PhD
41544

Diversity of Thermophilic Bacteria in Hot Springs and Desert Soil of Pakistan and Identification of Some Novel Species of Bacteria

By

ARSHIA AMIN BUTT

Department of Microbiology
Quaid-i-Azam University
Islamabad, Pakistan
2017

Diversity of Thermophilic Bacteria in Hot Springs and Desert Soil of Pakistan and Identification of Some Novel Species of Bacteria

By

ARSHIA AMIN BUTT

Thesis Submitted to
Department of Microbiology
Quaid-i-Azam University, Islamabad
In the partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Microbiology

Department of Microbiology
Quaid-i-Azam University
Islamabad, Pakistan
2017

IN THE NAME OF ALLAH, THE MOST COMPASSIONATE, THE MOST MERCIFUL,

"And in the earth are tracts and (Diverse though) neighboring, gardens of vines and fields sown with corn and palm trees growing out of single roots or otherwise: Watered with the same water. Yet some of them We make more excellent than others to eat. No doubt, in that are signs for wise people."

(Sura Al Ra'd, Ayat 4)

Author's Declaration

I **Arshia Amin Butt** hereby state that my PhD thesis titled

A **"Diversity of Thermophilic Bacteria in Hot Springs and Deserts Soil of Pakistan and Identification of Some Novel Species of Bacteria"** is my own work and has not been submitted previously by me for taking any degree from this University

(Name of University) **Quaid-e-Azam University Islamabad.**

Or anywhere else in the country/world.

At any time if my statement is found to be incorrect even after my Graduate the university has the right to withdraw my PhD degree.

Name of Student: **Arshia Amin Butt**

Date: **27th March 2017.**

Plagiarism Undertaking

I solemnly declare that research work presented in the thesis titled **"Diversity of Thermophilic Bacteria in Hot Springs and Deserts Soil of Pakistan and Identification of Some Novel Species of Bacteria"**
is solely my research work with no significant contribution from any other person. Small contribution/help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and University **Quaid-e-Azam University Islamabad** towards plagiarism. Therefore I as an Author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of PhD degree, the University reserves the rights to withdraw/revoke my PhD degree and that HEC and the University has the right to publish my name on the HEC/University Website on which names of students are placed who submitted plagiarized thesis.

Student /Author Signature:

Name: Arshia Amin Butt

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled **"Diversity of Thermophilic Bacteria in Hot Springs and Desert Soil of Pakistan and Identification of Some Novel Species of Bacteria"** was conducted by **Ms. Arshia Amin Butt** under the supervision of **Dr Iftikhar Ahmed**

No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the **Department of Microbiology Quaid-e-Azam University Islamabad** in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Field of **Microbiology**

Department of **Microbiology**

University of**: Quaid-e-Azam University Islamabad**

Student Name: Arshia Amin Butt Signature: _____

Examination Committee:

a) External Examiner 1: Name: **Dr Ghazala Kaukab**
Signature:_____
(HOD- Department of Biochemistry, PMAS Arid Agriculture University Rawalpindi)

b) External Examiner 2: Name **Dr. Allah Nawaz**
Signature:_____
(Principal Clinical Bio Chemist Capital Hospital Islamabad)

c) Internal Examiner: Name
Signature:_____

Co-Supervisor Name: Dr. Javid Iqbal Dasti ~~**Dr Iftikhar Ahmed**~~ Signature:_____

Name of Dean/HOD: **Dr Fariha Hassan** Signature:_____

DEDICATED

To

My dearest Husband

Malik JAWAD Saboor

For his endless support, encouragment and making me believe in myself,

and

to my Lifeline, my son,

Aali Jawad.

My parents and parent in laws

There is no doubt in my mind that without their continued support and counseling I could not have completed this process.

CERTIFICATE

This thesis, titled "Diversity of Thermophilic Bacteria in Hot Springs and Desert Soil of Pakistan and Identification of Some Novel Species of Bacteria." submitted by **Ms. Arshia Amin Butt** to the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Doctor of Philosophy in Microbiology.

Supervisor: _____

Dr. Iftikhar Ahmed
Principal Scientific Officer / Director (IMCCP, NARC)

Co-Supervisor: _____

Dr. Javed Iqbal Dasti
Assistant Professor

External Examiner: _____

External Examiner: _____

Chairperson: _____

Dr. Fariha Hassan
~~Associate~~ Professor

Dated: 15-03-2017

Acknowledgments

First of all, all praises to ALLAH SUBHANAWATALLA, the Almighty, the creator and sustainer of all life, the origin of all power, knowledge and wisdom. His will is final. It was He who made me capable enough o understand the work, He who gave the command and the initiative to conclude this study. All praises to our beloved Prophet Hazrat Muhammad (SAWW) who got us out of the blinding ignorance and showed us the true path of life.

I would like to convey my earnest gratitude to my husband, parents and parent in laws. They were always there to help, support, and share, and guide me every step of the way. To other family members **Miss Aabaa, Zartashia Amin, Aisha Amin, Tayyaba Amin, Mehroze Amin, Tahir Butt, Fateh, Taymoor, Mansoor, Amlash, Shamil, Rehmat, Rafeh**, who were always there to share my burdens. My family taught me about life and gave so many sacrifices for me just so I can be where I am today. May Allah shower His blessings upon them always Amen!

I further express my deepest appreciation to my supervisor **Dr Iftikhar Ahmed**. His presence itself gave me hope and his open optimism towards the work gave me courage. He allowed me a free hand to pursue what I planned for the work and guided me accordingly. I believe it's his pain staking efforts and devotion towards this field is what made me carry out my PhD in the first place. I feel honoured to be his doctoral student.

My appreciation is also due to my co supervisor, **Dr. Javed Iqbal Dasti**. He guided me about everything related to him so I can easily carry out my work. I don't want to Miss **Dr Nauman Khalid** who was very helpful whenever I asked him for guidance and discussed with me every bit of ambiguity.

My special thanks to **Dr Abdul Hameed, Dr Safia Ahmed, Dr Fariha Hassan, Dr Muhammad Imran, Dr Aamer Ali Shah** who supported my work in every way possible and most importantly provided me with a platform for the study. Their advice and guidance was most candid and frank helping me ease my work whenever any hurdle came.

I am extremely grateful to my supervisor in China, **Prof. Dr. Wen Jun Li**, at Sun Yat-Sen University, Guangzhou, who was Director of Yunnan institute of

Microbiology during my entire stay in China, I was guided by him at each step. He understood my problems and went out way to support me. Consequently, his treatment of me was more as of a friend than a student which helped me enormously. My experience of travelling abroad and working in his well-equipped lab has been most fruitful due to his support. He guided me about the taxonomy as well as offered pyrosequencing study otherwise this work wouldn't have reached a conclusion. I also appreciate him for introducing me with **Dr Byung-Yong Kim** and **Dr Dharmesh** Singh from Chun Lab, Seoul National University and **Dr Xiao-Yang Zhi** from YIM institute of Microbiology who gave me lot of time for guiding about pyrosequencing studies.

I am greatly thankful to **Mrs Xiao Min** whose attitude was always extremely friendly and I am very grateful for all the help she provided me. Thanks, are also due to all my friends and research colleagues **Miss Nadia, Saira, Safina, Yin Yrui, Liu Lan, Jhao Jhang yu, Xiao Mei, Ming Hong, Bing Bing, Wen, Jhan Jhao, Zhuging, Xian Wen Dong** for all the general help and support with the lab work. My special gratitude goes off to **Neeli Habib** for taking time off in working with me, attending to my work and providing me the support whenever I needed it. She is one of those persons whose company gave me courage to complete my experiments. Great thanks to **Firasat Hussain, Ghani Jan Usman** and **Inam** who were a big moral support to stay in a new environment.

I am very thankful to all funding agencies which were supporting us.

Arshia Amin Butt

Table of Contents

LIST OF TABLES

LIST OF FIGURES

Abbreviations

YE Yeast extract

TEM Transmission electron microscopy

SEM Scanning electron Microscopy

HPLC High Performance Liquid Chromatography

TLC Thin layer chromatography

ML Maximum likelihood

MP Maximum parsimony

NJ Neighbour joining

XYL medium Xylitol medium

CMC medium Sodium carboxymethyl cellulose

MSC medium Microcrystalline cellulose

R2-A medium Reasoner's 2A agar medium

ISP2 medium International Streptomyces project

SCA Starch casein agar

CCA Canonical correlation analysis

PCO Principle coordinate analysis

UPGMA Unweighted pair-group method with arithmetic mean

MEGA Molecular evolutionary genetic analysis

VB solution Vitamin B solution

PABA paraaminobenzoicacid

NCCP National culture collection of Pakistan

TP Tatta Pani

TF Tato Field

MA Murtazaabad

KCTC Korean Collection for Type Cultures

CGMCC China General Microbiological Culture Collection *Center*

API Analytical profile index

MK Menaquinone

DPG Diphosphatidyl glycerol; PG Phosphatidylglycerol; GL Glycolipids;

DMG Dimannosyl- diacylglycerol

CHAPTER 1

Executive Summary

Diversity of Thermophilic Bacteria in Hot Springs and Desert Soil of Pakistan and Identification of Some Novel Species of Bacteria

Microorganisms that grow in hostile or extreme environments are currently a popular and an emerging subject for study/scientific research. These microorganisms have been recognized as 'extremophiles', which inhabit environments with extreme levels of salinity, pH, pressure, heat, cold, and even radiation. These extremophiles, having many interesting biological secrets, provide a novel source of scientific discoveries in the basic and applied sciences. In this thesis, we reported microbial diversity of thermophilic bacteria in hot springs and desert soil of Pakistan. During these studies, several novel bacteria were also identified and were delineated as novel species of bacteria.

1.1 Actinobacterial and Bacterial diversity in hot water springs soil and desert soil

Samples for the isolation of strains were collected from hot water spring sites in Tatta pani Kotli Kashmir (74°32'46.10"E, 35°28'30.40"N ASL 3841 ft), Murtaazabad (74°36'19.77"E, 36°16'30.34"N 6809 ft ASL), Tato field hot water spring (74°32'49.10"E, 35°28'30.40"N ASL 3861 ft) and Cholistan desert Bahawalpur Pakistan (29°23'43"N 71°41'1"E ASL 291.9948ft).Various enrichment and selective media were used. Different substrates were used in media to select various bacteria. These isolates were purified and stored as slants (Short term preservation), and as 30% glycerol stocks or lyophilized cultures (Long term preservation). These strains were identified based on16SrRNA gene sequence analysis by using standard PCR conditions and primers. Based on the marker gene sequence similarities it was observed that, 248 isolated strains belong to 37 genera and 3 major phyla which were *Proteobacteria, Firmicutes* and *Actinobacteria.*

1.2. Novel taxa from hot water spring soil

Of the potentially novel species of *Actinobacteria* and *Bacteria* two were characterized by polyphasic taxonomy. These strains were characterized as novel species of the genera *Nocardioides* and *Streptomyces*.

1.2.1. *Nocardioides pakistanensis* sp. nov.

NCCP-1340^T, which belongs to *Nocardioides* genus was isolated and described as a new species. Optimum requirements for pH, temperature and salt were recorded according to standards protocols. Sequence similarity of 16SrRNA gene tells that NCCP-1340^T be in the right place with the genus *Nocardioides* and was most closely related to *Nocardioides iriomotensis* IR27-S3^T. DNA–DNA affinity value was less than 53%. After following the minimal criteria for identification of novel species which included physiological features, biochemical characteristics and the phylogenetic studies, strain NCCP-1340^T signifies a novel species of genus *Nocardioides,* and we propose the name *Nocardioides pakistanensis* sp. nov.

1.2.2. *Streptomyces caldifontis* sp. nov.

An *Actinobacterium*, NCCP-1331^T, was isolated during diversity study for which ideal temperature, pH and salt requirement was 28°C, 7.0 and 2 % NaCl (w/v). Maximum parsimony maxmimum likelihood and neighbor joining procedures for the 16SrRNA gene sequence confirmed that strain NCCP-1331^T belonged to the genus *Streptomyces* and with closest affinity with *Streptomyces brevispora* BK160^T, 97.9 % nucleotide similarity, DNA–DNA similarity values of strain NCCP-1331^T with *S. brevispora* KACC 21093^{T}was 42.7 %. After following the minimal criteria for description of a novel species it was indicated that strain NCCP-1331^T belongs to the genus *Streptomyces*, for which we propose the name *Streptomyces caldifontis* sp. nov.

1.3. Novel taxa from desert soil

Of the potentially novel species of *Actinobacteria* and *Bacteria* two which were characterized by polyphasic taxonomic approach belongs to genus *Microvirga* and novel genus *Zafaria solitudinis* gen. nov. sp. nov..

1.3.1. *Microvirga pakistanensis* sp. nov

A Gram negative strain labelled as NCCP-1258^T, from Cholistan desert soil, Bahawalpur, Pakistan for which optimum growth conditions were determined for important parameters like, media optimization, growth conditions for pH, temperature and salt concentration. Phylogenetic studies were performed with three algorithms based on 16srRNA gene sequence was aligned against sequences present in datab base of Ezbiocloud and comparison revealed that strain NCCP-1258^T is in right place with genus *Microvirga* and its closest relative is *Microvirga lotononidis*. DNA-DNA similarity values of NCCP-1258^T was less than the 42% that is enough to delineate a new species. According to the criteria for describing a new species that belongs to *Microvirga* genus and other rhizobium species 16SrRNA gene sequence do not give accurate resolution and concatenated sequences of housekeeping gene (*dnaK*, *gyrB*, *recA* and *rpoB*) should also be amplified and compared for similarity. While following all criteria's strain NCCP-1258^T was placed as a new species of genus *Microvirga*, for which the name *Microvirga pakistanensis* sp. nov. was proposed.

1.3.2. *Zafaria solitudinis* gen. nov. sp. nov.

A new bacterium from phylum *Actinobacteria*, NCCP-1664^T, was isolated from Cholistan desert, Pakistan which was most closely related to *Arthrobacter halodurans* (97.70 %) but DNA–DNA relatedness value with above mentioned type strain was less than 54 %. Later on the strain was studied with polyphasic taxonomic approach. After following all criterias to delineate a strain as a new species strain it was revealed that NCCP-1664^T could not be placed in any existing *Arthrobacter* group be distinguished from the closely related taxa and, represented a novel species of the genus novel genus *Zafaria* gen., nov.for which we propose the name *Zafaria solitudinis* gen. nov. sp. nov.

1.4. Diversity and distribution of bacterial diversity by 454 pyrosequencing approach and correlation between physical parameters and species distribution

Pyrosequencing approach was used targeting the V3 region of rRNA gene and microbial community was determined.

1.4.1. Hot water Springs bacterial diversity of Pakistan

Samples from nine hot water springs which were distributed over three geothermal fields namely, Tato field (also called Tatta Pani Chilas in local language), Tatta pani and Murtazaabadalong the MKT, were collected. Physical parameters considered were

were N2, CO, electrical conductivity, HCO3, NH4, pH, Temp, SiO2, SO4-2, Ca, K+ and Na levels. Shifts in bacterial community were observed with variation in the level of temperature, sulfate, nitrate and carbonate level.

Major phyla observed were *Proteobacteria, Chloroflexi, Thermotoga, Bacteriodetes, Deinococcus thermus, Nitrospirae, Acidobacteria* and others well reported thermal spring phyla which are still unclassified were UT06, OP11, BRC1, OD1, OP8, OP1, OP3, OP9, OMAN, NKB19 (Figure 1). Presence of 40.1 % unclassified OTUs clearly suggest the presence of many undiscovered and unexplored unique microbiota within these sites. Innovative visions into the nature of the ecological connections among central taxa in these communities, were determined which will help in determining the future course of studies of these sites.

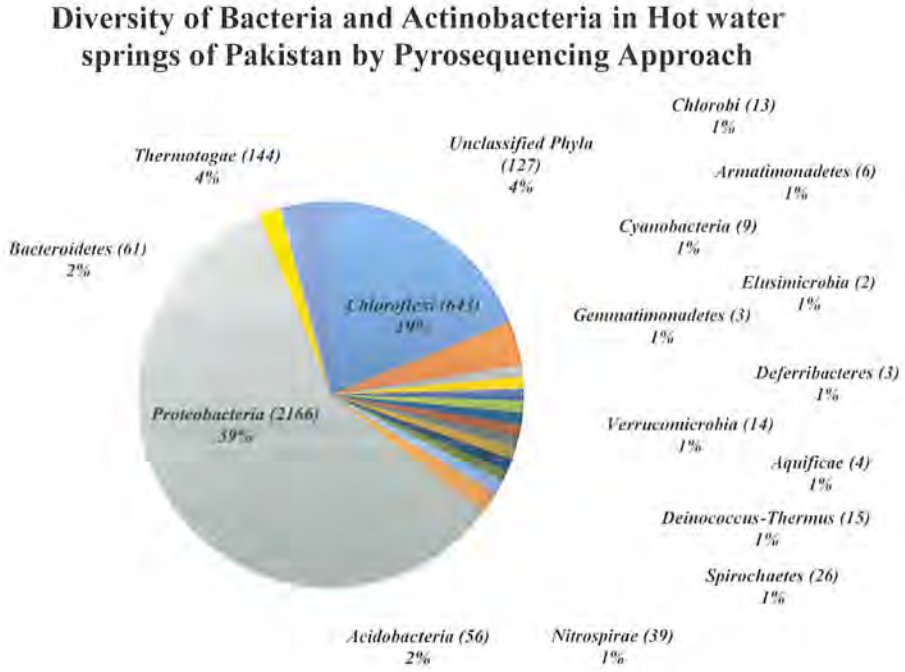


Figure 1 Pie Chart showing distribution of Bacterial phyla and Actinobacteria in Hot water springs of Pakistan by Pyrosequencing Approach of 16S rRNA gene. Values in brackets are showing number of average number of OTUs (operational taxonomi units). Unclassified OTUs belongs to UT06, Caldithrix_p, BRC1, OP8, OP11, MATCR, OMAN, Bacteria_uc, NKB19, JX105615_p, WS1 and OD1.

1.4.2. Desert samples bacterial diversity of Pakistan

The Cholistan desert part, situated in Pakistan, supports specific flora that grows in the large area that is covered in dust. We studied eight different samples, two from the

rhizosphere of forerunner plants, two from surface at 50 cm depth and two from 100cm depth and control samples and used pyrosequencing of V1-V3 sections to compare the bacterial population diversity of the samples.

The results disclosed a total of 4,692 OTUs in the seven samples, calculated. OTUs (Operational taxonomic units) related to Phylum *Proteobacteria, Deinococcus thermus, Chloroflexi* and *Actinobacetria* were dominant in rhizosperic desert samples (NCCP-D1-a, NCCP-D1-b), while in other samples instead of *Actinobacteria* number of OTUs for *Thermotogae* and *Chloroflexi* were in significant in number. In NCCP-D3-a and NCCP-D3-b *Bacteroidetes* and *Acidobacteria* was also in considerable number (Figure 2).

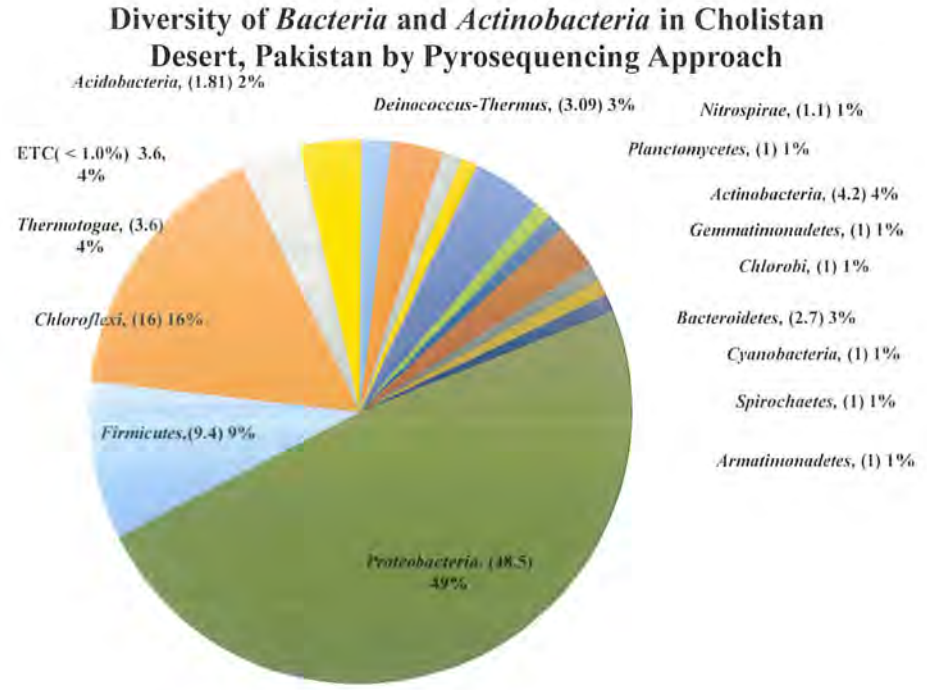


Figure 2 Pie Chart showing distribution of Bacterial phyla and Actinobacteria in Cholistan desert soil of Pakistan by Pyrosequencing Approach of 16 vS rRNA gene. Values are showing number of average number of OTUs (operational taxonomic units).

This work showed that the Cholistan desert area of Pakistan contain varied bacterial communitues in the vicinityof roots of forerunner desert plants. It also showed that the microbiota of deserts depended on availability of physiochemical resources e.g., precipitation, nutrients etc.

CHAPTER 2

Introduction and Review of Literature

2.1 Extremophile originality

It is well established that a variety of archaeal and bacterial species animate under environmental conditions, which include pressure, temperature, UV light, very low levels of nutrients and low or high levels of pH. (Gerday & Glansdorff, 2007). (Cavicchioli, 2002) suggested the possibility that while considering these extremophiles as models we can get insights into the life style at celestial habitat.

It would be highly significant to establish identification between ancient and primitive organisms. It has been observed that cladistically ancient organisms are positioned near the base of rRNA based trees but they neither possess primeval molecular genetic elements, nor they are more rudimentary in their metabolic abilities than their aerobic counterparts (Islas, Velasco, Becerra, Delaye, & Lazcano, 2003). Pre-RNA worlds are the source of primitive living systems, in which life may have been founded on polymers using backbones other than ribose phosphate and possibly the nitrogenous bases different from guanine, adenine, uracil and cytosine (Levy & Miller, 1998), followed by a stage in which life was based on RNA both as the genetic material and as catalysts (Joyce, 2002).

2.2 Nascent earth and extreme environment

Only very few facts support hyper-thermophilic origin of life. Firstly, the base of rRNA-based molecular phylogenies are full of hyperthermophiles (Pace, 1991, 1997). Secondly, instantaneously after earths creation, the exterior of the earth was very warm and planet persisted to be smelted for some time after its formation. Life on earth appeared during that time and only hyperthermophilic life was possible (Wetherill, 1990); (Juergen Wiegel, 1998b). The biphasic temperature–growth curves of many thermophiles growing at elevated temperatures and the existence of cryptic thermophiles are considered as additional arguments for the start of life in the range of

60–90°C and that hyperthermophiles as well as mesophiles and psychrophiles are adaptations to changed environments.

While some antagonists say that earth's surface speedily lost temperature to provide mesophilic origin of life (Wilde, Valley, Peck, & Graham, 2001). Chemical decomposition of recognized biochemical compounds i.e.; amino acids, nucleobases, RNA and thermolabile molecules have half-lives for decomposition at temperatures between 250°C and 350°C are at the most a few minutes (S. L. Miller & Bada, 1988). Another theory that supports mesophilic origin of life came from (Gulen, Petrov, Okafor, Vander, O'Neill, Hud & Williams, 2016) and (Petrov, Gulen, Norris, Kovacs, Bernier, Lanier & Hud, 2015) believes that characteristic of ribosome that armour it in high temperature e.g., RNA folding are changed gradually during evolution. Hyperthermophilic microbial routines are the artefact of secondary adaptations that settled during early stages of cell evolution. But we do not have an information on the alignment of the native stratosphere during the period of the beginning of life or on environmental conditions that were central for the development of living structures (Lazcano & Bada, 2003). (Delaye, Becerra, & Lazcano, 2005) believes that the source of the mutant sequences inherited to those establish in all prevailing species and the deviance of the bacteria, archaea, and eucarya did not happened at the same time, i.e. the parting of the principal dominions took place later, perhaps even much later, then the advent of the genetic constituents of their least common ancestors. The last universal ancestor is the last evolutionary conclusions of a sequence of familial proceedings, including paralogous duplications that took place earlier to the parting of Bacteria, Archaea, and Eucarya. (Dworkin, Lazcano, & Miller, 2002) and (Forterre, Brochier, & Philippe, 2002) believes that if hyperthermophily is not truly primeval, then heat-loving lives may be remnants of an edition that progressed after the beginning of life and afore or soon after the parting of the main ancestries. (Forterre et al., 2002) believes in lateral transfer of reverse gyrase. (Dworkin et al., 2002) have faith in the fact that are adapter to live at high temperatures due to some stresses other than temperatures.

2.3 Thermal environments and biodiversity

Wilson (1992) created the term biodiversity and wrote "The Diversity of Life". At that time there were 4,800 species described in the "kingdom" Monera Recently,

Hug, Baker, Anantharaman, Brown, Probst, Castelle, Ise, (2016) gave new tree of life (Figure 3) in which 92 phyla which are representing total Bacterial Eukaryotic and

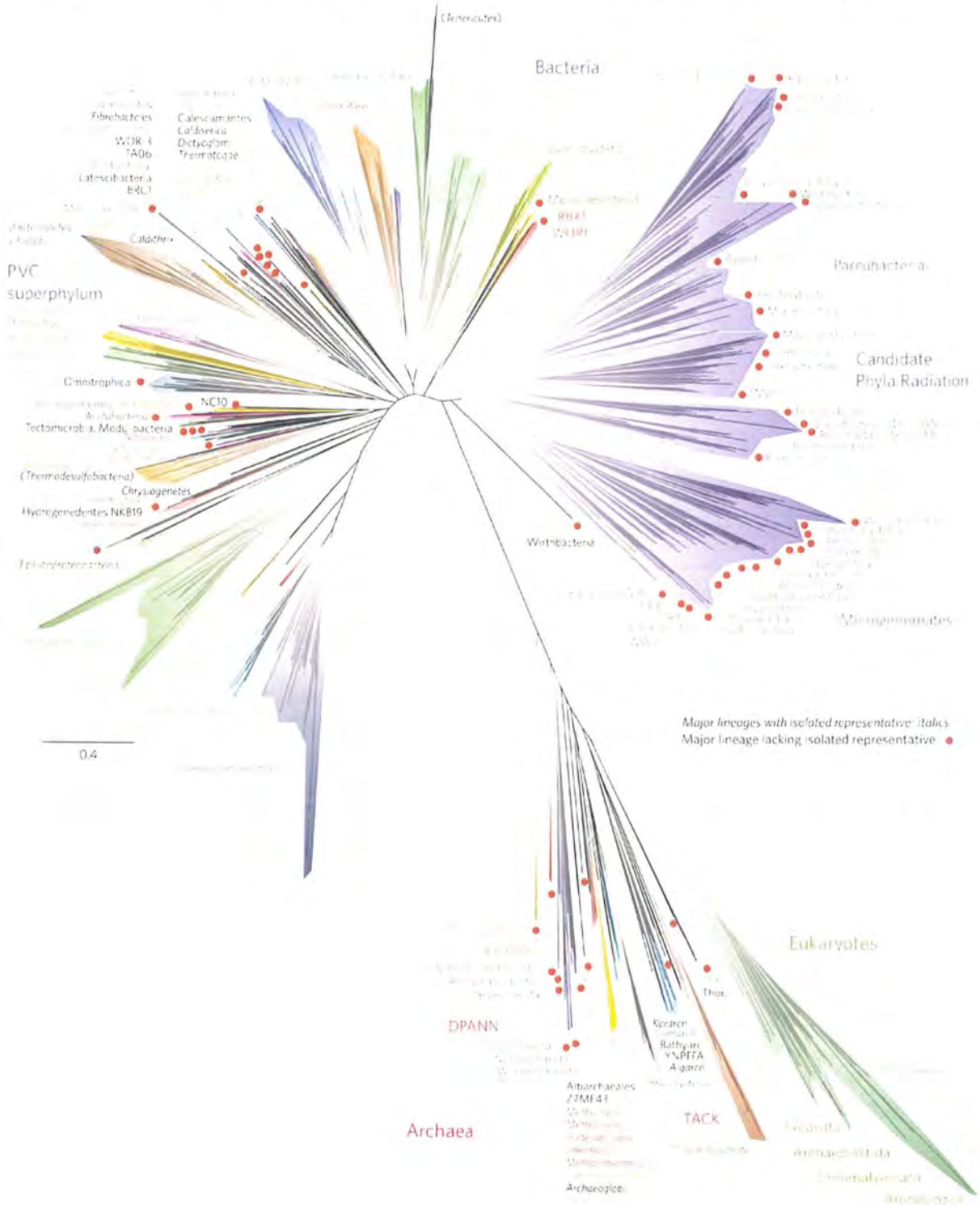


Figure 3 Tree of Life adopted from (Hug et al., 2016)

Currently published names includes; 11,305 species, 520 sub-species, 2,103 genera, 320 families, 137 orders, 20 sub-orders, 87 class, 7 subclass of which 31 are later homotypic synonyms, 1,257 are new combinations (http://www.bacterio.cict.fr/number.html#total, May 2012) and huge numbers of aligned 16S rRNA sequences are deposited in the Ribosomal Database Project (RDP) database, according to 2006 statistics 210,000 sequence were deposited (http://rdp.cme.msu.edu/misc/ news.jsp#mar0206, March 2006), and every month 30–50 more are added.What makes them popular models to test biogeographical hypotheses is the Island-like nature of thermal environments. Using similar strains of the thermophilic archaeon Sulfolobus originating from hot springs in Yellowstone National Park and Italy. (Zillig, Stetter, Wunderl, Schulz, Priess & Scholz, 1980) formulated the hypothesis that "geographical barriers between habitats of the same type do not exist for microorganisms." This hypothesis also corresponds to the oft-quoted hypothesis that "everything is everywhere and the environment selects" (Beijerinck, 1913). However, (Whitaker, Grogan, & Taylor, 2003) attribute genetic divergence detected by multilocus sequence analysis of strains of *Sulfolobus solfataricus* from five sites to geographic isolation.

2.4 General features and geography of Pakistan Hot water springs

There are seven hot springs of the Murtazabad which lye along the Main Karakoram Thrust in Northern Areas of Pakistan with the surface temperature range of 47 to 92°C. All the thermal waters of Pakistan formulated from $NaHCO_3$. Tatta Pani and Tato thermal springs along the Main Mantle Thrust have the surface temperature from 48 to 92°C. These are also $NaHCO_3$ type. Geothermal springs of Chagai are related to the youngest volcano (Koh-I-Sultan) of Pakistan. The Northern Areas having geothermal fields of Tatta Pani, Tato and Murtazabad are located between the latitudes 35° 20′ N to 36° 30′ N and longitudes 74°E to 76°E .

Important mountain ranges of the area are the Rakaposhi and Karakoram etc. Rainfall in these areas are light and the continental drift at the these areas of Pakistan occurs during later on. The formation involved three elements which control property of earth, i.e. Indian mass, the rocks placed on the southern part of the the tectonic plate covering Eurasia, and the Kohistan Island Arc System (Todaka et al., 1988); (M Ahmad et al., 2005; Powell, 1979); (Khan, Khan, Leghari, & Khan, 1987).

2.5 Chemical composition of Hot springs of Pakistan.

Among various hot water springs present in Pakistan, (Chang, Gachal, Qadri, & Sheikh, 2013) has reported the chemical composition of Manghopir thermal spring for the year 2008.

Table 1 Chemical composition of hot water spring Manghopir (Chang et al., 2013)

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Date	5	12	23	14	10	5	15	12	16	9	10	12
Time	12:00	12.10	12:20	12:00	12:40	11:30	1:00	1:40	1:10	12:30	1:30	2:30
Temp: air° C	22	23	27	26	30	32	33	28	28	27	24	23
Temp: H_2O °C	19	20	24	23	27	28	29	25	25	24	20	20
pH	7.9	7.5	7.0	7.09	7.2	7.4	7.1	7.62	7.3	7.05	7.08	7.09
EC mu/scm	3750	3840	4030	3614	3845	4420	3184	2778	2465	4790	3842	3250
TDS mg/L	1910	2148	2619	1952	2135	2373	1898	1659	1872	2580	2278	1947
Turbidity	58	60	62	58	62	60	68	70	62	56	58	60
Ca mg/L	400	180	80	92	80	68	76	84	70	64	137	379
Mg meq/L	74	76	78	73	58	43	48	56	90	153	110	85
Hardness mg/L	1305	720	420	530	475	350	738	1298	960	790	986	1298
HCO_3 ppm	450	410	335	350	585	700	558	378	595	960	676	472
Alkalinity mg/L	9.00	7.50	6.80	7.0	12.4	14.00	11.5	7.60	9.40	19.2	15.3	9.4
Cl mg/L	319	435	553	534	590	646	545	443	476	512	532	569
Na meq/L	58	165	733	458	684	855	498	207	518	680	518	233
K mg/L	19	18	17	14	20	21	22	21	24	19	21	23
SO_4 mg/L	480	570	860	427	496	576	438	336	475	684	515	240
Co_3 ppm	0	0	0	0	0	0	0	0	0	0	0	0
BOD mg/L	4.2	4.0	3.1	3.3	3.2	2.8	2.5	2.3	2.4	3.5	3.8	3.0
DO mg/L	3.8	4.2	5.6	5.1	4.8	5.2	5.0	5.1	4.9	4.3	4.4	5.3

2.6 Thermophiles

Temperature, as an environmental factor, compels all living microorganisms. In contrast to the upper temperature boundaries, the lower temperature boundaries for growth among microorganisms are not well defined (Russell et al., 1990). Thermophiles are the microorganisms that "love" heat. A word of caution is necessary regarding the use of the term thermophilic. The term means different temperature ranges for different groups of microorganisms. For example, *Candida thermophile* is described as a thermophilic yeast with a maximum growth temperature (T max) of 51°C. The optimal growth temperature (T opt) for this microorganism is 30–35°C (Shin, Shin, Yoon, & Park, 2001). Among Bacteria, this would be a thermotolerant species.

The record for the widest temperature span for growth, 50°C, is held by *Methanothermobacter thermautotrophicus* , able to grow from 22°C to 75°C (Gerday & Glansdorff, 2007). Strain 121, a Fe(III)-reducing Archaea isolated from a hydrothermal vent along the Juan de Fuca Ridge, is reported to have a doubling time of 24h at 121°C and remains viable after exposure to temperatures as high as 130°C (Kashefi & Lovley, 2003). The most heat-resistant spore is held by *Moorella thermoacetica* strain JW/DB-4. Under autotrophic conditions at 60°C, this bacterium forms spores with a decimal reduction time, 2h at 121°C. A subpopulation of spores apparently requires 1h at 100°C to become fully activated before germinating (Byrer, Rainey, & Wiegel, 2000). *Thermus aquaticus*, an aerobic, thermophilic bacterium, was isolated from Yellowstone National Park, Wyoming, in the late 1960s (Brock & Freeze, 1969), and the microorganism's Taq DNA polymerase has become an essential component of molecular biology. Cosmopolitan microorganisms from thermal environments include *Methanothermobacter thermautotrophicus, Thermanaerobacter thermohydrosulfuricus, Thermoanaerobacterium thermosaccharolyticum,* and *Geobacillus stearothermophilus.*

2.7 The interaction correlation between biogeography and biogeochemistry

The interaction correlation between biogeography and biogeochemistry in thermal environments is also worthy. As an example, three combinations can be defined by (Engle, Li, Woese, & Wiegel, 1995). Relaxed biogeography and biogeochemistry;

e.g., *Thermoanaerobacterium thermosaccharolyticum* and *Thermobrachium celere* have a relaxed biogeography and biogeochemistry. They have been isolated from a variety of environments from several locations including thermobiotic, mesobiotic, slightly alkaline and acidic environments. Relaxed biogeography and restricted biogeochemistry, e.g., *Clostridium paradoxum* and *Clostridium thermoalcaliphilum*, isolated from sewage sludge on four different continents, but only from sewage sludge. In all these sewage sludge facilities environment is mesobiotic and pH is nearly neutral. Motility of spores in these two strains is dependent on pH, temperature and presence of glucose. Both strains are peptidolytic and caseinolytic (Juergen Wiegel, 1998a). Restricted biogeography and relaxed biogeochemistry i.e., *Anaerobranca horikoshii* has only been isolated from a specific area behind the old faithful ranger station in Yellowstone National Park, but from several pools in that area, representing a spectrum of pH values from acidic (pH 5) to alkaline (pH 8.5). Although relatively easy to isolate, strains of *A. horikoshii* have not been obtained from other areas of Yellowstone National Park or other countries, nor has its sequence been found in environmental 16S rRNA gene libraries.

2.8 Survival Mechanisms at thermophilic environment

Survival of bacteria (Figure 4) at high temperatures is because of various strategies. It involve amino acid substitutions (Arnórsdóttir, Sigtryggsdóttir, Thorbjarnardóttir, & Kristjánsson, 2009), hydrophobic cores (Bezsudnova et al., 2012), interactions among subunits (Pang & Allemann, 2007), inactivation of spores by high hydrostatic pressure (Sarker, Akhtar, Torres, & Paredes-Sabja, 2015) and by adjusting membrane fluidity after adjusting membrane fatty acid composition (Y. Yoon, Lee, Lee, Kim, & Choi, 2015) and also by maintaining membrane fluidity using various thermosensors e.g., DesK (Cybulski et al., 2015)

2.9 Types of thermal environments

Various types of thermal environments exist including terrestrial, solar heated, marine, subsurface, anthropogenic, temporary and mesobiotic environments, a brief overview of these environments were reviewed below

Figure 4 Survival mechanisms of bacteria for survival at high temperatures (Q. Wang, Cen, & Zhao, 2015).

2.9.1 Terrestrial thermal environments

The Northern areas of Pakistan have many hot springs in the Gilgit, Hunza, Yasin and Murtazabad (M), valleys. The Tatta Pani (TP) hot springs are positioned on the Karakoram Highway These springs are placed at the height of 1200m to 4650m from the sea level (S. Ahmad et al., 2013). (Shuja, 1986) and (Malik Sikander Bakht, 2000) have also studied hot springspresent beside the same range. Hot springs are dispersed and their temperature ranges up to 91°C. Three parts of Pakistan i.e. Kashmir, North West Frontier Province and Balochistan would-be regions where geothermal possessions are situated. Major events which shaped earth during the Cenozoic and Mesozoic age have their role in designing these structures in Pakistan.

Worldwide these environments are found at geysers, solfatares (mud or paint pots), and mud or paint pots in volcanically active regions throughout the world, including Iceland, Western North America, New Zealand, Japan, Eastern Russia, and the rest of the so called Pacific Ring of Fire, major examples includes: Yellowstone National Park at North America which is being studied dating back to 1897 (Reysenbach & Shock, 2002). Neutral to alkaline areas richer in chloride salts or carbonate were observed in areas of terrestrial environments (Zhao, Romanek, Mills, Wiegel, & Zhang, 2005).

2.9.2. Solar-heated environments

Solar-heated environments may occur anywhere on earth receiving solar energy inputs. Such environments are likely inhabited by mesophilic, thermotolerant, and

thermophilic microorganisms because solar energy can heat some soils to 60°C and shallow waters to 50°C at certain times of the day or year, as pointed out by (Brock, 2012). Thermal environments on Earth's surface also experience evaporation, and thus many environments have elevated salinity and, therefore, halophilic inhabitants. For example, *Thermohalobacter berrensis*, a thermophilic and halophilic bacterium, was isolated from a solar slattern in France (Cayol et al., 2000). Halo-alkali-thermophiles, halophilic (up to 25% NaCl 4.5 M Sodium ion as $NaCl/Na_2CO_3$), thermophilic (up to 75°C), and alkaliphilic (up to pH 25°C 10.5) triple extremophiles, coined, have been isolated from dry salts from salt flats in Nevada and from sediments of athalassohaline lakes in Wadi An Natrun, Egypt (N. M. Mesbah & Wiegel, 2005).

2.9.3. Marine environments

Marine thermal environments may occur at Beaches: Hot Water Beach (Whitianga, New Zealand), Pozzuol: (Italy), Savusavu (Fiji Island) (Elizabeth Adrienne Burgess, 2009). Under 8m of water: Vents off the coast of Mílos Island, Greece (Sievert, Kuever, & Muyzer, 2000). Under 2,500m: Abysmal of water, deep-sea hydrothermal vents first discovered in 1977 near the Galápagos Islands (Corliss, Dymond, Gordon, & Edmond, 1979).

