Isolation and Characterization of Novel Bacteria and Bacteriophages from the Haloalkaline Lake Elmenteita

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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To my parents who made great sacrifices to make me who I am today, for their advice and guidance. To all my family members for moral support all through my studies. God bless you all.

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ABBREVIATIONS AND ACRONYMS

µg	Microgram
µl	Microliter
µm	Micrometer
AFLP	Amplified Fragment Length Polymorphism
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BCRC	Bioresource Collection and Research Center
BLAST	Basic Local Alignment Search Tool
bps	Basepairs
cAMP	Cyclic Adenosine Monophosphate
CECT	Spanish Type Culture Collection
CFB	Cytophaga Flavobacterium Bacteroides
DAAD	German Academic Exchange Service
DDH	DNA DNA Hybridization
DNA	Deoxyribonucleic Acid
DPG	Diphosphatidylglycerol
dsDNA	Double Stranded Deoxyribonucleic Acid
DSMZ	German Collection of Microorganisms and Cell Cultures
fem.	Feminine
G + C	Guanine and Cytosine
gen.	Genetive
GLC	Gas Liquid Chromatography
Gr.	Greek
HPLC	High Perfomance Liquid Chromatography
ICTV	International Committee on the Taxonomy of Viruses
IJSEM	International Journal of Systematic and Evolutionary Microbiology
INSDC	International Nucleotide Sequence Database Collaboration
ISP	International Streptomyces Project
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KACC	Korean Agricultural Culture Collection

kb	Kilo Basepair
KCTC	Korean Collection for Type Cultures
KDa	Kilo Daltons
KOH	Potasium Hydroxide
kV	Kilovolt
KWS	Kenya wildlife services
LB	Luria Bertani
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization -Time of Flight
MB	Marine Broth
mg	Milligram
MK	Menaquinone
ml	Milliliter
MLSA	Multi Locus Sequence Analyses
mM	Millimolar
mm	Millimeter
MS	Mass Spectra
n.	Noun
N. L.	Neo Latin
NACOSTI	National Commission for Science Technology and Innovation
$NaHCO_3$	Sodium Hydrogen Carbonate
NCBI	National Center for Biotechnology Information
NEMA	National Environmental Management Authority
nm	Nanometer
OH	Hydroxyl
opm	OmniLog Phenotype Microarray
ORFs	Open Reading Frames
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
PFGE	Pulsed Field Gel Electrophoresis
PFU	Plaque Forming Unit
PG	Phosphatidylglycerol

PI	Phosphatidylinositol
PIM	Phosphatidylinositolmannosides
PME	Phosphatidylmethylethanolamine
r.p.m	Rotations per Minute
RAPD	Randomly Amplified Polymorphic DNA
rep-PCR	Repetitive Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sp. nov.	Novel species
TE	Tris-EDTA
TEM	Transmission Electron Microscope
TLC	Thin Layer Chromatography
tRNA	Transfer Ribonucleic Acid
UV	Ultra Violet
V/cm	Volt per Centimeter
v/v	Volume per Volume
w/v	Weight per Volume

ABSTRACT

There is need to investigate novel cultivation approaches and develop new equipment to recover many previously uncultured microbes, classify and catalogue them as they provide a valuable resource for research and the development of modern biotechnology. A combination of both culture-dependent and culture-independent methods has been previously used to document the microbial diversity of Lake Elmenteita. The aim of this study was to isolate novel microorganisms from the sediments of the haloalkaline lake Elmenteita using media supplemented with signal molecule cAMP and characterize by polyphasic taxonomy. The novel bacteria's colony characteristics, features of the cell, biochemical, physiological and chemotaxonomic markers were determined. Genotypic characteristics were also studied. Bacteriophages were also isolated from sediments using indigenous bacterial hosts. The resultant bacteriophages were characterized by morphology, growth characteristics, host range, structural proteins and genomes by restriction endonuclease analysis and pulsed-field gel electrophoresis. The bacteriophages were further sequenced, annotated and analysed using various bioinformatic tools. Three novel bacterial strains designated No.164^T, No.7^T and No.156^T were isolated, characterized and identified as *Belliella kenyensis*, *Streptomyces alkaliphilus* and *Nocardiopsis mwathae,* respectively. The 16S rRNA gene sequences were deposited in GenBank (KF976732, KF976730 and KF976731). Different phage morphotypes were isolated. They all could be assigned to order *Caudovirales* and Families *Siphoviridae, Myoviridae and Podoviridae*. Only 14 phages with clean cultures were further characterized. The phages were stable over a wide range of temperature with optimum at 30-35°C. pH range was optimum at 8.0-10.0.Protein profiles, restriction fragment length polymorphism analysis were distinct for each of the phages. The complete nucleotide sequences of the bacteriophages varied in size from 30, 926 bp-145, 844 bp and had a G + C content of between 39.84-52.08 %. 56-192 potential open reading frames were identified and annotated. Majority had ATG as start codon.The genome sequences gave insight into genome architecture and content in terms of gene function and also the relatedness of the phages. The phage genomes also significantly add to our understanding of phage diversity in the Lake.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

Lake Elmenteita is situated on the floor of the Kenyan Rift Valley at 1776 m above sea level and has no direct outlet (Melack, 1988). The region is characterized by a hot, dry and semi-arid climate with a mean annual rainfall of 700 mm. Due to the high temperatures of between 30-40^{o}C very high evaporation rates occur during the dry seasons leading to a reduction in the total surface area. The size of Lake Elmenteita is approximately 20 km^{2} and the depths rarely exceed 1.0 m (Mwaura, 1999). The alkalinity of the water is high (1 200 mg $CaCO_3$/l) the water temperature ranges between 30 and 40^{o}C and the pH is above 9 with a high concentration of carbonates, chlorides and sulphates (Mwirichia et al., 2010a). The groups of bacteria able to grow under such alkaline conditions in the presence of salt are referred as haloalkaliphiles. They require special adaptation mechanisms to survive and grow under salinity and alkaline pH. These properties of dual extremity of halophiles and alkaliphiles make them interesting from both, fundamental research and biotechnological points of view.

The ability to recover and analyse 16S rRNA genes directly from environmental DNA provides a means to investigate the taxonomic composition of microbial populations in any environment (Dojka et al., 2000) and expand our knowledge about diversity but do not unravel their metabolic capabilities (Mwirichia et al., 2011) In order to be able to link the structure and function, cultivation approaches need to be directed toward the isolation of characteristic members. It is essential that organisms be isolated in pure culture and their characteristic features be tested under standard conditions (Stackebrandt et al., 2002). The significant contribution made by microorganisms in ecosystem sustainability as well as the industrially important biomolecules obtained from them: antibiotics, anti-cancer drugs, enzymes, biofuel and various other compounds, implies that cataloging them is imperative. Effective microbial identification system is pertinent to catalog their diversity as it exists (Sarethy et al., 2014). For prokaryotes, taxonomy plays an essential role in enabling

the reliable identification of microbial strains (Moore et al., 2010). Collectively phenotypic, chemotaxonomic and genotypic methods for determining taxonomic position of microbes constitute what is known as the 'polyphasic approach' for bacterial systematics. This approach is currently the most popular choice for classifying bacteria and several microbes (Prakash et al., 2007). Taxonomic information is essential, as it enables scientists to understand the biodiversity and relationships among living organisms from different ecosystems (Gevers et al., 2005).

Most studies on Lake Elmenteita have however, been performed on Prokaryotes (*Archaea* and *Bacteria)* and *Eucarya* but far fewer on viruses. Bacteriophages are viruses of prokaryotes and are the most abundant and diverse group of biological entities on the planet (Pedulla et al., 2003). Apart from being ubiquitous and numerous, bacteriophages also play fundamental roles in structuring the microbial food webs, primarily by killing microbes (Fuhrman, 1999), by governing microbial diversity and diversification (Weinbauer, 2004) and to a lesser extent, by being a potential food source for protists (Gonzalez & Suttle, 1993; Bettarel et al., 2005). Natural phages are presumed to be the largest reservoir of uncharacterized genetic diversity on earth (Suttle, 2005; Hambly & Suttle, 2005) and theories regarding their role in shaping the diversity of prokaryotes have been proposed (Thingstad & Ligell, 1997; Winter et al., 2005). A comprehensive picture of their diversity will therefore help to understand the role of viruses in microbial ecology and the entire ecosystem. A study on phages in Lake Elmenteita will increase our knowledge of phage diversity, biology and genetic relationship in the lake. Insight into bacteriophage genomics will further knowledge of gene function and how they may be exploited for biological and biotechnological ends.The aim of this study was to isolate novel bacteria from the haloalkaline lake Elmenteita using media supplemented with signal molecule cAMP and characterize by polyphasic taxonomy. The study also sought to isolate bacteriophage using indigenous bacteria and characterise by structural morphology, plaque morphology, host range, protein analysis, restriction fragment length polymorphism and genome size estimation. Further, sequencing, annotation and analysis of the phage genomes were undertaken.

1.1 PROBLEM STATEMENT

The use of molecular techniques has shown that so far only a small fraction of microbial diversity from Lake Elmenteita has been cultivated. (Mwirichia et al., 2010b; Mwirichia et al., 2011). There is still an enormous unexplored reservoir of bacteria from this Lake that could be potential source of natural compounds to be used as a resource for the development of new antibacterial, antifungal, antitumour drugs and enzymes for industrial use (Keller & Zengler, 2004). Unfortunately, only a small percentage of bacteria can be cultivated using traditional cultivation methods (Zengler et al., 2002). New methods and techniques are needed to access this vast reservoir of microbial diversity.

Bacteriophages are a rich source of genetic material with potential for biological and biotechnological use (Bench et al., 2007). However, knowledge of bacteriophage diversity in Lake Elmenteita is evidently incomplete and therefore studies on phage diversity and genome sequencing in Lake Elmenteita is required.

1.2 JUSTIFICATION

The sustenance of life on earth depends on maintaining the diversity of microorganisms. Climate change and human intervention is resulting in depletion of biodiversity and many hotspots are also fast losing their endemic biodiversity. It is likely that loss of macro life forms also results in loss of the associated microbial species hence isolating and cataloging novel microorganisms is important (Sarathey et al., 2014).

Accessing novel and uncultured bacterial majority is of significant interest and has been recognized as one of the principal challenges for microbiology today (Joseph et al., 2003; Hurst, 2005). New enrichment and novel cultivation technologies are therefore important to offer considerable advantages for recovering many previously uncultured microbes.

Classification of microorganisms based on rRNA analysis has shown that the majority of microbes present in nature have no counterpart among previously cultured organisms (Mwirichia et al., 2010b; Mwirichia et al., 2011; Ráppe & Giovannoni, 2003). Establishing metabolic properties and potential of these diverse organisms in the absence of pure culture presents an immense challenge for microbial ecologists (Kaeberlein et al., 2002). A comprehensive understanding of the physiology of these organisms and of the complex environmental processes in which they engage, what secondary metabolites might be released or biotransformations might be possible undoubtedly require their cultivation.

The significant contribution made by microorganisms in ecosystem sustainability as well as the industrially important biomolecules obtained from them: antibiotics, anti-cancer drugs, enzymes, biofuel and various other compounds (Moore et al., 2010), implies that cataloging them is imperative. Cultures remain an essential requirement, not only for biodiscovery but also for a full taxonomic characterisation and to give a name to an organism. Taxonomy plays an essential role in enabling the reliable identification of microbial strains (Prakash et al., 2007).

Phages have drawn recent attention and re-appraisal as they are the most common DNA-containing entities on earth and provide a valuable resource to the development of modern biotechnology (Marks & sharp, 2000). Despite their relative abundance and importance, little is known about their diversity in natural ecosystems (Bench et al., 2007) such as in Africa (Ackermann, 2011). Lake Elmenteita is specifically unexplored and studies on the Lake should be initiated.

1.3 HYPOTHESIS

There are novel species of bacteria and bacteriophages in the haloalkaline lake Elmenteita

1.4 GENERAL OBJECTIVE

To isolate and characterize novel bacteria and bacteriophages from the haloalkaline Lake Elmenteita.

1.5 SPECIFIC OBJECTIVES

1) To isolate, characterize and identify novel bacteria from haloalkaline lake Elmenteita

3) To isolate, characterize and identify novel bacteriophages from Lake Elmentaita

3) To sequence, annotate and analyse the bacteriophage genomes

4) To delineate a possible role for the novel isolates in the soda lake ecosystem

1.6 EXPECTED OUTPUT

This study is intended to provide novel bacteria, bacteriophages and bacteriophage genomic data for further exploitation

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 INTRODUCTION

Over 1.8 million eukaryotic species are recognized, while estimates indicate that our planet hosts 5 to 10-fold more species. The lack of an extensive and accurate picture of the microbial diversity is partly due to inadequate technical advances in the microbial cultivation field (Alain & Querellou, 2009). Over the past three decades, molecular biology was an enormous driving force in microbiology in uncovering the microbial diversity (Curtis et al., 2002). Molecular ecology and metagenomics have increased significantly our knowledge of the genetic diversity and have led to interesting hypotheses (Hugenholtz & Tyson, 2008). Culture-independent 16S rRNA-based studies indicate that the previously uncultured fraction comprises numerous unknown eukaryotes and entirely novel phylogenetic groups (Glöckner et al., 2000; Gich et al., 2001; Urbach et al., 2001; Zwart et al., 2002; Warnecke et al., 2004). Considering that many of the genes stored in the databases have unknown functions or are incorrectly annotated, it is probable that metagenomes alone will not offer sufficient knowledge to cultivate all organisms (Leadbetter, 2003). In order to be able to link the structure and function of microbes, cultivation approaches thus need to be directed toward the isolation of characteristic members.

Majority of microbial species do not grow on synthetic media *in vitro* and remain unexplored (Nicholas et al., 2010). Accessing this “missing” microbial diversity is of significant interest for both basic and applied sciences and has been recognized as one of the principal challenges for microbiology today (Joseph et al., 2003; Hurst, 2005). Learning how to grow the uncultured majority is an important step towards understanding the full range of taxonomic and metabolic biodiversity in nature as well as a pathway to biotechnological discovery (Stott et al., 2008).

2.2 STRATEGIES FOR ISOLATING NOVEL BACTERIA

Species that would otherwise be culturable, fail to grow because the microbial species that dominate in natural settings are not adapted for growth in media containing high concentrations of complex organic carbon and the artificial conditions inherent in most culture media for example, extremely high substrate concentrations or the lack of specific nutrients required for growth (Ferris et al., 1996; Demming et al., 2000). Efforts to grow the uncultured bacterial majority have employed new enrichment and cultivation protocols such as diffusion chambers that allow growth *in situ* (Bollmann et al., 2007) and high-throughput systems using flow cytometry or dilution techniques to separate mixed cultures into individual cells that can then be incubated under a range of conditions (Connon & Giovannoni, 2002; Zengler et al., 2002; Bruns et al., 2003). At the same time, traditional cultivation methods of plating and batch liquid culture have continued to be effective for isolating new species, especially where media compositions, gelling agents and energy sources are diversified and optimized to particular environment. Combining new media with extended incubation times for slow-growing species, dilute media with low nutrient concentrations and low pH values, complex carbon substrates and rapid polymerase chain reaction (PCR)-based screening of bacterial colonies has led to the discovery of many novel species (Keller & Zengler, 2004; Janssen, 2006). Other media additives used to stimulate growth include signal molecules such as cAMP and homoserine lactones (Guan et al., 2000; Bruns et al., 2002). An additional strategy for obtaining new bacteria in culture is to start from unusual ecosystems as they offer a great diversity as compared to anywhere else in the biosphere. Bacteria from remote areas still remain virtually unexplored and there is no doubt that extreme environments are a rich source of microorganisms of biotechnological importance (Keller & Zengler, 2004).

2.3 PHYSICO-CHEMICAL PROPERTIES OF SODA LAKES

Soda lakes in the Kenya's Great Rift Valley include Lake Elmenteita, Magadi, Bogoria, Nakuru and Sonachi. Their development is a consequence of geological and topological factors (Mwatha, 1991). Soda lakes were formed by combination of environmental factors, which result in large amount of sodium carbonate and have very high concentration of Ca^{2+} and Mg^{2+} (Grant & Mwatha, 1989; Jones et al., 1994), while the salinity of these lakes ranges from around 5% total salts (W/V) in Lake Bogoria, Nakuru, Elementaita and Sonachi but saturated in Lake Magadi with roughly equal proportions of Na_2CO_3 and Nacl as major salts (Mwatha, 1991; Lanzén et al., 2013).

The Great Rift Valley running through Eastern Africa is a semi-arid tropical zone where evaporation exceeds the rate of inflow of water. Dissolved minerals concentrate into alkaline brines with carbonate as the major anion, forming shallow soda lakes (Jones et al., 1994). Studies on these lakes have shown that they are habitats of novel species of bacteria and archaea (Duckworth et al., 1996). They are extremely productive because of high ambient temperatures, high light intensities and unlimited supplies of CO_2 (Grant, 1992) hence feature considerable microbial diversity (Zarvarzin et al., 1999). Lakes such as Bogoria and Elmenteita are characterized by hot springs which host both hyperthermophilic and haloalkalithermophilic microorganisms. Hyperthermophilic bacteria and archaea represent the organisms at the upper-temperature limits of life (Stetter & Zillig, 1985; Brock, 1986; Stetter, 1996). They grow fastest between 80 and 105°C and are unable to grow below 60°C. Their adaptations towards high pH and elevated temperature draw attention not only as a source of industrially valuable enzymes but also for studying adaptive mechanisms to extreme environmental parameters.

2.4 PREVIOUS STUDIES ON SODA LAKES

The Rift Valley soda lakes have been subject to various studies. Cultivation-dependent and independent surveys of the soda lakes in the Rift valley has resulted in the isolation of several hundred strains of aerobic, heterotrophic and (halo) alkaliphilic bacteria and archaea (Duckworth et al., 1996; Grant and Sorokin, 2011) and the detection of several novel lineages of putative Bacteria and Archaea (Grant et al., 1999; Rees et al., 2004).

2.4.1 Bacterial diversity in Kenyan soda lakes

Studies on the low saline lakes of the Kenyan Rift Valley such as Bogoria, Crater Lake Sonachi, Elementeita and Nakuru revealed the presence of diverse populations of aerobic sulfur oxidizing bacteria of genera *Thioalkalimicrobium* and *Thioalkolivibrio* (Sorokin et al., 2001). Anaerobic alkalithermophiles from Lake Bogoria include *Thermosyntropha lipolytica* (Svetlitshnyi et al., 1996). A large amount of research involving Lake Magadi to try to purify and culture novel forms of bacteria able to live in alkaline lakes saw two haloalkaliphilic strains *Spirochaeta alkalica* and *S. africana* isolated (Zhilina et al., 1997). In 2004, eight new strains of denitrifying bacteria were found in a lagoon with a pH of 10 (Boltianskaya et al., 2004). Another research in 2007 described experiments that isolated a new genus and species of bacteria known as *Methylohalomonas lacus* (Sorokin et al., 2007). Organotrophic bacteria of the phylum Actinobacteria, namely *Bogoriella caseilytica* (Groth et al., 1997) and Cellulomonas *bogoriensis* (Brian et al., 2005) have been described from Lake Bogoria. Others are members of the genus *Dietzia natronolimnaea* (Duckworth et al., 1998), the genera *Arthrobacter* and *Terrabacter* (Duckworth et al., 1998) from Lake Oloiden, Kenya. Phototrophic eukaryotes of the diatoms belonging to the genera *Nitzchia* and *Navicula* are predominant in these ecosystems (Tindall et al., 1984). Other groups represented include alkaliphilic anoxygenic phototrophic bacteria, mainly of the genera *Ectothiorhodospira* and Halorhodospira (Melack & Kilham, 1974). Anoxygenic phototrophic bacteria are also capable of forming visible blooms in soda lakes and members of the genera

Ectothiorhodospira and *Halorhodospira* provide substantial contributions to primary production (Grant et al., 1990; Grant & Horikoshi, 1992; Grant, 2006). The genera *Ectothiorhodospira* and *Halorhodospira* are able to oxidise sulphide to sulphate, depositing extracellular elemental sulphur (Hecky & Kilham, 1973). Remarkable primary productivity supports a diverse and stable population of aerobic organotropic bacteria in soda lakes (Tindall et al., 1980). Anaerobic groups consist of the acetogenic ammonifiers and alkaliphilic hydrogenotrophic sulphate reducers *Desulfonatronovibrio* and *Desulfonatronum*, obligately autotrophic sulphur-oxidizing bacteria, methane-oxidizing *Methylobacter alcaliphilus* and alkaliphilic *Methylomicrobium* sp. able to oxidize methane and ammonia (Zarvazin et al., 1999).

Kenyan soda lakes have revealed a typical predominance of dense blooms of Cyanobacteria in less saline alkaline lakes (Mwirichia et al., 2010b). The predominant filamentous species are *Spirulina platensis, Spirulina maxima* and *Cyanospira* (Anabaenopsis) (Melack & Kilham, 1974; Tindall et al., 1984; Florenzano et al., 1985). The unicellular species Chorococcus *spp., Synechococcus sp.* or *Synechocystis* have also been found, and in some cases they may be the dominant primary producers (Grant et al., 1990; Mwatha & Grant, 1993; Grant, 2006).

In an attempt to isolate novel groups of bacteria from Lake Elmenteita, different media with filter-sterilised water from the lake was used. The majority of the resultant isolates were affiliated to the class Gammaproteobacteria and to the genus *Bacillus*. Other groups recovered were related to *Marinospirillum, Idiomarina, Vibrio, Enterococcus, Alkalimonas, Alkalibacterium, Amphibacillus, Marinilactibacillus* and the actinobacteria *Nocardiopsis* and *Streptomyces*. Novel taxa were identified which had not been isolated previously from the soda environment (Mwirichia et al., 2010a). Further, a culture-independent approach was also used to study the bacterial diversity. The results indicated the presence of 37 orders in the Domain bacteria. *Cyanobacteria* and members of the phylum *Firmicutes* group were the most represented. 93.1% of the sequenced clones had similarity values below 98% to both cultured and as yet uncultured bacteria

(Mwirichia et al., 2011). In a study to isolate alkaliphilic bacteria from Lake Magadi, Kambura et al. (2012) used different media prepared with filter-sterilised water from the lake. Analysis of partial sequences of 16S rRNA genes using showed 80% of the isolates were affiliated to the genus *Bacillus* and 20% were affiliated to members of *Gammaproteobacteria*. Culture-independent 16S rRNA-based studies indicate that the previously uncultured fraction comprises numerous unknown bacteria and entirely novel phylogenetic groups.

2.4.2 Mycological diversity in Kenyan soda lakes

Despite the general preference of fungi to grow at neutral or slightly acidic pH, some have been shown to grow at high pH too (Steiman et al., 2004). However, the diversity of filamentous fungi that can grow at high ambient pH values (8-11) remains largely understudied in the soda lakes of the Kenyan Rift Valley. Some of the studies to isolate and characterize Fungi from these environments saw the isolation of genera, *Acrimonies*, *Scopulariopsis*, *Verticilium*, *Fusarium* and *Paecilomyces* from lake Sonachi (Ndwiga et al., 2014) and microorganisms belonging to the genus *Penicillium*, *Aspergillus*, *Polyzellus*, *Fusarium*, *Neurospora* while others clustered closely with uncultured fungus, from lake Magadi (Salano, 2011). A recent study to systematically characterize the alkaliphilic and alkalitolerant filamentous fungi isolated from alkaline (soda) soils resulted to isolation of two new obligate alkaliphilic species of *Sodiomyces* from lake Magadi (Grum-Grzhimaylo et al., 2015).

2.4.3 Archael Diversity in Kenyan soda lakes

Kenyan Rift Valley lakes extreme environments have been shown to harbor a high archaeal diversity affiliated to a wide range of genera including the genera *Natronococcus*, *Halovivax*, *Halobiforma*, *Halorubrum*, and *Halalkalicoccus* (Mwirichia et al., 2010b), genera *Natronobacterium* and *Natronococcus* (Tindall et al., 1984; Grant et al., 1999). Several 16S rRNA gene sequences related to putative novel Archaea (Euryarchaeota) have been retrieved from the alkaline saltern at Lake

Magadi, Kenya (Grant et al., 1999). Haloalkaliphilic Archaea related to *Natronomonas*, *Natrialba*, *Natronolimnobius* and *Halorubrum* spp. have also been isolated from Lake Magadi (Grant & Sorokin, 2011). A metagenomic study on lake Magadi showed dominamce of Archaea. The closest match was generally to *Natronobacterium* (now proposed as *Natronomonas*) *pharaonis* (Kamekura et al., 1997), with identities to species such as *Natronococcus occultus*, *Natrobacterium* (now *Natrialba*) *magadii*, or *Natronobacterium* (now *Halorubrum*) *vacuolatum*. The study showed the highest percentage of the clones belonged to uncultured members of the Domain Archaea in the order *Halobacteriales* (Mwirichia et al., 2010b).

2.4.4 Phage Diversity in Kenyan soda lakes

Viruses from these environments are particularly understudied. Previous virus studies on the soda lakes in the Kenyan Rift Valley include isolation of a phage infecting a haloalkaliphilic bacteria from Lake Magadi by Muruga et al. (2013), isolation and study of a bacteriophage infecting an alkaliphilic *Vibrio metschnikovii* from Lake Magadi (Moulton et al., 2011) that saw the isolation of a bacteriophage Ø1M3-16, tailed bacteriophage with a morphotype consistent with the family Siphoviridae. Electron microscopic study of cyanophages that affect African flamingo population in Lake Nakuru (Peduzzi et al., 2014) revealed the highest numbers of viruses ever reported in any natural aquatic environment (up to $7.0x10^{9}$ ml^{-1}). This strongly supported the significant role that viruses may have in such an environment, for example, as important mechanism of mortality in prokaryotes. Study on viruses (bacteriophage, cyanophage and virus of archaea) inhabiting soda lakes in Kenyan Rift Valley is quite limited and much needs to be done.

CHAPTER THREE

3.0 *BELLIELLA KENYENSIS* SP. NOV., ISOLATED FROM THE HALOALKALINE LAKE ELMENTEITA IN THE AFRICAN RIFT VALLEY

ABSTRACT

A red-pigmented, Gram-reaction-negative, aerobic bacterial strain, designated No.164^T, was isolated from sediment sample from the alkaline Lake Elmenteita located in the Kenyan Rift Valley. Results of 16S rRNA gene sequence analysis indicated that the isolate belonged to the genus *Belliella*, with the highest sequence similarity (97%) to *Belliella pelovolcani* DSM 46698^T. Optimal growth temperature was 30-35°C, at pH 7.0-12.0 in the presence of 0-4% (w/v) NaCl. Flexirubins were absent. The respiratory menaquinone (MK-7), predominant cellular fatty acids (*iso*-$C_{15:0}$, *anteiso*-$C_{15:0}$ and a mixture of $C_{16:1\omega}$7c and/or *iso*-$C_{15:0}$ 2-OH) and DNA G + C content (38.1 mol%) of strain No.164^T were consistent with those of other members of the genus *Belliella*. The polar lipids consisted of phosphatidylethanolamine, eight unspecified lipids and one unspecified phospholipid. Several phenotypic characteristics can be used to differentiate this isolate from those of other *Belliella* species. The polyphasic data presented in this study indicated that this isolate should be classified to represent a novel species in the genus *Belliella*. The name *Belliella kenyensis* sp. nov. is therefore proposed; the type strain is strain No.164^T (= DSM 46651 = CECT 8551). The INSDC accession number for the 16S rRNA gene sequence of strain No.164^T is KF976732

3.1 INTRODUCTION

A globally distributed, environmentally abundant group of microorganisms, the phylum *Bacteroidetes* or CFB group (Ludwig & Klenk, 2001) is considered to be a bacterial group of special relevance for aquatic environments (Brettar, 2004). *Bacteroidetes* constitute a significant proportion of marine microbial communities (Glöckner et al., 1999) and are known to play an important role in the biogeochemical cycling and for the degradation of organic matter such as complex polysaccharides (Höfle, 1992; Pinhassi et al., 1999; Cottrell & Kirchman, 2000). The family *Cyclobacteriaceae* (a member of the class *Cytophagia,* phylum '*Bacteroidetes*') was proposed (Ludwig et al., 2008) and comprises six genera which includes the genus *Belliella.* The genus *Belliella* to which this novel strain will be assigned currently comprises two species; *B. pelovolcani* isolated from a rare mud-volcano in Wandan, Pingtung County, Taiwan (Arun et al., 2009) and *B. baltica* isolated from the Baltic sea (Brettar et al., 2004). In this paper we describe morphological, physiological and chemical data which support the identification of isolate No.164^T, as representing a novel species of the genus *Belliella* isolated from a soda lake, Lake Elmenteita in the Kenyan Rift Valley.

3.2 SAMPLING SITE

Lake Elmenteita is situated at 0^{o}27´S, 36^{o}15´E on the floor of the Kenyan Rift Valley at 1776 m above sea level and has no direct outlet (Melack, 1988). The region is characterised by a hot, dry and semi-arid climate with a mean annual rainfall of about 700 mm (Mwaura, 1999). Due to the high temperatures there are very high evaporation rates during the drier seasons leading to a seasonal reduction in the total surface area. The size of Lake Elmenteita is roughly 20 km^2 and the depths rarely exceed 1.0. The alkalinity of the water is high with a high concentration of carbonates (1200 mg Na_2CO_3/l), chlorides and sulphates (Mwirichia et al., 2010b). The water temperature ranges between 30-40^{o}C and the pH is above 9.

3.3 MATERIALS AND METHODS

Strain No.164^T was isolated from a sediment sample collected from Lake Elmenteita. The sediment sample (2 g) was suspended in filter sterilised lake water (10 ml) and solution serially diluted using the same water. Aliquots (100 µl) of appropriate dilutions were plated onto R2A agar (DSMZ medium 830), at pH 8 supplemented with cAMP (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 10 µM (Bruns & Cypionka, 2002). The plates were incubated at 28ºC for 14 days with constant monitoring. Colonies appearing on the plates were streaked again on to R2A agar for purification. Stocks of the isolate were frozen at -20ºC in R2A broth to which 15% glycerol (v/v) was added.

All physiological tests for this study were performed at 28ºC using strain No.164^T and cultures of *B. pelovolcani* DSM 46698^T, *B. baltica* DSM 15883^T and *Nitritalea halalkaliphila* DSM 46695^T in parallel assays. Cultural characteristics were tested on marine agar (DSMZ medium 123), R2A agar (DSMZ medium 830), LB agar (DSMZ medium 381) and nutrient agar (DSMZ medium 1) for 7 days. Colony features were observed after 5 days under a binocular microscope according to Pelczar (1957). Exponentially growing bacterial cultures were observed with an optical microscope (Zeiss AxioScope A1) with a 1000-fold magnification and phase-contrast illumination. Photomicrographs of bacterial cells of strain No.164^T grown in marine broth after 3 days were taken with a field-emission scanning electron microscope (FE-SEM Merlin, Zeiss, Germany). Gram reaction was performed using the KOH test described by Gregersen (1978). Motility was determined on wet mounts under phase contrast microscopy (magnification, 400-fold). Growth was assessed at 10-55ºC (in increments of 5ºC), pH values from 5.0-13.0 (in increments of 1.0 pH unit) and 0, 1-15% (w/v) NaCl (in increments of 2 units). Biochemical characteristics such as hydrolysis of casein, xanthine, tyrosine, starch, DNA, sensitivity to different antibiotics were determined by previously described methods (Smibert & Krieg, 1994). Anaerobic growth was checked using the Anaerocult system (Merck) on marine agar. For the detection of flexirubin-type pigments, bacterial cell mass was

exposed to a 20% (w/v) KOH solution, as described by Reichenbach (1989); this was then examined for an eventual colour shift. Cellular pigments were extracted with acetone/methanol (7:2, v/v) (Arun et al., 2009) from cultures grown on marine agar. Absorption spectra were determined with a scanning UV/visible spectrophotometer (BioMATE 6) at 350-700 nm. Strains were additionally characterized using API 20 NE and API ZYM (bioMérieux) identification systems according to manufacturer's instructions.

For chemotaxonomic studies, strain No.164^T and reference strains were grown in LB broth in a shaking incubator at 150 r.p.m, 28^{o}C for 4 days. The cell material was harvested by centrifugation and washed twice with 0.9% NaCl solution and freeze dried. All chemotaxonomical analyses were conducted under standardized conditions with strain No.164^T and cultures of the same set of reference strains as listed above for the phenotypic characterizations. The extraction and analysis of cellular fatty acids was carried out from biomass grown on marine agar plates at 28^{o}C for 3 days and harvested from the last quadrant streak. Analysis was conducted using the Microbial Identification System (MIDI) Sherlock Version 6.1 (method TSBA40 database) as described by Sasser (1990). Fatty-acid patterns were visualized as heat map using the lipid extensions of the opm package (Vaas et al., 2013). Polar lipids were extracted, separated by two-dimensional TLC and identified according to procedures outlined by (Minikin et al., 1984) with modifications proposed by (Kroppenstedt & Goodfellow 2006). Menaquinones (MK) were extracted from freeze-dried cell material using methanol as described by Collins et al. (1977) and analysed by high-performance liquid chromatography (HPLC) (Kroppenstedt, 1982).

G + C content of chromosomal DNA of strain No.164^T was determined by HPLC according to Mesbah (1989). Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR products was carried out as described by Rainey et al. (1996). The resulting sequence was compared with sequences of other type strains using the EzTaxon server (Kim et al., 2012). The 16S rRNA gene sequences closely related to strain No.164^T were downloaded from INSDC. Phylogenetic analysis was based on alignment of 16S rRNA gene sequences

from closely related type strains with validly published names inferred as described by Montero-Calasanz. (2013a). Rooting was done using the midpoint method (Montero-Calasanz et al., 2013a). Pairwise similarities were calculated as recommended by Meier-Kolthoff et al. (2013) and Montero-Calasanz et al. (2013b). DDH experiments were not carried out between strain No.164^{T} and its closest phylogenetic neighbours *B. pelovolcani* (DSM 46698^{T}) and *B. baltica* (DSM 15883^{T}) as the level of 16S rRNA gene sequence similarity between the strains was less than the cutoff value recommended for genomic distinction of species by Meier-Kolthoff et al. (2013) and Stackebrandt & Goebel (1994).

3.4 RESULTS AND DISCUSSION

Strain No.164^{T} was Gram-reaction-negative, aerobic, pleomorphic rods (Plate 3.1), and showed no motility. It grew very well in marine medium, moderately in R2A and LB. On MB agar, the isolate formed small red colonies with entire margins and were non adherent to the agar. The culture grew over a temperature range of 20-40ºC (optimal growth temperature was 30-35ºC), in the presence of 0–5% (w/v) NaCl (optimal range 0-4% (w/v) and between pH 7.0-12.0 (optimal range 8-10). The strain was positive for catalase and DNase activity, degradation of casein and hydrolysis of starch but negative for xanthine and tyrosine degradation.

