USE OF MOLECULAR APPROACHES TO STUDY THE OCCURRENCE OF EXTREMOPHILES AND EXTREMODURES IN NON-EXTREME ENVIRONMENTS

By

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2011

DEDICATION

To the memory of my father, loving mother, wife “Muneera” and son “Anas”, brothers and sisters.

ACKNOWLEDGEMENTS

Above all, I thank Allah for helping me in completing this project. I wish to express my thanks to my supervisor Professor Milton Wainwright, for his guidance, supervision, support, understanding and help in this project. In addition, he also stood beside me in all difficulties that faced me during study. My thanks are due to Dr. D. J. Gilmour for his co-supervision, technical assistance, his time and understanding that made some of my laboratory work easier.

In the Ministry of Regional Municipalities and Water Resources, I am particularly grateful to Engineer Said Al Alawi, Director General of Health Control, for allowing me to carry out my PhD study at the University of Sheffield.

My deepest and special thanks are due to my beloved mother and wife for their support, patience and continuous encouragement throughout the period of my study.

Finally, I would like to extend my sincere thanks to colleagues in Wainwright's laboratory (G10) for constant encouragement and support. Special thanks to my best friend in MBB department Suleiman Alnaimat who was accompanying me thought-out the period of my study as well as Miss Reda Amasha for her encouragement and support, mainly making "Du'a".

ABSTRACT

A number of samples were collected from various extreme and non-extreme environments. A range of molecular approaches, in addition to classical microbiology techniques, were used to isolate microorganisms, mainly archaea and bacteria from unusual environments. Halo-bacteria and archaea from non-saline environments and alkaliphilic bacteria and archaea capable of withstanding low pH values were isolated and identified using 16S rRNA gene cloning, PCR amplification and phylogenetic analysis. Detailed analysis of their halophilic and alkaliphilic physiology for adaptation in the environment was investigated using nuclear magnetic resonance spectroscopy (NMR) to detect the compatible solutes and atomic emission spectophotometery (AES) to measure the cellular potassium ions. In addition, ultraviolet-type C tolerant bacteria were isolated from terrestrial environments using phylogenetic analysis of the 16S rRNA gene sequences.

A selection of other molecular techniques were used in this thesis. Fluorescent *in situ* hybridisation (FISH) allowed simultaneous identification, enumeration and visualization of microbial cells of the two domains; Archaea and Bacteria within a community in their natural habitat. In addition, using EZ-PCR test, culturable-independent *Mycoplasma* DNA in environmental samples was detected.

Further investigation, using other advanced molecular techniques, into the physiology of extremophilic microorganisms found in non-extreme environment may provide a better understanding of the microbial interactions and the essential roles which different species play in the environment.

SUMMARY

Summary of Aims

The aims of this study were to:

a) Determine if halophilic and halotolerant bacteria as well as archaea can be isolated from non-saline environments and if so to identify them using molecular methods.

b) Determine how halophilic and halotolerant bacteria and archaea, isolated from non-saline environments, can survive extreme saline conditions.

c) Conduct studies on the screening and isolation of alkaliphilic and alkalitolerant bacteria and archaea which are also able to grow in acidic media.

d) Conduct studies on the question-how do presumptive alkalitolerant and alkaliphiles survive in such alkaline as well as low pH conditions?-

e) Conduct studies on the isolation of ultraviolet-type C tolerant bacteria from terrestrial environments using phylogenetic analysis of the 16S rRNA gene sequences.

f) The FISH technique was also used to allocate the isolated microorganisms to either archaea or bacteria.

g) Finally, studies were conducted to determine (using a PCR detecting method) if mycoplasmas can be isolated from extreme and non-extreme environments.

The first aim of this study was to determine if halophilic and halotolerant bacteria as well as archaea could be isolated from non-saline environments and if so to identify them using molecular methods; the following results were obtained:

1. Some 96% of the non-saline soil samples contained halophilic bacteria capable of growing at 1.0M , while 54% grew at 2.0M NaCl and 16 % were able to grow at 3.0M NaCl; only 6% of the isolates however, grew at 4.0M NaCl.
2. Some 12% of isolates from saline environmental samples yielded microorganisms capable of growing at 4.0M NaCl. Whereas, all samples

produced growth at salt concentrations up to 2.0M and 62% of them produced bacteria capable of growing at 3.0M NaCl.

3. None of the air samples or single rain sample yielded any salt tolerant isolates. Two bacteria capable of growing at 2.0M NaCl only, were however, isolated from a hailstone sample.
4. The number of colony forming units isolated from both non-saline and saline soil decreased with increasing concentrations of NaCl added to plates containing Plate Count Agar.
5. The majority of the isolates obtained in this study were representatives of previously isolated organisms.
6. The isolates obtained from non-saline soils were *Bacillus* species, and analysis of partial sequences of their 16S rRNA genes showed that some of them exhibited similarities higher than 99% with those of *Bacillus subtillis, Paenibacillus ehimensis* and 100 % with *Bacillus weihenstephanensis*. No bacteria other than species of the genus *Bacillus* were isolated from non-saline samples.
7. Two isolates were obtained from hailstones on media containing 2M NaCl, one was identified as *Pantoea dispersa* (99.12 similarity) and the other as *Staphylococcus sciuri sciuri* (99.94 similaritiy). *Bacillus pumilus* isolated from the stratosphere grew in up to 3M NaCl.
8. The data presented here show that Bacilli are dominant among halophilic bacteria in non-saline environments; halophilic bacteria, in general, are however, not restricted to this genus.
9. The two archaeal isolates obtained from desert sands and desert varnish collected from Oman, were mixed with bacterial colonies and it was not

possible to separate them even with the use of antibiotics and as a result it was impossible to obtain valid sequence data for the identification of these isolates.

10. All previously mentioned isolates were isolated from non-saline environments which would be expected to act as a source of halotolerant but not halophilic bacteria. On the other hand, a strain of bacteria isolated from saline soil, showed strict obligate halophilic characteristics as it was not capable of growing, or even surviving without salt; optimum growth occurring at 1.0M and 2.0M NaCl as well as 4M salt.

The study also focused on the question of how halophilic and halotolerant bacteria and archaea, isolated from non-saline environments, can survive extreme saline conditions. The following results were obtained:

1. In most isolates, the results for intracellular glycine betaine levels above 1.0M NaCl showed large variability, a general trend towards an increase in glycine betaine level with increasing salt concentration was observed.
2. NMR analyses of compatible solutes in all halotolerant isolates showed the accumulation of glycine betaine is important for osmotic adaptation of this isolate when growing in the presence of high NaCl concentrations (except when grown in M9 medium). The fact that betaine was the main osmoprotectant present in most isolates depended on the composition of the medium (i.e. LB medium contains yeast extract) showing that the presence of betaine in the cells was definitely not synthesized by the cells (e. g. *de novo*) and it appears that betaine is absolutely required as an organic osmolyte for these isolates when grown under stressed conditions.
3. The compatible solutes reported in this study mostly correspond with those reported in the literature and the results show that the halophilic isolates usually accumulated amino acids and polyols and their derivatives such as, glycerol,

ectoine, glutamate, proline and lysine, as well as quaternary amines such as glycine betaine which have no net charge at physiological pH and do not disturb metabolic processes.

4. This study also demonstrated that glycine betaine and glutamate were the main compatible solutes which were accumulated at high concentration in halobacteria.
5. Potassium accumulation was not found in halotolerant and halophilic bacteria, either when exposed to adaptative salt concentrations or following osmotic shock.
6. Halophilic archaea are presumably maintaining the osmotic equilibrium between their cytoplasm and the hypersaline surroundings by accumulating high salt concentrations.
7. The total cellular K^+ concentration increased for, presumptive archaea (DSRT3), when the cells were grown with increasing NaCl concentration (w/v), when the cells were subjected to osmotic shock or when trained to tolerate a range of NaCl concentrations.
8. This study has shown that most halotolerant and halophilic bacteria, HL1, STRA2 and HSO, do not utilise the "salt-in-cytoplasm" strategy. This strategy has long been assumed to be used by most halophilic archaea (such as the DSRT3 isolate in this study), and certain halophilic eubacteria. The findings of this study show that halophilic isolates accumulate glycine betaine, proline and glutamate instead of potassium ions. These isolates also accumulated different organic osmolytes when exposed to different salinities.

Studies were also conducted on the screening and isolation of alkaliphilic and alkalitolerant bacteria and archaea which are also able to grow in acidic media. The following results were obtained:

1. All of the organisms isolated from different Redcar lagoon samples, with pH ranging from 8.6 to 12.0, were shown to be different genera and species except two samples; the lake sample with a pH of 12 and the soil sample with pH 8.6. Although these two samples were different in pH value they gave the same isolate, namely a bacterium which was 100% similar to *Lactococcus lactis*. Only *L. lactis* that has been isolated from the lake sample with a pH of 12 and the other one which was isolated strain from Redcar-soil with a pH of 12 (95% similar to *Bacillus massiliensis*) were the only bacteria able to grow in medium at a pH value of 4.5. All of the other isolates were able to grow only at pH 7.0 and above.
2. Six alkaliphilic and alkalitolerant bacteria were isolated from the alkaline samples and two of them were presumptively acid-tolerant. All of the isolates were capable of growth on high pH medium as well as at neutral pH. A very narrow range of growth media was used in attempts to isolate bacterial and archaeal microorganisms, therefore, the proportion of organisms present in samples which could grow using the media employed was probably significantly reduced compared to the total microbial population. Since all attempts to culture these microorganisms were made using aerobic conditions micro-aerophilic organism and anaerobes were obviously excluded.

The work conducted here focused on the question-how do presumptive alkalitolerant and alkaliphilic bacteria survive in such alkaline as well as low pH conditions?- produced the following results:

1. Three alkaliphilic isolates were selected for the characterization of the osmo-adaptation mechanisms; RS-12 (*Alkalibacterium kapii*; grows at pH range from 7 to 12) and RS12-4 *(Bacillus massiliensis*; grows at pH range from 4.5 to 12) which were isolated from Redcar-Soil with pH value of 12, in addition to

RR12-4 *(Lactococcus lactis*; grows at pH range from 4.5 to 12) which were isolated from Redcar-root with pH value of 12.

2. The mechanisms of compatible solute accumulation reported in this study generally agre with what has been reported elsewhere, namely that low-salt tolerant alkaliphiles and also halotolerant and halophiles (reported above) usually accumulate amino acids and polyols and their derivatives such as, glycerol, ectoine as well as quaternary amines such as glycine betaine.

A study was made of the occurrence of ultraviolet-C tolerant bacteria in terrestrial environments using phylogenetic analysis of the 16S rRNA gene sequences. The following results were obtained:

1. The experimental isolation approach used in this study has the advantage that the bacteria were exposed to UV while they were still in their natural oligtrophic growth state, so that their normal physiology was not altered by growth on high nutrient media, before exposure to UV.
2. The desert soil (sampled near Nizwa city, Oman) was the only soil sample to yield a UV-C resistant isolate and this was shown to be the spore forming bacterium, *Paenibacillus ehimensis*. The other UV-C resistant organisms were archaea isolated from desert sand (Waheeba sands, Oman) and desert varnish (sampled near Alashkhara city, Oman); all of these locations can be considered to be hyper-arid environments.
3. No UV-C resistant bacteria were isolated from the wide range of soils sampled from the UK, all of which contain large numbers of bacteria (ranging from $1x10^5$ to $1x10^8$, (as determined by dilution plating on Plate Count Agar). The soil suspension exposed to UV-C contained a maximum of $1x10^6$ bacteria; no isolated members of this population were resistant to UV-C.

4. However, from one of the three UK hailstone samples, a single UV-C resistant bacterium (*Staphylococcus sciuri sciuri*) was also isolated. Additionally, two UV-C resistant isolates were obtained from Sheffield rain water; these were independently identified as isolates *Exiguobacterium sp* and *Bacillus pumilus*.
5. A single UV-C resistant strain was isolated from the stratosphere sample following exposure to UV-C; this was identified a *B. pumilus*.
6. No UV-C resistant bacteria were obtained from a range of seawater samples, or from the hypersaline soil or from the building surface sample from Oman.
7. The resistant isolates were exposed to UV-C and the number of cells before and after exposure were determined by plate counting. Non-irradiated samples contained bacterial concentrations ranging from 3.7×10^5 to 8.1×10^5 CFU/ml, while the number of bacteria following radiation was 1.1×10^5 to 7.8×10^5 CFU/ml, i.e. a slight but not statistically significant difference.
8. In an attempt to correlate the possible link between desiccation resistance and ionizing-radiation resistance, dried strains including; *E.coli* 0127, *Streptococcus thermophilus* 10387, *Bacillus subtilus* 3106, *Serratia marcescens* 1981 and *Streptococcus viridochromogenes* were sieved by 25µm aperture sieve and exposed to UV-C radiation. The majority of the dried strains tested did not grow on nutrient agar or liquid medium. *Streptococcus thermophilus* was the only bacterium which showed resistance to UV-C radiation.

The FISH technique was used to allocate the isolated microorganisms to either Archaea or Bacteria, the following results were obtained:

1. In early attempts to use FISH epi-fluorescence microscopy revealed high levels of background autofluorescence due to the presence of minerals in the sediment/soil-extract. These minerals also demonstrated non-specific binding to the oligonucleotide probes.

2. As a result, a confocal laser scanning microscope was used instead of the more commonly used epifluorescence microscope. Although, PerfectHyb Plus Hybridization Buffer contained formamide the concentration was found to be insufficient during the hybridisation step for FISH; higher concentrations of formamide were therefore added to decrease non-specific binding of fluorescent oligonucleotide probes to the minerals present in the sediment/soil-extract medium and to optimise the probe specificity. Both Archaea (stained red with Cy5) and bacteria (stained green with Alexa Fluor 488) were observed without attached minerals.
3. FISH allowed the detection of microorganisms, depending on the labeled probes used, in their natural habitat. The use of FISH confirms the presence of bacteria and archaea in both environmental samples with perhaps some room for doubt in the rock varnish sample. FISH, using confocal microscopy revealed the presence of archaea and bacteria among the microbial community of desert sand which also confirms our previous findings. Fish confirmed the presence of both archaea and bacteria in the sand. By employing bacterial and archaeal primers; FISH confirmed that few archaea were present in desert varnish.
4. Archaea accounted for more than 90% of the population in desert sand isolates whereas they made up only a small part of the population in rock varnish isolates. Interestingly, archaea were not isolated from hypersaline soil with 10% salt concentration although extremophilic halobacterium was isolated. FISH however, revealed the presence of both extremophilic halo-archaea and bacteria in this soil sample. Enrichment cultures of the hypersaline soil analysed by confical microscopy consisted of approximately 70% Archaea and 30% bacteria following culture at 45°C.

5. The alkaline soil sample with pH value of 12 is an extreme environment but the use of FISH, plus confocal microscopy, revealed a small number of archaea among microbial communities.
6. Agricultural and garden (non-extreme) soil samples were tested for the presence of archaea among the bacterial community. All of them failed to show any archaeal fluorescence, a finding which was which was confirmed by the 90 to 100% of the DAPI-stained cells hybridized to the bacterial probe. The majority of microorganisms in these soils detected by FISH analysis were therefore bacteria, a fact which agrees with the findings obtained using culture approaches.

Studies aimed at detecting Mycoplasmas in extreme and non-extreme environments showed the following:

1. *Mycoplasma* was detected by PCR in two environmental samples, i.e. for the hailstone sample and the other for the park grass sample, both of which were collected in Sheffield.
2. Although, only viable Mycoplasmas should be considered as potential source of infection, EZ-PCR- *Mycoplasma* detects non-viable Mycoplasmas.

LIST OF CONTENTS

"Thus many extremophiles are an excellent source of hyperstable macromolecules, and there is no doubt that the discovery of extremophiles, and especially of archaea, has stimulated a wealth of fundamental and applied research into these organisms and their cellular constituents"

Centre for Extremophile Research

University of Bath

2010

LIST OF FIGURES

LIST OF TABLES

APPENDIX A: CTAB BUFFER AND SOC MEDIUM

CTAB buffer
2% CTAB (hexadecyltrimethylammonium bromide)
100 mM TrisHCl [pH=8]
20 mM EDTA,
1.4 M NaCl
2% β-mercaptoethanol [added just before use]

SOC medium

1. Add the following to 900ml of distilled H_2O
    - 20g Bacto Tryptone
    - 5g Bacto Yeast Extract
    - 2ml of 5M NaCl.
    - 2.5ml of 1M KCl.
    - 10ml of 1M $MgCl_2$
    - 10ml of 1M $MgSO_4$
    - 20ml of 1M glucose
2. Adjust to 1L with distilled H_2O
3. Sterilize by autoclaving

APPENDIX B: DETAILS OF OLIGONUCLEOTIDES (PRIMERS) SYNTHESIS

eurofins mwg|operon

Oligonucleotide Synthesis Report

Mr. Khalid AlAbri
University of Sheffield

Order ID: 2288208
Customer ID: 923032
Your Order ID (PO#): 4500288978

Order Date: 25/10/2010
Lab No.: 1123
No. of Oligos: 1/4

Eurofins MWG Operon
Anzingerstraße 7a
D- 85560 Ebersberg

No.	Oligo Name	Sequence (5' -> 3')	Yield [OD]	Yield [µg]	Yield [nmol]	Concentration [pmol/µl]	Vol. for 100pmol/µl	Tm [°C]	MW [g/mol]	GC-Content	Synthesis Scale	Purification	Modification	Barcode ID	QC Report
1	A571F	GCYTAAAGSR6CCGTAGC (18)	4.3	126	22.8	-	228	56.0	5514	55.6 %	0.05 µmol	HPSF	6-INO	012370917	-
2	UA1204R	TTMGGGGCATRC8KACCT (18)	5.8	178	32.2	-	322	54.8	5508	52.8 %	0.05 µmol	HPSF	6-INO	012370918	-
3	16SUNI.FOR	CCGAATTCGTCGACAACA GAGGATCCTGGCTCAG (34)	10.4	290	27.8	-	278	73.1	10436	55.9 %	0.05 µmol	HPSF	-	012370919	-
4	16SUNI.REV	CCCGGGATCCAAGCTTA CGGCTACCTTGTTACGAC TT (37)	22.4	659	58.4	-	584	73.9	11276	54.1 %	0.05 µmol	HPSF	-	012370920	-

Z = Inosine

eurofins mwg|operon

Oligonucleotide Synthesis Report

Mr. Khalid AlAbri
University of Sheffield

Order ID: 2225723
Customer ID: 923032
Your Order ID (PO#): 4500267539

Order Date: 22/07/2010
Lab No.: 4490
No. of Oligos: 4/4

Eurofins MWG Operon
Anzingerstraße 7a
D- 85560 Ebersberg

No.	Oligo Name	Sequence (5' -> 3')	Yield [OD]	Yield [µg]	Yield [nmol]	Concentration [pmol/µl]	Vol. for 100pmol/µl	Tm [°C]	MW [g/mol]	GC-Content	Synthesis Scale	Purification	Modification	Barcode IDO	QC Report
1	A571F	GCYTAAAGSR6CCGTAGC (18)	1.5	44	8.0	-	80	56.0	5514	55.6 %	0.05 µmol	HPLC	6-INO	012050822	-
2	UA1204R	TTMGGGGCATRC6KACCT (18)	7.6	233	42.3	-	423	54.8	5508	52.8 %	0.05 µmol	HPLC	6-INO	012050823	-
3	16SUNI.FOR	CCGAATTCGTCGACAACA GAGGATCCTGGCTCAG (34)	13.6	379	36.4	-	364	73.1	10436	55.9 %	0.05 µmol	HPSF	-	012050824	-
4	16SUNI.REV	CCCGGGATCCAAGCTTA CGGCTACCTTGTTACGAC TT (37)	8.9	262	23.2	-	232	73.9	11276	54.1 %	0.05 µmol	HPSF	-	012050825	-

Z = Inosine

APPENDIX C: THE PHYLOGENETIC ANALYSIS AND POSITION OF HALOPHILIC ISOLATES

> |FN582329.1| *Paenibacillus ehimensis* partial 16S rRNA gene, strain IB-739
Length=1486

Score = 1026 bits (555), Expect = 0.0
Identities = 557/559 (99%), Gaps = 0/559 (0%)
Strand=Plus/Minus

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Query  1     GGCTCCTTGCGGTTACCCCACCGACTTCGGGTGTTGTAAACTCTCGTGGTGTGACGGGCG  60
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Sbjct  1428  GGCTCCTTGCGGTTACCCCACCGACTTCGGGTGTTGTAAACTCTCGTGGTGTGACGGGCG  1369

Query  61    GTGTGTACAAGACCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCAAT  120
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Sbjct  1368  GTGTGTACAAGACCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCAAT  1309

Query  121   TCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGACCGGCTTTCTTAGGA  180
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Sbjct  1068  CGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCTCC  1009

Query  421   TCTGTCCCGAAGGCCTACCCTATCTCTAGGATATTCAGAGGGNTGTCAAGACCTGGTAAG  480
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Sbjct  1008  TCTGTCCCGAAGGCCTACCCTATCTCTAGGATATTCAGAGGGATGTCAAGACCTGGTAAG  949

Query  481   GNTCTTCGCGTTGCTTCGAATTAAACCACATACTCCACTGCTTGTGCGGGTCCCCGTCAA  540
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Sbjct  948   GTTCTTCGCGTTGCTTCGAATTAAACCACATACTCCACTGCTTGTGCGGGTCCCCGTCAA  889

Query  541   TTCCTTTGAGTTTCACTCT  559
             ||||||||||||||||||
Sbjct  888   TTCCTTTGAGTTTCACTCT  870
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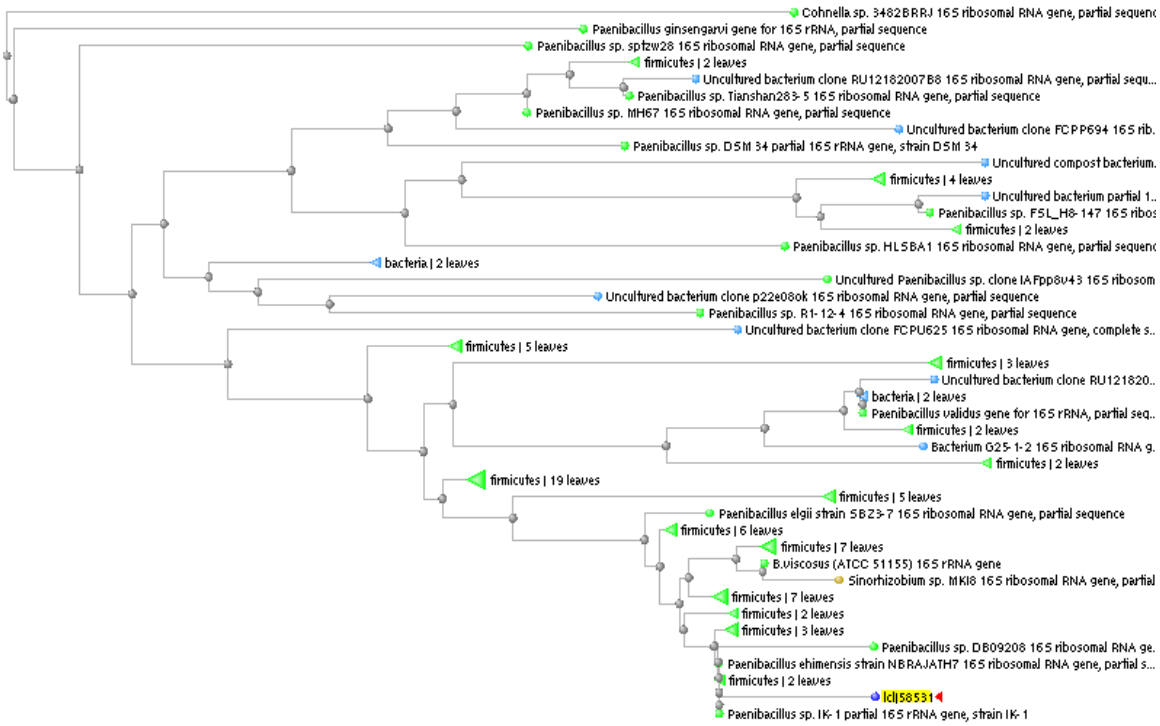

>[|EF101710.1|](#) *Bacillus subtilis* strain HU60 16S ribosomal RNA (rrnE) gene, partial sequence
Length=1426

Score = 1166 bits (631), Expect = 0.0
Identities = 640/646 (99%), Gaps = 0/646 (0%)
Strand=Plus/Minus

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Query  75    GTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCA  134
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Sbjct  1333  GTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCA  1274

Query  135   GCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGC  194
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Sbjct  1273  GCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGC  1214

Query  195   TTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGT  254
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Sbjct  1213  TTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGT  1154

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Sbjct  1153  CATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTC  1094

Query  315   ACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGA  374
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Sbjct  913   TCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCT  854

Query  555   TTGAGTTTCAGTCTTGCGACCGTANTCCCCAGGCGGAGCGCTTAATGCGTTATCTGCAGC  614
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Query  615   ACTAAGGGGCGGAAACCCNCTAACACTTAGCACTCATCGTTTACGG  660
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Sbjct  793   ACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGG  748
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sequence
Length=721

Score = 270 bits (146), Expect = 2e-69
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Strand=Plus/Minus

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Sbjct  575  TGGTCCGCGATTACTAGCGA-TTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGA  517

Query  58   ACTGAGAACGG-TTTATGAGA-TAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTACCGGC  115
            |||||||||| ||||||||| |||||||||||||||||||||||||||||||||||| |
Sbjct  516  ACTGAGAACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTACCGTC  457

Query  116  CATTGTAGCACGGGTGTAGCCCAGGTCATAA-GGGCATGATGATTTGACGTCATCCCCAC  174
            |||||||||||| |||||||||||||||||| |||||||||||||||||||||||||||
Sbjct  456  CATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCAC  397

Query  175  CTTCCTCCGGTTTGTC  190
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Sbjct  396  CTTCCTCCGGTTTGTC  381
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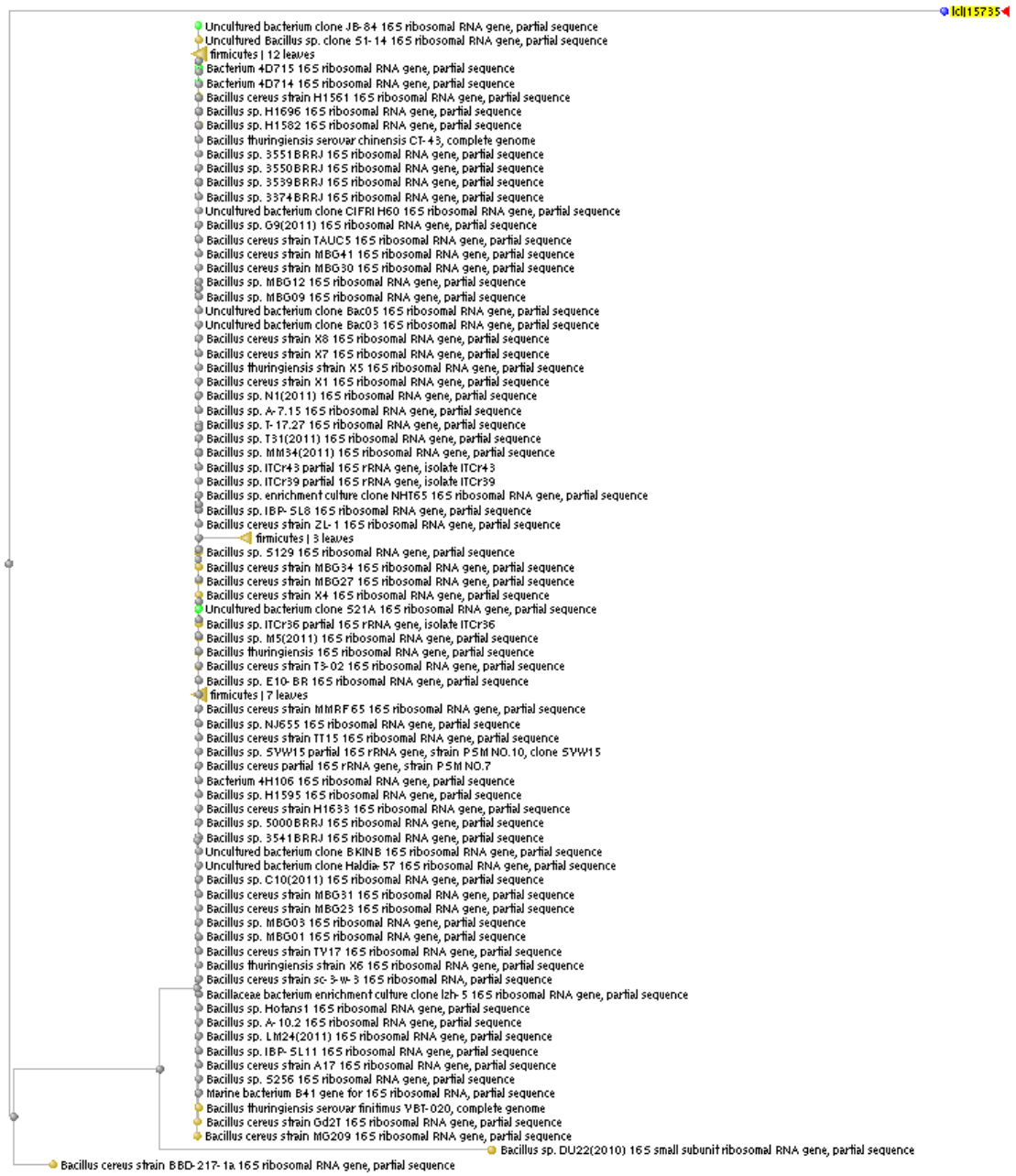

> |AY461753.2| *Bacillus* sp. H-12 16S ribosomal RNA gene, partial sequence
Length=1512

Score = 547 bits (296), Expect = 1e-152
Identities = 301/303 (99%), Gaps = 2/303 (1%)
Strand=Plus/Plus

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Query  1    TATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGAT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  88   TATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGAT  147

Query  61   AACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCAT-GTTCGAAATTGA  119
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  148  AACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGA  207

Query  120  AAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGT  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
Sbjct  208  AAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGT  267

Query  180  AACGGCTCACCAAGGCAACGATGCGTAGCGAACCTGAGAGGGTGATCGGCCACACTGGGA  239
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  268  AACGGCTCACCAAGGCAACGATGCGTAGCGA-CCTGAGAGGGTGATCGGCCACACTGGGA  326

Query  240  CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  327  CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA  386

Query  300  AAG  302
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Sbjct  387  AAG  389
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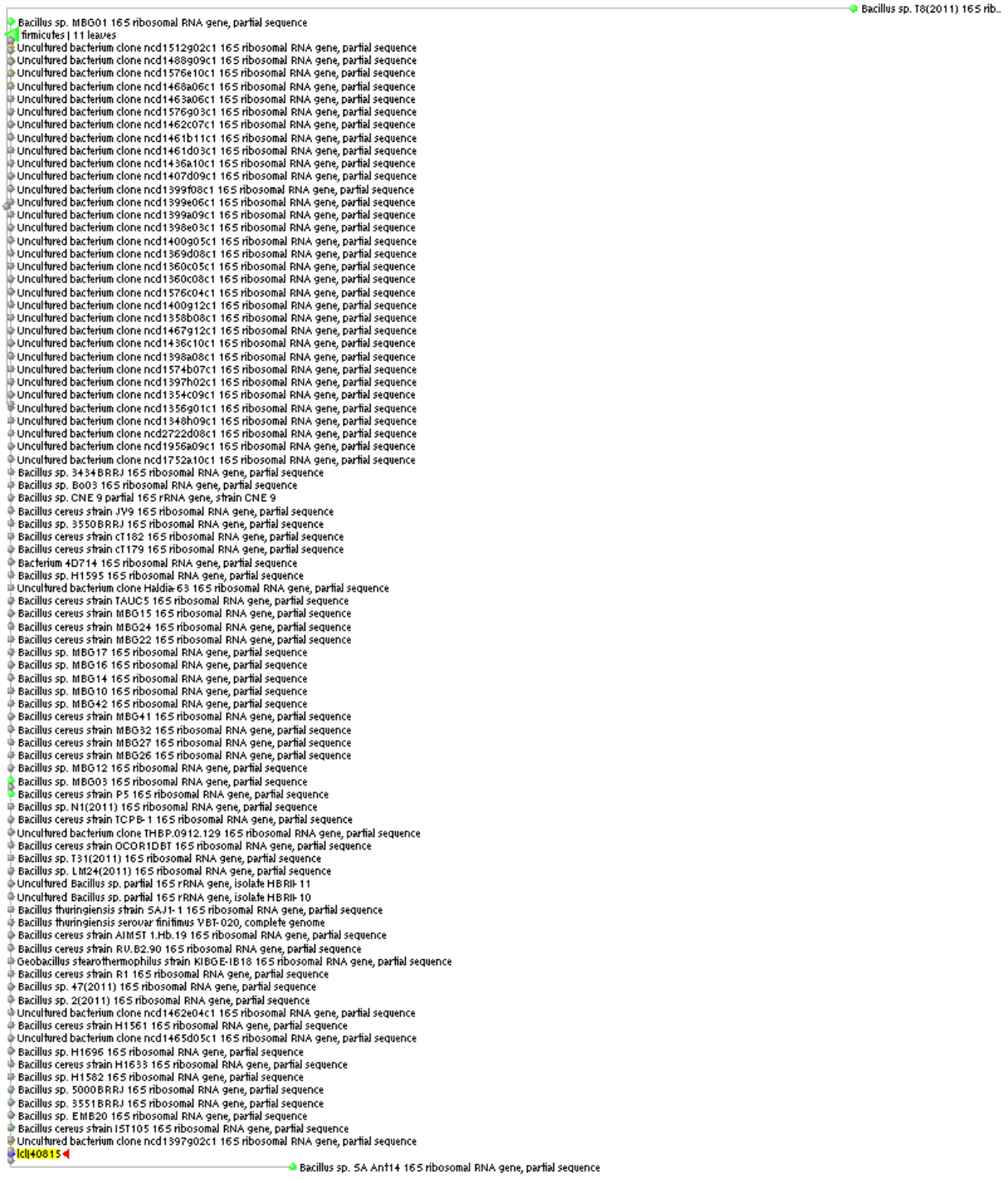

> |GQ413935.1| *Bacillus subtilis* strain RV3 16S ribosomal RNA gene, partial sequence
Length=1516

Score = 1031 bits (558), Expect = 0.0
Identities = 566/570 (99%), Gaps = 1/570 (0%)
Strand=Plus/Minus

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Query  35    GAGGTTACCTCACCGACTTCGGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTA  94
             |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1451  GAGGTTACCTCACCGACTTC-GGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTA  1393

Query  95    CAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCT  154
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1392  CAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCT  1333

Query  155   TCACGCAGNCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTA  214
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1332  TCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTA  1273

Query  215   ACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCAT  274
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1272  ACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCAT  1213

Query  275   AAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACC  334
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1212  AAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACC  1153

Query  335   TTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTT  394
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1152  TTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTT  1093

Query  395   AACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCC  454
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Sbjct  1092  AACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCC  1033

Query  455   GAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGAAAGGTTCTTCG  514
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1032  GAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCG  973

Query  515   CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTG  574
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Sbjct  972   CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTG  913

Query  575   AGTTTCAGCCTTGCGACCGTACTCCCCAGG  604
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Sbjct  912   AGTTTCAGTCTTGCGACCGTACTCCCCAGG  883
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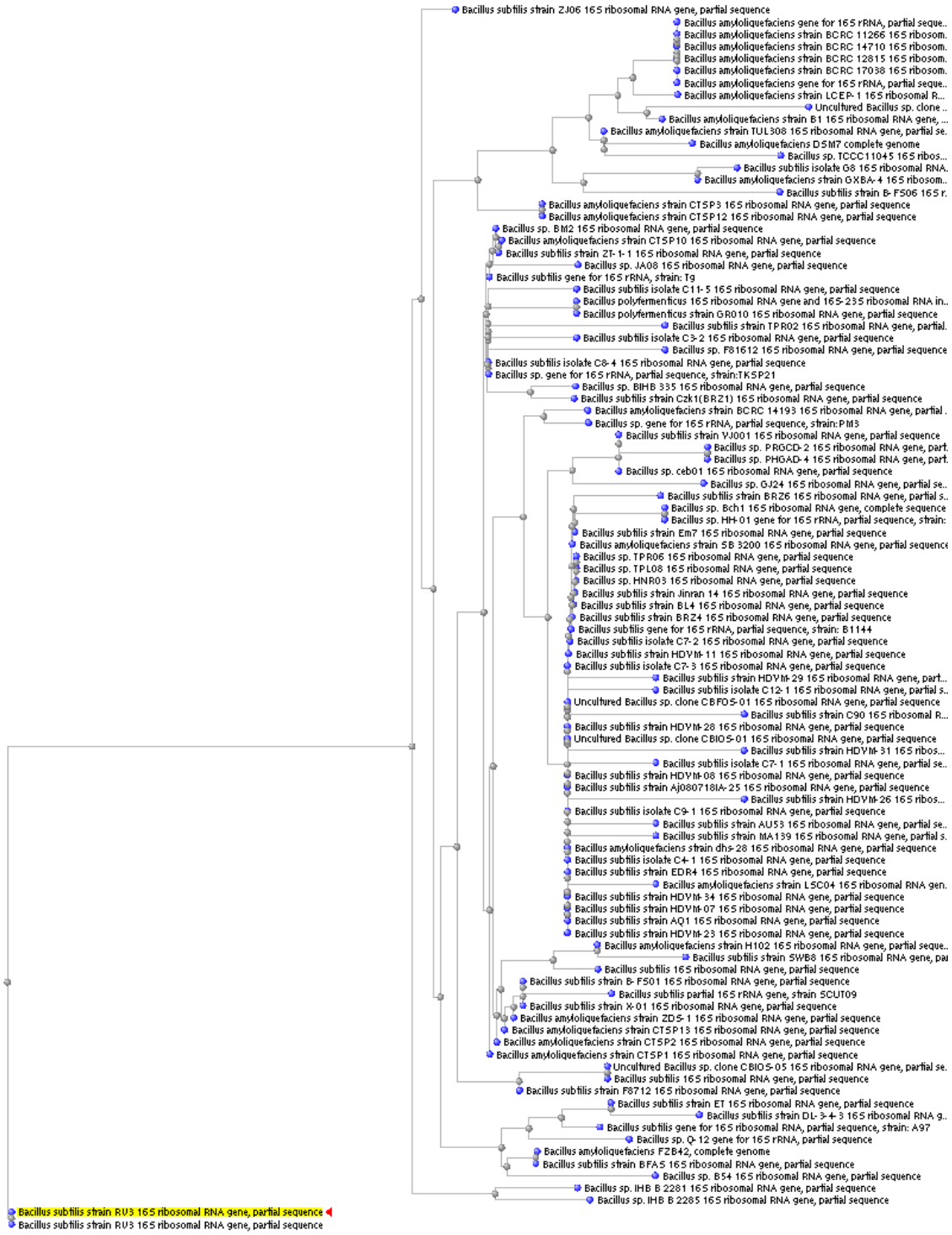

>|JF276896.1| *Bacillus weihenstephanensis* strain BW70UT1570 16S ribosomal RNA
gene, partial sequence
Length=1060

Score = 784 bits (424), Expect = 0.0
Identities = 424/424 (100%), Gaps = 0/424 (0%)
Strand=Plus/Plus

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Query  1    GAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCAT  60
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Sbjct  48   GAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCAT  107

Query  61   AAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  108  AAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATAG  167

Query  121  TTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  168  TTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTA  227

Query  181  GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  228  GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGG  287

Query  241  CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  288  CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC  347

Query  301  GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  348  GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTA  407

Query  361  AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACC  420
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Sbjct  408  AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACC  467

Query  421  TAAC  424
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Sbjct  468  TAAC  471
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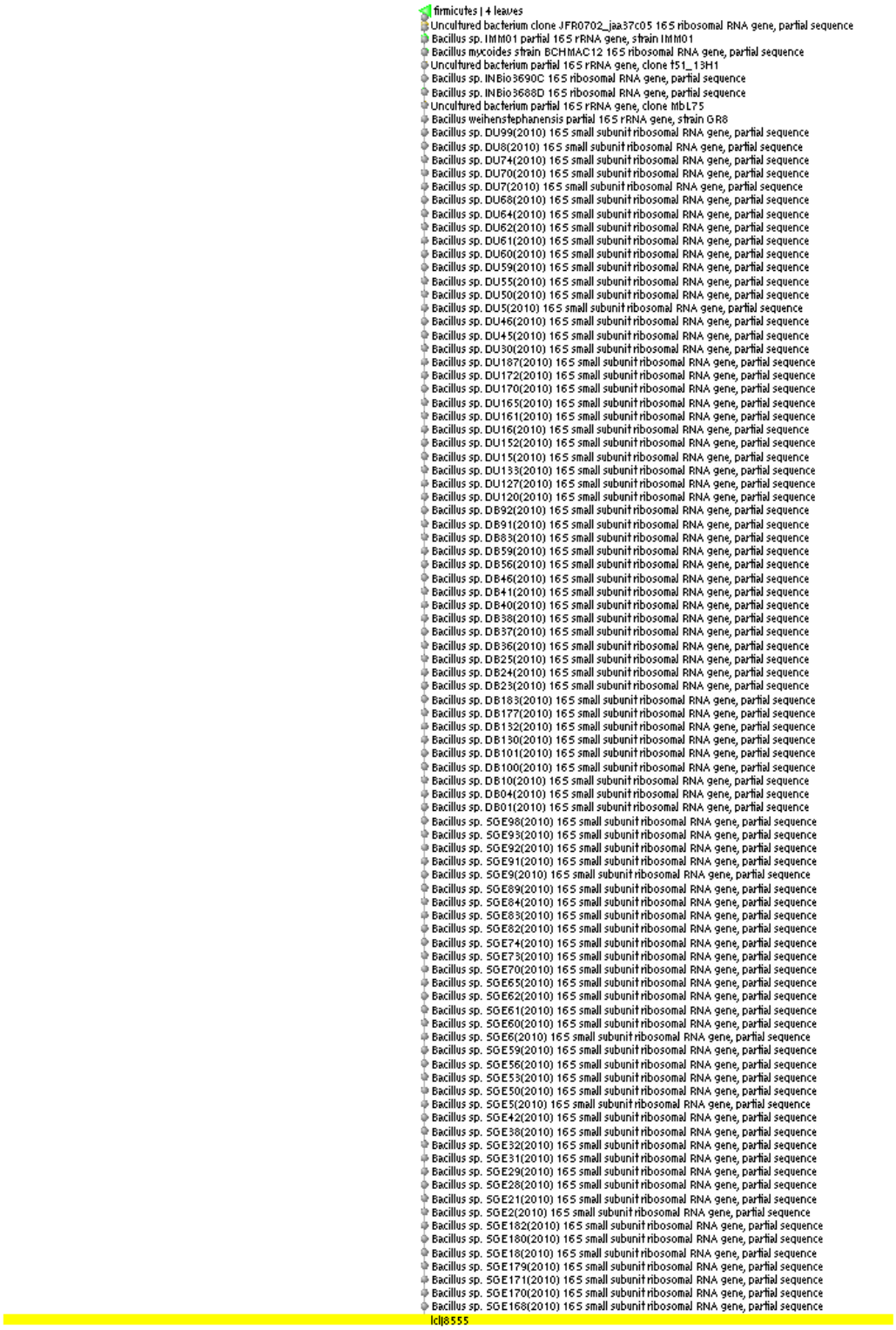

> |AM982536.2| Uncultured bacterium partial 16S rRNA gene, clone PIST-AEB02
Length=874

Score = 985 bits (533), Expect = 0.0
Identities = 539/542 (99%), Gaps = 1/542 (0%)
Strand=Plus/Minus

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Query  1    CCTTCGACGGCTAGCTCCTAATAAAAGGTTACTCCACCGGCTTCGGGTGTTACAAACTCT  60
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Sbjct  835  CCTTCGACGGCTAGCTCCTAATAAAAGGTTACTCCACCGGCTTCGGGTGTTACAAACTCT  776

Query  61   CGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGTAGCATGCTGATC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  775  CGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGTAGCATGCTGATC  716

Query  121  TACGATTACTAGCGATTCCAGCTTCATGTAGTCGAGTTGCAGACTACAATCCGAACTGAG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  715  TACGATTACTAGCGATTCCAGCTTCATGTAGTCGAGTTGCAGACTACAATCCGAACTGAG  656

Query  181  AATAATTTTATGGGATTTGCTTGACCTCGCGGGTTCGCTGCCCTTTGTATTATCCATTGT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  655  AATAATTTTATGGGATTTGCTTGACCTCGCGGGTTCGCTGCCCTTTGTATTATCCATTGT  596

Query  241  AGCACGTGTGTAGCCCAAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  595  AGCACGTGTGTAGCCCAAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCT  536

Query  301  CCGGTTTGTCACCGGCAGTCAACCTAGAGTGCCCAACTTAATGATGGCAACTAAGCTTAA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  535  CCGGTTTGTCACCGGCAGTCAACCTAGAGTGCCCAACTTAATGATGGCAACTAAGCTTAA  476

Query  361  GGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCAT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  475  GGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCAT  416

Query  421  GCACCACCTGTCACTTTGTCCCCCGAAGGA-AAAACTCTATCTCTAGAGCGGTCAAAGGA  479
            ||||||||||||||||||||||||||||||  ||||||||||||||||||||||||||||
Sbjct  415  GCACCACCTGTCACTTTGTCCCCCGAAGGGGAAAACTCTATCTCTAGAGCGGTCAAAGGA  356

Query  480  TGTCAAGATTTGGNAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTT  539
            |||||||||||||  ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  355  TGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTT  296

Query  540  GT  541
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Sbjct  295  GT  294
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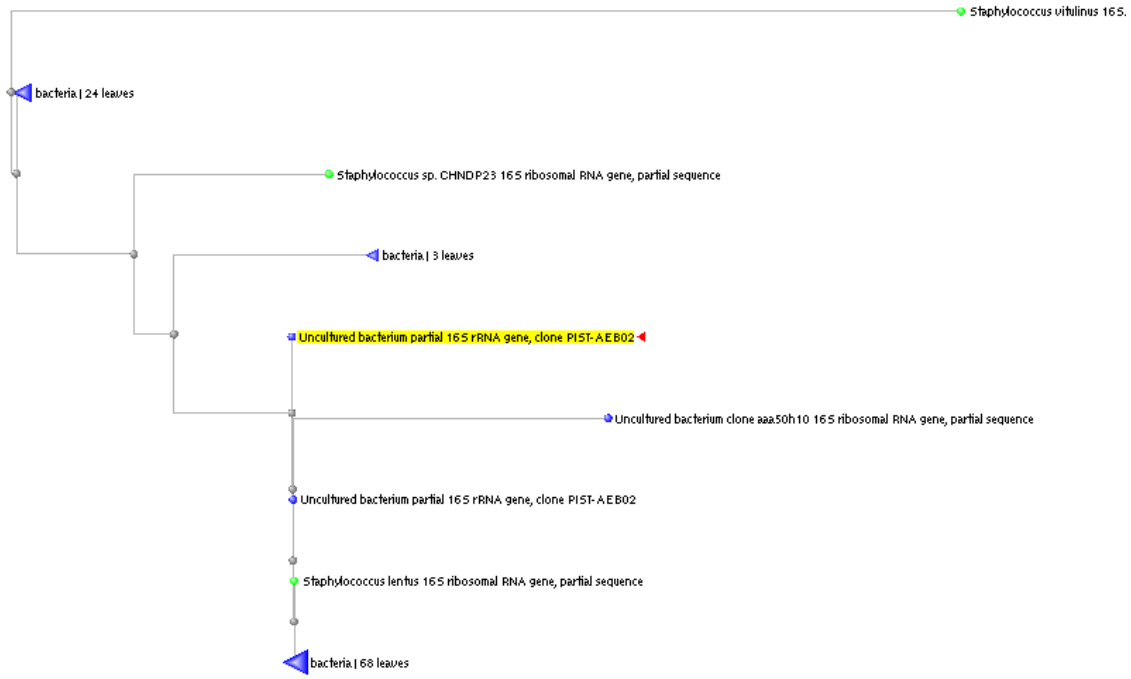

APPENDIX D: THE PHYLOGENETIC ANALYSIS AND POSITION OF ALKALIPHILIC ISOLATES

>  CP002365.1|  *Lactococcus lactis* subsp. lactis CV56, complete genome
Length=2399458

Score = 878 bits (475), Expect = 0.0
Identities = 484/484 (100%), Gaps = 2/484 (0%)
Strand=Plus/Minus

```
Query  1       GGTTTTAAGAGATTAAGCTAAACCATCACTGTCTCGCGACTCGTTGTACCATCCATTGTA  60
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Sbjct  471878  GGTTTTAAGAGATT-AGCTAAA-CATCACTGTCTCGCGACTCGTTGTACCATCCATTGTA  471821

Query  61      GCACGTGTGTAGCCCAGGTCATAAGGGGCATGANGATTTGACGTCATCCCCACCTTCCTC  120
               ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  471820  GCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTC  471761

Query  121     CGGTTTATCACCGGCAGTCTCGTTAGAGTGCCCAACTTAATGATGGCAACTAACAATAGG  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  471760  CGGTTTATCACCGGCAGTCTCGTTAGAGTGCCCAACTTAATGATGGCAACTAACAATAGG  471701

Query  181     GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATG  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  471700  GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATG  471641

Query  241     CACCACCTGTATCCCGTGTCCCGAAGGAACTTCCTATCTCTAGGAATAGCACGAGTATGT  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  471640  CACCACCTGTATCCCGTGTCCCGAAGGAACTTCCTATCTCTAGGAATAGCACGAGTATGT  471581

Query  301     CAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTG  360
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Sbjct  471580  CAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTG  471521

Query  361     CGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGC  420
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Sbjct  471520  CGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGC  471461

Query  421     TTATTGCGTTAGCTGCGATACAGAGAACTTATAGCTCCCTACATCTAGCACTCATCGTTT  480
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  471460  TTATTGCGTTAGCTGCGATACAGAGAACTTATAGCTCCCTACATCTAGCACTCATCGTTT  471401

Query  481     ACGG  484
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Sbjct  471400  ACGG  471397
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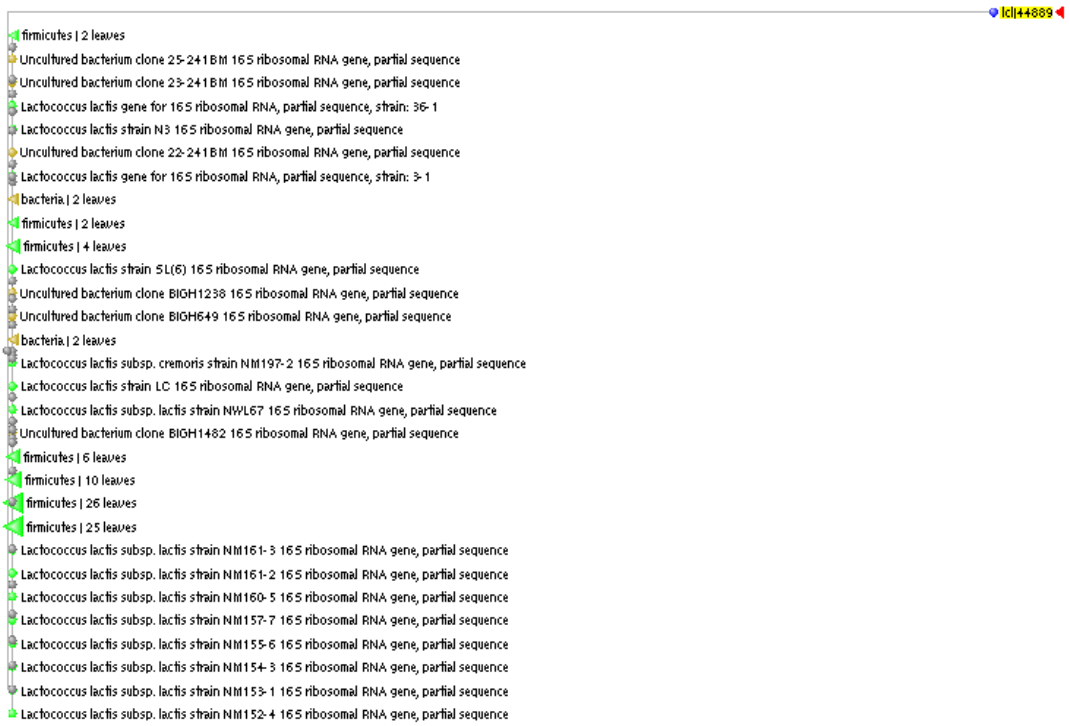

> |HQ397583.1| *Bacillus horikoshii* strain BSCS7 16S ribosomal RNA gene, partial sequence
Length=1417

Score = 569 bits (308), Expect = 3e-159
Identities = 318/323 (98%), Gaps = 2/323 (1%)
Strand=Plus/Minus

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Query  1     TACTAGCGATTCCGGCTTCATGCAGGCGAGTTGGAGCCTGC--TCCGAACTGAGAACGGT  58
             |||||||||||||||||||||||||||||||| |||||||  ||||||||||||||||
Sbjct  1300  TACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAACGGT  1241

Query  59    TTTCTGGGATTGGCTCGACCTCGCGGTTTTGCTGCCCTTTGTACCGTCCATTGTAGCACG  118
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1240  TTTATGGGATTGGCTCGACCTCGCGGTTTTGCTGCCCTTTGTACCGTCCATTGTAGCACG  1181

Query  119   TGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTT  178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1180  TGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTT  1121

Query  179   TGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTG  238
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Sbjct  1120  TGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTG  1061

Query  239   CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCA  298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1060  CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCA  1001

Query  299   CCTGTCACTCTGTCCCCNGAAGG  321
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Sbjct  1000  CCTGTCACTCTGTCCCCCGAAGG  978
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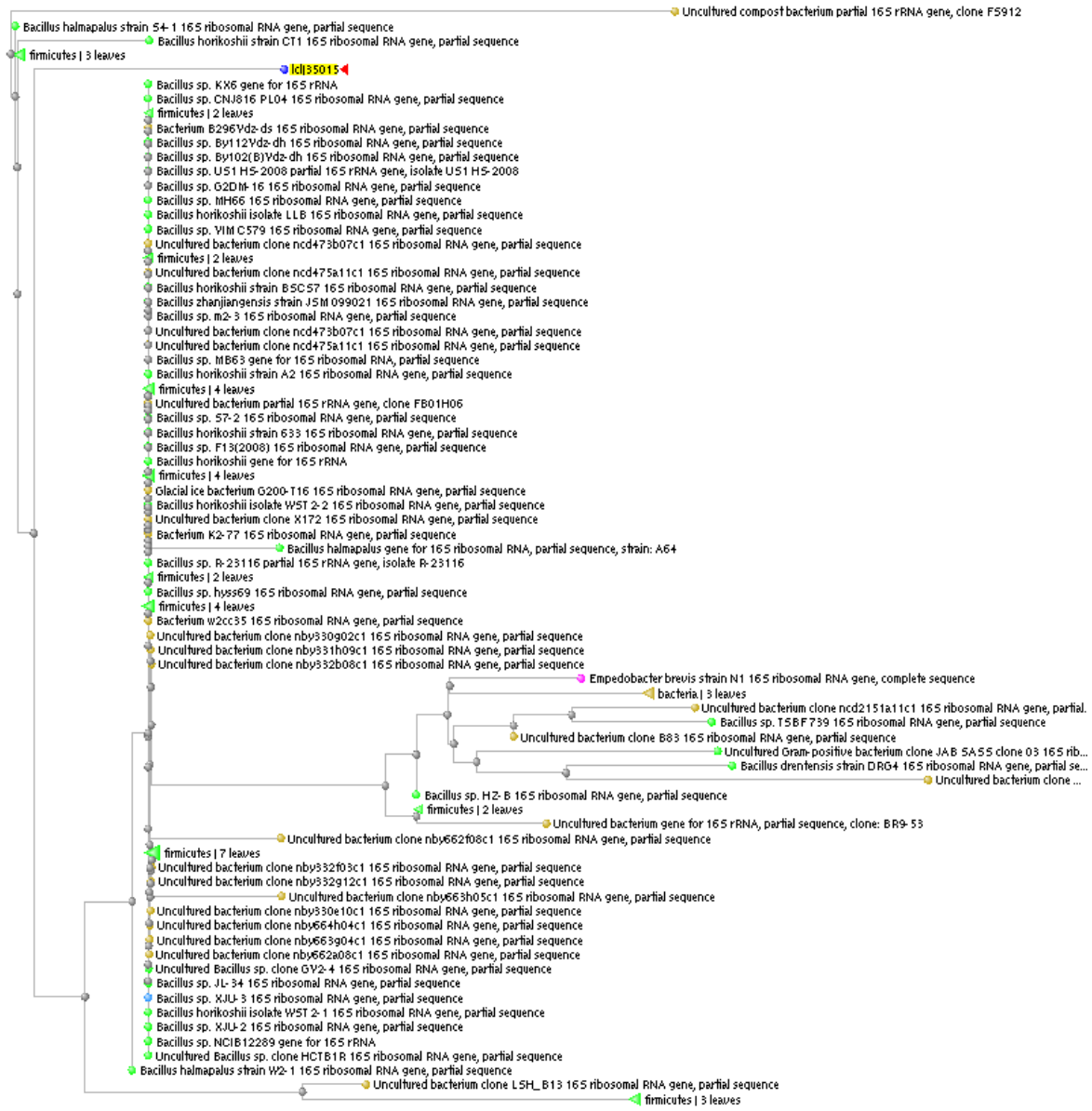

> |AB593337.1| *Carnobacterium* sp. cG53 gene for 16S ribosomal RNA, partial sequence
Length=1447

Score = 686 bits (371), Expect = 0.0
Identities = 371/371 (100%), Gaps = 0/371 (0%)
Strand=Plus/Minus

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Query  1     AACTGAGAATGGCTTTAAGAGATTAGCTTGGCCTCGCGACCTTGCGACTCGTTGTACCAT  60
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Sbjct  1274  AACTGAGAATGGCTTTAAGAGATTAGCTTGGCCTCGCGACCTTGCGACTCGTTGTACCAT
1215

Query  61    CCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCA  120
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Sbjct  1214  CCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCA
1155

Query  121   CCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAACTAAATGCTGGCAACTA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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```
Sbjct  1154  CCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAACTAAATGCTGGCAACTA
1095

Query  181   GTAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGA  240
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Sbjct  1094  GTAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGA
1035

Query  241   CAACCATGCACCACCTGTCACTTTGTCCCCGAAGGGAAAGCTCTATCTCTAGAGTGGTCA  300
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Sbjct  1034  CAACCATGCACCACCTGTCACTTTGTCCCCGAAGGGAAAGCTCTATCTCTAGAGTGGTCA  975

Query  301   AAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCAC  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  974   AAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCAC  915

Query  361   CGCTTGTGCGG  371
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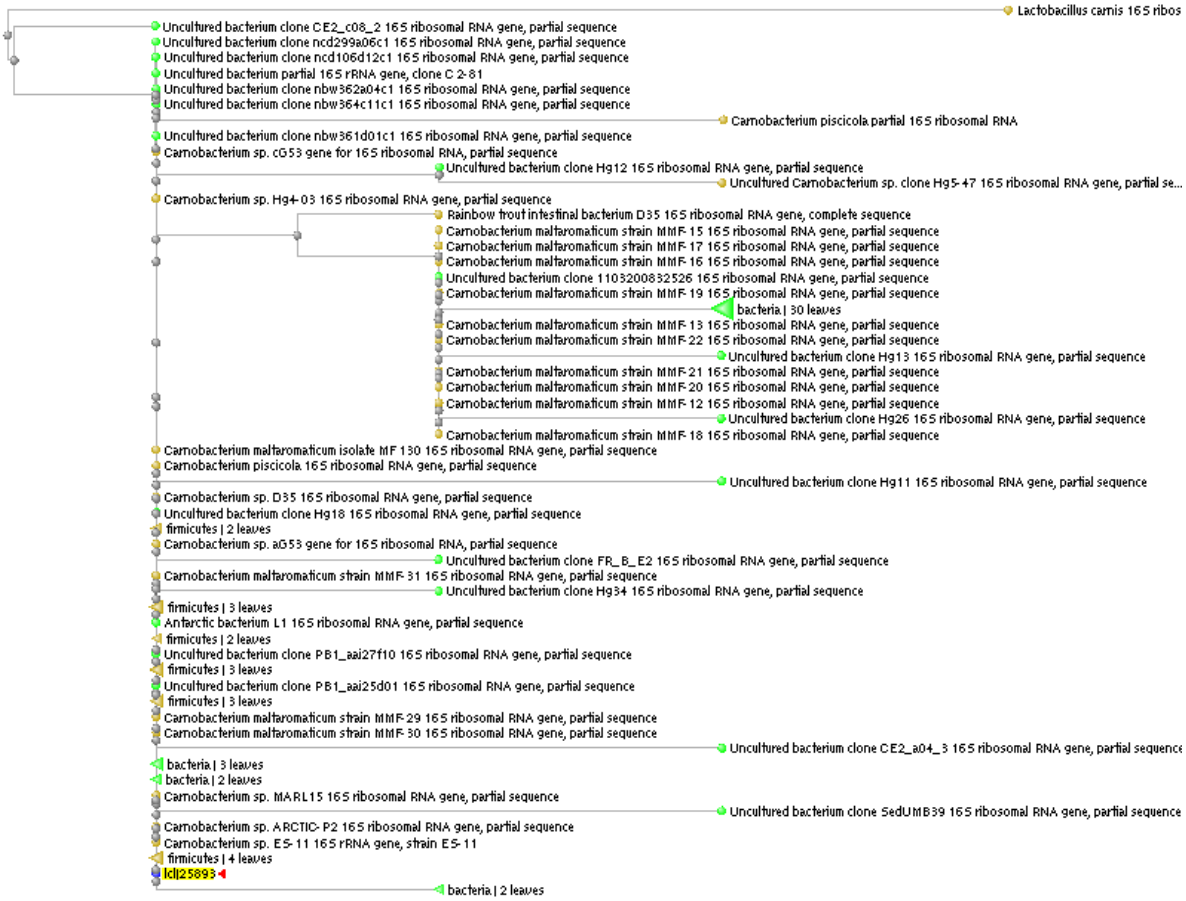

> CP002365.1| *Lactococcus lactis* subsp. lactis CV56, complete genome
Length=2399458

Score = 1092 bits (591), Expect = 0.0
Identities = 599/603 (99%), Gaps = 2/603 (0%)
Strand=Plus/Minus

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Query  1       GATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAAN  60
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Sbjct  471938  GATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAAT  471879

Query  61      GGTTTTAAGAGATTAGCTAAACATCACTGTCTCGCGACTCGTTGTACCATCCATTGTAGC  120
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Sbjct  471878  GGTTTTAAGAGATTAGCTAAACATCACTGTCTCGCGACTCGTTGTACCATCCATTGTAGC  471819

Query  121     ACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCG  180
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Sbjct  471818  ACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCG  471759

Query  181     GTTTATCACCGGCAGTCTCGTTAGAGTGCCCAACTTAATGATGGCAACTAACAATAGGGG  240
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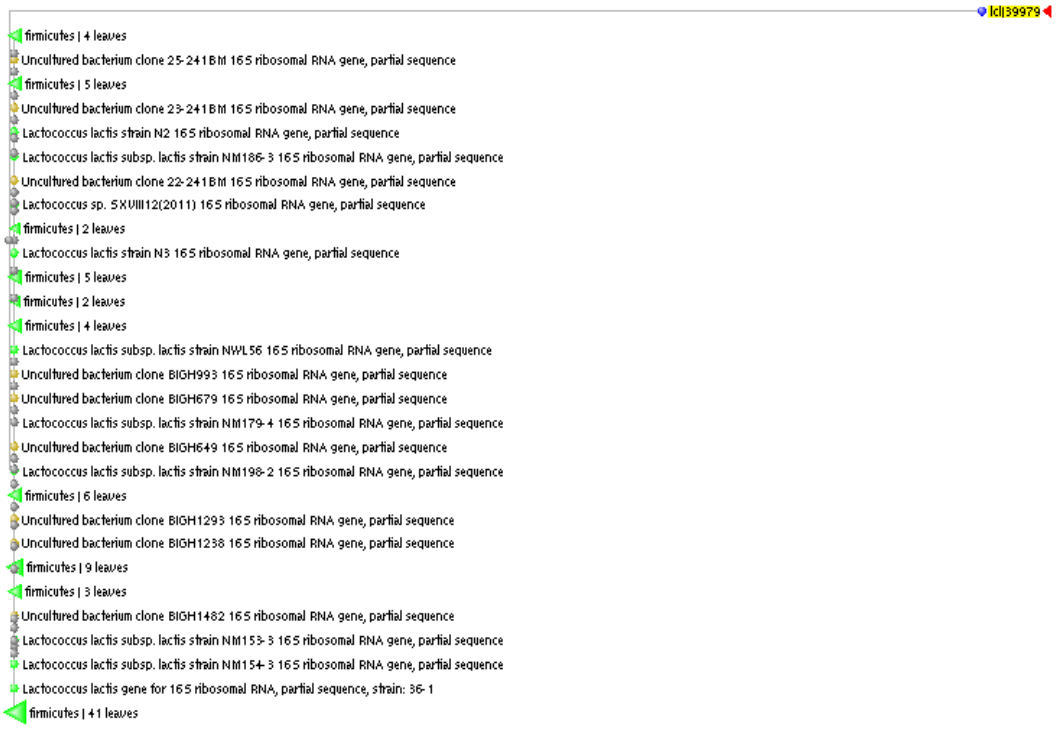

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Strand=Plus/Minus

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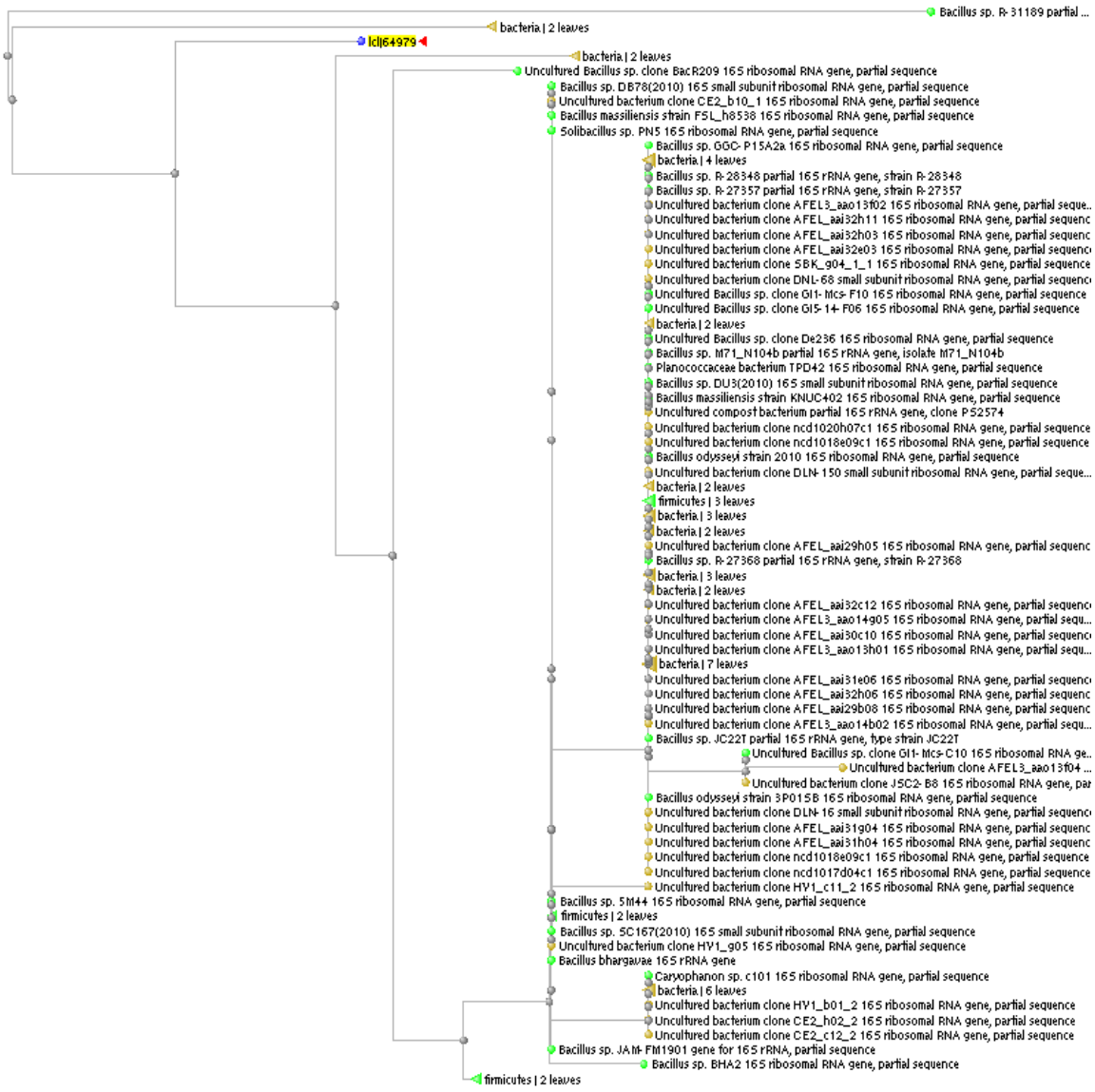

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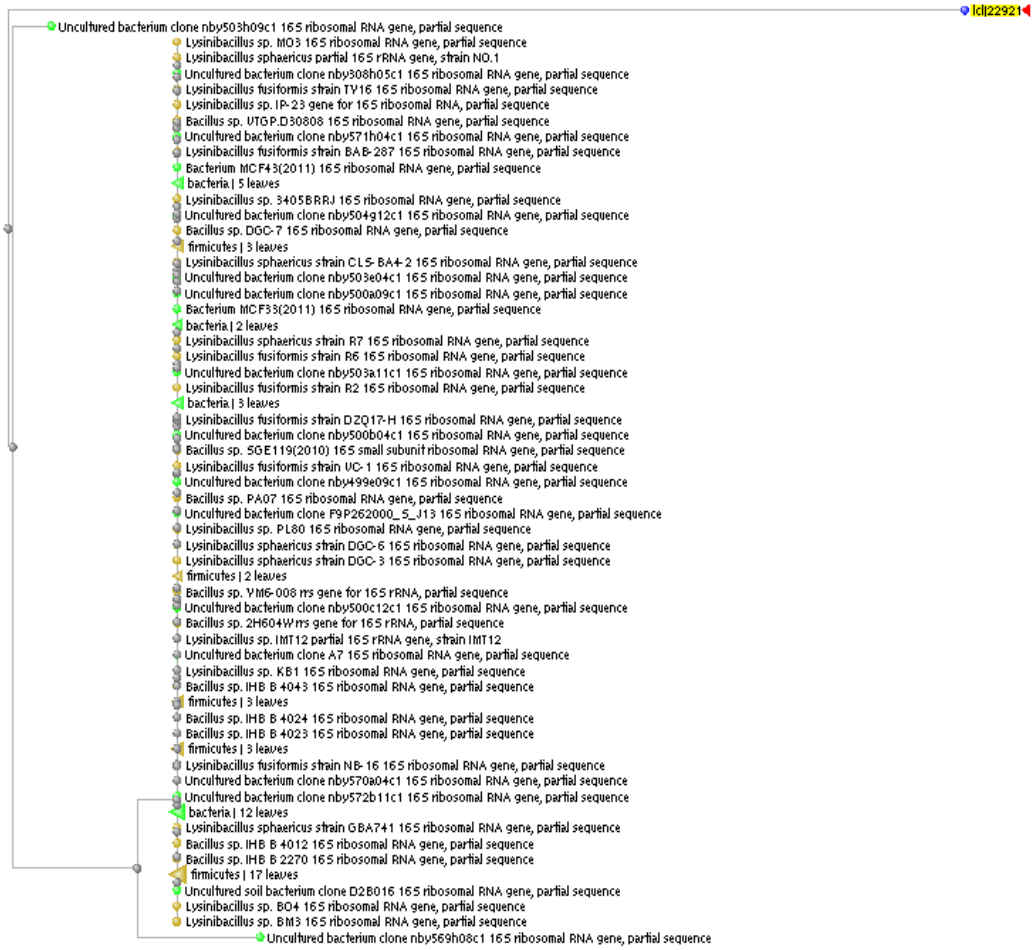

> |AB294174.1| *Alkalibacterium kapii* gene for 16S rRNA, partial sequence, strain: T171-1-1
Length=1490

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Identities = 329/332 (99%), Gaps = 2/332 (1%)
Strand=Plus/Minus

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Sbjct  1029  GCACCACCTGTCACTCTGTCCCCGAAGGGAAA  998
```

APPENDIX E: MANUFACTURER INSTRUCTIONS OF UV LAMPS

EL Series Ultraviolet Hand Lamps
User's Guide

Introduction

The EL Series of Ultraviolet Lamps offer a uniform and intense source of ultraviolet light (radiation). The lamps emit one of three UV wavelengths or white light in four watt, six watt and eight watt configurations for exposure/illumination of materials. All EL Series Lamp models can be used with the universal J138 Lamp Stand. The four watt models can be used with C-10E Cabinet for viewing materials in a darkroom environment. Eight watt lamps can be used with the C-65 Cabinet.

Important Safety Information

Caution: Shortwave and midrange UV radiation will cause damage to unprotected eyes and skin. Before operating any unit, be sure all personnel in the area are properly protected. UV Blocking Eyewear should be worn as well. UVP has a complete line of UV Blocking Eyewear: Spectacles, Goggles and Faceshield designed for this purpose.

Operation

Plug power cord into a properly grounded electrical outlet. The proper voltage of the lamp is found on the product information label.

A rocker switch is conveniently located on top of the unit to turn the lamp on or off. For models with multiple wavelengths, the rocker switch accommodates for the selection of wavelength or to shut the unit off.

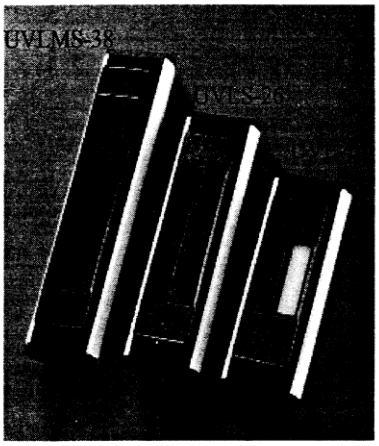
Each lamp comes with two UV tubes or one UV and one white light tubes; the UVLSM-38 is equpped with three tubes. Models with two tubes of the same wavelength operate with both tubes on at same time. Other models operate with only a single tube at one time.

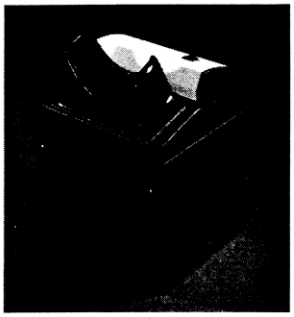
Specifications

Physical dimensions for four, six and eight watt lamps:

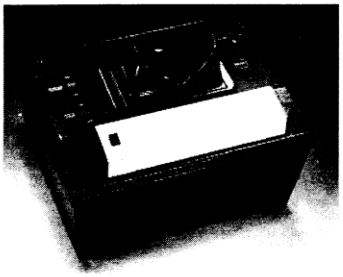
Four watt lamps:	9.8"L x 2.5"H x 3.8"W (249 x 64 x 97 mm)
Six watt lamps:	11.9"L x 2.5"H x 3.8"W (302 x 64 x 97 mm)
Eight watt lamps:	14.9"L x 2.5"H x 3.8"W (376 x 64 x 97 mm)

UVP
ULTRA-VIOLET PRODUCTS

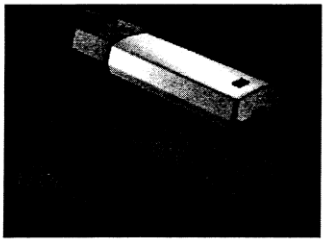




C-10E Cabinet with
four watt lamp



C-65 Cabinet with
eight watt lamp



Universal stand for all
EL Series Lamps

Four Watt Lamp Specifications

Model	Wavelength	Part Number	Nominal Volts/Hz/Amp
UVS-14	254nm/White Light	95-0266-01 95-0266-02 95-0266-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVL-14	365nm/White Light	95-0264-01 95-0264-02 95-0264-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVS-24	254nm	95-0269-01 95-0269-02 95-0269-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVL-24	365nm	95-0267-01 95-0267-02 95-0267-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVLS-24	365nm/254nm	95-0271-01 95-0271-02 95-0271-03	115/60/0.16 230/50/0.16 100/50-60/0.16

Six Watt Lamp Specifications

Model	Wavelength	Part Number	Nominal Volts/Hz/Amp
UVS-16	254nm/White Light	95-0274-01 95-0274-02 95-0274-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVL-16	365nm/White Light	95-0272-01 95-0272-02 95-0272-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVM-16	302nm/White Light	95-0273-01 95-0273-02 95-0273-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVS-26	254nm	95-0277-01 95-0277-02 95-0277-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVL-26	365nm	95-0275-01 95-0275-02 95-0275-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVM-26	302nm	95-0276-01 95-0276-02 95-0276-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVLS-26	365nm/254nm	95-0279-01 95-0279-02 95-0279-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVLM-26	365nm/302nm	95-0278-01 95-0278-02 95-0278-03	115/60/0.16 230/50/0.16 100/50-60/0.16

Eight Watt Lamp Specifications

Model	Wavelength	Part Number	Nominal Volts/Hz/Amp
UVS-18	254nm/White Light	95-0200-01 95-0200-02 95-0200-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVL-18	365nm/White Light	95-0198-01 95-0198-02 95-0198-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVM-18	302nm/White Light	95-0199-01 95-0199-02 95-0199-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVS-28	254nm	95-0249-01 95-0249-02 95-0249-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVL-28	365nm	95-0248-01 95-0248-02 95-0248-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVM-28	302nm	95-0250-01 95-0250-02 95-0250-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVLS-28	365nm/254nm	95-0201-01 95-0201-02 95-0201-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVLM-28	365nm/302nm	95-0251-01 95-0251-02 95-0251-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVLMS-38	365/302/254nm	95-0252-01 95-0252-02 95-0252-03	115/60/0.16 230/50/0.16 100/50-60/0.16

Replacement Tubes and Switches

Replacement Part	Part Number
Switch, On/Off	53-0134-01
Switch, On/Off/On	53-0135-01
Tube, 4 watt, 365nm	34-0005-01
Tube, 4 watt, 254nm	34-0066-01
Tube, 4 watt, white light	34-0003-01
Tube, 6 watt, 365nm	34-0034-01
Tube, 6 watt, 302nm	34-0044-01
Tube, 6 watt, 254nm	34-0015-01
Tube, 6 watt, white light	34-0063-01
Tube, 8 watt, 365nm	34-0006-01
Tube, 8 watt, 302nm	34-0042-01
Tube, 8 watt, 254nm	34-0007-01
Tube, 8 watt, white light	34-0056-01

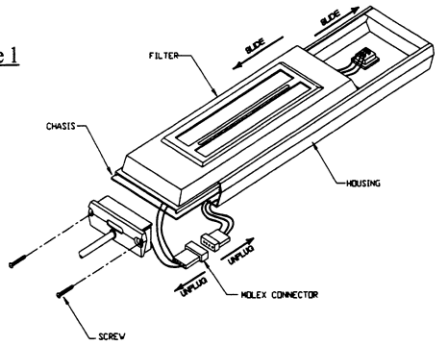
Accessories

Accessories	Part Number
J138 Lamp Stand	18-0063-01
C-65 Cabinet	95-0257-01
C-10E Cabinet	95-0072-08
Spectacles	98-0002-01
Goggles	98-0002-02
Faceshield	98-0002-04

Changing the Replacement Tubes

Always disconnect the lamp from the electrical power source prior to replacing tubes. Remove the two Phillips Head Screws located in the End Cap. Grasp the extruded housing and slide the chassis and filter out together away from the switch. Disconnect the interior Molex Connector (see Figure 1) and slide chassis and filter out of the housing completely. Once out of the extrusion, the chassis and filter will come apart. Carefully grasp the tube ends and twist one quarter turn until it works free (see Figure 2). Insert the new tube, giving it a twist to lock in place. Reassemble the lamp. Be careful not to pinch wires during assembly.

Figure 1



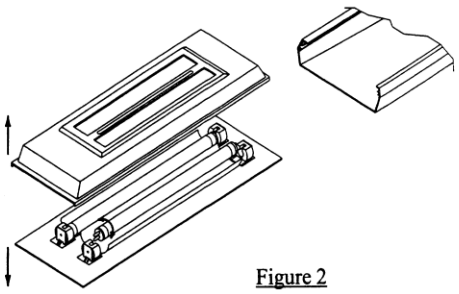


Figure 2

Cleaning the EL Series Lamps

The painted surfaces and filter areas of the lamp should cleaned with a damp sponge or cloth towel and mild soap. Never use abrasive cleaners, solvent based cleaners or scouring pads.

ALWAYS DISCONECT THE LAMP FROM THE ELECTRICAL POWER PRIOR TO CLEANING.

Maintenance/Repair/Technical Assistance

UVP offers technical support for all of its products. If you have any questions about the product's use, operation or repair, call or fax UVP Customer Service at the following offices:

In the US: Tel: (909)946-3197 or toll free (800)452-6788; Fax (909)946-3597; E-Mail uvp@uvp.com
Europe/UK: Tel: +44(0)1223-420022; Fax: +44(0)1223-420561; E-Mail: uvp@dial.pipex.com

A **Returned Goods Authorization (RGA)** number must be obtained from UVP Customer Service before returning any products.

Warranty

UVP, Inc. warrants its EL Series Lamps to be free of defects in materials and workmanship for a period of one (1) year from the date of purchase. Tubes and filters are warranted for a period of 90 days. If equipment failure or malfunction occurs durng the warranty period, UVP shall examine the inoperative equipment and have the option of repairing or replacing any part(s) which, in the judgement of UVP, were originally defective or became so under conditions of normal usage and service.

No warranty shall apply to this instrument, or part thereof, that has been subject to accident negligence, alteration, abuse or misuse by the end user. Moreover, UVP makes no warranties whatsoever with respect to parts not supplied by UVP or that have been installed, used and/or serviced other than in strict compliance with the instruments appearing in this manual.

In no event shall UVP be responsible to the end user for any incidental or consequential damges, whether foreseeable or not, including but not limited to property damage, inability to use equipment, lost business, lost profits, or inconvenience arising out of or connected with the use of instruments produced by UVP. Nor is UVP liable or responsible for any personal injuries occurring as a result of the use, installation and/or servicing of equipment.

This warranty does not supersede any statutory rights that may be available in certain countries.