Organisms inhabiting such environments face multiple challenges i.e.; Venting water can exceed 300°C, but in deep-sea vents it cools quickly upon mixing with cold, deep-sea water, and habitat types range from those preferred by hyperthermophiles to temperatures habitable by psychrophiles (Kelley, Baross, & Delaney, 2002) i.e.; Black smoker chimneys, associated with volcanic psychrophiles activity, plate spreading zones generally are fueled by high concentrations of sulfides (Kelley et al., 2002). Serpentinite-hosted systems, like the Lost City hydrothermal field, are enriched in hydrogen and methane as energy sources (Kelley et al., 2005). *Thermococcus barophilus*, obtained from the Snake Pit region of the Mid-Atlantic Ridge, requires elevated pressure for growth at or above 95°C (Marteinsson et al., 1999). *Pyrococcus* strain ES4 shows an extension of T_{max} under increased pressure (Pledger, Crump, & Baross, 1994; Summit, Scott, Nielson, Mathur, & Baross, 1998).

(Jolivet, Corre, L'Haridon, Forterre, & Prieur, 2004; Jolivet, L'Haridon, Corre, Forterre, & Prieur, 2003) reported that at hydrothermal vents, the level of natural radioactivity can be 100 times greater than that at Earth's surface because of increased occurrence of elements such as Pb, Po, and Rn. For example, Archaea *Thermococcus*

gammatolerans, isolated from a hydrothermal site in Guaymas Basin. *Thermococcus marinus* from the Snake Pit hydrothermal site on the Mid-Atlantic Ridge, *Thermococcus radiotolerans* from a hydrothermal site in the Guaymas Basin. Additionally, all organisms existing in marine environments also have some tolerance i.e; *Marinococcus halophilus* if incubated at 20° C requires only 0.01M NaCl but if growth temperature raises upto 25°C minimal salt requirement for growth is 0.5M (Ventosa, Nieto, & Oren, 1998).

2.9.4 Subsurface environment

Subsurface thermal environments include petroleum reservoirs and geothermally heated lakes and aquifers. Activity in subsurface environments varies with the availability of nutrients, water, energy, depth, surrounding matrix and source materials. Lethal temperatures may not occur until as much as 10,000m below the surface (Pedersen, 2000) with few exceptions e.g., Uzon Caldera, temperatures well above 100°C can occur at depths of only a few meters (Elizabeth Adrienne Burgess, 2009). A depth record for culturable life has been established at 5,278m (Szewzyk, Szewzyk, & Stenström, 1994). Elevated temperatures found within petroleum reservoirs can be up to 130°C (Grassia, McLean, Glénat, Bauld, & Sheehy, 1996). The geochemical conditions in reservoirs are variable because of age, source material, and surrounding geology, and prokaryote communities (Orphan, Goffredi, Delong, & Boles, 2003). (Takahata, Nishijima, Hoaki, & Maruyama, 2000) have proposed that microorganisms in these environments may face oligotrophic conditions. Subsurface geothermal aquifers such as the well- known and expensive Great Artesian Basin of Australia are non-volcanically heated but experience temperatures up to nearly 103°C.

2.9.5 Anthropogenic environments

Anthropogenic habitats include household and water heaters and industrial process environments and thermal effluent from power plants (Brock, 2012). One of the earliest well known anaerobic thermophiles, *Thermoanaero bacter* (*basonym Clostridium*) *thermohydro sulfuricus*, was isolated from an Austrian sugar factory (Y.-E. Lee, Jain, Lee, & Zeikus, 1993). Other thermophiles have been isolated from thermally polluted effluent from a carpet factory (Carreto et al., 1996), the smoldering slag heap of a uranium mine (Fuchs, Huber, Teiner, Burggraf, & Stetter, 1996), and mushroom compost (Korn-Wendisch et al., 1995) . Strains of *Themus aquaticus* have

been isolated from various anthropogenic thermal environments including hot tap water and greenhouse soil (Brock & Freeze, 1969).

2.9.6 Temporary environments and mesobiotic environments

Thermophiles can be isolated from various environments, such as animal droppings, manure piles and compost, temporarily heated by biodegradation of organic material, sun-heated soils, sediments at the edges of lakes and puddles which can have temperatures up to 50°C but are frequently around 35–45°C. Whereas most of the thermophiles isolated from these environments are *Firmicutes*. One example is the archaeon *Methanothermobacter thermautotrophicus.* This species can be easily isolated from sun-heated black sediments of lakes and mesobiotic sewage plants, but it also has been isolated from sun-heated wood stumps in Georgia, United States (G. Luo, Wang, & Angelidaki, 2013) and mesobiotic environments such as cold stream sediments in Germany (Jürgen Wiegel, Braun, & Gottschalk, 1981) or sediments of Lake Mendota, Wisconsin, for which temperatures have never been measured 16°C. In contrast, thermophiles living in steady thermal environments.

2.9.7 Cultural diversity

Most of the microorganisms from nearly all environments inhabit are presently uncultured (P. Hugenholtz, 2002). Considering the extreme conditions in which most thermophiles thrive, some require special handling or novel approaches for their enrichment, culturing, and isolation (Mesbah and Wiegel, 2005).

2.9.8 Phylogenetic and genetic diversity

Amplification of 16S rRNA genes directly from environmental DNA has shown intense variation in amount of diversity (Barns, Fundyga, Jeffries, & Pace, 1994);(P. Hugenholtz, Pitulle, Hershberger, & Pace, 1998) (H. Kimura et al., 2005); (Elizabeth Adrienne Burgess, 2009; Elizabeth A. Burgess, Wagner, & Wiegel, 2007). Sequences from deep-sea hydrothermal vents led to the identification of novel lineages among Archaea and Bacteria (Reysenbach, Longnecker, & Kirshtein, 2000) but some thermal environment communities may contain only a few phylogenetic types e.g., (Reysenbach & Shock, 2002) identified only three major phylogenetic groups out of 35 clones analyzed during a study on Yellowstone National Park.

Table 2 : Few of the new species of thermophilic and mesophilic bacteria isolated and identified during 2016.

Isolated strains	Temp.	Reference
Athalassotoga saccharophila gen. nov., sp. nov.	30-60	(Itoh et al., 2016)
Lampropedia cohaerens sp. nov.	45	(Tripathi et al., 2016)
Bacillus licheniformis RBS 5 sp. nov.	65	(Salem et al., 2016)
Caldimicrobium thiodismutans sp. nov.	75	(Kojima, Umezawa, & Fukui, 2016)
Inmirania thermothiophila gen. nov.	35-68	(Slobodkina, Baslerov, et al., 2016)
Herbinix luporum SD1D sp. nov.	40-65	(Koeck, Hahnke, & Zverlov, 2016)
Amycolatopsis deserti sp. nov	20-60	(Busarakam et al., 2016)
Tepidibacillus decaturensis sp. nov.	20-60	(Dong et al., 2016)
Chelatococcus thermostellatus sp. nov.	50	(Ibrahim, Lebbe, Willems, & Steinbüchel, 2016)
Deferrisoma palaeochoriense sp. nov.	30-70	(Pérez-Rodríguez, Rawls, Coykendall, & Foustoukos, 2016)
Streptomyces sp. Al-Dhabi-1 sp. nov.	55	(Al-Dhabi, Esmail, Duraipandiyan, Arasu, & Salem-Bekhit, 2016)
Thermostilla marina gen. nov., sp. nov	30-68	(Slobodkina, Panteleeva, Beskorovaynaya, Bonch-Osmolovskaya, & Slobodkin, 2016)
Dethiosulfatarculus sandiegensis gen. nov., sp. nov.	30-37	(Davidova et al., 2016)
Risungbinella massiliensis sp. nov.	25-37	(Dubourg et al., 2016)
Brevibacillus gelatini sp. nov.	45	(Inan, Ozer, Guler, Belduz, & Canakci, 2016)
Sulfurovum riftiae sp. nov.	25-45	(Giovannelli et al., 2016)
Lascolabacillus massiliensis gen. nov., sp. nov.	37	(Beye, Bakour, Traore, Raoult, & Fournier, 2016)
Flexilinea flocculi gen. nov., sp. nov.	25-43	(Sun, Toyonaga, Ohashi, Matsuura, et al., 2016)
Lentimicrobium saccharophilum gen. nov., sp. nov.,	20-40	(Sun, Toyonaga, Ohashi, Tourlousse, et al., 2016)
Massilibacterium senegalense gen. nov., sp. nov.,	37	(Alou et al., 2016)
Altererythrobacter terrae sp. nov.,	15-37	(Srinivasan et al., 2016)
Phycicoccus endophyticus sp. nov.,	20-42	(William D Orsi et al., 2016)
Bacillus gobiensis sp. nov.,	15-40	(B. Liu et al., 2016)
Cyanobacterial strains	26-58	(Bravakos, Kotoulas, Skaraki, Pantazidou, & Economou-Amilli, 2016)

2.9.9. Metabolic diversity

Among all types of microbial metabolism from thermal environments, chemolithotrophy either autotrophy or heterotrophy is a foundation of hyperthermophilic communities in sunless and too hot environments which are not suitable for photoautotrophic production. Few chemolithoautotrophs e.g., bacteria of the order Aquificales are considered as primary producers in these ecosystems (Carrine E Blank, Sherry L Cady, & Norman R Pace, 2002). Among Bacteria are anaerobic Firmicutes such as the facultative chemolithoautotrophs *Moorella thermacetica* that undergoes homoacetogenic fermentations from carbohydrates, and the anaerobe *Ammonifex degensii* , capable of forming ammonium from nitrate via chemolithoautotrophic growth (Huber et al., 1996), Fe(III) reducers *Thermolithobacter ferrireducens, Thermolithobacter carboxydivorans*, a hydrogenic CO utilizer (Juergen Wiegel, Hanel, & Aygen, 2003). Photoheterotrophic *Chloroflexus aggregans*, *Chloroflexus aurantiacus*, *Heliobacterium modesticaldum* and *Roseiflexus castenholzii* (Hanada, Takaichi, Matsuura, & Nakamura, 2002). In some examples, in situ geochemistry of thermal environments may be shaping the dominant metabolisms or perhaps is shaped by the dominant metabolisms (Orphan et al., 2003). Many thermal environments are enriched in elements that are toxic to humans, such as As and selenium (Se), and some microorganisms in these habitats use toxic, redox-active elements to gain energy, via either oxidation or reduction (Donahoe-Christiansen, D'Imperio, Jackson, Inskeep, & McDermott, 2004).

2.9.10 Ecological diversity

The thermophilic prokaryotes have introduced us to novel modes of life because of biological interactions in geothermally heated environments. The discovery of deep-sea hydrothermal vent communities demonstrated that life can exist at temperatures 100°C as well as at 2°C on the basis of associated microbial vent community (Corliss et al., 1979). Novel symbioses between eukaryotes and prokaryotes have been identified at deep-sea vents, such as the association between the tube worm *Riftia pachyptila* and chemosynthetic, sulfur-oxidizing bacteria (Cavanaugh, Gardiner, Jones, Jannasch, & Waterbury, 1981) or the thermotolerant Pompeii worm, which utilize eurythermal enzymes of a community of prokaryotes living on its back (Chevaldonné et al., 2000). Fledgling field of microecology is rapidly expanding, and thermal environments are exemplary systems for it (Magurran, 2013), For example, In hot water

springs the diversity of microorganisms within mats of *cyanobacteria* has been examined and the importance of a prokaryote species is determined based on its role in their environments (Ward, Ferris, Nold, & Bateson, 1998) and the effect of temperature, on structuring community of prokaryotes through genetical parameters and the distribution of different metabolic types (Norris, Wraith, Castenholz, & McDermott, 2002); (Orphan et al., 2003). FISH is also helpful method that enable us to examine the structural distribution of microorganism of known phylogenetic affiliations (Nübel et al., 2002). Lipids present within the membranes of prokaryotes can be diagnostic for various types of microorganisms and have provided insight into the distribution of microorganisms among different environments. For example, analysis of glycerol dialkyl glycerol tetraethers (GDGTs) from selected hot springs in Nevada exposed the presence of the archaeal lipid crenarchaeol, which was believed to present in low-temperature and marine environments. Second evidence came from presence of DGGE band sequences of 16S rRNA genes from these springs which were related to thermophilic Crenarchaeota and confirmed that the presence of crenarchaeol is not exclusive to the cold-adapted, marine branch of the Crenarchaeota (Pearson et al., 2004).

2.10 Bacterial diversity in hot springs of Pakistan

All over the world thermal springs have been studied thoroughly, bacterial ecologists have great interest in discovering community structure of thermal springs. Studies includes microbial composition (Carrine E Blank et al., 2002), bioenergetics in the ecosystem (Spear, Walker, & Pace, 2005a), biogeochemistry of geothermal system (Spear et al., 2005a) Tibetan and Tengchong thermal spring community structures (Briggs et al., 2014; Jiang et al., 2016; J. Li, Peng, Zhang, Jiang, & Chen, 2016), hot spring, Japan (Kojima et al., 2016), hot water spring Kenya (Kambura et al., 2016), Amazonian hot water spring (Paul, Cortez, Vera, Villena, & Gutiérrez-Correa, 2016), Bulgaria hot water spring (Stefanova et al., 2016), US Hot springs (Nobu et al., 2016) etc. Unfortunately, a big series of hot water springs we were aiming to discuss is still undiscovered. Geographically Pakistan is situated at latitude 24° to 37° North and longitude 62° to 75° East. It is geothermally rich area. Hot springs are scattered along Main Mantle Thrust and Main Karakoram Thrust in Chilas and Hunza (Malik Sikander Bakht, 2000) areas in the Northern areas of Pakistan. Their temperature reaches up to 91°C (Javed, Zahoor, Sabar, Haq, & Babar, 2012a). Tibet, occupies more or less the

same geological position in Himalayan mountain ranges as Pakistan, (Zaigham, Nayyar, & Hisamuddin, 2009) .

In Pakistan to date, not a single study has been reported for bacterial diversity of hot water springs. Various other studies in which selective bacteria were isolated from hot water springs of Pakistan includes isolation of strain *Ralstonia sp.* MRL-TL from hot water spring to check its ability to degrade poly(ε-caprolactone) (PCL) (Shah et al., 2015), Analysis of Power Generation from Geothermal Resources (Ahsan Mustaqeem, Qureshi, & Waqar), Euthermal hot water spring mango Pir was studied for physico-chemical and biological studies and Cyanophyta, Zooplankton, Bacillarophyta and Nematoda were isolated (Jahangir, Khuhawar, Leghari, & Leghari, 2001), Fresh water spring was studied from Kohat, Pakistan and the quality assessments of the drinking water were carried out by determining, total coliform bacteria, total plate count, total fecal coliform and E. coli (S. Ahmad et al., 2013).

At the same time, if desert sites are considered than from last 3-5 years various episodes of epdemic diseases appeared among local populations of desert areas. We selected these desert sites to give insight into the local microbiota of these problem sites. These extreme dry soils harbor unique microbiota. The objective of our study was to discover unique and undiscovered microbiota Tatta pani, Tato Field and Murtazaabad hot water springs and cholistan desert, to find any new species of bacteria and characterize these candidate strains using polyphasic taxonomic approach for validation at species level, to study microbial diversity and community structure of TattaPani, Murtazabad and Tato Field hot springs and Cholistan desert soil by second generation pyrosequencing and to compare the gap between cultured and non cultured microbiota and to compare the microbial composition of different sites among the hot springs and to assess the relationships between microbial community compositions and environmental conditions (e.g. water geochemistry).

CHAPTER 3

Isolation, Identification and Sequencing

3.1. Sampling, Isolation and Preservation

Samples for the isolation of strains were collected from hot water spring sites in Tatta pani Kotli Kashmir (74°32'46.10"E, 35°28'30.40"N ASL 3841 ft), Murtaazabad (74°36'19.77"E, 36°16'30.34"N 6809 ft ASL), Tato field hot water spring (74°32'49.10"E, 35°28'30.40"N ASL 3861 ft) and Cholistan desert Bahawalpur Pakistan(29°23'43"N 71°41'1"E ASL 291.9948ft).

Table 1 Media optimization using different media.

Medium	Composition (g L^{-1})
XYL	Xylan-10 g, tryptone-0.5, trace salt-1ml, $CaCO_3$ -0.5
CMC	CMC-Na-1, yeast extract-0.01 g, Na_2NO_3-0.5 g, KH_2PO_4 -1g, $MgSO_4.7H_2O$-0.5 g, trace salt-1ml, $CaCO_3$-0.5 g
MSC	MSC -1g, yeast extract-0.01 g, Na_2NO_3-0.5 g, KH_2PO_4-1 g, $MgSO_4.7H_2O$-0.5, trace salt-1ml, $CaCO_3$-0.5 g
T5	Glucose-1 g, lotus root starch-1 g, yeast extract-2 g, tryptone-0.5 g, trace salt-1 ml, $CaCO_3$-0.5 g
R2A	Glucose-0.6, soluble starch-0.6, yeast extract-0.6 g, caseinoacids-0.6 g, sodium pyruvate -0.3 g, K_2HPO_4-0.3 g, $MgSO_4.7H_2O$ -0.05
ISP2	Malt extract, 10.0g; yeast extract, 4.0g; glucose, 4.0g
Starch/casein agar	Starch-10g, casein-0.3g, KNO_3-2 g, NaCl-2 g, K_2HPO_4-2g, $MgSO_4.7H_2O$-0.05 g, $CaCO_3$-0.2 g, $FeSO_4.7H_2O$-0.01g
TSA	Tryptone 0.5g, glucose 1.0 g, yeast extract 2.0g, $CaCO_3$ 1g, trace salt 1ml,
HV agar	Humic acid 1g, KCl- 1.7g, Na_2HPO_4-0.5g, $MgSO_4$-0.5g, $CaCO_3$-0.02g, $FeSO_4$-0.01g, vitamin B complex stock solution 1mL, agar, 1.5~1.8% (Vitamine B solution: 5.0mg each of VB1, 2, 6 and niacin, D-calcium pantothenate, inositol, and PABA (para amino acid), 2.5mg biotin, and 10mL dd water).
ISP3	Oat meal 20g, trace salt solution (ml) 1.00, ferric sulphate heptahydrate 0.001g, manganese chloride tetrahydrate 0.001g, zinc sulphate heptahydrate 0.001g
ISP7	L-Asparagine 1g, L-tyrosine 0.5g, dipotassium phosphate 0.5g, magnesium sulphate. $7H_2O$ 0.5g, sodium chloride 0.5g, trace salt solution (ml)
GA agar	Asparagine 1g, glycerol 10g, K_2HPO_4 1g, trace salt solution 1mL.
Note: CMC (Sodium carboxymethyl cellulose) and MSC (Microcrystalline cellulose) were added to check enzyme production and ability to degrade cellulose, Glycerol Asparagine (GA), agar, 1.5~1.8% is added	

Various enrichment and selective media were used. Different substrates were used in media to select various bacteria. List of different media with recepies are given in Table 3. Total 248 strains were isolated. These isolates were purified and stored as slants (Short term preservation), and as glycerol stocks or lyophilized cultures (Long term preservation).

3.2. 16srRNA Gene Sequence Analysis

These strains were identified based on 16SrRNA gene sequence analysis by using standard PCR conditions and primers (27F 1492 R). Amplified product was isolated by loading in 1% agarose gel for gel electrophoresis. Bromophenol blue was used as loading dye.

3.3. Sequencing Profile of Isolates from Hot Water Spring Sites and Cholistan Desert Site

It was revealed that these 248 strains belong to 37 genera and 3 major Phyla; *Proteobacteria*, *Actinobacteria* and *Firmicutes*.

Table 2 List of Strains Isolated from Cholistan Desert Soil and Identified by 16SrRNA Gene Sequence Similarity.

Strain ID	Accession #	Texonomy	Similarity	Length
NCCP-1101	LC065128	*Achromobacter dolens* LMG 26840^T	98.47	1112
NCCP-1102	LC065129	*Achromobacter marplatensis* B2^T	99.64	1426
NCCP-1103	LC065130	*Mirovirga flocculans* ATCC BAA-817^T	98.5	869
NCCP-1104	LC065131	*Arthrobacter cryotolerans*	100	1364
NCCP-1105	LC065132	*Arthrobacter cupressii*	100	1465
NCCP-1107	LC065134	*Arthrobacter defluvii* 4C1-a^T	98.35	1539
NCCP-1108	LC065135	*Arthrobacter halodurans* JSM 078085^T	97.36	1545
NCCP-1109	LC065136	*Microvirga subterranea* DSM 14364^T	98.86	1052
NCCP-1112	LC065139	*Arthrobacter rhombi* F.98.3HR.69^T	97.15	668
NCCP-1113	LC065140	*Bacillus aerophilus* 28K^T	100	904
NCCP-1115	LC065142	*Bacillus invictus* Bi.FFUP1^T	99.37	1139
NCCP-1117	LC065144	*Bacillus licheniformis* ATCC 14580^T	96.06	1443
NCCP-1120	LC065147	*Bacillus methylotrophicus* CBMB205^T	96.74	1468
NCCP-1121	LC065148	*Bacillus methylotrophicus* CBMB205^T	99.23	1445
NCCP-1122	LC065149	*Bacillus safensis* FO-36b^T	100	814
NCCP-1123	LC065150	*Bacillus siamensis* KCTC 13613^T	100	787
NCCP-1124	LC065151	*Bacillus sonorensis* NBRC 101234^T	96.45	1071

NCCP-1126	LC065153	*Bacillus sporothermodurans* M215 ^{T}	100	1496
NCCP-1127	LC065154	*Bacillus sporothermodurans* M215 ^{T}	94	1440
NCCP-1129	LC065156	*Bacillus tequilensis* KCTC 13622 ^{T}	98.49	1135
NCCP-1135	LC065162	*Bacillus tequilensis* KCTC 13622 ^{T}	99.93	1550
NCCP-1136	LC065163	*Bacillus tequilensis* KCTC 13622 ^{T}	99.89	884
NCCP-1138	LC065165	*Bacillus tequilensis* KCTC 13622 ^{T}	95.46	1169
NCCP-1139	LC065166	*Brevibacillus brevis* NBRC 15304 ^{T}	99.9	
NCCP-1140	LC065167	*Cupriavidus gilardii* CIP 105966 ^{T}	99.67	633
NCCP-1143	LC065170	*Cupriavidus gilardii* CIP 105966 ^{T}	97.12	1050
NCCP-1145	LC065172	*Cupriavidus metallidurans* CH34 ^{T}	98.87	1059
NCCP-1146	LC065173	*Massilia flava* Y9 ^{T}	97.57	948
NCCP-1147	LC065174	*Massilia flava* Y9 ^{T}	99.27	1102
NCCP-1148	LC065175	*Massilia suwonensis* 5414S-25 ^{T}	99.88	856
NCCP-1149	LC065176	*Massilia umbonata* LP01 ^{T}	94.85	996
NCCP-1150	LC065177	*Microvirga lotononidis* WSM3557 ^{T}	97.98	1499
NCCP-1151	LC065178	*Microvirga aerilata* 5420S-16 ^{T}	98.38	927
NCCP-1153	LC065180	*Microvirga aerilata* 5420S-16 ^{T}	99.29	845
NCCP-1154	LC065181	*Microvirga aerilata* 5420S-16 ^{T}	98.85	870
NCCP-1155	LC065182	*Microvirga flocculans* ATCC BAA-817 ^{T}	98.41	1459
NCCP-1157	LC065184	*Microvirga lotononidis* WSM3557 ^{T}	96.27	968
NCCP-1158	LC065185	*Microvirga lotononidis* WSM3557 ^{T}	97.02	940
NCCP-1159	LC065186	*Microvirga lotononidis* WSM3557 ^{T}	97.67	945
NCCP-1161	LC065188	*Microvirga lupini* Lut6 ^{T}	98.7	1082
NCCP-1163	LC065190	*Microvirga lupini* Lut6 ^{T}	98.94	1040
NCCP-1164	LC065191	*Streptomyces kasugaensis* M338-M1 ^{T}	100	1202
NCCP-1165	LC065192	*Microvirga lupini* Lut6 ^{T}	98.67	1051
NCCP-1166	LC065193	*Microvirga vignae* BR3299 ^{T}	98.25	916
NCCP-1169	LC065196	*Microvirga vignae* BR3299 ^{T}	98.66	820
NCCP-1170	LC065197	*Microvirga vignae* BR3299 ^{T}	98.67	831
NCCP-1171	LC065198	*Microvirga zambiensis* WSM3693 ^{T}	98.44	834
NCCP-1172	LC065199	*Nocardia alba* YIM 30243 ^{T}	98.79	915
NCCP-1174	LC065201	*Nocardia grenadensis* GW5-5797 ^{T}	98.88	893
NCCP-1175	LC065202	*'Phenylobacterium zucineum'* HLK1 ^{T}	92.78	1282
NCCP-1177	LC065204	*Pseudomonas geniculata* ATCC 19374 ^{T}	100	1088
NCCP-1178	LC065205	*Pseudomonas geniculata* ATCC 19374 ^{T}	100	1016
NCCP-1181	LC065208	*Pseudoxanthomonas mexicana* AMX 26B ^{T}	44.99	1192
NCCP-1182	LC065209	*Pseudoxanthomonas mexicana* AMX 26B ^{T}	99.69	965
NCCP-1184	LC065211	*Pseudoxanthomonas mexicana* AMX 26B ^{T}	100	1535

NCCP-1186	LC065213	*Rhizobium selenitireducens* ATCC BAA-1503 ^{T}	94.71	1200
NCCP-1187	LC065214	*Rhizobium selenitireducens* ATCC BAA-1503 ^{T}	97.71	967
NCCP-1190	LC065217	*Streptomyces flavidovorans*	97.95	735
NCCP-1191	LC065218	*Streptomyces gancidicus* NBRC 15412 ^{T}	99.87	780
NCCP-1192	LC065219	*Streptomyces glauciniger* NBRC 100913 ^{T}	98.69	998
NCCP-1193	LC065220	*Streptomyces griseostramineus* NBRC 12781	97.89	1000
NCCP-1201	LC065228	*Streptomyces hyderabadensis* OU-40 ^{T}	96.26	860
NCCP-1202	LC065229	*Streptomyces minutiscleroticus* NBRC 13000	96.65	778
NCCP-1203	LC065230	*Streptomyces mutabilis* NBRC 12800 ^{T}	99.88	855
NCCP-1204	LC065231	*Streptomyces netropsis* NBRC 3723 ^{T}	99.72	1409
NCCP-1206	LC065233	*Streptomyces phaeogriseichromatogenes* NRRL 2834 ^{T}	95.73	1430
NCCP-1207	LC065234	*Streptomyces pratensis* ch24 ^{T}	98.09	1088
NCCP-1208	LC065235	*Streptomyces somaliensis* NBRC 12916 ^{T}	99.93	1350
NCCP-1212	LC065239	*Streptomyces yanii* NBRC 14669 ^{T}	97.12	1064
NCCP-1213	LC065240	*Streptosporangium canum* HBUM 170018 ^{T}	99.9	967
NCCP-1258	LC065285	*Microvirga lotononidis* WSM3557 ^{T}	97.98	1499
NCCP-1286	LC065313	*Paenisporosarcina quisquiliarum* SK 55 ^{T}	99.76	830
NCCP-1293	LC065320	*Rhizobiumselenitireducens*ATCCBAA-1503	98.45	840
NCCP-1316	LC065343	*Xanthobacter tagetidis* DSM 11105 ^{T}	99.89	924
NCCP-1317	LC065344	*Xanthobacter tagetidis* DSM 11105 ^{T}	99.89	919
NCCP-1321	LC065348	*Rhizobium selenitireducens* ATCC BAA-1503	98.45	840
NCCP-1329	LC065356	*Microvirga vignae* BR3299 ^{T}	98.29	818
NCCP-1664	LC065376	*Arthrobacter halodurans* JSM 078085 ^{T}	97.7	1539

Table 3 List of Strains Isolated from Tatta Pani Hot Water Spring Soil and Identified by 16SrRNA Gene Sequence Similarity

Strain ID	Accession #	Texonomy	Similarity	Length
NCCP-1199	LC065226	*Streptomyces hyderabadensis* OU-40 ^{T}	97.07	1475
NCCP-1160	LC065187	*Hydrogenophaga defluvii* BSB 9.5 ^{T}	97.8	955
NCCP-1214	LC065241	*Achromobacter spanius* LMG 5911 ^{T}	98.63	879
NCCP-1215	LC065242	*Alcaligenes aquatilis* LMG 22996 ^{T}	97.26	1014
NCCP-1216	LC065243	*Alcaligenes faecalis subsp. parafaecalis* G ^{T}	94.09	1229
NCCP-1217	LC065244	*Aneurinibacillus migulanus* DSM 2895 ^{T}	98.88	1433
NCCP-1218	LC065245	*Anoxybacillus kamchatkensis* JW/VK-KG4 ^{T}	99.23	913
NCCP-1219	LC065246	*Anoxybacillus salavatliensis* A343 ^{T}	99.5	1442

NCCP-1220	LC065247	*Arthrobacter globiformis* NBRC 12137 ^{T}	99.77	888
NCCP-1221	LC065248	*Arthrobacter globiformis* NBRC 12137 ^{T}	99.79	940
NCCP-1222	LC065249	*Bacillus acidiceler* CBD 119 ^{T}	97.19	1000
NCCP-1223	LC065250	*Bacillus acidiceler* CBD 119 ^{T}	99.17	845
NCCP-1224	LC065251	*Bacillus aerius* 24K ^{T}	95.35	1446
NCCP-1225	LC065252	*Bacillus aerophilus* 28K ^{T}	99.6	1000
NCCP-1231	LC065258	*Bacillus invictus* Bi.FFUP1 ^{T}	99.89	935
NCCP-1232	LC065259	*Bacillus licheniformis* ATCC 14580 ^{T}	98.8	1087
NCCP-1233	LC065260	*Bacillus methylotrophicus* CBMB205 ^{T}	98.3	1070
NCCP-1234	LC065261	*Bacillus methylotrophicus* CBMB205 ^{T}	98.04	1084
NCCP-1235	LC065262	*Bacillus safensis* FO-036b ^{T}	100	940
NCCP-1236	LC065263	*Bacillus siamensis* KCTC 13613 ^{T}	99.79	971
NCCP-1237	LC065264	*Bacillus subterraneus* DSM 13966 ^{T}	99.89	889
NCCP-1238	LC065265	*Bacillus subtilis subsp. subtilis* NCIB 3610 ^{T}	98.6	1000
NCCP-1239	LC065266	*Bacillus tequilensis* 10b ^{T}	99.88	860
NCCP-1240	LC065267	*Bacillus tequilensis* 10b ^{T}	99.74	779
NCCP-1241	LC065268	*Bacillus tequilensis* 10b ^{T}	99.77	870
NCCP-1242	LC065269	*Bacillus tequilensis* KCTC 13622 ^{T}	99.35	1072
NCCP-1243	LC065270	*Bacillus toyonensis* BCT-7112 ^{T}	99.89	871
NCCP-1244	LC065271	*Brevibacillus brevis* NBRC 100599 ^{T}	99.79	932
NCCP-1246	LC065273	*Brevibacillus choshinensis* DSM 8552 ^{T}	100	879
NCCP-1247	LC065274	*'Brevundimonas olei'* MJ15 ^{T}	100	973
NCCP-1248	LC065275	*Cupriavidus gilardii* CIP 105966 ^{T}	96.1	1146
NCCP-1249	LC065276	*Cupriavidus gilardii* CIP 105966 ^{T}	100	176
NCCP-1251	LC065278	*Cupriavidus gilardii* CIP 105966 ^{T}	98.51	960
NCCP-1252	LC065279	*Fontibacillus aquaticus* GPTSA19 ^{T}	96.41	1172
NCCP-1253	LC065280	*Geobacillus thermodenitrificans subsp. calidus* F84b ^{T}	98.29	909
NCCP-1254	LC065281	*Herbaspirillum massiliense* JC206 ^{T}	95.4	1069
NCCP-1255	LC065282	*Hydrogenophaga atypica* BSB 41.8 ^{T}	98.21	837
NCCP-1256	LC065283	*Hydrogenophaga atypica* BSB 41.8 ^{T}	99.4	1009
NCCP-1257	LC065284	*Hydrogenophaga defluvii* BSB 9.5 ^{T}	98.21	780
NCCP-1260	LC065287	*Hydrogenophaga defluvii* BSB 9.5 ^{T}	97.05	1121
NCCP-1261	LC065288	*'Kocuria assamensis'* S9-65 ^{T}	99.88	849
NCCP-1262	LC065289	*'Kocuria assamensis'* S9-65 ^{T}	100	951
NCCP-1263	LC065290	*Kocuria palustris* DSM 11925 ^{T}	99.89	930
NCCP-1264	LC065291	*Leucobacter tardus* K 70/01 ^{T}	98.72	1418
NCCP-1266	LC065293	*Leucobacter tardus* K 70/01 ^{T}	98.82	1110

NCCP-1268	LC065295	*Microbacterium aerolatum* V-73 ^{T}	99.45	921
NCCP-1269	LC065296	*Microbacterium resistens* DMMZ 1710 ^{T}	97.97	1085
NCCP-1270	LC065297	*Microbacterium resistens* DMMZ 1710 ^{T}	99.67	924
NCCP-1271	LC065298	*Micromonospora chokoriensis* 2-19/6 ^{T}	99.65	867
NCCP-1273	LC065300	*Nocardioides daedukensis* MDN22 ^{T}	99.75	1205
NCCP-1274	LC065301	*Nocardioides luteus* KCTC 9575 ^{T}	99.88	841
NCCP-1275	LC065302	*Nocardioides jensenii* ^{T}	99.61	1059
NCCP-1276	LC065303	*Nocardioides luteus* KCTC 9575 ^{T}	99.03	1029
NCCP-1277	LC065304	*Nocardioides mesophilus* ^{T}	100	1206
NCCP-1279	LC065306	*Nonomuraea jabiensis* A4036 ^{T}	99.77	1327
NCCP-1280	LC065307	*Nonomuraea muscovyensis* FMN03 ^{T}	99.66	1179
NCCP-1281	LC065308	*Ochrobactrum-pseudogrignonense*CCUG 30717 ^{T}	98.31	717
NCCP-1283	LC065310	*Paenibacillus dendritiformis* CIP 105967 ^{T}	100	1471
NCCP-1284	LC065311	*Paenibacillus puldeungensis* CAU 9324 ^{T}	100	1409
NCCP-1285	LC065312	*Paenibacillus thailandensis* S3-4A ^{T}	96.8	1000
NCCP-1287	LC065314	*Phenylobacterium lituiforme* FaiI3 ^{T}	98.75	1281
NCCP-1288	LC065315	*Phenylobacterium lituiforme* FaiI3 ^{T}	98.62	940
NCCP-1289	LC065316	*Pseudoxanthomonas mexicana* AMX 26B ^{T}	98.11	795
NCCP-1294	LC065321	*'Roseomonas alkaliterrae'* YIM 78007 ^{T}	96.49	1000
NCCP-1295	LC065322	*Sphingomonas cynarae* SPC-1 ^{T}	98.69	839
NCCP-1296	LC065323	*Staphylococcus saprophyticus subsp. bovis* GTC 843 ^{T}	98.92	1117
NCCP-1298	LC065325	*Stenotrophomonas maltophilia* ATCC 13637	99.68	941
NCCP-1306	LC065333	*Streptomyces flavidovorans* NBRC13039 ^{T}	98.21	838
NCCP-1307	LC065334	*Streptomyces canarius* NBRC 13431 ^{T}	96.91	1036
NCCP-1308	LC065335	*Streptomyces capoamus* JCM 4734 ^{T}	97.71	1181
NCCP-1310	LC065337	*Streptomyces cinerochromogenes* NBRC 13822 ^{T}	98.62	867
NCCP-1313	LC065340	*Microvirga lotononidis* WSM3557 ^{T}	98.07	880
NCCP-1315	LC065342	*Xanthobacter tagetidis* DSM 11105 ^{T}	99.37	800
NCCP-1318	LC065345	*Xylanimicrobium pachnodae* VPCX2 ^{T}	97.94	831
NCCP-1331	LC065358	*Streptomyces brevispora* BK160 ^{T}	97.94	1541
NCCP-1340	LC065367	*Nocardioides iriomotensis* IR27-S3 ^{T}	96.81	1498
NCCP-1345	LC065372	*'Roseomonas alkaliterrae'* YIM 78007 ^{T}	97.76	760
NCCP-1346	LC065373	*Bacillus invictus* Bi.FFUP1 ^{T}	99.69	989
NCCP-1347	LC065374	*Streptomyces griseostramineus* NBRC 12781 ^{T}	97.89	1000
NCCP-1348	LC065375	*Arthrobacter oryzae* KV651 ^{T}	100	1465

Table 4 . List of Strains Isolated from Tato Field Hot Water Spring Soil and Identified by 16srRNA Gene Sequence Similarity.

Strain ID	Accession #	Texonomy	Similarity	Length
NCCP-1226	LC065253	*Bacillus anthracis* ATCC 14578 ^{T}	98.51	1052
NCCP-1227	LC065254	*Bacillus anthracis* ATCC 14578 ^{T}	99.66	910
NCCP-1228	LC065255	*Bacillus aryabhattai* B8W22 ^{T}	99.77	880
NCCP-1229	LC065256	*Bacillus cereus* ATCC 14579 ^{T}	99.89	929
NCCP-1230	LC065257	*Bacillus invictus* Bi.FFUP1 ^{T}	100	818
NCCP-1245	LC065272	*Brevibacillus choshinensis* DSM 8552 ^{T}	99.89	924
NCCP-1250	LC065277	*Cupriavidus gilardii* CIP 105966 ^{T}	97.91	671
NCCP-1259	LC065286	*Hydrogenophaga defluvii* BSB 9.5 ^{T}	97.89	900
NCCP-1292	LC065319	*Pseudoxanthomonas mexicana* AMX 26B ^{T}	99.9	967
NCCP-1305	LC065332	*Streptomyces aureofaciens* NBRC 12843	98.53	815
NCCP-1309	LC065336	*Streptomyces kasugaensis* M338-M1 ^{T}	100	1269
NCCP-1265	LC065292	*Leucobacter tardus* K 70/01 ^{T}	99.77	863
NCCP-1272	LC065299	*Micromonospora chokoriensis* 2-19/6 ^{T}	99.39	816
NCCP-1282	LC065309	*Ochrobactrum pseudogrignonense* CCUG 30717 ^{T}	100	958
NCCP-1291	LC065318	*Pseudoxanthomonas mexicana* AMX 26B ^{T}	99.47	1127
NCCP-1297	LC065324	*Staphylococcus saprophyticus subsp. saprophyticus* ATCC 15305 ^{T}	99.38	1445
NCCP-1311	LC065338	*Streptomyces kasugaensis* M338-M1 ^{T}	100	1073
NCCP-1312	LC065339	*Streptomyces drozdowiczii* NBRC 101007 ^{T}	97.85	1400
NCCP-1319	LC065346	*Pseudoxanthomonas mexicana* AMX 26B ^{T}	100	980
NCCP-1320	LC065347	*Microvirga lotononidis* WSM3557 ^{T}	97.97	1280
NCCP-1322	LC065349	*Paenibacillus puldeungensis* CAU 9324	96.14	956
NCCP-1323	LC065350	*Microvirga zambiensis* WSM3693 ^{T}	98.43	830
NCCP-1324	LC065351	*Paenibacillus dendritiformis* CIP 105967	100	919
NCCP-1325	LC065352	*Leucobacter tardus* K 70/01 ^{T}	97.46	1067
NCCP-1326	LC065353	*Bacillus sporothermodurans* M215 ^{T}	96.54	1019
NCCP-1327	LC065354	*Streptomyces celluloflavus* NBRC 13780	98.81	1096
NCCP-1328	LC065355	*Microvirga zambiensis* WSM3693 ^{T}	97.98	991
NCCP-1330	LC065357	*Massilia flava* Y9 ^{T}	98.17	929
NCCP-1332	LC065359	*Bacillus taquilensis* KCTC13622 ^{T}	100	1059
NCCP-1333	LC065360	*Bacillus aerophilus* 28K ^{T}	99.09	1106
NCCP-1334	LC065361	*Streptomyces caeruleatus* GIMN4 ^{T}	96.51	1197
NCCP-1335	LC065362	*Nonomuraea jabiensis* A4036 ^{T}	98.7	1545
NCCP-1336	LC065363	*Paenibacillus puldeungensis* CAU 9324	95.73	1535

NCCP-1337	LC065364	*Nonomuraea jabiensis* A4036 ^{T}	99.28	971
NCCP-1338	LC065365	*Hydrogenophaga atypica* BSB 41.8 ^{T}	99.51	1031
NCCP-1339	LC065366	*Nonomuraea jabiensis* A4036 ^{T}	98.55	901
NCCP-1341	LC065368	*Massilia flava* Y9 ^{T}	98.35	1091
NCCP-1342	LC065369	*Nocardia alba* YIM 30243 ^{T}	99.89	903
NCCP-1343	LC065370	*Bacillus taquilensis* KCTC13622 ^{T}	98.46	918
NCCP-1344	LC065371	*Bacillus taquilensis* KCTC13622 ^{T}	99.93	1408

Table 5 List of Strains Isolated from Murtazaabad Hot Water Spring Soil and Identified by 16SrRNA Gene Sequence Similarity.