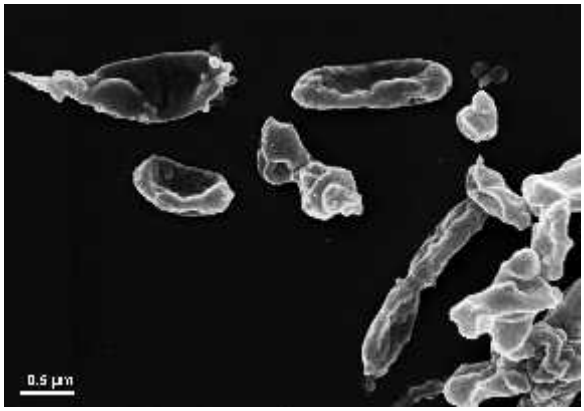

Plate 3.1: Scanning electron micrograph of strain No. 164^{T} grown in marine broth at 28ºC for 3 days

In the flexirubin test, the strain did not show a colour shift after alkalinization with 20% KOH. The isolate produced a red coloured pigment, a feature characteristic of the order *Cytophagales* (Reichenbach, 1992), to which the strain belongs. The spectrum of the pigment extract of the strain showed a broad peak with a maximum around 475 nm typical for carotenoids (Figure 3.1). The strain contained carotenoids but no flexirubins as often observed for marine CFB organisms (Reichenbach et al., 1981).

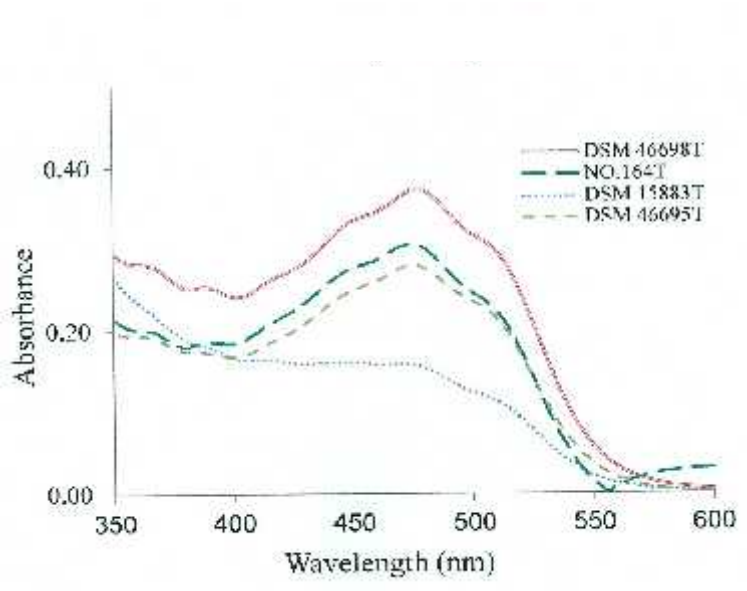

Figure 3.1: Absorption spectra of pigments extracted from strains: 1, *Belliella kenyensis* sp. nov. No. 164^{T}; 2, *B. pelovolcani* DSM 46698^{T}; 3, *B. baltica* DSM 15883^{T} and 4, *Nitritalea halalkaliphila* DSM 46695^{T}.

Strain No.164^{T} did not grow anaerobically on marine agar. The fatty acid profiles were dominated by branched-chain fatty acids (*iso*-$C_{15:0}$ (30.6%), *anteiso*-$C_{15:0}$ (14.3%), Summed feature 3 comprising $C_{16:1\_\omega7c}$ and/or *iso*-$C_{15:0}$2-OH (8.1%) and *iso*-$C_{17:0}$3-OH (7.7%) (Table 3.1) which is similar to those observed in other representatives of the genus but differs in quantities (Arun et al., 2009; Brettar et al., 2004) and fits to the features reported by Anil Kumar et al. (2010) in their emended description of the genus. Fatty-acid patterns were also visualized as heat map (Figure 3.2).

Table 3.1: Cellular fatty acid compositions of strain No.164^T and the reference strains.

Values are (≥1) percentages of total fatty acids. Strain 1, Belliella kenyensis sp. nov. No.164^T; 2, B. pelovolcani DSM 46698^T; 3, B. baltica DSM 15883^T and 4, Nitritalea halalkaliphila DSM 46695^T.

Fatty acid	No.164^T	*B. pelovolcani*	*B. baltica*	*Nitritalea halalkaliphila*
Straight chain				
C 15:0	1.7	6.2	1.8	-
Branched chain				
iso-C 14:0	-	-	-	2.2
iso-C 15:0	30.6	22.3	23.5	31.6
iso-C 16:0	2.0	1.6	-	8.6
iso-C 17:0	-	-	1.0	-
anteiso-C 15:0	14.3	2.3	7.2	5.1
iso-C 15:1 G	1.9	7.3	12.4	-
iso-C 16:1 H	2.3	2.0	-	7.1
iso-C 17:1_ω9c	6.2	7.0	9.9	4.5
anteiso-C 17:1_ω9c	1.1	-	-	-
Unsaturated				
C 15:1_ω6c	1.8	5.6	-	1.7
C 16:1_ω5c	1.2	1.0	3.0	1.4
C 17:1_ω6c	4.4	10.3	4.2	4.2
C 17:1_ω8c	-	1.7	-	-
Hydroxylated				
iso-C 15:0 3-OH	1.5	2.7	3.1	2.6
iso-C 16:0 3-OH	1.7	1.2	-	6.2
iso-C 17:0 3-OH	7.7	6.9	8.0	7.3
C 17:0 2-OH	1.7	-	1.0	-
Unknown fatty acids				
ECL 14.959	1.3	2.6	-	1.2
ECL 16.582	1.3	1.1	1.1	-
Summed features[a]				
Summed feature 3	8.1	9.0	5.8	5.1
Summed feature 4	4.6	4.7	5.3	6.1

All of the data are from the present study. [a] Summed feature are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 1 comprises *iso*-C 15:1 H and/or C 13:0 3-OH. Summed feature 3 comprises C 16:1_ω7c and/or *iso*-C 15:0 2-OH. Summed feature 4 comprises *iso*-C 17:1 I and/or *anteiso*-C 17:1 H.

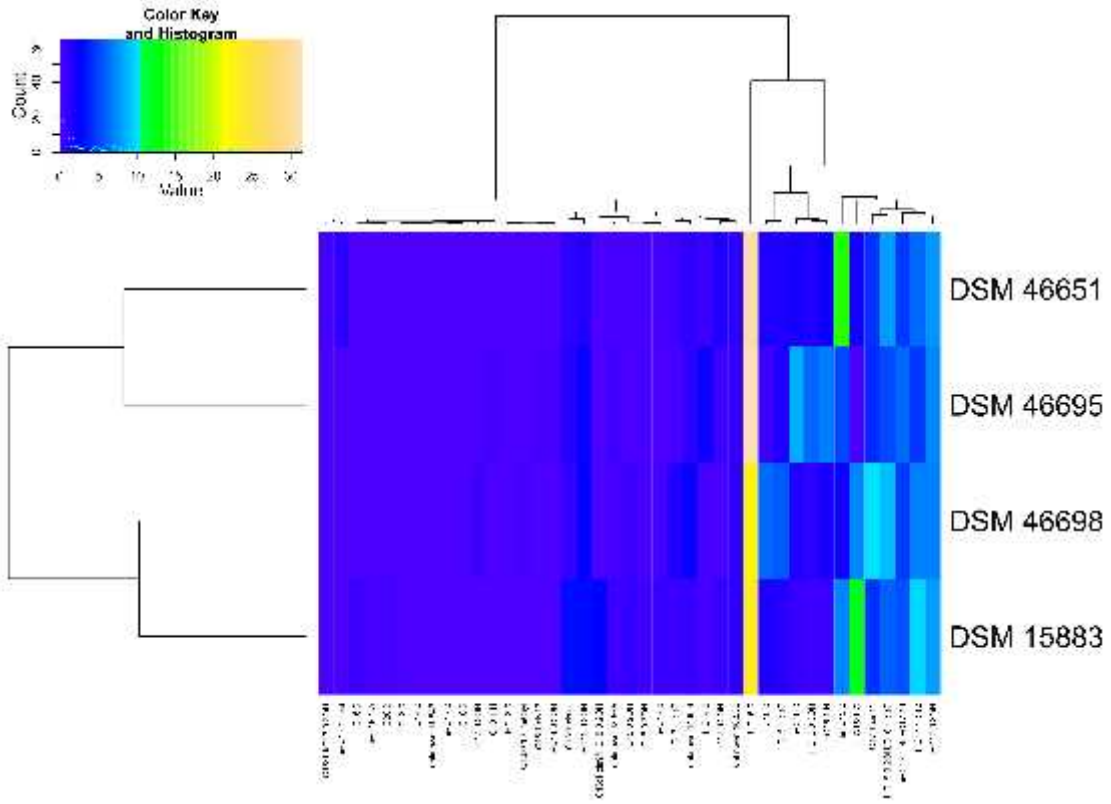

Figure 3.2: Heat map generated using the lipid extensions of the opm package under default settings after importing the files output by the MIDI system.
1, *Belliella kenyensis* sp. nov. No. 164^T DSM 46651^T; 2, *B. pelovolcani* DSM 46698^T; 3, *B. baltica* DSM 15883^T and 4, *Nitritalea halalkaliphila* DSM 46695^T

Strain No.164^T displayed menaquinone MK-7 as the predominant respiratory quinone. The phospholipid pattern consisted of phosphatidylethanolamine (PE), one unspecified phospholipid (PL) and eight unspecified lipids (NL1-8) (Plate 3.2) which fits very well to previous reports on representatives of the genus, such as *B. pelovolcani* by Arun et al. (2009), *B. baltica* by Brettar et al. (2004) and *Nitritalea halalkaliphila* by Anil Kumar et al. (2010).

Plate 3.2: Polar lipids profile of *Belliella kenyensis* sp. nov. strain No.164^T, after separation by two-dimensional TLC. Plate was sprayed with molydatophosphoric acid for detection of total polar lipid. PE, phosphatidylethanolamine; PL, unspecified phospholipid and NL, unspecified lipid.

Strain No.164^T was sensitive to (µg per disc) amikacin (30), ampicillin (10), aztreonam (30), cephalothin (30), chloramphenicol (30), erythromycin (15), lincomycin (15), norfloxacin (10), penicillin (10) tetracyclin (30), vancomycin (30), but resistant to colistin sulphate (10), gentamycin (10), kanamycin (30) and oxacillin (5). In API ZYM tests strain No.164^T was positive for alkaline phosphotase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, acid phosphatase, phosphoamidase, α-galactosidase, α-glucosidase, β-glucosidase and N-Acetyl-β-glucosaminidase. Negative activity was observed for esterase C4, lipase C8, lipase C14, β-glucuronidase, α-mannosidase and α-fucosidase. In API 20 NE positive for cytochrome oxidase, urease hydrolysis, assimilation of arginine dihydrolase, β-glucosidase, glucose, arabinose, mannose, mannitol, maltose and fermentation of glucose. Positive for reduction of nitrates to nitrites, indole production, esculin and gelatin hydrolysis. As a general rule, phenotypic features were rated as positive when weak or more pronounced signal was obtained. A summary of selected differential phenotypic characteristics are presented in Table 3.2.

Table 3.2: Cultural and phenotypic characteristics that differentiate strain No.164^T from the type strains of closely related species.

Strains: 1, *Belliella kenyensis* sp. nov. No.164^T; 2, *B. pelovolcani* DSM 46698^T; 3, *B. baltica* DSM 15883^T and 4, *Nitritalea halalkaliphila* DSM 46695^T.

Characteristic	1.	2.	3.	4.
Cultural characteristics Cell size (µm) Colony colour	 0.3-0.4 wide, 1.5-2.0 long red	 0.3 wide, 2.0-5.0 long[b] Red	 0.3-0.5 wide, 0.9-3.0 long[b] Pink	 0.7-0.8 wide, 2-3µm long[b] pink
Antibiotic resistance (µg per disc)* Kanamycin (30) Colistin Sulphate (10) Cephalothin (30) Oxacillin (5) Gentamycin (10) Amikacin (30) Aztreonam (30)	 - - + - - + +	 + - + + - - +	 - - - - - - -	 - + + + + + +
Enzymatic activity Esterase C4 Lipase C8 Lipase C14 β-glucosidase N-Acetyl-β-glucosaminidase α-mannosidase	 - - - + + -	 + + + - - -	 + + - + + +	 + + - + - -
Degradation of Tyrosine Starch Casein	 - + +	 - + -	 + - +	 + - +
API 20NE Esculin Nitrate reduction to nitrite Gelatin	 - + -	 - - -	 + + -	 - - +
Optimum growth Temperatures (^{0}C) pH NaCl% (w/v)	 30-35 7-12 0-4	 25-35 6-9 0-3	 20-35 6-10 0-5	 25-35 6-12 1-15
Predominant menaquinone (% of total) MK-7	97.4	89.1	97.3	94.3
Polar lipids	PE, PL, NL1-7	PE, PL1-2, NL1-9[b]	PE, PL1-2, NL1-9[b]	PE, PL, NL1-6[b]
DNA G+C content (mol%)	38.1	40.0[a]	35.4[a]	49.0[a]

Key: +, positive reaction; –, negative reaction; MK, menaquinone; PE, phosphatidylethanolamine; PL, unspecified phospholipid; NL, unspecified lipid. [b], data was obtained from Anil Kumar et al. (2012). [a], data for *B. pelovolcani*, *B. baltica* and *Nitritalea halalkaliphila* was obtained from Arun et al. (2009), Brettar et al. (2004) and Anil Kumar et al. (2010) respectively. All data are from the present study, unless indicated otherwise.

The almost complete (1451 bps) 16S rRNA gene sequence showed the greatest degree of similarity with the type strains of *B. pelovolcani* (97.0%), *B. baltica* (95.7%) and *Nitritalea halalkaliphila* (93.3%). Strain No.164^T clustered within the same phylogenetic group with the type strains of *B. pelovolcani* (DSM 46698^T) and *B. baltica* (DSM 15883^T) but formed a distinct phyletic line by both maximum likelihood and maximum-parsimony estimations (Figure 3.3).

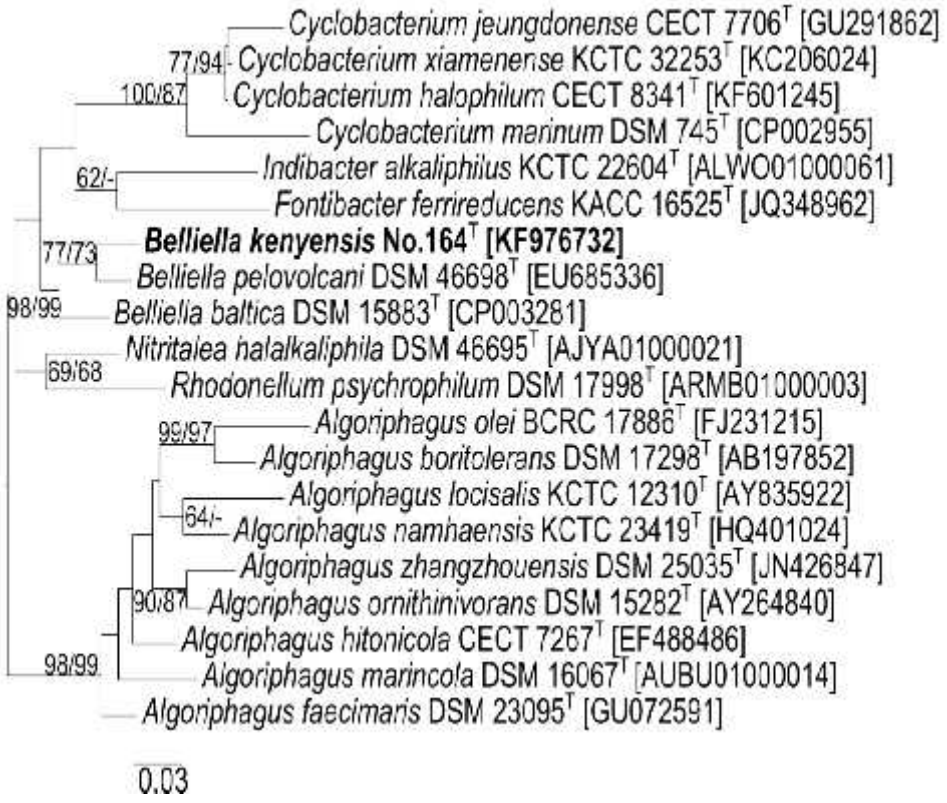

Figure 3.3: Maximum likelihood phylogenetic tree inferred from 16S rRNA gene sequences, showing the phylogenetic position of strain No.164^T relative to the type strains within the genus *Belliella* and species of other closely related genera.
The branches are scaled in terms of the expected number of substitutions per site (see scale). Support values from maximum-likelihood (left) and maximum-parsimony (right) bootstrapping are shown above the branches if equal to or larger than 60%.

The type strain has a genomic DNA G + C content of 38.1 mol%. The phylogenetic analysis further supported the assignment of strain No.164^T to the genus *Belliella* as a novel species. Strain No.164^T could be differentiated from *B. pelovolcani* (DSM 46698^T) and *B. baltica* (DSM 15883^T) according to both phenotypic and genotypic features and therefore a third representative of the genus *Belliella*, for which the name *Belliella kenyensis* sp. nov. is proposed. The INSDC accession number for the 16S rRNA gene sequences of the type strain No.164^T (= DSM 46651 = CECT 8551) is KF976732.

3.5 CONCLUSION

Belliella kenyensis (ke.ny.en'sis. N.L. fem. Adj. kenyensis pertaining to Kenya, the country from which the sample was collected).

Cells are Gram-reaction-negative, aerobic, pleomorphic-rods and non-motile. Grow very well in marine medium, moderately in R2A and LB. Form small, red, non-adherent colonies with entire margins on marine agar. Optimal growth temperature is 30-35ºC in the presence of 0-4% (w/v) NaCl and pH range of 8-10. Positive for catalase and DNase activity, degradation of casein and hydrolysis of starch. Negative for xanthine and tyrosine degradation. Does not grow anaerobically on marine agar. Contain carotinoids but no flexirubins. The main cellular fatty acids are branched-chain fatty acids *iso*-$C_{15:0}$, *anteiso*-$C_{15:0}$ and summed feature 3 comprising $C_{16:1\_\omega7c}$ and/or *iso*-$C_{15:0}$ 2-OH. A menaquinone with seven isoprene units (MK-7) is the predominant respiratory quinone. The polar lipids consisted of phosphatidylethanolamine, seven unspecified glycolipids and one unspecified phospholipid. Sensitive to amikacin, ampicillin, aztreonam, cephalothin, chloramphenicol, erythromycin, lincomycin, norfloxacin, penicillin, tetracyclin, vancomycin but resistant to colistin sulphate, gentamycin, kanamycin and oxacillin. In API ZYM tests, positive for alkaline phosphotase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, acid phosphatase, phosphoamidase, α-galactosidase, α-glucosidase, β-glucosidase and N-Acetyl-β-glucosaminidase. Negative activity was observed for esterase C4, lipase C8, lipase C14, β-glucuronidase, α-mannosidase and α-fucosidase. In API 20 NE positive for cytochrome oxidase, urease hydrolysis, assimilation of arginine dihydrolase, β-glucosidase, glucose, arabinose, mannose, mannitol, maltose and fermentation of glucose. Positive for reduction of nitrates to nitrites, indole production, esculin and gelatin hydrolysis. The type strain has a genomic DNA G + C content of 38.1 mol%. The INSDC accession number for the 16S rRNA gene sequences of the type strain No.164^T (= DSM 46651 = CECT 8551) is KF976732.

CHAPTER FOUR

4.0 *STREPTOMYCES ALKALIPHILUS* SP. NOV., ISOLATED FROM SEDIMENTS OF LAKE ELMENTEITA IN THE KENYAN RIFT VALLEY

ABSTRACT

A novel strain designated No.7^T was isolated from a sediment sample collected from the alkaline, saline Lake Elmenteita located in the Kenyan Rift Valley. The optimal growth temperature was 30-35ºC, at pH 8.0-12.0 in the presence of 7.0-10.0% (w/v) NaCl. The culture formed a light green beige abundant aerial mycelium on Hirokoshi 1 agar and was found to have morphological and chemotaxonomic characteristics typical of members of the genus *Streptomyces*. The peptidoglycan contained LL-diaminopimelic acid as diamino acid, with no diagnostic sugars identified. The predominant menaquinone was MK-9(H_6). The main polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and an unknown phospholipid. Cellular fatty acids consisted of saturated branched-chain acids with *iso*-$C_{15:0}$, *anteiso*-$C_{15:0}$, *iso*-$C_{16:0}$ and ante*iso*-$C_{17:0}$ acids predominating. The type strain had a genomic DNA G + C content of 72.8 mol % and formed a distinct phyletic line within the *Streptomyces*. Based on the chemotaxonomic results, 16S rRNA gene sequence analysis and low DNA-DNA hybridization value with the type strain of *Streptomyces calidiresistens*, it is proposed that strain No.7^T (= DSM 42118 = CECT 8549) represents a novel species, *Streptomyces alkaliphilus*. The INSDC accession number for the 16S rRNA gene sequence of strain No.7^T is KF976730.

4.1 INTRODUCTION

Culture-independent studies on the lakes of the East African Rift Valley in Kenya demonstrated that they are promising habitats for novel species of *Bacteria* and *Archaea* (Mwirichia et al. 2010a). They are naturally eutrophic reservoirs capable of high primary productivity and feature considerable microbial diversity (Zavarzin et al., 1999).

In the search for new bioactive compounds it is most promising to screen novel isolates in uncharted habitats (Antony-Babu & Goodfellow, 2008). *Streptomycetes* are well-known as rich sources of novel and clinically significant bioactive compounds and have been extensively studied over many decades (Santhanam et al., 2012). These bioactive secondary metabolites represent an array of different and structurally diverse chemical classes such as polyketides, peptides, macrolides, indoles, aminoglycosides and terpenes (Blunt et al., 2012; Fenical & Jensen, 2006; Strohl, 1997). The genus proposed by Waksman and Henrici (1943) has the highest number of species with validly published names. At the time of writing this thesis, the taxon comprised approximately 560 recognized species (Parte, 2014).

The subgeneric classification of the genus has been clarified by the application of genotypic and phenotypic procedures (Goodfellow et al., 2007; Rong & Huang, 2010) that have also been used to circumscribe novel species isolated from environmental sources (Nagai et al., 2011; Zucchi et al., 2012). The genus *Streptomyces* is characterised by branched substrate mycelia and aerial hyphae, have cell walls that contain LL-diaminopimelic acid but no characteristic sugars (wall chemotype I; Lechevalier & Lechevalier, 1970), straight chain, as well as *iso*- and *anteiso*-branched chain fatty acids with chain-length of 14-18 carbon atoms (Kroppenstedt, 1990), major menaquinones are MK-9(H_6) and MK-9(H_8), a complex polar lipid patterns that typically contains diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol mannosides (Collins et al., 1977; Minnikin et al., 1984) and a DNA G + C content of 69-78 mol % (Korn-Wendisch & Kutzner, 1992). In this paper we describe morphological, physiological and chemical

data which support the identification of isolate No.7^T, representing a novel species of the genus *Streptomyces* isolated from a soda lake in the Kenyan Rift Valley.

4.2 ISOLATION AND PHENOTYPIC CHARACTERIZATION

Lake Elmenteita is situated at 0^{o}27´S, 36^{o}15´E on the floor of the Kenyan Rift Valley at 1776 m altitude and has no direct outlet (Melack, 1988). The region is characterised by a hot, dry and semi-arid climate with a mean annual rainfall of about 700 mm (Mwaura, 1999). Due to the high temperatures there are very high evaporation rates during the drier seasons leading to a seasonal reduction in the total surface area of the lake. The size of Lake Elmenteita is roughly 20 km^2 and the depth rarely exceeds 1.0 m (Mwirichia et al,. 2010b). The alkalinity of the water is high (1200 mg Na_2CO_3/l) with a high concentration of carbonates, chlorides and sulphates (Mwirichia et al., 2010b). The water temperature ranges between 30-40^{o}C and the pH is above 9.

Strain No.7^T was isolated from a sediment sample collected from Lake Elmenteita. The sediment sample (2 g) was suspended in filter sterilised lake water (10 ml) and the solution was serially diluted using the same water. Aliquots (100 µl) of appropriate dilutions were plated onto solid basal medium for alkaliphilic micro-organisms, Horikoshi 1 (Glucose, 10.0 g; Polypepton, 5.0 g; Yeast extract, 5.0 g; K_2HPO_4, 1.0 g; $MgSO_4 \times 7\ H_2O$, 0.2 g; Agar, 15.0 g; Distilled water, 900.0 ml; after autoclaving, 100 ml of sterilized 10% Na_2CO_3 solution was added to the medium) (Horikoshi 1999), supplemented with cAMP (Sigma Chemical Co., St. Louis, MO.) to a final concentration of 10 µM (Bruns & Cypionka, 2002). The plates were incubated at 28^{o}C for 14 days. Colonies appearing on the plates were streaked again on to Horikoshi l agar for purification. The isolate was stocked in Horikoshi l broth with 15% glycerol (v/v) at -20^{o}C.

Physiological tests for this study were performed at 28^{o}C using strain No.7^T and cultures of *Streptomyces marinus* (Khan et al., 2010) DSM 41970^T, *S. qinglanensis* (Hu et al., 2012) DSM 42035^T, *S. tateyamensis* (Khan et al., 2010) DSM 41969^T, *S.*

hebeiensis (Xu, 2004) DSM 41837^T, *S. erringtonii* (Santhanam et al., 2013) DSM 42088^T, *S. glauciniger* (Huang et al., 2004) DSM 41867^T and *S. calidiresistens* (Duan et al., 2014) DSM 42108^T in parallel assays. Cultural characteristics were tested on ISP 1, 4, 5, 6 and 7 agar (Shirling & Gottlieb 1966), Horikoshi l agar (Horikoshi, 1999), modified Bennett's agar (Jones, 1949) and nutrient agar (DSMZ medium 1) for 21 days. Colony features were observed after 21 days under a binocular microscope according to (Pelczar, 1957) and colony colour determined by comparing with the RAL Classic K5 colour chart.

Exponentially growing bacterial cultures were observed with an optical microscope (Zeiss AxioScope A1) with a 100-fold magnification and phase-contrast illumination. Pictomicrographs of bacterial cells of strain No.7^T grown on Horikoshi 1 agar for 21 days were taken with a field-emission scanning electron microscope (FE-SEM Merlin, Zeiss, Germany). Gram reaction was performed using the KOH test described by (Gregersen, 1978). Growth was assessed at 10 to 55^{o}C (in increments of 5^{o}C) on modified Bennett's agar for 14 days. Tolerance to a range of NaCl concentrations 0, 1 to 15%, w/v (in increaments of 2) was tested on nutrient agar medium as the basal medium by incubating the cultures for 14 days at 28^{o}C. Growth at pH values from 5.0 to 13.0 (in increments of 1.0 pH unit) was assessed on modified Bennett's agar for 14 days at 28^{o}C by adding NaOH or HCl, respectively, since the use of a buffer system inhibited growth of the cultures. Oxidase activity was analysed using filter-paper disks (Sartorius grade 388) impregnated with 1% solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (Sigma Chemical Co., St. Louis, MO.); a positive test was defined by the development of a blue-purple colour after applying biomass to the filter paper. Catalase activity was determined based on formation of bubbles following the addition of 1 drop of 3% H_2O_2. Degradation of specific substrates was examined using agar plates with various basal media: casein degradation was tested on plates containing milk powder (5% w/v), NaCl (0.5%) and agarose (1%); tyrosine degradation was determined as previously described (Gordon and Smith, 1955) on plates containing peptone (0.5%), beef extract (0.3%), L-tyrosine (0.5%) and agarose (1.5%); xanthine decomposition (0.4%) was examined using the same basal medium; starch degradation was tested on plates containing

nutrient broth (0.8%), starch (1%) and agarose (1.5%), then developed by flooding in 1% iodine solution. Other phenotypic characteristics were tested using standard procedures (Shirling & Gottlieb 1966; Williams et al., 1983; Smibert & Krieg, 1994). The utilization of carbon compounds and acid production were tested using API 50CH strips (bioMérieux). Enzymatic activities were tested using API ZYM galleries according to the instructions of the manufacturer (bioMérieux). Phenotypic features were rated as positive when a signal was obtained either weak or more pronounced.

4.3 CHEMOTAXONOMIC PROCEDURES

For chemotaxonomic studies, cultures of strain No.7^T were grown in Horikoshi l broth in a shaking incubator at 150 r.p.m and 28^{o}C for 7 days. The mycelia were harvested by centrifugation and washed twice with 0.9% NaCl solution and freeze dried. Amino acids and whole-cell sugars were prepared according to (Lechevalier & Lechevalier 1970), followed by thin layer chromatography (TLC) analysis (Staneck & Roberts 1974). Polar lipids were extracted, separated by two-dimensional TLC and identified according to procedures outlined by (Minikin et al., 1984) with modifications proposed by (Kroppenstedt & Goodfellow, 2006).The composition of peptidoglycan hydrolysates (6 N HCl, 100^{o}C for 16 h) was examined by TLC as described by (Schleifer & Kandler 1972). Menaquinones (MK) were extracted from freeze-dried cell material using methanol as described by Collins et al. (1977) and analysed by high-performance liquid chromatography (HPLC) (Kroppenstedt, 1982). The extraction and analysis of cellular fatty acids was carried out from biomass grown in a shaking incubator at 150 r.p.m. for 3 days at 28^{o}C. Analysis was conducted using the Microbial Identification System (MIDI) Sherlock Version 6.1 (method TSBA40 database) as described by (Sasser, 1990).

4.4 GENETIC AND PHYLOGENETIC ANALYSIS

The G + C content of the chromosomal DNA of strain No.7^T was determined by HPLC according to (Mesbah, 1989). Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product was carried

out as described by (Rainey et al., 1996). The resultant sequence was compared with the corresponding sequences of type strains of members of the genus *Streptomyces* using the EzTaxon server (Kim et al., 2012). The 16S rRNA gene sequences closely related to strain No.7^T were downloaded from EzTaxon. Phylogenetic analysis was based on alignment of 16S rRNA gene sequences from closely related type strains of species with validly published names in the genus *Streptomyces* inferred as described by Montero-Calasanz et al. (2013a). Rooting was done using the midpoint method. Pairwise similarities were calculated as recommended by Meier-Kolthoff et al. (2013) and Montero-Calasanz et al. (2013b). DNA-DNA hybridization between strain No.7^T and its closest phylogenetic neighbour *Streptomyces calidiresistens* DSM 42108^T (Duan et al., 2014) was performed as described by De Ley et al. (1970) with the modifications suggested by Huss et al. (1983) using a Cary 100 Bio UV/VIS.

4.5 RESULTS AND DISCUSSION

Strain No.7^T was Gram-reaction-positive, aerobic, formed abundant light green beige aerial mycelium and brown substrate mycelia on Hirokoshi 1 agar. The strain formed an extensively branched substrate mycelia that carries aerial hyphae which differentiate into straight chains of smooth-surfaced oval spores (0.9-1.0 × 0.7-0.8 µm) on Horikoshi 1 agar after 21 days of incubation (Plate 4.1).

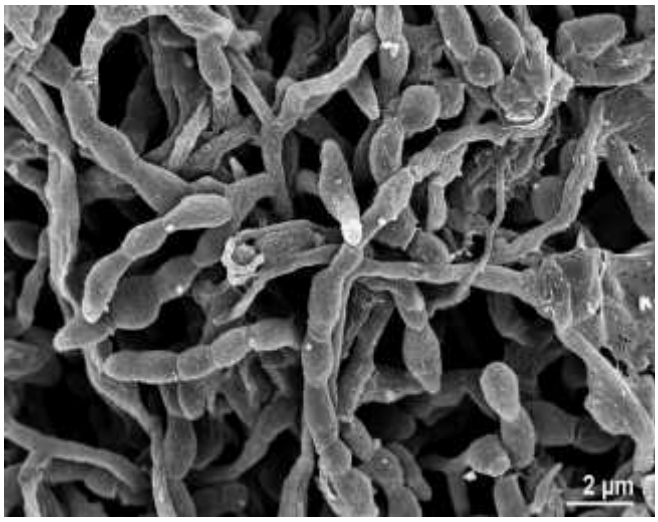

Plate 4.1: Scanning electron micrograph of strain No.7^T grown on Horikoshi 1 agar after 21 days at 28°C showing chains of smooth oval spores.

Table 4.1: Differential cultural characteristics of strain No.7^T and the type strains of closely related Streptomyces species.

Strains were examined after growing on each of eight different agars for 21 days at 28^{o}C. Strains: 1, *S. alkaliphilus* sp. nov. No.7^T; 2, *S. qinglanensis* DSM 42035^T; 3, *S. marinus* DSM 41970^T; 4, *S. tateyamensis* DSM 41969^T; 5, *S. hebeiensis* DSM 41837^T; 6, *S. erringtonii* DSM 42088^T; 7, *S. glauciniger* DSM 41867^T and 8, *S. calidiresistens* DSM 42108^T. All data are from the present study unless indicated otherwise.

Cultural characteristics	**1**	**2**	**3**	**4**	**[a]5**	**[a]6**	**[a]7**	**[a]8**
Horikoshi 1 agar								
Growth	+++	+	-	-	nd	nd	nd	nd
Aerial mycelium	light green	cream	-	-				
Substrate mycelium	beige	cream	-	-				
Modified Bennett's agar	brown							
		+++	+++	++	nd	nd	+++	nd
Growth	++	cream	cream	beige			dark greyish	
Aerial mycelium	light green	cream	cream	cream			brown	
Substrate mycelium	beige						greenish black	
ISP 1	brown	++	+	+	nd	nd		nd
Growth		cream	pure white	pure white			nd	
Aerial mycelium	+	cream	cream	cream				
Substrate mycelium	dusty grey							
ISP 4	agate grey	++	+	+	++	+++		Grow well
Growth		pure white	pure white	aluminium	deep yellow	grayish	++	
Aerial mycelium	++	cream	cream	grey	grey	white	light greyish	
Substrate mycelium	pure white			agate grey	deep grey	white	brown	
ISP 5	light green	+	+		yellow		yellowish white	Grow
Growth	beige	pure white	cream	++		+++		weakly
Substrate mycelium		cream	cream	cream	++	grayish	+	
Aerial mycelium	+			cream	white	white	greyish brown	
ISP 6	pure white	++	-		yellow white	gray white	greyish olive	
Growth	cream	beige	-	+				nd
Aerial mycelium		cream	-	beige	nd	+	+++	
Substrate mycelium	++			cream		grayish	grey	
ISP 7	beige	+	+			white	yellow	
Growth	cream	pure white	beige	++		cream		nd
Aerial mycelium		cream	beige	aluminium	nd		+++	
Substrate mycelium	+			grey		++	greyish cream	
Nutrient agar	light green	+	++	sepin brown		grayish	yellowish cream	
Growth	light green	pure white	pure white			white		Grow well
Aerial mycelium		cream	cream	+	+	brown	+++	
Substrate mycelium	++			pure white	yellow grey		greyish white	
	grey			cream	light grey	nd	yellow	
	brown green				reddish			

Key: -, no growth; +, poor; ++, moderate; +++, abundant; nd, not determined. [a], data for *S. hebeiensis*, *S. erringtonii*, *S. glauciniger* and *S. calidiresistens* was obtained from Xu (2004), Santhanam et al., (2013), Huang et al., (2004) and Duan et al., (2014) respectively.

