glucose | + | - |
| α-Methyl-D-Glucoside | + | - |
| β-Methyl-D-Glucoside | - | - |
| α-Methyl-D-Mannoside | ± | - |
| Palatinose | - | - |
| D-Psicose | - | - |
| D-Raffinose | - | - |
| L-Rhamnose | ± | - |
| D-Ribose | - | ± |
| Salicin | - | - |
| Sedoheptulosan | - | - |
| D-Sorbitol | - | ± |
| Stachyose | + | - |
| Sucrose | + | ± |
| D-Tagatose | - | - |
| D-Trehalose | - | - |
| Turanose | ± | - |
| Xylitol | - | - |
| D-Xylose | - | - |
| Acetic acid | ± | - |
| α-Hydroxybutyric acid | + | - |
| β- Hydroxybutyric acid | + | ± |
| γ- Hydroxybutyric acid | + | + |
| p-Hydroxy-Phenylacetic acid | - | ± |
| α-Ketoglutaric acid | + | ± |
| α-Ketovaleric acid | + | + |
| Lactamide | ± | - |
| D-Lactic acid Methyl Ester | + | - |
| L-Lactic acid | + | - |
| D-Malic acid | + | - |
| L-Malic acid | + | - |
| Pyruvatic acid Methyl Ester | ± | - |
| Succinic acid Mono-methyl Ester | + | ± |
| Propionic acid | + | + |

| | | |
|---|---|---|
| Pyruvic acid | + | + |
| Succinamic acid | + | - |
| Succinic acid | + | - |
| N-Acetyl-L-Glutamic acid | + | ± |
| L-Alaninamide | ± | - |
| D-Alanine | ± | - |
| L-Alanine | ± | - |
| L-Alanyl-Glycine | + | - |
| L-Asparagine | - | - |
| L-Glutamic acid | + | - |
| Glycyl-L-Glutamic acid | ± | - |
| L-Pyroglutamic acid | + | ± |
| L-Serine | + | + |
| Putrescine | + | - |
| 2,3-Butanediol | + | + |
| Glycerol | + | + |
| Adenosine | - | - |
| 2-Deoxy Adenosine | ± | - |
| Inosine | ± | - |
| Thymidine | ± | - |
| Uridine | ± | - |
| Adenosine-5-Monophosphate | + | + |
| Thymidine-5-Monophosphate | ± | + |
| Uridine-5-Monophosphate | + | + |
| D-Fructose-6-Phosphate | + | + |
| α-D-Glucose-1-Phosphate | + | + |
| D-Glucose-6-Phosphate | + | + |
| D-L-α-Glycerol Phosphate | + | + |

**Table 4.6.** Carbon utilization tests carried out on GP2 Biolog 96 well plates. The reduction of the tetrazolium dye was measured at $OD_{595}$. For AL2, values between 0.35 and 0.4 were rated weak growth (±) and values above 0.4 were rated as good growth (+). For ABr1, values between 0.4 and 0.45 were rated as weak growth (±) and values above 0.45 were rated as good growth (+). This was based on the OD value in the presence of water only.

### 4.2.7 Determination of Compatible Solutes (Osmolytes) by Nuclear Magnetic Resonance Spectroscopy (NMR)

Compatible solutes are normally synthesized or accumulated in response to salinity increases (see section 1.4). In this experiment, the idea was to look for compatible solute production in response an increase in pH. The two *Exiguobacterium* strains (ABr1 and AL2) were grown in pH 7 and pH 10 Horikoshi medium and treated as described in section 2.17 to release the compatible solutes for identification using NMR. Figure 4.3 shows the compatible solutes accumulated by *Exiguobacterium* AL2 and ABr1 when grown in Horikoshi medium at pH 7 and 10. From the spectra it is clear that glycine betaine was accumulated by *Exiguobacterium* AL2 at pH 10, but was absent from pH 7 grown AL2 cells. No betaine was detected at either pH value for *Exiguobacterium* ABr1, but a sugar similar to glucose was accumulated at pH 10 by ABr1. It is interesting that a known compatible solute (betaine) was accumulated at high pH by one strain and it is also interesting that the two strains behaved differently in this NMR experiment.

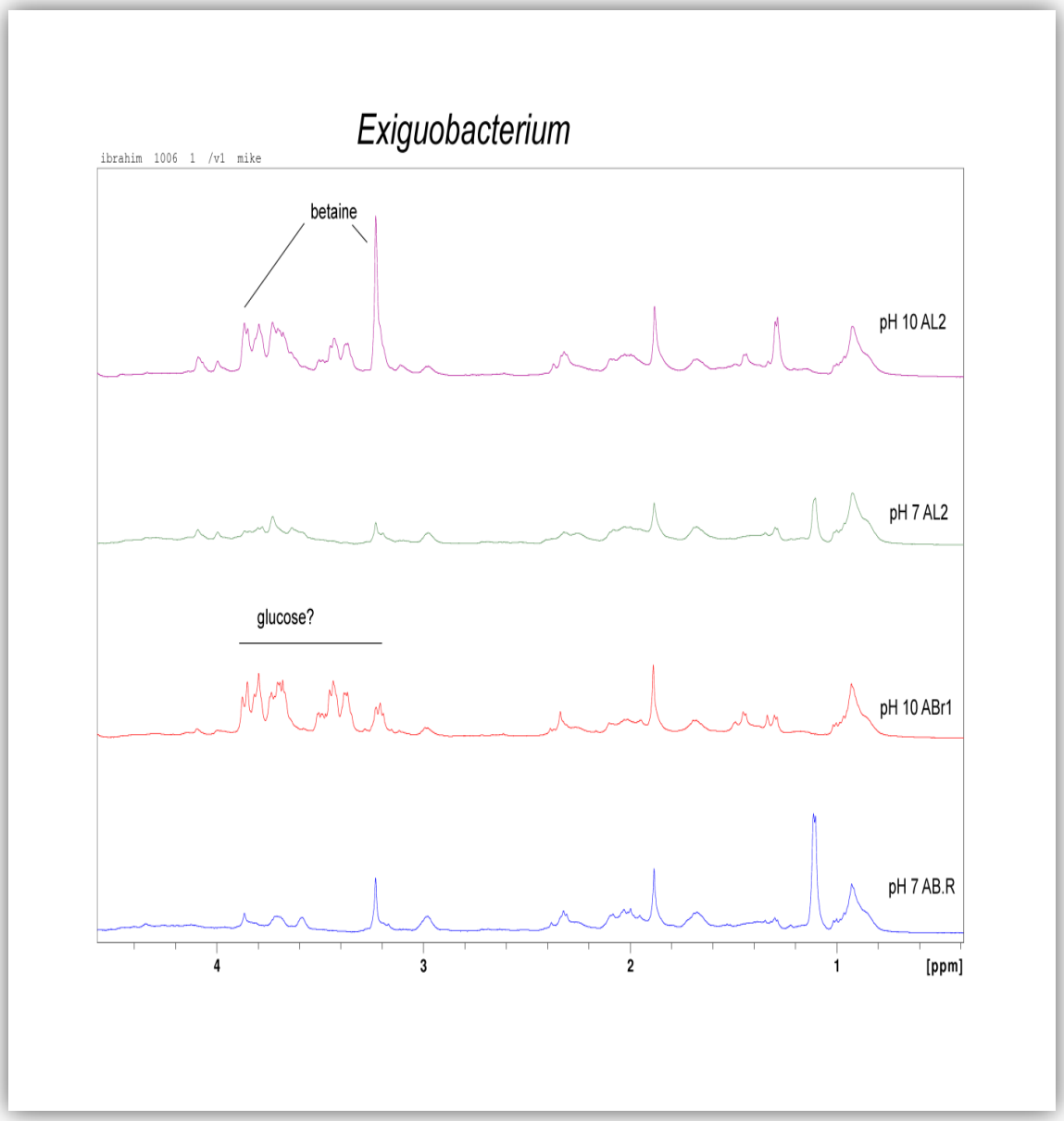



**Figure 4.3.** One-dimensional 1H-NMR spectra of cell extracts derived from *Exiguobacterium* AL2 and ABr1 cells grown in Horikoshi medium with adjusted pH of 7 and 10. Clear peaks associated with betaine are seen at pH 10 for AL2.

## 4.3 Conclusions

As described in the introduction to this chapter, a growing number of isolates are being identified as members of the *Exiguobacterium* genus (section 4.1). The basic characteristics of the two strains (AL2 and ABr1) isolated from the Lathkill and Bradford rivers respectively agree well with the description for the *Exiguobacterium* genus i.e. Gram –positive rods, facultative anaerobes, with orange/red colonies that grow well at pH values up to pH 10. The antibiotic tests and the Biolog GP2 plate tests suggest that AL2 and ABr1 are potentially new species within the *Exiguobacterium* genus (Vishnivetskaya *et al*., 2009). However, more information is required to validly publish the strains as new species of *Exiguobacterium* including details of the major fatty acids found in AL2 and ABr1 plus longer sequences of the 16S rRNA genes from both strains (Singh *et al*., 2013).

The identification of the compatible solute betaine in *Exiguobacterium* AL2 cells grown at pH 10 is the first time that a compatible solute has been described in an *Exiguobacterium* species. The results described in this chapter also make it clear that the two strains are likely to be separate species within the genus *Exiguobacterium*.

# Chapter Five

# PHYSIOLOGICAL CHARACTERISTICS OF *Halobacillus blutaparonensis* and *Staphylococcus warneri*

## 5.1. Introduction

Microbial life can be found in a wide range of fresh water and marine environments as diverse fresh water ponds/rivers and hypersaline lakes. Microorganisms can thrive in these environments including the saturated salt hypersaline environments. Microorganisms show a wide range of salt tolerance and some demonstrate a requirement for salt (Gilmour, 1990).

Extremophilic microorganisms are those microbes which can be found in extreme environments, but extremophiles can also be isolated from non-extreme environments such as soil and fresh water, where they may not be expected to grow well (Echigo *et al*., 2005). Halophilic and halotolerant bacteria are one type of extremophilic microorganisms. Halophilic microorganisms are able to grow and survive in hypersaline environments by their ability to maintain osmotic balance. Halophilic microorganisms can adapt to saline environments in two ways. The first way is to maintain cytoplasmic osmotic balance with the external environment by accumulating high concentrations of various organic osmotic compatible solutes e.g. ectoine or betaine. The second way is to maintain cytoplasmic osmotic balance with the external hypersaline environment by accumulating high concentrations of NaCl (see section 1.4). Osmotic regulation by the second method requires special adaptations of the intracellular enzymes that have to function in the presence of salt (Margesin and Schinner, 2001). Therefore halophilic microbes can thrive in environments that contain a wide range of NaCl concentrations. Slightly halophilic or halotolerant organisms require 1-6% (0.2-1M) NaCl, while moderate halophiles require 6-15% (0.5-2.5 M) NaCl. Extreme halophiles require 15-30% (2-5.2 M) NaCl, where it reaches saturation level (Gilmour, 1990, Oren, 2008).

In Chapter 3, three strains of bacteria, which were isolated from the Al-Asfar fresh water lake in Hassa Saudi Arabia, were identified using 16S rRNA gene sequencing as *Staphylococcus warneri* (4cFLTR), *Halobacillus blutaparonensis* (4M6) and *Halomonas venusta* (6aFLTR). It was decided not to study *H. venusta* in further detail, because the genus *Halomonas* has been very well studied and characterised in the literature (Arahal *et al*., 2007). The two other strains, *H. blutaparonensis* and *S. warneri* are the subject of further characterisation as described in this chapter.

*H. blutaparonensis* is a Gram-positive rod spore forming bacterium, motile that thrives in moderately halophilic environments. *H. blutaparonensis* was first isolated from *Blutaparon portulacoids* roots in Brazil, the plant *B. portulacoids* is a succulent herb

found on sand dunes and beaches of the Atlantic coast of Brazil (Barbosa *et al.*, 2006). Apart from this publication, no other work has been published on *H. blutaparonensis.*

*S. warneri* is a Gram-positive, coccus-shaped bacterium, coagulase – negative, considered one of the most dangerous nosocomial pathogenic agent in hospitals (Kamath *et al*., 1992). However it was believed for a long time that *S. warneri* bacteria are harmless common commensals inhabiting the skin and nasal cavities of humans and animals (Kassem, 2009). High risk of *S. warneri* infection was found in immunocompromised patients (Ivić *et al*.) , 2013). *S. warneri* can be isolated from many environments e.g. as ponds, rivers, food premises, computer surfaces and hospitals. More than one route of transmission of *S. warneri* between hosts is known (zoonoses/anthroponosis) and between hosts and their environment have contributed to the spread of infection beyond the boundaries of health care facilities and into the community (Kassem, 2009). It has also been found that this microorganism is industrially useful, because it can convert oleic acid to 10-ketostearic acid (Lanser and Nakamura, 1996).

## 5.2 Results and Discussion

### 5.2.1 Isolation and Selection of Microorganisms

The aim of this part of the work was to isolate and characterise extremophilic microorganisms from fresh water samples collected from Hassa, Saudi Arabia. A total of 24 flasks were filled with about 50 ml of LB medium containing different concentrations of NaCl (0.5, 1 and 2 M) and inoculated with 10 ml of water samples which were collected from the Al-Asfar lake and drainage canals, Saudi Arabia. All flasks were incubated at 37°C with shaking. Table 5.1 shows the extent of bacterial growth after 24 hours incubation.

| Sample no. | 0.5 M | 1M | 2M |
|---|---|---|---|
| 1 | - | - | - |
| 2 | +++ | ++ | + |
| 3 | - | - | - |
| 4 | +++ | +++ | - |
| 5 | +++ | +++ | +++ |
| 6 | +++ | +++ | +++ |
| 7 | +++ | ++ | +++ |
| 8 | +++ | +++ | - |

**Table 5.1.** Growth of bacteria in LB medium containing 0.5, 1 or 2 M NaCl after inoculation with water from the eight samples taken from the Hassa region in Saudi Arabia. Sample 1 was treated water used for irrigation, samples 2 and 3 were taken near the treatment plant, samples 4 and 5 were taken from drainage canals and samples 6, 7 and 8 were taken from the lake itself. Full details of the sampling sites are given in section 2.2.2.

To isolate halophilic bacteria from Saudi samples, serial sub-culture and streak plating techniques were used repeatedly, using Lurea Bertani LB media (liquid and solid plates). Single colonies were selected according to the findings of best growth different molarities. Cells were checked in terms of morphology and motility and were examined microscopically using Gram stain reaction and motility test respectively to find out more information about the bacterial isolates. The two strains studied further in this chapter emerged from this process - 4M6 strain came from sample 4 (*Halobacillus blutaparonensis*) and 4cFLTR came from sample 4 (*Staphylococcus warneri*). The basic characteristics of both strains are listed in Table 5.2.

| Name of Bacterium | Gram Stain Reaction | Motility | Cell Shape | Colour |
|---|---|---|---|---|
| *Halobacillus blutaparonensis* | Positive | positive | rod shape | creamy to faint yellow |
| *Staphylococcus warneri* | Positive | positive | cocci | turbid white |

**Table 5.2.** The basic characteristic features of *H. blutaparonensis* (4M6) and *S. warneri* (4cFLTR) strains. Cells were grown in LB medium and were incubated at 37ºC with continuous shaking at 250 rpm overnight.

### 5.2.2 Growth of *H. blutaparonensis* at Different Salinities

Growth curves were carried out for *H. blutaparonensis* to observe the effect of different salinities (0.17 M, 1 M, 2 M, 3 M and 4 M NaCl) on growth rate in rich LB medium. Bacterial growth was quantified and monitored using direct optical density (OD) measurements at 600 nm using the Unicam Helisα spectrophotometer (Figure 5.1). It appears that *H. blutaparonensis* grew very well up to 1 M NaCl and good growth was also found at 2 and 3 M NaCl, but it was decreased compared to growth at 1 M NaCl. No growth was found at 4 M NaCl (Figure 5.1).

### 5.2.3 Growth of *S. warneri* at Different Salinities

Growth curves were carried out for *S. warneri* to find out the effect of media that contain different molarities of NaCl (0.17 M, 1 M, 2 M, 2.5 M and 3 M) on growth rate in rich LB medium. Bacterial growth was quantified and monitored using direct optical density (OD) measurements at 600 nm using the Unicam Helisα spectrophotometer (Fig 5.2). Unlike, *H. blutaparonensis* (Fig 5.1), *S. warneri* growth decreased at all salinities above 0.17 M NaCl. However, good growth takes place up to 2.5 M NaCl, but at 3 M NaCl there was only very weak growth (Fig 5.2).

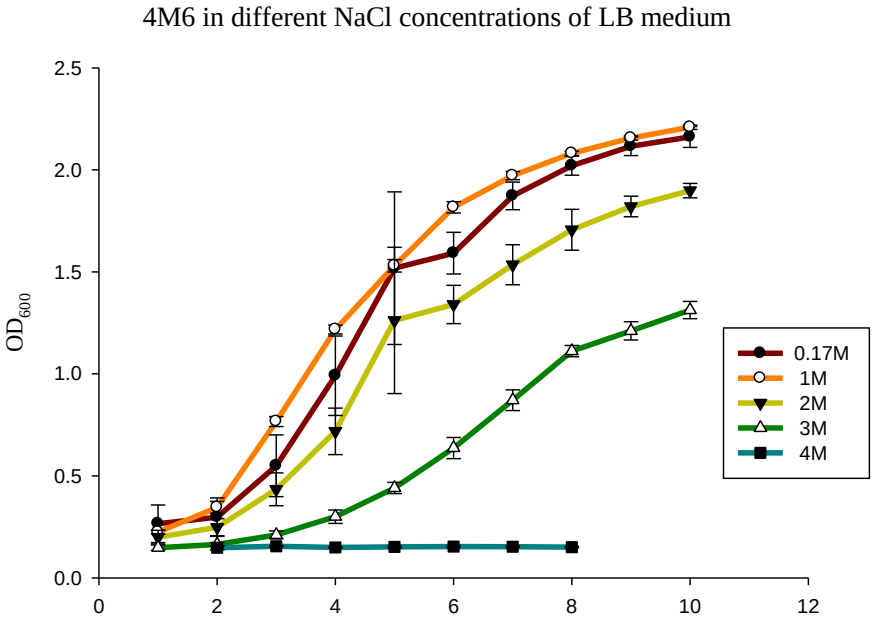



**Figure 5.1.** Growth curves for *H. blutaparonensis* cells growing in LB medium with different molarities of NaCl (0.17 M, 1 M, 2 M, 3 M and 4 M), incubated overnight at 37ºC on an orbital shaker at 250 rpm. The OD was measured at 600 nm against a medium blank. Data points are the means of three replicates plus or minus standard deviation. The x axis shows time in hours.