Strain ID	Accession #	Texonomy	Similarity	Length
NCCP-1267	LC065294	*Leucobacter tardus* K 70/01 ^{T}	98.28	1104
NCCP-1106	LC065133	*Arthrobacter defluvii* 4C1-a ^{T}	99.56	907
NCCP-1110	LC065137	*Arthrobacter humicola* ^{T}	100	1463
NCCP-1111	LC065138	*Arthrobacter pascens* DSM 20545 ^{T}	97.88	947
NCCP-1114	LC065141	*Bacillus aerophilus* 28K ^{T}	98.29	1465
NCCP-1116	LC065143	*Bacillus invictus* Bi.FFUP1 ^{T}	98.44	1112
NCCP-1118	LC065145	*Bacillus methylotrophicus* CBMB205 ^{T}	96.39	1000
NCCP-1119	LC065146	*Bacillus methylotrophicus* CBMB205 ^{T}	95.38	1142
NCCP-1125	LC065152	*Bacillus sonorensis* NBRC 101234 ^{T}	99.3	1449
NCCP-1128	LC065155	*Bacillus sporothermodurans* M215 ^{T}	99.19	1527
NCCP-1130	LC065157	*Bacillus tequilensis* KCTC 13622 ^{T}	98.35	1032
NCCP-1131	LC065158	*Bacillus tequilensis* KCTC 13622 ^{T}	97.84	1118
NCCP-1132	LC065159	*Bacillus tequilensis* KCTC 13622 ^{T}	98.25	1036
NCCP-1133	LC065160	*Bacillus tequilensis* KCTC 13622 ^{T}	99.88	860
NCCP-1134	LC065161	*Bacillus tequilensis* KCTC 13622 ^{T}	99.87	792
NCCP-1137	LC065164	*Bacillus tequilensis* KCTC 13622 ^{T}	98.94	850
NCCP-1141	LC065168	*Cupriavidus gilardii* CIP 105966 ^{T}	97.93	930
NCCP-1142	LC065169	*Cupriavidus gilardii* CIP 105966 ^{T}	98.78	1557
NCCP-1144	LC065171	*Cupriavidus gilardii* CIP 105966 ^{T}	100	1139
NCCP-1152	LC065179	*Microvirga aerilata* 5420S-16 ^{T}	98.76	970
NCCP-1156	LC065183	*Microvirga flocculans* ATCC BAA-817	96.88	1154
NCCP-1162	LC065189	*Microvirga lupini* Lut6 ^{T}	88.5	880
NCCP-1167	LC065194	*Microvirga vignae* BR3299 ^{T}	97.03	1044
NCCP-1168	LC065195	*Microvirga vignae* BR3299 ^{T}	98.06	926
NCCP-1173	LC065200	*Nocardia asteroides* NBRC 15531 ^{T}	100	1050
NCCP-1176	LC065203	*'Phenylobacterium zucineum'* HLK1 ^{T}	99.02	1121
NCCP-1179	LC065206	*Pseudomonas geniculata* ATCC 19374 ^{T}	99.57	927

NCCP-1180	LC065207	*Pseudomonas geniculata* ATCC 19374 ^{T}	99.5	811
NCCP-1183	LC065210	*Pseudoxanthomonas mexicana* AMX 26B ^{T}	99.21	1075
NCCP-1185	LC065212	*Rhizobium selenitireducens* ATCC BAA-1503 ^{T}	97.25	1026
NCCP-1188	LC065215	*Rhizobium selenitireducens* ATCC BAA-1503 ^{T}	98.89	1491
NCCP-1189	LC065216	*Rhizobium selenitireducens* ATCC BAA-1503 ^{T}	98.48	857
NCCP-1194	LC065221	*Streptomyces griseostramineus* NBRC 12781 ^{T}	99.07	966
NCCP-1195	LC065222	*Streptomyces griseus subsp. griseus* KCTC 9080 ^{T}	99.04	732
NCCP-1196	LC065223	*Streptomyces hyderabadensis* OU-40 ^{T}	98.75	1364
NCCP-1197	LC065224	*Streptomyces hyderabadensis* OU-40 ^{T}	98.48	1415
NCCP-1198	LC065225	*Streptomyces hyderabadensis* OU-40 ^{T}	97.83	1141
NCCP-1200	LC065227	*Streptomyces hyderabadensis* OU-40 ^{T}	94.8	1210
NCCP-1205	LC065232	*Streptomyces netropsis* NBRC 3723 ^{T}	99.89	904
NCCP-1209	LC065236	*Streptomyces tauricus* JCM 4837 ^{T}	100	1341
NCCP-1210	LC065237	*Streptomyces thermoviolaceus subsp. thermoviolaceus* DSM 40443 ^{T}	100	880
NCCP-1211	LC065238	*Streptomyces violascens* ISP 5183 ^{T}	97.08	1000
NCCP-1278	LC065305	*Nonomuraea jabiensis* A4036 ^{T}	99.73	1483
NCCP-1290	LC065317	*Pseudoxanthomonas mexicana* AMX 26B ^{T}	98.08	1156
NCCP-1299	LC065326	*Stenotrophomonas maltophilia* ATCC 13637 ^{T}	99.35	770
NCCP-1300	LC065327	*Streptomyces albidoflavus* DSM 40455 ^{T}	99.89	874
NCCP-1301	LC065328	*Streptomyces anulatus* NRRL B-2000 ^{T}	99.66	876
NCCP-1302	LC065329	*Streptomyces atratus* NRRL B-16927 ^{T}	99.38	815
NCCP-1303	LC065330	*Streptomyces atrovirens* NRRL B-16357	99.66	895
NCCP-1304	LC065331	*Streptomyces aurantiogriseus* NBRC 12842 ^{T}	98.25	862
NCCP-1314	LC065341	*Xanthobacter tagetidis* DSM 11105 ^{T}	98.73	1105

Murtazaabad hot water spring possessed diverse *Bacteria* and *Actinobacteria*. *Streptomyces* genus was most dominant at this site. Species belonging to 14 genera were isolated from this site. Tato Field hot water spring possessed diverse *Bacteria* and *Actinobacteria*. *Streptomyces* genus was most dominant at this site. Species belonging to 14 genera were isolated from this site. Most diverse site among all hot water spring sites was Tatta pani with 31 different genera. Among Bacteria most dominant were *Bacillus* sp. and *Hydrogenophaga* sp. While among *Actinobacteria*

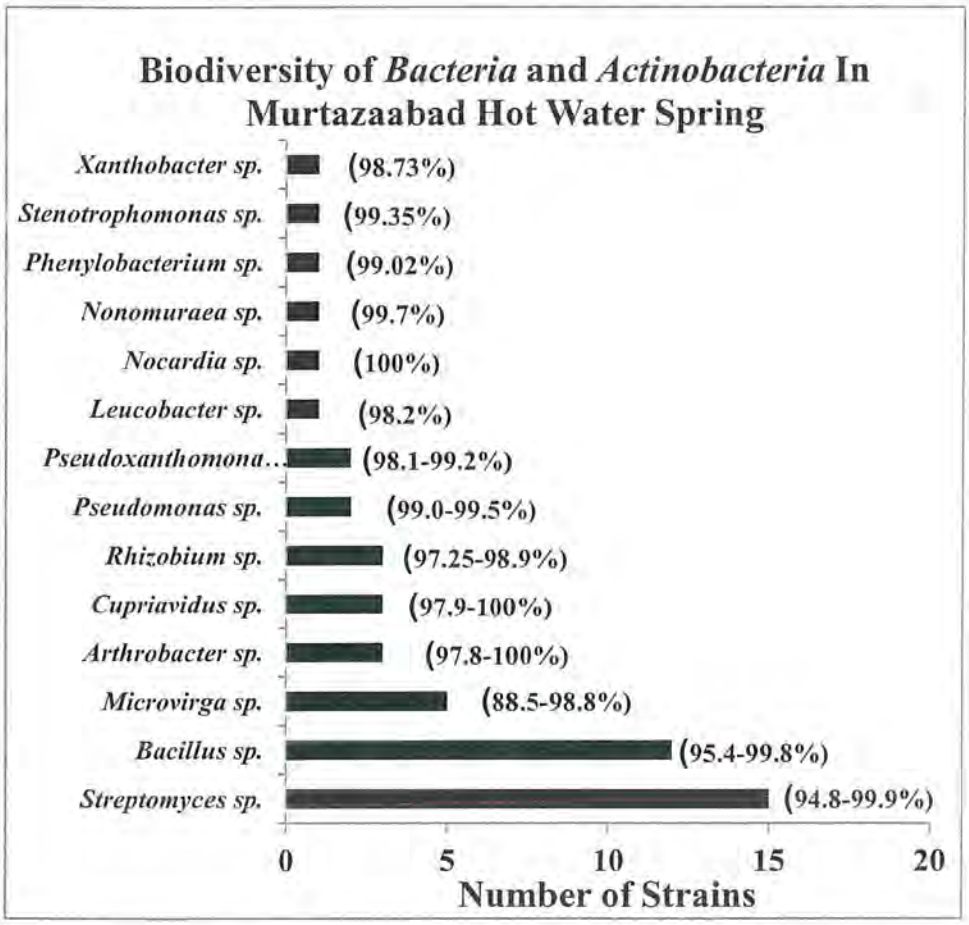


Figure 5 Diversity of Bacteria and Actinobacteria In Murtazaabad Hot Water Spring

Various species belonging to *Arthrobacter*, *Nocardioides*, *Streptomyces*, *Nonomuraea*, *Leucobacter* and *Microbacterium* genera were present. Cholistan desert soil possess *Microvirga* genus in most dominant number that is followed by *Bacillus* genus and *Streptomyces* genus. Partial 16SrRNA gene sequence similarity of isolated strains showed that the selected sites were abundant in candidate novel strains of *Bacteria* and *Actinobacteria.* Various candidate novel strains whose partial 16SrRNA gene sequence similarity was ranging from 93% to 98.2% were selected and their full length 16SrRNA similarity was confirmed by cloning method. Late on four candidate novel strains, two from hot water springs and two from desert sites were studied by polyphasic taxonomic approach.

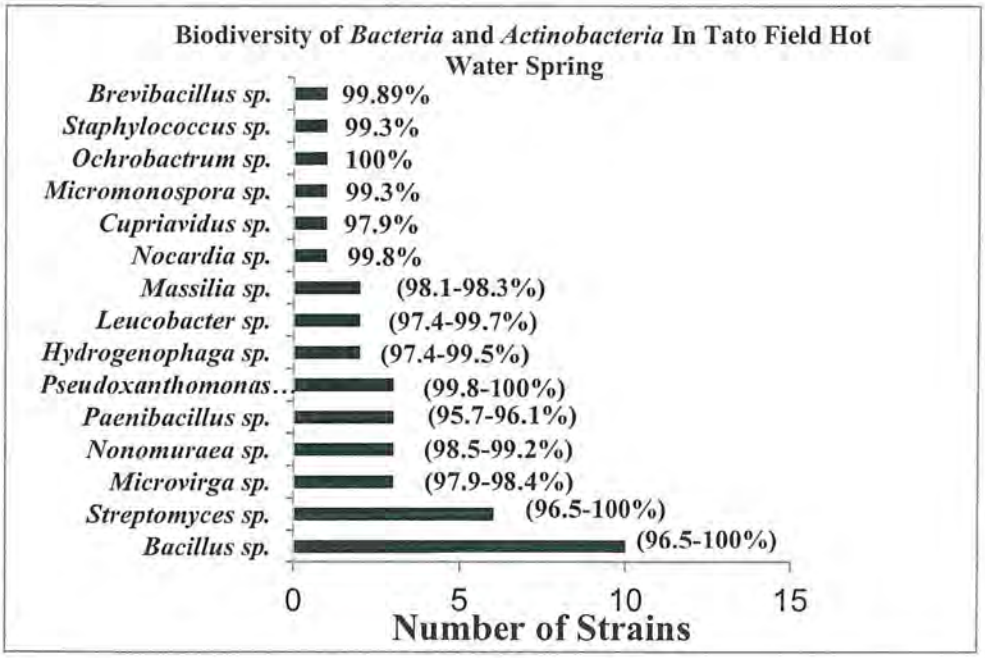


Figure 6 Diversity of Bacteria and Actinobacteria in Tato Fiels Hot Water Spring

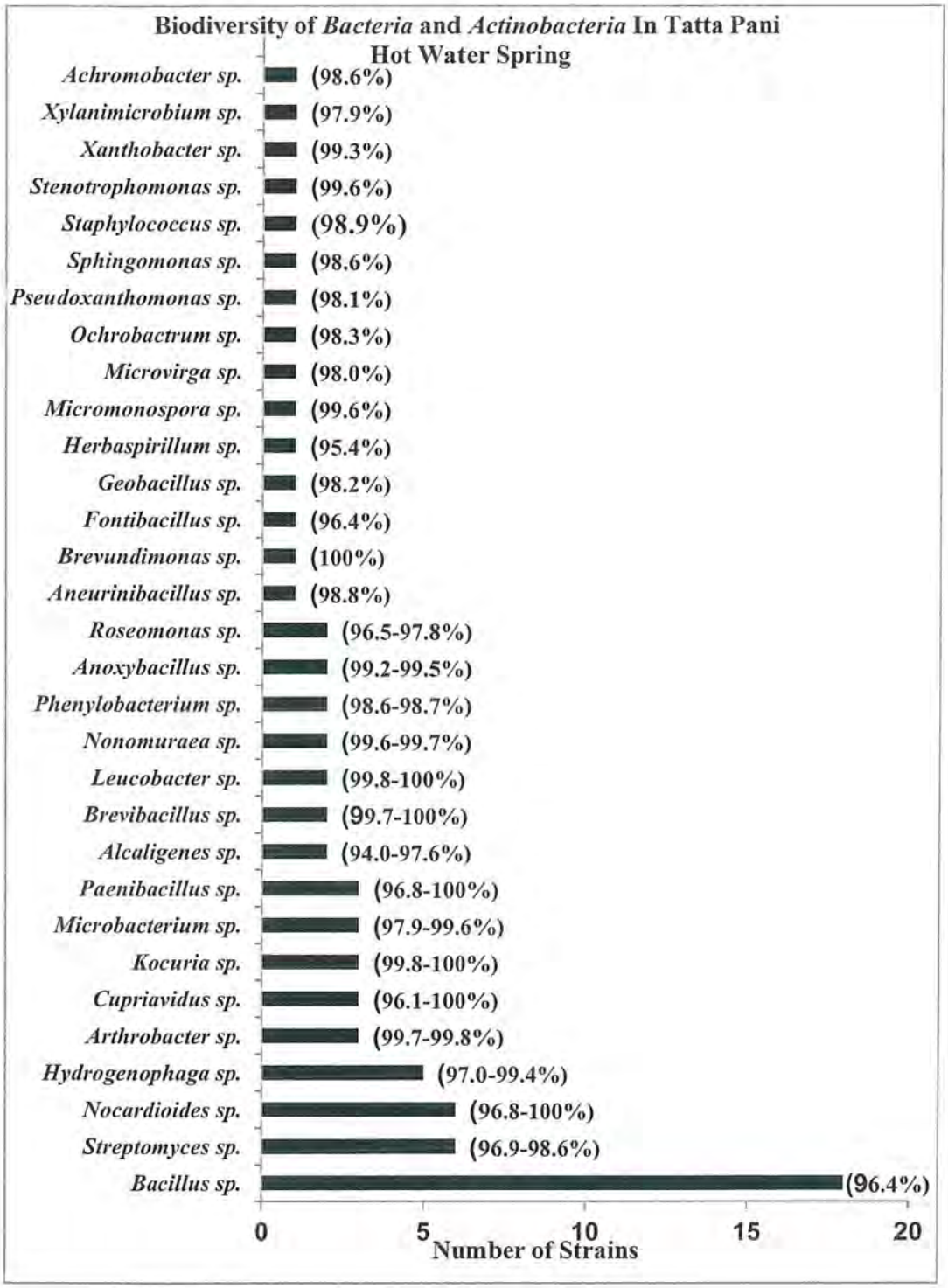


Figure 7 Diversity of Bacteria and Actinobacteria In Tatta Pani Hot Spring

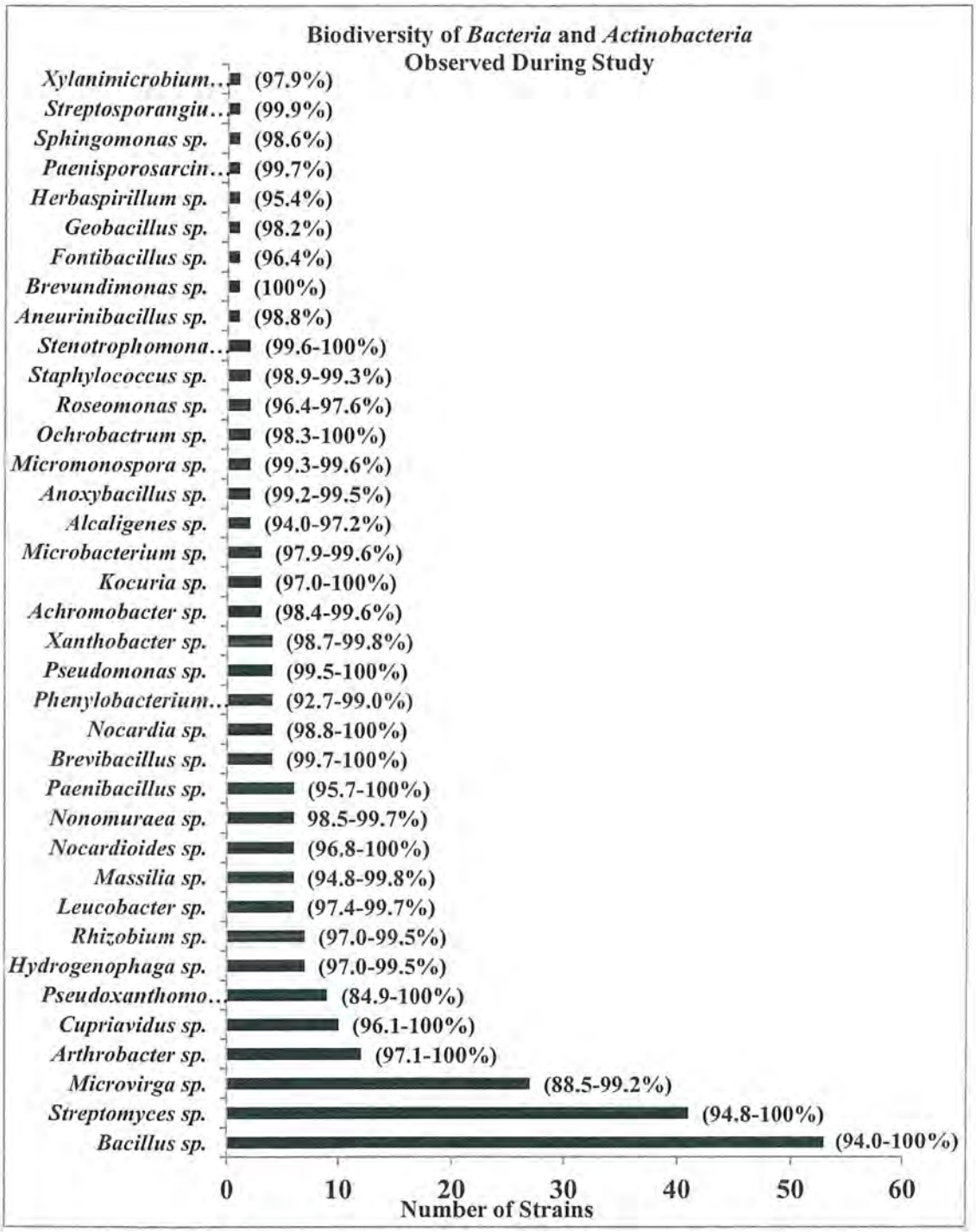


Figure 8 Diversity of Bacteria and Actinobacteria in Hot water springs of Pakistan

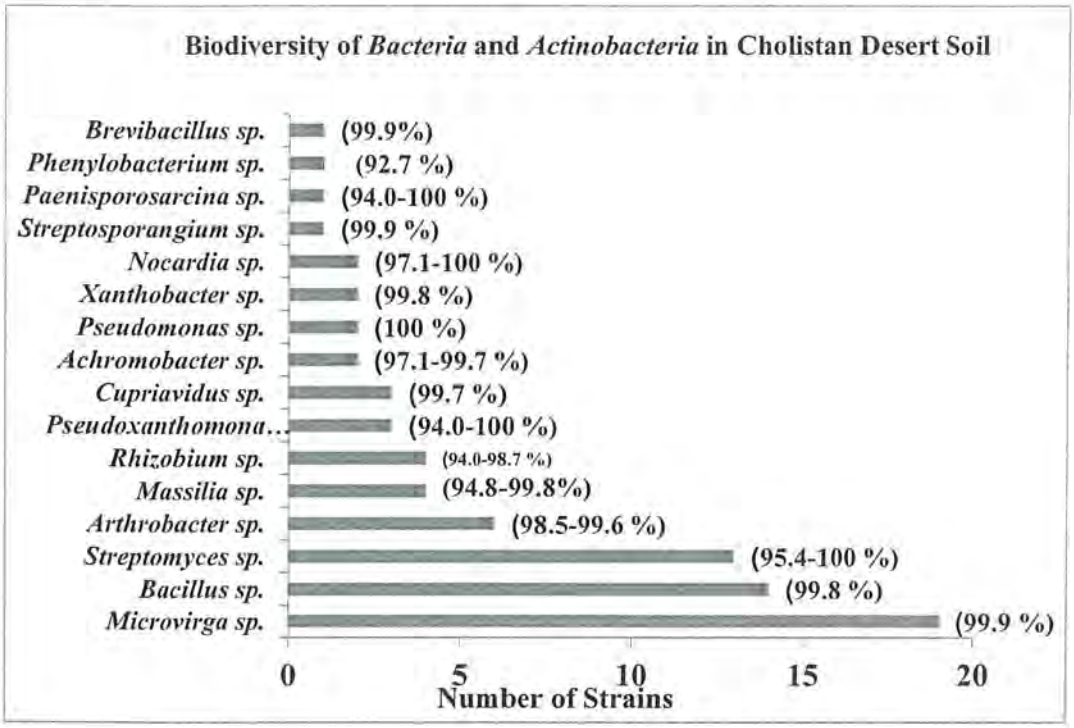


Figure 9 Diversity of Bacteria and Actinobacteria in Cholistan Desert Soil

3.4. Protocols of Major Techniques for Polyphasic Texonomic Study

Following protocols were used for performing major experiments:

DNA Extraction was done by following procedure : Added 500ulTE buffer in cells and 20ul Lysis buffer, left overnight, added 50ul SDS buffer and 5ul phosphokinase, boiled at 55^{0}C for 1 hour, added 550ul of Tris phenol: Chloroform: isoamylalchohol in the ratio of 25:24:1 shaked, centrifuged at 12,000rpm for 10 minutes, took supernatant, repeated process, again took supernatant, added 800ul of ethanol and 80ul of Na-acetate, kept at room temperature for 10 minutes, centrifuged at 12,000rpm for 10 minutes, discarded supernatant, washed pellet with 70% ethanol, discarded ethanol, kept at 50°C for drying, dissolved in TE buffer 50ul and stored at 4°C, after dissolution confirmed by running on 1% agarose gel. 3ul loading buffer (Bromophenol blue) was used. PCR amplification of extracted DNA was done by using universal primers for 16S rRNA gene. Conditions for PCR were adusted at 95^{0}C (4 minutes), 95°C (45sec.), 55^{0}C (45sec.), 72^{0}C(1:30min.), 72^{0}C(10 min.) and 32 cycles were run. Again PCR product ws confirmed by running in 1% agarose gel according to intructions by W. J. Li et al. (2007). The 16S rRNA gene sequence was purified by using a PCR purification kit (Sangon Biotech, China) and cloned by pEASY-T1 cloning kit (Transgen Biotechnology) to obtain a complete sequence. Took 100ul PCR product, Run gel of

all product in wide wells of agarose gel, check the bands under UV light and fine bands were cut and store in Eppendorf tubes, added 500ul of binding solution in the cut gel sections, boiled at 550C for 10 minutes until all jel melted, transferred melted jel mixture into spin filter tube, centrifuged for 1 min at 10, 000rpm, discarded effluent, used 600ul washing solution to wash DNA, centrifuged for 1 minute at 10, 000rpm, discarded effluent, washed again with 600ul washing buffer, centrifuged at 10, 000 rpm for 1 minute, discarded effluent, centrifuged again for 4-5 minutes to remove traces of all washing buffer from binded DNA, transferred the spin filter into a new Eppendorf, added 20-30ul of elution buffer, left at room temperature for 1-2 minutes, centrifuged for 1 minute at 12,000rpm, repeated process, discarded the spin filter and saved DNA at -20^{0}C. In the next phase added 3ul of extracedDNA in PCR tubeand 0.3ul of Peasy T1 simple cloning vector and left it at 25°C in each new Eppendorf along with DNA and vector mixture, all process will be done in ice, left in ice box at room temperature for 20-30 minutes, boiled at 42°C for 1:30 minutes, left in ice for 2 minutes, took eppendorfs filled with 300-500ul of LB broth and added 200ul of mixture in it, shaked for 2 hours at 37°C, spreaded on plates of LB agar and incubated at 37oC until growth appeared, poured LB broth in eppendorfs with 100ul/100ml kanamycin, took PCR grade white tips and picked the colonies with tip and added in the LB broth+ kanamycin eppendorfs, shaked for 24 hours at 37°C, and then subjected to sequencing.

DNA-DNA hybridization was performed by following steps:

Following steps were followed: DNA was extracted and washed with 0.25 ml each of RNase A solution (2mg/ml+0.15M NaCl, pH5.0) and RNase T1 solution (800 units/1ml of 0.1Mtris HCl), left at 30°C for 30 minutes and at 80°C for 10 minutes to deactivate DNase, shifted in ice and added 2ml phenol, shaked swiftly, precipitated DNA with propanol and spooled well, washed DNA, air dried washed DNA with 0.1x SSC solution and checked optical density at 260nm. Added 0.1x SSC solution, incubated at 100°Cfor 10 minutes, shifted in ice for 5 minutes, added 1x PBS-MgCl2 buffer to dilute upto 0.2OD and poured 100ul of DNA into 96 well microtitre plate, incubated at 30°C for 4 hours, , added 300ul of 1X PBS into DNA, after 10 minutes discarded PBS buffer, incubated at 45°C for 15 minutes added 200ul of DNA hybridization solution(40ml 10xSSc buffer, 20ml 50x Denhardt's solution, 2ml modified salmon(10m/ml), 38 ml dd H2O, 100 ml formamide), after 4 hours of incubation at 37°C added 1x PBS (300ul) per well and kept at 45°C, added 200up pf

prehybridizarion mixture tomeach well, kept for 4 hours at strain specific temperature, after 2 hours added 10OD DNA 10ul photobiotin, kept samples in icebox in front of UV light at 20cm distance form light for 90 minutes, all assembly was in a dark room.. Aded 185ul of Tris HCl(pH9,0.1M), 200ul of Tris Hcl-secondary butyl alcohol(0.1M) and shaked with hands. Centrifuged and transferred the lower layer 200ul in a new eppendorf repeated the step until it becomes color less, again centrifuged, and mixed properly with 30-50 times pipetting, added 950 ul of prehybridization solution and 50ul of DNA(10OD), mixed well. Discarded liquid with pressure from microtitre plates, transferred 10OD DNA to each well according to arrangement. Covered plates with parafilm and aluminium foil and incubated at hybridization temperature of 40 °C and the hybridizations were performed with eight replications in all the eight wells in a row. After 12 hours discarded the liquid and added 1xsSC solution, 300ul in each well and kept at 40°C for 15 minutes. Repeated process 5 times and liquid was removed from well everytime with pressure, added alkaline phosphatase 100ul in each well and kept at 37°C for 1 hour, added 100ul of 4-MUP solution and incubated at 37°C for 1 hour. And results were checked.

For detection of aminoacids following steps were followed: took strains in milk tubes of same size for DNA extraction, added 400ul of 6N HCl in each tube and sealed by Bunsen burner, kept the milk tubes overnight in sand at 121°C, broke the milk tubes and took the strains in a new Eppendorf, centrifuged at 12,000 rpm for 10 minutes, took dried TLC plates and loaded 15ul strain by PCR tip drop by drop and dried it thoroughly prepared chromatogram with methanol: pyridine: glacial acetic acid:H2O in the ratio of 10ml:1ml:0.25ml:5ml. placed TLC plate in chromatogram for 4 hours, dried at room temperature overnight, left TLC plate in in fresh chromatogram with same solution for 4 hours, dried plates with cold dryer sprayed with ninhydrin(0.4%) and heated at 100-110°C for 2-3 minutes. Results were observed.

CHAPTER 4

Description of Novel Species and Genus Proposed During Study

Following novel genus and species will be decribed in chapter 4.

4.1 Zafaria solitudinis gen. nov. sp. nov.
4.2. Microvirga pakistanensis sp.nov.
4.3. Streptomyces caldifontis sp. nov.
4.4. Nocardioides pakistanensis sp. nov.

4.1 Proposal of *Zafaria solitudinis* gen. nov. sp. nov. Moderately Thermophilic, Isolated from Cholistan desert, Pakistan

4.1.1. Abstract

A Gram-staining positive, non-spore forming, non-pigmented and non-motile bacterium, designated strain as NCCP-1664^T, was isolated from Cholistan desert, Pakistan. Cells of strain NCCP-1664^T were observed to be strictly aerobic with rod to coccus growth cycle, which were catalase positive and oxidase negative. The growth was observed at pH 6.0-9.0 (optimum pH 7-8) at 28-45 °C (optimum 37 °C) and could tolerate 0-16% NaCl (optimum 2%). Phylogenetic analyses based on 16S rRNA gene sequence revealed that, strain NCCP-1664^T belongs to family *Micrococcaceae* and was closely related to the members of genus *Arthtobacter* having highest sequence similarity with *Arthrobacter ginkgonis* (98.9 %), *A. halodurans* (97.7 %) and *A. oryzae* (97.3 %) and less than 97 % with other closely related taxa. DNA–DNA relatedness values of strain NCCP-1664^T with above mentioned type strains was found to be less than 54 %. DNA G+C content of strain NCCP-1664^T was 67.5 mol%. Recent reclassification of *Arthrobacter* species in the genera *Paenarthrobacter*, *Pseudarthrobacter*, *Glutamicibacter*, *Paeniglutamicibacter* and *Pseudoglutamicibacter* supported that the chemotaxonomic data of strain NCCP-1664^T (peptidoglycan type as A3α L-Lys – L-Ala; menaquinones as MK-9(H_2) (82.4 %), MK-8(H2) (17.5 %), major fatty acids as anteiso -$C_{15:0}$ (51.2 %), $C_{18:1}\omega9$ *c* (16.9 %), $C_{18:0}$ (12.9 %) and anteiso-$C_{17:0}$ (9.6 %) and polar lipids profile comprising of phosphatidylglycerol, diphosphatidylglycerol, glycolipids and unknown phospholipids) along with physiological, biochemical characteristics and the phylogenetic analyses allowed to describe it in to a novel genus, for which the name *Zafaria solitudinis* gen. nov. sp. nov. is proposed with the type strain NCCP-1664^T (=DSM 29936^T = KCTC 39549^T).

Keywords. Cholistan desert, peptidoglycan type A3α , polyphasic taxonomic approach

4.1.2. Introduction

The genus *Arthrobacter* was first established by Conn and Dimmick (1947) within the family *Micrococcaceae,* by describing three species, including type species *Arthrobacter globiforme,* for which later on (SKERMAN, McGowan, & Sneath,

1980)proposed name *Arthrobacter globiformis.* Classification of genus *Arthrobacter* recently has undergone tremendous change when (Busse, 2016) reclassified selected species of the genus *Arthrobacter* into novel genera, namely *Glutamicibacter* gen. nov. (*Glutamicibacter protophormiae* group Basonym: *Arthrobacter protophormiae* (Erko Stackebrandt, Fowler, Fiedler, & Seiler, 1983), *Paeniglutamicibacter* gen. nov. (*Paeniglutamicibacter sulfureus* Basonym: *Arthrobacter sulfureus* (Erko Stackebrandt et al., 1983), *Pseudoglutamicibacter* gen. nov. (*Pseudoglutamicibacter cumminsii* group Basonym: *Arthrobacter cumminsii* (Funke et al., 1996)), *Paenarthrobacter* gen. nov. (*Paenarthrobacter aurescens* group Basonym: *Arthrobacter aurescens* (H. C. Phillips, 1951) and *Pseudarthrobacter* gen. nov. (*Pseudarthrobacter polychromogenes* group Basonym: *Arthrobacter polychromogenes* (Schippers-Lammertse, Muijsers, & Klatser-Oedekerk, 1963)) and compared the characteristics with *Arthrobacter sensu stricto* (Includes*A. globiformis, A. pascens, A. oryzae* and *A. humicola*).

Members of this genus are aerobic, catalase-positive, Gram-staining positive bacteria that show a rod–coccus growth cycle, having lysine in the peptidoglycan as a diagnostic amino acid and a high DNA G+C content (Y. G. Chen et al., 2009). Species of the genus *Arthrobacter,* have been isolated from varying sources, which includes human specimens (Mages, Frodl, Bernard, & Funke, 2008; Yassin, Spröer, Siering, Hupfer, & Schumann, 2011),filtration substrates (Ding, Hirose, & Yokota, 2009), poultry litter (Tvrzova et al., 2005), the surfaces of cheese (Irlinger, Bimet, Delettre, Lefevre, & Grimont, 2005), soil and sediment (Kageyama, Morisaki, Omura, & Takahashi, 2008; Westerberg, Elvang, Stackebrandt, & Jansson, 2000), sea water (Y. G. Chen et al., 2009), rice rhizosphere (Krishnan et al., 2016), alpine soil (D.-C. Zhang et al., 2010) as well as sewage and wastewater reservoir sediments (K. K. Kim et al., 2008; Roh et al., 2008). Some isolates are able to degrade complex organic compounds like 4-chlorophenol (4-CP) (Westerberg et al., 2000) and some produce plant growth promoting hormones e.g; indole acetic acid(IAA) and siderophores (Krishnan et al., 2016).Over the last decade, several novel species belonging to the genus *Arthrobacter* have been isolated from cold environments such as six cold tolerant *Arthrobacter* spp. including *A. psychrolactophilus* from Pennsylvania Farmlands of Antarctica(Loveland-Curtze, Sheridan, Gutshall, & Brenchley, 1999),*A. cryotolerans* and *A. livingstonensis*from Antarctic soil(Ganzert, Bajerski, Mangelsdorf, Lipski, & Wagner, 2011),*A. ardleyensis*from Antarctic lake sediments (M. Chen, Xiao, Wang, Zeng, &

Wang, 2005), A. gangotriensis and A. kerguelensis from penguin rookery soil sample Antarctica(Gupta, Reddy, Delille, & Shivaji, 2004) *A. psychrophenolicus* from an alpine ice cave(Margesin, Schumann, Sproer, & Gounot, 2004).

4.1.3. Materials and Methods

Isolation, morphology and phenotypic Characterization

During the investigation of the microbial diversity of Cholistan desert, Bahawalpur Pakistan, several strains were isolated. In this study, a novel halotolerant bacterium, designated NCCP-1664^T, was characterized using polyphasic taxonomic approach. Based on phenotypic and phylogenetic analyses together with biochemical characterization, NCCP-1664^T strain can be considered to represent a novel genus *Zafaria* gen.,nov., for which the name *Zafaria solitudinis* gen. nov. sp. nov. has been proposed.

Soil samples were collected in sterilized NUNC tubes from the deep sediments of Cholistan desert, Pakistan (29°23′43″N 71°41′1″E ASL 291.9948ft) and kept at 4 °C before usage. The samples were serially diluted to isolate bacterial strains using International *Streptomyces* Project (ISP) 2 medium (Shirling & Gottlieb, 1966). Among various colonies, a strain, designated as NCCP-1664^T, was recovered after 48 hours on ISP 2 agar medium plate incubated at 37 °C. The strain was streaked repeatedly on ISP 2 medium (Shirling & Gottlieb, 1966) untill pure colonies were obtained. Purified strain was stored in 25%v/v glycerol at -80 °C for further studies.The type strains of closely related species, *Arthrobacter halodurans* JSM 078085^T, *A. cupressi* D48^T, *A. oryzae* KV-651^T, *A. humicola* KV-653^T, *A. cryotolerans* LI3^T, were used as reference strains in these studies.

To optimize the growth on various media, strain NCCP-1664^T was grown at pH7.0 on ISP 2g/L(Malt extract 10.0g, yeast extract 4.0g, glucose 4.0g and agar 15g), R2A g/L (Glucose-0.6g, soluble starch-0.6g, yeast extract-0.6 g, caseinoacids-0.6 g, sodium pyruvate -0.3 g, K_2HPO_4-0.3g, $MgSO_4 \cdot 7H_2O$ -0.05 and agar- 15g), ISP 3(Oat meal 20g, trace salt solution (ml) 1.00, ferric sulphate heptahydrate 0.001g, manganese chloride tetrahydrate 0.001g, zinc sulphate heptahydrate 0.001g and agar 15g), TSA (tryptone 0.5g, glucose 1.0 g, yeast extract 2.0g, CaCO3 1g, trace salt 1ml and agar 15g) and ISP 7(L-Asparagine 1g, L-tyrosine 0.5g, dipotassium phosphate 0.5g, magnesium sulphate. 7H2O 0.5g, sodium chloride 0.5g, trace salt solution (ml) and agar 15g). The colony morphology of strain NCCP-1664^T was observed on ISP 2 media

at 37 °C after 3 days. The cells were observed using phase-contrast microscopy (BH-2; Olympus) and further detailed morphology was examined under scanning electron microscopy (QUANTA 200; FEI). Gram staining was carried out using the standard Gram reaction. Growth at various temperatures (4, 10, 15, 20, 28, 30, 33, 37, 40, 45, 50, 55 and 60 °C) was observed on ISP2 medium for 1 week. The pH range for growth was tested at pH 4, 5, 6, 7, 8, 9, 10, and 11 using the buffer system described by (Ping Xu et al., 2005) at 37 °C for 4 days by culturing the strain in trypticase soy broth (TSB; Becton Dickinson). Tolerance to NaCl (0, to 20 %, w/v, with 1 % increment) was investigated on ISP 2 medium by incubating at 37 °C for 10 days. Catalase and oxidase activities were determined as described previously by (Kovacs, 1956). Growth under anaerobic conditions was determined on nutrient agar supplemented with or without 0.1% nitrate by using the GasPak Anaerobic Systems (BBL) according to the manufacturer's instructions.

The biochemical, enzymatic activities and utilization of sole carbon and nitrogen source were determined according to the manufacturer's instruction by using API ZYM, API 20E, API 50CH, API 20NE strip (bioMérieux, France). Biolog GN III microplates™ were also used for characterization of single-carbon source assimilation and other biochemical tests, by incubating the plates at 37°C according to the manufacturer's instructions.

16S rRNA gene amplification, sequencing and phylogenetic analysis

The preliminary identification of strain NCCP-1664^T was done based on 16S rRNA gene sequence. Extraction of genomic DNA and PCR amplification of the 16S rRNA gene of the strain NCCP-1664^T was performed using protocol described previously by (W. J. Li et al., 2007). The sequence obtained was compared with available 16S rRNA gene sequences of cultured species from the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/)(O. S. Kim et al., 2012a). Phylogenetic analysis was performed using the software package MEGA 6 software package (Tamura et al., 2013) after multiple alignment of the sequences using CLUSTAL_X program (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). Neighbour-joining (Saitou & Nei, 1987) tree was constructed using Kimura's two parameter model as a distance matrices (M. Kimura, 1980). To determine the support of each clade, bootstrap analysis was performed with 1000 replications (Joseph Felsenstein, 1985). The validity of the

neighbour-joining tree was evaluated with maximum-likelihood (ML) and maximum parsimony (MP) algorithms.