Strain No.7^{T} grew very well on Horikishi 1 medium, moderately on modified Bennett's agar, nutrient agar, ISP 4 and sparsely on ISP 1, 5, 6 and 7. A summary of selected differential cultural characteristics are presented in Table 4.1.

Strain No.7^{T} grew over a temperature range of 25-40^{o}C (optimal growth temperature was 30-35^{o}C). Growth was observed in the presence of 0-10% (w/v) NaCl (optimal range 7-10% (w/v) and between pH 7.0-13.0 (optimal range 8-12). Analysis of cell-wall components revealed the presence of LL-diaminopimelic acid (Cell wall type I), which is consistent with other representatives of the genus *Streptomyces* (Hu et al., 2012; Khan et al., 2010). Whole-cell sugar analysis showed no diagnostic sugars as typical for the genus (Lechevalier and Lechevalier 1970) but showed presence of glucose and ribose. Strain No.7^{T} contained menaquinone MK-9(H_6) 57.0%, but also 28.6% MK-9(H_8) and 7.6% MK-9(H_4). The main cellular fatty acids (>10%) were saturated branched-chain acids *anteiso*-$C_{15:0}$ (32.9%), *iso*-$C_{16:0}$ (25.7%), *anteiso*-$C_{17:0}$ (12.3%) and *iso*-$C_{15:0}$ (10.4%). Fatty acid patterns were also visualized as heat maps (Figures 4.1 and 4.2).

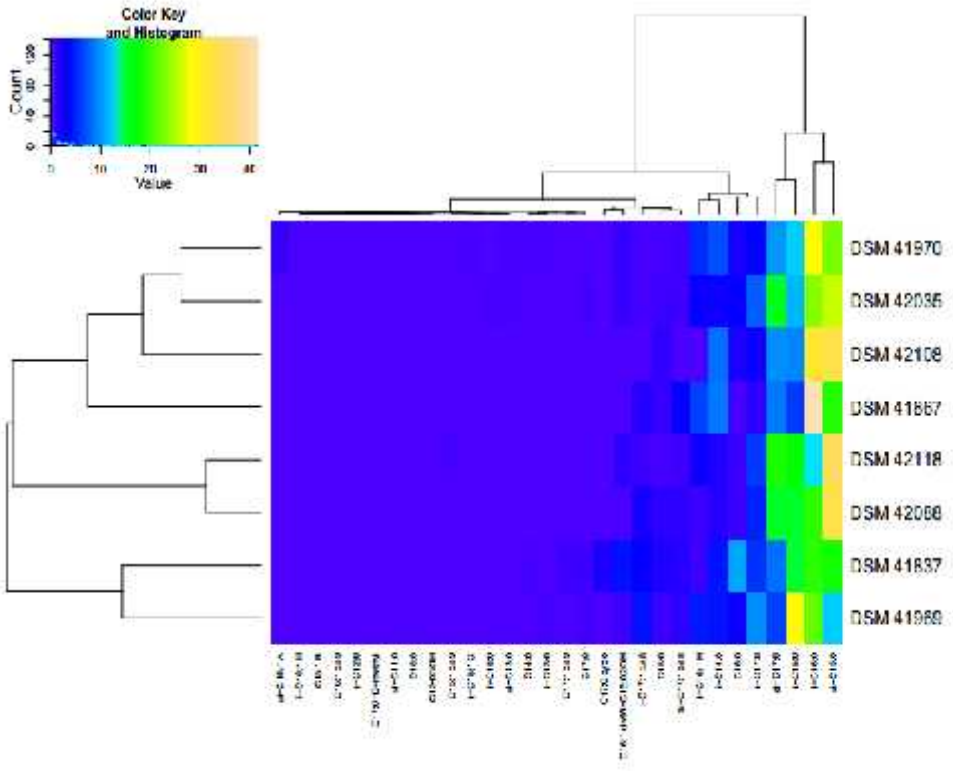

Figure 4.1: Heatmap generated using the lipid extensions of the opm package showing un-transformed measurements under default settings after importing the fatty acids result files from the MIDI system**.**

Strains: *Streptomyces alkaliphilus* sp. nov. No.7^{T} DSM 42118^{T}; *S. qinglanensis* DSM 42035^{T}; *S. marinus* DSM 41970^{T}; *S. tateyamensis* DSM 41969^{T}; *S. hebeiensis* DSM 41837^{T}; *S. erringtonii* DSM 42088^{T}; *S. glauciniger* DSM 41867^{T} and *S. calidiresistens* DSM 42108^{T}.

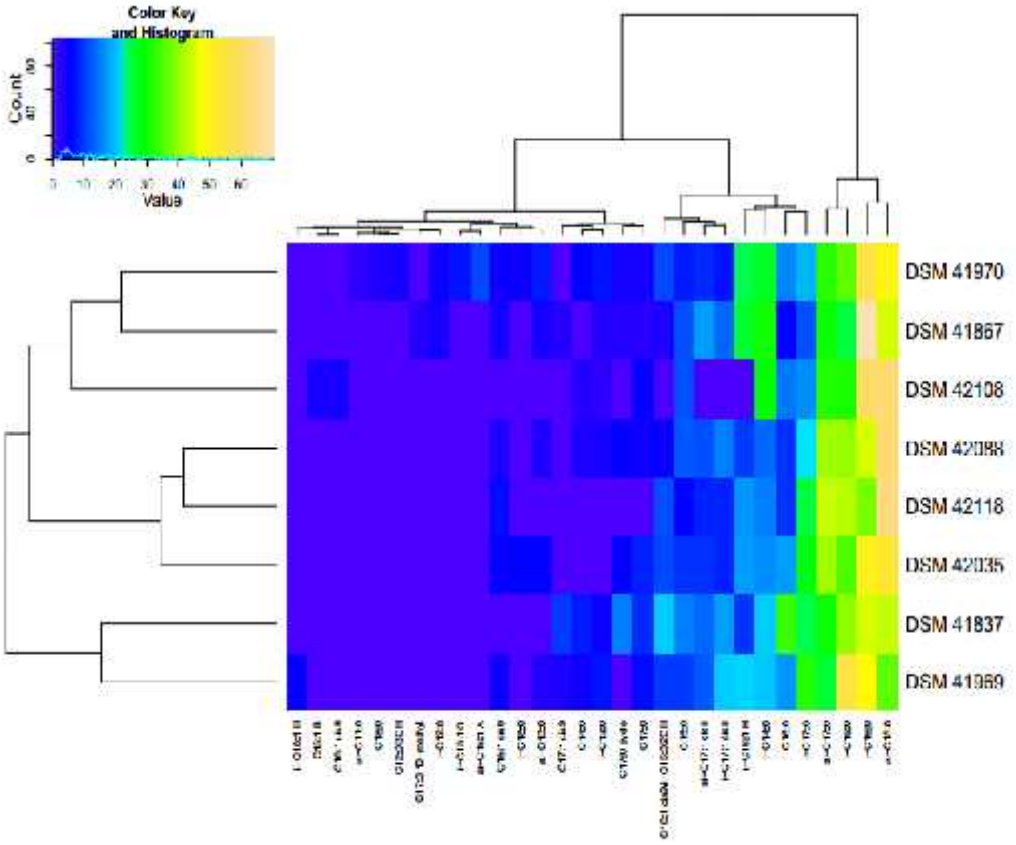

Figure 4.2: Heatmap generated using the lipid extensions of the opm package showing un-transformed and log-transformed measurements respectively, under default settings after importing the result files from the MIDI system.

Strains: *Streptomyces alkaliphilus* sp. nov. No.7^{T} DSM 42118^{T}; *S. qinglanensis* DSM 42035^{T}; *S. marinus* DSM 41970^{T}; *S. tateyamensis* DSM 41969^{T}; *S. hebeiensis* DSM 41837^{T}; *S. erringtonii* DSM 42088^{T}; *S. glauciniger* DSM 41867^{T} and *S. calidiresistens* DSM 42108^{T}.

The phospholipid pattern consisted of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and an unknown phospholipid (PL) (Plate 4.2).

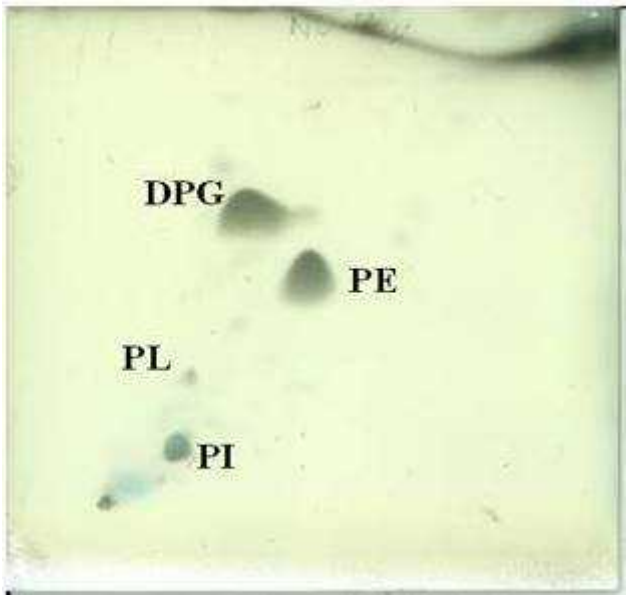

Plate 4.2: Polar lipids profile of *Streptomyces alkaliphilus* sp. nov. No.7^{T}, after separation by two-dimensional TLC.

Plate was sprayed with molydatophosphoric acid for detection of total polar lipid. DPG, diphosphadidylglycerol; PE, phosphatidylethanolamine; PI,

The genomic G + C content was 72.8 mol %. Several phenotypic properties distinguished the novel strain No.7^{T} from other closely related type strains of the species (Table 4.2). These characteristics are in line to the assignment of isolate No.7^{T} in the genus S*treptomyces* (Khan et al., 2010; Hu et al., 2012; Xu, 2004; Santhanam et al., 2013; Huang et al., 2004).

Table 4.2: Differential physiologic and chemotaxonomic characteristics of strain No.7^{T} and the type strains of closely related Streptomyces species.

Strains order as above. All data are from the present study unless indicated otherwise.

Characteristic	1	2	3	4	5	6	7	8
Carbon utilization								
Mannitol	+	+	+	+	+	-	+	-
Sucrose	+	-	+	+	+	+	+	+
Glucose	+	+	+	+	+	-	-	-
Sorbitol	+	-	-	+	+	-	-	+
D-Fructose	+	+	+	-	+	-	+	+
L-Arabinose	-	+	-	-	+	-	+	-
Raffinose	+	+	+	-	+	+	+	+
Rhamnose	+	-	-	-	+	-	+	-
D-Xylose	+	+	+	-	+	+	+	-
Enzymatic activity								
Alkaline phosphatase	-	+	+	+	+	+	+	+
Cystine aminopeptidase	+	-	+	-	+	+	+	+
Trypsin	+	-	+	-	-	+	+	+
Chymotrypsin	+	-	-	+	+	+	+	-
α-Galactosidase	+	-	-	-	+	-	+	-
β-Glucuronidase	-	+	-	-	+	+	+	+
β-Glucosidase	-	+	+	+	-	-	-	+
N-acetyl-β-Glucosaminidase	-	+	+	+	+	+	+	-
α-Mannosidase	-	-	+	+	+	+	+	-
Degradation of								
Aesculin	-	-	-	-	+	-	+	-
Xanthine	-	-	-	-	+	+	+	-
Tyrosine	-	-	-	-	+	+	-	-
DNA	+	+	-	+	+	-	+	-
Starch	+	+	-	-	+	-	+	+
Gelatin	-	-	+	-	+	-	+	+
Urea	+	+	-	-	+	+	+	-
Casein	+	+	+	+	+	-	+	+
Tweens 20, 40, 60	+	+	+	+	+	-	+	+
[a]Optimum growth								
Temperatures (°C)	30-35	30-35	25-30	25-30	27-37	10-35	10-35	37-45
pH	8-12	7-9	7-9	6-8	nd	5-10	5-10	8-9
NaCl%(w/v)	7-10	1-3	2-7	2-7	4-7	4	0	1-3
[a]Predominant menaquinones	MK-9(H_6) MK-9(H_8) MK-9(H_4)	MK-9(H_6) MK-9(H_8) MK-9(H_4)	MK-9(H_6) MK-9(H_8) MK-9(H_4)	MK-9(H_6) MK-9(H_8) MK-9(H_4)	MK-9(H_6) MK-9(H_8) MK-9(H_4)	MK-9(H_6) MK-9(H_8) -	MK-9(H_6) MK-9(H_8) -	MK-9(H_6) - MK-9(H_4)
[a]Polar lipids	DPG, PE, PI, PL	DPG, PC, PE, PI, PIM	DPG, PE, PI	PE, PI, PC, PIM	PE	nd	DPG, PI, PE	DPG, PE, 4PL, 2NL, AL
Major fatty acids (% of total)[b]	*ai*-$C_{15:0}$(32.9), *i*-$C_{16:0}$(25.7), *ai*-$C_{17:0}$(12.3), *i*-$C_{15:0}$(10.4)	*i*-$C_{16:0}$(34.1), *ai*-$C_{15:0}$(23.8), *ai*-$C_{17:0}$(11.6),	*i*-$C_{16:0}$(40.4), *i*-$C_{14:0}$(19.0), *ai*-$C_{15:0}$(16.4)	*i*-$C_{16:0}$(33.1), *ai*-$C_{15:0}$(15.7), *i*-$C_{15:0}$(13.4)	*ai*-$C_{15:0}$(23.2), *i*-$C_{16:0}$(17.2), *i*-$C_{15:0}$(10.9), *i*-$C_{14:0}$(10.4)	*ai*-$C_{17:0}$(33.2), *ai*-$C_{15:0}$(29.0), *i*-$C_{16:0}$(19.20), *i*-$C_{15:0}$(15.8)	*i*-$C_{16:0}$(25.4), *ai*-$C_{17:0}$(17.4), *ai*-$C_{15:0}$(16.7)	*ai*-$C_{15:0}$(33.4), *i*-$C_{16:0}$(31.7), *ai*-$C_{17:0}$(10.0)

Key: +, positive reaction; –, negative reaction; nd, not determined; MK, menaquinones; *i*-, *iso*-branched, *ai*-, *anteiso*-branched; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PIM, phosphatidylinositolmannosides; PI, phosphatidylinositol; PL, unknown phospholipid; NL, unspecified aminolipid. AL; unspecified lipid. [b], only components making up ≥ 10 % peak area ratio are shown. [a], data for *S. hebeiensis*, *S. erringtonii*, *S. glauciniger* and *S. calidiresistens* was obtained from Xu (2004), Santhanam et al. (2013), Huang et al. (2004) and Duan et al. (2014) respectively.

The almost complete (1466 bp) 16S rRNA gene sequence showed the highest degrees of sequence similarity with the type strains of *S. calidiresistens* (99.4%; Duan et al., 2014), *S. marinus* (97.1%), *S. qinglanensis* (97.0%) and *S. tateyamensis* (96.9%). Strain No.7^T however, clustered phylogeneticaly with the type strains of *S. calidiresistens* (99.4%; Duan et al., 2014), *S. glauciniger* (96.9%; Huang et al., 2004), *S. erringtonii* (96.7%; Santhanam et al., 2012) and *S. tateyamensis* (96.9%; Khan et al., 2010) in both, maximum likelihood and maximum-parsimony inferred phylogenies (Figure 4.2). The 16S rRNA gene sequence analysis thus supports the assignment of strain No.7^T to the genus *Streptomyces*. These data shows that strain No.7^T is closely related to Streptomyces clade 126/127 which includes members like *S. glauciniger* and *S. tateyamensis*as as delineated by Labeda et al. (2012).

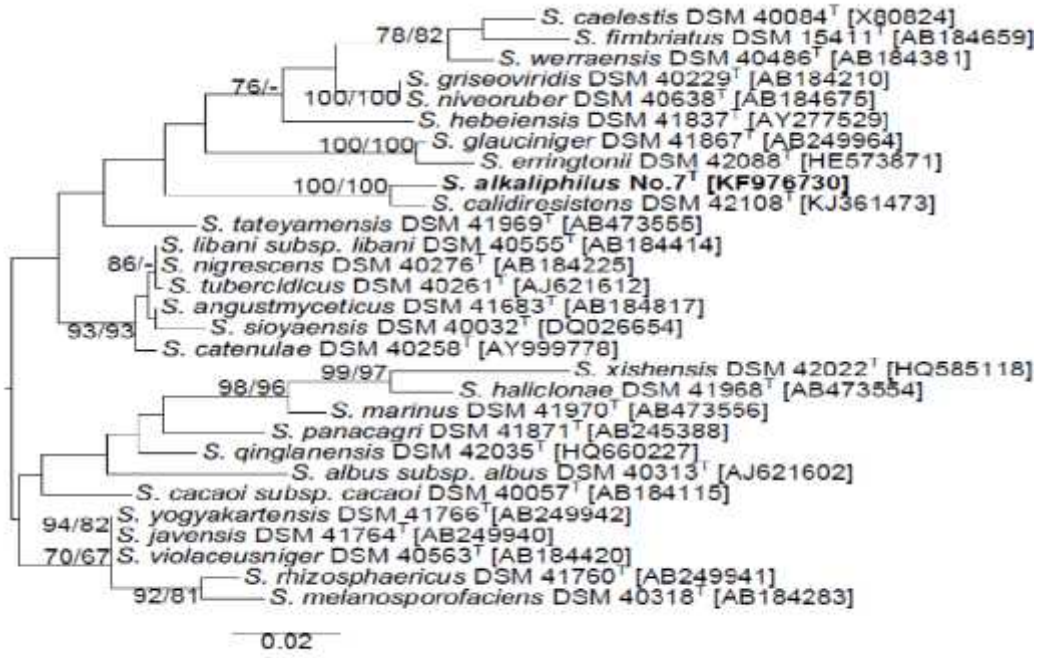

Figure 4.3: Maximum likelihood phylogenetic tree inferred from 16S rRNA gene sequences, showing the phylogenetic position of strain No.7^T relative to the type strains within the genus Streptomyces.

The branches are scaled in terms of the expected number of substitutions per site (see size bar). Support values from maximum-likelihood (left) and maximum-parsimony (right) bootstrapping are shown above the branches if equal to or larger than 60 %.

Strains No.7^T and *S. calidiresistens* DSM 42108^T shared 42.1±1.0% DNA-DNA relatedness. This value of genomic relatedness is far below the threshold value of 70% recommended by Stackebrandt & Goebel (1994) and Meier-Kolthoff et al. (2013) as indicating species status. Therefore, strain No.7^T is considered to represent a novel species in the genus *Streptomyces*.

On the basis of the difference shown in the 16S rRNA gene sequence analysis and the phenotypic differences between strain No.7^T and its closest phylogenetic neighbours, we propose that strain No.7^T should be classified as a novel species of the genus *Streptomyces.* Therefore we propose that strain No.7^T should be classified as a novel species of the genus *Streptomyces*, for which the name *Streptomyces alkaliphilus* sp. nov. is proposed.

4.6 CONCLUSION

Streptomyces alkaliphilus (al.ka.li'phi.lus. N.L. n. *alkali* alkali; Gr. adj. *philos* loving; N.L. adj. alkaliphilus alkali-loving)

Aerobic, Gram-reaction-positive streptomycete that forms light green beige abundant aerial mycelium and brown substrate mycelia on Hirokoshi 1 agar. Forms extensively branched substrate mycelium that carries aerial hyphae which differentiate into straight chains of smooth-surfaced oval spores (0.9-1.0 × 0.7-0.8 µm) on Horikoshi 1 agar after 21 days of incubation. Grows well on Horikishi 1 medium, moderately on modified Bennett's agar, nutrient agar, ISP 4 and sparsely on ISP 1, 5, 6 and 7. The optimal growth temperature is 30-35ºC, at pH 8.0-12.0 in the presence of 7-10% (w/v) NaCl. Metabolises D-fructose, D-glucose, mannitol, D-raffinose, Rhamnose, sucrose, sorbitol and D-xylose as sole carbon sources for energy and growth. In API ZYM tests, positive for esterase C4, lipase C8, lipase C14, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, acid phosphatase, phosphoamidase, α-galactosidase, β-galactosidase, α-glucosidase, and α-fucosidase. Negative activity was observed for alkaline phosphatase, β-

glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase and α-mannosidase. Positive in tests for urease and catalase production, degradation of casein, Tween 20, 40 and 60, hydrolysis of starch. Negative for gelatin liquefaction, nitrate reduction and aesculin hydrolysis. The peptidoglycan in the cell wall contains LL-diaminopimelic acid as the diamino acid, with no diagnostic sugar. The predominant menaquinones are MK-9(H_6) and MK-9(H_8). The main polar lipids were diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol. Cellular fatty acids consist of saturated branched-chain acids with *iso*-$C_{15:0}$, *anteiso*-$C_{15:0}$, *iso*-$C_{16:0}$ and *anteiso*-$C_{17:0}$ predominating. The type strain has a genomic DNA G + C content of 72.8 mol %. The INSDC accession number for the 16S rRNA gene sequences of the type strain No.7^T (= DSM 42118 = CECT 8549) is KF976730.

CHAPTER FIVE

5.0 *NOCARDIOPSIS MWATHAE* SP. NOV., ISOLATED FROM THE HALOALKALINE LAKE ELMENTEITA, IN THE AFRICAN RIFT VALLEY

ABSTRACT

Strain No.156^T isolated from a sediment sample from the haloalkaline lake Elmenteita in the African rift valley was studied by a polyphasic taxonomic approach. The strain produced yellow aerial and substrate mycelia, grew best over a temperature range of 30-35^{o}C in salt concentrations of 6-9% (w/v) and at pH 7-9. The DNA G+C contents of the novel strain was 71 mol%. Analysis of 16S rRNA sequences indicated that the isolate belonged to the genus *Nocardiopsis* with a sequence similarity below 98% to the type strains of all other representatives of the genus. Mycolic acids were not detected in whole cell methanolysates. The cell wall contained meso-diaminopimelic acid without diagnostic sugars. Major phospholipids included phosphatidylmethylethanolamine, phosphatidylcholine, phosphatidylglycerol and phosphatidylinositol but no diphosphatidylglycerol. The predominant menaquinones were MK-11(H_8), MK-11(H_6), MK-10(H_8) and MK-10(H_6). The fatty acid composition included *iso*- and *anteiso*-branched acids combined with tuberculostearic acid (Me18:0), straight-chain saturated (16:0, 18:0) and unsaturated fatty acids. These characteristics match those of the genus *Nocardiopsis*. Based on 16S rRNA gene sequence analysis and phenotypic characteristics, a novel species with the name *Nocardiopsis mwathae* is proposed. The type strain is No.156^T (= DSM 46659 = CECT 8552). The INSDC accession number for the 16S rRNA gene sequence is KF976731.

5.1 INTRODUCTION

The genus *Nocardiopsis* was first described by Meyer (1976) based on morphological characteristics and the chemical composition of cells. Members of the genus *Nocardiopsis* have been isolated from wide range of sources; alkaline slag dump (Schippers et al., 2002), indoor environments (Peltola et al., 2001), the atmosphere of a composting facility (Kämpfer et al. 2002) clinical material (Bernatchez & Lebreux, 1991; Yassin et al., 1997) household waste (Yassin et al., 2009) but predominantly reported from saline or alkaline soils (Yassin et al., 1993a; Al-tai & Ruan, 1994; Al-Zarban et al., 2002; Tang et al., 2003; Li et al., 2004, 2006; Yang et al., 2008). The genus *Nocardiopsis* was proposed to include organisms with the following salient chemotaxonomic characteristics: cell wall chemotype III C (meso isomer of diaminopimelic acid and no characteristic sugars in whole-cell hydrolysates) (Lechevalier & Lechevalier, 1970), phospholipid type PIII (phosphatidylcholine and phosphatidylmethylethanolamine as diagnostic phospholipids), lack of mycolic acids (Rainey et al., 1996), menaquinone MK-10 with variable degrees of saturation as the major isoprenoid quinones (Collins et al., 1977), the fatty acids include *iso*-branched, *anteiso*-branched, and 10-methyl-branched fatty acids (Rainey et al., 1996) and a DNA G + C content ranging between 64 and 71 mol% (Grund & Kroppenstedt, 1990). At the time of writing the genus contained 45 species (with five subspecies) and the genomic analysis which generates a more in-depth insight into its genetic mechanisms of environmental adaptability covered already about half of the type strains (Li et al., 2013). Members of the genus *Nocardiopsis* are known to produce bioactive metabolites (Li et al., 2012) hence isolation from different environments may provide access to new bioactive products and contribute to an understanding of their ecological roles. In this paper we describe morphological, physiological, and chemical data which support the identification of strain No.156^{T} isolated from a sediment sample from Lake Elmenteita, a soda lake in the African Rift Valley, as representative of a novel species of the genus *Nocardiopsis*. The name *Nocardiopsis mwathae* sp. nov. is proposed for this species.

5.2 ISOLATION AND PHENOTYPIC CHARACTERIZATION

Strain No.156^T was isolated from sediment collected from Lake Elmenteita in March 2013. Sediment samples (2 g) were suspended in filter sterilised Lake water (10 ml) and serially diluted using the same water. Aliquots (100 µl) of appropriate dilutions were plated onto marine agar (DSMZ medium 123), at pH 8 supplemented with cAMP (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 10_µM (Bruns & Cypionka 2002). The plates were incubated at 28ºC for 14 days. Colonies appearing on the plates were streaked again on to fresh marine agar (DSMZ medium 123) for purification. The purified strain was stocked in marine broth with 15% glycerol (v/v) at -20ºC.

All physiological tests were performed at 28°C using strain No.156^T and cultures of *N. rosea* DSM 44842^T, *N. gilva* DSM 44841^T, *N. baichengensis* DSM 44845^T, *N. rhodophaea* DSM 44843^T, *N. chromatogenes* DSM 44844^T (Li et al., 2006), *N. halophila* DSM 44494^T (Al-tai & Ruan 1994) and *N. composta* DSM 44551^T (Kämpfer et al., 2002) as reference strains, in parallel assays. Aerial spore mass and substrate mycelia coloration were tested on marine medium (DSMZ medium 123), ISP2 (DSMZ medium 5318), ISP3 (DSMZ medium 609), ISP4 (DSMZ medium 547), Czapek medium (DSMZ medium 83), Sabouraud medium (DSMZ medium 1429), potato dextrose medium (DSMZ medium 129), CASO medium (DSMZ medium 220) and nutrient agar (DSMZ medium 1) for 21 days. Colony features were observed after 21 days under a binocular microscope according to (Pelczar, 1957). Exponentially growing bacterial cultures were observed with an optical microscope (Zeiss AxioScope A1) with a 1000-fold magnification and phase-contrast illumination. Colours were determined using the RAL Classic K5 colour chart. Pictomicrographs of bacterial cells of strain No.156^T grown on marine agar for 21 days were taken with a field-emission scanning electron microscope (FE-SEM Merlin, Zeiss, Germany). Gram reaction was performed using the KOH test described by (Gregersen, 1978).

Growth was assessed at 10-55^{o}C (in increments of 5^{o}C) for 14 days on marine agar, pH values from 5.0-13.0 (in increments of 1.0 pH unit) for 14 days at 28^{o}C using the buffer system described by Xu et al. (2005) and 0, 1-15% (w/v) NaCl (in increments of 2 units) for 14 days on nutrient agar at 28^{o}C. Biochemical characteristics such as hydrolysis of casein, degradation of xanthine, tyrosine, starch, and catalase and DNase activity were determined using standard procedures (Shirling & Gottlieb 1966; Williams et al., 1983; Smibert & Krieg, 1994). Strains were additionally characterized using API 20NE and API ZYM (bioMérieux) identification systems according to manufacturer's instructions. Phenotypic features were rated as positive when signal obtained was either weak or more pronounced.

5.3 CHEMOTAXONOMIC PROCEDURES

For chemotaxonomic studies, strain No.156^{T} and the reference strains were grown in marine broth in a shaking incubator at 150 r.p.m, 28^{o}C for 4 days. The mycelia were harvested by centrifugation and washed twice with 0.9% NaCl solution and freeze dried. All chemotaxonomical analyses were conducted under standardized conditions with strain No.156^{T} and cultures of the same set of reference strains as listed above for the phenotypic characterisations. The presence of mycolic acids was checked by the acid methanolysis method described by Minnikin et al. (1980). Amino acids and whole-cell sugars were prepared according to (Lechevalier & Lechevalier, 1970), followed by thin layer chromatography (TLC) analysis (Staneck & Roberts, 1974). Polar lipids were extracted, separated by two-dimensional TLC and identified according to procedures outlined by (Minikin et al., 1984) with modifications proposed by (Kroppenstedt and Goodfellow 2006). The composition of peptidoglycan hydrolysates (6 N HCl, 100^{o}C for 16 h) was examined by TLC as described by (Schleifer & Kandler, 1972). The extraction and analysis of cellular fatty acids was carried out from biomass grown in shaking incubator at 150 r.p.m for 3 days held at 28^{o}C. Analysis was conducted using the Microbial Identification System (MIDI) Sherlock Version 6.1 (TSBA40 database) as described by (Sasser, 1990). Fatty-acid patterns were visualized as heat map using the lipid extensions of the opm package (Vaas et al., 2013).

5.4 GENETIC AND PHYLOGENETIC ANALYSIS

G + C content of chromosomal DNA of strain No.156^T was determined by HPLC according to (Mesbah, 1989). Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product was carried out as described by (Rainey et al., 1996). The resulting sequence was compared with 16S rRNA gene sequences of other type strains using the EzTaxon server (Kim et al., 2012). Closely related 16S rRNA gene sequences to strain No.156^T were downloaded from EzTaxon. Phylogenetic analysis was based on alignment of 16S rRNA gene sequences from closely related type strains with validly published names in the genus *Nocardiopsis* inferred as described by Montero-Calasanz. (2013a). Rooting was done using the midpoint method (Montero-Calasanz et al., 2013a). Pairwise similarities were calculated as recommended by Meier-Kolthoff et al. (2013) and Montero-Calasanz et al. (2013b). DNA-DNA relatedness experiments were not carried out between strain No.156^T and its closest phylogenetic neighbours as the level of 16S rRNA gene sequence similarity between the strains was less than the cut-off value recommended for genomic distinction of species by Stackebrandt and Goebel (1994) and Meier-Kolthoff et al. (2013).

5.5 RESULTS AND DISCUSSION

Strain No.156^T was found to be aerobic, Gram-reaction-positive and formed a well-branched substrate mycelium with long, densely branched hyphae (Plate 5.1). Fragmentation of substrate mycelia did not occur as is typical of *Nocardiopsis* species (Labeda et al., 1984).

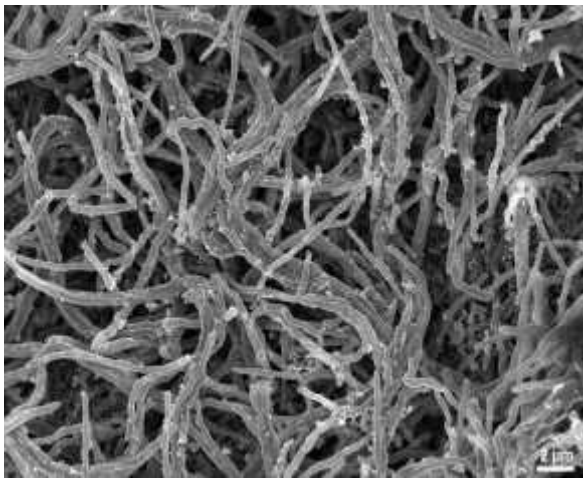

Plate 5.1: Scanning electron micrograph of strain No.156^{T} grown on marine agar for 21 days at 28°C.

Strain No.156^{T} grew abundantly on marine and Sabouraud agar, moderately on CASO agar, Czapek agar, nutrient agar and ISP3 agar, sparsely on ISP2 agar and potato dextrose agar, but did not grow on ISP4 agar. The substrate mycelium was yellow and formed white aerial mycelia on ISP3, ISP4 and marine agar, but not on the other media tested. No diffusible pigments were produced (Table 5.1).

Table 5.1: Differential cultural characteristics of strain No.156^T and the reference strains:

1, *Nocardiopsis mwathae* sp. nov. No.156^T; 2, *N. rosea* DSM 44842^T; 3, *N. gilva* DSM 44841^T; 4, *N. baichengensis* DSM 44845^T; 5, *N. rhodophaea* DSM 44843^T; 6, *N. chromatogenes* DSM 44844^T; 7, *N. halophila* DSM 44494^T and 8, *N. composta* DSM 44551^T. All strains were examined after growing on each of nine different media for 21 days at 28^{o}C. All data are from the present study, unless indicated otherwise.

Cultural characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**[a]8**
CASO								
Growth	++	++	+++	+++	+++	++	++	
Aerial spore-mass	-	-	-	-	white	-	-	
Substrate mycelium	yellow	light red	yellow	yellow	light red	light red	yellow	
Czapek								
Growth	++	++	++	+++	++	+++	+++	
Aerial spore-mass	-	white	-	white	white	white	white	
Substrate mycelium	yellow	light red	yellow	yellow	light red	beige red	yellow	
ISP 2								
Growth	+	++	++	+++	++	++	+++	
Aerial spore-mass	white	white	white	white	white	white	white	
Substrate mycelium	yellow	light red	yellow	cream	light red	black red	pale yellow	
ISP 3								
Growth	++	++	++	++	++	+	+	
Aerial spore-mass	white	-	white	-	white	-	-	
Substrate mycelium	yellow	light red	yellow	yellow	light red	beige red	yellow	nd
ISP 4								
Growth	-	++	+	++	++	+	-	
Aerial spore-mass	-	-	-	white	-	-	-	
Substrate mycelium	-	light red	yellow	yellow	light red	light red	-	
Marine agar								
Growth	+++	++	+++	+++	+++	++	++	
Aerial spore-mass	white	white	-	white	-	white	white	
Substrate mycelium	yellow	light red	yellow	yellow	light red	light red	yellow	
Nutrient agar								
Growth	++	++	++	+++	++	++	++	
Aerial spore-mass	-	-	-	white	-	-	-	
Substrate mycelium	yellow	light red	yellow	yellow	light red	beige red	yellow	
Potato dextrose								
Growth	+	++	++	+++	++	++	++	
Aerial spore-mass	-	-	white	white	-	white	white	
Substrate mycelium	yellow	light red	yellow	yellow	light red	black red	pale yellow	
Sabouraud								
Growth	+++	+++	++	++	++	+++	++	
Aerial spore-mass	-	-	white	white	-	-	-	
Substrate mycelium	yellow	light red	yellow	yellow	light red	black red	Yellow	

-, no growth; +, sparse; ++, moderate; +++, abundant. All data are from this study. [a]; data obtained from Kämpfer et al. (2002)

Strain No.156^T grew over a temperature range of 25-45^{o}C (optimum, 30-35^{o}C), pH 7.0-12.0 (optimum, pH 7.0-9.0) and at NaCl concentrations of 1-9% (w/v) (optimum, 6-9% (w/v)). The strain was positive for catalase, DNase activity and degradation of casein but negative for xanthine, starch and tyrosine degradation. No mycolic acids were detected in whole cell methanolysates which is in line with earlier report by Rainey et al. (1996). Analysis of cell-wall components revealed the presence of meso-diaminopimelic acid and no diagnostic sugars (Lechevalier et al., 1971; Labeda et al., 1984). The polar lipid pattern revealed the presence of the diagnostic components phosphatidylmethylethanolamine (PME) and phosphatidylcholine (PC), as well as phosphatidylglycerol (PG), phosphatidylinositol (PI) and four unspecified glycolipids (GL1-4). Strain No.156^T did however not have diphosphatidylglycerol (DPG) and other additional phospholipids, which is unique for this genus (Plate 5.2).