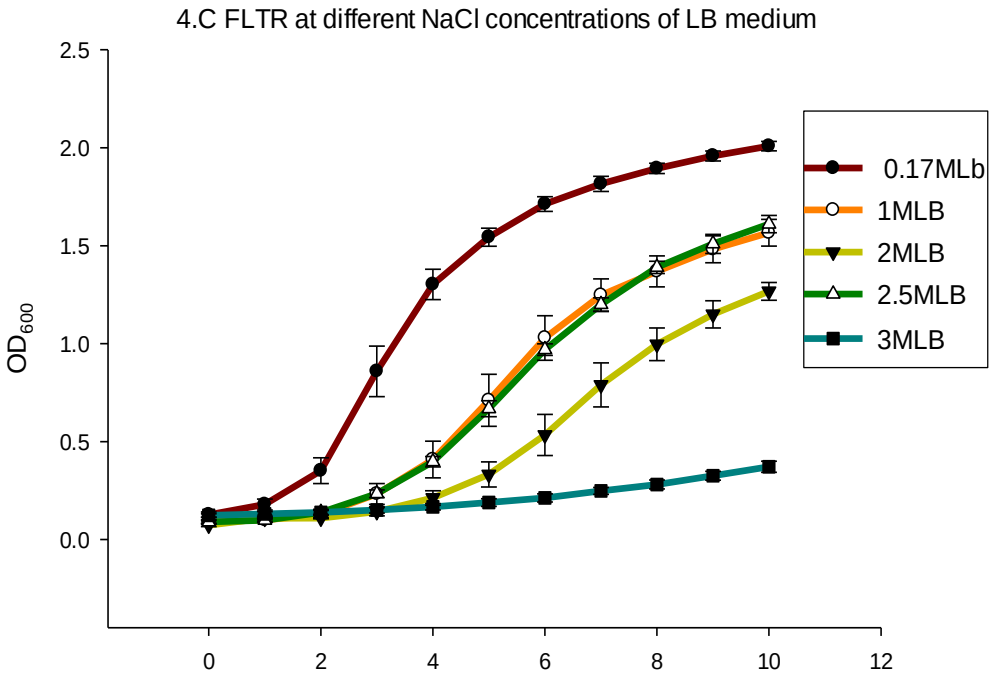



**Figure 5.2.** Growth curves for *S. warneri* cells growing in LB medium at different molarities of NaCl (0.17 M, 1 M, 2 M, 2.5 M and 3 M) in LB medium, incubated overnight at 37ºC on an orbital shaker at 250 rpm. The OD was measured at 600 nm against a medium blank. Data points are the means of three replicates plus or minus standard deviation. The x axis shows the time in hours.

### 5.2.4 Antimicrobial susceptibility tests

Antibiotic resistance tests were carried out on *H. blutaparonensis* using the standard disk method as described in Section 2.9. Bacterial cells were spread on agar plates of LB solid medium containing different concentrations of NaCl. Antibiotic discs were placed on plates and inhibition zones were calculated after overnight incubation. It was decided to perform antimicrobial susceptibility tests for *H. blutaparonensis* only because of the lack of information in this regard in the literature. In contrast the antibiotic sensitivity of *S. warneri* has already been widely studied (Center *et al*., 2003). The results for *H. blutaporensis* are shown in Table 5.3.

**Table 5.3.** Antibiotic sensitivity tests for *Halobacillus blutaparonensis* to antibiotics. Data points are the means of three replicates plus or minus standard deviations.

| Antibiotic | 0.17MLB | 1MLB | 2MLB | 2.5MLB | 3MLB |
|---|---|---|---|---|---|
| AML | 4.5± 0.12 | 3.9± 0.12 | 4.3± 0.15 | 4.3± 0.26 | 4.6± 0.21 |
| VA | 2.1± 0.11 | 1.9± 0.05 | 2.1± 0.05 | 2.2± 0.10 | 2.4± 0.05 |
| TE | 3.2± 0.15 | 3.1± 0.10 | 3.3± 0.17 | 3.5± 0.10 | 3.9± 0.31 |
| E | 3.4± 0.08 | 3.1± 0.05 | 3.3± 0.26 | 3.3± 0.11 | 4.1± 0.30 |
| P | 4.4± 0.20 | 3.8± 0.25 | 4.4± 0.20 | 4.2± 0.25 | 4.6± 0.23 |
| FOX | 3.5± 0.10 | 3.4± 0.05 | 4.2± 0.15 | 4.1± 0.12 | 3.8± 0.35 |
| MY | 2.2± 0.12 | 2.4± 0.05 | 2.7± 0.10 | 2.8± 0.05 | 3.4± 0.05 |
| S | 1.7± 0.05 | -ve | -ve | -ve | -ve |
| IPM | 4.4± 0.42 | 4.1± 0.36 | 4.3± 0.05 | 4.2± 0.09 | 4.5± 0.20 |
| C | 2.7± 0.10 | 2.8± 0.05 | 2.9± 0.05 | 3.3± 0.10 | 3.7± 0.42 |
| CN | 2.5± 0.36 | 2.1± 0.05 | 1.6± 0.05 | 1.6± 0.23 | 1.6± 0.05 |
| SXT | 1.7± 0.20 | 1.7± 0.05 | 2.3± 0.10 | 2.4± 0.20 | 2.3± 0.10 |

In 4M6 *Halobacillus blutaparonensis*, it is found that the sensitivity to antibiotics may be affected by the concentration of NaCl present. The inhibition zone may differ when the salinity is increased as in lincomycin MY and erythromycin E or may be decreased as in the case of gentamycine CN. However, most antibiotic inhibition zones were only slightly affected or not affected at all when exposed to higher or lower salinities (e.g penicillin P). However for streptomycine S increasing the salinity lead to the complete absence of inhibition zones.

### 5.2.5 Biolog Plate Results for *H. blutaparonensis* 4M6 and *S. warneri* 4cFLTR

Cells of *H. blutaparonensis* and *S. warneri* were grown in LB medium, in 250 ml flasks and incubated at 37°C overnight. The bacteria were then washed with M9 minimal medium (without any carbon source) three times to avoid any carryover of carbon sources from the LB medium. Biolog GP2 plates were inoculated with washed bacterial cells to give an $OD_{600}$ of 0.2 and incubated overnight at 37°C. The results in Table 5.4 show that the carbon utilization patterns for *H. blutaparonensis* and *S. warneri* are very different as would be expected since they are closely related organisms. Both organisms are capable of utilizing a wide range of carbon sources, which helps them to colonise a range of natural habitats.

| **GP2 BIOLOG** | ***H. blutaparonensis* 4M6** | ***Staph. warneri* 4.C FLTR** |
|---|---|---|
| Water | 0.986 | 0.492 |
| α- cyclodextrin | - | - |
| β- cyclodextrin | - | ± |
| Dextrin | - | - |
| glycogen | + | ± |
| inulin | ± | - |
| mannan | - | + |
| tween 40 | + | + |
| tween 80 | + | + |
| N-acetyl-D-Glucosamine | + | ± |
| N-acetyl- β-D-Mannosamine | + | ± |
| Amygdalin | ± | - |
| L-Arabinose | - | - |
| D-Arabitol | - | ± |
| Arbutin | - | - |
| D-cellobiose | ± | - |

| | | |
|---|---|---|
| D-fructose | - | - |
| L-fucose | ± | - |
| D-galactose | + | - |
| D-galacturonic acid | + | + |
| Gentiobiose | + | + |
| D-Gluconic acid | + | + |
| α-D-Glucose | + | ± |
| m-Inositol | + | ± |
| α-D-Lactose | ± | ± |
| Lactulose | ± | - |
| Maltose | - | - |
| Maltotriose | ± | - |
| D-Mannitol | ± | - |
| D-Mannose | ± | - |
| D-Melezitose | + | - |
| D-Melibiose | + | ± |
| α-Methyl-D-Galactoside | + | ± |
| β-Methyl-D-Galactoside | + | ± |
| 3-Methyl Glucose | + | - |
| α-Methyl-D-Glucoside | ± | ± |
| β-Methyl-D-Glucoside | ± | + |
| α-Methyl-D-Mannoside | ± | + |
| Palatinose | - | ± |
| D-Psicose | - | + |
| D-Raffinose | ± | + |
| L-Rhamnose | + | + |
| D-Ribose | ± | + |
| Salicin | ± | + |
| Sedoheptulosan | + | + |
| D-Sorbitol | + | + |
| Stachyose | + | + |
| Sucrose | + | + |
| D-Tagatose | - | ± |
| D-Trehalose | - | + |
| Turanose | - | + |
| Xylitol | ± | + |
| D-Xylose | - | + |
| Acetic acid | ± | + |
| α-Hydroxybutyric acid | + | + |
| β- Hydroxybutyric acid | + | + |
| γ- Hydroxybutyric acid | + | + |
| p-Hydroxy-Phenylacetic acid | + | ± |
| α-Ketoglutaric acid | + | + |
| α-Ketovaleric acid | + | + |
| Lactamide | - | + |
| D-Lactic acid Methyl Ester | - | + |
| L-Lactic acid | ± | + |
| D-Malic acid | - | + |
| L-Malic acid | - | + |
| Pyruvatic acid Methyl Ester | ± | + |
| Succinic acid Mono-methyl | ± | + |

| | | |
|---|---|---|
| Ester | | |
| Propionic acid | + | + |
| Pyruvic acid | ± | + |
| Succinamic acid | + | + |
| Succinic acid | + | + |
| N-Acetyl-L-Glutamic acid | ± | + |
| L-Alaninamide | - | + |
| D-Alanine | - | + |
| L-Alanine | - | + |
| L-Alanyl-Glycine | ± | + |
| L-Asparagine | ± | + |
| L-Glutamic acid | - | + |
| Glycyl-L-Glutamic acid | + | + |
| L-Pyroglutamic acid | + | + |
| L-Serine | + | + |
| Putrescine | ± | + |
| 2,3-Butanediol | ± | + |
| Glycerol | ± | + |
| Adenosine | - | + |
| 2-Deoxy Adenosine | - | + |
| Inosine | - | + |
| Thymidine | - | + |
| Uridine | - | + |
| Adenosine-5-Monophosphate | - | + |
| Thymidine-5-Monophosphate | - | + |
| Uridine-5-Monophosphate | - | + |
| D-Fructose-6-Phosphate | - | + |
| α-D-Glucose-1-Phosphate | - | + |
| D-Glucose-6-Phosphate | ± | + |
| D-L-α-Glycerol Phosphate | ± | + |

**Table 5.4.** Carbon utilization tests carried out on GP2 Biolog 96 well plates. The reduction of the tetrazolium dye was measured at $OD_{595}$. For 4M6 (*H. blutaparonensis*), values between 1.0 and 1.25 were rated weak growth (±) and values above 1.25 were rated as good growth (+). For 4cFLTR (*S. warneri*), values between 0.5 and 0.75 were rated as weak growth (±) and values above 0.75 were rated as good growth (+). This was based on the OD value in the presence of water only.

### 5.2.6 Determination of Compatible Solutes (Osmolytes) by Nuclear Magnetic Resonance Spectroscopy (NMR)

In order to identify the compatible solutes accumulated by *Halobacillus blutaparonensis* and *Staphylococcus warneri*, their cells were grown in different salinities and the compatible solutes were extracted from the cells and detected by NMR as described in section 2.23.

The spectra of *Halobacillus blutaparonensis* cells contain signals from normal cellular metabolites (e.g. signals in the range of 0.5 – 3 ppm). There was some change in these signals with NaCl concentration: signals between 2 and 2.5 ppm decreased gradually in intensity from 2 to 3.5 M NaCl. These were mainly from glutamate, showing that glutamate is being used as an osmolyte at low salt concentrations. The most obvious signals in the spectrum were from betaine, which is present at 2 M salt and increases up to 3 M salt. Therefore, the glutamate is being replaced as an osmolyte by betaine as the salt concentration increases. This is a common observation for many moderately halophilic bacteria.

At 3.5 M NaCl, the concentration of betaine decreased (Figure 5.3). This is not what one would expect if it is being used as the osmolyte. However, it is a very common observation that the osmolyte concentration is unpredictable at very high salt concentration, when the cells are struggling to grow.

Figure 5.4 shows *S. warneri* samples behaved in a consistent manner. There are signals from normal cell metabolites (0.5 – 3 ppm) which did not change much with NaCl concentration. The only signals that changed significantly are from the osmolyte betaine, which increased steadily as the salt concentration increases. It is concluded that betaine is the only compatible solute that increased with increasing salinity in *S. warneri cells.*

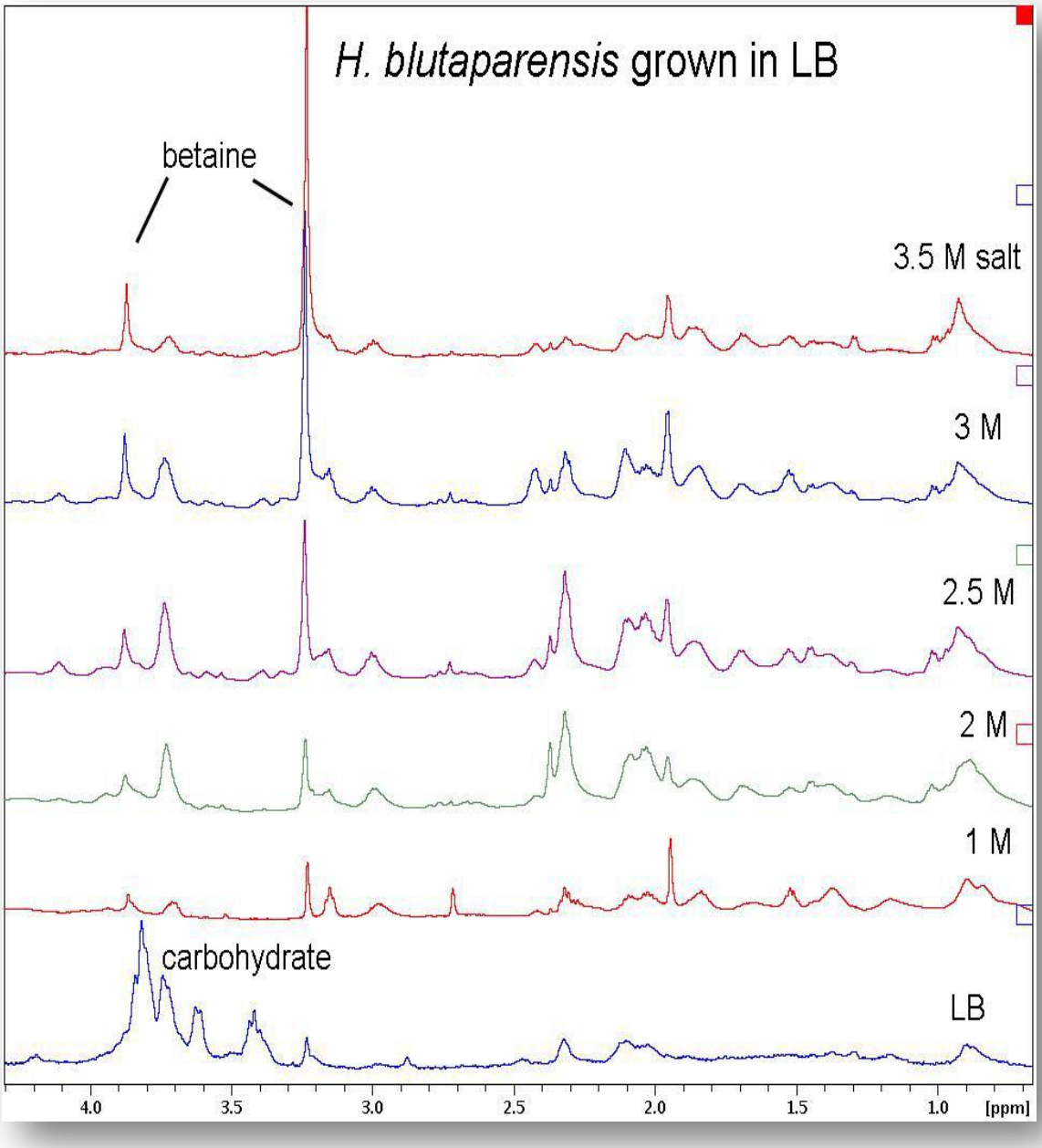



**Figure 5.3.** One-dimensional 1H-NMR spectra of cell extracts derived from *Halobacillus blutaparonensis* cells grown in LB medium with different concentrations of NaCl from 0.17 (normal LB medium) to 3.5 M NaCl.

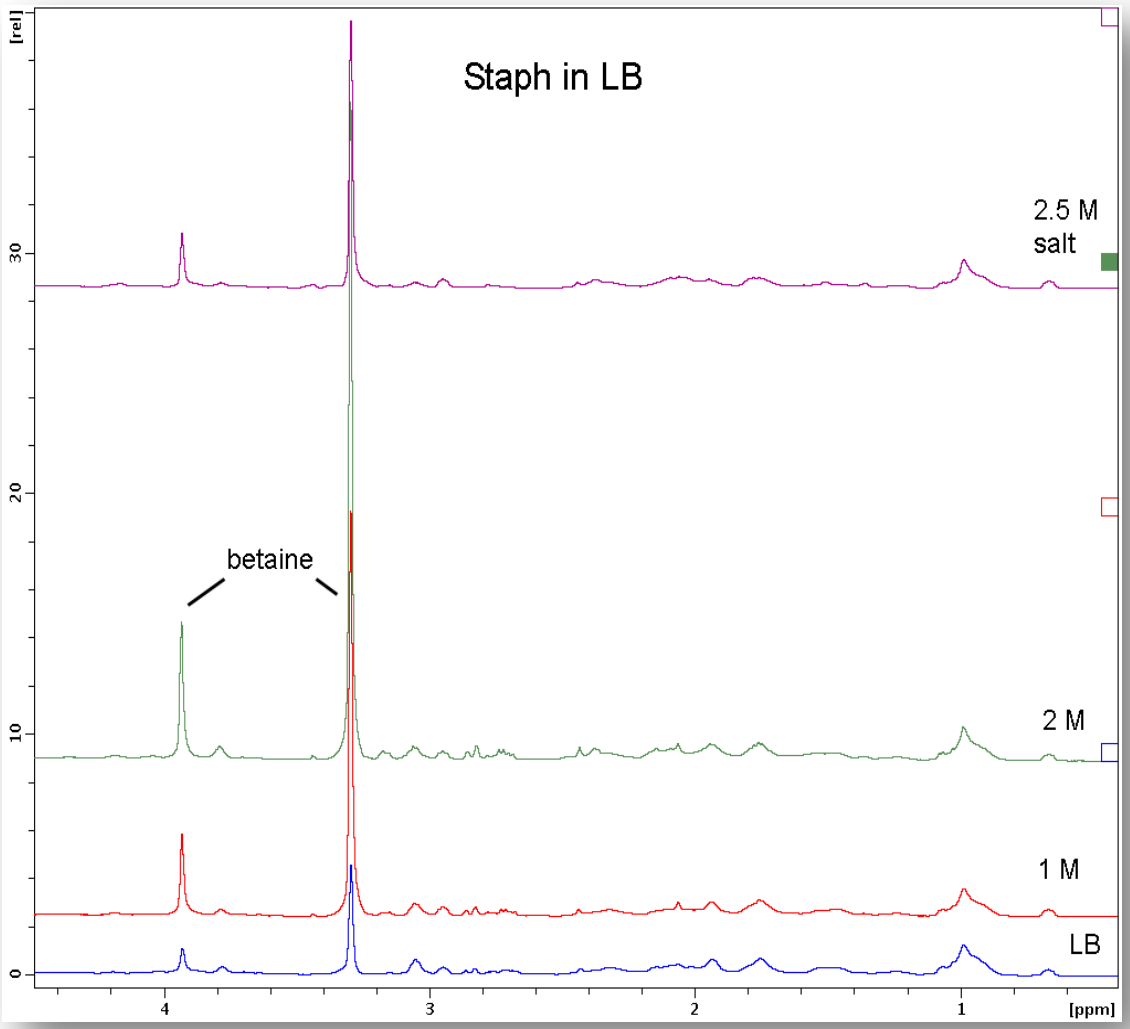



**Figure 5.4**. One-dimensional 1H-NMR spectra of cell extracts derived from *Staphylococcus warneri* cells grown in LB medium with different concentrations of NaCl from 0.17 M (normal LB medium) to 2.5 M NaCl.