DNA–DNA hybridizations and DNA base composition

DNA-DNA relatedness among NCCP-1664^T and reference strains *Arthrobacter oryzae* KV-651^T and *A. halodurans* JSM 078085^T were determined. Total genomic DNA was prepared using protocols of Marmur (1963) andPitcher, Saunders, and Owen (1989) as described previously (Goris et al., 1998). DNA–DNA hybridizations were performed with biotin-labelled probes in 96-micro-wells plate (NUNC) according to Ezaki et al. (1989); Christensen, Angen, Mutters, Olsen, and Bisgaard (2000a) with modifications by Goris et al. (1998) and fluorescence measurements were performed using an HTS7000 Bio Assay Reader (Perkin Elmer). The hybridization temperature was 40 °C and the hybridizations were performed with eight replications. The G+C content of the genomic DNA was determined by using reversed phase HPLC using DNA of *Escherichia coli* DH5α as the reference (M. Mesbah et al., 1989).

Chemotaxonomy characterization

For chemotaxonomic analysis of strains NCCP-1664^T and its reference strains, several characteristics were investigated using standard methods. Peptidoglycan analysis was done with the help of commercial facility of DSMZ. Polar lipids were extracted and separated by two-dimensional thin layer chromatography using standard procedures by Minnikin et al. (1979); (M. Collins & Jones, 1980). Menaquinones were extracted by following protocol described by (M. D. Collins et al., 1977) and analyzed using HPLC(Kroppenstedt, 1982). Fatty acid methyl esters were analysed by using the Microbial Identification System (Sherlock Version 6.1; MIDI database: TSBA6; (Sasser, 1990). Biomass for fatty acid analysis was obtained from cells grown on tryptic soy agar (Difco) at 37°C for 2 days.

4.1.4. Results and discussion

Isolation, morphology and phenotypic Characterization

Cells of strain NCCP-1664^T were observed to be Gram-staining positive, strictly aerobic, non -motile and non- spore forming with rod to coccus life cycle (Figure 10).

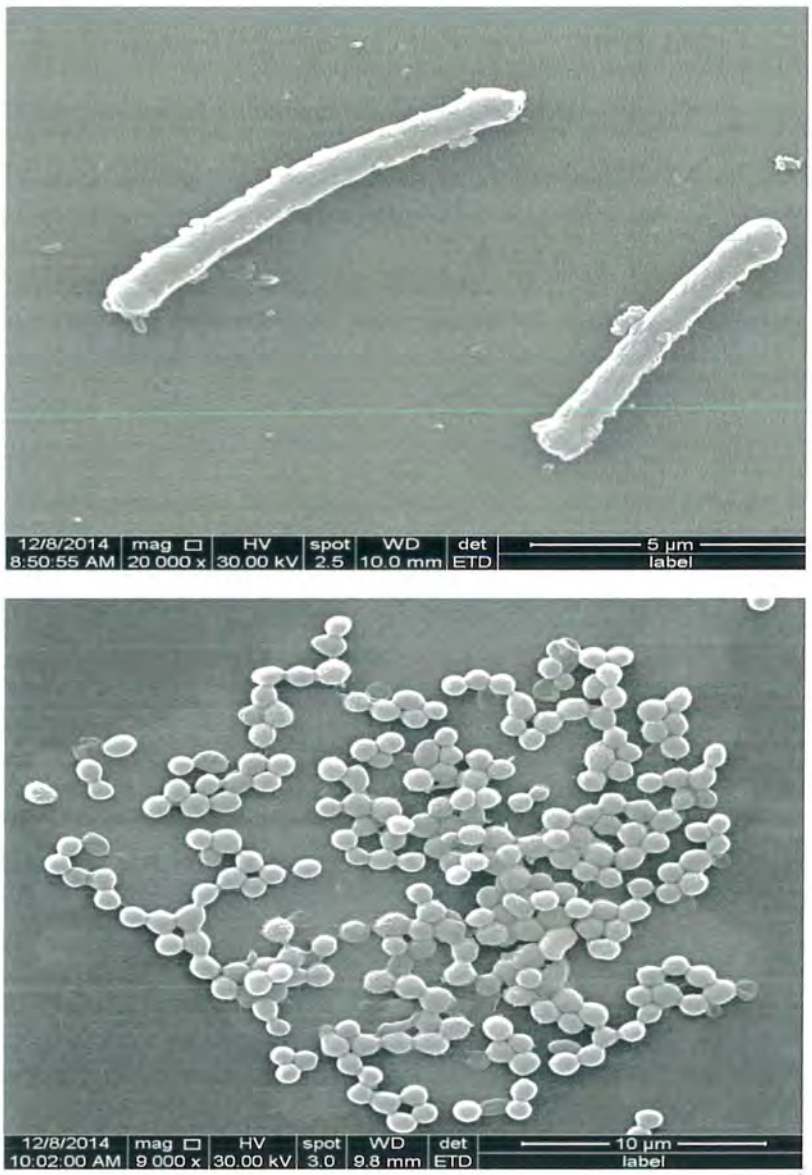


Figure 10 Scanning electron micrographs of cells of strain NCCP-1664 ^{T}(a–b) grown on TSA agar medium at 37 °C for (a) 11 h; (b) 60 h

Colonies are round in shape and off white in color. Strain NCCP-1664^{T}can grow at temperature range of 28- 45 ºC with optimum at 37 ºC. No growth was observed at temperature lower than 28 ºC and there was also slow growth at 45 ºC. This temperature range is towards higher side than that the reference type strains can grow at (Ganzert et al., 2011), (Kageyama et al., 2008), (J. Zhang, Ma, & Yu, 2012) and (Y. G. Chen et al., 2009).Optimum growth of strain NCCP-1664^{T} was observed at pH 7.0-8.0 but can grow at pH range 6.0-9.0. No growth was observed above or below this pH

range. Cells tolerated a NaCl concentrations between 0-16 % (optimum growth was observed with 2% NaCl (w/v). Strain NCCP-1664^T exhibited many phenotypic features that were similar to that of the closely related taxa, *Arthrobacter oryzae* KV-651^T, *A. cryotolerans* LI3^T, *A. halodurans* JSM 078085^T, *A. cupressi* D48^T and *A. humicola* KV-653^T (given in below text); but many biochemical and physiological characteristics also differentiated these species (Table 8 and are also summarized in the species description). Of these, the most notable differentiating characteristics were positive for catalase and negative for oxidase and urease production. Strain NCCP-1664^T showed growth at 45°C, which is unusual for other species of the genus *Arthrobacter*.

There were several phenotypic characteristics that differentiate strain NCCP-1664^T from the closely related references species. Among these involved positive reaction for acid production with D-cellobiose while all the other reference species exhibited negative reaction. The strain under study also exhibited negative reaction for β-galactosidase, assimilation of maltose, acid production with D-fructose, enzyme reaction of cystein arylamidase, glucose fermentation and utilization of L-alanine and L-alanyl-glycin as carbon sources while all other reference strains were positive. NCCP-1664^T was observed to be negative for H_2S production, hydrolysis of esculin and gelatin while reference strain *A. cupressi* D48^T was positive for these reactions. Nitrate reduction was positive in NCCP-1664^T while negative in *A. cupressi* D48^T, *A. humicola* KV-653^T and *A. cryotolerans* LI3^T. Several other phenotypic characteristics have been summarized in Table 8

Table 8. Differentiating physiological and biochemical characteristics of strain NCCP1664^T with the type strains of closely related reference species of the genus *Arthrobacter*.

*1 A. haloduranss*JSM 078085^T
2 A. cupressi
*3 A. oryzae*KV-651^T
*4 A. humicola*KV-653^T
*5 A. cryotolerans*LI3^T

Characteristics	**NCCP-1664^T**	***1***	***2***	***3***	***4***	***5***
NaCl range (%)w/v (optimum)	0-16 (2)	0-12 (0)	0-2 (0)	0-2 (0)	0–3 (0-1)	0-10 (0-1)
Temperature range (°C) (optimum)	28-45 (37)	4-35 (30)	4-35 (30)	4-35 (30)	4-35 (30)	4-24 (16)
pH range (optimum)	6.0-9.0 (7.0-8.0)	6.0-9.0 (7.0)	6.0-10.0 (7.0)	6.0-11.0 (7.0)	6.0-10.0 (7.0)	4.0-9.0 (6.0)
β-galactosidase (ONPG)	–	+	+	+	+	+
H_2S production	–	–	+	–	–	+
Nitrate reduction	+	+	–	+	–	–
Hydrolysis of esculin, gelatin	–	+	+	–	–	–
Assimilation of:						
Arabinose	–	–	–	–	–	+
Maltose	–	+	+	+	+	+
Mannitol	–	–	+	–	–	–
N-acetyl glucosamine	+	+	+	–	–	–
Mannose, Phenyl acetic acid	–	–	w+	–	–	–
Acid production from:						
Glycerol	+	–	+	–	–	–
D-galactose, D-melezitose, D-rafinose, D-turanose	–	–	+	–	–	–
D-fructose	–	+	+	+	+	+
L-rhamnose	–	–	–	–	–	w+
D-lactose, D-lyxose	–	+	–	–	–	–
D-melibiose	–	–	+	–	+	–
D-trehalose	–	+	+	–	–	w+
Inulin	+	–	+	–	–	–
Oxidation / fermentation of:						
D-sorbitol	+	–	w+	+	+	–
Glucose fermentation	–	+	+	+	+	+
D-sucrose	–	–	+	–	+	–
Enzyme activity						
Alkaline phosphatase	+	–	–	w+	–	–

Esterase (C 4), α-galactosidase, valine arylamidase	–	–	+	–	+	–
Esterase lipase (C 8)	+	+	+	–	w+	+
Lipase (C 14)	–	–	–	w+	w+	–
Leucine arylamidase	+	–	+	+	+	+
Cystein arylamidase	–	+	+	+	+	+
Trypsin	+	–	+	–	w+	–
α –chymotrypsin	–	–	+	–	–	–
Acid phosphatase	+	–	+	+	+	+
Napthol-As-BI-phosphohydrolase	+	–	+	–	+	–
β-galactosidase	–	+	+	+	+	+
α- glucosidase	+	+	+	w+	+	–
β-glucosidase, N-acetyl-*β*-glucosaminidase, α-mannosidase	–	–	–	–	+	–
Oxidation of carbon substrates:						
Glycogen	–	–	+	–	–	+
D-galactose	+	+	+	–	w+	w+
α-Hydroxy butyric acid	+	–	w+	–	–	–
α-Ketoglutaric acid	+	+	+	w+	–	+
D,L-lactic acid and Succinamic acid	w+	–	w+	–	–	–
Malonic acid	+	–	+	–	–	–
Propionic acid	w+	–	w+	+	–	w+
L-alanine	–	+	+	+	+	+
L-alanyl-glycine	–	+	+	+	+	+
L-asparagine	+	–	+	+	–	–
L-glutamic acid	w+	+	+	w+	–	–
Glycyl-L-glutamic acid	+	+	+	–	–	–
L-proline	+	–	w+	+	–	–
L-pyroglutamic acid	+	–	+	–	–	–
L-serine	+	+	+	+	–	–
Putrescine	+	+	–	+	–	+

All data are taken from current study.

+, Positive; w+, weakly positive; –, negative.

16S rRNA gene amplification, sequencing and phylogenetic analysis

The comparison of 16S rRNA gene sequence (1539 nucleotides; DDBJ/EMBL/GenBank accession number LC065376) of strain NCCP-1664^T with sequences of the type strains of the genus *Arthrobacter* on EzTaxon Server database

showed the highest sequence similarity of 97.70 % with *A. halodurans* JSM 078085^T and 97.27 % with *A. oryzae* Kv651^{T}and less than 97% with the other species of the genus *Arthrobacter* and other taxa of the related genera. All the three computation methods for phylogenetic analysis clearly determines that NCCP-1664^T does not belong to any of the recently described groups in the *Arthrobacter* genus by (Busse, 2016). It forms cluster with *A.halodurans* that is member of *Arthrobacter sensu latu* group yet. Maximum likelihood tree (Fig. 20) showed that strain NCCP-1664^T formed a monophyletic cluster with *Arthrobacter halodurans* JSM 078085^T, at a high bootstrap value (82%) and these nodes are reproduced by Neighour joining and Maximum parsimony methods (Filled spots at nods of Fig. 12) suggesting a strong coherence of strain NCCP-1664^T with *A. halodurans* JSM 078085^T (EU583729).

DNA–DNA hybridizations and DNA base composition

Since the 16S rRNA gene sequence similarities of NCCP-1664^T were higher than 97 % with two species *Arthrobacter halodurans* and *A. oryzae*, therefore DNA–DNA hybridization was carried out with type strains of two closely related species. DDH values showed 54.1 ± 2.8 % relatedness with *Arthrobacter halodurans* JSM 078085^T and 47.9 ± 3.5 % with *A. oryzae* KV-651^T. As strain NCCP-1664^T shared low level of sequence similarity with closely related species of the genus *Arthrobacter*, hybridization values are less than the 70% threshold recommended for delineation of any strain to a novel species (E. Stackebrandt & Goebel, 1994; Wayne et al., 1987) it is suggested that strain NCCP-1664^T belongs to a novel genus *Zafaria* gen., nov.,
The DNA G+C content of strain NCCP-1664^T was determined to be 67.5 mol%, which was within the range of the other members of the genus *Arthrobacter* (Y. G. Chen et al., 2009; Ganzert et al., 2011; Kageyama et al., 2008; J. Zhang et al., 2012)

Chemotaxonomy characterization

Fatty acid profile of NCCP-1664^T shows that major fatty acids present were anteiso -$C_{15:0}$ (51.2%), $C_{18:1}\omega 9c$ (16.9%), $C_{18:0}$ (12.9%) and anteiso-$C_{17:0}$ (9.6%) (Table 9). Fatty acid profile was almost similar to most closely related reference strain *A. halodurans* except $C_{14:0}$ which is absent in NCCP-1664^T. High percentage of $C_{18:1}\omega 9c$ is observed in comparison to *A.halodurans* JSM 078085^T as well as from *A.oryzae* KV-651^T, which is 2nd closest relative of NCCP-1664^T and also member of '*A. globiformis* group' / *Arthrobacter sensu stricto* which includes type species of the genus, *A. globiformis* too (Busse, 2016). It gave a clear evidence that NCCP-1664^T belongs to a

separate genus,inspite of relevance with closely related groupsit doesn't satisfy core characteristics of any group.

Table 9. Cellular fatty acid profile (%) of strain NCCP-1664^T in comparison to the closely related type strains

Characteristics	**NCCP-1664^T**	*1**	*2***	*3****
iso-$C_{14:0}$	1.7	1.3	1.5	1.4
$C_{14:0}$	–	1.4	1.1	1.2
iso-$C_{15:0}$	4.5	3.6	4.8	4.7
anteiso -$C_{15:0}$	51.2	51.0	50.1	51.2
iso-$C_{16:0}$	4.4	3.2	4.5	4.0
$C_{16:0}$	3.2	-	1.8	5.1
anteiso-$C_{17:1}\omega 9c$	1.6	1.8	1.7	3.0
anteiso-$C_{17:0}$	9.6	9.1	14.9	6.0
$C_{18:1}\omega 9c$	16.9	10.6	10.2	10.0
$C_{18:0}$	12.9	8.1	7.6	13.3

data are obtained in this study.
Values are percentages of total fatty acid detected. –, not detected
**Arthrobacter halodurans* JSM 078085^T
***Arthrobacter oryzae* KV-651^T
****Paeniglutamicibacter cryotolerans* comb. nov.Basonym: *Arthrobacter cryotolerans* LI3^T

Strain NCCP-1664^T was observed to possess MK-9(H_2) (82.4%), MK-8(H2) (17.5%) as a major components of isoprenoid quinones. As described by (Busse, 2016), presence of MK-9(H_2) as the major isoprenoid quinone is characteristic chemotaxonomic feature of genus *Arthrobacter*, *Arthrobacter sensu stricto* ('*A. globiformis* group') also possess MK-9(H_2) as predominant menaquinone. The polar lipids profile comprised of diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), glycolipid (GL) and unknown polar lipid (PL) (Figure 11). Polar lipid profile of NCCP-1664^T is distinct from all closely related genera, as shown in Table 10.

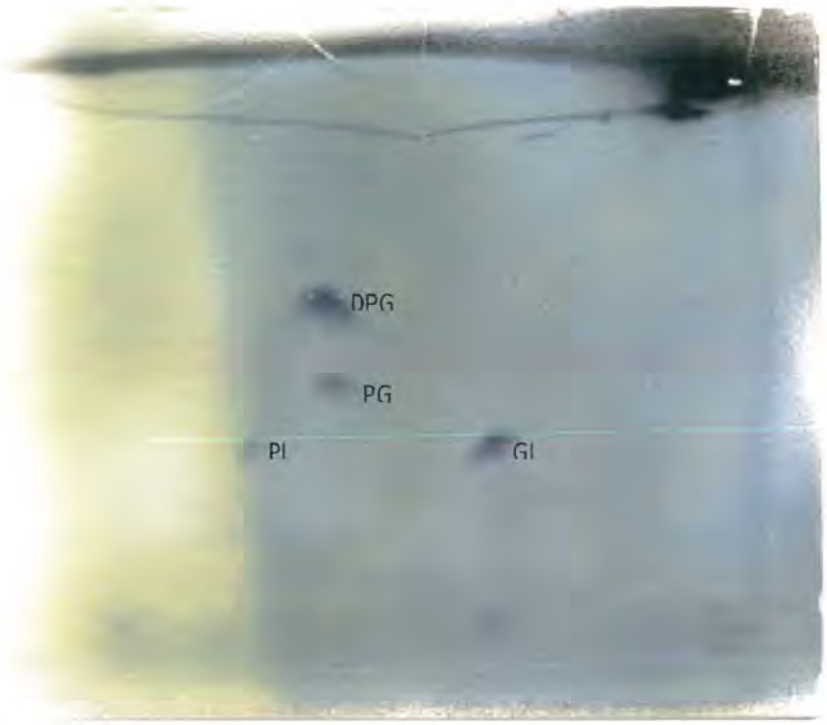


Figure 11 Polar lipid profile of strain NCCP1664^T after separation by two-dimensional thin layer chromatography

The peptidoglycan type of strain NCCP-1664^{T}comprised of the amino acids alanine, glutamic acid and lysine in the following approximate molar ratio: 2.7 Ala : 1.0 Glu : 0.9 Lys (0.4 D-Ala, 0.8 L-Ala, 1.0 D-Glu, 1.5 L-Lys), L-Glu was not detected. The hydrolysate contained additionally to the amino acids the peptides L-Ala – D-Glu, L-Ala – D-Ala, L-Lys – D-Ala, L-Lys – L-Ala and D-Ala – L-Lys – L-Ala, alanine represented the N-terminus of the interpeptide bridge. From these data it was concluded that NCCP-1664^T DSM 29936 displayed the peptidoglycan type A3α L-Lys – L-Ala, type A11.4. The peptidoglycan structure matches partially with *Arthrobacter* sensu lato (*Arthrobacter psychrolactophilus* group) and *Citricoccus* genus which possess A3α type 11.4 but difference in peptidoglycan structure of NCCP-1664^T lies in absence of threonine as compared to *Arthroacter* sensu lato (*Arthrobacter psychrolactophilus* group) and absence of L-Glu in comparison to *Citricoccus* genus.

The peptidoglycan, polar lipids, fatty acid and respiratory quinones profiles of strain NCCP-1664^T clearly shows that it belongs to a distinct genus.

4.1.5. Description of *Zafaria* gen. nov

Zafaria i (Zafai'ri.a N.L. masc. gen. n. Zafaria after the name of Dr. Yousuf Zafar, a distinguished professor of biotechnology in Pakistan). We propose that based on the phylogenetic analysis, quinones MK-9(H_2) (82.4%), MK-8(H2) (17.5%), and polar lipids diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), glycolipid (GL), unidentified phospholipids, peptidoglycan type A3α L-Lys – L-Ala , type A11.4 which contained amino acids alanine, glutamic acid and lysine in the molar ratio: 2.7 Ala : 1.0 Glu : 0.9 Lys (0.4 D-Ala, 0.8 L-Ala, 1.0 D-Glu, 1.5 L-Lys) and absence of L-Glu, NCCP-1664^T DSM 29936 belongs to a distinct genus *Zafaria* gen. nov.

4.1.6. Description of *Zafaria solitudinis* gen. nov. sp. nov.

Solitudinis (cho.lis.tan.en'sis. N.L. fem. adj. solitudinis is pertaining to Cholistan desert, from where the type strain was isolated).

In addition to the characteristics described for the genus, the species has the following features. Cells are aerobic and non -motile. Colonies are round and off white in color. The optimum temperature, pH and NaCl concentration for growth are 37 °C, 7.0-8.0 and 2 % (w/v), respectively. Positive for assimilation of D-sorbitol, *N*-acetyl glucosamine and nitrate reduction but negative for glucose fermentation, acidification of D-sucrose, hydrolysis of esculin and gelatin; assimilation of arabinose, mannose, phenyl acetic acid, maltose and mannitol. Positive for acid production from inulin and glycerol but negative from D-galactose, D-melezitose, D-raffinose, D-turanose, D-lactose, D-lyxose, D-fructose, L-rhamnose, D-melibiose and D-trehalose. Strong enzyme activity is observed for esterase lipase (C 8), alkaline phosphatase, leucine arylamidase, trypsin, acid phosphatase, napthol-As-BI-phosphohydrolase, α-glucosidase but no enzyme activity for esterase (C 4), α-galactosidase, valine arylamidase, lipase (C 14), cystein arylamidase, α –chymotrypsin, β-glucoronidase β-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase. positive for utilization of substrates as carbon source: D-galactose, α-hydroxy butyric acid, α-ketoglutaric acid, malonic acid, L-asparagine, glycyl-L-glutamic acid, L-proline, L-pyroglutamic acid, L-serine and putrescine; weakly positive for D,L-lactic acid, succinamic acid, propionic acid, L-glutamic acid but negative for glycogen L-alanine L-alanyl-glycine (Biolog GN III microplates™). Cell-wall peptidoglycan contained amino acids: alanine, glutamic acid and lysine and absence of L-Glu, representing the cell wall

peptidoglycan type A3α. The major whole-cell sugars are galactose and glucose. Major fatty acids are anteiso-$C_{15:0}$, $C_{18:1}\omega 9c$, $C_{18:0}$ and anteiso-$C_{17:0}$. In addition to polar lipids described in genus description it also unknown phospholipids (PL). The DNA G+C content is 67.5 mol%.

The type strain is NCCP-1664^T (=DSM 29936^T = KCTC 39549^T), which was isolated from soil sample collected from Cholistan desert, Bahawalpur, Pakistan. The DDBJ/EMBL/GenBank accession number for 16S rRNA gene sequence of strain NCCP-1664^T is LC065376.

Table 10 Differentiation of NCCP-1664T DSM 29936 from closel y related genera.

Trait	NCCP-1664T	*Arthrobacter deserti* DSM 29935T	*Arthrobacter halodurans* DSM 21081T	*Arthrobacter* sensu stricto (*'A. globiformis* group')*	*Paenarthrobacter* gen. nov. (*'A. aurescens* group')*	*Pseudarthrobacter* gen. nov. (*'A. oxydans* group')*	*Glutamicibacter* gen. nov. (*'A. protophormiae* group')*	*Paeniglutamicibacter* gen. nov. (*'A. sulfureus* group')*	*Pseudoglutamicibacter* gen. nov. (*'A. albus/cumminsii* group')*	*Arthrobacter* sensu lato*						*Sinomonas* genus***	*Citricoccus* genus****	*Zhihengliuella* genus*****	*Micrococcus* genus******	*Kocuria* genus*******
										Arthrobacter woluwensis	*Arthrobacter nasiphocae*	*Arthrobacter agilis* group'	*Arthrobacter citreus* group'	*Arthrobacter psychrolactophilus* group'	*Arthrobacter pigmenti* group					
Peptidoglycan	A3α (L-Lys – L-Ala)	A4 α(L-Lys – L-Ala - L-Glu)	A4 α (L-Lys – L-Ala - L-Glu)	A3a (Lys–Ala2–3)	A3a (Lys–Ala–Thr–Ala)	A3a (Lys–Ser–Thr–Ala)	A4a (Lys–Ala–Glu)	A4a (Lys–Glu)	A4a (Lys–Ala–Glu or Lys–Ser–Glu)	A4a (Lys–D–Asp Gly)	A3a (Lys–Ala2–Gly2–3–Ala (Gly)	A3a (Lys–Thr–Ala2–3)	A3a (Lys–Thr–Ala2)	A3a (Lys–Thr–Ala1–3 or Lys–Ala1–4)	A3a (Lys – Ala–Ser–Ala3 or Lys–Ala4)	A3 α (gal. man. Rib)	A4a (Ala/Gly/Glu/Lys Lys±Gly ±Glu)	A4a (L-Lys–L-Ala–L-Glu)	A2 or A4a (L-lys)	A3a (L-Lys-Ala3-4)
Type/ & Molar ratio	A11.4	A11.35	A11.35	A11.5 or A11.6	A11.17	A11.23	A11.35	A11.54	A11.35 or A11.58	A11.60	A11.62	A11.27 or A11.28	A11.27	A11.25, A11.27, A11.28 or	unspecified or A11.7	A11.22	A11.40/56	A11.35	A11.pep or A11.31	A11.6 or A11.7
Quinones	MK9 (H_2) MK-8(H2)	MK-9 (H2)	MK-9(H2)	MK-9(H2)	MK-9(H2)	MK-9(H2)	MK-8. MK-9	MK-8, MK-9 orMK-10	MK-8(H2)	NA	MK8(H2), MK-9(H2)	MK-9(H2)	MK-9(H2)	MK-9(H2)	MK-9(H2)	MK-9(H2)	MK-9(H2)	MK-9, MK-10	MK-8. MK-7(H2)	MK7(H2) orMK8(H2)MK9(H2)
Polar Lipids	DPG, PG, GL. Unknown PL	DPG PG PI	DPG, PG, PI	MGDG, DGDG/V, DMG, TMDG, PI	MGDG, DMG TMDG, PI	MGDG, DMG/V TMDG, PI	MGDG /V, DMG, TMDG /V	DGDG	MGDG, DMG, TMDG PI, PLI-5	NA	NA	DMG, TMDG, PI	DMG TMDG PI	DMG, TMDG/V PI	PI	DMG, PI	DMG, TMDG PI		DMG, Pl	Pl/V

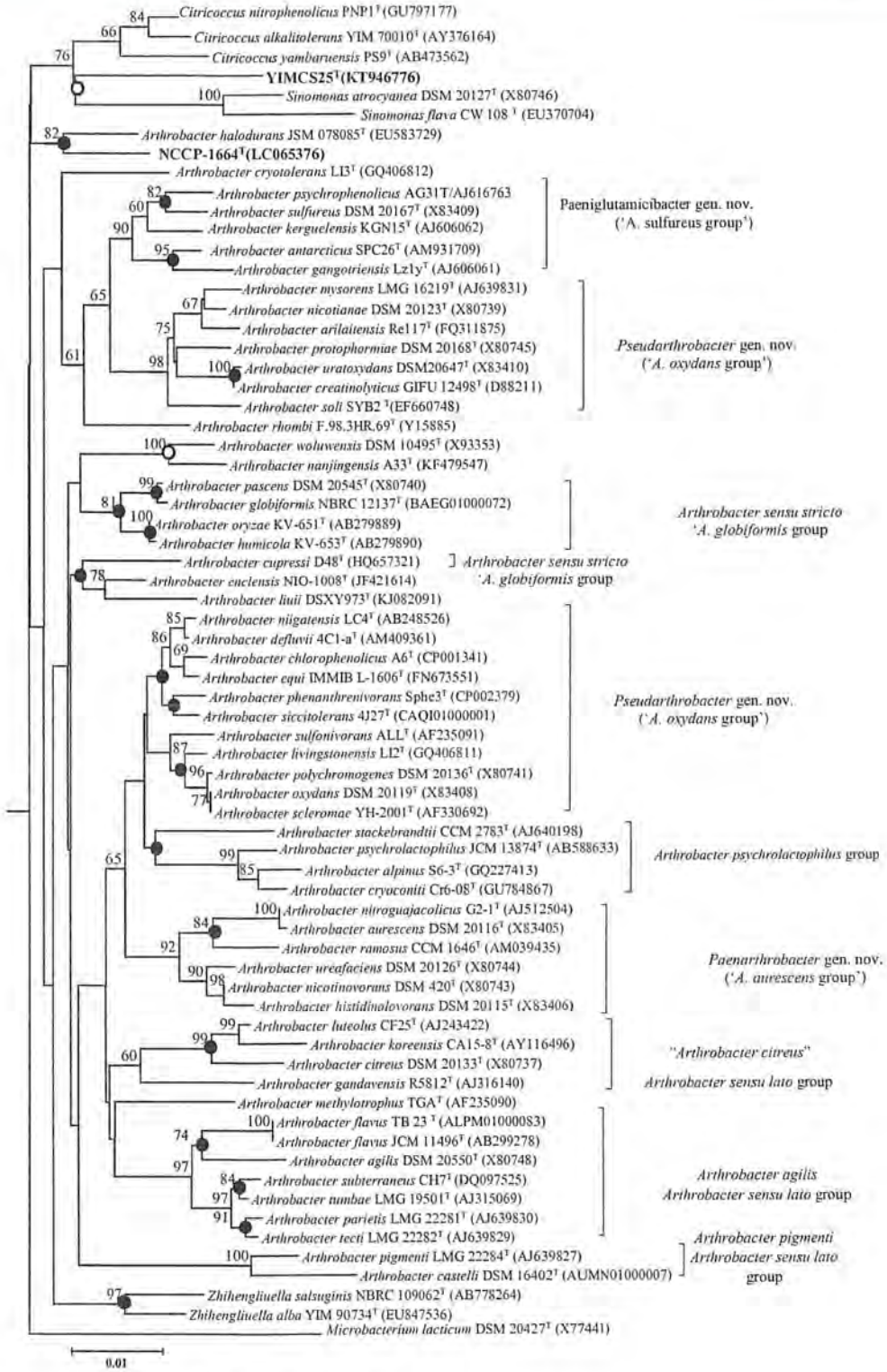


Figure 12. Maximum likelihood tree

4.2. *Microvirga pakistanensis* sp. nov.,

4.2.1 Abstract

A Gram-negative, non-spore forming, non-pigmented, strictly aerobic and non-motile short rod bacterium, designated NCCP-1258^T, was isolated from Cholistan desert soil, Bahawalpur, Pakistan. Growth of strain NCCP-1258^T was observed at pH range 6.5-9.5 (optimum 7.5-8.5), temperature range of 20-45 °C (optimum 40 °C) and it tolerated 0-2 % NaCl (optimum 0.5 %, w/v). Phylogenetic analysis based on 16S rRNA gene sequence comparison revealed that strain NCCP-1258^T belongs to genus *Microvirga* and is most closely related to *Microvirga lotononidis* (98.0 %), *Microvirga vignae* (97.4 %), *Microvirga lupini* (97.2 %), *Microvirga zambiensis* (97.2 %) and *Microvirga flocculans* (97.1 %). Analysis of the concatenated sequences of four housekeeping gene loci (*dnaK*, *gyrB*, *recA* and *rpoB*) also confirmed the placement of strain NCCP-1258^T within the genus *Microvirga*. DNA-DNA relatedness values of NCCP-1258^T with above mentioned type strains were less than 42 %. The DNA G+C content of strain NCCP-1258^T was 64.3 mol %. Chemotaxonomic data (predominant menaquinone system was Q-10; major fatty acids were $C_{16:0}$, $C_{18:1}$ ω7*c* and $C_{19:0}$ cyclo ω8*c*; the polar lipid profile contained diphosphatidylglycerol, phosphatidylcholine, phosphatidyl dimethyl ethanolamine and phosphatidyl ethanolamine) also supported the affiliation of strain NCCP-1258^T to the genus *Microvirga*. On the basis of physiological and biochemical characteristics, the phylogenetic analyses and DNA–DNA relatedness, strain NCCP-1258^T can be distinguished from the closely related taxa and thus represents a novel species of the genus *Microvirga*, for which the name *Microvirga pakistanensis* sp. nov. Is proposed with the type strain NCCP-1258^T (= CGMCC 1.15074^T = KCTC 42496^T).

Keywords:

Microvirga pakistanensis, Cholistan desert, Moderately thermotolerant

4.2.2 Introduction

The genus *Microvirga* was established by Kanso and Patel (2003), which describes aerobic, Gram-negative, non-sporulating and rod-shaped bacterium, which required yeast extract for growth. The first species described in the genus *Microvirga* was *Microvirga subterranea* (Kanso & Patel, 2003); the genus was assigned to the class *Alphaproteobacteria*, phylum *Proteobacteria*. At the time of writing this manuscript, the genus *Microvirga* comprised 9 species (LPSN, http://www.bacterio.net/microvirga.html). Members of *Microvirga* have been isolated from various habitats, e.g. Japanese hot spring (Takeda, Suzuki, & Koizumi, 2004), Australian geothermal waters (Kanso & Patel, 2003), Chinese rice field soil (Jianli Zhang, Song, Xin, Zhang, & Fang, 2009), Korean atmospheric samples (Weon et al., 2010), cow pea grown in semi arid Brazil (Radl et al., 2014). and nitrogen fixing *Lupinus texensis* from Texas, USA (Ardley et al., 2012). Recently, another one as-yet not validly named new species, *Microvirga massiliensis* sp. nov., was isolated in Marseille from a stool sample collected in Senegal, and it had the human commensal with the largest genome (Caputo et al. 2016).

During investigation of the microbial diversity of desert soil of Cholistan, Bahawalpur, Pakistan, several strains including pink coloured strain designated NCCP-1258^T were isolated. Based on 16S rRNA gene sequence analysis, strain NCCP-1258^T was most closely related to *Microvirga lotononidis* (type strain WSM3557^T) (Ardley et al., 2012), which was isolated from native legumes *Listia angolensis* (in Zambia) and *Lupinus texensis* (Texas, USA). In this study, a bacterium, designated NCCP-1258^T, was characterised by polyphasic taxonomic approach to delineate its exact taxonomic position. Further, phenotypic and biochemical characterization was performed along with phylogenetic relationships of 16S rRNA gene and four housekeeping genes.

4.2.3 Materials and methods

Isolation, morphology and phenotypic characterisation

During a study of bacterial diversity from desert soil of Cholistan, Bahawalpur, Pakistan, (lat/lon 29°23′43″N 71°41′1″E) a soil sample was serially diluted in sterilized water and inocula from 10^{-3} and 10^{-4} dilutions were spread on R2A agar medium (containing yeast extract 0.1 %, peptone 0.1 %, casein hydrolysate 0.1 %, soluble starch 0.1 %, glucose 0.1 %, sodium pyruvate 0.06 %, KH_2PO_4 0.06 % and bacto agar 1.5 %) and incubated at 40 °C. During isolation, a pink coloured colony of strain NCCP-1258^T was recovered after 3 days of incubation on R2A agar medium at 40 °C. For further purification, the strain was streaked repeatedly. The purified cells of strain NCCP-1258^T were maintained on R2A medium and stored in glycerol stocks at -80°C as well as in lyophilized ampules. The type strains of closely related species, *Microvirga lotononidis* WSM3557^T (= LMG 26455^T), *M. lupini* Lut6^T (= LMG 26460^T), *M. zambiensis* WSM3693^T (=LMG 26454^T) and *M. flocculans* ATCC BAA-817^T (= JCM 11936^T) were used as reference strains in all the characterization experiments unless otherwise mentioned.

Growth of strain NCCP-1258^T was tested on various media, such as ISP 2, oat meal agar (ISP 3), TSA, R2A and nutrient agar media at 40 °C. The colony morphology of strain NCCP-1258^T was observed on R2A agar at 40 °C after 3 days of incubation. Cells grown on R2A agar for 24 hrs were observed using phase-contrast microscopy (BH-2; Olympus) and further detailed morphology under scanning electron microscopy (QUANTA 200; FEI). Gram staining was carried out using the standard Gram reaction Gregersen (1978). Growth at various temperatures (4, 10, 15, 20, 28, 30, 33, 37, 40, 45, 50, 55 and 60 °C) was observed on R2A agar for 1 week. The pH range for growth was tested at pH between 4.0 and 11.0 (with 0.5 pH value increments) using the buffer system described by P. Xu et al. (2005) at 40 °C for 4 days in R2A broth and the growth was determined using a spectrophotometer at OD_{600} nm. Tolerance to NaCl (0, to 20 %, w/v, with 1 % increment) was investigated on R2A agar by incubation at 40 °C for 10 days. Catalase and oxidase activities were determined as described previously (Kovacs,

1956). Growth under anaerobic conditions was determined on R2A agar supplemented with or without 0.1 % nitrate by using the GasPak Anaerobic Systems (BBL) according to the manufacturer's instructions.

The biochemical, enzymatic activities and utilization of sole carbon and nitrogen sources were determined using API 20E, API 50CH, API 20NE and API ZYM strips according to the manufacturer's instructions (bioMérieux, France). Further physiological and biochemical features of strain NCCP-1258^T were determined using Biolog GN III microplate^{TM} by incubating at 40 °C according to the manufacturer's instructions.

Phylogenetic analyses

PCR amplification and sequencing of 16S rRNA gene of strain NCCP-1258^T were performed using the protocol described previously (W. J. Li et al., 2007). The phylogenetic position of strain NCCP-1258^T was identified based on 16S rRNA gene sequence and by comparison with the sequences of type species on EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/, (O. S. Kim et al., 2012a) and BLAST search on the DDBJ/NCBI servers. To clarify the taxonomic status of the strain, housekeeping genes [gyrase subunit B (*gyrB*), RNA polymerase beta subunit (*rpoB*), deoxyribonucleic acid subunit K (*dnaK*) and recombination protein subunit A (*recA*)] were also amplified and sequenced using the primers and annealing temperature conditions as described by Ardley et al. (2012). The sequences were submitted to DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/) and are listed in respective dendrograms.

Phylogenetic analyses were performed using MEGA 6 software package (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) based on 16S rRNA gene sequences of strain NCCP-1258^T and its closely related taxa. Phylogenetic trees were constructed using three algorithms: maximum parsimony (MP), neighbour joining (NJ) and maximum likelihood (ML) methods. The phylogenetic relationship was also confirmed using housekeeping loci. The sequence similarities of the housekeeping genes were estimated with the available sequences of closely related validly published species using the Kimura 2-parameter model. The concatenated data set was created by combining the

nucleotide sequences of the four housekeeping genes and phylogenetic trees were re-constructed using this concatenated data set with the concatenated sequences of related species of the genus *Microvirga* and other closely related genera. The stability of the relationship was assessed using bootstrap analysis for all the phylogenetic trees with 1,000 re-samplings for the tree topology.

DNA base composition, DNA–DNA hybridization

DNA-DNA hybridisation was performed between strain NCCP-1258^T and the reference strains. Total genomic DNA was extracted using a combination of the protocols of Marmur (1963) as described previously (Goris, Suzuki, Vos, Nakase, & Kersters, 1998). DNA–DNA hybridizations were performed with biotin-labelled probes in micro-well plate (NUNC) according to the method of Ezaki, Hashimoto, and Yabuuchi (1989) with modifications by Goris et al. (1998) and fluorescence measurements were conducted using Bio Assay Reader (model HTS7000, Perkin Elmer). The hybridization was performed at 40 °C with eight replications. The DNA G+C content of the genomic DNA of strain NCCP-1258^T was determined on reversed phase HPLC of enzymatically degraded DNA of *Escherichia coli* DH5α as the reference (M. Mesbah, Premachandran, & Whitman, 1989).

Chemotaxonomy

Chemotaxonomic characteristics of strains NCCP-1258^T and its reference strains were determined under the same experimental conditions. Cellular fatty acid analysis was performed by growing strain NCCP-1258^T and all reference strains on TSA at 33 °C for 3 according to the recommendation given by Sasser (1990) using Microbial Identification System (Sherlock Version 6.1; MIDI database: TSBA6). Menaquinones were extracted and analyzed using HPLC by following the procedures of M. D. Collins, Pirouz, Goodfellow, and Minnikin (1977) and Kroppenstedt (1982). Polar lipids were extracted and identified by two-dimensional thin layer chromatography by following procedures of (M. Collins & Jones, 1980; Minnikin, Collins, & Goodfellow, 1979).