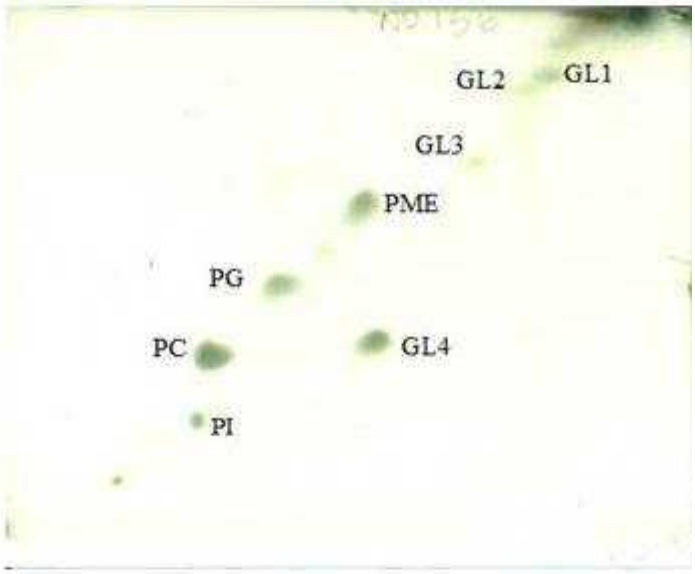

Plate 5.2: Polar lipids profile of Nocardiopsis mwathae sp. nov. No.156^T, after separation by two-dimensional TLC.

Plate was sprayed with molydatophosphoric acid for detection of total polar lipid. PC, phosphatidylcholine; PME, phosphatidylmethylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol and GL, unspecified glycolipids

The predominant menaquinones were MK-11(H_8) (23%), MK-11(H_6) (22%), MK-10(H_8) (20%) and MK-10(H_6) (17%) as previously described for the reference strains *N. rosea* DSM 44842^T, *N. gilva* DSM 44841^T and *N. rhodophaea* DSM 44843^T whose predominant menaquinones consist of 11 isoprene units in the side chain and a variable degree of saturation. This quinone system is unique among species of the genus and has also been reported for the type strains of *N. composta* (Kämpfer et al. 2002) and *N. potens* (Yassins et al., 2009) while other representatives of *Nocardiopsis* are reported to contain a quinone system dominated by menaquinone MK-10 and a variable degree of saturation of the isoprenoid side chain (Collins et al., 1977). The following fatty acids were detected (>2%): $C_{18:1\omega9c}$ (22.2%), *iso*-$C_{16:0}$ (19.7%), 10-methyl-$C_{18:0}$ (tuberculostearic acid, 12.5%), *iso*-$C_{16:0}$ (7.6%), *anteiso*-$C_{17:1}$ (7.1%), *anteiso*-$C_{17:0}$ (4.6%), $C_{16:1\omega9c}$ (3.3%) and 10-methyl-$C_{16:0}$ (2.9%) (Table 5.2). Fatty acid patterns were also visualized as heat maps (Figures 5.1-5.2). This combination of fatty acids with *iso*/*anteiso*-branched fatty acids, smaller amounts of 10-methyl-branched and unbranched fatty acids is characteristic for *Nocardiopsis* species (Fischer et al., 1983; Kroppenstedt, 1992).

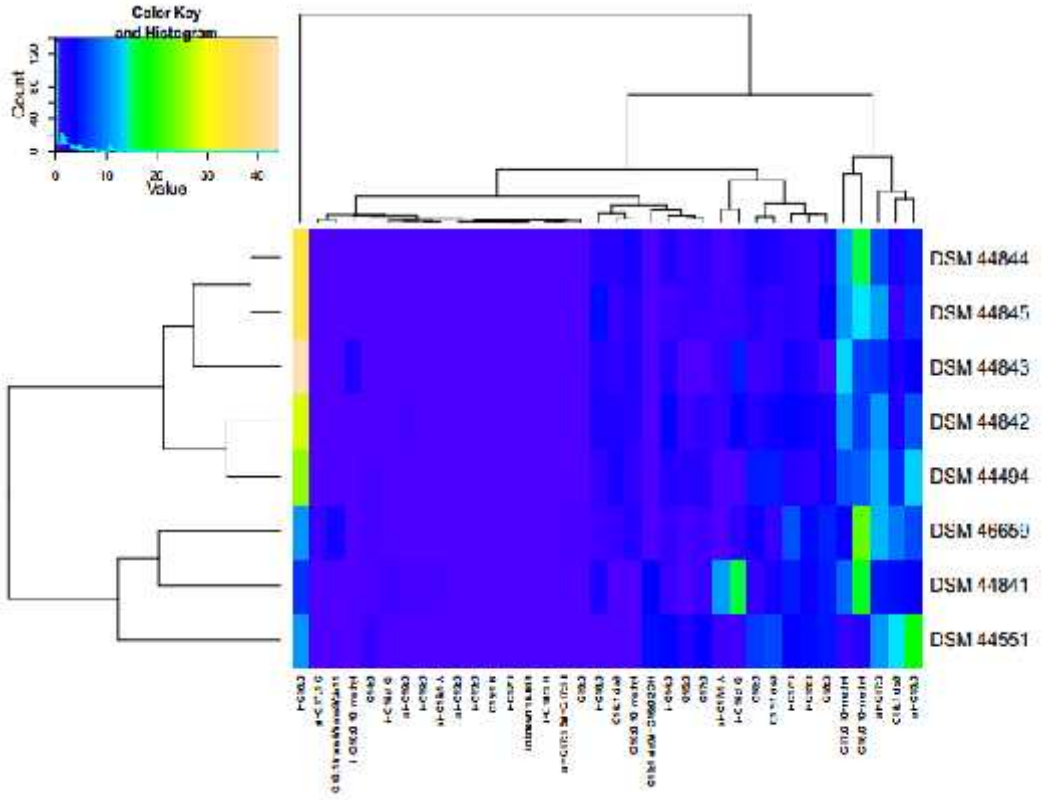

Figure 5.1: Heatmaps generated using the lipid extensions of the opm package showing un-transformed measurements under default settings after importing the fatty acids result files from the MIDI system**.**

Strains: *Nocardiopsis mwathae* sp. nov. No.156^{T} DSM 46659^{T}, *N. rosea* DSM 44842^{T}, *N. gilva* DSM 44841^{T}, *N. baichengensis* DSM 44845^{T}, *N. rhodophaea* DSM 44843^{T}, *N. chromatogenes* DSM 44844^{T}, *N. halophila* DSM 44494^{T} and *N. composta* DSM 44551^{T}.

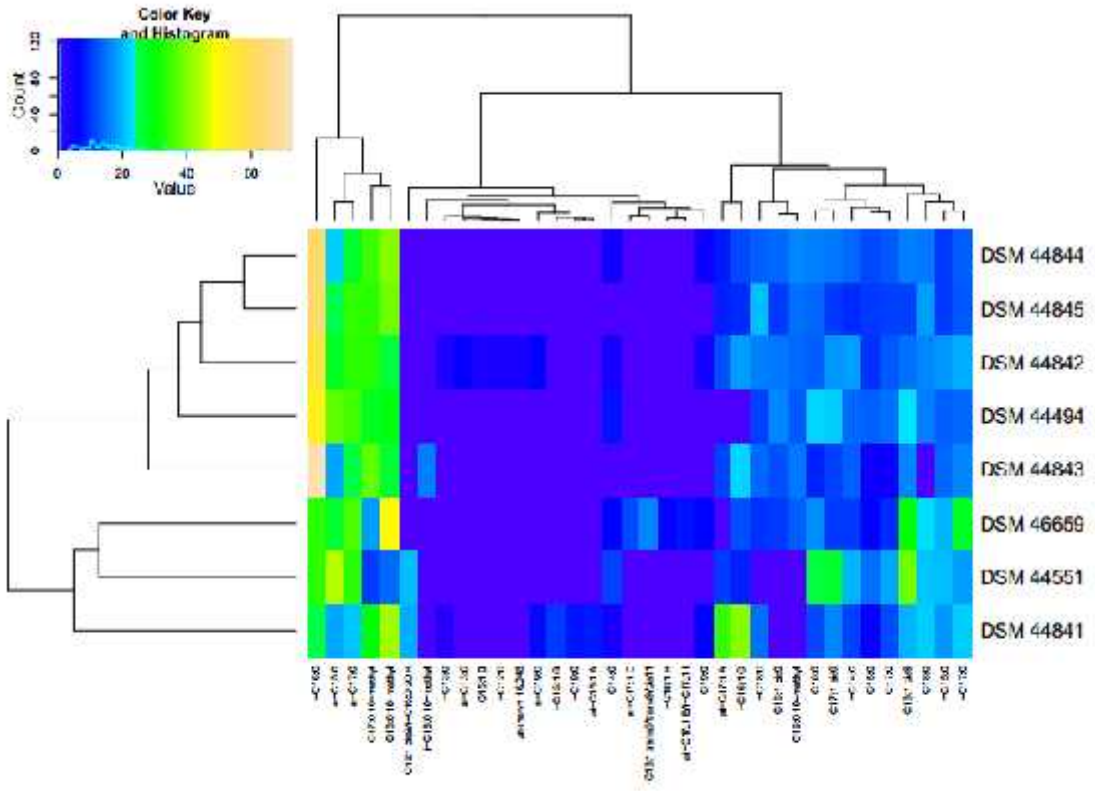

Figure 5.2: Heatmaps generated using the lipid extensions of the opm package showing log-transformed measurements under default settings after importing the fatty acids result files from the MIDI system.

Strains: Nocardiopsis mwathae sp. nov. No.156^{T} DSM 46659^{T}, *N. rosea* DSM 44842^{T}, *N. gilva* DSM 44841^{T}, *N. baichengensis* DSM 44845^{T}, *N. rhodophaea* DSM 44843^{T}, *N. chromatogenes* DSM 44844^{T}, *N. halophila* DSM 44494^{T} and *N. composta* DSM 44551^{T}.

Table 5.2: Cellular fatty acid compositions of strain No.156^T and the reference strains.

Strain order as above. Values are (>1) percentages of total fatty acids.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Saturated (straight chain) fatty acids								
15:0								1.84
16:0	1.43	1.05		1.65	1.48	3.44	1.59	4.32
17:0					1.56			4.46
18:0	2.01	2.09	2.76	2.63	3.62	2.87	2.56	6.35
Saturated (branched chain) fatty acids								
*i*14:0		1.33			1.47	1.01		2.47
*i*15:0		1.87	1.46		3.85			2.43
*i*16:0	7.59	27.10	5.33	22.74	16.25	28.66	33.55	11.68
*i*17:0	1.60	3.94	3.29	2.83	7.47	1.95	2.06	2.12
*i*18:0	1.30	3.15	3.65	1.63	2.43	1.69	1.51	
*ai*15:0	2.64	5.71	2.80	3.87	10.52	5.59	4.60	12.18
*ai*17:0	4.55	14.76	4.87	19.11	20.29	13.97	16.43	13.23
*ai*17:1c			10.68	2.53			2.34	
*ai*17:1a	7.14	1.30			1.18	1.34		1.91
16:0 10METHYL	2.93		5.06	1.96		1.70	2.12	
17:0 10METHYL	2.09	10.96	8.52	7.03	4.05	5.09	5.01	3.04
TBSA 18:0 10METHYL	12.47	7.94	9.15	22.94	2.48	22.20	20.16	5.74
monounsaturated fatty acids								
16:1 *iso* G	19.68	2.57	20.99	1.44		1.38	2.05	1.03
18:1 *iso* G	2.67		2.21					
16:1 ω_9c	3.28		2.18	1.70	2.15	2.18	1.74	3.12
17:1 ω_9c		3.22	3.84	1.09	6.06			9.00
18:1 ω_9c	22.19	9.52	7.97	3.84	11.62	3.23	1.92	11.85
Sum in feature 7	2.51							

i-, *iso*-branched, *ai*-, *anteiso*-branched; Sum in feature 7: 18:1cis 11/4 9/t 6.

Genomic G + C content was 71.0 mol%. In API ZYM tests the culture was positive for alkaline phosphotase, lipase C14, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, acid phosphatase, phosphoamidase, α-glucosidase, and N-Acetyl-β-glucosaminidase, but no enzymatic activity was observed for α-galactosidase, β-glucosidase esterase C4, lipase C8, β-glucuronidase, α-mannosidase and α-fucosidase. In API 20 NE the culture was positive for cytochrome oxidase, urea and aesculin hydrolysis L-arginine, β-glucosidase β-galactosidase, D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose and fermentation of glucose. Reduction of nitrates to nitrites, indole production and gelatin hydrolysis was not observed. The chemotaxonomic properties of strain No.156^T were consistent with its classification in the genus *Nocardiopsis* (Meyer, 1976; Kroppenstedt, 1992). A summary of selected differential phenotypic characteristics are presented in Table 5.3.

Table 5.3: Differential phenotypic characteristics of strain No.156^{T} and the reference strains.

Order of strains as above. All data are from the present study, unless indicated otherwise.

Phenotypic characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Optimum growth Temperatures (^{0}C) pH NaCl% (w/v)	30-35 7-9 6-9	30-40 7-8 5-7	30-40 7-8 6-8	35-40 7-8 6-8	30-40 7-8 5-8	30-40 7-8 4-9	25-30 7-9 6-12	[a]30-40 [a]7-9 [a]5-10
Degradation of DNA Xanthine	+ -	+ -	+ -	+ -	+ -	- -	+ +	- -
Enzymatic activity Lipase C14 Trypsin α-galactosidase β-galactosidase β-glucosidase	+ + - - -	+ + - - -	- + - - +	+ - + + +	- + - - +	+ - + + +	+ - + + +	+ - - + -
API 20NE Aesculin Nitrate reduction Gelatin Urea	+ - - +	+ - - -	- + - +	- - + +	+ - - +	+ - - +	- - - +	+ - + -
Polar lipids (*Plll)	Plll; PME, PC, PG, PI, GL1-4	[b]Plll; DPG, PME, PG, PC, PI, PE, PL, GL	[b]Plll; DPG, PME, PG, PC, PI, PE, PL, GL	[b]Plll; DPG, PME, PG, PC, PI, PIM, PL, PE, GL	[b]Plll; DPG, PME, PG, PC, PI, PIM, PL, PE, GL	[b] Plll; DPG, PG, PC, PI, PME, PL, PIM, PE, GL	[b]Plll	[a]Plll; PME, PC, DPG, PG, PL1, PL2
Major menaquinones	MK-11($H_{6,}$ H_{8}) MK-10($H_{6,}$ $H_{8,}$)	[b]MK-11($H_{0,}$ $H_{2,}$ H_{4})	[b]MK-11($H_{4,}$ $H_{6,}$ $H_{8,}$)	[b]MK-10($H_{2,}$ $H_{4,}$ $H_{6,}$)	[b]MK-11($H_{6,}$ H_{8})	[b]MK-10($H_{0,}$ $H_{2,}$ $H_{4,}$)	[b]MK-10($H_{6,}$ $H_{8,}$)	[a]MK-11(H_{8}) MK-10($H_{6,}$ $H_{8,}$)
DNA G+C content (mol%)	71.0	[b]67.9[b]	[b]68.1	[b]73.2	[b]69.0	[b]71.8	[b]71.5	[a]74.7

+, positive reaction; –, negative reaction; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PME, phosphatidylmethylethanolamine; PC, phosphatidylcholine; PIM, phosphatidylinositolmannosides; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, unspecified phospholipid; GL, unspecified glycolipid; [a]; data obtained from Kämpfer et al. 2002, [b]; data obtained from Li et al. (2006). *; PIII refers to the phospholipid pattern as described by Lechevalier et al (1977).

The almost complete (1463 bp) 16S rRNA gene sequence revealed the strain was closely related to the representatives of the genus *Nocardiopsis* and showed the highest degree of similarities with the type strains of *N. rosea* (97.9%), *N. composta* (97.7%), *N. gilva* (97.5%), *N. rhodophaea* (97.3%) and *N. halophila* (97.2%). Strain No.156^T and the closely related type strains clustered with members of the genus *Nocardiopsis* by both maximum likelihood and maximum-parsimony estimations (Figure 5.1). Based on its 16S rRNA sequences, it is clear that the isolate strain No.156^T belongs to the genus *Nocardiopsis* and represents a novel species.

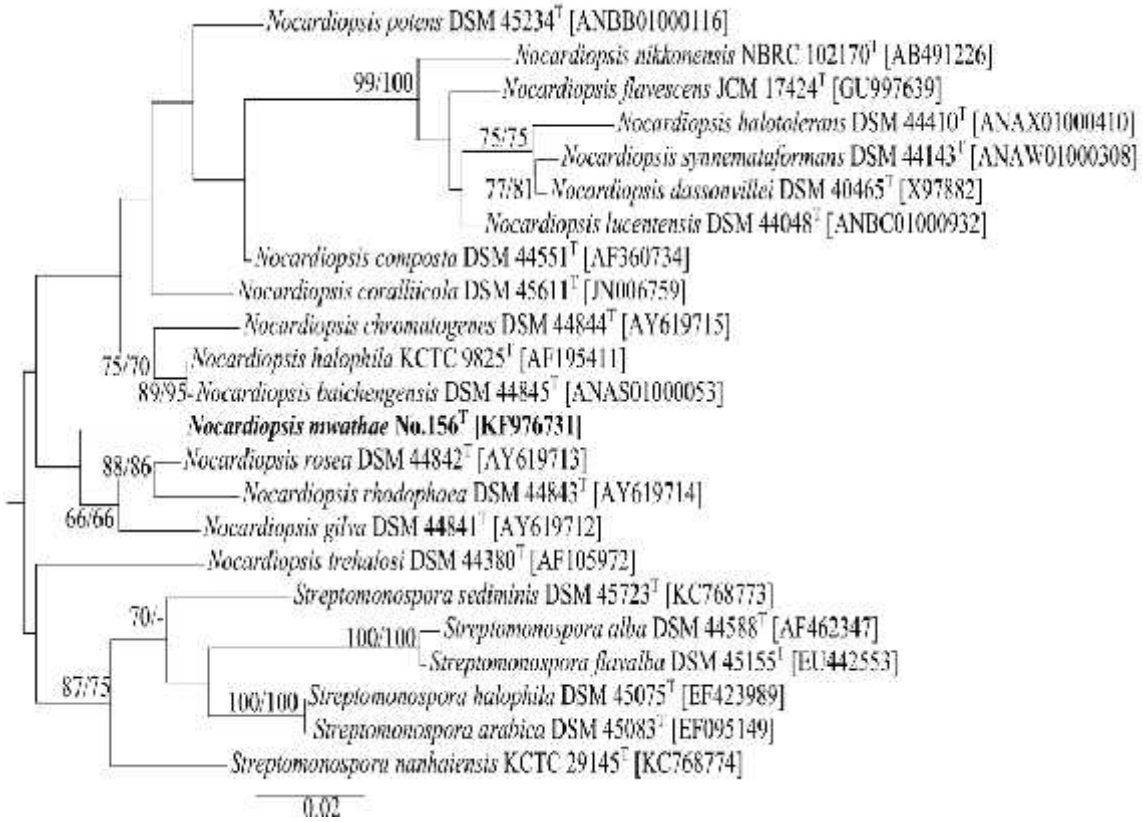

Figure 5.3: Maximum likelihood phylogenetic tree inferred from 16S rRNA gene sequences, showing the phylogenetic position of strain No.156^T relative to the type strains within the genus Nocardiopsis.

The branches are scaled in terms of the expected number of substitutions per site (see size bar). Support values from maximum-likelihood (left) and maximum-parsimony (right) bootstrapping are shown above the branches if equal to or larger than 60%.

The presented data demonstrates that strain No.156^T is a representative of a novel species of the genus *Nocardiopsis.* The strain can be differentiated from other *Nocardiopsis* species by cultural and phenotypic characteristics given in Table 1 and 2 respectively. The distinctness of this organism is further seen by the distinct phylogenetic position within the genus *Nocardiopsis* (Figure 5.1). The name *Nocardiopsis mwathae* is therefore proposed for the novel species with strain No.156^T as the type strain.

5.6 CONCLUSION

Nocardiopsis mwathae (N.L. gen. fem. n. *mwathae*, of mwatha, named after Kenyan microbial ecologist, the late Professor Wanjiru Mwatha in recognition of her pioneering work on Kenyan soda lakes).

Aerobic, Gram-reaction-positive bacterium. Forms well-branched substrate mycelium on marine agar. Grows abundantly on marine and Sabouraud agar, moderately on CASO, Czapek, nutrient and ISP3 agar, sparsely on ISP2 agar and potato dextrose agar but not on ISP4 agar. Forms yellow substrate mycelia on all media tested and white aerial mycelia on ISP3, ISP4 and marine agar. Diffusible pigments are not produced. Optimum growth is at 30-35ºC, pH 7.0-9.0 with 6-9% (w/v) NaCl. Positive for catalase, DNase activity and degradation of casein, negative for starch, xanthine and tyrosine degradation. Mycolic acids were not detected. Cell wall contains meso-diaminopimelic acid and no diagnostic sugars. The main polar lipids are phosphatidylmethylethanolamine, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol and four unspecified glycolipids, but no diphosphatidylglycerol and uncharacterised phospholipids. The predominant menaquinones are MK-11(H_8), MK-11(H_6), MK-10(H_8) and MK-10(H_6). Major cellular fatty acids are $C_{18:1\omega9c}$ (22.2%), *iso*-$C_{16:0}$ (19.7%), 10-methyl-$C_{18:0}$ (tuberculostearic acid, 12.5%). The type strain has a genomic DNA G + C content of 71.0 mol %. In API ZYM tests, the culture is positive for alkaline phosphotase, lipase C14, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, acid phosphatase, phosphoamidase, α-glucosidase and N-

Acetyl-β-glucosaminidase. No enzymytic activity is observed for α-galactosidase, β-glucosidase, esterase C4, lipase C8, β-glucuronidase, α-mannosidase and α-fucosidase. In API 20 NE, the culture is positive for cytochrome oxidase, urea and aesculin hydrolysis, L-arginine, β-glucosidase, β-galactosidase, D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose and fermentation of glucose. Reduction of nitrates to nitrites, indole production and gelatin hydrolysis is not observed. The INSDC accession number for the 16S rRNA gene sequences of the type strain No.156^{T} (= DSM 46659 = CECT 8552) is KF976731.

CHAPTER SIX

6.0 ISOLATION, CHARACTERIZATION AND COMPARATIVE ANALYSIS OF BACTERIOPHAGES FROM THE HALOALKALINE LAKE ELMENTEITA, KENYA.

ABSTRACT

Viruses that infect eubacteria called bacteriophage are known to exist in essentially every possible niche where bacteria reside. Phages provide a valuable resource to the development of modern biotechnology. Effective phage identification system is essential as it enables scientists to understand the biodiversity and relationships among phages in ecosystems. Viruses from the soda lakes in the Kenyan Great rift valley are under-studied at present hence rich reservoirs of enormous genetic and biological diversity remain to be mined and analyzed. As a step towards better understanding of the diversity and biology of phages and their hosts in haloalkaline Lake Elmenteita, bacteriophage were isolated from sediment sample using indigenous bacteria as hosts. Comparative study of the phages was conducted in order to determine their morphologies, growth characteristics, host ranges, structural proteins and genomes by restriction fragment length polymorphism and pulsed-field gel electrophoresis analysis. Nine bacteria were found to be susceptible. They were all Gram-reaction-positive, grew well on alkaline nutrient, Horikoshi-1 and LB media, over a temperature range of 25-45ºC (optimum, 30-35ºC) and pH range of 7.0-12.0 (optimum, pH 9.0-11.0). They showed different morphologies and belong to the order *Bacillales*. 18 seemingly different phage morphotypes with different dimensions were revealed by electron microscopy. They all could be assigned to the order *Caudovirales* and three common virus families; Myoviridae, Siphoviridae and Podoviridae. 14 clean virus cultures were further characterized. The phages were stable over a wide range of temperature, 25-45°C with optimum at 30-35°C. pH range was 7.0-12.0 with optimum at 8.0-10.0 The bacteriophage exhibited high specificity for their host bacteria. Protein profiles, restriction fragment length polymorphism analysis were distinct for each of the phages. According to pulsed-field gel electrophoresis, the 14 phage genomes could be divided into two groups. Group l; had genomes of ~37, 500 to ~58, 500 bp and group ll; with the largest genome sizes of ~145, 000 to ~200, 000 bp. The results showed that the 14 phages were distinct and confirmed the presence and diversity of bacteriophage in the haloalkaline Lake Elmenteita. The results set the stage for future studies aimed at better understanding of virus/host relationships.

6.1 INTRODUCTION

Soda lakes are strongly alkaline lakes, typically with a pH of 8.5 to >12, high concentrations of carbonate ions and with salinities ranging from brackish to hypersaline (Lanzén et al., 2013). The Kenya's Great Rift Valley contains this type of Lakes namely Elmenteita, Magadi, Bogoria, Nakuru and Sonachi (Rees et al., 2004). They are extremely productive because of high ambient temperatures, high light intensities and unlimited supplies of CO_2 (Grant, 1992) hence feature considerable microbial diversity that is of significant scientific interest (Zarvarzin et al., 1999). The groups of microbes able to grow under alkaline conditions in the presence of salt are referred as haloalkaliphiles. They possess special adaptation mechanisms to survive and grow under salinity and alkaline pH. These properties of dual extremity of halophiles and alkaliphiles make them interesting from both fundamental research and biotechnological points of view (Feng et al., 2005; Singh et al., 2010). Studies on diversity and isolation of bacterial species from these lakes has been highly documented (Rees et al., 2004; Mwirichia et al., 2010a; Mwirichia et al., 2010b; Mwirichia et al., 2011). However, viruses from these environments are particularly under-studied at present hence rich reservoirs of enormous genetic and biological diversity remain to be mined and analysed. Previous virus studies on these lakes include isolation of phages from Lake Magadi by Jamison et al. (2010) and Muruga et al. (2013), isolation and study of a bacteriophage infecting an alkaliphilic *Vibrio metschnikovii* from Lake Magadi (Moulton et al., 2011) and electron microscopic study of cyanophage that affect African flamingo population in Lake Nakuru (Peduzzi et al., 2014).

Phage ecology has essentially been investigated in North America, Europe and the oceans. Research into phage ecology in other parts of the world is almost non existent. As a result the phage flora of vast geographic areas, namely most of South America, Africa, Australia, China, India, all of Middle America, the Middle East and Siberia remains essentially unknown (Koko et al., 2011). The only reports on phages from African environments are limited to tailed phages in the subsoil of the Moroccan and Tunisian Sahara (Prigent et al., 2005) and the Namibian desert (Prestel et al., 2008), RNA phages in sewage in South Africa (Grabow et al., 1993;

Schaper et al., 2002), and unknown phages of non identified bacteria in Senegal (Bettarel et al., 2006). The Saharan phages were observed by electron microscopy, but not propagated or identified. The Namibian phages were observed by electron microscopy in bulk cultures, but they were not isolated and only one of the morphotypes present was identified. As a step towards better understanding of the diversity and biology of phages and their hosts in haloalkaline lake Elmenteita, bacteriophage were isolated from sediment sample using indigenous bacteria as hosts and characterized by electron microscopy, host ranges, protein structures, restriction fragment length polymorphism and pulsed-field gel electrophoresis analysis. To our knowledge, these experiments represent the first report of isolation and characterization of bacteriophage from this lake.

6.2 MATERIALS AND METHODS

6.2.1 Study site and sampling

The sampling site Lake Elmenteita is situated at 0º27´S, 36º15´E on the floor of the Kenyan Rift Valley at 1776 m altitude and has no direct outlet (Melack, 1988). The region is characterised by a hot, dry and semi-arid climate with a mean annual rainfall of about 700 mm (Mwaura, 1999). The size of Lake Elmenteita is roughly 20 km^2 and the depth rarely exceeds 1.0 m (Mwirichia et al., 2010b). The alkalinity of the water is high (1200 mg Na_2CO_3/l) with a high concentration of carbonates, chlorides and sulphates (Mwirichia et al., 2010b). The water temperature ranges between 30-40°C and the pH was above 9. Sediment sample plus the overlying water were collected (March, 2013) into sterile jars, capped on site and preserved in cooled boxes for transportation to the molecular laboratory in Jomo Kenyatta University of Agriculture and Technology (JKUAT). In the laboratory the samples were packaged for transfer to DSMZ in Braunschweig, Germany and stored at 8°C.

6.2.2 Isolation of bacterial host strains

Approximately 2 g of sediment was used to make a mastermix using filter sterilised

water (10 ml) from the lake and the solution serially diluted using the same water. Aliquots (100 µl) of appropriate dilutions were plated onto solid LB medium adjusted to approximately pH 9.5 with Sodium sesquicabornate (4.3 g $NaHCO_3$/5.2 g $NaCO_3$/100 ml distilled water). The plates were incubated at 28°C for 3 days. Colonies appearing on the plates were purified by three consecutive single colony passages. Isolated bacterial strains were used as hosts for the detection and enrichment of lytic bacteriophages from the same lake. Susceptible strains were stocked in LB broth (pH 9.5) with 15% glycerol (v/v) at -20°C.

6.2.3 Morphology, physiology and phylogenetic analysis of host bacteria

Growth of the strains on different media (LB, nutrient agar and Horikoshi-1) at 28°C was assessed. Growth was also assessed at temperature 20-45°C (in increaments of 5°C), pH values from 5.0-13.0 (in increments of 1.0 pH unit) using LB as the basal medium. Cell morphology (size, shape, arrangement) was determined by phase-contrast microscopy (magnification, 400×) after 3 days of incubation at 28°C. Motility was determined on wet mounts under phase contrast microscopy (magnification, 400×). Gram reaction was performed using the KOH test described by (Gregersen, 1978). Strains were additionally characterized using API 20NE and API ZYM (bioMérieux) identification systems according to manufacturer's instructions.

Bacterial hosts' genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR products was carried out as described by (Rainey et al., 1996). Identification of phylogenetically closest neighbours and calculation of pairwise 16S rRNA gene sequence similarity was achieved using the EzTaxon server (http://www.eztaxon.org) (Kim et al., 2012). The genomic homogeneity of the strains was also examined in comparison with their close relatives by Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra (MS) and analysis performed according to Töth et al. (2008).

6.2.4 Isolation of Bacteriophages

Medium: LB medium supplemented with 2mM $CaCl_2$ (Sigma-Aldrich, St. Louis, MO) adjusted to approximately pH 9.5 using 1M Sodium-Sesquicarbonate (4.3 g $NaHCO_3$, 5.2 g $NaCO_3$, 100 ml distilled water;1 ml in 10 ml medium) was used, unless indicated otherwise.

Approximately 1 g of sediment sample was suspended in 9 ml LB broth in sterile 15 ml centrifuge tube and mixed thoroughly on an overhead shaker for 1 h at room temperature. The sample was centrifuged at 7 500 r.p.m for 15 minutes then filtered through a 0.45 µm pore size syringe filter (Millipore corp, Billerica, MA). The supernatant (5 ml) was added to equal amount of double strength LB broth and inoculated with an early log-phase (0.1 ml) host culture. After overnight enrichment at 28°C with gentle shaking, the culture was centrifuged at 7500 r.p.m for 15 minutes (Twest and Kropinski, 2009). This enrichment procedure was repeated thrice. The supernatant obtained from the final enrichment step was filter sterilized through a 0.45 µm pore size syringe filter and verified for the presence of phages by the soft agar overlay method (100 µl phage lysate was added to 200 µl of an overnight culture of indicator strain and mixed with 5 ml of liquid soft agar at 45°C). This mixture was spread on solid LB medium, incubated overnight at 28°C and controlled for the presence of plaques (Carlson, 2005). Uninfected host strain was used as negative control for checking bacteriocin reactions to confirm the validity of plaques (Liu et al., 2006). Underlay procedure for phage purification was adopted (Twest & Kropinski, 2009). The procedure was repeated three times.

6.2.5 Bacteriophage amplification and purification

To recover phages, confluent lysis was generated and the phages eluted by transferring agar overlayer aseptically into 10 ml of mid-log host cell culture in LB broth and incubated at 28°C with gentle shaking (overnight) (Yamamoto & Alberts, 1970). The phage supernatant was collected by centrifugation at 7,500 r.p.m for 15 minutes, filtered (0.45 µm) and the phage stock stored at 4°C. The titre of the stock was determined by the overlay method.

One ml of the phage lysate was transferred aseptically to 10 ml of the mid-log host cell culture in LB broth and incubated at 28°C with gentle shaking until clearing was observed (overnight). The phage supernatant was collected by centrifugation at 7500 r.p.m for 15 minutes (Sorvall RC6, F10S-6×500y rotor). The fresh lysate (10 ml) was added to 200 ml of mid-log host cell culture and repeated as above. Phages were concentrated by centrifugation at 12,000 r.p.m for 2 h (Sorvall RC6, F21S-8×50 rotor). The phage pellet was resuspended in 1 ml of TE buffer (20 mM Tris [pH 7.5], 50 mM NaCl) (Beilstein & Dreiseikelmann, 2006).

One and a half ml concentrated phage suspension was overlaid onto a four-step Caesium Chloride (CsCl) gradient containing 0.7 ml each of 1.7 g/ml, 1.5 g/ml, 1.4 g/ml and 1.3 g/ml CsCl (Optical grade, Gibco) in a 4.3 ml ultracentrifuge tube (Beckman Coulter). Phages were centrifuged for 2 h at 20°C and 22, 000 r.p.m in ultracentrifuge (Beckman Coulter, Optima^{TM} L-XP; SW 60 Ti 12E873 rotor). Phage-containing bands (white-to-grey) were extracted by puncturing the wall of the ultracentrifuge tube using a needle and the CsCl removed by dialysis (visking dialysis tubing: Type (inch) 8/32, wall thickness (mm) 0.050, width (mm) 10, Ø (mm) 6.3; ROTH) for 15 h with two changes of TE buffer (10 mM Tris [pH 7.5], 50 mM NaCl) (Beilstein & Dreiseikelmann, 2006).

6.2.6 Negative staining and electron microscopy of bacteriophages

Thin carbon support films were prepared by sublimation of a carbon thread onto a freshly cleaved mica surface. Phages were adsorbed onto the carbon film and negatively stained with 2% (w/v) aqueous uranyl acetate, pH 5.0, according to the method of Valentine et al. (1968) Samples were examined in a TEM 910 transmission electron microscope (Carl Zeiss, Oberkochen) at an acceleration voltage of 80 kV. Images were taken at calibrated magnifications using a line replica. Images were recorded digitally with a Slow-Scan CCD-Camera (ProScan, 1024×1024,

Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany). (Manfred Rohde, Helmholtz Centre for Infection Research Inhoffenstraße 7, 38124 Braunschweig, Germany). The phenotypic diversity of the bacteriophages was determined using the morphological criteria outlined by the International Committee of Taxonomy of Viruses (Murphy et al., 1995) and Ackermann (1996).