UVP
ULTRA-VIOLET PRODUCTS

Internet: http://www.uvp.com

Corporate Headquarters: UVP, Inc.
2066 W. 11th Street, Upland, CA 91786 USA
Tel: (909)946-3197 or toll free in US/Canada (800)452-6788
Fax: (909)946-3597 E-Mail: uvp@uvp.com

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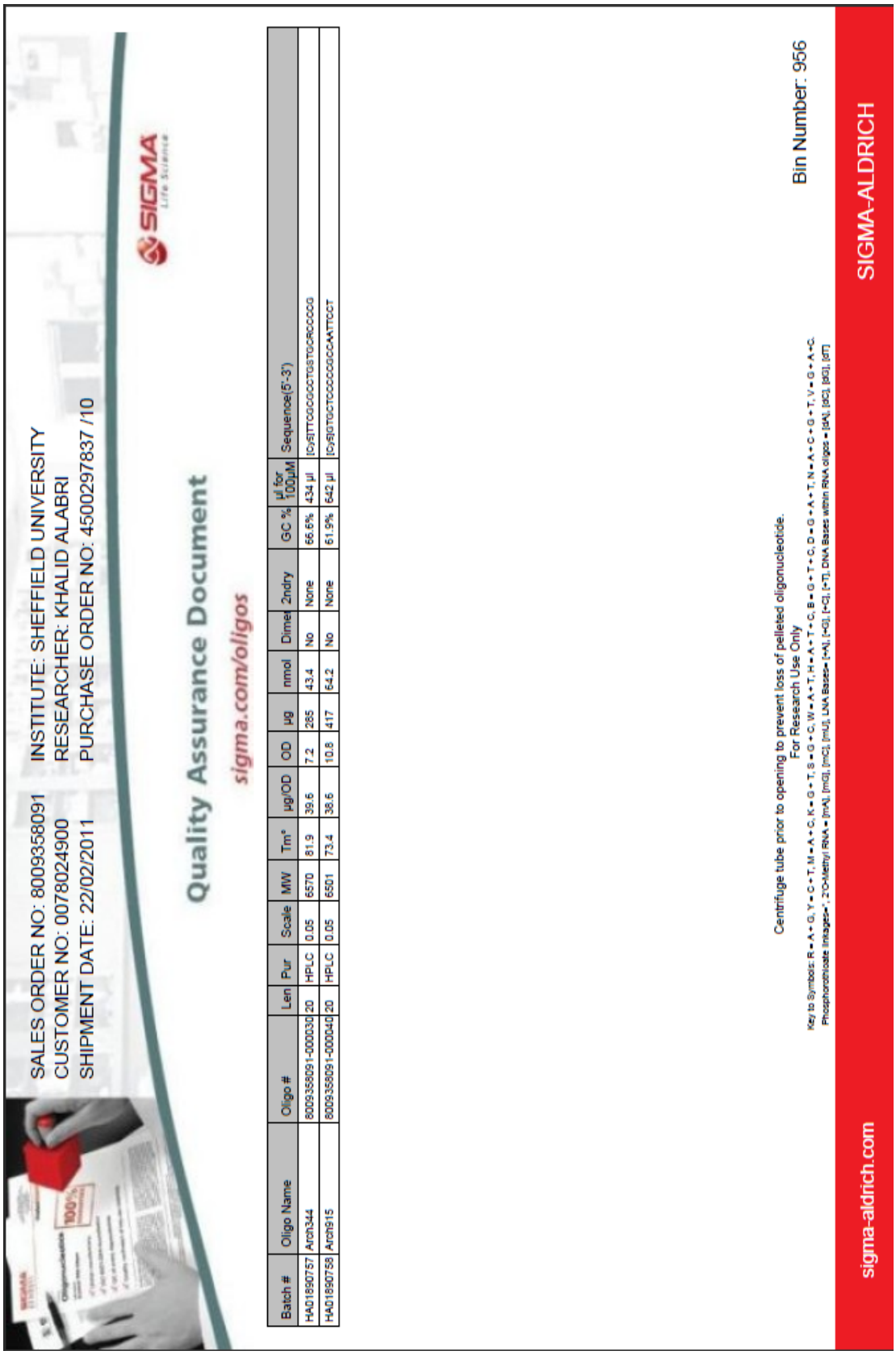
E-mail: uvpuk@uvp.com

81-0117-01 Rev. A

APPENDIX F: DETAILS OF FLURESCENT PROBES SYNTHESIS



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CUSTOMER NO: 0078024900
SHIPMENT DATE: 22/02/2011

INSTITUTE: SHEFFIELD UNIVERSITY
RESEARCHER: KHALID ALABRI
PURCHASE ORDER NO: 4500297837 /10

SIGMA Life Science

Quality Assurance Document

sigma.com/oligos

Batch #	Oligo Name	Oligo #	Len	Pur	Scale	MW	Tm°	µg/OD	OD	µg	nmol	Dimer	2ndry	GC %	µl for 100µM	Sequence(5'-3')
HAD18990757	Arch344	8009358091-000030	20	HPLC	0.05	6570	81.9	39.6	7.2	285	43.4	No	None	65.6%	434 µl	[Cy5]TTCGCGCCTCGTGCACCCCG
HAD18990758	Arch915	8009358091-000040	20	HPLC	0.05	6501	73.4	38.6	10.8	417	64.2	No	None	61.9%	642 µl	[Cy5]GTGCTCCCCCGCCCAATTCCT

Centrifuge tube prior to opening to prevent loss of pelleted oligonucleotide.
For Research Use Only

Bin Number: 956

Key to Symbols: R = A + G, Y = C + T, M = A + C, K = G + T, S = G + C, W = A + T, H = A + T + C, B = G + T + C, D = G + A + T, N = A + C + G + T, V = G + A + C.
Phosphorothioate linkage = *; 2'O-Methyl RNA = [mA], [mG], [mC], [mU], LNA Bases= [+A], [+G], [+C], [+T], DNA Bases within RNA oligos = [dA], [dC], [dG], [dT]

sigma-aldrich.com

SIGMA-ALDRICH

SALES ORDER NO: 8009690569
CUSTOMER NO: 0024968206
SHIPMENT DATE: 21/02/2011

INSTITUTE: SHEFFIELD UNIVERSITY
RESEARCHER: KHALID ALABRI
PURCHASE ORDER NO: XK0/4500309129

SIGMA Life Science

Quality Assurance Document

sigma.com/oligos

Batch #	Oligo Name	Oligo #	Len	Pur	Scale	MW	Tm°	µg/OD	OD	µg	nmol	Dimer	2ndry	GC %	µl for 100µM	Sequence(5'-3')
HA02068005	Arch1060	8009690569-000010	18	HPLC	0.05	5912	66.1	38.4	6.2	238	40.3	No	None	63.1%	403 µl	[Cy5]GGCCATGCACCWCCTCTC

Centrifuge tube prior to opening to prevent loss of pelleted oligonucleotide.
For Research Use Only

Key to Symbols: R = A + G, Y = C + T, M = A + C, K = G + T, S = G + C, W = A + T, H = A + T + C, B = G + T + C, D = G + A + T, N = A + C + G + T, V = G + A + C.
Phosphorothioate linkages=*, 2'O-Methyl RNA = [mA], [mG], [mC], [mU], LNA Bases= [+A], [+G], [+C], [+T], DNA Bases within RNA oligos = [dA], [dC], [dG], [dT]

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Batch #	Oligo Name	Oligo #	Len	Pur	Scale	MW	Tm°	µg/OD	OD	µg	nmol	Dimer	2ndry	GC %	µl for 100µM	Sequence(5'-3')
EV00055846	Eub338I	8009358091-000010	19	HPLC	0.05	6199	64.4	37.7	1.83	69.3	11.1	No	Strong	63.1%	111 µl	[A488]GCTGCCTCCCGTAGGAGT
EV00055847	Eub338II/III	8009358091-000020	19	HPLC	0.05	6204	69.0	37.9	1.35	51.4	8.3	No	Strong	63.1%	83 µl	[A488]GCWGCCACCCGTAGGTGT

Centrifuge tube prior to opening to prevent loss of pelleted oligonucleotide.
For Research Use Only

Key to Symbols: R = A + G, Y = C + T, M = A + C, K = G + T, S = G + C, W = A + T, H = A + T + C, B = G + T + C, D = G + A + T, N = A + C + G + T, V = G + A +C.
Phosphorothioate linkages=*, 2'O-Methyl RNA = [mA], [mG], [mC], [mU], LNA Bases= [+A], [+G], [+C], [+T], DNA Bases within RNA oligos = [dA], [dC], [dG], [dT]

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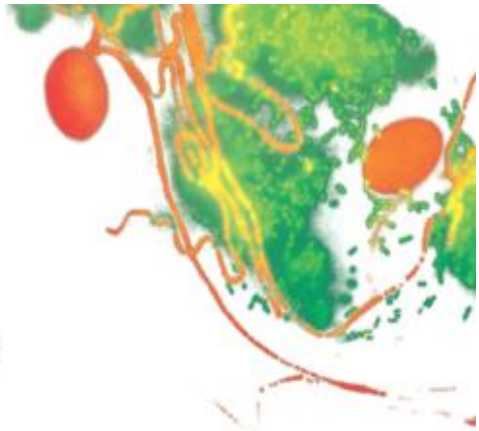
APPENDIX G: QIAGEN BUFFERS AND CULTURING MEDIA

Buffer	Composition	Storage
Buffer B1 (Bacterial Lysis Buffer)	50 mM Tris·Cl, pH 8.0; 50 mM EDTA, pH 8.0; 0.5% Tween®-20; 0.5% Triton X-100	2–8°C or room temp.
Buffer B2* (Bacterial Lysis Buffer)	3 M guanidine HCl; 20% Tween-20	2–8°C or room temp.
Buffer C1 (Cell Lysis Buffer)	1.28 M sucrose; 40 mM Tris·Cl, pH 7.5; 20 mM $MgCl_2$; 4% Triton X-100	2–8°C
Buffer G2* (Digestion Buffer)	800 mM guanidine HCl; 30 mM Tris·Cl, pH 8.0; 30 mM EDTA, pH 8.0; 5% Tween-20; 0.5% Triton X-100	2–8°C or room temp.
Buffer Y1 (Yeast Lysis Buffer)	1 M sorbitol; 100 mM EDTA; 14 mM β-mercaptoethanol	2–8°C
Buffer QBT (Equilibration Buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol, 0.15% Triton X-100	2–8°C or room temp.
Buffer QC (Wash Buffer)	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol	2–8°C or room temp.
Buffer QF(Elution Buffer)	1.25 M NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol	2–8°C or room temp.
PBS	Standard phosphate-buffered saline, pH 7.4	room temp.
TE	10 mM Tris·Cl, pH 8.0; 1 mM EDTA, pH 8.0	room temp.
Culture media		
LB (Bacterial culture medium)	10 g/liter tryptone; 5 g/liter yeast extract; 1 g/liter NaCl; pH 7.0	room temp.
YPD (Yeast culture medium)	10 g/liter yeast extract; 20 g/liter peptone; 20 g/liter dextrose	room temp.

** Not compatible with disinfection reagents containing bleach. Contains guanidine HCl, which is an irritant. Take appropriate safety measures, and wear gloves when handling.*

APPENDIX H: LIVE/DEAD BacLight Bacterial Viability Kits



Cell Viability

LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits

Sensitive, single-step, fluorescence-based assays

- → Complete in 15 minutes with no wash steps
- → Compatible with fluorescence-based microscopes and microplate readers, flow cytometers, and fluorometers
- → Discriminate as little as 1–10% live or dead cells in a mixed population

Rapid, reliable assessment of bacterial viability

LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits provide sensitive, single-step, fluorescence-based assays for bacterial cell viability.[1-4] These assays can be completed in minutes, require no wash steps, and can be applied to bacteria in suspension or trapped on filters. The *Bac*Light™ kits are well suited for use with a fluorescence microscope or in quantitative assays employing a fluorescence microplate reader, flow cytometer, or fluorometer.

The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits employ two nucleic acid stains—green-fluorescent SYTO® 9 stain and red-fluorescent propidium iodide stain. These stains differ in their ability to penetrate healthy bacterial cells. When used alone, SYTO® 9 stain labels both live and dead bacteria. In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO® 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red (Figures 1 and 2). Live and dead bacteria can be viewed

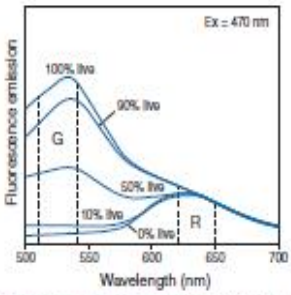


Figure 1—Viability analysis of bacterial suspensions. The viability of different proportions of live and isopropanol-killed *Escherichia coli* was assessed using the reagents in the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits. Live bacteria are stained fluorescent green (G) by SYTO® 9 stain, and dead bacteria are stained fluorescent red (R) by propidium iodide. Bacterial suspensions simultaneously incubated in the two stains and then excited at 470 nm exhibit a fluorescence spectral shift from green to red as the percentage of live bacteria in the sample is decreased.

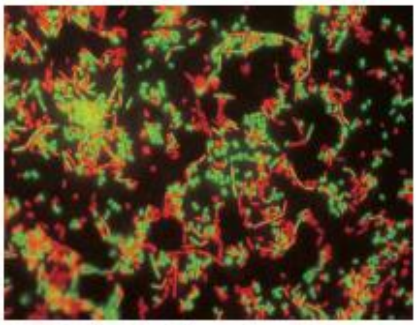


Figure 2—Viability of a mixture of *Micrococcus luteus* and *Bacillus cereus* assessed using reagents in the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits.

invitrogen™

separately or simultaneously by fluorescence microscopy with suitable optical filter sets. Mounting oil is supplied for viewing bacteria on filter membranes. The *BacLight*™ assay has been used on many gram-negative and gram-positive bacteria, mycoplasmas, yeasts, biofilms, and protozoa.

Four kits for greater flexibility

LIVE/DEAD® *BacLight*™ Bacterial Viability Kits contain the SYTO® 9 and propidium iodide nucleic acid stains supplied either premixed at two different proportions in solution (L7007) or as separate vials of the two stains in solution (L7012). For added convenience, a third kit format (L13152) contains the same two nucleic acid stains dried and premeasured into separate polyethylene transfer pipet applicator sets. This kit does not require organic solvents or refrigerated storage. A fourth kit format (L34856) is specially designed for flow cytometry use, and allows researchers to reliably distinguish and quantitate live and dead bacteria even in a mixed population containing a range of bacterial types. In addition to SYTO® 9 stain and propidium iodide, this kit includes a calibrated suspension of microspheres for accurate sample volume measurements.

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Ordering information

Product	Quantity	Cat. no.
LIVE/DEAD® *BacLight*™ Bacterial Viability Kit, for microscopy	1,000 assays	L7007
LIVE/DEAD® *BacLight*™ Bacterial Viability Kit, for microscopy and quantitative assays	1,000 assays	L7012
LIVE/DEAD® *BacLight*™ Bacterial Viability and Counting Kit, for flow cytometry	100 assays	L34856
LIVE/DEAD® *BacLight*™ Bacterial Viability Kit, 10 applicator sets	500 assays	L13152
Related products		
LIVE/DEAD® *FungaLight*™ Yeast Viability Kit, for flow cytometry	1 kit	L34952
LIVE *BacLight*™ Bacterial Gram Stain Kit, for microscopy and quantitative assays	1,000 assays	L7005
SYTO® 9 Green Fluorescent Nucleic Acid Stain	100 µl	S34854

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SUGGESTIONS FOR FUTURE WORK

1. It would be useful to use some of the novel culture-independent techniques which are now available in future work relating the isolation of extremophiles from non-extreme environments Further investigation into the physiology of extremophilic microorganisms found in non-extreme environment may also provide a better understanding of the microbial interactions and the essential roles which different species play in the environment.
2. All attempts made here to isolate and culture microorganisms were carried out under aerobic conditions. It would be interesting however, to use micro-aerophilic or even anaerobic media in the future for the isolation of extremophiles for example do anaerobic, salt tolerant bacteria exist in non-extreme soils?
3. It would be interesting to conduct work on balancing the cytoplasmic pH of alkalitolerant and alkaliphilic isolates in order to answer the questions raised regarding how these isolates can grow in such an extreme environment and to determine if there are any differences in physiological and structural aspects between alkaliphilic, alkalitolerant and neutrophilic microorganisms?
4. Attempts might be made to isolate bacteria tolerant to UV-C from different environment samples from both the Earth and the different areas of the stratosphere to further characterize the sources of selection pressure on UV-C resistant microorganisms, possibly in relation to the origin of life.
5. Viable *Mycoplasma* species could be detected in the environment using the developed reverse transcription-polymerase chain reaction (RT-PCR) assay, because of its short half-life, which indicates where cells are viable or were recently viable.

CHAPTER 9

GENERAL DISCUSSION

Few studies have been reported on the ability of halophiles to grow in non-saline environments. Usami *et al*. (2007) studied halophiles from a non-saline soil in Japan and isolated two strains of halophilic bacteria, designated $BM2^T$ and HN2. The $BM2^T$ cells, which were shown to be Gram-positive, rod-shaped, aerobes grew at 5 - 25% (w/v) NaCl, with an optimum at 10-15%, (w/v) NaCl at 20–50 °C and pH of 7-10. Molecular method of 16S rRNA gene sequence was used to analyze their phylogeny and it was found that strain $BM2^T$ showed 98% sequence similarity related to *Alkalibacillus haloalkaliphilus* DSM 5271^T.

Some 96% of the non-saline soil samples were found in this study to contain halophilic bacteria capable of growing at 1.0M, while 54% grew at 2.0M NaCl and 16% were able to grow at 3.0M NaCl; only 6% of the isolates however, grew at 4.0M NaCl. Echigo *et al.*, (2005) reported isolating moderate halophiles from non-saline soils from Tokyo, Japan, using agar plates containing 20% NaCl (3.4 M). In our study, some 12% of isolates from saline environmental samples yielded microorganisms capable of growing at 4.0M NaCl. Whereas, all samples produced growth at salt concentrations up to 2.0M and 62% of them produced bacteria capable of growing at 3.0M NaCl. None of the air samples or single rain sample yielded any salt tolerant isolates. Two bacteria capable of growing at 2.0M NaCl only, were however, isolated from the hailstone sample. The number of colony forming units isolated from both non-saline and saline soil decreased with increasing concentrations of NaCl added to plates containing Plate Count Agar.

The majority of the isolates obtained in this study were representatives of previously isolated organisms. The isolates obtained from non-saline soils were

Bacillus species, and analysis of partial sequences of their 16S rRNA genes showed that some of them exhibited similarities higher than 99% with those of *Bacillus subtillis, Paenibacillus ehimensis* and 100% with *Bacillus weihenstephanensis*. No bacteria other than species of the genus *Bacillus* were isolated from non-saline samples. These isolates have not yet been reported as halotolerant or halophilic and there is no published work on their growth at different sodium chloride concentration.

Echigo *et al*. (2005) have demonstrated that halophilic bacteria capable of growing in the presence of 20% NaCl inhabit non-saline environments such as ordinary garden soils, fields and roadways in an area surrounding Tokyo, Japan. Analyses of partial 16S rRNA gene sequences of 176 isolates showed that they were halophiles belonging mainly to genera of the family *Bacillaceae*, *Bacillus* (11 isolates), *Filobacillus* (19 isolates), *Gracilibacillus* (6 isolates), *Halobacillus* (102 isolates), *Lentibacillus* (1 isolate), *Paraliobacillus* (5 isolates) and *Virgibacillus* (17 isolates). Sequences of 15 isolates showed similarities less than 92%, suggesting that they may represent novel taxa within the family Bacillaceae.

An isolate was obtained here from hailstones on media containing 2M NaCl but then able to grow at 4M NaCl and was identified as *Staphylococcus sciuri sciuri* (99.94 similaritiy). *Bacillus pumilus* isolated from stratosphere grow up to 3M NaCl; these isolates are not well known as halophilic bacteria.

It is generally believed that halophiles able to grow in media containing more than 20% (3.4M) are restricted to saline environments, and no reports have been published on the isolation of halophiles from stratospheric samples including rain and hailstone.

The data presented here show that bacilli are dominant among halophilic bacteria in non-saline environments; halophilic bacteria, in general, are however, not restricted to this genus.

The two archaeal isolates obtained from desert sands and desert varnish collected from Oman. They were mixed with bacterial colonies and it was not possible to separate them even with the use of antibiotics and as a result it was impossible to obtain valid sequence data for the identification of these isolates.

Echigo *et al*. (2005) used agar plates containing 20% NaCl and Medium No. 168 which is recommended by the JCM (Japan Collection of Microorganisms) for the cultivation of haloarchaea. All of the pink to brown colonies that considered to be presumptive haloarchaea were picked up, but they turned out to be members of the family Bacillaceae. In order to confirm that haloarchaea were not present in the soil samples, at least to the limits of detection, soil samples were spread on agar plates containing 20% NaCl, (pH 7.0 and 9.0) and supplemented with 30 $\mu g.ml^{-1}$ ampicillin. Generally, numbers of colonies decreased to less than one tenth of those obtained on plates without ampicillin. All of them yielded amplification bands only with bacterial primers, showing that no haloarchaea but only halophilic bacteria were present.

Another study, conducted by Savage *et al*. (2008), reported the occurrences of halophilic archaeon, from a Zodletone spring in south-western Oklahoma, United States, which was characterized by its low-salt and rich-sulphide content. A novel strain $BZ256^T$ of halophilic archaeon was isolated and the cells were shown to be non-flagellated cocci, non-motile and forming *Sarcin*a-like clusters. This strain grew at 1.3 to 4.3M salt concentrations and optimally at almost 3.5M NaCl, a minimum of 1 mM Mg^{+2} was necessary for growth. Growth was optimal at 25–50 °C and pH range from 5 to 9; strain $BZ256^T$ was related to *Halogeometricum borinquense*.Based on phenotypic and phylogenetic analyses, strain $BZ256^T$ was characterized as a novel species within the genera of Halobacteriaceae, for which the *Halosarcina pallida* gen. nov., sp. nov. has been proposed**.**

All previously mentioned isolates were obtained from non-saline environments which would be expected to act as a source of halotolerant but not halophilic bacteria. On the other hand, a strain of bacteria isolated from saline soil, showed strict obligate halophilic characteristics as it not capable of growing, or even surviving without salt; optimum growth occurring at 1.0M and 2.0M NaCl as well as 4M salt. This bacterium was first isolated from hypersaline sediments of the Great Salt Lake in Utah. It grows in media containing 0.5 to 30% (w/v) salt, and optimum growth occurs in medium containing around 10% salt (Spring *et al*., 1996).

Part of the work presented in this Thesis focused on the question of how halophilic and halotolerant bacteria and archaea, isolated from non-saline environments, can survive extreme saline conditions. In most isolates, the results for intracellular glycine betaine levels above 1.0M NaCl showed large variability, a general trend towards an increase in glycine betaine level with increasing salt concentration was observed. NMR analyses of compatible solutes in all halotolerant isolates showed the accumulation of glycine betaine is important for osmotic adaptation of this isolate when growing in presence of high NaCl concentrations (except when grown in M9 medium). The fact that betaine was the main osmoprotectant present in most isolates depended on the composition of the medium (i.e. LB medium contains yeast extract) showing that the presence of betaine in the cells was definitely not synthesized by the cells (e. g. *de novo*) and it appears that betaine is absolutely required as a organic osmolytes for these isolates when grown under stressed conditions. It is likely that only extreme halotolerant cyanobacteria accumulate glycine betaine or glutamate (Page-Sharp *et al.*, 1999). It has been reported that glycine betaine suppresses *de novo* ectoine synthesis completely or partially, depending on the concentration of NaCl which is present in the growth media (Calnovas *et al*., 1996). Recently, Kurz *et al.* (2010) also found that betaine suppresses the production of many of these compounds including ectoine,

thereby suggesting that the production of the ectoine is generally inhibited during the period when glycine betaine (choline) uptake is possible.

The compatible solutes reported in this study mostly correspond with those reported in the literature and the results show that the halophilic isolates usually accumulated amino acids and polyols and their derivatives such as, glycerol, ectoine, glutamate, proline and lysine, as well as quaternary amines such as glycine betaine which have no net charge at physiological pH and do not disturb metabolic processes. This study also demonstrated that glycine betaine and glutamate were the main compatible solutes which were accumulated at high concentration in halobacteria. Many halophilic and halotolerant bacteria accumulate glycine betaine and ectoine, while haloarchaea accumulate glycine betaine and other amino acids (Galinsk, 1993; Demirjian *et al.*, 2001). On the other hand, in this study, potassium accumulation was not found in halotolerant and halophilic isolates, either when exposed to adaptative salt concentrations or following osmotic shock.

True obligately halophilic organisms are known to require high intracellular potassium concentrations for optimal growth and thus for cellular function. However, Kokoeva *et al.* (2002) reported that halobacterial cells accumulate glycine betaine and at the same time reduce their glutamate levels. Thus, a requirement for high intracellular concentrations of potassium ions does not seem to be necessary for maintaining halophilic enzyme stability

Halophilic archaea are presumably maintaining the osmotic equilibrium between their cytoplasm and the hypersaline surroundings by accumulating high salt concentrations. The total cellular K^+ concentration increased for, presumptive archaea (DSRT3), when the cells were grown with increasing NaCl concentration (w/v) when the cells were subjected to osmotic shock or when trained to tolerate a range of NaCl concentrations.

This study has shown that most halotolerant and halophilic bacteria, HL1, STRA2 and HSO, do not utilise the "salt-in-cytoplasm" strategy. This strategy has long been assumed to be used by most halophilic archaea (such as the DSRT3 isolate in this study), and certain halophilic eubacteria. The findings of this study show that halophilic isolates accumulate glycine betaine, proline and glutamate instead of potassium ions. These isolates also accumulated different organic osmolytes when exposed to different salinities.

These findings agree with other reports showing that haloarcheon *Halobacterium salinarium*s accumulates K^+ as a response to increases in external osmotic stress (Vreeland, 1987; Brown, 1990). *Halobacterium salinarium* cells were only analysed when grown in 15%, 20%, 25 and 30% NaCl concentrations (w/v) as they failed to grow in media with NaCl concentration less than 15% (w/v). The accumulation of intracellular potassium ions is an osmo-adaptation strategy which, to date, has mainly been found to be characteristic for the halophilic archaea (Galinski, 1995; Martin *et al.*, 1999). This view has been further extended by the finding of a specific KCl uptake system in *H. salinarum*, including a potassium ion driven uniporter and the unique light-driven chloride pump halorhodopsin (Kolbe *et al.*, 2000; Ng *et al.*, 2000).

Studies were also conducted in the work reported in this thesis on the screening and isolation of alkaliphilic and alkalitolerant bacteria and archaea which are also able to grow in acidic media. The following results were obtained: Six alkaliphilic and alkalitolerant bacteria were isolated from the alkaline samples and two of them were presumptively acid-tolerant. All of the isolates were capable of growth on high pH medium as well as at neutral pH. A very narrow range of growth media were used in attempts to isolate bacterial and archaeal microorganisms, therefore, the proportion of organisms present in samples which could grow using the media employed was probably significantly reduced compared to the total microbial population. Since all

attempts to culture these microorganisms were made using aerobic conditions micro-aerophilic organism and anaerobes were obviously excluded. This is in contrast to the findings of Grant and Horikoshi (1989) who showed that Horikoshi's medium plates in its original form, with a glucose carbon source, yielded high numbers of alkaliphilic bacteria including *Bacillus* sp. and actinomycetes.

The majority of the alkaliphiles were present on the plates, were Gram positive rods and a number of these possessed endospores, showing them to be *Bacillus* species. *Bacillus* species have also been reported to be the most commonly isolated alkaliphilic bacteria (Grant and Horikoshi, 1989; Horikoshi, 1991; Jones *et al.*, 1994).

All of the organisms isolated from different Redcar lagoon samples, with pH ranging from 8.6 to 12.0, were shown to be different genera and species except two samples; the lake sample with a pH of 12 and the soil sample with pH 8.6. Although these two samples were different in pH value they gave the same isolate, namely a bacterium which was 100% similar to *Lactococcus lactis*. Only *L. lactis* that has been isolated from the lake sample with a pH of 12 and the other one which was isolated strain from Redcar-soil with a pH of 12 (95% similar to *Bacillus massiliensis*) were the only bacteria able to grow in medium at pH value of 4.5. All of the other isolates were able to grow only at pH 7.0 and above. Two alkaliphilic moderate halophiles have also been isolated from non-saline soils in an area surrounding Tokyo, Japan. *Bacillus oshimensis* was isolated from a soil sample obtained in Hokkaido, (Yumoto *et al.*, 2005a), and *Oceanobacillus oncorhynchi* was isolated from the skin of a freshwater rainbow trout (Yumoto *et al.*, 2005b). Both strains grew in medium containing 0–20 % (w/v) NaCl, and optimal growth occurred at pH 9–10.

The work conducted here focused on the question-how do presumptive alkalitolerant and alkaliphiles survive in such alkaline as well as low pH conditions?-produced the following results: Three alkaliphilic isolates were selected for the

characterization of the osmo-adaptation mechanisms (1) RS-12 (*Alkalibacterium kapii*; grows at pH range from 7 to 12). *A. kapii* was proposed as new species by Ishikawa *et al.* (2009). Cells are Gram-positive, non-sporulating, straight rods and the optimum pH for their growth is 8.5–9.0, with a range of 6.0 to 10.0. Our findings show that the optimum pH was above neutral (Figure 4.5) findings which correspond with the findings of Ishikawa *et al* found. (2) RS12-4 *(Bacillus massiliensis*; grows at pH range from 4.5 to 12) which was isolated from Redcar-Soil with pH value of 12. Glazunova *et al.,* (2006) isolated *B. massiliensis* from a sample of cerebrospinal fluid and it has a temperature range for growth is 25–45^{o}C, but not a published pH growth optimum. Here, we found that it was capable of growing at pH ranges of 4.5 and 10.0; optimum growth occurs at pH 10.0 (Figure 4.9). (3) RR12-4 *(Lactococcus lactis*; grows at pH range from 4.5 to 12) which were isolated from Redcar-root with pH value of 12. *L. lactis* cells are reported to grow optimally at pH 6.0 (Serna Cock and Rodríguez de Stouvenel, 2006); no published work is available on its ability to grow at high pH. Here, we report its ability to grow over at pH range of 4.5 and 10.0; with an optimum at pH 7.0 (Figure 4.10). The mechanisms of compatible solute accumulation reported in this study generally agrees with the data reported elsewhere, namely that low-salt tolerant alkaliphiles (Zhilina *et al*., 1997; Pikuta *et al*., 1998; Pikuta *et al*., 2003; Sorokin *et al*., 2005; Zhilina *et al*., 2005) and also halotolerant and halophilic isolates (reported above) usually accumulate amino acids and polyols and their derivatives such as, glycerol, ectoine as well as quaternary amines such as glycine betaine.

A study was made here of the occurrence of ultraviolet-C tolerant bacteria in terrestrial environments using phylogenetic analysis of the 16S rRNA gene sequences. The following results were obtained:

The experimental isolation approach used in this study has the advantage that the bacteria were exposed to UV while they were still in their natural oligtrophic growth

state, so that their normal physiology was not altered by growth on high nutrient media, before exposure to UV.

The desert soil (sampled near Nizwa city, Oman) was the only soil sample to yield a UV-C resistant isolate and this was shown to be the spore forming bacterium, *Paenibacillus ehimensis* the other UV-C resistant organism were archaea isolated from desert sand (Waheeba sands, Oman) and desert varnish (sampled near Ashkhara city, Oman); all of these locations can be considered to be hyper-arid environments. The findings of this study thus show that UV-C resistant bacteria can be isolated from a narrow range of environments on the Earth studied here. Kuhlman *et al*. (2005) similarly reported the isolation of UV-C tolerant bacteria from a desert varnish. No UV-C resistant bacteria were isolated from the wide range of soils sampled from the UK, all of which contain large numbers of bacteria (ranging from $1x10^5$ to $1x10^8$, (as determined by dilution plating on Plate Count Agar). The soil suspension exposed to UV-C contained a maximum of $1x10^6$ bacteria; no isolated members of this population were resistant to UV-C. However, from one of the three UK hailstone samples, a single UV-C resistant bacterium (*Staphylococcus sciuri sciuri*) was also isolated. Additionally, two UV-C resistant isolates were obtained from Sheffield rain water; these were independently identified as isolates *Exiguobacterium sp* and *Bacillus pumilus*. Our null hypothesis stated that such UV-C resistant bacteria which are isolated from Earth environments must have originated from space or the stratosphere, because UV-C is not known to reach the surface of the Earth, these are the only available regions where a selective pressure would produce UV-C resistant bacteria. The most resistant isolate was obtained from the stratosphere which survived 60 min of UV-C exposure (Table 6.1). The new domain Archaea isolate also showed the ability to resist UV-C in this case from 15 to 30 min. A single UV-C resistant strain was isolated from the stratosphere sample following exposure to UV-C; this was identified a *B. pumilus*.

It is noteworthy that three of the four UV-C resistant bacteria were isolated from environments associated with the atmosphere i.e. rain, hail and the stratosphere. *Bacillus pumilus* has been found to be highly resistant to UV in clean rooms (Link *et al.*, 2004, La Duc *et al*., 2007) and also in space (Newcombe *et al*, 2005) in addition to the stratosphere (Shivaji *et al*., 2006). The isolation of a species of UV-C resistant *Staphylococcus* that is resistant to UV-C might at first sight appear strange, since this property is not generally associated with members of this genus. However, it is noteworthy that Staphylococci have been isolated from the stratosphere (Wainwright *et al*, 2003). The existence of UV-C resistant isolates which are non-spore forming bacteria is confirmed by the isolation of a species of *Exiguobacterium*. La Duc *et al.* (2007) isolated members of this genus from extreme clean rooms exposed to UV-C. The spore forming *Paenobacillus* isolate, however, is more readily acceptable as being UV-C resistant and its closeness to Bacilli is emphasized by the fact that *Paenobacillus ehimensis* was initially referred to as *Bacillus ehimensis.*

No UV-C resistant bacteria were obtained from a range of seawater samples, or from the hypersaline soil or from the building surface sample from Oman.

The resistant isolates were exposed to UV-C and the number of organism before and after exposure were determined by plate counting. Non-irradiated samples contained bacterial concentrations ranging from 3.7×10^5 to 8.1×10^5 CFU/ml, while the number of bacteria following radiation was 1.1×10^5 to 7.8×10^5 CFU/ml, i.e. a slight but not statistically significant difference. In an attempt to correlate the possible link between desiccation resistance and ionizing-radiation resistance, dried strains including; *E.coli* 0127, *Streptococcus thermophilus* 10387, *Bacillus subtilus* 3106, *Serratia marcescens* 1981 and *Streptococcus viridochromogenes* were sieved by 25µm aperture sieve and exposed to UV-C radiation. The majority of the dried strains tested did not grow on nutrient agar or liquid medium. *Streptococcus thermophilus* was the

only bacterium which showed resistance to UV-C radiation. Maxcy and Rowley (1978) were the first to report experimental evidence that there could be a link between the ability of bacteria to survive dehydration and ionizing-radiation resistance. They demonstrated that by selecting for desiccation tolerance in natural microflora, it was possible to simultaneously isolate radiation resistant species. Subsequently, detailed evaluations of members of the radiation resistant bacteria supported the view that these phenotypes could be interrelated by demonstrating that ionizing radiation and desiccation can produce similar types of DNA damage (Mattimore and Battista, 1996; Billi *et al*., 2000) and that the loss of DNA repair capacity in a radiation-resistant species can result in a strain which is no longer able to survive dehydration (Battista, 1996).

It should be noted that only one sample was taken from each of these environments; such limitation of sample replication is not regarded as significantly important in this study because we are simply attempting to bacteria tolerant to UV-C from the Earth environments. The fact that no such bacteria were isolated from a single sample of such environments does not mean that they are absent; it maybe that an extensive ecological examination of the environmental studied here would produce UV-C resistant bacteria, other than the ones isolated here.

Early attempts to use FISH epi-fluorescence microscopy revealed high levels of background autofluorescence due to the presence of minerals in the sediment/soil-extract. These minerals also demonstrated non-specific binding to the oligonucleotide probes.

The cultured microbial communities of different environmental materials including ordinary, saline and alkaline soil samples were mainly analysed using confocal laser scanning microscopy. However, non-specific binding of fluorescence probes to minerals and background fluorescence of the soil extract caused difficulties in some

cases of detection of hybridised microbial cells using epi-fluorescent microscopy. Moter and Gobel (2000) and Caracciolo *et al.* (2005) also reported that the analysis of microorganisms by epi-fluorescent microscopy was interfered with by tiny inorganic particles and minerals which have a high affinity to nucleic acid labeled probes. As a result, a confocal laser scanning microscope was used instead of the more commonly used epifluorescence microscope. Although, PerfectHyb Plus Hybridization Buffer contained formamide the concentration was found to be insufficient during the hybridisation step for FISH; higher concentrations of formamide were therefore added to decrease non-specific binding of fluorescent oligonucleotide probes to the minerals present in the sediment/soil-extract medium and to optimise the probe specificity. Both archaea (stained red with Cy5) and bacteria (stained green with Alexa Fluor 488) microorganisms were observed without attached minerals.

FISH allowed for the detection here of microorganisms, depending on the labeled probes used, in their natural habitat. The use of FISH confirms the presence of bacteria and archaea in both environmental samples with perhaps some room for doubt in the rock varnish sample. FISH, using confocal microscopy revealed the presence of archaea and bacteria among the microbial community of desert sand which also confirms our previous findings. Fish confirmed the presence of both archaea and bacteria in the sand. By employing bacterial and archaeal primers; FISH confirmed that few archaea were present in desert varnish.

Archaea accounted for more than 90% of the population in desert sand isolated in Chapter 2 whereas they made up only a small part of the population in rock varnish sample. Interestingly, archaea were not isolated from hypersaline soil with 10% salt concentration although extremophilic halobacterium was isolated. FISH however, revealed the presence of both extremophilic halo-archaea and bacteria in this soil sample. Enrichment cultures of the hypersaline soil analysed by confocal microscopy

consisted of approximately 70% archaea and 30% bacteria following culture at 45°C. The alkaline soil sample with pH value of 12 is an extreme environment but the use of FISH, plus confocal microscopy, revealed a small number of archaea among microbial communities.

Agricultural and garden (non-extreme) soil samples were tested for the presence of archaea among the bacterial community. All of them failed to show any archaeal fluorescence, a finding which was confirmed by the 90 to 100% of the DAPI-stained cells hybridized to the bacterial probe. The majority of microorganisms in these soils detected by FISH analysis were therefore bacteria, a fact which agrees with the findings obtained using culture approaches. Ibáñez-Peral (2008) used the FISH technique to analysis the microbial diversity in an environment in White Island, New Zealand. When the cultures were cultivated at 60°C the enrichment cultures from the sample sites, (when analysed by confocal microscopy), consisted of approximately 70% bacteria and 30% archaea. Archaeal species were not uniformly present in cultures incubated at 37°C. These results reflected the culturable representatives of the microbial communities of White Island at the three locations studied. Cottrell and Kirchman (2000) determined the compositions of bacterioplankton communities in surface waters of coastal California using clone libraries of 16S rRNA genes and fluorescence *in situ* hybridization (FISH) in order to compare the community structures inferred from these two culture-independent approaches. Pernthaler *et al*. (2002) also used FISH and Catalysed Reporter Deposition (CARD) for the identification of marine bacteria. These methods help in the determination of the detection rates of coastal North Sea bacterioplankton by CARD-FISH with a general bacterial probe (EUB338-HRP) which they were significantly higher (mean, 94% of total cell counts; range, 85 to 100%) than that with a monolabeled probe (EUB338-mono; mean, 48%; range, 19 to 66%).

FISH and rRNA-targeted oligonucleotide probes were used by Pernthaler *et al*. (1999) to investigate the phylogenetic composition of bacterioplankton communities in several freshwater and marine samples. An average of about 50% of the cells was detected by probes for the domains Bacteria and Archaea; of these, about half could be identified at the subdomain level with a set of group-specific probes. The wide applicability of FISH to studies of bacterioplankton composition has been previously reported in the litertature. FISH can also be used to observe the development of specific genera or species within the bacterioplankton (Pernthaler *et al*., 1999; Pernthaler *et al*., (2002).

Studies aimed at detecting Mycoplasmas in extreme and non-extreme environments showed that *Mycoplasma* was detected by PCR method technique in only two environmental samples, i.e. for the hailstone sample and the other from a park grass soil sample, both of which were collected in Sheffield. Unfortunately, there have been few published works reported the environmental distribution of Mycoplasmas. These organisms have however, been shown to survive for periods of a week on experimentally located human hair, feathers, straw and timber inside a poultry house environment (Christensen *et al*., 1994) and examination of a wide range of *Mycoplasma* species found considerable variation in their virulence mechanisms and methods of persistence in the host (McAuliffe *et al*., 2006).

CHAPTER 8

DETECTION OF *MYCOPLASMA* IN EXTREME AND NON-EXTREME ENVIRONMENTS USING EZ-PCR TEST

8.1 Introduction

The name *Mycoplasma* is derived from the Greek *mykes*, for fungus, and *plasma*, for formed. Frank was first user of this term by in 1889, thinking that he had isolated a fungus. Mycoplasmas were previously called "Pleuropneumonia-Like Organisms (PPLO)", due to their ability to cause contagious bovine pleuropneumonia (CBPP).

Mycoplasma is a genus of bacteria which are characterized by their small genome size which results in significantly reduced biosynthetic capabilities and explains their dependence on a host. They are thought to have undergone reductive evolution, losing several genes possessed by more complex bacteria in the process; therefore, they lack many genes. Some of these lost genes includes those for cell wall synthesis and also for the production of all 20 amino acids, in addition to genes encoding enzymes of the citric acid cycle and the majority of all other biosynthetic genes (Razin *et al*., 1998; McAuliffe *et al*., 2006).

Although Mycoplasmas have a very limited genome, little is known about their mechanisms of virulence and persistence methods in the host. Regardless of their small genome size, Mycoplasmas are known as a causative agent of a wide range of disease in animals as well as humans. They are associated with reproductive disorders and often cause disease of a chronic and persistent nature such as arthritis, and pneumonia. However, little is known about their mechanisms of persistence or their pathogenicity factors in the host. There are only a few characterized virulence factors of Mycoplasmas; these include, in certain *Mycoplasma* species, the production of a carbohydrate capsule (Tajima *et al*., 1982; Almeida and Rosebush, 1991), hydrogen peroxide (Brenan and Feinstein, 1969; Miles *et al*., 1991), the ability to scavenge

arginine from host cells (Sasaki *et al*., 1984). It is difficult to explain how Mycoplasmas manage to cause such chronic and severe infection given their scarcity of virulence factors and the fact that they lack a cell wall.

However, studies have reported that these microorganisms can be isolated from the environment, so a search for them may yield information that is of considerable interest especially when techniques can be applied using molecular methods of identification. The successful detection of different species of *Mycoplasma*, by culture and PCR, from samples collected in the environment of experimentally infected turkeys and chickens, or under field conditions, has been described by Marois *et al.* (2000). They showed that samples of drinking water, food, feathers, droppings or dust were positive for the presence of these organisms, by culture and *Mycoplasma*-PCR- the percentage of positive results for environmental samples, in field conditions, were 25 to 50 %. There have been relatively few other reports of the isolation of Mycoplasmas from a few other environments; such as poultry waste samples (Marois *et al*., 2000), bedding sand used in dairy units (Justice-Allen *et al*., 2010) and a swine waste disposal system (Orning *et al*., 1978). It has also been shown that *Mycoplasma* species may persist on environmental materials, such as drinking water and food, and that they can be detected by PCR-based techniques in environmental samples (Marois *et al*., 2000).

The aim of the work described in this Chapter was to search for the presence of Mycoplasmas in extreme and non-extreme environments.

8.2 Materials and Methods

8.2.1 Samples description

The following types of environmental samples have been tested here for the presence of Mycoplasmas. They are a) the upper atmosphere, i.e. the stratosphere, b) rainwater and hailstone, c) saline samples, i.e. seawater d) saline soil and e) alkaline samples with a pH 8.6–12, including soda lake sample, an alkaline soil, and f) ordinary

soil samples material such as agricultural and garden soils, park grass, desert sand and rocks.

8.2.2 Detection of *Mycoplasma*

All environmental samples were analyzed to detect the presence of Mycoplasmas in this study using an EZ-PCR *Mycoplasma* Test Kit (Geneflowltd, Cat No.20-70-20).

8.2.2.1 Test sample preparation for PCR

An amount of 1.0 ml of sample of test solution was transferred to a 2ml microcentrifuge tube and was centrifuged at 1000 rpm for 1min, to form a pellet. The supernatant was then transferred into a fresh sterile tube and centrifuged at 15000 rpm for 10 min to sediment the *Mycoplasma*. The supernatant was carefully discarded and the pellet was re-suspended in 50µl of buffer solution and mixed thoroughly with a micropipette (the pellet was not always visible). The test sample was then heated at 95^{o}C for 3mins, and immediately stored at -20^{o}C.

8.2.2.2 PCR amplification

PCR amplification of *Mycoplasma* DNA was performed as described by manufacturer. For the test sample, positive and negative control, a reaction mixture of the following was prepared (on ice in a PCR tube) by the addition of the PCR mixture containing: sdH_2O, reaction mix and the test sample (Table 8.1).The tube was placed in a Thermal Cycler and the *Mycoplasma* programme was run. After PCR amplification, the PCR tube was removed from the thermal cycler and 4ul of 5x loading dye was added to each tube. For the positive control, the DNA template control (270bp) was provided by manufacturer whereas for the negative control, the DNA template was replaced with double-distilled water.

Table 8.1 PCR reaction mixtures for amplification *Mycoplasma* DNA fragment.

Reagent/Component	Volume
Sterile deionized water (sdH_2O)	35 µl
Reaction Mix	10 µl
Test sample **Positive control** **Negative control**	5 µl

The reaction procedure (Table 8.2) consisted of an initial denaturation step at 94^{o}C for 30sec, followed by 35 cycles of denaturation at 94^{o}C for 30 sec., primer annealing at 60^{o}C for 120 sec., and extension at 72^{o}C for 60 sec., and was ended by 1 cycle of denaturation at 94^{o}C for 30 sec., primer annealing at 60^{o}C for 120 sec, and extension at 72^{o}C for 5 min.

Table 8.2 PCR amplification protocol of *Mycoplasma* DNA fragment.

Steps	Time	Temperature	Number of cycle
Initial denaturation	30 sec	94^{o}C	1
Denaturation	30 sec	94^{o}C	35
Annealing	2 min	60^{o}C	
Extension/Elongation	1 min	72^{o}C	
Final denaturation	30 sec	94^{o}C	1
Final annealing	2 min	60^{o}C	
Final extension	5 min	72^{o}C	
Hold		4^{o}C	

8.2.2.3 Agarose gel electrophoresis

The amplified products (10µl), including positive and negative control, with (2µl) 6X loading dye were separated in a 2% agarose gel in TAE buffer for 45 minutes at a constant voltage of 80 V. Amplified products were detected by UV transillumination

with ethidium bromide staining. A 200 bp Ladder (Bioline, UK) was used as a molecular size standard.

8.3 Results and Discussion

8.3.1 The occurrence of *Mycoplasma* in various environments

The use of PCR allowed for the detection of Mycoplasmas or *Mycoplasma* DNA in various environmental samples. The results of the *Mycoplasma* testing obtained from different environments are shown in Table 8.3. The samples represented saline environments, such as sea water and hypersaline soil, and alkaline environments, such as soda lake and high alkaline soils. All were negative for Mycoplasma

Table 8.3 The presence or absence of *Mycoplasma* in various environments.

Sample materials		Results
Upper atmosphere environments	Stratosphere	Negative
	Rain	Negative
	Hailstone	**Positive**
Saline environments	Horn Sea	Negative
	Oman Gulf	Negative
	Hypersaline soil (Oman)	Negative
Alkaline environments	Redcar (pH 12) soil	Negative
	Redcar (pH 6.8) soil	Negative
	Alkaline sediment (pH 12)	Negative
	Alkaline plant root (pH 12)	Negative
	Turkey soda lake (pH 9.0)	Negative
Ordinary environments	Garden soil	Negative
	Park grass	**Positive**
	Desert sands	Negative
	Agricultural soil	Negative
	Desert Rock varnish	Negative

Mycoplasma was however, detected by PCR method technique in two environmental samples, i.e. for the hailstone sample and the other for the park grass sample, both of which were collected in Sheffield. The reason for the appearance of two positives against a general trend of negatives for the isolation of environments Mycoplasmas is not immediately obvious. The presence of Mycoplasmas in the park grass sample may reflect a level of pollution from animal faeces, mainly birds and dogs and associated bacteria in such environments, but this explanation cannot apply to hailstones, which would be expected to be pristine except for atmospheric dust. The presence of Mycoplasmas in hailstones is of considerable potential importance and the main source needs to be verified, this is because Mycoplasmas from this source may indicate their presence in rainwater and generally in the upper atmosphere, from where they could undergo intercontinental transfer; this would be of importance and of particular interest should these organisms be plant, human or animal pathogens.

8.3.2 PCR detection techniques of *Mycoplasma*

The technique of EZ-PCR-detecting *Mycoplasma* used in the present study allowed us to detect culturable-independent *Mycoplasma* DNA in environmental samples. The specificity of this PCR methods has not been checked and indeed this test kit detects various types of *Mycoplasma* species *(M. fermentans, M. hyorhinis, M. arginini, M. orale, M. salivarium, M. hominis, M. pulmonis, M. arthritidis, M. bovis, M. pneamoniae, M. pirum and M. capricolum*), as well as *Acholeplasma* and *Spiroplasma* species, with high sensitivity and specificity.

The principle of this method is that rRNA gene sequences of prokaryotes, including Mycoplasmas, are well conserved, whereas, the sequences and lengths of the spacer region in the rRNA operon (e.g. the region between 16S and 23S gene) differ from species to other. The detection procedure utilizing the PCR process with this primer set consists of: a) amplification of a specific and conserved 16S rRNA gene region of

Mycoplasma using two primers b) detection of the amplified fragment by agarose gel electrophoresis.

Practically, this system does not let the amplification of DNA originating from other sources, such as bacteria or tissue samples, which would affect the detection result. Therefore, amplification of the gene sequence with PCR using this primer set enhances not only the sensitivity, but also the specificity of detection. Amplified products are then detected by agarose gel electrophoresis (Figure 8.1).

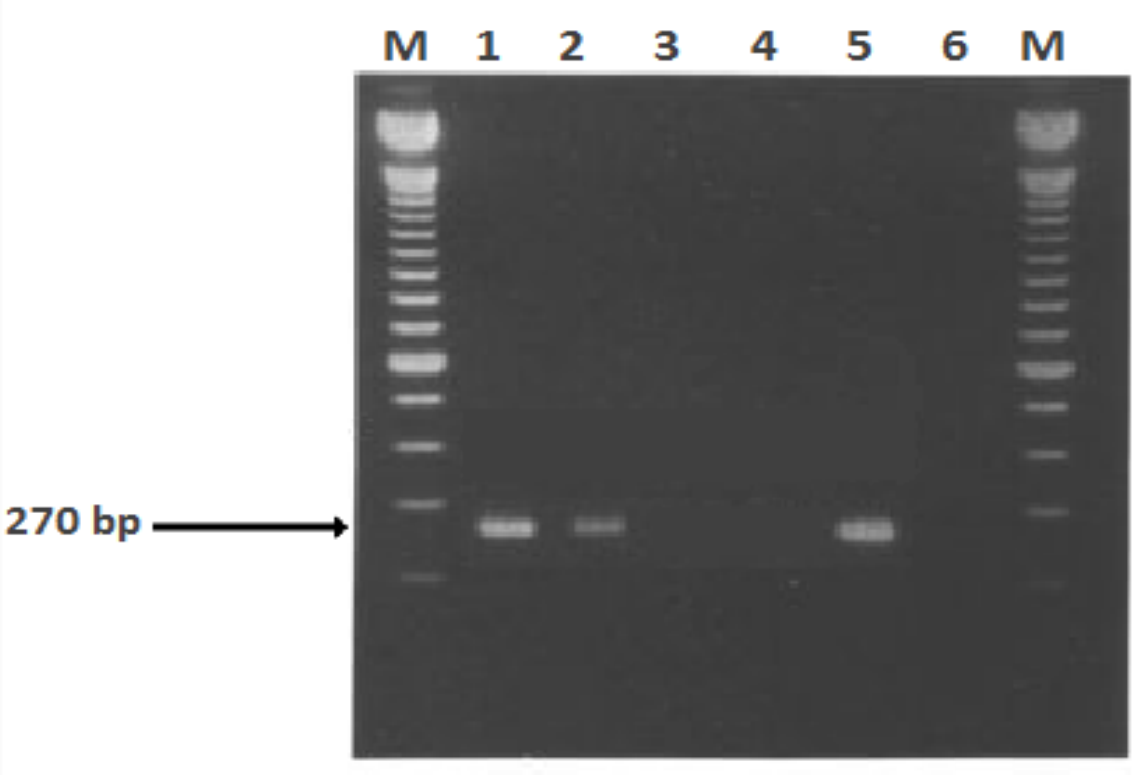


Figure 8.1 Polymerase chain reaction (PCR) detection of *Mycoplasma* species in environmental samples. EZ-PCR-detecting Mycoplasma test analyzed by electrophoresis in 2% agarose gel; (lane 1); positive control; (lane 2); hailstone sample, (lane 3 and 4); negative result, (lane 5); park grass sample and (lane 6) negative control in addition to (M); 1kb hyperladder.

Although, only viable Mycoplasmas should be considered as potential source of infection, EZ-PCR-detecting *Mycoplasma* method is in the fact detecting DNA from viable and non-viable Mycoplasmas. Marois *et al*. (2002) developed a reverse

transcription-polymerase chain reaction (RT-PCR) assay which was developed to detect viable *Mycoplasma* in environment. It was basically developed to extract and analyse RNA by (RT-PCR) since, because of its short half-life, its detection indicates that cells are viable or were viable very recently.

8.4 Conclusions

Unfortunately, there have been few published works reporting the environmental distribution of Mycoplasmas. They have however, been shown to survive for periods of a week on experimentally located human hair, feathers, straw and timber inside a poultry house environment (Christensen *et al*., 1994) and examination of a wide range of *Mycoplasma* species found considerable variation in their virulence mechanisms and methods of persistence in the host (McAuliffe *et al*., 2006).

Thus in this study, the use of PCR Mycoplasma test, enabled us to investigate the presence of Mycoplasmas in the environmental samples used here. This study showed that Mycoplasmas were rarely isolated from the environment, two examples of the isolation of environmental Mycoplasmas were found but further research is clearly needed to extend this work to determine the distribution of these organisms in a wider number of environmental samples and to determine how they can grow and exist without apparent hosts, or else locate the hosts in such samples.

CHAPTER 7

FLUORESCENT *IN SITU* HYBRIDISATION (FISH), AS A CULTURE-INDEPENDENT TECHNIQUE

7.1 Introduction

Fluorescent *in situ* hybridisation (FISH) is a hybridisation method which can be used to identify the presence, or absence, of a specific microorganism or a phylum. Fluorescently-labelled oligonucleotide probes bind to specific target sequences within the cell which can then be visualised by epifluorescence microscopic techniques. The most commonly used probe for *in situ* hybridisation is a fluorescently labelled sequence targeted to 16S rRNA. Oligonucleotide probes can be designed to detect and visualize a specific species, genus or domain. A major advantage of the FISH technique is that it can be carried out *in situ,* so no bias is established as a result of DNA preparation method or even PCR. FISH can also be semi-quantitative if the number of cells which was fluoresced from a specific probe is compared to the total number of cells stained by DAPI (4',6-diamidino-2-phenylindole). It is also possible to detect microorganisms which make up a very low proportion of a microbial population, especially if the probe is chosen carefully or is well designed. FISH has been applied to many environmental samples including acidic environments such as acid mine drainage (Hallberg *et al*., 2006) and to the Rio Tinto (Garcia-Moyano *et al.*, 2007) and Iron Mountain in California (Baker *et al*., 2004).

The aim of the work reported in this Chapter was to allocate the isolated microorganisms from different environments including ordinary and extreme sample materials to either archaea or bacteria.

7.2 Materials and Methods

7.2.1 Sample description

The samples were obtained from different environments including agricultural and, gardens soils, marine sediment, desert varnish and sands in addition to hypersaline soil with a 10% salt concentration (see Chapter 2). Alkaline sediment samples were tested including sample with pH value of 12 and 8.6 (see Chapter 4).

7.2.2 Isolation conditions

7.2.2.1 Sediment/soils and media

Sediment/soil extracts were obtained by mixing 150 g of each sample from the different sampling sites with 1 L of ddH_2O. The mixture was boiled for at least 2 hours with occasional stirring. Then, the boiled extract was cooled for 30 min to settle and remove large particles in the mixture. Smaller particles were then removed by filtering the mixture solution through Whatman No.1 filter paper. The final sample-extracts were, then, autoclaved at 121°C for 20 min and stored at 4°C.

Sediment/soil-extract liquid medium was supplemented with 10 g yeast extract, 10-100g NaCl (depending on the microorganism of interest), 1.0 ml of 500 mM of $FeCl_2.6H_2O$ (sterilised by filtration through a 0.22 µm filter) and 1.0 ml of trace elements including: (900 mg) $MnCl_2.4H_2O$, (2.25 mg) $Na_2B_4O_7.10H_2O$, (110 mg) $ZnSO_4.7H_2O$, (25 mg) $CaCl_2.2H_2O$. The final pH was adjusted to 7.20±0.2; for alkaliphile and alkalitolerant microorganisms the pH ranged from 5.0 up to 10.0 and finally stored at 4°C.

7.2.2.2 Enrichment cultures

For enrichment of microorganisms directly from the environment, approximately 0.5 g of each sample material was transferred aseptically to a test tube containing 10 ml of sediment/soil-extract and/or nutrient liquid media. Cultures were incubated aerobically overnight at 25ºC, 37ºC and 45ºC with shaking (150 rpm). Microbial

growth was monitored using light microscopy. Subcultures of actively growing cultures were then carried out by transferring aseptically 500 µl to fresh liquid media.

7.2.3 Microscopy

7.2.3.1 Epi-fluorescence microscopy

The basis of the epi-fluorescence microscope is to illuminate a particular sample at a specific wavelength which is then absorbed by the fluorophore resulting in light emission at a longer wavelength than the excitation light. The excitation light of an epi-fluorescent microscope is passed from above through the objective and then onto the sample. Epi-fluorescence microscopy observation was conducted using a Nikon SMZ1500 microscope (Nikon Corporation, Tokyo, Japan) equipped with epi-fluorescence attachment. It was connected with a universal condenser for Nomarski differential interference contrast (DIC) and a mercury burner for broad band excitation. The microscope characterizes diachronic mirrors with various sets of barrier and excitation filters (which reflect short radiation wavelengths towards the objective) to illuminate the specimen, while passing longer wavelengths (selection of filters in Table 7.1). Images were captured and processed using a Nikon DXM 1200F digital camera (Nikon Corporation, Tokyo, Japan) and linked with Nikon ACT-1 software package v.2.62.

Table 7.1 Filter set used in the epi-fluorescence microscope.

Fluorochrome	Dichroic mirror	Excitation filter	Emission filter
Alexa fluor 488	DM500	20 BP490	17 O 515
Cy5	DM570	20 BP 545	17 O 590
DAPI	DM400	20 UD 1	17 < 420

7.2.3.2 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is used mainly to increase micrograph contrast and to reconstruct three-dimensional (3D) images by using a spatial pinhole to eliminate (out of focus) light or flare in specimens that are thicker than the focal plane (Sugita and Tenjin, 1993; Pawley and Masters, 2008).

CLSM was performed using an Olympus FluoView FV1000 (Olympus Corporation, Japan) equipped with an inverted microscope with a universal condenser and three lasers (Melles Griot, Carlsbad, USA) (Table 7.2): a Helium Neon red laser (633 nm excitation laser), a Helium Neon green laser (543 nm excitation wavelength) and Argon laser (488 nm excitation wavelength). Samples slides were examined using a citiflour mounting solution objective with numeric aperture 1.35. Images were captured and processed using Olympus software Fluoview v.4.3.

Table 7.2 General characteristics of the confocal laser scanning microscope.

Fluorochrome	Excitation wavelength	Filter
Cy5	543 nm- HeNe laser	BA 510 IF
Alexa Flour 488	488 nm- Argon laser	BA 510 IF and BA530 RIF

7.2.4 Fluorescent *in situ* hybridisation (FISH)

FISH technique was performed as described by Langendijk *et al*. (1995); Pernthaler *et al*. (2002) and Ibáñez-Peral (2008).

7.2.4.1 Design of oligonucleotide fluorescent probes

Labeled oligonucleotide probes were synthesised and purified by Sigma, Aldrich-Proligo (Sigma-Aldrich, France and UK) (Appendix F). Lyophilised probes were dissolved as per manufacturer procedure in sterile Tris-acetate-EDTA (TAE) 1x solution (Fisher BioReagent buffer, UK) and stocks solutions were stored at -20ºC.

Working solutions were diluted using (TAE) 1x solution to a final concentration of 50 and/or 100 ng/µl and then stored at -20ºC.

In general, domain specific probes were used as mixtures, called ArchMix (Arch344, arch915 and Arch1060, 50 ng/µl each) and EubMix (Eub338I, 50 ng/µl and Eub338II/III, 100 ng/µl). ArchMix was labelled with Cy5 (Cyanine) while, EubMix was labelled with Alexa Fluor 488 (Table 7.3).

Table 7.3 Binding position, sequence and specificity of oligonucleotide probes.

Oligonucleotide	Position*	Sequence 5'-3'	Specificity	Reference
Arch344	344	TTCGCGCCTGSTGCRCCCCG	Archaea	(Moissl *et al*., 2003)
Arch915	915	GTGCTCCCCCGCCAATTCCT	Archaea	(Stahl and Amann,1991)
Arch1060	1044	GGCCATGCACCWCCTCTC	Archaea	(Moissl *et al*., 2003)
Eub338I	338	GCTGCCTCCCGTAGGAGT	Bacteria	(Amann *et al*., 1990)
Eub338II/III	338	GCWGCCACCCGTAGGTGT	Bacteria	(Amann *et al*., 1990)

*Position relative to the *E.coli* 16S rRNA gene

7.2.4.2 Control organisms and culture conditions

Microbial strains used as a positive control for FISH were the archaoen; NCIMB 14054 unnamed archaea and the bacterium *E. coli* DH5α (Moissl *et al.* 2003). NCIMB 14054 Archaea was purchased from the National Collection of Industrial, Marine and Food Bacteria (NCIMB, Aberdeen, UK) and *E. coli* strain DH5α from Life Technologies (Invitrogen, UK). NCIMB 14054 archaea was grown in Payne Seghal and Gibbons medium containing 20% NaCl (Chapter 2, Section 2.2.2) at 45^{o}C and *E.*

coli was grown in LB liquid medium at 37ºC with shaking at 250 rpm under aerobic conditions. Archaeal and bacterial growth was observed by light microscopy and cell numbers were estimated and recorded as cells per field of view. 150-200 µl of actively growing archaeal and bacterial control cultures were subcultured and transferred aseptically to 15-20 ml of fresh media (1:100 dilutions).

7.2.4.3 Preparation of microbial samples

Actively growing microbial cultures (1ml) of samples and control microorganisms were fixed with 1/10 volume (~110 µl) of 30% (w/v) paraformaldehyde stock solution directly to the culture (end concentration 3% (w/v)). The cells were incubated at 4ºC for 10 min or at room temperature for 1 h and then centrifuged at (~11340 g) for 10 min and the supernatant was removed completely. For cell wall permeabilisation, cells were incubated in a lysozyme solution (10 mg ml^{-1} in 0.05 M EDTA, 0.1 M Tris-HCl [pH 7.5]; Sigma) at 37°C for 20 min. The cell pellet was re-suspended in 1 ml of 1x of phosphate buffer saline (PBS), centrifuged at (~ 11340 g) for 10 min and the supernatant discarded. This step was repeated twice and the final pellet was re-suspended in 25-50µl of 1x PBS. Finally, one volume of absolute ethanol (100% EtOH) was added to the samples and the fixed cells were stored at -20ºC.

7.2.4.4 Preparation of microscope slides

Slides with epoxy resin colour mask with 6 wells of 8 mm diameter (Figure 7.1),were used (these are also called Diagnostic glass slides with an epoxy-resin mask) (Paul Marienfeld GmbH all Co. KG, Lauda-Königshofen, Germany). These slides were cleaned several times by washes with detergent followed by rinsing with ddH_2O and then wiping with acetone. After air-drying at room temperature, slides were briefly dipped into hot gelatine solution (0.01% $KCr(SO_4)_2$, 0.1% gelatine w/v) at approximately 70°C. Left in a vertical position, the slides were allowed to air-dry at

room temperature. This prepared slide which is now referred to as a "coated slide", was finally stored in a dust-free environment.

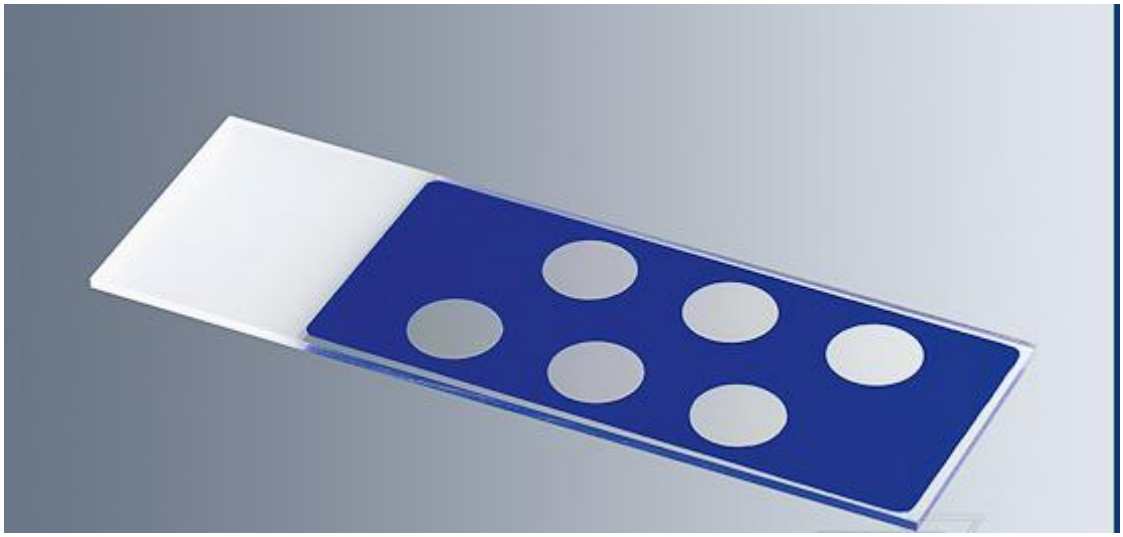


Figure 7.1 Microscope slides with well-wettable reaction wells (6 x 8 mm) are used for diagnosis. The epoxy resin colour masks feature remarkably high resistance against solvents.

7.2.4.5 Hybridisation conditions and probe specificity.

A fixed culture (10µl) was spotted into the wells of the coated slides (pre-treated microscope slides) without scratching the coated surface. After air-drying, the cells were dehydrated in an increasing ethanol concentration (starting from 50%, 80% and ended with 99% (v/v) in dH_2O) for 2 min for each concentration. The slides were dried at room temperature and then, 8 µl of 1x FISH hybridisation buffer (PerfectHyb Plus Hybridization Buffer, Sigma-Aldrich, UK; It was left out at >30^{o}C to avoid precipitation) was added to each well. The slides were then placed horizontally in a hybridisation chamber (completely closed chamber containing moist tissues) and pre-hybridised for 15 min at 48ºC. Subsequently, the slides were placed on a heating block of 37^{o}C and the labeled; oligonucleotide probes were then added to each well. Each probe (100ng) was applied for the samples and 50 ng of each probe was used to the

control cultures. The slides were covered with a cover slip and then hybridised for 20 min at 48ºC.

After hybridization, 3 ml of (preheated at 48ºC) FISH washing buffer (0.23 M NaCl, 10 mM Tris-HCl pH 7.2 and 0.25% SDS) was used to briefly rinse the slides before they were placed in hybridisation chambers containing the same buffer. The slides were washed with FISH washing buffer for 15 min at 48ºC (rotating at 50 rpm), rinsed with cold dH_2O and air-dried at room temperature in the dark. An aliquot (10 µl) of (1 µg/ml in 0.01 M Tris/HCl, pH 7.2) DAPI solution (4',6-diamidino-2-phenylindole, Sigma), for DNA-specific counterstaining, was applied to each well and incubated in a moist chamber for at least 5 min. Finally, the slides were rinsed with cold dH_2O, dried, mounted in Citifluor AF-1 (Citifluor Ltd, London, United Kingdom) mounting solution and stored at room temperature in the dark.

In each hybridisation reaction, positive controls for every probe or probe mixture were included. Samples and control slides were observed by epi-fluorescence microscopy (Section 7.2.3.1) and Confocal laser scanning microscopy (Section 7.2.3.2) using appropriate filters for Alexa 488 and Cy5 visualisation.

7.3 Results and discussion

In this study, fluorescent *in situ* hybridization technique was mainly used to investigate the microbial interaction between the microorganisms cultured in the sediment/soil-extract medium. FISH was carried out with domain-specific probes to assign the microorganisms to either archaea or bacteria. The controls for FISH were NCIMB 14054 archaea and *E. coli* stained with Cy5 and Alexa Fluor 488, respectively (Figure 7.2).

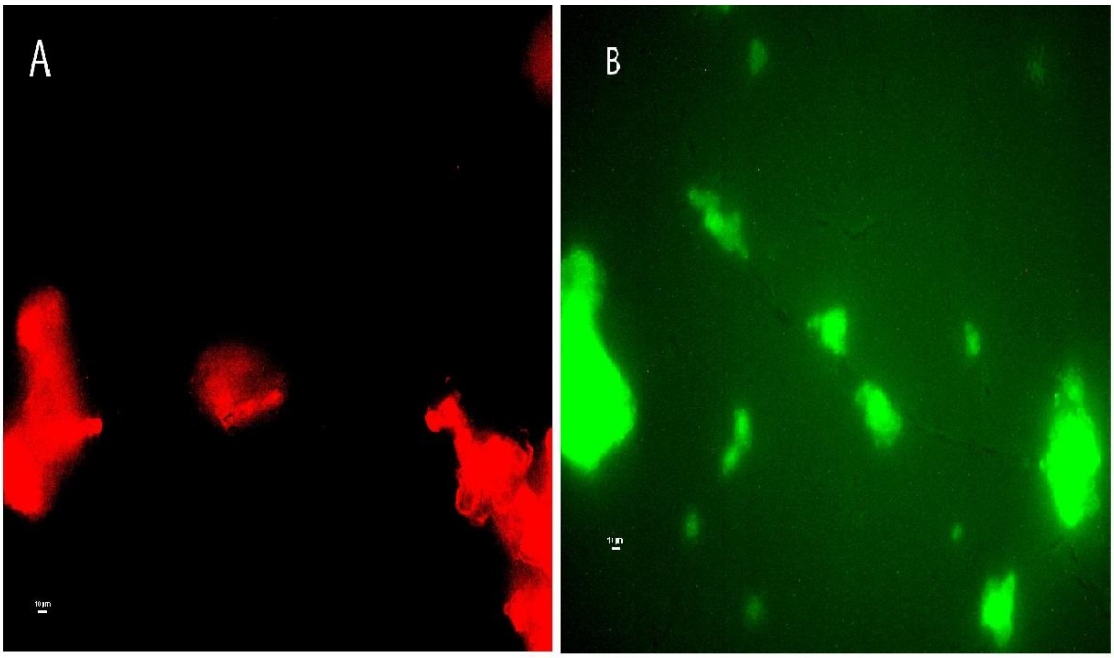


Figure 7.2 NCIMB 14054 archaea and *E. coli* DH5α stained with domain-specific probes used as a control for FISH. Images were taken by epifluorescence microscope. A: red fluorescence showing Archaea control stained with Cy5. B: Green fluorescence showing *E. coli* control stained with Alexa Fluor 488.

In early attempts to use FISH in these studies, epi-fluorescence microscopy revealed high levels of background autofluorescence due to the presence of minerals in the sediment/soil-extract. These minerals also demonstrated non-specific binding to the oligonucleotide probes. A control was run for the medium extract which confirmed that the intensive background from the sample materials interfered with the technique's ability to distinguish microorganisms from these minerals (Figure 7.3).

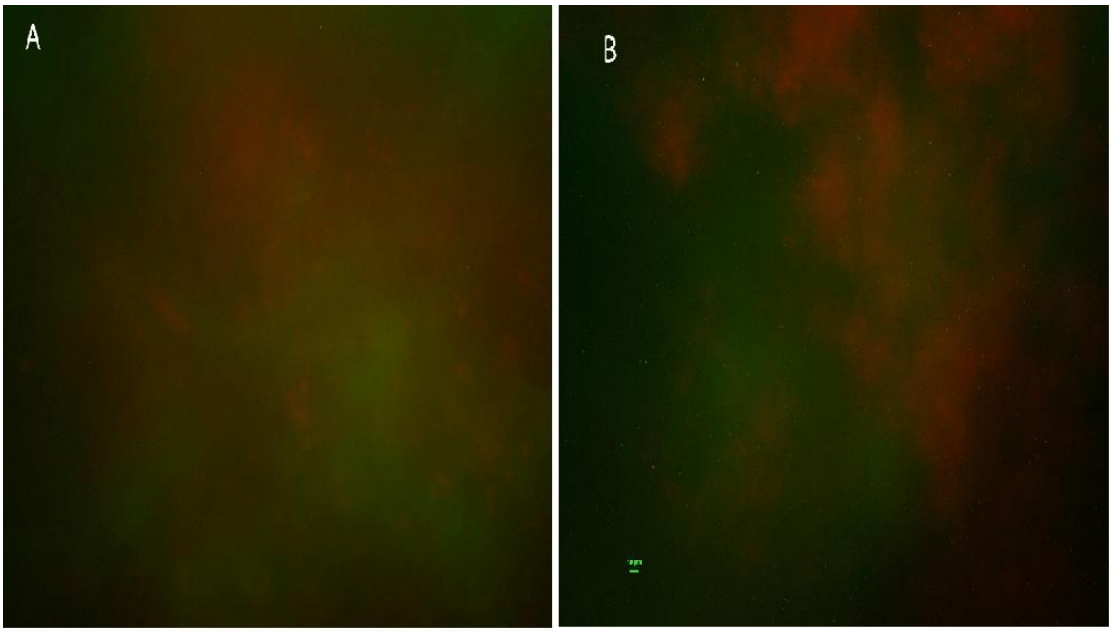


Figure 7.3 FISH images by epi-fluorescence microscopy of enrichment cultures from hypersaline soil (10% salt concentration) growing at 37^{o}C – 40^{o}C. A: control medium without culture, B: fluorescent archaea and bacteria which interfered with the particles in the medium extract.

The use of a higher formamide concentration optimises the probe specificity and enhances the loss of the reliability of the cell walls. The concentration of formamide is optimized by varying the quantity (%) of it within the hybridization buffer from 0 to 60% (Harmsen *et al*., 1997). Therefore, the cells were hybridized and washed at 48°C with the addition of formamide. However, mineral contamination still resulted in high levels of fluorescence making it difficult to differentiate microbial cells under the epi-fluorescence microscope (Figure 7.4). The use of 30-35% formamide was found to be the optimum concentration to manage this problem.

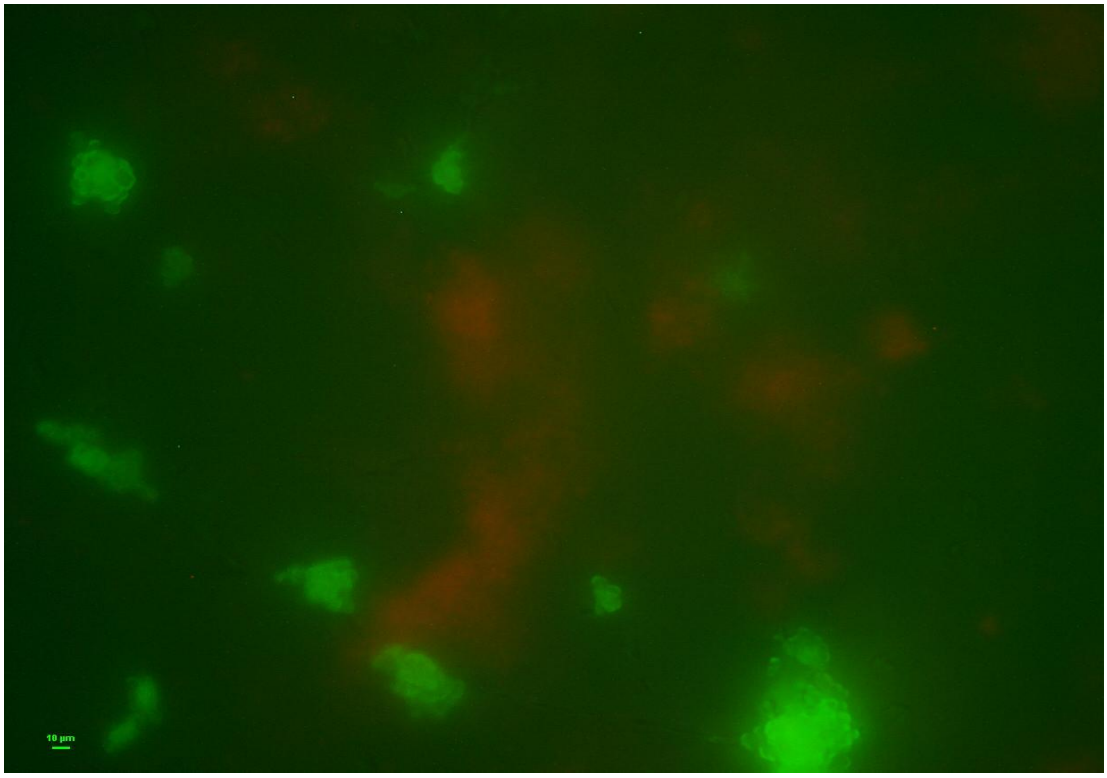


Figure 7.4 FISH images obtained by epi-fluorescence microscopy after addition of 35% of formamide in hybridization of enrichment cultures from hypersaline soil (10% salt concentration) growing at 37^{o}C – 40^{o}C.

The *in situ* probing application combined with conventional fluorescence microscopy for the analysis of complex microbial interactions can also be weakened by background fluorescence caused by humic substances or detritus, biofilms, and the inherent autofluorescence of images. Hybridization assays using fluorescently labeled oligonucleotide probes in conjunction with confocal laser microscopy offer the possibility of eliminating these difficulties (Wagner *et al*., 1994). Unlike epi-fluorescence microscopy which captures the background of the samples that stick on the sample, confocal laser scanning microscope can decrease interference from non-specific background fluorescence, permitting FISH studies in various environmental materials (Bloem *et al.*, 1995). As a result, a confocal laser scanning microscope was

used instead of the more commonly used epifluorescence microscope. Although, PerfectHyb Plus Hybridization Buffer contained formamide, the concentration was found to be insufficient during the hybridisation step for FISH; higher concentrations of formamide were therefore added to decrease non-specific binding of fluorescent oligonucleotide probes to the minerals present in the sediment/soil-extract medium and to optimise the probe specificity. In all cultures, both archaea (stained red with Cy5) and bacteria (stained green with Alexa Fluor 488) microorganisms were observed as clear communities without attached minerals. Confocal laser scanning microscopy was used to avoid the autofluorescence materials by applying point illumination and a pinhole in an optically conjugate plane to lead to only the detection of fluorescence in the focal plane (Figure 7.5).

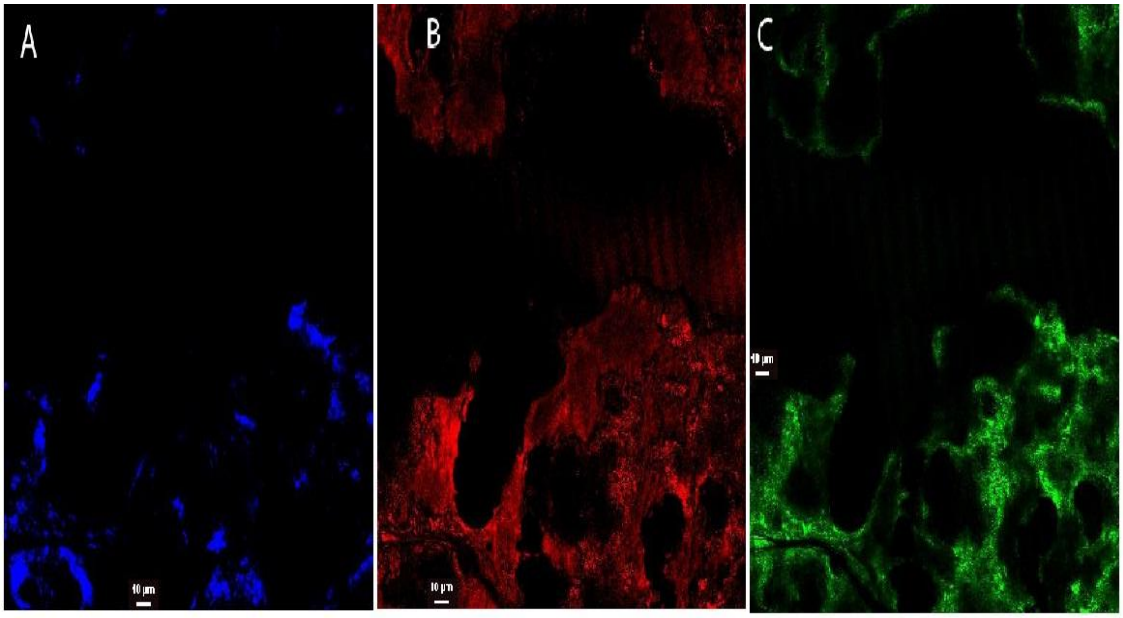


Figure 7.5 FISH images by confocal microscopy of NCIMB 14054 archaea and *E. coli* DH5α stained with domain-specific probes used as a control. A: whole microbial communities showing DAPI fluorescence, same image stained with fluorescent labeled probe B: Red fluorescence showing archaea control stained with Cy5. C: green light showing *E. coli* control stained with Alexa Fluor 488.

FISH allowed the detection of microorganisms, depending on the labeled probes used, in their natural habitat. The use of FISH confirms the presence of bacteria and archaea in both environmental samples with perhaps some room for doubt in the rock varnish sample. This was achieved by testing the isolated cultures from rock varnish which were stuck with *Bacillus* sp. (Chapter 2) due to the difficulty in making the extract medium. FISH using confocal microscopy reveals the presence of archaea and bacteria among the microbial community of desert sand which also confirms our previous findings (Chapter 2). Fish confirmed the presence of both archaea and bacteria in the sand which confirms the results obtained in Chapter 2, using bacteria and archaeal primers; the use of FISH confirms that few archaea were present in desert varnish. Archaea were observed to account for more than 90% of the population in desert sand sample (Figure 7.6) whereas Archaea comprised only a small part of the population in rock varnish sample (Figure 7.7), despite this it was obviously detected using molecular identification of 16S rRNA in Chapter 2.

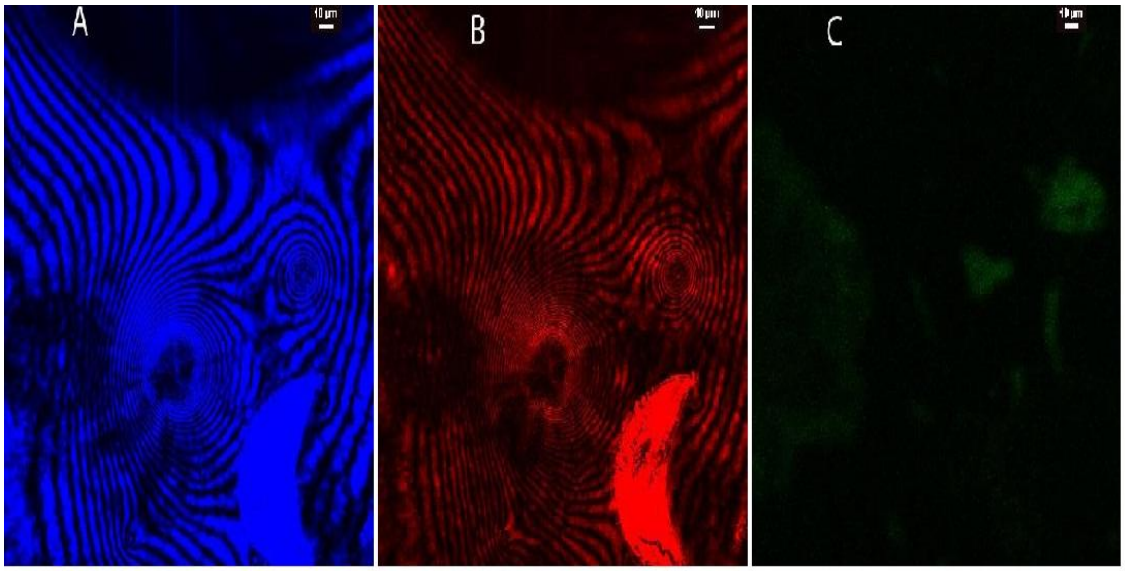


Figure 7.6 FISH images by confocal microscopy of halophilic isolates cultured in Payne Seghal and Gibbons medium containing 20% NaCl from desert sand and stained with domain-specific probes. A: whole microbial communities showing DAPI fluorescence, same image stained with fluorescent labeled probe B: Red fluorescence showing archaeal population stained with Cy5. C: Green light showing bacterial population stained with Alexa Fluor 488.

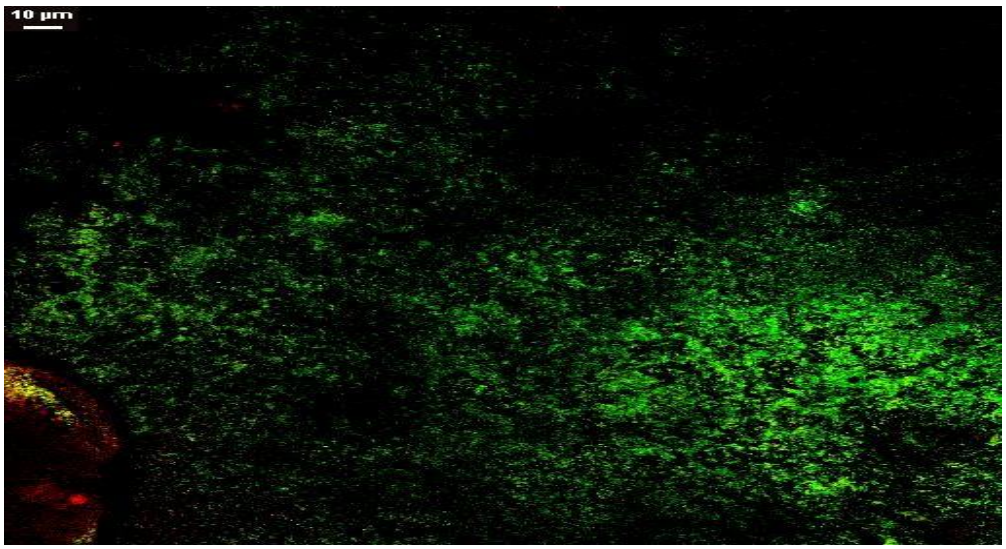


Figure 7.7 FISH images by confocal microscopy of halophilic isolates cultured in Payne Seghal and Gibbons medium containing 20% NaCl from rock desert varnish and stained with domain-specific probes. The two-colour image reveal mixed populations of both archaea-Cy5 (red) and bacteria-Alexa Fluor 488 (green).

Interestingly, in Chapter 2, archaea were not isolated from hypersaline soil with 10% salt concentration although extremophilic halobacterium was isolated. FISH however, reveals the presence of both extremophilic halo-archaea and bacteria in this soil sample (Figure 7.8). This could be due to the miss-isolating the archaea or because they were unculturable in media used. The enrichment cultures of hypersaline soil analysed by confocal microscopy consisted of approximately 70% Archaea and 30% bacteria following culture at 45°C.

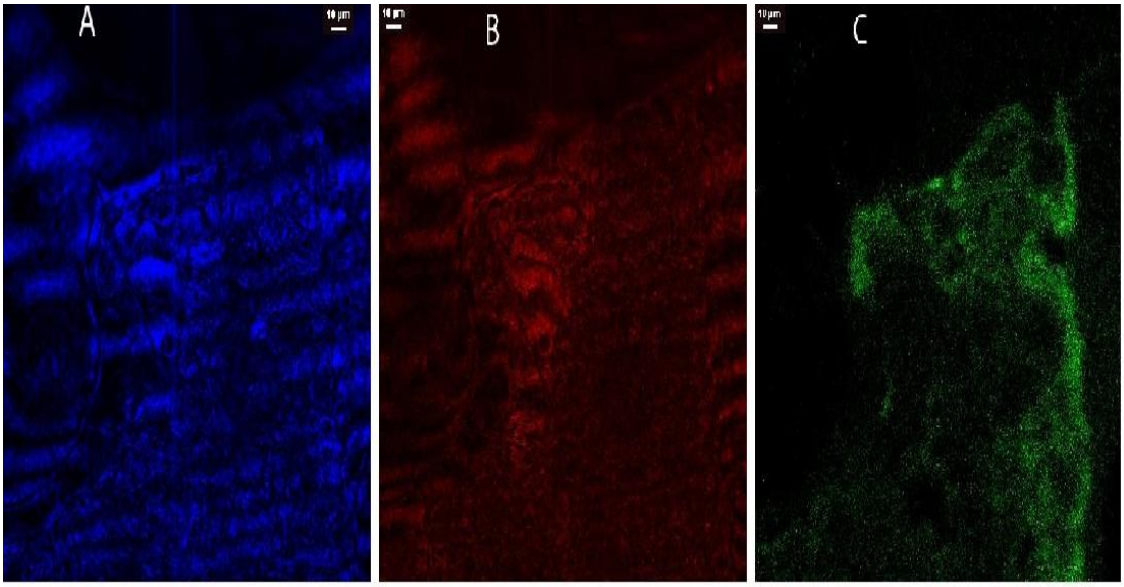


Figure 7.8 FISH images by confocal microscopy of hypersaline soil with 10% salt concentration cultured in soil extract medium and stained with domain-specific probes. A: whole microbial communities showing DAPI fluorescence, same image stained with fluorescent labeled probe B: Red fluorescence showing archaeal population stained with Cy5. C: Green light showing bacterial population stained with Alexa Fluor 488.

The alkaline soil sample with pH value of 12 is an extreme environment but the use of FISH plus confocal microscopy revealed a very small number of archaea among the microbial communities (Figure 7.9). This is could be due to the specific probes was not good enough to differentiate the targeted species from the rest of the population in specifically alkaline sample.

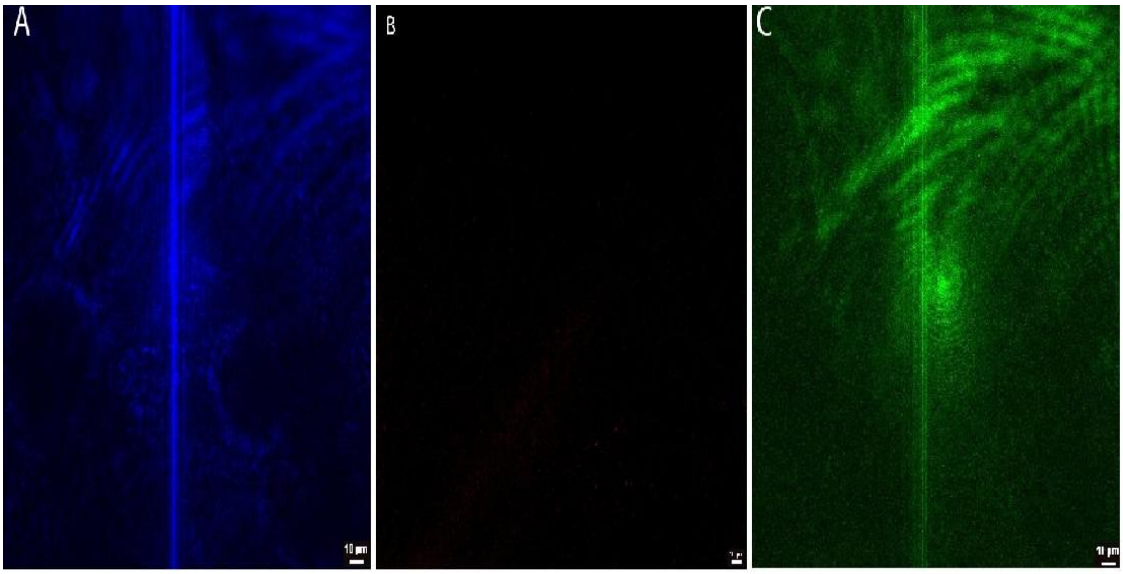


Figure 7.9 FISH images by confocal microscopy of alkaline soil sample with pH 12 cultured in sediment extract medium at 40^{o}C and stained with domain-specific probes. A: whole microbial communities showing DAPI fluorescence, same image stained with fluorescent labeled probe B: Red fluorescence showing archaeal population stained with Cy5. C: Green light showing bacterial population stained with Alexa Fluor 488.