4.2.4 Results and discussion

Morphology and phenotypic characterization

The 3-days old cells of strain NCCP-1258^T were pleomorphic with round ends, which occurred singly, in pairs or in small chains or clusters (Figure 13). Optimum growth of cells was observed in R2A medium with pH 7.5-8.5 (range 6.5-9.5), at 40 °C (range 20-45 °C) and could tolerate 0-2 % NaCl (optimum 0.5 %, w/v). Cells of strain NCCP-1258^T were non-motile, which differentiated strain NCCP-1258^T from the closely related reference species: *M. flocculans*, *M. zambiensis* and *M. lotononidis* that are reported to be motile by polar flagella (Ardley et al., 2012; Takeda et al., 2004).

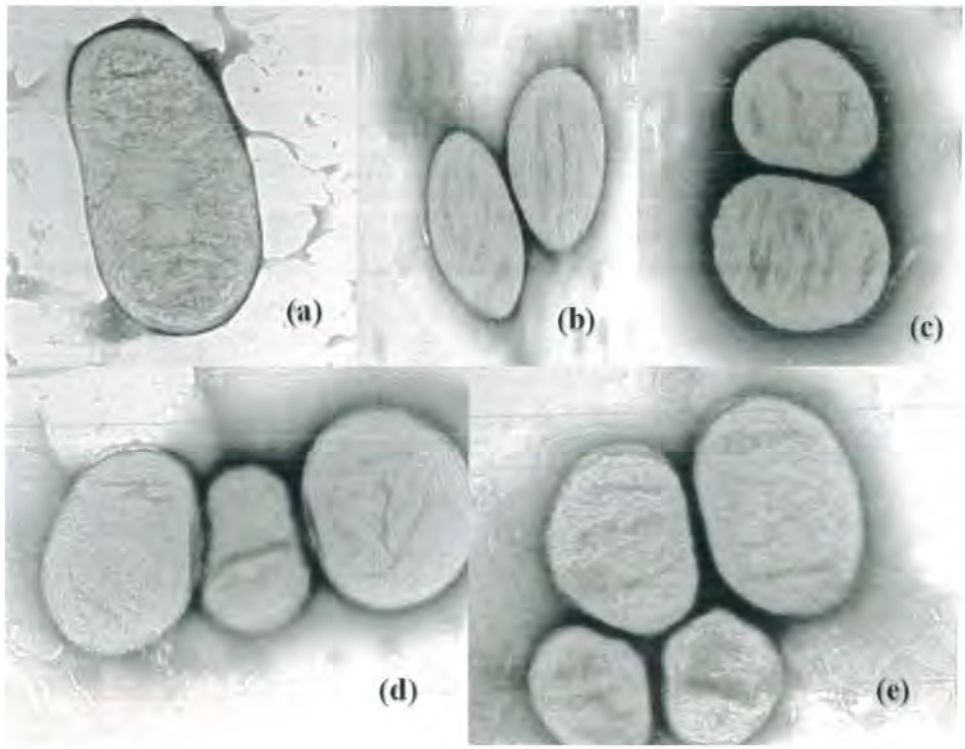


Figure 13 Transmission electron micrograph of cells of strain NCCP-1258^T grown on R2A agar (a) after 24 hours; (b-e) polymorphic cells observed after three days showing arrangements in pairs, tetrads and quadrats.

Strain NCCP-1258^T was positive for oxidase and urea hydrolysis, unlike *M. flocculans* and *M. subterranean* which did not hydrolyse urea, *M. zambiensis*, *M. lupini*, *M. lotononidis* and *M. subterranean* were oxidase negative. NCCP-1258^T was positive for tryptophan deamination, whereas the closely related strains were either

weakly positive or negative (Ardley et al., 2012; Kanso & Patel, 2003; Takeda et al., 2004). Strain NCCP-1258^T hydrolysed esculin and gelatine (weak) and produce acid from arbutin and D-melibiose but the closely related strains did not (Ardley et al., 2012). Other detailed reactions which differentiated strain NCCP-1258^T from the closely related species of *Microvirga* are enlisted in Table 11 and/or described in species description.

Table 11: Differentiating phenotypic and biochemical characteristics of strain NCCP-1258^T with the type strains of closely related species of genus *Microvirga.*

All strains are negative for lysine and ornithine decarboxylases, citrate utilization, H_2S production, indole production and arginine dihydrolase. No acid is produced from glycerol, erythritol, D-ribose, D- and L-xylose, D-adonitol, methyl-βD-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, amygdalin, D-maltose, D-lactose, D-trehalose, Inulin, D-melezitose, D-raffinose, amidon (starch), glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate, D-mannitol, inositol, D-sorbitol, L-rhamnose, D-sucrose and amygdalin. All strains are negative for assimilation of N-acetyle glucosamine, maltose, potassium gluconate, capric acid, adipic acid, malate, trisodium citrate and phenyl acetic acid (API 50CH, API 20NE and API20E bioMérieux, France)

Strongly positive enzyme reaction was observed for alkaline phosphatase whereas negative for valine arylamidase, cystein arylamidase, trypsin, acid phosphatise, α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, β-glucosidase, N-acetyl- β-glucosaminidase, α-mannosidase and α-fucosidase (API ZYM, bioMérieux, France).

All strains were observed to utilize succinic acid and succinamic acid as carbon source but do not utilize dextrin, glycogen, Tweens 40 and 80, L-erythritol, D-galactose, α-D-lactose, lactulose, methyl pyruvate, mono-methyl-succinate, cis-aconitic acid, citric acid, formic acid, α-β-γ-hydroxy butyric acid, α-keto butyric acid, α-ketoglutaric acid, α-ketovaleric acid, D- and L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, bromo succinic acid, L-alaninamide, D- and L-alanine, L-alanyl-glycine, L-asparagine, L-glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-, L-serine, L-threonine, D- and L-carnitine, γ-amino butyric acid, urocanic acid, inosine, phenyl ethylamine, putrescine, D- and L-α-glycerol phosphate, glucose-1 and glucose-6-phosphate (Biolog GN III microplates^{TM}).

All data are from current study.+, Positive; –, Negative; W+, weakly positive

Characteristics	**NCCP-1258^T**	***M. lotononidis* WSM3557^T**	***M. lupini* Lut6^T**	***M. zambiensis* WSM3693^T**	***M. flocculans* ATCC BAA-817^T**
Motility	Non motile	Motile, polar flagella	Non-motile	Motile, polar flagella	Motile, Polar flagella
NaCl optimum Range (w/v)	0.5 (0-2.0)	0–1.0 (0-2.0)	0–0.5 (0-1.5)	0–0.5 (0-2.0)	0 (0-1.0)
Temperature optimum Range °C	40 (20-45)	40 (15-45)	40 (10-43)	35 (15-35)	40–45 (20-45)
pH optimum Range	7.5-8.5 (6.5-9.5)	7.0–8.5 (5.5-9.5)	7.0–8.5 (5.5-9.5)	7.0– 8.5 (6.0-9.5)	7.0 (6.0-9.0)
Oxidase	+	–	–	–	+
Urease	+	+	+	+	–
Tryptophane deaminase	+	W+	W+	–	–
Acetoin production	–	W+	W+	W+	–
Nitrate reduction	+	+	+	+	–
Hydrolysis of:					
Gelatin	W+	–	–	–	+
Esculin	+	–	–	–	+
Acid production from:					
Arbutin, D-melibiose	+	–	–	–	–
Salicin	W+	+	+	+	–
D-cellobiose	–	+	+	+	–
Glucose fermentation	+	+	+	+	–
Assimilation of:					
Mannose	+	–	–	–	–
D-arabinose	+	+	+	+	–
L-arabinose	–	+	+	+	–
D-mannitol	–	+	+	+	–
Enzyme activity:					
Esterase (C 4)	+	–	–	–	–
Esterase lipase (C 8)	+	–	–	+	–
Lipase (C 14)	+	–	–	–	+
Leucine arylamidase, α – chymotrypsin	–	–	–	+	–
Napthol-As-BI-phosphohydrolase	–	–	–	+	–
Carbon sources utilized:					

D-fructose	+	–	+	–	–
L-fucose	+	–	W+	–	–
L-glutamic acid	+	+	+	+	–
Acetic acid	–	+	+	+	–

Phylogenetic analysis, DNA base composition and DNA–DNA hybridization

The comparison of 16S rRNA gene sequence (1499 nucleotides; DDBJ/EMBL/GenBank accession number LC065285) of strain NCCP-1258^T showed the highest similarity of 98.0 % with *Microvirga lotononidis* WSM3557^T, 97.4 % with *M. vignae* BR3299^T, 97.2 % with *M. lupini* Lut6^T and *M. zambiensis* WSM3693^T and 97.1 % with *M. flocculans* ATCC BAA-817^T and less than 97 % with the other species of genus *Microvirga* and other taxa of the related genera on the EzTaxon Server database. A neighbour-joining phylogenetic trees based on 16S rRNA gene and concatenated sequences of four housekeeping genes (Figure 15, Figure 18) revealed that strain NCCP-1258^T fell within the radiation of a cluster comprised of *Microvirga lotononidis*, *M. vignae*, *M. lupini*, *M. zambiensis* and *M. flocculans* with a bootstrap value of 72 %. A similar tree topology was also observed when the phylogenetic analyses were performed using MP and ML algorithms (Figs 16, 17, 19 and 20). The sequence similarity value of the *gyrB*, *rpoB*, *dnaK* and *recA* housekeeping genes was also highest with *M. lotononidis* (93.6, 91.4, 88.2 and 83.4 %, respectively) (Figure 14). This low sequence similarity of the four housekeeping genes also supported the hypothesis that strain NCCP-1258^T belongs to a novel species.

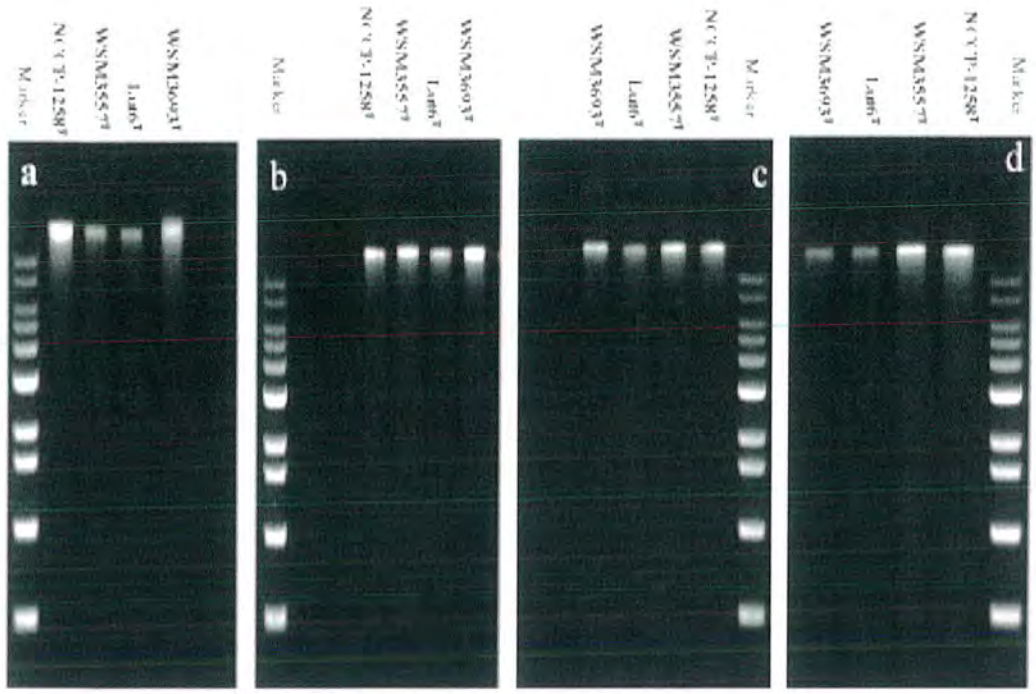


Figure 14 Seperation of amplified house keeping genes of NCCP-1258^T and its closely related genes for all four house keeping genes.

Table 12 PCR conditions for amplification of house keeping genes

Locus	Sequence (5′–3′)	Length	Annealing Temp. (°C)	Accession No.
dnaK -F *dnaK* -R	GAGATCGGCGACGGCGTGTTC GATGCGGATCTGSTGCTCCTTG	762bp	56	LC085514
gyrB -F *gyrB*-R	GGCATGTATATCGGCGACAC GCTGCGGGATGTTGTTGGTGAA	714bp	56	LC085516
recA -F *recA*-R	CAGATCGAGCGCGCCTTCGGCAA ATCTGGTTGATGAAGATCACCAT	581bp	55	LC085515
rpoB-F *rpoB* -R	CGTATCGCGGYTCCTGGCTC CGAGGCGCATGTTCATCTTGAC	548bp	55	LC085517

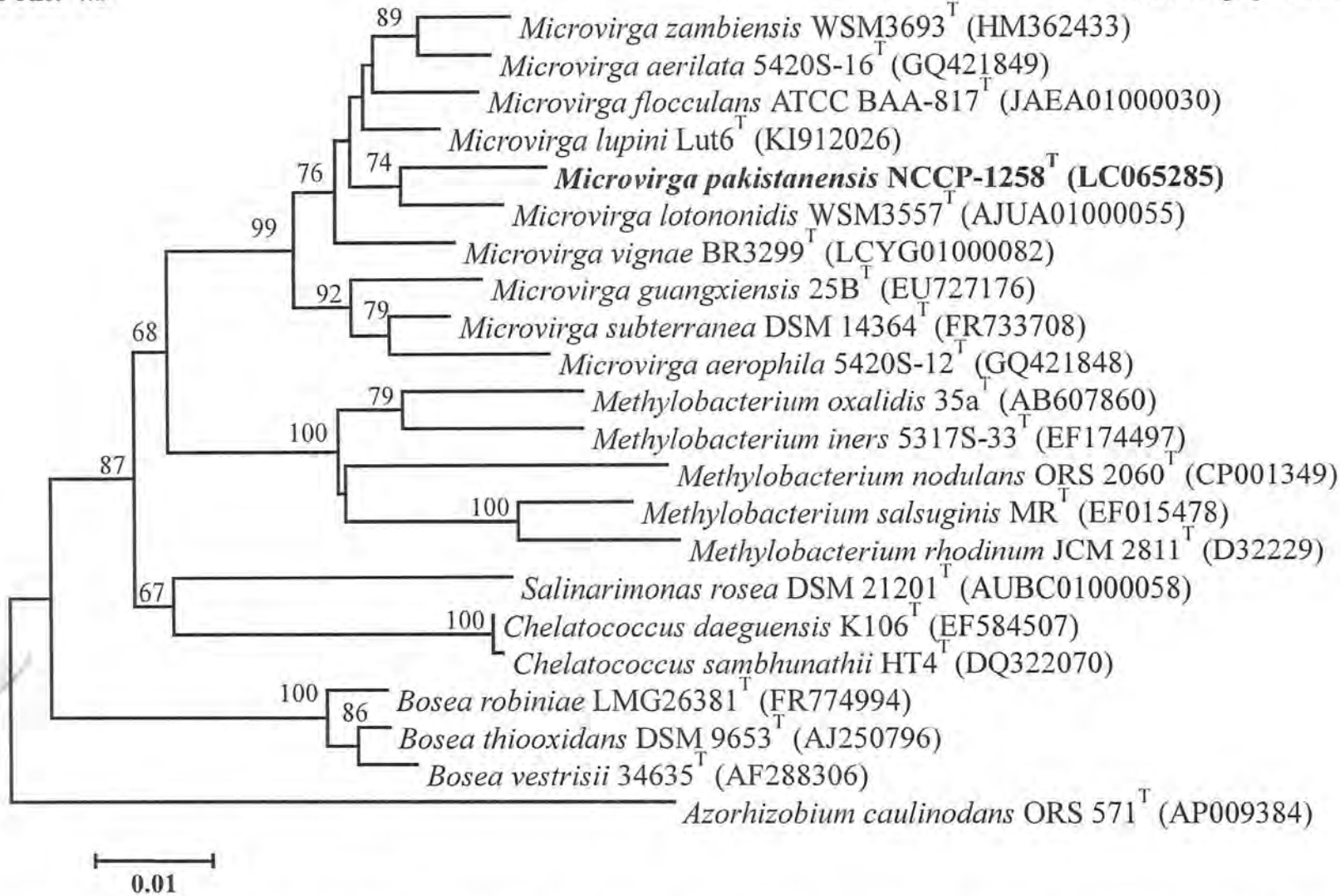


Figure 15 Neighbour-joining phylogenetic tree inferred from 16S rRNA gene sequences (1,304 bp) showing inter relationship of strain NCCP-1258^T with the members of genus *Microvirga* and other closely related genera. Bootstrap value (>60%) expressed as percentages of 1,000 replications, are shown at the branch points.. *Azorhizobium caulinodans* ORS 571^T (AP009384) was rooted as outgroup. The length of the bar represents 1% nucleotide sequence divergence.

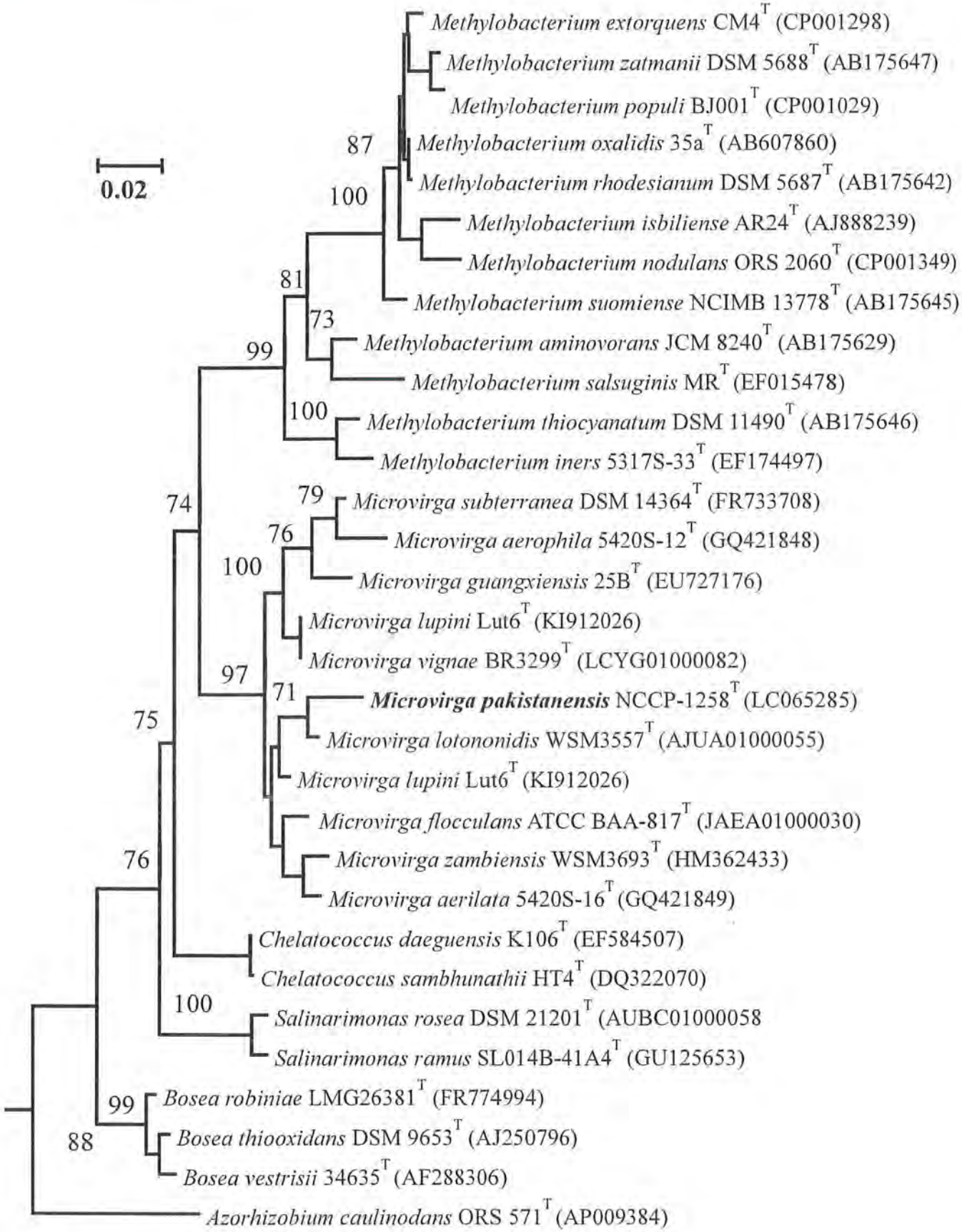


Figure 16. Maximum likelihood phylogenetic tree of 16S rRNA gene sequence of NCCP-1258^T

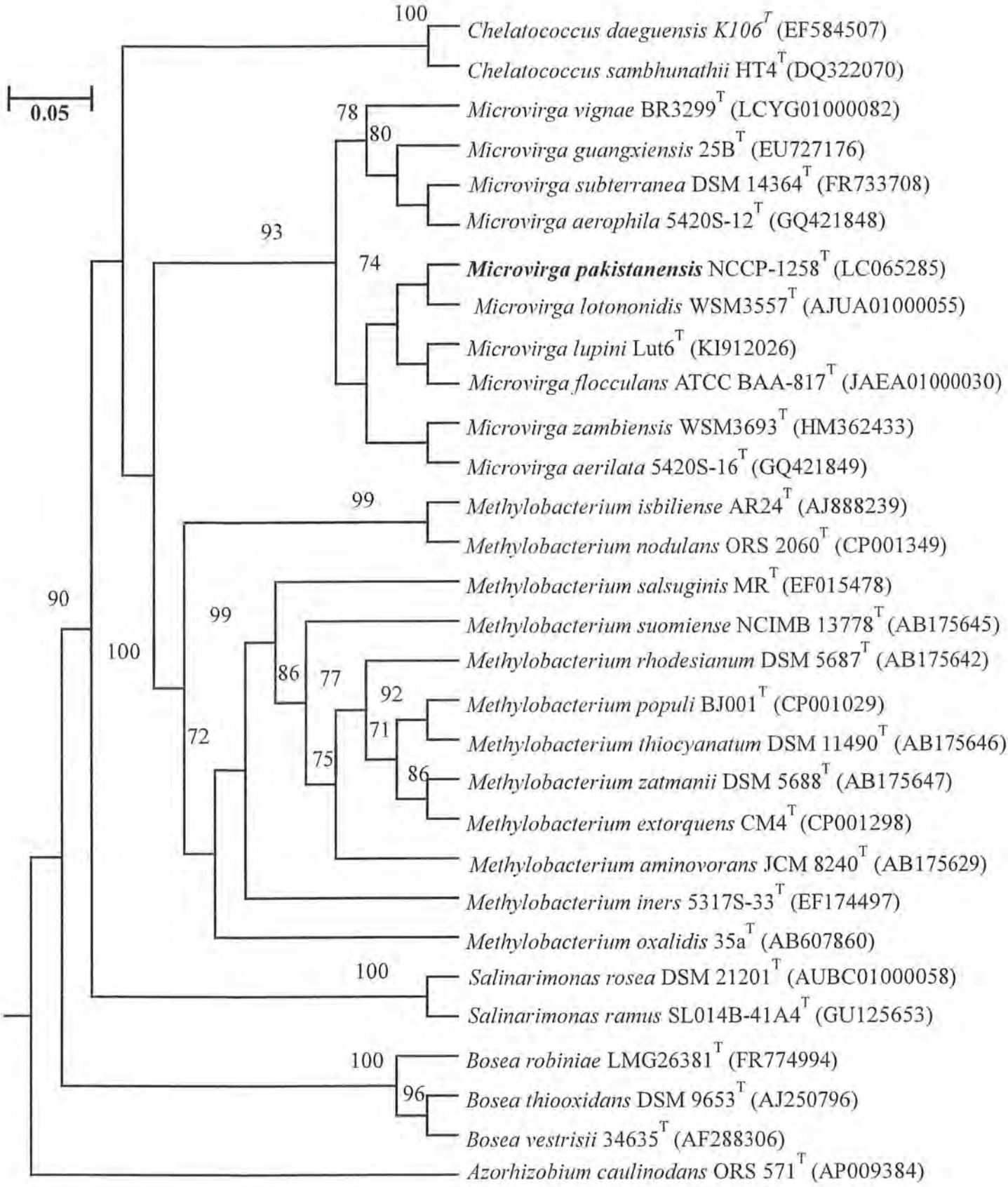


Figure 17. Maximum Parsimony phylogenetic tree of 16S rRNA gene sequences of NCCP-1258^T

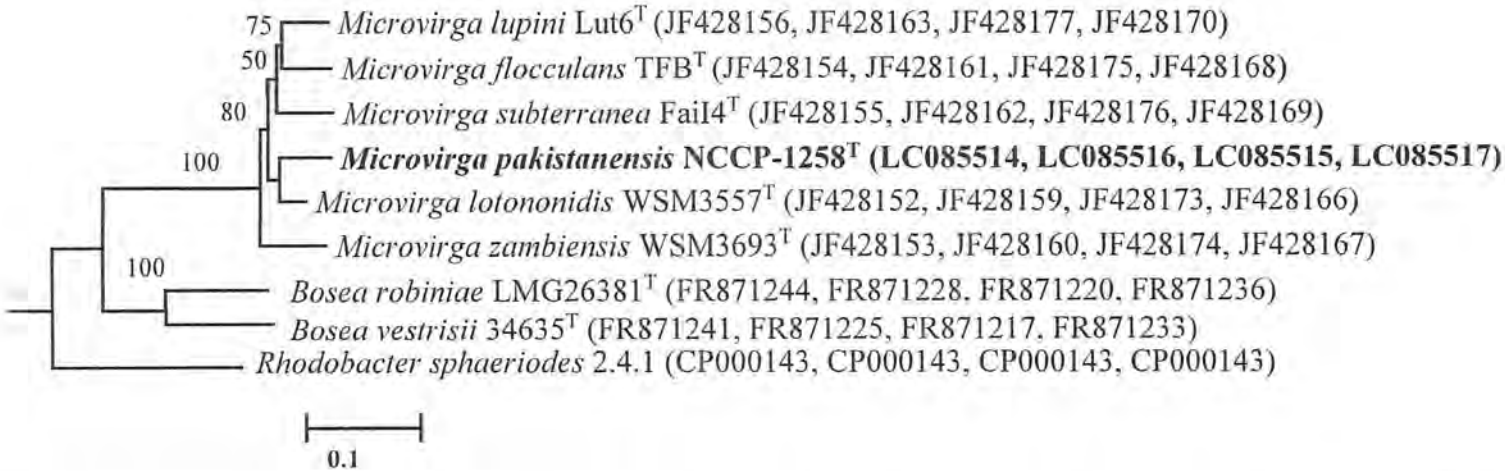


Figure 18 Neighbour-joining phylogenetic tree inferred from 1886bp concatenated sequence of the *rpoB, gyrB, dnaK and recA* showing inter-relationship of strain NCCP-1258^T with the closely related type strains of genus *Microvirga* and closely related genera. *Rhodobacter sphaeriodes* 2.4.1 was rooted as an out-group. The bootstrap values (only >60% are shown), expressed as a percentage of 1000 replications, are given at the branching points. Accession numbers are mentioned in sequence *dnaK*, *gyrB*, *recA*, *rpoB*. All nodes were also recovered by maximum-liklihood and maximum parsimony algorithms. The length of the bar represents 10 % nucleotide sequence divergence.

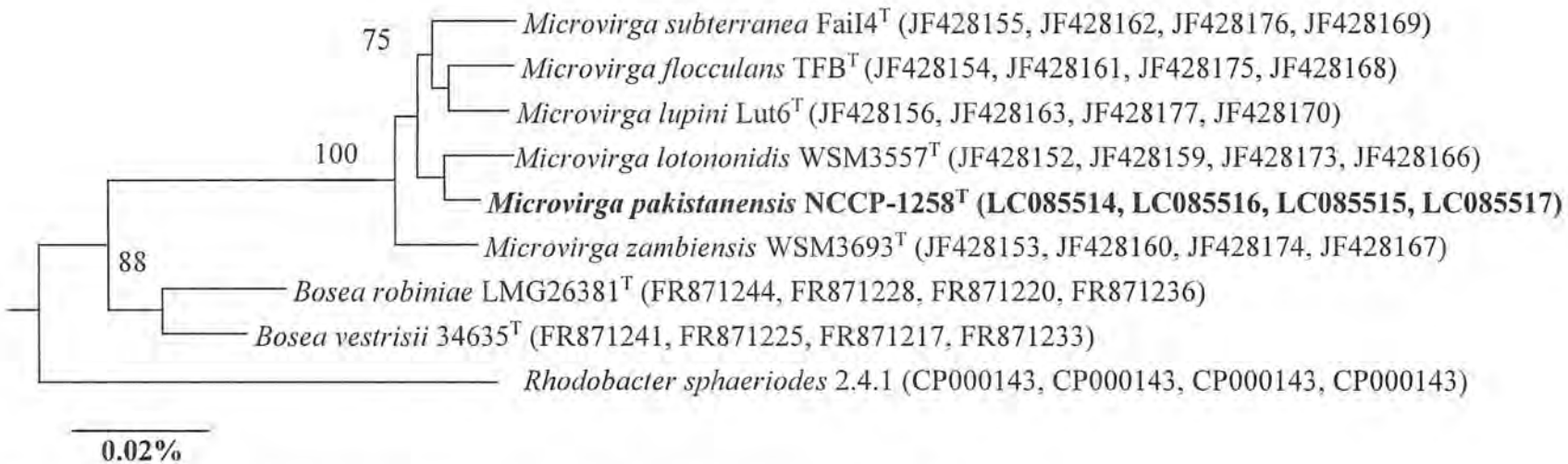


Figure 19 . Maximum-likelihood phylogenetic tree of *rpoB, gyrb, dnak* and *reca* of NCCP-1258^T

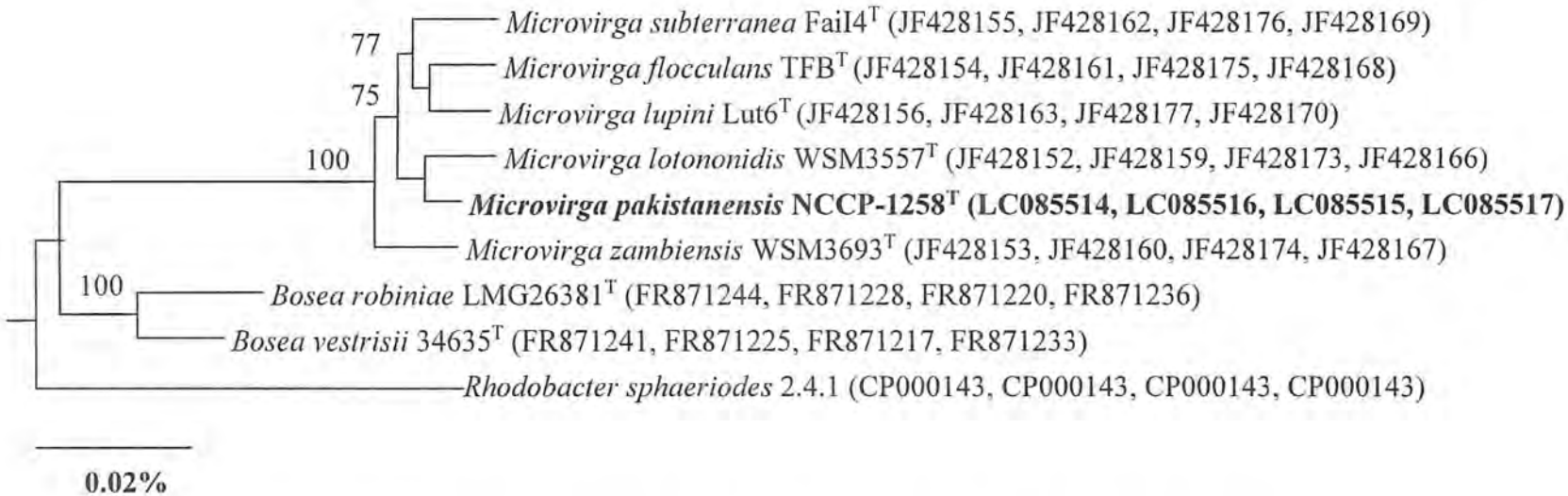


Figure 20 Maximum parsimony phylogenetic tree of 1886bp sequence of *rpoB, gyrb, dnak* and *reca* sequence of NCCP-1258^T

Since the 16S rRNA gene sequence similarity of strain NCCP-1258^T is higher than 97 % with four closely related type strains DDH was carried out as suggested by E. Stackebrandt and Goebel (1994). The results revealed that DDH values of strain NCCP-1258^T were 41.6 % with the type strain *Microvirga lotononidis* WSM3557^T, 39.2 % with *M. lupini* Lut6^T, 33.6 % with *M. zambiensis* WSM3693^T and 15.4 % with *M. flocculans* ATCC BAA-817^T. These values are less than the 70 % threshold that is indicative of the presence of new species (Wayne et al., 1987). The DNA G+C content of strain NCCP-1258^T was determined to be 64.3 mol %, which is within the range of the members of genus *Microvirga* (Ardley et al., 2012; Weon et al., 2010).

Chemotaxonomic analyses

The cellular fatty acid profile of strain NCCP-1258^T comprised predominantly of $C_{18:1}$ ω7*c* (54.0 %), $C_{19:0}$ cyclo ω8*c* (21.4 %) and $C_{16:0}$ (14.5 %), was similar to the profiles obtained for the reference strains, although significant variation in the values of these components clearly differentiates our strain from the closely related reference strains (Table 13).

Table 13. Cellular fatty acid profile (%) of strain NCCP-1258^T in comparison to closely related type strains of the genus *Microvirga*.

Characteristics	NCCP-1258^T	*M. lotononidis* WSM3557^T	*M. lupini* Lut6^T	*M. zambiensis* WSM3693^T	*M. flocculans* ATCC BAA-817^T
$C_{12:0}$	0.9	0.6	1.9	1.8	0.7
$C_{14:0}$	1.1	2.8	0.9	3.1	3.2
$C_{14:0}$ 3-OH	6.6	22.4	28.8	22.3	11.0
$C_{16:0}$	14.5	10.6	10.4	9.0	13.2
$C_{17:1}$ ω8*c*	0.7	0.6	1.7	1.8	0.8
$C_{17:0}$	2.3	0.9	0.5	1.7	1.2
$C_{17:0}$ cyclo	6.4	1.2	2.6	2.3	1.4
$C_{18:0}$	3.3	2.0	1.0	1.1	–
$C_{18:1}$ ω9*c*	–	2.1	2.5	3.3	7.3
$C_{18:1}$ ω7*c*	54.0	60.0	55.4	55.1	53.1
$C_{19:0}$ cyclo ω8*c*	21.4	27.6	7.6	22.4	22.8
Summed features 2	10.2	12.3	14.8	14.4	12.0
Summed features 3	2.8	2.8	6.1	7.8	3.6

Summed feature 2 comprise of one or both of $C_{14:0}$ 2OH/iso-$C_{16:1}$ I and Summed feature 3 comprise of one or both of $C_{16:1}$ ω6*c* / $C_{16:1}$ ω7*c*, which cannot be separated by MIDI system.

It was noted that $C_{17:0}$ cyclo and $C_{18:1}$ ω7*c* were present in higher amounts, while $C_{18:1}$ ω9*c* is absent in the profile of strain NCCP-1258^T. Strain NCCP-1258^T contained Q-10 as a sole menaquinone system. The closely related type strains were also reported to have Q-10 as the major menaquinone, Q-8 and Q-9 were present as minor components in these closely related reference strains but absent in strain NCCP-1258^T. The polar lipids profile comprised of diphosphatidyl glycerol (DPG), phosphatidyl choline (PC), phosphatidyl dimethyl ethanolamine (PDE) and phosphatidyl ethanolamine (PE) (Figure 21).

Figure 21 Two dimensional TLC of polar lipid profile of strain NCCP-1258^T. DPG, diphosphatidyl glycerol; PC, phosphatidyl choline; PDE, phosphatidyl dimethyl ethanolamine and PE, phosphatidyl ethanolamine.

Polar lipid profiles of the closely related strains *M. lotononidis* WSM3557^T, *M. lupini* Lut6^T and *M. zambiensis* WSM3693^T were also reported to contain these four components (Ardley et al., 2012). However, phosphatidyl glycerol (PG) is absent in strain NCCP-1258^T which differentiated this isolate from the closely related taxa. On the basis of physiological, chemotaxonomic, phylogenetic and genomic data, strain NCCP-1258^T is considered to be a new member of the genus *Microvirga.*

4.2.5 Description of *Microvirga pakistanensis* sp. nov.

Microvirga pakistanensis (pa.kis.tan.en'sis. N.L. fem. adj. pakistanensis is pertaining to Pakistan, where the type strain was isolated).

Cells are Gram-negative, aerobic, non-motile and non-spore forming short rods (~2 μm in length). Colonies are small, round with entire margins, shiny surface, concave and pink in colour. The optimum temperature, pH and NaCl concentration for growth are 40 °C, 7.5-8.5 and 0.5% (w/v), respectively. Positive for tryptophan deamination, nitrate reduction, oxidase and hydrolysis of esculin, urea and gelatine (weak), and fermentation of glucose but negative for Voges-Proskauer test. Acid was produced from arbutin, D-melibiose, salicin (weak), but not from D-cellobiose. Positive for assimilation of D-mannose, D-arabinose but negative for assimilation of L-arabinose and D-mannitol. Strong enzyme activity for esterase (C 4), esterase lipase (C 8) and lipase (C 14) but negative for leucine arylamidase, α-chymotrypsin and napthol-As-BI- phosphohydrolase. D-fructose, L-fucose and L-glutamic acid were utilized as carbon sources but negative for utilization of acetic acid. The major cellular fatty acids are $C_{16:0}$, $C_{18:1}$ ω7*c*, $C_{19:0}$ cyclo ω8*c* and summed feature 2 ($C_{14:0}$ 2-OH / iso-$C_{16:1}$ I). The predominant menaquinone system is Q-10. The polar lipid profile comprises of diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidyl dimethyl ethanolamine (PDE) and phosphatidyl ethanolamine (PE). The DNA G+C content is 64.3 mol%.

The type strain NCCP-1258^T (= CGMCC 1.15074^T = KCTC 42496^T) was isolated from desert soil of Cholistan, Bahawalpur, Pakistan. GenBank accession numbers for type strain NCCP-1258^T are LC065285 (16S rRNA gene); LC085517 (*rpoB* gene), LC085516 (*gyrB* gene), LC085515 (*recA* gene) and LC085514 (*dnaK* gene).

4.3 *Streptomyces caldifontis* sp. nov., isolated from a hot water springe of Tatta Pani, Kotli, Pakistan

4.3.1. Abstract

A Gram-staining positive, non-motile, rod-shaped, catalase-positive and oxidase-negative bacterium, designated NCCP-1331^T, was isolated from a hot water spring soil collected from Tatta Pani, Kotli, Azad Jammu & Kashmir, Pakistan. The isolate grew at a temperature range of 18-40 °C (optimum 30°C), pH 6.0-9.0 (optimum 7.0) and with 0–6 % NaCl (optimum 2 % NaCl (w/v)). The phylogenetic analysis based on 16S rRNA gene sequence comparison with MP, ML and NJ algorithms shown that strain NCCP-1331^T belongs to the genus *Streptomyces* and was closely related to *Streptomyces brevispora* BK160^T with 97.9 % nucleotide similarity, followed by *Streptomyces drosdowiczii* NRRL B-24297^{T}with 97.88% nucleotide similarity. The DNA–DNA relatedness values of strain NCCP-1331^T with *S. brevispora*KACC 21093^{T}and *S. drosdowiczii* CBMAI 0498^{T}were 42.7 % and 34.7 %, respectively.LL-DAP was detected as diagnostic amino acid along with, alanine, glycine, leucine and glutamic acid. The isolate contained MK-9(H_8) as the predominant menaquinone. Major polar lipids detected in NCCP-1331^T were phosphatidylethanolamine, phosphatidylinositol and unknown phospholipids.Major fatty acids were iso-$C_{16:0}$and anteiso-$C_{15:0}$, $C_{16:0}$. The genomic DNA G+C content was 69.8 mol%. On the basis of phylogenetic, phenotypic and chemotaxonomic analysis, it is indicated that strain NCCP-1331^T represents a novel species of the genus *Streptomyces*, for which we propose the name *Streptomyces caldifontis* sp. nov.. The type strain is NCCP-1331^T(= KCTC 39537^T = CPCC204147^T).