6.2.7 Phage titering and plaque morphology

Serial dilutions of phage stock lysates were prepared and aliquots of these dilutions were used to determine the phage titer by the overlay method. Plates were incubated at 28°C overnight and the resulting PFUs were quantified to determine phage titer. Plaque morphologies of the phages were determined and plaque diameters measured.

6.2.8 Thermal and pH stability tests

The thermal stability of phages was examined by pre-incubating phage suspensions at different temperatures (20, 25, 30, 35, 40, 45 and 50°C respectively) at pH 7.0 for 6 hours. The phage suspensions were immediately cooled in ice water and the surviving phages were titered by the double agar layer method. The pH stability of phages was examined by pre-incubating the phage suspensions at different pH levels (2, 4, 6, 8, 10 and 12 respectively) at 25°C for 6 h. The surviving phages were immediately counted by the double agar layer method (Yu et al., 2013).

6.2.9 Host range analysis of phages

To ascertain the range of infectiveness of the obtained bacteriophages, all the susceptible bacterial strains isolated in this study were used. Double layer agar plates with different bacterial strains were prepared. The lysis spectrum of isolated phages was determined by spotting 10 µl of phage lysate on each agar plate with different bacterial strains. The plates were incubated at 28°C overnight and examined for clearing (Stenholm et al., 2008).Observed inhibition of growth as marked by clearing where the lysate was spotted was denoted as susceptibility of the bacteria.

6.2.10 Protein profiles

SDS-PAGE was performed by the method of Laemmli (1970). A sample of 50 µl purified virus particles (5×10^{10}/ml) was dissolved in 50 µl loading buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (50 µl Mecarptoethanol, 950 µl Laemmli sample buffer (2×) for SDS-PAGE; SERVA electrophoresis). After heating at 95°C for 5 min, the samples were subjected to electrophoresis in 12% SDS-PAGE gel along with protein marker (PageRuler^{TM} Broad Range Unstained protein ladder; Thermo Scientific) with Tris-glycine as running buffer. After electrophoresis proteins were visualized by staining with Coomassie Brilliant Blue R250 dye (Sigma).

6.2.11 DNA extraction

DNA was extracted from CsCl purified high-titre stocks of phage using phage DNA isolation kit (Norgen Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. The purity and the concentration of the DNA were determined using spectrophotometer (Invitrogen Qubit).

6.2.12 Restriction digestion patterns

For comparison of DNA fragment patterns, phage genomic DNA was digested with different restriction endonucleases according to the instructions of the manufacturer (Fermentas life sciences, UK). A total of five restriction endonuclease enzymes were used namely; *Dra*I, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I and *Bam*H1. Restriction fragments were separated by electrophoresis for one hour on 1.0% agarose (Sigma, USA) gel stained with ethidium bromide. DNA molecular weight marker (mi-1Kb DNA Marker; Metabion, Germany) was used for size determination of DNA fragments (Mitra and Ghosh, 2007).

6.2.13 Genome estimation

Pulsed field gel electrophoresis (PFGE) was used to estimate the genomes size for the 14 bacteriophage isolates according to the protocol published by Linghor et al (2009). Plugs were prepared and gel run at 5 V/cm for 24 h at 14°C with initial switch at 5s and final switch at 15s.

6.3 RESULTS AND DISCUSSION

6.3.1 Isolation and characterization of bacterial host strains

Nine bacteria from Lake Elmenteita were found to be susceptible. They all were Gram-reaction- positive, grew well on alkaline nutrient (DSMZ medium 31), basal media for alkaliphilic micro-organisms; Horikoshi-1 (DSMZ medium 1081) and LB (DSMZ medium 381) media, over a temperature range of 25-45^{o}C (optimum, 30-35^{o}C) and pH range of 7.0-12.0 (optimum, pH 9.0-11.0). The bacteria showed different morphologies. The partial (approximately 900 bp) 16S rRNA gene sequences comparative analysis of the isolates revealed they belong to the order *Bacillales*. The level of similarity between the isolates and their closest known relatives was between 98-100%. This was supported by MALDI-TOF protein spectra analysis which showed high similarity of the isolates with previously described strains in the order *Bacillales* (Table 6.1). A summary of selected physiological properties to further characterize the isolates, as indicated by API 20NE and API ZYM (bioMérieux) identification systems are presented in Table 6.2.

Table 6.1: Host bacteria, % 16 S rRNA gene sequence similarity, there characteristic morphologies and motilities as observed under phase contrast microscope (400×).

	Host	**Closest relative**	**% 16S rRNA gene sequence similarity**	**Motility and morphology**
1	HS32	*Vibrio metschnikovii*	98.85	transitional motility, vibrio
2	HS61	*Bacillus pseudofirmus*	99.85	non-motile, short rods
3	HS123	*Exiguobacterium aurantiacum*	99.78	non-motile, cocci
4	HS125	*Bacillus bogoriensis*	98.19	non-motile, rods
5	HS126	*Bacillus horikoshii*	99.87	gliding motility, long rods
6	HS132	*Exiguobacterium alkaliphilum*	100.00	motile, cocci
7	HS136	*Bacillus cohnii*	99.64	non-motile, short rods
8	HS140	*Bacillus pseudalcaliphilus*	98.18	gliding motility, long-rods
9	HS171	*Bacillus halmapulus*	98.87	gliding motility, long rods

Table 6.2: A summary of selected phenotypic characteristics of the host bacteria as indicated by API 20NE and API ZYM (bioMérieux) identification systems at 28ºC according to manufacturer's instructions.

Substrate	**Test organisms**								
	HS32	**HS61**	**HS123**	**HS125**	**HS126**	**HS132**	**HS136**	**HS140**	**HS171**
API 20NE									
Indole production	+	++	-	+	++	+	++	+	+
Glucose fermentation	+	-	+	-	+	+	-	-	+
Arginine dihydrolase	+	-	+	+	+	++	-	+	+
Urease production	+	-	+	+	-	+	-	+	++
Esculin hydrolysis	++	++	++	++	++	+	+	++	++
Gelatin hydrolysis	++	+	-	+	+	+	-	+	++
API ZYM									
Alkaline phosphatase	+	+	++	++	++	++	++	++	++
Esterase C4	+	+	+	++	++	+	+	+	+
Lipase C8	-	+	++	++	++	+	+	+	+
Leucine aminopeptidase	+	++	++	++	++	++	++	++	++
Valine aminopeptidase	+	++	-	++	++	+	+	+	++
Cystine aminopeptidase	-	++	-	+	++	+	-	+	-
Trypsin	-	+	-	++	++	-	+	+	++
α -galactosidase	++	-	+	++	-	-	++	-	-
β -galactosidase	++	-	++	++	+	++	-	-	-
α-glucosidase	+	++	++	++	+	++	++	++	-
β -glucosidase	++	++	++	++	++	++	+	-	-

Key: -, no reaction; +, good; ++, excellent.

6.3.2 Bacteriophage isolation and morphological characterization

The most important criterion for phage taxonomy is ultrastructure (Ackermann et al., 1992). A total of 18 seemingly different phage morphotypes were isolated and

phenotypes examined by electron microscopy. Transmission electron microscope revealed a variety of structural features. Both tailed and non-tailed forms are present in this Lake. According to Ackermann (1996) classification, they all could be assigned to the order *Caudovirales* and three common virus families; *Myoviridae, Siphoviridae and Podoviridae* (Plates 6.1-6.18). Among them, nine (K, J, M, I, H, F, C, P and R) belonged to Family *Siphoviridae* (icosahedral head with long, flexible, non-contractile tail), seven (A, L, D, G, Q, B and E) to Family *Myoviridae* (icosahedral head and contractile thick tail with defined base plates) and two (N and O) to the Family *Podoviridae* (short non-contractile tail). *Siphoviridae* and *Myoviridae* phages were prevalent morphotypes compared to *Podoviridae* suggesting that most common viruses in the Lake are tailed phages. This supports earlier reports by Ackermann (1999) that *Siphoviridae* is by far the most frequent phage group, followed by the *Myoviridae* and *Podoviridae*.

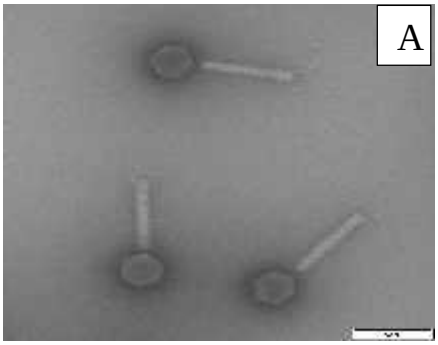

Plate 6.1: Transmission electron micrograph of phage A (vB_EauM-23).

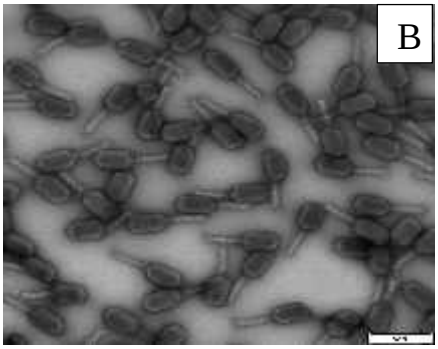

Plate 6.2: Transmission electron micrograph of phage B (vB_VmeM-32).

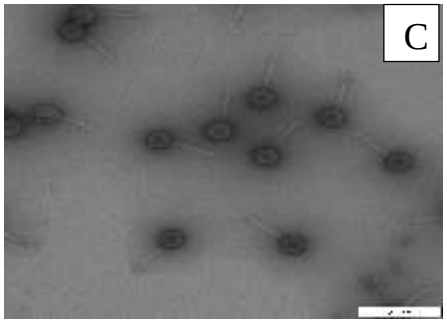

Plate 6.3: Transmission electron micrograph of phage C (vB_BpsS-36).

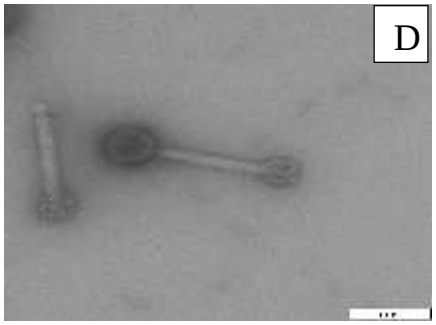

Plate 6.4: Transmission electron micrograph of phage D (vB_BpsM-61).

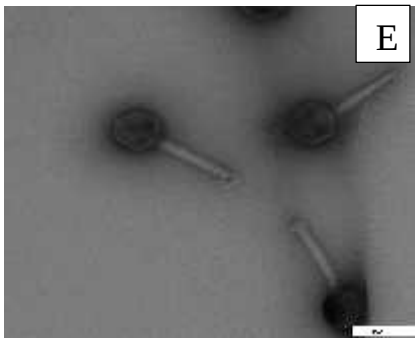

Plate 6.5: Transmission electron micrograph of phage E (vB_VmeM-196).

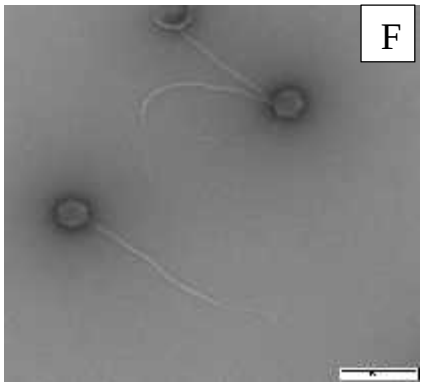

Plate 6.6: Transmission electron micrograph of phage F (vB_BpsS-140).

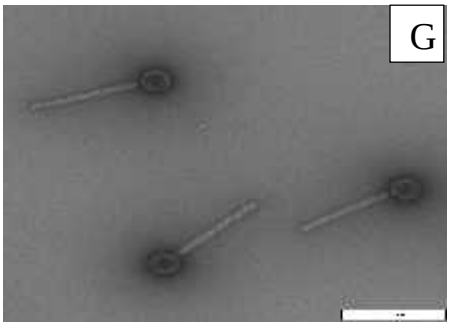

Plate 6.7: Transmission electron micrograph of phage G (vB_EalM-137).

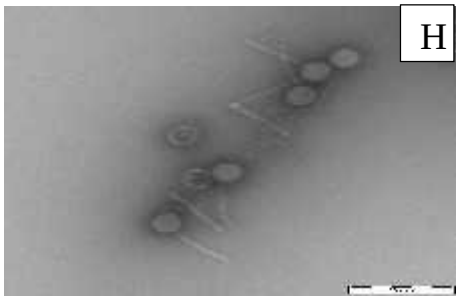

Plate 6.8: Transmission electron micrograph of phage H (vB_BcoS-136).

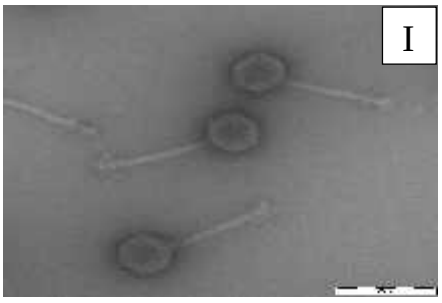

Plate 6.9: Transmission electron micrograph of phage I (vB_BhaS-171).

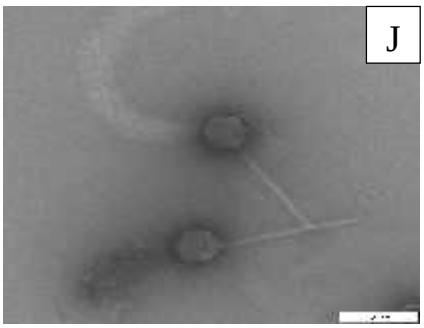

Plate 6.10: Transmission electron micrograph of phage J (vB_BboS-125).

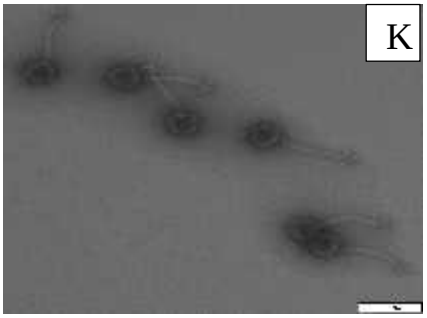

Plate 6.11: Transmission electron micrograph of phage K (vB_BboS-125).

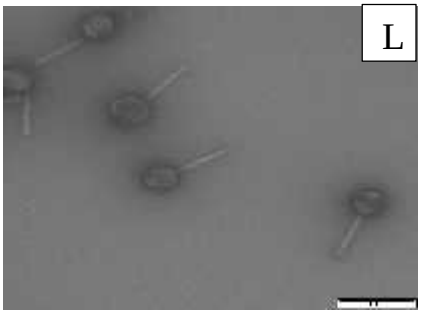

Plate 6.12: Transmission electron micrograph of phage L (vB_EalM-132).

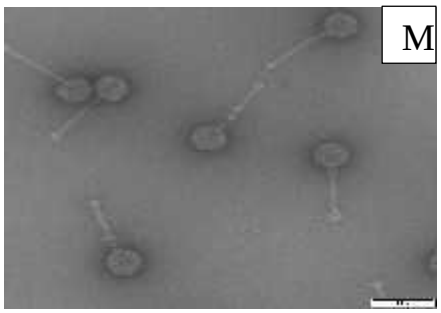

Plate 6.13: Transmission electron micrograph of phage M (vB_BhoS-126a).

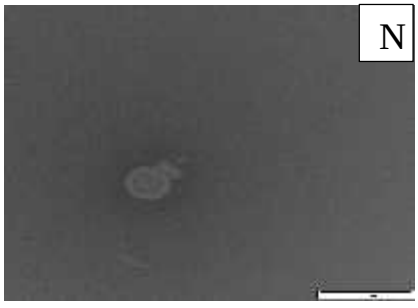

Plate 6.14: Transmission electron micrograph of phage N (vB_BhoP-126b).

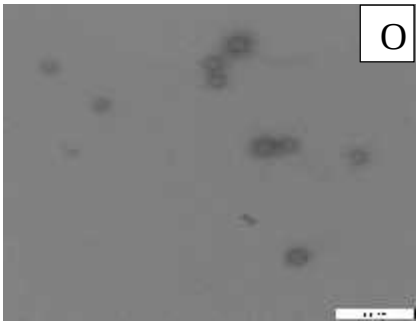

Plate 6.15: Transmission electron micrograph of phage O (vB_EauL-123c).

Plate 6.16: Transmission electron micrograph of phage P (vB_EauS-123b).

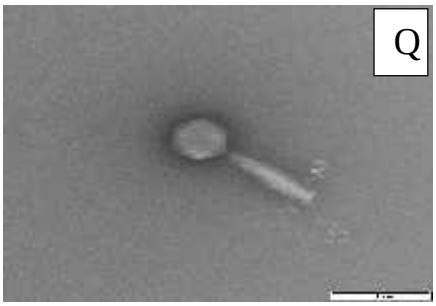

Plate 6.17: Transmission electron micrograph of phage Q (vB_EalM-137a).

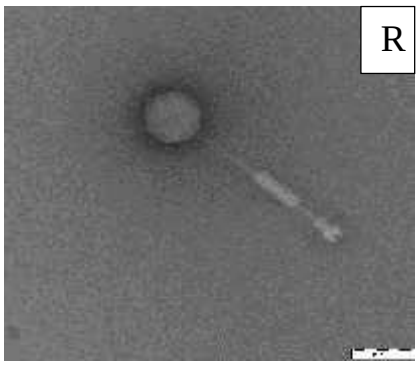

Plate 6.18: Transmission electron micrograph of phage R (vB_EalS-137b).

Most of the phages isolated in Lake Elmenteita have morphological structures that have been described previously from a marine environment (Frank & Moebus, 1987). Few showed unique structures that have not been reported previously for haloalkaliphilic bacteriophage. Among the Siphoviruses, K; has nodule like structures at the end of the tail with a distal pin, M; has ring like structure both at the neck and tail end with a hook like structure at the tail, and F; is a typical syphovirus with an extremely long flexible tail with an additional extremely long tail fibre. Among the Myoviruses, A; appeared to have a constriction at connection of head with tail, D; has a unique knob-like structure at the end of the tail, Q; has a thick tail with three ribbon like tail-fibres that seem to detach easily and B; with an elongated head. Podovirus N has a collar like structure between head and the short tail. The dimensions of the phages were also determined. The capsid diameters ranged between 47.3-130.4 nm and tail lengths measured from the bottom of the neck to the base plate ranged between 37.4-546.3 nm. The unique structural features and different phage dimensions further indicated diversity within the various families. The bacteriophages were further named according to the recommendations outlined by Kropinski et al. (2009) (Table 6.3). Out of the 18 bacteriophage isolates, only 14 with clean culture were further characterized.

Table 6.3: A summary of the phages obtained in this study from Lake Elmenteita showing the respective host bacteria, family, naming according Kropinski et al. (2009) recommendations and the dimensions.

Reference		Host	Family	Name	Phage size (nm)		
					Head diameter	Tail length	Total size
1	D	HS61	*Myoviridae*	vB_BpsM-61	66.2	191.6	257.8
2	A	HS123	*Myoviridae*	vB_EauM-23	60.0	124.5	184.5
3	K	HS123	*Siphoviridae*	vB_EauS-123	48.7	138.0	186.7
4	P	HS123	*Siphoviridae*	vB_EauS-123b	-	-	-
5	O	HS123	*Podoviridae*	vB_EauP-123c	-	-	-
6	J	HS125	*Siphoviridae*	vB_BboS-125	60.8	178.9	239.7
7	M	HS126	*Siphoviridae*	vB_BhoS-126a	57.4	119.5	176.9
8	N	HS126	*Podoviridae*	vB_BhoP-126b	47.3	37.4	84.7
9	L	HS137	*Myoviridae*	vB_EalM-132	84.5	161.2+160.6	406.3
10	H	HS136	*Siphoviridae*	vB_BcoS-136	59.3	144.9	204.2
11	Q	HS137	*Myoviridae*	vB_EalM-137a	-	-	-
12	R	HS137	*Siphoviridae*	vB_EalS-137b	-	-	-
13	G	HS137	*Myoviridae*	vB_EalM-137	61.6	214.2	275.8
14	F	HS140	*Siphoviridae*	vB_BpsS-140	83.3	546.3+178.9	808.5
15	I	HS171	*Siphoviridae*	vB_BhaS-171	58.1	116.5	174.6
16	C	HS140	*Siphoviridae*	vB_BpsS-36	57.4	109.6	167.0
17	B	HS32	*Myoviridae*	vB_VmeM-32	130.4	108.8	239.2
18	E	HS32	*Myoviridae*	vB_VmeM-196	77.2	158.9	236.1

Key: -, (not measured)

6.3.3 Phage titering and plaque morphology

Titers of isolated phages ranged from 10^4 to 10^{10} PFU/ml following overnight

incubation with the host bacteria. All the phages displayed plaques that varied from turbid to very clear with characteristically variable sizes from pinpoint to 0.4 mm (Table 6.4). According to plaque morphology the Siphoviruses generally displayed minute clear plaques (pinpoint-0.2 mm), while the Myoviruses displayed large plaques that ranged between 0.3-0.4 mm. The plaques were categorized as clear, moderately clear and turbid.

Table 6.4: Plaque morphologies and diameter of the phages determined on their respective indicator bacterial strains.

	Phage	**Lawn indicator**	**Plaque morphology**	**Plaque diameter (mm)**
1	vB_BpsM-61	*Bacillus pseudofirmus*	Clear plaques, entire edge	0.3-0.4
2	vB_EauM-23	*Exiguobacterium aurantiacum*	Clear plaques	0.2-0.3
3	vB_EauS-123	*Exiguobacterium aurantiacum*	Tiny clear plaques	0.1-0.2
4	vB_BboS-125	*Bacillus bogoriensis*	Turbid, plaques with clear halo at the centre, diffuse edge	0.3-0.4
5	vB_BhoS-126a	*Bacillus horikoshii*	Turbid plaques with halo at the centre	0.2-0.3
6	vB_BhoP-126b	*Bacillus horikoshii*	Turbid plaques with halo at the centre	0.3-0.4
7	vB_EalM-132	*Exiguobacterium alkaliphilum*	Clear plaques	0.2-0.3
8	vB_BcoS-136	*Bacillus cohnii*	Clear plaques	Pinpoint-0.1
9	vB_EalM-137	*Exiguobacterium alkaliphilum*	Clear plaques	0.1-0.2
10	vB_BpsS-140	*Bacillus pseudalcaliphilus*	Clear plaques	0.3-0.4
11	vB_BhaS-171	*Bacillus halmapulus*	Clear plaques	0.1-0.2
12	vB_BpsS-36	*Bacillus pseudalcaliphilus*	Clear plaques	0.1-0.3
13	vB_VmeM-32	*Vibrio metschnikovii*	Clear plaques	0.2-0.3
14	vB_VmeM-196	*Vibrio metschnikovii*	Clear plaques	0.2-0.3

6.3.4 Thermal and pH stability tests

The phages were stable over a wide range of temperature and pH. Growth at 20^{o}C generally yielded no plaques. The Plaque forming units however increased exponentially from 25^{o}C with maximum at 35^{o}C. At 45^{o}C the plaques reduced with none at 50^{o}C. Optimal temperature for plaque formation was at 30-35°C. At pH 2 and 4 no plaques were observed. Plaques formed from pH 7 and increased with increasing pH values with maximum at 10. Optimal pH was at 8-10.

6.3.5 Host range analysis

Host range analysis tested against the nine bacterial hosts isolated in this study revealed that the bacteriophage exhibited high specificity for their host bacteria. This characteristic has been previously reported for marine bacteriophages (Coetzee, 1987; Børsheim, 1993). Because of their specificity the phages can be applied to map the distribution of bacteria, a very sensitive tool for tracing specific groups of bacteria compared to using taxonomy of bacteria (Børsheim, 1993).

6.3.6 Protein profiles

The structural proteins were analyzed by SDS-PAGE. Protein profiles assessment exhibited variations (Figure 6.19). The results revealed that the protein patterns of the phages contained: (1) vB_BpsM-61; 3 minor bands, (2) vB_EauM-23; 7 minor bands and 1 major band (20 KDa), (3) vB_EauS-123; 4 minor bands and 1 major band (20 KDa), (4) vB_BboS-125; 1 minor band, (5) vB_BhoP-126b; 5 minor bands and 1 major band (20 KDa), (6) vB_BhoS-126a; 1 minor band, (7) vB_EalM-132; 2 minor bands and 1 major band (20 KDa), (8) vB_BcoS-136; 1 minor band and 2 major bands (15 and 30 KDa), (9) vB_EalM-137; 2 minor bands and 3 major bands (15, 20 and 25 KDa), (10) vB_BpsS-140; 3 minor bands and 1 major band (20 KDa), (11) vB_BhaS-171; 3 minor bands and 1 major band (20 KDa), (12) vB_BpsS-36; 3 minor bands and 3 major bands (15, 20 and 30 KDa), (13) vB_VmeM-32; 6 minor bands and 1 major band (40 KDa), (14) vB_VmeM-196; 5 minor bands and 1 major

band (40 KDa). The molecular weights of the structural polypeptides ranged from 10 to 100 KDas. Most phages had the major band at 20 KDa. The minor bands were varied and might be responsible for host-specificity or the characteristics specific to a particular phage (Hantke, 1978). It is common to have wide variations in the banding pattern of proteins of different phages (Surekhamol et al., 2014). For instance, Barrangou et al. (2002) observed the presence of 25 bands in six *Leuconostoc fallax* phages, and Nasu et al. (2000) found a single 5 KDa band in a phage of *Vibrio parahaemolyticus*. The protein profiles show that protein patterns can be used for phage characterization and differentiation.

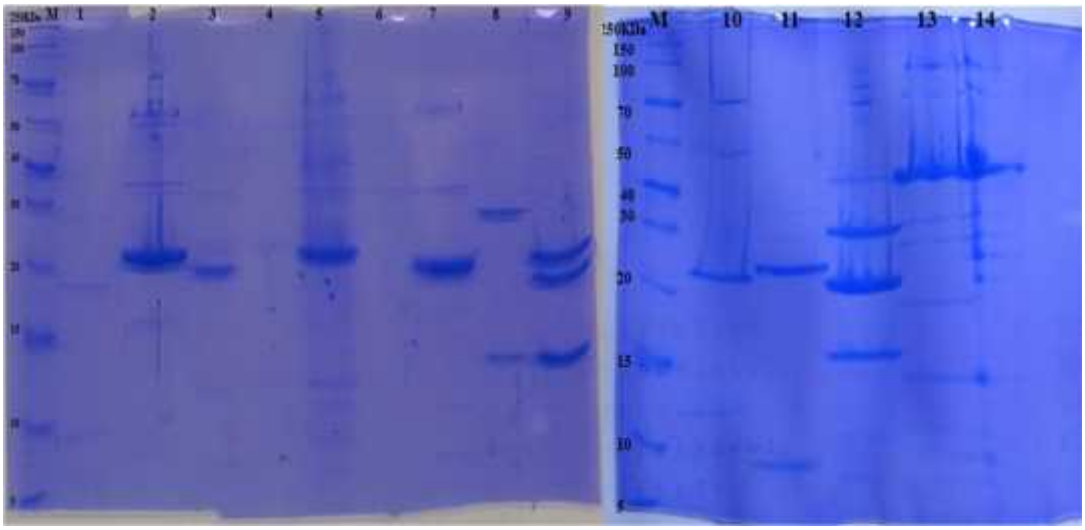

Figure 6.11: SDS-polyacrylamide gel electrophoretic profiles of phage structural proteins. (M) broad range Page Ruler^{TM} protein molecular weight marker (Thermoscientific), (1) vB_BpsM-61, (2) vB_EauM-23, (3) vB_EauS-123, (4) vB_BboS-125, (5) vB_BhoP-126b, (6) vB_BhoS-126a, (7) vB_EalM-132, (8) vB_BcoS-136, (9) vB_EalM-137, (10) vB_BpsS-140, (11) vB_BhaS-171, (12) vB_BpsS-36, (13) vB_VmeM-32, (14) vB_VmeM-196, Numbers to the left indicate band size in KDa.

6.3.6 Restriction digestion patterns

Previously, the prime use for restriction digestion of bacteriophage DNA has been for restriction mapping of the bacteriophage genome (Strauch et al., 2001). However, restriction digestion patterns have been used to differentiate between bacteriophages (Ogunseitan et al., 1992). In this study, the patterns of restriction digest profiles for each phage were different (Figure 6.20). The restriction analysis also indicated that all phages were dsDNA viruses. Endonuclease *Eco*RI was able to digest all genomes. *Dra*I also digested all the genomes apart from vB_BpsS-140 (Table 6.5). Both *Eco*Rl and *Hind*lll yielded similar patterns for vB_BhoS-126a and vB_BhoP-126b, phages with similar host, though morphologically different have almost similar genome. Most phages showed insensitivity to restriction endonucleases *Pst*I (all but vB_EauM-23, vB_BboS-125, vB_BhoS-126b and vB_BpsS-36) and BamH1 (all but vB_BpsM-61).

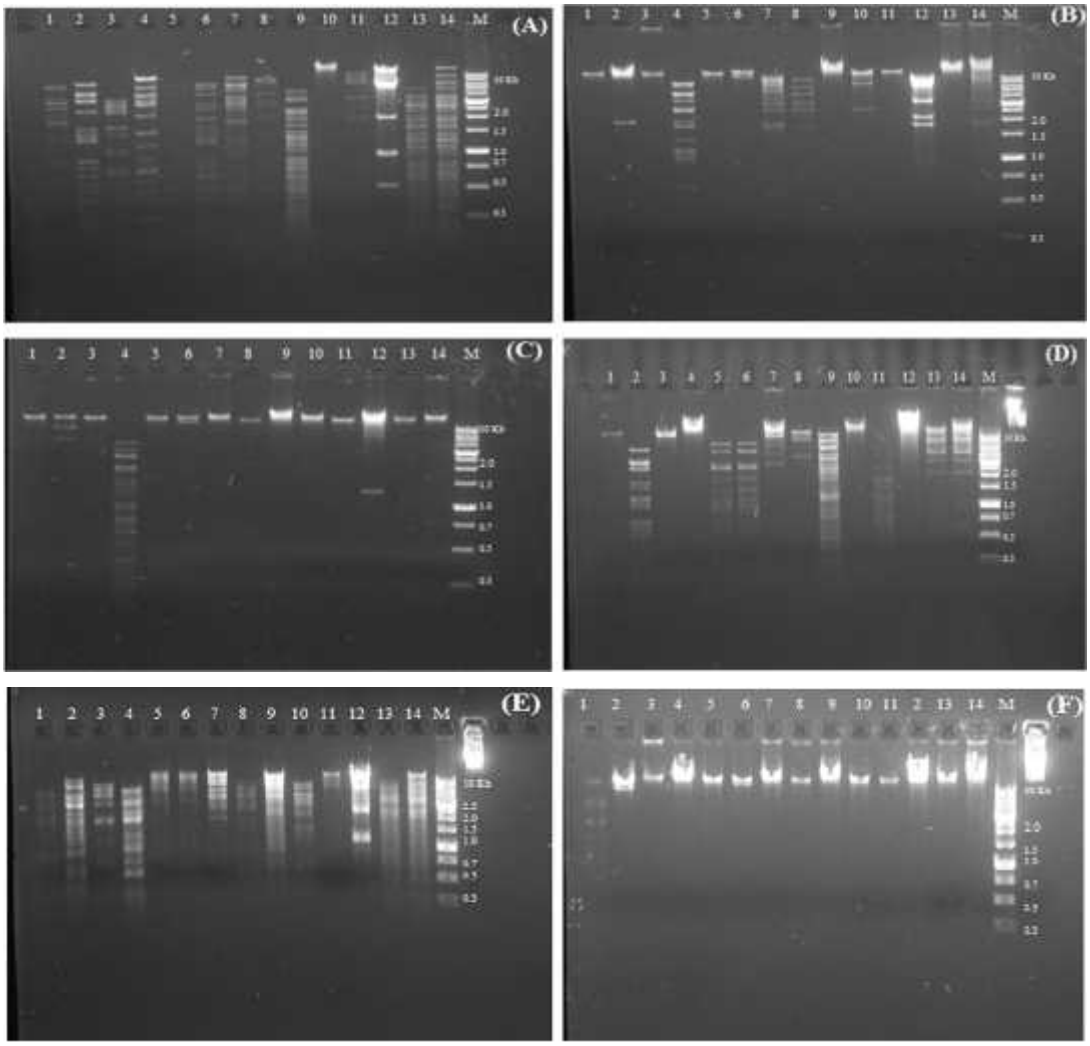

Figure 6.2: Restriction profiles of the phages after digestion of DNA with restriction enzymes, overnight at 37ºC and electrophoresed on 1% agarose gel.

Different restriction enzymes were used which cut wherever the recognition sequence was present. (A) *DraI,* (B) *KpnI,* (C) *PstI* (D) *HindIII* (E) *EcoRI* and (F) *BamH1* all from Fermentas. Lane (1) vB_BpsM-61, (2) vB_EauM-23, (3) vB_EauS-123, (4) vB_BboS-125, (5) vB_BhoP-126b, (6) vB_BhoS-126a, (7) vB_EalM-132, (8) vB_BcoS-136, (9) vB_EalM-137, (10) vB_BpsS-140, (11) vB_BhaS-171, (12) vB_BpsS-36, (13) vB_VmeM-32, (14) vB_VmeM-196, (M) 1kb DNA marker (Metabione). Numbers to the right indicate band size in kb

Bacteriophage resistance to restriction enzymes is common and has been reported previously. Sixteen *Campylobacter* phages have been shown to be refractory to digestion by a number of commonly used restriction enzymes (Sails et al., 1998) also, some *Lactococcus lactis* bacteriophages have been found to be highly refractory to digestion by several restriction enzymes (Prevots et al., 1990). Several explanations have been proposed to explain phage DNA resistance to restriction enzymes referred to as antirestriction mechanisms. Among these explanations is the presumably elimination of restriction sites as an evolutionary response of the phages to pressures from their host restriction enzymes (Moineau et al., 1993). Another explanation for the phage DNA insensitivity is integration of unusual bases in the viral DNA, such as hydroxymethyl uracil or hydroxymethyl cytosine, that make the DNA somewhat refractory to endonuclease cleavage (Jensen et al., 1998). Alternatively, phage genomes may encode methyltransferases that modify specific nucleotides within the recognition site of one or more of the restriction endonucleases (Kruger et al., 1983; Hill et al., 1991). By this method, a high similarity was found between phages vB_BhoS-126a and vB_BhoP-126b and much less similarity was found among the other phages. The varied distribution of restriction endonuclease sites indicate different phage genomes

Table 6.5: Grouping of restriction endonucleases by cutting pattern.
Non cutters are those which produced only one high molecular weight band by gel electrophoresis. Poor cutters produced few bands, good cutters produced five or more bands and complete cutters caused complete digestion of DNA.