Four of the agricultural and garden (non-extreme) soil samples were tested for the presence of archaea among the bacterial community. All of them failed to show any archaeal fluorescence, a finidng which was confirmed by the 90 to 100% of the DAPI-stained cells hybridized to the bacterial probe (Figure 7.10). The majority of microorganisms in these soils detected by FISH analysis were therefore bacteria, a fact which reflects findings using culture approaches.

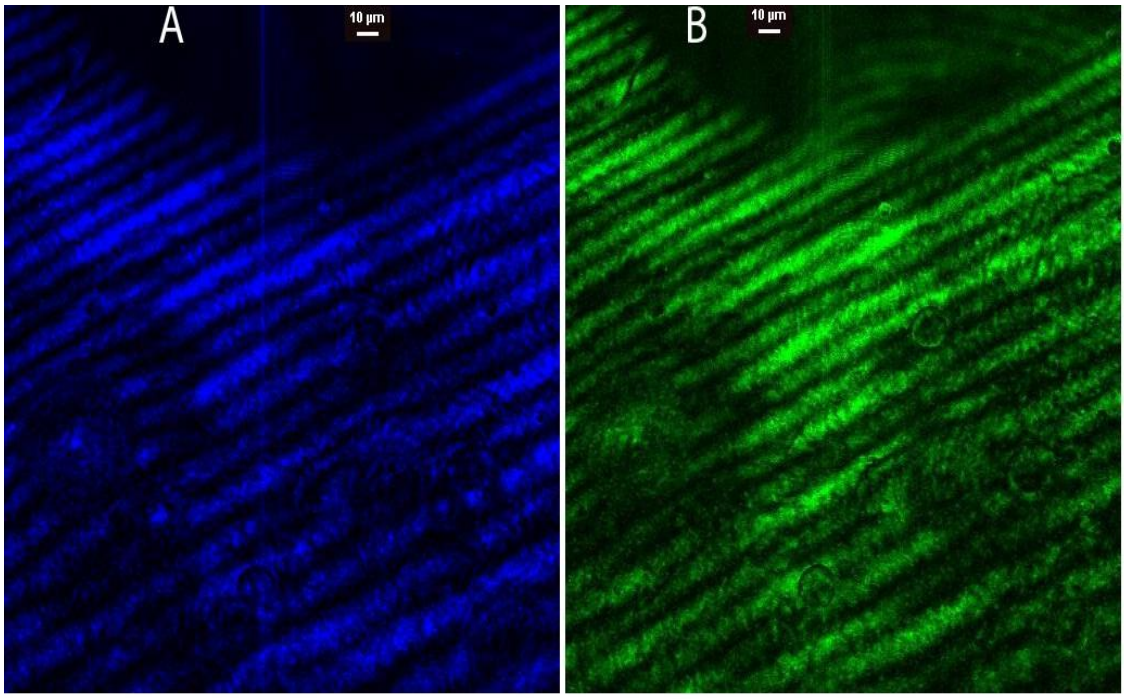


Figure 7.10 FISH images by confocal microscopy of ordinary soil (garden soil) cultured in soil extract medium and stained with domain-specific probes. A: whole microbial communities showing DAPI fluorescence, same image stained with fluorescent labeled probe B: green light showing bacterial population stained with Alexa Fluor 488.

7.4 Conclusions

The fluorescent *in situ* cell hybridisation (FISH) technique is applied to the identification, enumeration and monitoring of microorganisms. Designing the specific probes and transposing these probes to the targeted sites inside the cells are the two most critical points of this method. Species specific probes must be specific enough to differentiate the targeted species from the rest of the population in a particular community. Group specific probes need to act as universal probes to all members of this group. For this reason, a database with a large number of nucleic acid sequences is required to make sure that the designed probe is truly unique or truly universal as required. Therefore, the rRNA genes are commonly chosen so as to assist probe design. In addition, FISH targeting of the rRNA is chosen due to the availability of large numbers of rRNAs in active cells. However, the specific nucleotide positions in the

genes produce different intensities of detectable labeling. This variation takes place because rRNAs are found inside the cells in association with other molecules such as ribosomes, mRNA and ribosomal protein. As a result, these associations may prevent the access of the designed probes to their binding sites in the rRNAs. This assumption was demonstrated by an experiment conducted by Fuchs *et al.* (1998) involving probing of *E. coli* with 200 different fluorescence-labelled probes targeting specific nucleotides less than 10 nucleotides at room temperature throughout its 16S rRNA gene. The results of this experiment showed good detectable fluorescence intensity between probes. Since the structure of ribosomes and rRNAs are much conserved because of their functional roles, the nucleotide position that provides good detectable fluorescence probes in *E.coli* could be useful for designing probes for other species. The probes, once designed and synthesised, must be transported to the targets inside the cells. To do this, cells should be made permeable to the probes, a process referred to as "cell fixation". This fixation is achieved by treating the cells with chemicals such as formalin and paraformaldehyde, which act to cross- link the cell wall and membrane. The cells can be, then, hybridised to the fluorescence-labelled probes and they can be viewed and counted under an epifluorescence microscope fitted with appropriate filters. It is also possible to detect more than one species at the same time using multiple fluorescence-labelled probes with different dyes (Amann, 1995; Amann and Schleifer, 2001).

In summary, the cultured microbial communities of different environmental materials including ordinary, saline and alkaline soil samples were analysed by confocal laser scanning microscopy. However, non-specific binding of fluorescence probes to minerals and background fluorescence of the soil extract caused difficulties in some cases of detection of hybridised microbial cells using epi-fluorescent microscopy. Moter and Gobel (2000) and Caracciolo *et al* (2005) reported these observations and

found that the analysis of microorganisms by epi-fluorescent microscopy was interfered with by tiny inorganic particles and minerals with high affinity to nucleic acid labeled probes. Finally, Lenaes *et al.* (2007) recommended the use of molecular beacons to reduce the fluorescent background produced by the non-specific binding of labeled oligonucleotide probes to the minerals.

CHAPTER 6

THE ISOLATION OF ULTRAVIOLET-C RESISTANT BACTERIA AND ARCHAEA FROM THE EARTH AND ITS ATMOSPHERE

6.1 Introduction

Radiation on the short-wavelength end of the electromagnetic spectrum, such as high frequency ultraviolet, gamma rays and x-rays, is found to be ionizing; radiation with lower-energy, such as infrared, visible light and radio waves, in contrast, are non-ionizing radiation. The range of wavelengths of UV radiation reaching the Earth's surface is from 290 to 400 nm which is shorter than the wavelength of visible light (from 400 to 700 nm) (Gascn *et al.*, 1995). UV-C consists of wavelengths shorter than 286 nm which means that it does not reach the Earth's surface.

Extreme ionizing-radiation resistance has been reported in members of the domains Archaea and Bacteria. Of the genera containing ionizing-radiation-resistant microorganisms, *Deinococcus* and *Rubrobacter* show the highest resistance levels and all species of these two genera have been shown to be either UV radiation resistant or gamma radiation resistant or both (Yoshinaka *et al*., 1973; Suzuki *et al*., 1988; Murray 1992; Carreto *et al*., 1996; Battista 1997; Ferreira *et al*., 1997; Ferreira *et al*., 1999; Fredrickson *et al*., 2004; Suresh *et al*., 2004). The genus *Deinococcus*, which corresponds to a deeply branching lineage within the bacteria, comprises the following 11 described species, *D. frigens, D. geothermalis, D. grandis, D. indicus, D. marmoris, D. murrayi, D. proteolyticus, D. radiodurans, D. radiophilus, D. radiopugnans, and D. saxicola* (Brooks and Murray 1981; Oyaizu *et al.*, 1987; Ferreira *et al*., 1999; Hirsch *et al.*, 2004; Suresh *et al*., 2004). There are also some other ionizing radiation- resistant bacteria which have been isolated and described including species of the genera *Acinetobacter, Chroococcidiopsis, Hymenobacter, Kineococcus*, *Kocuria*, and *Methylobacterium* (Ito and Iizuka, 1971; Brooks and Murray, 1981; Green and

Bousfield, 1983; Nishimura *et al*., 1988; Grant and Patterson, 1989; Nishimura *et al*., 1994; Billi *et al*., 2000; Collins *et al*., 2000; Phillips *et al*., 2002). Moreover, hyperthermophilic euryarchaeote species of the genera Thermococcus and Pyrococcus have also been found to contain ionizing radiation resistant strains (DiRuggiero *et al*., 1997; Jolivet *et al*., 2004; Jolivet *et al*., 2003). Strains of the species *Acinetobacter radioresistens, Hymenobacter actinosclerus, Kineococcus radiotolerans, Methylobacterium radiotolerans, Pyrococcus furiosus, Pyrococcus abyssi, Thermococcus gammatolerans, Thermococcus marinus,* and *Thermococcus radiotolerans* (Ito and Iizuka, 1971; Grant and Patterson, 1989; DiRuggiero *et al*., 1997; Phillips *et al*., 2002; Jolivet *et al*., 2004; Jolivet *et al*., 2003) are less resistant than species of the genera *Deinococcus* and *Rubrobacter (*Yoshinaka *et al*., 1973; Murray, 1992; Carreto *et al*., 1996; Ferreira *et al*., 1997; Ferreira *et al*., 1999; Battista and Rainey, 2001;) but nevertheless survive exposure to much lower levels of radiation.

The origin of radiation resistance in these prokaryotes is not clear because it cannot be regarded as an adaptation to naturally occurring environmental radiation. This is because naturally occurring sources of ionizing radiation, particularly UV-C, on Earth occur at very low levels (Scott, 1983; Karam and Leslie, 1999), making it unlikely that exposure has led to acquired resistance.

Many of the environments from which radiation-resistant microorganisms have been isolated can be considered to be desiccated or dried and it has been reported that many of these microorganisms are also resistant to desiccation (Maxcy and Rowley, 1978; Sanders and Maxcy, 1979; Dose *et al*., 1992; Murray, 1992; Mattimore and Battista, 1996; Billi *et al*., 2000). As a result, it has been suggested that the ability of organisms to resist ionizing radiation is linked to desiccation resistance, rather than radiation exposure.

6.2 Materials and Methods

6.2.1 Sample description and sampling sites

Soil samples were collected from the UK (a garden soil, an agricultural soil (previous crop wheat); soils from under oak (*Quercus robur*), beech (*Fagus silvaticus*), sycamore (*Acer pseudoplatnaus),* and pine (*Pinus* sp), and a saline soil and a desert soil from Oman. In addition to other environmental samples were: stratosphere (as described by Wainwright *et al*., 2003), rain and hailstones (collected from the roof of Firth Court, University of Sheffield), a building surface sample exposed to high levels of sunshine in Oman and a seawater sample from the Oman Gulf. Soil samples, arid and non-arid, were collected using a sterile scoop and were stored at the ambient temperature until they were processed.

6.2.2 UV exposure and culturing methods

The UV-C exposure apparatus (Figure 6.1) consisted of the sample holder at 12 cm above which was suspended the UV lamp which worked as the source of UV-C, all these contents were placed inside the laboratory hood. The 8 W UV was provided by a commercial low-pressure mercury lamp (model UVGL-25; UV Products, San Gabriel, CA) (Appendix E). The inside of the hood was sterilized, including the air, using the built in UV light and also by leaving the UV-C lamp switched on for a period of 2 hours prior to sample exposure. Soil samples (1g) were suspended in 10ml autoclave-sterilised deionised water in a sterile container and shaken for 1 hour. Inorganic particles were then removed from the filtrate by passing the extracts through a sterile micropore filter (0.45 micron).

Aliquots of the soil suspension (10 µl) were then aseptically transferred onto the surface of a sterile, UV transparent, fused quartz or silica quartz glass cover slip (2.5cm × 2.5cm), allowed to air dry and then exposed to UV-C. In the case of hailstones (after thawing) and rain water, the sample (0.45 micron filtered, 10 µl) was directly applied to

the cover slip. The sample on the cover slip was then exposed both sides to UV-C for 20 minutes which correspond to a total UV dose of 19230 J m^2. After the exposure the cover slip was aseptically transferred to 50 ml nutrient liquid media and incubated overnight at 25^{o}C. Where bacterial growth occurred a sample was then streaked onto nutrient agar media and incubated overnight at 25^{o}C.

A sterile fused quartz cover slip without a sample was exposed, as a control, to show that the tested cover slips were not contaminated with UV-C resistant bacteria from the laboratory air or the experiment. A second control consisted of a separate fused quartz cover slip on which a bacterial culture of *E. coli* was placed which was determined before the experiment to be killed by UV-C after 10 minutes of exposure on both sides of the cover slip to show that the UV lamp was effectively producing sterilising UV-C. In addition to these two controls, the J-225 meter (Figure 6.1) was used to demonstrate that the sample was receiving UV with wavelength of 254nm radiation over the entire exposure period. The resistant bacterial isolates were independently identified using 16S rRNA (Section 6.2.8).

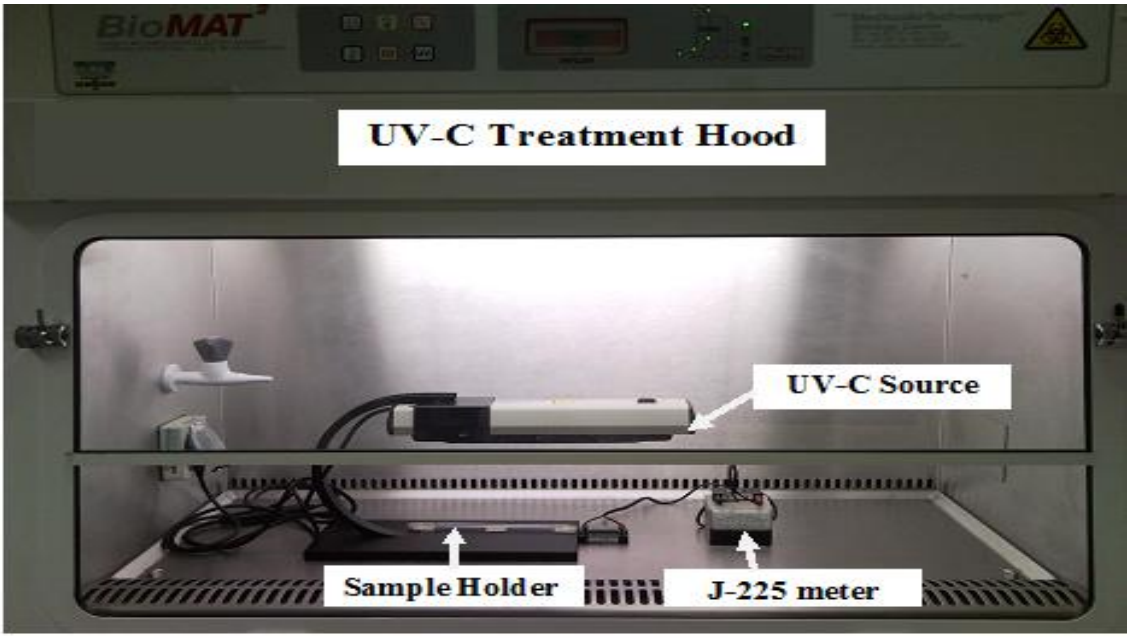


Figure 6.1 UV exposure hood with the shortwave UV (254nm) source, samples holder, which is positioned 12 cm way from UV source, and the J-225 meter (UV-C meter).

6.2.3 Conformation of the UV-C resistance of the isolates

The isolates were grown in LB liquid media (overnight at 25^{o}C). The resultant cultures were then serially diluted in sterile, distilled water (sdH_2O) and an aliquot was transferred to LB liquid medium which was incubated overnight at 25^{o}C. Samples of the dilutions in which no bacterial growth was obvious by eye were then placed on an autoclave-sterilized quartz cover slip and examined under the microscope (x100, oil). A dilution was the chosen in which the bacterial cells were shown not to form clumps, or overlay one another and a sample was transferred to a sterile fused quartz cover slip. The cover slip was exposed to UV-C radiation as described above for the different environmental samples. After 20 minutes both sides UV-C exposure, the quartz cover slip was transferred to sterile distilled water and shaken. The water was then plated onto LB agar media and evidence of growth of only the bacterium used was checked. This experiment was repeated three times and bacteria on cover slips which were not exposed to UV-C were included as controls.

6.2.4 Pure cultures and Gram staining

When growth was observed by turbidity, cultures were spread or streaked on the same medium containing 1.5% agar. Petri dishes were incubated at 25°C for 24 hours and pure cultures were obtained by serial plating and repeated sub-culture on the same agar medium plates. As described in Chapter 2 (Section 2.2.2.2), single colonies were picked off and streaked onto a fresh plate in order to obtain pure culture. Pure culture was obtained by serial plating and repeated sub-culture on the same agar medium plates which then was confirmed by Gram staining.

Gram staining was carried out on each strain, in order to characterize them prior to identification. The staining procedure was carried out as the following; 1µl of the each culture was used to prepare a smear on a microscope slide with a droplet of water. The procedure was conducted as described in Chapter 2 (Section 2.2.4).

6.2.5 Total count (before/after) UV exposure

The number of colony forming units was determined as a measure of the number of culturable microorganisms (viable) present in the solutions obtained from various UV-exposed surfaces. Samples were serially diluted on sterile Plate Count Medium in order to obtain between 30 and 300 colonies per plate. The sample (0.1 ml) was then aseptically transferred to the surface of a sterile fused quartz silica quartz glass cover slip (2.5cm×2.5cm), allowed to air dry and then exposed to UV-C. The exposed cover slip was then washed with 0.9 ml of autoclaved H_2O and then the resultant aliquot was plated onto plate count agar (PCA) (Difco). Also samples, 0.1 ml were directly spread on agar plates without exposing them to UV-C but with addition of 0.9 ml of sdH_2O. Triplicate samples were plated in agar media and colonies were counted after 24 hours of incubation at 25^{o}C. The results are expressed as number of colony forming units (cfu), (more detailed methodology presented in Chapter 2, Section 2.2.3).

This method provided information regarding the numbers of microorganisms that were capable of growing on this media after UV-C exposure in addition to the total count before radiation exposure.

6.2.6 Live/Dead staining

Bacterial cells of various samples materials were stained using the (LIVE/DEAD *Bac*Light Bacterial Viability Kit L7007, Molecular Probes Invitrogen, UK) (Biggerstaff *et al*., 2006). The staining procedure was performed as stated by manufacturer (Appendix H). 3 µl of the dye mixture was directly applied to the slide with bacterial cells and incubated in a dark place at room temperature for 15 minutes. The slide was then covered with cover slips, and observed with Nikon SMZ1500 microscope (Nikon Corporation, Tokyo, Japan) equipped with epi-fluorescence attachment, using an oil immersion lens, giving a total magnification of x100. Living bacteria are fluorescent green, whereas dead cells are stained fluorescent red.

6.2.7 Determination of desiccation effect on the UV resistance

In order to determine the survival of dried isolates following exposure to doses of UV-C radiation, *E.coli* 0127, *Streptococcus thermophilus* 10387, *Bacillus subtillis* 3106, *Serratia marcescens* 1981 and *Streptococcus viridochromogenes* were tested. All these strains were freeze dried. In the freeze dried state, each strain was sieved using a sterile 25µm aperture stainless steel sieve, Figure 6.2, (FB68870 - Sieve 100mm diameter, FisherScientific, UK) and were then aseptically transferred to the silica quartz glass cover slip (2.5cm×2.5cm).

The cover slip was then exposed to UV-C for 1-2 hrs from both sides. After the exposure, the cover slip was then aseptically transferred to 50 ml of nutrient liquid media and incubated overnight at 25^{o}C; each strain was also checked for growth in nutrient media without UV exposure. Where bacterial growth occurred a sample was then transferred to nutrient agar and incubated at 25^{o}C for 24hrs. The strains were independently identified using 16S rRNA to confirm the presence of the tested strain and not contamination.

Figure 6.2 Sieve 100mm diameter stainless steel 25µm aperture.

6.2.8 Molecular identification techniques

Extraction of genomic DNA for 16S rRNA gene sequence was carried out using a KeyPrep bacterial DNA extraction kit, PCR amplification of the 16S rRNA gene, and sequencing of the purified PCR products was carried out as described in Chapter 2 (Section 2.2.7). The purified reaction mixtures were electrophoresed in 1% agarose gels (2.2.7.2). The identity of the 16S rRNA gene sequences examined in this study was determined using the BLASTN facility at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST/). Sequences were then aligned with representative reference sequences of members of the lineage to which the BLAST search data assigned them. (see Chapter 2, Section 2.2.7.5 for details).

6.3 Results and discussion

6.3.1 The isolation of UV-C resistant bacteria from environmental samples

The isolation of ionizing resistant bacteria, particularly UV-C, was conducted using a small volume of soil-water extract, rain or hailstone melt-water dried onto a fused quartz glass cover slip which was alternatively exposed to 20 minutes of UV-C light from both sides; the UV permeability of the quartz glass meant that the bacteria in the water extracts received exposure to UV-C from both sides of the cover slip, thus ensuring complete exposure of any bacteria in the air dried sample to UV-C. This is an unusual approach to exposing bacteria to UV as most studies on the effects UV on bacteria have involved isolating bacteria from the environment straight away onto nutrient-rich agar medium and exposing them under these conditions to UV while growing on a bacterial growth medium. No UV-C resistant bacteria were isolated, from the first control cover slip, lacking a sample, and the *E. coli* culture used in the second control was killed after 10 minutes exposure, showing that the UV-C lamp was working properly. The experimental isolation approach used in this study has the advantage that the bacteria were exposed to UV while they were still in their natural

oligtrophic growth state, so that their normal physiology has not been altered, before exposure to UV by growth on rich nutrient media. Only the desert soil (sampled near Nizwa city, Oman), using this approach, yielded a UV-C resistant isolate and this was isolated as the spore forming bacterium, *Paenibacillus ehimensis* and archaea from desert sand (Waheeba sands, Oman) and desert varnish (sampled near Ashikhara city, Oman) which can be considered to be hyper-arid environmental sample materials (Table 6.1). From the wide range of soils sampled from the UK, all of which contain large numbers of bacteria (ranging from $1x10^5$ to $1x10^8$ (as determined by dilution plating on Plate Count Agar), no UV-C resistant bacteria were isolated. The soil suspension exposed to UV-C contained a maximum of $1x10^6$ bacteria; of this number, none were resistant to UV-C radiation. However, from one of the three UK hailstone samples, a single UV-C resistant bacterium (*Staphylococcus sciuri sciuri*) was also isolated. In addition, two UV-C resistant isolates were obtained from Sheffield rain water; these were independently identified as isolates *Exiguobacterium sp* and *Bacillus pumilus* (Table 6.1). Finally, a single UV-C resistant strain was isolated from the stratosphere sample following exposure to UV-C; this was also identified as *B. pumilus*. The isolation of the stratosphere sample bacterium was done in a different laboratory used to analyse the soil sample, so the two isolates of *B. pumilus* were not the result of cross contamination. Table 6.2 and Figure 6.3 show the result of stratosphere isolate which has also been sent to NCIMB, Aberdeen for confirmation of the identity after being identified in this lab. No UV-C resistant bacteria were obtained from different seawater samples, from the hypersaline soil or from the building surface sample from Oman. It should be noted that only one sample was taken from each of these environments; such limitation of sample replication is not regarded as significantly important in this study because we are simply attempting to isolate bacteria tolerant to UV-C from the Earth environments. The fact that no such bacteria

were isolated from a single sample of such environments does not mean that they are absent; it may be that an extensive ecological examination of the environments studied here would produce UV-C resistant bacteria, other than the ones isolated here.

Table 6.1 UV-C resistant bacteria isolated from the environments studied.

Sources of samples	UV tolerant isolates	UV-C killing time (minutes)
Stratosphere	*B. pumilus*	>60
Rain	*B. pumilus* *Exiguobacterium sp.*	>45 >15
Hailstone	*Staphyloccus sciuri sciuri*	>30
Sea sample (Arabian Sea)	Nil	-
A building surface frequently exposed to sunlight (Oman)	Nil	-
UK soil samples (6 in total)	Nil	-
Desert soil (Oman)	*Paenibacillus ehimensis*	>30
Saline Soil (Oman)	Nil	
Desert varnish (Oman)	Archaea	>15
Desert sands (Oman)	Archaea	>30

The findings of this study show that UV-C resistant bacteria can be isolated from a narrow range of environments on the Earth studied here. Kuhlman *et al*. (2005) similarly reported the isolation of UV-C tolerant bacteria from a desert varnish.

Our null hypothesis stated that such UV-C resistant bacteria which are isolated from Earth environments must have originated from space or the stratosphere, because UV-C is not known to reach the surface of the Earth, these are the only available regions where a selective pressure would produce UV-C resistant bacteria. The most resistant isolate was obtained from the stratosphere which survived 60 min of UV-C

exposure (Table 6.1). The new domain Archaea isolate also showed the ability to resist UV-C in this case from 15 to 30 min.

6.3.2 Analysis of cultures and isolates using molecular identification techniques

A group of five UV-C radiation-resistant bacteria and two archaea were recovered in this study. Partial 16S rRNA gene sequences were determined for these isolates recovered from different environmental samples including arid soil and rock samples. Comparison of these 16S rRNA gene sequences to the public databases using the BLASTN facility (www.ncbi.nlm.nih) made it possible to assign each isolate to a taxonomic group at the family or genus level and in some cases at the species level.

Complete sequences were determined for representative isolates; as a result phylogenetic dendrograms could be made and constructed. Since the microorganisms, particularly archaea and bacteria that survived UV-C radiation were of special interest, a number of isolates sequenced were isolates recovered from the soil sample after exposed to UV-C radiation. The seven isolates whose taxonomic identities were determined based on partial 16S rRNA gene sequence comparisons (800 nucleotide positions) fell into different taxonomic groups based on their closest relatives (Table 6.2).

Some isolates were assigned to taxonomic groups that have previously been shown to contain UV radiation-resistant bacteria. It is noteworthy that three of the four UV-C resistant bacteria were isolated from environments associated with the atmosphere i.e. rain, hail and the stratosphere. *B. pumilus* has been found to be highly resistant to UV in clean rooms (Link *et al.*, 2004, La Duc *et al*., 2007) and also in space (Newcombe *et al.*, 2005) in addition to the stratosphere (Shivaji *et al*., 2006). The isolation of a species of UV-C resistant *Staphylococcus* might at first sight appear strange, since this property is not generally associated with members of this genus. However, it is noteworthy that staphylococci have been isolated from the stratosphere (Wainwright *et al.*, 2003). The

existence of UV-C resistant isolates which are non-spore forming bacteria is confirmed by the isolation of a species of *Exiguobacterium*. La Duc *et al.* (2007) isolated members of this genus from extreme clean rooms exposed to UV-C. The spore forming *Paenobacillus* isolate, however, is more readily acceptable as being UV-C resistant and its closeness to bacilli is emphasized by the fact that *Paenobacillus ehimensis* was initially referred to as *Bacillus ehimensis.*

The isolates recovered from the arid soil from different regions of Oman after UV-C irradiation doses were limited. 16S rRNA gene sequence analysis of these isolates showed that they are members of archaea and the genera *Bacillus*. These isolates were studied using both universal archaea and bacteria primers (Table 6.3). This study expands our knowledge of the diversity of UV-C radiation resistant bacteria. Maxcy and Rowley (1978) were the first researchers to report experimental evidence that there could be a link between the ability of bacteria to survive dehydration and ionizing-radiation resistance. They demonstrated that by selecting for desiccation tolerance in natural microflora, it was possible to simultaneously isolate radiation resistant species. Subsequently, detailed evaluations of members of the radiation resistant bacteria supported the notion that these phenotypes could be interrelated by demonstrating that ionizing radiation and desiccation can cause similar forms of DNA damage (Mattimore and Battista, 1996; Billi *et al.*, 2000) and that the loss of DNA repair capacity in a radiation-resistant species can result in a strain that is no longer competent to survive dehydration (Battista, 1996).

Table 6.2 The top ten highest identities confirmed stratosphere isolate 16S rRNA by NCIMB, Aberdeen.

STRAT1 TOP 10 HITS MicroSeq™ 500

Sequence Name	% Match	Sequence Name	% Match
Bacillus pumilus	100	*Bacillus subtilis spizizenii*	94.04
Bacillus atrophaeus	94.26	*Bacillus subtilis spizizenii*	93.94
Bacillus vallismortis	94.26	*Bacillus subtilis subtilis*	93.94
Bacillus amyloliquefaciens	94.25	*Bacillus mojavensis*	93.73
Bacillus benzoevorans	94.23	*Bacillus licheniformis*	92.72

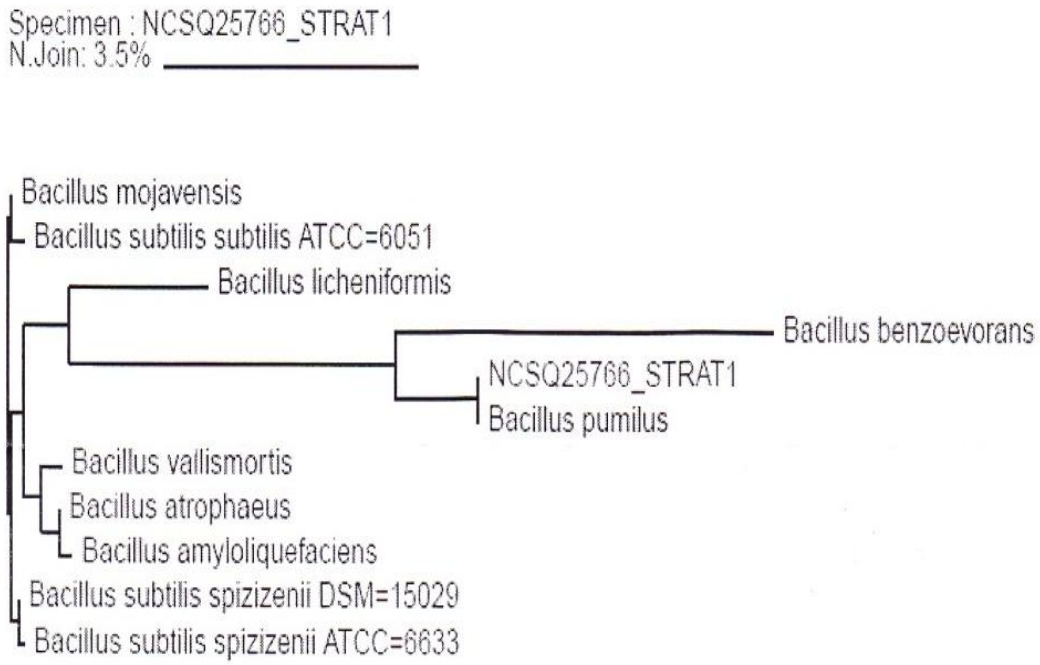


Figure 6.3 The phylogenetic tree of stratosphere isolate done at NCIMB, Aberdeen.

6.3.3 Total count before/after UV exposure

The non-irradiated samples contained bacterial concentrations ranged from 3.7 x 10^5 to 8.1 x 10^5 CFU/ml. In contrast, the plates containing the irradiated samples ranged

from 1.1 x 10^5 to 7.8 x 10^5 CFU/ml. There was no significant decrease in the number of CFU/ml recovered from each isolate after irradiation with UV-C, and this was observed for most of strains (Table 6.3). It should be noted that the values below 3.0 x 10^5 CFU/ml derived from less than 30 colonies on an agar surface which were obtained for samples exposed to UV-C are outside the statistical limits of the dilution plating technique used but still does not provide an indication of the decrease in the number of colonies with UV-C radiation dose.

The data demonstrated that there are microorganisms in natural environmental samples, such as rain, hailstone and the arid samples examined here, that comprise populations of UV-C radiation resistant bacteria. These organisms are resistant to levels of radiation that far exceed the background levels in the natural environment.

Table 6.3 Total count of radiation resistant bacteria isolated from the environments studied before and after UV-C exposure.

Sources of samples	**UV tolerant isolates**	**C.F.U. of UV resistant**	
		Before UV-C exposure	**After UV-C exposure**
Stratosphere	*B. pumilus*	8.1 x 10^5	7.8 x 10^5
Rain	*B. pumilus* *Exiguobacterium sp.*	5.4 x 10^5 3.7 x 10^5	2.2 x 10^5 1.1 x 10^5
Hailstone	*Staphyloccus sciuri sciuri*	6.8 x 10^5	2.5 x 10^5
Desert soil (Oman)	*Paenibacillus ehimensis*	5.6 x 10^5	4.7 x 10^5
Desert varnish	Archaea	7.3 x 10^5	6.2 x 10^5
Desert sands	Archaea	5.7 x 10^5	4.9 x 10^5

6.3.4 The potential problem of shading

It is of interest to discuss the origin of the UV-C resistant bacteria isolated in these studies. However, before that, it is necessary to discuss a potential procedural problem, namely shading, a factor which may have had a major influence on the results. Samples of environmental materials obviously contain a wide range of particulates which could shade microorganisms, particularly bacteria, from the UV-C applied here. In a similar way, bacteria may be protected from getting irradiated by UV wavelengths by forming clumps, the innermost of which are not exposed to UV radiation. This problem, which is often overlooked in studies in which the effect of UV on bacteria is examined, was avoided by a) filtering out all particles from the soil suspension, rain water and other sample materials prior to exposure to UV radiation, b) applying of a small volume of liquid on the fused quartz cover slip which was allowed to naturally dry to form a very thin layer, and c) by exposing this sample layer to UV radiation from two sides, above and below (i.e. through the UV-C transparent fused quartz glass cover slip used in this study). Finally, d) cultures of the putative UV-C resistant strains were exposed to at least 40 minutes exposure to UV-C radiation and their resistance to this radiation was confirmed by culturing them onto LB liquid medium; growth occurred in the non-exposed cultures, but not in those cultures exposed to UV-C. In addition to these shading control measures, UV-C resistant strain isolated from stratosphere was exposed to at 20 minutes exposure to UV-C radiation and stained with LIVE/DEAD *Bac*Light bacterial viability stain and observed under the fluorescent microscope. Figure 6.4 shows that far fewer dead cells (which stain red) were present compared to the high number of living cells (staining green). This means there was no potential shading otherwise we will find a number of dead cells (red colour) have shaded the live cells (green colour). As a result, it is concluded that shading did not produce false positive and that the putative UV-C isolates were indeed resistant to UV-C.

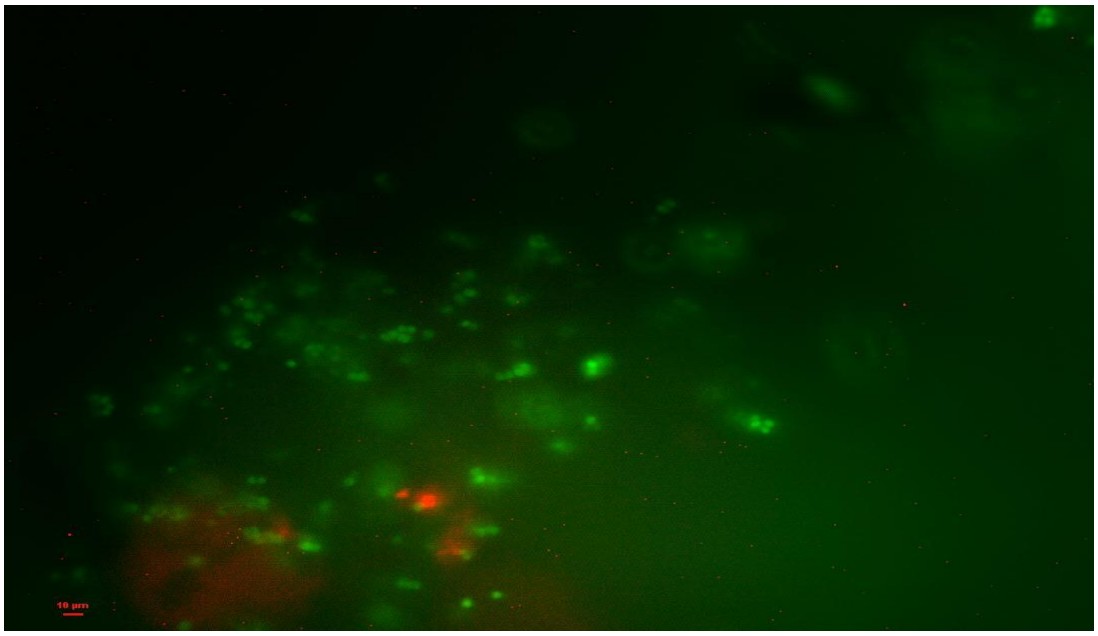


Figure 6.4 Live and dead *B. pumilus* cells as seen under the fluorescent microscope after 60 mins UV-C exposure and using (live/dead) bacterial stain.

6.3.5 Determination of levels of ionizing-radiation and desiccation resistance.

Arid lands of different degrees cover more than 35% of the Earth's land surface and can be considered to represent natural environments that are desiccated (McGinnies, 1988). Little is known about the microbial diversity of hyperarid or arid environments, and there are no data on the abundance or diversity of UV-C radiation-resistant microorganisms in these habitats.

In an attempt to correlate the possible link between desiccation resistance and ionizing-radiation resistance, dried strains including; *E.coli* 0127, *Streptococcus thermophilus* 10387, *Bacillus subtilus* 3106, *Serratia marcescens* 1981 and *Streptococcus viridochromogenes* were sieved by 25µm aperture sieve and exposed to UV-C radiation. The majority of the dried strains tested did not grow on nutrient agar or liquid medium. *Streptococcus thermophilus* was the only bacterium which exhibited resistant to UV-C radiation. Due to difficulties in using standard bacterial isolation procedures, media, such as lactose medium, were used which were better suited for the

isolation of the tested strains. Portions of dried tested strains were grown before exposure in order to check the ease of resuscitating freeze-dried bacteria.

Beside the isolation of UV-C radiation-resistant bacteria from arid samples, this study provided further insight into dried UV radiation-resistant microorganisms.

6.4 Conclusions

A paradox immediately arises however, due to the fact that vast amounts of cosmic dust is continually reaching the Earth's surface, and based on the theory of neopanspermia a significant proportion of the materials will consist of microorganisms, notably bacteria. Such bacteria might be expected to have probably been exposed to high dose of UV-C radiation and, as a result, UV-C resistant bacteria should be common on the Earth and should be readily isolated from all different environments. The fact that UV-C radiation resistant bacteria are not ubiquitous on the Earth can be explained by the fact that any bacterium reaching the Earth from space will have been exposed to a vast amounts of environmental insults, such as freezing desiccation and only a few will be have survived the journey to the Earth. In addition, as it has been reported in this study and it is known from laboratory studies that it is difficult to resuscitate freeze-dried bacteria using standard bacterial isolation procedures, so that space-derived, and thereby freeze dried, bacteria would not be readily isolated when using the standard isolation techniques used in this study. It is well known that only a small fraction of the viable bacteria present on the Earth can be isolated using the techniques in current use; the possibility exists that such viable, but apparently unculturable bacteria originate from space and the fact that they are difficult to culture is due to them having survived the extreme rigours of the space environment. The results show that UV-C resistant bacteria do exist on the Earth and these isolated bacteria may have come or originated from the UV-C rich space environment.

There exists however, a UV-C rich environment which is nearer to the Earth surface than deep space, namely the stratosphere (at a height of 41km) (Wainwright, 2008) and resistant bacteria to UV-radiation have been isolated from this region (Yang *et al*., 2008). While bacteria have been isolated from the layer of stratosphere, it is possible that the resistant bacteria to UV-C which have isolated in this study on the Earth originated from this region. Although, it is still difficult to explain how bacteria might arrive in the high stratosphere, it is likely that some do reach the lower, UV radiation-rich areas of this region (Wainwright, 2008). It has been suggested by Wainwright (2008) that the layer of stratosphere may act as a massive natural UV "mutation laboratory" in which bacteria from the Earth which are normally protected from exposure to UV-C by the Earth's atmosphere can be exposed to all different forms of UV radiations. Mutations emerging from such ionizing radiation exposure may then have played an important role in bacterial evolution and in the evolution of life in general. As a result, the UV-C resistant bacteria isolated on the Earth in this and other studies (such as desert varnish by Kuhlman *et al*., 2005), may therefore have originated from the Earth–derived bacteria which have been transported to the low stratosphere and here exposed to the selection pressure of UV-C, before being returned to the Earth under the effect of gravity.

An alternative explanation is that the UV-C resistant bacteria isolated in this study were produced as an incidental result of the bacterium acquiring resistance to other selection pressures either in the Earth or the top layer of space, such as desiccation as is seen for the development of resistance by bacteria to ionizing radiation (Mattimore and Battista, 1996). However, if this is the case, it begs the question-why are not UV-C resistant bacteria readily isolated and extensively distributed over the Earth's deserts or rocks and other desiccated regions? It has been suggested that DNA repair mechanisms may have evolved not to counter the damage of ionizing radiation but rather to

compensate for desiccation, another naturally occurring stress that generates a pattern of DNA damage similar to that produced by ionizing radiation (Mattimore and Battista, 1996). The process of desiccation is inherently DNA damaging and results in DNA double-strand breaks (Dose *et al*., 1992; Mattimore and Battista, 1996), the primary lethal lesions resulting from exposure to ionizing radiation, and it is assumed that desiccation-tolerant species, as well as ionizing-radiation-resistant species, can avoid or effectively repair these lesions.

Finally, the small number of UV-C resistant isolates obtained here might have been produced by other selection pressure on the Earth imposed by the industrial use of UV-C as a sterilizing agent in for example hospitals (Andersen *et al*., 2006).; a prosaic source of such UV-C resistant bacteria.

CHAPTER 5

OSMO-ADAPTATION MECHANISMS OF ALKALITOLERANT AND ALKALIPHILIC BACTERIA AND ARCHAEA ISOLATED FROM ALKALINE SAMPLES

5.1 Introduction

Extremely alkaliphilic bacteria, mainly genera of *Bacillus* species, are the most broadly characterized bacteria that grow optimally at pH 10 and above. Numerous mechanisms have been suggested to explain alkaliphily, including elevation of cytoplasmic buffering capacity at highly alkaline growth pH (Krulwich *et al*., 1985a) and the production of cell wall macro-molecules which afford a special barrier to the flux of relevant ions (Aono and Ohtani, 1990; Ito *et al*., 1994; Aono *et al*., 1995). Other suggested mechanisms include the use of Na^+/H^+ antiporters that, energatical by the primary electrochemical proton gradient (Δp) (i.e. respiration or the proton-translocating F_1F_O-ATPase), catalyze net proton accumulation (Krulwich, 1995). It is not clear whether the cytoplasmic buffers, cell wall components or antiporters used by alkaliphiles are essentially the same as those used in neutralophiles, or whether, being present in larger amounts, they are necessary to meet the demands of alkaliphily.

The relative importances of the various mechanisms which are used in cytoplasmic pH regulation have yet to be determined. However, from non-alkaliphilic mutant strains and studies of the antiporter-related Na^+ -dependence of alkaliphily, there is compelling evidence that active mechanisms are involved (Krulwich *et al*., 1996; Kitada *et al*., 1994). There is also evidence that at least some of the other mechanisms are involved and even essential (Aono and Ohtani, 1994). It seems, for example, that passive mechanisms are more important than active ones when alkaliphilic bacteria are growing on fermentative growth substrates.

The work reported in this Chapter focuses on the question-how do presumptive

alkalitolerant and alkaliphiles survive in such alkaline as well as low pH conditions.

5.2 Materials and Methods

5.2.1 Identification and quantification of intracellular organic solutes by NMR spectroscopy

As described previously in Chapter 3 (Section 2.3.1) 5.0 ml sample of each cell culture was harvested at 5000 xg for 10 min and the supernatant was discarded. Organic solutes from the cell pellets were extracted by sonicating (2 x20 sec) at 15 amplitude microns, then centrifuged and freeze dried. Dried material was subsequently dissolved in 500 µl of D_2O. Analysis of the organic solutes present in the bacterial isolates was carried out using ^{1}H and ^{13}C NMR on total extracts in D_2O. NMR spectra were recorded with Bruker DRX-500 spectrometer operating at 500 MHz for 1H, 125.8 MHz for ^{1}H-^{1}H HSQC (Cummings, 1993) and a Bruker Bruker DRX-600 at 600 MHz for two dimension. (see Chapter 3, (Section 3.2.1) for detailed information)

5.3 Results and Discussion

Three presumptive alkaliphilic and alkalitolerant bacteria were selected in the isolation procedures. Table 4.2 (Chapter 4, Section 4.3.2) provides a summary of the growth of these isolates in addition to Section 4.3.3. Table 5.1 lists the presumptive alkaliphilic isolates and shows the results of testing the ability of the isolates to tolerate high and neutral pH conditions.

Table 5.1 Selected alkaliphilic and alkalitolerant bacteria cultivated from alkaline environment to study the osmo-adaptation mechanisms.

Source	Source pH	Isolating pH	Representative sequence	Closest described microorganism
Redcar-Soil 1	12	12	RS12	(100%) *Alkalibacterium kapii*
		4.5	RS12-4	(95%) *Bacillus massiliensis*
Redcar-Root	12	4.5	RR12-4	(100%) *Lactococcus lactis*

5.3.1 Strategies for adapting to an alkaline environment

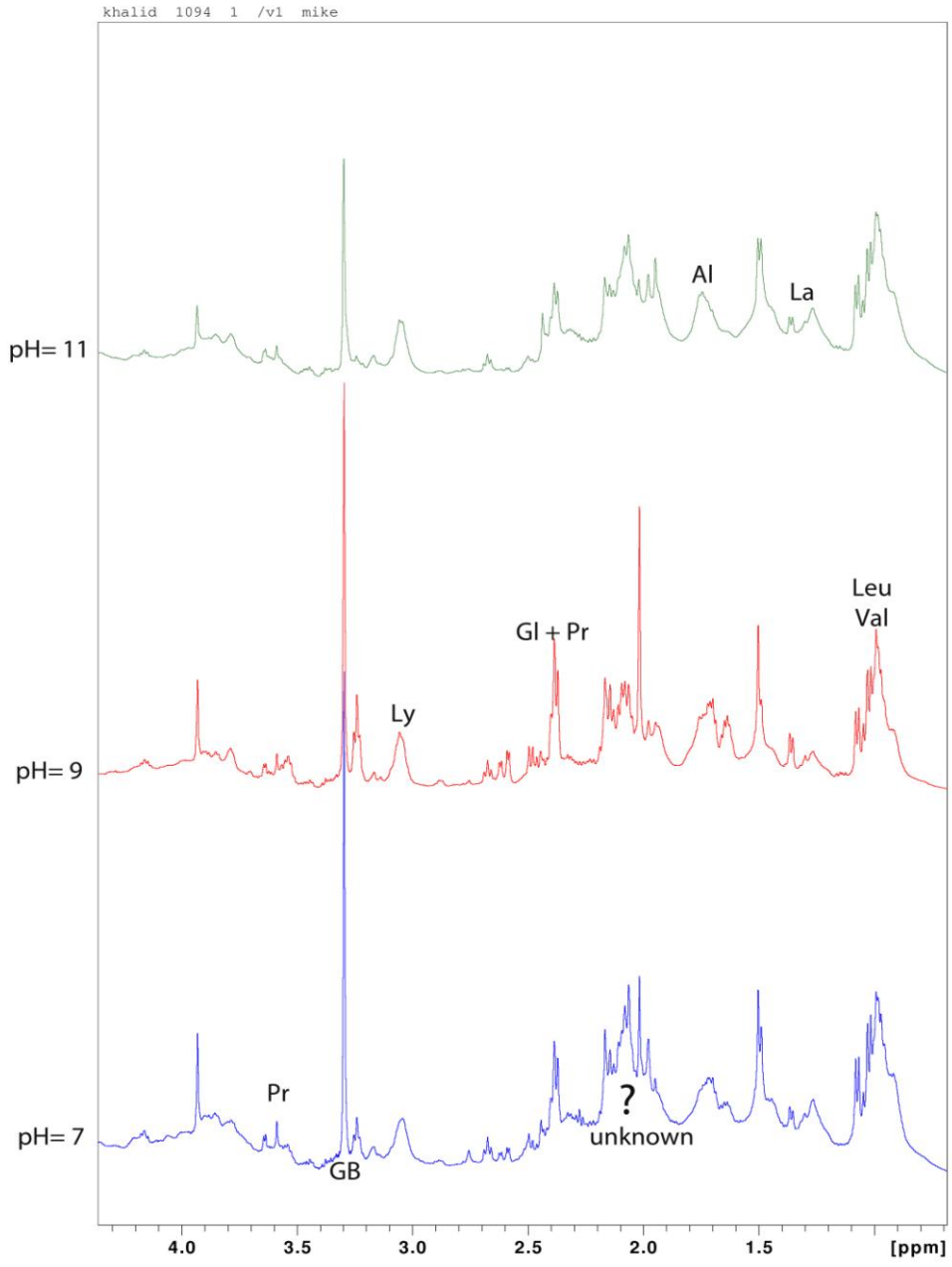
5.3.1.1 Osmoregulatory solutes in alkalitolerant and alkaliphilic isolates (accumulation of compatible solutes).

Not all the groups of alkaliphiles, particularly species of alkaliphilic *Bacillus*, have been examined for the occurrence and distribution of organic solutes. For example, no information is available on the composition and concentrations of intracellular solutes within the recently characterized *Alkalibacterium* species. Therefore, this study gives significant information on the osmoprotectants required by bacteria isolated from alkaline environments.

The main compatible solute in the *Alkalibacterium* species, RS12, was glycine betaine, the content of which increased when the pH value in the LB medium (Figure 5.1 (a)) was decreased or close to neutral. This strain behaves exactly as a strict alkaliphile which cannot tolerate neutral pH unless it generates a very high concentration of compatible solutes. Many different amino acids such as glutamate, proline and lysine were shown to be involved in osmoregulation of RS12 when strain growing in LB medium.

On the other hand, when this strain was grown in M9 minimal medium (Figure 5.1 (b)), glycine betaine was no longer the sole organic molecule. The concentration of this compound was very low (when grown at pH 11) and had no significant effect on the osmoregulatory mechanisms of the strict alkaliphile RS12. Since M9 minimal medium does not contain any betaine derivatives, this strain must accumulated glycine betaine following *de novo* synthesis

(a)


khalid 1094 1 /v1 mike
pH= 11
Al
La
pH= 9
Gl + Pr
Leu
Val
Ly
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Pr
GB
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2.5
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(b)

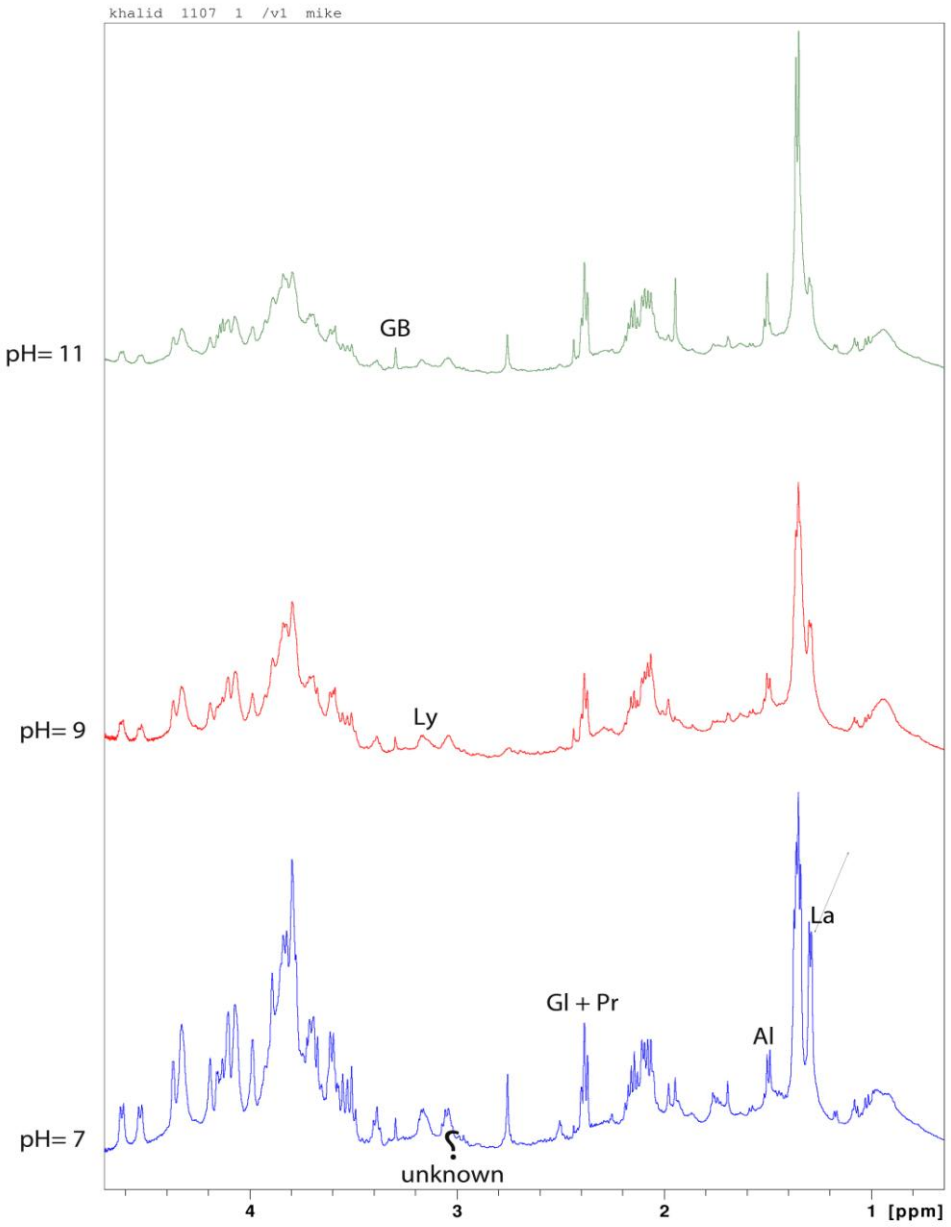
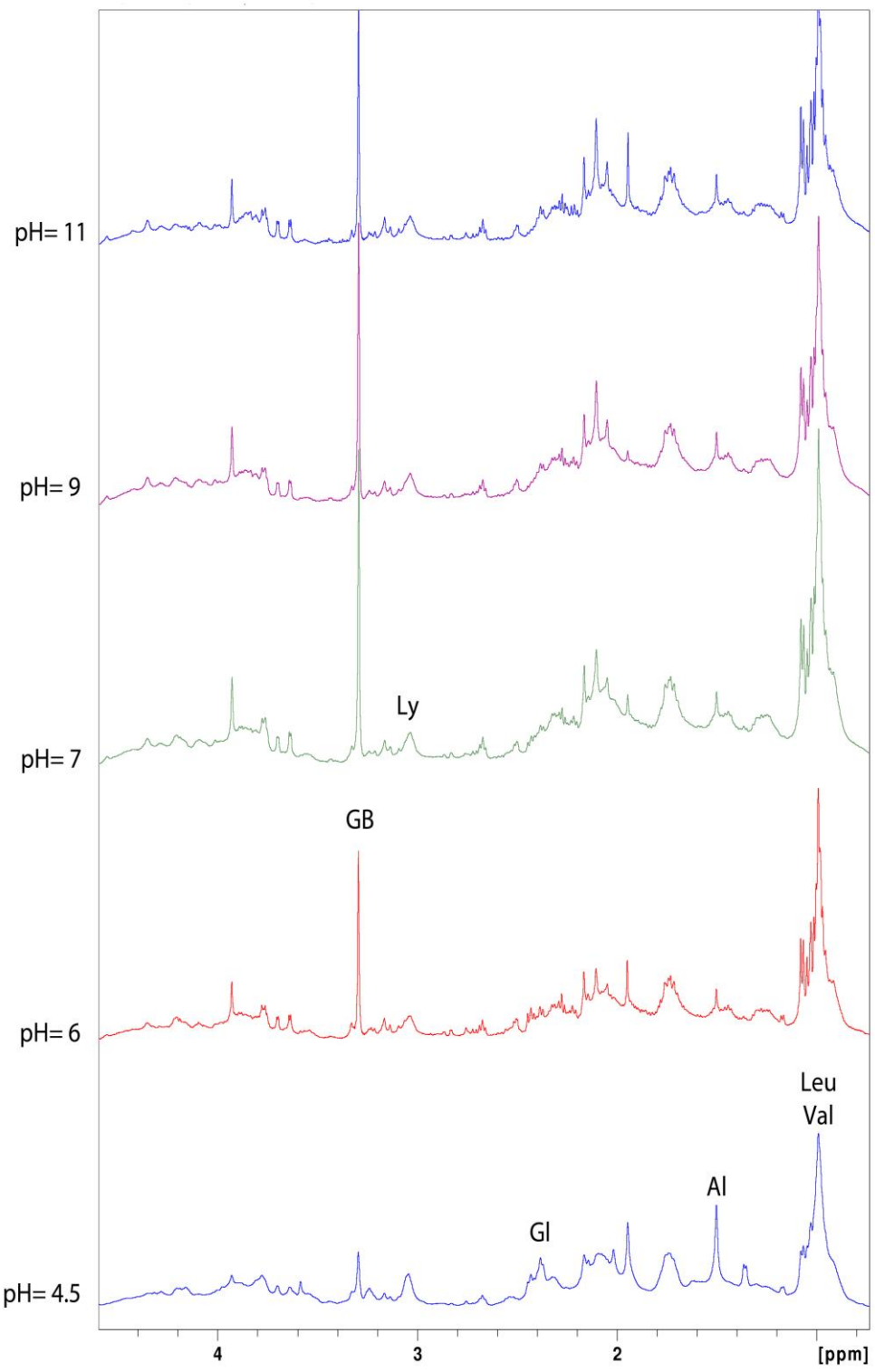


Figure 5.1 NMR ^{1}H spectra of cell extracts from RS12 strain at different values of pH; pH of 7.0, pH of 9.0 and pH of 11.0. Spectra are showing the fractions in (a) LB medium and (b) in M9 minimal medium. The ^{1}H spectra identified, GB; glycine betaine, Pr; proline, Gl; glutamate,; Ly; lysine, Al; alanine, La; lactate, Leu; leucine, Val; valine, as the most abundant compatible solutes.

Strain RR12-4 which was 100% similar to *Lactococcus lactis*, glycine betaine was again the main organic compatible solute when this strain grown in LB media as shown in Figure 5.2 (a). This Figure also shows that the minimum production of glycine betaine was at minimum when grown at pH value of 4.5, but other compatible solutes were accumulated at this pH value with glutamate and alanine increasing gradually until the highest at pH 11.0. These results suggest that RR12-4 was not stressed at pH value of 4.5, which tend to be more acidotolerant despite the fact that this isolate was obtained from plant roots growing in a soil with a pH of 12.0. *Lactococcus lactis* was also isolated from the plant roots grown in alkaline lake sediment, pH 12 and also from a soil sample at pH 8.6; the former isolate could grow at low pH, while the latter isolate could not.

However, this strain behaved completely different when grown in M9 minimal medium as shown in Figure 5.2 (b), glycine betaine was not the sole organic molecule at pH 6.0 and 7.0 whereas it was the main at pH value of 4.5 and again at pH 11.0. Other amino acids, such as glutamate and proline were involved in osmoregulation of this strain at pH 9.0. Peaks in Figure 5.2 (b) suggest that RR12-4 strain grown in M9 minimal medium was not stressed at pH value of 4.5 and 9.0 whereas they were stressed at neutral pH.

(a)



(b)

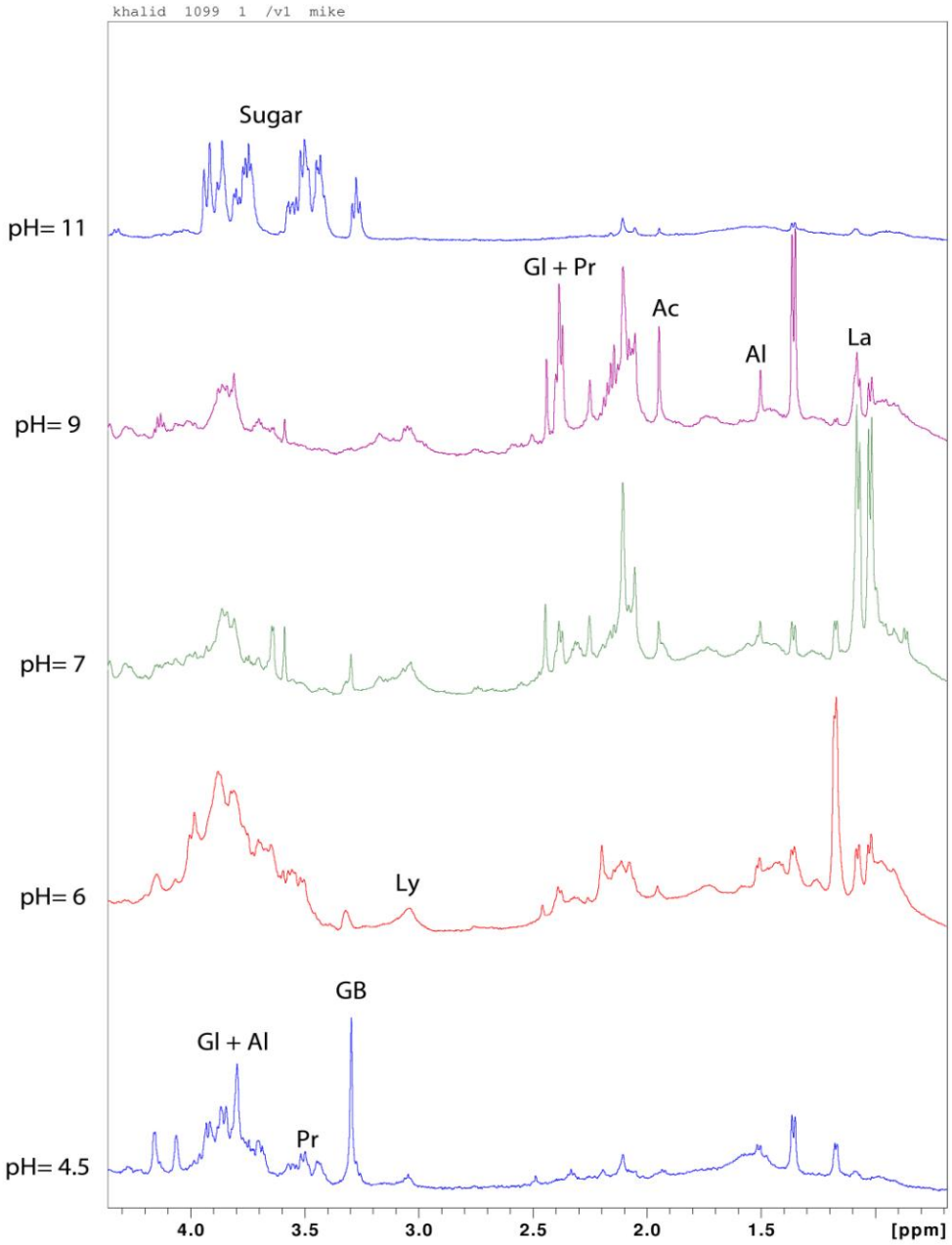
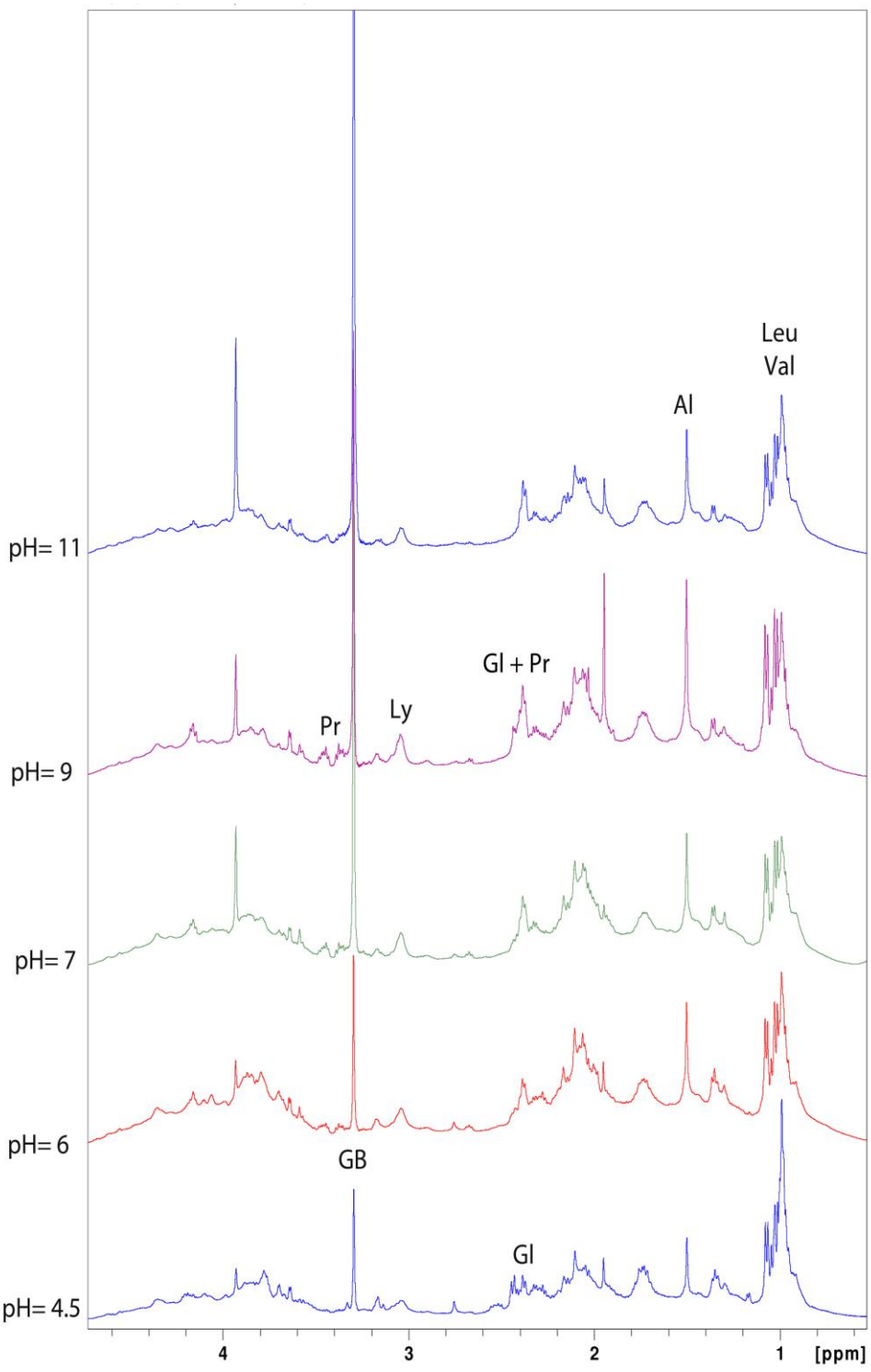


Figure 5.2 NMR ^{1}H spectra of cell extracts from RR12-4 strain at different values of pH; pH 4.5, pH of 6.0, pH of 7.0, pH of 9.0 and pH of 11.0. Spectra are showing the fractions in (a) LB medium and (b) in M9 minimal medium. The ^{1}H spectra identified, GB; glycine betaine, Pr; proline, Gl; glutamate, Ly; lysine, Ac;acetate, Al; alanine, La; lactate, Leu; leucine, Val; valine, as the most abundant compatible solutes.

Again, in RS12-4, which was 95% similar to *Bacillus massiliensis,* glycine betaine was the main organic compatible solute (when grown in LB medium) (Figure 5.3 (a)). As in RR12-4, the production of glycine betaine in RS12-4 strain was at a minimum when grown at pH value of 4.5 and kept increasing gradually until the highest pH of 11.0. These results suggest that RS12-4 was stressed as pH value increased from 4.5 onward, which shows that it probably prefers acidic condition although it was isolated from a very alkaline soil at pH 12.0. Again, this strain behaved completely differently when grown in M9 minimal medium as shown in Figure 5.3 (b); glycine betaine was not the main compatible solute at pH 6.0 and 7.0 whereas it was the main one produced at a pH value of 4.5 and again at pH 11.0. Unlike RR12-4, RS12-4 strain was less stressed at pH 11.0 than pH 4.5 when growing in M9 minimal medium**.** At pH 4.5, amino acids, such as proline, alanine and lactate, were involved in osmo-adaptation of RS12-4 strain. The NMR peaks in Figure 5.3 (b) suggesting that RS12-4 grown in M9 minimal medium is more likely to be an alkalitolerant strain.

(a)



(b)

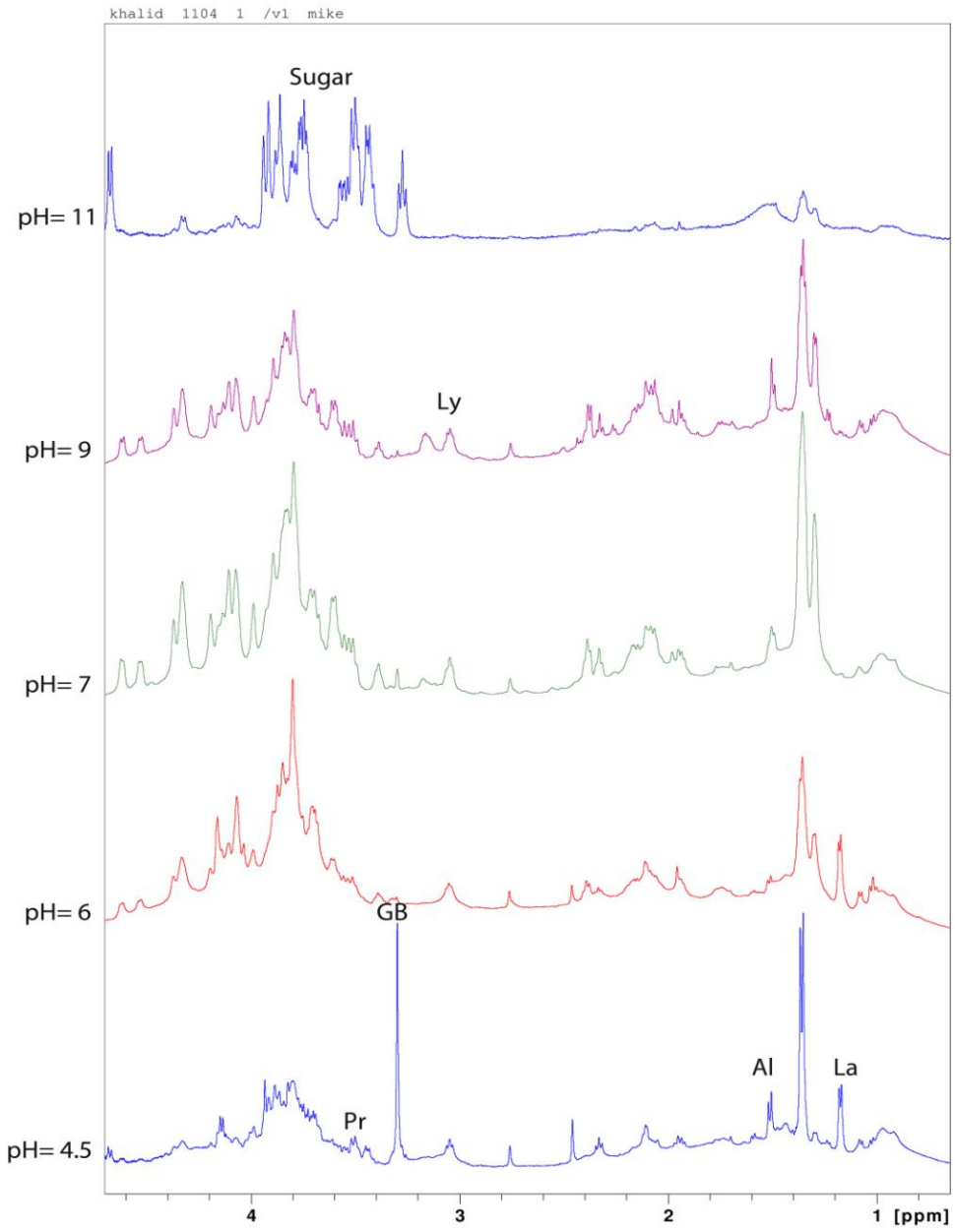


Figure 5.3 NMR ^{1}H spectra of cell extracts from RS12-4 at different values of pH; pH 4.5, pH of 6.0, pH of 7.0, pH of 9.0 and pH of 11.0. Spectra are showing the fractions in (a) LB medium and (b) in M9 minimal medium. The ^{1}H spectra identified, GB; glycine betaine, Pr; Proline, Gl; Glutamate, Ly; Lysine, Al; alanine, La; lactate, Leu; leucine, Val; valine, as the most abundant compatible solutes.

It has been reported that high concentrations of compatible solutes do not greatly interfere with normal enzymatic activity. Therefore, few adaptations of the cells proteomes are required. The list of organic compounds that have been shown to serve as osmotic solutes in alkaliphilic and haloakaliphiles microorganisms, prokaryotic as well as eukaryotic, is extensive. Most organic osmolytes are based on amino acids, as well as amino acid derivatives, sugars, or sugar alcohols (Galinski, 1995; Roberts, 2005; Oren, 2008). Analysis of intracellular compatible solutes in all three studied isolates grown in LB medium showed the presence of glycine betaine at a high and at a concentration specific to each salt concentration.

Extra spectra were run to determine the exact nature of the compounds, which proved worthwhile particularly in those signals which were present between chemical shifts. In the RS12-4 isolate (*Bacillus massiliensis*) grown in LB medium at pH 4.5, a ^{1}H-^{1}H 2D spectrum (Figure 5.4) was run to connect together ^{1}H signals within a series of 3-bond steps of each other (i.e., that are J-coupled). RS-12 (*Alkalibacterium kapii*) cells were grown at pH 9 in LB medium and another spectrum was run, i.e. C-H HSQC (Figure 5.5) to correlate ^{1}H chemical shifts with ^{13}C shifts and thereby provide a much more secure identification of compatible solutes present. However, from these two spectra it became clear there were osmolytes present that confirm NMR ^{1}H spectra for each strain, and correspond with the most common compatible solute identified in halotolerant organisms, i.e. glycine betaine and glutamate (Chapter 3).

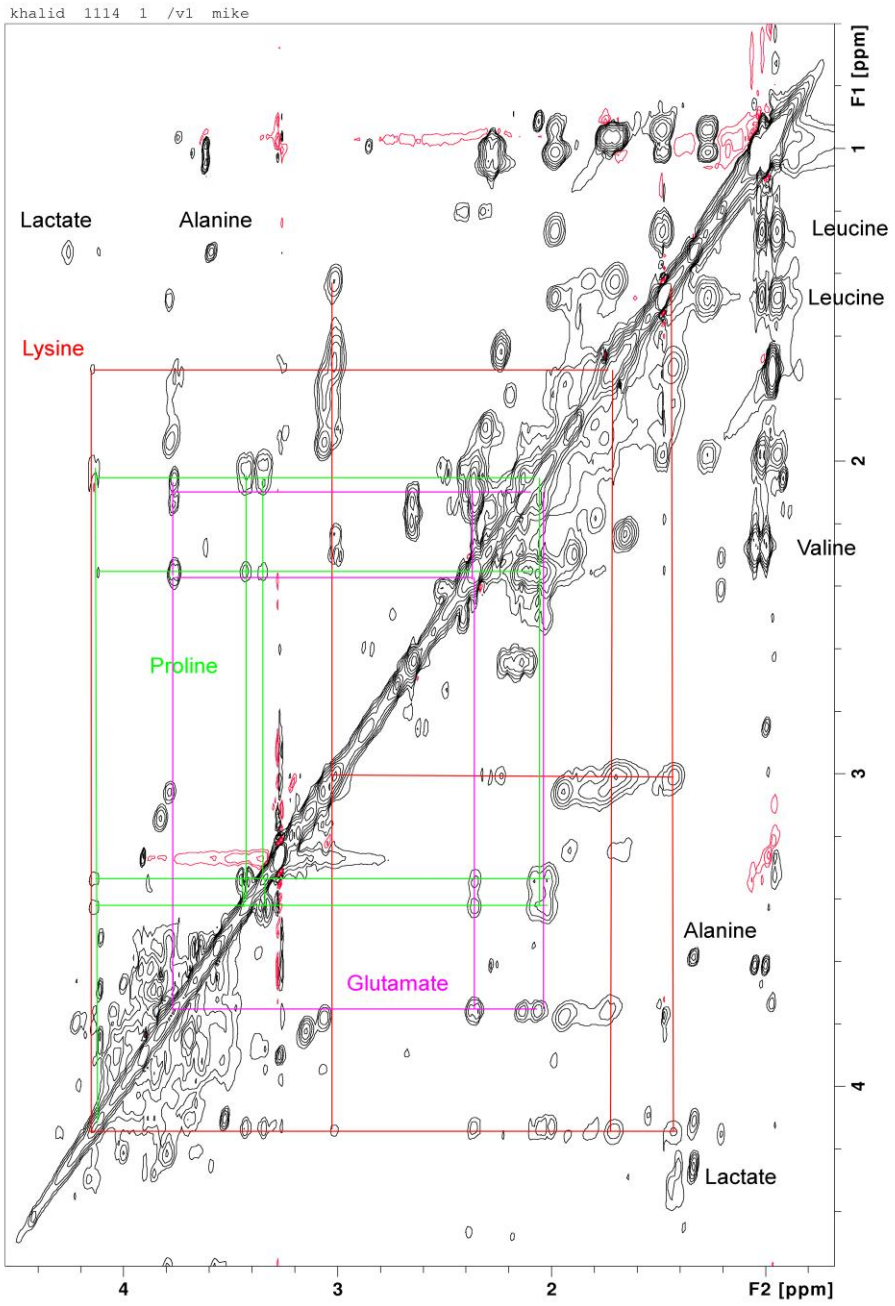


Figure 5.4 A 500 MHz ^{1}H-^{1}H 2D HSQC spectrum of cell extract from the RS12 cells grown at pH 9.0 in LB medium. The two dimensions are labeled with the chemical shift in ppm from TSP.

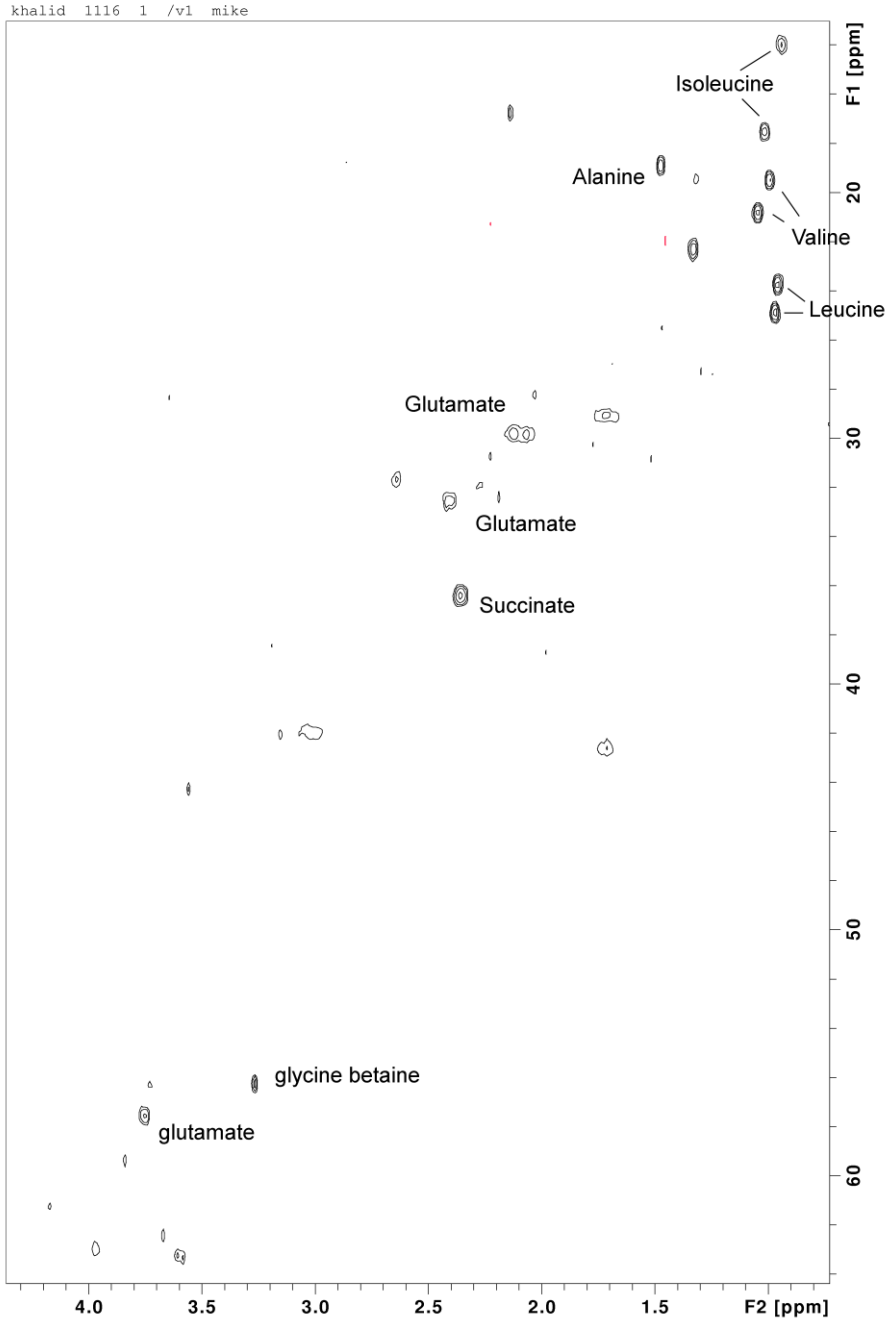


Figure 5.5 2D C-H HSQC spectrum of cell extract from the RS12-4 cells grown at pH 4.5 LB medium. This correlates ^{1}H chemical shifts with ^{13}C shifts and therefore gives a much more secure identification.

5.4 Conclusions

Soda lakes are well known to represent extreme environments characterized by alkaline pH (9–11) and moderate to high concentrations of salts such as Na_2CO_3/$NaHCO_3$. The availability of these sodium carbonates in these soda lakes leads to a highly buffered alkaline environment (Grant and Tindall, 1986). Microorganisms which survive in such environments are in general obligately haloalkaliphilic, requiring at least pH 9 and the presence of Na^+ for their growth; their biology and diversity has been widely reviewed (Grant *et al.*, 1990; Duckworth *et al*., 1996; Khmelenina *et al*., 1997; Oren, 2002; Yoshimune *et al*., 2010).

This strategy of accumulation of compatible solutes has not yet studied specifically in alkaliphile microorganisms. However, studies on halo-alkaliphilic sulfur-oxidizing (SOB) have been conducted on the Gammaproteobacteria belonging to the genera *Ectothiorodospira*, *Thioalkalivibrio*, *Thioalkalimicrobium* and *Thioalkalispira* (Zhilina *et al*., 1997; Pikuta *et al*., 1998; Pikuta *et al*., 2003; Sorokin *et al*., 2005; Zhilina *et al*., 2005) which are all low-salt tolerant alkaliphiles. Different types of organic osmolytes (compatible solutes) are involved and utilized in their stress-regulation, including sugar derivatives, glycerol, glycine betaine and ectoine. Glycine betaine is one of the main compatible solutes and synthesized *de novo* in both *Thioalkalimicrobium aerophilum* strain and *Thioalkalivibrio versutus* strain but it can also be accumulated from the surrounding environment (Galinsk, 1995; Banciu *et al*., 2004).

The mechanisms of compatible solute accumulation reported in this study generally agrees with what has been reported in the quoted literatures, namely that low-salt tolerant alkaliphiles and also halotolerant and halophiles (Chapter 3) usually accumulate amino acids and polyols and their derivatives such as, glycerol, ectoine and quaternary amines such as glycine betaine.

On the other hand, cytoplasmic pH is an important aspect of bacterial cell physiology, as it controls the permeability of the cell membrane to protons by controlling the activity of ion transport systems which facilitate proton entry. Most alkaliphiles have an optimal growth pH at around 10, this being the most significant difference from well-investigated neutrophilic microorganisms. Therefore, it was intended that an experiment be conducted on balancing cytoplasmic pH of alkalitolerant and alkaliphilic isolates. However, due to technical difficulties and demands from other experiments, the experiment was not conducted. Future work is desirable to provide answers to the questions raised regarding how these isolates can grow in such an extreme environment and to determine what are the differences in the physiological and structural aspects between alkaliphilic, alkalitolerant and neutrophilic microorganisms?

CHAPTER 4

THE ISOLATION OF ALKALITOLERANT AND ALKALIPHILIC BACTERIA AND ARCHAEA FROM ALKALINE SAMPLES

4.1 Introduction

Microorganisms can grow over the pH range from less than pH l.0 to approximately pH l3.0 (Kroll, 1990). Alkaline adapted microorganisms can be classified into two main categories, alkaliphiles and alkalitolerants. Many microorganisms exhibit optimal growth at more than one pH depending on their growth conditions, particularly nutrients, temperature and the presence of metal ions. The term alkaliphile is generally restricted to those microorganisms that actually require alkaline media for growth. The optimum growth rate of these microorganisms is observed to be at least two pH units above neutrality. Therefore, the term alkaliphile is used for microorganisms that grow optimally at pH values above 9.0, often between 9.0 and 10.0, but cannot grow at the pH value less than 7.0 (Horikoshi, 1999). Microorganisms which can grow at pH values more than 9 or 10, but with optimum growth rates at around neutrality or less, are referred to as alkalitolerant (Jones *et al.*, 1994; Grant *et al.*, 1990). Most non-extremophilic microorganisms grow often within the external pH values that range from 5.5 to 9, but their optimal growth normally falls within the narrow range of pH 7.2 ±0.5 (Padan *et al*., 2005).

One of the most fundamental factors affecting the growth and reproduction of microorganisms is the hydrogen ion concentration (Kroll, 1990; Padan *et al*., 2001). This influences the ionic state of several metabolites and it also affects inorganic ion-availability for example in relation to Ca^{+2}, Fe^{+2} and Mg^{+2}, ions which may become insoluble and precipitate particularly at alkaline pH. Moreover, the concentration of

hydrogen ion significantly affects the stability of macromolecules and how they function in biological processes (Kroll, 1990; Hicks and Krulwich, 1995)

As stated in Chapter 1 (Section 1.3.5), microorganisms that live at extreme values of pH either alkaliphiles or acidophiles must maintain an internal (cytoplasmic) pH that is compatible with optimal functional and structural integrity of the cytoplasmic proteins which help these microorganisms to grow (Padan *et a1*., 2005). Therefore, it is very likely that the cytoplasmic pH of such alkaliphiles and acidophiles is closer to pH 7 than is the existing external pH. Acidophilic microorganisms are usually isolated from acidic environments and are widely distributed; workers have also reported the isolation of acidotolerant bacteria from environments which are not acid, for example, pH neutral environments such as garden soil and other sediments (Horikoshi, 1996 and 1999; Grant *et al.*, 1990).

These observations show a strong correlation between temporary increases in alkalinity and biological activity of alkaliphiles including sulphate reduction, ammonification, and photosynthesis (Grant *et al.*, 1990). Alkaliphilic microorganisms living in extreme environments have been intensively studied (Yumoto *et a1*., 2000). In this chapter, the isolation of alkaliphilic bacteria and archaea able to grow at a pH less than 5 was demonstrated using alkaline samples from the Redcar, soda lake soils and Turkish soda lake sediment.

Mayes *et al*. (2008) detailed how Redcar alkaline steel slag leachates are a by-product of iron and steel production and are made up mainly of Ca and Mg silicates and alumino-silicates. The weathering of steel slag gives rise to a high pH (pH 9–13) leachate. The hydrolysis of lime (CaO) in this slag to portlandite, $Ca(OH)_2$, and subsequent dissociation in solution, creates the hydroxyl ion (OH^-) which raises solution pH (Eq. 1).

$$Ca(OH)_2 \rightarrow Ca^{2+} + 2OH^- \qquad (1)$$

4.2 Materials and Methods

4.2.1 Description and sample sites and sampling procedure

The main sampling site used here was alkaline steel slag dumps at Redcar, UK (OS 589249) (Figure 4.1). A total of 5 alkaline areas of the tips collected from this sampling site. Each sample was taken from 10 cm deep and comprised: soil with pH value of 12, lake sediment (pH 12), roots (pH 12) and another nearby lake sediment sample with pH value of 8.6). A soda lake sediment sample from Turkey (pH 9.3) was also examined in this study. In addition to alkaline environment, one sample from acidic environment in Spain (with a pH of 2.7) was examined to look for the presence of alkaliphile or alkalitolerant microorganisms.