## 5.3 Conclusions

The work described in this chapter shows that moderately halophilic (*H. blutaparonensis*) and halotolerant (*S. warneri*) microorganisms can be isolated from freshwater samples – in this case from the Al-Asfar Lake and surrounding drainage canals in Hassa, Saudi Arabia.

Figure 5.1 shows that *H. blutaparonensis* is moderately halophilic, because growth at 1 M NaCl is virtually identical to growth in normal LB medium i.e. 0.17 M NaCl. *H. blutaparonesis* could grow at 3 M NaCl, but no growth was found at 4 M NaCl (Fig 5.1). This agrees well with the original description of *H. blutaparonensis* by (Barbosa *et al*., 2006) who found that the maximum salinity for growth was 20% NaCl, which equals 3.4 M NaCl. The response of *H. blutaparonensis* to salinity makes sense in the context of the Brazilian shoreline where it was isolated by Barbosa *et al*. (2006), since this habitat is subject to fluctuating salinity as the tide ebbs and flows, but it is less obvious why the organism is well suited to grow in the freshwater Al-Asfar Lake. In both the current study and the published work by Barbosa, LB medium was used to isolate and grow *H. blutaparonensis* in varying levels of NaCl. Interestingly, the current work showed that *H. blutaparonensis* would not grow in high salinity M9 minimal medium, it appears to require rich medium to grow at high salinity. This requirement for high levels of nutrients also fits well with its original isolation from the roots of the *Blutaparon* plant, but not so well for its isolation from the lake. However, other work from our laboratory plus the published work of (Echigo *et al*., 2005) clearly demonstrates that halophilic bacteria can be found in ordinary garden soils and freshwater river samples. Interestingly, the opposite is also true i.e. bacteria isolated from salt fields in Japan can also grow well at low salinities (Fukushima *et al*., 2007). Therefore, the main conclusion from this part of the work is that salt tolerant or moderately halophilic bacteria can be found in freshwater environments.

The original work on *H. blutaparonensis* by (Barbosa *et al*., 2006) did not address how the organism copes with high salinities. In the current work, *H. blutaparonensis* was shown to accumulate the compatible solute betaine at high salinities using NMR analysis (Fig 5.3). Betaine is a common compatible solute and is often used in rich LB medium where choline can be transported into the cells at high salinities and converted to betaine by a two-enzyme pathway (Cummings and Gilmour, 1995). This is a very efficient way of accumulating compatible solute and allowing growth at high salinity.

In M9 minimal medium, many moderate halophiles switch to ectoine as their compatible solute and most organisms will accumulate some ectoine at high salinities even when grown in LB medium (Kunte, 2006). However, *H. blutaparonensis* does not accumulate ectoine at high salinities in LB medium (Fig 5.3) and it does not grow in minimal medium at high salinity. This suggests that *H. blutaparonensis* cannot synthesise ectoine and is thus dependent on choline-driven betaine synthesis for growth at high salinities. It thus differs from another species of *Halobacillus*, *H. halophilus*, which is known to synthesise ectoine (Saum and Müller, 2008).

The further characterization carried out on *H. blutaparonensis* in the current work included antibiotic sensitivity assays (Table 5.3) and also carbon source utilization (Table 5.4). This information shows that *H. blutaparonensis* is sensitive to a range of antibiotics and it utilizes a wide range of carbon sources. The information gathered for *H. blutaparonensis* agrees well with the published description by (Barbosa *et al*., 2006) and it seems highly likely based on the 16S rRNA gene sequencing (Chapter 3) and the physiological characterization in this chapter that isolate 4M6 is a strain of *H. blutaparonensis*.

The other strain studied in this chapter, *S. warneri*, is of interest due to its potential pathogenicity as described in section 5.1. It is halotolerant rather than moderately halophilic, because the best growth was at 0.17 M NaCl (Fig 5.2). Only very slow growth was seen at 3 M NaCl. NMR analysis showed that *S. warneri* also accumulated betaine as its compatible solute at high salinities (Fig 5.4). No data have been published concerning compatible solute production by *S. warneri*, but it is known that *S. aureus* accumulates betaine and proline as its main compatible solutes (Miller *et al*., 1991). In the current work, only betaine was found to be accumulated by *S. warneri* (Fig 5.4).

Table 5.4 shows that *S. warneri* utilized a very wide range of carbon sources and the organism is clearly well adapted to grow in environments with different mixtures of carbon sources, which may help to explain the presence of this pathogenic organism in the Al-Asfar Lake water.

# Chapter 6

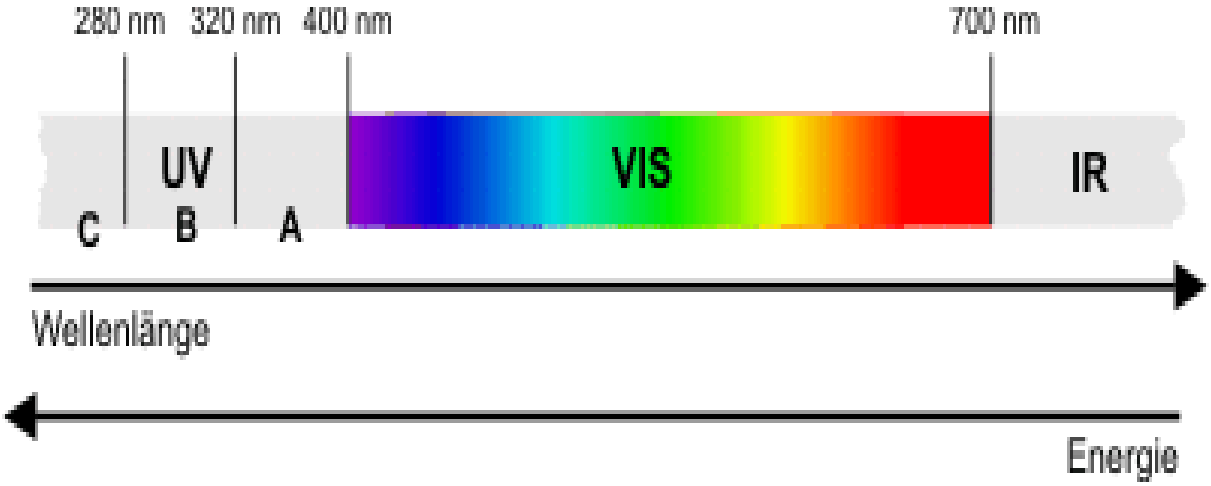
# EFFECT OF UV RADIATION ON THE SURVIVAL OF *Exiguobacterium, Halobacillus blutaparonensis and Staphylococcus warneri*

## 6.1 Introduction

Solar radiation in the environment is potentially destructive. Ultra Violet (UV) light or radiation is one type of solar radiation that can cause serious damage to a variety of organisms from bacteria to humans due to its deleterious effect on DNA (Sinha and Häder, 2002, Kumari *et al*., 2008). DNA is the store of genetic information in living cells, its integrity is essential to life. DNA, however, is not inert; rather, it is a chemical entity subject to assault from the environment. Any UV damage, if not repaired, will lead to serious damage, mutation and possibly disease (Rodriguez-Rocha *et al.*, 2011). UV light is classified into three types: UVA, UVB and UVC electromagnetic radiation (Table 6.1). UV light has wavelengths less than that of visible light (400 nm) and more than that of X-ray radiation (100 nm). UVA and UVB are naturally found in the environment on Earth and cause many health hazards especially skin lesions and skin cancer. UVC is almost all absorbed by the ozone layer, but it is commonly used in laboratories and hospitals for the purpose of disinfection and decontamination due to its germicidal action where it is produced using special lamps (Gascon *et al.*, 1995).

**Table 6.1.** Different types of UV radiation.

| | | | | |
|---|---|---|---|---|
| Ultraviolet A | UVA | 315 – 400 nm | long wavelength | black light |
| Ultraviolet B | UVB | 280 – 315 nm | medium wavelength | |
| Ultraviolet C | UVC | 100 – 280 nm | short wavelength | germicidal |



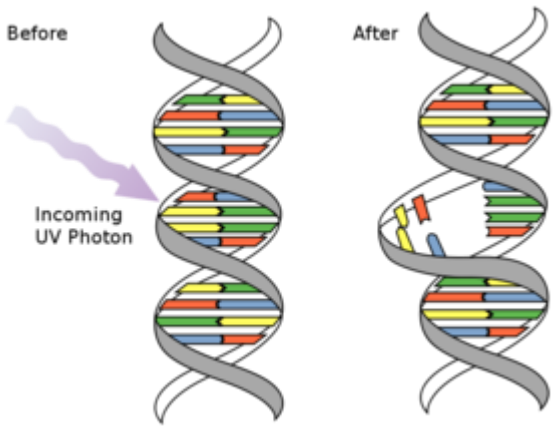

http://www.imb-jena.de/www_kog/research/dnadamage/uv.html

**Figure 6.1.** Illustrates the spectrum of UV light wavelength which is less than that of visible light (400 nm) and more than that of X-ray radiation (100 nm). UVA and UVB are the natural source of UV radiation in the environment. UVC is the shortest wavelength and can be produced synthetically.

Exposure of bacterial cells to UVC (245 nm) results in a variety of DNA lesions. UV can cause two of the most abundant mutagenic and cytotoxic DNA photolesions i.e. cyclobutane-pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs). These pyrimidine dimers can block DNA replication unless repaired (Kielbassa *et al*., 1997). Pyrimidine dimers form when the carbon-carbon double bonds within individual pyrimidines break and become bonded with the carbons of the adjacent nucleotide. The most frequent occurrence of this type of UV damage occurs between adjacent thymines (Fig 6.2).

Many ways of making genetic repairs exist, among them the autogenic DNA repair mechanism in which accurate transmission of genetic information occurs from one cell to its daughters and this is the key factor in survival of the organisms. This transmission requires extreme accuracy in DNA replication and precision in chromosome distribution (Reed *et al.*, 1996). Among the repair mechanisms that overcome the UV induced damage are photoreactivation, nucleotide excision repair (NER) and base excision

repair (BER) (Friedberg *et al*., 1995). NER has a multienzyme repair complex which can remove a wide variety of types of base damage as oligonucleotides (Reed *et al*., 1996). While BER is more selective in combating UV damage and repair is by recognition of the type of lesion or its chemical by-products. The damage is removed in the form of a single base (Doetsch and Cunningham, 1990).




http://en.wikipedia.org/wiki/Pyrimidine_dimers

Figure 6.2. The effect of UV radiation on thymine or cytocine causing molecular base lesions and pyrimidine dimers via photochemical reactions.

Since microorganisms vary in their tolerance of UV radiation in terms of the time of exposure, it was decided to test the resistance to UV radiation of various strains to shed some light on participation of environmental microbes in public health. Three strains were chosen, particularly relevant to those bacteria of medical or public health significance; *Staphylococcus warneri* in comparison to a non pathogenic environmental strain; *Halbacillus blutaparonensis*, in addition to *Exiguobacterium* which represents a genus of alkaliphilic bacteria that can sometimes be pathogenic.

Experimental design was such that the bacterial samples were divided into liquid cultures for the purpose of evaluating the effect of UV on DNA and agar plate cultures to find out the effect of UV on colony formation. In both cases, the effect of UVC exposure was tested after various times of exposure at a wavelength of 254 nm (power =

40 Joule). The UV lamp was set at a height of 25 to 30 cm above the bacterial cultures. To look for the presence or absence of DNA regenerating agents such as 6-4 photoproducts and cyclobutane-pyrimidine dimers that prove the presence or onset of DNA regeneration, a period of light and dark treatment was applied post UV radiation exposure. Finally the Electron Microscope facility was used to examine the effect of UV light on the morphology of cells.

## 6.2 Results and Discussion

The effects of exposure to Ultra Violet radiation were examined using cells incubated on agar plates (solid medium) and in liquid cultures.

### 6.2.1 Agar Plates

To study the effects of UV light on bacterial cells incubated on agar plates, dilutions of overnight cultures were made ($10^{-4}$, $10^{-5}$, and $10^{-6}$) which were streaked on LB agar plates and subjected to UV light for 90 seconds, 105 seconds, 120 seconds, 135 seconds, 150 seconds and 165 seconds for *Exiguobacterium* (Fig 6.3). For *H. blutaparonensis*, the same dilutions of overnight cultures were subjected to UV light for 110 seconds, 115 seconds, 120 seconds, 125 seconds and 130 seconds (Fig 6.4). Finally dilutions of ($10^{-3}$, $10^{-4}$, and $10^{-5}$) for *S. warneri* were exposed to UV radiation for 60 seconds, 90 seconds, 105 seconds, 110 seconds, 120 seconds, 135 seconds and 150 seconds (Fig 6.5). The different times were based on preliminary experiments. In addition, control plates that were not subjected to UV radiation for each dilution were counted. The results in Figures 6.3 to 6.5 suggest that on agar plates, all three organisms are susceptible to the killing effect of UV light. It appears that the pathogenic strain, *S. warneri*, is most sensitive, with *H. blutaparonensis* being marginally the most resistant.

Figures 6.6 and 6.7 show examples of plates exposed to UV light for *Exiguobacterium* (Fig 6.6) and *S. warneri* (Fig 6.7). It is very clear that *S. warneri* is much less resistant to UV exposure than *Exiguobacterium.* Figures 6.6 and 6.7 also show a flaw in the experimental protocol (i.e. the control plates and plates exposed to UV for short time periods had confluent growth and individual colonies could not be counted). For this reason, it was decided to conduct all future experiments by exposing liquid cultures to the UV light.

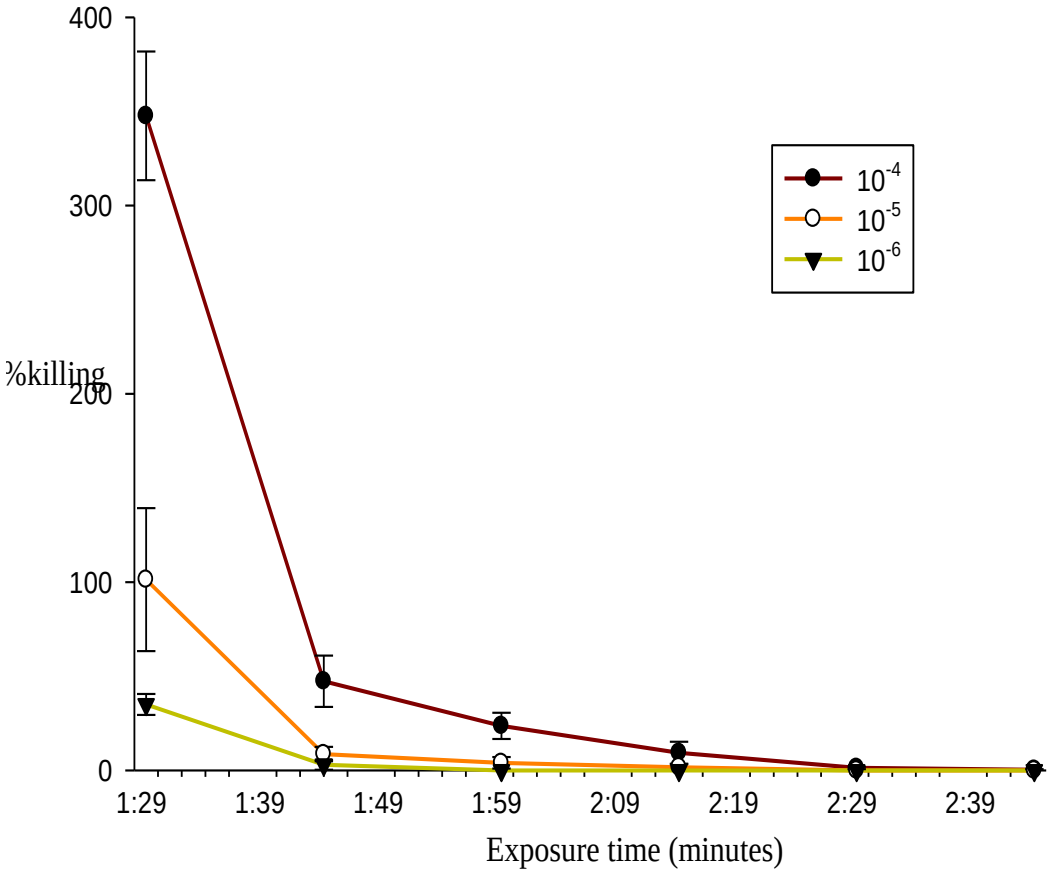



**Figure 6.3.** Effect of UV exposure on *Exiguobacterium* colony % killing. Dilutions ($10^{-4}$, $10^{-5}$ and $10^{-6}$) of overnight cultures were spread on plates and then exposed to UV light. After overnight incubation, the numbers of colonies on each plate were counted. Each point represents mean ± standard error. Note that the x axis does not start at zero.