4.3.2. Introduction

The genus *Streptomyces* was proposed by Waksman and Henrici (Williams, 1989), which belongs to phylum *Actinobacteria,* one of the major taxonomic units amongst the 18 major lineages presently documented within the domain Bacteria(Gao & Gupta, 2012). According to latest updates, the phylum comprises of 6 classes (*Acidimicrobiia, Actinobacteria, Coriobacteriia, Nitriliruptoria, Rubrobacteria* and *Thermophilia*), 50 families and 221 genera. Recently more than 30 new genera have been added and787 species as well as 38 sub-species have been cited in *Streptomyces* files (http://www.bacterio.net/streptomycesa.html).The members of this phylum are well

known for their high DNA G+C content and secondary metabolites(Gao & Gupta, 2012; Verma et al., 2013). The DNA G+C content ranges from 42% (*Gardnerella vaginalis*,(Greenwood & Pickett, 1980) to 74.4% (*Kineococcus radiotolerans*,(R. W. Phillips et al., 2002).Type species *Streptomyces albus* also possess high DNA G+C content, i.e. 73.3 mol% (Zaburannyi, Rabyk, Ostash, Fedorenko, & Luzhetskyy, 2014). The technical hitches related to classification of the genus *Streptomyces* at sub-generic level have been resolved by the solicitation of progressive genotypic and phenotypic methods(Goodfellow, Kumar, Labeda, & Sembiring, 2007; Rong & Huang, 2010),which resulted in reassignment of misclassified species of the genus *Streptomyces* i.e.*Streptomyces hygroscopicus* strains as *Streptomyces aldersoniae* sp. nov., *Streptomyces angustmyceticus* sp. nov., comb. nov., *Streptomyces ascomycinicus* sp. nov., *Streptomyces decoyicus* sp. nov., comb. nov., *Streptomyces milbemycinicus* sp. nov.,*Streptomyces wellingtoniae* sp. nov.(Y. Kumar & Goodfellow, 2010).

Recently, there are few other species of the genus *Streptomyces*which are published, i.e.; *Streptomyces indoligenes*(X. Luo, Sun, Xie, Wan, & Zhang, 2016), *Streptomyces andamanensis* (Sripreechasak, Tamura, Shibata, Suwanborirux, & Tanasupawat, 2016), *Streptomycesrhizosphaerihabitans, Streptomyces adustus*(H.-J. Lee & Whang, 2016)and *Streptomycesverrucosisporus*(Phongsopitanun et al., 2016), *Streptomyces scabiei*(Labeda, 2016), *Streptomycesbryophytorum* (C. Li et al., 2016)and*Streptomyces xinjiangensis*(Cheng et al., 2016),but these are still not validated names. During the course of a study on microbial diversity of Tatta Pani hot water spring, Kotli, Azad Jammu & Kashmir, Pakistan, a strain, designated NCCP-1331^T, was isolated. The results of polyphasic taxonomic studies indicated that strain NCCP-1331^T is a novel species of genus *Streptomyces*, for which we propose the name *Streptomyces caldifontis* sp. nov.

4.3.3. Materials and Methods

4.3.3.1. Isolation, morphology and phenotypic Characterization

Strain NCCP-1331^T was isolated from soil sample collected from hot water spring of Tatta Pani, Kotli (Azad Jammu & Kashmir), Pakistan (74°32'46.10"E, 35°28'30.40"N, ASL 3841 ft). About 2 g of soil sample, pre-heated at 55 °C for 20 min, was incubated at 28 °C for 21 days and then diluted with distilled water to 10^{-3} and 10^{-4} dilution. The supernatant was spread on starch casein agar

medium supplemented with 25 μg mL^{-1} nystatin. The agar plates were checked after two weeks at 28 °C (Williams & Davies, 1965). Colonies were picked and further purified by subcultural several times on same medium. The purified strain was preserved in glycerol suspensions (25 % (w/v) at -80 °C and was also maintained on oatmeal agar slopes (International *Streptomyces* Project - ISP medium 3; (Shirling & Gottlieb, 1966)at 4 °C and as mixtures of mycelial fragments and spores in 20 % (w/v) glycerol at -80 °C. Closely related strains*Streptomyces brevispora*KACC 21093^{T}and *Streptomyces drosdowiczii* CBMAI 0498^{T}were used as reference strains.

For optimization of growth on various media, cells were streaked on different such as XYL, CMC, MSC, T5, R2A, ISP2, Starch/casein(Table 14).

Table 14 Growth optimization of strain NCCP-1331^T using different media.

Medium	Composition (gL^{-1})	Growth after 7 days
XYL	Xylan-10 g ; tryptone-0.5 ; trace salt-1ml ; $CaCO_3$ -0.5 ; agar- 1.5~1.8%	Moderate
CMC	CMC-Na-1 ; yeast extract-0.01 g ; Na_2NO_3-0.5 g ; KH_2PO_4 -1g ; $MgSO_4 \cdot 7H_2O$-0.5 gtrace salt-1ml ; $CaCO_3$-0.5 g ; agar- 1.5~1.8%	Excellent
MSC	MSC -1g ; yeast extract-0.01 g ; Na_2NO_3-0.5 g ; KH_2PO_4-1 g ; $MgSO_4.7H_2O$-0.5 ; trace salt-1ml ; $CaCO_3$-0.5 g ; agar- 1.5~1.8%	Good
T5	Glucose-1 g ; lotus root starch-1 g ; yeast extract-2 g ; tryptone-0.5 g ; trace salt-1 ml ; $CaCO_3$-0.5 g ; argar-1.5~1.8%	Moderate
R2A	Glucose-0.6 ; soluble starch-0.6 ; yeast extract-0.6 g ; caseinoacids-0.6 gSodium pyruvate -0.3 g ; K_2HPO_4-0.3 g ; $MgSO_4 \cdot 7H_2O$ -0.05 ; agar-1.5~1.8%	Moderate
ISP2	Malt extract, 10.0g; yeast extract, 4.0g; glucose, 4.0g and agar, 15g	Good
Starch/casein agar	Starch-10g, casein-0.3g, KNO_3-2g, NaCl-2g, K_2HPO_4-2g, $MgSO_4.7H_2O$-0.05g, $CaCO_3$-0.2g, $FeSO_4.7H_2O$-0.01g, agar-15g, pH 7.5	Excellent

Note: CMC (Sodium carboxymethyl cellulose) and MSC (Microcrystalline cellulose) were added to check enzyme production and ability to degrade cellulose.

Sodium carboxymethyl cellulose(CMC) and microcrystalline cellulose(MSC) were added to checkcellulase activity.Cell morphology of strain NCCP-1331^T was observed under a light microscopy (model BH2; Olympus, Japan) and further detailed with scanning electron microscopy (ESEM-TMP, Philips) using cells grown at 37 °Cfor 3 days on ISP2 medium.Gram staining was performed according to standard Gram staining procedure and was confirmed by KOH lysis test (Gregersen, 1978). Cell motility was studied using hanging-drop technique(Bernardet, Nakagawa, & Holmes, 2002). The temperature range for growth was investigated on ISP2 agar at different temperatures (4, 10, 15, 18, 20, 28, 30, 37, 40, 45, 50, 55 and 60°C). NaCl tolerance at various concentrations (0–10% (w/v) at intervals of 1%) were tested on ISP2 agar at pH 7.5 at optimum growth temperature 30 °C for NCCP-1331^T. Growth at pH 4.0-10.0 (in 0.5 pH unit intervals) was examined in ISP2 agar medium with pH adjusted as described by P. Xu et al. (2005). Spores were examined under scanning electron microscope according to procedure described byO'Donnell, Falconer, Goodfellow, Ward, and Williams (1993). Cellulolytic activity was determined according to protocol explained by(L. Semêdo et al., 2004; L. T. A. S. Semêdo et al., 2000).Growth under anaerobic conditions was determined on ISP2 agar supplemented with or without 0.1 % nitrate by using the GasPak Anaerobic Systems (BBL) according to the manufacturer's instructions.

Utilization of different carbon sources were examined by using GNIII MicroPlates (Biolog, USA). Catalase activity was detected by production of bubbles on addition of 3% (v/v) H_2O_2, whereas oxidase activity was tested using 1% N,N,N',N'-tetramethyl-p-phenylenediamine. Hydrolysis of starch, gelatin, casein, urea and Tweens (20, 40, 60, 80) were determined as described byCowan and Steel (1974). Nitrite reduction and H_2S production were performed as recommended byTindall, Sikorski, Simbert, and Krieg (2007). Other physiological and biochemical properties were analysed using API 50CH/B, API 20E, API ZYM, API-ATB Vetstrips (bioMérieux, France) and Biolog GN III microplates^{TM} according to the manufacturers' instructions.

4.3.3.2. 16S rRNA gene amplification, sequencing and phylogenetic analysis

Almost complete sequence of 16S rRNA gene was determined using commercial service of Sangon Biotech (Shanghai, China). The sequences of closely related validly published species were retrieved from on EzTaxon-e server (O. S. Kim

et al., 2012b). Multiple alignment of 16S rRNA gene sequences were carried out using the CLUSTALX program (Thompson et al., 1997) and evolutionary distances were calculated using Kimura's two-parameter model (M. Kimura, 1980). Phylogenetic analyses were performed using neighbour-joining (Saitou & Nei, 1987),maximum-likelihood(J.Felsenstein,1981) and maximum-parsimony (Fitch, 1971) methods with MEGA version 6.0 (Tamura et al., 2013) software. Bootstrap analysis with (Joseph Felsenstein, 1985) was performed using 1000 replications to assess topology of phylogenetic trees.

4.3.3.3. DNA–DNA hybridizations and DNA base composition

For DNA-DNA relatedness of strain NCCP-1331^{T}was performed with the reference strains, *S. brevispora* KACC 21093^{T}and *S. drosdowiczii* CBMAI 0498^T with biotin-labelled probes in 96-micro-well plate (NUNC) and fluorescence measurements were carried out using an HTS7000 Bio Assay Reader (Perkin Elmer) according to Ezaki et al. (1989) and Christensen et al. (2000a) with some modifications by Goris et al. (1998). Marmur (1963), Pitcher et al. (1989), (Goris et al., 1998). Recipes and details of these procedures are already published as references have been mentioned earlier. The G+C content was determined by following same procedure for purification of DNA as explained in DDH experiment and OD was checked to ensure the quality of DNA, lateron reversed phase HPLC using DNA of *Escherichia coli* DH5α as the reference was performed (M. Mesbah et al., 1989).

4.3.3.4. Chemotaxonomy characterization

For chemotaxonomic analyses, freeze-dried cells of strain NCCP-1331^T and the reference strains were prepared by growing biomass in tryptoneyeast extract broth (ISP medium 1,(Shirling & Gottlieb, 1966) for 7 days at 37°C. Polar lipids were extracted and seperated by two-dimensional thin layer chromatography using standard procedures by Minnikin et al. (1979); (M. Collins & Jones, 1980). Menaquinones were extracted by following protol described by (M. D. Collins et al., 1977)and analyzed using HPLC(Kroppenstedt, 1982). Fatty acid methyl esters were analysed by following steps: harvesting biomass for fatty acid analysis from cells grown on tryptic soy agar (Difco) at 37°C for 2 days from 4th quadrant, saponification by using methanolic base and heat, methylation and finally extraction.and estimation by using the Microbial Identification System (Sherlock Version 6.1; MIDI database: TSBA6; (Sasser, 1990).

4.3.4. Results and discussion

4.3.4.1. Morphology and phenotypic characterization

Strain NCCP-1331^T was aerobic, Gram-stain positive, catalase-positive and oxidase-negative. Cells were non-motile, formed a branched substrate mycelium andrectiflexibilis aerial hyphae, white coloured smooth-surfaced spores appear on oatmeal agar (Figure 22).

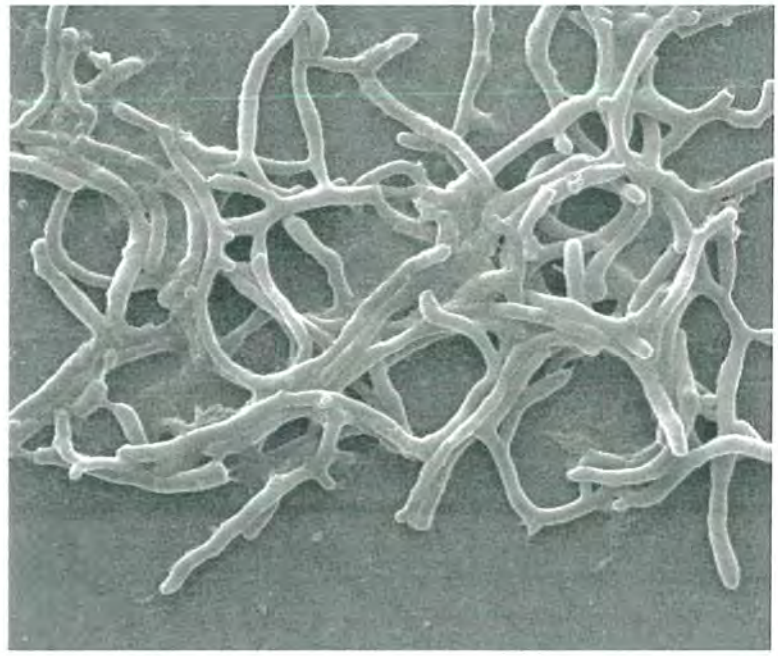


Figure 22 Scanning electron micrograph of strain NCCP-1331^T grown on ISP2 medium for 7 days at 30°C

Table 15 Differential physiological characteristics of strain NCCP-1331^T and its closest phylogenetic neighbours in the genus *Streptomyces*.

All strains are positive for hydrolysis of glycerol and esculin, acid production from:D-ribose, methyl-αD-glucopyranoside, D-arabinose, D-sorbitol, D-fucose, D-arabitol, potassium 5-ketogluconate, N-acetyl glucosamine, D-cellobiose, potassium 2-ketogluconate, glycogen and negative for erythritol, gentibiose, dulcitol, methyl-αD-mannopyranoside, methyl-βD-xylopyranoside, D-adonitol,L-xylose, L-sorbose,amygdalin, arbutin, D-lactose, D-trehalose, xylitol, D-melezitose, D-raffinose, D-turanose, D-lyxose, and potassium gluconate(API 50CH bioMérieux, France)

All strains were positive for arginine dihydrolase, citrate utilization, tryptophane deamination and acetoin production and fermentation of amygdalin but negative for fermentation ofL-rhamnose (API 20E bioMérieux, France)

All strains were resistant to (μg ml^{-1}) penicillin G(10), cefoperazon (4), lincomycin (2), pristinamycin (2), tylosin (2), metronidazol (4), streptomycin sulfate(8),, nalidixic acid, vancomycin (40),oxacillin (2),nitrofurantoin (25) and fusidic acid (2) but sensitive to spectinomycin (64), gentamicin (4), apramycin (16), cephalothin (8), doxycyclin (4), colistin (4), cotrimoxazol (2), flumequin (4), oxolinic acid (2), enrofloxacin (0.5),chloramphenicol (8),tetracycline (4),niaproof 4 (API-ATB Vet).

Enzyme activity was positive for alkaline phosphatase,α- glucosidase,N-acetyl- β-glucosaminidase, leucine, arylamidase and α –chymotrypsin but negative for valine arylamidase,L-histidine,cystein arylamidase, β-galactosidase, α- galactosidase, α-fucosidase and α-mannosidase(API ZYM, bioMérieux, France)

All strains were positive for utilization of pectin, Tween 40, L-lactic acid, β-hydroxy-D, L-butyric acid, α-hydroxy-butyric acid, citric acid, D-gluconic acid, D-glucose-6-phosphate L-glutamic acid, acetoacetic acid, acetic acid, D-fructose-6-phosphate, propionic acid, quinic acid, D-galacturonic acid, 3-methyl glucose, L-aspartic acid, L-galactonic acid, lactone, D-lactic acid, methyl ester, α-keto-butyric acid, α-keto-glutaric acid as carbon source but do not utilize D-sorbitol, N-acetyl-β-D-mannosamine, D-salicin, gelatin, dextrin, methyl pyruvate, myoinositol, N-acetyl-D-glucosamine, stachyose, D-serine, N-acetyl neuraminic acid and D-melibiose. All strains were resistant to sodium bromate, sodium butyrate, potassium tellurite,1% sodium lactatebut sensitive to fusidic acid, tetrazolium blue,D-serine, tetrazolium violet, guanidine HCl, minocycline,troleandomycin andrifamycin SV(Biolog GN III microplates™).

Characteristics	**NCCP-1331^T**	***S. brevispora* KACC 21093^T**	***S. drosdowiczii* CBMAI 0498^T**
Temperature range °C (Optimum)	18-40 (30)	4 -37 (28)	4 -37 (28)
pH range (Optimum)	6.0-9.0 (7.0)	5.0-9.0 (7.0)	5.0-9.0 (7.0)
NaCl tolerance (%) (Optimum)	0-8 (0-2)	0-7 (0-2)	0-6 (0-2)
Spore mass colour	white	colourless	grey
Melanin production	–	–	+
Nitrate reduction	–	+	+
H_2O_2 production	+	+	–
Hydrolysis of gelatin, glycerol and urea	+	+	–
Indole production	+	+	–

Fermentation of D-glucose	+	+	–
Acid production from:			
Starch, inulin, D-tagatose	–	+	–
D-galactose	w	+	–
D-fructose,L-arabitol, D-saccharose (sucrose), salicin	+	+	–
Oxidation of:			
D-mannose	w	–	+
D-maltose, D-melibiose, inositol	–	+	–
D-mannitol	+	–	–
D-sorbitol	–	+	–
Enzyme activity:			
Lysine- andornithine- decarboxylases	+	+	–
Acid phosphatase, β-glucosidase	+	w	+
Napthol-As-BI- phosphohydrolase	+	+	–
β-glucoronidase	+	+	–
Trypsin	+	+	–
Utilize nitrogen source:			
Hydroxy-L-proline, L-histidine	+	–	+
Utilize carbon source:			
p-Hydroxy-phenylacetic acid, L-alanine, glycyl-L-proline and γ-amino-butryric acid	+	–	+
L-Malic acid	+	–	w
β-Methyl-D-glucoside	-	w	–
N-acetyl-D-galactosamine, D-aspartic acid, L-serine, D- and glucuronic acid	+	–	–
L-Arginine	–	w	+
L-fucose	+	w	–
Glucuronamide, mucic acid, L-pyroglutamic acid, D-saccharic acid and formic acid	+	+	–
Bromo-succinic acid	–	+	+
Antibiotic and chemical resistance / susceptibility (ug ml^{-1}):			
Amoxycilin (4) , Rifamcin (4)	S	R	S
Amox-clav. acid ((4/2)	S	w	S
Erythromycin (1)	w	S	R
Sulphamethizole (100)	S	S	w
Kanamycin sulphate (10)	R	R	S
Aztreonam	S	S	R
Lithium Chloride	S	R	R

Strain NCCP-1331^T efficiently degraded cellulose at 45°C and was observed to show cellulolytic activity at high temperature condition (upto 60°C). The organism grew well on ISP 7, but did not grow on GYM agar and grew poorly on all other tested media.Growth occurred at temperatures ranging from 18 to 40°C (optimum 30°C). The pH range for growth was observed to be 6.0-9.0 (optimum pH 7.0). Strain NCCP-1331^T was able to grow in presence of 0-8% (optimum 0-2%) (w/v)

NaCl. Strain NCCP-1331^T could be distinguished from closely related reference species, *Streptomyces brevispora*KACC 21093^{T}and *S. drosdowiczii* CBMAI 0498^{T}by having negative reaction for nitrate reduction, melanin production, and positive for H_2O_2 production. In contrast to reference species, strain NCCP-1331^T can utilize *p*-hydroxy-phenylacetic acid, L-alanine, glycyl-L-proline and γ-amino-butryric acid as carbon source and hydroxy-L-proline andL-histidine as nitrogen source. The detailed differentiating characteristics are listed in Table 15 and further described in species description.

4.3.4.2 Phylogenetic analysis, DNA–DNA hybridization and DNA base composition

The DDBJ/EMBL/GenBank accession number for NCCP-1331^{T}is LC065358. Nearly complete 16S rRNA gene sequence showed that strain NCCP-1331^T (1541bp) belonged to genus *Streptomyces* and shared 97.94 and 97.88 % sequence similarities with *S. brevispora* BK160^T and *S. drosdowiczii* NRRL B-24297^T, respectively. Neighbour-joining phylogenetic tree (Figure 23) delineated that strain NCCP-1331^T clustered with *S. brevispora* BK160^T and *S. drosdowiczii* NRRL B-24297^{T}at high bootstrap value (87 % and 72 %, respectively). This phylogenetic relationship was also confirmed in phylogenetic trees generated with maximum-parsimony and maximum-likelihood algorithms (Figure 31), suggesting that strain NCCP-1331^T is a member of genus *Streptomyces*. To confirm a separate taxonomic position of strain NCCP-1331^T, DNA-DNA hybridization (DDH) was performed with closely related reference species. The results showed thatstrain NCCP-1331^{T}had 42.7 ± 1.6 % DDH value with *S. brevispora*KACC 21093^{T}and 34.7 ±2.3% with *S. drosdowiczii* CBMAI 0498^T. These values are less than the threshold of 70 % to describe the strain as a member of new species (E. Stackebrandt & Goebel, 1994). DNA base composition is an importantindicator at the species level.The results showed that strain NCCP-1331^T was found to have 69.8 mol%of genomic DNA G+C content, which was within the range reported for members of the genus *Streptomyces* (69-78 mol%) (Kämpfer, 2012; Ludwig et al., 2012).

4.3.4.3. Chemotaxonomic analyses

Strain NCCP-1331^T had cellular fatty acids profile predominantly comprised of iso-$C_{16:0}$ (19.5 %), anteiso-$C_{15:0}$ (12.7 %) and $C_{16:0}$(10.8 %) followed by iso-$C_{15:}$

$_{0}$, anteiso-$C_{17:0}$, iso-$C_{14:0}$ and some other minor components including summed features 3 and 8 (Table 16). This profile is similar to other members of the genus *Streptomyces,* especially with closely related reference strains having all the components which are slight different in amounts, except summed feature 3 and 8 ($C_{18:1}$ ω7*c* /$C_{18:1}$ ω6*c*), which were exclusively present in strain NCCP-1331^T. Members of the genus *Streptomyces* have straight chain, iso- and anteiso-branched chain fatty acids (Kämpfer, 2012).

Table 16 Cellular fatty acid profile (%) of strain NCCP-1331^T in comparison to type strains of closely related species of genus *Streptomyces*

Characteristics	**NCCP-1331^T**	***S.brevispora*KACC 21093^T**	***S. drosdowiczii* CBMAI 0498^T**
iso-$C_{14:0}$	4.7	4.9	6.0
iso-$C_{15:0}$	6.3	5.9	19.0
anteiso-$C_{15:0}$	12.7	36.4	18.0
iso-$C_{16:1}$ H	1.8	1.3	1.6
iso-$C_{16:0}$	19.6	19.9	22.0
$C_{16:0}$	10.8	8.5	6.0
anteiso-$C_{17:0}$ ω 9*c*	2.5	2.6	2.3
iso-$C_{17:0}$	2.1	3.2	10.0
anteiso-$C_{17:0}$	5.6	12.6	7.0
$C_{17:1}$ ω 8*c*	2.2	2.6	2.3
Summed Feature 3*	4.1	–	–
Summed Feature 8*	2.	–	–

*Summed feature3 and summed feature 8 were either $C_{16:1}$ ω 7*c* or $C_{16:1}$ ω 6*c*and $C_{18:1}$ ω 7*c* or $C_{18:1}$ ω6*c*, respectively that could not have been separated by MIDI system.

The phylogenetic position of strain NCCP-1331^T together with similarity of cellular fatty acids profile clearly indicated that the isolate belonged to genus *Streptomyces*(Kämpfer, 2012; Ludwig et al., 2012; L. Semêdo et al., 2004; Zucchi et al., 2012).

MK-9(H_8)(84%) was determined to be the predominantrespiratory quinone system in the novel strain; however, MK-9(H_6) (15%); was also detected as minor component. The presence of MK-9(H_8)is common to the members ofthe genus *Streptomyces*. Strain NCCP-1331^T contained LL-diaminopimelic acid (DAP),along with, alanine, glycine,leucine and glutamic acid as the diagnosticamino acids, representing peptidoglycan type LL-DAP-Gly (which seems very likely to be cross-linkage of LL-DAP by glycine residues at position 3(Schleifer & Kandler, 1972). Our results also showed that whole cell sugars were absent in whole-organism hydrolysate of strain NCCP-1331^T (wall chemotype I sensu (Lechevalier & Lechevalier, 1970). Presence of LL- diaminopimelic acid, glycine, lack of distinguishing sugars are characteristic of this cell wall type(Williams, 1989). Major polar lipids profile ofstrain NCCP-1331^{T}comprised of phosphatidylethanolamine, phosphatidylinositol and unknown phospholipids.The presence ofphosphatidylethanolamine is reported to be the major polar lipid in all the members of *Streptomyces* (Kämpfer, 2012). On the basis of phylogenetic, phenotypic and chemotaxonomic analysis, theisolated strain is distinct from other related species within the genus*Streptomyces*. It is suggested that strain NCCP-1331^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces caldifontis* sp. nov. is proposed.

4.3.5. Description of *Streptomyces caldifontis* sp. nov.

Streptomyces caldifontis (cal.di.fon'tis. L. adj. *caldus*, hot; L. masc. n. *fons*, *fontis*, a spring; N.L. gen. n. *caldifontis*, of a hot spring).

Cells are Gram-staining-positive, aerobic, non-motile and exhibits branched substrate mycelium that bears aerial hyphae. Colonies on T5 agar are pointed, white-coloured. Growth occurred at 18-40°C (optimum30°C), at pH 6.0-9.0 (optimum 7.0) and in presence of 0-8 %(w/v) NaCl (optimum 0-2%). Does not produce any diffusible pigment (melanine) and forms white colored spore mass. Negative for nitrate reduction. Positive for lysine-and ornithine-decarboxylases, hydrolysis of gelatin, glycerol and urea, indole production, H_2O_2 production and fermentation of D-glucose. Positive for

acid production with D-fructose,L-arabitol, D-saccharose (sucrose), salicin, D-galactose (weak),but negative fromL-arabinose,starch, inulin, D-tagatose, Positive for oxidation of D-mannitol and D-mannose (weak) but negative for D-maltose, D-melibiose, inositol and D-sorbitol (API 50CH, and API20E bioMérieux, France). Strong Enzyme activity for, acid phosphatase, *β*-glucosidase, napthol-As-BI-phosphohydrolase, *β*-glucoronidase and trypsin (API ZYM, bioMérieux, France).Can utilize hydroxy-L-proline and L-histidine as nitrogen source. Positive for utilization of *p*-hydroxy-phenylacetic acid, L-alanine, glycyl-L-proline and γ-amino-butyric acid L-malic acid *N*-acetyl-D-galactosamine, D-aspartic acid, L-serine, D- glucuronic acid,glucuronamide, mucic acid, L-pyroglutamic acid, D-saccharic acid, formic acidand L-fucose as carbon source but donot utilize *β*-methyl-D-glucoside L-arginine bromo-succinic acid as carbon source (Biolog GN III microplates™, API-ATB Vet). The major menaquinone is MK-9(H_8) with minor amount of MK-9 (H_6). Main fatty acids were iso-$C_{16:0}$ and anteiso-$C_{15:0}$ and $C_{16:0}$. Peptidoglycan was LL-diaminopimelic acid. The peptidoglycan type contained glycine, alanine, leucine and glutamic acid. Major polar lipids detected in NCCP-1331^T were phosphatidylethanolamine, phosphatidylinositol and unknown phospholipids. The genomic DNA G+C content is 69.8 mol%.

The type strain NCCP-1331^T (=KCTC 39537^T = CPCC204147^T) was isolated from soil sample collected fromhot water spring, located at Tatta Pani, Kotli Azad Jammu & Kashmir, Pakistan.

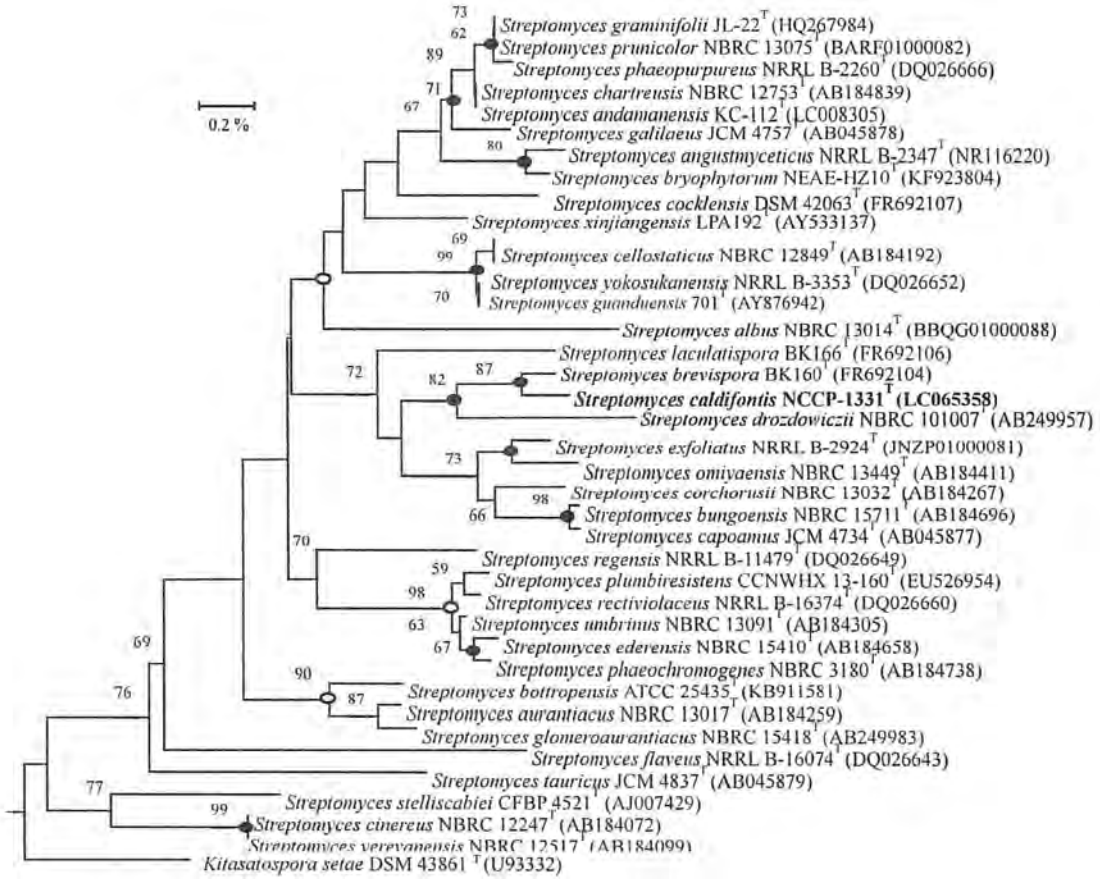


Figure 23 Neighbour-joining tree for strain NCCP-1331T

4.4 Description of *Nocardioides pakistanensis* sp. nov.,

4.4.1 Abstract

A Gram-staining positive, non-spore forming, non-pigmented and non-motile bacterium, designated as NCCP-1340^T, was isolated from hot water spring, Tatta Pani Pakistan. Cells of strain NCCP-1340^T were observed to be aerobic, rod shaped, catalase and urease positive but H_2S production and oxidase negative. The growth was observed at pH 6.0-8.0 (optimum pH 7.0) and at 20-40°C (optimum 37°C). The strain could tolerate 0-8 % NaCl (optimum 2 %, v/v). Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain NCCP-1340^T belongs to the genus *Nocardioides* and was most closely related to *Nocardioides iriomotensis* IR27-S3^T (96.8%), *Nocardioides daedukensis* MDN22^T (96.6%), *Nocardioides jensenii* DSM 20641^T (96.1%) and *Nocardioides daejeonensis* MJ31^T (96.1 %). The DNA–DNA relatedness values of strain NCCP-1340^T with *Nocardioides iriomotensis* IR27-S3^T (=JCM 17985^T), *Nocardioides daedukensis* MDN22^T (=KCTC 19601^T) and *Nocardioides jensenii* DSM 20641^T (=KCTC 9134^T) were found to be less than 53 %. The DNA G+C content of strain NCCP-1340^T was determined to be 71.8 mol%. The affiliation of strain NCCP-1340^T to the genus *Nocardioides* was further supported by chemotaxonomic data which showed presence of MK-8(H_4) as major menaquinone system; iso-$C_{16:0}$, $C_{17:0}$, $C_{16:0}$ 10-methyl, iso-$C_{15:0}$ and $C_{15:0}$ as major cellular fatty acids; and diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and unidentified glycolipids (GLs) and polar lipids (PLs) in polar lipids profile. Cell wall peptidoglycan contained LL-diaminopimelic acid as the diagnostic amino acid. On the basis of physiological and biochemical characteristics and the phylogenetic analyses, strain NCCP-1340^T can be distinguished from the closely related taxa and thus, it represents a novel species of genus *Nocardioides,* for which the name *Nocardioides pakistanensis* sp. nov. is proposed with the type strain NCCP-1340^T (= DSM 29942^T = JCM 30630^T).

Keywords: *Nocardioides pakistanensis* sp. nov., Hot water spring, Tatta Pani Pakistan

4.4.2 Introduction

The generic name *Nocardioides* was first introduced by Prauser (1976) with the type species *Nocardioides albus* forming its phylogenetically evolutionary lineage within the family *Nocardioidaceae*. Soon after the genus published, another two species *Pimelobacter jensenii* and *Arthrobacter simplex* were reclassified to the genus *Nocardioides* as *Nocardioides jensenii* and *Nocardioides simplex*, respectively (M. Collins, Dorsch, & Stackebrandt, 1989; O'Donnell, Goodfellow, & Minnikin, 1982). At the time of writing this manuscript, the genus *Nocardioides* is comprised of over 79 species with validly published names (http://www.bacterio.net/nocardioides.html). Members of this genus are Gram-staining positive, non-acid-fast, aerobic and motile or non motile actinomycetes (Evtushenko, Krausova, & Yoon, 2015). The genus *Nocardioides* is versatile, being isolated not only from soil (Ahn et al., 2014; Prauser, 1976), but also from sea water (H. M. Kim, Choi, Hwang, & Cho, 2008), volcanic ash (S. D. Lee & Lee, 2014), sea food samples (Lin et al., 2015), desert (Tuo et al., 2015), ground water (J. H. Yoon, Kim, Kang, Oh, & Park, 2004), beach sand (D. W. Lee, Hyun, & Lee, 2007) and sediment sample (Dastager, Lee, Ju, Park, & Kim, 2009). Different species of the genus *Nocardioides* have the ability to grow from psychrophilic (D. C. Zhang et al., 2012) to mesophilic (Dastager, Lee, Pandey, & Kim, 2010) and thermophilic. These can also be halophilic and alkaliphilic in nature (Dastager, Lee, Ju, Park, & Kim, 2008b; P. Xu et al., 2005). Some strains of this genus were also isolated from the medicinal plants (Qin et al., 2012). Members of *Nocardioides* genus are considered to be biotechnologically important because of their ability to degrade phenol, p-nitrophenol (J. H. Yoon et al., 1999), dibenzofuran (Kubota et al., 2005), *N*-acylhomoserine lactone (J. H. Yoon et al., 2006), crude-oil (Schippers, Schumann, & Sproer, 2005) and agar (Dastager, Lee, Ju, Park, & Kim, 2008a). In view of above biotechnological applications of various members of the genus *Nocardioides*, a novel strain belonging to the genus *Nocardioides* that was isolated from hot water spring located at Tatta Pani, Kotli, Pakistan, was characterized in the present study. The polyphasic taxonomic results delineated that strain NCCP-1340^T belongs to a new species of genus *Nocardioides,* for which the name *N. pakistanensis* sp. nov. is proposed.

4.4.3 Materials and methods

Isolation, morphology and phenotypic characterization

Water samples were collected in sterilized NUNC 50 ml tubes from the hot water spring located at Tatta Pani, Kotli, Pakistan (35°47'N, 74°54'E) and kept at 4 °C before usage. The samples were serially diluted to isolate bacterial strains. Among various colonies, a strain, designated as NCCP-1340^T, was recovered on International *Streptomyces* Project (ISP) 2 agar medium (Shirling & Gottlieb, 1966) at 37 °C after 48 h. The strain was streaked repeatedly on ISP 2 medium until pure colonies were obtained. Purified strain was stored in glycerol at -80 °C for further work. *N. daedukensis* MDN22^T (=KCTC 19601^T), *N. jensenii* DSM 20641^T (=KCTC 9134^T) and *N. mesophilus* MSL-22^T (=KCTC 19310^T) were obtained from Korean Collection for Type Cultures (KCTC, Republic of Korea), *Nocardioides iriomotensis* IR27-S3^T (=JCM 17985^T) was obtained from Japan Collection of Microorganisms (JCM, Japan), and they were used as reference strains in these studies.

To optimize the growth on various media, strain NCCP-1340^T was grown on ISP 2, oatmeal agar (ISP 3), tryptic soy agar (TSA, Becton Dickinson), R2A (Becton Dickinson) and nutrient agar (Becton Dickinson). The colony morphology of strain NCCP-1340^T was observed on ISP 2 medium at 37 °C after 3 days. The cells were observed using phase-contrast microscopy (BH-2; Olympus) and further detailed morphology under scanning electron microscopy (QUANTA 200; FEI). Gram staining was carried out using the standard Gram reaction. Growth at various temperatures (4, 10, 15, 20, 28, 30, 33, 37, 40, 45, 50, 55 and 60 °C) was observed on ISP 2 medium for 2 weeks. The pH range for growth was tested at pH 4.0-11.0 (in 1.0 pH unit intervals) using the buffer system described by P. Xu et al. (2005) at 37 °C for 4 days in tryptic soy broth (TSB; Becton Dickinson). Tolerance to NaCl (0 to 20 %, w/v, with 1 % increment) was investigated on ISP 2 medium by incubating at 37 °C for 10 days. Catalase and oxidase activities were determined as described by Kovacs (1956). Growth under anaerobic conditions was determined on nutrient agar supplemented with or without 0.1 % nitrate by using the GasPak Anaerobic Systems (BBL) according to the manufacturer's instructions.

The biochemical, enzymatic activities and utilization of sole carbon and nitrogen source were determined according to the manufacturer's instruction by using

API ZYM, API 20E, API 50CH, API 20NE strip (bioMérieux, France), and also Biolog GEN III microplates™, at 37 °C according to the manufacturer's instructions.

16S rRNA gene sequencing and phylogenetic analyses

The preliminary identification of strain NCCP-1340^T was done based on 16S rRNA gene sequencing. Extraction of genomic DNA and PCR amplification of the 16S rRNA gene of strain NCCP-1340^T was performed using protocol described previously (W. J. Li et al., 2007). The sequence obtained was compared with the sequences of cultured species from the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) (O. S. Kim et al., 2012a). Phylogenetic analysis was performed using the MEGA version 6 software package (Tamura et al., 2013) after multiple alignment of the sequences using CLUSTAL X program (Thompson et al., 1997). Neighbour-joining (Saitou & Nei, 1987) tree was constructed using Kimura-2 parameter model for evolutionary distance matrices (M. Kimura, 1980). To determine the support for each clade, bootstrap analysis was performed with 1000 replications (Joseph Felsenstein, 1985). The validity of the neighbour-joining tree was evaluated using other algorithms: maximum-likelihood (J. Felsenstein, 1981) and maximum parsimony (Fitch, 1971).

DNA-DNA hybridizations and DNA base composition

DNA-DNA relatedness of strain NCCP-1340^T with the reference strains *Nocardioides iriomotensis* IR27-S3^T, *N. daedukensis* MDN22^T and *N. jensenii* DSM 20641^T was determined. Total genomic DNA was prepared using the protocols of Marmur (1963) and Pitcher et al. (1989) as described previously (Goris et al., 1998). DNA–DNA hybridization was performed with biotin-labelled probes in 96-micro-well plate (NUNC) and fluorescence measurements were carried out using an HTS7000 Bio Assay Reader (Perkin Elmer) according to Ezaki et al. (1989) and Christensen, Angen, Mutters, Olsen, and Bisgaard (2000b) with modifications by Goris et al. (1998). The hybridization temperature was 40 °C and the hybridizations were performed with eight replications.