Figure 4.1 Sampling site -alkaline lakes, Redcar, UK

4.2.2 pH readings:

The pH values of samples were taken in the field using a digital handheld pH probe (ExStick™ PH110, EXTECH® Instruments, Waltham, USA). The pH probe was calibrated prior to use using pH 4.0, 7.0 and 10.0 buffer solutions (BDH) following the

manufacturer's instruction, applying the required temperature compensation. The pH probe was directly placed onto the water and sediment sample materials until a constant value was displayed. After each reading, it cleaned with deionised water and re-calibrated. A follow-up reading was taken in the lab using a bench top pH meter (3310, JENWAY LTD, UK) in addition to indicator strips (WHATMAN-BDH, England) for moist sediment and water samples.

4.2.3 Culture and isolation of alkiphilic bacteria

Isolation of alkaliphilic microorganisms was carried out using Horikoshi medium (liquid and plates). For 1 litre of the Horikoshi medium at different pH values, the ingredients shown in Table 4.1 were added and dissolved in 750 ml of distilled water, with the exception of glucose, KH_2PO_4 and Na_2CO_3; a separate solution of the following was then prepared: 10 g of glucose to 100 dH_2O, 1g of KH_2PO_4 was added to 50 ml dH_2O and 10 g of Na_2CO_3 to 80 ml dH_2O. The pH of the final solution was adjusted as necessary using either 1M HCL and or 1M NaOH and all solutions were separately autoclaved to avoid the precipitation and caramelisation (brown colour) of the media at high pH. The two solutions were then left to cool at approximately 50^{o}C and aseptically combined and mixed. The pH value of liquid media was checked after autoclaving and was shown to alter only by ±0. 05 pH units.

Table 4.1 Composition of Horikoshi medium as described by Horikoshi (1999).

Ingredient	Gram per liter
Glucose	10
Na_2CO_3	10
Tryptone	5
Yeast extract	5
KH_2PO_4	1
$Mg_2SO_4.7H_2O$	0.2

In addition to this alkaline selective medium (Horikoshi medium), 25% nutrient and M9 minima media were used to isolate alkaliphile and alkalitolerant bacteria. These media were prepared as described in Table 2.1, Section (2.2.2), but without NaCl. Five different pH values were used (4.5, 6.0, 7.0, 9.0 and 11.0). The pH value of liquid media was rechecked after autoclaving and shown not alter by more than ±0.05 pH units.

For the isolation of archaea, a modified medium was based on Payne Seghal and Gibbons medium described in Chapter 2, Section (2.2.2), using solidified DSM97 medium (DasSarma *et al.*, 1995; Rob, 1995) with the same components listed in Table 2.1 but with the addition of Na_2CO_3 at the same concentration used in Horikoshi medium and without NaCl including: casamino acids, yeast extract, trisodium citrate,; KCI, $MgCI_2.6H_2O$, $MgSO_4.7H20$, $FeSO_4.7H_20$, $MnSO_4.H_2O$ and calcium chloride salts. Three antibiotics (streptomycin, penicillin and ampicillin) at 100 $\mu g.ml^{-1}$ were also added in order to prevent the overgrowth of bacteria (Wais, 1988).

For all media, the pH was adjusted at 5 different values; 4.5, 6.0, 7.0, 9.0 and 11.0. Different pH values were obtained using 1.0M NaOH (BDH) and 1.0M HCL (Sigma). The pH value of the suspension was measured using a bench top pH meter (described above) which had been standardised using pH 10.0 and pH 4.0 buffers (BDH).

4.2.3.1 Enrichment cultures

The initial isolation of alkaliphilic microorganisms was carried out as follows: Five grams of each soil sample were added aseptically to flask containing 50 ml (w/v) of liquid media and 1.0 ml of water samples was plated directly on agar medium. As described in Chapter 2 (Section 2.2.2.1), cultures were incubated aerobically at (25ºC and 37ºC) for bacteria and (37ºC and 45ºC) for archaea, with shaking (100 rpm) for 48 to 72 hours, with an additional one week incubation if no growth was observed. Microbial

growth was monitored by turbidity and then actively growing cultures were subcultured.

4.2.3.2 Pure cultures

When growth was observed by turbidity, cultures were spread or streaked on the same medium containing 1.5% agar. Petri dishes were incubated at 25°C and/or 37°C (bacteria) and 37ºC and/or 45ºC (archaea) for 2-3 days. As described in Chapter 2 (Section 2.2.2.2), single colonies were picked off and streaked onto fresh plates to obtain pure culture. Pure culture was obtained by serial plating and repeated sub-culture on the same agar medium plates and confirmation using by light microscopy.

4.2.4 Counts of colony forming units (CFU)

Colony forming units were determined in order to measure the number of culturable microorganisms (viable) present in samples obtained from the various environmental sample. For the estimation of the numbers of colony forming units and alkaliphilic bacteria present in the soil samples. 5 g of soil samples were suspended in 95 ml of distilled, sterile water and shaken at 250 rpm for 2 hours and then diluted serially with the same solution, Aliquots of 1.0 and 0.1 ml each were spread on agar plates with pH value of 4.5, 6.0, 7.0, 9.0 and 12.0. A standardized test portion of one-tenth decimal dilutions of soil samples were inoculated by the standard surface spread method using the Horikishi agar media and triplicate samples were plated and incubated at temperatures ($25^{o}C \pm 0.5^{o}C$) for 48 hours to enumerate the bacteria. Results are expressed as the number of colony forming units (cfu) in a given volume of the sample (Feldsine *et al*., 2002).

4.2.5 Microscope examination

Gram staining was carried out on the isolated alkaliphilic and alkalitolerant strains. The staining procedure was carried out as the following; an inoculating loop, 1µl of the

alkalibacterial culture was used to prepare a smear on a microscope slide with a droplet of water. The procedure was run as described in Chapter 2 (Section 2.2.4).

4.2.6 Long-term storage of cultures

A11 isolates were maintained by adding 1.0 ml of overnight culture (active inoculum) to 1.0 ml of sterile glycerol (50% v/v) and immediately frozen at -80ºC. Frozen stocks were slowly thawed on ice for recovery of cultures, inoculated with sterile swap to fresh Horikoshi (for alkaliphile and alkalitolerant) and/or M9 minimal (for alkalitolerant) media of the same pH.

In addition, each strain of alkalophilic bacteria was subcultured routinely once every two weeks to minimize contamination over time. This was performed by either streaking overnight colonies on Horikoshi and M9 minimal plates or by overnight culture into Horikoshi and M9 minimal broth with the optimal pH and under suitable conditions and then stored in a fridge (at 4^{o}C) until required.

4.2.7 Molecular identification techniques

4.2.7.1 Genomic DNA extraction

Nucleic acids from alkalitolerant bacterial and archaea were extracted using the ANACHEM KeyPrep protocol described previously in Chapter 2, (Section 2.2.7.1.3). As was stated in the discussion section of Chapter 2, this is the most efficient and quickest method of genomic DNA extraction. Briefly, cells were lysed by the addition of lysozyme and other chemical buffer supplied by the manufacturer. The protein precipitation reagent was added to soluble fraction. The soluble fraction was then passed through a spin filter with a silica membrane to adsorb DNA. The DNA was purified with ethanol and buffer washes and eluted with 100µl of elution buffer. This elution of the DNA sample was collected and examined by agarose gel electrophoresis (see Chapter 2, Section 2.2.7.2 for details).

4.2.7.2 Amplification, extraction and purification of 16S rRNA

After the extraction of genomic DNA from a particular microorganism, 16S rRNA gene was amplified from the total DNA extracted using the polymerase chain reaction (PCR) as described in Chapter 2, Section (2.2.7.3). Bacterial and archaeal universal primers were used for amplification or sequencing of 16S rRNA genes as described previously and listed in Table 2.3. Amplification of 16S rRNA was performed in a total volume of 50 µl containing (as listed in Table 2.2) a DNA template, reaction buffer, 20 mM $MgCl_2$, 25 mM dNTPs (5 mM dATP, 5 mM dGTP, 5 mM dCTP, and 5 mM dTTP; BIOLINE, UK), forward and reverse primer, 0.2 U of Taq DNA polymerase and sterile deionised water (sdH_2O). The reaction mixtures were cycled 30 times through the temperature profile listed in Table 2.4 for denaturation, annealing and extension. Then, 10 µl of each amplification mixture was then analyzed by agarose gel electrophoresis. The PCR product was purified through QIA quick® PCR Purification kit as per manufacturer's instruction (Qiagen, UK).

PCR products were then sent to Medical School, the University of Sheffield, for DNA sequencing. If the sequenced 16S rRNA was not matched with BLASTN or the sequence were not sufficient to match, the 16S rRNA then gene cloning and library construction was performed.

4.2.7.3 The 16S rRNA gene cloning and library construction

The purified PCR products were ligated into pCR2.1-TOPO vector according to the manufacturer's instructions (Invitrogen, UK). The procedures for this part of cloning and library construction are described in Chapter 2 (Section 2.2.7.4.2) but the following is a brief summary of the protocol used. The ligation mix was transferred to a 2.0 ml sterile tube and high efficiency competent *E. coli* cells were added. The mixture was chilled on ice before heat shocking at 42^{o}C. The cells were then immediately suspended to SOC medium and incubated at 37^{o}C for 90 min with shaking. Next, transformation

mixtures were plated onto LB agar plates supplemented with isopropyl-β-D-galactopyranoside (IPTG), ampicillin, and 5- bromo-4-chloro-3-inolyl-β-D-galactoside (X-Gal) and incubated at 37^{o}C overnight. Successful transformants (positive) containing recombinant plasmids appeared as white colonies while failed transformants (negative) was blue. The positive transformants with white colonies were randomly picked and grown overnight at 37^{o}C on LB agar plates containing ampicillin.

Plasmid DNA was extracted from cultures and purified using QIAprep Spin Miniprep Kit (Quigen, UK) according to the manufacturer's instructions as described in (Section 2.2.7.4.3). The quality and quantity of plasmid was checked using agarose gel electrophoresis (Section 2.2.7.2). Restriction digests were carried out as described in (Section 2.2.7.4.4)

4.2.7.4 Oligonucleotide primer construction

The probe (primer) construction was completed and tested to confirm the sequenced gene from the Medical School, it was also used to complete the 16S rRNA gene sequences (1542 bp), alternative to the 16S rRNA gene cloning and library construction.

In order to isolate a fragment of 16S rRNA gene from particular alkaliphilic isolates, a PCR strategy using degenerate primers was designed. Forward primer: 5'→3' and reverse primer: 3'→5' were used for the amplification of the gene fragment of one isolate. These primers that were obtained from each isolate were sequenced for 16S rRNA. The optimal length of a particular alkaliphilic designed PCR primers as 18-22 bp. This length was found to be sufficient for adequate specificity and short enough for the primers to easily bind to the template at the annealing temperature. The PCR was carried out under the conditions described in Chapter 2 (Section 2.2.7.1.3), and Table 2.4.

4.2.8 Effect of medium pH on the growth of bacteria and growth curve determination

The effect of pH on the growth of isolates was determined using overnight cultures in nutrient liquid medium (best grown medium) with the addition of KH_2PO_4 and Na_2CO_3. 1.0 ml of active inoculum from each isolate was inoculated into nutrient liquid medium pH 4.5, pH 7 and pH 10 and the optical density (OD) at 600 nm was measured using a Unicam Heliso, spectrophotometer against the medium blank uisng l ml plastic cuvettes. Flasks, 4 x 250 ml conical flasks (the fourth flask was used to control the pH over the time for each strain) containing 50 ml of each pH value nutrient medium were inoculated with 1.0 ml of the same pH adapted cells from an overnight culture. The pH was controlled manually during batch culture growth by monitoring the fourth flask and adding the appropriate acid/alkali to all four flasks. The OD_{600} was measured against a medium blank immediately after inoculation and then every one hours over an incubation period at 25^{o}C. To determine the growth curves, the optical density, growth, were plotted against time of incubation.

4.2.9 Gel electrophoresis of whole-cell proteins

SDS-PAGE of whole-cell proteins and sodium dodecyl sulphate (SDS) gel electrophoresis was conducted as described elsewhere (Stan- Lotter *et al.*, 1989, 1993, 2002). Briefly, approximately 1.0 ml of overnight liquid culture was centrifuged at (~11340 g) for 5 min. The resulting pellet (~ 50 mg.ml^{-1} wet weight) was then re-suspended in 100µl of SDS buffer and the cells were lysed by boiling for 10 minutes (Laemmli, 1970) and then centrifuged at (~11340 g) for 1 min, to remove any precipitates. Gels were stained with Coomassie Brilliant Blue R250 to visualize the proteins and run for 5 hours in order to separate the proteins. Marker proteins of 10 to 170 kDa in size (Fermentas, UK) (Figure 4.2) were used and the gel was washed in

acrylamide for 16 hrs and finally scanned for further evaluation (Stan- Lotter *et al*., 2002).

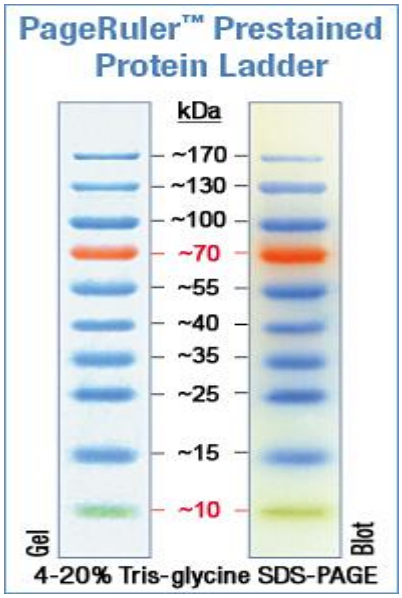


Figure 4.2 PageRuler prestained protein ladder (10 – 170 kDa).

4.3 Results and Discussion

4.3.1 The presence and occurrence of alkalitolerant and alkaliphilic isolates in alkaline samples and measurements of total cells

The isolation of bacteria from unusual environments is of interest as novel organisms may be found which may produce novel biotechnological compounds; this includes the isolation of microorganisms from alkaline environment which can survive at very low pH. The aim of the work covered in this Chapter was the screening and isolation of alkaliphilic and alkalitolerant bacteria and archaea which are also able to grow in acidic media with low pH. This was achieved by using different media including selective media for alkaliphiles such as Horikoshi medium. A number of alkaliphilic microorganisms, mainly bacteria were isolated in this study from alkaline environments. Interestingly, some of these microorganisms were also able to survive, and more significantly grow, at low pH values such as 4.5. One sample from acidic environment in Spain (with a pH of 2.7) was examined and no visible growth was found using all different ranges of pH values.

Alkaliphilic microorganisms have been isolated in alkaline media containing magnesium sulphate, sodium bicarbonate and potassium carbonate such as Horikoshi medium. Other general media such as nutrient and M9 minimal media also produced significant growth.

Suspensions of the sample materials were used to prepare serial dilutions which were plated onto a selection of isolation media but mainly Horikoshi media. Figure 4.3 shows the distribution of microorganisms from alkaline environment at different pH's including moderate acidic pH value such as 4.5. The same results were found independent of the medium chosen.

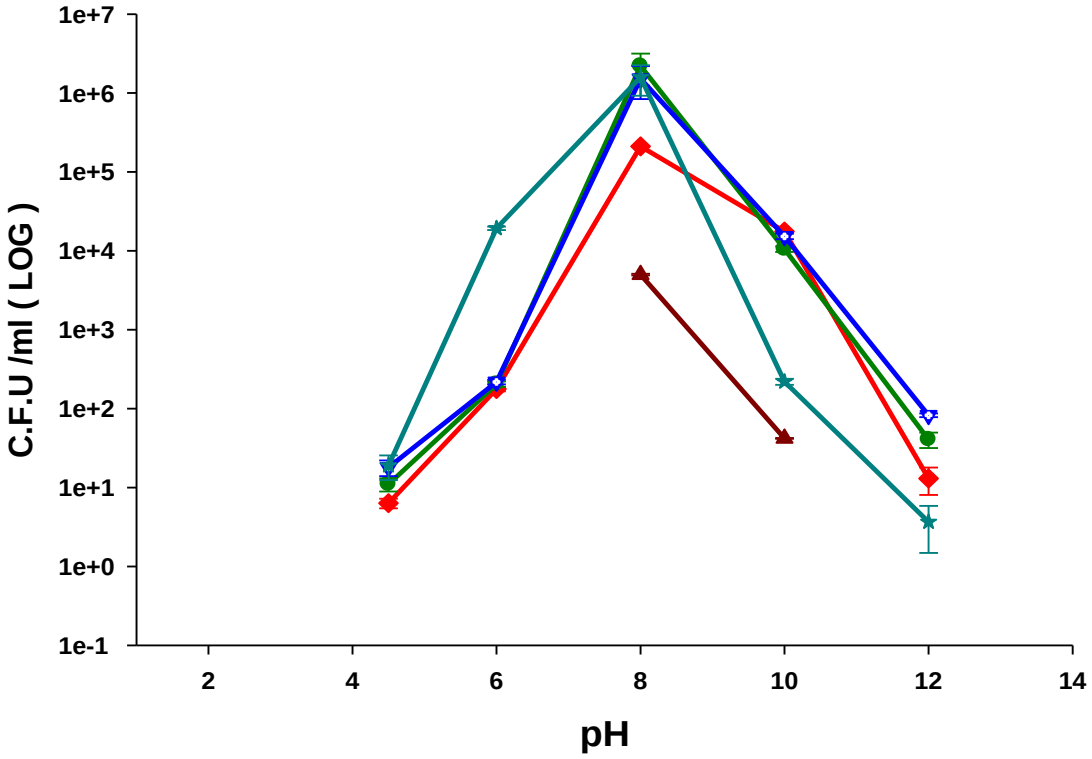


Figure 4.3 The total bacterial cells count from different alkaline environments on agar plates with different pH values (12 -4.5) using Horikoshi medium. The total cell count from samples represent alkalitolerant and alkaliphilic microorganisms including; Root with pH 12 ( ), Lake with pH12 ( ), Soil with pH12 ( ) Lake with pH8.6 ( ) and Turkey sediment with pH9.3 ( ). (I) refers to Standard Deviation (SD).

The results of these studies agreed with the work of Horikoshi *et al*. (1999) (see Chapter 1, Section 1.3.2, Figure 1.4 for details). They reported typical pH dependency of the growth of neutrophilic and alkaliphilic bacteria which correspond with our findings as shown in Figure 4.3**.**

The presumptive alkalitolerant isolates subcultured from the soil-dilution-plates and the enrichment culture were tested for growth on low, neutral and high pH agars. Initially attempts were made to use pH gradient plates where an alkaline agar was overlaid with a acidic agar as shown in Figure 4.4. These were prepared in 25 cm x 25 cm Nunc bioassay dishes; however, this method was abandoned as the pH of the large surface area plates was observed to drop rapidly.

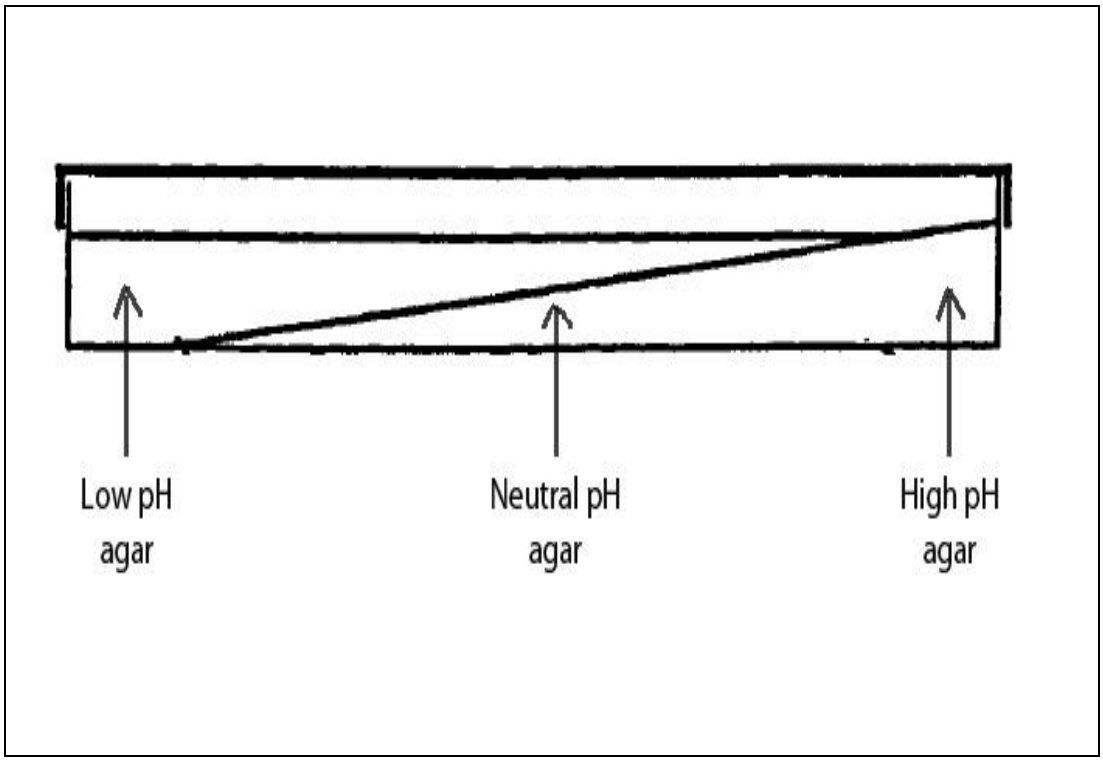


Figure 4.4 A pH gradient plate showing the overlay of an alkaline agar medium with a neutral and acidic agar medium.

The isolations were performed using fresh plates as the pH of alkaline medium can drop with time due to carbon dioxide absorption and colony metabolism, thus allowing the growth of organisms which could not tolerate the initial high pH conditions (Grant and Tindall, 1986; Grant and Horikoshi, 1989). When stored at 4°C, however, no appreciable drop in pH from pH 10.5 was observed in plates of Horikoshi medium, over a seven day period. As a result, these plates were not used routinely.

Microbial populations in the very low pH value plates were frequently too low to be counted. Only counts of 3.0×10^5 colony forming units per gram of soil (cfu/g) and over were statistically reliable. Counts lower than 3.0×10^5 have been included in Figure 4.3 as they gave an indication of the broad observable trends, but these have to be accepted as preliminary results on which further work needs to be done. Where the average values were above 3.0×10^5 colony forming units per g soil the spread of counts rarely exceeded plus or minus 15%.

Visible examination showed that only a few types of bacteria were isolated and this limited variety decreased with pH. A number of the isolates were picked off and sub-cultured. Only two alkalitolerant organisms were isolated. This is in contrast to Grant and Horikoshi (1989) who suggested that Horikoshi's medium plates in its original form, with a glucose carbon source, yielded high numbers of alkaliphilic bacteria including *Bacillus* sp. and actinomycetes.

Gram staining and/or the observation of different colony types on the isolation plates showed that only a narrow range of alkalitolerant and alkaliphilic microorganisms were present in the environmental samples. These results do not correspond with the observation of Horikoshi (1991) that a wide range of alkaliphilic microorganisms, which include the Gram negative and positive bacteria, can be readily isolated from high pH environments.

The majority of the alkaliphiles present on the plates, were Gram positive rods and a number of these possessed endospores, showing them to be *Bacillus* species. *Bacillus* species have also been reported to be the most commonly isolated alkaliphilic bacteria (Grant and Horikoshii, 1989; Horikoshii, 1991; Jones *et al.*, 1994).

4.3.2 Analysis of cultures and isolates using molecular identification techniques

The purity of each culture was confirmed by streaking the cultures on fresh agar media. The colony and cell morphology was examined using phase contrast microscopy. The pH growth ranges were checked for all the isolates by streaking colonies onto pH gradient plates and incubating them at 25^{o}C and 37^{o}C for 24 h. Genomic DNA was extracted from cells using the commercially available kit mentioned above in (Section 4.2.9.1). The phylogenies of isolates were then studied by sequencing and analysis of 16S rRNA gene clone libraries.

The results of BLASTN on the NCBI website revealed a limited variety of microorganisms present in Redcar samples, including no species of archaea. Each isolate produced few matches with percentage identities greater than 95%, with most isolates having 99% or 100% identity (Table 4.2) over the 800-1000 bp sequenced region to other sequences in the NCBI database. The phylogenetic analysis and position of the alkaliphilic bacteria are plotted in Appendix D.

A total of six microorganisms were isolated and identified from the soda lake and soil samples from Redcar, which indicates the presence of a very low level of microbial diversity (Table 4.2).

The almost complete 16S rRNA sequence (1542 bp) of strain RS12, RS12-4 and RR12-4 was determined using probe construction (please refer to Section 4.2.9.4). Alignments of this sequence with sequences available from the GenBank database (BLASTN) showed that the closest relative of strain RS12 was *Alkalibacterium sp*, with a sequence similarity of 100%, RS12-4 was *Bacillus massiliensis*, with a sequence

similarity of 95%, and RR12-4 was *Lactococcus lactis*, with a sequence similarity of 100%. The probe construction method (Table 4.3) was conducted as a confirmation of sequenced 16S rRNA gene analysis.

Table 4.2 Summary of 16S rRNA sequence analyses (representative BLASTN matches) of microorganisms cultivated from alkaline environment.

Sample	pH reading	Representative sequence	Best blast match database	% sequence identity	Closest described microorganism	NBCI (Accession no.)
Redcar-Soil 1	12	RS12 RS12-4	*Alkalibacterium sp.* *Bacillus sp.*	100 95	*Alkalibacterium kapii.* *Bacillus massiliensis*	AB555563.1 EU147196.1
Redcar-Sediment	12	RSd12	*Bacillus sp.*	98	*Bacillus horikoshii*	HQ397583.1
Redcar-Root	12	RR12 RR12-4	*Lysinibacillus sp*. Uncultured *Lactococcus sp.*	96 100	*Lysinibacillus sp*. *Lactococcus lactis*	HM057842.1 CP002365.1
Redcar-Lake 1	12	RL12	*Carnobacterium sp*. cG53 gene	100	*Carnobacterium sp*.	AB593337.1
Redcar-Soil 2	8.6	RS8.6	Uncultured *Lactococcus* sp.	99	*Lactococcus lactis*	CP002365.1
Redcar-Lake 2	8.6	RL8.6	*Bacillus sp.*	95	*Bacillus massiliensis*	EU147196.1

Table 4.3 Summary of constructed oligonucleotide primers for some isolates of interest synthesized by Eurofins (mwg/operon), UK.

Oligonucleotide	Sequence, 5'-3'	Sequence, 3'-5'	Specificity for isolate
RS12	GGCGCTAATGATCGCTAA	TTTCCTTCGGGGACAGAG	RS12
RR12-4	CCAAAATTCTCTCCTTCGAT	CCGTAAACGATGAGTGCTAG	RR12-4
RS12-4	GGCGCTAATGATCGTAA	CCGCAACGAGCGCAACCC	RS12-4

All of the organisms isolated from different Redcar lagoon samples, with pH ranging from 8.6 to 12.0, were shown to be different genera and species except the lake sample with a pH of 12 and the soil sample with pH 8.6. Although these two samples were different in pH value they gave the same isolate, namely a bacterium which was 100% similar to *Lactococcus lactis*. This strain and the other one which was isolated strain from Redcar-soil 1 (95% similar to *Bacillus massiliensis*) were the only bacteria able to grow in medium at pH value of 4.5. All of the other isolates were able to grow only at pH 7.0 and above.

4.3.3 Effects of pH stress on the alkalitolerant and alkaliphilic bacterial isolates

All of the presumptive alkalitolerant and alkaliphiles isolates selected were capable of growth on high pH (pH 10.0). The isolates were also able to grow well on neutral nutrient broth and some of the isolates grew equally well under both conditions although some performed better under alkaline conditions, whilst others preferred neutral conditions. These preferences are shown in Figures (Figure 4.6, Figure 4.7, Figure 4.8, Figure 4.9, and Figure 4.10). All of the six isolates tested were tolerant of high pH conditions. This suggests that these isolates were at least alkalitolerant. Differences in the performance of the isolates on the three different pH media (pH 10.0, pH 7.0 and pH 4.5) were recorded. Better performance on the high pH medium might indicate that the isolates were alkaliphiles, examples are shown in Figure 4.5, Figure 4.6, Figure 4.7 and Figure 4.8; better growth on the neutral plates in contrast indicates an alkalitolerant species, an example is shown in Figure 4.10. Interestingly, the growth curve of the strains (Figure 4.9) isolated from alkaline soil with pH 12 showed they were able to grow optimally at high pH medium, as well as being able to grow at pH of 4.5.

From soil with pH 12 a strain was isolated which was 100% similar to *Alkalibacterium kapii*. Figure 4.5 shows the effect of different pH values on this isolate

growth. The genus *Alkalibacterium* currently comprises five species: *A. kapii* (Ishikawa *et al*., 2009), *A. indicireducens* (Yumoto *et al*., 2008), *A. iburiense* (Nakajima *et al*., 2005), *A. psychrotolerans* (Yumoto *et al*., 2004) and *A. olivapovliticus* (Ntougias and Russell, 2001). All of these bacteria were isolated from artificial alkaline environments, and all have an optimum pH for growth between values of 9 and 11.5 and halophilic properties (the optimal NaCl concentrations are 3–13%; they also produce lactic acid as the main product of glucose fermentation (Ishikawa *et al*.,2009).

Alkalibacterium kapii [kapi.i. N.L. n. kapium ka-pi (a fermented shrimp paste in Thailand); was proposed as new species by Ishikawa *et al.* (2009). Cells are Gram-positive, non-sporulating, straight rods and the optimum pH for their growth is 8.5–9.0, with a range of 6.0 to 10.0. Our finding, the optimum pH was above neutral, in this study, agrees with what Ishikawa *et al* found.

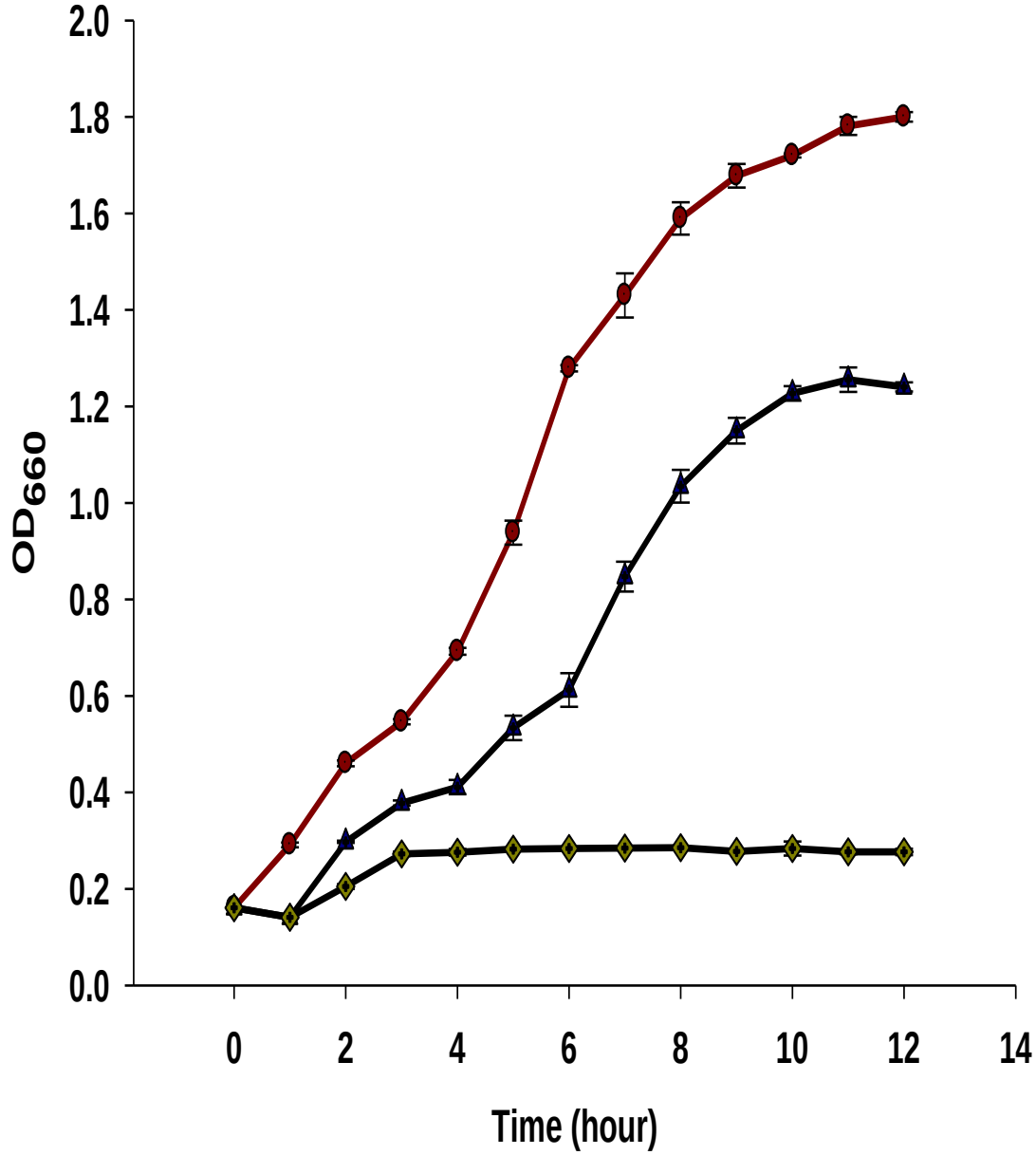


Figure 4.5 Growth curves of *Alkalibacterium kapii* grown at different pH values; pH 10.0 ( ), pH 7.0 ( ) and pH 4.5 ( ) measured at optical density of 660 nm. (I) refers to Standard Deviation (SD).

From sediments with a pH 12, a strain was isolated which is 98% similar to *Bacillus horikoshii*. Figure 4.6 shows the effect of different pH values on the growth of this isolate. *B. horikoshii*; (named *horikoshi* after the Japanese microbiologist Koki Horikoshi, who has made seminal contributions to the study of alkaliphilic bacteria). *B. horikoshii* was proposed as new species by Nielsen *et al* (1995) and was isolated from an alkaline soil sample. It is typified by having rod-shaped cells with ellipsoidal spores located sub-terminally in a sporangium which may be slightly swollen; growth was observed at pH 7.0, with an optimum at about pH 8.0 and temperatures between 10 and 40^{o}C. Salt tolerance is moderate, with a maximum at 8-9 % salt concentration (Nielsen *et al.,* 1995); the optimum pH for growth was above neutral which agrees with the findings of Nielsen *et al.* (1995).

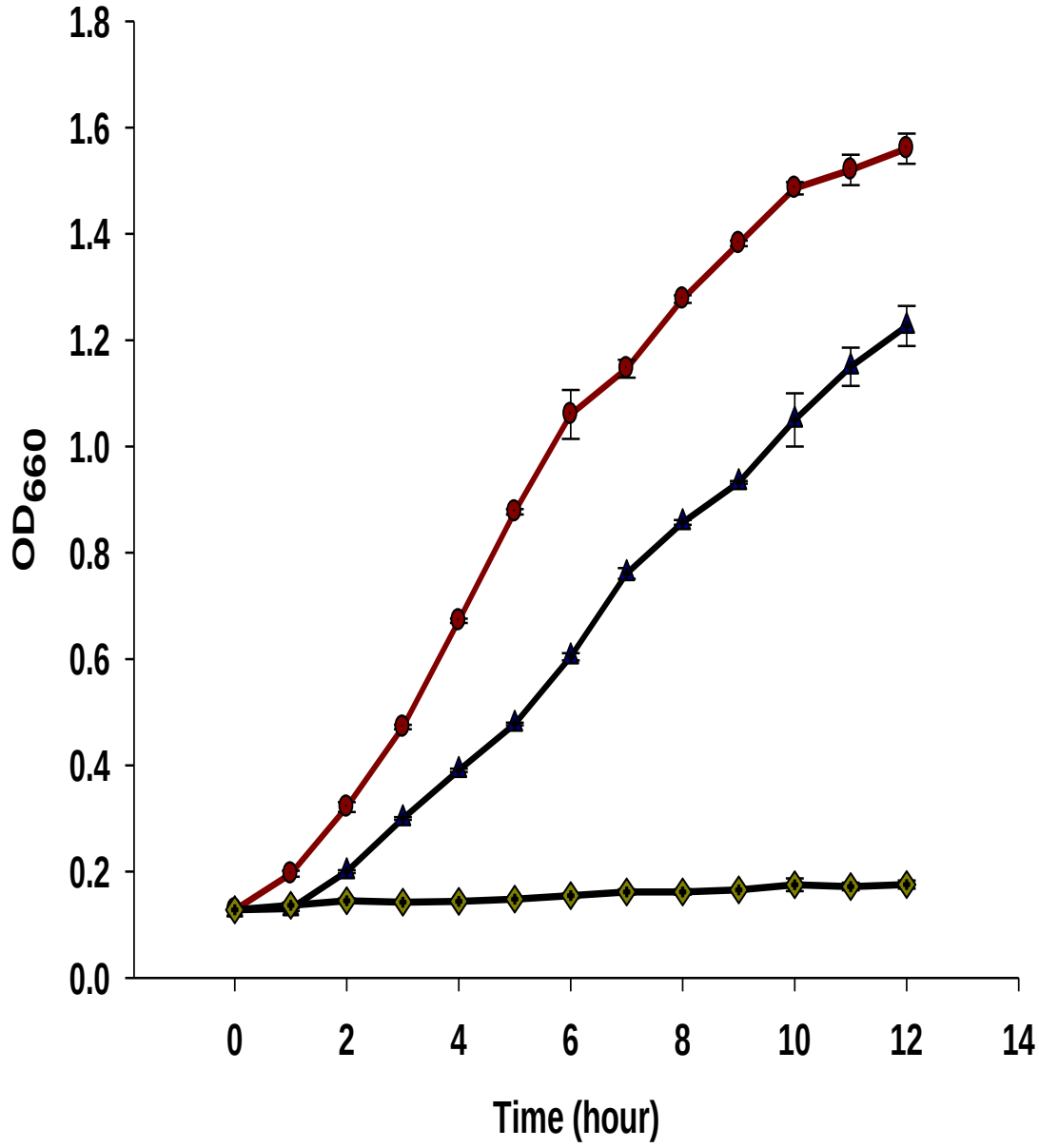


Figure 4.6 Growth curves of *Bacillus horikoshii* grown at different pH values; pH 10.0 ( ), pH 7.0 ( ) and pH 4.5 ( ) measured at optical density of 660 nm. (I) refers to Standard Deviation (SD).

From an alkaline lake, pH 12, a strain was isolated having 100% similarity to *Carnobacterium maltaromaticum.* Figure 4.7 shows the effect of different pH values on the growth of this isolate. *Carnobacterium* species belong to the family Lactobacillaceae and are not considered to be human pathogens, although they are known to cause disease in fish; strains are typically Gram-positive (Hoenigl *et al*., 2010).

The genus *Carnobacterium* comprises 11 species, but only two of these, *C. maltaromaticum* (formerly *Carnobacterium piscicola*) and *C. divergens* have been isolated frequently from the environment and from foodstuffs (Hoenigl *et al*., 2010). *C. maltaromaticum* has been isolated from live/diseased fish, moth larval midgut, polar sea, deep sea, cold and alkaline tufa columns, Japanese lakes and *Sphagnum* ponds (Leisner *et al*., 2007; Walker *et al*., 2006). Large numbers of *C. maltaromaticum* are also found in salted lumpfish roe, and frozen, smoked mussels (Basby *et al*., 1998; Tahiri *et al*., 2004). No previous study has reported the pH optimum of this strain; our findings show a pH value for growth of this isolate between 7 and above10, with an optimum at pH 10.0

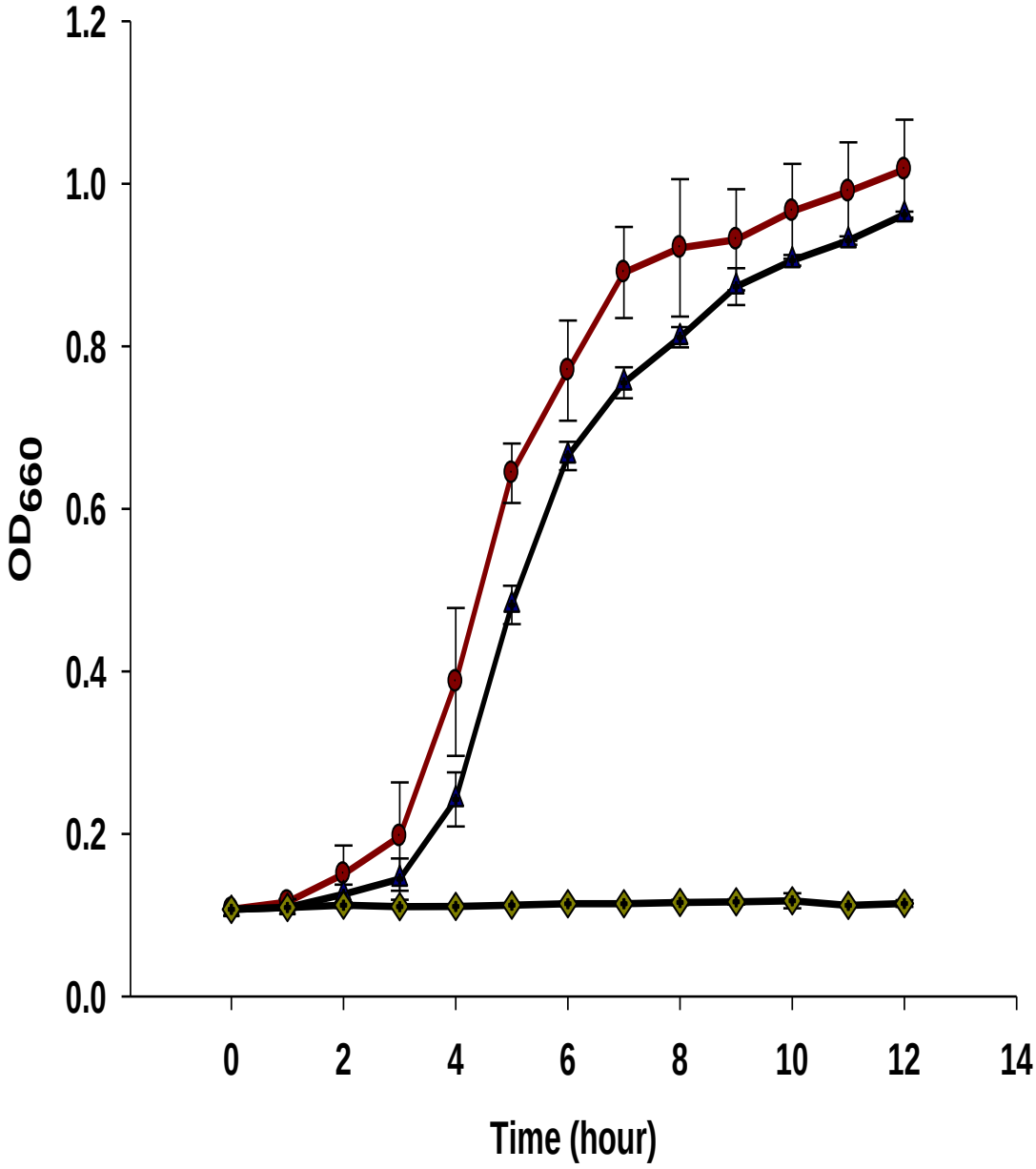


Figure 4.7 Growth curves of *Carnobacterium maltaromaticum* grown at different pH values; pH 10.0 ( ), pH 7.0 ( ) and pH 4.5 ( ) measured at optical density of 660 nm. (I) refers to Standard Deviation (SD).

From roots planted in alkaline lake with pH 12, a strain was isolated which was 96% similar to *Lysinibacillus sp*. Figure 4.8 shows the effect of different pH values on this isolate growth. *Lysinibacillus* sp is Gram-positive, rod-shaped, endospore-forming bacteria. *Lysinibacillus* sp. reported to grow at pH ranges of 5.0-10.0; optimum growth occurs at pH 7.0, at temperature of 15-45°C and optimally at 30°C and in 0-5% NaCl (w/v) but not in 6% salt (Lee *et al.*, 2010; Jung *et al.*, 2010). The findings of the present study correspond with the published work on the pH optimum for this organism.

Jung *et al.* (2010) isolated *Lysinibacillus* sp from the samples of sediment in the Yellow Sea. Molecular methods of 16S rRNA gene sequence analysis demonstrated that this genus is in fact *Bacillus* and is closely related to *Bacillus massiliensis* (97.4%), *Bacillus odysseyi* (96.7%), *Lysinibacillus fusiformis* (96.2%) and *Lysinibacillus boronitolerans* (95.9%). Recently, based on genotypic analysis and other chemotaxonomic data, some spore-forming species within the genus *Bacillus* (Ash *et al*., 1991) were reclassified into new genera, i.e. *Lysinibacillus sphaericus* and *L. fusiformis* (Ahmed *et al*., 2007). This new genus *Lysinibacillus* is made up of four species: *L. parviboronicapiens* (Miwa *et al*., 2009), *L. boronitolerans*, *L. fusiformis* and *L. sphaericus* (Ahmed *et al*., 2007). As for the taxonomic position of *B. massiliensis* and *B. odysseyi*, these species seem to be distantly phylogenetically related to *Solibacillus silvestris* or *Rummeliibacillus pycnus* (Ahmed *et al*., 2007; Krishnamurthi *et al.*, 2009).

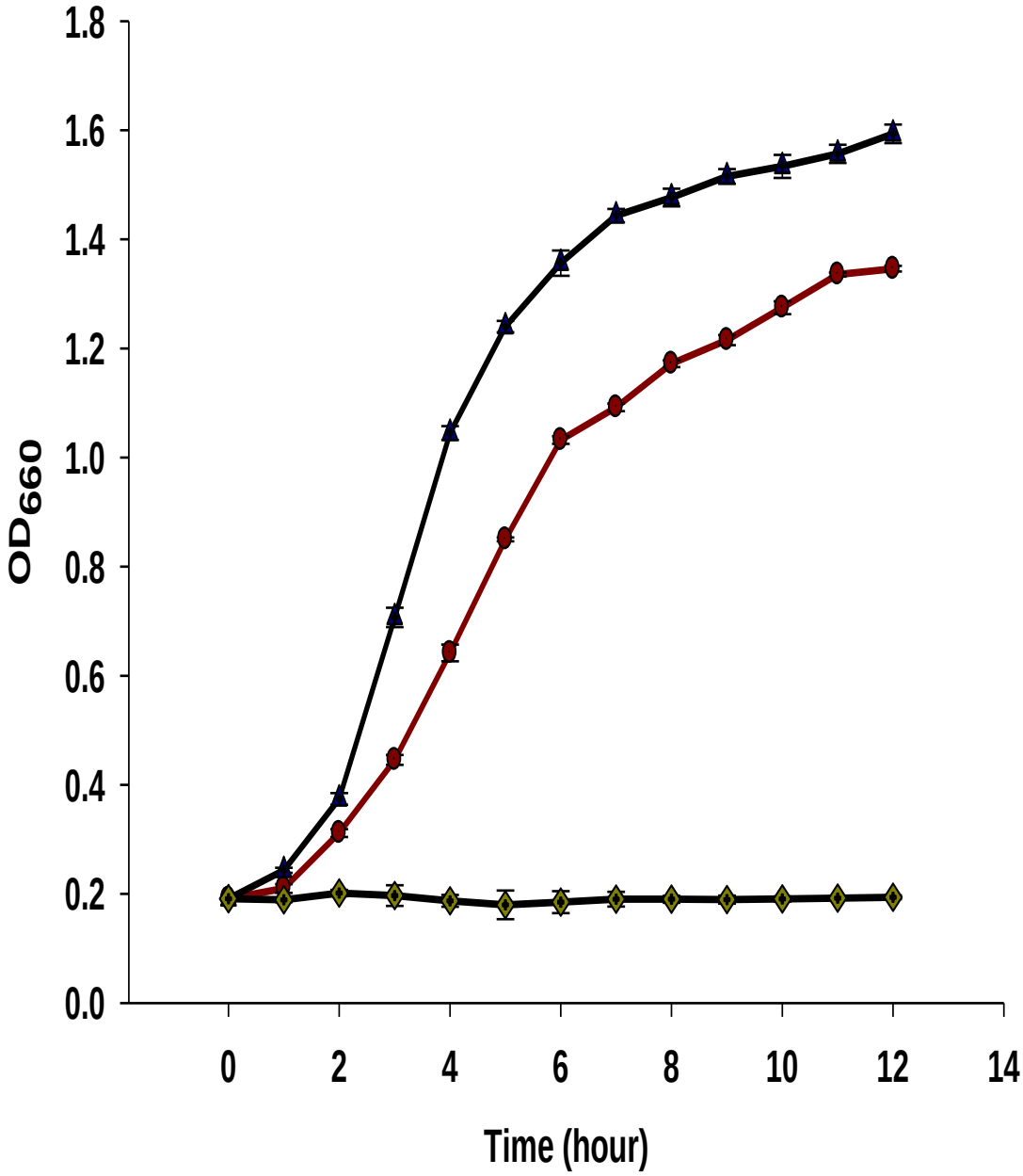


Figure 4.8 Growth curves of *Lysinibacillus sp.* grown at different pH values; pH 10.0 ( ), pH 7.0 ( ) and pH 4.5 ( ) measured at optical density of 660 nm. (I) refers to Standard Deviation (SD).

A strain which was 95% similar to *Bacillus massiliensis* was also isolated. Cells are Gram-positive, aerobic, rod-shaped, motile, endospore -forming. It was originally isolated from a sample of cerebrospinal fluid (Glazunova *et al.,* 2006). It has a temperature range for growth is 25–45^{o}C, but not a published pH growth optimum. Here, we found it was capable of growing at pH ranges of 4.5 and 10.0; optimum growth occurs at pH 10.0 (Figure 4.9).

Lactococcus lactis is a Gram-positive coccoid bacterium used widely in the production of cheese and buttermilk (Madigan and Martinko, 2005), but has recently also become well-known as the first genetically modified organism to be used in the treatment of human diseases (Braat *et al.,* 2006). *L. lactis* cells grow in pairs and in short chains, are non-motile and do not produce spores and non-motile and are reported to grow optimally at pH 6.0 (Serna Cock and Rodríguez de Stouvenel, 2006); no published work is available on its ability to grow at high pH. Here, its ability to grow over at pH range of 4.5 and 10.0; with an optimum at pH 7.0 is reported (Figure 4.10).

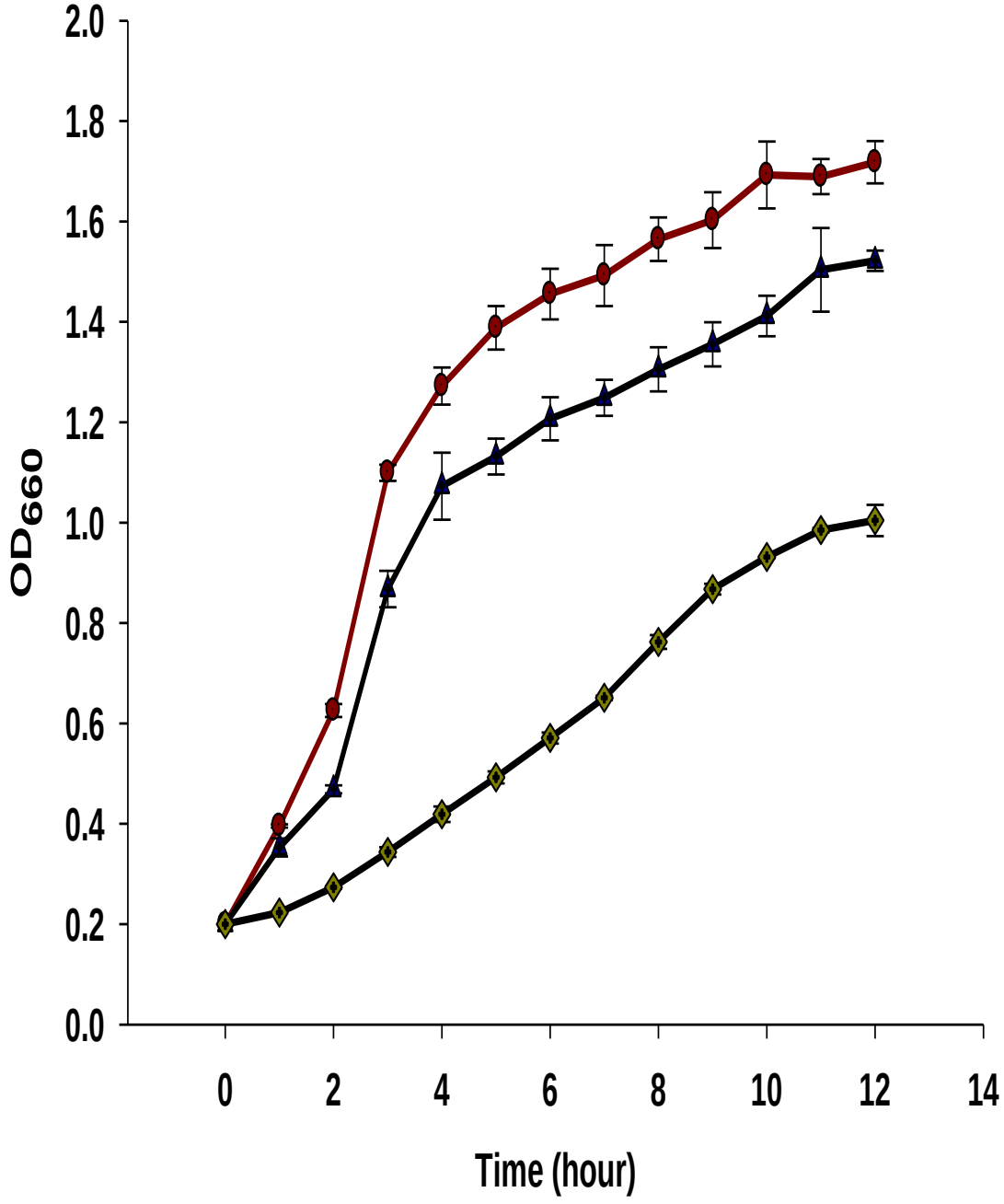


Figure 4.9 Growth curves of *Bacillus massiliensis* grown at different pH values; pH 10.0 ( ), pH 7.0 ( ) and pH 4.5 ( ) measured at optical density of 660 nm. (I) refers to Standard Deviation (SD).

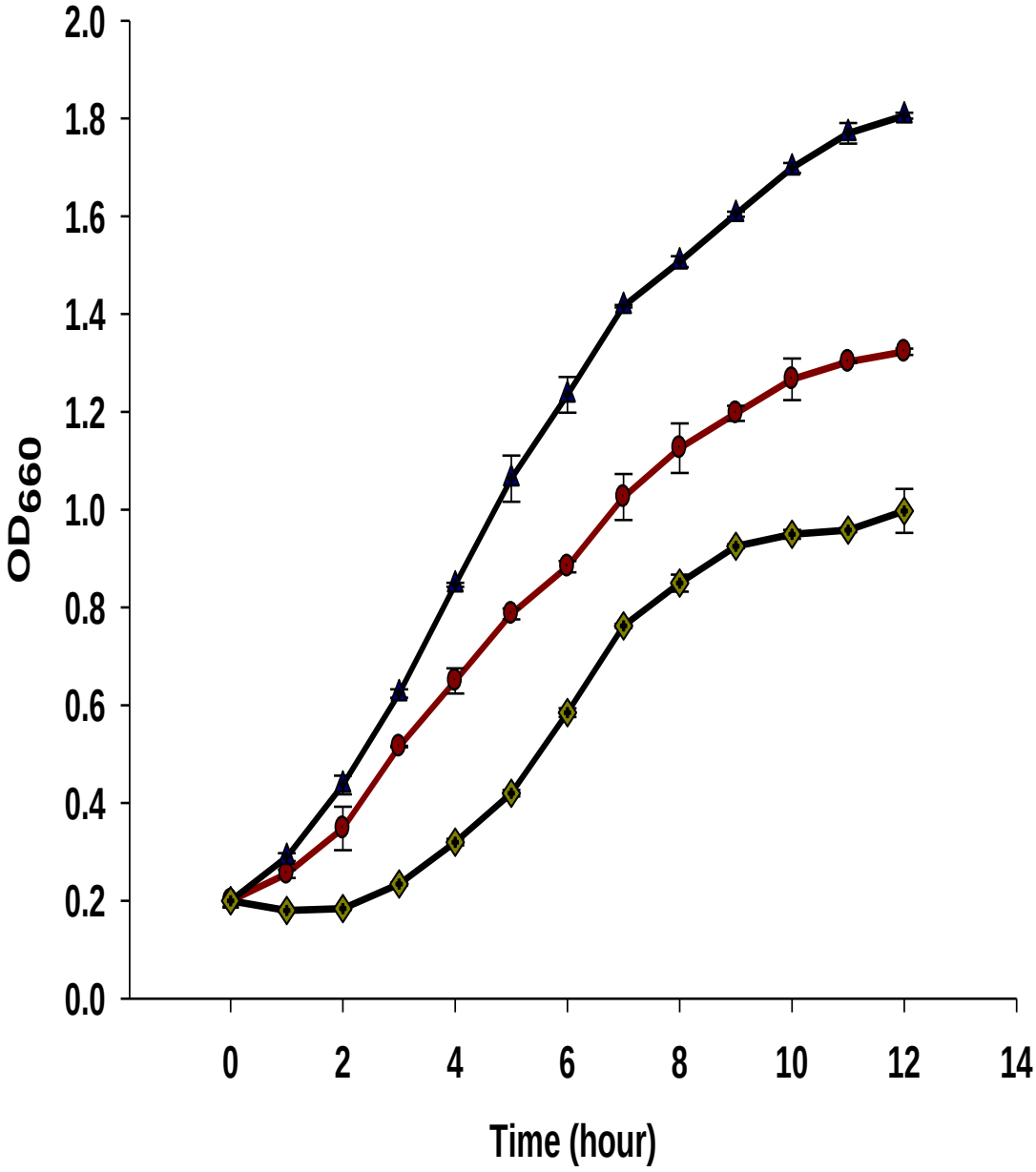


Figure 4.10 Growth curves of *Lactococcus lactis* grown at different pH values; pH 10.0 ( ), pH 7.0 ( ) and pH 4.5 ( ) measured at optical density of 660 nm. (I) refers to Standard Deviation (SD).

Some of these isolates grew very much slower under low pH conditions than neutral conditions. These included isolates RR12-4 and RS8.6 (see Figure 4.9 and Figure 4.10, respectively. The onset of growth, in the case of these isolates, under low pH value appeared to be delayed.

Interestingly, two isolates from two different soil samples with different pH values were isolated as *L. lactis* (Table 4.2). One isolate was obtained from plant roots grown in the soda lake with pH of 12.0 (RR12-4) and the other was obtained from soil sample with pH 8.6 (RS8.6). However, each behaved differently at pH values; strains RR12-4 was able to survive a wide range of pH's (4.5-11); strain RS8.6 in contrast, survived only at pH (6-9).

Sodium dodecyl sulphate (SDS-PAGE) of whole-cell proteins was performed as a rapid method for distinguishing between bacterial species (Jackman, 1987) as described in (Section 4.2.9) with different SDS to protein dilution (e.g. 1:10, 1:20 and 1:30). Strains RR12-4 and RS8.6 exhibit protein profiles that are identical to each other (Figure 4.11). This result was similar to the 16S rRNA gene analysis which indicated that strains RR12-4 and RS8.6 were similar to strains of *L. lactis* although, the NBCI Accession numbers were different (Table 4.2)**.**

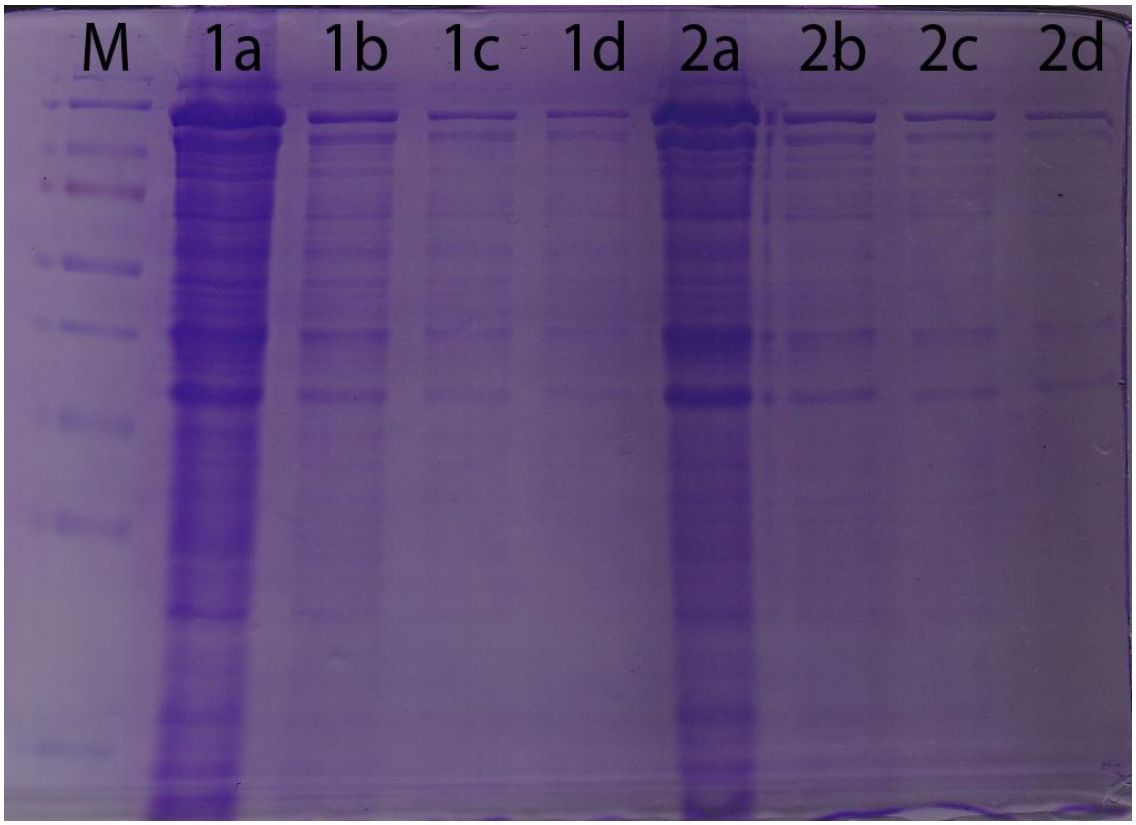


Figure 4.11 Whole-cell proteins from two alkaliphilic bacterial strains isolate RR12-4 and isolate RS8.6 following separation by SDS-PAGE. Following lysis of cells, approximately 20 μg of protein was applied, diluted to different concentrations and were stained with Coomassie blue. Lane 1, molecular mass marker; Lane 1a, 1b, 1c and 1d, *Lactococcus lactis* (RR12-4) with different SDS dilution a; no dilution, b; (1:10), c; (1:20) and d; (1:30) and Lane 2a, 2b, 2c and 2d, *Lactococcus lactis* (RS8.6) with different SDS dilution as former strain.

4.4 Conclusions

The aim of this study was to determine whether alkalitolerant and alkaliphilic bacteria exist in alkaline environments which can grow along different pH gradient including at acidic pH, and if so to determine if they belong to the domain-Bacteria or Archaea. Various methodologies were successfully used to identify the bacterial communities using three different pH isolation and growth media (see Section 4.2.3). Six alkaline resistant (i.e. alkaliphilic and alkalitolerant) bacteria were isolated from the alkaline samples and two of them were presumptively acid-tolerant. All of the isolates

were capable of growth on high pH medium as well as at neutral pH; 3 isolates were selected for further characterization; the osmo-adaptation mechanisms they employ which will be discussed in Chapter 5.

Horikoshi (1991) stated that alkaliphiles are not confined to soda lakes but may be found almost anywhere even in deep-seas trenches and in soils whose bulk pH measurement would not be suggestive of the presence of alkaliphiles. However, it is equally clear that some organisms are unique to soda lakes. Jones *et al*. (1998) on the other hand claim that extremely halophilic alkaliphlic microorganisms are confined to the hypersaline alkaline lakes, This is obviously not the case, since halotolerant and alkalitolerant bacteria can be found in less saline soda lakes. Alkaliphilic Gram-positive bacteria of the genus *Bacillus,* are ubiquitous and occur in both natural and man-made alkaline environment. For example, an alkaliphilic strain isolated from olive-processing effluent was found to be closely related to a Gram-positive bacterium isolated from Lake Nakuru. Jones *et al.* (1998**)** have assumed that many of the non-exacting alkaliphiles have spread outside the border of the soda lake environment. This is not surprising since soda lakes all around the world are obviously not entirely closed systems and instead suffering considerable disturbance from wildlife, in particular, the vast flocks of flamingos that feed in *Spirulina* and return their faeces to the nutrient cycle.

Soda lakes are typified by the presence of a high concentration of sodium carbonate formed by evaporative concentration, and are also associated with varying degrees of salinity and a low concentration of both magnesium and calcium ions (Grant and Tindall, 1986; Grant and Horikoshi, 1992; Grant and Jones, 2000).

A very narrow range of growth media was used in attempts to isolate bacterial and archaeal microorganisms, therefore, the proportion of organisms present in samples which could grow using the media provided was probably significantly reduced

compared to the total microbial population. All attempts to culture these microorganisms were carried out under aerobic conditions, thus excluding a further proportion of the microbial population which was micro-aerophilic or even anaerobes.

The question obviously arises- how do these alkaliphilic microorganisms grow in such an extreme environment and yet manage to grow at pH's less than neutral? Is there any difference in physiological and structural characteristics between alkaliphilic, alkalitolerant and neutrophilic microorganisms? The data presented in Chapter 5 will hopefully help, answer these questions.

CHAPTER 3

OSMO-ADAPTAION MECHANISMS OF HALOPHILIC AND HALO-TOLERANT BACTERIA AND ARCHAEA ISOLATED FROM NON-EXTREME ENVIRONMENTAL SAMPLES

3.1 Introduction

It is useful, at this point in the thesis, to distinguish genotypic from phenotypic adaptation. Genotypic adaptation corresponds to a long-term evolutionary change in the genetic structure of the microorganism permitting it to grow over a certain range of salinity. Phenotypic adaptation on the other hand, takes place within a population during their lifetime (i.e., it is the changes that take place within cells), and does not result from the selection of genetic variants. Halophiles display both genotypic and phenotypic adaptations; the halotolerants adapt phenotypically, often in a very similar manner to halophiles, but it is not clear whether they display genotypic adaptation (Russell, 1989).

Many extremophilic microorganisms have evolved unique properties of considerable biotechnological and, thus, commercial significance. The taxonomy, biochemistry, and physiology of these microorganisms, partially including industrial applications, have been reviewed by Galinski and Tindall (1992), Eisenberg *et al*. (1992), Grant *et al*. (1998), and Oren (1999). Briefly, two mechanisms are used to cope with environmental salinity. Halophilic archaea maintain their internal osmotic balance (cytoplasmic balance) with the hypersaline environment by accumulating high concentrations of a salt, often potassium. This strategy of osmoregulation requires particular adaptation of the intracellular enzymes that have to properly function in the presence of the salt. Halotolerant or halophilic eubacteria are characterized by a very high metabolic diversity. Thus, their intracellular

salt concentration is low and they manage an osmotic balance of their cytoplasm with the external medium by accumulating high concentrations of various compatible solutes; their intracellular enzymes have no special salt tolerance. Those microorganisms are able to develop in the presence of different concentrations of salt are found in all three domains of life: Archaea, Bacteria, and Eukarya. Those non-halophilic microorganisms which can grow in the absence and also in the presence of salt, are designated halotolerant; those halotolerants that have the ability to grow above approximately 15% (w/v) (2.5 M) of NaCl are considered to be extremely halotolerant. Microorganisms which require salt to grow are defined as halophiles. According to the most commonly used definition (Kushner, 1978), one can distinguish between slight halophiles (including many marine organisms; seawater contains about 3% (w/v) NaCl), moderate halophiles (with optimal growth at 3–15% (w/v) salt), extreme halophiles (with optimal growth at 25% (w/v) NaCl and above; halococci and halobacteria), and borderline extreme halophiles (these require at least 12% (w/v) salt) (Margesin and Schinner, 2001).

The aim of the work described in this Chapter focuses on how halophilic and halotolerant bacteria and archaea, isolated from non-saline environments, manage to grow under extreme saline conditions.

3.2 Materials and Methods

3.2.1 Identification and quantification of organic composition of intracellular solutes using NMR spectroscopy

Compatible solutes are best described as organic osmolytes which are responsible for osmotic balance and at the same time compatible with the cell metabolism (Galinski, 1993). A comprehensive survey of these solutes has been conducted using high-performance liquid chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) methods.

3.2.1.1 Sample preparation

A sample (5 ml) of each of the cell cultures was harvested at 5000 x g for 10 min and the supernatant was discarded. The resulting pellets were suspended in 1 ml of ddH_2O and vortexed for 1 minute at room temperature and then were sonicated 2 x20 sec (using Soniprep 15, SANYO) at 15 amplitude microns. The sonicated samples were then centrifuged at 5000 x g for 10 mins and the supernatant was transferred into microcentrifuge tubes. The resulting solutions were frozen at $^{-}80$ for 2h and then dried (Heto PowerDry PL9000), at -91.4^{o}C condenser and 0.36 hPa pressure, for two days.

Finally, ^{1}H in addition to 2D ^{1}H-^{1}H HSQC and 2D C-H HSQC NMR spectra were obtained by re-dissolving the dried cells in 500 µl of D_2O to the microcentrifuge tube and then 5 µl of trimethyl siylpropionate (TSP) added to the NMR tube (Frings *et al.*,1993). The dissolved samples were finally analyzed using the NMR.

3.2.1.2 NMR spectroscopy

NMR analyses were carried out on a Bruker DRX-500 spectrometer operating at 500 MHz for ^{1}H and 125.8 MHz for ^{1}H-^{1}H HSQC (Cummings, 1993). All spectra were obtained at a sample temperature of 25^{o}C using a 5 mm NMR tube (SIGMA). Sodium trimethyl silylpropionate (TSP) was used as an internal standard for both chemical shift referencing and concentration. Chemical shifts are listed in ppm (frequency relative to TSP x10^{6}).

For ^{1}H, spectra were acquired using a 90^{o} pulse width (9-20 µs, depending on salt concentration) in to 4k complex points over a spectral width of 12500Hz. This gave an acquisition time of 330 ms, and a 1s relaxation delay was used between scans, typically acquiring 128 transients depending on signal intensity in the sample.

The two-dimensional TOCSY (total correlation spectroscopy) spectrum was acquired on a Bruker DRX-600 at 600 MHz using an 80 ms spin-lock mixing time, a spectral width

of 15015 Hz in the direct dimension and 7507.5 Hz in the indirect dimension, 4k complex points in the direct dimension and 400 in the indirect dimension, and a spin-lock field of 8.4 kHz. Two-dimensional ^{1}H-^{1}H and ^{1}H correlated spectra (C-HSQC) were acquired using 2k real points over a spectral width of 6266 Hz, with a relaxation delay of 2s. ^{1}H garp decoupling was applied during the acquisition time.

The magnetisation pathway was selected using gradients, and the magnetisation transfer delay was optimised for a ^{1}JCH (3-bond steps J-coupling) coupling constant of 145 Hz. In the indirect (^{1}H) dimension, 64 complex points were acquired over a spectral width of 8818 Hz. All spectra were transferred to a UNIX computer and processed using FELIX (Accelrys, San Diego, CA). Assignments were made by spiking the sample with authentic samples, and by comparison to published data (e. g. Nagata *et al*., 1996).

3.2.1.3 Quantification of compatible solutes

Quantification was performed by ^{1}H NMR using lysine as an internal concentration standard. Peak integrals were used to quantify proline, glutamate and glycine betaine concentrations (most common compatible solutes in halophiles) by comparison with the integrals obtained for standard solutions.

3.2.2 Determination of intracellular potassium ion concentration

3.2.2.1 Sample preparation

Total cellular potassium ion concentration was determined by atomic emission spectrophotometry (AES). Based on OD_{600} at 1.5, 10 ml of cells at the various NaCl concentrations were harvested at late log phase from the four halophilic bacteria strains for the adaptive growth experiments. The resulting pellets were washed by ddH_2O (10 ml x3).

The washed pellets were suspended in 4 ml (standard volumes to use in AES) of ddH_2O and vortexed for 1 minute at room temperature and then sonicated (2 x20 sec). The

sonicated samples were centrifuged at 3000 x g for 10 mins and then the supernatant was transferred into (15ml) falcon tube. Then, the resulting solutions were sent to the Kroto Research Institute, University of Sheffield for AES.

3.2.2.2 Atomic emission spectrophotometer (AES)

The analytical quality control (AQC) values should have been 5.00mg/l. All the samples were diluted to bring them in to the calibration range 1 to 10 mg/l; this dilution factor has been taken in to account. The ddH_2O was used as a blank and to zero the machine.

3.3 Results and Discussion

3.3.1 Accumulation of compatible solutes as strategies for adapting to a high salt stress

3.3.1.1 Osmoregulatory solutes in halotolerant and halophilic isolates

In nuclear magnetic resonance (NMR), the chemical shift expresses the dependence of the levels of nuclear magnetic energy on the electronic environment in a molecule. The chemical shift is important in NMR analysis as a technique to investigate molecular properties by looking at nuclear magnetic resonance spectra. In absolute terms, the chemical shift is defined by the frequency of the resonance expressed with reference to a standard compound which is defined to be at 0 ppm. Chemical shifts (δ) are relevant and significant in NMR spectroscopy performance such as proton (^{1}H) NMR and carbon-13 (^{13}C) NMR and is generally expressed in parts per million (p.p.m) by frequency of a signal (Derome, 1987; Wishart *et al*., 1992; Chylla and Markley, 1995)

The tested sample is positioned in the magnetic field and excited using pulsations in the radio frequency input circuit. The realigned magnetic fields stimulate a radio signal in the output circuit which is used to produce the output signal. Instant analysis of the

complex output generates the actual spectrum. The pulse action is repeated as many times as required in order to allow the signals to be recognized from the background noise.

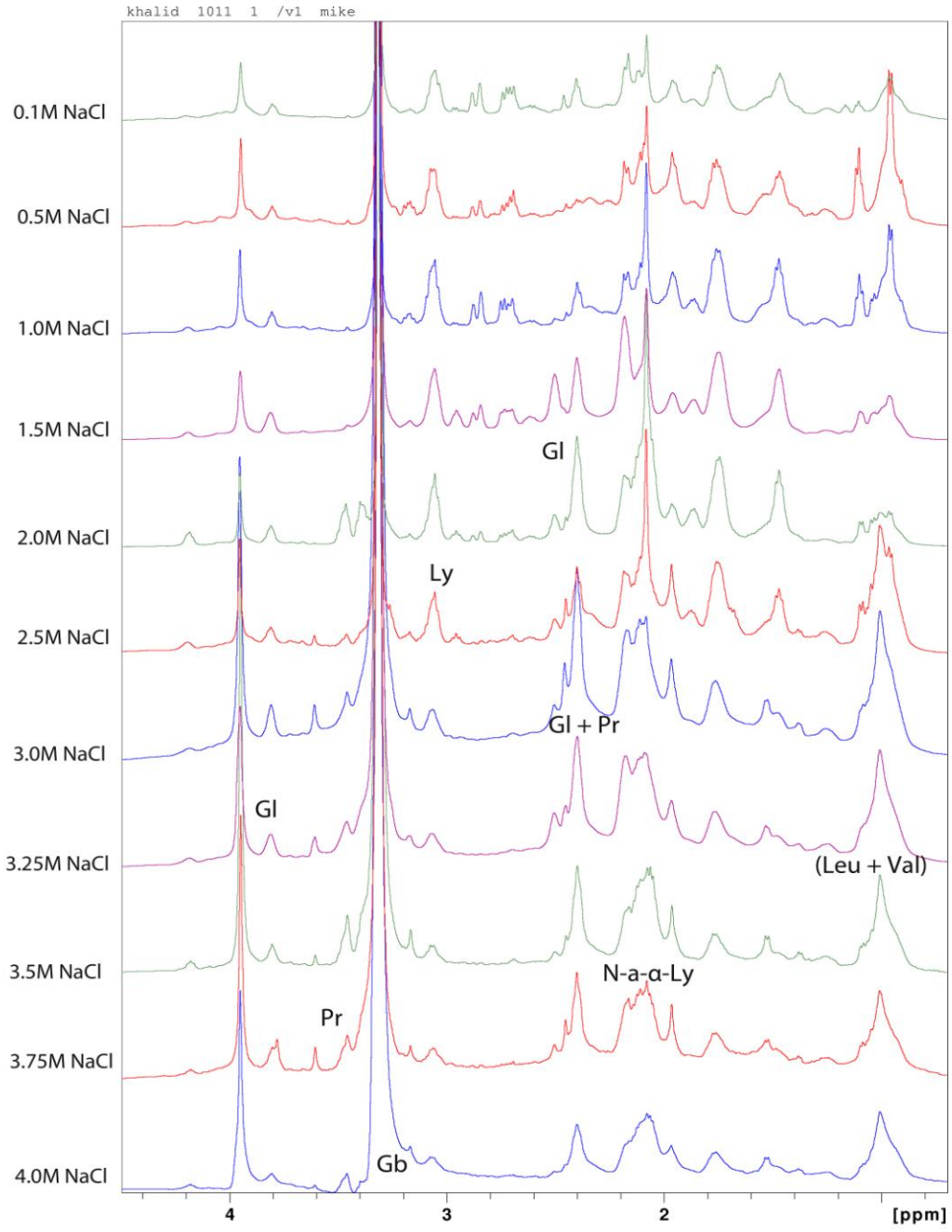
Most of isolated halotolerant and halopilic isolates grew better in rich media than minimal media. Compatible solutes present in the halotolarant isolates, HL1 and STRA2, in addition to DSRT3 and haliphilic isolate HSO grown at the various NaCl concentrations were analysed after growth in LB media containing yeast extract and M9 minimal media with 0.5% LB; limited growth generally occurred in the latter.

The effect of increasing salinity on the quantitative and qualitative content of compatible solutes in these four isolates was investigated. The resultant NMR spectra show relative concentrations of the analysed and common compatible solutes determined in these isolate when grown under various salinities.

In HL1 isolate, glycine betaine was shown to be the main organic solute present in the LB media using ^{1}H NMR spectra (Figure 3.1 (a)) at all NaCl concentrations. The glycine betaine concentration increased significantly in this isolate grown in NaCl concentration ranging from 0.1M to 4.0M NaCl concentration. In M9 minimal medium however, glycine betaine was found in low concentrations until 0.5% of LB medium (at 3.5M) had to be used to enhance the growth of this isolate (Figure 3.1 (b)). Several different amino acids, notably glutamate, proline and lysine were also identified. As the osmotic stress was increased, the production of these amino acids was also increased.

Interestingly, N-acetyl-α-lysine has been identified in this isolate following growth in both media which is not very commonly the case for halotolerant or halophilic bacteria. The concentration of this compound was constant at all NaCl concentrations in minimal media but varied in LB media increasing from 0.1 M to 2.5 M NaCl, followed by a decrease.

(a)


khalid 1011 1 /v1 mike
0.1M NaCl
0.5M NaCl
1.0M NaCl
1.5M NaCl
2.0M NaCl
2.5M NaCl
3.0M NaCl
3.25M NaCl
3.5M NaCl
3.75M NaCl
4.0M NaCl
Gl
Ly
Gl + Pr
Gl
(Leu + Val)
N-a-α-Ly
Pr
Gb
4
3
2
[ppm]

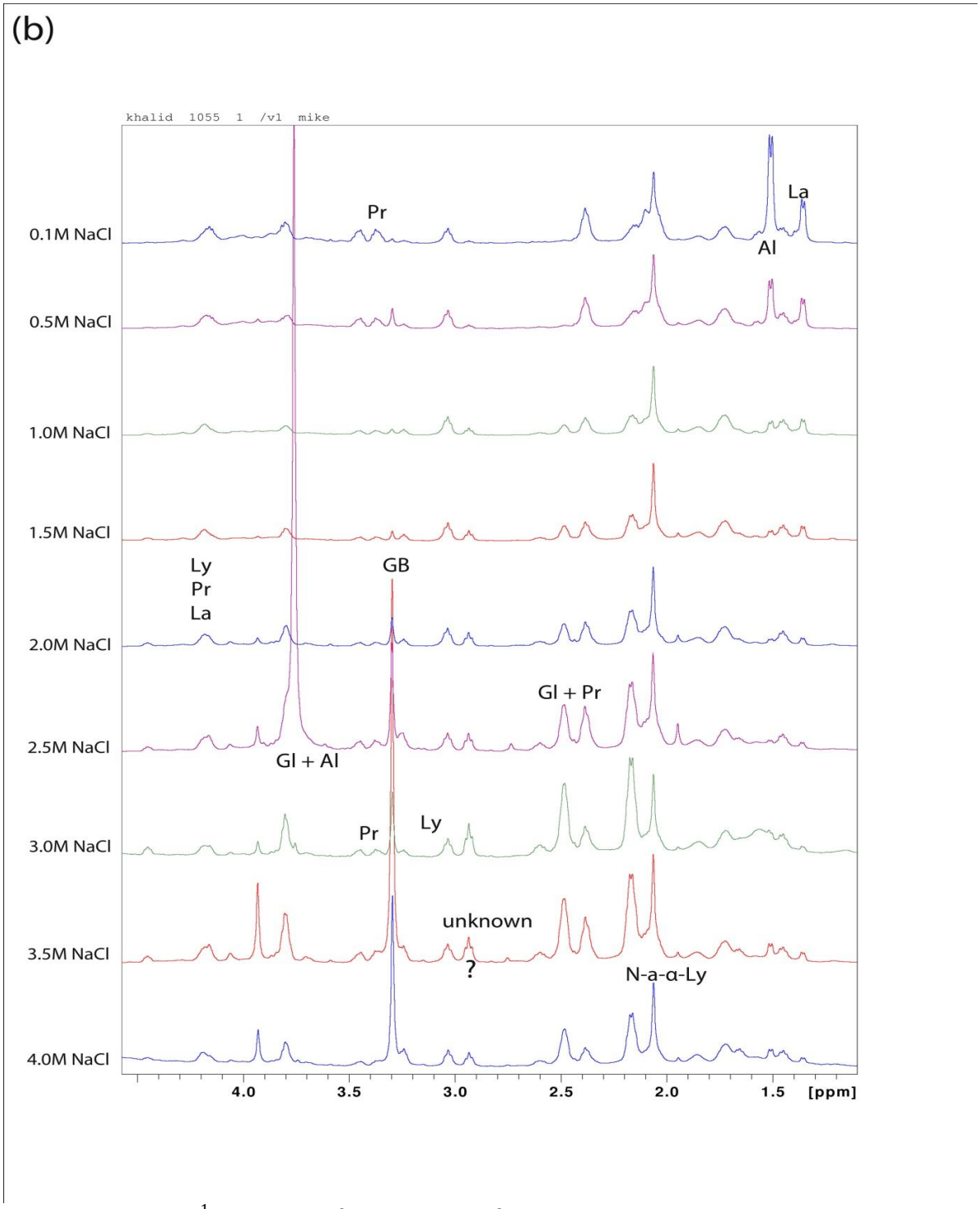
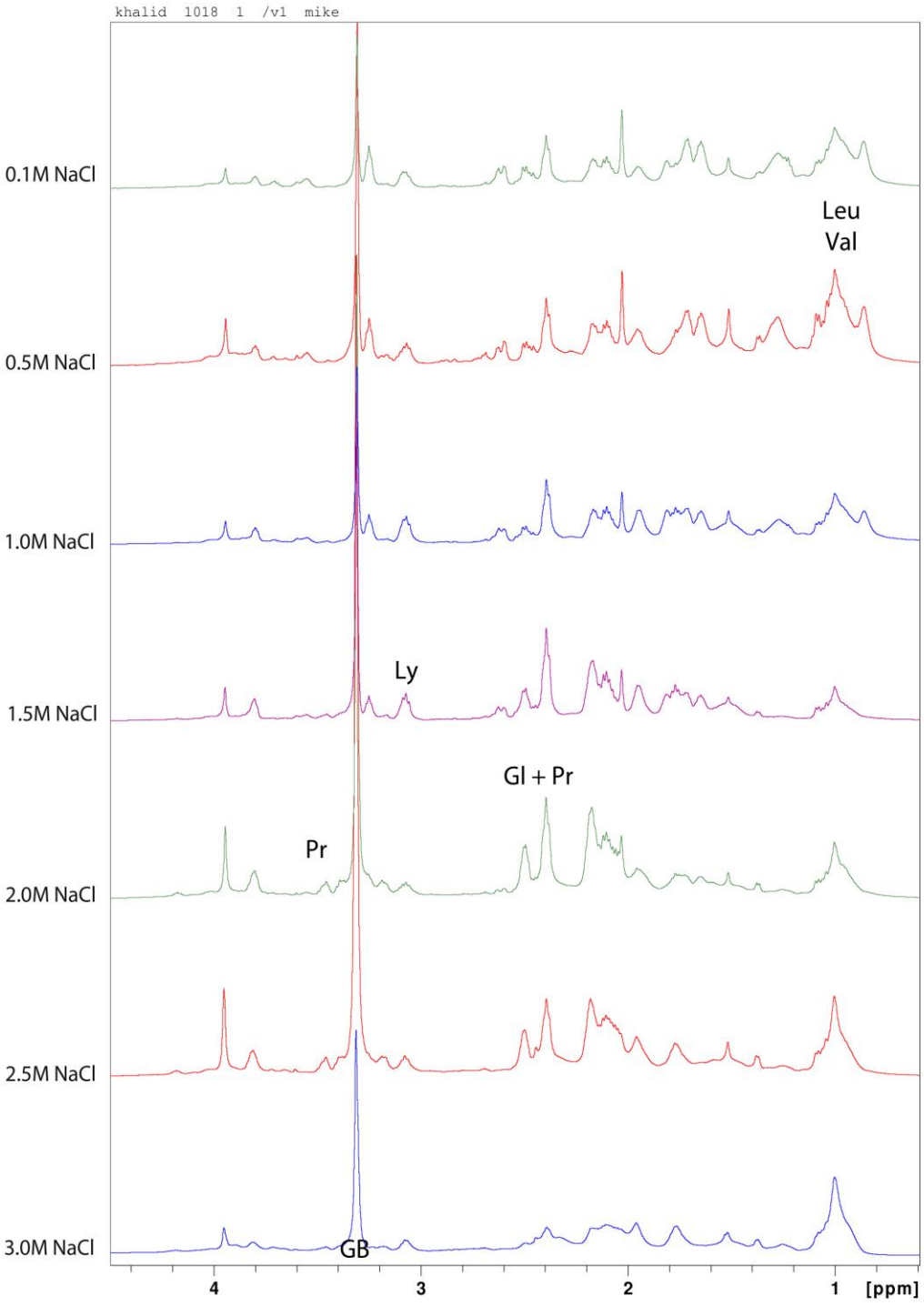


Figure 3.1 NMR ^{1}H spectra of cell extracts from HL1 at 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 NaCl (M); **(a)** in LB medium and **(b)** in M9 minimal salt medium spectra. The ^{1}H spectra identified, GB; glycine betaine, Pr; proline, Gl; glutamate,; Ly; lysine, N-a-α-ly; N-acetyle-α-lysine; Al; alanine, La; lactate, Leu; leucine, Val; valine, as the most abundant compatible solutes.