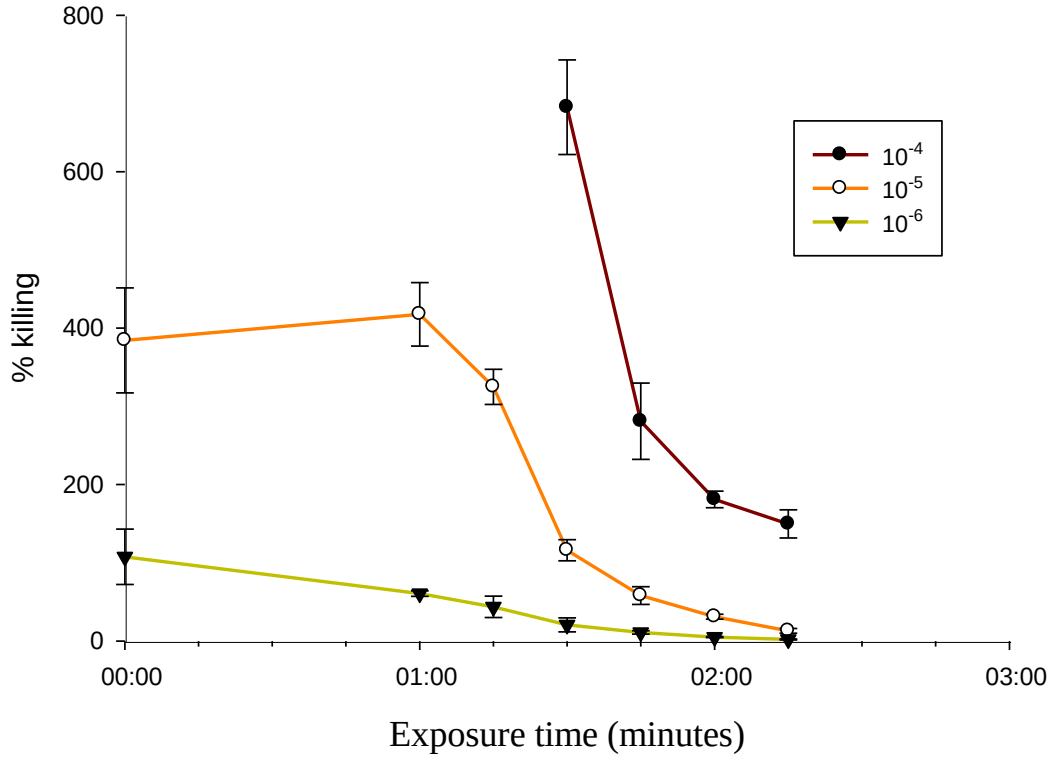
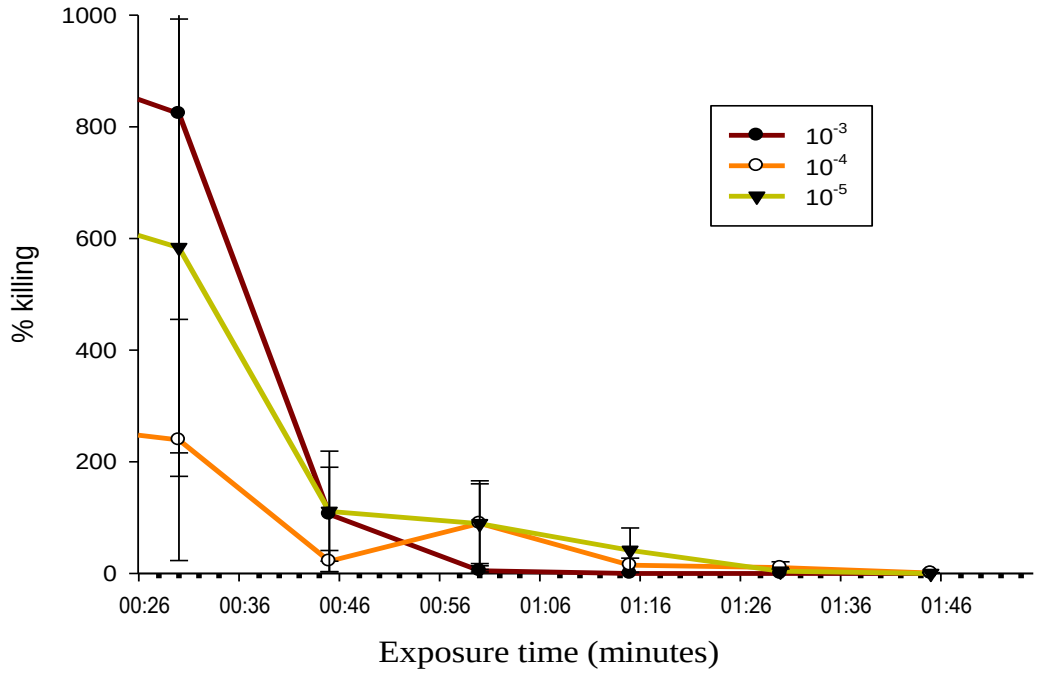



**Figure 6.4.** Effect of UV exposure on *H. blutaparonensis* colony % killing. Dilutions ($10^{-4}$, $10^{-5}$ and $10^{-6}$) of overnight cultures were spread on plates and then exposed to UV light. After overnight incubation, the numbers of colonies on each plate were counted. Each point represents mean ± standard error.




UV exposed *Staphylococcus warneri*

**Figure 6.5.** Effect of UV exposure on pathogenic *Staphylococcus warneri*, colony % killing. Dilutions ($10^{-3}$, $10^{-4}$ and $10^{-5}$) of overnight cultures were spread on plates and then exposed to UV light. After overnight incubation, the numbers of colonies on each plate were counted. Each point represents mean ± standard error. Note the x axis does not start at zero.

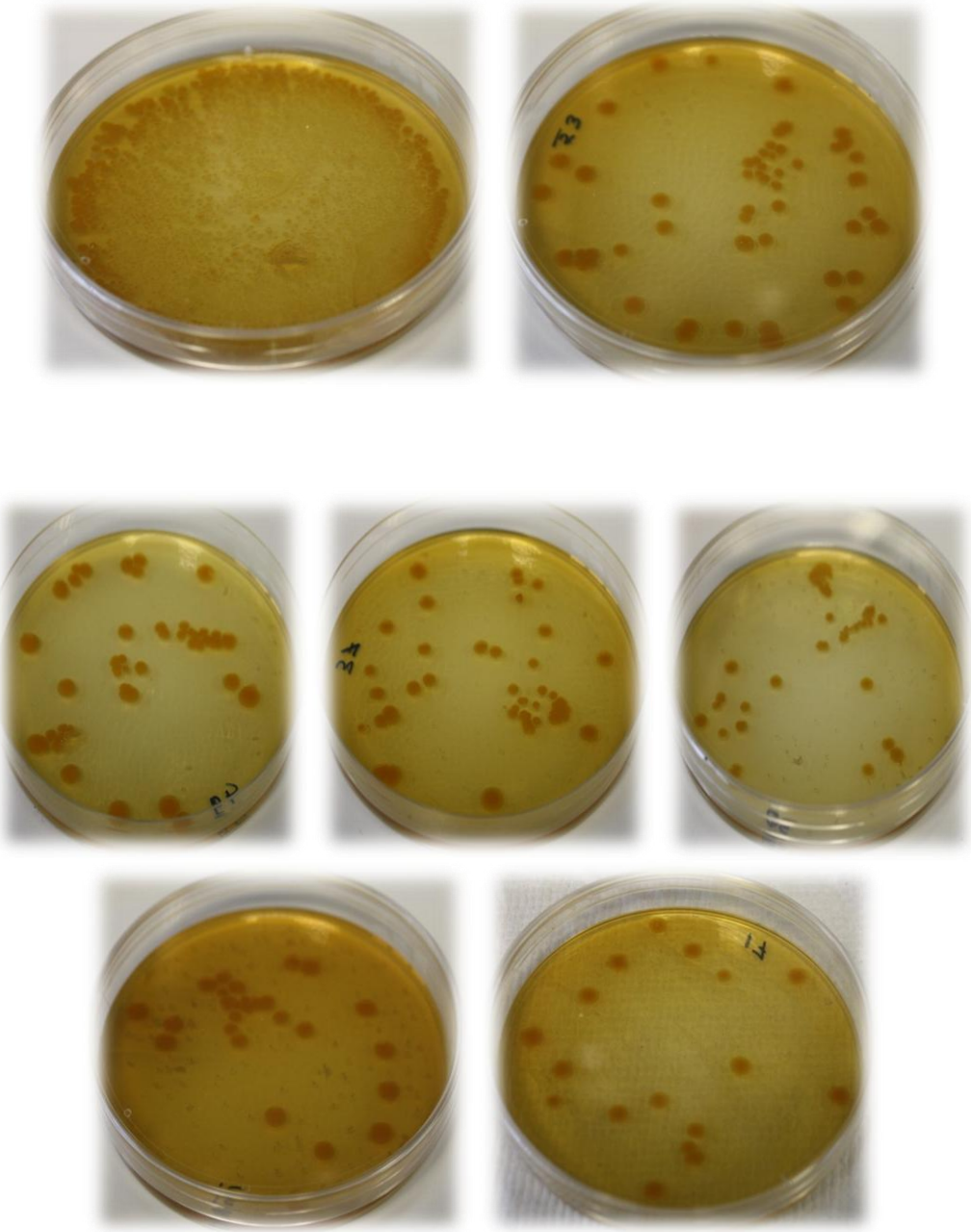


**Figure 6.6.** Examples of *Exiguobacterium* plates where colonial growth has been affected after different times of UV exposure. The top left plate is the control (i.e no exposure to UV) and all other plates show the effect of increasing times of UV exposure.

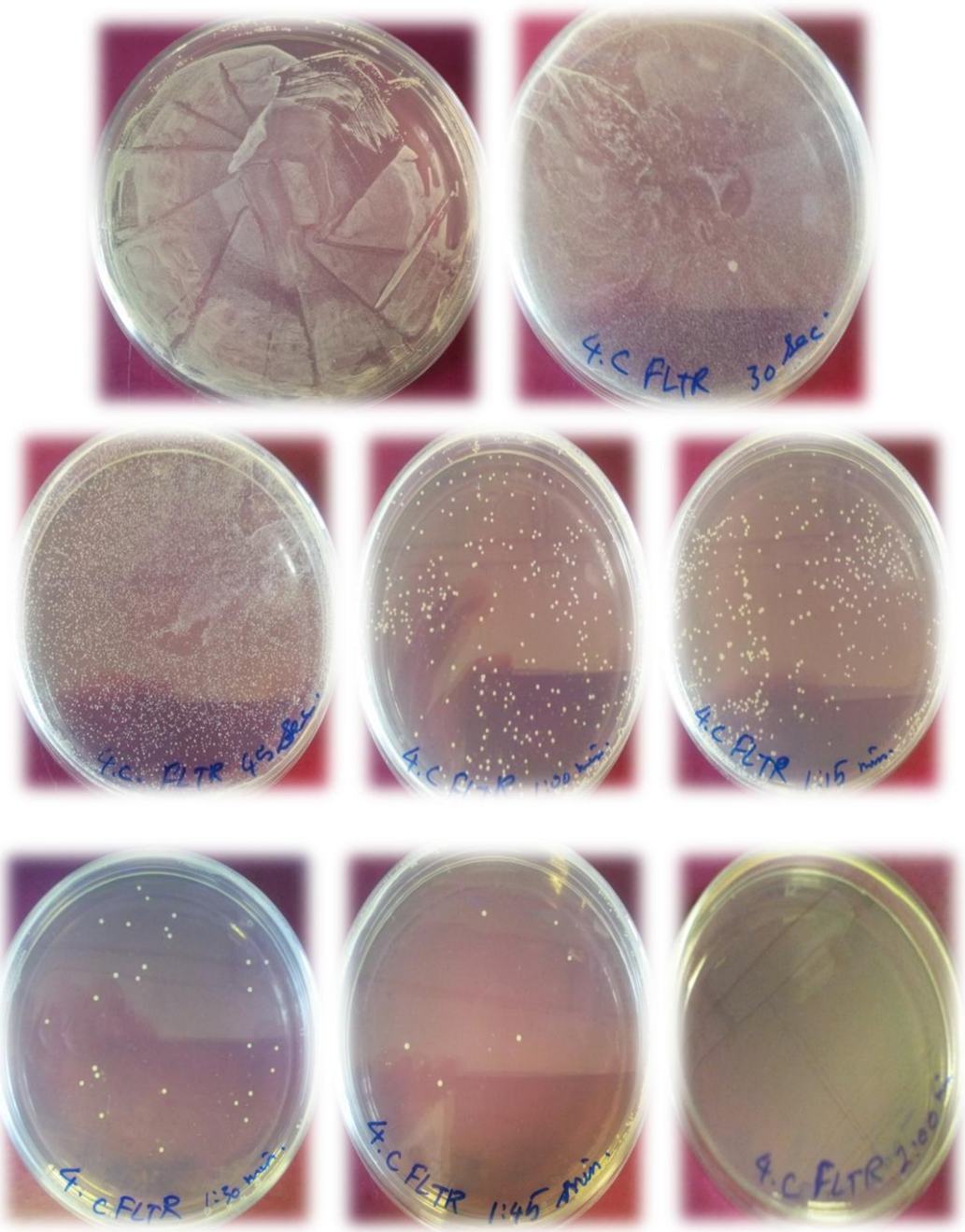



**Figure 6.7.** Examples of *S. warneri* plates after exposure to UV light. The top left plate is the control (no exposure to UV), but the other plates show the clear effect UV light has on colony formation starting from 30 second UV exposure with increment in radiation of 15 seconds per exposure. Eventually few or no colonies are visible.

### 6.2.2 Liquid Samples

Liquid samples were put in glass Petri dishes and exposed to UVC light at a height of 25 - 30 cm. On the basis of preliminary experiments, all *Exiguobacterium* liquid samples were exposed to UV light for long time periods of 10, 20, 30, 45 and 60 minutes. *H. blutaparonensis* liquid samples were exposed to UVC light for 60 seconds, 75 seconds, 90 seconds, 105 seconds, 120 seconds and 135 seconds. *S. warneri* liquid samples were exposed to UVC radiation for 60 seconds, 90 seconds, 105 seconds, 120 seconds, 135 seconds and 150 seconds. The UV treated samples were divided into two groups, one group was kept in the light for 1 hour (light treatment) and the other group was kept in the dark for 1 hour (dark treatment). Then genomic DNA was extracted from all liquid samples exposed to UVC light, plus controls that were not subjected to UVC exposure.

Before loading on gels to see the effect of UVC on the genomic DNA, the amount of DNA in each sample was quantified using the spectrophotometer and the results are shown in Tables 6.2 to 6.5. In all cases the concentration of genomic DNA in the UVC exposed samples has been significantly reduced. Figures 6.8 to 6.10 show the degenerative changes to the genomic DNA extracted from the three organisms. The degenerative effects are most clearly seen in Figure 6.8 (*Exiguobacterium*) and Figure 6.9 (*H. blutaparonensis*) where the degraded DNA is seen as streaks down the lane of the gel. Figure 6.10 does not show this effect as clearly for *S. warneri*, because of the low amounts of DNA loaded on to the gel (Table 6.4).

Figures 6.11 and 6.12 show the effect of light and dark treatment on the recovery of UV damaged DNA from *Exiguobacterium* cells. In Figure 6.11, lanes 1 to 7 show DNA exposed to increasing lengths of exposure to UV light and lanes 2 to 7 show DNA after increasing exposure times. It seems clear that dark treatment is more effective at allowing recovery of the DNA. A similar conclusion can be drawn from Figures 6.13 and 6.14 for *S. warneri* DNA (i.e. that incubation in the dark significantly enhanced the recovery of DNA from UV induced damage).

**Table 6.2.** DNA quantification of *Exiguobacterium* samples after light and dark treatment before being loaded on to the gel. The control (not exposed to UVC) *Exigubacterium* genomic DNA concentration was 0.036 µg/ml.

| Duration of UVC Exposure (min.) | Dark Treatment (µg/ml) | (Light Treatment µg/ml) |
|---|---|---|
| 10 | 0.016 | 0.010 |
| 20 | 0.014 | 0.015 |
| 30 | 0.023 | 0.029 |
| 45 | 0.025 | 0.016 |
| 60 | 0.015 | 0.015 |

**Table 6.3.** DNA quantification of *H. blutaparonensis* samples after light and dark treatment before being loaded on to the gel. The control (not exposed to UVC) *H. blutaparonensis* genomic DNA concentration was 0.244 µg/ml.

| Duration of UVC exposure (min.) | Dark Treatment (µg/ml) | Light Treatment (µg/ml) |
|---|---|---|
| 1:00 | 0.003 | 0.023 |
| 1:15 | 0.004 | 0.002 |
| 1:30 | 0.004 | 0.002 |
| 1:45 | 0.004 | 0.005 |
| 2:00 | 0.004 | 0.014 |
| 2:15 | 0.004 | 0.003 |

**Table 6.4.** DNA quantification of *S. warneri* samples after light and dark treatment before being loaded on to the gel. The control (not exposed to UVC) *S. warneri* genomic DNA concentration was 0.007 µg/ml.

| Duration of UVC Exposure (minutes/seconds) | Dark Treatment µg/ml | Light Treatment µg/ml |
|---|---|---|
| 1:00 | 0.007 | 0.003 |
| 1:30 | 0.002 | 0.001 |
| 1:45 | 0.003 | 0.003 |
| 2:00 | 0.002 | 0.002 |
| 2:15 | 0.005 | 0.004 |
| 2:30 | 0.009 | 0.001 |

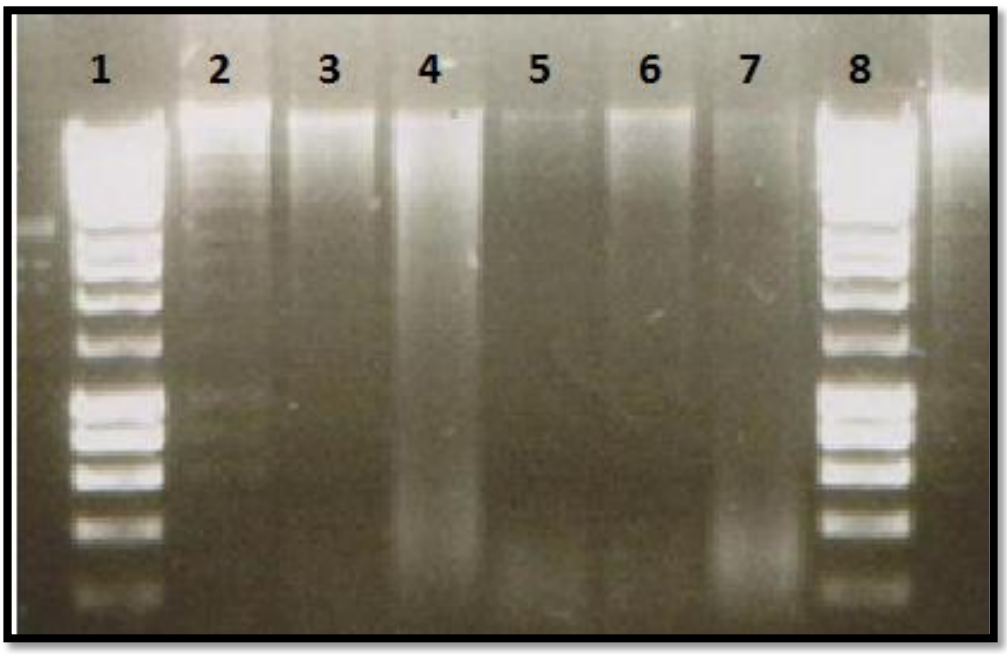



**Figure 6.8.** Effect of UVC radiation on *Exiguobacterium* strain. Lanes 3 to 7 show degenerative changes to the DNA due to UVC light exposure, lane 2 is the least UV exposed lane while it increased till it reaches the maximum radiation on lane no. 7. Lane no. 1 and 8 are ladders

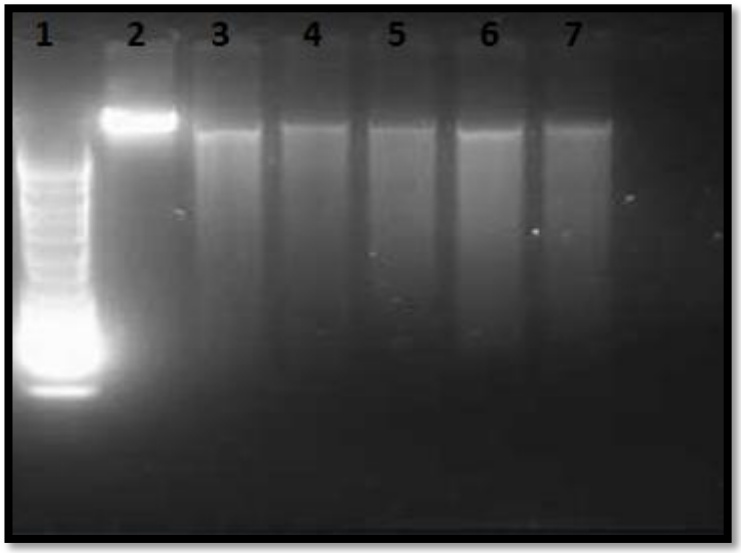



**Figure 6.9.** Effect of exposure to UV radiation on *H. blutaparonensis.* Lanes 3 to 7 show degenerative changes to the DNA due to the effect of increasing exposure to ultraviolet light. Time of UV exposure started at 1 minute 30 seconds in lane 3 and then increased by 15 seconds for each lane until lane 7. Lane 2 is the control (not exposed to UV), while lane 1 is the ladder.