The G+C content of the genomic DNA was determined using reversed phase HPLC and DNA of *Escherichia coli* DH5α was used as the reference (M. Mesbah et al., 1989).

Chemotaxonomy characterization

Chemotaxonomic characteristics of strain NCCP-1340^T and the reference strains were determined using standard methods under the same conditions. The whole-

cell hydrolysate was analysed for sugars and diagnostic amino acids in cell wall peptidoglycans according to the procedures described by Hasegawa, Takizawa, and Tanida (1983); Lechevalier and Lechevalier (1970) and S. K. Tang et al. (2009). Polar lipids were extracted as described by Minnikin et al. (1979) and identified by two-dimensional thin layer chromatography (M. Collins & Jones, 1980). Menaquinones were extracted and analyzed using HPLC (M. D. Collins et al., 1977; Kroppenstedt, 1982). Cellular fatty acid analysis was performed by using the Microbial Identification System (MIDI) Sherlock software version 6.1 (method TSBA6 database, (Sasser, 1990). Biomass for cellular fatty acid analysis was obtained from cells grown on tryptic soy agar (BD) at 37 °C for 2 days

4.4.4 Results and discussion

Morphology and phenotypic characterization

Cells of strain NCCP-1340^T were observed to be Gram-staining positive, strictly aerobic, non-motile and non-spore forming rods (Figure 24).

Figure 24. Scanning electron micrograph of cells of strain NCCP-1340^T grown on TSA agar medium at 37 °C for 48 hours.

Colonies of strain NCCP-1340^T on ISP 2 medium grew round in shape and off white in colour. Growth of strain NCCP-1340^T occurred at a temperature range of 20-40 °C with an optimum at 37 °C. No growth was observed at 45 °C or less than 20 °C.

Strain NCCP-1340^T could grow at a pH range of 6.0-8.0 with an optimum pH of 7.0, but no growth was observed at pH 5 or 9. Cells could tolerate 0-8% NaCl concentrations (w/v) with optimum growth at 2 % NaCl. Strain NCCP-1340^T was observed to be negative for arginine dihydrolase, acid production with D-lactose, utilization of mono-methyl-succinate, methyl pyruvate, succinamic acid, L-alaninamide and L-glutamicacid substrates as carbon source whereas all the reference strains were found to be positive under same growth conditions for these characteristics except *N. Jensenii* DSM 20641^T and *N. daedukensis* MDN22^T which were weakly positive for utilization of mono methyl-succinate, succinamic acid, L- alaninamide and methyl pyruvate respectively. In contrast to the reference strains, NCCP-1340^T was observed to be positive for α- glucosidase and *β*-glucosidase. It was also positive for assimilation of mannitol, inositol, sorbitol and showed positive enzyme activity for lipase (C 14), *β*-galactosidase and *N-acetyl- β*-glucosaminidase but all the reference strains (except *N. irimotensis* IR27-S3^T) were found negative(Dastager et al., 2010; Yamamura et al., 2011; J. H. Yoon et al., 2010). Strain NCCP-1340^T also distinguished for positive enzyme activity of alkaline phosphatase, acid phosphatase and trypsin, which were negative in *N. irimotensis* IR27-S3^T. Our isolate showed negative results for indole production and acid production with glycerol in contrast to *N. daedukensis* MDN22^T and *N. irimotensis* IR27-S3^T, which produced positive results for indole production and acid production with glycerol (weak). Several other characteristics which distinguished strain NCCP-1340^T from the reference strains have been enlisted in table 17, while other phenotypic results are mentioned in species description.

Table 17 Differentiating phenotypic and biochemical characteristics of strain NCCP-1340^T in comparison with the type strains of closely related *Nocardioides* species

Characteristics	**NCCP-1340^T**	***N. iriomotensis***	***N. daedukensis***	***N. jensenii***	***N. mesophilus***
NaCl range optimum (w/v)	0-8 (2)	0-7 (2)	0-1 (0)	0-5 (2)	0-20 (2)
Temperature range (optimum)˚C	20- 40 (37)	12-37 (30)	4-37 (30)	18-37 (30)	20-37 (28)
Motility	Non-motile	Non-motile	Non-motile	Non-motile	Motile, without flagella
Arginine dihydrolase	–	+	+	+	+
Nitrate reduction	–	+	+	+	–
Indole production	–	+	+	–	–
Hydrolysis of:					
Urea, esculin	+	+	+	+	–
Gelatin	+	+	w+	+	–
Assimilation of:					
N-acetyl glucosamine	w+	+	+	+	+
Mannitol, inositol, sorbitol	+	+	–	–	–
Amygdalin	+	–	w+	–	+
Acid production:					
Glycerol	–	w+	w+	–	–
Methyl-α-D-glucopyranoside, Arbutin	w+	–	–	–	–
D-lactose	–	+	+	+	+
Fermentation of D-glucose	–	+	+	–	+

Enzyme activity:					
Alkaline phosphatase, acid phosphatase, trypsin	+	–	+	+	+
Esterase (C 4)	+	+	+	–	–
Lipase (C 14)	+	+	–	–	–
Leucine arylamidase	+	+	–	+	+
Napthol-As-BI phosphohydrolase	+	–	–	+	+
β-galactosidase, N-acetyl-*β*-glucosaminidase	+	+	–	–	–
α- glucosidase, *β*-glucosidase	+	–	–	–	–
Substrates utilized as C source:					
Tween 80	+	+	+	–	–
Maltose	+	+	+	+	–
D-mannitol	+	+	–	+	–
D-mannose	–	+	–	+	–
Methyl pyruvate	–	+	w+	+	+
Mono-methyl-succinate, Succinamic acid, L-alaninamide	–	+	+	w +	+
L-glutamic acid	–	+	+	+	–
Glycyl-L-aspartic acid	w+	+	+	+	+

+, Positive; w+, weakly positive; –, negative.. All data was collected from this study

Phylogenetic analysis, DNA base composition and DNA–DNA hybridization

The comparison of 16S rRNA gene sequence (1498 nucleotides; DDBJ/EMBL/GenBank accession number LC065367) of strain NCCP-1340^T with sequences of the type strains of closely related species of genus *Nocardioides* showed the highest similarity to *Nocardioides iriomotensis* IR27-S3^T (96.8%) followed by *N. daedukensis* MDN22^T (96.6%), *N. jensenii* DSM 20641^T (96.1%) and *N. daejeonensis* MDN22^T (96.1%), and less than 95% with the other species of genus *Nocardioides* and other taxa of the related genera. A maximum-likelihood phylogenetic tree (Figure 25) showed that strain NCCP-1340^T was closely related to the cluster comprised of *N. iriomotensis* IR27-S3^T, *N. daedukensis* MDN22^T, *N. jensenii* DSM 20641^T, and *N. daejeonensis* MDN22^T with a high bootstrap value (78 %).

Although 16S rRNA gene sequence similarity of NCCP-1340^T was less than 97 %, however, to confirm that strain NCCP-1340^T belongs to a novel species, DNA–DNA hybridizations (DDH) were carried out with type strains of three closely related species. DDH values were 53.2±3.1% with *N. irimotensis* IR27-S3^T, 46.3±2.2% with *N. daedukensis* MDN22^T and 33.1±2.1% with *N. jensenii* DSM 20641^T, which were found to be less than 70 % to support the conclusion that it represents a separate species from the recognized species of genus *Nocardioides* (E. Stackebrandt & Goebel, 1994; Wayne et al., 1987). The DNA G+C content of strain NCCP-1340^T was determined to be 71.8 mol%, which was within the range of other members of the genus (M. Collins et al., 1989; Dastager et al., 2010; Yamamura et al., 2011; J. H. Yoon et al., 2010)).

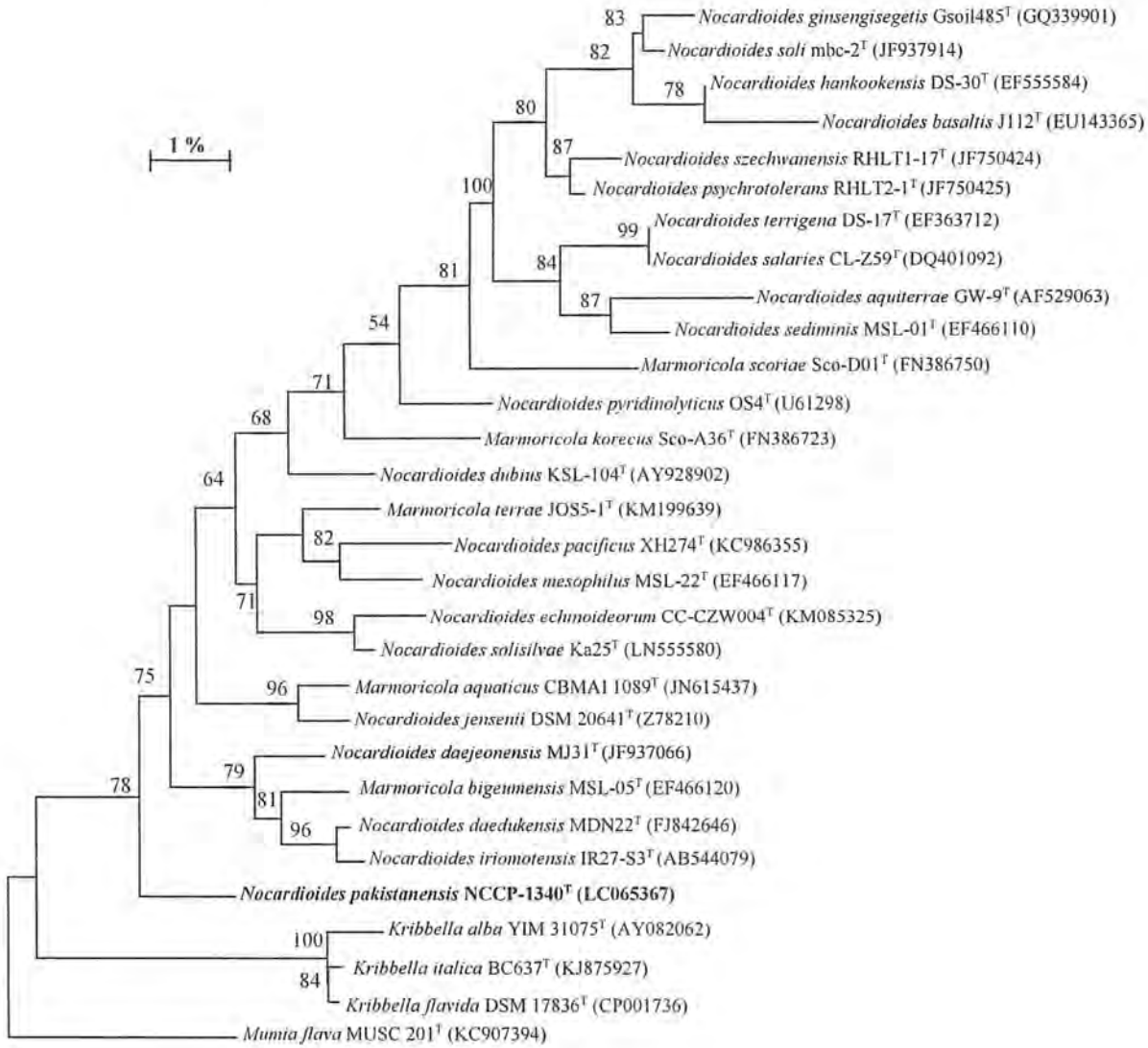


Figure 25. Maximum-likelihood phylogenetic tree showing the inter-relationships of strain NCCP-1340^T with the closely related type strains of the genus *Nocardioides* and the other related genera, inferred from the 16S rRNA gene sequences. Data with gaps and ambiguous nucleotides were removed from the alignment for construction of the tree, which is rooted using using *Mumia flava* MUSC 201^T (KC907394) as an out-group. The tree was generated using the MEGA 6 software package (Tamura et al., 2013) based on a comparison of 1098 nucleotides in the alignment. Bootstrap values (>50 % is shown), expressed as a percentage

Chemotaxonomic analyses

The major cellular fatty acids of strain NCCP-1340^T were determined to be iso-$C_{16:0}$ (23.2%), $C_{17:0}$ (18.3 %), $C_{16:0}$ 10-methyl (10.7 %), iso-$C_{15:0}$ (9.7 %) and $C_{15:0}$ (9.6 %), which are typical cellular fatty acids profile for the members of genus *Nocardioides.*

Table 18 Cellular fatty acid profile (%) of strain NCCP-1340^T in comparison to the type strains of closely related species of the genus *Nocardioides.*

Characteristics	NCCP-1340^T	*N. iriomotensis*	*N. daedukensis*	*N. jensenii*	*N. mesophilus*
$C_{15:0}$	9.6	9.4	6.4	8.5	8.6
iso-$C_{15:0}$	9.7	13.2	8.4	15.3	9.7
$C_{15:1}$ ω6*c*	1.7	1.2	2.6	1.1	1.7
anteiso-$C_{15:0}$	1.7	2.5	3.8	3.1	2.7
iso-$C_{16:0}$	23.2	17.1	20.4	10.6	21.2
$C_{16:0}$ 10-methyl	10.7	4.2	14.7	10.8	16.6
$C_{17:1}$ *cis*-9	6.4	14.0	13.2	12.2	10.7
anteiso-$C_{17:1}$ ω9*c*	3.2	2.9	1.7	1.7	1.2
iso-$C_{17:0}$	5.7	3.8	4.6	5.3	3.8
$C_{17:0}$	18.3	10.2	10.1	8.3	7.4
$C_{18:1}$ ω9*c*	3.9	2.6	4.7	3.5	5.5
Summed Feature 3†	1.2	1.4	1.7	1.6	1.9

All data are obtained in this study. Values are percentages of total fatty acid detected † Summed feature 3 contains one or more of $C_{16:1}$ ω 7*c* / $C_{14:0}$ that cannot be separated by GLC with the MIDI system.

Strain NCCP-1340^T was found to possess a higher amount of $C_{17:0}$ (18.3%) as compared to the reference strains (Table 18). It was also noted that $C_{17:1}$ *cis*-9 (6.4 %) was detected less in the profile of strain NCCP-1340^T, compared to the closely related reference strains (Table 18). Nevertheless, the cellular fatty acids profile obtained for strain NCCP-1340^T is similar to those of other members of the genus *Nocardioides* (Dastager et al., 2010; Yamamura et al., 2011; J. H. Yoon et al., 2010), except some difference of values, which establishes that the strain NCCP-1340^T belongs to a novel species. Major menaquinone system of strain NCCP-1340^T was found to be MK-8(H_4) (98.4 %), whereas minor amount (1.6 %) of MK-9 (H_2) was also observed The presence of MK-8(H_4) as the major isoprenoid quinone, is the characteristic chemotaxonomic feature described for the genus *Nocardioides* (Evtushenko et al., 2015).

The polar lipids were diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and unidentified glycolipids (GL) and polar lipids (PL)

(Fig. S3). Overall, the polar lipids, whole cell sugar content, fatty acid and respiratory quinones of strain NCCP-1340^T are consistent with those of the other members of the genus *Nocardioides* (Dastager et al., 2010; Yamamura et al., 2011; J. H. Yoon et al., 2010). On the basis of physiological, phylogenetic, chemotaxonomic and genomic characteristics, strain NCCP-1340^T is considered to be a new member of the genus *Nocardioides.*

4.4.5 Description of *Nocardioides pakistanensis* sp. nov.

Nocardioides pakistanensis (pa.kis.tan.en'sis. N.L. fem. Adj. pakistanensis pertaining to Pakistan, where the type strain of this organism was isolated).

Cells are Gram-staining positive, aerobic, non-motile, non-spore forming short rods. Colonies are round and off-white in colour. The optimum temperature, pH and NaCl concentration for growth is 37 °C, 7.0 and 2 % (w/v), respectively. Positive for hydrolysis of urea, esculin and gelatine and also assimilation of mannitol, sorbitol, inositol amygdalin and *N*-acetyl glucosamine (weak). Negative for arginine dihydrolase, nitrate reduction, indole production and fermentation of D–glucose. High enzyme activity is observed for alkaline phosphatase, acid phosphatase, esterase (C 4), lipase (C 14), leucine arylamidase, trypsin, napthol-As-BI phosphohydrolase, *β*-galactosidase, *N*-acetyl-*β*-glucosaminidase, α- glucosidase and *β*-glucosidase. Weakly positive for acid production with methyl-α-D-glucopyranoside and arbutin but negative for D- lactose and glycerol. Positive for utilization of Tween 80, maltose, D-mannitol and glycyl-L-aspartic acid (weak) as carbon source and negative for mono-methyl-succinate, D-mannose, succinamic acid, L-alaninamide, L-glutamic acid and methyl pyruvate. The cell wall peptidoglycans contain LL-diaminopimelic as the diagnostic amino acid. The predominant menaquinone is MK-8(H_4). The major cellular fatty acids are iso-$C_{16:0}$, $C_{17:0}$, $C_{16:0}$ 10-methyl, $C_{15:0}$ and iso-$C_{15:0}$. Major polar lipids are diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) phosphatidylinositol (PI) and unidentified glycolipids (GL) and polar lipids (PL). The DNA G+C content is 71.8 mol%.

The type strain is NCCP-1340^T (= DSM 29942^T = JCM 30630^T) which was isolated from soil sample collected from Tatta Pani hot water spring, Kotli, Azad Jammu & Kashmir, Pakistan. GenBank accession number for 16S rRNA gene sequence of strain NCCP-1340^T is LC065367.

CHAPTER 5

Bacterial Community Structure in the Cholistan Desert, Pakistan: Comparative Analysis between Rhizospheric and Non-Rhizospheric Soil Samples

5.1. Abstract

Life is scarce in arid regions and hot deserts due to harsh environmental conditions such as large fluctuations in temperature, high UV irradiation and low precipitation. These extreme environments can influence the microbial community structure in desert ecosystems. In this study, we attempt to study and compare the distribution of bacteria between the rhizospheric and non-rhizospheric soils of the Cholistan desert located in Pakistan. The study involved seven different samples collected from depth of 50 cm and 100 cm from the rhizosphere of pioneer plants and surface sand. Pyrosequencing based on V1-V3 regions of 16S rRNA gene was employed to reveal the bacterial diversity of the samples. The results showed a total of 4,698 OTUs in the seven samples, calculated using ≥ 97% sequence similarity levels. The distribution of OTUs ranged from 437 in NCCP-D2-b to 1124 in NCCP-D1-a. Species richness as indicated by Chao 1 indices ranged from 976 (NCCP-D2-a) to 2,483 (NCCP-D1-a). Besides OTUs belonging to phyla *Proteobacteria* and *Chloroflexi*, the rhizospheric soil samples containing root-remains (NCCP-D1-a/b) were dominated by *Firmicutes, Deinococcus-Thermus Actinobacetria* and *Acidobacteria*, while the phylum *Thermotogae* was present in significant quantity in samples without root-remains (NCCP-D2-a/b). In non-rhizospheric samples (NCCP-D3-a/b), OTUs belonging to phyla *Proteobacteria*, *Chloroflexi. Bacteroidetes* and *Acidobacteria* were present in considerable number. Our study shows that the Cholistan desert area of Pakistan contained a diverse bacterial community, but its distribution is controlled by several edaphic environmental factors.

Keywords: Bacterial community, desert sand, pyrosequencing, rhizosphere, 16S rRNA.

5.2. Introduction

Diverse populations of archaea and bacteria form an integral component of microbial communities in any environment on earth, particularly soils (Fierer & Lennon, 2011; Kuske et al., 2002; Roesch et al., 2007). Their diversity and distributions are dependent upon various factors constituting the environment (Kuske et al., 2002). Edaphic factors such as moisture content and pH of the soil can be correlated with the richness of the environment (Reth, Reichstein, & Falge, 2005). On the other hand, plant communities influence the spatial structure of soil by the growth of their roots and shape the chemical composition of the rhizosphere (Angers & Caron, 1998; Philippot, Raaijmakers, Lemanceau, & van der Putten, 2013). These interactions subsequently enhance the rhizophere mineral flows, thereby linking it to microbial community dynamics (Lu, Murase, Watanabe, Sugimoto, & Kimura, 2004; R. Zhang & Wienhold, 2002). Despite our understanding on plant and soil interactions, it is difficult to interpret and exactly classify all soil microbial communities (Berg & Smalla, 2009; Buée, De Boer, Martin, van Overbeek, & Jurkevitch, 2009). One reason for this is majority of soil microbes are intractable to conventional isolation and culturing methods. The development of next-generation, multiplexed pyrosequencing techniques (Micah Hamady, Walker, Harris, Gold, & Knight, 2008) has partly enable characterizations and comparisons of microbial communities in diverse ecosystems (Lauber, Hamady, Knight, & Fierer, 2009; Roesch et al., 2007), and thereby provide a means for understanding the microbial community structures of complex and underexplored environment such as deserts.

When surface area is concerned than deserts comprises of principal terrestrial ecosystem which are characterized by several environmental extremes such as high aridity, elevated UV radiations and low moistures (Pointing and Belnap, 2012). In desert ecology, environmental stresses limit life to typically the poikilohydric life forms (Angel, Soares, Ungar, & Gillor, 2010; Bates et al., 2011; Fierer & Jackson, 2006; Marschner, Yang, Lieberei, & Crowley, 2001; Neilson et al., 2012; Osman et al., 2016; Salama, EL-Ghani, & El-Tayeh, 2013). The survival of these communities is, therefore, highly depended on the interactions between various biotic and abiotic factors (Cary, McDonald, Barrett, & Cowan, 2010; Jones & Lennon, 2010). Numerous hypotheses suggested rich microbial communities near water and nutrient limited environments because of gathered nutrients between root and soil (Herman, Provencio, Herrera-

Matos, & Torrez, 1995; Schlesinger, Raikes, Hartley, & Cross, 1996). Desert plants may also exert direct influence on microbial communities by providing valuable nutritive resources. These effects, however, tend to be either species-specific (Marschner et al., 2001) or cultivar-specific (van Elsas et al., 2012).

In this study, we employed a multiplexed second generation pyrosequencing technique to estimate the bacterial community structure in the Cholistan desert, Pakistan. Comparison was made between the microbial community in sandy desert soil and rhizosphere associated with desert plants. We further analyzed the influence of physicochemical parameters of soil on distribution of different bacteria at all taxonomic levels. In addition, a brief analysis for the distribution of the phylum *Actinobacteria* among the Cholistan Desert samples is given.

5.3. Materials and Methods

5.3.1. Study Site and Sample Collection

The sampling site for the present study is located on Cholistan desert, Bahawalpur, Pakistan (29.395556 N 71.750278 E; Figure 26).

Figure 26 Map indicating Cholistan desert, Bahawalpur, Pakistan

Soil and rhizosphere samples were collected aseptically in triplicates from a randomly chosen 100 m^2 following standard protocols (S. Wang et al., 2013). The samples collected were transported to laboratory in liquid nitrogen and stored at -80 °C until further processing. A description of the samples is given in Table 19

Table 19 Description of the samples

Sample	Sample type	Sequence Read Archives	Accession number
NCCP-D-0	Wet soil around desert area	SRS822652	SAMN03284242
NCCP-D1-a	Rhizosphere sample (with remains of roots) at depth of 100 cm	SRS825421	SAMN03284243
NCCP-D1-b	Rhizosphere sample (with remains of roots) at depth of 50 cm	SRS825422	SAMN03284244
NCCP-D2-a	Rhizosphere sample (without plant roots) at depth of 100 cm	SRS825423	SAMN03284245
NCCP-D2-b	Rhizosphere sample (without plant roots) at depth of 50 cm	SRS825424	SAMN03284246
NCCP-D3-b	Dry soil from vegetation free desert area at depth of 100 cm	SRS825425	SAMN03284247
NCCP-D3-b	Dry soil from vegetation free desert area at depth of 50 cm	SRS825426	SAMN03284248

Wet soil from 3 different locations around the vicinity of the desert area was collected, mixed and treated as the control sample (NCCP-D-0) for comparative purpose.

5.3.2. Physicochemical Analysis

The soil-pH values and electrical conductivity were calculated by diluting the soil sample with water at a 1:1 (w/v) ratio. pH was confirmed by using a 1:1 (w/v) mixture with 0.01 M $CaCl_2$ soln. The use of 0.01 M $CaCl_2$ tend to mask small differences in salt contents accumulated under limited rainfall in arid regions and under restricted drainage (McLean, 1982). Other physical parameters calculated were carbon and nitrogen percentage, which were done by estimating amount of released CO_2, and NO_2 at 900°C. The cation exchange capacity (CEC) was calculated according to protocols described by (Andrew et al., 2012; Rhoades, 1982a, 1982b). which involved estimation of decrease in Mg^{+2} conc. of a soln. of $MgSO_4$ when it got equilibration with the soil after exchange with Ba^{+2}.

5.3.3. DNA extraction, Amplification and Pyrosequencing

DNAs were extracted using PowerSoil® DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instruction. Described procedures of Hur et al. (2011) were adopted for pyrosequencing analyses of the genomic DNA extracts. Amplifications of the V1-V3 region of the bacterial 16S rRNA gene were done using a C1000 Touch Thermal Cycler (Bio-Rad, CA, USA) and bar-coded fusion primers (8 nucleotide long barcodes) (Table S1). The PCR mix

contained 100 ng template DNA, 5 µL 10x *ExTaq* buffer, 0.2 mM of each dNTPs, 0.5 µM of each primer, and 2 units *ExTaq DNA Polymerase* (Takara, Otsu, Japan) in a 50 µl reaction. Denaturation was done at 94 °C for 5 min, the amplification reaction was carried out using a Touch-down program for 10 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 45 s) and extension (72 °C, 90 s) with subsequent decrease in annealing temperature by 0.5 °C, followed by an additional 20 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 45 s), extension (72 °C, 90 s). The amplified products were checked on 2 % agarose gel electrophoresis and visualized using the Gel Doc system (Bio-Rad, USA). Amplicons were purified using a QIAquick PCR purification kit (Qiagen, CA, USA) and quantified using a PicoGreen dsDNA Assay kit (Invitrogen, CA, USA). Equimolar concentrations of each amplicon from different samples were pooled, purified using AMPure bead kit (Agencourt Bioscience, MA, USA) and amplified on sequencing beads by emulsion PCR. Pyrosequencing reactions were performed using a Roche GS FLX Titanium system at ChunLab, Inc (Seoul, Korea) according to the manufacturer's instructions.

5.3.4. Statistical Analysis

All reads shorter than 300 bp or those containing any ambiguous base or incorrect primer sequences were excluded from the dataset. Operational taxonomic units (OTUs) were defined by using the CD-HIT program (W. Li & Godzik, 2006) at a similarity cut-off level of ≥97 %. Two-way analysis of variance (ANOVA) on microbial diversity indices and relative sequence abundances of important phyla against physicochemical properties was performed. For significant difference among the different sites, pairwise test was performed using SPSS statistics (IBM SPSS version 20.0 software, IBM Corp).

Species richness was estimated by employing Chao 1 and ACE methods, while quantitative species richness/evenness by nonparametric Shannon and Simpson's indices in Mothur package v.1.8.0 (Schloss et al., 2009). Each pyrosequencing read was taxonomically assigned by using a combination of BLASTN-based searches (Altschul, Gish, Miller, Myers, & Lipman, 1990) and pairwise similarity comparisons in EzTaxon database (O. S. Kim et al., 2012b). Relative abundance of each taxon was clustered by using Bionumerics version 6.0 software (Applied Maths, Sint Martens Latem, Belgium) while unweighted UniFrac Matrix (M. Hamady, Lozupone, & Knight, 2010) was used

to visualize bacterial community in a Principal Coordinates Analysis (PCoA) plot. An Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram was generated using Fast UniFrac analysis (M. Hamady et al., 2010). Canonical correspondence analysis (CCA) was used to visualize the pattern of soil bacterial community variation and distribution along the measured environmental variables.

5.4. Results

5.4.1. Physicochemical Characteristics of the Samples

Detailed description of the physicochemical parameters of the samples is provided in Table 20. All the samples showed a low concentration of C, N and K. Carbon level within similar samples were same (NCCP-D1-a/b and NCCP-D3-a/b), except for rhizospheric samples without root remains (NCCP-D2-a and NCCP-D2-b). Ammonia concentration is lowest in control (0.1μg/L) and highest in NCCP-D1-a/b. Nitrite concentration is highest in NCCP-D1-a/b and lowest in control. Potassium and Magnesium concentration are highest in NCCP-D3-a/b and NCCP-D2-b respectively. The pH values for all the samples are slightly alkaline and ranged from 7.1 in NCCP-D3-b to 7.7 in NCCP-D2-b.

Table 20 Physicochemical parameters of the sampling sites

Sample	C mg L^{-1}	NH_4 μg g^{-1} dry soil	NO^{2-} μg g^{-1} dry soil	Mg mg L^{-1}	K mg L^{-1}	pH
NCCP-D-0	10	0.1	0.4	2.1	33	7.1
NCCP-D1-a	15	2.3	2.6	2	44	7.5
NCCP-D1-b	15	2.3	2.6	2	44	7.4
NCCP-D2-a	15	1.3	1.6	2	44	7.5
NCCP-D2-b	12	1.1	2.5	3	50	7.7
NCCP-D3-a	14	1.1	1.4	1.7	52	7.4
NCCP-D3-b	14	1.2	1.6	1.5	52	7.1

5.4.2. Pyrosequencing Data and Rarefaction Analysis

The pyrosequencing data was based on the amplifications of V1-V3 region of 16S rRNA genes from the total DNA extracted from desert samples. After excluding 20,952 low quality reads and 8,092 dropped reads, total number of sequences obtained

from the seven samples were 20,952 (Table 21) with an average length ranging from 470 to 560 bases after trimming.

Table 21 Metadata for total number of reads and dropped reads of all samples

Samples*	Quality Reads	Dropped Reads	Chimeric score of dropped reads
NCCP-D-0	4190	851	2918
NCCP-D1-a	2912	1095	2223
NCCP-D1-b	2911	1197	2506
NCCP-D2-a	2677	1243	3098
NCCP-D2-b	2784	1313	3708
NCCP-D3-a	2683	1158	2479
NCCP-D3-b	2795	1235	3741

NCCP = National culture collection of Pakistan
Amplification of the V1-V3 region of the 16S rRNA genes were done using the primer pairs: GAGTTTGATCMTGGCTCAG (forward) and WTTACCGCGGCTGCTGG (reverse)

If the control is excluded, the highest number of sequences was obtained in NCCP-D1-a (2,912 sequences) and the least in NCCP-D2-a (2,677). Rarefaction curves for all the samples fail to reach an asymptote and therefore more samples will be necessary to determine complete microbial diversity of the samples.

5.4.3 Bacterial Diversity and Species Richness

Measurement of sample richness was based on OTUs (sequences with similarity cut-off level of $\geq$ 97%) that represent the potential number of species present in a community. The OTUs, ranging from 437 (NCCP-D2-b) to 1124 (NCCP-D1-a), were discerned in the six samples, while average number of OTUs for control sample was 385 (Table 22). The alpha diversity indices of the desert samples were calculated using the Chao1 estimator, Jackknife, ACE, Simpson and the Shannon diversity indices (Table 22).

Table 22 Alpha diversity indices of the Cholistan desert samples

Samples	Valid reads	OTUs	Chao 1	Jackknife	ACE	Shannon	Simpson
NCCP-D-0	4190	385	799.306	1036.922	1122.164	3.96	0.06
NCCP-D1-a	2912	1124	2482.953	3684.148	4044.267	6.14	0.01
NCCP-D1-b	2911	1014	2235.706	2806.511	3474.43	5.93	0.01
NCCP-D2-a	2678	442	976.234	1201.685	1584.542	4.55	0.03
NCCP-D2-b	2784	437	1102	1453.315	1702.534	4.50	0.03
NCCP-D3-a	2685	744	1642.193	2277.132	2505.786	5.66	0.01
NCCP-D3-b	2795	552	1182.373	1504.435	1613.255	5.18	0.01

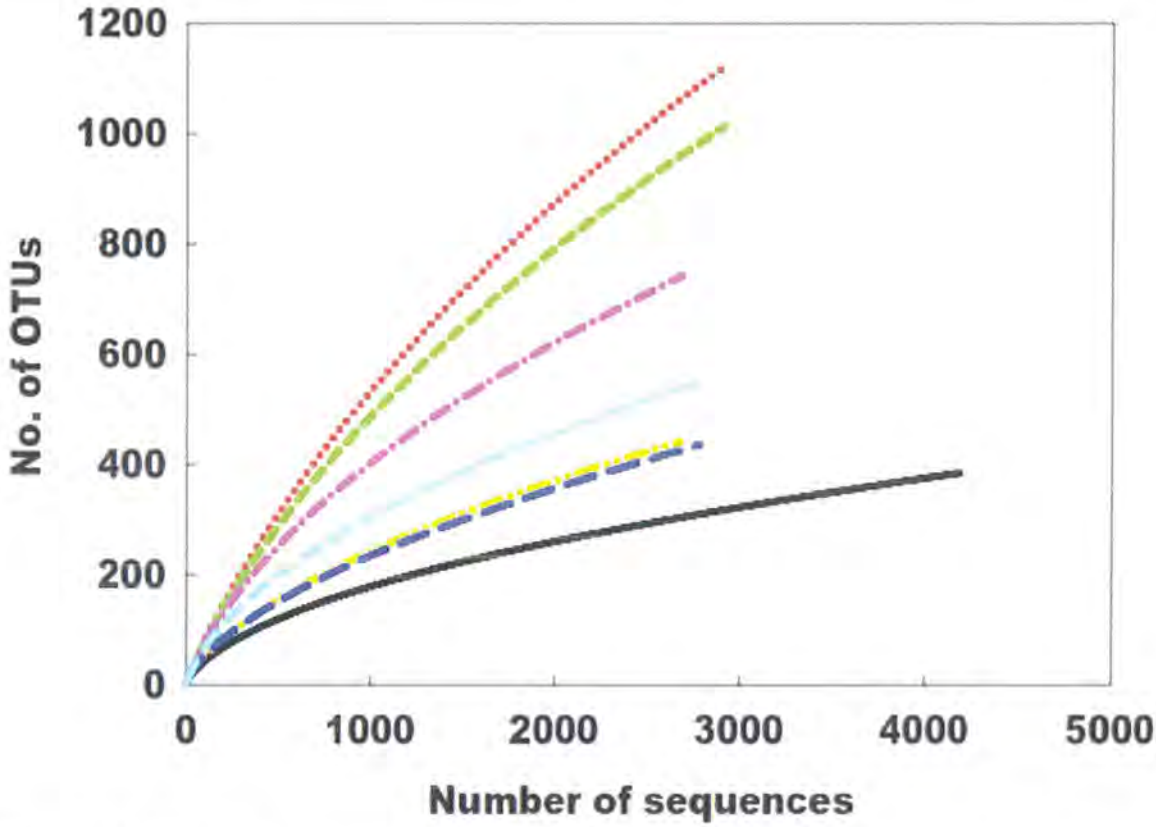


Figure 27 Rarefaction curve determined by CD-HIT method showing sampling accuracy. Pooled samples showed relatively even sampling of rhizospheric soils (NCCP-D1-a/b; NCCP-D2-a/b) and desert soil samples (NCCP-D3-a/b). (———) denote NCCP-D1-0, (.....) NCCP-D1-a, (— — —)NCCP-D1-b, (——) NCCP-D2-a, (– – – –) NCCP-D2-b, (–·–·–) NCCP-D3-a and ( ) NCCP-D3-b.

The richness, assessed with the Chao1 estimator among the sequences, showed that the diversity can vary significantly among rhizospheric and non-rhizospheric soil samples in the same desert. The richness, estimated by Chao1, ranged from 976.234

(NCCP-D2-a) to 2,482.953 (NCCP-D1-a). The highest diversity level according to Shannon indices is observed in NCCP-D1-a (6.14) followed by NCCP-D1-b (5.93) indicating maximum diversity in rhizospheric samples with root-remains. Most dominant phyla among all the rhizospheric and non-rhizospheric samples were *Proteobacteria* and *Chloroflexi*. Besides these two phyla, the prominent phyla present in samples NCCP-D1-a and NCCP-D1-b were *Firmicutes, Deinococcus-thermus*, *Actinobacteria* and *Acidobacteria* (Figure 27).

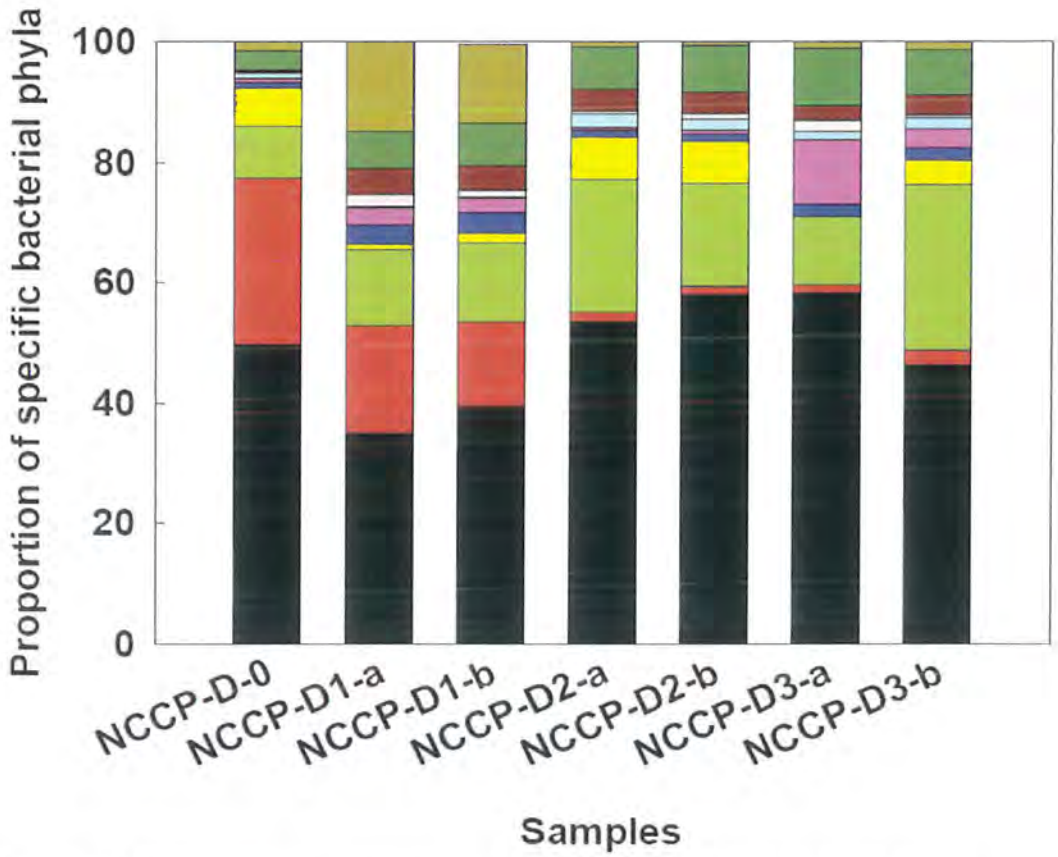


Figure 28 Taxonomic classification of different sites at phylum level. (▬) *Proteobacteria*, (▬) *Firmicutes*, (▬) *Chloroflexi*, (▬) *Thermotogae*, (▬) *Acidobacteria*, (▬) *Bacteroidetes*, (▬) *Chlorobi*, (▬) *Planctomycetes*, (▬) *Deinococcus-Thermus*, (▬) *Actinobacteria* and (▬) remaining phyla (<1%).

5.4.4. Beta Diversity Analysis for Bacterial Community

Pairwise comparison between different samples showed significant difference among diversity of different sites (Table 23).