Compatible solutes present in the STRA2 isolate grown at the various NaCl concentrations were also analysed. As was the case in HL1, glycine betaine was shown to be the main organic solute present in the LB medium using ^{1}H NMR spectra (Figure 3.2 (a)) at all NaCl concentrations. Different amino acids such as glutamate, proline and lysine were, however, were involved in the osmoregulation of this isolate. The concentration of these compatible solutes increased significantly in this isolate when it was grown in NaCl concentrations ranging from 0.1M to 3.0M NaCl. Again, the production of glycine betaine using M9 minimal medium was found at very low concentrations; as a result, 0.5% of LB medium (at 3.0 M NaCl) had again to be used to enhance the isolate's growth (Figure 3.2 (b)).

(a)


khalid 1018 1 /v1 mike
0.1M NaCl
0.5M NaCl
1.0M NaCl
1.5M NaCl
2.0M NaCl
2.5M NaCl
3.0M NaCl
Leu
Val
Ly
Gl + Pr
Pr
GB
4
3
2
1
[ppm]

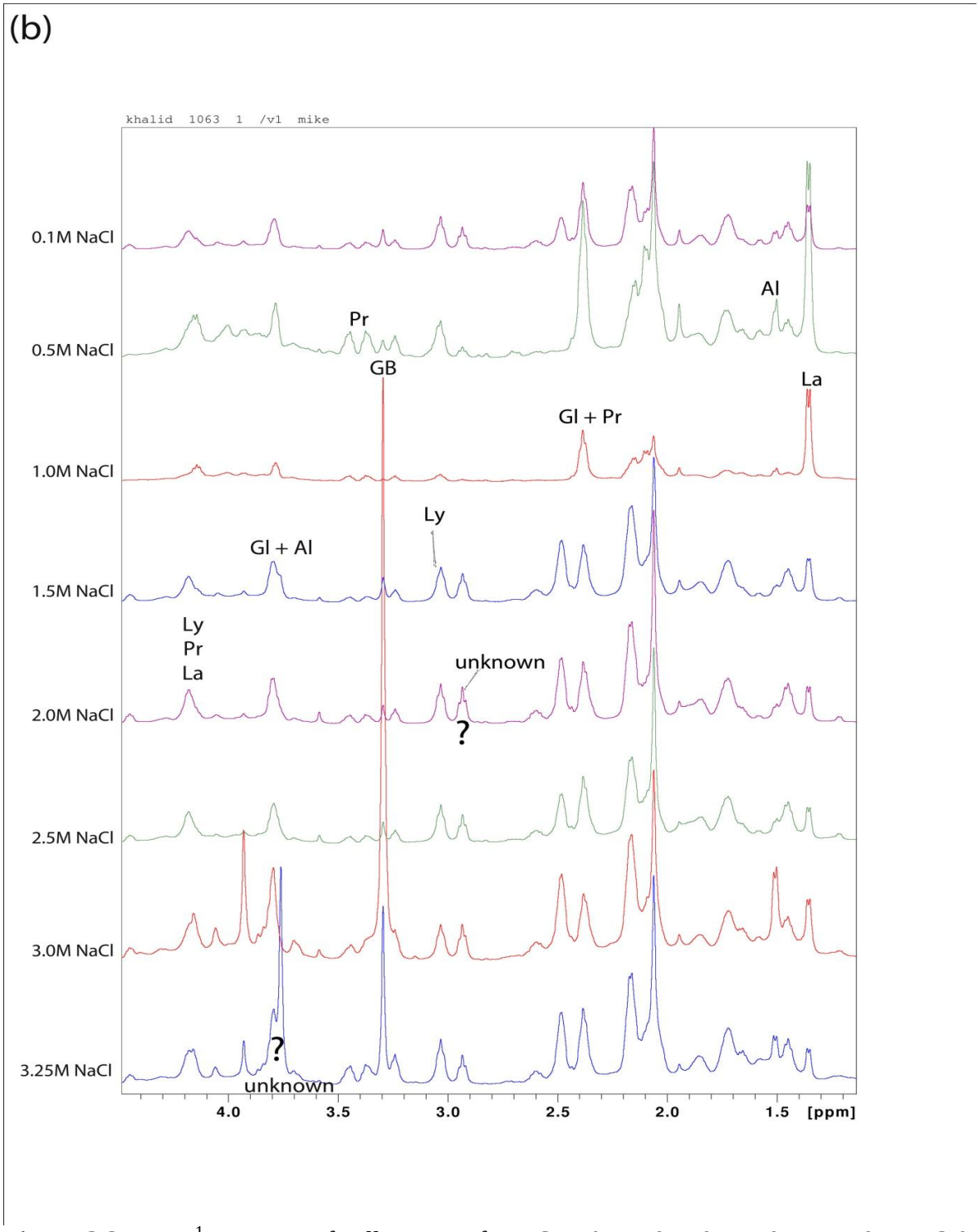
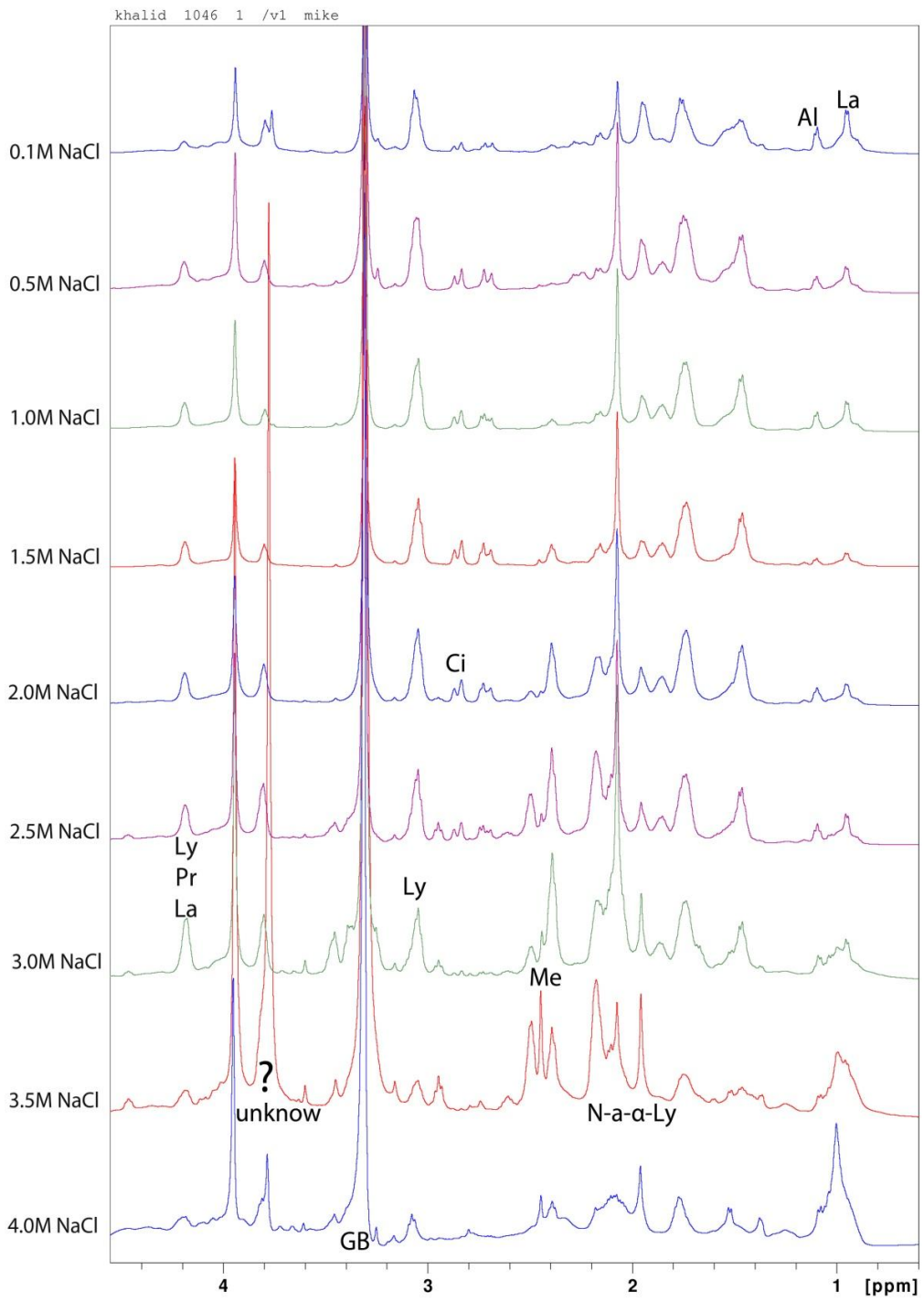


Figure 3.2 NMR ^{1}H spectra of cell extracts from STRA2 at 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 NaCl (M); **(a)** in LB medium and **(b)** in M9 minimal salt medium spectra. The ^{1}H spectra identified, GB; glycine betaine, Pr; proline, Gl; glutamate,; Ly; lysine, Al; alanine, La; lactate, Leu; leucine, Val; valine, as the most abundant compatible solutes.

The ^{1}H NMR spectra of isolate DSRT3 showed the presence of a mixture of different compounds and this complicated their identification. Again, when grown in LB medium, this isolate used glycine betaine as its main organic solute. Unlike the former two isolates, however, several different compatible solutes, one of which is citrate, methyl, N-acetyl-α-lysine and lysine were involved in osmo-adaptaion in this isolate (Figure 3.3 (a)).

The production of glycine using M9 minimal medium was not a major organic solute used in osmo-adaptaion of DSRT3 isolate, especially at 3.5 M NaCl although, 0.5% of LB medium was used to enhance the isolate growth at 3.0 M NaCl (Figure 3.3 (b)). This isolate synthesized glycine betaine at low salt concentration, particularly at 0.5-1.5 (M).

(a)


khalid 1046 1 /v1 mike
0.1M NaCl
0.5M NaCl
1.0M NaCl
1.5M NaCl
2.0M NaCl
2.5M NaCl
3.0M NaCl
3.5M NaCl
4.0M NaCl
Al
La
Ci
Ly
Pr
La
Ly
Me
?
unknow
N-a-α-Ly
GB
4
3
2
1
[ppm]

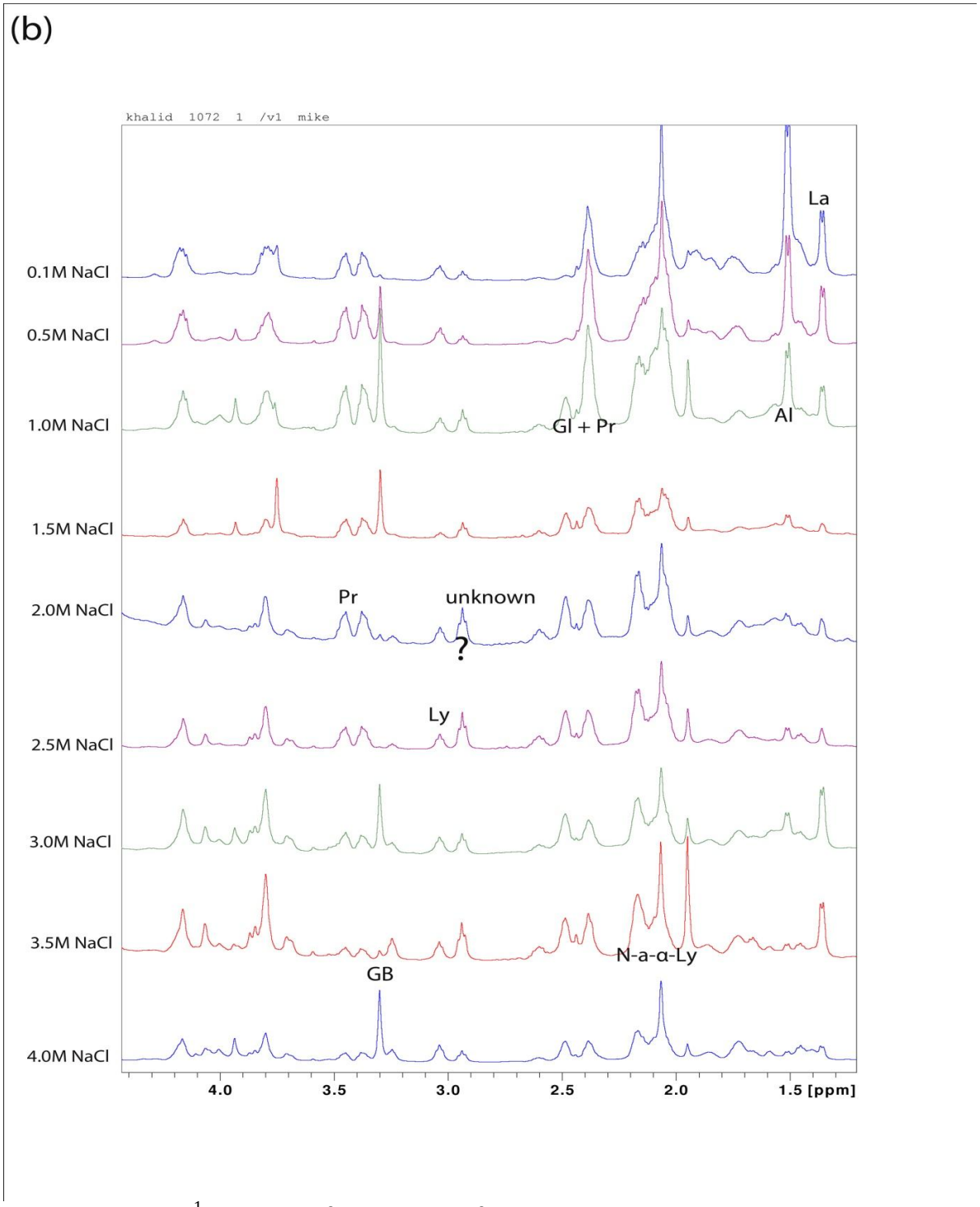
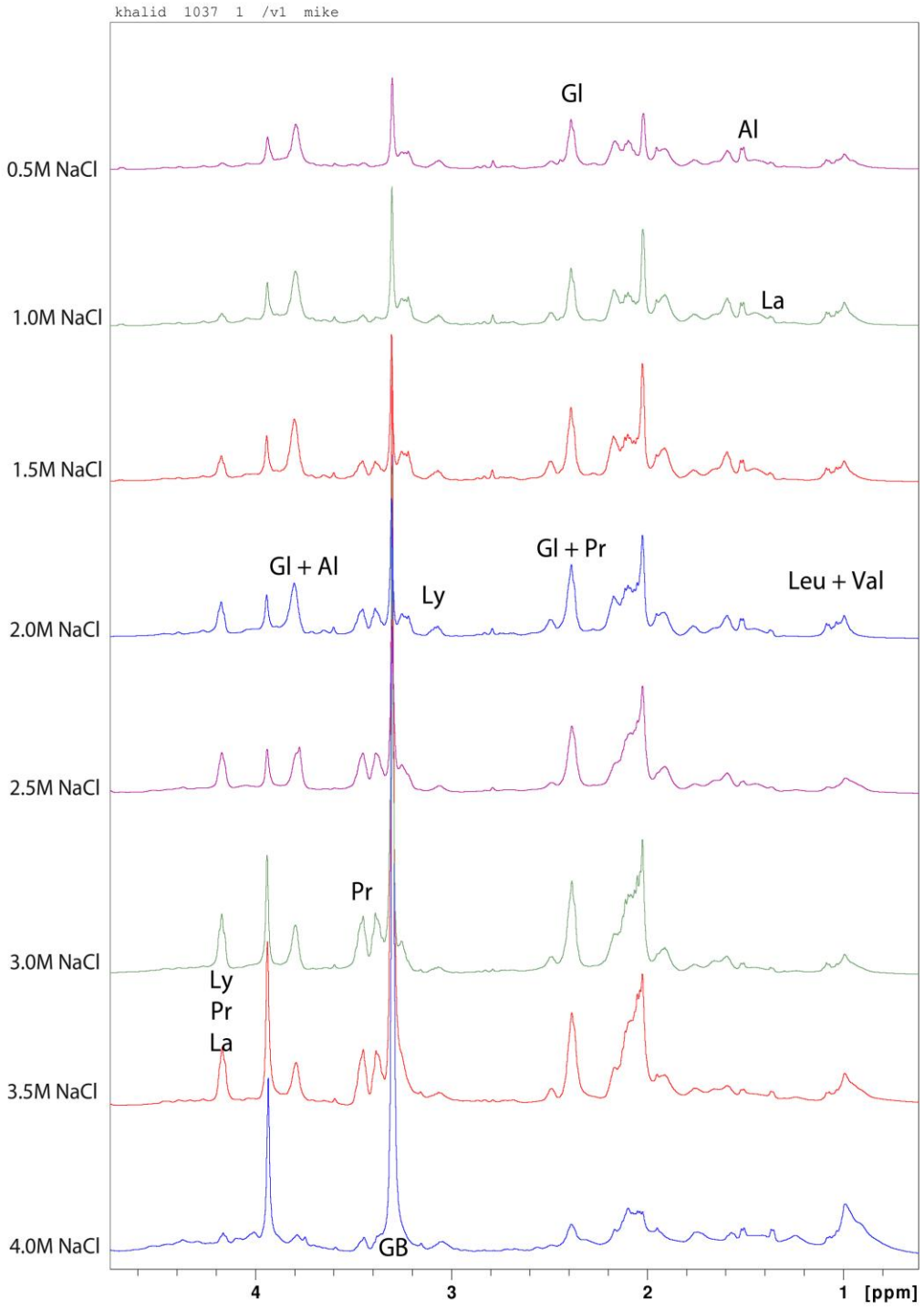


Figure 3.3 NMR ^{1}H spectra of cell extracts from DSRT3 at 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 NaCl (M); **(a)** in LB medium and **(b)** in M9 minimal salt medium spectra. The ^{1}H spectra identified, GB; glycine betaine, Pr; proline, Gl; glutamate, Ly; Lysine, N-a-α-ly; N-acetyle-α-lysine; Al; alanine, La; lactate, Ci; citrate, Me; methyl, as the most abundant compatible solutes.

The HSO isolate was identified as being a moderate halophilic bacterium. The analysis of ^{1}H NMR spectra of this isolate showed the presence of a mixture of different compounds which were also found in a different pattern of compatible solutes concentration to that seen in the other isolates. All of the isolates seen so far increased the production of compatible solutes as the osmotic stress (M NaCl) increased. In halophilic HSO isolate, however, the production of compatible solutes was essentially constant at all NaCl concentrations (Figure 3.4 (a)) except at 3.0M and 4.0 M NaCl where the production was high, showing that this halophilic isolate became stressed at 3.0 M and above.

Generally, glycine betaine, glutamate, proline and lysine were identified. Extreme halophilic isolates are unique in producing ectoine as a main compatible solute. Interestingly, the HSO isolate produce ectoine only when grown in M9 minimal medium, Figure 3.4 (b), and only at 1.0, 1.5 and 2.0 M NaCl where its growth was at optimum (Chapter 2, Session 2.3.5). It has been reported that glycine betaine suppresses *de novo* ectoine synthesis completely or partially, depending on the concentration of NaCl which is present in the growth medium (Calnovas *et al*., 1996). Recently, Kurz *et al.* (2010) have also found that betaine suppresses the production of many of these compounds including ectoine, thereby suggesting that the production of the osmolyte itself is generally inhibited when osmolyte uptake is possible.

(a)


khalid 1037 1 /v1 mike
Gl
Al
La
Gl + Al
Ly
Gl + Pr
Leu + Val
Pr
Ly
Pr
La
GB
0.5M NaCl
1.0M NaCl
1.5M NaCl
2.0M NaCl
2.5M NaCl
3.0M NaCl
3.5M NaCl
4.0M NaCl
4
3
2
1 [ppm]

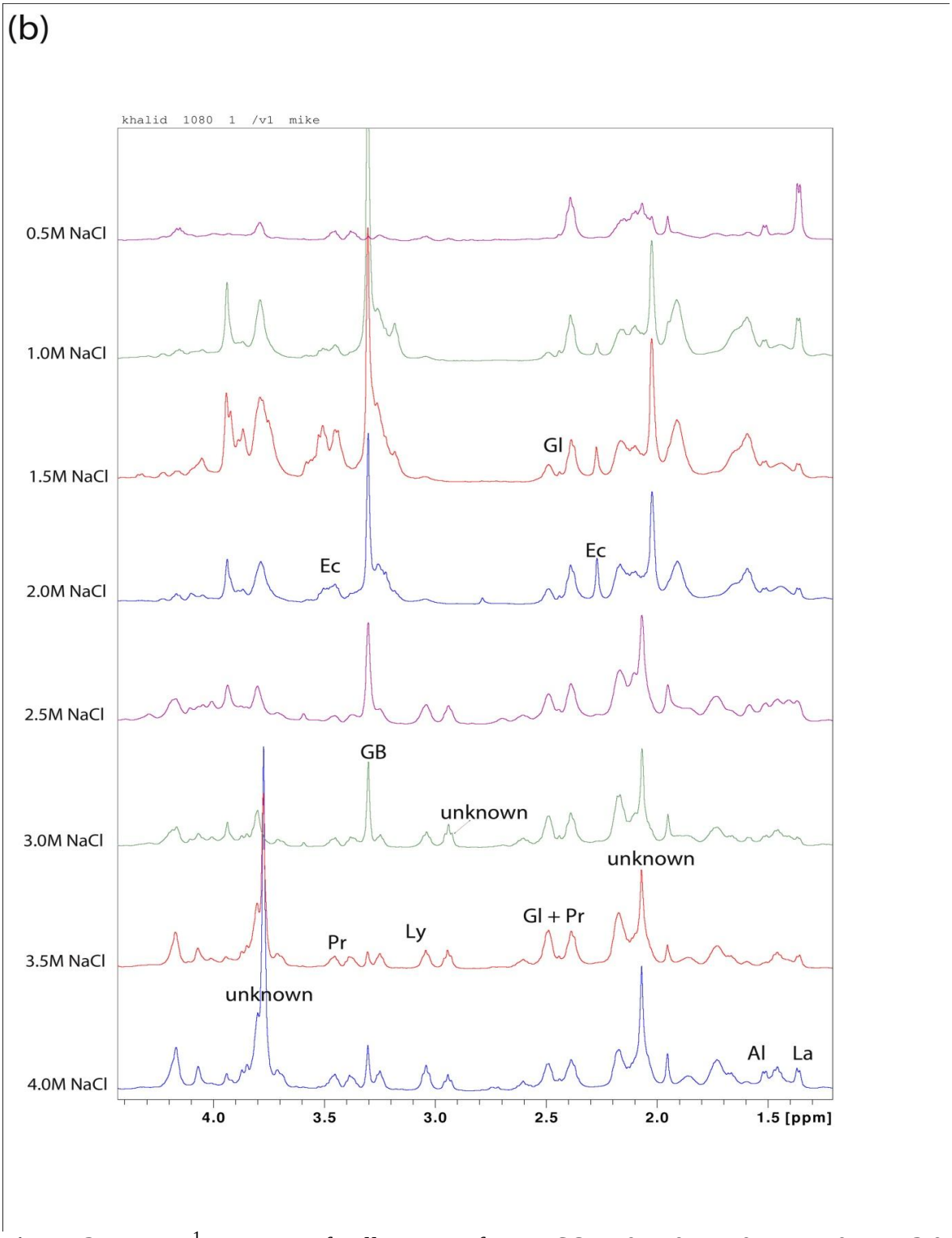


Figure 3.4 NMR ^{1}H spectra of cell extracts from HSO at 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 NaCl (M); **(a)** in LB medium and **(b)** in M9 minimal salt medium spectra. The ^{1}H spectra identified, GB; glycine betaine, Pr; proline, Gl; glutamate,; Ec; ectoine, Ly; lysine, Al; alanine, La; lactate, Leu; leucine, Val; valine, as the most abundant compatible solutes.

Extra spectra were run to determine the most common compounds, which proved worthwhile particularly in relation to the signals which were present between chemical shifts. A 2D C-H HSQC spectrum was run and this was to correlate ^{1}H chemical shifts with ^{13}C shifts and therefore provided a much more secure identification of what is present. However, it became clear there were some osmolytes present, and this spectrum fails to differentiate between them. Another spectrum, was therefore run, i.e. a ^{1}H-^{1}H 2D spectrum (Figure 3.5), and it connected together ^{1}H signals within a series of 3-bond steps of each other (i.e. that are J-coupled). This means that it could be determined which set of signals come from the same molecule as each other. One set has been highlighted in green and one in red, i.e. the same colour coding as used for the C-H HSQC (Figure 3.6). As a result, a list of H and C chemical shifts was produced for each molecule. A search of various databases was made. The best fit for the red signal turned out to match exactly with proline.

The green signals shown in Figure 3.7, matched closely to lysine, except that there was also a sharp intense signal at about 2.05 ppm which is typical for an N-acetyl group; the compound was N-acetyl lysine, a well-known osmolyte in archaea. However, what was in fact found here was clearly not beta-lysine, but alpha-lysine, a compound which appears not to have previously been identified as an osmolyte. Glycine betaine is also present (blue signals in HSQC), and this is a compound which is almost universally present when cells are grown on LB medium in the presence of salt.

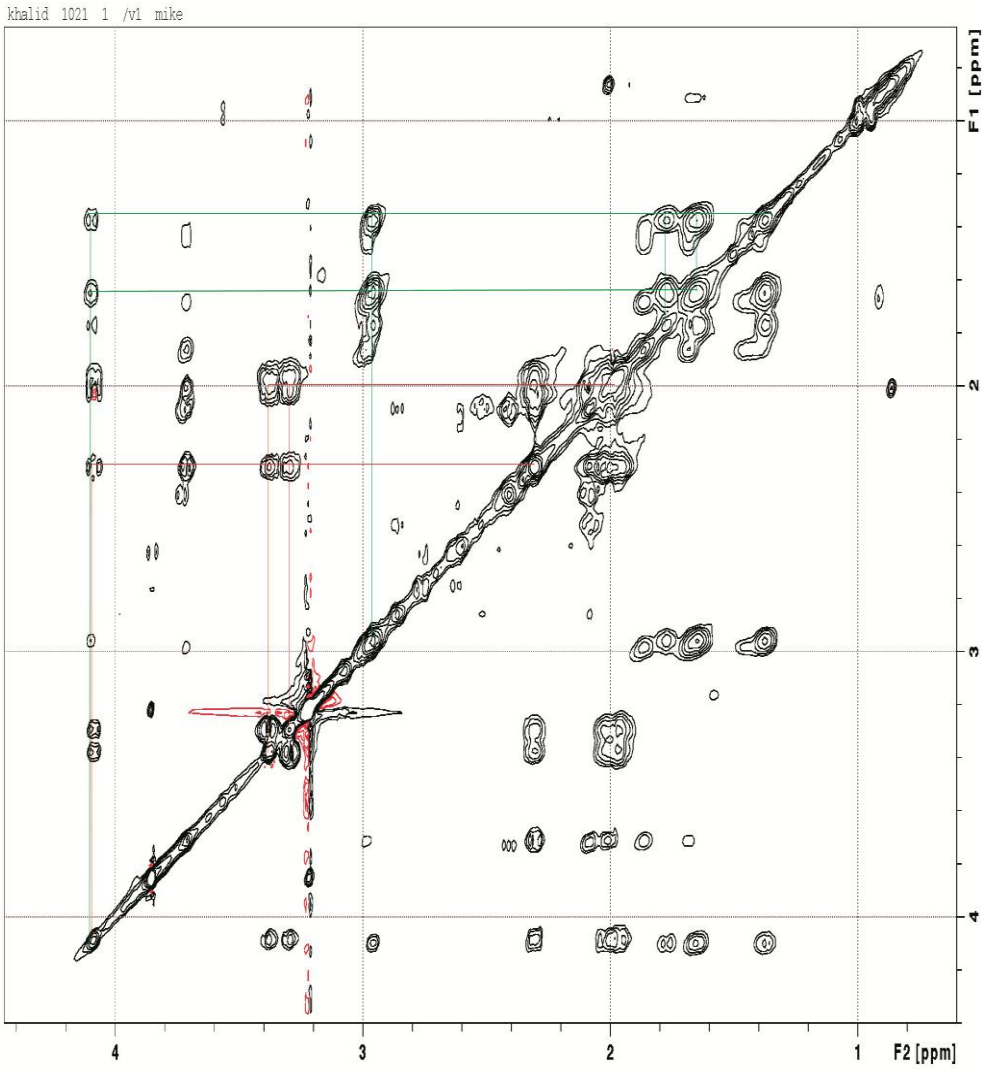


Figure 3.5 A 500 MHz ^{1}H-^{1}H 2D HSQC spectrum of cell extract from the HL1 cells grown at 3.0M NaCl in LB medium. The two dimensions are labeled with the chemical shift in ppm from TSP.

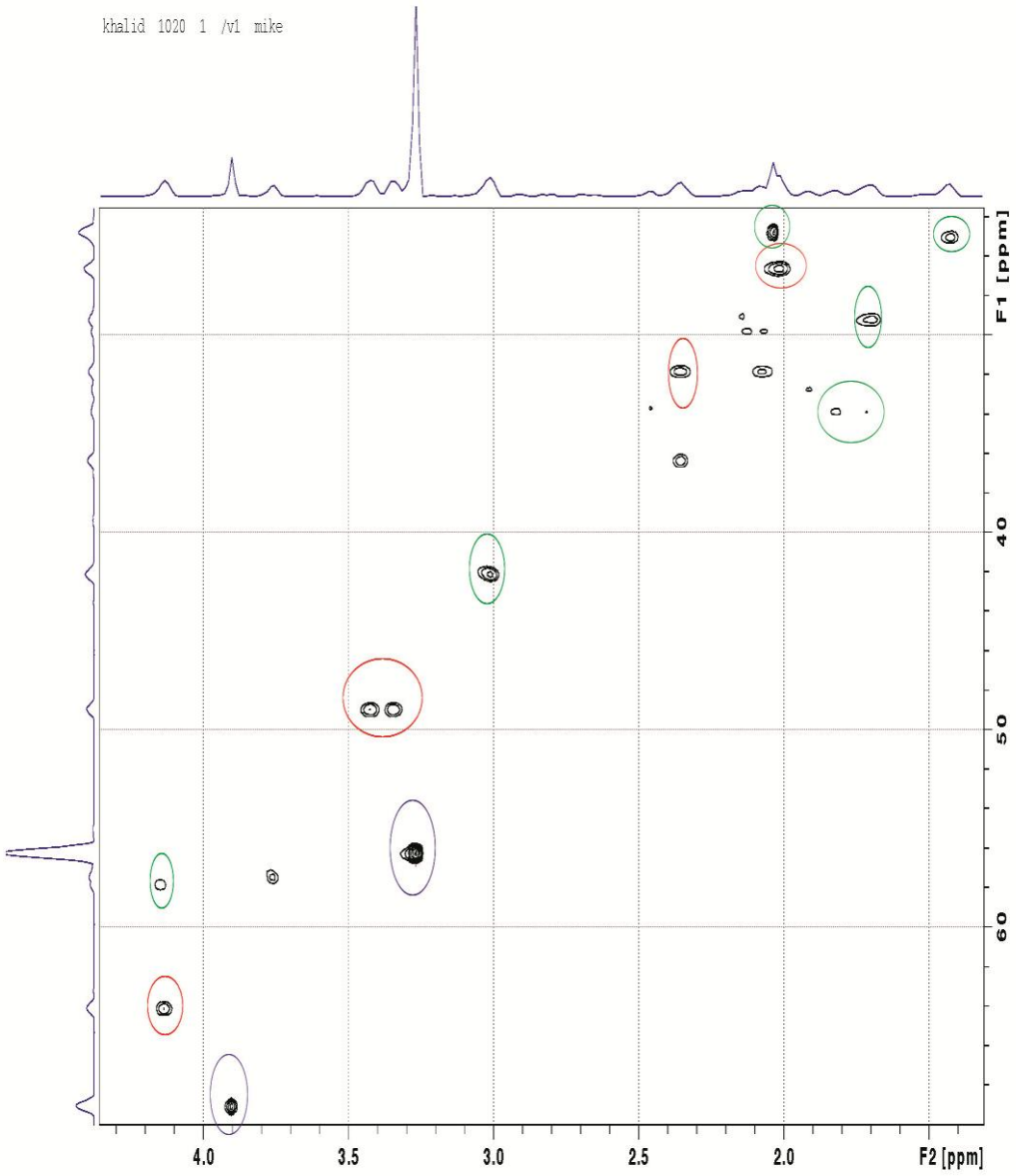


Figure 3.6 2D C-H HSQC spectrum of cell extract from the HL1 cells grown at 3.0M NaCl in LB medium. This correlates ^{1}H chemical shifts with ^{13}C shifts and therefore gives a much more secure identification

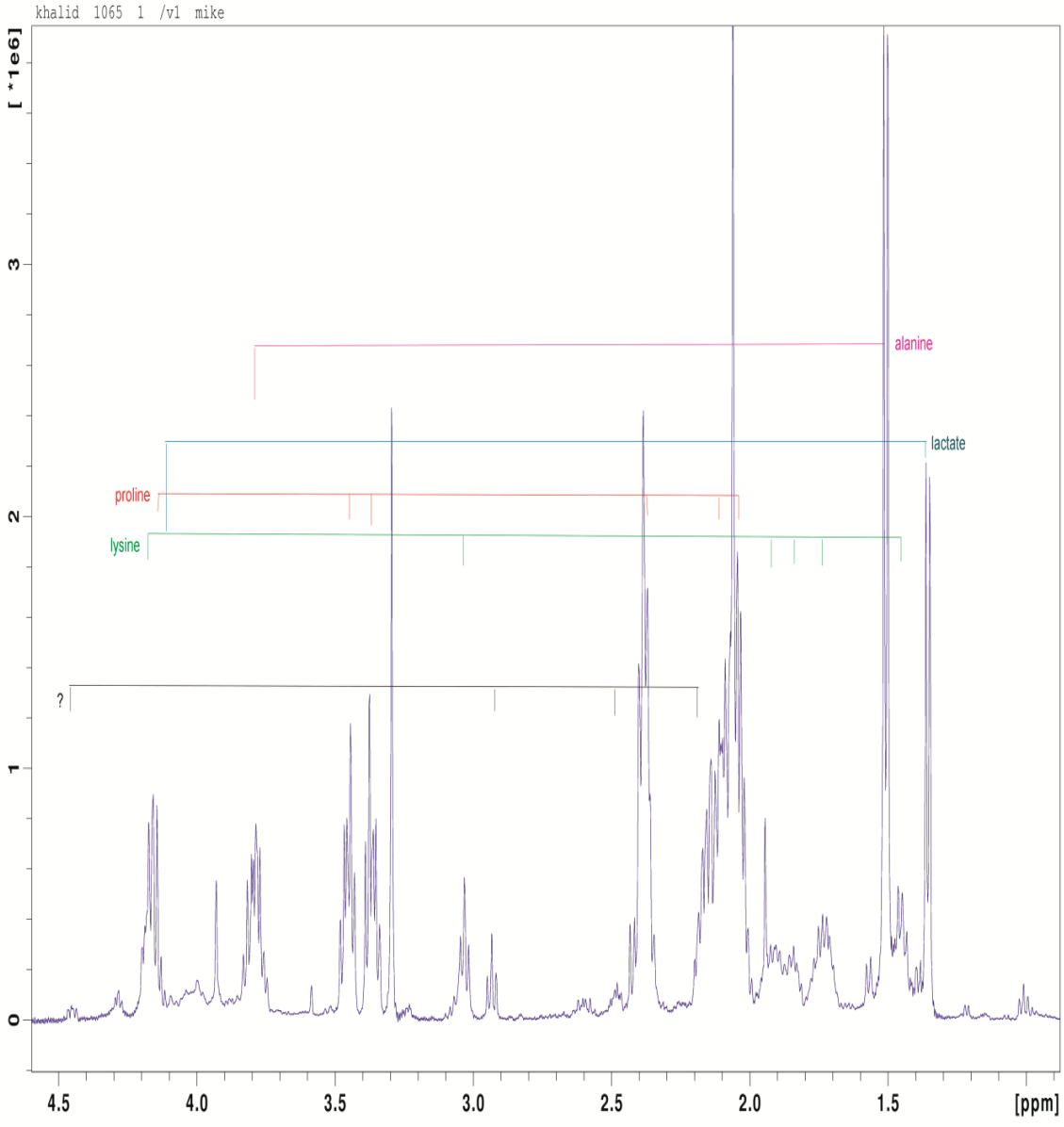


Figure 3.7 Reports NMR ^{1}H spectra of common compatible solutes determined in these isolates, HL1, STRA2, DSRT3 and HSO, grown under various salinities.

Although, in most isolates, the results for intracellular glycine betaine levels above 1.0M NaCl showed large variability, a general trend towards an increase in glycine betaine level was observed.

Generally, in some isolates, the ^{1}H NMR analysis showed the presence of a mixture of different compounds which complicated their identification. As result, the extract was further partially purified by fractionation into basic fractions, thereby enabling the identification of the compounds present using ^{1}H NMR. In the basic fractions, glycine betaine was identified from its resonances at 3.2 ppm and 3.9 ppm (Figure 3.7). Several other amino acids, one of which is glutamate, were also identified. Glutamate, proline and some other organic osmolytes were further identified using 2 dimensional HSQC NMR (Figure 3.8). Identification of these compounds was further confirmed by comparison to the known proton chemical shifts of glycine betaine, glutamate and proline. These compounds were also identified when NMR analysis was carried out on yeast extract and casamino acid, both of which were included in the medium used to grow the bacteria.

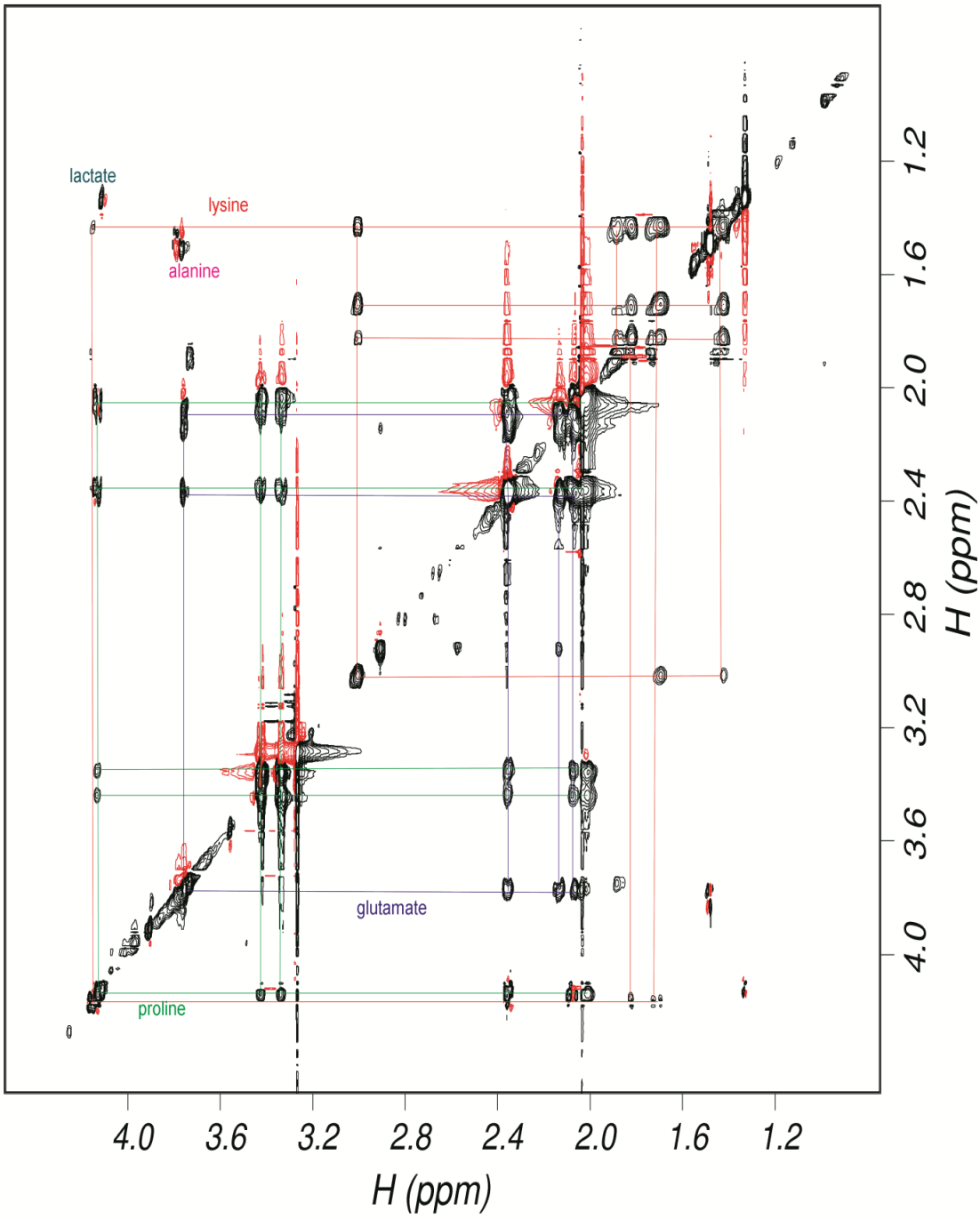


Figure 3.8 A 500 MHz ^{1}H-^{1}H 2D HSQC spectrum of cell extract from the STRA3 cells grown at 3.0M NaCl in M9 minimal media. (The two dimensions are labeled with the chemical shift in ppm from TSP. Assignments are marked on the spectrum: proline, glutamate, alanine, lactate and lysine.

NMR analyses of compatible solutes in all halotolerant isolates showed the accumulation of glycine betaine is important for osmotic adaptation of this isolate when growing in presence of high NaCl concentrations (except when grown in M9 medium). The fact that betaine was the main osmoprotectant present in most isolates depended on the composition of the medium (i.e. LB medium contains yeast extract) indicates that the presence of betaine in the cells was definitely not synthesized by the cells (e. g. *de novo*) and it might be that betaine is absolutely required as a organic osmolytes for these isolates when grown under stressed conditions; it is likely that only extreme halotolerant cyanobacteria synthesise glycine or glutamate betaine *de novo* (Page-Sharp *et al.*, 1999).

Like halotolerant strains, the halophilic isolate isolated from hypersaline environments, HSO, accumulate betaine as the sole osmoprotectant to adapt to high salinities when grown in LB medium. As a moderate halotolerant, HSO should accumulate ectoine, which happened when it grew in minimal salt, not betaine.

3.3.1.2 Estimation of the quantity of compatible solutes

Due to the wide mixture of compounds present the only compounds which could be quantified were proline, glutamate and glycine betaine. However, quantification by NMR is often not accurate due to the effects of temperature and pH changes during analysis and extraction procedures that bring about the loss of these compounds. Nevertheless, the results obtained revealed a trend which furthers our understanding of adaptation in these microorganisms. Quantification of glycine betaine and proline at different NaCl concentrations was performed in halophilic HSO isolate. As shown in Figure 3.9, as NaCl concentration increases, glycine betaine and proline levels in the cells also increase (up to almost 7.0 µmole/g).

The above can be explained on the basis that either the HSO isolate was only able to synthesise glycine betaine under low or optimal growth conditions in minimal medium and

the unknown or unable to identified compatible solute was produced in response to this deficit. Alternatively this isolate may only be able to produce unknown compatible solute at high salinities and the glycine betaine level decrease as a result from the production of this unknown compound. A final possibility is that ectoine in HSO cells increased as the salinity increased at 1-2 M NaCl, but was undetectable beyond a concentration of 2M salt**.**

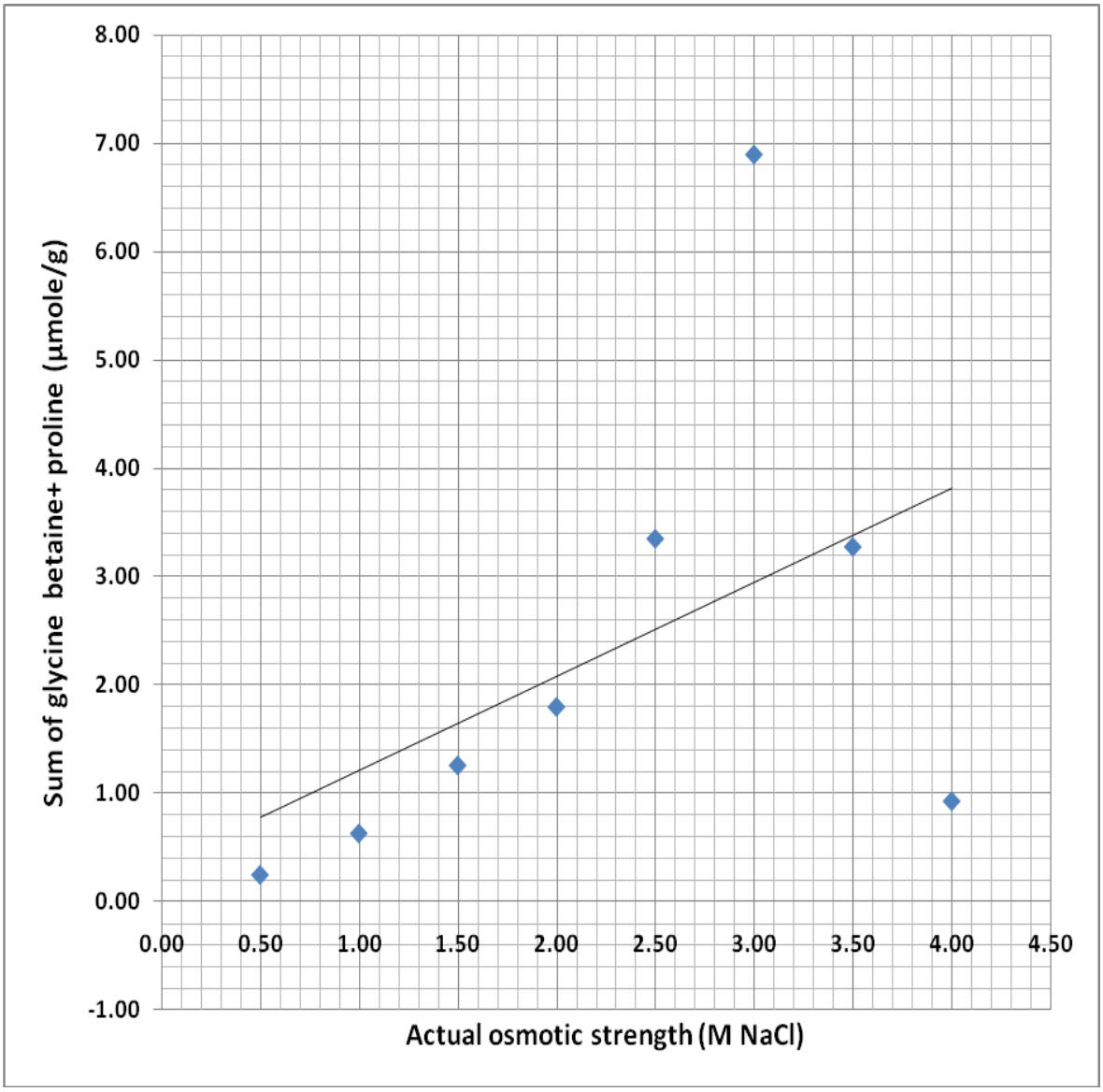


Figure 3.9 The sum of glycine betaine and proline concentrations measured by ^{1}H NMR against dry weight of halophilic HSO isolate grown under 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 NaCl (M) in LB medium. (R^2 value; y= 0.989x)

Quantification of glycine betaine and methyl at different NaCl concentrations was performed using the DSRT3 isolate when growing in LB medium. As shown in Figure 3.10, as NaCl concentration increased, glycine betaine and methyl levels in the cells also increased. As with the halophilic HSO isolate, for some unknown reasons the concentration of these compatible solutes then fell (4.8 µmole/g) in 4M NaCl after having reached the peak (5.6 µmole/g) at 3.5M.

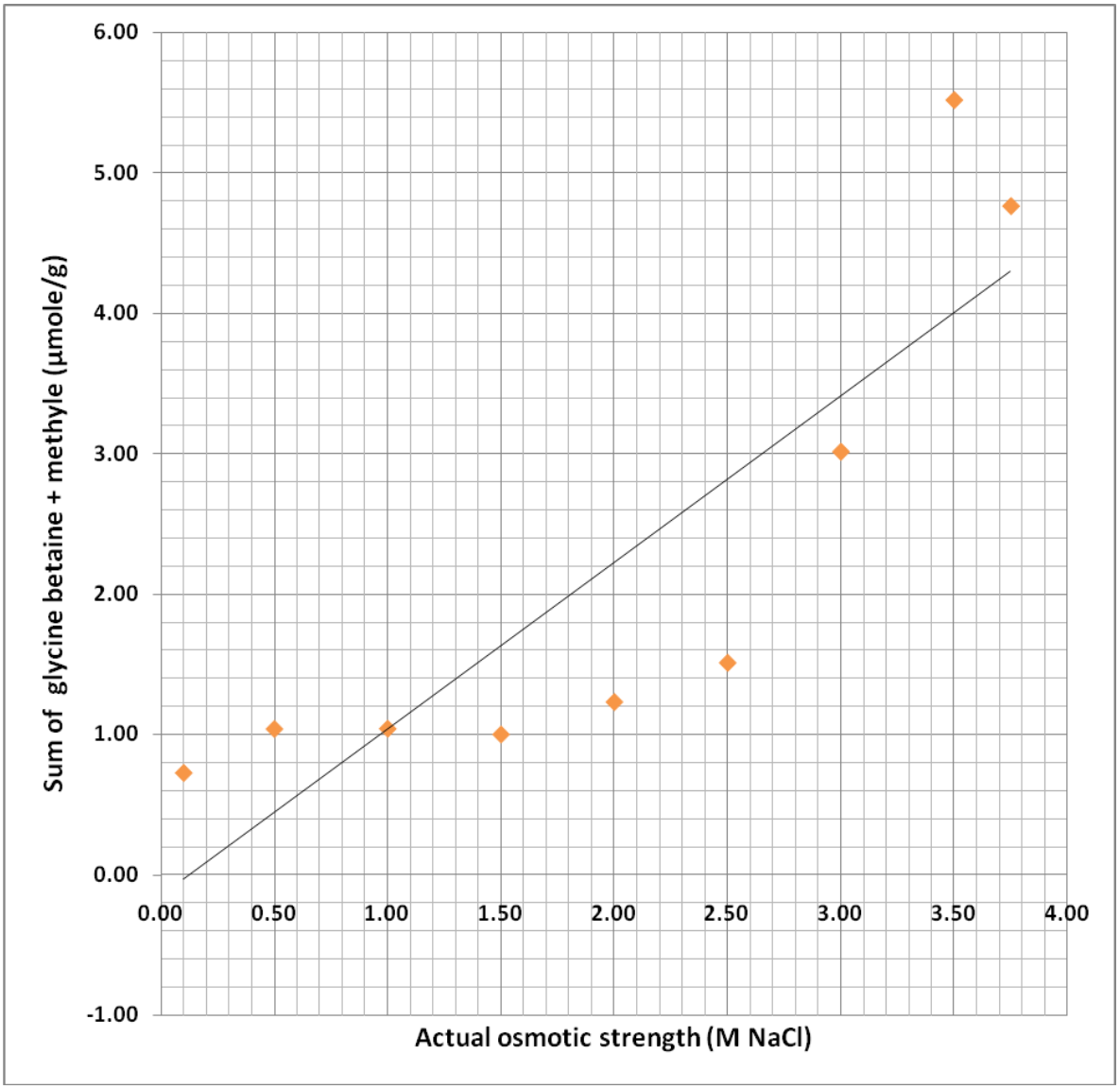


Figure 3.10 The sum of glycine betaine and methyl concentrations measured by ^{1}H NMR against dry weight of DSRT3 isolate grown under 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 NaCl (M) in LB medium. (R^2 value; y= 1.1318x)

Quantification of glycine betaine and glutamate at different NaCl concentrations was performed in HL1 isolate in M9 minimal medium. As previously mentioned, isolates and as shown in Figure 3.11, when NaCl concentration increases, glycine betaine and glutamate levels in the cells increase. Again, as in the strict halophilic isolate (HSO) and the DSRT isolate, the concentration of glycine betaine and glutamate fell (3.2 µmole/g) at 4M NaCl after reaching a peak (5.2 µmole/g) at 3.5M.

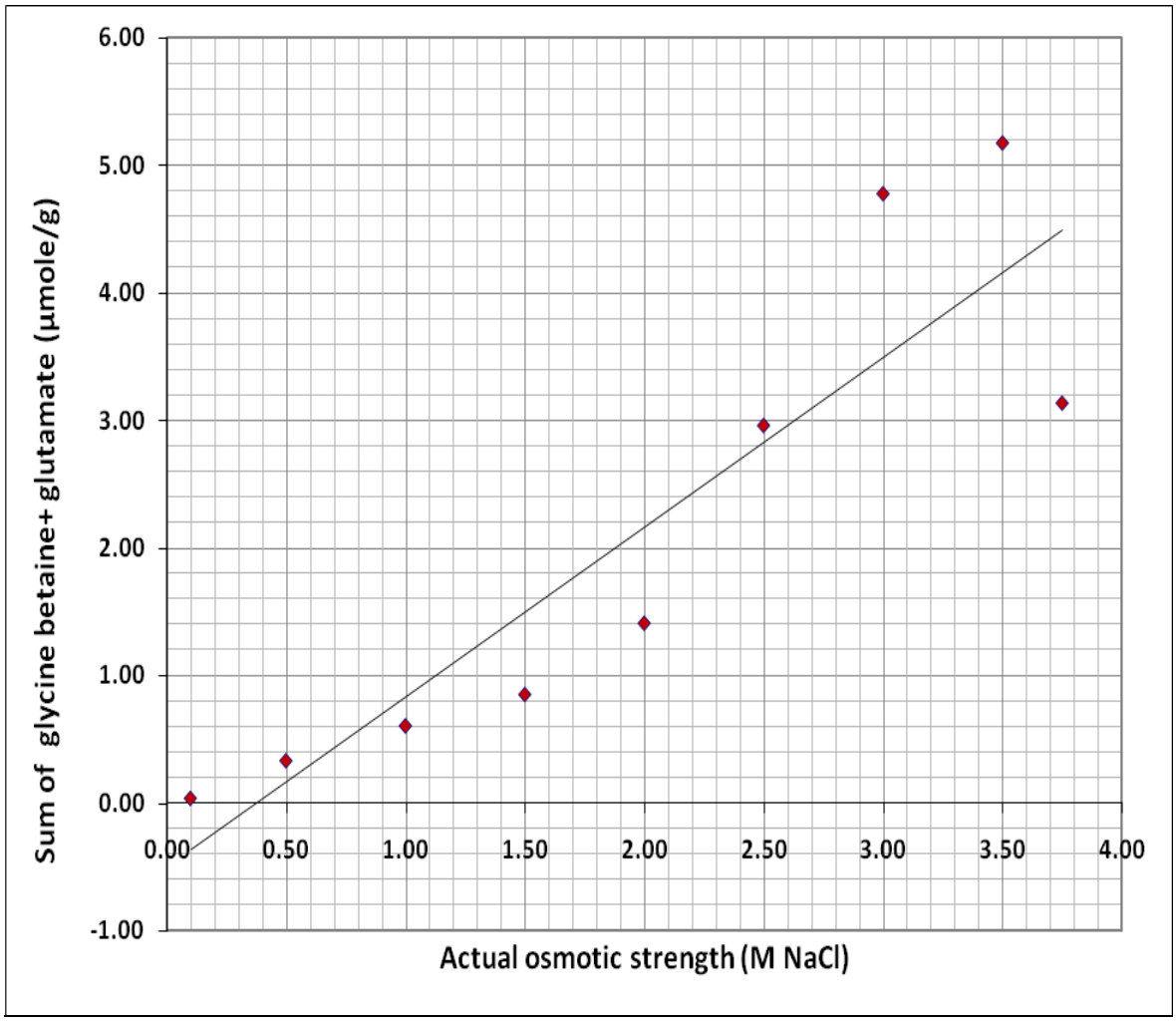


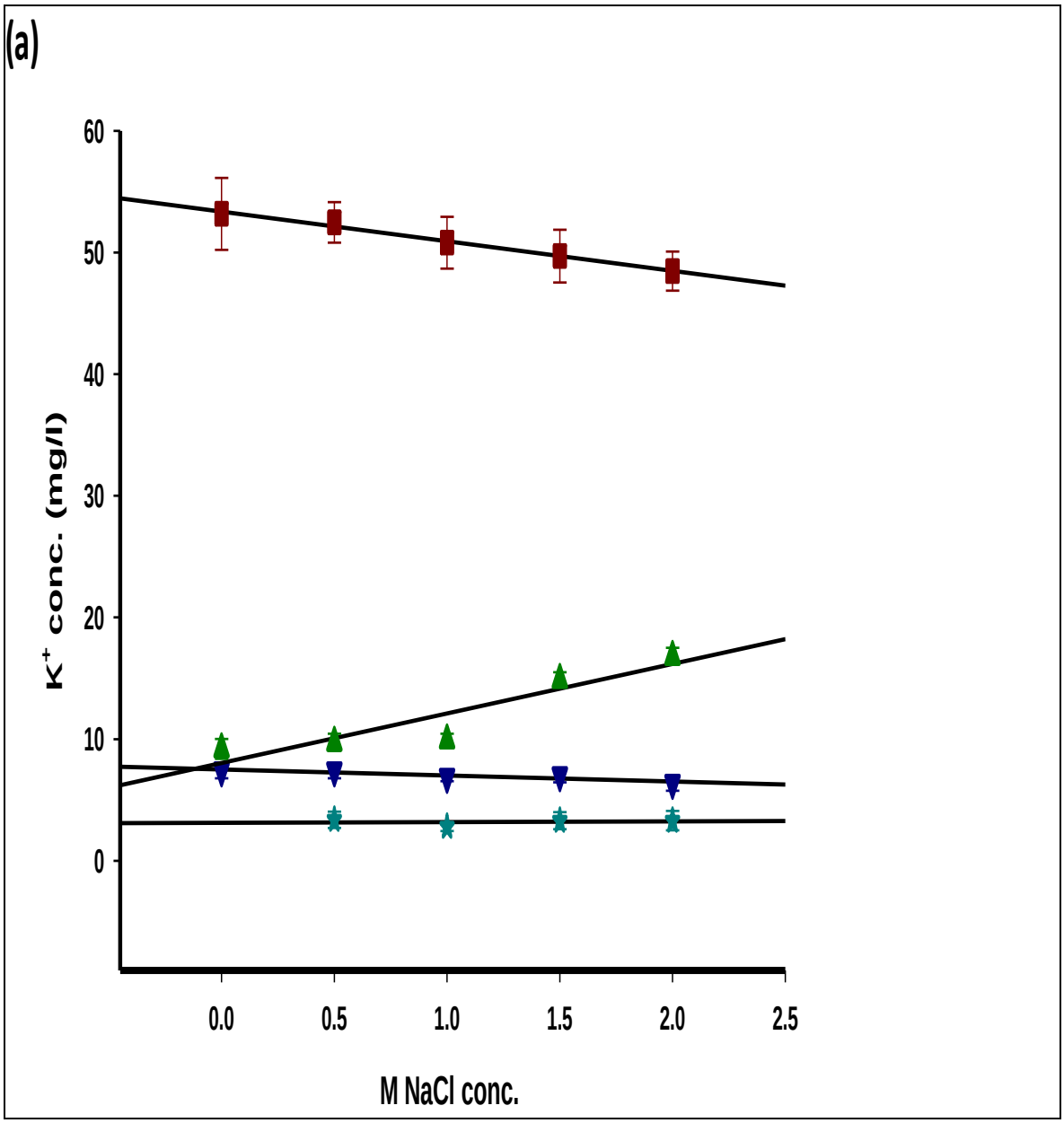
Figure 3.11 The sum of glycine betaine and glutamate concentrations measured by ^{1}H NMR against dry weight of HL1 isolate grown under 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 NaCl (M) in M9 minimal medium. (R^2 value; y= 1.1.078x)

The results described in this section (i.e. accumulation of compatible solutes) generally show that three main osmolytes act as osmoprotectant in these isolates, namely: glycine betaine, proline and glutamate. The highest concentration was obtained for glycine betaine under all growth conditions tested, the relative concentration of which increased as salinity increased. However, the concentration of glycine betaine dropped slightly when the salinity was increased to 4M NaCl. Interestingly, its concentration steadily increased even at extreme salinities (i.e. above optimum salinity). In contrast, the synthesis of proline, glutamate and methyl compounds occurred only at high salt concentrations. The concentration of the other amino acids produced as a substitute for glycine betaine was also reduced at high salinities.

3.3.2 Total cellular potassium ions as a strategy for adapting to a high salt stress in halotolerant isolates (Accumulation of potassium ions)

Levels of potassium ions (K^+) in HL1, DSRT3, HSO and STRA2 isolates were monitored under the various conditions described in section 3.2.2 by atomic emission spectophotometer. Total cellular K^+ concentrations of these isolates at various NaCl concentrations were analysed under two conditions, i.e. when the cells were subjected to osmotic shock and when they were trained to osmotic stress (Figure 3.12 and Figure 3.13).

There was no indication of potassium ion accumulation in halophilic HSO and halotolerant STRA2 isolates (Figure 3.12 (a)) when the cells were subjected to osmotic shock over a range of NaCl concentrations. Figure 3.12 (b) and Figure 3.13 show there was no significant change in the total cellular K^+ concentration for these two isolates when the cells were trained to osmotic stress. The results obtained here show that halophilic HSO isolates along with halotolerant STRA2 do not accumulate potassium when the cells are exposed to osmotic shock and also when the cells were grown with increasing NaCl concentrations.


(a)
60
50
40
30
20
10
0
K+ conc. (mg/l)
0.0
0.5
1.0
1.5
2.0
2.5
M NaCl conc.

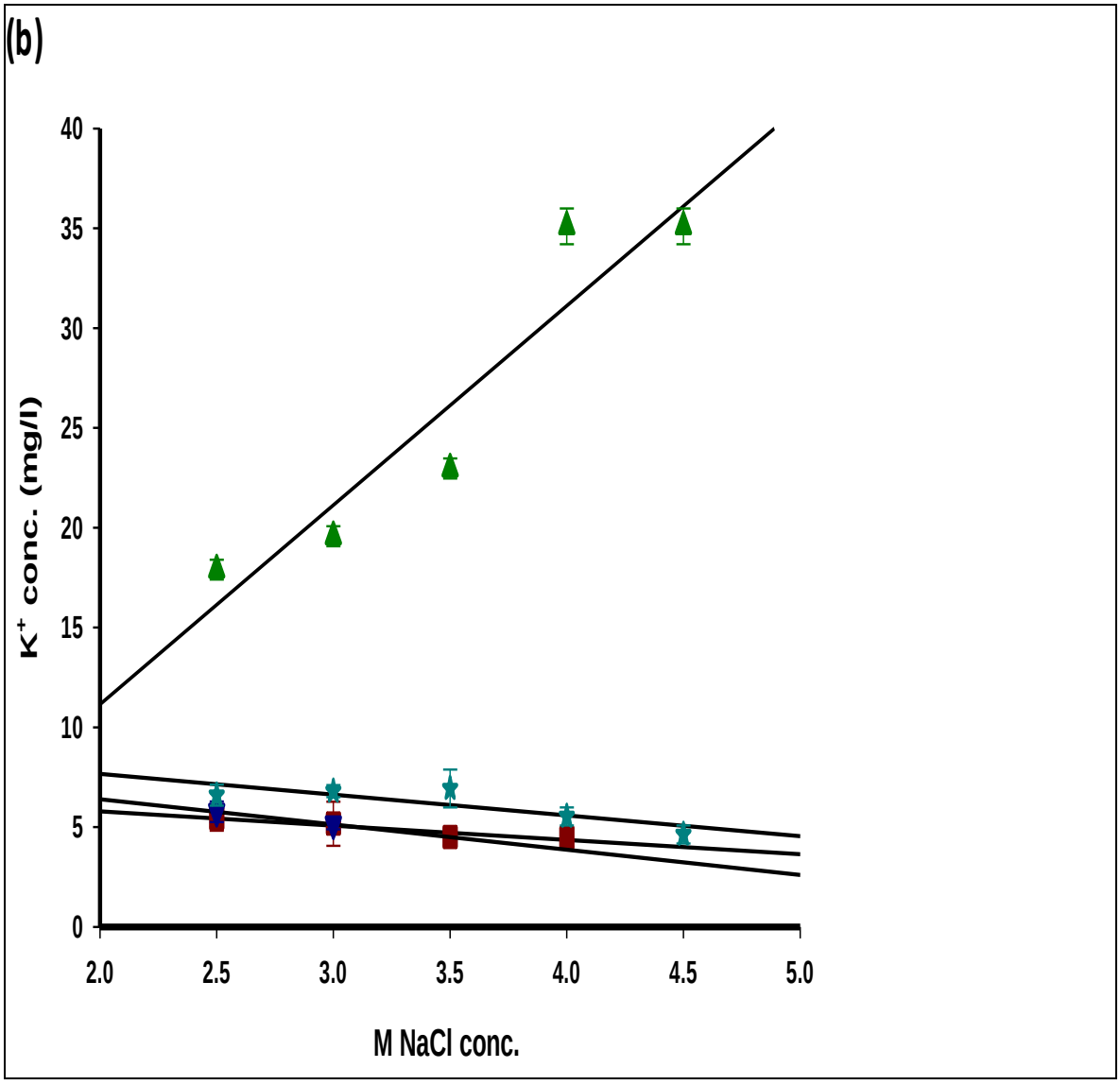


Figure 3.12 Total cellular K^+ concentrations measured by atomic emission spectrophotometer in HL1 ( ), DSRT3 ( ), HSO ( ) and STRA2 ( ) isolates. **(a)** K^+ concentrations measured when cells undergo osmotic shock at various NaCl concentrations (0.0, 0.5, 1.0, 1.5 and 2.0 M) in 24 hours. **(b)** K^+ concentrations measured when cells have grown under osmotic shock at 2.0 M NaCl and then adapted to the NaCl concentration at (2.5, 3.0, 3.5, 4.0 and 4.5 M) in 24 hours.

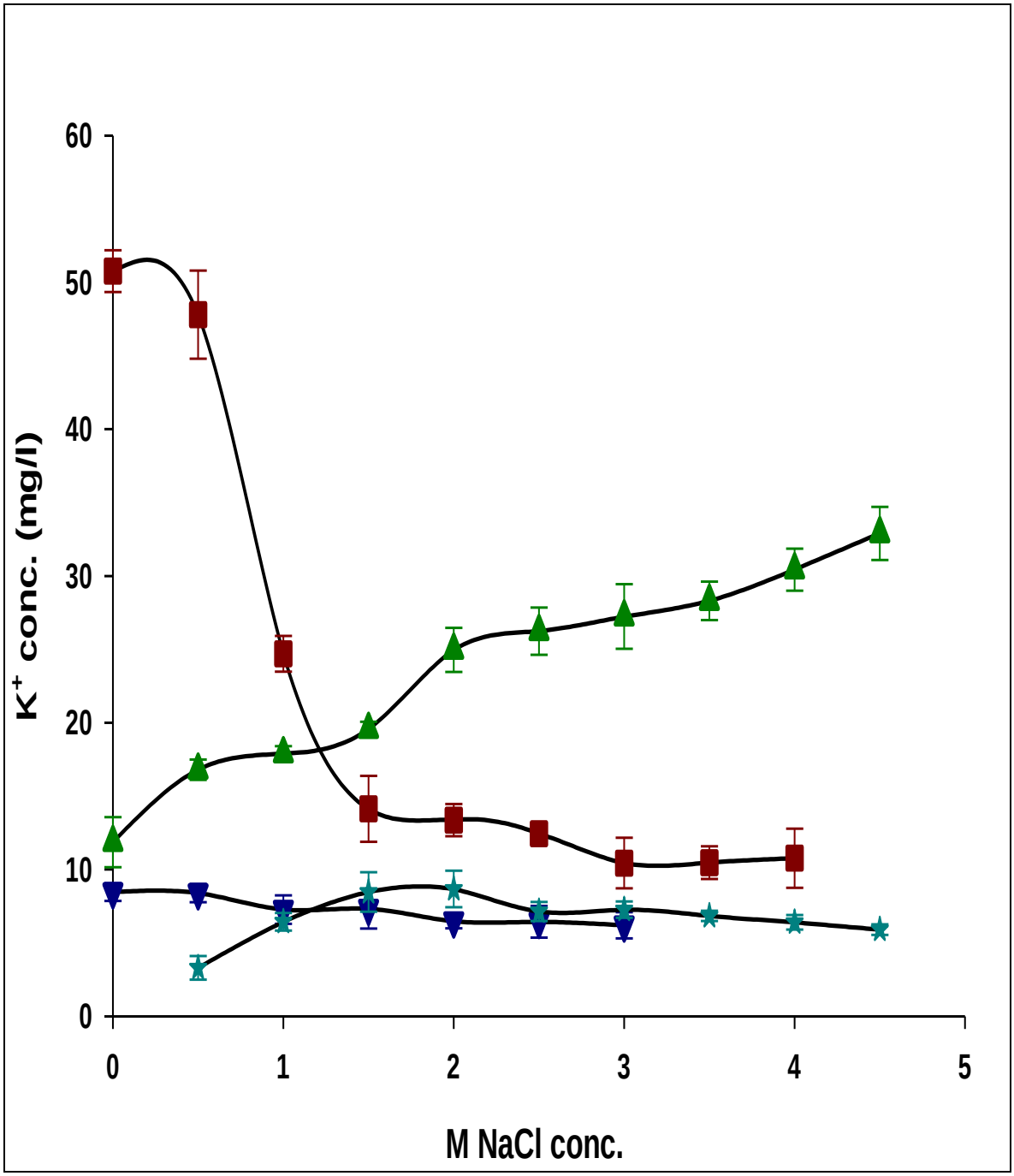


Figure 3.13 Total cellular K^+ concentrations measured by Atomic emission spectrophotometer in HL1 ( ), DSRT3 ( ), HSO ( ) and STRA2 ( ). K^+ concentrations measured when cells have grown and undergo osmotic stress adapted to the NaCl concentration at (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 M) in 24 hours.

For unknown reasons, HL1 strain showed a high K^+ concentration when it was subjected to osmotic shocks but not when trained to osmotic stress (Figure 3.12 (a)). The intracellular concentration of this ion was optimal, i.e. above 50 mg/l, a relatively high concentration compared to that found for the other isolate although DSRT3 isolate came close to producing this concentration but only at the highest osmotic stress. Whereas, when this isolate was trained to different concentrations of NaCl (Figure 3.12 (b) and Figure 3.13), there was no significant internal accumulation of total cellular K^+.

However, the total cellular K^+ concentration increased for DSRT3 when the cells were grown with increasing NaCl concentration (w/v) and in both cases, when the cells were subjected to osmotic shock or when trained at different NaCl concentrations (Figure 3.12 and Figure 3.13). These findings agree with other reports showing that haloarcheon *Halobacterium salinarium* accumulates K^+ as a response to increases in external osmotic stress (Brown, 1990; Vreeland, 1987). *Halobacterium salinarium* cells were only analysed when grown in 15%, 20%, 25 and 30% NaCl concentrations (w/v) as they failed to grow in media with NaCl concentrations less than 15% (w/v).

The accumulation of intracellular potassium ions is an osmo-adaptation strategy that to date has mainly been found to be characteristic for the halophilic archaea (Galinski, 1995; Martin *et al.*, 1999). This has been further extended by the finding of a specific KCl uptake system in *H. salinarum*, including a potassium ion driven uniporter and the unique light-driven chloride pump halorhodopsin (Kolbe *et al.*, 2000; Ng *et al.*, 2000).

However, in this study no potassium accumulation was observed in halotolerant and halophilic isolates either under adaptative salt concentrations or following osmotic shock. While, there have been reports of some inconsistency with the analyses using flame

photometry (Roberts, 2005), comparing the analysis of *Halococcus hamelinensis* to that of *Halobacterium salinarium* NRC-1 which accumulates potassium, the results presented clearly showed that *H. hamelinensis* does not accumulate potassium. As a result, potassium accumulation does not appear to be a universal primary response to salt stress. Future studies in halotolerent isolates and particularly *Halobacillus* isolates (such as HSO isolate) need to be conducted to determine whether genes encoding K^+ uniporters, and the unique light-driven chloride pump halorhodopsin, are absent, or present but not expressed in the HSO isolate.

Due to their accumulation of potassium ions, halophilic archaea require salt tolerant enzymes containing a large excess of acidic amino acid residues. This reduces the hydrophobicity of halophilic archaeal proteins in addition prevents the salting-out effects of K^+ and allows the cellular protein to retain its flexibility under extreme salinity. However, this also imposes a limit on the effectiveness of cellular potassium ions as a compatible solute (Yancey *et al.*, 1982). High production of glycine betaine also enables halophilic enzymes to stabilize in the absence of high salt levels (Vuillard *et al.*, 1995). Thus, the supposed glycine betaine uptake mechanisms identified, indicate that halotolerant organism need not accumulate potassium ions. Glycine betaine has also been detected in all halotolerant and halophilic isolates when grown in rich media. Accumulation of potassium ions is therefore not a mechanism used for osmoregualtion in most of the isolates obtained and identified here and it is therefore proposed that the accumulation of compatible solutes is a major means for survival in response to salinity.

3.4 Conclusions

From the osmo-adaptation point of view, halotolerant and halophilic microorganisms are able to remain alive in hypersaline environments due to their facility to manage the osmotic equilibrium. They have the ability to accumulate potassium chloride and sodium

chloride to achieve the concentrations of environmental ions .Consequently, halophilic proteins can also cope with extreme hypersaline environment including sodium chloride concentrations of beyond 5M and potassium chloride concentrations of almost 4M (Demirjian *et al.*, 2001).

This study confirmed what has been reported by Galinsk, (1993), namely that halophiles also accumulate high concentrations of organic solute within the cytoplasm in order to prevent any loss of cellular water under these conditions; cell volume is maintained when an iso-osmotic balance with the medium is achieved. The compatible solutes reported in this study mostly correspond with those reported in the literature and the results show that the halophilic isolates usually accumulated amino acids and polyols and their derivatives such as, glycerol, ectoine, glutamate, proline and lysine and quaternary amines like glycine betaine which have no net charge at physiological pH and do not disturb metabolic processes. Many halophilic and halotolerant bacteria accumulate glycine betaine and ectoine, while halotolerant bacteria accumulate glycine betaine and other amino acids (Galinsk, 1993; Demirjian *et al*, 2001).

This study also demonstrated that glycine betaine and glutamate were the main compounds which are accumulated at high concentration in halobacteria. It has previously been shown by von Blohn *et al.* (1997) that the presence of compatible solutes in the growth medium reduces the transcription of osmotically induced genes encoding components for uptake and synthesis of compatible solutes. Osmoprotectants are preferentially taken up from the culture medium (Martin *et al.*, 1999), and for compatible solutes such as glycine betaine, high affinity transport systems exist to scavenge any extracellular compatible solutes. These transport systems reduce the amount of energy used in the synthesis of compatible solutes when they are readily available in the surrounding environment (Oren, 1999). As a result of this adaptation to extreme environments, many

halophilic strains have developed other exclusive characteristics which may be of significant biotechnological potential, a possibility which has been reviewed by Galinski and Tindall (1992), Eisenberg *et al*. (1992), Grant *et al*. (1998), Ventosa *et al*. (1998), and Oren (1999).

Microorganisms can use two approaches to deal with a hypersaline environment. The osmotic equilibrium of halophilic archaea can be maintained between their cytoplasm and the hypersaline surroundings by accumulating high salt concentrations. In this environmental stress, this mechanism of osmotic equilibrium or osmo-regulation needs unique intracellular enzymes adaptations that can function in the presence of salt. Such halophilic enzymes are adapted by obtaining a quite large amount of negative charged residues of amino acid on the surfaces in order to avoid precipitation. As a result, in conditions with low concentrations of salt, the solubility of enzymes is often very weak, in which case their applicability might be limited (Madern *et al.,* 2000). On the other hand, this feature has been beneficially employed by using enzymes from halophiles in both aqueous and non-aqueous media (Klibanov, 2001).

In contrast, halotolerant or halophilic bacteria are typified by exhibiting a very high metabolic variety. These organisms have low intracellular concentration of salt, and are able to manage an osmotic equilibrium between their cytoplasm and the surrounding medium by accumulating high concentrations of organic osmotic solute concentrations, while their intracellular enzymes are not efficient in terms of salt tolerance (Ventosa *et al*., 1998).

On the other hand, true halophilic organisms are known to require high intracellular potassium concentrations for optimal growth and thus for cellular function. However, Kokoeva *et al.* (2002) reported that halobacterial cells accumulate glycine betaine and at the same time reduce their glutamate levels. Thus, a requirement of high intracellular

concentrations of potassium ions for maintaining halophilic enzyme stability does not seem to be necessary. This study has shown that most halotolerant and halophilic bacteria, HL1, STRA2 and HSO, also does not utilise this "salt-in-cytoplasm" strategy. This strategy has long been assumed to be used by most halophilic archaea, such as the DSRT3 isolate in this study, and certain halophilic eubacteria. The findings of this study show that halophilic bacterial isolates accumulate glycine betaine, proline and glutamate instead of potassium ions. These isolates also accumulated different organic osmolytes when exposed to different salinities. In addition, it has been reported that halophiles do not appear to synthesise any compatible solutes, a process that depletes cellular energy (Oren, 1999), but instead possess three putative glycine betaine transporters with highest sequence homology to bacterial osmolyte transporters.

The osmoregulation mechanism involving potassium ion uptake does not require a large amount of energy. As a result, it is unusual for halophilic bacterial isolates not to accumulate potassium, a potentially energy-saving mechanism used to allow them to adapt to high salinity. Based on the present data it is concluded that transport systems have evolved to incorporate glycine betaine and amino acids, such as glutamate, proline and lysine in halophilic bacterial isolates. The presence of transporters for these compatible solutes would permit scavenging of these solutes and provide adaptation to salinity.

Based on the evidence presented in this study, the "salt-in-cytoplasm" strategy assumed to be employed by all halophilic archaea should be reconsidered and the alternate mechanisms of osmoadaptation in halophilic archaea should be further investigated. Further investigation into the physiology of halophilic microorganisms found in non-saline environment may provide a better understanding of the microbial interactions and the essential roles which very different species play in the environment.

CHAPTER 2

THE ISOLATION OF HALOPHILIC AND HALOTOLERANT BACTERIA AND ARCHAEA FROM NON EXTREME ENVIRONMENTAL SAMPLES

2.1 Introduction

Microorganisms are well known for their ability to live in a wide range of environments, both Man-made and natural; saline environments offer a well documented example of such environments commonly referred to as called “extreme”, although the term ‘extreme’ is misleading as will be explained below.

A distinction must be made between "requirement for salt" and "tolerance for salt" (Larsen, 1986). Halotolerant microorganisms have no specific requirement for salt, other than the usual 100 – 200 mM NaC1 required by all non-halotolerant organisms. Bacteria can to grow in up to ~1.20 M NaC1 i.e. slightly halotolerant, up to ~3.0 M NaCl which is moderately halotolerant, or up to saturated (5.0 M) NaCl, i.e. extremely halotolerant. A distinction can be made within those halotolerant groups between those for which growth rate is decreased by any addition of salt, and those for which the growth rate achieves an optimum with the addition of some salt, but then declines at higher salinities.

Microorganisms which require salt for growth are called halophiles, and they are classified in a similar manner as halotolerant species into three main groups; slight, moderate, and extreme halophiles (Kushner, 1978). There is an important distinction between the slight and moderate halophiles, on the one hand and extreme halophiles, on the other. The slight and moderate halophiles, which include algae, fungi and bacteria, have a requirement for 0.2 - 0.5 M NaCl and will grow in up to ~ 1.0 M and 3.0-4.0 M NaC1, respectively. In contrast, extreme halophiles include only bacteria and a few fungi, and have a much higher salt requirement, depending on the species, some will

grow at 2.0-4.0 M NaCl, and will also grow in up to saturated NaC1. Slight and moderate halophiles tend to be eubacteria, including species of cyanobacteria, while extreme halophiles are mainly archaea (Margesin and Schinner, 2001).

This has important consequences as far as adaptation to salinity (haloadaptation) is concerned, because the archaea possess a somewhat distinctive physiology/biochemistry, particularly in their membrane-lipid-composition (as has been described in Chapter 1).

Therefore, as a group, the halophilic and halotolerant microorganisms cover the entire salinity range, from a few millimolar up to saturated. Some species of algae, such as *Dunaliella*, and bacteria, such as *Micrococcus varians* and *M.halophilus*, can grow across the entire range (Ventosa *et al*., 1998). This is a significant level of adaptation, not only in relation to the direct effects of increasing concentration of NaCl, but also to the inevitable high level of osmotic pressure which results. Since the cell membrane is the semi-permeable barrier between the external medium and the cytoplasm, it is perhaps expected that this membrane displays adaptation in the face of changed salinity, particularly in the composition of its lipid.

The aim of the work discussed in this Chapter of study is to account for the presence of halophilic and halotolerant bacteria and archaea in non-saline environment and identify them using a molecular approach.

2.2 Materials and Methods

The basic microbiological methods used in all parts of this Thesis are detailed in the general microbiological methods sections given below; modifications are referred to where appropriate. All chemical reagents and media used were of analytical grade. They were prepared according to manufacturer's instructions using distilled water. Media were autoclaved at 121^{o}C for 15 min and reagents were stored at 2-8 ^{o}C until used.

2.2.1 Sample description and sampling sites

A total of 55 samples were collected in an area surrounding Sheffield, United Kingdom including; Hull (Hornsea beach) and Beverly and Oman including; Waheeba sands, Nizwa and Muscat. Each soil sample was taken from surface areas, such as under trees, fields, gardens, cliff of the beach, and also desert varnish (Figure 2.1 and Figure 2.3(a)) and desert sands (Figure 2.3 (b)) in addition to soils sampled from Oman, at a depth of 3m. The other environmental samples were: obtained from the stratosphere collected as described by Wainwright *et al*. (2003) and also rain and hail (Figure 2.2) that were collected from the roof of Firth Court, University of Sheffield. The rain and hail samples were collected in sterile glass beakers and a control sample of surrounding air has been collected in each collection of rain and hail samples. Six different air samples have also been collected to test the presence of halophilic or halotolerant microorganisms. These samples were collected in sterile autoclavable bags by exposing them to air in United Kingdom and Oman for almost 2 hours and then flushing with autoclaved water.

For comparison, seven samples were collected from saline environments, 6 were sea waters (Horn Sea, Arabian Sea and Gulf of Oman) and 1 hypersaline soil with 10% salt concentration collected from Ashikhara City in Oman (Figure 2.3 (b)).

Figure 2.1 Desert varnish on a rock collected from Ashikhara, Oman.

Figure 2.2 Small hailstones approximately 1cm in diameter collected from Sheffield, UK.

Figure 2.3 Sampling site of (a) desert varnish and hypersaline soil, Ashikhara, Oman (b) desert sands; Waheeba sands, Oman.

2.2.2 Culturing and isolation

The growth medium used (for both nutrient liquid and agar medium) contained the following ingredients (per liter); 5.0 g yeast extract (Difco), 15.0 g peptone, (1.0M, 2.0M, 3.0M and 4.0M) NaCl and 20.0 g Bacto-agar (Difco), pH 7.2 (Table 2.1).

In addition to nutrient medium, M9 minimal salt medium (Sigma) was used and prepared by dissolving l0.0 g of M9 minimal salts and 1.0M to 4.0M of NaCl in 900 ml distilled water. The volume was then made up to 980 ml with distilled water and autoclaved. The other ingredients were also separately dissolved in distilled water as shown in Table 2.1 and then autoclaved. After autoclaving, all solutions were allowed to cool to approximately 50^{o}C, combined aseptically and gently inverted to mix and give 1.0 litre of basal M 9 minimal medium. Table 2.1 shows different basal media for the different halophilic bacteria and archaea.

For the isolation of archaea, Payne Seghal and Gibbons medium (www.ncimb.com) or also known as DSM97 medium (DasSarma *et al.*, 1995; Robb, 1995) was used; it contained the following: casamino acids, 7.50 g; yeast extract, 10.0 g *tri*sodium citrate,

3.00 g; NaCl, 250 g; KCl, 2.00 g; $MgCl_2$.6H2O, 7.23 g; $MgSO_4$.7H20, 20.0 g; $FeSO_4$.7$H_2$0, 0.05 g; $MnSO_4$.H_2O, 0.20 mg and with the addition of calcium chloride ($CaCl_2$.2H_2O, 2.70 g). The addition of three antibiotics (streptomycin, penicillin and ampicillin) at 100 μg ml^{-1} prevented the overgrowth of bacteria (Wais, 1988).

Table 2.1 The basal media used for isolating microorganisms.

Ingredient	Archaea HP-DSM97* g/L	Plate Count g/L	Nutrient g/L	Luria-Bertani (LB) g/L	M9 Minimal #
Tryptone		5.0		10.0	
Peptone			15.0		
Glucose		1.0			9.0 ml of (15g in 45ml)
Yeast extract	10.0	2.5	5.0	5.0	
NaCl	250.0	as required	as required	10.0	as required
KCl	2.0				
Mg_2SO_4. 7H_2O	20.0				1.0 ml of 1M Mg_2SO_4.7H_2O
$MgCl_2$. 6H_2O	7.23				
$CaCl_2$. 2H_2O	2.70				1.0 ml of 1mM $CaCl_2$.2H_2O
$MnSO_4$. H_2O	0.2 mg				
$FeSO_4$. H_2O	0.05				
Casamino acid	7.50			7.50	
trisodium citrate	3.0				
NH_4Cl					9.0 ml of (5g in 45ml)
M9 minimal salt					10g in 980ml distilled water
Agar	20.0	9.0	20.0	15.0	20.0
Final pH	7.4 ±0.2	7.0 ±0.2	7.2 ±0.2	7.2 ±0.2	7.2 ±0.2

***** Casamino acid and yeast extract were autoclaved separately to prevent precipitation of salt.

Each ingredients were separately dissolved in distilled water as shown in the table and autoclaved and after autoclaving, all solutions were allowed to cool to approximately 50^oC, combined aseptically and gently inverted to mix and give 1.0L.

2.2.2.1 Enrichment cultures

For the enrichment of microorganisms directly from the environment, approximately 5.0 g of sample material was transferred aseptically to flask containing 50 ml (w/v) of liquid media. Cultures were incubated aerobically at (25ºC and 37ºC) for bacteria and (40ºC and 50ºC) for archaea, with shaking (100 rpm) for 48 to 72 hours, with an additional one week when no growth was observed. Microbial growth was monitored by turbidity and then actively growing cultures, 500 µl was transferred aseptically to fresh media.

2.2.2.2 Pure cultures

Solid media (1.5% agar) containing a range of concentrations of salt were inoculated with 500 µl of each liquid medium culture and incubated aerobically at 25°C and/or 37°C (bacteria) and 40ºC and/or 50ºC (archaea) for up to 3 days. Single colonies were picked off and streaked onto fresh plates to obtain pure cultures. Pure cultures were obtained by serial plating and repeated sub-culture on the same agar medium plates which were then confirmed by light microscopy.

2.2.3 Counting of colony forming units (CFU)

Colony forming units were used to measure the number of culturable microorganisms (viable) present in solution and harvested from various environmental samples. For the determination of numbers of colony forming units and halophilic bacteria present in the soil samples, 5.0 g of soil samples were suspended in 95 ml of distilled, sterile water, shaken at 250 rpm for 2 hours and then diluted serially with the same solution, and 1.0 and 0.1 ml each was spread on agar plates with 0.0M, 1.0M, 2.0M and 3.0M of NaCl concentration. A standardized test portion of one-tenth decimal dilutions of soil samples was inoculated by the standard surface spread (SS) method using plate count agar (PCA, Oxoid, UK) (Table 2.1) and triplicate samples were plated and incubated at temperatures (25^0C and 37.0 ± 0.5^oC) for 48 hours to enumerate the

microorganisms. Results are expressed as a number of colony forming units (cfu) in a given volume of the sample (Feldsine *et al*., 2002).

2.2.4 Microscope examination

The direct microscopic examination of Gram-stained smears of isolates was done in order to differentiate the total Gram-positive cocci or bacilli and the Gram-negative cocci or bacilli (Ikram and Hill, 1991). The standard Gram stain was used.