No	Phage	Non cutter	Poor cutter	Good cutter	Complete cutter
1	**vB_BpsM-61**	*Kpn*I, *Pst*I, *Hind*III,	*Bam*H1		*Dra*I, *Eco*RI
2	**vB_EauM-23**	*Bam*H1	*Kpn*I, *Pst*I		*Dra*I, *Hind*III, *Eco*RI
3	**vB_EauS-123**	*Kpn*I, *Pst*I, *Hind*III, *Bam*H1			*Dra*I, *Eco*RI
4	**vB_BboS-125**	*Hind*III, *Bam*H1			*DraI*, *Kpn*I, *Pst*I, *Eco*RI
5	**vB_BhoS-126a**	*Kpn*I, *Pst*I, *Bam*H1			*Dra*I, *Hind*III, *Eco*RI
6	**vB_BhoP-126b**	*Bam*H1	*Kpn*I, *Pst*I		*Dra*I, *Hind*III, *Eco*RI
7	**vB_EalM-132**	*Pst*I, *Bam*H1			*DraI*, *Kpn*I, *Hind*III, *Eco*RI
8	**vB_BcoS-136**	*Pst*I, BamH1		*Hind*III	*DraI*, *Kpn*I, *Eco*RI
9	**vB_EalM-137**	*Kpn*I, *Pst*I, *Bam*H1			*Dra*I, *Hind*III, *Eco*RI
10	**vB_BpsS-140**	*Dra*I, *Pst*I, *Hind*III, *Bam*H1	*Kpn*I		*Eco*RI
11	**vB_BhaS-171**	*Kpn*I, *Pst*I, *Bam*H1	*Eco*RI		*Dra*I, *Hind*III
12	**vB_BpsS-36**	*Hind*III, *Bam*H1	*PstI*	*DraI*, *Kpn*I	*Eco*RI
13	**vB_VmeM-32**	*Kpn*I, *Pst*I, *Bam*H1			*Dra*I, *Hind*III, *Eco*RI
14	**vB_VmeM-196**	*Pst*I, *Bam*H1		*Kpn*I	*Dra*I, *Hind*III, *Eco*RI

6.3.7 Genome size estimation

The genome size of all the 14 phages estimated by Pulsed-field gel electrophoresis of purified DNA, revealed phage genomes ranging in size from ~37, 500 bp to ~200, 000 bp. The 14 phages could be divided into two groups according to genome size. Group l; Bacteriophage 32, 132, 136 and 196 with the largest genome sizes of

~145,000 to ~200,000 bp and group ll; the rest of the phages with genome sizes of ~37, 500 to ~58, 500 bp (Figure 6.21). vB_BhoS-126a and vB_BhoP-126b bands co-migrated to the same position in same pattern, suggesting that the two genomes are very similar. The presence of distinct bands suggests the presence of putative dsDNA. Phages 123, 126a, 126b, 171 and 196 also had other bands appear in there lanes. Potentially, this could indicate a segmented genome (Snyder, 2012). These results coupled with the similar data from the prior characterization work further support the genetic diversity among the phages.

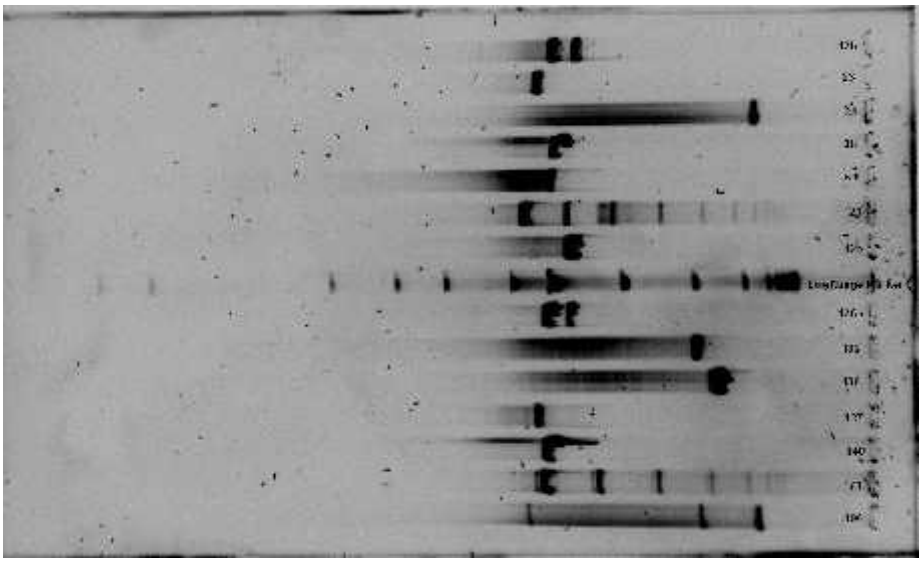

Figure 6.32: Pulsed-field gel of the 14 phage genomes.
Lane (12b) vB_BhoP-126b, (23) vB_EauM-23, (32) vB_VmeM-32, (36) vB_BpsS-36, (61) vB_BpsM-61, (123) vB_EauS-123, (125) vB_BboS-125, (126a) vB_BhoS-126a, (132) vB_EalM-132, (136) vB_BcoS-136, (137) vB_EalM-137, (137) vB_EalM-137, (140) vB_BpsS-140, (171) vB_BhaS-171, (196) vB_VmeM-196 with Low range PFG DNA marker in Kb (Biolabs, England).

Phage vB_BhoS-126a (Siphovirus) and vB_BhoP-126b (Podovirus) that have a common host bacteria *Bacillus horikoshii*, have distinctively different morphotypes but almost similar characteristics including plaque morphology, restriction endonuclease patterns, same genome size and co-migrated in the same pattern. Such viruses are referred to as “ecoviriotypes” which are viruses with different but very

closely related genomes (like single nucleotide polymorphisms-SNPs) (Li et al., 2013). High mutation rates in viruses, reported to be between 10^{-8} to 10^{-6} substitutions per nucleotide (Sanjuán et al., 2010) may contribute to the generation of phenotypic variation in viruses (e.g., tail proteins), with variant forms selected in response to host evasion mechanisms, such as hosts expressing variant forms of cell envelope proteins (Santos et al., 2010). This is an extension to the concept of ecotypes and niche adaptation which allows adaption to differences or changes in environmental parameters (Luk et al., 2014).

6.4 CONCLUSION

The effective use of bacteriophage in all applications must be supported by detailed understanding of the bacteriophage themselves and their physiologic characteristics. Isolation, characterization and comparative analysis of phages were the main accomplishments of this study, as an outcome the phages turned out to be different in identity from each other. The taxonomic grouping based upon ultrastructural characteristics, structural proteins, restriction endonuclease and genome size analysis is therefore an effective approach to the classification of the phages. Although we investigated only a small part of the viral community, we established that there is great morphologic and genetic variation in the bacteriophages which leads to high levels of species and strain diversity.

CHAPTER SEVEN

7.0 ANNOTATION AND GENOME ANALYSIS OF BACTERIOPHAGES FROM HALOALKALIPHILE LAKE ELMENTEITA

ABSTRACT

Natural phage communities are reservoirs of considerable uncharacterized genetic diversity on earth. Phage taxonomy has classically depended on definitions outlined by the International Committee on Taxonomy of Viruses which groups phages based on morphological and behavioral phenotypes. This approach lacks a direct connection to phage genome which is the most incredible tool in tackling phage diversity from the context of their sequences. Phage genomics have advanced the use of phages for development of genetic, biotechnological and clinical tools, and a large variety of approaches and utilities. The goal of this research was to sequence, annoatate and analyse genomes of bacteriophages isolated from the haloalkaline Lake Elmenteita using the various available bioinformatic tools. A total of 13 DNA phage genomes were sequenced. All the genomes were circular apart from vB_EauS-123 and vB_BpsS-36 that were linear. The complete nucleotide sequences of the genomes varied in size from 30, 926 bp - 199, 467 bp and the G + C content of between 32.16-52.08 %. A range of 56-260 potential ORFs were identified and annotated by different bioinformatic tools with majority having ATG as start codon. The ORFs are located on different strands with ORFs on the forward strand transcribed from left to right and on reverse strand transcribed from right to left. vB_EauM-23, vB_BboS-125 and vB_BhoP-126b ORFs are all located on forward strand while vB_BhoS-126a ORFs all located on reverse strand. Genome-wide coding density ranged between 86.0-93.5 %. No tRNA genes were predicted in most genomes apart from vB_VmeM-32 with 3, vB_EalM-132 with 2 and vB_BcoS-136 with 17 tRNA gene sequences. Genome wide comparisons showed no significant sequence similarity to other genomes in GenBank which revealed the phages are novel. The genome sequences give insight into genome architecture and content in terms of gene function and also the relatedness of the phages and other phages.

7.1 INTRODUCTION

Natural phage communities are reservoirs of considerable uncharacterized genetic diversity on Earth (Hambly & Suttle, 2005). Phages are relatively simple in genetic organization and have smaller genomes compared to bacteria. This relative simplicity combined with the ease and rapidity at which large numbers can be generated has made them a model to better understand molecular processes (Hatfull, 2009). They are tremendously diversified with genome sizes from as low as 17 kbp up to 0.5 Mbp with high frequency of novel genes found in newly characterized phage genomes (Oliveira et al., 2013). Phage taxonomy has classically depended on the definitions outlined by the International Committee on the Taxonomy of Viruses-ICTV (Murphy et al., 1995; Büchen-Osmond, 1996) which groups phages based on morphological and behavioral phenotypes (capsid size, shape, structure, plaque morphology, resistance to organic solvents, differences in latent period, burst sizes, host range, genome size and nucleic acid properties). All these fields of analysis merit attention and are actively pursued. However, these approaches lack a direct connection to the phage genome sequence which is the most credible in tackling phage diversity (Hatfull, 2009) and provides information necessary to classify phages into groups that reflect their biology (Paul et al., 2002). Phage genomics has advanced the use of phages for development of genetic, biotechnological, clinical tools and a large variety of approaches and utilities (Hatfull, 2009). Complete phage genomes help to identify conserved sequences referred to as 'signature genes' (Rohwer & Edwards, 2002) that facilitate studies of phage evolutionary history and relationships, biodiversity, biogeography, identification of novel phage taxa (Paul et al., 2002; Fuller et al., 1998; Rohwer et al., 2000; Hambly et al., 2001). Studies trying to unravel unknown gene functions and ultrastructural studies elucidating the roles of individual particle proteins in phage virions can lead to the discovery of novel natural products following genome-mining approaches. Identifying genes producing these proteins and being able to recombinantly produce these proteins has potential benefits for biotechnology (Cheng et al., 2005b). Genomic sequence information enables construction of primers and probes to detect specific phage in the environment (Paul et al., 2002). In an attempt to understand the diversity and

biogeography of viruses infecting eukaryotic algae Chen et al. (1996) designed PCR primers to selectively amplify part of the DNA polymerase genes from viruses that infect two eukaryotic algae, an endosymbiont *Chlorella*-like alga and *Micromonas pusilla*. These primers were used to amplify sequences from environmental samples for phylogenetic analyses and to examine biodiversity using denaturing gradient gel electrophoresis (Short & Suttle, 2002; Short et al., 2000). Sequencing and annotating phage genomes presents valuable information about the phage structure and content of bacteriophage genomes. The functions of genes, whose presence might otherwise have gone unnoticed, can be more fully explored (Hatfull, 2009). With the introduction of new sequencing technologies, the number of completely sequenced phage genomes has increased from 40 in January 1997 to 552 in August 2009 (Ceyssens, 2009) and now 2, 184 complete virus genome sequences in GenBank (http://www.ncbi.nlm.nih.gov/nuccore). The goal of this research was to sequence 13 bacteriophages from the haloalkaline Lake Elmenteita, annotate and analyse the genomes using the various available bioinformatic tools. It is expected that the genome sequences will give insight into genome architecture and content in terms of gene function and also the relatedness of the phages. The phage genomes will also significantly add to our understanding of phage diversity in the Lake.

7.2 MATERIALS AND METHODS

7.2.1 DNA extraction

DNA was extracted from CsCl purified high-titre stocks of phage using phage DNA isolation kit (Norgen Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. The purity and the concentration of the DNA were determined using spectrophotometer (Invitrogen Qubit).

7.2.2 Genome sequencing and analysis

All phage genomes described here were sequenced in-house (DSMZ) using a SMRT

sequencing approach (Pacific Biosciences). Prediction of potential ORFs and annotation was done using PROKKA annotation pipeline (Seemann, 2014) after removal of terminal redundancies identified using r2cat (Husemann & Stoye, 2009). Translated ORFs were compared with known protein sequences using Blastp, using the non-redundant public GenBank database (Altschul et al., 1997) and by manual curation of the outputs of a variety of similarity searches. Protein e-values provided in BLAST were used to determine how likely the gene encoded the same function as the homologs identified in the search. The intergenic genome regions of the phages were searched for transcriptional regulation elements, tRNA genes and terminators. tRNA gene sequences were searched using the tRNAscan-SE v1.2.1 (Lowe & Eddy, 2009) and ARAGORN v1.2.36 (Laslett & Canback, 2004). Terminators were identified based on homology to other phages in the database. Genome size, G+C % content, coding density, total number of ORFs and additional elements such as inspection of the sequence to search ATG, GTG and TTG as start codons was determined using ARTEMIS tool for sequence visualization (Carver et al., 2008). Genome wide BLAST search was performed against NCBI GenBank database to find sequence homology to other available phage genomes (Altschul et al., 1997). DNAPlotter (Carver et al., 2009) was used to generate circular DNA maps to display regions and features of interest. Linear comparison of genomic regions and visualization was achieved by Easyfig (Sullivan et al., 2011).

7.3 RESULTS AND DISCUSSION

The complete nucleotide sequences of the bacteriophages varied in size from 30, 926 bp-199, 467 bp and had a G + C content of between 32.16-52.08 %. A range of 56-260 potential ORFs were identified and annotated with majority having ATG as start codon (Table 7.1). All the genomes were circular apart from vB_EauS-123 and vB_BpsS-36 that were linear. The ORFs are located on different strands with ORFs on the forward strand transcribed from left to right and on reverse strand transcribed from right to left. vB_EauM-23, vB_BboS-125 and vB_BhoP-126b have all ORFs located on the forward strand while vB_BhoS-126a all ORFs located on the reverse strand. Coding density ranged between 86.0-93.5 %. tRNA gene sequences were predicted in vB_VmeM-32 (3), vB_EalM-132 (2) and vB_BcoS-136 (17) (Table

7.2). The circular DNA maps displaying regions and features of interest in the phage genomes were generated (Figures 7.1-7.14, apart from Figure 7.10). First and second tracks show forward (blue) and reverse (grey) transcribed ORFs respectively. Third track shows terminators (green) and fourth tRNA (green). Moving inward, the track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

Table 7.11: A summary of the sequenced genomes showing some of the architectural features as indicated by the various bioinformatics tools.

	Phage	Genome size (bp)	G +C % content	Coding %	ORFs	tRNAs	Terminators	Nature of genome	Start Codon		
									ATG	GTG	TTG
1	vB_EauM-23	37, 660	52.08	91.7	66	-	6	Circular	62	2	2
2	vB_VmeM-32	199, 912	36.25	91.2	260	3	48	Circular	246	3	11
3	vB_BpsS-36	50, 485	41.14	91.6	68	-	6	Linear	62	5	1
4	vB_BpsM-61	48, 160	43.48	93.0	75	-	8	Circular	64	11	-
5	vB_EauS-123	30, 925	47.73	91.5	56	-	6	Linear	52	2	1
6	vB_BboS-125	58, 528	48.57	92.2	81	-	6	Circular	81	-	-
7	vB_BhoS-126a	30, 926	41.48	93.5	66	-	7	Circular	49	8	9
8	vB_BhoP-126b	38, 893	41.47	92.4	64	-	7	Circular	46	9	9
9	vB_EalM-132	145, 844	40.61	86.0	192	2	55	Circular	181	10	1
10	vB_BcoS-136	160, 590	32.16	88.5	240	17	15	Circular	202	17	21
11	vB_EalM-137	41, 601	50.94	91.2	64	-	8	Circular	60	2	2
12	vB_BpsS-140	55, 091	39.84	91.0	68	-	4	Circular	64	2	2
13	vB_BhaS-171	38, 975	40.82	91.6	67	-	5	Circular	50	8	9

Phage vB_EauM-23; host, *Exiguobacterium auranticum* has a circular genome of 37, 660 bp with a G+C content of 52.0 % and coding density of 91.7 %. The phage has total of 66 ORFs all transcribed on the forward strand in the forward direction with ATG as start codon for all apart from ORFs No. 23, 44 (GTG) and No. 29, 39 (TTG). The phage genome with 6 terminators showed no significant similarity with other phage genomes in GenBank.

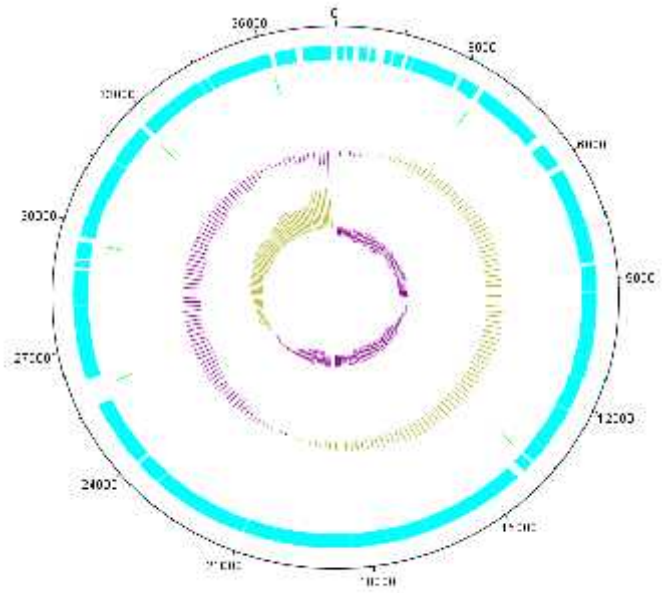

Figure 7.1: Genome map of vB_EauM-23 drawn to scale.

The outer two tracks show ORFs transcribed on forward strand (blue) and terminators (green). Moving inward, the track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C])

Phage vB_VmeM-32; host *Vibrio metchinkovii* has the biggest genome in this study of 199, 912 bp with a G+C content of 36.25 % and coding density of 91.2 %. The phage has total of 260 ORFs transcribed on both the forward and reverse strands. 246 of the proposed genes begin with ATG, 11 start with TTG and three start with GTG. 3 tRNA gene sequence with GC range between 42.9-55.3 % were identified using a tRNA scanning program. tRNA-Asn(gtt) (location 28049, 28124), tRNA-Arg(tct) (location 27971, 28047) and tRNA-Met(cat) (location 27879, 27954). 48 promoters were also identified. The phage genome showed no significant similarity with other phage genomes in GenBank.

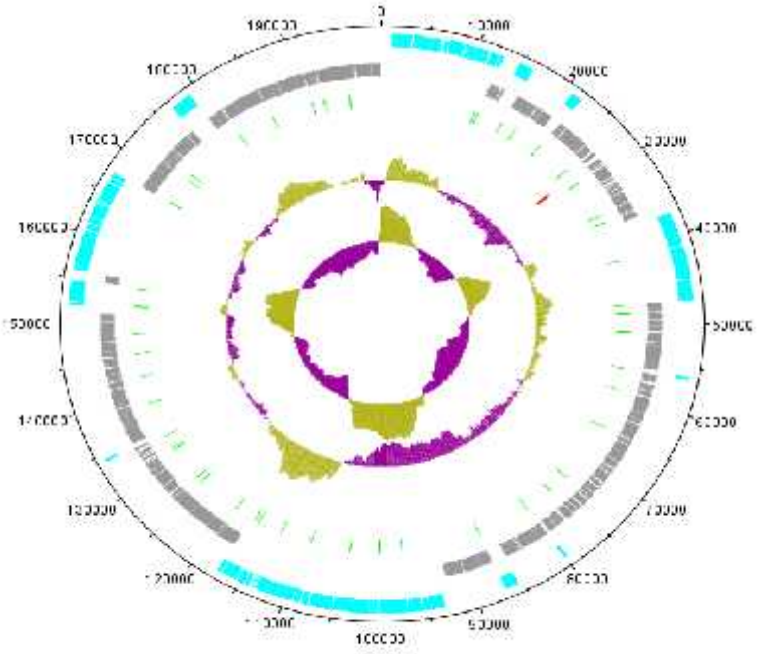

Figure 7.2: Genome map of vB_VmeM-32 drawn to scale.

First and second tracks show forward and reverse transcribed ORFs respectively. Third track shows terminators and fourth tRNA. Moving inward, the track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

Phage vB_BpsS-36; host, *Bacillus pseudalcaliphilus* has a circular genome of 50, 485 bp with a G+C content of 41.14 % and a coding density of 91.6%. The phage genome with six terminators has a total of 60 ORFs transcribed on both the forward and reverse strand with ATG as start codon for most, apart from ORFs No. 21, 26, 29, 66, 67 (GTG) and No. 20 (TTG).

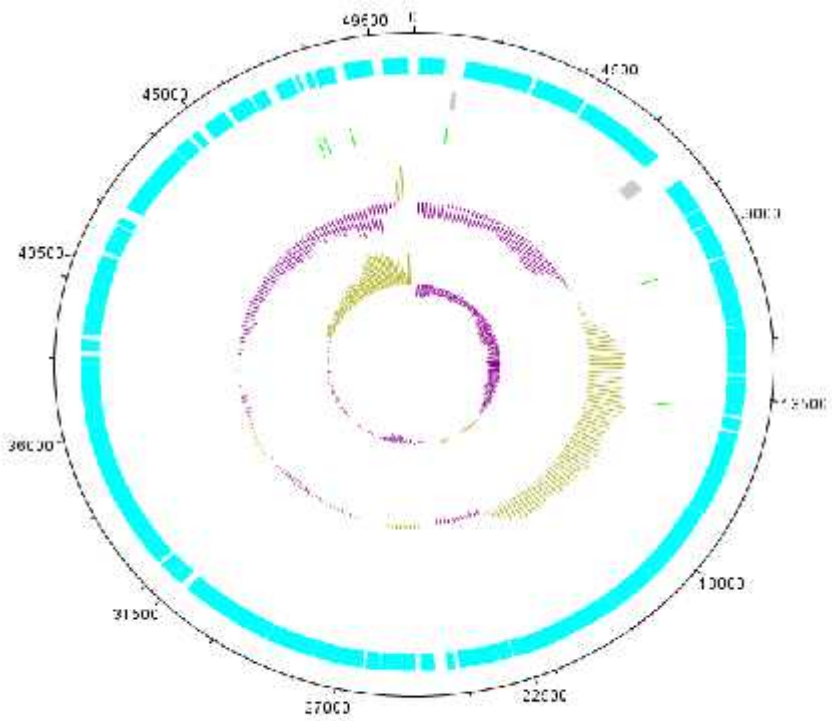

Figure 7.33: Genome map of vB_BpsS-36 drawn to scale.

First and second tracks show forward (blue) and reverse (grey) transcribed ORFs respectively. Third track (green) shows terminators. Forth track show the %GC content (purple=low %GC) and innermost of the genome

Phage vB_BpsM-61; host, *Bacillus pseudofirmus* has a circular genome of 48, 160 bp with 43.48 % G+C content and a coding density 93.0%. The genome has a total of 75 ORFs most transcibed on the forward strand. All apart from 11 (GTG) ORFs had ATG as start codon. 8 terminators were identified.

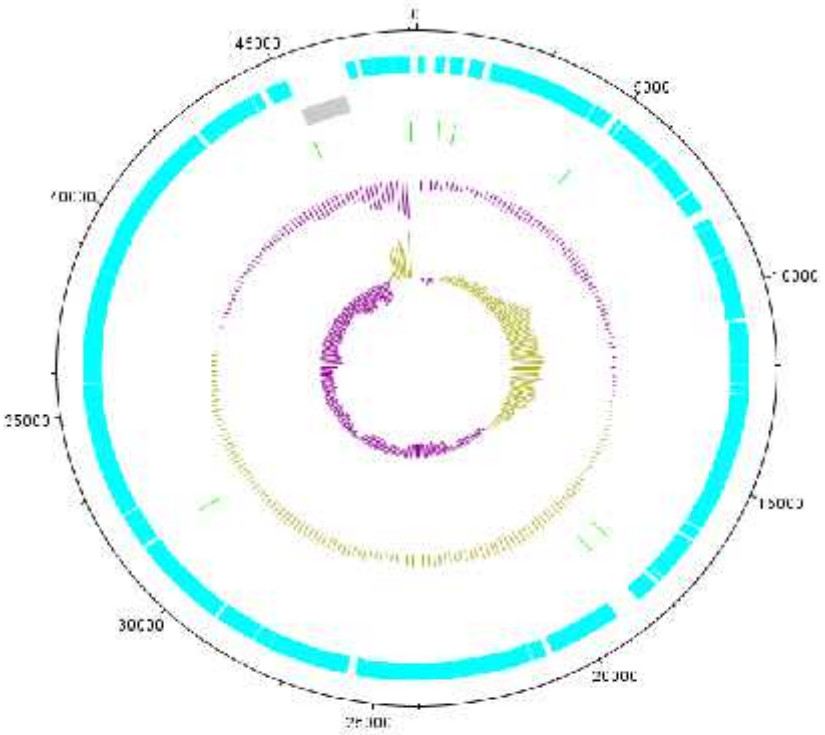

Figure 7.4: Genome map of vB_BpsM-61 drawn to scale.
First and second tracks show forward (blue) and reverse (grey) transcribed ORFs respectively. Third track shows terminators (green). Forth track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

Phage vB_EauS-123; host, *Exiguobacterium aurantiacum* a linear genome with the smallest size in this study of 30, 925 bps with a G+C content of 47.73 % and a coding density of 91.5%. The genome has a total of 56 ORFs with 6 terminators. All apart from 10, 47 (GTG) and 54 (TTG) had ATG as start codon. Most of the ORFs were transcribed on the reverse strand.

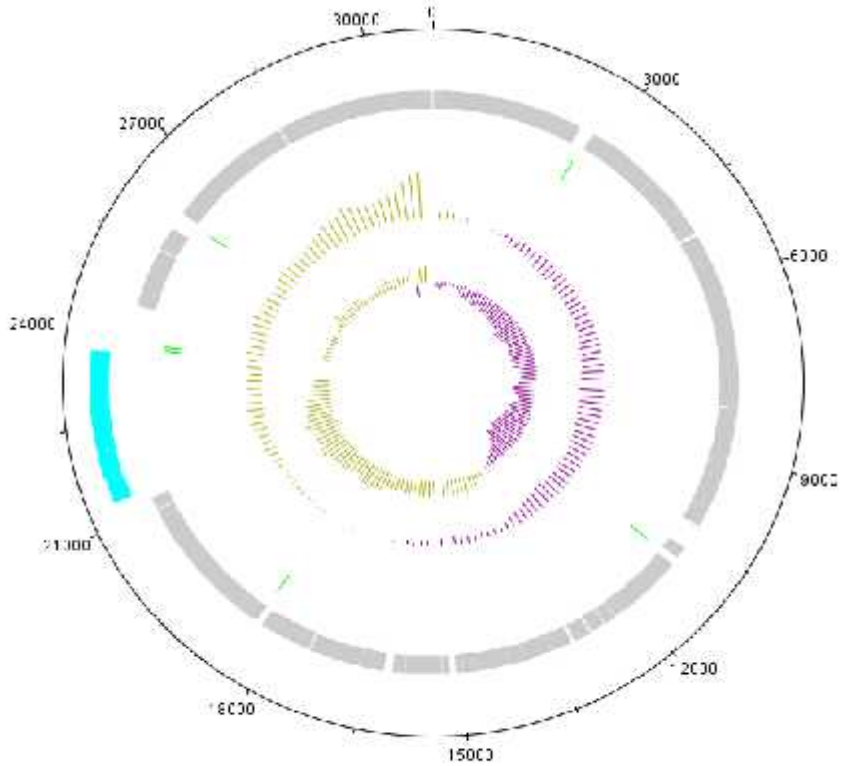

Figure 7.54: Genome map of vB_EauS-123 drawn to scale.

First and second tracks show forward (blue) and reverse (grey) transcribed ORFs respectively. Third track (green) shows terminators. Forth track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

Phage vB_BboS-125; host, *Bacillus bogoriensis* has a circular genome of 58, 528 bp with G+C content of 48.57 % and a coding density of 92.2 %. The genome has a total of 81 ORFs all transcribed on the forward strand with ATG as the start codon. 6 terminators were identified.

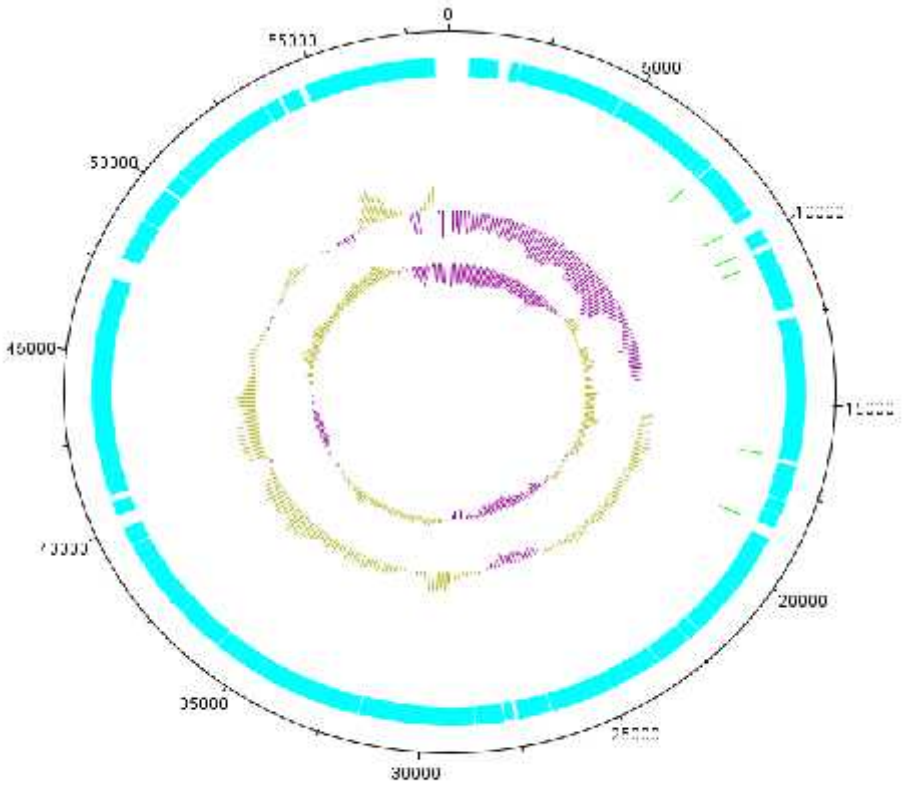

Figure 7.65: Genome map of vB_BboS-125 drawn to scale.
First track show forward (blue) transcribed ORFs and second track shows terminators (green) respectively. Moving inward, the track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

Phage vB_BhoS-126a; host, *Bacillus horikoshii* is circular genome of 30, 926 bp with a G+C content of 41.48 %, a coding density of 93.5 % with 7 terminators. Has a total of 66 ORFs with start codon of ATG (49), GTG (8) and TTG (9). All the ORFs are transcribed on the forward strand.

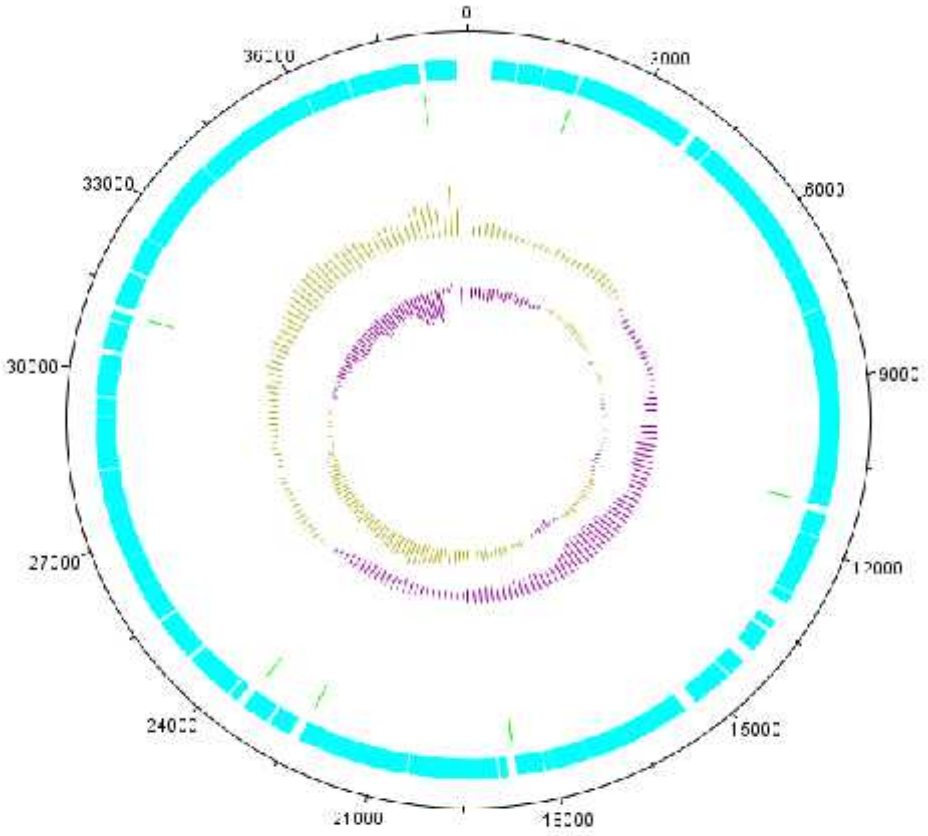

Figure 7.7: Genome map of vB_BhoS-126a drawn to scale.

First track show forward transcribed ORFs and second track shows terminators respectively. Moving inward, the track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

Phage vB_BhoP-126b; host, *Bacillus horikoshii* is circular genome of 38, 893 bp with a G+C content of 41.47 %, a coding density of 92.4 % with 7 terminators. Has a total of 64 ORFs with start codon of ATG (46), GTG (9) and TTG (9). All the ORFs are transcribed on the reverse strand.