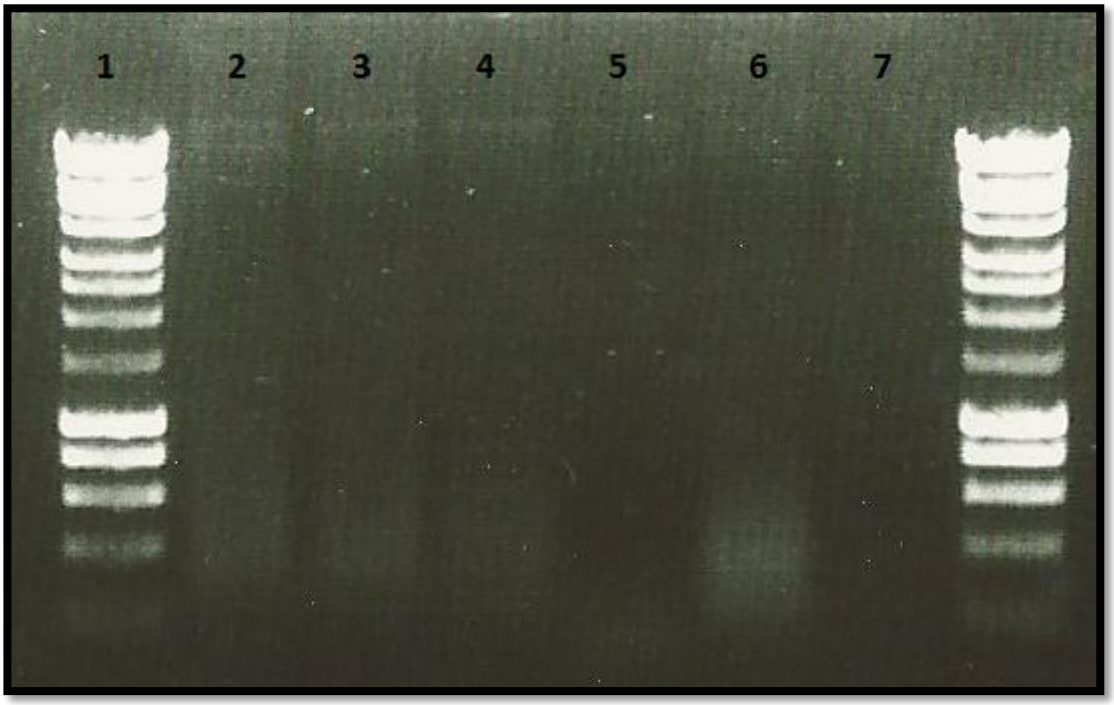



**Figure 6.10.** Effect of UVC exposure on *S. warneri*. Lanes 2 to 7 show degenerative changes of DNA due to exposure to UV radiation. Time of UV exposure increased gradually in lanes starting from lane 2 till it reaches its maximum at lane 7. Lanes 1 and 8 contain DNA ladder.

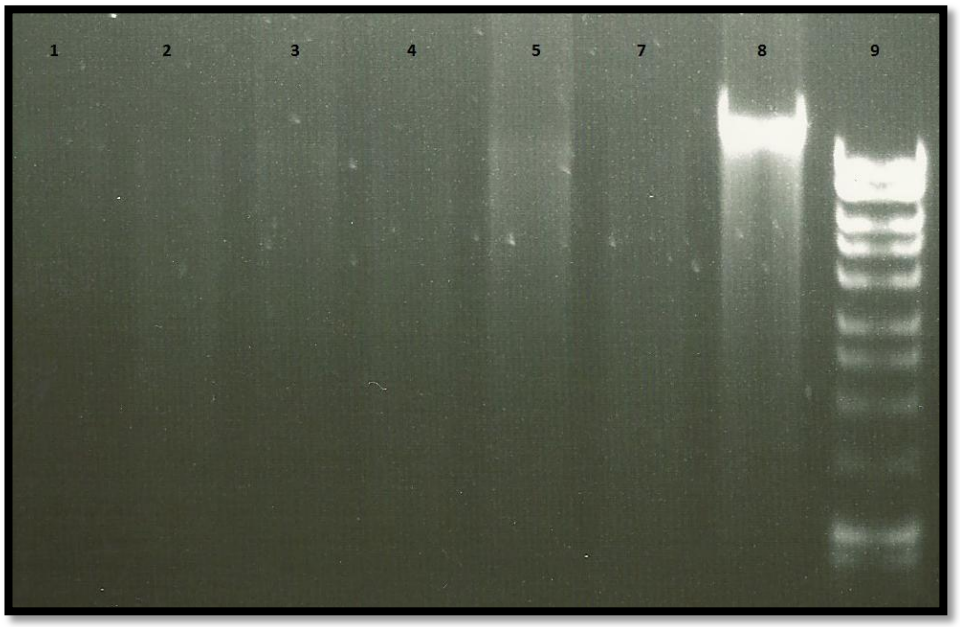



**Figure 6.11.** The effect of light treatment (1 hour) on *Exiguobacterium* after UV exposure which starts in lane 1 and increased till it reaches its maximum at lane 7. Lane 8 is a non UV treated control.

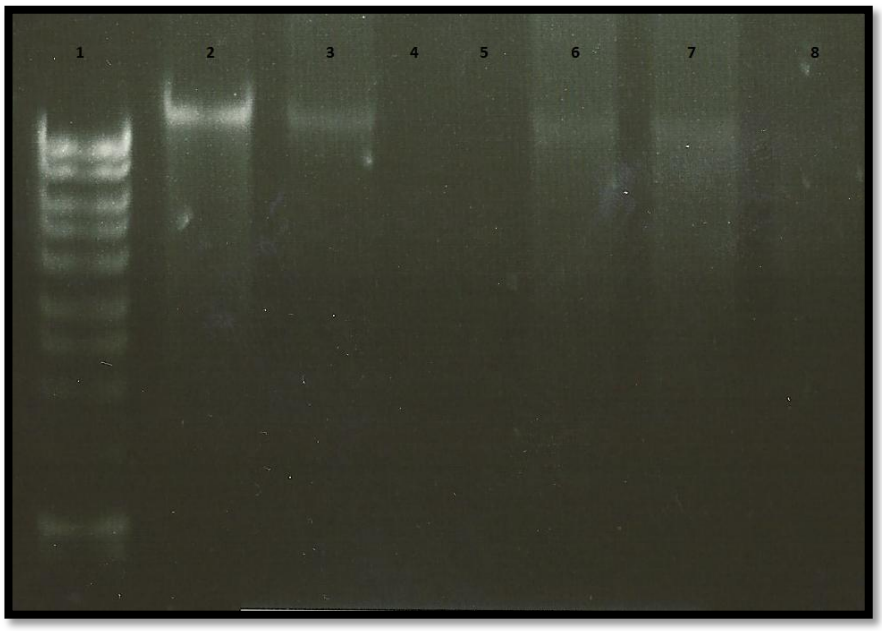



**Figure 6.12.** The effect of dark treatment (1 hour) on *Exiguobacterium* after UV exposure which starts in lane 3 and increased till it reaches its maximum at lane 7. Lane 1 is a DNA ladder. Show different degrees of degenerative changes beside autogenic repair after prolonged period of dark treatment adopted to *Exiguobacterium*.

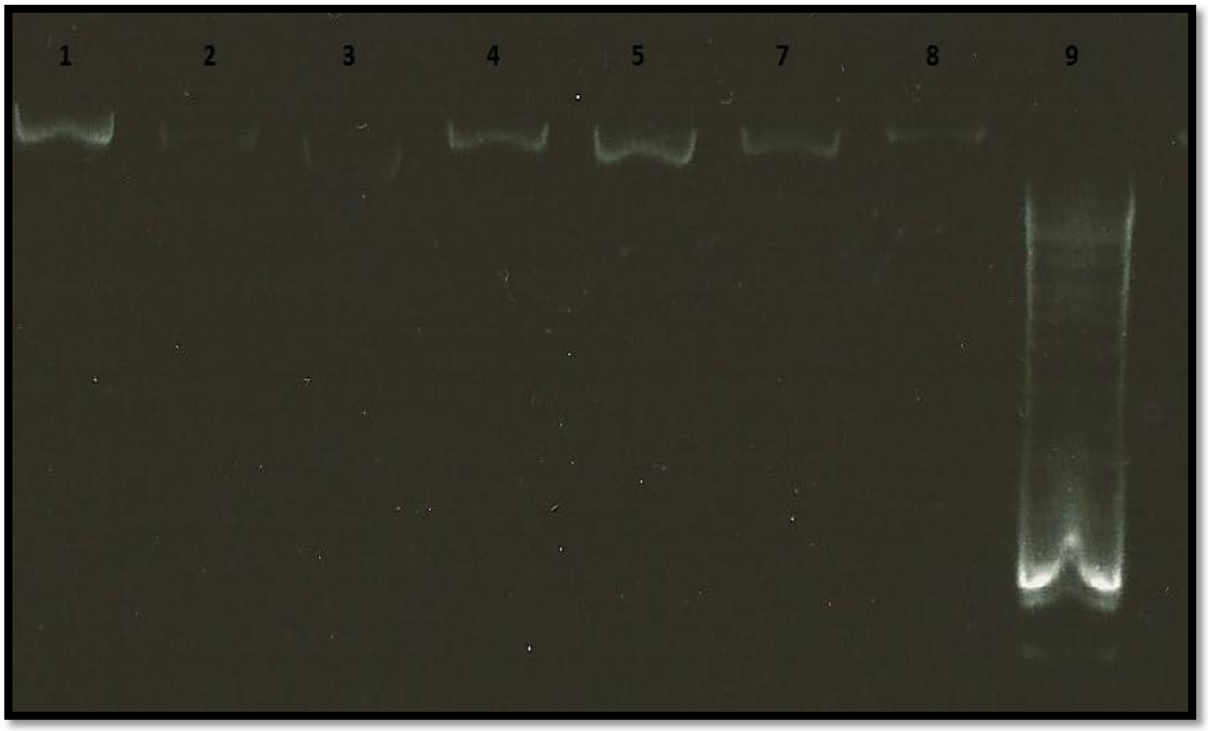



**Figure 6.13.** The effect of light treatment (1 hour) on *S. warneri* after UV exposure which starts in lane 2 and increased till it reaches its maximum at lane 7. Lane 8 is a non UV treated control. Lane 9 is a DNA ladder.

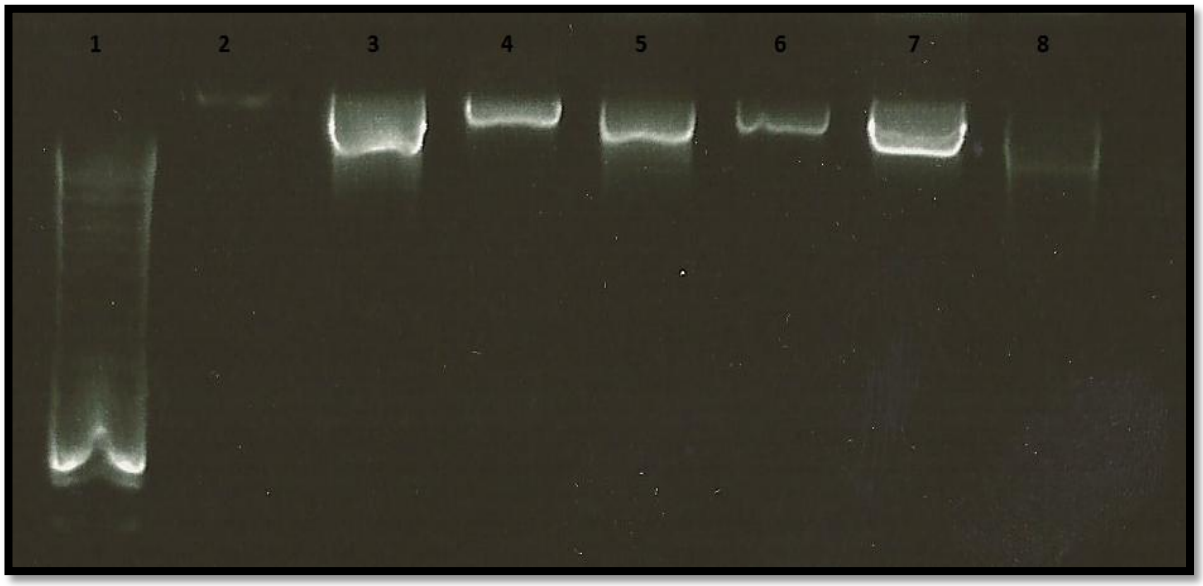



**Figure 6.14.** The effect of dark treatment (1 hour) on *S. warneri* after UV exposure which starts in lane 3 and increased till it reaches its maximum at lane 8. Lane 2 is a non UV treated control.

To attempt to further investigate the degenerative DNA effects seen in Figures 6.11 to 6.14, quantification of DNA was carried out and unified amounts of DNA (200 ng) were loaded in the wells. After running the gels, all lanes were analysed using soft gel analysis data base software (Quantity One, Quantitation Software BioRad). To help see the trends, all curves were normalized to the maximum value to account for any remaining differences in DNA content between lanes.

Figures 6.15B and 6.16B show the normalized results for *Exiguobacterium* DNA subjected to UV exposure and then allowed to recover in the dark or the light respectively. The first peak corresponds to the DNA near the well and the second double peak shows the main concentration of DNA in the lane. The tail beyond the double peak shows the amount of DNA in the smear following the main bands of DNA. For the dark treated samples, the largest smear is shown in Lane 6 (Fig 6.15B), which would suggest the most DNA damage in the sample exposed for the longest time to UV light. However, the opposite effect is seen after light treatment, where the largest smear is seen in the control sample not exposed to UV light (Fig 6.16B).

Figures 6.17B and 6.18B show the normalized results for *S. warneri* DNA subjected to UV exposure and then allowed to recover in the dark or the light respectively. The DNA smear data after the major peak of DNA shows the largest amount of DNA (i.e. the most obvious smear) in the control samples (not exposed to UV light) after both light and dark treatment (Figs 6.17B and 6.18B). These results and the result for *Exiguobacterium* DNA after light treatment (Fig 6.16B) seem counterintuitive (i.e. more DNA damage in the control than in the UV exposed samples). One possible explanation is that more of the UV exposed DNA has been completely degraded and is not seen on the gel and cannot be detected by the soft gel analysis software.

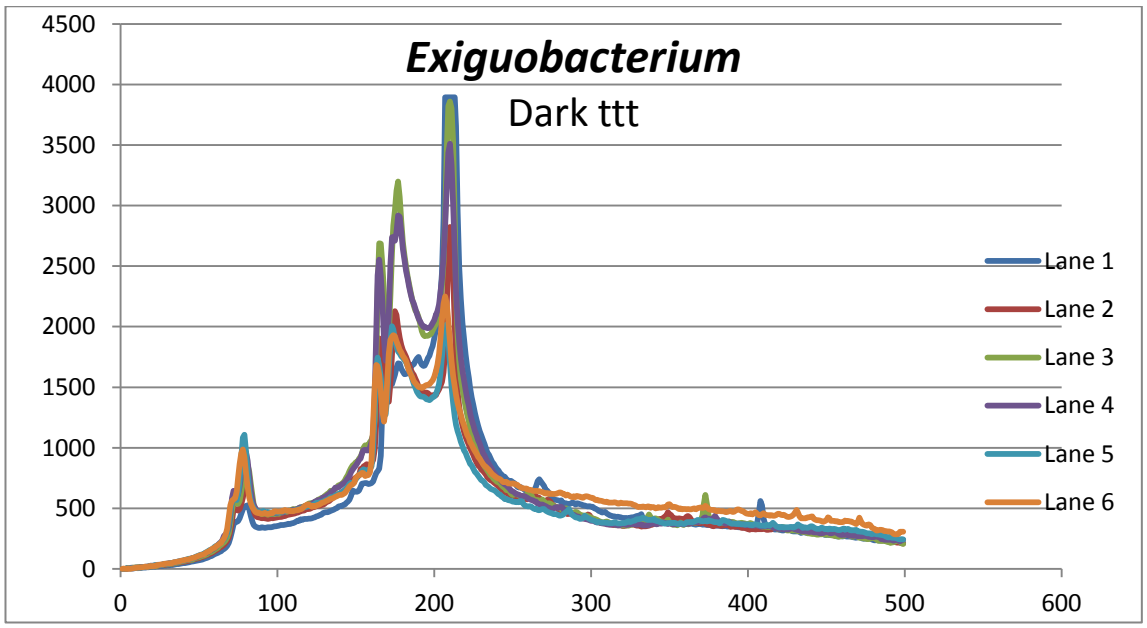



**Figure 6.15A**. Soft Gel Analysis of dark treated *Exiguobacterium* DNA samples. The software scans the gel and produces a curve for each lane starting at the top of the gel and working down.