2.2.5 Measurement of NaCl contents and pH of samples

Ten grams of each soil sample was suspended in 10 ml of ddH_2O (ultrapure water), shaken for 1 h, and supernatants were obtained by centrifugation. The content of NaCl in soil samples was determined with NaCl meter (Figure 2.4) and the pH of the soil extract was determined by using a bench top pH meter (3310, JENWAY LTD, UK).

Figure 2.4 The salinity meter used for measuring salinity at sites. A Salinity Meter (Oaklon, Eutech), a microprocessor based, hand held meter which gives +/- 0.5% full scale accuracy and measures NaCl in either percentage or ppm. It includes a conductivity meter (cell constant, K=1), with built in temperature sensor.

2.2.6 Long-term storage of cultures

All isolated strains were maintained by adding 1.0 ml of overnight culture (active inoculum) to 1 ml of sterile glycerol (50% v/v) and immediately frozen at -80^{o}C. Frozen stocks were slowly thawed on ice for recovery of cultures, inoculated with a sterile swab to fresh, liquid LB medium with the optimal NaCl concentration and incubated under suitable conditions when required.

In addition, each strain of halophilic isolates was subcultured routinely once every two weeks to minimize contamination. This was performed by streaking overnight colonies on LB agar plates overnight culture into LB broth with the optimum NaCl concentration and under suitable conditions and then stored in fridge (at 4^{o}C) until required.

2.2.7 Molecular identification techniques

2.2.7.1 Genomic DNA extraction

There are several molecular techniques used to extract bacterial DNA. Some of them required advanced prepared buffers and solutions and some of them are commercially available kits. In this research, three different methods were used, based on their speed, ease and effeciency of use in addition to availabily of kits at the time of identification.

2.2.7.1.1 CTAB method

The CTAB method as described by Chen *et al*. (2001) was usedas follows:-

Samples (2.0ml) of bacterial cells from an ovemight culture were harvested by centrifugation at 10,000 rpm (~11340 g) for l0 minutes. The supernatant was poured off immediately and then each pellet was resuspended in 500 µl of CTAB buffer [2%, (w/v) cetyltrimethylammonium bromide (CTAB), 2% (v/v) β-mercaptoethanol, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, l0 mM EDTA (ethylene diamine tetraacetic acid)] (Appendix A).

The samples were incubated in a bench hot block at 65^{o}C for 1 h. The genomic DNA was extracted using an equal volume (500 µl) of phenol-chloroform-isoamylalcohol (25:24:1, v/v) and then centrifuged at 11340 g for 5 min at the room temperature. The supernatant (aqueous layer) was carefully transferred into a clean 1.5 ml microcentrifuge tube and 500 µl of chloroform was added into the same tube and centrifuged at 11340 g for 5 minutes at room temperature. The supernatant was then transferred into a clean microcentrifuge tube and then 1/10 total volume of 3.0 M sodium acetate (pH 5.2) was added into the same tube. DNA was precipitated with 2.5 volumes of 100% (v/v) cold ethanol and incubated at $^{-}$20^{o}C for 15 minutes and then the pellet was harvested by centrifugation at 11340 g for 15 minutes at 4^{o}C, and the supernatant was discarded immediately. The pellet was gently washed with 1.0 ml of 70% cold ethanol and precipitated by centrifugation at 11340 g for 15 minutes at 4^{o}C and the supernatant was carefully poured off immediately. The genomic DNA pellet was then air dried for 10 min and then was resuspended in 50 µl of ddH_2O and incubated in a hot block at 55^{o}C for 2 hours. Successful extraction of genomic DNA was tested using gel electrophoresis (Section 2.2.7.2) and then PCR reaction was carried out (Section 2.2.7.3).

2.2.7.1.2 The QIAGEN kit

QIAGEN genomic-tip protocol (Genomic-tip 20/G) is designed to prepare up to 20µg of genomic DNA from Gram-negative and Gram-positive bacteria and the purified genomic DNA ranges in size from 20 to 160 kb. All buffers including B1, B2, QBT, QC, and QF were prepared, stored and used according to the manufacturer's protocol.

An OD_{600} reading of overnight culture was between 0.5 and 1.0 to ensure significance. A sample with readings above 1.0 was diluted so that the readings fall within this range. (Stock culture in glycerol has been made for each strain, section

2.2.6). A bacterial pellet from an appropriate volume of culture was collected by centrifugation at 3000–5000 x *g* for 5–10 min and the supernatant discarded. The bacterium-pellet was re-suspended by 1 ml aliquot of Buffer B1 with 2.0 µl of RNase A solution (100 mg/ml) (this aliquot, B1 and RNase, was prepared individually in microcentrifuge tube). 20.0 µl of 100 $mg.ml^{-1}$ lysozyme stock solution (preferably fresh lysozyme dissolved in distilled water to a concentration of 100 mg/ml), and 45.0 µl of QIAGEN Protease or Proteinase K stock solution were added and then incubated at 37^{o}C for at least 30 min. During incubation, the lysozyme enzymatically breaks down the bacterial cell wall, while the detergents in buffer B1 ensure complete lysis of the bacteria and degradation of RNA by RNase A ensures complete removal during column procedure (loading onto QIAGEN Genomic-tip).

After incubation of the suspension, 350 µl of buffer B2 was added to denature proteins such as nucleases and DNA-binding proteins. The excess QIAGEN Protease digests the denatured proteins into smaller fragments, facilitating efficient removal during purification. The suspension was mixed by inverting the tube several times or by vortexing for a few seconds and incubated at 50^{o}C for 30 min.

Before loading the sample, an amount of 1.0 ml of buffer QBT was applied to equilibrate a QIAGEN Genomic-tip 20/G (it is also called column), and allowed to empty by gravity flow. The sample was then loaded onto the equilibrated QIAGEN Genomic-tip and left to enter the resin by gravity flow. The QIAGEN Genomic-tip was washed with 3 x 1.0 ml of buffer QC. This buffer was used to remove contaminants in the majority of DNA preparations.

In a clean 10.0 ml collection tube, the genomic DNA was eluted with 2 x 1.0 ml of buffer QF and precipitated by adding 1.4 ml of room temperature isopropanol and immediately mixed and centrifuged at >5000 x *g* for at least 15 min at 4°C. Then, the supernatant was carefully removed and the centrifuged DNA pellet was washed with

1.0 ml of cold 70% ethanol which then was briefly mixed and centrifuged at >5000 x *g* for 10 min at 4°C. The supernatant was then carefully removed without disturbing the pellet and applied to air-dry for 5–10 min. The DNA pellet was re-suspended in 100 µl of TE buffer and dissolved overnight at room temperature. Then, DNA was ready for gel electrophoresis (Section 2.2.7.2) and PCR reaction (Section 2.2.7.3).

2.2.7.1.3 ANACHEM keyprep

The KeyPrep bacterial DNA extraction kit (ANACHEM, labstore, UK) is also designed for rapid and efficient purification of up to 20 µg of high molecular weight genomic DNA from both Gram-negative and Gram-positive bacteria. This kit applies the principle of a mini column spin technology and the use of optimized buffers ensure only DNA is isolated, while cellular proteins, metabolites, salts and other low molecular weight impurities are subsequently removed during the washing steps. All buffers including BG wash and elution buffers were prepared, stored and used according to the manufacturer's protocol.

A volume of 1.5 ml of bacteria culture grown overnight or culture grown to log phase was centrifuged at 5,000 x g for 2 min at room temperature and the supernatant completely decanted. The bacterial pellet was suspended with 100 µl of buffer R1 and the cells mixed completely by pipetting up and down. 20.0 µl of (50 $mg.ml^{-1}$) lysozyme was add into the cell suspension with thoroughly vortexing and then incubated at $37^{o}C$ for 20 min. After incubation, the pellet of digested cells was harvested by centrifugation at 11340 g for 3 min and the supernatant was decanted completely.

For protein denaturation, the pellet was re-suspended in 180 µl of buffer R2 and added 20.0 µl of proteinase K. After mixing thoroughly, the suspension was incubated at $65^{o}C$ for 20 min in a shaking water bath. If lysate was not clear at the end of incubation, the incubation time was extended to 30 min. 4.0 µl of 100 $mg.ml^{-1}$ RNase A

(DNase-free) was added into the lysate to get RNA-free DNA and incubated at 37^{o}C for 5 min (Residual RNA fragments was completely removed during column washing).

For homogenization, 2 volumes (~410 µl) of buffer BG was added into the sample and mixed thoroughly by inverting tube several times until a homogeneous solution was obtained. The homogeneous solution was then incubated for 10 min at 65^{o}C. After the incubation, 200 µl of absolute ethanol was added and mixed, immediately and thoroughly, to prevent uneven precipitation of nucleic acid due to high local ethanol concentrations.

The sample was then transferred into a column assembled in a clean collection tube and centrifuged at 11340 g for 1 min. The column was washed with 750 µl of wash buffer (wash buffer, concentrate, was diluted with absolute ethanol before use) and centrifuged at 11340 g for 1 min.

The flow through liquid was discarded and one more centrifugation was applied to the column at 11340 g for 1 min to remove residual ethanol which affects the quality of DNA and may subsequently inhibit enzymatic reactions. The final step, involved transfer to a clean microcentrifuge tube and 100 µl of preheated elution buffer (at 65^{o}C) was added, left standing for 2 min and then centrifuged at 11340 g for 1 min to elute DNA which was ready for gel electrophoresis (Section 2.2.7.2) and PCR reaction (Section 2.2.7.3).

2.2.7.2 Agarose gel electrophoresis

All genomic DNA in addition to PCR products and recombinant plasmid (will be described later) were analyzed by gel electrophoresis. In all procedures, unless it is stated otherwise, 10.0 µl of sample was mixed with 2.0 µl of (6x) Orange G loading dye and run on a 1% agarose gel (w/v). Each gel was loaded in 1x TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH8.0, Fisher Scientific, cat.BP1332).

Due to the small size of the BioRad Subcell GT electrophoretic tank with only 6 or 15 well comb, 0.5 g low melting point agarose was added to 250 ml flask containing 1 ml of 5X TAE and 40 ml distilled water. This mixture was then heated in the microwave until the agarose was dissolved. The solution was allowed to cool before 2.5µl ethidium bromide (Fisher Scientific, cat.E/P800/03), stain to visualize DNA, was added and then the gel poured to set in the BioRad Subcell GT electrophoretic tank.

The gel was subjected to electrophoresis for 40 min at 80 V using the BioRad Power Pack 300. Visualisation of gel was performed in the Uvitec "Uvidoc" mounted camera system to confirm the presence of the extracted genomic DNA and the absent of RNA, the absence of RNA was indicated by no smearing on the loaded gel, otherwise, it was removed by adding extra 1.0 µl of RNAse to the whole extracted genomic DNA and incubated at 4^{o}C ovemight.

2.2.7.3 Amplification of 16S rRNA

2.2.7.3.1 PCR amplification of 16S rRNA gene

Following the extraction of whole genomic DNA from a particular microorganism, 16S rRNA gene was amplified from the whole DNA extracted using polymerase chain reaction (PCR).

PCR reactions were performed using distilled water with extracted genomic DNA in a reaction mix of 25 mM $MgCl_2$, 10x *Taq*-buffer (Bioline, UK), 2.5 mM dNTPs (Fischer Biotech, UK), *Taq* polymerase (Bioline, UK) and 10 $pmole.l^{-1}$ of each primer. Table 2.2 illustrates different amount of components used for amplification of 16S rRNA gene of bacteria and archaea.

Table 2.2 PCR reaction mixtures for bacterial (Jungblut *et al.*, 2005) and archaeal (DeLong, 1992) 16S rRNA amplification.

Component	Bacterial 16S rRNA	Archaeal 16S rRNA
Sterile Milli-Q water	27.0 – 31.0 µl	29.0 – 33.0 µl
10x Taq buffer	5.0 µl	5.0 µl
$MgCl_2$ solution (50 mM)	6.0 µl	4.0 µl
dNTP mix (2.5 mM each)	5.0 µl	5.0 µl
Forward primer (10 ppmole.l^{-1})	0.5 µl	0.5 µl
Reverse primer (10 ppmole.l^{-1})	0.5 µl	0.5 µl
AmpliTaq polymerase (5 U/µl)	0.5 µl	0.5 µl
DNA template (10 – 100 ng)	1.0 – 5.0 µl	1.0 – 5.0 µl

Thermal cycling was performed in a MyCycler thermal cycler (BioRad Laboratories, inc., USA). Primers used for the amplification of the bacterial (Weisburg *et al*., 1991) and archaeal 16S rRNA (Baker *et al*., 2003) gene were fD1 and rD1, A571F and UA1204R and, A751F and UA1406R, respectively (Table 2.3). These primers were synthesised and purified by Eurofins (mwg/operon), Germany (Appendix B).

Table 2.3 Oligonucleotide primers used in this study. Primers were synthesized by Eurofins (mwg/operon), Germany.

Primer name	Sequence (5'-3')	Target Gene	Source
fD1	CCGAATTCGTCGACAACAGAGGATCCT GGCTCAG	Bacterial 16S rRNA	Weisburg *et al*., 1991
rD1	CCCGGGATCCAAGCTTACGGCTACCTT GTTACGACTT	Bacterial 16S rRNA	Weisburg *et al*., 1991
A751F	CCGACGGTGAGRGRYGAA	Archaeal 16S rRNA	Baker *et al*., 2003
UA1406R	ACGGGCGGTGWGTRCAA	Archaeal 16S rRNA	Baker *et al*., 2003
A571F	GCYTAAAGSRICCGTAGC	Archaeal 16S rRNA	Baker *et al*., 2003
UA1204R	TTMGGGGCATRCIKACCT	Archaeal 16S rRNA	Baker *et al*., 2003

Thermal cycling conditions for amplification of bacterial and archaeal 16S rRNA genes were as follows: initial denaturation step at 94°C for 3 min was followed by 30 cycles of DNA denaturation at 94°C for 1min, primer annealing at 50°C (55°C for archaea) for 1 min, strand extension at 72°C for 2 min and a final extension step at 72°C (74°C for archaea) for 5 min. Thermal cycling conditions for amplification of bacteria and archaeal 16S rRNA genes were as per standard protocols and stated in Table 2.4 (Jungblut *et al.*, 2005; DeLong, 1992). Each run of PCR contained a negative control (2.0 µl sdH_2O instead of template DNA) and a positive control (2 µl of *E. coli* instead of template DNA).

Table 2.4 PCR amplification protocol for bacterial and archaeal 16S rRNA.

Steps	Time	Temperature for bacterial PCR	Temperature for archaeal PCR	Number of cycle
Initialization	3 min	94^oC	94^oC	1
Denaturating	1 min	94^oC	94^oC	30
Annealing	1 min	50^oC	55^oC	
Extension/Elongation	2 min	72^oC	72^oC	
Final elongation	5 min	72^oC	74^oC	1
Hold		4^oC	4^oC	

2.2.7.3.2 Extraction and purification of PCR Products

After the amplification was completed, the PCR reactions were pooled and electrophoresed in 1% agarose gels. 10µl of PCR reaction was added to 2.0 µl of Orange G loading dye and run on agarose gel (described in Section 2.2.7.2) and the correct sized amplified of gene confirmed by 5.0 µl of 1 kb hyper ladder I (Figure 2.1).

The band of the desired PCR products was about 1.5 kb (16S rRNA gene) to be used for cloning were purified by using the QiaQuick Gel Purification Kit (Qiagen Ltd., UK) according to the manufacturer's instructions, which was designed to purify single or double stranded DNA fragments from PCR and other enzymatic reactions. Five

volumes of buffer (PBI) were added to l volume of PCR sample in sterile 1.5 ml tube (500 µl of PBI buffer were pipetted into 100 µl of the DNA). Then, it was pipetted into a QiaQuick spin column and placed in a 2.0 ml collection tube which was then centrifuged at 11340 g for 60 seconds and the flow through was discarded and the QiaQuick spin column was placed again in the same tube. The PCR product was washed with 750 µl of buffer PE in the QiaQuick spin column and centrifuged at 11340 g for 60 seconds and then the flow through was discarded. The QiaQuick spin column was moved to a clean 1.5 ml microcentrifuge tube and then purified products were eluted with 50 µl of buffer EB (10 mM Tris-Cl, pH 8.5) to the centre of the QiaQuick membrane and centrifuged at 11340 g for 60 seconds. The product was run on a 1% agarose gel and 5.0 µl of 1 kb Hyperladder I as a marker ladder to ensure that the correct sized product had been purified. Then, it was ready to be used in cloning or stored at -20^{o}C.

Alternatively, the purified PCR products were also sent directly to Medical School, the University of Sheffield, for sequencing and then phylogenetic analysis was performed as will be described in (Section 2.2.7.5). Most of the samples tested were also cloned as will be described in (Section 2.2.7.4)

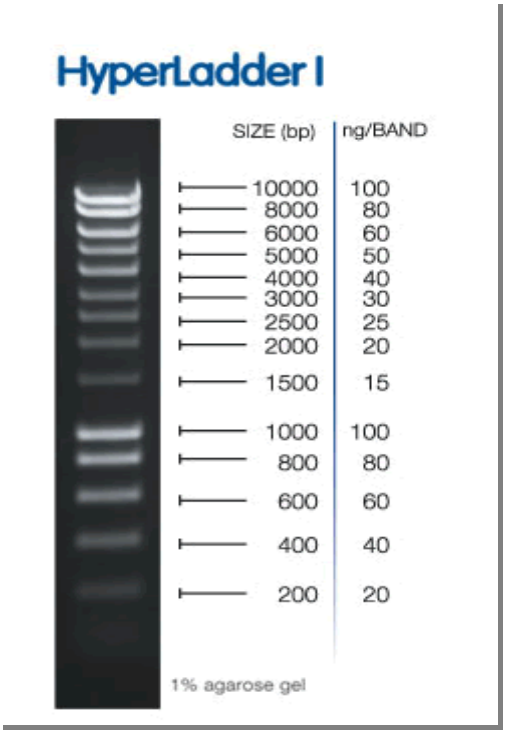


Figure 2.5 Standard hyperladder I with 14 lanes indicates higher intensity bands, 1000 and 10,000 and each lane (5µl) provides 720ng of DNA (BIOLINE supplier).

2.2.7.4 The 16S rRNA gene cloning and library construction

2.2.7.4.1 Preparation of competent cells

However, due to the very low transformation rate of competent cells from *E. coli* DH5α (those I have made), mainly commercially available competent cells (Promega) were used in transformation following the protocol recommended by the manufacturer.

Initially, competent cells were prepared from *E. coli* DH5α as described by Dower *et al*. (1998). Briefly, LB medium (50 ml) was inoculated with a single well-isolated colony of *E. coli* DH5α grown for 24 hours at 37^{o}C in an LB agar plate. The inoculated cultures were incubated overnight at 37^{o}C with shaking at 200 rpm. Each 25 ml was used to inoculate 500 ml LB medium pre-warmed to 37^{o}C in a 1.0 L flask. The cultures were grown at 37^{o}C with shaking until the OD_{600} reading reached 0.4 (about 3-4 hours). Then, the cultures were transferred into sterile ice-cold 500 ml centrifuge bottles and chilled on ice for 20 min before centrifuged at 3000 g for 10 min at 4^{o}C to

precipitate the cells. Each cell pellet was washed once by re-suspending in 250 ml ice-cold ddH_2O and the supernatant was removed. The suspended pellet was centrifuged at 3000 x g for 10 min at 4^oC, and then washed again by re-suspending in 40 ml in the same manner (the cell pellets were re-suspended by gentle agitation, not vortexing). Then, the cell pellets were re-suspended in 20 ml ice-cold 10% glycerol and centrifuged again at 3000 g for 5 min at 4^oC. The supernatant was removed as completely as possible, and the cells were pooled into 2.0 ml ice-cold 10% glycerol. The cells were distributed into 50 µl single-use aliquots in sterile 1.5 ml centrifuge tubes, snap frozen in liquid nitrogen and stored at –80^oC for not more than 6 months without a significant loss of competence.

2.2.7.4.2 Ligation and transformation of *E.coli*

Purified PCR product was ligated with the cloning vector pCR2.l, Figure 2.6, following the manufacturer's instructions (Invitrogen). The purified PCR products were ligated into the cloning vector using the following reaction: 4.0 µl insert, 1.0 µl PCR2.1 vector ligase and 1.0 µl ligase buffer (salt solution). The ligation mixture was incubated at room temperature for 5 min and used to transform competent *E. coli* DH5α cells. 4.0 µl of ligation mixture was added to 50 µl aliquots of competent *E. coli* DH5α cells, which were defrosted on ice for 30 minutes before use, and very gently mixed. The mixture was incubated on ice for 15 minutes and then heat shocked at 42^oC for 30 seconds followed by a 5 minute incubation period on ice. 100 µl SOC medium (cat.BIO-86033, bioline, UK) (Appendix A), at room temperature, was added and the mixture was incubated at 37^oC with gentle shaking for 1h. 40 µl of 40 mg.ml^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 40 µl of 100mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and l0 µl of 100 mg.ml^{-1} ampicillin were added to molten LB agar at approximately 50^oC and then was poured onto individual petri dishes which were incubated at 37^oC overnight. Blue-white screening on the plates was

used as a selective marker for successful ligation and transformation. Positive transformants containing recombinant plasmids appeared as white colonies while negative transformants were blue. Individual white colonies were picked off and grown overnight at 37^{o}C with shaking at 250 rpm on an LB agar plate containing ampicillin (50 µg.ml^{-1}). This plate was regarded as a master plate. Replica plates were made from the master plate and grown overnight for rapid screening for the desired recombinant plasmids.

2.2.7.4.3 Extraction of recombinant plasmid DNA from the *E.coli* host

Plasmid DNA was prepared from broth cultures, grown overnight with shaking at 37^{o}C in LB medium containing 100 g ml^{-1} ampicillin, using a QIAprep Spin Miniprep Kit (Qiagen, UK) according to the recommended protocol by manufacturer. The culture was centrifuged at 5000 g for 10 min. The supernatant was then discarded. The pellet was re-suspended in 250 µl of buffer P1 and transferred to a 1.5 ml microcentrifuge tube. Then, 250 µl of buffer P2 was added and mixed thoroughly by inverting the tube 6 times. 350 µl of buffer N3 was added and mixed immediately and thoroughly by inverting the tube 6 times and then, centrifuged for 10 min at (~17,900 g). After centrifugation, the supernatants was applied to the QIAprep spin column by decanting or pipetting and centrifuged for 1 min and the flow-through was discarded. The QIAprep spin column was washed by adding 500 µl of buffer PB and centrifuged for 1 min. After discarding the flow-through, the QIAprep spin column was washed by adding 750 µl of buffer PE and centrifuged for 1 min and again, after discarding the flow-through, an additional 1 min was imposed to remove residual wash buffer. In clean 1.5 ml microcentrifuge tube, the plasmid was eluted with 50 µl of buffer EB (10 mM Tris pH 8.5), 1 min stand, and 1 min centrifugation.

The presence, size, quantity and quality of the plasmid were determined using agarose gel electrophoresis (Section 2.2.7.2).

2.2.7.4.4 Digestion of recombinant plasmid DNA with restriction endonucleases

Restriction digests were carried out for 2 hours at 37^{o}C to test when the insert had ligated into the vector, using: 2.0 µl of purified plasmid DNA, 1.0 µl of *Eco*RI enzyme 1.0 µl of 10x reaction buffer and 6.0 µl of MilliQ water in PCR tube.

The mixture was analysed by gel electrophoresis as described in (Section 2.2.7.2). Only digests giving two products (at 1.5 kb and 3.9 kb) were regraded as successful. The plasmid preparations containing the correct sized insert (vectors containing the PCR inserts) were sent to the Medical School, the University of Sheffield, for DNA sequencing.

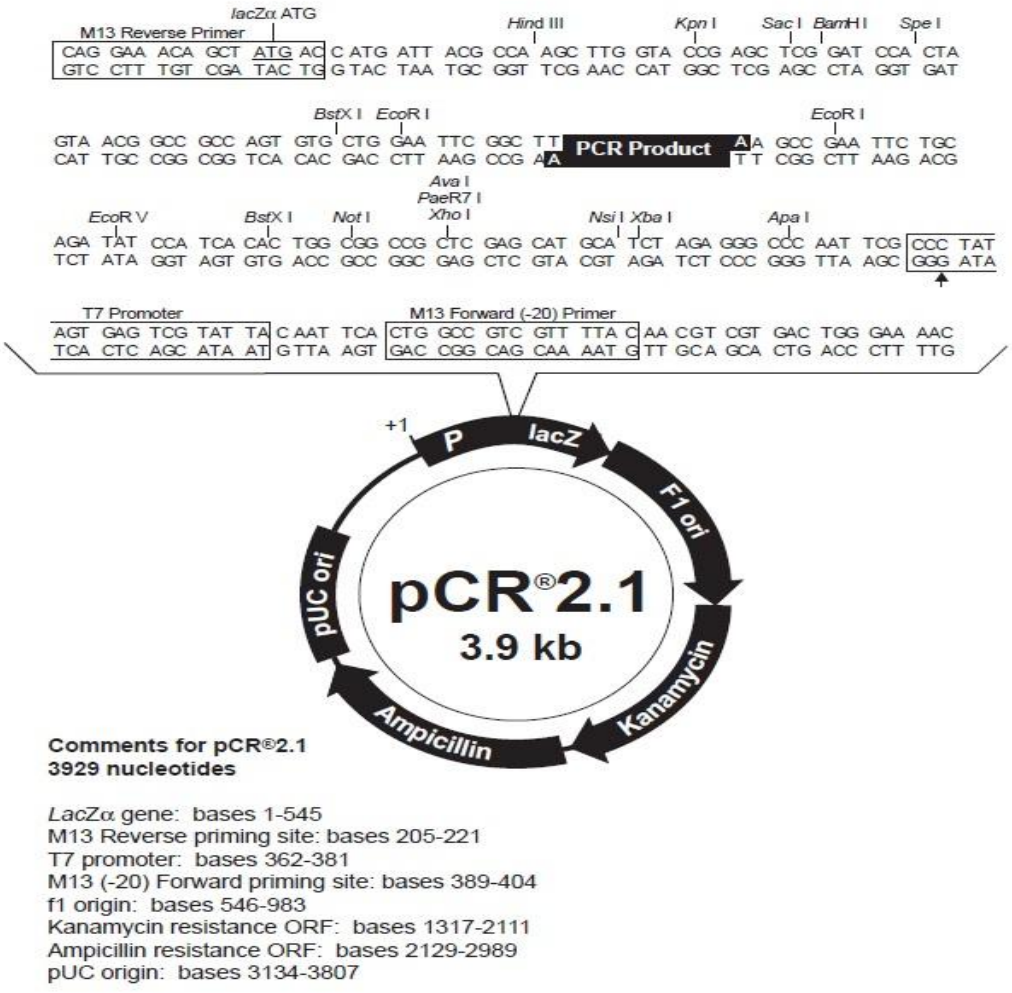


Figure 2.6 The vector pCR2.1-TOPO is designed to accept PCR products made with Taq DNA polymerase and therefore having 3' overhanging nucleotides. (supplier; Invitrogen http://www.invitrogen.com/content/vectors/pcriitopo_map.pdf)

2.2.7.5 Phylogenetic (DNA sequence) analysis

For the phylogenetic placement, 16S rRNA gene sequences were processed by the National Collection of lndustrial, Marine and Food Bacteria (NCIMB) using the MicroSeq database and the EMBL public database. Partial sequences generated here were assembled and the errors of consensus sequences were corrected manually using FinchTV software (Figure 2.7). The most closely related sequences against NCBI database were identified using BLAST (Altschul *et al*., 1997) and the sequences extracted, aligned and manually adjusted according to the 16S rRNA secondary structure using ClustalW (1.83) (Chenna *et al*., 2003).

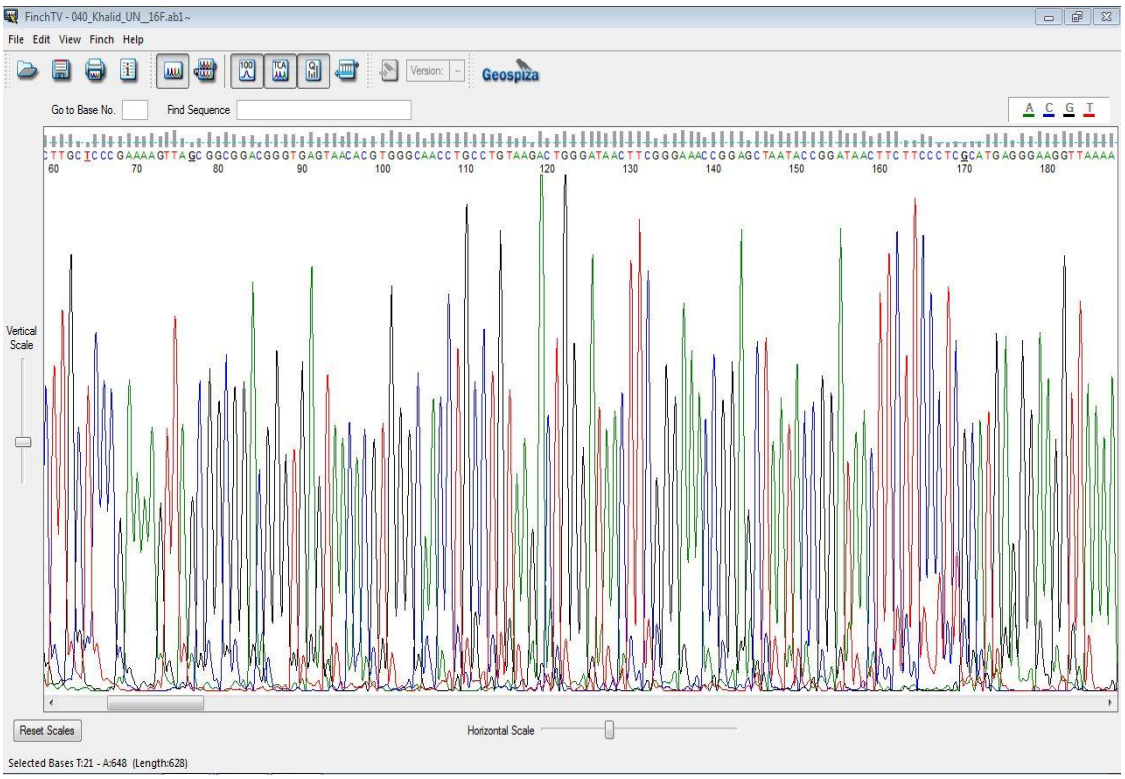


Figure 2.7 FinchTV software that manually adjusts errors of consensus sequences before BLASTn NCIMB database**.**

2.2.8 Effect of NaCl concentration on growth of bacteria and preparation of growth curves

The effect of NaCl concentration on the growth of isolated strains (HL1, STRA2, DSRT3 and HSO) was determined at different concentrations of (0, 1.0, 2.0, 3.0 and

4.0M) NaCl. This was achieved by running a growth curve experiment for each isolate. An inoculum for each isolate, was prepared by growing overnight to give a high cell density and then the OD_{600} was measured using a Unicam Heliso, spectrophotometer, against a medium blank, in l ml plastic cuvettes. Then, 50 ml of medium was added to each flask (3x 250 ml conical flasks; A, B and C) using a sterile serological pipette. 1.0 ml of inoculum culture was added to each triplicate flask and the initial OD_{600} of the culture was adjusted between 0.1 and 0.2. The OD_{600} was measured against a medium blank immediately after inoculation then every one hour over an incubation period at 25^{o}C or 37^{o}C (depending on the isolate optimum growth) on a rotary shaker at 250 rpm (the flasks were immediately returned to shaker before measuring the ODs). Growth curves were then plotted against time of incubation.

2.2.9 Quality control

All batches of media were tested to fulfill four main parameters: sterility, support of bacterial growth, selective inhibition of bacterial growth and appropriate response of biochemical indicators (Feldsine *et al*., 2002). A representative portion of each new batch was incubated overnight at 37^{o}C to check for sterility before use. In order to detect contamination, a few milliliters of the final product were inoculated into 10 times its volume of sterile broth so as to dilute out any inhibitory substances.

The ability of each batch of new media to support growth and exhibit the required differential and selective quality was determined using stock strains from a recognized culture collection NCTC (National Collection of Type Cultures),(Barrow and Feltham, 1993).

Working solutions and stains were tested weekly for their required purpose and chemical reagents were checked for performance when new batches of media were prepared (Claney, 1994).

2.3 Results and Discussion

2.3.1 The presence and occurrence of halobacteria and archaea in non-saline and saline samples

The aim of the work reported in this Chapter was to provide a general picture of the occurrence of halophilic or halotolerant bacteria and archaea in non- saline environments and to determine the habitat of halophiles. Knowledge of the specific characteristics of the environment help to account for the composition of the microbial populations and their interactions with the habitat in future studies; it has been claimed that only 1% of microorganisms (Torsvik *et al.*, 1996; Hugenholtz, 1998) can be cultured.

Media described in Section (2.2.2) were used. In the low nutrient medium (25% w/v) there was observable growth at concentrations of NaCl up to 2.0M and 3.0M and rarely at 4.0M. Similarly, there was observable growth in the M9 minimal medium up to 2.0M NaCl and some at 3.0M, but no visible growth in the highest (4.0M) salt concentration. The salinity meter (Figure 2.4) was used to measure the salt concentration of all of the non-extreme soil samples as well as the hailstone and rain samples and the concentration of salt found was zero. Whereas samples taken from near marine beach (as well as seawater) areas were as high as 2.0% and hypersaline soil containing 10% salt. The pH of the soil extract ranged from 5.5 to 8.5. There was no obvious relationship between pH and the occurrences of halophiles in the soil samples**.** Initially, 96% of the non-saline soil samples contained halophilic bacteria at 1.0M, 54% of growth occurred at 2.0M NaCl and 16 % of them contained growth up to 3.0M NaCl whereas, only 6% of samples showed growth at 4.0M NaCl (Figure 2.8). Six different saline samples were examined to identify halophilic bacteria, surprising, only 12% of them showed visible growth at 4.0M NaCl, compared to non-saline samples. Whereas,

all of them produced growth at salt concentrations up to 2.0M (Figure 2.9) and 62% of them was able to grow at 3.0M NaCl. On the other hand, none of the air samples produced any salt tolerant isolates.

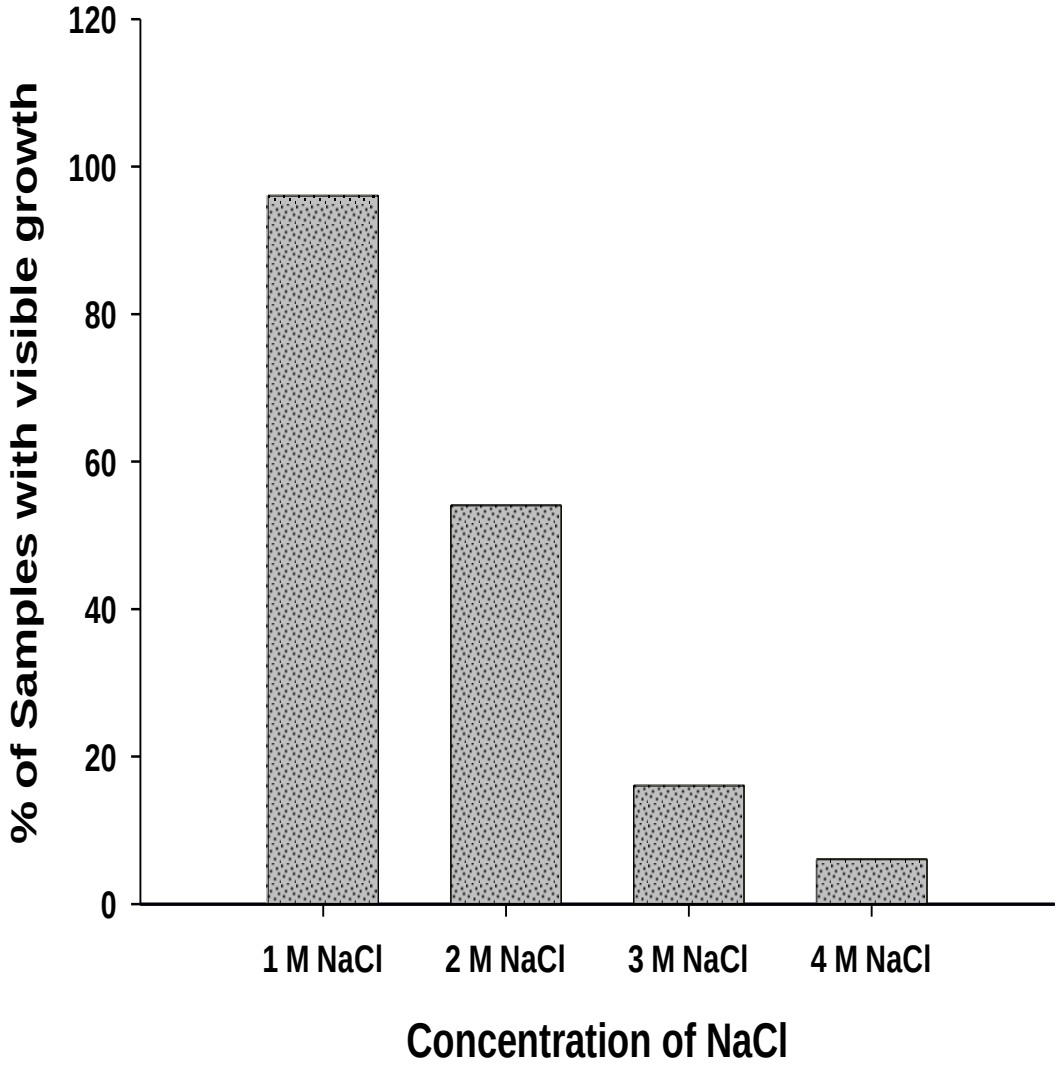


Figure 2.8 Halophilic microbial growth from non-saline samples on 25% nutrient agar plates containing 1.0 to 4.0 M NaCl.

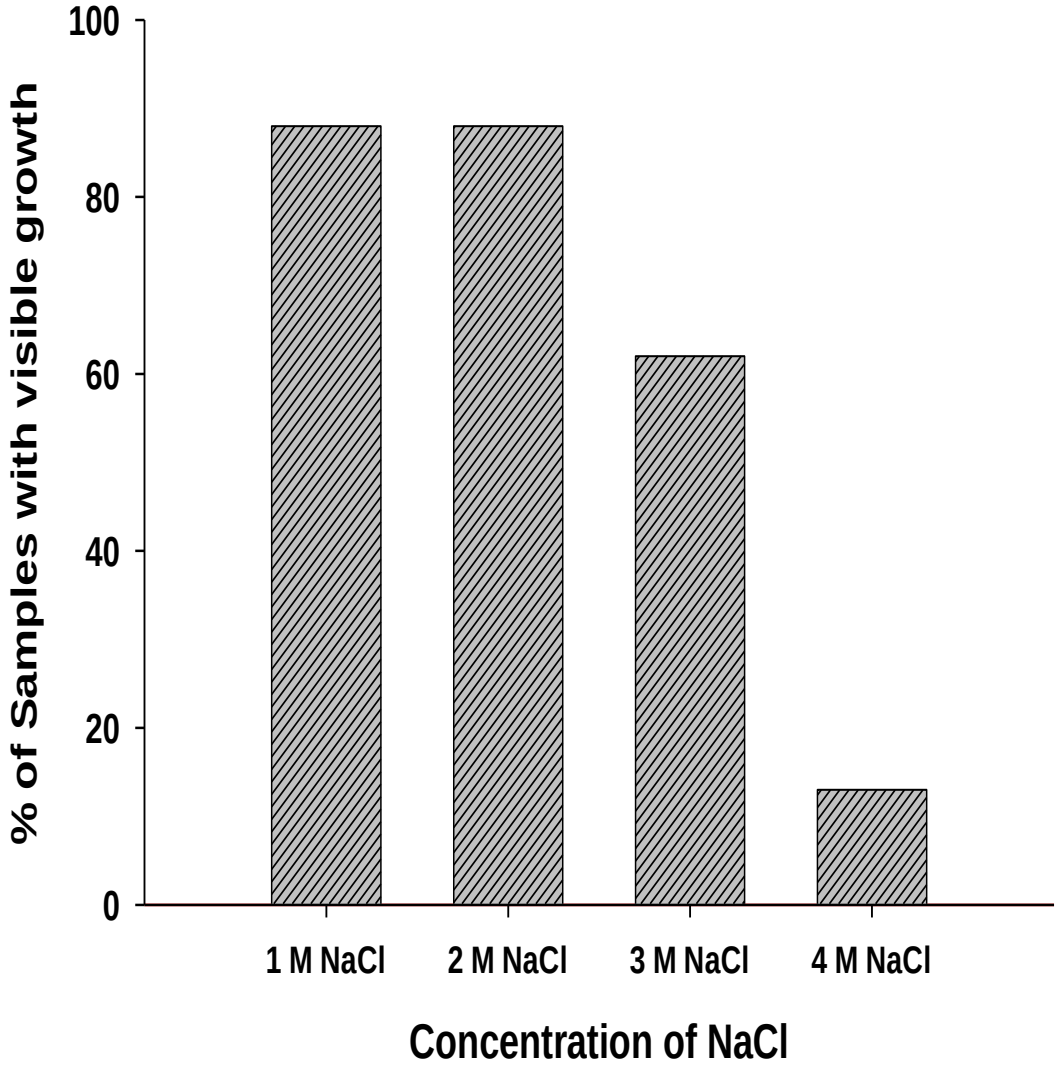


Figure 2.9 Halophilic microbial growth from saline samples on 25% nutrient agar plates containing 1.0 to 4.0 M NaCl.

Most of the species which have been isolated from non-saline environments, were found to be halotolerant (as will be discussed in Section 2.3.5) and capable of growth at 3.0M NaCl. For unknown reason(s), cells of some colonies on the initial culturing plates failed to grow when re-cultured onto fresh plates, but grew on plates with lower NaCl concentrations. A soil extract medium was prepared and using the soil extract as a medium, there was a visible growth again at high salt concentration. Therefore, it seems that some unknown growth factors present in soil might be responsible for this incidence. There could be a possibility, however, that halophilic bacteria on liquid medium or agar plates escaped from being picked up for purification. One more possibility is that those halophilic strains lost the ability to form colonies on the particular agar plates we used during repeated transfers in the purification procedures, or that they simply did not form colonies because the composition of agar plates is not suitable for them. These findings correspond somehow with the results found by Echigo *et al*. (2005) who have reported that halophilic bacteria lost the ability to form colonies on the agar plates they used during repeated transfers in the purification procedures.

In addition, rain water samples and hailstone sample were examined to identify the presence and the distribution of these halophiles. There was no visible halophile in one sample of rain water whereas the other sample and the hailstone showed halophilic growth up to 2.0M NaCl only.

An attempt was made to determine other possible sources of halophiles. One of these trails was to study the presence of these microorganisms in hailstone and rainwater. Hailstones form in storm clouds, due to chilled water drips solidifying and freezing on contact with condensed dirt or dust. They are blown to the upper part of the cloud by the storm's updraft. This updraft dispels and the hailstones drop down, return again into the updraft and are picked up. As a hailstone becomes intense and too heavy

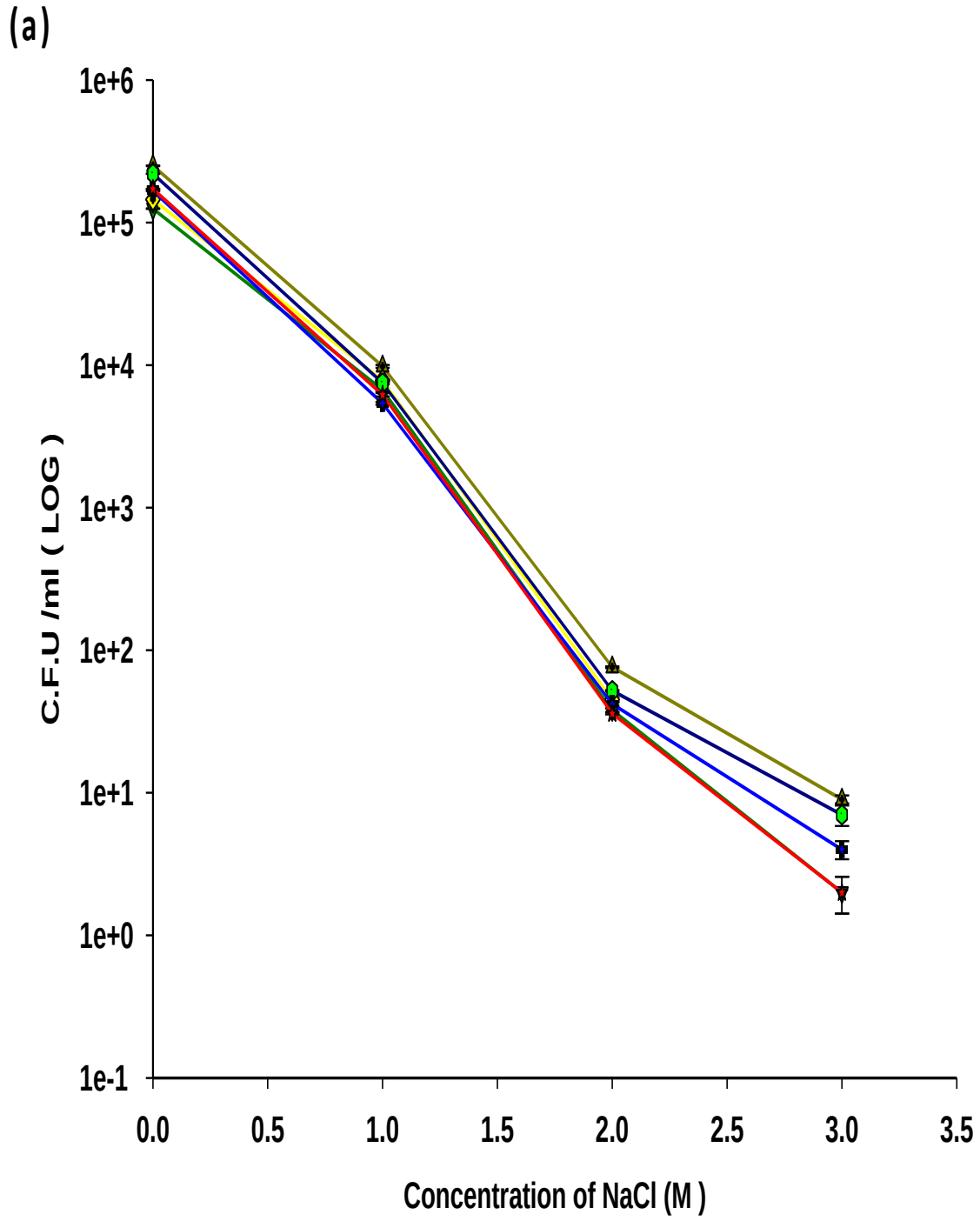
for the storm's updraft to keep it up, it falls down from the cloud (Kang *et al*., 2007). However, the tested hailstone sample showed halophilic growth up to 2.0M NaCl as did one rain water sample, whereas the other rain water sample did not expose any visible halophilic growth.

2.3.2 Measurement of the number of total halobacterial cells

The total plate of halophilic counts was determined in non-saline samples at 0, 1.0, 2.0 and 3.0M NaCl using plate count media incubated at 25^{o}C and 37^{o}C. It was found that the viable counts of bacteria of the different non-saline samples which were able to grow in different salt concentrations behaved similarly, exhibiting an initially maximum count and then decreasing with high salt concentration (Figure 2.10 (a) and (b)). The viable counts of halophilic bacteria decreased with increasing salt and maximum counts obtained were of the order of 10^6 per gram of soil with 1.0M NaCl, while no counts were obtained at concentrations of 3.0M, although growth was obtained during isolation studies at 3.0 M in some samples.

The viable counts of bacteria in the non-saline samples were similar to those found in saline environments; the highest count was found at low salt concentration and then decreased but less dramatically than in non-saline soil samples (Figure 2.11). The viable counts of bacteria able to grow in 1.0M and 2.0M NaCl and highest viable counts of halophilic bacteria were recorded as low salt concentration. The total numbers of isolates were more than 1000 times smaller than those of non-saline soil samples in media without NaCl. Non-saline soil samples have shown that increasing concentrations of NaCl of agar plates for colony just decreased the number of c.f.u. On agar plates containing 3.0M NaCl, numbers of colonies per plate ranged from 0 to 9 colonies which were below the statistically reliable level of 30 to 300 colonies per plate. Numbers of halophilic bacteria, however, were almost the same as those of non-saline soil samples. These data show that halophilic bacteria which can grow at 3.0M

NaCl can be isolated from all of the samples studied here at a minimum level of 1.0 c.f.u. (colony forming unit)/g soil.



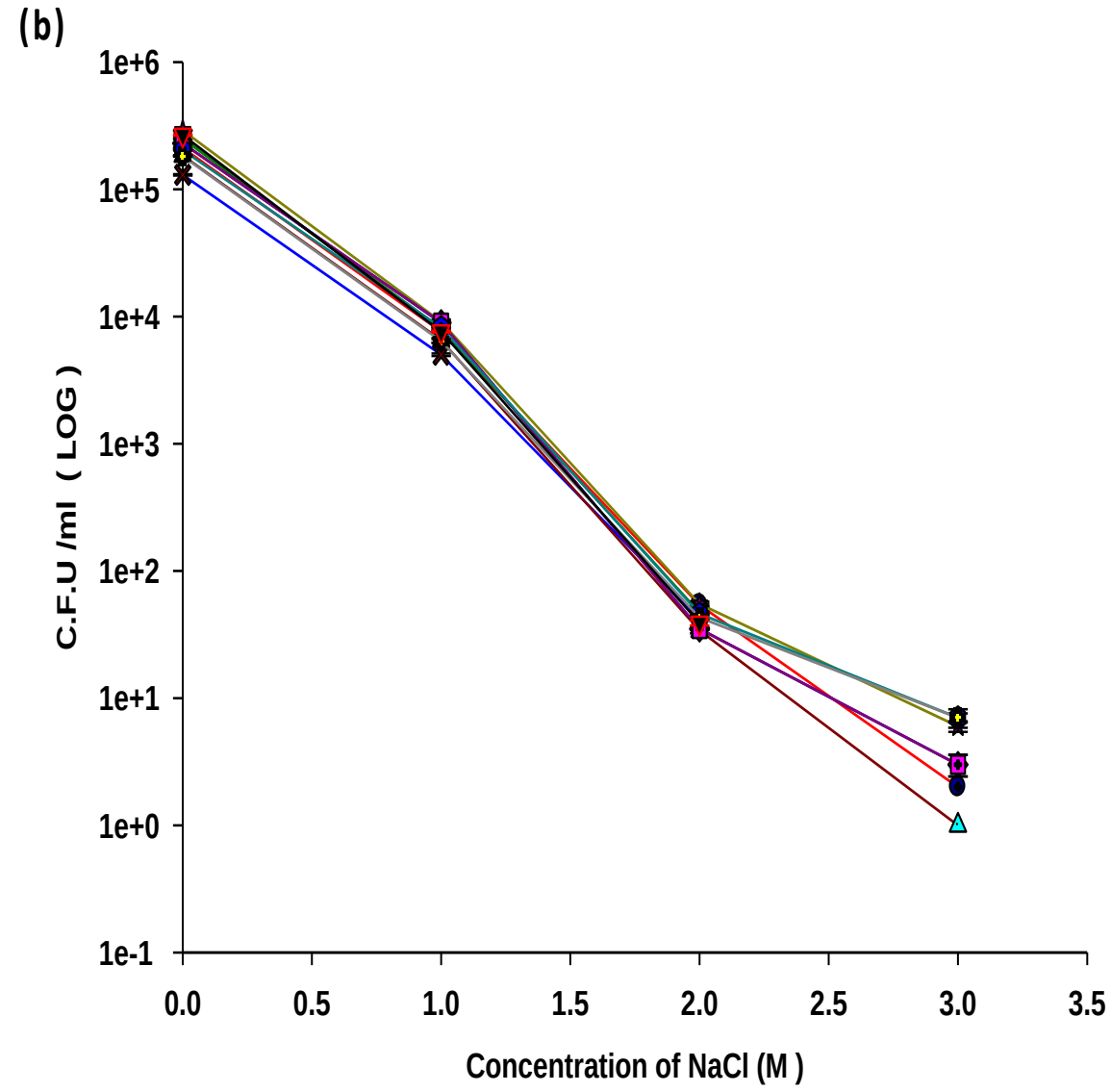


Figure 2.10 The total bacterial count from non-saline soil samples on agar plates containing 0.0 up to 3.0 M NaCl; (a) the total cell count from samples represent the first attempt on isolating halophiles including; soil from garden (), cliff (), city of Beverley () and soil under trees such as beech (), sycamore (), pine (), (b) the total cell count from samples represent the conformational attempts on isolating halophiles, all were different garden soils including soil from a near road which had been treated with salt grit during periods of snow. (I) refers to Standard Deviation (SD).

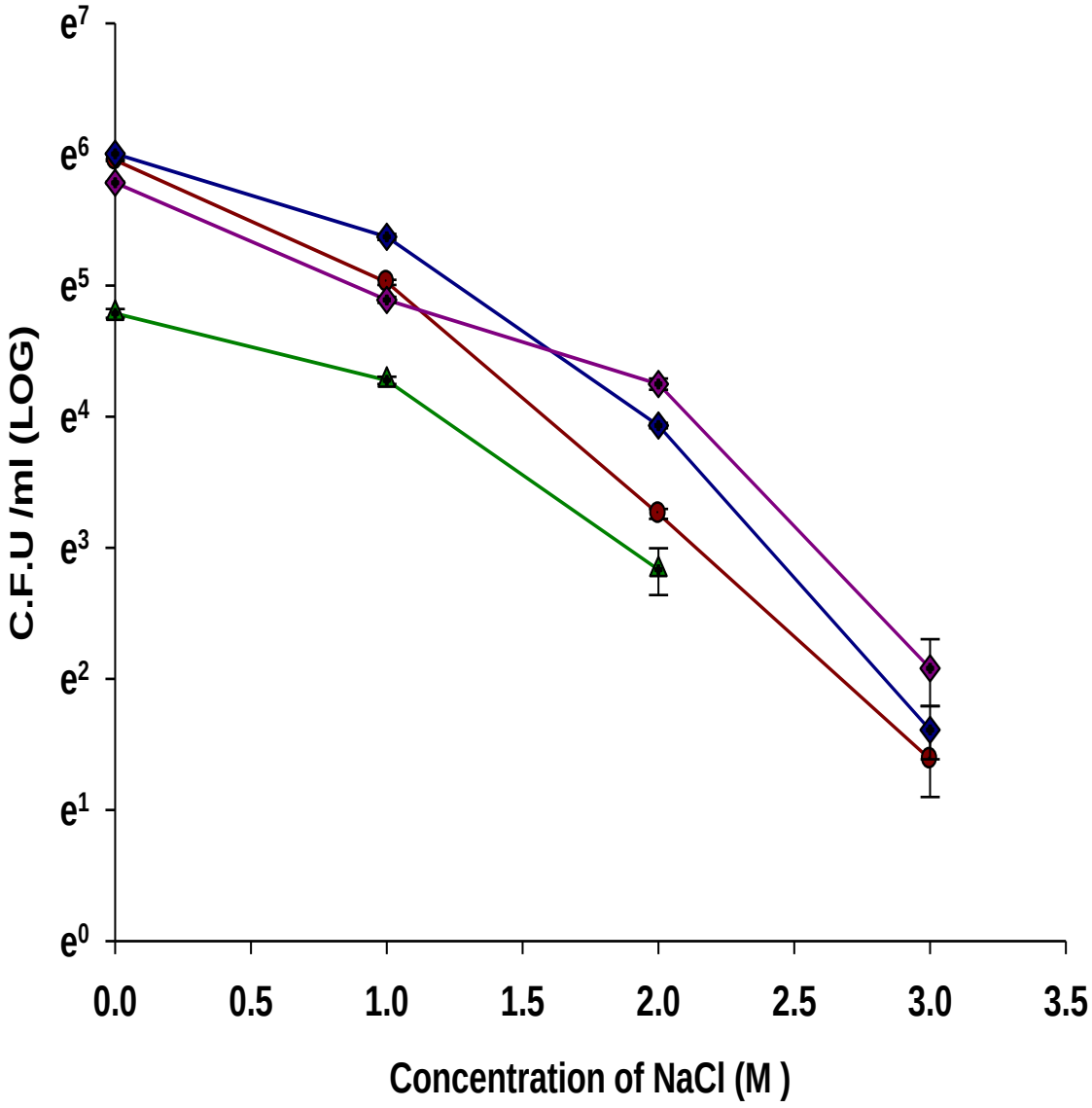


Figure 2.11 The total bacterial cells count from saline samples on agar plates containing 0.0 to 3.0 M NaCl. These samples have included; beach sand (), sea water (), wash () and cliff (). (I) refers to Standard Deviation (SD).

2.3.3 DNA extraction, cloning and sequencing based on 16S rRNA

This part of the Chapter focuses on the cultivation and isolation of halophilic bacteria and archaea from the non-saline environment (ordinary environment). Several different conventional liquid media were used for cultivation purposes. The lack of success using standard methods led to the development of a new liquid medium based on a soil-extract which mimicked the natural habitat and supplied the essential metabolic trace elements required for microbial growth. Molecular analyses revealed that the dominant culturable bacterial species present in the mixed cultures belonged to the Firmicutes, Bacteroidetes, and α-Proteobacteria groups. In some case, pure isolates were recovered using agarose-based solid medium based upon soil-extract. Microorganisms live in complex communities and networks that interact with each other and their natural habitats (Watnick and Kolter, 2000).

2.3.3.1 Genomic DNA extraction

Considering *E.coli* as a typical bacterium, the *E.coli* chromosome is 4,638,858 bp long and this comes to roughly 0.005 picograms per cell. In a typical overnight culture started from a single colony, the number of viable bacteria is around $1\text{-}2\times10^9$ bacteria/ml which means that 1 ml of culture should yield about 5 µg of genomic DNA per 10^9 bacteria.

In this study, an initial attempt to use the CTAB method of extracting DNA did not show any DNA band in gel electrophoresis which means that it failed to extract DNA. Although the ANACHEM KeyPrep Kit was much easier and cheaper and less time consuming, both Qiagen and ANACHEM kits of whole genomic DNA extraction method (described in Section (2.2.7.1)) resulted in high quality and high molecular weights of genomic DNA (Figure 2.12).

Figure 2.12 The whole genomic DNA in agarose gel analysis isolated from environmental sample. M stands for the l-kb DNA ladder, lanes 1 - 8 represent genomic DNA with band size over 10 kb.

2.3.3.2 PCR amplification of extracted DNA

16S rRNA gene sequences contain hypervariable regions that can provide specific signature sequences useful for archaeal and bacterial identification. Comparative analysis of our gene of interest, the 16S rRNA sequences, was done with the help of universal primer. The amplified gene was banded in between 1 and 2 kb (Figure 2.13), specifically 1,542 in length**.**

Sometimes, a band was produced that gave a completely unexpected and nonsensical sequence and at other times, sequencing with one of our PCR primers gave a completely blank lane. It was quite possible to sequence a PCR product straightforwardly without initially cloning the fragment. However in some cases, direct PCR sequencing was successful when a PCR reaction was purified prior to sequencing. At this stage, the PCR product was sent for direct sequencing to the Medical School,

University of Sheffield, however in most cases 800-1000 bp provided good sequencing data.

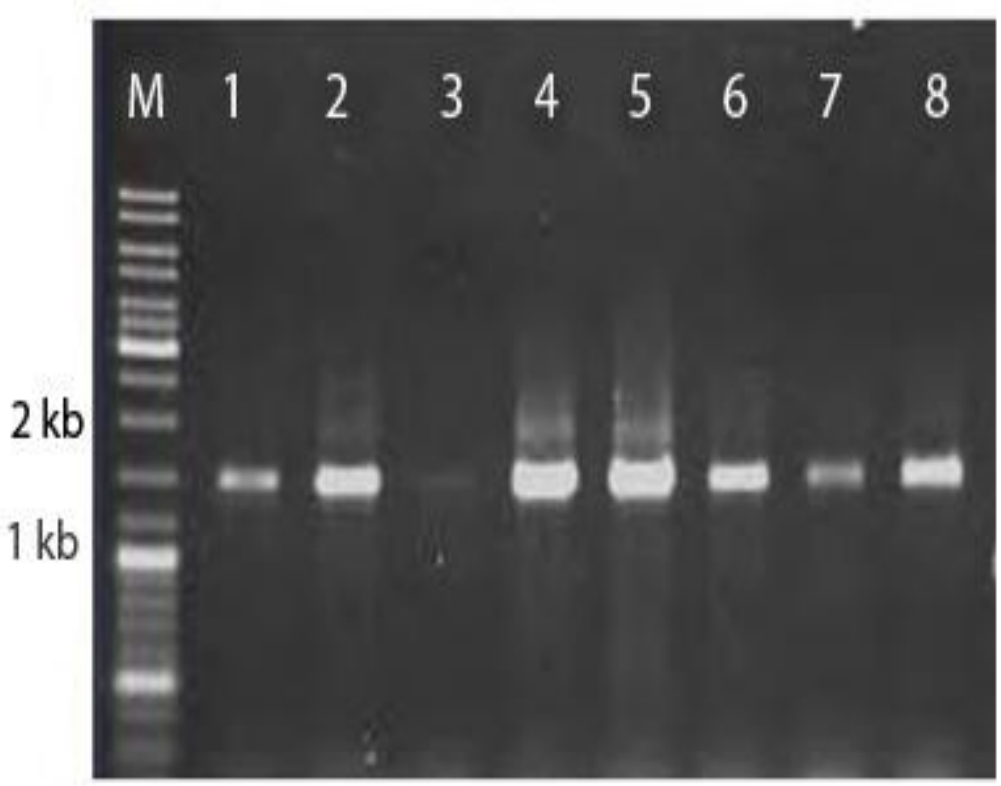


Figure 2.13 Product of 16S rRNA gene (1.5 kb) from the polymerase chain reaction (PCR) on agarose gel (1%) electrophoresis with ethidium bromide. M stands for the l-kb DNA ladder, lanes 1 and 2 represent 16S rRNA gene involving the universal archeal primers, lane 3 negative PCR reaction using archaeal primers and lane 4-8 represents 16S rRNA gene involving the bacterial primers.

2.3.3.3 Clones and sequence analysis of clones

Although, 800-1000 bp was good sequencing data, in order to obtain a full length of (1.5kb) read, 16S rRNA was ligated into plasmid (vector). In Wainwright and Gilmour laboratory, the vector pCR2.1 was most commonly used. This vector was 3.9 kbp in length and had *EcoR*I sites either side of the region where the 16S rRNA have been

inserted. The transformation of the *E.coli* with the vector containing (ligated) the 16S rRNA insert was successsful (Figure 2.14).

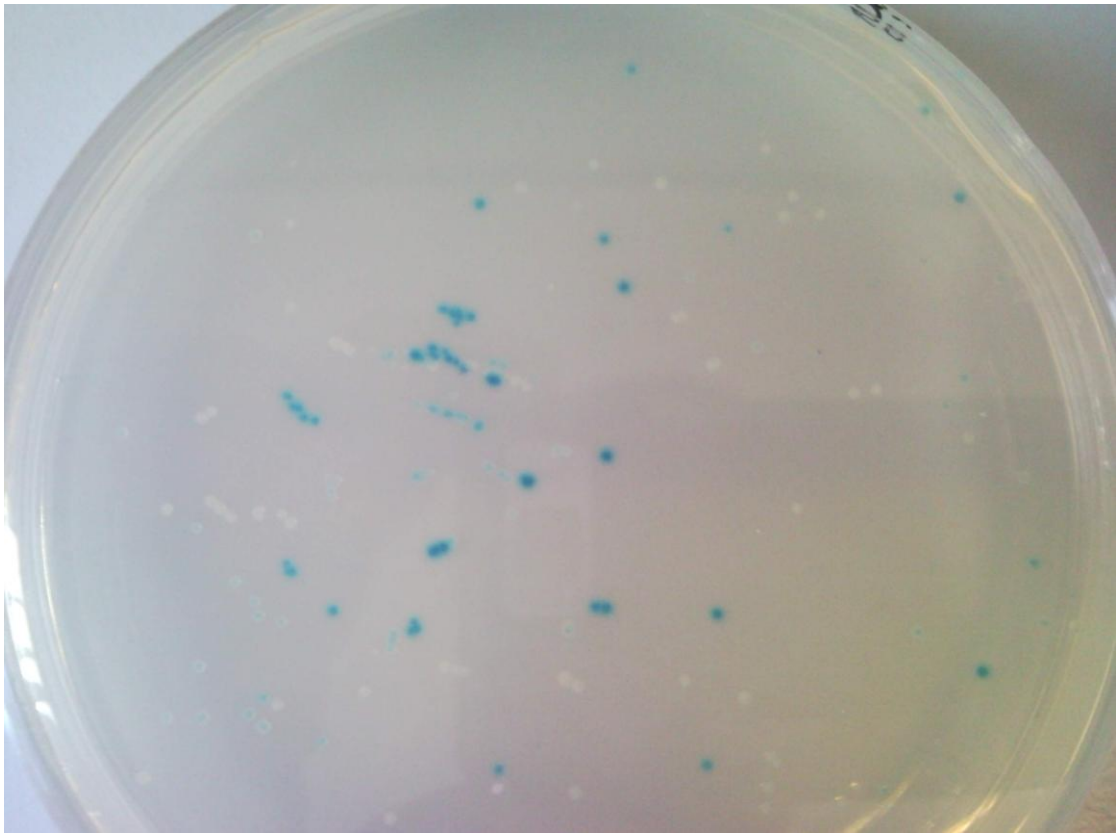


Figure 2.14 An LB agar plate showing the result of a blue - white screen for the detection of successful ligations in vector-based gene cloning. The ligation was successful when the bacterial colony was white; if not, the colony was blue.

Plasmid DNA is typically about 3-5 kb and the size is increased based on the insert. A typical high copy number of our plasmid, which was pCR 2.1 vector, should yield between 4-5 µg of DNA per ml of LB culture. For isolating high yields of plasmid DNA, the culture was in late log phase or early stationary phase and the culture was prepared using fresh single colonies from plates and the antibiotic was fresh and in a correct strength to maintain the plasmid during growth. Also, care was taken not to overgrow the culture which could result in genomic DNA contamination in the plasmid prep. Then, restriction digest was carried out to confirm if the plasmid has ligated with the correct insert which was shown in (1%) gel electrophoresis (Figure 2.15).

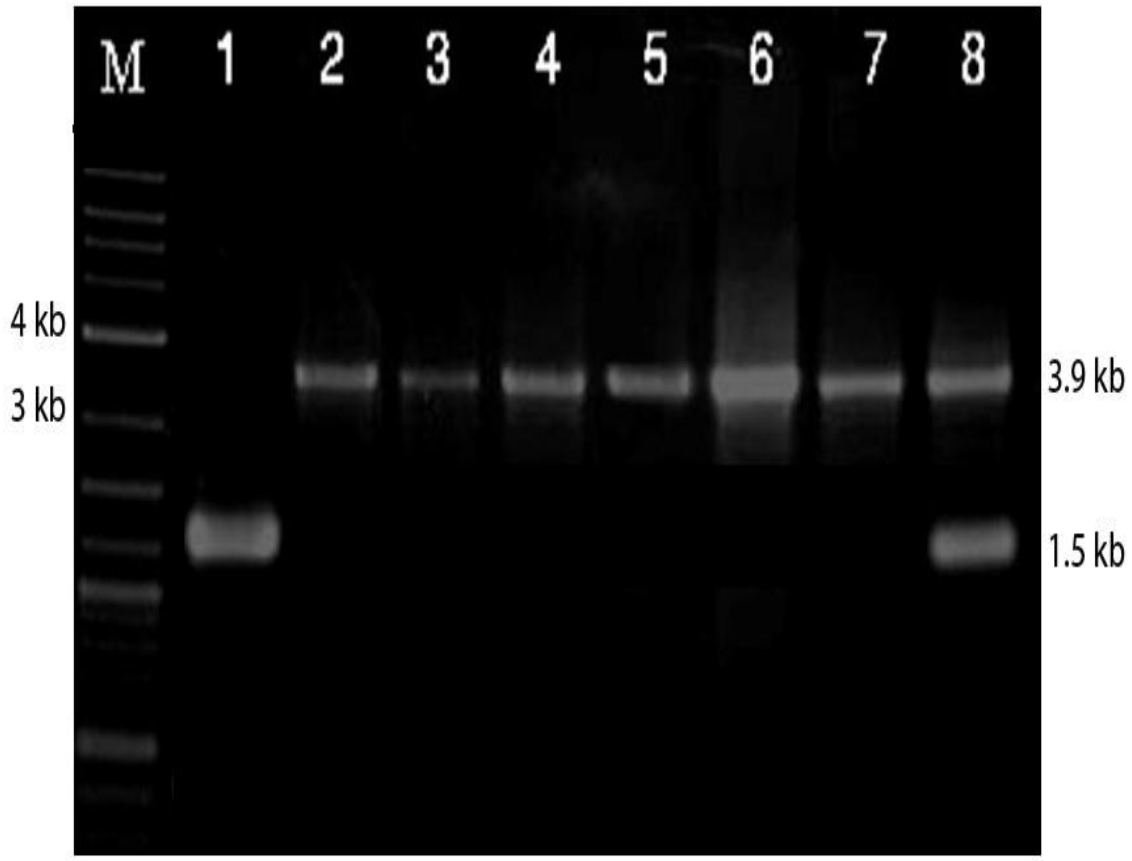


Figure 2.15 Representative figure of positive clones screened by colony PCR. Lane 1: gene control without vector. Lanes 2-7: negative clones from transformation of pCR 2.1 vector (Plasmid) with size 3.9 kb in *E. coli*. Lane 8: positive clone of the pCR 2.1 vector (Plasmid) with size 3.9 kb and the lower band indicates the 16S rRNA gene with size 1.5 kb. Lane M contains 50 ng DNA ladder (Bioline).

After analysis of vector, those containing the correct inserts were sent away for sequencing again at the medical school which then produced the full length sequence. This sequence has given the best blast match database or the closest described microorganisms and percentage of sequence identity (Table 2.5).

2.3.3.4 Phylogenetic analysis

Bacterial and archaeal phylogenetic trees were generated from the sequences obtained from the recombinant libraries. All the sequences obtained from the clone

library were deep branching and were affiliated (NCBI BLASTN) with environmental sequences obtained from non saline-related or saline-related samples. All sequences were closely related to cultured microorganisms, showing more than 99% sequence identity with their corresponding closest BLAST relatives and two not closely related to any cultured microorganisms.

Here is an example of phylogenetic analysis for HSO isolate whereas the rest of isolates are plotted in (Appendix C). Figure 2.16 shows the phylogenetic position of the halophilic bacterium. Phylogenetic analysis of isolate using the BLASTN algorithm at NCBI showed a 100 % identity to *Halobacillus sp*., particularly *H. trueperi* (Figure 2.17) small subunit rRNA, (NCBI accession number GQ903458.1).

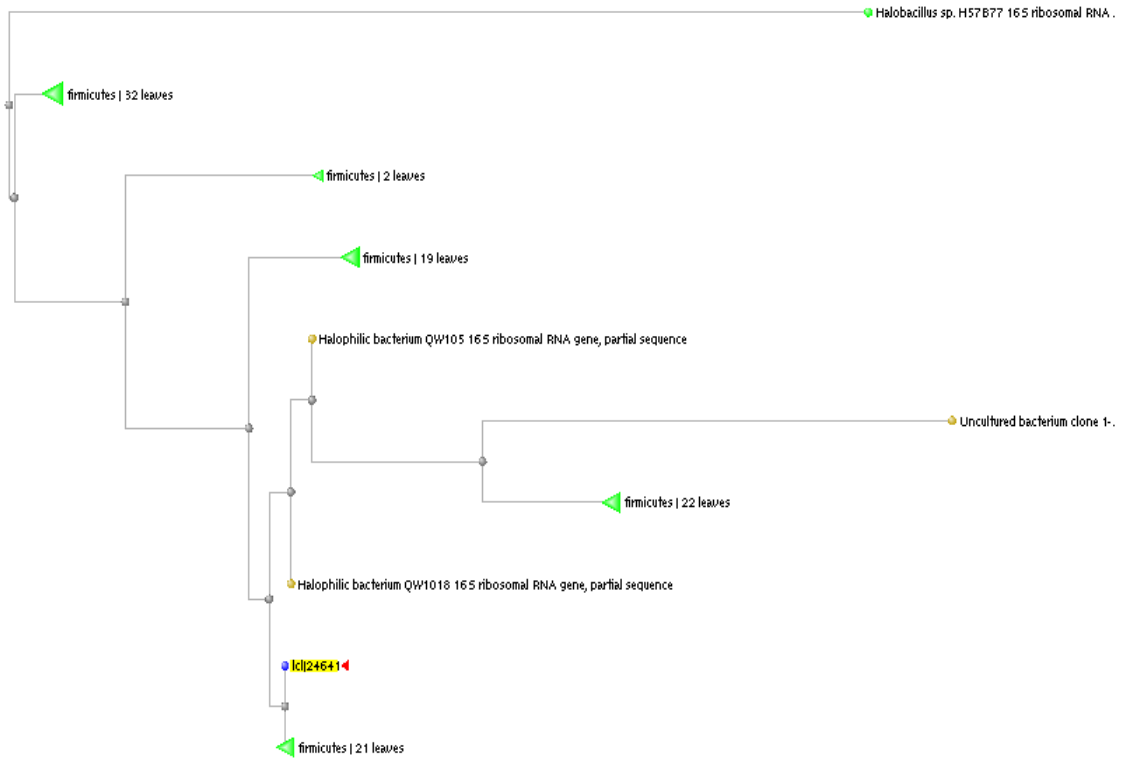


Figure 2.16 Neighbour joining phylogenetic tree of the 16S rRNA gene of HSO isolate, with a maximum sequence difference of 0.05. The colour Green represents firmicutes, brown represents bacteria and yellow represents unknowns. The tree shows that this isolate was a halophilic bacterium and been isolated from saline environment the closest known relative.

```
>  gb|GQ903458.1|  Halobacillus trueperi strain XJSL8-9 16S ribosomal
RNA gene,
partial sequence
Length=1529

 Score = 1171 bits (634),  Expect = 0.0
 Identities = 634/634 (100%), Gaps = 0/634 (0%)
 Strand=Plus/Minus

Query  1     AAGGTTACCTCACCGACTTCGGGTGTTGCCAACTCTCGTGGTGTGACGGGCGGTGTGTAC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1466  AAGGTTACCTCACCGACTTCGGGTGTTGCCAACTCTCGTGGTGTGACGGGCGGTGTGTAC  1407

Query  61    AAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1406  AAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTT  1347

Query  121   CATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAATGGTTTTATGGGATTTGCTACA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1346  CATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAATGGTTTTATGGGATTTGCTACA  1287

Query  181   CCTCGCGGCTTCGCTGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1286  CCTCGCGGCTTCGCTGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATA  1227

Query  241   AGGGGCATGATGATTTGACGTCATCCCCGCCTTCCTCCGGTTTGTCACCGGCAGTCACCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1226  AGGGGCATGATGATTTGACGTCATCCCCGCCTTCCTCCGGTTTGTCACCGGCAGTCACCT  1167

Query  301   TAGAGTGCCCAACTGAATGCTGGCAACTAAGATTAGGGGTTGCGCTCGTTGCGGGACTTA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1166  TAGAGTGCCCAACTGAATGCTGGCAACTAAGATTAGGGGTTGCGCTCGTTGCGGGACTTA  1107

Query  361   ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTTGGTCCCCG  420
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Sbjct  1106  ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTTGGTCCCCG  1047

Query  421   AAGGGAAAGCCCTATCTCTAGGGATGTCCAAGGATGTCAAGACCTGGTAAGGTTCTTCGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1046  AAGGGAAAGCCCTATCTCTAGGGATGTCCAAGGATGTCAAGACCTGGTAAGGTTCTTCGC  987

Query  481   GTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  986   GTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGA  927

Query  541   GTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  926   GTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTA  867

Query  601   AGGGGTGGAAGCCCCCTAACACCTAGCACTCATC  634
             |||||||||||||||||||||||||||||||||
Sbjct  866   AGGGGTGGAAGCCCCCTAACACCTAGCACTCATC  833
```

Figure 2.17 The highest percentage identity matches following BLASTN comparison of *Halobacillus* sp 16S rRNA and the NCBI nucleotide collection (nr/nt) database. The "Query" line refers to the input sequence, *Halobacillus* sp. G-12 16S ribosomal RNA gene, partial sequence whilst the "Subject" line refers to the matching sequence, in this instance *Halobacillus trueperi* small subunit rRNA.

2.3.4 Analysis of cultures and isolates

16S rRNA genes were amplified from total DNA of isolates which was cultured from non-saline environments. A plasmid mini-prep was prepared from the clones obtained and representative clone was sequenced. Twelve clones were obtained from the non-saline and saline archaeal and bacterial 16S rRNA gene libraries. BLAST analysis of the clones identified 10 different species with two of these groups belonging to the archaea.

2.3.4.1 Bacterial 16S rRNA clone libraries

Bacteria belonging to the genera *Bacillus* (5 isolates), one isolate of following species; *Staphyloccocus, Exiguobacterium, Pantoea* and two isolates with the closest match to the unculturable bacteria, were also isolated from desert sand and desert varnish rock samples (Table 2.5).

Table 2.5 Summary of 16S rRNA sequence analyses (Representative BLASTN matches) of microorganisms (bacteria and archaea) cultivated from non saline environment and from hypersaline soil.

Samples	Salt concentration (%)	Representative sequence	Best blast match database	% sequence identity	Closest described microorganism	NBCI (Accession no.)
Stratosphere	0	STRA2	*Bacillus sp.*	99	*Bacillus pumilus*	NCIMB
Hailstone	0	HL1 HL2	*Staphylococcus sp.* *Pantoea sp.*	99.94 99.12	*Staphylococcus Sciuri sciuri* *Pantoea dispersa*	NCIMB
Desert sands	0	DSRT3	Uncultured bacterium partial 16S rRNA gene	99	Uncultured bacterium clone	AM982536.2
Desert varnish	0	DV1 ArchDV	*Bacillus sp.* Uncultured bacterium partial 16S rRNA gene	99 92	*Bacillus sp.* Uncultured bacterium clone	AY461753.2 HQ902650.1
Garden soil	0	QDF	*Bacillus sp.*	99	*Bacillus subtillis* strain RV3	GQ413935.1
Beech soil	0	BES	*Bacillus sp.*	100	*Bacillus weihenstephanensis* strain BW70UT1570	JF276896.1
Sycmour soil	0	SYCS	*Bacillus sp.*	99	*Bacillus subtillis* strain HU60	EF101710.1
Hypersaline soil	10	HSO	*Halobacillus* sp. G-12	100	*Halobacillus trueperi*	GQ903458.1
Oman Soil	0	NIZ	*Paenibacillus* sp.	99	*Paenibacillus ehimensis*	FN582329.1

All isolates reported so far are obligate extreme halophiles involving a minimum of 10% (w/v) salt concentrations for growth (Oren, 2000), and most growing optimally at 20%-25% (w/v) NaCl. It is generally believed that habitats of halophiles able to grow in media containing more than 20% are restricted to saline environments (Saiz-Jimenez and Laiz, 2000).

In this study, the strains isolated from the ordinary soils were *Bacillus* species, and analysis of partial sequences of their 16S rRNA genes showed that some of them possessed similarities higher than 99% with those of *Bacillus subtillis, Paenibacillus ehimensis* and 100% with those *Bacillus weihenstephanensis*. No bacteria other than species of the genus *Bacillus* were isolated from ordinary soil samples.

Two isolates were obtained from hailstones on media containing 2M NaCl, one was *Pantoea dispersa* (99.12% similarity) and the other was *Staphylococcus sciuri sciuri* (99.94% similarity). The latter was isolated by culturing the hailstone directly to low nutrient agar containing a range of concentrations of salt up to 4M. Initially, visible growth was seen at concentrations up to 2M NaCl, but in the second re-culture using the same type of medium, growth only occurred up to 4M NaCl. The ability of staphylococci to grow in a wide range of salt concentrations is well documented (Graham and Wilkinson, 1992; Amann *et al*., 1995; Clements and Foster, 1999; Garzoni and Kelley, 2009; Morikawa *et al*., 2010) but *Staphylococcus sciuri sciuri* has not yet been reported halotolerant or halophilic and there is no published work on its growth at different sodium chloride concentrations.

Bacillus pumilus isolated from the stratosphere (41 km biosphere obtained using a balloon lofted cryosampler (Wainwright *et al*, 2003)) grew up to 3M NaCl (first and second re-cultures). This strain is also not well known as a halophilic bacterium and is also used as a soil inoculant in agriculture and horticulture (Gutiérrez-Mañero *et al*., 2001).

A halophilic bacterium was also isolated from a hypersaline soil in Oman; it was used to compare with the two bacteria isolated from non-saline samples and also for further study of osmo-adaptation. Using 16S rRNA method, it was shown to have a 100% similarity with *Halobacillus trueperi*. This bacterium was first isolated from hypersaline sediments of the Great Salt Lake in Utah. It grows in media containing 0.5 to 30% (w/v) salt, and optimum growth occurs in medium containing around 10% salt (Spring *et al*., 1996).

Since halophilic bacteria have been isolated from the Dead Sea (Larsen, 1986), numerous halophilic and halotolerant microorganisms, particularly bacteria and archaea have been isolated from saline environments (Oren, 2002), The data presented here show that bacilli are dominant among halophilic bacteria in non-saline environments although it is known that halophilic bacteria, in general, are not restricted to this genus. Oren (2002) defined a "halophile" as an organism having tolerance to 10% (2M or 100 g/L) NaCl, while some halophilic microorganisms are able to grow in the presence of more than 15% NaCl. It is also known that all microorganisms that have to date been isolated as halophiles are inhabitants of saline environments. On the other hand, some bacteria isolated from soil are able to tolerate high NaCl concentrations. For example, *Bacillus agaradhaerens*, *B. pseudofirmus*, and *B. clarkii* are tolerant up to 2M or 2.5M NaCl (Nielsen *et al*., 1995). Some microorganisms have been reported to grow at 3M NaCl or lower concentrations from non-saline soil samples (Saiz-Jimenez and Laiz, 2000; Echigo *et al*., 2005; Usami *et al*. 2007). Halophiles are able to grow in media containing 3M NaCl, but are generally believed to be restricted to saline environments (Saiz-Jimenez and Laiz, 2000).

Some of the isolates obtained here were sent to (NCIMB, Aberdeen) for further confirmation of the identification obtained here; the identity of the HL1 strain which

has been isolated from hailstone, for example, was confirmed in this way (Table 2.6 and Figure 2.18).

Table 2.6 The top ten highest identities confirmed HL1isolate 16S rRNA by NCIMB, Aberdeen.

Sequence Name	% Match	Sequence Name	% Match
Staphylococcus sciuru sciuri	99.94	*Staphylococcus fleurettii*	98.7
Staphylococcus sciuri carnaticus	99.9	*Staphylococcus epidermidis*	95.46
Staphylococcus vitulinus	99.0	*Staphylococcus epidermidis*	95.46
Staphylococcus lentus	98.91	*Staphylococcus hyicus*	95.44
Staphylococcus vitulinus	98.9	*Staphylococcus muscae*	95.42

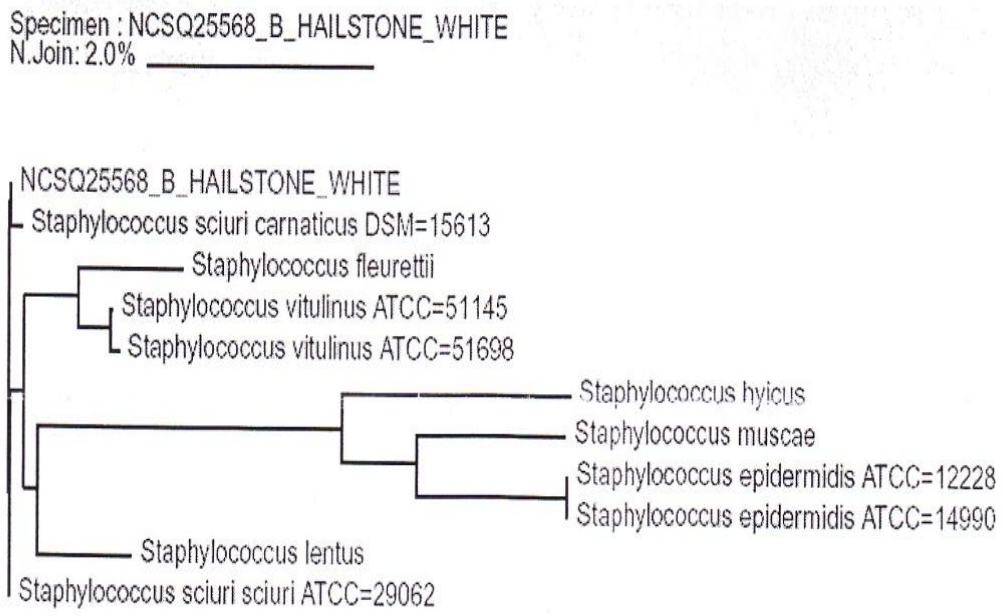


Figure 2.18 The phylogenetic tree of HL1 isolate from hailstone, identified at NCIMB, Aberdeen.

2.3.4.2 Archaeal 16S rRNA clone library

The two archaeal isolates were mixed with bacterial colonies and were not possible to separate them even with the use of different antibiotics and therefore not possible to get the right sequence data and identification. These two isolates with highest identity to the haloarchaea were isolated from desert sand and desert varnish collected from Oman (Table 2.5) which were under other osmotic stresses available such as dryness and lack of nutrition. All these strains could grow in solutions saturated with NaCl and showed optimal growth above 40°C.

Until relatively recently, the use of 16S rRNA was regarded as the "gold standard" for the identification of bacteria. No publication was acceptable without"16S" -based identification and traditional identification techniques, based for example on the Bergey Manual, fell into non-use. The situation is now changing, in that a number of problems are seen to be arising with regard to the use of this technique. For example, it is limited by the quality of data which is placed into the databases it requires, and much of this information turns out to be of dubious quality. It appears to be particularly problematic for identifying members of the genus *Bacillus* to the species level. This of course was how it was applied in the work described here in this Thesis. The use of this technique here was essentially as a training exercise to obtain expertise in the use of 16S rRNA and any reference to isolates at the species level needs to be considered in regard to the limitations of the technique; this would be especially the case if the data were offered for publication.