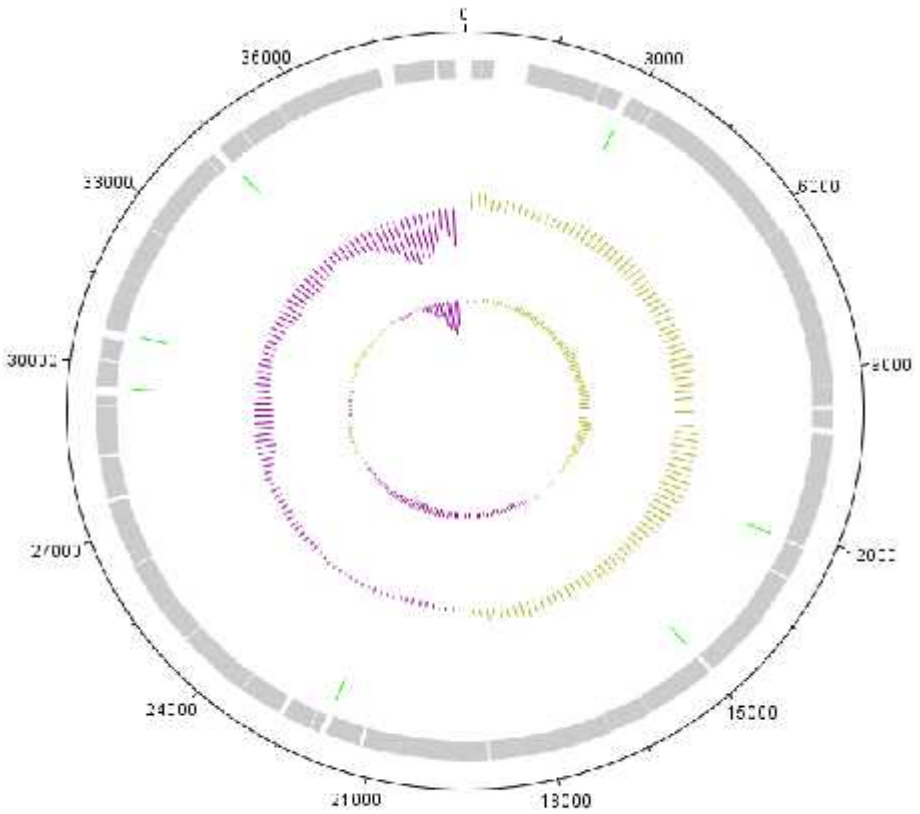

Figure 7.86: Genome map of vB_BhoP-126b drawn to scale.
First track show reverse transcribed ORFs and second track shows terminators respectively. Moving inward, the track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

Phage vB_EalM-132; host, *Exiguobacterium alkaliphilum* is among the big genomes of this study. Circular genome of 145, 844 bp with a G+C content of 40.61 % and a coding density of 86.0 %. The genome has a total of 192 ORFs transcribed both on the forward and reverse strands. 181 ORFs have ATG as start codon, 10 with GTG and 1 with TTG. 55 terminators and 2 tRNA gene sequences were identified. The phage had the highest number of terminators in this study. tRNA-Arg(tct) (location 33244,33319) with 76 bases and %GC of 46.1 and tRNA-Asn(gtt) (location 33473,33545) with 73 bases and %GC of 49.3.

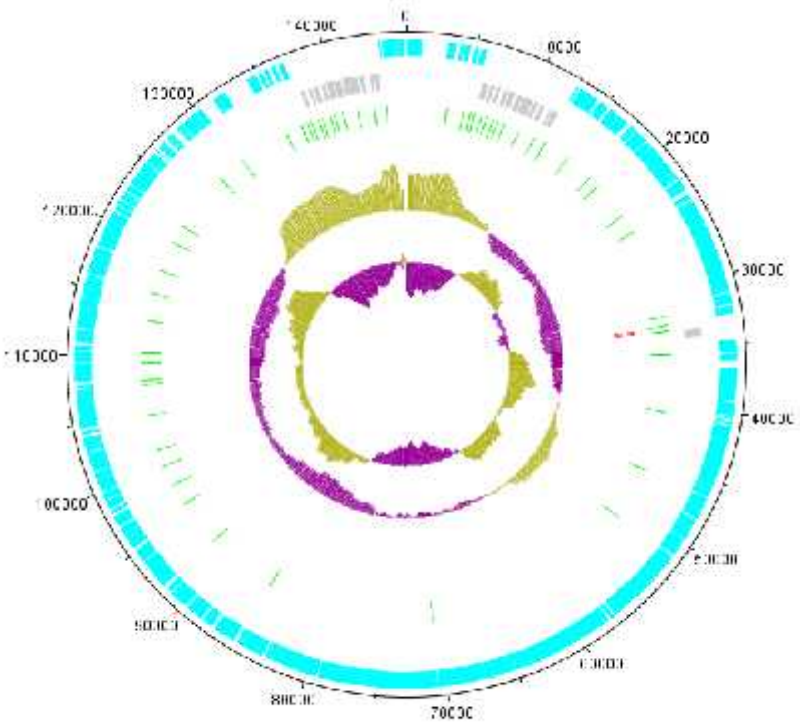

Figure 7.9: Genome map of vB_EalM-132 drawn to scale.

First and second tracks show forward and reverse transcribed ORFs respectively. Third track shows terminators and fourth tRNA. Moving inward, the track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

Phage vB_BcoS-136; host, *Bacillus cohnii* is also among the big genomes in this study. The phage has a circular genome of 160, 590 bp, G+C content of 32.16 with 88.5 % of the sequence predicted to encode proteins. The genome has a total of 240 ORFs. 202 ORFs had ATG as start codon, 17; GTG and 21; TTG. 17 tRNA gene sequences with GC range of between 37.3%-58.7% (Table 1) and 15 terminators were identified with.

Table 7.2: A Summary of the tRNA gene sequences predicted in the genome of phage vB_BcoS-136

No.	**tRNA**	**Position**	**bases**	**%GC**
1	tRNA-Asn(gtt)	54444,54518	75	49.3
2	tRNA-Asp(gtc)	54522,54596	75	49.3
3	tRNA-Ala(tgc)	54841,54912	72	45.8
4	tRNA-Gln(ttg)	54925,55001	77	46.8
5	tRNA-Trp(cca)	55006,55079	74	45.9
6	tRNA-His(gtg)	55246,55316	71	49.3
7	tRNA-Glu(ttc)	55653,55727	75	48.0
8	tRNA-Ile(gat)	55729,55803	75	37.3
9	tRNA-Tyr(gta)	55807,55891	85	49.4
10	tRNA-Leu(tag)	56065,56139	75	52.0
11	tRNA-Pro(tgg)	56144,56221	78	50.0
12	tRNA-Thr(tgt)	56223,56297	75	42.7
13	tRNA-Arg(acg)	56315,56388	74	47.3
14	tRNA-Ser(tga)	56488,56581	94	48.9
15	tRNA-Ser(gct)	56588,56679	92	58.7
16	tRNA-Arg(tct)	56681,56754	74	50.0
17	tRNA-Gly(tcc)	56758,56828	71	53.5

Close phylogenetic neighbour to the genome of phage vB_BcoS-136 is a previously published novel giant Siphovirus phage vB_BanS-Tsamsa, ACC_KC481682.1, a temperate phage of *Bacillus anthracis* the causative agent of anthrax infections in wildlife, livestock and humans (Carter, 1988), obtained from a carcass site in Etosha, national park in northern Namibia. The phage has a genome size of 168, 876 bp, GC content of 34 % with 272 ORFs, 17 tRNAs and 2 pseudo-tRNA genes (Ganz et al., 2014).

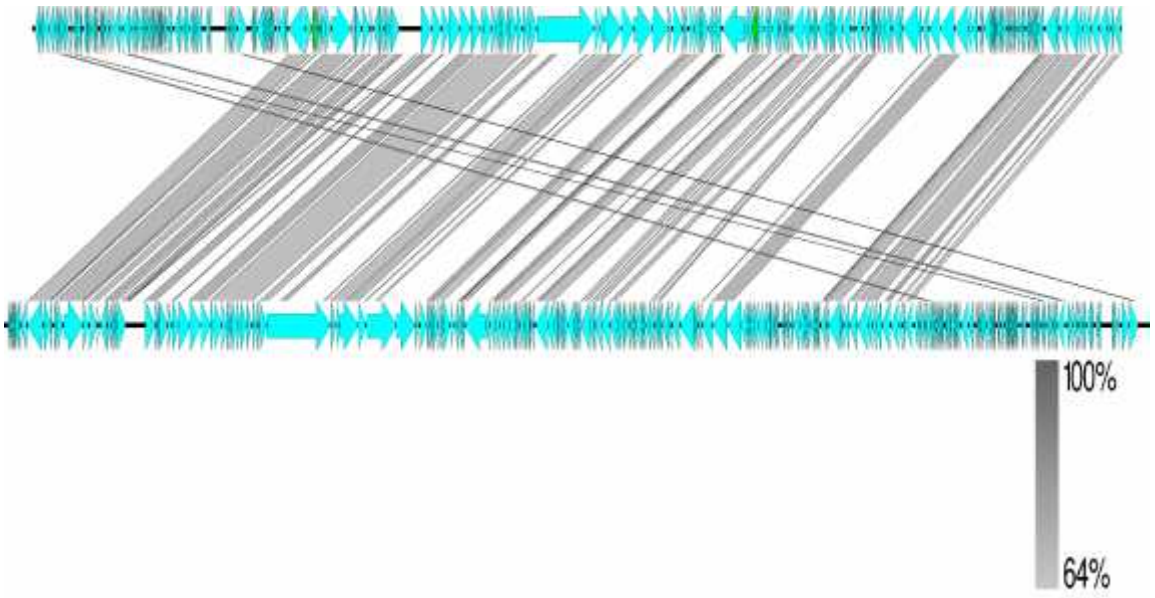

Figure 7.107: Linear comparison between the genomic regions of phage vB_BcoS-136 (top) and Phage vB_BanS-Tsamsa (bottom).

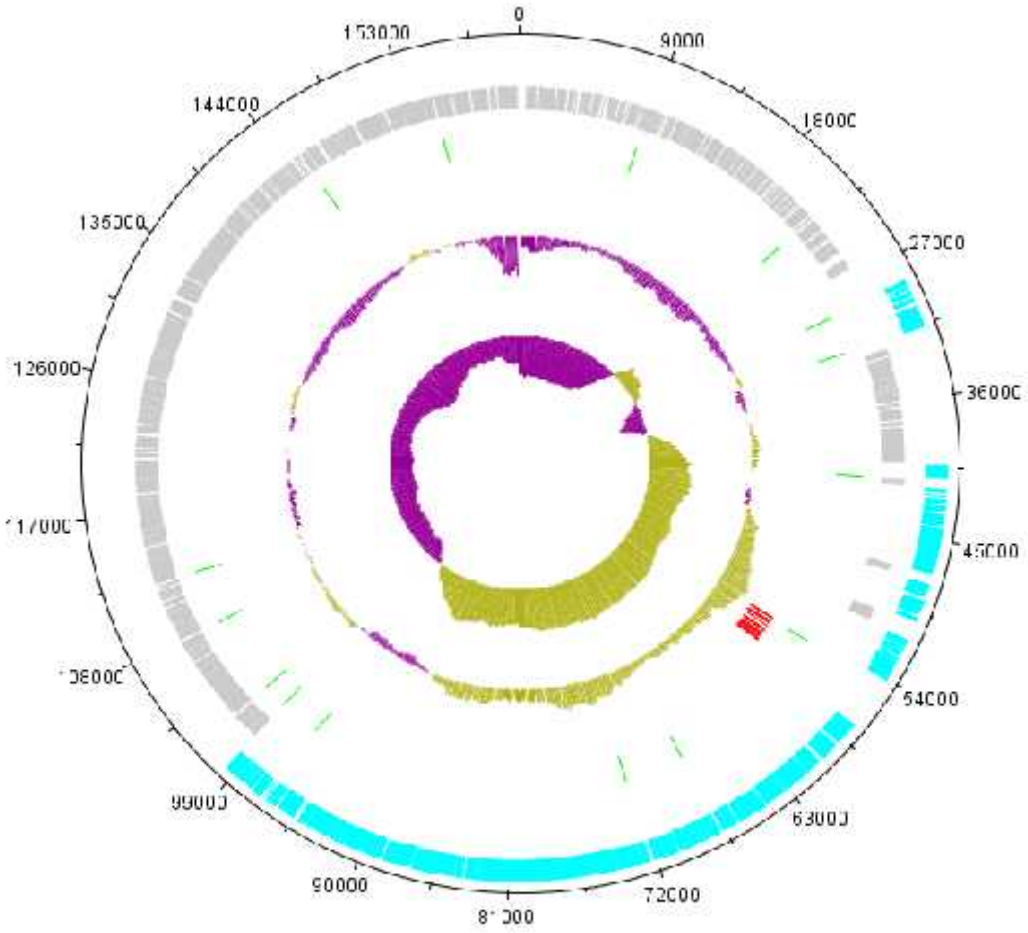

Figure 7.118: Genome map of vB_BcoS-136 drawn to scale.

The outer two tracks show open reading frames. First and second tracks show forward and reverse transcribed ORFs respectively. Third track shows terminators and forth track tRNAs. Moving inward, the track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

Phage vB_EalM-137; host, *Exiguobacterium alkaliphilum* has a circular genome of 41, 601 bp, G+C content of 50.94 and coding density of 91.2 %. The genome has a total of 64 ORFs majority transcribed on the forward strand. Majority (60) of the ORFs had ATG as start codon with few (No.40, 49) having GTG and (No.14, 61) with TTG. 8 terminators were identified.

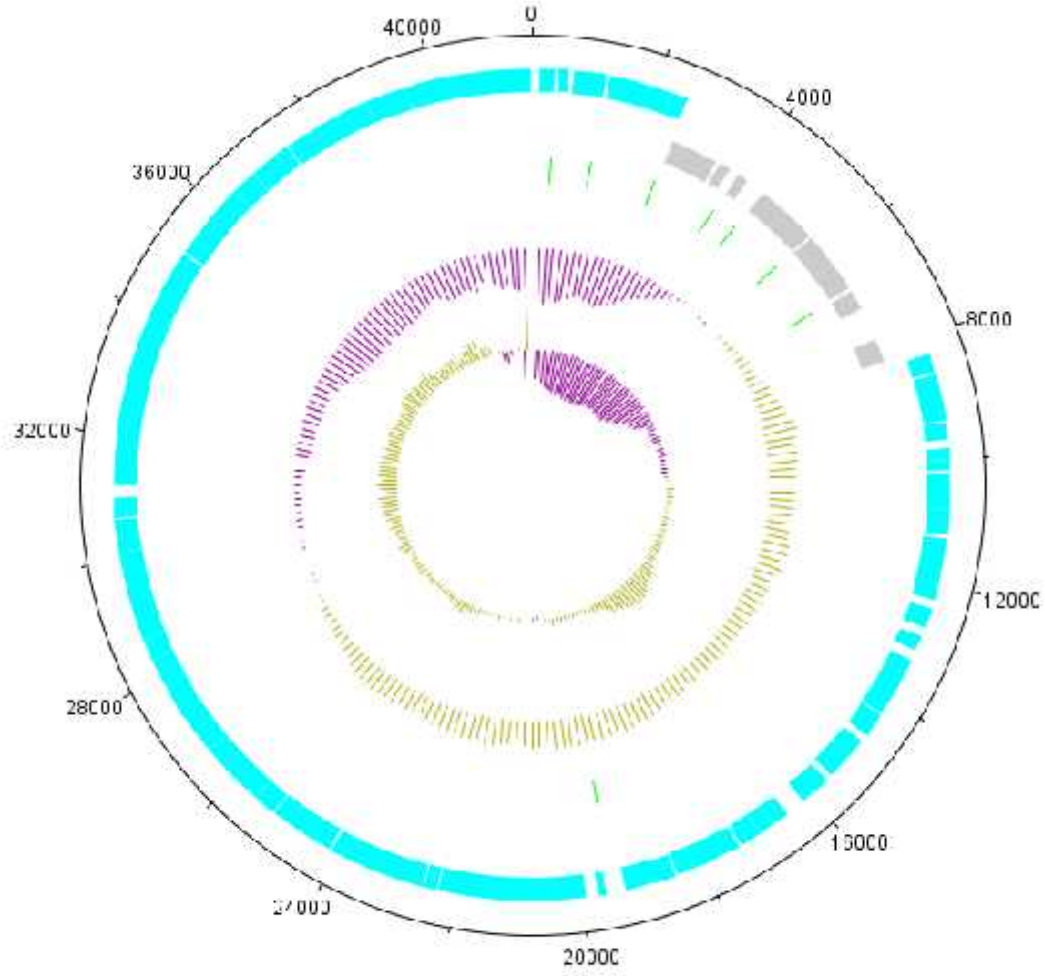

Figure 7.129: Genome map of vB_EalM-137 drawn to scale.

First and second tracks show forward and reverse transcribed ORFs respectively. Third track shows terminators. Forth track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

Phage vB_BpsS-140; host, *Bacillus pseudalcaliphilus* a circular genome with size of 55, 091 bp, G+C content of 39.84 and coding density of 91.0 %. The genome has a total of 68 ORFs majority transcribed on the forward strand. Majority (64) ORFs had ATG as start codon with few (No.22, 60) having GTG and (10, 54) with TTG. 4 terminators were predicted.

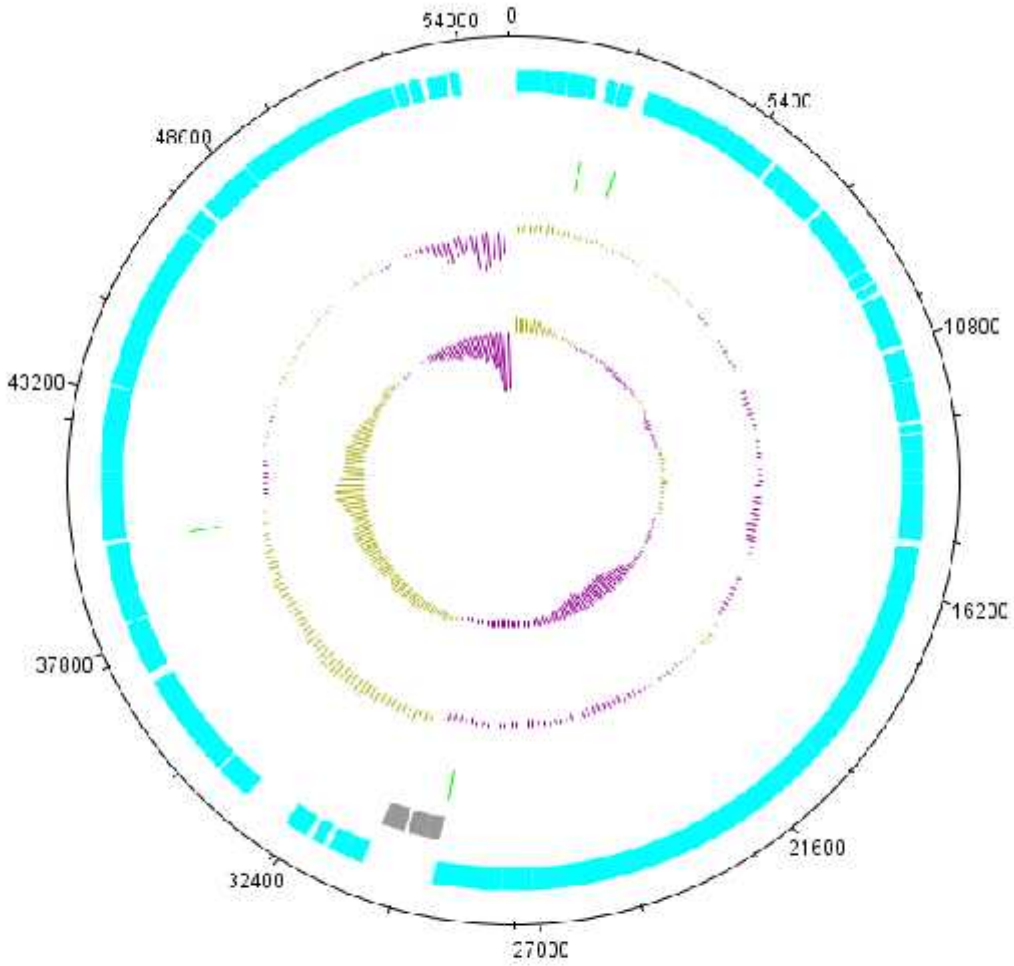

Figure 7.1310: Genome map of vB_BpsS-140 drawn to scale.

First and second tracks show forward and reverse transcribed ORFs respectively. Third track shows terminators. Forth track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

Phage vB_BhaS-171; host, *Bacillus halmapulus* has a circular genome of 38, 975 bp, G+C content of 40.82 and coding density of 91.6 %. The genome has a total of 67 ORFs majority transcribed on the reverse strand. Majority (50) ORFs had ATG as start codon with few (8) having GTG and (9) with TTG. 5 terminators were predicted.

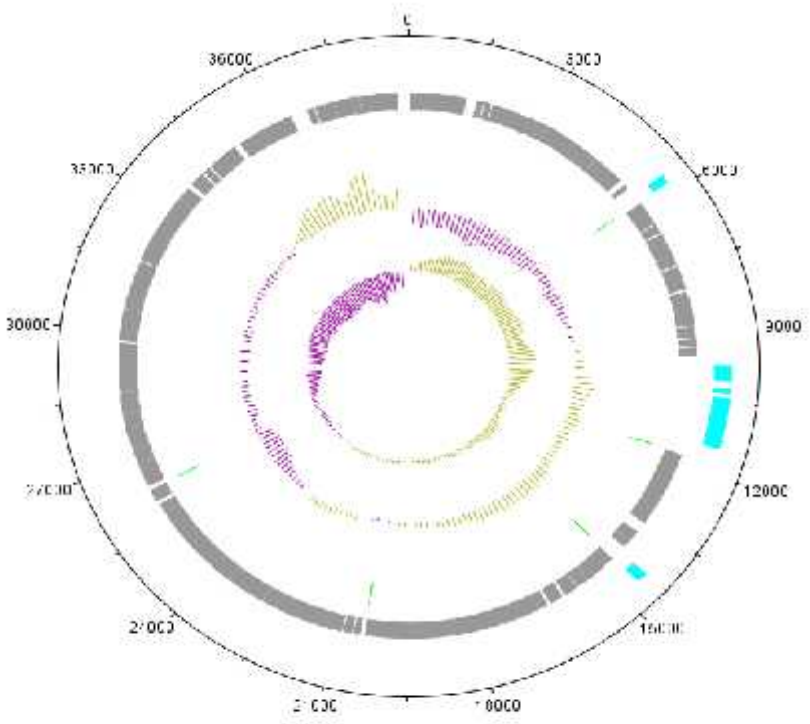

Figure 7.1411: Genome map of vB_BhaS-171 drawn to scale.

The outer two tracks show open reading frames. First and second tracks show forward and reverse transcribed ORFs respectively. Third track shows terminators. Forth track show the %GC content (purple=low %GC) and innermost of the genome map GC skew ([G-C]/[G+C]).

The results of these study reveal Lake Elmenteita has both the small (30, 926 bp) and the giant (199, 912 bp) bacteriophages. Comparison of the 13 genomes with other phage genomes in Genbank database showed pronounced genetic differences in the genotypic composition of the phages hence novel. Comparative analyses amongst the phages in this study show each phage genome to be distinct in structure and composition. There is also no uniform distribution of architectural themes across the genome spectrum or among phages in the same family. Transfer RNA (tRNA) genes play an essential role in protein translation in all living cells. tRNAs function in translation, viral replication, amino acid biosynthesis, cell wall remodeling, among others. It has also been suggested that these highly stable RNA molecules can provide a structural framework that regulate other cellular processes (Geslain & Pan, 2011). tRNA gene sequences in this study together with other genome features like terminators therefore are an important information resource to the genomics research communities (Chan & Lowe, 2009).

7.4 CONCLUSION

Sequencing, annotation and genome analysis was the main accomplishment of this study. The unraveling of each novel phage genome has provided a direct supply of potentially interesting proteins ready for further exploitation for biological and biotechnological ends. Our research also contributes to the diversity of phage sequences in the DNA database and make their respective genomes useful as comparisons for future gene annotations. The genome sequences showed diversity among the phages. A large proportion of the predicted genes are unknown or 'hypothetical' and therefore require experimental characterization. A useful endeavor would therefore be the determination of currently unknown gene functions through study of bacteriophage gene expression. By connecting genes with structure and function, we would be able to better understand phage biology. To this avail, the continued pursuit of phage whole genome sequencing will increase the value of the virome data and offer profuse insights into the diversity of phages in the haloalkaline lake Elmenteita.

CHAPTER EIGHT

8.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

8.1 GENERAL DISCUSSION

The main goal of this study was to isolate novel bacteria and bacteriophages from the haloalkaline lake Elmenteita. Previous studies on the lake have shown that the lake supports a dense and diverse population of aerobic, organotrophic, halophilic, alkaliphilic and alkalitolerant representatives of major bacterial and archaeal phyla (Grant & Sorokin, 2011). Culture-independent 16S rRNA-based studies however indicate that the previously uncultured fraction comprises numerous unknown phylogenetic groups (Mwirichia et al., 2011). Majority of microbial species do not grow on synthetic media *in vitro* and remain unexplored. Several approaches have been employed including different types of media (Mwirichia et al., 2010a), use of filter-sterilised water from the lakes to enrich media (Mwirichia et al., 2010a; Kambura et al., 2012) among other methods. The above traditional cultivation approaches though important in isolating haloalkaliphilic microbes for further biotechnological exploitation, majority of the resultant isolates often obtained are affiliated to the class *Gammaproteobacteria* and to the genus *Bacillus*.

Classical cultivation strategies have traditionally supplied excessive nutrients to a system, resulting in the enrichment of fast-growing bacteria that are capable of colony or biofilm formation. New approaches to the cultivation of bacteria that rely on growth in dilute nutrient media or simulated natural environments are beginning to address this issue (Keller & Zengler, 2004; Davis et al., 2004). Ferrari et al. (2005) described a novel micro-cultivation method for soil bacteria that mimics natural conditions. The slurry membrane system employed by this group combines the use of a polycarbonate membrane as a growth support and soil extract as the substrate. The result is abundant growth of uncharacterized bacteria as micro-colonies. Koepke et al. (2005) applied different cultivation methods to coastal subsurface sediments

finding that, in general, no group of bacteria was retrieved by more than a single method. This result supports the view that no single method or medium is suitable for isolating the vast diversity of microorganisms within a single sample. By combining low nutrient enrichments and molecular methods, a high diversity of new amylase genes was detected in a neutral sulfide-rich hot spring in Iceland. Enrichments based on hot spring water, low concentrations of starch and long incubation times were used to select slow-growing, starch-degrading microorganisms (Hobel et al., 2005).

In this study, in an attempt to access some of the yet uncultured bacteria in the soda lakes Elmenteita, media supplemented with signal molecule cAMP which increases cultivation efficiency of bacteria was used (Bruns et al., 2002). Three novel bacterial species; Strain No.164^{T}, *Belliella kenyensis*, Strain No.7^{T}, *Streptomyces alkaliphilus* and Strain No. 156^{T}, *Nocardiopsis mwathae* were isolated. These results show that use of new enrichment and novel cultivation techniques can help to both assess and access microbial diversity that has resisted cultivation hence establish metabolic properties and biotechnological potential of these diverse microorganisms.

Microbial communities in natural alkaline environments such as soda lakes have attracted attention as a possible source of novel enzymes and metabolites for use in biotechnology. Most of the microorganisms isolated are able to produce hydrolytic enzymes such as lipase, amylase, cellulase, hemicellulase, esterase and proteases at alkaline pH. The stability of these enzymes at alkaline pH is attributed to their habitat (alkaline lake) and growth profile in a wide range of pH (Kieser et al., 2000). The ability of the three novel microorganisms isolated in this study to produce exoenzymes indicated by there activity against several tested substrates is a characteristic that confirm their role in the decomposition of organic matter in the habitats. Further more, many currently employed alkaliphiles enzymes are very useful as tools for biotechnological exploitation. Research has revealed a great diversity of bacterial extremophiles that could produce a large pool of enzymes to choose from for developing new biotechnological applications such as medicine, food, and research reagents. The stability and activity of thermophilic enzymes can be controlled by separate molecular determinants. These enzymes can be used as

molecular templates to design highly stable enzymes that have high activity at high temperatures (Vieille and Zeikusl, 2001).

Phage ecology has essentially not been investigated in Lake Elmenteita and generally under-studied in the Kenyan Rift Valley lakes. Research in other parts of the world like most of South America, Africa, Australia, China, India, all of Middle America, the Middle East and Siberia (Koko et al., 20ll; Ackermann, 2011) is almost nonexistent as a result the phage flora of vast geographic areas remains unknown. An attempt to isolate phages from the haloalkaline Lake Elmenteita resulted in 18 different morphotypes that belonged to the order *Caudovirales.* Thirteen of the phages' genomes were fully sequenced and annotated. Genome wide BLAST search against NCBI GenBank database showed no significant similarity with other phage genomes in GenBank indicating all the phages were novel. This study has added to the biodiversity of isolated phages, and annotated genomes found within GenBank therefore offering greater insight into both gene function and genome evolution. By isolating and analyzing more bacteriophages, the breadth of phage research will expand, hence giving insight into phage diversity.

8.2 CONCLUSIONS

The study had demonstrated that the haloalkaline lake habours diverse groups of yet uncultured novel bacteria and that there are possibilities of isolating these novel species of bacteria in the lake. The use of new enrichment and novel cultivation technologies can help capture these novel bacteria. In this study, the use of signal molecule cAMP and different isolation media, enabled capturing of 3 novel bacteria.

Full taxonomic characterization, reliable identification, comprehensive understanding of the physiology of novel bacteria and to give a name requires all possible methods that inform on the biological nature of the strain. Polyphasic approach based on phenotypic, biochemical and phylogenetic data was used to distinguish the novel bacterial stains from close relatives and to determine the genus and species.

The isolated bacteria grew well at high alkalinity of pH ranging from 7-12, temperature range of 30-35^{0}C and varying NaCl concentrations. The above conditions are therefore to be adopted if the isolates are to be exploited industrially.

The bacterial isolates hydrolyzed skim milk, starch, aesculin, urea, gelatin and had positive activity for enzymatic tests of trypsin, lipase alkaline phosphatase, phosphoamidase among others. This indicates that they could be a potential source of enzymes at alkaline pH.

The results of this study also confirm the presence of novel bacteriophages from Lake Elmenteita. The phage morphotypes, structural proteins, RFLP patterns showed high diversity among the phages. The phage genomes also provide useful information about the diversity of the phages in the lake and also provide potentially interesting proteins ready for further exploitation for biological and biotechnological purposes.

8.3 RECOMMENDATIONS

1. Lake Elmenteita together with other soda lakes habour novel uncultured bacteria. New enrichment and novel cultivation technologies are therefore recommended for future research as they offer considerable advantages for recovering many previously uncultured microbes.

2. Classification of newly isolated bacterial strains on the basis of the polyphasic approach is recommended. Also previously classified organisms, as and when required, can be re-classified on this ground to obtain information about their accurate position in the microbial world and enable microbiologists to decipher the natural phylogenetic relationships between microbes.

3. The full extent of phage biodiversity in the Haloalkaline Lake Elmenteita remains unknown and unexplored. It is recommended to continue isolating additional novel bacteriophages to add to our phage knowledge and the phage database.

4. Concerted effort to sequence additional phage genomes, identify unexplored phage genes with biotechnological potential and designate biological functions to each of these genes is recommended.

5. Future studies should move away from standard isolation techniques and/or well-studied bacterial host organisms. Skipping the culturing step and assembling whole phage genomes directly from metagenomic sequence data to offer a look at the uncultivated phage community genome (metavirome) and enable indirectly assess phage diversity and abundance in Lake Elmenteita and all the Soda in the Kenyan Rift valley.

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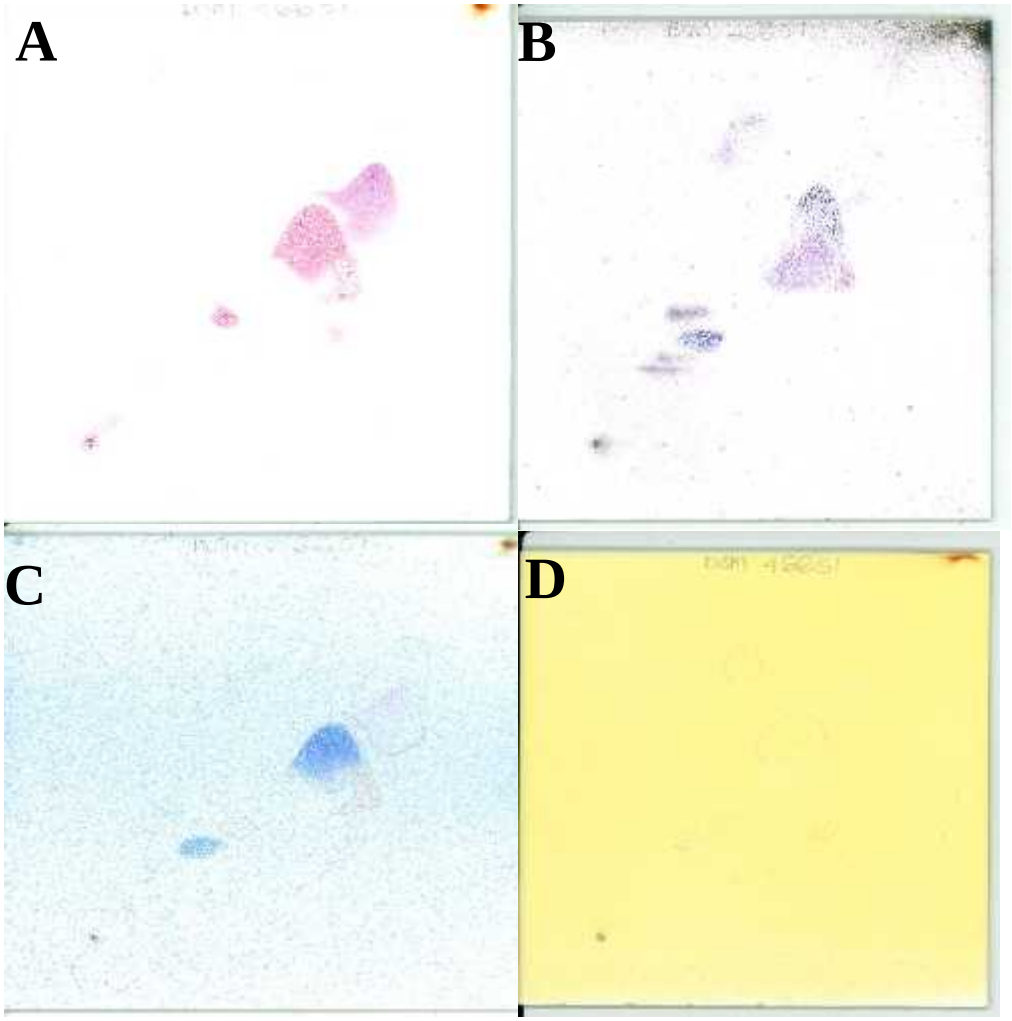
APPENDICES

Appendix 1: Polar lipids profile of *Belliella kenyensis* sp. nov. strain No.164^{T}, after separation by two-dimensional TLC.

Plate A sprayed with ninhydrin for the detection of amino groups. Plate B sprayed with molybdenum blue for the detection of phosphate containing substances. Plate C sprayed with anisaldehyde for the detection of mannose containing substances. Plate D sprayed with Dragendorf reagent for the detection of Phosphatidylcholine.