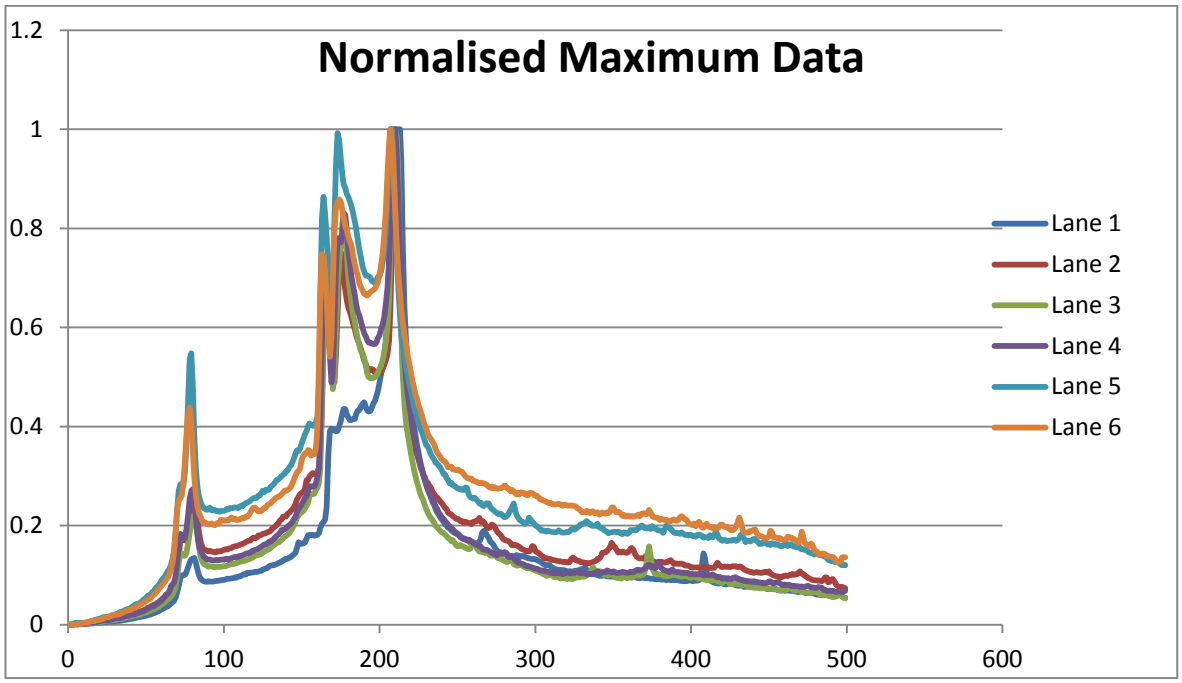



**Figure 6.15B.** *Exiguobacterium* DNA samples from Figure 6.15A, the data have been normalised to the maximum value.

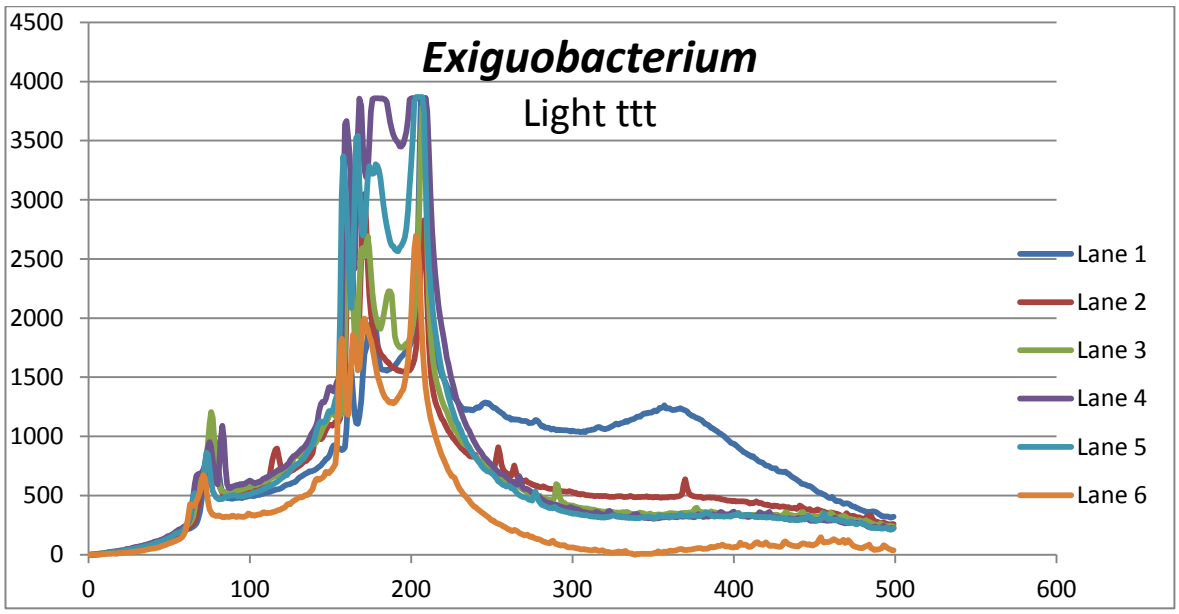



**Figure 6.16A.** Soft Gel Analysis of light treated *Exiguobacterium* DNA samples**.** The software scans the gel and produces a curve for each lane starting at the top of the gel and working down. Lane 1 is the control (no exposure to UV light) and Lanes 2 – 6 are increasing times of UV exposure.

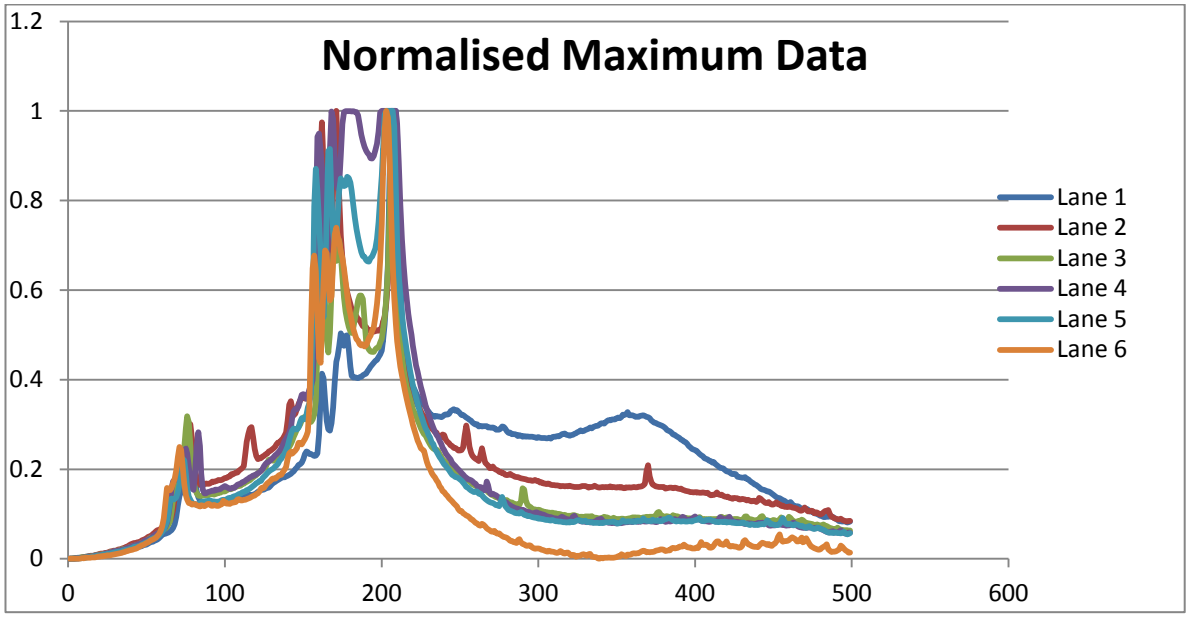



**Figure 6.16B.** *Exiguobacterium* DNA samples from Figure 6.16A, the data have been normalised to the maximum value.

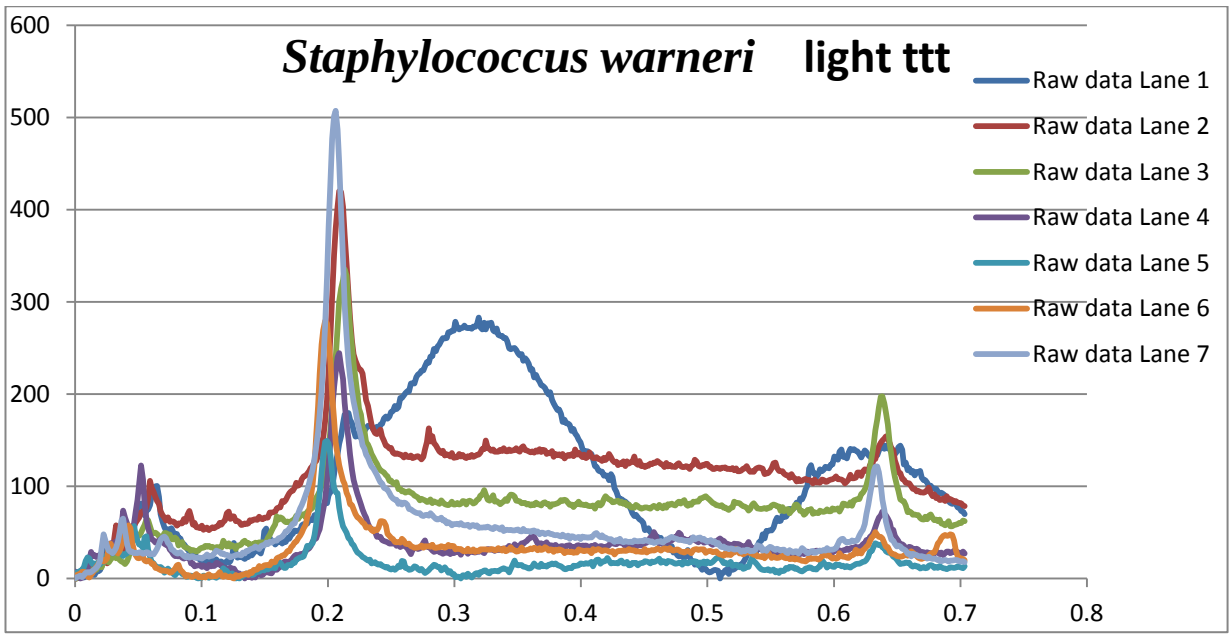



**Figure 6.17A.** Soft Gel Analysis of light treated *S. warneri* DNA samples**.** The software scans the gel and produces a curve for each lane starting at the top of the gel and working down. Lane 1 is the control (no exposure to UV light) and Lanes 2 – 7 are increasing times of UV exposure.

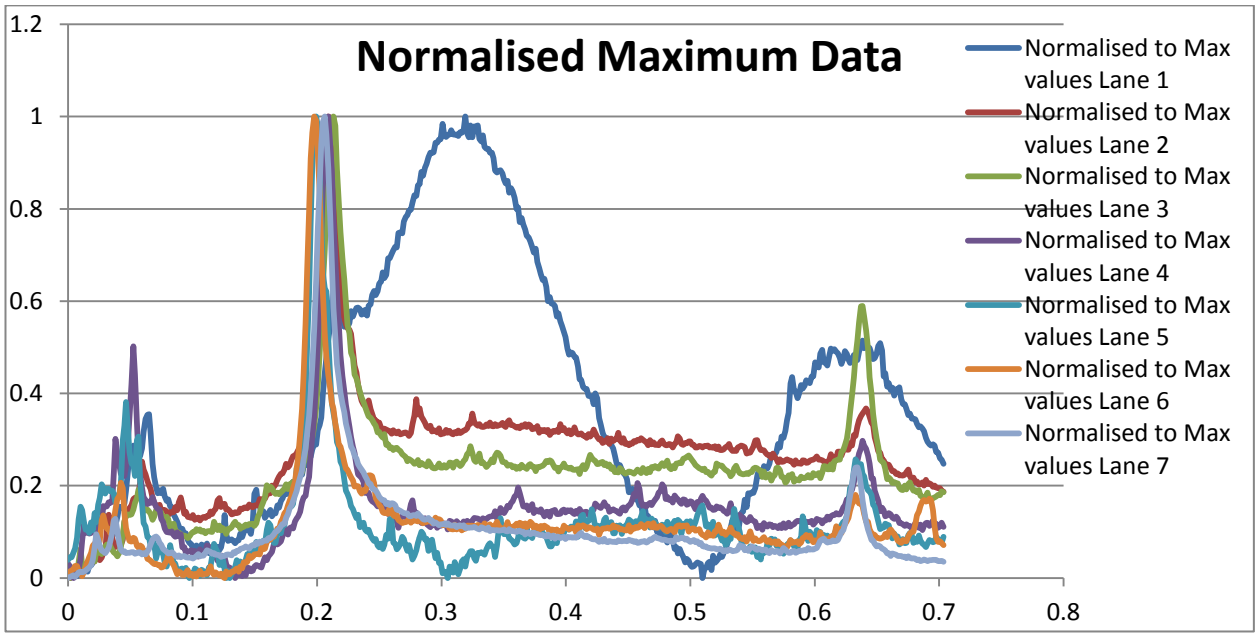



**Figure 6.17B.** *S. warneri* DNA samples from Figure 6.17A, the data have been normalised to the maximum value.

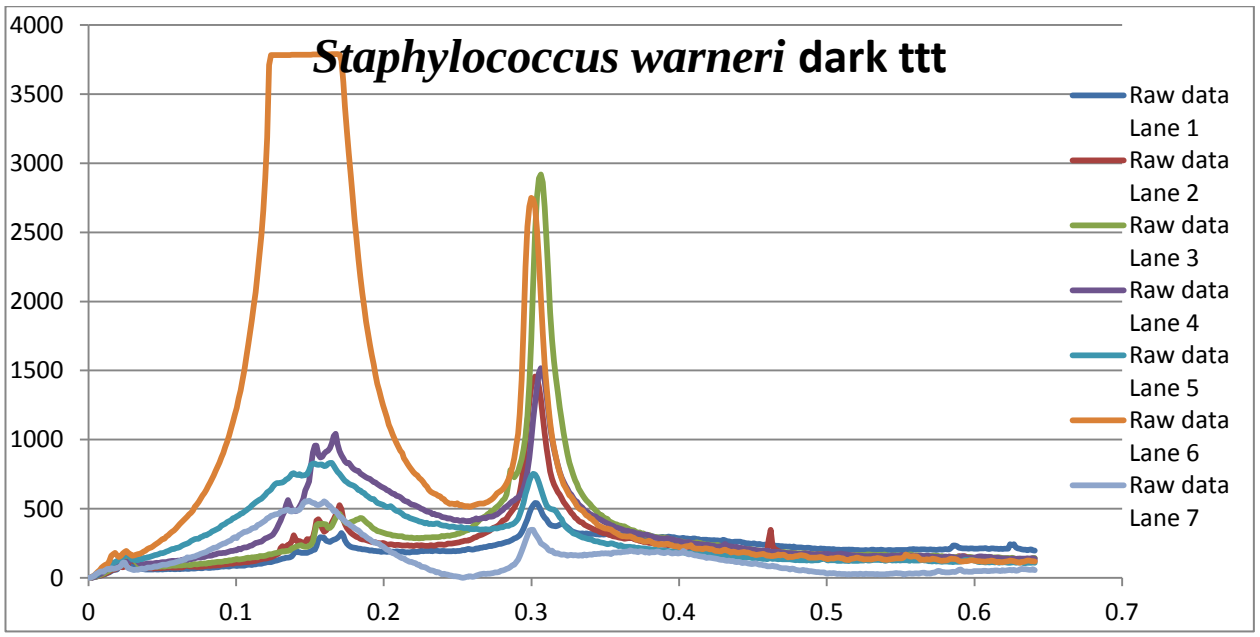



**Figure 6.18A.** Soft Gel Analysis of dark treated *S. warneri* DNA samples**.** The software scans the gel and produces a curve for each lane starting at the top of the gel and working down. Lane 1 is the control (no exposure to UV light) and Lanes 2 – 7 are increasing times of UV exposure.

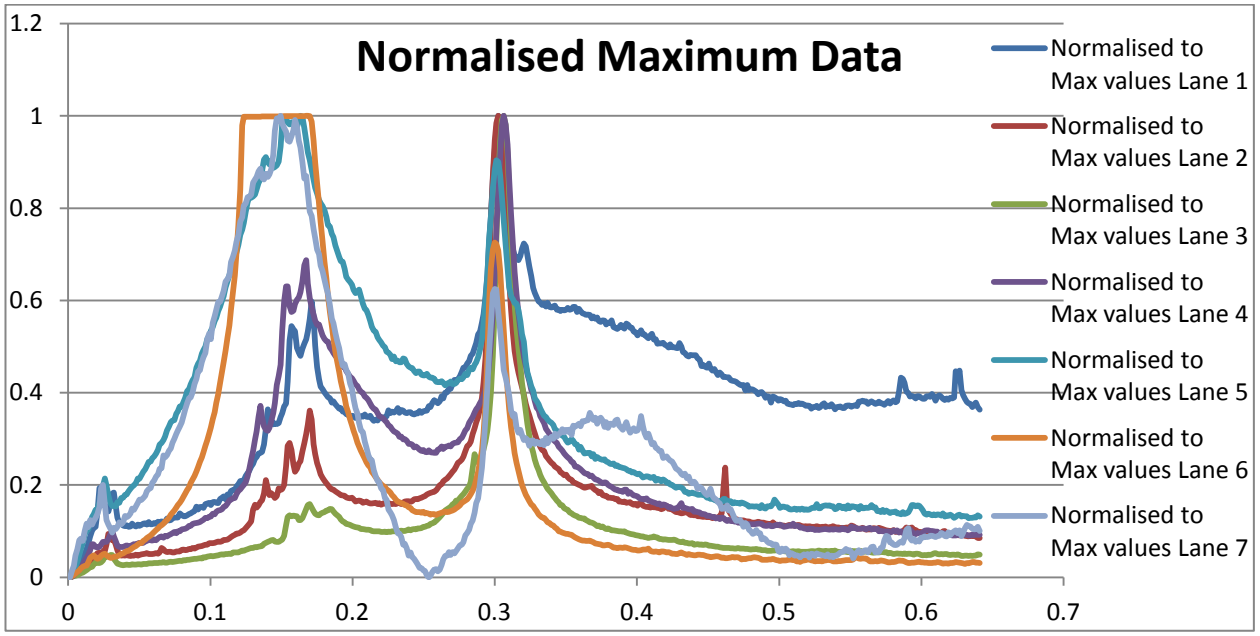



**Figure 6.18B.** *S. warneri* DNA samples from Figure 6.18A, the data have been normalised to the maximum value.

### 6.2.3 Electron Microscope Analysis of *Exiguobacterium and S. warneri* cells exposed to UV light

Scanning and transmission electron microscope experiments were carried out to examine the effect of UV radiation on internal structure and shape of *Exiguobacterium* and *S. warneri* cells. The effects of light and dark treatment post UV exposure on cell structure and shape were also studied. The analyses were carried out in the Biomedical Science Department (The University of Sheffield) as described in section 2.22.

Figures 6.19 and 6.20 show the SEM images of *Exiguobacterium* cells exposed to UV light for increasing time periods up to 60 minutes and then being allowed to recover in the light (Fig 6.19) or the dark (Fig 6.20) for one hour. There are no obvious changes to the cell morphology in response to exposure to UV radiation. Figures 6.21 and 6.22 show TEM images for *Exiguobacterium* cells exposed to UV as described above. Again, there are no obvious differences in the cell structure after UV exposure.

Figures 6.23 and 6.24 show the SEM images for *S. warneri* cells exposed to UV light for up to 150 seconds and then being allowed to recover in the light (Fig 6.23) or the dark (Fig 6.24) for one hour. There are no obvious changes to the cell morphology in response to exposure to UV radiation. Figures 6.25 and 6.26 show TEM images for *S. warneri* cells exposed to UV as described above. Again there are no obvious differences in cell structure after UV exposure and indeed many cells are in the process of cell division indicating that they were actively growing after the UV exposure.