2.3.5 The effects of salt stress on halotolerant isolates

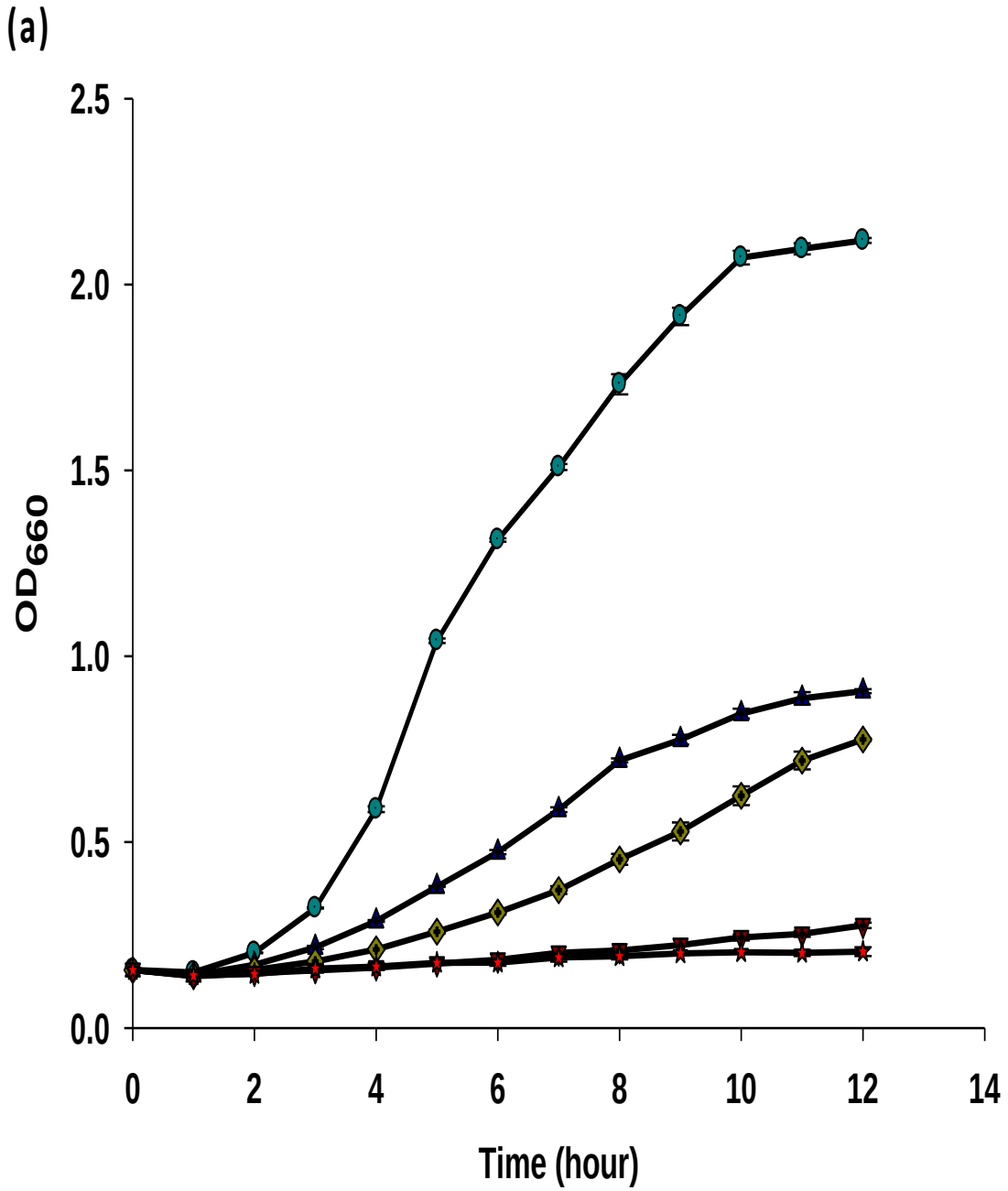
Four of the presumptive halophilic and halotolerant isolates were selected for further characterisation. One isolate was (STRA2) a strain of *Bacillus* and closely related to those of *B. pumillus*. It was isolated from the stratosphere. One other isolate, (HL1) was 99% related to *S. sciuri sciuri* and was isolated from a hailstone in

Sheffield. The third isolate was (HSO), which was 100% related to *H. trueperi* isolated from hypersaline soil in Oman. The forth isolate was (DSRT3) which gave positives with both universal archaeal and bacterial primers. It was 100% related to an unculturable bacterium which was obtained from desert sand in Oman.

The growth of these halotolerant and halophilic isolates were studied under different NaCl concentrations (0 to 4.0M). Two ways of measuring the pattern of the growth of these isolates was used. One approach was to shock each isolate at a range of concentrations of NaCl, while in the second approach the isolate was trained to grow at the maximum concentration of NaCl in which visible growth occurred and then the growth curve experiment was run on trained isolates obtained from the highest salt concentration used.

As shown in Figure 2.19 (a), shocked isolate HL1 grew well at 1.0M NaCl. However, the strain was able to tolerate salt concentrations higher than 2.0M NaCl. This isolate was tolerant to very high salt concentration but grew optimally without NaCl; its growth at 1.0M salt concentration was fifty percent lower than in the absence of salt.

The trained isolate HL1 grew, (as shown in Figure 2.19 (b)) very well in all salt concentrations. The isolate was also able to tolerate salt concentrations lower than 1.5M and higher than 2.0M NaCl. The growth curves at stress of 1.0 and 2.0M NaCl were very close to the optimum growth in zero stress. HL1 cells had a longer lag phase when grown above 3.0 and 4.0M NaCl when grown under shocked conditions than shown by the trained isolate.


(a)
2.5
2.0
1.5
1.0
0.5
0.0
OD660
0
2
4
6
8
10
12
14
Time (hour)

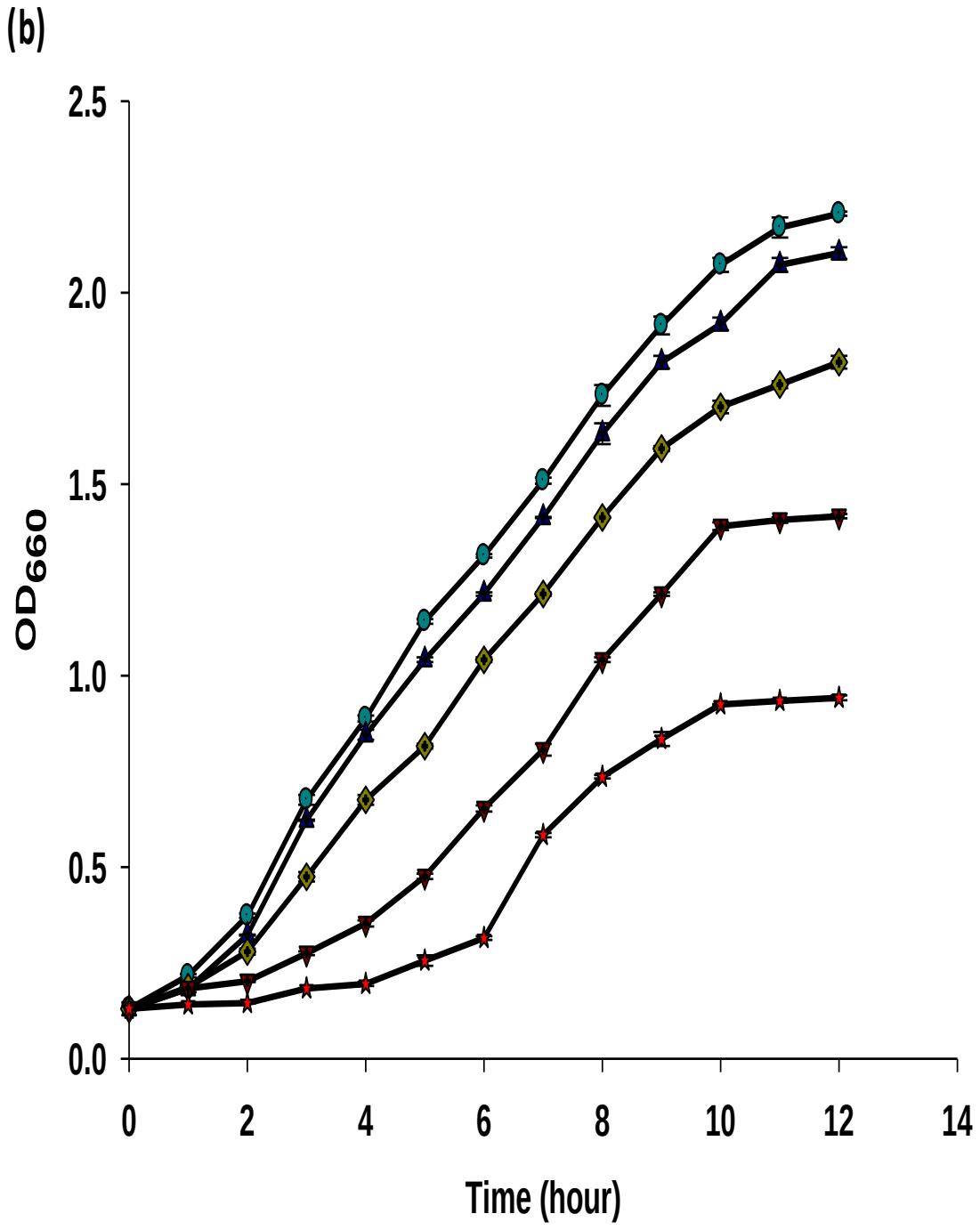
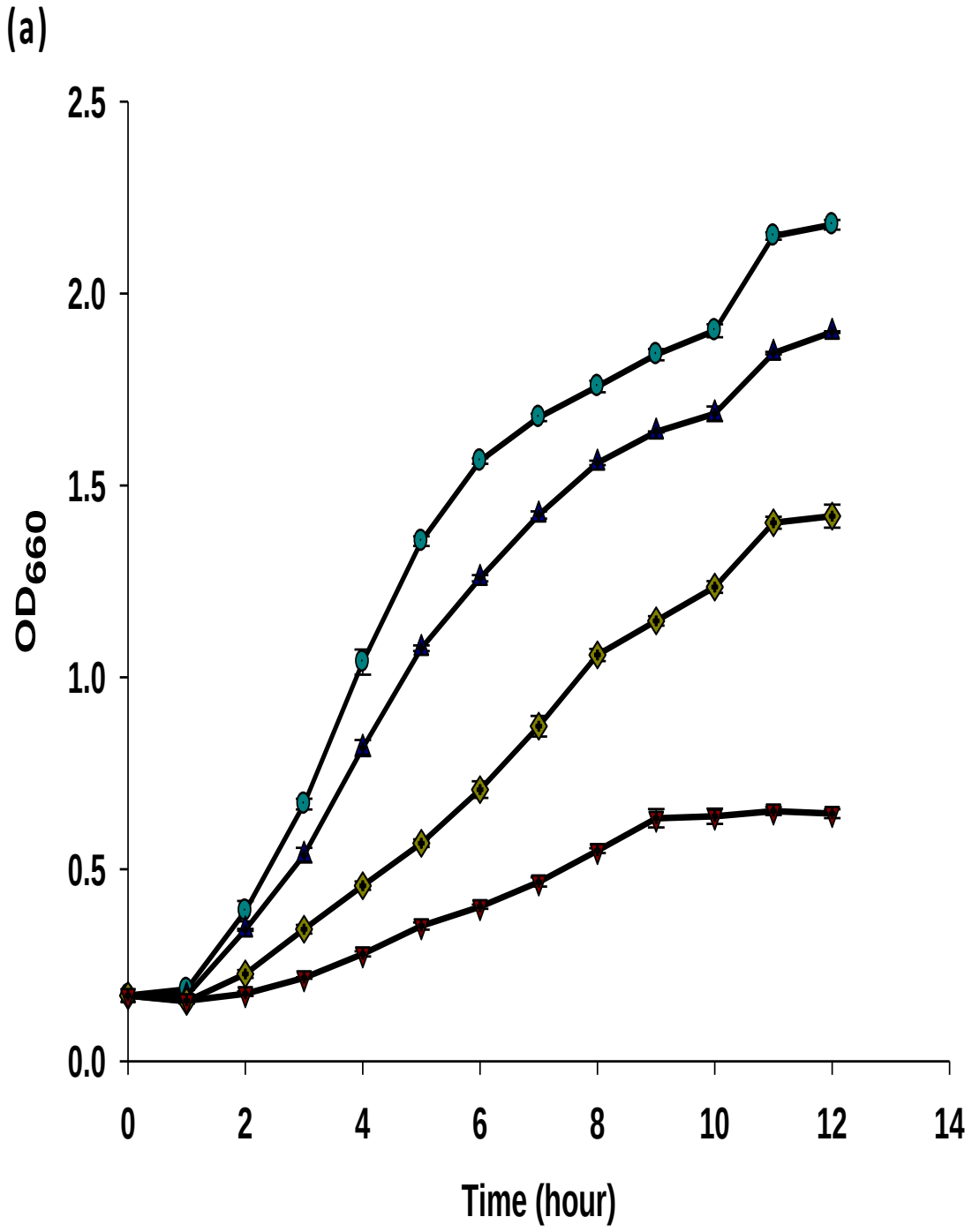


Figure 2.19 Growth curves of HL1 isolate grown under different NaCl (M) conditions; 0M (), 1.0M (), 2.0M (), 3.0M (), 4.0M () taken at optical density of 660 nm. (a) When cells undergo osmotic shock at various NaCl concentrations. (b) When cells trained up to 4M NaCl and then inoculated at various concentrations. (I) refers to Standard Deviation (SD).

On the other hand, the pattern of growth of shocked STRA2 isolate, shown in Figure 2.20 (a), was somehow different from HL1 isolate. Although HL1 isolate was able to survive at 4.0M of NaCl, STRA2 showed good growth at 2.0M NaCl and also grew optimally at 0M and at 1.0M NaCl.

The trained isolate STRA2 grew, as shown in Figure 2.20 (b), with the same pattern of growth when was it was shocked in all different salt concentration without exception. However, the strain was able to tolerate salt concentrations at 3.0M NaCl. STRA2 isolate was a spore forming bacterium which is known to form these spores once it is under stress (trained or shocked) better than it could under rich conditions (Figure 2.21). Therefore, this could explain no significant difference in growth pattern when it was shocked or trained. STRA2 cells in 0M NaCl and all other NaCl stress had a longer doubling time when grown under trained conditions than shocked.


(a)
2.5
2.0
1.5
1.0
0.5
0.0
OD660
0
2
4
6
8
10
12
14
Time (hour)

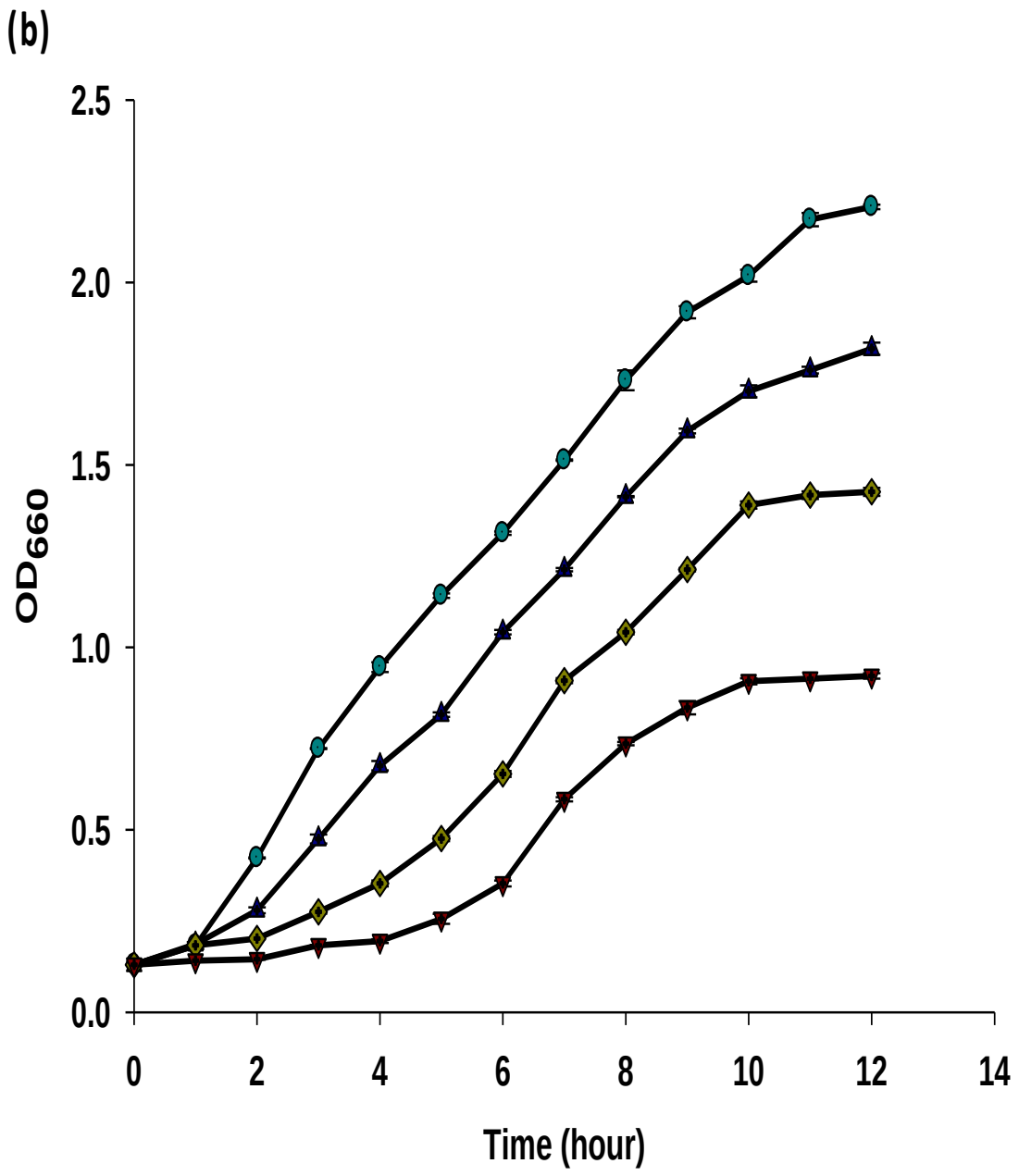


Figure 2.20 Growth curves of STRA2 isolate grown under different NaCl (M) conditions; 0.0M (), 1.0M (), 2.0M (), 3.0M () taken at optical density of 660 nm. (a) When cells undergo osmotic shock at various NaCl concentrations. (b) When cells trained up to 3.0M NaCl and then inoculated at various concentrations. (I) refers to Standard Deviation (SD).

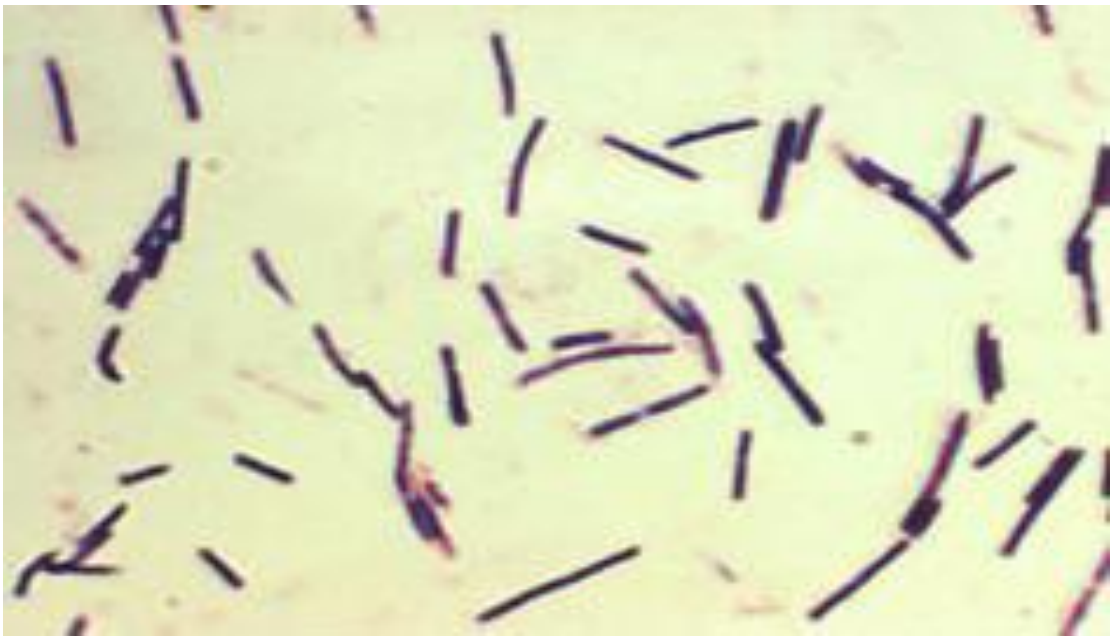
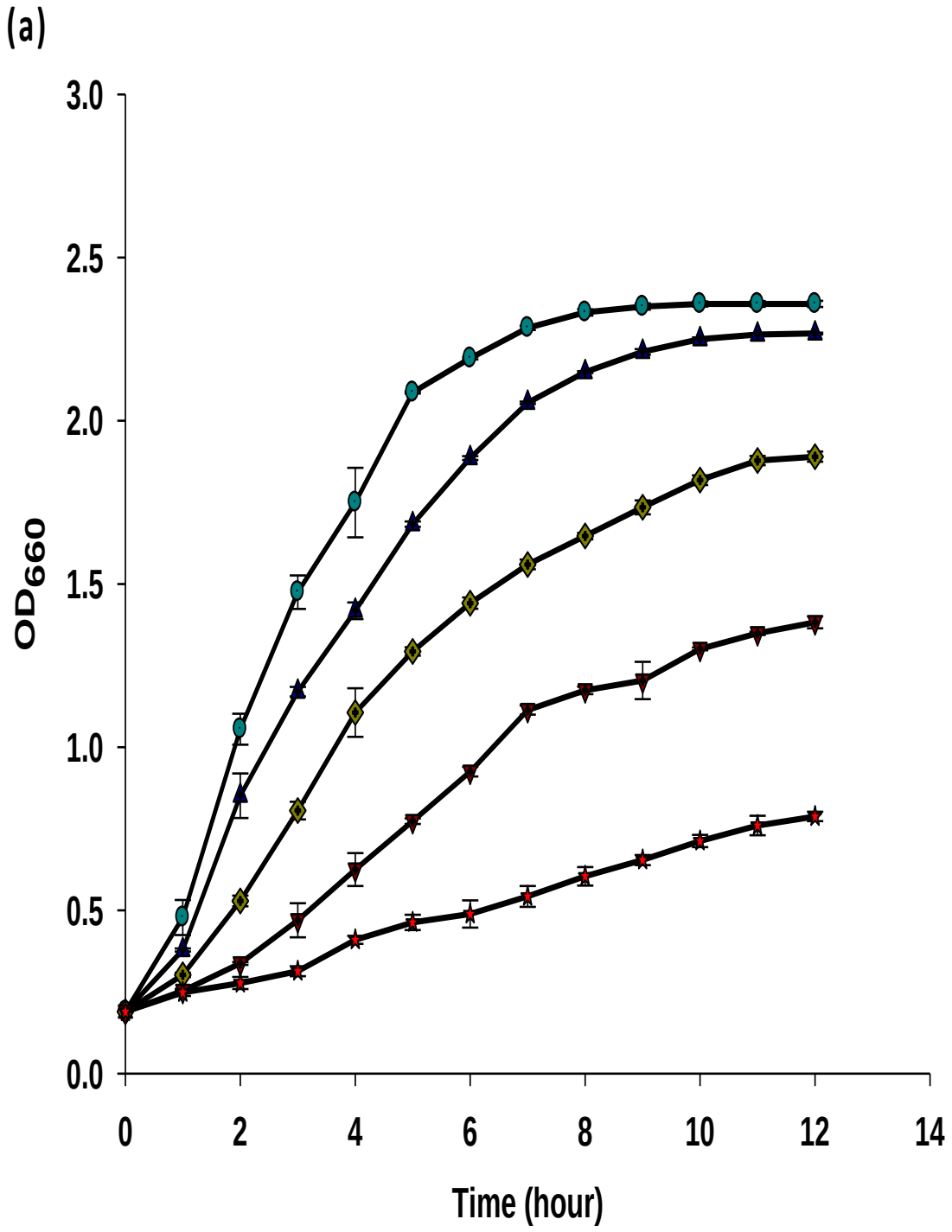


Figure 2.21 STRA2 isolate is a rod-shaped, aerobic and spore forming bacterium; it formed spores when it was stressed by NaCl.

HL1 and STRA2 isolates were more likely to have behaved as halotolerant (grow optimally without salt stress). DSRT3 was significantly different, and grew well in all of the different salt concentration over both conditions (trained and shocked) (Figure 2.22 (a) and (b)). Interestingly, the growth was similar at 0M and 1M when it was trained and 2M NaCl was close to the optimum for growth.

DSRT3 cells grown in all of the NaCl stress conditions showed a longer doubling time when grown under trained conditions than when shocked and had a longer lag phase when grown at 3.0 and 4.0M NaCl; the lag phase being shorter in trained conditions than in shocked. Furthermore, as shown in Figure 2.23, changes in the morphology of the DSRT3 isolate were observed when cells were grown in NaCl concentrations of 1.0M or higher. As NaCl concentration increased from 1.0M to 3.0M, cells increased in size at the highest levels of salt.


(a)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
OD660
0
2
4
6
8
10
12
14
Time (hour)

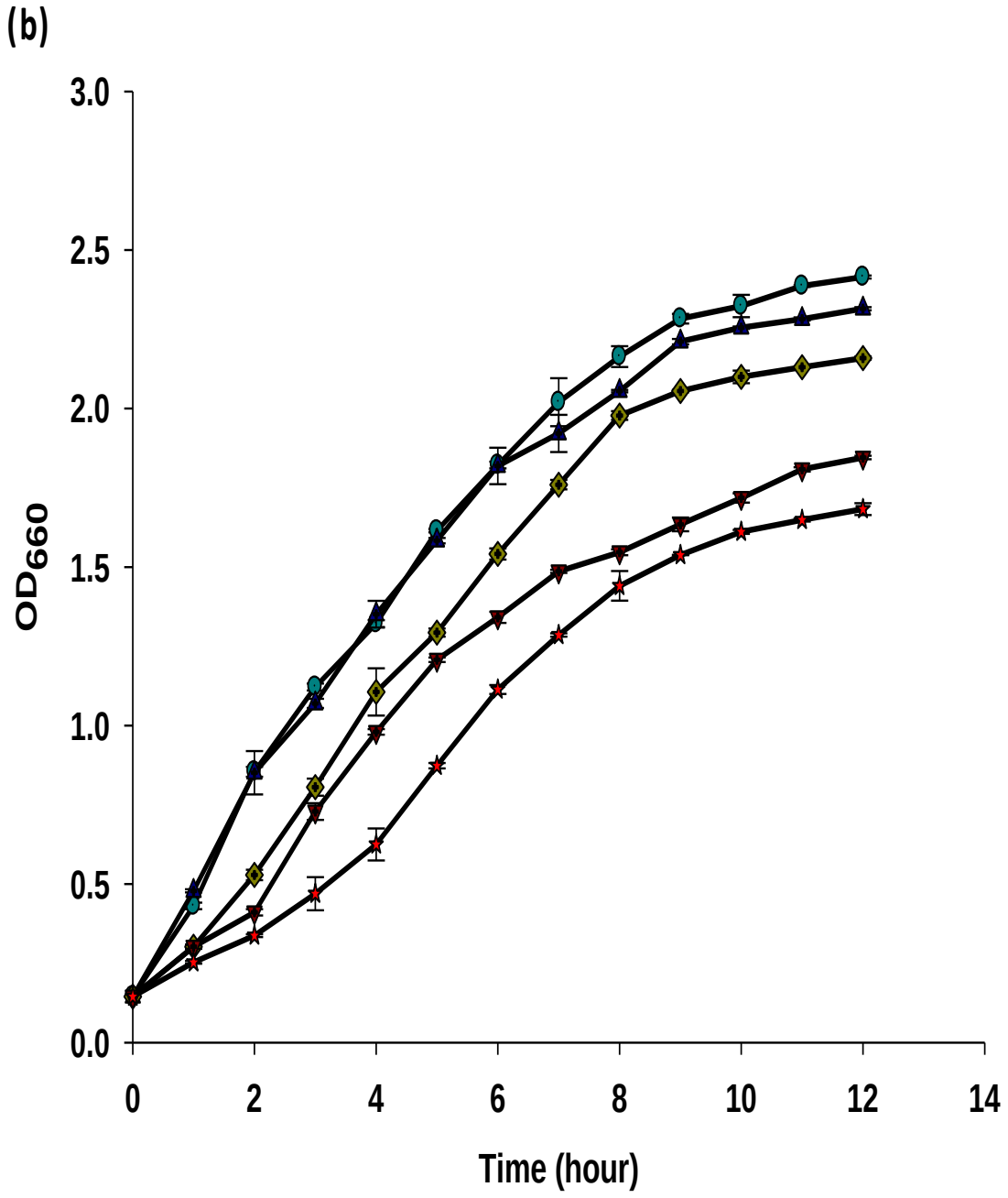


Figure 2.22 Growth curves of DSRT3 isolate grown under different NaCl (M) conditions; 0M (), 1.0M (), 2.0M (), 3.0M (), 4.0M () taken at optical density of 660 nm. (a) When cells undergo osmotic shock at various NaCl concentrations. (b) When cells trained up to 4M NaCl and then inoculated at various concentrations. (I) refers to Standard Deviation (SD).

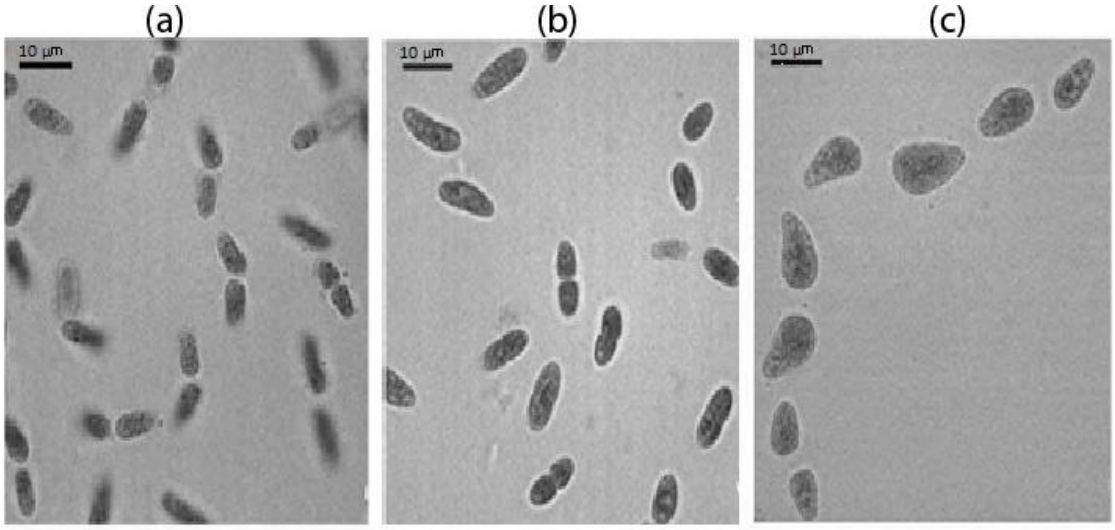
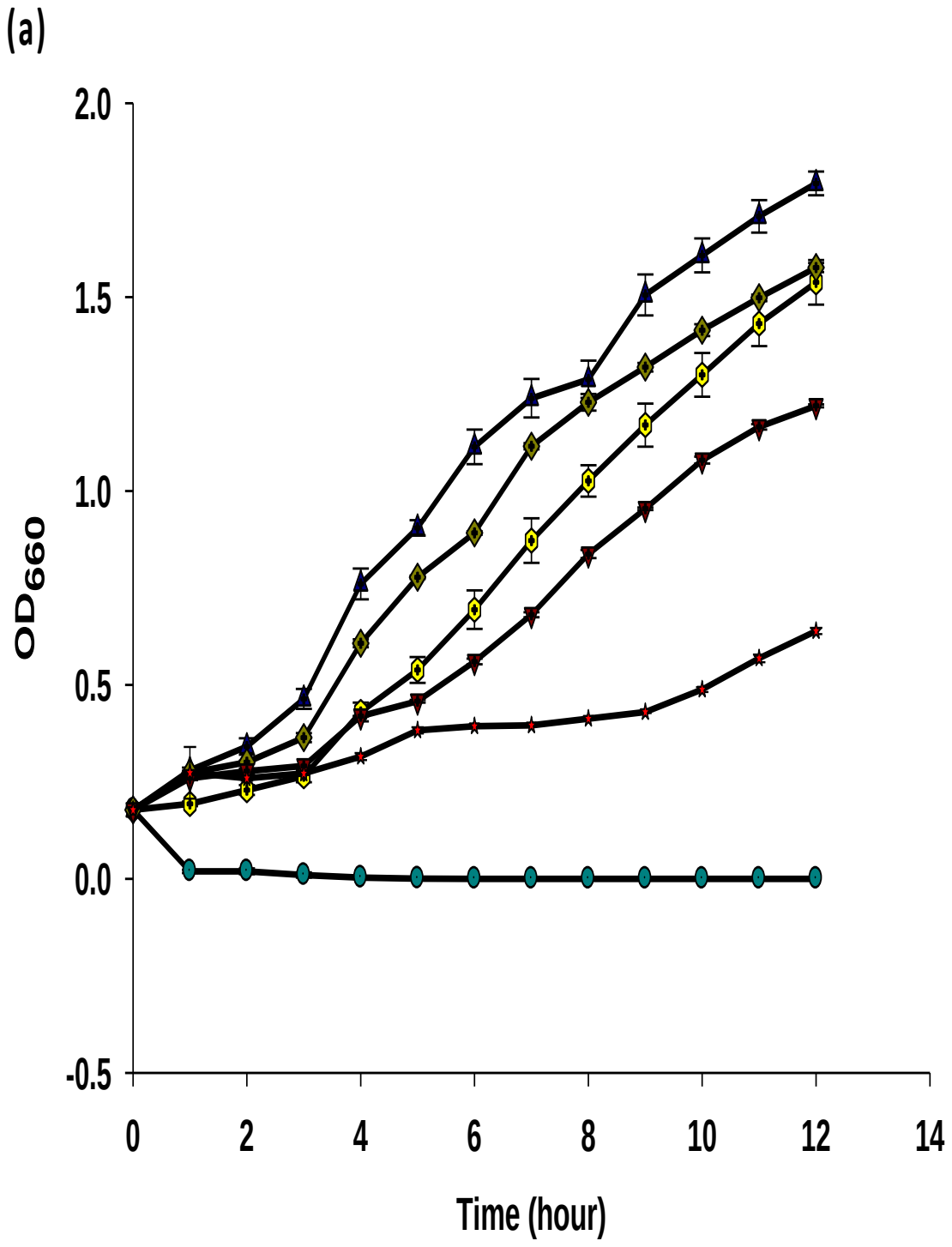


Figure 2.23 Light microscopy of the halotolerant isolate DSRT3 grown at (a); 0.0M NaCl, (b); 1.0M NaCl, (c): 3.0M NaCl.

All previously mentioned isolates HL2, STRA2 and DSRT3 were isolated from non-saline environment which would be expected to act as a source of only halotolerant bacteria. HSO isolate which has been isolated from saline soil, showed halophilic behaviour. It was not able to grow or even survive without salt and the optimum growth was at 1.0M and 2.0M NaCl (Figure 2.24 (a) and (b)). Interestingly, its growth at 3.0M and 4.0M NaCl was similar to the growth of halo-tolerant (above mentioned) isolates.

Another significant difference, its growth at 0.5M NaCl was the same as its growth at 4M (Figure 2.24 (b)). Like the DSRT3 isolate, HSO cells grown in all of the NaCl stress conditions showed a longer doubling time when grown under trained conditions than when shocked and had a longer lag phase when grown at 3.0 and 4.0M NaCl; the lag phase being shorter in shocked conditions than in trained. Furthermore, as shown in Figure 2.25, changes in morphology of the HSO isolate were observed when cells were grown in NaCl concentrations of 3.0M. In addition, orange pigmentation made this strain (Figure 2.26) different from all other mentioned isolates (all other isolates formed creamy to white colonies).


(a)
2.0
1.5
1.0
0.5
0.0
-0.5
OD660
0
2
4
6
8
10
12
14
Time (hour)

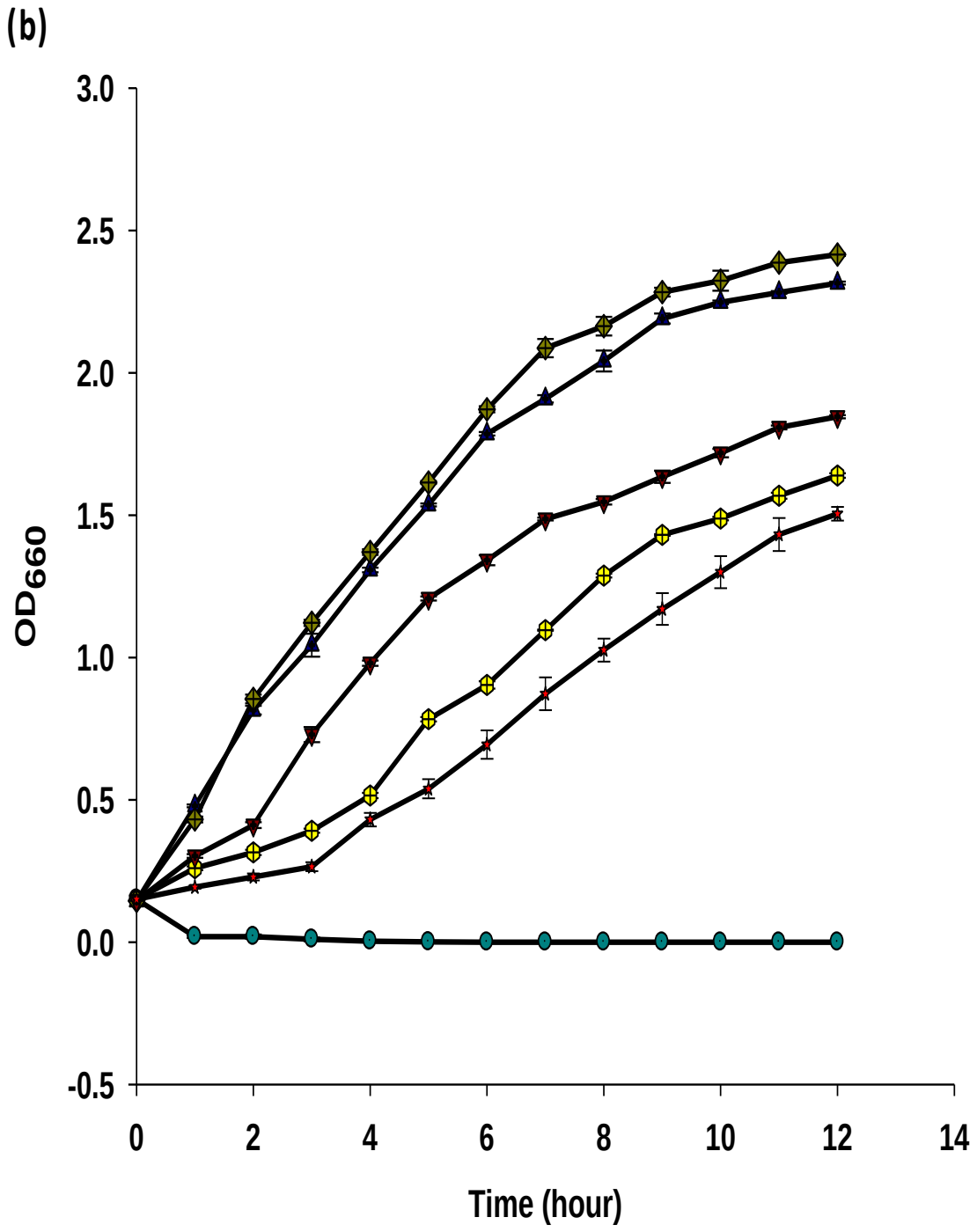


Figure 2.24 Growth curves of HSO isolate grown under different NaCl (M) conditions; 0M (), 0.5M () 1.0 M (), 2.0M (), 3.0M (), 4.0M () taken at optical density of 660 nm. (a) When cells undergoing osmotic shock at various NaCl concentrations. (b) When cells trained up to 4M NaCl and then inoculated at various concentrations. (I) refers to Standard Deviation (SD).

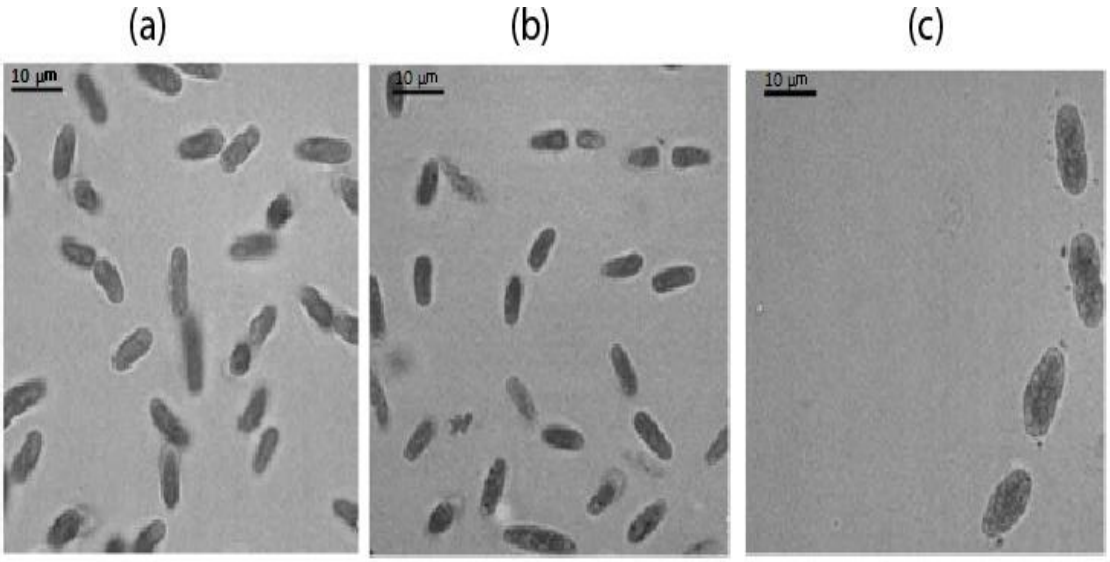


Figure 2.25 Light microscopy of the halophilic HSO isolate grown at (a); 0.5M NaCl, (b); 1.0M NaCl, (c): 3.0M NaCl.

Figure 2.26 An agar plate of a halophilic growth of HSO strain isolated from hypersaline soil. Specifically, it formed orange colonies in M9 minimal medium.

2.4 Conclusion

This work described in this Chapter was aimed at investigating the occurrence of halophilic bacteria and archaea in non-extreme environments. Most of the isolate in this study have previously been isolated and identified cultured microorganisms or showed close matches to previously uncultured microorganisms.

However, culture-independent techniques alone are insufficient for the study of environmental microbial occurrences as the sequences obtained from 16S rRNA gene libraries of natural microbial communities frequently contain many novel sequences, which often do not match with the sequences of cultivated isolates from the same samples (Suzuki *et al.,* 1997; Felske *et al.,* 1999). Moreover, cultivation of microorganisms is required in order to understand the interactions between microbes in their environment and to provide access to genes encoding metabolic pathways (Keller and Zengler, 2004) which may be dispersed throughout the genome, i.e to gain a comprehensive understanding of the microbial physiologies. Most microbial communities in the environment are not isolated using traditional cultivation methods, which required complex media as the specific requirements of growth, and as a result many uncultured microorganisms are unknown (Leadbetter, 2003; Ferrari *et al*., 2004). It has been reported by Green and Keller (2006) that no single method or medium is suitable for the cultivation of the majority of microorganisms from environmental samples as they prefer stable, nutrient-poor environments. In another study, Ferrari *et al*. (2005) suggested that it is essential to understand the physical and chemical characteristics of each particular environment being studied in order to isolate any microbial communities and to develop a cultivation method which can simulate the natural habitat. Halophilic microorganisms inhabiting non saline environments have been difficult to cultivate due to their fastidious requirements. This study has revealed that there is a need to improve traditional techniques with new and novel approaches.

Frohlich and Konig, (2000), Reysenbach *et al*., (2000), Kaeberlein *et al*., (2002) and Svenning *et al*., (2003) have developed several cultivation techniques in recent years to mimic the natural habitat which have resulted in the successful cultivation of microorganisms from diverse phyla.

The development of a culturing medium based upon, for example, the use of sediment-extract provided the essential nutrients and trace elements required for successful microbial growth. This may aid, in future studies, the isolation of additional novel halophilic strains. Additionally, techniques such as extinction dilution extinction, or novel approaches can be used, such as the use of membranes or chambers to isolate microorganisms from unexpected communities (Janssen *et al*., 2002; Kaeberlein *et al*., 2002); these approaches would likely improve the number of isolates from unexpected environments.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction to extremophiles and their environment

1.1.1 Terminology and definition

1.1.1.1 Extreme environments

The study of life in extreme environments is one of the most exciting fields of current research, studies which have led to the development of the new science of astrobiology, i.e. the search for life on other planets. The life of microorganisms is largely dictated by their environment and thereby relies upon specific environmental factors such as pH and temperature. Environments which are within the temperature range 20-35°C and pH 5-7 are regarded as being non-extreme. In fact the concept of "extreme environment" is a human construct since it relates to conditions which humans find optimal. In contrast, an anaerobe for example would find our aerobic environment and life-style to be extreme. Natural environments which are considered to be extreme include for example, alkaline, acidic and saline lakes, hot springs, deserts and the deep ocean. Other extremes are provided by high radiation, high salinity, low or high pH, low or high temperature, very low water activity, high metal concentrations, very low nutrient content, high pressure or low oxygen tension (Albers *et a1*.,2001; Gomes and Steiner, 2004; Redecke *et a1*.,2007). The range of these environments include those with high salinity containing 2-5M NaCl, low temperatures between -2 to 10°C, high temperatures between 55 to 12l°C and high acidity having pH values lower than 4 or high alkalinity environments that have pH values above 9 (Hough and Danson, 1999; van den Burg, 2003; Gomes and Steiner, 2004). Environments with high pressure having hydrostatic pressures up to 1400 atmospheres are also considered to be

extreme. Extreme environments also often result from human activity, e.g. acid mine waters (Satyanarayana *et al*., 2005).

1.1.1.2 Extremophilic microorganisms

Extreme environments are occupied by many groups of microorganisms which are exclusively adapted to survive and grow under extreme conditions such as those referred to above (Horikoshi, l99la). Extreme microorganisms can grow and reproduce optimally when one or several stress conditions are in the extreme range (Edwards 1990; Albers *et al*., 2001). Microbiologists use the term extremophiles to describe organisms which live in extreme conditions in which other forms of life cannot withstand, while Wainwright (Wainwright, 2003) suggested the term "extremodure" to describe microorganisms which can survive, but not grow, in extreme environments. Most extremophiles are found among the domain archaea (Woese *et al*., 1990; Albers *et al*., 2001), however, some bacteria and also eukaryotes can tolerate extreme conditions (Figure 1.1) (Albers *et a1*., 2001; Konings *et al*.,2002).

Life in extreme environments has been intensively studied, mainly in relation to the diversity of organisms and the regulatory and molecular mechanisms which allow them to grow in such environments. Extremophilic microorganisms are structurally adapted at the molecular level to withstand such extreme conditions (Gomes and Steiner, 2004); moreover, some of these microorganisms are polyextremophiles, i.e. they can withstand more than one type of extreme environment (multiple extremes). For example, acidothermophiles can grow at pH3 and 80^{o}C (e.g. *Sulfulobus sofataricus* and *Sulfolobus acidocaldarius*) (Irwin, 2010); thermophilic alkalitolerant bacteria such as *Anaerobranca* sp. in contrast grow at high temperatures under extremely alkaline conditions (Engle *et al*., 1995).

Depending on the environments in which they live, extremophilic microorganisms are classified as being: acidophiles, alkaliphiles, halophiles, psychrophiles,

thermophiles, radioresistant, osmophiles and oligotrophs (Edwards, 1990; Gilmour, 1990; Jennings, 1990; Horikoshi, l991a; Ulukanli and Digrak, 2002). It is noteworthy that many extremophiles are among the most primitive of bacteria, as suggested by their position near the bottom of the universal phylogenetic tree (Figure 1.1) (Madigan *et al*., 1997; Hough and Danson, 1999; Oren, 2008).

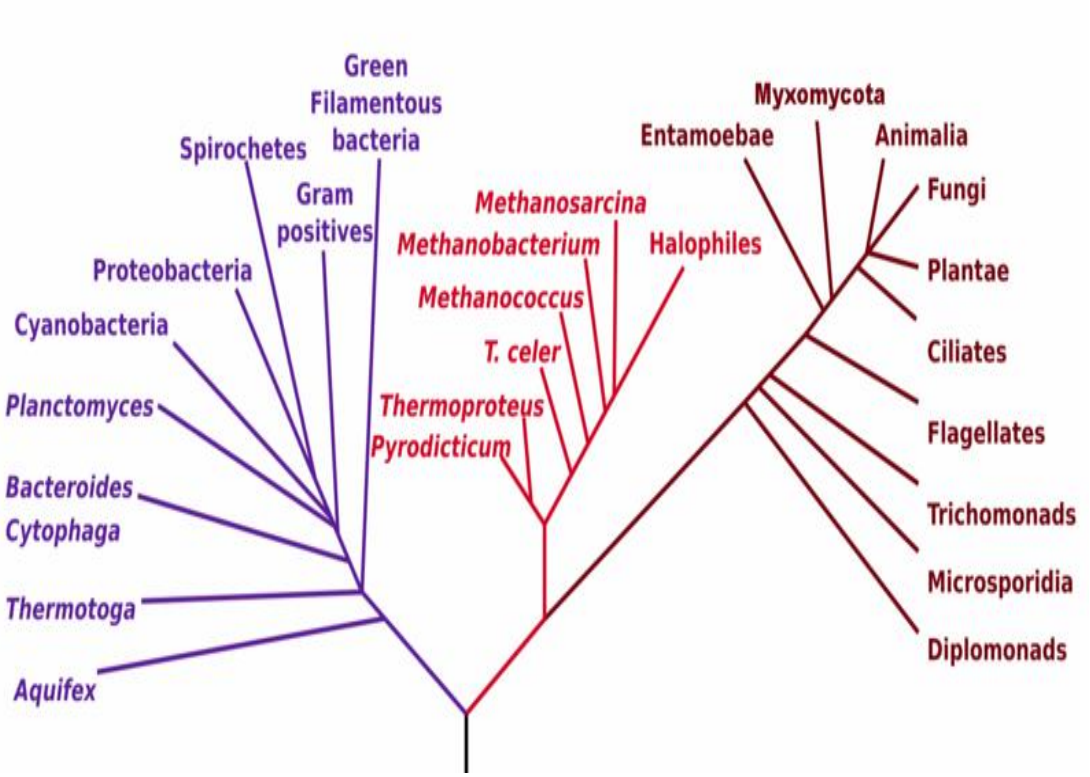


Figure 1.1 The Universal phylogenetic tree of life based on comparative sequence data from 16S or 18S rRNA. The colours highlight the three domains; Bacteria are shown in purple, Archaea in red and Eukarya in dark brown. (The tree is based on Figure 11.16 in Madigan and Martinko, 2006 and Oren 2008).

Table 1.1 shows the types of extreme environments recognized and the representative organisms which grow under the relevant extreme conditions (Madigan *et al.*, 1997).

Table 1.1 Characteristics of different groups of extremophiles and their environments. (Modified from, Hough and Danson, (1999) and van den Burg, (2003))

Environmental Factor		Category	Definition	Major Microbial Habitats	Living organisms
Temperature		hyperthermophile thermophile mesophile psycrophile	growth >80°C 60-80°C 15-60°C <15°C	hot springs and vents, sub-surface ice, deep ocean, arctic	*Pyrolobus fumarii*, *Synechococcus lividis* *Homo sapiens* *Psychrobacter*, some insects
Salinity		Halophile	salt-loving (2-5M NaCl)	salt lakes, solar salterns, brine	*Halobacteriacee, Dunaliella salina*
Pressure		Piezophile	pressure-loving (<1000 atm.)	deep-sea (e.g. Mariana Trench), sub-surface	
pH	Low	Acidophile	pH < 2	acidic hot springs, sulfide mines	*Cyanidium caldarium, Ferroplasma sp.* (both pH 0)
	High	Alkaliphile	pH > 9	soda lakes/ deserts	*Natronobacterium, Bacillus firmus*OF4, *Spirulina spp.* (all pH 10.5)
Radiation		Radioresistant		soil, contaminated areas	*Deinococcus radiodurans*
Toxic heavy metals		Metalophiles	tolerate high concentrations metal	contaminated areas	*Ferroplasma acidarmanus*(Cu, As, Cd, Zn); *Ralstonia* sp. CH34 (Zn, Co, Cd, Hg, Pb)

Extremophiles are in the main microorganisms which can tolerate a much wider range of environmental extremes than other life forms. Microbes can grow at the following extremes: a) from (-12°C) up to more than (+100°C); salt concentrations of

saturated brine, hydrogen ion concentration from acid (pH = 0) to alkaline (pH= 12) and hydrostatic pressures equipped to 131,722,500 Pascal (Prescott *et al*., 2002).

For some time now, microbiologists have recognized that extremophiles can be isolated from non-extreme environments, although this ability has not been generally emphasized.

Until recently, novel extremophile-archaea has not yet been grown in culture. Halophilic archaea (haloarchaea) are often the dominant heterotrophic organisms in areas such as salt lakes, salterns and salt deposits. All such isolates which have been reported so far are obligate extreme halophiles involving minimum of 10% (w/v) salt concentrations for growth (Oren, 2000), and most growing optimally at 20%-25% (w/v) NaCl. However, the existence of haloarchaea capable of growth at lower salt levels has been suggested due to diverse 16S rRNA gene sequences being found in low salt environments (Munson *et al*., 1997)

1.1.2 Phylogeny, taxonomy, and physiology of extremophiles

Microorganisms have been conventionally classified based on their physiology and morphology, but it has generally not been possible to establish the evolutionary characteristics and relationships between different microbial groups. The establishment of natural phylogeny into microbial systematics according to the sequence data of nucleic acid has enabled microorganisms to be classified in evolutionary terms as well as allowing for the determination of phylogenetic lineages between them (Pace, 1997). As a result, distinctive phylogenetic groups could be determined and identified at each taxonomic level (such as: species, genus, family, etc.) using comparative analysis of molecule using DNA sequence databases. A major scientific breakthrough in the late 1970's came when Carl Woese recognized, using molecular phylogenetics, the Archaea, a third domain of life in addition to Bacteria and Eukarya (Woese and Fox ,1977; Woese *et. al*., 1990) (Figure 1.1). The prokaryotic Bacteria and Archaea

domains show common phenotypic characteristics but also exhibit a number of specific features which are listed in Table 1.2.

Table 1.2 Comparison of Bacteria and Archaea (modified from Kristjansson and Stetter, 1992)

Character	Bacteria	Archaea
Cell wall components	Murein	Pseudomurein proteins, polysaccharides
Membrane lipids	Glycerol fatty acid esters	Glycerol isopranyl ethers
Square and flat structures	-	+
Endospores	+	-
tRNA -common arm- contains	Ribothymidine	Pseudo-uridine or 1-methylpseudo-uridine
Methyonyl initiator tRNA formylated	+	-
Introns in genes	-	+
Eukaryotic RNA polymerase	-	+
Special coenzymes	-	+
Max growth temperature	95°C	121°C
Complete photosynthesis	+	-
Methanogenesis	-	+
Calvin cycle used in $C0_2$ fixation	+	-

The phylogeny of extremophilic microorganisms has been linked to questions regarding the early evolution and origin of life on Earth. Many thermophiles, hyperthermophiles and halophiles appear to have very deep lineages (Figure 1.1), a fact which is particularly evident among the archaeal halophiles and thermophiles (Madigan *et. al*,. 1997)

The currently accepted strains of thermophilic bacteria which grow at ≥ 60°C are distributed across some 52 genera, whereas the thermophilic archaea cover 33 genera. Physiologically, the anaerobic and aerobic archaea are differentiated by their optimal

pH, the aerobic being extremely acidophilic, while both anaerobic and aerobic Bacteria are generally neutrophilic or slightly acidophilic (Figure 1.2).

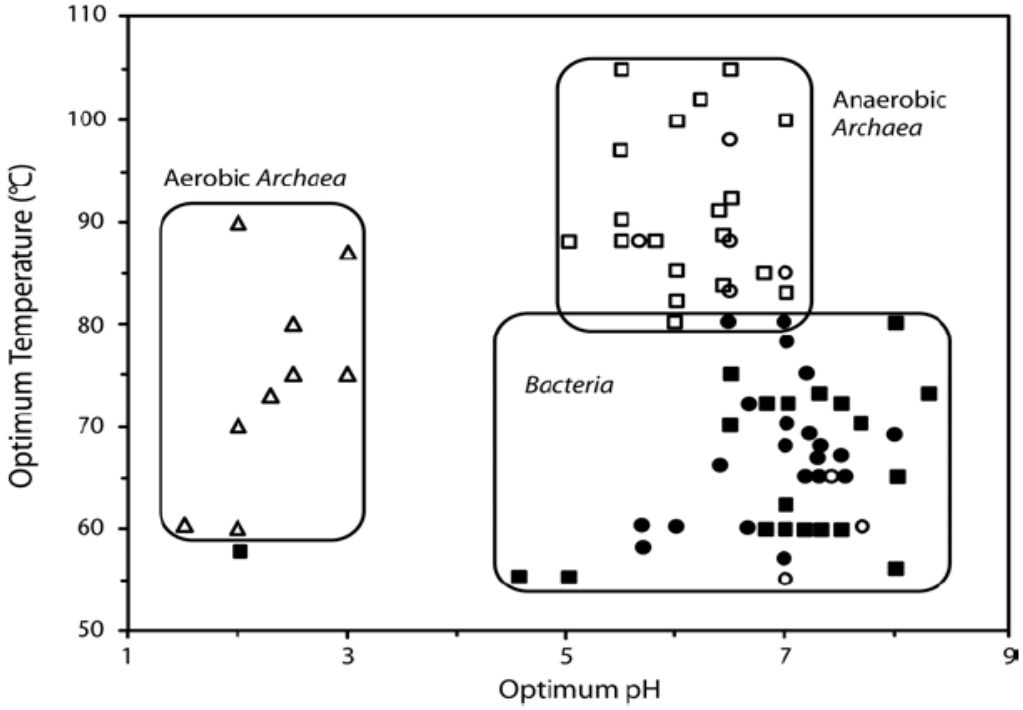


Figure 1.2 The distribution profile of thermophiles based on their optimum pH and temperature. Bacteria are marked with closed symbols and Archaea with open signs. Legend ●: anaerobic Bacteria, ■: aerobic Bacteria, □: anaerobic Archaea, Δ: aerobic Archaea and ○: methanogens (Taken from Kristjansson and Stetter, 1992).

1.1.2.1 A new domain; Archaea

The Archaea (in Greek archaios, ancient or archaic) are a quite diverse group, both in terms of their physiology and morphology. They stain either Gram negative or positive and can be rod-shaped, spherical, spiral, plate-shaped, lobed, irregularly shaped, or pleiomorphic. Some archaea are single cells, while others form aggregates or filaments (Chaban *et al*., 2006). They vary in diameter from 0.1 to 14 μm, and some filaments can grow up to 200 μm in length. They multiply by budding and, binary fission (Prescott *et. al*., 2002) and all known archaea belong to four taxonomic groups (phyla), namely: Crenarchaeota, Korarchaeota, Euryarchaeota and Nanoarchaeota;

three major physiological and/or metabolic groups are reported: extreme halophiles, extreme thermophiles and methanogens (Figure 1.1), (Huber *et al*., 2002; Hohn *et al*., 2002).

The Euryarchaeota (in Greek eurus, means wide, and archaios) include the greatest phenotypic diversity among known culturable strains within the halophiles, some hyperthermophiles and some thermoacidophiles, and the methanogens (Forterre *et al*., 2002). The extreme halophiles or particularly halobacteria contain 14 known genera and to date, no thermophilic halophiles have been isolated. The methanogens are obviously strict anaerobes which obtain energy by converting H_2, CO_2 or simple methyl compounds into CH_4. Five orders compose the Methanobacteriales, the Methanopyrales, the Methanogens, the Methanococcales, the Methanosarcinales and the Methanomicrobiales. The first three orders are totally hyperthermophilic or contain both thermophilic and mesophilic genera, although no thermophilic Methanosarcinales or Methanomicrobiales are known. All of these five orders can reduce CO_2 as the carbon source for the production of CH_4 (Boone *et al*., 2001). The order Thermoplasmatales, which contains two genera, *Picrophilus* and *Thermoplasma* represent the thermoacidophiles. *Thermoplasma* grows on coal mine refuse, under extreme acidic and moderately hot conditions (pH 1-2, 55-65°C). *Thermoplasma* species when growing at 9°C, form irregular filaments, while they are spherical at lower temperatures (Prescott *et al*., 2002). In relation to acidity, *Picrophilus* is the most extreme archaeon and can grow only below pH 3.5, with an optimum at pH of 0.7 and is still able to grow at pH 0 (Boone *et. al*., 2001; Schleper *et al*., 1995).

Halobacterium is an archaeon group that has a high tolerance for high levels of salinity. The genus *Halobacterium* consists of many species of aerobic archaea which grow in environments containing high salt concentration. The cell walls of these species of archaea need to be quite different from those of bacteria, as

regular lipoprotein membranes fail to function in high salt concentrations. They can grow as either cocci or rods, and in colour, either purple or red. The genus *Halobacterium* grows optimally in environment with 42°C (NCBI, 2007).

Of the two subdomains of archaea, the Euryarchaeota, comprise extreme halophiles, sulphur reducers, sulphate reducers, thermophilic heterotrophs, and methanogens while the Crenarchaeota are thermophiles. Recent molecular phylogenetic studies have shown that archaea can be more widespread and diverse than is represented by the currently cultured members of the Domain. Two new phyla, Nanoarchaeota and Koryarchaeota have recently been identified (Bintrim *et al*., 1997; Brochier, *et al*., 2005; Auchtung *et al*., 2006).

1.1.3 Extremophilic microorganisms and their habitats

Extreme environments are related to geochemical extremes such as salinity and pH as well as to physical extremes such as temperature, pressure and radiation. Extremophiles are able to grow in non-conventional environments under non-standard and unusual conditions due to their structural and functional properties and mechanisms including their adapted organelles and enzymes, these being optimized for growth under such extreme conditions (Herdy, 2006).

1.1.3.1 Extremes of temperature

1.1.3.1.1 Thermophiles

Examination of extreme environments has led to a range of microorganisms being isolated which are capable of growing and surviving a variety of extreme environmental conditions. Temperature is an important factor which determines where bacteria and archaea grow. Microorganisms can be classified on the basis of temperature into: thermophiles, mesophiles and psychrophiles; additionally, thermophiles can be classified into obligate or facultative. Obligate thermophiles require high temperatures to live, while facultative thermophiles can grow at low

(below 50°C) or high temperatures; hyperthermophiles in contrast, are found only in environments higher than 80°C. Thermophilic bacteria live at temperatures between 55°C to 65°C such as in hot springs (Brock *et al*., 1972; Kumar *et al*., 2004), hydrothermal vents (Slobodkin *et al*., 2001; Sako *et al*., 2003), solfataric fields (Goorissen *et al*., 2003), hot water heaters (Brock and Boylen, 1973) and compost heaps (Strom, 1985; Bae *et al*., 2005, Kim *et al*., 2001). Research on thermophilic microorganisms has recently been highlighted by an increase in interest in thermophilic archaea, which were originally thought to be only found at temperatures of 100°C and above. Most archaea have been isolated from extreme environments or specialized ecological niches including hot springs (Barns *et al*., 1994; González *et al*.,1999) and hydrothermal vents (Canganella *et al*., 1998; González *et. al*., 1998; Takai *et al*., 2001;). However, distant relatives of this group have recently also been found in marine and lake sediments (DeLong, 1992; Schleper *et al*., 1997), terrestrial environments (Jurgens *et al*., 1997; Mcmullan *et al*., 2004), oil reservoirs (Miroshnichenko *et al*., 2001), temperate ocean water (Vetriani *et al*., 1999) and polar seas (DeLong *et al*., 1994). They have been reported to remain alive for long periods at low temperatures where they may grow slowly or remain inactive (Rahman *et al*., 2004).

A diversity of high temperature habits, both Man-made and natural, exist, although high temperature habitats are generally not as prevalent as cold habitats. Such hot environments include geothermal and volcanic regions where temperatures are frequently higher than boiling, sun-heated sediments, soil and composts which can reach 70°C. In hot springs, the temperature is higher than 60°C, and remains regular due to frequent volcanic and geothermal activity (Kristjansson and Hreggvidsson, 1995). In addition to temperature, other parameters related to the environment, such as available energy and nutrient sources, ionic force and pH affect the ability of thermophiles to grow. Geothermal environments are typified by low or high pH. The

best known and studied geothermal regions are in Russia, Italy, New Zealand, Yellowstone National Park in North America, Iceland, and Japan (Shi *et al*., 1997).

Neutral/akaline hot water springs are situated outside of volcanic active zones. Other types of geothermal regions are characterized by areas with acidic soils containing sulphur, acidic hot water springs and hot mires. These areas are situated within the zone of volcanic activities, which are normally called "high temperature areas or fields" (Marteinsson *et al*., 1999, Stetter, 1988).

Several species of both bacteria and archaea have been classified, on the basis of their optimum temperature requirements, as thermophiles including *Thermophilus*, *Thermus aquaticus, Thermodesulfobacterium commune*, *Thermomicrobium roseum, Sulfulobus acidocaldarius, Methanococcus vulcanicus. Dictyoglomus thermophilum*, *Thermotoga maritima* and *Sulfurococcus mirabilis* and as hyperthermophilic microorganisms include, *Acidianus infernos*, *Methanococcus jannaschii*, *Archaeoglobus profundus*, *Pyrobaculum islandicum, Methanopyrus kandleri*, *Pyrococcus furiosus*, *Pyrolobus fumarii, Pyrodictium occultum*, *Thermococcus littoralis*, *Nannoarchaeum equitans* and *Ignicoccus islandicum* (Ghosh *et al*., 2003; Satyanarayana *et al*., 2005).

A few thermophilic fungi belonging to the Zygomycetes (e.g. *Rhizomucor pusillus*, *R. miehei*) and Hyphomycetes (e.g. *Acremonium alabamensis*) and Ascomycetes (e.g. *Chaetomium thermophile*) have been isolated from soils, composts, nesting materials of birds, wood chips and many other sources. Furthermore, some algae such as *Cyanidium caldarium* and protozoa including *Oxytricha falla*, *Cothuria* sp., *Naegleria fowleri* and *Cyclidium citrullus* have been reported to grow at high temperatures (Satyanarayana *et al*., 2005).

1.1.3.1.2 Psychrophiles

Psychrophiles (cold loving) are extremophiles which can grow and reproduce in temperatures, ranging from -15°C to +10°C (Morita, 1975, Irwin, 2010; Zecchinon *et a1*., 2001). Cold environments exist in many soils and water bodies (fresh, marine, polar and high), and glaciers. Freezers and refrigerators are the major Man-made cold environments. Of the total earth's surface, approximately 70 to 72% is made up of oceans and almost 90% of this water is at a temperature of 4°C and below. At heights above 3000 m, the atmospheric temperature is permanently below 4°C. It has been recorded that the temperature decreases with height increases to reach -40°C in the stratosphere (Ravenschlag *et al*., 2001). Mountains where ice or snow remain laying all year around are characterized by low temperatures. Low temperature environments therefore largely control the biosphere. Such cold environments can be divided into two categories: one is psychrotrophic, which is seasonally cold and alternates with mesophilic temperatures and the other is psychrophilic, i.e. environments which are cold all the time (Morita, 2000).

The majority of psychrophilic microorganisms are bacteria or archaea, although recent studies have found novel groups of psychrophilic fungi living in oxygen deficient areas under alpine snowfields (Chaturvedi *et al*., 2010); snow algae from these areas represent a novel group of eukaryotic cold- adapted microorganisms.

Generally, psychrophiles possess lipid cell membranes which are chemically resistant to the stiffening resulting from extremely low temperature; they often synthesize antifreeze protein in order to protect their DNA, even at temperatures below the freezing point of water. In addition, psychrophiles have to generate cold-adapted enzymes showing high catalytic activity in extreme cold; a result, psychrophilic microorganisms have been known as sources of enzymes with potential for use in extreme cold catalysis (Gerday *et al*., 2000).

A distinctive feature of psychrophilic extremozymes is the correlation of high catalytic activity and low thermal stability at moderate temperatures (Bentahir *et al*., 2000; D'Amico *et al*., 2003). Van den Burg (2003) found that many psychrophilic extremozymes are potentially useful for industrial applications, examples including commercially available proteases, lipases or amylases.

Many microbial genera have been reported to be psychrophilic including: *Psychrobacter* sp. *Arthrobacter* sp., and members of the genera *Pseudomonas, Bacillus, Halomonas, Methanogenium* , *Micrococcus, Sphingomonas, Hyphomonas, Polaribacter, Psychroserpens, Polaromonas* and *Vibrio* (Satyanarayana *et al*., 2005).

1.1.3.2 Extremes of salinity

Halophiles are extremophilic microoorganisms which can grow and reproduce in environments containing very high concentrations of salt; they include species of Bacteria, Archaea and some Eukaryota e.g. the alga *Dunaliella salina*. Some well-known species contain bacteriorhodopsin, a purple to red coloured pigment derived from carotenoid compounds. Halophilic microorganisms can be been divided into three groups by the extent of their halotolerance: i.e. slight, moderate or extreme. These microorganisms can be found anywhere having a salt concentration five times greater than the salt concentration of the sea and oceans. Such areas include: the Dead Sea, Owens Lake, California and the Great Salt Lake in Utah (Margesin and Schinner, 200l; Madem and Zaccai, 2004).

Although seas and oceans are the main saline source of water, hypersaline environments are classified as any water body containing salt concentrations higher than seawater (the percentage of dissolved salt in seawater being around 3 to 3.5% (w/v)). Many hypersaline areas are formed via a process called thalassic; seawater evaporation, producing a salt concentration of around 2.5 to 3M NaCl salt

concentration (Grant, 1991). The Dead Sea (thalassic) and Great Salt Lake (thalassic) are the best known and most studied hypersaline lakes. The former is acidic to some extent, while the Great Salt Lake is alkaline (Sleator and Hill, 2002).

Halophilic microorganisms adapt to saline environments in two ways. The most common mechanism to maintain an osmotic balance of cytoplasm with the external environment is to accumulate high concentrations of various compatible solutes e.g. betaine and ectoine. Halophiles or halotolerant microorganisms that can accumulate these organic solutes have low intracellular concentrations of salt and therefore their intracellular enzymes have no need for additional mechanisms of salt tolerance (Margesin and Schinner, 2001). The accumulation of high concentrations of potassium chloride is another means of maintaining an osmotic balance between the cytoplasm in halophilic microorganisms and the external hypersaline environment.

Halophilic microorganisms will be discussed in detail in section 1.2 of this Chapter.

1.1.3.3 Extremes of pH

1.1.3.3.1 Acidophiles

Acidophiles are organisms that able to live and reproduce under highly acidic conditions usually with a pH value of 4 or below. These microorganisms are defined as extreme acidophiles if their optimal growth at pH 2 or below and moderate acidophiles if their optimal growth at pH 3-5. Acidophlies have been reported in different branches of the tree of life, including Bacteria, Archaea, and Eukaryotes (Johnson and Hallberg, 2003; Baker-Austin and Dopson, 2007).

Acidophiles have been isolated from acid mine drainage and geothermal vents (Futterer *et al*., 2004; Johnson and Hallberg, 2003) and microbes such as archaea (*Picrophilus oshimae* and *P. torridus*) have been found optimally grow at pH 0.7 and at 60°C (Gomes and Steiner, 2004). Most acidophiles develop efficient mechanisms to pump out intracellular protons in order to keep their cytoplasm at or near neutral pH.

Therefore, the intracellular proteins of these microorganisms do not need to develop mechanism for maintaining acid stability. On the other hand, other acidophilic microorganisms, such as *Acetobacter aceti*, have an acidified cytoplasm which pushes out almost all genomic proteins in order to achieve stability (Menzel and Gottschalk, 1985).

1.1.3.3.2 Alkaliphiles

Alkaliphiles are microorganisms which are classified as extremophiles which can live and reproduce in alkaline environments with a pH value above 9, including carbonate-rich soils and playa lakes. Alkaliphiles maintain a relatively low level of alkalinity within the cells of about pH 8 by constantly pumping hydrogen ions (H^+), across their cell membranes into their cytoplasm, in the form of hydronium ions (H_3O^+) (Horikoshi, 1999; Kitada *et al*., 1997; Ma *et al*, 2004a). Many bacterial species have been reported as alkaliphilic microorganisms, including *Geoalkalibacter ferrihydriticus, Alkalibacterium iburiense and Bacillus okhensis.*

The best known and most studied alkaline environments are mainly found in both soda deserts (e.g. East African Rift valley) and soda lakes (e.g. Sambhar Lake in India). Both of these are typified by an abundance of Na_2CO_3, depletion of calcium and magnesium cations, due to carbonate precipitation and a salinity range of 5% to 30% (w/v) (Rees *et al*., 2004).

Alkaliphilic microorganisms will be discussed in detail in section 1.3 of this Chapter.

1.1.3.4 Extremes of radiation

Microorganisms that can live in environments with very high levels of radiation, including ionizing and ultraviolet (UV) radiation, are known as radioresistance or UV resistant microorganisms, respectively. Many cellular mechanisms of radioprotection

may be involved, in this resistance, such as increased gene expression, DNA repair and alterations in the levels of cytoplasmic and nuclear proteins.

One of the best known radioresistant organisms is *Deinococcus radiodurans* which can survive acids, dehydration and exposure to cold and vacuum, and is therefore known as a polyextremophile; it has been listed in *The Guinness Book of World Records* as the world's toughest bacterium (DeWeerdt, 2002).

UV resistant microorganisms will be discusses in more detail below (section 1.4).

1.1.3.5 Extremes of nutrient availability

An oligotroph is defined as an organism that can survive and grow in an environment providing very low levels of nutrients; oligotrophs are characterized by fast growth but with low metabolism rates and generally low population density (Prescu *et. al*., 2005).

Oceans, seas and desert soils are typically characterized as being depleted in nutrients i.e. they are oligotrophic. Surface seawater contains very small amounts of organic substance with dissolved organic carbon (DOC) ranges from 0.1 to 1.0 mg carbon 1^{-1} but, deep sea water contains much less than surface sea water. Most soils are regarded as being oligotrophic and desert soils, because they lack vegetation are particularly low in available nutrients (Satyanarayana *et al*., 2005).

Pelagibacter ubique is an example of an oligotrophic organism. It is the most abundant organism in the oceans with a total estimated population of $1x10^{27}$ individual cells (Prescu *et. al*., 2005).

1.1.3.6 Extremes of pressure

Barophiles (also called piezophiles**)** are extremophilic microorganisms that can survive and grow in environments exposed to high pressure. These microorganisms are either barotolerant or obligate barophiles which cannot survive in a low pressure environment. For example, in order to grow, *Halomonas* species, particularly,

Halomonas salaria require a pressure value of 101,325000 Pascal and a temperature of 3°C (Nogi and Kato, 1999). Most piezophiles can grow in darkness and are also usually sensitive to UV radiation because they lack mechanisms for DNA repair.

Elevated hydrostatic pressure environments are naturally found in the deep sea as well as in sulphur and oil wells. Microorganisms which can survive in this environment are known as barophiles and almost all have been isolated from the deep sea at a depth of from 1500 to 2000 meters. High pressure environments are also found in soils where other parameters such as high salt concentration, elevated temperature, and limited amount of available nutrient may apply added stress to microorganisms. Some barophiles can withstand temperatures at 100°C or beyond and hydrostatic pressure around or beyond 253,31250 Pascal (Nogi and Kato, 1999).

1.1.3.7 Other extreme conditions

A water activity (a_w) of 0.85 is the limit, under which microorganisms tolerant high osmotic stress and can survive in highly intense syrups. Dehydration is a consequence of low aw values and extreme osmotic pressure. Some microorganisms are very tolerant to desiccation, including members of the bacterial genus, *Deinococcaceae*. This is thought to be largely due to the thickness of their cell walls, which aid in the protection of membrane integrity. Microbial exposure to organic solvents is another, often overlooked environmental stress (Rainey *et al.*, 1997).

1.1.4 Extremophilic microorganisms in non- extreme environments

It has been observed that some extremophiles are able to live in non-extreme environments (Marchant *et al*. 2002). Thermophiles for example, have been isolated from non-thermophilic as well as thermophilic environments (Sneath, 1986). Similarly, alkaliphiles have been isolated from acidic soil (with a pH of 4.0) (Horikoshi, 1999). Obligate anaerobic bacteria like methanogens, homoacetogens, and sulfate-reducers have also been routinely isolated from arable and desert soils; species isolated include

Moorella thermoautotrophica (formally *Clostridium thermoautotrophicus*) and *Geobacillus stearothermophilus* (formally *Bacillus stearothermophilus*) (Peters and Conrad, 1995).

Therefore, it is believed that microorganism culturing and isolation of microoganisms from a certain environment does not necessarily mean that the microorganism is able to grow and reproduce but able just to survive in that environment (Echigo *et al*. 2005)**.**

1.2 Introduction to halophiles and halotolerant microorganisms

Microorganisms which can grow, reproduce and survive in hypersaline conditions are known as halophiles, i.e. salt-loving organisms. These are mostly eukaryotic and prokaryotic microorganisms, which have the ability to manage the equilibrium of environmental osmotic pressure and withstand the denaturing reactions of salts (Le Borgne *et al*., 2008). The most studied examples of extreme halophilic organisms include archaeal *Halobacterium* sp., *Dunaliella salina* of the green algae, and *Aphanothece halophytica* of cyanobacteria genera.

The classification of halophilic microorganisms is based on NaCl requirement into three major groups; slight (optimum growth at (1–5% (w/v) or from 0.2 to 1 M NaCl), moderate (optimum growth at (5–20% (w/v) or 1 to 3 M NaCl) and extreme (optimum growth at (20–30% (w/v) or from 3 to 5 M NaCl) halophiles. In contrast, non-halophilic microorganisms can only grow at the optimum level when the concentration of NaCl is less than 0.2 M (Saiz-Jimenez and Laiz 2000). Microorganisms adapted to growth in saturated levels of salt are mainly extremely halophilc archaea. Interestingly, these microorganisms have novel molecular features, novel enzymes which can function in saturated level of salts, gas vesicles that support the floating characteristic of the cell and sensory rhodopsins that promote phototactic reactions. Their novel

characteristics and capacity for large-scale culturing make halophiles potentially valuable for biotechnology (Kirkwood *et al*. 2008).

1.2.1 Diversity of halophiles in extreme environments

Most of the extremophilic microorganisms, that have been reported so far, belong to the domain of the Archaea. However, many other extremophiles from the kingdoms of eubacteria and eukaryote have also been recently reported and identified (Hendry, 2006).

In hypersaline environments, high osmolarity can be harmful to microbial cells due to the fact that water is lost to the outside medium until osmotic equilibrium is achieved. Generally, halophiles accumulate high concentrations of solute in the cytoplasm in order to avoid cellular water loss under these conditions (Galinski, 1993). Microbial cell volume is sustained when the osmotic equilibrium with the medium is accomplished. The osmolytes or compatible solutes, which accumulate in halophilic microorganisms, are generally sugars polyols, amino acids and amino acids derivatives, such as ectoine, betaine, trehalose, sucrose, glycerol and glycine. Some extreme halophilic microorganisms, especially the genera of halobacteria, accumulate potassium chloride equivalent to the external sodium chloride concentration. Halophilic fungi accumulate polyols, whereas many halophilic bacteria accumulate ectoine, glycine and betaine. Compatible solutes may be accumulated from the surrounding medium or following biosynthesis.

The diversity of halophilic bacteria and archaea has been reviewed by Grant (1998). In hypersaline microbial mats, phototrophic bacteria occur anaerobically below the illuminated zones and cyanobacterial layers. *Thiocapsa halophila* produces the compatible solutes known as *N*-acetylglutaminylglutamine amide and glycine betaine, both used for osmotic protection. The moderate halophile *Chlorobium limnicola*

accumulates the compatible solute glycine betaine from the surrounding medium and also produces trehalose in order to make use of it as an osmolyte (Grant *et al*., 1998).

Species of genera *Pseudomonas, Vibrio, Acinetobacter, Flavobacterium, Alteromonas, Marinomonas* and *Deleya*, which are Gram-negative aerobic organotrophic bacteria, are commonly found in hypersaline environments. In solar saltern conditions, Gram-negative bacteria are more common than aerobic heterotrophs, although similar species of *Bacillus, Salinococcus*, *Marinococcus*, and *Sporosarcina* have been reported from hypersaline soils. Globally, neutral hypersaline waters contain halophilic bacteria belonging to genera of archaea such as *Halococcus, Halorubrum, Halobacterium, Haloarcula, Haloferax, Haloterrigena, Halobaculum* and *Halorubrum.* Salted foodstuffs have also been found to contain proteolytic *Halobacterium salinarum* and Raghavan and Furtado (2004) report that a total of around 5.5×10^3 cells of halophilic archaea can be isolated from each gram of Indian Ocean sediments.

The concentration of salt inside the cells of Halophilic archaea is extremely high, for examples, potassium accumulates internally at concentration of 4 to 5 mol l^{-1} while sodium accumulates in lower concentrations. Halophiles contain purple membranes, which have "crystalline lattice of chromo-protein, known as bacteriorhodopsin", which play a main role in light dependent trans-membrane proton force. This membrane potential which is generated is used to maintain a phase of phototrophic growth and generate ATP. Halo-methanogenic archaea such as *Methanohalophilus evestigatum, Methanohalophilus halophilus* and *Methanohalophilus mahii* have been isolated from hypersaline and alkaline saline environments (Koch, 2001; Boone *et al*., 2001). To date, no thermophilic halophiles have been reported.

Green algae of the genus *Dunaliella* such as *Dunaliella. viridis, Dunaliella. parva and Dunaliella. salina*, are found at moderate level of salinity (NaCl at 1 to 3.5 M). This genus mainly uses the polyols glycerol as the compatible solutes (Satyanarayana

et al., 2005). A selection of diatoms species such as *Amphora Navicula, Amphora Nitzschia* and *Amphora coffeaeformis* have also been isolated from environments containing around 2M NaCl. Some diatoms also accumulate oligosaccharides and proline for the purpose of osmolarity regulation, as do some protozoa like *Fabrea salina* and *Porodon utahensis*. The halophilic yeast, *Cladosporium glycolicum* has been found in the Great Salt Lake, while another halophilic yeast, *Debaryomyces hansenii* has been isolated from seawater. Halotolerant fungi such as *Basipetospora halophila* and *Polypaecilum pisce* have been isolated from salted sea foods. Buchalo *et al.* (2000**)** have isolated 26 species of fungi from the Dead Sea and reported that they represent genera of fungi such as *Ulocladium chlamydosporum, Stachybotrys chartarum* and *Acremonium persicinum*, genera of Ascomycotina such as *Eurotium amstelodami Chaetomium aureum, Emericella nidulans, Chaetomium flavigenum, Thielavia terricola and Gymnoascella marismortui,* and genera of Zygomycotina such as *Absidia glauca*.

Cyanobacteria are common in the planktonic biomass of hypersaline ponds and lakes. *Aphanothece halophytica,* which is unicellular, has been shown to grow over a broad range of salt concentrations. The major compatible solute was glycine betaine which is produced from choline or obtained from the environment. The cyanobacterium, *Dactylococcopsis salina* and the filamentous *Oscillatoria salina, Oscillatoria neglecta, Oscillatoria limnetica, Phormidium ambiguum* and *Microcoleus chthonoplastes* have been reported from hypersaline lakes, particularly from the green layer of mats (Cavicchioli and Thomas, 2004).

1.2.2 Occurrences of halophiles in non-extreme environments

Generally, halophiles are microorganisms which are adapted to grow in hypersaline environment and for their optimum growth, obviously, require a certain salt concentration (Oren, 2002). For optimal growth, extremely halophilic microorganisms

require (15–30%) NaCl 3-5 M. An example of such halophile is *Halobacillus salinus*, which has been isolated from a salt lake in Korea and can grow at 4 M of NaCl (Yoon *et al*., 2003).

The majority of isolates which have been reported so far are strict halophiles which require at least of 8% (w/v) NaCl for their growth, and for optimal growth in the range of 15% and 23% (w/v) NaCl (Saiz-Jimenez and Laiz, 2000, Oren, 2000).

Few studies have been reported on the ability of halophiles to grow in non-saline environments. Usami *et al*. (2007) conducted a study to isolate halophiles from non-saline soil in Japan and isolated two strains of halophilic bacteria, designated $BM2^T$ and HN2. The $BM2^T$ cells were found to be Gram-positive, rod-shaped and aerobic. They grew at 5 - 25% (w/v) NaCl, with an optimum at 10-15 %, (w/v) NaCl at 20–50 °C and pH of 7-10. Molecular method of 16S rRNA gene sequencing has been used to analyze their phylogeny and strain $BM2^T$ had 98% sequence similarity to *Alkalibacillus haloalkaliphilus* DSM 5271^T. Another study, conducted by Savage *et al*. (2008), reported the occurrences of halophilic archaean, from a Zodletone spring in south-western Oklahoma, United States, which was characterized by its low-salt and rich-sulphide content. A novel strain $BZ256^T$ of halophilic archaeon was isolated and the cells were shown to be non-flagellated cocci, non-motile and forming *Sarcin*a-like clusters. This strain grew at 1.3 to 4.3 M salt concentrations and optimally at almost 3.5 M NaCl, a minimum of 1 mM Mg^{+2} was necessary for growth. Growth was optimal at 25–50 °C and pH range from 5 to 9; strain $BZ256^T$ was related to *Halogeometricum borinquense*. Based on phenotypic and phylogenetic analyses, strain $BZ256^T$ was characterized as a novel species within the genera of *Halobacteriaceae*, for which the name *Halosarcina pallida* gen. nov., sp. nov. has been proposed**.**

1.2.3 Strategies used for osmo-adaptation in halophilic bacteria and archaea

Microorganisms present in dynamic environments, such as ocean and sea, require rapid cellular adaptation to external changes. For example, offshore sediments are often exposed to desiccation at low tide but are submerged at high tide. This decreases and increases, respectively, the osmotic stress in such environment which already has twice the salinity levels of seawater. Rainfall and evaporation are also a source of drastic changes in the osmolarity in these surroundings and saline ponds and as a result the environment is subjected to rapidly changing osmotic conditions. As a result, the microbial communities that exist in such environments must possess regulatory mechanisms for surviving such extreme and varying osmotic stress.

Two osmoadaptation mechanisms are known in halophilic microorganisms, called a) "salt-in cytoplasm mechanism" and b) accumulation of organic osmolytes.

1.2.3.1 Salt-in cytoplasm mechanisms

The "salt-in cytoplasm mechanism" involves the accumulation of high concentration of intracellular ions, commonly potassium, and is a strategy employed by both halophilic archaea and bacteria to overcome osmotic pressure. These prokaryotes acquire physiologies which are adapted to a hypersaline environment (Galinski and Truper, 1994; Ventosa *et al.*, 1998). Potassium ions are the predominant solutes accumulated in extremely halophilic archaea (Brown, 1990; Vreeland, 1987). These microorganisms keep their cytoplasm largely free of sodium by accumulating potassium and expelling sodium ions. The levels of internal potassium ions in this group of halophilic prokaryotes can be five times more than that of the internal sodium, an ability which allows them to maintain osmotic balance in the environment in which they usually survive (Perez-Fillol and Rodriguez-Valera, 1986). The gradient of potassium occurring in the cells is made by both the potassium ion uniporter and a sodium ion/proton antiporter (DasSarma *et al.*, 1995). *Halobacterium salinarium* is

able to generate an electrochemical proton gradient across the membrane either by aerobic respiration or by the light-driven proton pump. This results in the conversion of light to energy for driving essential processes using a proton gradient and in the generation of a pH difference and a membrane potential which works as a strong driving force to draw out chloride ions as well as driving potassium uptake (Michel and Oesterhelt, 1980a). Furthermore in *H. salinarium*, no membrane potential was measured when the extracellular sodium chloride concentration was reduced (Michel and Oesterhelt, 1980b). The possession of bacteriorhodopsin in addition to the potassium ions accumulation through uniporters, helps halophilic archaea to survive in a hypersaline environment.

Mechanism of adaptation of halophiles to different osmolarities was also investigated in another halophilic archaeon, *Haloferax volcanii*, which was able to grow in a wide range of sodium chloride concentrations, (Daniels *et al.*, 1984; Mojica *et al.*, 1993; Ferrer *et al.*, 1996). This adaptation to low salinity conditions in *Haloferax volcanii* involved wider, more active and more specific responses in protein synthesis than exposure to high salt conditions, mainly via the production of general stress proteins (Mojica *et al.*, 1993; Ferrer *et al.*, 1996). However, the cells experienced slower growth when salinities increased. This growth lag matched with the time required for the cells to accumulate sufficient intracellular potassium through an energy-dependent potassium uptake system, coped with the increases in sodium chloride concentration, until an osmotic balance was achieved (Mojica *et al.*, 1997). When salt concentration decreased, the potassium extrusion was almost immediate and osmotic equilibrium was rapidly achieved. A critical point for the cells is the ability to stand the unexpected change in cell wall turgour pressure during these changes. Osmoregulation through the acquisition of sufficient solutes must then be the main limiting factor for the growth and recovery of the cell after up shifts in external salinity.