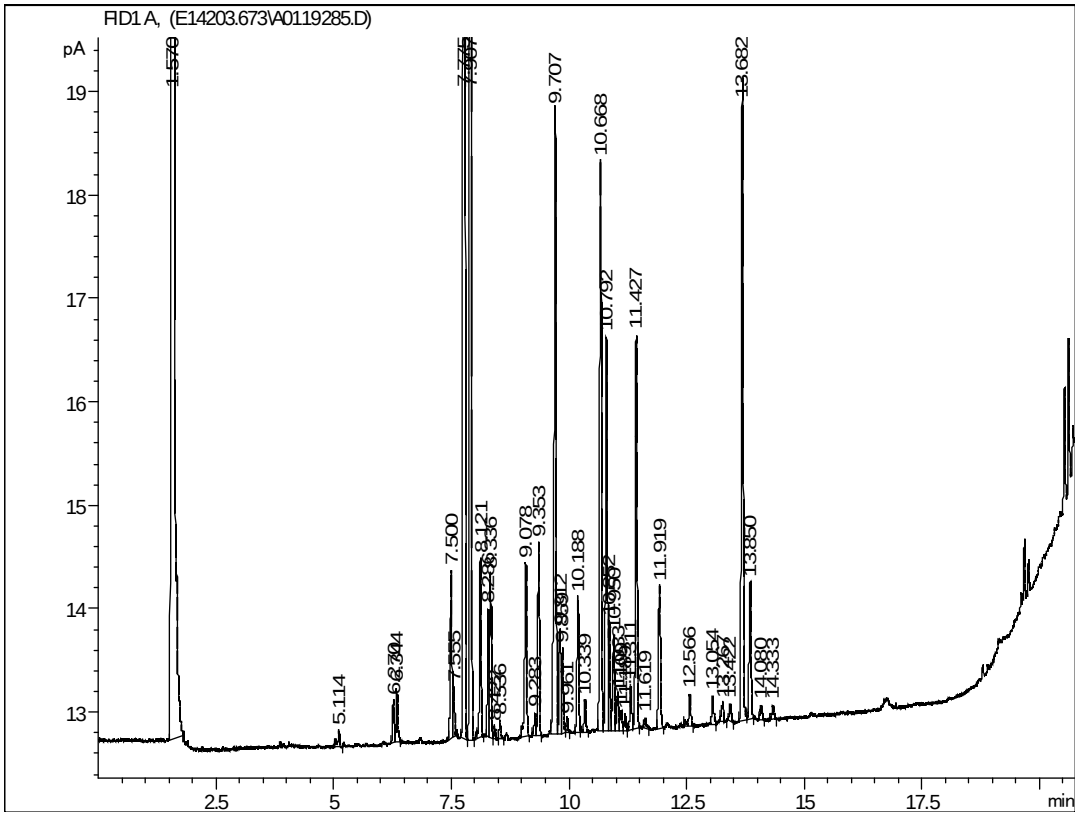
Appendix 2: Chromatographic run and composition reports of cellular fatty acids of strain No.164T, *Belliella kenyensis* sp. nov.

Volume: DATA File: E142036.73A Samp Ctr: 11 ID Number: 29285
Type: Samp Bottle: 17 Method: TSBA40
Created: 2/3/2014 8:20:24 PM
Sample ID: UN-JULIAH-4E/46651

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.570	3.638E+8	0.024	----	7.042	SOLVENT	----	< min rt	
5.114	764	0.036	1.040	12.613	13:0 ISO	0.19	ECL deviates -0.001	Reference -0.006
6.270	1907	0.035	1.010	13.562	unknown 13.565	0.45	ECL deviates -0.003	
6.344	2586	0.038	1.008	13.619	14:0 ISO	0.61	ECL deviates 0.000	Reference -0.005
7.500	8042	0.039	0.986	14.440	15:1 ISO G	1.87	ECL deviates 0.000	
7.555	2178	0.035	0.985	14.477	Sum In Feature 1	0.51	ECL deviates -0.001	15:1 ISO I/13:0 3OH
7.775	132416	0.035	0.981	14.625	15:0 ISO	30.60	ECL deviates 0.002	Reference -0.003
7.907	62029	0.036	0.979	14.713	15:0 ANTEISO	14.30	ECL deviates 0.000	Reference -0.004
8.121	7944	0.035	0.976	14.857	15:1 w6c	1.83	ECL deviates 0.001	
8.286	5507	0.035	0.974	14.967	unknown 14.959	1.26	ECL deviates 0.008	
8.336	7401	0.037	0.973	15.001	15:0	1.70	ECL deviates 0.001	Reference -0.004
8.422	500	0.031	----	15.054		----		
8.536	885	0.036	0.970	15.124	14:0 ISO 3OH	0.20	ECL deviates 0.005	
9.078	9915	0.043	0.963	15.457	16:1 ISO H	2.25	ECL deviates -0.004	
9.283	1174	0.043	----	15.583		----		
9.353	8833	0.038	0.960	15.626	16:0 ISO	2.00	ECL deviates -0.001	Reference -0.005
9.707	35815	0.045	0.955	15.844	Sum In Feature 3	8.06	ECL deviates -0.008	15:0 ISO
9.812	5230	0.039	0.954	15.908	16:1 w5c	1.18	ECL deviates -0.001	
9.859	4114	0.036	----	15.937		----		
9.961	765	0.041	0.953	16.000	16:0	0.17	ECL deviates 0.000	Reference -0.004
10.188	6841	0.040	0.950	16.133	15:0 ISO 3OH	1.53	ECL deviates -0.001	
10.339	1663	0.041	0.949	16.221	15:0 2OH	0.37	ECL deviates 0.002	
10.668	27908	0.041	0.945	16.415	ISO 17:1 w9c	6.21	ECL deviates -0.001	
10.792	20587	0.042	0.944	16.488	Sum In Feature 4	4.58	ECL deviates 0.002	17:1 ANTEISO B/i I
10.852	5015	0.037	0.943	16.523	ANTEISO 17:1	1.11	ECL deviates -0.001	
10.950	5616	0.045	0.942	16.581	unknown 16.582	1.25	ECL deviates -0.001	
11.033	1943	0.042	0.942	16.629	17:0 ISO	0.43	ECL deviates -0.001	Reference -0.003
11.109	1132	0.045	----	16.674		----		
11.189	839	0.037	0.940	16.721	17:0 ANTEISO	0.19	ECL deviates -0.002	Reference -0.005
11.311	2409	0.044	0.939	16.792	17:1 w8c	0.53	ECL deviates 0.000	
11.427	20088	0.042	0.938	16.860	17:1 w6c	4.44	ECL deviates 0.000	
11.619	566	0.040	----	16.973		----		
11.919	7761	0.046	0.934	17.146	16:0 ISO 3OH	1.71	ECL deviates -0.004	
12.566	1876	0.046	0.928	17.516	16:0 3OH	0.41	ECL deviates -0.003	
13.054	1617	0.046	----	17.796		----		
13.267	1039	0.043	0.923	17.918	18:1 w5c	0.23	ECL deviates -0.001	
13.422	905	0.043	0.922	18.007	18:0	0.20	ECL deviates 0.007	Reference 0.007
13.682	35382	0.045	0.920	18.156	17:0 ISO 3OH	7.66	ECL deviates -0.005	Reference -0.005
13.850	7704	0.045	0.918	18.252	17:0 2OH	1.67	ECL deviates -0.002	
14.080	732	0.036	0.917	18.385	TBSA 10Me18:0	0.16	ECL deviates -0.007	
14.333	786	0.039	0.915	18.529	17:0 3OH	0.17	ECL deviates -0.007	
----	2178	---	----	----	Summed Feature	0.51	15:1 ISO H/13:0	13:0 3OH/15:1 i I/H
----	-----	---	----	----		----	15:1 ISO I/13:0 3OH	
----	35815	---	----	----	Summed Feature	8.06	16:1 w7c/15 iso	15:0 ISO
----	20587	---	----	----	Summed Feature	4.58	17:1 ISO I/ANTEI B	17:1 ANTEISO B/i I

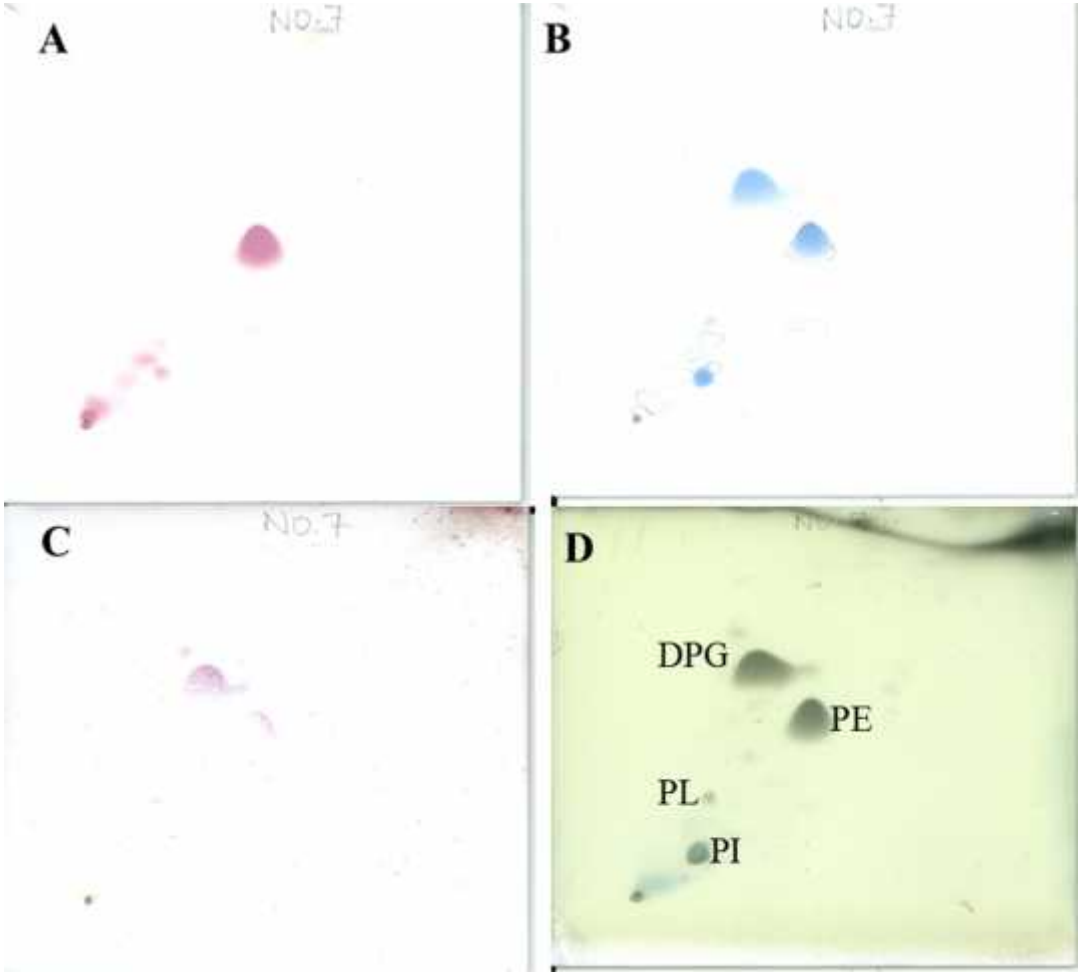
ECL Deviation: 0.004 Reference ECL Shift: 0.005 Number
Reference Peaks: 11
Total Response: 450415 Total Named: 441311
Percent Named: 97.98% Total Amount: 424670
Matches:

Library	Sim Index	Entry Name
TSBA40 4.10	0.054	Flavobacterium-johnsoniae* (Cytophaga johnsonae)



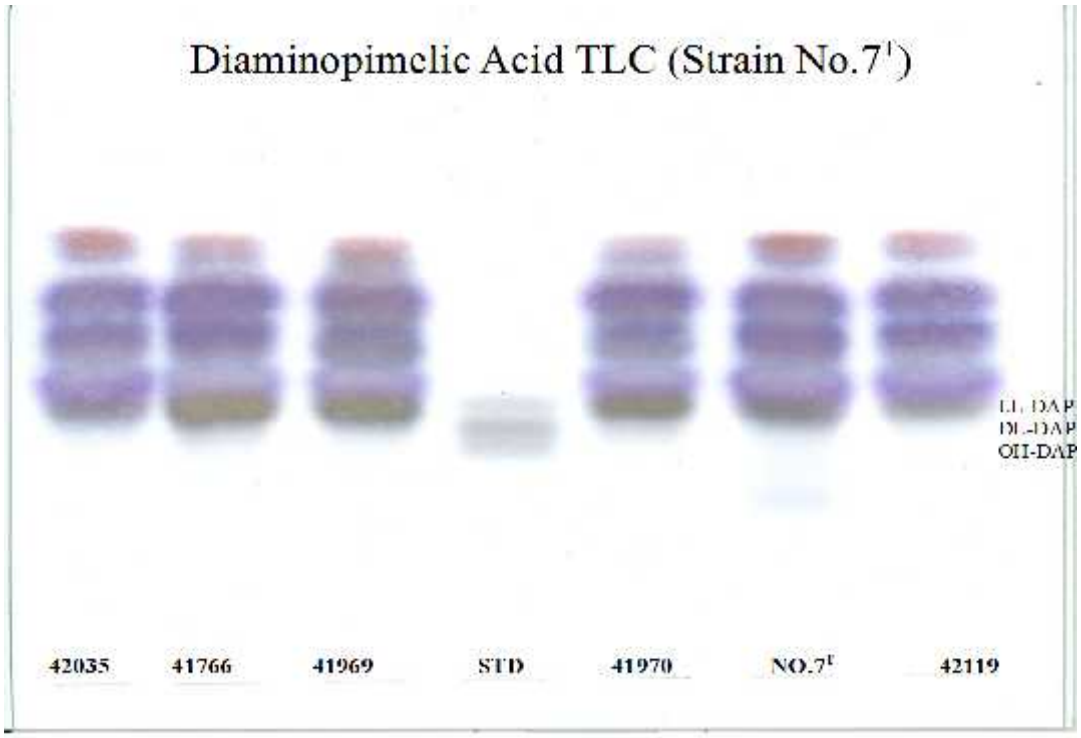
Appendix 3: Polar lipids profile of *Streptomyces alkaliphilus* sp. nov. No.7^{T}, after separation by two-dimensional TLC.

Plate A sprayed with ninhydrin for the detection of amino groups. Plate B sprayed with molybdenum blue for the detection of phosphate containing substances. Plate C sprayed with anisaldehyde for the detection of mannose containing substances. Plate D sprayed with molydatophosphoric acid for detection of total polar lipid. DPG, diphosphadidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol and PL; unknown phospholipid



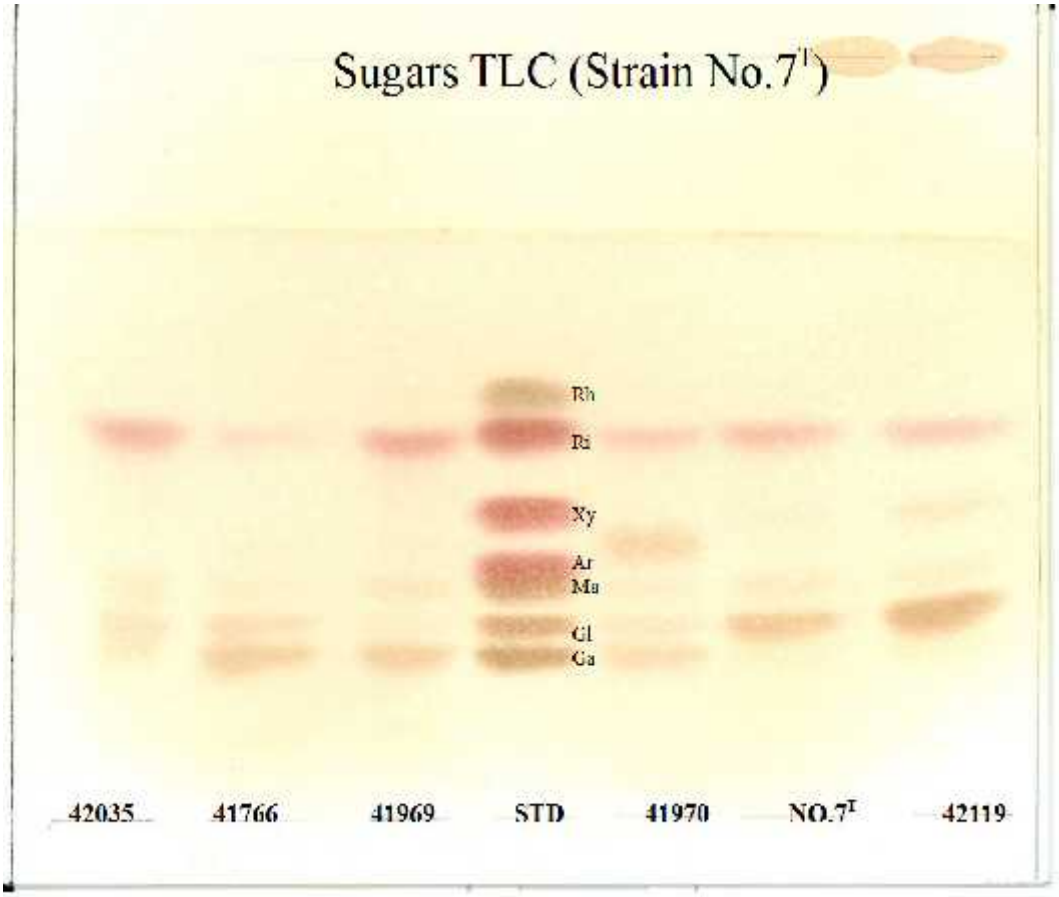
Appendix 4: Seperation of amino acids from whole cell hydrolysates by cellulose thin layer chromatography (TLC) of strain No.7T and type strains of closely related species.

DSM 42035^{T} *Streptomyces qinglanensis*, DSM 41766^{T} *S. yogyakartensis*, DSM 41969^{T} *S. tateyamensis*, STD standard, DSM 41970^{T} *S. marinus*, No.7^{T} sp. nov., DSM 42119^{T} *Streptomyces sp.* The standard indicates the seperation of the three stereoisomers of 2, 6-Diaminopimelic acid (DAP).



Appendix 5: Seperation of sugars by thin layer chromatography (TLC) of whole cell hydrolysates of strain No.7T and type strains of closely related species.

DSM 42035^{T} *Streptomyces qinglanensis*, DSM 41766^{T} *S. yogyakartensis*, DSM 41969^{T} *S. tateyamensis*, STD standard, DSM 41970^{T} *S. marinus*, No.7^{T} sp. nov. DSM 42119^{T} *Streptomyces* sp. Standard: Rh; Rhamose, Ri; Ribose, Xy; xylose, Ar; Arabinose, Ma; Maltose, Gl; Glucose and Ga; Galactose.



Appendix 6: Chromatographic run and composition reports of cellular fatty acids of strain No.7^{T} *Streptomyces alkaliphilis* sp. nov.

Volume: DATA7 File: E13B216.39A Samp Ctr: 20 ID Number: 28853

Type: Samp Bottle: 37 Method: TSBA40
Created: 11/21/2013 11:08:15 PM
Sample ID: STMY-SPEC-42118(ndK,FS-SCH,3d)

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.569	3.62E+8	0.023	----	7.038	SOLVENT	----	< min rt	
2.399	489	0.021	----	8.821		----	< min rt	
6.355	2072	0.032	1.002	13.619	14:0 ISO	2.32	ECL deviates 0.000	Reference -0.003
7.784	15902	0.035	0.977	14.622	15:0 ISO	17.32	ECL deviates -0.001	Reference -0.003
7.919	32059	0.037	0.975	14.713	15:0 ANTEISO	34.84	ECL deviates 0.000	Reference -0.002
8.132	430	0.037	0.972	14.855	15:1 w6c	0.47	ECL deviates -0.001	
8.351	342	0.029	0.969	15.002	15:0	0.37	ECL deviates 0.002	Reference 0.000
9.092	2674	0.038	0.960	15.458	16:1 ISO H	2.86	ECL deviates -0.003	
9.365	12923	0.038	0.957	15.626	16:0 ISO	13.79	ECL deviates -0.001	Reference -0.003
9.675	1317	0.037	0.954	15.816	Sum In Feature 3	1.40	ECL deviates -0.006	16:1 w7c/15 iso
9.975	871	0.036	0.951	16.001	16:0	0.92	ECL deviates 0.001	Reference -0.001
10.685	709	0.038	0.944	16.418	ISO 17:1 w9c	0.75	ECL deviates 0.002	
10.866	715	0.038	0.943	16.524	ANTEISO 17:1	0.75	ECL deviates 0.000	
11.045	5609	0.040	0.941	16.629	17:0 ISO	5.89	ECL deviates -0.001	Reference -0.003
11.204	17484	0.040	0.940	16.723	17:0 ANTEISO	18.33	ECL deviates 0.000	Reference -0.002
----	1317	---	----	----	Summed Feature	1.40	16:1 w7c/15 iso	15:0 ISO

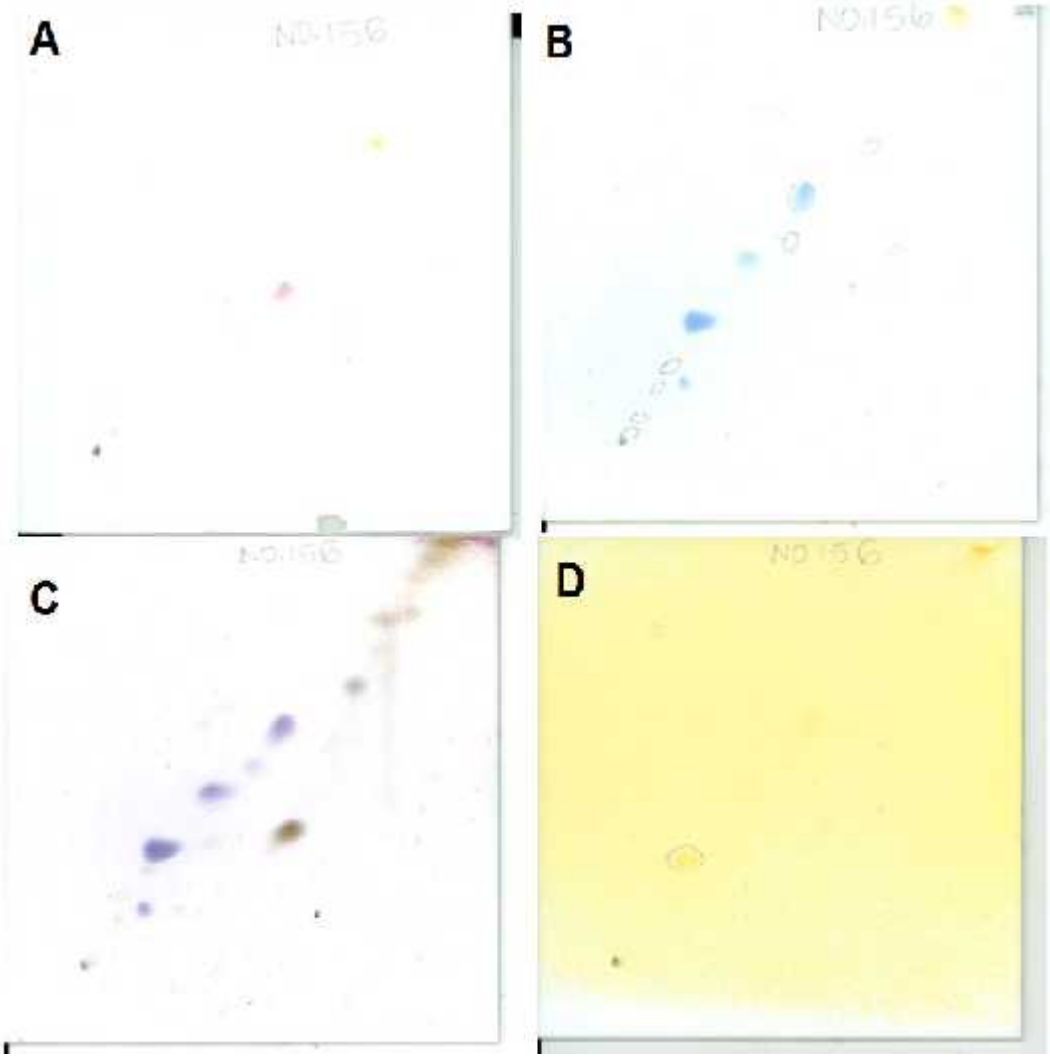
ECL Deviation: 0.002 Reference ECL Shift: 0.002 Number Reference Peaks: 8
Total Response: 93108 Total Named: 93108
Percent Named: 100.00% Total Amount: 89680

Matches:

Library	Sim Index	Entry Name
TSBA40 4.10	0.263	Bacillus-oleronius**
	0.193	Brevibacterium-mcbrellneri**
	0.164	Virgibacillus-pantothenticus* (Bacillus)
	0.164	Corynebacterium-aquaticum (not an approved name)
	0.158	Micrococcus-luteus-GC subgroup B*

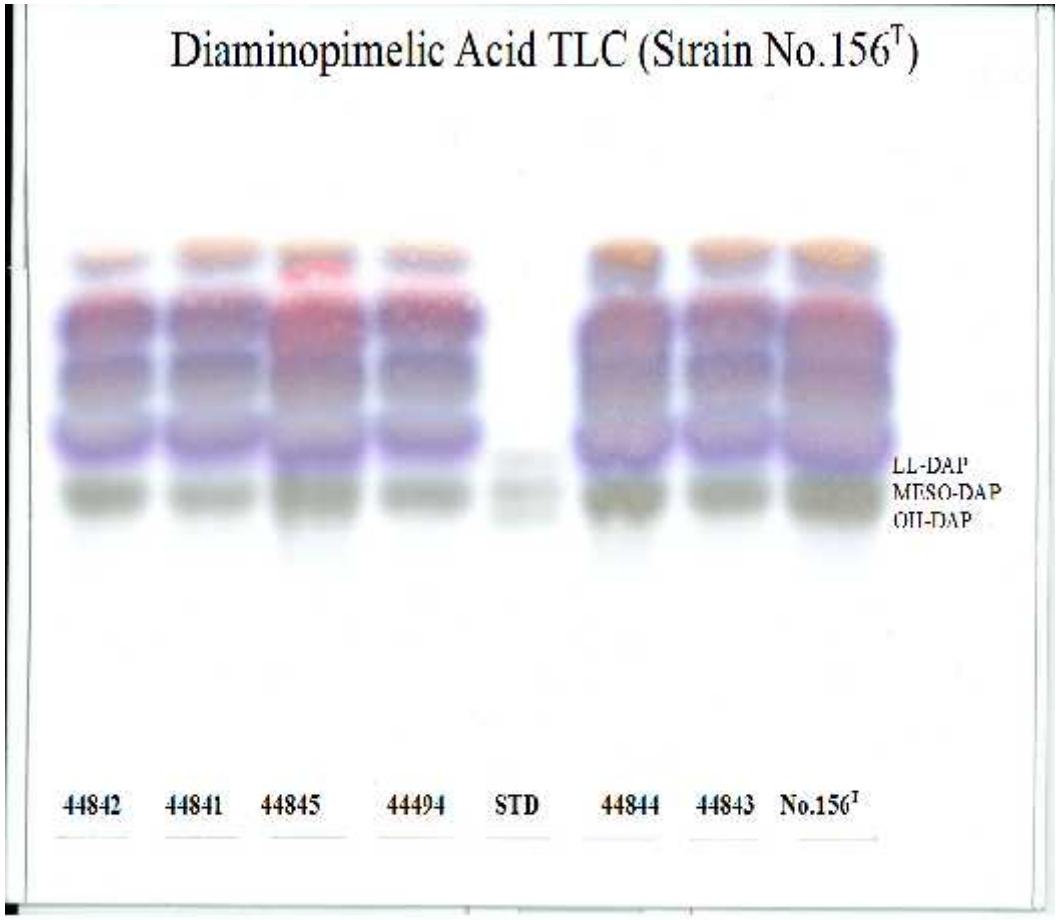
Appendix 7: Polar lipids profile of *Nocardiopsis mwathae* sp. nov. No.156^{T}, after separation by two-dimensional TLC.

Plate A sprayed with ninhydrin for the detection of amino groups. Plate B sprayed with molybdenum blue for the detection of phosphate containing substances. Plate C sprayed with anisaldehyde for the detection of mannose containing substances. Plate D sprayed with Dragendorf reagent for the detection of Phosphatidylcholine



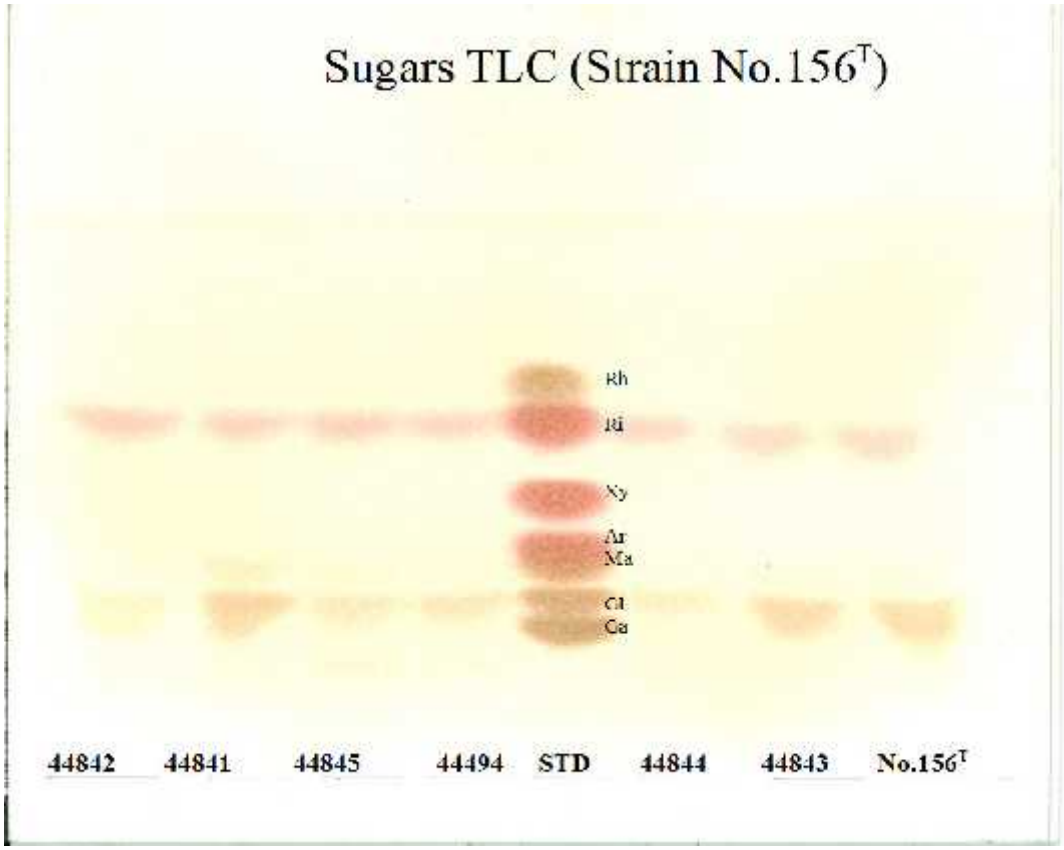
Appendix 8: Seperation of amino acids from whole cell hydrolysates by cellulose thin layer chromatography (TLC) of strain No.156T and type strains of closely related *Nocardiopsis* species.

DSM 44842^{T} *Nocardiopsis rosea*, DSM 44841^{T} *N. gilva*, DSM 44845^{T} *N. baichengensis*, DSM 44494^{T} *N. halophila*, STD standard, DSM 44844^{T} *N. chromatogenes*, DSM 44843^{T} *N. rhodophaea* and No. 156^{T} sp. nov. The standard indicate the seperation of the three stereoisomers of 2, 6-Diaminopimelic acid (DAP).



Appendix 9: Seperation of sugars by thin layer chromatography (TLC) of whole cell hydrolysates of strain No.156T and type strains of closely related Nocardiopsis species.

DSM 44842^{T} *Nocardiopsis rosea*, DSM 44841^{T} *N. gilva*, DSM 44845^{T} *N. baichengensis*, DSM 44494^{T} *N. halophila*, STD standard, DSM 44844^{T} *N. chromatogenes*, DSM 44843^{T} *N. rhodophaea* and No. 156^{T} sp. nov. Standard: Rh; Rhamose, Ri; Ribose, Xy; xylose, Ar; Arabinose, Ma; Maltose, Gl; Glucose and Ga; Galactose.



Appendix 10: Chromatographic run and composition reports of cellular fatty acids of strain No.156^{T} *Nocardiopsis mwathae* sp. nov. DSM 46659^{T}.

Volume: DATA File: E13A076.68A Samp Ctr: 16 ID Number: 28556
Type: Samp Bottle: 22 Method: TSBA40
Created: 10/7/2013 10:15:33 PM
Sample ID: UN-JULIAH-NORC-156-DSM 46659

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.570	4.108E+8	0.025	----	7.035	SOLVENT	----	< min rt	
6.363	1511	0.032	1.004	13.619	14:0 ISO	0.92	ECL deviates 0.000	Reference -0.003
6.863	460	0.033	0.993	14.000	14:0	0.28	ECL deviates 0.000	Reference -0.003
7.649	353	0.033	0.980	14.527	15:1 ANTEISO	0.21	ECL deviates 0.000	
7.794	1385	0.033	0.978	14.624	15:0 ISO	0.82	ECL deviates 0.001	Reference -0.002
7.927	4581	0.037	0.976	14.713	15:0 ANTEISO	2.71	ECL deviates 0.000	Reference -0.003
9.074	34753	0.038	0.960	15.442	16:1 ISO G	20.22	ECL deviates 0.000	
9.375	13459	0.038	0.957	15.626	16:0 ISO	7.80	ECL deviates -0.001	Reference -0.004
9.678	5841	0.039	0.953	15.812	Sum In Feature 3	3.37	ECL deviates -0.010	16:1 w7c/15 iso
9.983	2545	0.039	0.950	15.999	16:0	1.47	ECL deviates -0.001	Reference -0.004
10.734	5262	0.046	0.943	16.441	16:0 10 methyl	3.01	ECL deviates 0.009	
10.893	12851	0.041	0.941	16.534	17:1 ANTEISO	7.33	ECL deviates -0.006	
11.055	2878	0.040	0.940	16.629	17:0 ISO	1.64	ECL deviates -0.001	Reference -0.003
11.213	8224	0.040	0.939	16.722	17:0 ANTEISO	4.68	ECL deviates -0.001	Reference -0.003
11.331	1765	0.043	0.938	16.792	17:1 w8c	1.00	ECL deviates 0.000	
11.691	586	0.040	0.935	17.003	17:0	0.33	ECL deviates 0.003	Reference 0.001
12.393	3805	0.039	0.930	17.406	17:0 10 methyl	2.14	ECL deviates -0.003	
12.460	4865	0.042	----	17.444		----		
12.686	841	0.038	0.928	17.574	18:3 w6c	0.47	ECL deviates -0.003	
12.786	2383	0.048	0.927	17.632	18:0 ISO	1.34	ECL deviates 0.000	Reference -0.003
13.026	40641	0.045	0.926	17.769	18:1 w9c	22.80	ECL deviates 0.000	
13.109	4595	0.041	0.925	17.816	18:1 w7c	2.58	ECL deviates -0.007	
13.427	3690	0.044	0.923	17.999	18:0	2.07	ECL deviates -0.001	Reference -0.004
14.110	22986	0.043	0.920	18.392	TBSA 10Me18:0	12.81	ECL deviates 0.000	
----	5841	---	----	----	Summed Feature	3.37	16:1 w7c/15 iso 2OH	15:0 ISO

ECL Deviation: 0.004 Reference ECL Shift: 0.003 Number Reference Peaks: 11
Total Response: 180260 Total Named: 175395
Percent Named: 97.30% Total Amount: 164990

*** No Matches found in TSBA40