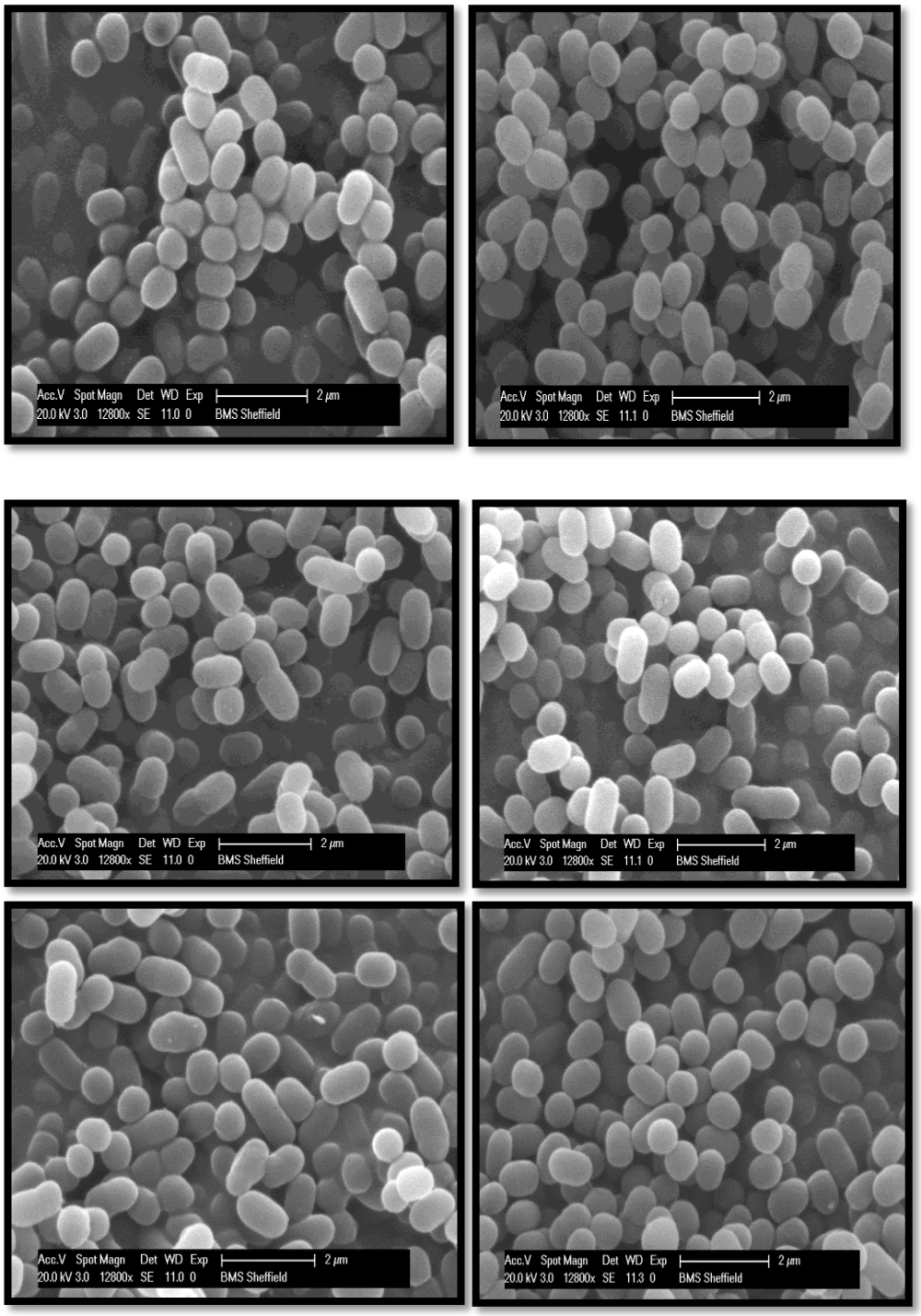



**Figure 6.19.** Scanning electron microscope images of *Exiguobacterium* cells exposed to UV light and then allowed to recover in the light. Top left is control (not exposed to UV light) and then cells exposed to UV light for 10, 20, 30, 45 and 60 mins. All cells were allowed to recover in the light for 1 hour.

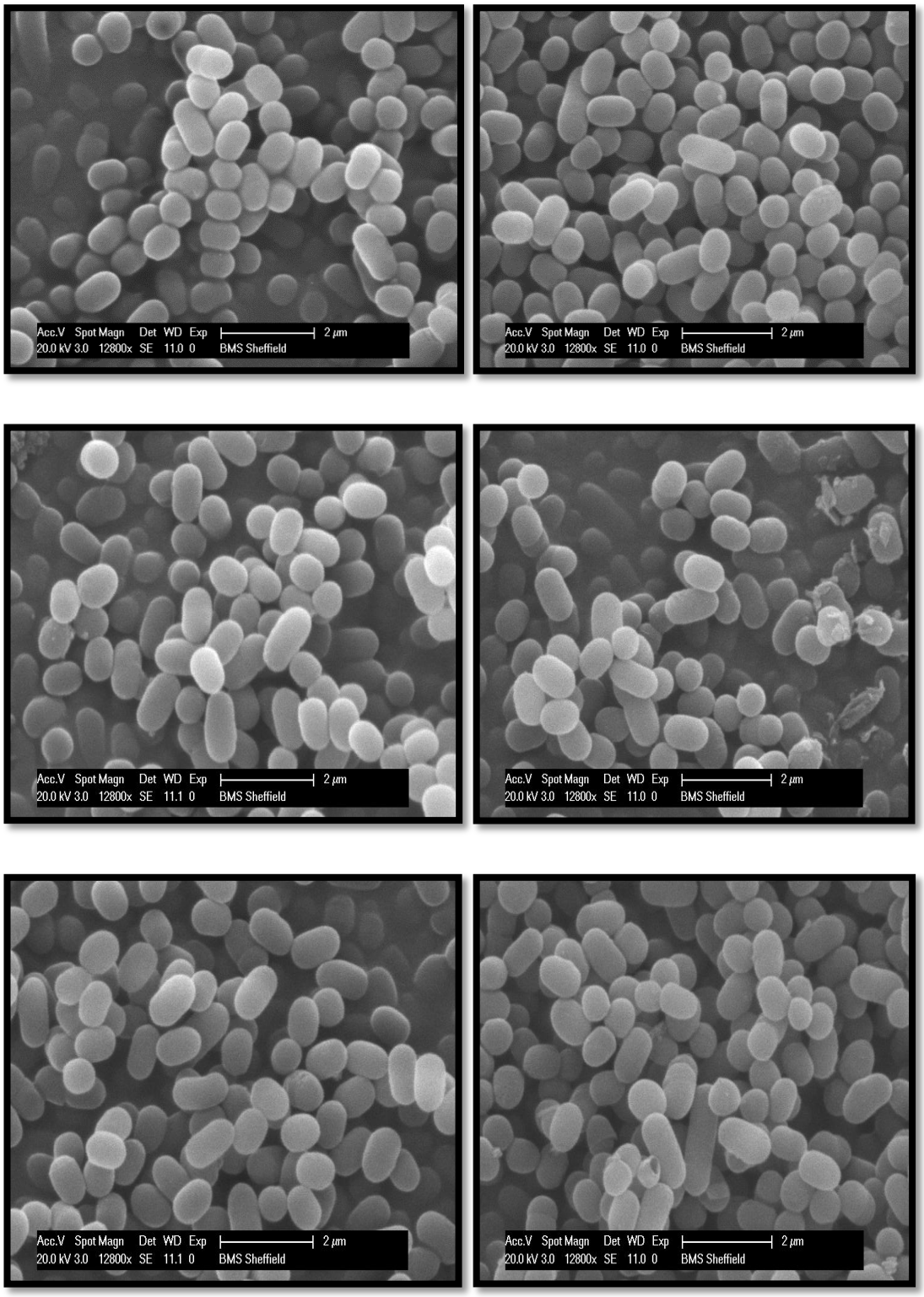



**Figure 6.20.** Scanning electron microscope images of *Exiguobacterium* cells exposed to UV light and then allowed to recover in the dark. Top left is control (not exposed to UV light) and then cells exposed to UV light for 10, 20, 30, 45 and 60 mins. All cells were allowed to recover in the dark for 1 hour.

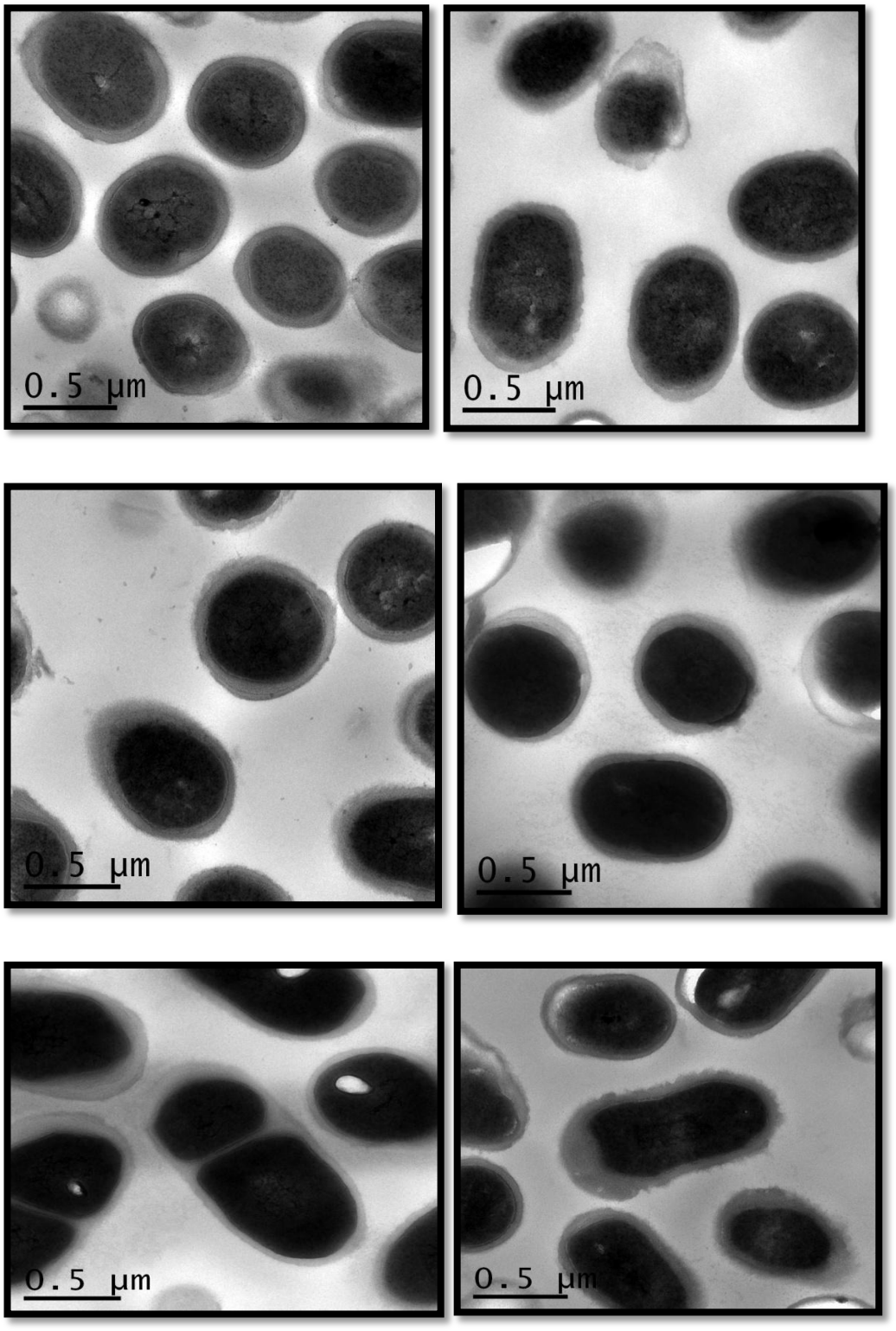



**Figure 6.21.** Transmission electron microscope images of *Exiguobacterium* cells exposed to UV light and then allowed to recover in the light. Top left is control (not exposed to UV light) and then cells exposed to UV light for 10, 20, 30,45 and 60 mins. All cells were allowed to recover in the light for 1 hour.

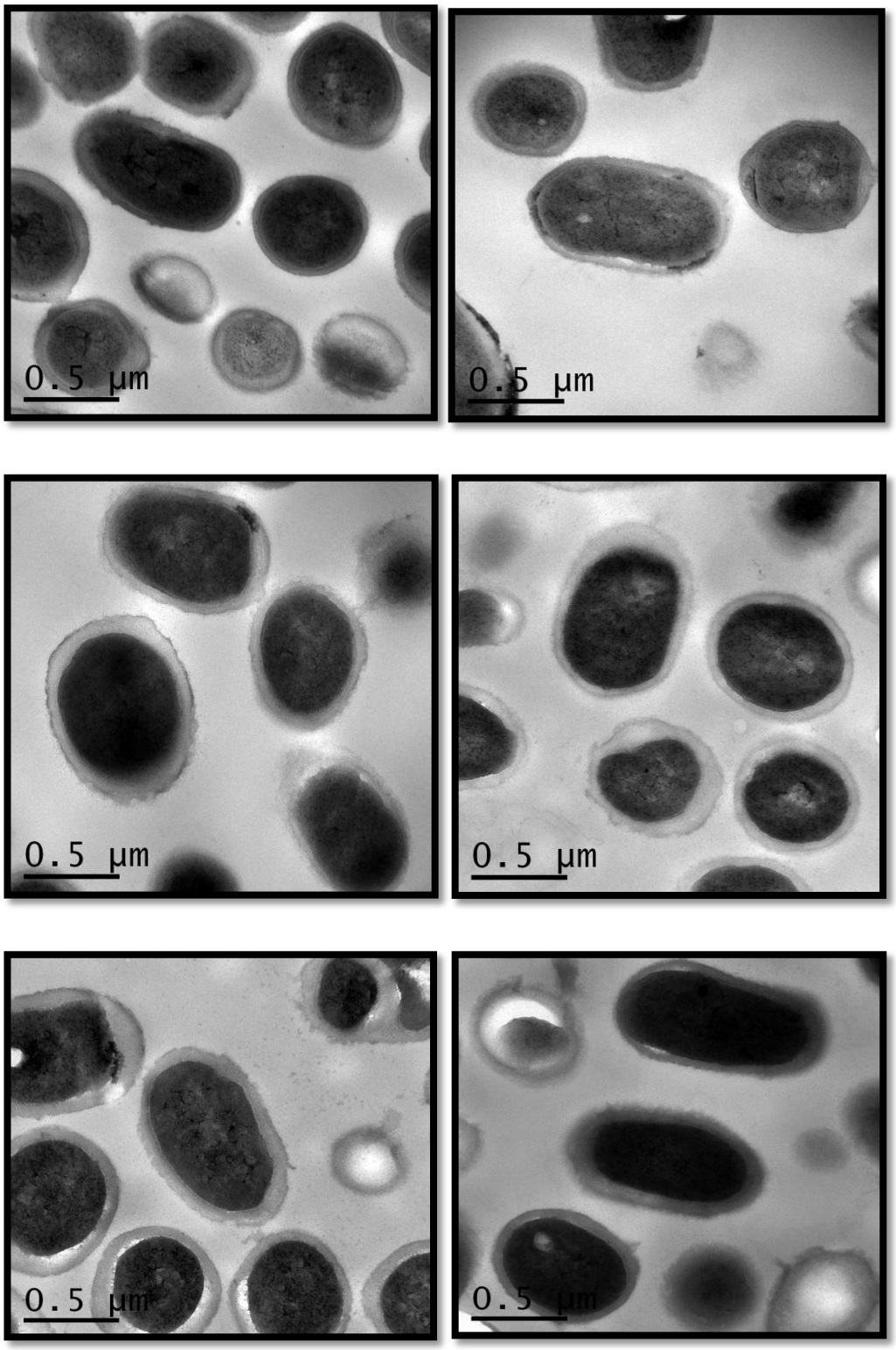



**Figure 6.22.** Transmission electron microscope images of *Exiguobacterium* cells exposed to UV light and then allowed to recover in the dark. Top left is control (not exposed to UV light) and then cells exposed to UV light for 10, 20, 30, 45 and 60 mins. All cells were allowed to recover in the dark for 1 hour.

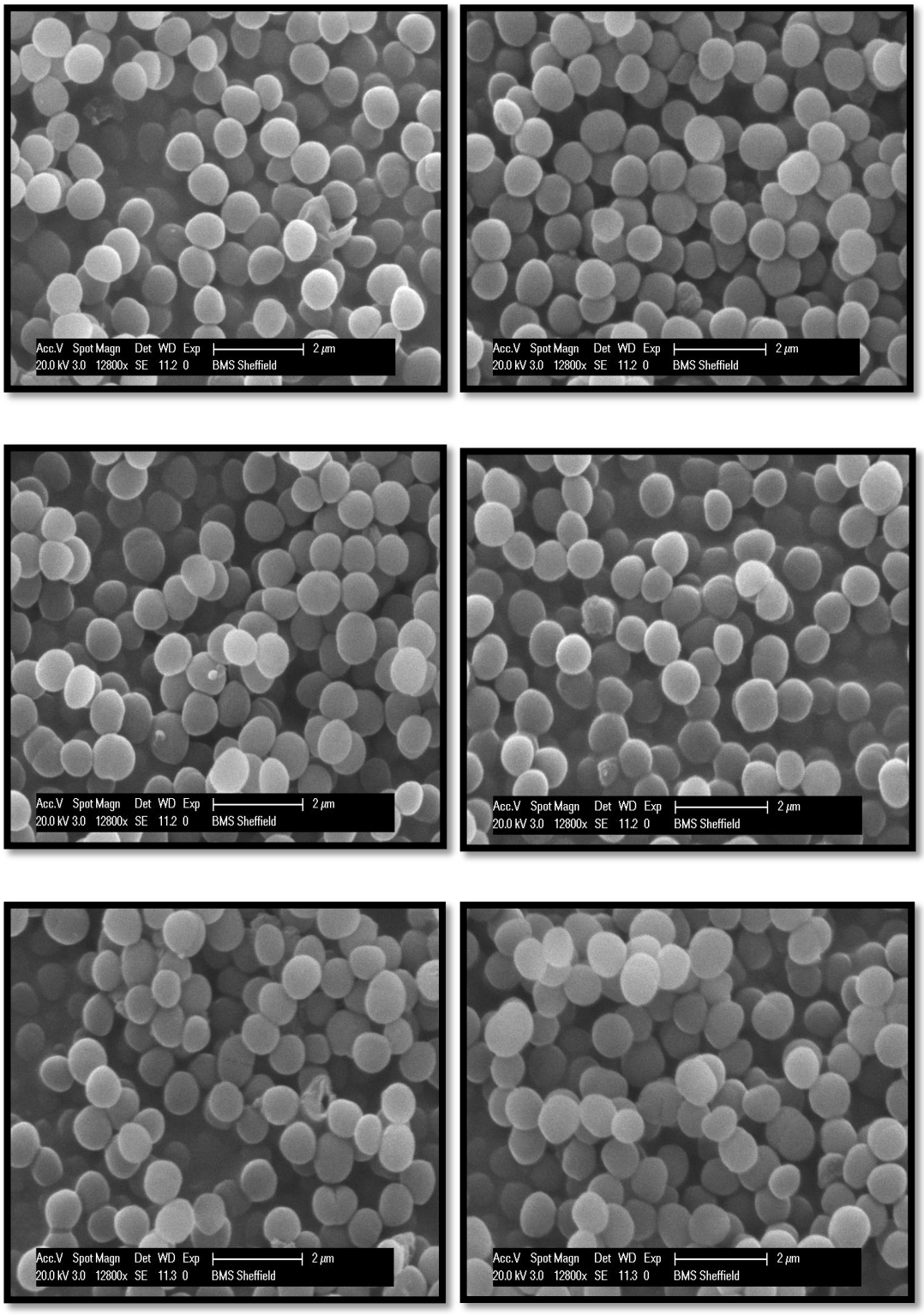



**Figure 6.23.** Scanning electron microscope images of *S. warneri* cells exposed to UV light and then allowed to recover in the light. Top left is control (not exposed to UV light) and then cells exposed to UV light for 60, 90, 120, 135 and 150 seconds. All cells were allowed to recover in the light for 1 hour.