For the halophilic archaea studied to date, this osmoregulation is achieved through the accumulation of potassium. However, another way of achieving this osmoregulation is through the accumulation of organic solutes, as will be described in the next section.

1.2.3.2 Accumulation of compatible solutes

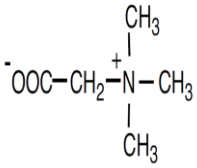
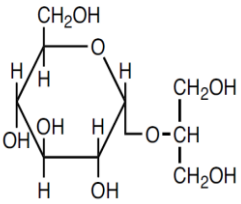
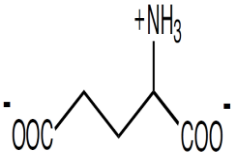
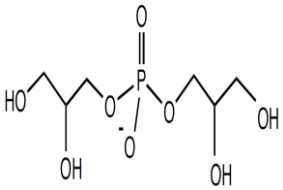
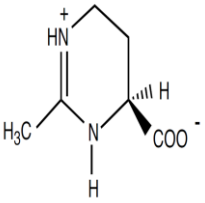
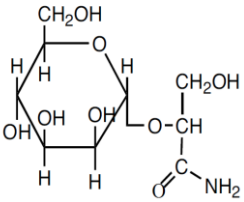
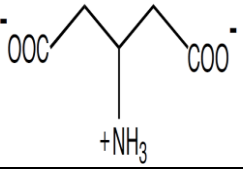
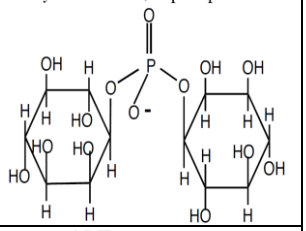
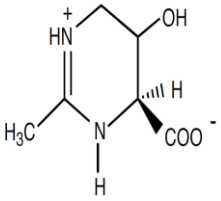
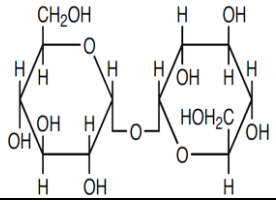
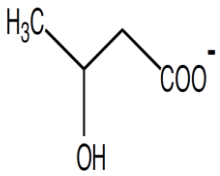
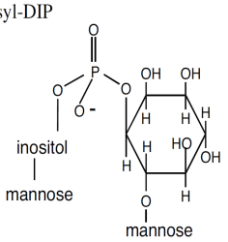
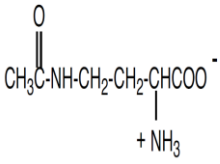
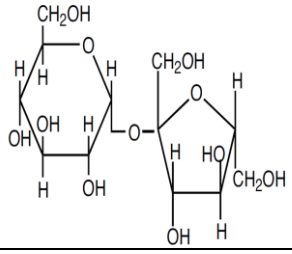
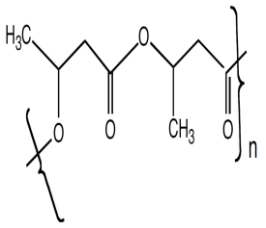
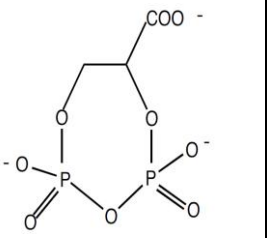
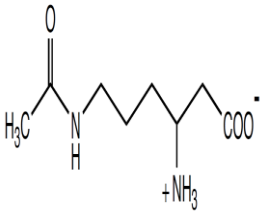
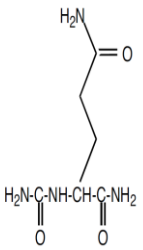
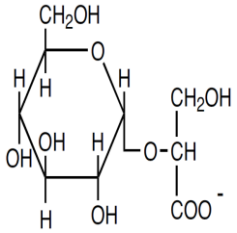
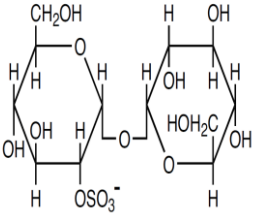
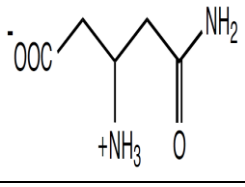
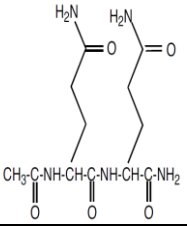
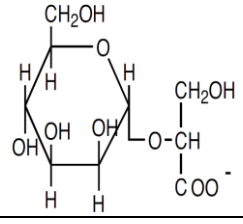
Cells growing in hypersaline environments accumulate organic solutes which do not interfere with their cellular metabolism to revoke or neutralize the drastic external osmotic stress which is encountered (Brown, 1976). These organic solutes are commonly known as compatible solutes or osmolytes; these are usually organic compounds such as zwitterionic solutes, uncharged solutes and anionic solutes (phosphates, sulphates, carboxylates) (Table 1.3). The compatible solutes are accumulated in the cells either by *de novo* synthesis or they can be taken up from the environment by transport systems. Halophilic or halotolerant microorganisms using this mechanism are more flexible in adapting to osmotic pressure than those employing the "salt-in-cytoplasm strategy", in that they are able to grow in a wider range of salinities (Kunte *et al.*, 2002).

1.2.3.2.1 Synthesis of compatible solutes

Halophilic or halotolerant microorganisms which use this strategy usually synthesize nitrogen-containing compounds such as ectoine and glycine betaine as their major compatible solutes (Galinski and Truper, 1994). Sugars including sucrose or trehalose are also common as osmolytes but are typically limited to salt tolerant microorganisms. Other compatible solutes such as free amino acids (e.g. proline and glutamate), polyols (e.g. glycerol and glucosylglycerol) and their derivatives, quaternary amines and their sulphate esters (e.g. choline-O-sulphate), sulfonium analogues (e.g. dimethylsulphoniopropionate and carnitine), and N-acetylated diamino acids and small peptides (e.g. N-acetylglutaminylglutamine amide and Nδ-acetylornithine) have also been identified in halophilic microorganisms (Kempf and

Bremer, 1998). Examples of the structure of some compatible solutes are shown in Table 1.3.

Table 1.3 Examples of some compatible solutes categorized under zwitterionic, uncharged and anionic (carboxylates and phosphates, sulphate) solutes (modified from Roberts, 2005).

Zwitterionic solutes	Uncharged solutes	Anionic solutes (carboxylates)	Anionic solutes (phosphate, sulfate):
betaine 	α-glucosylglycerol 	L-α-glutamate 	α-diglycerol phosphate 
ectoine 	α-mannosylglyceramide 	β-glutamate 	di-*myo*-inositol-1,1'-phosphate 
hydroxyectoine 	trehalose 	hydroxybutyrate 	mannosyl-DIP 
Nγ-acetyldiaminobutyrate 	sucrose 	poly-β-hydroxybutyrate 	cyclic-2,3-diphosphoglycerate 
Nε-acetyl-β-lysine 	N-α-carbamoyl-L-glutamine 1-amide 	α-glucosylglycerate 	sulfotrehalose 
β-glutamine 	N-acetylglutaminylglutamine amide 	α-mannosylglycerate 	

Combinations of different compatible solutes are usually present in cells and these can vary in response to growth medium and growth phase (Galinski and Truper, 1994). The growth of *Escherichia coli*, a well-studied organism for osmoadaptation, provides an example of such a phenomenon. Unlike halophilic archaea which mainly accumulate potassium ions, in *Escherichia coli* potassium ions are initially taken up from the environment and glutamate synthesis then occurs in response to high osmotic stress (McLaggan *et al.*, 1994). This is followed by the accumulation of another compatible solute such as glycine betaine and proline (Dinnbier *et al.*, 1988). It is also recognized that *E. coli* can synthesize trehalose, although this is not used as an osmoprotectant when proline and glycine betaine are present in the medium (Cayley *et al.*, 1992).

Glycine betaine can be synthesized by a few bacteria by the direct methylation of glycine (Galinski and Truper, 1994). Glycine betaine, in *E. coli*, is synthesized by a two-step oxidation pathway using glycine betaine aldehyde as the intermediate and choline as the precursor (Lamark *et al.*, 1991). A three step series of methylations from glycine to sarcosine has been shown in the halotolerant cyanobacteria *Aphanothece halophytica* (Waditee *et al.*, 2003) and in the extreme halophilic bacteria *Ectothiorhodospira halochloris* and *Actinopolyspora halophila* (Nyyssola *et al.*, 2000; Nyyssola and Leisola, 2001). These three steps of methylation are catalysed by two methyltransferases, glycine sarcosine methyltransferase and dimethylglycine methyltransferase or sarcosine dimethylglycine methyltransferase. Glycine betaine, in archaea, is only known to be synthesized by a halophilic methanoarchaeon, *Methanohalophilus portucalensis* using the methylation of glycine, dimethylglycine and sarcosine with the methyl group derived from S-adenosylmethionine (Lai *et al.*, 1999; Lai *et al.*, 2006; Roberts *et al.*, 1992). This halophilic methanoarchaeon can also synthesize β-glutamate and N^{ε}-acetyl β-lysine in addition to glycine betaine (Lai *et al.*, 1991; Lai and Gunsalus, 1992).

Methanosarcina sp. is another methanoarchaeon in which synthesis of N^{ε}-acetyl β-lysine, α-glutamate and glycine betaine is regulated in response to changes in osmotic stress (Pfluger *et al.*, 2003; Sowers and Gunsalus, 1995). There is no evidence, to date, of synthesis of glycine betaine in halophilic archaea.

In addition to the glycine betaine biosynthesis, synthesis of ectoine has also been studied in many different bacteria. Ectoine has been synthesized by heterotrophic halophilic bacteria by a different pathway using diaminobutyric acid, aspartic-semialdehyde and N-acetyl-diaminobutyric acid as intermediates (Peters *et al.*, 1990). This synthesis pathway of ectoine has also been demonstrated in *Marinococcus halophilus* and *Chromohalobacter salexigens*. It has been found that ectoine synthesis is regulated at the transcriptional level and in these bacteria, ectoine synthesis is suppressed by the presence of glycine betaine (Calderon *et al.*, 2004; Louis and Galinski, 1997). Over the years, genes for these pathways of compatible solutes synthesis have been identified and characterized. As more comprehensive studies have been conducted on the biosynthesis and the regulatory pathway of these osmolytes, it has been revealed that different intracellular osmolytes function in combinations and are regulated by each other. Even when a microorganism is not accumulating osmolytes in response to osmotic pressure, homologues of these genes involved in osmolytes synthesis might still exist (Gadda and McAllister-Wilkins, 2003).

1.2.3.2.2 Transport systems

In addition to the *de novo* synthesis just described above, osmolytes can also be taken up from the environment using transport systems, and therefore osmolyte transporters can play an important role in the microbial osmotic balance. A number of these transporters can be solute specific or they can be developed to accumulate any osmolyte precursors from the surroundings. In microorganisms exposed to osmotic pressure, mechanisms for osmosensing are important for the activation of osmolyte

transporters (Heermann and Jung, 2004; Woods, 1999). Various stimuli can trigger these transporters such as cytoplasmic potassium ions, turgour pressure, and macromolecules (Figure 1.3). Some of these transporters also act as regulators and osmosensors. Non-halophiles can also gain salt tolerance using such a method of osmoregulation (Jebbar *et al.*, 1992; Robert *et al.*, 2000). In halophiles, the transport systems, just described, are used to recover osmolytes which leak from out of the cytoplasm as caused by the steep solute gradient across the membrane. Solute producers that lack a functional transporter probably lose significant quantities of solutes to the medium (Hagemann *et al.*, 1997), a fact which explains why halophilic microorganisms have transporters which are specific for their own synthesized osmolytes.

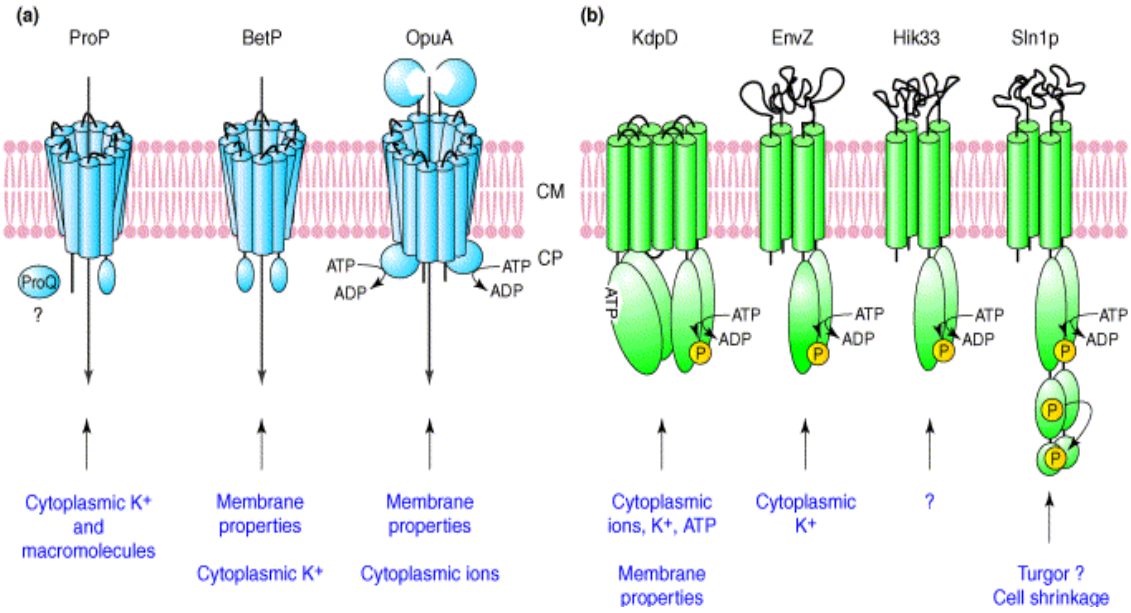


Figure 1.3 Schematic of stimuli perceived by osmosensory proteins. (a) osmoregulators and osmosensors responsible for the compatible solute transport. Examples of which are OpuA from *B. subtilis*, BetP from *C. glutamicum* and ProP from *E. coli*. These are compatible solute transporters capable of sensing extracellular osmotic changes. (b) Osmosensors that regulate gene expression encoding osmoregulators. These are generally kinases that sense extracellular osmotic changes but are not taking part in the actual compatible solutes transport or potassium ion uptake (Taken from Heermann and Jung, 2004).

1.2.3.2.2.1 ABC-type transporter

The ABC-transport systems contain a periplasmic substrate binding domain, a transmembrane unit and a cytoplasmic protein, which stimulates the transport through ATP hydrolysis. Osmoregulated compatible solute transporters belonging to the ABC-type transporters have been studied mainly in *Corynebacterium glutamicum*, *Escherichia coli*, and some other halotolerant bacteria such as *Bacillus subtilis* (Baliarda *et al.*, 2003; Kapfhammer *et al.*, 2005; Kempf and Bremer, 1998; Rubenhagen *et al.*, 2000; Tondervik and Strom, 2007; Wood *et al.*, 2005). The majority of such ABC-type uptake systems are also known as high affinity binding protein-dependent ABC-transporters. Some examples of these are OpuA, OpuB and OpuC in *Bacillus subtilis* and ProU in *E. coli*. High affinity transporters such as the ABC-type transporter have been also used by methanogens that can transport glycine betaine into the cell. For example, methanogens like *Methanosarcina mazei* has a salt induced primary transporter for glycine betaine (Roessler *et al.*, 2002).

1.2.3.2.2.2 Betaine Choline Carnitine Transporters (BCCT)

The BCCT group contains membrane protein transporting substrates including a quaternary ammonium group such as proline, glycine betaine, choline, and carnitine. The majority of BCCT transporters use electrochemical gradients generated by proton motive forces for substrate accumulation. The secondary ion coupled glycine betaine transporters is an example of BCCT transporters which is commonly found in osmotolerant microorganisms, such as methanogens. These glycine betaine transporters either belong to the sodium/solute symporter superfamily example the BetP from *C. glutamicum*, BetT and CaiT from *E. coli* and OpuD from *B. subtilis* or the major facilitator family of proteins e.g. ProP (*E. coli*) (Kappes *et al.*, 1996; Peter *et al.*, 1996). A high affinity and highly specific glycine betaine transport system belonging to the BCCT family of secondary transporters has also been identified in the halophilic

archaeon *Methanohalophilus portucalensis* (Lai *et al.*, 2000). This high affinity glycine betaine transporter involved in osmotic balance is stimulated by osmotic pressure across proton/sodium gradients (Proctor *et al.*, 1997).

The transporter BetP from *C. glutamicum* (Peter *et al.*, 1998; Rubenhagen *et al.*, 2000) and ProP from *E. coli* (Racher *et al.*, 1999) also function as sensors for osmotic changes. They can manage their own activity in response to osmotic pressure. As they are capable of functioning both as osmosensors and transporters, systems such as these influence the overall osmoregulation of the cell. These transport systems are independent of synthesis of compatible solutes. In addition to the glycine betaine transporters, a new type of osmoregulatory uptake system designated TeaABC has also been identified in halophilic heterotrophs (Grammann *et al.*, 2002). These transporters are osmoregulated and are also necessary for the uptake of ectoine leaking through the cytoplasmic membrane of the cells.

1.2.3.3 Other physiological mechanisms used for salt adaptation

1.2.3.3.1 Acidic Amino Acids

In addition to the accumulation of potassium ions in the cytoplasm and/or compatible solute accumulation, the presence of acidic amino acids is another unique feature of the halobacteria allowing them to survive in extreme salinity. In this case, the cytoplasm is exposed to an increase in ionic strength when potassium ions are accumulated in the cytoplasm. As a result, halophilic archaeal proteins have evolved to possess excess acidic amino acids to adapt to the changes in the cellular mechanisms (Fukuchi *et al.*, 2003; Lanyi, 1974; Rao and Argos, 1981). The proteins of these microorganisms withstand high salinity. However, they do not often show stability under low concentrations of salt and these proteins therefore function specifically under high salinity (Bonnete *et al.*, 1994; Eisenberg *et al.*, 1992). These halophilic proteins have extremely acidic amino acids compared to usual residues (Eisenberg, 1995) and

this feature is common to all halophilic archaeal proteins (Fukuchi *et al.*, 2003). The acidic amino acids produce negative charges at the surface, which is required for the halophilic protein solvation and to avoid denaturation, aggregation and precipitation of the proteins that happens in the non-halophilic counterparts following exposure to high concentration of salt (Dym *et al.*, 1995; Eisenberg *et al.*, 1992). In low salinity or osmolarity, the halophilic proteins are often denaturing and unfolding due to charge repulsion. In the halophilic archaea, the possession of acidic amino acids is a result of evolution and selection based on salinity (Dennis and Shimmin, 1997). Due to this unique feature of halophilic archaea, utilization of the strategy of potassium ions accumulation, these organisms usually exhibit a relatively narrow adaptation and as result, their growth is limited to hypersaline environments (Kushner and Kamekura, 1988).

1.2.3.3.2 Lipid Membranes

Lipid membranes composition is another feature of halophilic archaea allowing them to be adapted to saline environments compared to their non-halophilic counterparts. Lipid composition changes dependant on the salt concentration, have been well studied in halophilic bacteria (Kogut and Russell, 1984; Russell and Kogut, 1985; Russell, 1989). Extremely halophilic archaea membranes are characterized by the richness in a diacidic phospholipid, archaetidylglycerol methyl phosphate (PGP-Me). This kind of lipid is often stable at 3-5 M NaCl therefore contributing to the stability of membrane in high salt environments. In membranes of moderate halophiles and non-halophilies, this lipid was absent or replaced by other lipids (Tenchov *et al.*, 2006). In a study done in 1999 by van de Vossenberg and colleagues, membranes of halophilic archaea have shown to be adapted to low proton/sodium permeability at hypersaline conditions. When the concentration of NaCl increases, the membrane remained stable over a wide range of NaCl concentrations and at a high pH, indicating that halophilic

archaea are well adapted to hypersaline environments. Recently, *Haloarcula* strains have been reported to have high tolerance to organic solvents when grown at high concentrations of NaCl, and the lipid, PGP-Me, level in the membrane increased concomitantly (Usami *et al.*, 2005).

1.2.4 The potential biotechnology of halophiles

Compatible solutes are the most active substances involved in the process of maintaining osmotic balance. They are compatible with the cellular metabolism and keep up a positive balance of water in the halophilic cell. Due to their low molecular weight, these substances are highly water soluble amino acids and their derivatives, sugar alcohols as well as other alcohols and sugars (Galinski, 1995; da Costa *et al*., 1998; Ventosa *et al*., 1998).

From the biotechnology point of view, compatible solutes are becoming increasingly significant and of interest as biomolecules (such as DNA, enzymes, membranes) stabilizers as well as stress protective agents and salt antagonists. Ectoine is one of nature's most plentiful osmolytes and it is commonly found in aerobic heterotrophic bacteria (Galinski, 1995). Ectoines and hydroxyectoines can only be produced by a novel process of biotechnology called "bacterial milking". The compatible solutes can be produced due to the response of *Halomonas elongate* to the ambient salinity (Sauer and Galinski 1998).

Most of the enzymes that have been derived from extremely halophilic microorganisms that ulilize KCl as compatible solutes act effectively at concentrations above 1M salt whereas, they are denaturized and inactivated below 1M salt concentrations (Adams and Kelly 1995). In solutions with high salt concentration, the solubility of these enzymes is high, a fact which causes difficulties in using standard chromatographic procedures (Eisenberg *et al*. 1995).

Table 1.4 shows some of the various biotechnological products which have been produced or extracted from bacterial and archaeal halophiles.

Table 1.4 A range of novel biotechnological products produced or extracted from bacterial and archaeal halophiles, together with their uses.

Biotechnological products	Description	References
Hydrolases	– Derived from halophilic species of *Pseudomonas* (ATCC 55940). – Functional amino acids are broadly intended as intermediates for the production of pesticides, peptide hormones and antibiotics.	(Sudge *et al.* 1998; Joshi *et al*. (2000)).
Bacteriorhodopsin	– A selection of strict halophilic archaea are surrounded by pigmented membrane retina called halo-rhodopsin and bacteriorhodopsin (BR). – Isolated from *Halobacterium salinarum* strain S9 (in the past from *Halobacterium halobium*) – Some of its applications include use as an artificial retina, spatial light modulators and holography	(Oren, 1994; Lanyi 1995)
Liposomes	– Biopolymers extracted halophilic archaea such as *Halobacterium cutirubrum* produce "ether-linked lipids" – Used in cosmetics and medicines for the purpose of transporting the compounds to precise target sites of the body.	(Choquet *et al*. 1992; Galinski and Tindall 1992; Gambacorta *et al*. 1995)
Bio-surfactants	– Novel bio-surfactants known as "trehalose lipids" are produced by marine genera of rhodococci – Improve the efficiency of the remediation of soils and water contaminated by oil	(Yakimov *et al*. 1999; Banat *et al*. 2000)
Bioplastics	– Large quantities of poly (β -hydroxy butyric acid) (PHB) can be accumulated by halophile *Haloferax mediterranei.* – In some field, these compounds or plastics having biodegradation properties, might substitute for oil-derived thermo-plastics.	(Rodriguez-Valera and Lillo 1992; Ventosa and Nieto 1995; Steinbüchel *et al*. 1998)
Lectins	– Sugar-binding proteins obtained from halophilic archaea, have been used as tools in research on cells.	(Gilboa-Garber *et al.* 1998)
Poly(γ-D-glutamic acid)	– Produced from extreme archaeal halophile related to the genus *Natrialba.* – Used as drug carriers in the pharmaceutical and food industry	(Kunioka 1997; Hezayen *et al*. 2000)

1.3 Introduction to alkaliphilic and alkalitolerant microorganisms

1.3.1 Definition of alkaliphiles

Microorganisms, particularly bacteria, are broadly distributed in nature and the majority of them grow and reproduce optimally at just about neutral pH (close to pH 7). However, microorganisms have been reported to grow in extreme alkaline environments and these can be categorized into two main categories; alkaliphiles and alkalitolerant microorganisms (Krulwich and Guffanti, 1989a; Yumoto, 2002). Alkaliphiles (alkali from the Arabic for soda ash and phile- loving) have been further categorized into two main physiological categories, alkaliphiles and haloalkaliphiles. Alkaliphiles require an alkaline pH of 9 and pH 10 for optimal growth (i.e. obligate requirement for alkaline growth conditions). The growth requirements of haloalkaliphilic microorganisms are for both an alkaline pH of 9 and high salinity up to 4M NaCl (which is 8 times the salt content of normal sea water) (Horikoshi, 1999). *Bacillus alcalophilus* is an example of an alkaliphilic bacterium which retains its intracellular pH between 8.5 and 9.0 (Kroll, 1990; Krulwich, 1995; Jones and Grant, 2000).

Alkalitolerant microorganisms, on the other hand, can survive at pH 10 and sometimes above, but optimum growth takes place at pH of near neutral medium (in some published works they are called facultative alkaliphiles) (Krulwich and Guffanti, 1989a). *Bacillus firmus* OF4 is an example of the most common alkalitolerant bacterium that grows just about at pH 7.5 as well can survive at pH 10.5 in batch along with continuous culture in malate rich medium. The external pH effect on *B. firmus* OF4 growth has been studied. The growth rates of this isolate decreased above pH 11, which coincide with an obvious decline in its ability to maintain cytoplasmic pH and with the cells forming chains (Sturr *et al*., (1994).

1.3.2 Diversity of alkaliphilic microorganisms

The study of alkaliphilic microorganisms is fairly novel and when Horikoshi started experiments on alkaliphilic bacteria in 1968 (Horikoshi, 1996), there were very few published scientific papers which related to alkaliphiles. Alkaliphilic microorganisms have been commonly isolated from ordinary neutral environments such as garden soil, although in alkaline environments the total cell counts of the alkaliphilic bacteria is higher. Horikoshi has reported that alkaliphiles can also be isolated from acidic soil samples (Horikoshi, 1996 and 1999). Figure 1.4 illustrates the diversity of alkaliphilic bacteria.

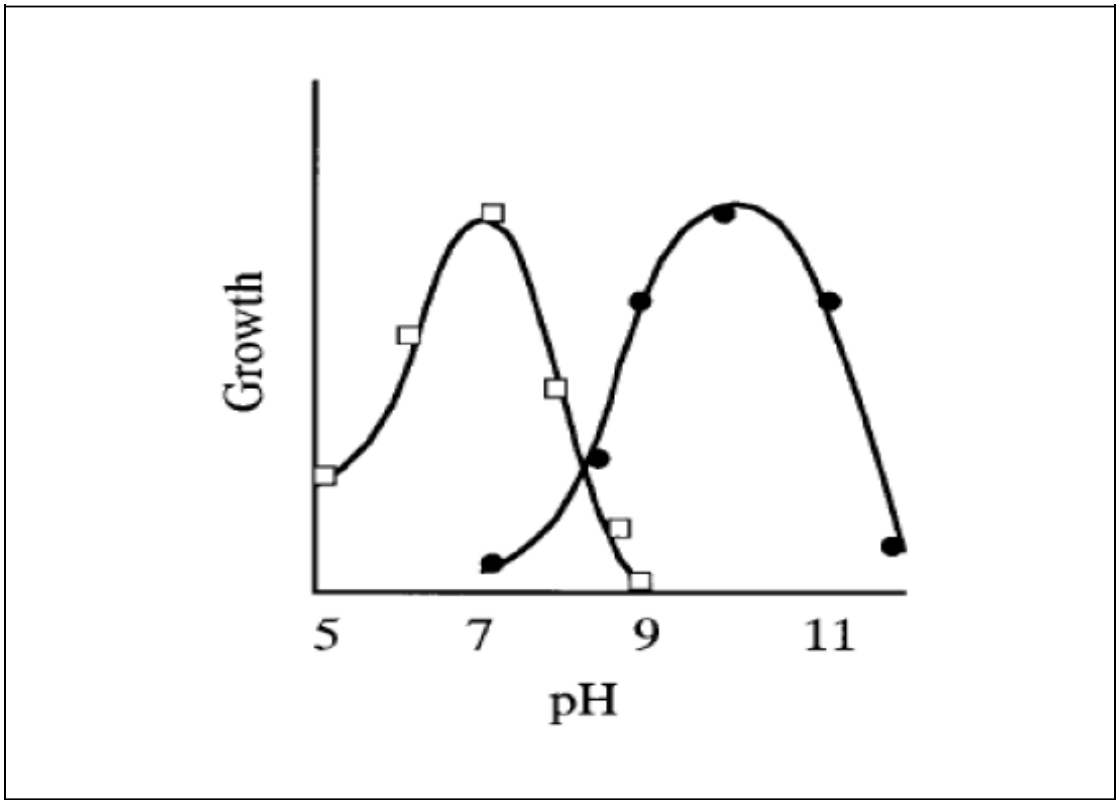


Figure 1.4 The pH dependency of alkaliphilic microorganisms. The typical pH dependency of the growth of neutrophilic and alkaliphilic bacteria is shown by open squares and solid circles, respectively (Taken from Horikoshi, 1999).

Figure 1.4 shows that there has been a rapid increase in the reported variety of alkaliphiles that have been isolated from different environments (Horikoshi, 1999**;**

Sorokin *et al*., 2001). Some of those environments were alkaline springs as well as garden soils, which are generally not mainly alkaline. It is also obvious that acidic soil samples are often good sources of alkaliphiles (Grant *et al*., 1990; Horikoshi, l99lb, 1996 and 1999; Goto *et al*., 2005).

Two isolate of Gram negative, non-motile, encapsulated cells have been found in the soda lakes of the southern Transbaikal region and have been reported to be alkalitolerant facultatively methylotrophic bacteria capable of growing well at pH values between 6.5 and 9.5 when methanol is provided as the source of carbon and energy; they optimally grew at pH between 8.0 and 8.5 (Doronina *et al*., 2001). From a sample of soil in Tochigi, Japan, an alkaliphllic *Bacillss* sp. called KSMKP43 was isolated and it was shown to grow well at pH range from 6.8 to 10 with an optimum at pH 9. Molecular methods of 16S rRNA gene sequence analysis showed the isolate as a cluster associated with *Bacillus halmapalus*. This isolate was reported as a Gram positive, spore forming, strictly aerobic, motile; it was also shown to have the ability to produce an unusual serine protease (Saeki *et al*., 2002). Another strain called YIM80379^{T} has been isolated from a soil sample collected from the eastern desert of Egypt and grown optimally at pH value of 9.5 - 10 and limited or no growth at pH 7. Furthermore, alkaliphilic endospore forming *Bacillus* sp. and non-endospore forming species of *Aeromonas, Actinopolyspora, Corynebacterium, Micrococcus, Pseudomonas* and *Paracoccus* have been isolated from neutral soils (Satyanarayana *et al*., 2005). On the other hand, *Chimaereicella alkaliphila*, a Gram-negative alkaliphilic bacterium has been isolated from highly alkaline groundwater at pH 11.4 and it does not show any growth when re-cultured in a medium at pH 7 (Tiago *et al*., 2006).

For the purpose of industrial applications, alkaliphilic microorganisms have also been isolated from a range of environments (Yumoto *et al*., 2000). An isolate called Vl-4 belonging to the genus *Bacillus*, was shown to grow in diluted Kraft black liquor at

pH value of 11.5 and is able to produce large amounts of xylanase when grown in alkaline medium at pH 9 and 10. Yang *et al*. (1995) reported that the maximal enzyme activity was obtained by cultivation in a defined alkaline medium with 1% corn steep liquor (pH 9) and 2% birchwood xylan.

A facultative alkaliphile referred to as PAT 05^T, has been isolated from the rhizosphere of the perennial shrub *Atriplex lampa* in northeastem Patagonia, Argentina. It is a Gram-positive, rod, spore-forming bacterium and it was identified, using 16S rRNA gene sequencing, as the type strain of *Bacillus patagoniensis*. This strain grows at pH between 7 and 10, while the optimum growth is at pH 8 and no growth was reported at pH 6 (Olivera *et al*., 2005).

Haloalkaliphilic microorganisms have often been isolated from extremely alkaline saline environments including soda lakes e.g. the Rift Valley lakes of East Africa (e.g. Lake Magadi), the western soda lakes of the United States and the Wadi Natrun lake in Egypt (Horikoshi, 1999; Rees *et al*., 2004; Satyanarayanaet *et al*., 2005). These lakes are often red in colour, due to the presence of large numbers of haloalkaliphilic archaea such as *Natronococcus occultus*, *Natronobacterium gregoryi* and *N. pharaonis* (Figure 1.5).

Figure 1.5 Kenya's Lake Magadi with red pigmentation due to haloalkaliphilic bacteria bloom. This lake is an extremely salty, alkali lake which contains little variety of life (photographed by Emory Kristof).

Cyanobacteria, such as *Cyanospira rippkae* and *Arthrospira (Spirulina) platensis* and anoxygenic phototrophic bacteria of the genera *Halorhodospira* and *Ectothiorhodospira* are the main agents of photosynthetic primary production in soda lakes (Jones *et al*, 1998; Milford *et al*., 2000).

Berber and Yenidunya have isolated haloalkaliphilic *Bacillus* species from the water and the surrounding soil of Lake Van (Berber and Yenidunya, 2005). A Gram-positive, facultative alkaliphile and non-motile organism, known as WW3-SN6 strain, has been isolated from alkaline wash waters resulting from edible olive preparation. This grows at pH of 7.0 and up to pH 10.5, with optimum growth at pH from 8.0 to 9.0 (and at a temperature from 27 to 32^{o}C); it could also grow in up to 15% (w/v) NaCl.

Duchworth *et al*. (1998**)** have isolated two strains of alkaliphilic aerobic organotrophs from a moderately saline and alkaline East African soda lake. The isolates were grown at pH between 6 and l0 and grew optimally at pH 9.0 and showing visible growth at salt concentration between 0% and 10%; both isolates are members of the monospecific genus *Dietzia maris*. However, since one of the isolates significantly varies in halotolerance and utilization of carbon source it appears to be a new species which has been designated, *Dietzia natronolimnaios*.

Two other strains of halotolerant alkaliphilic methanotrophic bacteria have been isolated from moderately saline soda lakes in Tuva, Central Asia. These strains are Gram negative and grow rapidly at pH values between 9.0 and 9.5, with only very slow growth at pH 7, while none occurred below this pH. Based on their alkaliphilic physiology, both of these strains were given the name, *Methylobacter alcaliphilus* sp. nov. (Khmelenina *et al*., 1997).

1.3.3 Habitats of alkaliphilic microorganisms

Natural alkaline environments including soda lakes and desert soils are mainly formed by the effects of climatic, geographical and topographical conditions (Grant and

Horikoshi 1992; Detkova and Pusheva, 2006; Tiago *et a1*., 2004). Industrial-derived waste waters (Ulukanli and Digrak, 2002) and industrial activities such as food processing (Grant *et al*., 1990; Jones *et al*., 1998) can also lead to the formation of alkaline environments.

Some biological activity, such as ammonification or sulphate reduction, can also lead to the formation of alkaline environments (Horikoshi, 1991a). In 1990, Grant and his colleagues reported the existence of two types of alkaline environments. High calcium (Ca^{+2}) environments are the first type, naturally represented by ground water bearing high levels of CaOH. Low Ca^{+2} environments are the second type and these include soda lakes and deserts that are generally dominated by the existence of high levels of sodium carbonate (Na_2CO_3) and low levels of magnesium (Mg^{+2}) ions. These two type of environments are the most stable and naturally occurring highly alkaline environments which exist in Earth, and here the pH is commonly at 10 and above (Ma *et a1*., 2004a; Tiago *et al*., 2004). The most frequently studied soda lakes are those of the East Africa Rift valley which have been known to science since 1930 (Grant *et al*., 1990; Jones *et al*., 1998). Central Asian soda lakes are other sites where microbiological studies of alkaliphiles have also been well documented (Zhilina and Zavarzin, 1994, Ma *et al*., 2004b).

Alkaline environments also occur as soda lakes containing high sodium carbonate concentrations and high concentration of other salts, including sodium chloride. This means that the haloalkaliphilic microorganisms of these lakes must be adapted to high values of pH and high concentration of sodium chloride (salinity); other stresses include low water activity and insufficiency of some micronutrients (Grant *et al*., 1990; Tiago *et al*., 2004). In environments such as soda lakes in the Rift Valley, pH values range from 8.5 to above 11.5 with the salinity ranging from 5% (w/v) to saturation (33%) (Rees *et al*., 2004). A soda lake in the Mongolia autonomous region of China,

called Lake Chahannor, has been reported to have pH values from 9.5 to 10.2 with high concentrations of both Cl^- and CO_3, up to 5M Na^+,; in addition it contains very low levels of Mg^{+2} and un-measurable amounts of Ca^{+2} (Ma *et al*., 2004a; Wei *et al*., 2007).

1.3.4 Compatible solute strategy in alkaliphiles

Sorokin and Kuenen (2005) reported that the main organic osmolytes in haloalkaliphilic sulphur-oxidizing bacterium, *Thioalkalimicrobium* species was found to be ectoine (2-methyl-4-pyrimidine carboxylic acid). In contrast, in *Thioalkalivibrio versutus* (grown in continuous culture), glycine betaine was the main organic osmolyte. Both these compatible solutes are well known as the dominant osmoprotectants among the halophilic microorganisms. Ectoine is found in *Halomonas* species, whereas glycine betaine is prevalent among all the members of these extremophilies (please refer to Section 1.2.3.2).

1.3.5 Mechanisms of cytoplasmic pH regulation in alkaliphiles

Every living cell has to manage its cytoplasmic pH to produce a compatible and optimal pH for cellular functions and stability of the proteins that maintain growth (Padan *et a1*., 2005). For these reasons, bacteria that can grow at an extreme levels of external pH values (above pH 9 for alkaliphiles and below pH 4 for acidophiles) must be able to retain their intracellular pH (cytoplasmic pH) within a reasonably narrow range, i.e. close to pH 7 (Booth, 1985; Cook, 2000; Yumoto, 2002). Generally, alkaliphiles exhibit cytoplasmic pH values in the range of 8.2 to 9, neutrophlies have cytoplasmic pH values between 7.5 and 8 and acidophiles show internal pH values in the range of 6 to 7 (Kashket, 1981; Hackstadt, 1983; Guffanti *et al*., 1984). Alkaliphiles use various cellular adaptations in order to avoid alkalization of their internal pH, so that their intracellular functions and activities (e.g. the replication of DNA and protein synthesis) continue working properly. The significance of maintaining the internal pH at around neutral pH values leads to decrease in the growth rates of alkaliphiles

associating with increasing internal pH (Sturr *et al*., 1994). Moreover, alkaliphiles have structures outside their cell membrane such as flagella, outer membrane and cell wall that indirectly make contact with the external pH and therefore must be functioning at high pH values (Kroll, 1990). The mechanisms used by alkaliphilic microorganisms to maintain their internal pH are well described and illustrated in the following figure (Figure 1.6).

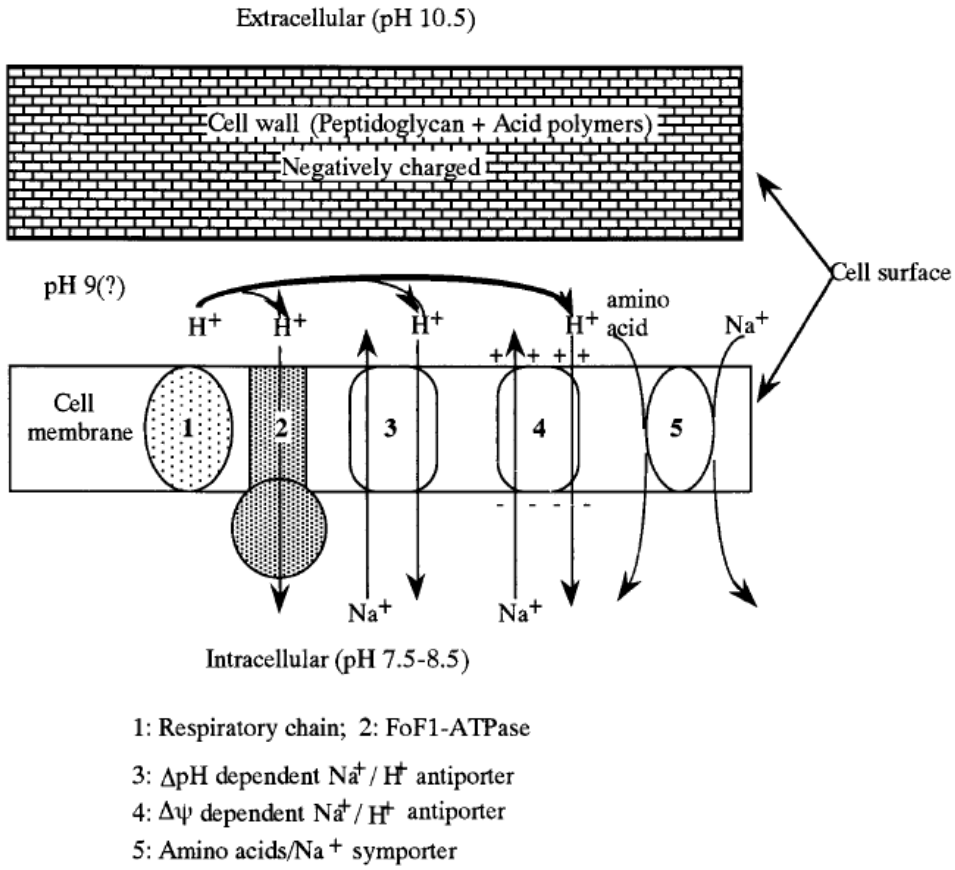


Figure 1.6 Schematic representation of cytoplasmic pH regulation (Taken from Horikoshi, 1999).

1.3.5.1 Passive mechanisms for cytoplasmic pH regulation in alkaliphiles

1.3.5.1.1 Anionic polymers

The cell walls of Gram positive alkaliphiles contain a peptidoglycan layer and acidic polymers (teichuronic acid and teichoic acid) which act as a negatively charged area on the cell surface, and then serve as a barrier which reduces the pH value at the cell surface (Horikoshi, 1999; Aono *et al*. 1999; Tsujii, 2002; Horikoshi, 2008). The

cell membrane surface must be kept below pH 9, because it very unstable at highly alkaline pH values (Horikoshi, 2008). In comparison with neutrophlilic Gram positive strains, the concentrations of anionic polymers are higher and this concentration in cell walls of alkaliphiles increases when the organism is grown at high external pH; a fact which indicates that the anionic polymers act as passive function in pH homeostasis. Figure 1.7 illustrates a schematic representation of the cell wall of a Gram-positive bacterium (Tsujii, 2002). The anionic polymer layer acts by fixing anions to the polymer chains and as a result positive ions are then bound in the aqueous part of the layer until the equilibrium point is achieved.

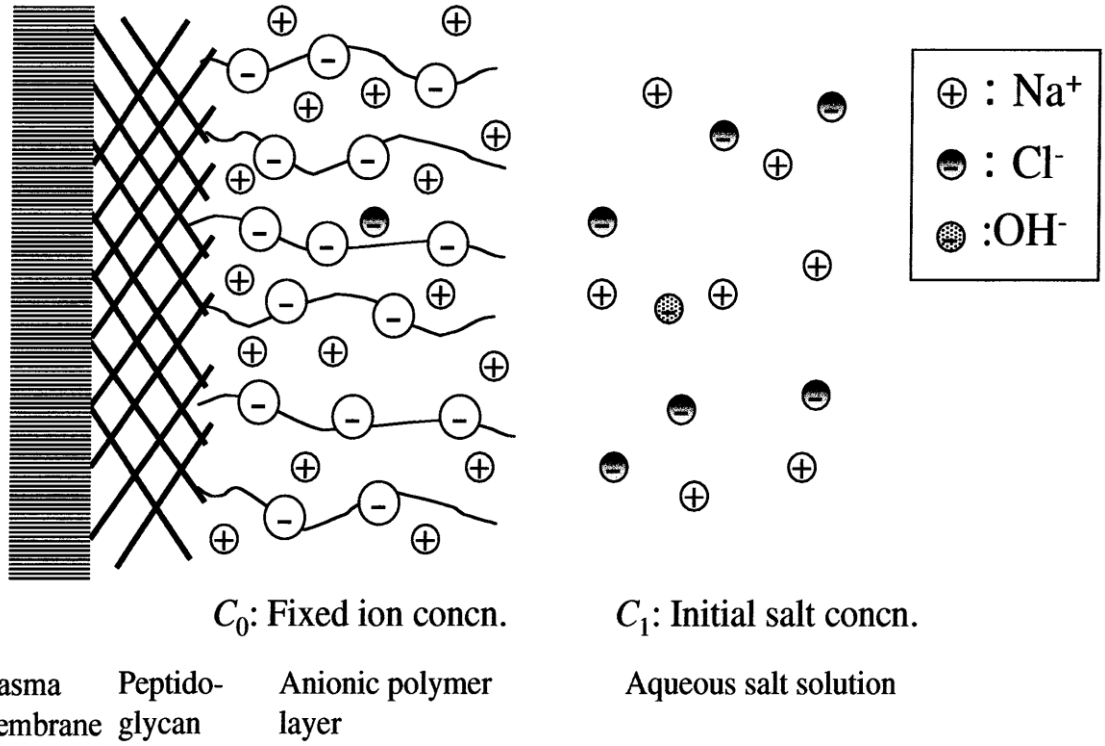


Figure 1.7 Structural model for the cell wall of Gram-positive bacteria. Many anionic polymer chains are situated in a brush-like shape on the surface of polymer hydrogel (peptidoglycan). Added salt in the bulk aqueous phase enters partly into the anionic polymer layer, and equilibration is attained. The equilibrated salt concentration inside the polymer layer is much smaller than that in bulk aqueous phase because of the very high concentration of cations (sodium ions) in the polymer layer (Taken from Tsujii, 2002).

1.3.5.1.2 Membrane fatty acids

The composition of the fatty acids in the cell membrane of bacteria plays an important role in protecting the cell from alkaline environments, and both Gram negative and Gram positive alkaliphilic bacteria have been shown to have very similar whole-cell fatty acid profiles (Hicks and Krulwich, 1995; Banciu *et al*., 2008). The production of saturated or mono-unsaturated fatty acids containing either 16 or 18 carbons is enhanced by the growth in alkaline conditions (Ma *et al*., 2004a).

1.3.5.2 Active mechanisms for cytoplasmic pH regulation in alkaliphiles

In pH homeostasis, the most important component is the function of cell membrane associated proteins which can catalyze inward proton transport. Cell membranes of alkaliphilic bacteria play a vital role in maintaining pH homoeostasis by using Na^+/ H^+ antiporter system ($\Delta\Psi$ dependent and ΔpH dependent), ATPase-driven H^+ expulsion and K^+/H^+ antiporter (Horikoshi, 2008; Krulwich *et a1*., 1997 and 1998; Wutipraditkul *et a1*., 2005)

1.3.5.2.1 Na^+/ H^+ antiporters

Harold and Papineau (1972) reported the first investigation of a bacterial Na^+/H^+ antiporter in *Streptococcus faecalis*. West and Mitchell (1974) then found that the addition of Na^+ to an anaerobic cell suspension of *E. coli* showed proton extrusion. Beck and Rosen (1979) then provided confirmation of Na^+ / H^+ antiporter activity as they established the obligate coupling between Na^+ and H^+ movements (i.e. Na^+ dependent translocation of H^+ and H^+ dependent translocation of Na^+). It is important to emphasize the characteristics and nature of cation/proton antiporters and Na^+ / H^+ antiporter in particular, as these play important physiological roles, including pH homeostasis, generation of a Na^+ motive force and the regulation of cell volume in both eukaryotes and prokaryotes under alkaline conditions (Krulwich *et al*., 1994; Brett *et al*., 2005; Padan *et al*., 2005). In pH homeostasis, the Na^+ / H^+ antiporter maintains the

internal pH of *Bacillus firmus* at 2 and 2.3 units below the external pH (Krulwich *et al*., 1998).

The Na^+ / H^+ antiporter which catalyzes net proton accumulation in the bacterial cytoplasm is powered by the ΔpH formed by respiratory electron transport and/or by the proton translocating F_1,F_0-ATPase localized in the cytoplasmic membrane (Figure 1.6). Alkaliphilic bacteria use an efficient way of cycling Na^+ back into the cell to keep the antiporter working and two important mechanisms, namely Na^+ channels and Na^+/solute symporters (Ito *et al*., 2004). The Na^+/solute symporters play a significant role in both in nutrient uptake and pH homeostasis, by providing a route for Na^+ re-entry to keep a Na^+/H^+ antiporter working, (Krulwich and Guffanti, 1989b). Na^+ channels mechanism exhibits non-pH related primary functions and is only important for pH homeostasis-related Na^+-uptake efficiency as Na^+ concentrations are low (Ito *et al*., 2004).

1.3.6 Industrial applications of alkaliphiles

Extremophilic microorganisms which are tolerant to extremes and able to survive in ecological niches such as at extremes of pH, high temperatures, high pressure and high salt concentrations have attracted considerable interest as a source of novel enzymes for industrial purposes (Niehaus *et al*., 1999). These kinds of microorganisms are known to produce exceptional biocatalysts (extremozymes) that work under extreme conditions (Margesin and Schinner, 200l; Rawlings, 2002). Microorganisms that can survive and grow at extreme pH values, for instance, produce extremozymes that are valuable for many industrial uses under either highly alkaline or even highly acidic conditions (Ulukanli and Digrak, 2002). Both acidophilic and alkaliphilic microorganisms maintain their cytoplasmic pH close to neutral and so their intracellular enzymes do not necessarily need to adapt to extreme pH. However, extracellular enzymes of

acidophiles and alkaliphiles have to functionally work at low or high pH respectively (van den Burg, 2003).

Some alkaliphilic bacteria contain unique enzymes which are stable at various pH values such as amylases, proteases, cellulases and lipases and these enzymes have been applied to several industrial applications and are being commercially produced (Table 1.5) (Horikoshi, 1999; Takami and Horikoshi, 2000; Ulukanli and Digrak, 2002; Horikoshi, 2008). For the laundry detergent market, the current proportion of total world enzyme production comfortably exceeds 30% (Horikoshi 1996; Grant *et al*., 1990) and there are also many similar applications for enzymes in the pharmaceutical, food and waste treatment industries, (Grant *et al*., 1990; Horikoshi, 1991b and 1996: Zhllina and Zavarzin, 1994; Enomoto and Koyama, 1999; Saeki *et al*., 2007).

Table 1.5 Enzymes produced by alkalophilic microorganism (Horikoshi, 1999; Takami and Horikoshi, 2000; Ulukanli and Digrak, 2002; Horikoshi, 2008).

Microorganism	Enzymes
Staphilothermus marinus	Amylase
Bacillus thermoleovocas	Lipase
Thermococcus litoralis	Pullulanase
Clostriridium absonum	Cellulase
Bacillus circulans	Xylanase
Fusarium proliferation	a-Galactosidase
Clostridium abosum	Penicillinase
Pyrococcus abyssi	Phosphatase

Currently, microbial proteases are being used because of their stability at high temperature and pH (Gupta *et al*., 2002; Joo *et al*, 2002; Kocabiyik and Erdem, 2002). They mostly find applications in the food industry, in the brewing, baking, meat tenderization process, dairy industry and peptide synthesis (Genckal and Tarib, 2006; Ibrahim *et al*., 2007); furthermore, they are used in pharmaceutical industry, medical

diagnosis, detergent industry, and in the dehairing process for leather production (Ito *et al*., 1998; Gessesse *et a1*.,2003; Saeki *et al*., 2007).

1.4 Introduction to UV-C tolerant microorganisms

Although a significant effort has been devoted, particularly by NASA and the Russians, to the study of space, little is known about the biology of the stratosphere and above in what is termed "near-space". It is surprising to find that we know little about the height to which the biosphere extends upwards into space; or put another way-what is the maximum height above the Earth at which microbes can be isolated? It might be imagined that microorganisms can simply drift from off the surface of the Earth into space. However this is not the case, because the tropopause, at a height of 17 km, acts as an effective barrier to the passage of particulates including microorganisms. Particulates can be ejected throughout the tropopause by volcanoes and possibly carried upwards by other phenomena such as "blue lightning" (Pasko *et al*. 2002). Additionally, any particles that can pass through the tropopause are subject to gravity. As a result, any bacteria which enter the stratosphere will be subjected to deposition. Consequently, it is to be likely that relatively few bacteria get to the stratosphere and those that do, will not remain more than about 12 months (depending on various assessments of the so-called residence time). Any bacteria that do get to the stratosphere will be subjected to some extreme conditions, particularly low nutrient status and temperature, and high levels of UV radiation. Unless such bacteria show a noticeable ability to survive such extremes, residence in the stratosphere will again result in a noticeable reduction in population density.

Recently, scientists in Indian have launched "balloon-borne cryosamplers" that were aseptically able to collect air samples from the stratosphere over the Indian Sub-Continent. Scientists here in this laboratory at the University of Sheffield and also at

the University of Cardiff were then able to demonstrate that microorganisms, including bacteria and fungi are present in the stratosphere (Wainwright *et al*., 2003).

Although the stratosphere is rich in UV-C, this type of radiation does not reach the Earth as a result it could be argued that any UV-C resistant microorganisms found on Earth must have come from the stratosphere and possibly beyond, since any Earth-derived organism would never have had the opportunity to evolve resistance to this type of radiation (Wainwright, 2003). Therefore, the possibility that the UV-C resistant microorganisms studied here might have originated from Earth or space will be considered.

1.4.1 General aspects of ultraviolet in the environment

Ultraviolet light is a part of the electromagnetic spectrum located outside the violet range of the visible spectrum. The UV spectrum ranges from 100 to 400 nanometers (nm). Energy in a wide range of wavelengths is radiated by the sun; most of these wavelengths are invisible to the human eye. The more energetic the radiation is from the shorter wavelength the more potential harm it can do to living systems. The range of wavelength of UV radiation that reaches the Earth's surface is from 290 to 400 nm which is shorter than wavelengths of visible light (from 400 to 700 nm) (Gascn *et al*, 1995).

It is generally accepted that approximately 3% of solar radiations is UV (i.e., radiation with a wavelengths shorter than 400 nm), 37% is visible light (with a wavelengths between 400 and 700 nm), and about 60% is infrared (longer than 700 nm). Since the Solar Constant is C = 1368.31 W m $^{-2}$ (Wilson *et al.*, 1981) a value of 123.15 J m $^{-2}$ s $^{-1}$ represents the UV radiated by the sun. Based on calculations, only 0.00028% of solar radiation is lethal to microorganisms, particularly bacteria (Pollard 1974). Three regions make up the solar spectrum of UV radiation namely: (1) ultraviolet A (UV-A), (2) ultraviolet B (UV-B) and (3) ultraviolet C (UV-C) radiation

(Figure 1.8). The wavelengths of UV-A radiation is between 320 and 390 nm which is just beyond the violet range of the visible spectrum. The wavelength of UV-B is between 286 and 320 nm, the latter wavelength being the shortest wavelength incident in sunlight reaching the Earth's surface. Ultraviolet-C consists of wavelengths shorter than 286 nm (Gascon *et al*., 1995).

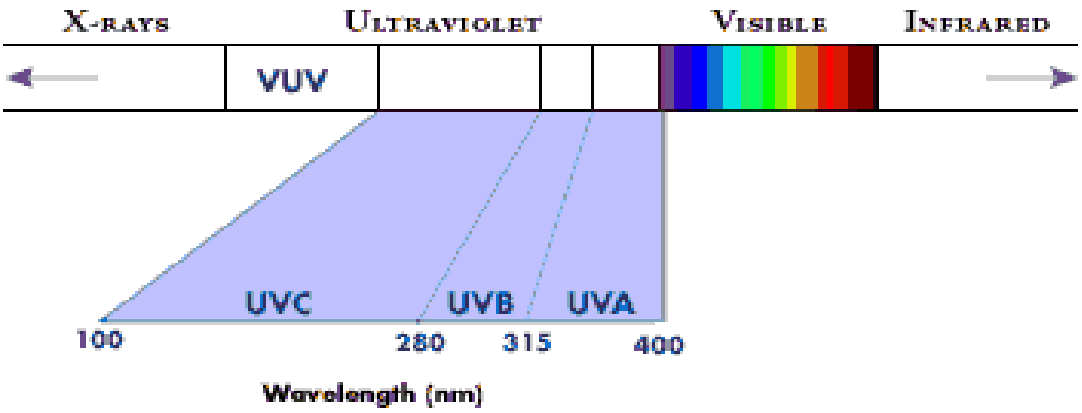


Figure 1.8 Electromagnetic radiation is found in a range of wavelengths. UV radiation, is harmful to most living organisms, corresponds to a small portion of the spectrum, wavelengths between 290 and 320 nm. ((Illustration by Simmon, 2001).

The occurrence of UV radiation in the upper part of atmosphere causes the oxygen molecules existing there to absorb all radiation with wavelength below 200 nm and thus to convert to ozone molecules. The ozone layer protects the Earth's surface from biologically harmful UV radiation having wavelengths of 300 nm and below. For this reason, the solar spectrum of UV radiation below 300 nm is extremely limited and non-existent at the Earth's surface. Oxygen and ozone molecules present in the stratosphere absorb 97 to 99% of the sun's high spectrum of UV radiation (with wavelengths from 150 to 300nm). Therefore, only 2.2% of the solar radiation outside the atmosphere is related to UV with wavelengths shorter than 320 nm, and 1% with UV wavelengths shorter than 300 nm (Cutchis, 1974).

1.4.2 The impact of UV on living organisms

Solar UV radiation affects almost all living organisms and biological behaviour in many ways has developed to deal with it. Different ranges of wavelength of UV radiation differ in their effects and all living organisms have to live with the harmful effects as well as the more beneficial ones. Longer UV wavelengths of UV-A play an essential and a helpful role in vitamin D formation by the skin and also exhibit harmful effects on humans by causing sunburn, skin cancer and cataracts. The incoming radiation at UV-B wavelengths causes harm at the molecular level i.e. to DNA (Kalimo *et al*., 1983).

Ultraviolet radiation inhibits the growth of microbes and disease causing oocysts such as *Giardia* and *Cryptosporidium.* Similarly enteric viruses, *Legionella pneumophila* and the hepatitis virus which can resist chlorine are eradicated by UV treatment. For most microorganisms such as bacteria and viruses, the elimination efficiency of UV treatment for microbiological contaminants often exceeds 99.99%.; the following organisms are eliminated at an efficiency of better than 99.99%: *Salmonella enteritidis* (Gastroenteritis), *E-coli*, *Salmonella typhi* (Typhoid fever), *Vibrio cholerae* (Cholera), *Legionella pneumophila* (Legionnaires' Disease), *Mycobacetrium tuberculosis* (Tuberculosis), the influenza virus, the hepatitus A virus (better than 90%) and the polio virus (Antopol and Ellner, 1979; Muraca *et al*., 1987).

1.4.2.1 Sensitivity and resistance of microorganisms to UV radiation

The environmental impacts of solar UV radiation are potentially destructive (Nasim and James 1978). Microbial cells, mainly bacteria, are subject to DNA damage from exposure to solar UV radiation (Miller and Kokjohn, 1990), and ultraviolet radiation is the main cause of the lethal and mutagenic effects of solar radiation. The main function of *recA* in the DNA repair suggests that this gene is basically responsible for the ability of bacteria to withstand such damage and, therefore, to exploit UV-exposed ecosystems

(Miller and Kokjohn, 1990). Mutations which are amongst the consequences of UV radiation paradoxically can have selective advantages. A balance between sensitivity and resistance to UV radiation could be advantageous from an evolutionary point of view, and it is possibly for this reason that protection against UV radiation is usually not absolute. This sensitivity-resistance balance is expected to vary from one organism to another and not surprisingly, there is a large diversity of resistance to both mutagenic and lethal effects of UV radiation (Gascn *et al*, 1995).

1.4.2.2 The effect of UV radiation on DNA

Ultraviolet radiation is mainly absorbed via the nucleotides in the DNA and DNA readily absorbs a range of UV-B wavelengths, leading to an alteration of the molecule's shape in one of several ways. Figure 1.9 illustrates one such alteration in shape as a result of exposure to UV-B radiation. Any change in the DNA molecule usually means that protein-building enzymes at that point on the molecule cannot "read" the DNA code. Consequently, proteins can be distorted, or the whole cell can die (Gelbart *et al*, 2002).

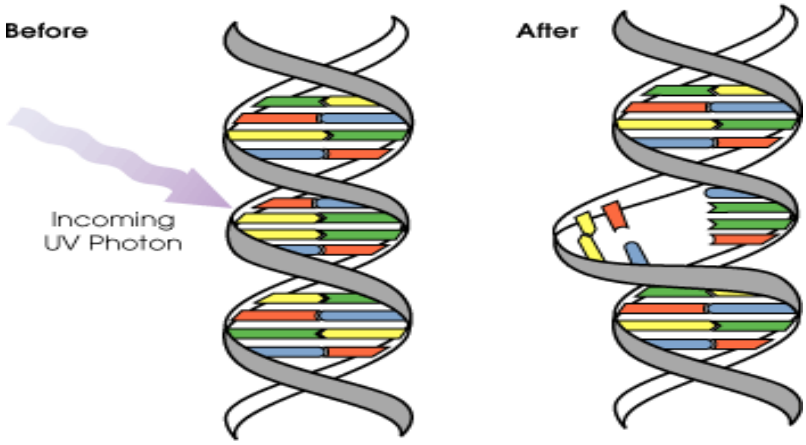


Figure 1.9 UV photon harms the DNA molecules of living organisms in one of several ways. The most common harmful event is that adjacent bases link and make bonds with each other, instead of crossways the "ladder." This creates a bulge, and the distorted DNA molecule does not functioning properly. (Illustration from Herring, 2011)

The effect of this absorption is to make a structure called a thymine dimer which is the bonding of two adjacent thymine bases on the same DNA strand. These dimers cause DNA- molecule distortion and, thereby cause failure in cell replication and ultimately death. In multicellular organisms, the damage due to exposure of UV radiation may also lead to cell death; however it can also cause diseases such as cancer (Gelbart *et al.*, 2002; Nicholson *et al.,* 1991).

1.4.2.3 Protection of microorganisms against the lethal effects of solar UV

Living organisms have developed repair mechanisms in order to protect themselves from UV radiation (Zion *et al*., 2006). For example, melanin in mammals and humans and anthocyanins in many plants act as external protection agents (Delpech, 2000). The molecules of these protection agents take up the UV radiation and stop it from gaining access to the cells genetic information. Gelbart *et al.* (2002) listed the major DNA repair mechanism in bacteria: as: photo-reactivating enzyme (photolyase), excision repair through the UV radiation, the ABC system (UvrABC), the SOS repair (uses the RecA protein) and post replication repair. *Serratia marcescens* produces a red pigment called prodigiosin (red pigment), which protects it from UV and has structural similarities to the side chain of human melanin molecule, another UV protective agent (Carter *et al.*, 1976; Kaidbey and Kligman, 1978).

Bacterial endospores are well known for their UV resistance which is based on structural proteins like *CotA.* Endospores also contain "light-absorbing pigments" in their coats (Nicholson, 2002) which absorb UV wavelengths (Nicholson and Galeano, 2003). Under the protein coat, there is an outer membrane and an extremely thick layer of peptidoglycan. Normally, peptidoglycan layers like this are found in the cell walls of bacteria; this adapted thick layer seems to play an important role in dehydrating the bacterial spores. Dehydration mechanisms are essential to spore survival since they

protect the cell from thermal harm to its DNA and proteins (Nicholson, 2002; Nicholson and Galeano, 2003; Moeller *et al*., 2009).

Endospores also contain dipicolinic acid (DPA) which makes up to 10% of an endospore dry weight and which protects against wet thermal damage, UV radiation and chemicals, such as hydrogen peroxide (Nicholson, 2002).

Bacterial spores, in the stratosphere and space, have to cope with solar UV-radiation which is the most deleterious factor, especially within the wavelength range between 220 nm and 260 nm (Taylor, 1974; Horneck *et al*., 1984), which is absorbed by the DNA molecules and therefore dangerously denaturing, in addition, solar UV and space vacuum work synergistically to decrease microbial viability (Horneck *et al*., 1984, Lindberg and Horneck, 1991; 1992). *Bacillus subtilis* has been exposed for almost 6 years to the space environment and, if shielded against solar UV radiation, some 80% of spores in multi-layers were able to stay alive in space (Lindberg and Horneck, 1991).

1.4.3 Biodiversity of UV tolerant microorganisms

1.4.3.1 The stratosphere

Microorganisms might be vertically carried into upper atmosphere by different mechanisms (Griffin, 2004). The comparatively low density and small size of microorganisms such as bacteria or spores allow them to stay airborne for some time before they sediment to the ground (Atlas and Bartha, 1997). Microorganisms that survive in the upper atmosphere must be resistant to ambient UV radiation. The earliest explorations were carried out in the late 1800's and early 1900's. Studies have confirmed that microbes exist in the stratosphere (Rogers and Meier, 1936; Bruch, 1967; Wainwright, 2003; Griffin, 2005), *Mycobacterium* and non-spore-forming Micrococci (Fulton, 1966; Bruch, 1967; Imshenetsky *et al*., 1977; Griffin, 2008); the question is how did they get there?

It is, in general, accepted that few particles of Earth source can pass the tropopause, a natural barrier existing at about 17 km beyond the Earth's surface. Volcanic eruptions loft particles beyond 30 km, particles larger than a few microns drop down quickly to the ground by gravity (Wainwright *et al*., 2003). Assuming Wainwright's collections on January 2001 provided representative stratospheric samples at 41 km no process that is only terrestrial appears to be able to sustain the high densities of bacterial clusters in the stratosphere.

Harris *et al*. (2002) reported the finding of clumps of cocci-shaped sub-micron-sized particles using micropore filters by which stratospheric air had been passed through (overall average radius of clumps 3.0μm up to 10μm). These clumps were shown to be bacteria (confirmed by using an epifluorescence microscope after using a scanning electron microscope). The epifluorescence microscope technique involved the use of carbocyanine (membrane potential sensitive dye) and fluorescence was taken to indicate the existence of viable cells.

Recently in 2009, Indian scientists have found three new species of bacteria that can survive UV radiation at a height of about 40km above the earth's surface. This finding could throw light on the origin of life on the Earth (Dailytech, 2009). These three new bacteria have been named: *Janibacter hoylei*, *Bacillus isronensis* and *Bacillus aryabhata*. These species of bacteria, which do not belong to any known strain on the Earth; the findings are essentially similar to those reported by Wainwright *et al*. (2003) who isolated two bacteria (*Bacillus simplex* and *Staphylococcus pasteuri*) and a single fungus, *Engyodontium album.*

1.4.3.2 The ambient environment of the Earth's surface

Within the UV spectrum of solar irradiation, UV-A predominates at the earth's surface with small amounts of UV-B. Keeping in our mind that UV-C (the shortest wavelength) radiation and high levels of UV-B are harmful to almost all forms of life.

UV-C and about 90% of UV-B radiation are absorbed by ozone layer, water vapor, carbon dioxide and oxygen in the atmosphere (World Health Organization, 2002), and therefore do not harm living organisms on the Earth's surface. UV exposure at higher altitude is severe due to the thinner layers of atmosphere (Figure 1.10). With every 1000 meters increase in height, levels of UV generally increase between 10 to 20%, depending on latitude, time of day and year and weather (Blumthaler, 1997; Seckmeyer, 1997; Schmucki and Philipona, 2002). The quantity of other cosmic radiation as well increases with the increase in altitude (Kendall, 2005), but these cosmic rays are less efficient than solar UV radiation in damaging life (Galante and Horvath, 2007).

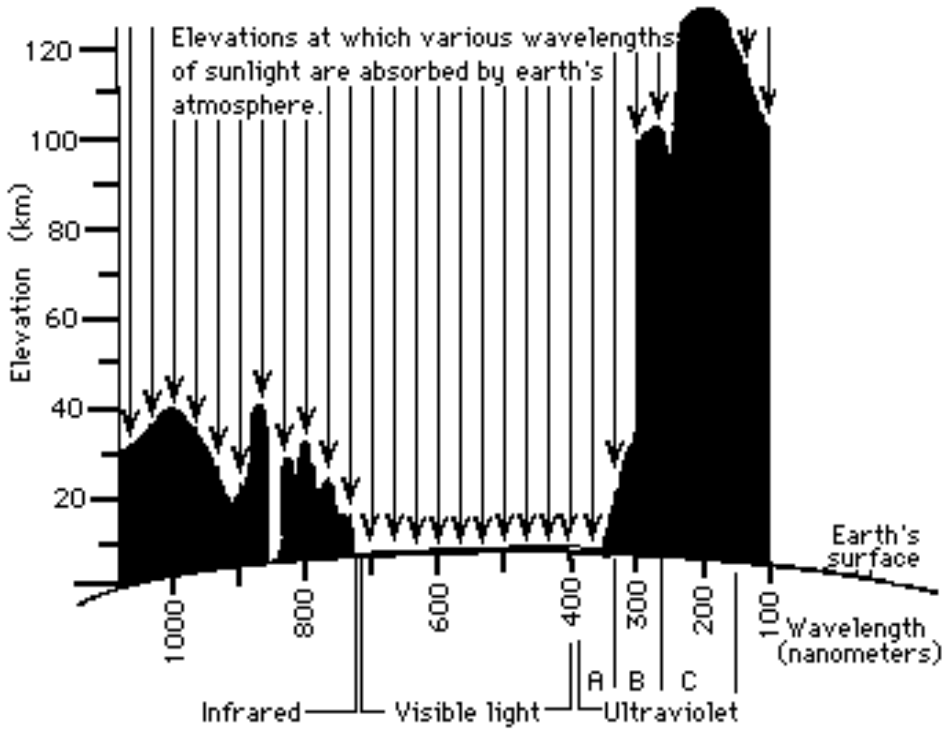


Figure 1.10 Latitude and elevation of the location with the absorption of different radiations. (http://www.nas.nasa.gov/About/Education/Ozone/radiation.html).

UV resistance is mediated by several well recognized pathways of enzymatic repair of DNA damage (Harm *et al*., 1980 and Van Houten, 1990), although overall UV tolerance may also depend on behavioral and physiological traits, such as cell pigmentation, morphology and phototaxis (Meltzer and Rice 1988).

1.5 Molecular biology techniques

The difficulty of assessing the true microbial communities by microscopic and cultural methods has hindered the investigations of prokaryotic biodiversity for some time. In pure culture, less than 0.1% of the total microbial population of a natural habitat has been successfully isolated, presumably because bacteria are extremely selective with their growth requirements; therefore, a wide variety of media must to be used to isolate microbes from the environment (Hill *et al*., 2000).

Molecular methods of identifying microorganisms have a high specificity and sensitivity (Martin *et al*., 2000). The identification of a microorganism and the determination of its characteristics by molecular techniques require only a gene sequence and the cell used can be non-functioning cell. The most commonly used technique is 16S rRNA genes or gene fragments which are selectively amplified by the Polymerase Chain Reaction (PCR) from the whole genomic DNA extracted directly from samples (either environmental samples or else other) with or without cultivating microorganisms. Figure 1.11 illustrates the main steps required to characterize an environmental sample by comparative rRNA analysis (Amann *et al*., 1995).

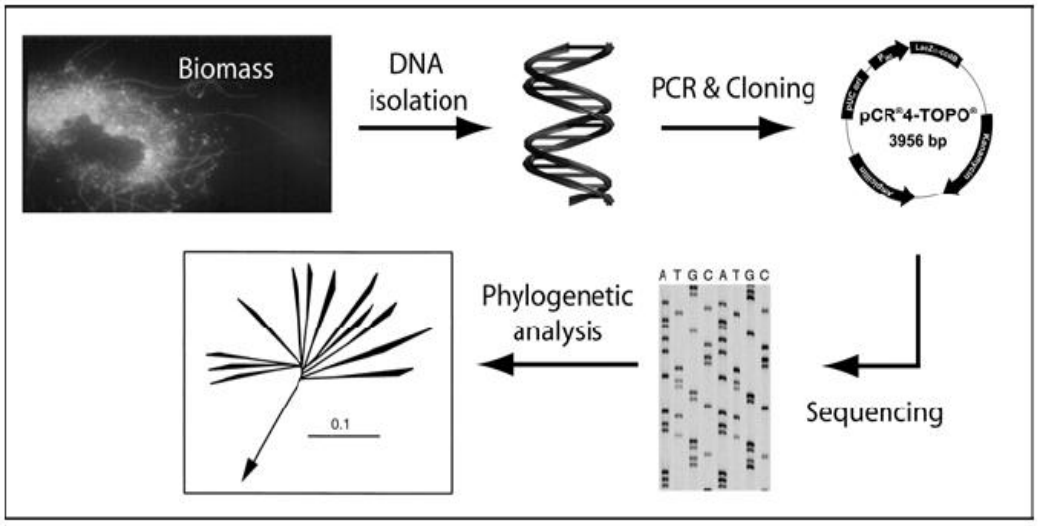


Figure 1.11 Flow chart showing different steps of identifying and characterizing any bacterium from a sample by 16S rRNA analysis. This technique starts from extracting the whole genomic DNA and ends up by sequencing and analyzing the gene (modified from Amann *et al*., 1995).

1.5.1 Deoxyribonucleic Acid (DNA)

DNA is the component of life, which passes genetic information from generation to generation in all living organisms. It is a nucleic acid that controls the genetic instructions used in the functioning and development of all known living organisms and contains the information needed to construct other cell components such as proteins and RNA molecules (van Holde, 1989). The DNA segments that contain these genetic instructions are known as genes.

Generally, DNA is a double stranded, threadlike, molecule made from deoxyribonucleotides and the backbone of the DNA strand consists of alternating sugar and phosphate residues linked by phosphodiester bonds between the 3'- hydroxyl of the sugar with 5'-hydroxyl of the adjacent sugar. The structure of the DNA helix is stabilized by van der Waals forces, hydrophobic interactions between the nitrogenous bases and the surrounding sheath of water and hydrogen bonds between complementary organic bases (a base pair). The alternating sugar and phosphate groups in each DNA strand form a backbone of DNA, and also confer directionality (Ratnayake 2004). Supposing that the double helical structure of DNA could be unwound and both strands of DNA laid side-by-side.

The four nitrogen bases found of the DNA chemical structure are adenine (A), thymine (T), cytosine (C) and guanine (G). These bases are linked to the phosphate/sugar to outline the whole nucleotide. In a double helix of the DNA, the direction of the nucleotides in one strand runs opposite to their direction in the other strand (the strands are antiparallel). However, the nitrogen bases of each strand forms the inner part of the double helix while the sugar-phosphate backbone is the outside part. The asymmetric ends of DNA strands are called the five prime (5′ end) and three prime (3′ end), with the 5' end there is terminal phosphate group and the 3' end there is a terminal hydroxyl group (van Holde, 1989).

1.5.2 Polymerase chain reaction (PCR) technique

In 1983, Kary Mullis invented and introduced the Polymerase Chain Reaction (PCR) (Saiki *et al*., 1992). PCR is a technical method used in molecular biology to exponentially amplify particular DNA sequences (either a single or few copies of a piece of DNA), generating thousands to millions of copies of a specific DNA sequences (Lexa *et al*., 2001; Fenollar *et al*., 2006; Yeung *et al*., 2009). Figure 1.12 shows how large amounts of target sequences can be amplified from few copies of a piece of DNA.

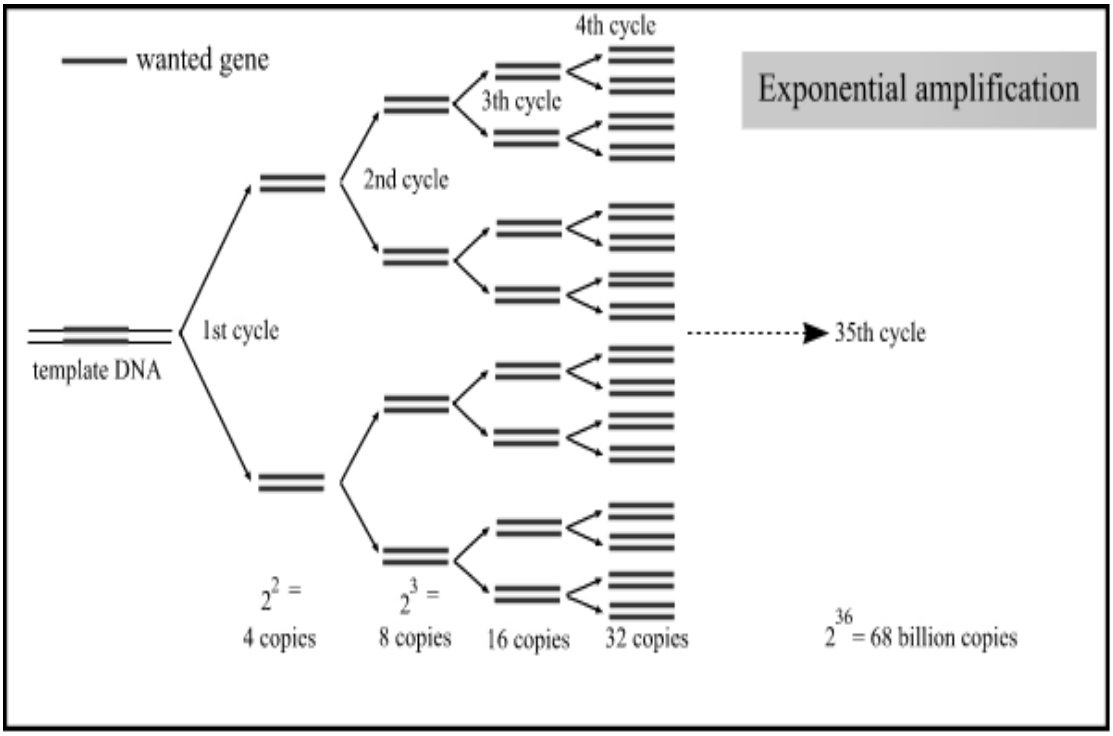


Figure 1.12 Scheme of the exponential amplification of the gene in polymerase chain reaction (modified from Vierstraete, 1999).

The PCR technique has a number of advantages compared to traditional diagnosis methods: one of these, PCR is a molecular method for amplifying target gene without using a living organism i.e. microorganisms that are not necessary to culture before their detection. Another advantage of PCR, this technique is that it is a highly precise and sensitive method for detection of nucleic acids and a practical tool for counting the

amount of particular nucleic acid present in a target sample. One more advantage is that PCR facilitates the identification of pathogens (Henson and French, 1993).

In the PCR technique, the first step is to create a mixture containing: genomic DNA template that contains the DNA region to be amplified (target), a heat-resistant DNA polymerase (commonly Taq polymerase; Taq stands for *Thermus aquaticus*), dNTPs (Deoxynucleoside triphosphates; the building blocks from which a new DNA strand is synthesized by the DNA polymerases), a pair of short DNA primers (forward and reverse primers) and a buffer solution which provides an appropriate chemical environment for best stability and possible activity of the DNA polymerase. In a computer-regulated heating block, a thermal cycler, the reaction is carried out and this thermal cycler permits rapid, controlled heating and cooling. Thermal cycles consists of a run of 20 to 40 repeated temperature (heating and cooling) changes and each cycle usually consist of 3 different temperature steps. In each cycle, the first reaction is the heating the mixture at 94^{o}C for denaturing the double stranded DNA into two single strands. Then, the reaction temperature is lowered to 45 to 65^{o}C for 20 to 40 seconds at which temperature the primers anneal to the single-stranded DNA template. In the final step of each cycle, the reaction is heated to a temperature which depends on the DNA polymerase used, commonly 72^{o}C at which Taq polymerase has its optimum activity temperature (Taq enzyme shows relative stability at DNA melting temperatures, which makes no need for enzyme replacement after each cycle and allows automated thermal cycling). Using dNTPs that are complementary to the template in 5' to 3' direction, at this step, Taq polymerase synthesizes a new DNA strand complementary to the DNA template strand. By the end of first cycle, the region between the two primers is being copied once which means making two copies of the original gene region. For each cycle of 20 to 40 cycles, there are three essential steps: denaturation step (melting of target DNA), annealing step (annealing of two

oligonucleotide primers to the denatured DNA strands) and extension/elongation step (primer extension by a thermo-stable DNA polymerase) (Henson and French, 1993).

The selection of appropriate primers is essential for the success of PCR analysis (Lexa *et al*., 2001). Practical primers must contain various specific characteristics and properties, for example: primers must be specific to the target (region that needs to be amplified), also to support the experimental conditions they must join with enough energy and thirdly, they must not lead to a weakening of the PCR reaction by forming structures.

1.5.3 16S ribosomal RNA gene (16S rRNA gene)

The gene sequencing technique called 16S rRNA has been used, since the 1980s, to detect possible phylogenetic relationships between different bacteria in order to make a molecular based-classification of bacteria from different sources, such as clinical or environmental specimens (Cai *et al*., 2003; Clarridge, 2004; Mignard and Flandrois, 2006). During the 1970's and 1980s, Carl Woese developed a scheme in which the molecular sequences of highly conserved molecules could be used to identify possible phylogenetic characteristics and relationships between bacterial species (Pace, 1997). Then Olsen *et al*. (1986) stated that three forms of rRNA molecules are found in the ribosomes of microorganisms, these include, 5S rRNA (120 nucleotides), 16S rRNA (1542 nucleotides) and 23S rRNA (3000 nucleotides). For the purpose of characterizing microorganisms, the first attempts to use rRNA were done by extracting the 5S rRNA molecules. However, the information content of 5S rRNA, which is approximately l20 - nucleotide long molecule, is too small and as a result it was neglected in favour of the ~ 1,500 nucleotide long 16S rRNA gene.

16S rRNA gene sequence is made of conserved and variable regions which are often very precise in term of species specificity. The 1,500 nucleotide long 16S rRNA gene is large enough, to contain statistically relevant sequence information. This type

and size of gene are also appropriate for phylogenetic studies of related microorganisms and for the phylogenetic relationship analysis between phyla and families (Amann *et al*., 1995).

The ends of the gene are well conserved among all domains of bacteria and archaea and so the whole gene can be amplified using PCR (Giovanonni *et al*., 1990). The 16S rRNA gene sequence has been established for many isolates; one of the biggest databank of nucleotide sequences is the Gene Bank which contains more than 20 million deposited sequences of which more than 90,000 are of 16S rRNA gene. This means that there are numerous formerly deposited sequences against which one can compare sequence of an unknown isolate (Clarridge, 2004).

1.5.4 Phylogenetic analyses

Phylogenetic trees construction has turned out to be a very practical tool for collecting all the necessary information concerning the evolutionary relationship between the recently obtained sequences and analysis of the historical evolutionary relationships among different groups of organisms (Olsen, 1987; Cantarel *et al*., 2006). Phylogenetic trees play an essential role in determining how close or distant a particular DNA sequence relates to other sequences, and permits the characterization of microorganisms on the basis of phylogenetic similarity (Bull and Wichman, 2001). Therefore, phylogenetic analyses are derived from the comparison of the ribosomal sequences with already identified ones, which are obtained from huge databases which are accessible worldwide (Maidak *et a1*., 2001).

1.5.5 Fluorescent *In Situ* hybridisation (FISH)

Fluorescent *in situ* hybridisation (FISH), as a culture-independent technique, allows the simultaneous identification, enumeration, visualization, and localization of individual microbial cells within a community in their natural habitat. This technique also provides insights into community diversity and structure, abundance of specific

types of microorganisms, and spatial distribution (DeLong *et al.* 1989; Amann *et al.* 1995). Recently, FISH has been used to visualize uncultured microbial cells from a wide range of environments (Eilers *et al.,* 2000; Gonzalez-Toril *et al*., 2003), in addition it has been applied to study microbial biofilms and communities (Bond *et al.,* 2000; Ferrari *et al.,* 2006). For example, Rudolph *et al*. (2001) showed that a microbial community comprising novel bacterial and archaeal species living in the cold sulphurous marsh water of Sippemauer Moor (Germany) were shown to consist of strings of pearls-like morphologies using FISH.

FISH relies mainly on the application of fluorescently-labelled oligonucleotide probes which specifically hybridised to complementary target sequences within an intact target cell. 16S rRNA is the most commonly used target molecule for FISH because of its genetic stability, its domain structure with variable and conserved regions and a high copy number (Woese, 1987). Oligonucleotide probes of this technique can be designed at a taxonomic level according to the region of the rRNA targeted (Amann *et al*., 1995) and new oligonucleotide probes targeting different phylogenetic levels are continually being designed (Nercessian *et al.,* 2004; Rusch and Amend, 2004) allowing assignation of the targeted organism to a phylogenetic group (Amann *et al*., 1990b).

However, FISH has also many limitations and the most common problem is autofluorescence of the organisms themselves or autofluorescence of sample detritus such as minerals and other soil particles which may interfere with target cell detection (Moter and Gobel, 2000; Bertaux *et al.,* 2007). Another limitation is the low fluorescent signal from the fluorescent probes which may be a consequence of the insufficient permeabilisation of cell walls using standard fixation methods, poor rRNA accessibility for the probes or low ribosomal content of the cells because of low metabolic activity (Poulsen *et al.,* 1993; Bhatia *et al.,* 1997). To overcome the limitations of FISH, several modifications and new technologies have been applied to it such as the use of

polynucleotide probes with multiple fluorescent labels and the use of peptide nucleic acids (DeLong and Taylor, 1999; Zimmermann *et al.*, 2001). These modified techniques have aided, for example, the detection of marine plankton previously undetectable with standard oligonucleotide probes (Pernthaler *et al.*, 2003). In comparison to traditional FISH, catalysed reporter deposition (CARD)-FISH (an *in situ* amplification method utilizing horseradish peroxidase, which enhances bacterial cell detection) results in increased signal intensity (Schönhuber *et al.*, 1997) and therefore allow for example, clear signal detection above the autofluorescent background in certain bacteria particularly cyanobacteria (Pernthaler *et al.*, 2002). Although these new and modified technologies can represent useful tools for the detection, as well as identification, of different microorganisms within environmental communities, these modifications are associated with very high costs and are time-consuming compared to the traditional FISH approach (Wagner *et al.*, 2003).

1.6 Aims of the project

A number of samples were collected from various extreme and non-extreme environments. A range of techniques, molecular biology in addition to classical microbiology techniques, were used to isolate microorganisms, mainly archaea and bacteria from unusual environments.

An effort was made here to isolate halo-bacteria and archaea from non-saline environments (Chapter 2) and alkaliphilic bacteria and archaea capable of withstanding low pH values (Chapter 4). In addition the isolation of ultraviolet-type C tolerant bacteria were isolated from terrestrial environments carried out (Chapter 6) using phylogenetic analysis of the 16S rRNA gene sequences. To understand how such microorganisms are adapted for growth (or perhaps merely survival), detailed analysis of their halophilic (Chapter 3) and alkaliphilic (Chapter 5) physiology for adaptation

in the environment was investigated using nuclear magnetic resonance spectroscopy (NMR) and atomic emission spectophotometery (AES).

A selection of other molecular techniques were used in this thesis. Fluorescent *in situ* hybridisation (FISH) was used to allow simultaneous identification, enumeration and visualization of individual microbial cells within a community in their natural habitat (Chapter 7). In addition, PCR was used to detect culturable-independent mycoplasma DNA in environmental samples (Chapter 8).

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> (2) Ultraviolet (UV) photons harm the DNA molecules of living organisms in different ways. In one common damage event, adjacent bases bond with each other, instead of across the â€œladder.â€ This makes a bulge, and the distorted DNA molecule does not function properly.
> which are published online at the following website (http://earthobservatory.nasa.gov/Features/UVB)
> I appreciate you great help
>
> Khalid Alabri
> PhD student
> Department of Molecular Biology& Biotechnology
> University of Sheffield
> Firth Court
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>
>
>
> User clicked from: http://earthobservatory.nasa.gov/Features/UVB/
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> NASA Earth Observatory
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Robert Simmon â€¢ NASA Earth Observatory
<http://earthobservatory.nasa.gov/> â€¢ (301) 614-6201