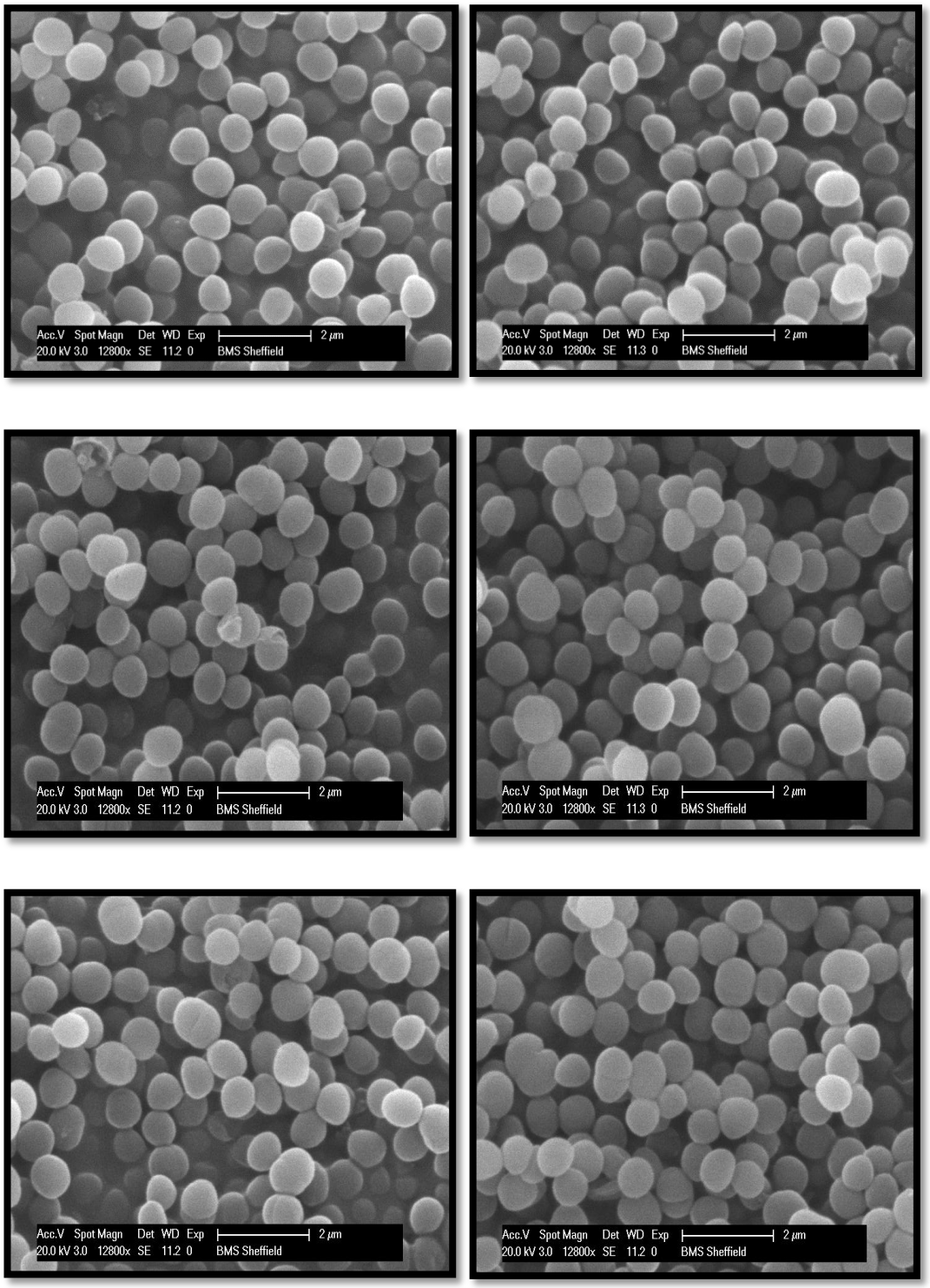



**Figure 6.24.** Scanning electron microscope images of *S. warneri* cells exposed to UV light and then allowed to recover in the dark. Top left is control (not exposed to UV light) and then cells exposed to UV light for 60, 90, 120, 135 and 150 seconds. All cells were allowed to recover in the dark for 1 hour.

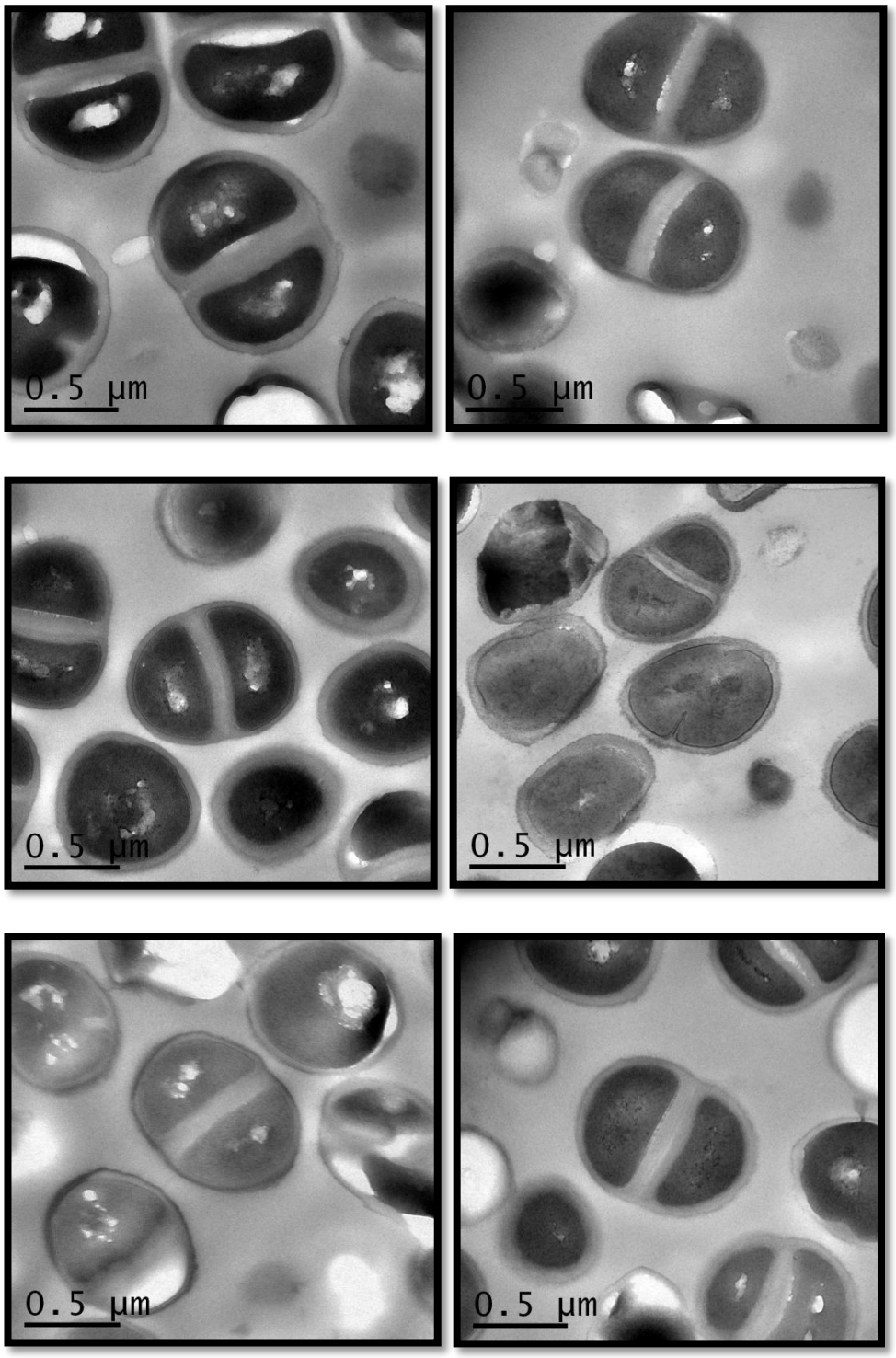



**Figure 6.25.** Transmission electron microscope images of *S. warneri* cells exposed to UV light and then allowed to recover in the light. Top left is control (not exposed to UV light) and then cells exposed to UV light for 60, 90, 120, 135 and 150 seconds. All cells were allowed to recover in the light for 1 hour.

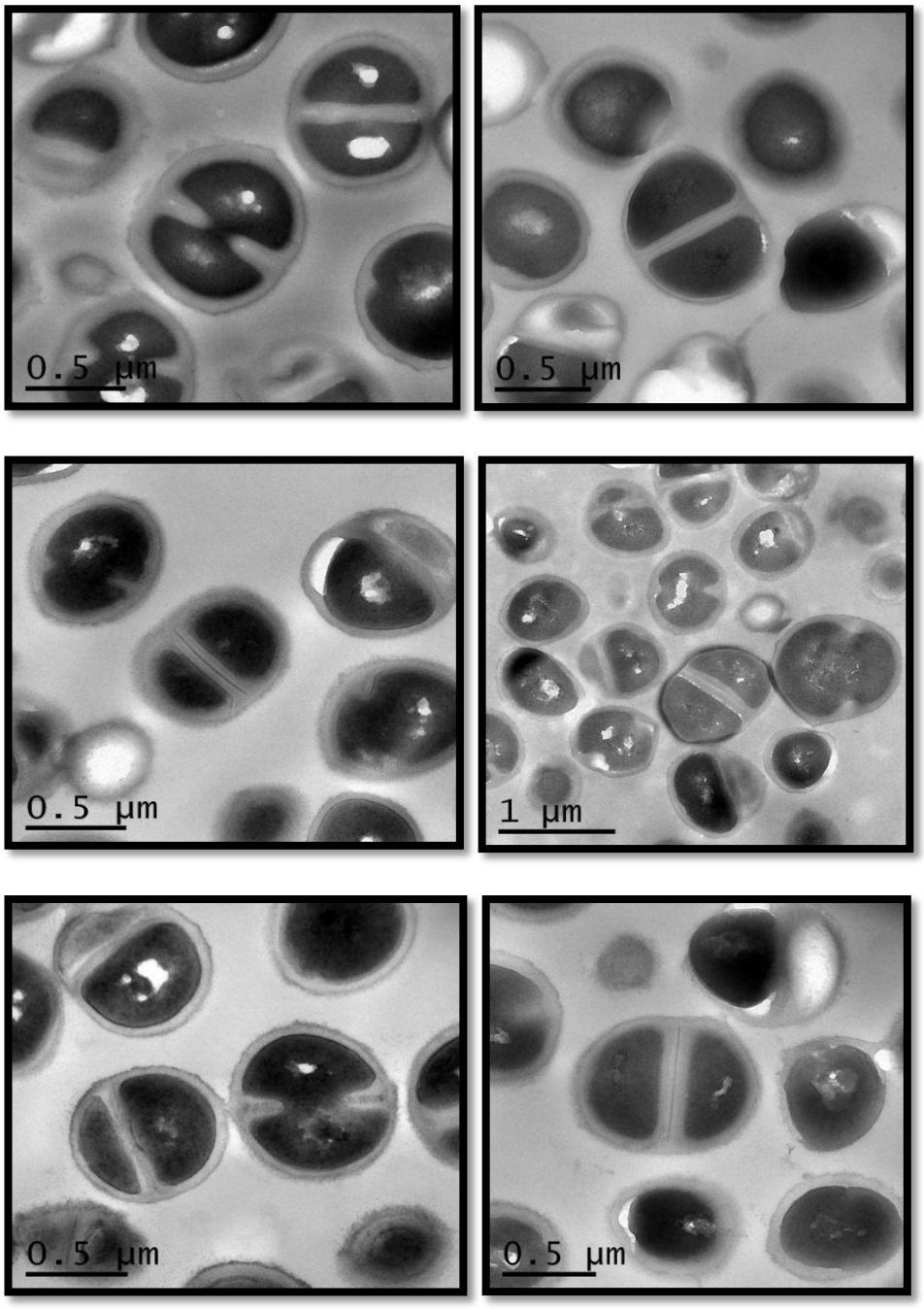



**Figure 6.26.** Transmission electron microscope images of *S.warneri* cells exposed to UV light and then allowed to recover in the dark. Top left is control (not exposed to UV light) and then cells exposed to UV light for 60, 90, 120, 135 and 150 seconds. All cells were allowed to recover in the dark for 1 hour.

## 6.3 Conclusions

The aim of the work described in this chapter was to investigate the response of *Exiguobacterium* and *S. warneri* to UVC light exposure. A few preliminary experiments were also carried out on *H. blutaparonensis.* Initial work on all three strains using cultures on agar plates showed that they were all susceptible to UVC radiation (254 nm) after a few minutes exposure (Figures 6.3 to 6.7).

Further experiments were carried out on liquid cultures and the exposure times for *Exiguobacterium* were greatly increased after preliminary experiments were carried out (data not shown). DNA was extracted from UV exposed cells and ran on gels to look for DNA degradation – smearing on the gel (Figures 6.8 to 6.14). The smearing was observed as was a large decrease in the amount of DNA extracted after UV exposure. The idea of allowing the cells to recover for one hour in the light and the dark was introduced into this set of experiments. It is known that light can stimulate photoreactivation events (Reed *et al*., 1996). The results were inconclusive when the gels were examined by eye, so it was decided to use a Soft Gel Analysis system to quantify the DNA in each lane of the gel.

The results of the Soft Gel Analysis are shown in Figures 6.15 to 6.18. The trends are not very clear, the simple expectation that the DNA would be more degraded (i.e. more smearing would be evident) was only seen for *Exiguobacterium* cells that had recovered in the dark. The other treatments: *Exiguobacterium* cells recovering in the light and *S. warneri* cells recovering in the light or dark, all showed the opposite effect – more DNA degradation in the controls.

Finally, the SEM and TEM images of *Exiguobacterium* and *S. warneri* (Figures 6.19 to 6.26) are very clear and differentiate nicely between the two species. However, no deleterious changes to cell morphology or internal cell structure can be seen. This is a puzzling result and more work is necessary to understand why the UVC radiation did not have more obvious effects on cell structure.

# Chapter 7

## General Conclusions

## and

## Future Work

## 7.1 General Conclusions

The major aim of the current work was to isolate bacteria from non-extreme environments using either high pH or high salinity media. The river samples from Derbyshire were subjected to pH 10 Horikoshi medium and two strains were isolated, both subsequently identified as members of the *Exiguobacterium* genus. Although only recognised as a separate genus as recently as 1983 (Collins *et al*., 1983), it now contains around 15 species and more seem to be added every year (Singh *et al*., 2013). *Exiguobacterium* strains have been found in a wide range of environments from garden ponds to lakes in Antarctica (Vishnivetskaya *et al*., 2009), so it is not surprising that *Exiguobacterium* strains have been found in the river samples. The major finding in the current work on *Exiguobacterium* is the presence of betaine acting as a compatible solute at pH 10. It is common for betaine to be found in cells stressed by high salinity, but it is unusual to find betaine synthesis/accumulation being induced by high pH.

The lake and drainage canal samples from Hassa, Saudi Arabia were subjected to high salinity medium (up to 3 or 4 M NaCl) to select for moderately halophilic or halotolerant microorganisms. Three strains were isolated and identified that can grow in high salinities: *Halomonas venusta, Halobacillus blutaparonensis* and *Staphylococcus warneri*. It was decided not to further characterise *H. venusta*, since the genus *Halomonas* has been well studied (Arahal *et al*., 2007). However, the other two organisms both merited further work.

*H. blutaparonensis* is little studied with only one paper published which describes its isolation from the roots of a succulent herbaceous plant found growing on sand dunes on Brazil's Atlantic coast. The physiological characterisation of *H. blutaparonensis* described in Chapter 5 agrees with the published description of this species (Barbosa *et al*., 2006). Little is known about how species of *Halobacillus* cope with high salinity, but NMR analysis in the current work shows that they do this by accumulating the compatible solute betaine. Unlike the better studied *Halomonas* species, *Halobacillus blutaparonensis* could not grow at high salinity on minimal medium and therefore appears not to be able to synthesise ectoine – another very common compatible solute. This is an additional important difference in the physiological make-up of the genera *Halomonas* and *Halobacillus* and may suggest that Gram-positive bacteria cannot synthesise ectoine.

*S. warneri* was of interest due to its pathogenic nature. It is mainly associated with hospital acquired infections and it is known to be present in many natural environments (Kassem, 2009). It is an important finding that it is present in the water samples from Hassa, Saudi Arabia, since this water is used for irrigation and recreation purposes. Betaine accumulation was shown to be the physiological mechanism used by this pathogenic bacterium to grow at high salinities and this has not previously been reported in the literature.

The final results chapter (Chapter 6) examined the effect of UVC light on the growth, cell morphology and DNA structure of *S. warneri* and *Exiguobacterium*. Experiments conducted on solid medium showed that UVC killed both organisms after a few minutes exposure. Further experiments were carried out on liquid cultures, where after exposure to UVC light, DNA was extracted from the cells and analysed by gel electrophoresis. In some experiments, Soft Gel analysis software was used to quantify the DNA in each lane of the gel, because it was difficult to judge accurate by eye. The results from the gel analysis were not clear cut, but DNA loss and degradation were evident for both organisms after exposure to UVC light. Samples were prepared for SEM and TEM to examine the effect of UVC light on cell shape and morphology. However, no obvious morphological changes were seen for either *Exiguobacterium* or *S. warneri*.

## 7.2 Future Work

The 16S rRNA gene sequencing work described in Chapter 3 successfully identified five strains of bacteria, three were identified to species level – *Halobacillus blutaparonensis*, *Staphylocoocus warneri* and *Halomonas venusta*. However, it should be noted that only 548, 747 and 479 bp of sequence were achieved for each stain respectively. This is some way below the maximum sequence length of the 16S rRNA gene of around 1500 bp. Therefore, future work would involve designing new 16S rRNA gene primers to lengthen the sequences achieved and consolidate the identifications found in the current work. New primers and better sequences are even more important for the *Exiguobacterium* strains (AL2 and ABr1), to potentially separate these strains as separate species and even possibly new species of *Exiguobacterium*.

Further work can also be done to follow up the effect of UVC light on *S. warneri* and *Exiguobacterium*. The results discussed in Chapter 6 have shown the development of sound methods to investigate the degradation of DNA due to UVC exposure. Future work would expand the times of UV exposure tested to ensure that the damage to DNA was repeatable and then the effect of recovery times in the light and dark could be clearly seen.

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