Table 23 Pair wise comparison between studied sites at phylum level

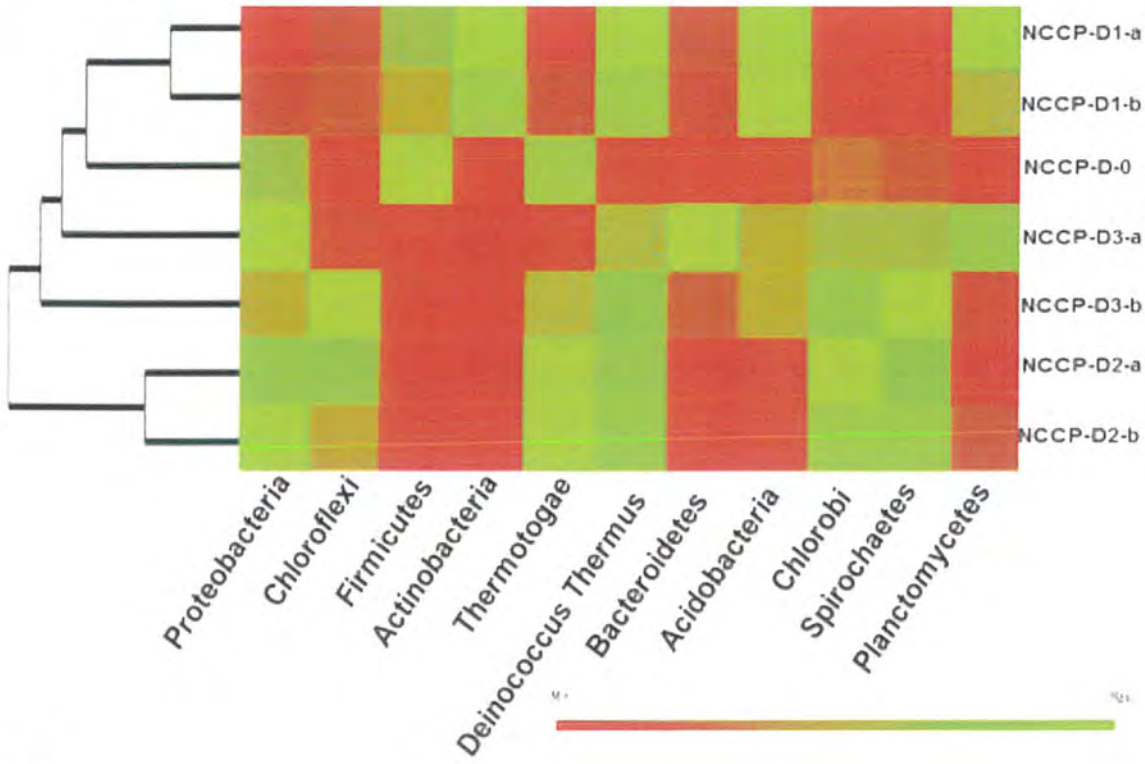
Sampling sites*		Mean Difference (I-J)	Significance	95% Confidence Interval for Difference	
(I)	(J)			Lower Bound	Upper Bound
NCCP-D1-a/b	NCCP-D2-a/b	-0.125	0.027	-0.655	0.425
	NCCP-D3-a/b	-0.171	0.010	-0.822	0.251
NCCP-D2-a/b	NCCP-D1-a/b	-0.155	0.024	-0.821	0.351
	NCCP-D3-a/b	0.205	0.027	-0.446	0.644
NCCP-D3-a/b	NCCP-D1-a/b	0.241	0.010	-0.310	0.621
	NCCP-D2-a/b	0.154	0.024	-0.358	0.706

*Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

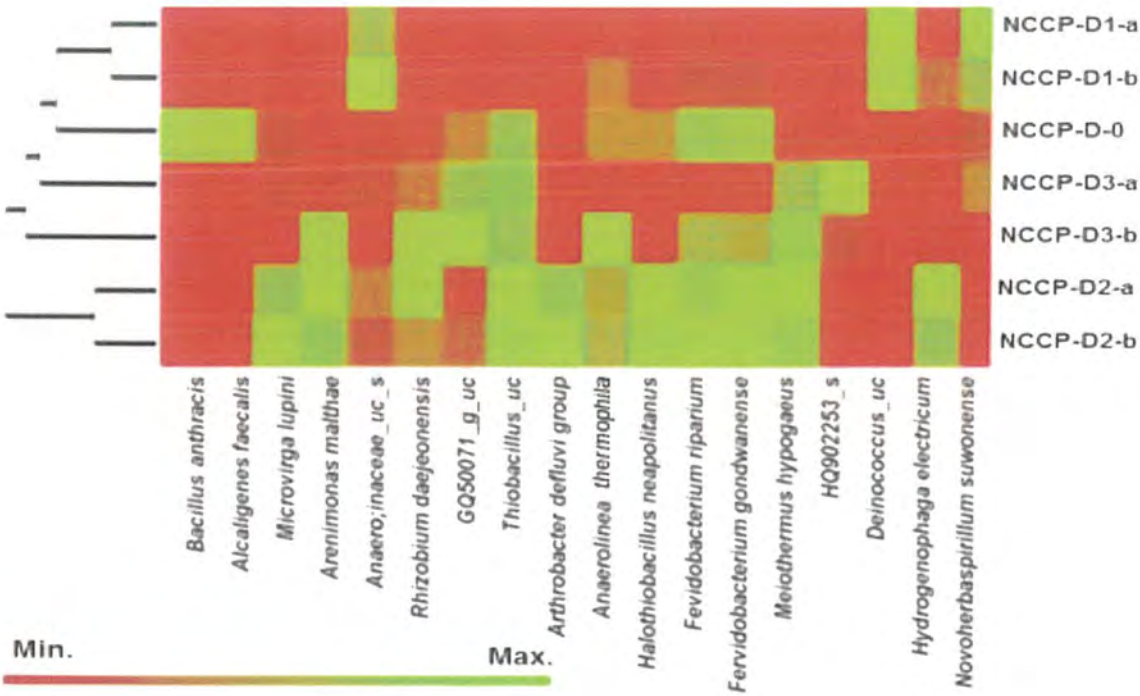
The difference in diversity of 11 dominant microbial phyla is represented in a heat map (Figure 29a). In samples NCCP-D1-a and NCCP-D1-b, the most dominant phyla were *Acidobacteria, Actinobacteria, Deinococcus- thermus* and *Planctomycetes*. In NCCP-D2-a and NCCP-D2-b, phyla *Thermotogae, Chlorobi, Deinococcus-thermus, Spirochaetes, Proteobacteria* and *Chloroflexi* represented the dominant groups. For the vegetation, free desert soils, the distribution of dominant phyla except *Spirochaetes, Chlorobi* and *Deinococcus-thermus* were not the same.

At NCCP-D3-a, phyla *Proteobacteria, Bacteroidetes* and *Planctomycetes* were present in considerable number, while *Chloroflexi* and *Thermotogae* were present abundantly in NCCP-D3-b. Heat maps generated at species level for most dominant species at all sites showed that for samples NCCP-D1-a and NCCP-D1-b, the dominant OTUs belonged to *Deinococcus*_uc, *Novoherbaspirillim suwonense* and *Anaeroliaceae*_uc_s (Figure 29b). While NCCP-D3-a possessed the unclassified species HQ902253_s as the most dominant OTUs, NCCP-D3-b was represented by *Anaerolinea thermophila, Arenimonas malthae* and *Rhizobium daejeonensis*. The species *Meiothermus hypogaeus, Thiobacillus_uc* and an unclassified species GQ50071_g_uc were common between NCCP-D3-a and NCCP-D3-b. Unlike the other two sites, samples NCCP-D2-a and NCCP-D2-b possessed various common dominant species including *Anaerolinea thermophile, Arenimonas malthae, Arthrobacter defluvi* group, *Fervidobacterium gondwanense, Fervidobacterium roparium, Halothiobacillus*

neapolitanus, *Hydrogenophaga electricum*, *Meiothermus hypogaeus*, *Microvirga lupine* and *Thiobacillus_uc* (Figure 29b).



(29a)



(29b)

Figure 29 (a) Heat Map Analysis at phylum level. (b) Heat Map Analysis at species level

5.4.5. Distribution of phylum *Actinobacteria* in Cholistan desert samples

The phylum *Actinobacteria* was considered for distribution analysis as there is much fluctuation in its distribution among the different desert samples while representing a dominant soil bacterial group (Figure 30).

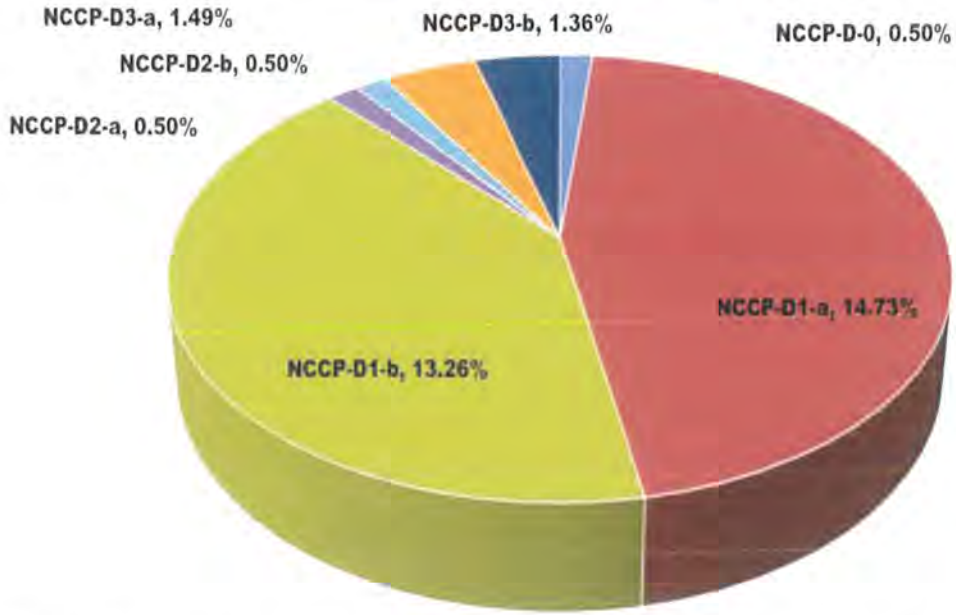


Figure 30 Percent distribution of OTUs belonging to phylum *Actinobacteria* in Cholistan desert samples

Highest number of OTUs for phylum *Actinobacteria* was observed in the rhizospheric samples NCCP-H1-a and NCCP-H1-b, representing 14.73 % and 13.26 % of total OTUs respectively. The distribution was however negligible in the remaining samples, i.e. 1.4% in NCCP-D3-a, 1.3% in NCCP-D3-b and 0.5% each in NCCP-D2-a, NCCP-D2-b. A review of the actinobacterial taxonomic groups distributed in sample NCCP-D1-a (with highest number of OTUs for actinobacteria) indicated that the major groups representing phylum *Actinobacteria* were from the class *Rubrobacteria* (183 OTUs) followed by class *Actinobacteria* (149) . Among the class *Rubrobacteria*, the highest number of OTUs was represented by genus *Rubrobacter* (94 OTUs) which are all unclassified at species level. The remaining OTUs of class *Rubrobacteria* are unclassified at various levels of bacterial hierarchy (unclassified order, family, genus or species). Among the class *Actinobacteria*, majority of the OTUs belonged to the order *Micrococcales* (61 OTUs) followed closely by *Propionibacteriales* (57). Unlike the class *Rubrobacter*, less number of OTUs in class *Actinobacteria* was unclassified. However, considering the overall distribution, the unclassified actinobacteria constituted a great bulk of the total OTUs (> 50%) (Table 24).

Table 24 List of the *Actinobacterial* taxonomic groups (out of 429 OTUs) classified to the species level, identified according to the results if the 16S rRNA pyrosequencing, in the rhizosphaeric desert so21dqil of Cholistan, Pakistan. Numbers within the parentheses indicate the number of OTUs obtained. Difference in total sum of OTUs is due to the omission of few OTUs which belonged to unclassified taxonomic group and therefore have not been mentioned in the list.

Class	Order	Family	Genus	Species
Actinobacteria_c (149)	*Propionibacteriales (57)*			
	Micrococcales (61)	*Micrococcaceae (54)*	*Arthrobacter (49)*	*Arthrobacter globiformis (16)*
				Arthrobacter humicola (10)
				Arthrobacter defluvii group (4)
				Arthrobacter pascens (2)
				Arthrobacter oryzae (5)
				Arthrobacter_uc (6)
				Arthrobacter flavus (2)
				Arthrobacter equi (1)
				Arthrobacter phenanthrenivorans (1)
				Arthrobacter oxydans group (1)
				Arthrobacter siccitolerans (1)
			Sinomonas (1)	*Sinomonas albida (1)*
			Micrococcaceae_uc (2)	
			Unclassified genus (2)	
		Microbacteriaceae (4)	*Naasia (3)*	*Naasia_uc (2)*
				Naasia aerilata (1)
		Microbacteriaceae_uc (1)	*Cellulomonadaceae (2)*	
		Cellulomonadaceae_uc (1)	*Cellulomonas (1)*	
			Cellulomonas_uc (1)	
		Intrasporangiaceae (1)	*Phycicoccus (1)*	*Phycicoccus_uc (1)*
	Streptosporangiales (7)	*Nocardiopsaceae (4)*	*Nocardiopsis (4)*	*Nocardiopsis terrae (4)*

		Streptosporangiaceae (2)	*Planobispora (1)*	*Planobispora rosea (1)*
			Streptosporangiaceae_uc (1)	
		Thermomonosporaceae (1)	*Thermomonosporaceae_uc (1)*	
	Frankiales (30)	*Geodermatophilaceae (29)*	*Blastococcus (16)*	*Blastococcus saxobsidens (3)*
				Blastococcus massiliensis (3)
				Unclassified specie (4)
				Blastococcus jejuensis (4)
				Blastococcus_uc (2)
			Geodermatophilus (12)	*Geodermatophilus obscurus (8)*
				Geodermatophilus_uc (2)
				Geodermatophilus ruber (1)
				Geodermatophilus tzadiensis (1)
			Modestobacter (1)	*Modestobacter marinus (1)*
		Frankiaceae (1)	*Frankiaceae_uc (1)*	
	Streptomycetales (7)	*Streptomycetaceae (6)*	*Streptomyces (6)*	*Streptomyces spinoverrucosus (2)*
				Streptomyces macrosporus (2)
				Streptomyces fradiae (1)
				Streptomyces cellulosae group (1)
		Streptomycetales_uc (1)		
	Corynebacteriales (15)	*Nocardiaceae (13)*	*Rhodococcus (13)*	*Rhodococcus globerulus (10)*
				Rhodococcus baikonurensis group (3)
		Mycobacteriaceae (2)	*Mycobacterium (2)*	*Mycobacterium moriokaense (1)*
				Mycobacterium tusciae (1)
	Micromonosporales (1)	*Micromonosporaceae (1)*	*Micromonosporaceae_uc (1)*	
	Kineosporiales (1)	*Kineosporiaceae (1)*	*Kineosporia (1)*	*Kineosporia_uc (1)*
	Pseudonocardiales (4)	*Pseudonocardiaceae (4)*	*Pseudonocardiaceae_uc (3)*	
			Lechevalieria (1)	*Lechevalieria deserti group (1)*
	Unclassified order (1)			
Rubrobacteria (183)	*Rubrobacterales (102)*	*Rubrobacteraceae (102)*	*Rubrobacter (94)*	*Unclassified species (94)*

			Rubrobacteraceae_uc (8)	
	Gaiellales (12)	*Gaiellaceae (11)*	*Gaiellaceae_uc (1)*	
			Gaiella (10)	*Unclassified species (10)*
		Gaiellales_uc (1)		
	Unclassified order (31)			
Thermoleophilia (26)	*Solirubrobacterales (22)*	*Unclassified family (8)*		
		Conexibacteraceae (4)	*Conexibacter (3)*	*Conexibacter_uc (3)*
			Conexibacteraceae_uc (1)	
		Unclassified family (4)		
		Solirubrobacteraceae (3)	*Solirubrobacter (2)*	*Solirubrobacter_uc (1)*
			Solirubrobacteraceae_uc (1)	
Acidimicrobiia (39)	*Acidimicrobiales (31)*	*Acidimicrobiaceae (25)*	*Unclassified genus (25)*	
		Iamiaceae (3)	*Unclassified genus (3)*	
		Ilumatobacter_f (2)	*Unclassified genus (2)*	
Nitriliruptoria (2)	*Euzebyales (2)*	*Euzebyaceae (2)*	*Unclassified genus (2)*	
FJ478799_c (27)				
Actinobacteria_uc (1)				
EU374107_c (1)				
OPB41 (2)				
Coriobacteriia (1)				

5.5. Discussion

Previous studies on deserts have underestimated the taxonomic diversity of microorganisms in desert soils (Connon, Lester, Shafaat, Obenhuber, & Ponce, 2007; Garcia-Pichel, Johnson, Youngkin, & Belnap, 2003; Nagy, Perez, & Garcia-Pichel, 2005; Navarro-Gonzalez et al., 2003; Price, Dehal, & Arkin, 2010). The main reason behind this underestimation was the dependence of diversity studies on standard cloning methods, thereby giving unassertive results (Garcia-Pichel et al., 2003; Navarro-Gonzalez et al., 2003; Price et al., 2010). During our study on Cholistan Desert, both alpha and beta diversity indices were much higher than previous evaluations for desert soil. Few recent studies also reported similar findings, and like

other extreme environments, deserts were found to possess both abundant and diverse bacterial diversity (An, Couteau, Luo, Neveu, & DuBow, 2013; Andrew et al., 2012; Prashar, Kapoor, & Sachdeva, 2014). Analysis of the rarefaction curve (Figure 27), however, indicated our speculated results comprised of less than half of the actual species diversity. These varied bacterial communities have possibly vital claims for the cornucopia of bacterial pathogens in desert soils (van Elsas et al., 2012) and are expected to play an important role in all biological mechanisms undergoing within desert soils e.g., soil formation, nutrient cycling, plant diversity, and primary productivity of desert environments (Puente, Li, & Bashan, 2009). Our strategy to study the samples at different depth proved to be very supportive and verified that the main driver for existence of a specific microbial diversity depended upon the physiological condition of the local environments (Figures 31).

Heat maps generated by unweighted-pair group method using arithmetic means (UPGMA) showed that OTUs were significantly associated with the principal location of samples e.g., the OTUs for species *Hydrogenophaga electricum* and *Anaerolinae thermophila* were abundantly present in NCCP-D1-b as compared to NCCP-D1-a. Similarly, OTUs belonging to *Arenimonas malthae*, *Rhizobium daejeonensis* and *Anaerolinae thermophile* were significant in number in NCCP-D3-b as compared to NCCP-D3-a. Similar observation was recorded by Andrew et al. (2012) where local soil characteristics shaped microbial communities in the bulk soil and rhizosphere. At phylum level, *Proteobacteria* was found to be most dominant group in all studied samples of Cholistan Desert. This observation was in contrast from the studies on Canadian, Alaskan and Siberian Arctic soils where *Acidobacteria* was most dominant phylum (Campbell, Polson, Hanson, Mack, & Schuur, 2010; Chu et al., 2010; Mannisto, Kurhela, Tiirola, & Haggblom, 2013; Neufeld & Mohn, 2005; Wallenstein, McMahon, & Schimel, 2009). The difference in the distribution might be related with the soil pH as our samples were neutral to slightly alkaline as compared to the acidic pH of the Canadian, Alaskan and Siberian soil. On the other hand, the dominance of phylum *Proteobacteria* over *Acidobacteria* was reported in the polar Arctic deserts (S. H. Lee, Jang, Chae, Choi, & Kang, 2013; McCann et al., 2016).

Copious of physical and chemical soil properties were associated with the variances between different bacterial communities as measured by CCA analysis (Figure 32).

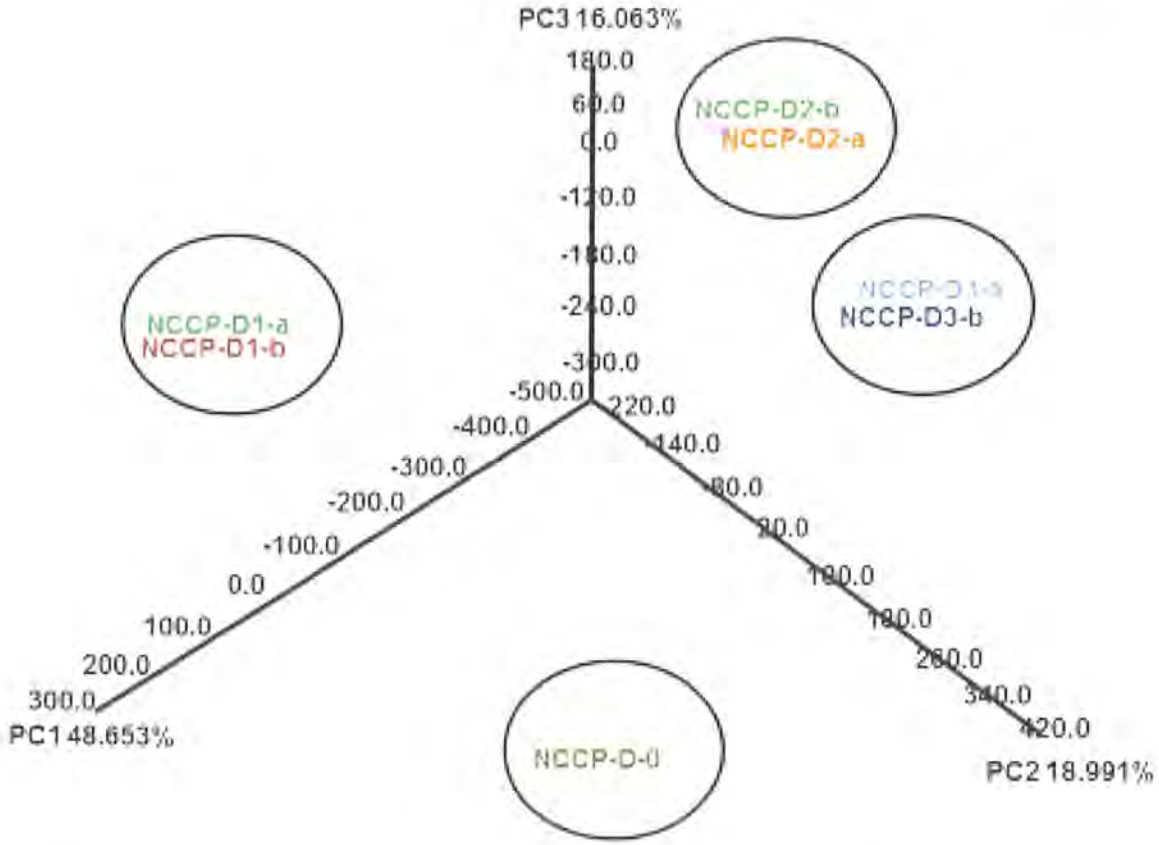


Figure 31 Three dimensional PCoA analysis (PC1 148.653%, PC2 18.991% and PC3 16.063%) showing clustering of sampling sites according to OTUs present at species level. Samples collected from rhizospheric soil with root-remains (NCCP-D1-a/b) do not share same cluster with rhizospheric soil without root-remains (NCCP-D2-a/b) or with native soil of desert (NCCP-D3-a/b). Control doesn't fall near any samples in the three-dimensional space.

Positive correlation of pH and bacterial diversity has been previously deliberated by Rao et al. (2016) and Chu et al. (2010). In our study, the most significant impact for the rhizospheric soils (NCCP-D1-a/b, NCCP-D2-a/b) was inserted by NO_2 and NH_4. Bacterial diversity at non-rhizospheric desert soils (NCCP-D3-a/b) was influenced by K^+ and Mg^{++} (Figure 32). Unlike the above parameters, percent carbon did not influence the selection of microbial population on either of the rhizospheric or non-rhizospheric samples. In other words, nitrogen didn't have any significant influence in designing bacterial community of native soil of desert (NCCP-D3-a/b) while K^+ and Mg^{++} didn't have significant impact on soil communities of rhizospheric soils (NCCP-D1-a/b or NCCP-D2-a/b).

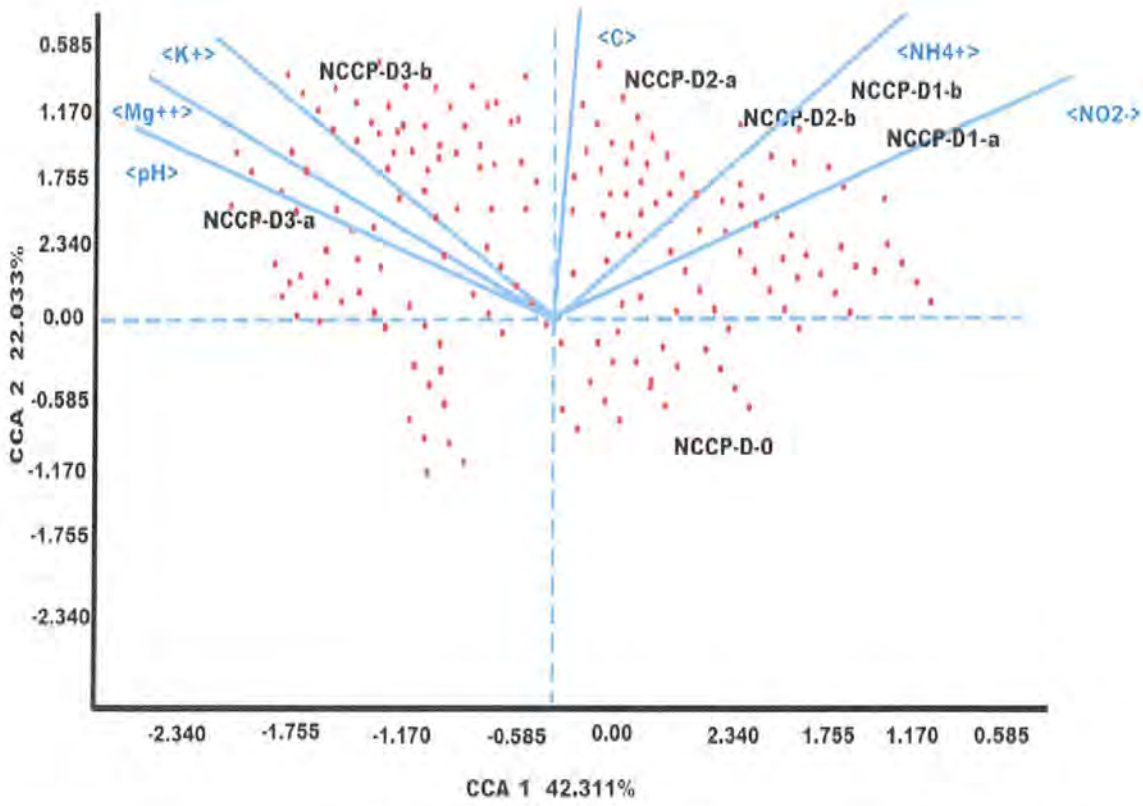


Figure 32 CCA plot showing effect of physicochemical parameters (pH, Mg, K, NH_4, NO_2 and C on distribution of different bacterial genera's.

In our study, the diversity of bacteria especially the phylum *Actinobacteria* was not significantly different at the different depth levels of the soil indicating that diversity in arid environments is not temperature-dependent. Bouizgarne and Aouamar (2014) reported that almost 30 % of rhizospheric microbial community comprises of phylum *Actinobacteria*. Similarly several strains of phylum *Actinobacteria* were isolated from Algerian Sahara desert (Goudjal et al. (2013). Trujillo, Riesco, Benito, and Carro (2015) also cited a list of rhizospheric, endophytic and plant associated Actinobacterial isolates and at the same time, W. D. Orsi et al. (2016) reported that pyrosequencing technique and omics should be utilized for easy understanding of actinobacterial diversity from habitats like deserts and others, which were also endorsed and reviewed by Qin, Li, Dastager, and Hozzein (2016). The study showed that available carbon and nitrogen are limiting factor in driving local microbial diversity in rhizospheric soils where as local soil flora is more influenced by other minerals.

CHAPTER 6

Diversity and distribution of thermophilic bacteria in Hot Springs of Pakistan

6.1. Abstract

Chilas and Hunza areas, located in the Main Mantle Thrust and Main Karakoram Thrust of the Himalayas, host a range of geochemically diverse hot springs. This Himalayan geothermal region encompassed sites ranging in temperature from 60 to 95 °C, in pH from 6.2 to 9.4, and in mineralogy from bicarbonates in Tato Field, sulfates in Tatta Pani to mixed type in Murtazaabad. Microbial diversity remained largely unexplored in these geothermal springs. In this study, we report a comprehensive, culture-independent survey of microbial communities in nine samples from these geothermal fields by employing a bar-coded pyrosequencing technique. The dominant taxa in these geothermal fields were depended on temperature, pH and physicochemical parameters of the geothermal sites. Murtazaabad hot springs with relatively higher temperature (90-95 °C) favored the growth of phylum *Thermotogae*, whereas Tatta Pani thermal spring site TP-H3-b (60 °C) favored the phylum *Proteobacteria*. At sites with low silica and high temperature, OTUs belonging to phylum *Chloroflexi* were dominant. Deep water areas of the Murtazaabad hot water spring favored the sulfur-reducing bacteria. Of the total OTUs obtained from these samples, 40.1 % was unclassified or uncharacterized suggesting the presence of many undiscovered and unexplored microbiota . This study has, therefore, provided novel insights into the nature of the ecological interactions among important taxa in these communities, which in turn will help in determining the future course of studies of these sites.

Key words: Main Karakoram Thrust, Hot springs, Pyrosequencing, 16S rRNA gene, Thermophilic bacterial diversity

6.2. Introduction

Bacteria inhabiting extreme environments play an important role in energy metabolism and matter cycling (L. Cai, Ye, Tong, Lok, & Zhang, 2013). Consistent efforts are still on to decipher the ecological role of microbial community in thermal springs(Carrine E Blank et al., 2002) (L. Liu et al., 2016). These include the community structure determination on Tibetan and Tengchong thermal springs (China), Nakabusa hot springs (Japan) (Kubo, Knittel, Amann, Fukui, & Matsuura, 2011), Siloam hot water springs (South Africa) Andean Mountain hot water springs (Colombia), Solfataric Fields (Iceland) (Skirnisdottir et al., 2000) Great Basin Hot springs and Yellowstone National Park, (United States) (L. Liu et al., 2016) (Leis et al., 2015). While some of these studies were performed to understand the geochemistry, geomicrobiology and bioenergetics of biogeothermal system (Spear, Walker, & Pace, 2005b), others were to determine its biotechnological potential (L. Liu et al., 2016). Interestingly, it was found that chemical type of hot springs influence the organization of microbial community (Berelson et al., 2011). In sulfur-rich environments with an anaerobic zones, sulfur bacteria were responsible for energy production which in turn help in maintenance of community structure (Zaigham et al., 2009).

Hot springs harbor abundant microbial temperature-tolerant enzymes that have many industrial implications (Mirete, Morgante, & González-Pastor, 2016; Unsworth, van der Oost, & Koutsopoulos, 2007). With advancing technology, more insight on thermostable enzymes have been provided with generation of metagenomic libraries of high temperature environment (Mirete et al., 2016; Unsworth et al., 2007). Studies of such extreme environments are necessary because different parameters (e.g. physiochemical and geological properties) affect microbial diversity in a specific environment (Lau, Aitchison, & Pointing, 2009; Mohanrao & Singh, 2016). Great plate count anomaly illustrates that less than 1 % of existing microorganisms are culturable. Under such conditions, culture-independent approaches facilitate the exploration of microbial diversity from diverse habitats (Hou et al., 2013). Most of the microbial diversity studies revealed that temperature, pH and biogeography are important factors which design microbial distribution (Huang et al., 2011).

The Main Mantle Thrust and the Main Karakoram Thrust (MKT) in Chilas and Hunza areas of northern Pakistan are host to many hydrothermal activity with numerous

thermal springs distributed between latitude 30° - 37° N and longitude 73° - 77° E (M.S. Bakht, 2000). While limited cultivation-based study of the geothermal springs in this region has been reported (Javed et al., 2012a), a cultivation-independent study which provide a more comprehensive assessment of microbial diversity was still lacking. The primary objective of this study was to census the hot springs in Tato Field, Tatta Pani and Murtazaabad and investigate the relationship between microbial communities and physicochemical parameters. The results of this study gives an insight about the microbiology of geothermal systems in the Himalayan region.

6.3. Material and Methods

6.3.1. Description of the Sampling Sites

The nine hot springs selected for the current study were distributed over three geothermal fields located along the MKT namely, Tato Field (also called Tatta Pani Chilas in local language), Tatta Pani and Murtazaabad (Figures 33 and 34).

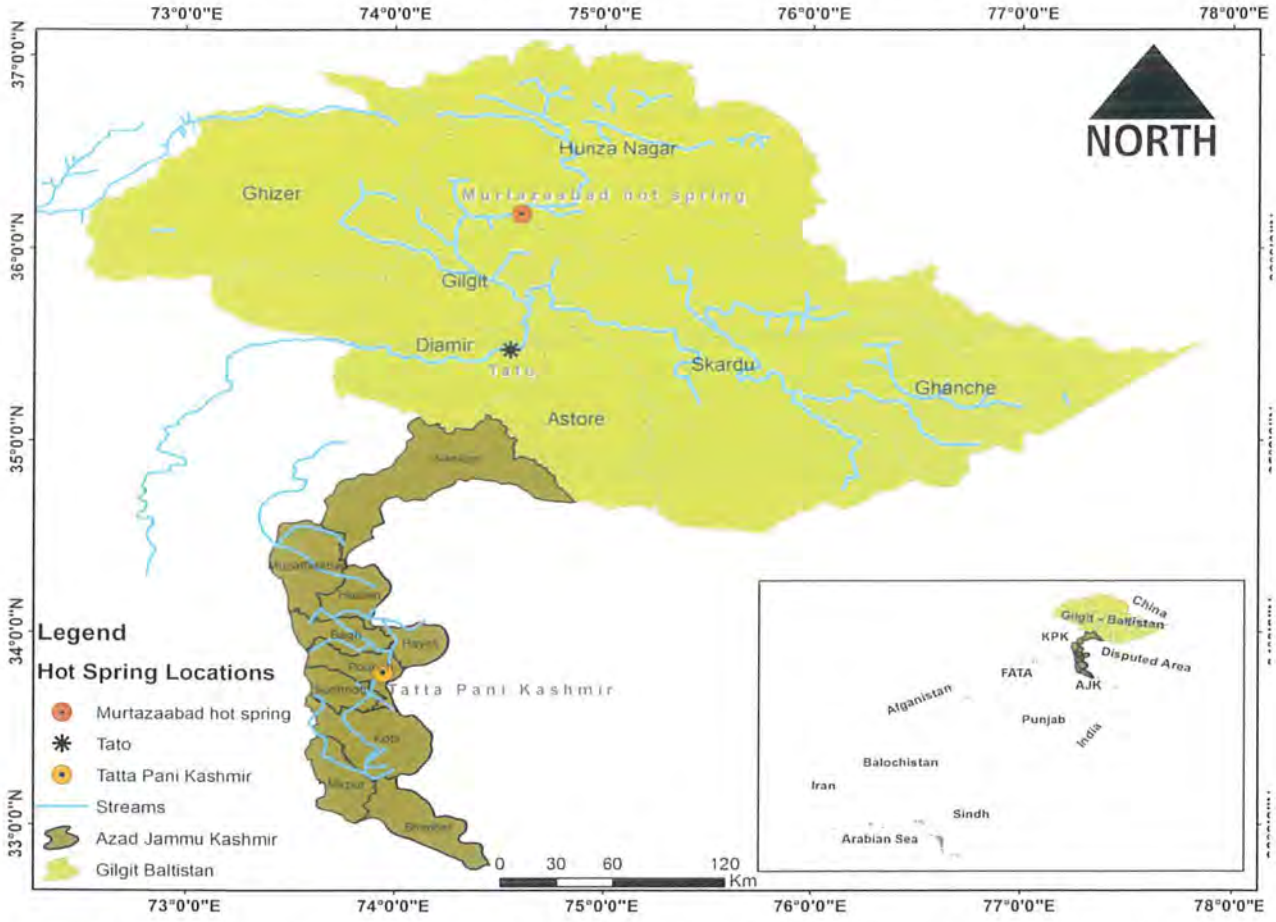


Figure 33 Geographic location of the sampling sites of Tato Field (also called as Tatta Pani Chilas), Tatta Pani and Murtazaabad in Pakistan.

a) Tato Field hot springs

b) Tatta pani hot spring

c) Murtazaabad hot springs

Figure 34 Illustration of the sampling site

These geothermal fields encompassed sites ranging in temperature from 60 to 98 °C and in pH from 6.2 to 9.4. The heat in these systems were said to be generated from the abrasions between the Indian and Eurasian plates (M. Ahmad, Akram, Hussain, Sajjad, & Zafar, 2001). The Tato Field springs are located at 1,200 m above Tatta Pani and are surrounded by Nanga Parbat Gneisses. Tatta Pani hot springs are reported to record a maximum temperature of 98 °C while Murtazaabad has a maximum recorded temperature of 90 °C (Dwivedi et al., 2012; Virk, Sharma, & Sharma, 2002). Murtazaabad and Tato Field hot springs flow from fluvial deposits or talus (M. Ahmad et al., 2001; Javed, Zahoor, Sabar, Haq, & Babar, 2012b). Murtazaabad springs stream from a sharp precipice and hard rocks, which are assigned to the lower Paleozoic to Precambrian age (Malik Sikander Bakht, 2000).

6.3.2. Sample Collection and Physicochemical Analysis

Soil samples were collected aseptically in sterilized 50 ml Falcon tubes following standard protocols (S. Wang et al., 2013). Care was taken during sampling to avoid exposure of the samples to environment. Additionally, one soil sample from vicinity of each spring was collected as control. The control sites have temperature ranging from 30 to 40 °C. Temperature, electrical conductivity and pH were measured at the sampling sites by a handheld pH meter (Whatman PHA 260) after calibrating with warmed buffer. Sample tubes were immediately transferred in to liquid nitrogen for transportation and further analysis in laboratory. UNEP analytical methods 2014 (protocol code 14031) using colorimetric Wagtech photometer was followed for measurement of silicates. Measurement of sulfates (SO_4^{-2}) concentration was done without filtrating the samples and that of sodium (Na^+), potassium (K^+) and calcium (Ca^{+2}) after filtration and acidification with HNO_3, N_2 and NH_4 and CO concentrations were also determined by standard procedures determined by APHA 1985 (APHA, 1985; Nickson, McArthur, Shrestha, Kyaw-Myint, & Lowry, 2005). Bicarbonates and silica contents were determined following the methods of Stumm et al. (Stumm, Morgan, & Drever, 1996) and White, Brannock, and Murata (1956) respectively.

6.3.3. DNA extraction, Amplification and Pyrosequencing

DNAs were extracted using PowerSoil® DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instruction, quality assessed and subsequently stored at -80 °C for further analysis. Described procedures

of Hur et al. Hur et al. (2011) were adopted for pyrosequencing analyses of the genomic DNA extracts. Amplifications of the V1~V3 region of the bacterial 16S rRNA gene (http://www.ezbiocloud.net/resource/M1001) were done using a C1000 Touch Thermal Cycler (Bio-Rad, CA, USA) and bar-coded fusion primers (8 nucleotide long barcodes) (Table 1). The PCR mix contained 100 ng template DNA, 5 µL 10x *ExTaq* buffer, 0.2 mM of each dNTPs, 0.5 µM of each primer, and 2 units *ExTaq DNA Polymerase* (Takara, Otsu, Japan) in a 50 µl reaction. After initial denaturation at 94 °C for 5 min, the PCR was carried out using a Touch-down program for 10 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 45 s) and extension (72 °C, 90 s) with subsequent decrease in annealing temperature by 0.5 °C, followed by an additional 20 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 45 s), extension (72 °C, 90 s). The amplified products were checked on 2 % agarose gel electrophoresis and visualized using the Gel Doc system (Bio-Rad, USA). Amplicons were purified using a QIAquick PCR purification kit (Qiagen, CA, USA) and quantified using a PicoGreen dsDNA Assay kit (Invitrogen, CA, USA). Equimolar concentrations of each amplicon from different samples were pooled, purified using AMPure bead kit (Agencourt Bioscience, MA, USA) and amplified on sequencing beads by emulsion PCR. Sequencing reactions were performed using a Roche GS FLX Titanium system at ChunLab Inc (Seoul, Korea) according to the manufacturer's instructions. Raw pyrosequencing reads were submitted to Sequence Read Archive (SRA), NCBI under accession numbers SAMN03284249-SAMN03284258 (SRA: SRS825427-SRS825436 BioProject PRJNA271634).

6.3.4. Statistical Analysis of Microbial and Physicochemical Data

All reads shorter than 300 bp or those containing any ambiguous base or incorrect primer sequences were excluded from the dataset (Data not shown). Operational taxonomic units (OTUs) were defined by using the CD-HIT program (W. Li & Godzik, 2006) at a similarity cut-off level of ≥97 %. Two-way analysis of variance (ANOVA) on microbial diversity indices and relative sequence abundances of important phyla against physiochemical properties was performed. Tukey's HSD (honest significant difference), Tamhane's and LSD (Fisher's Least Significance Difference) pairwise tests were performed to find significant difference within samples

of same sites. For significant difference among the different sites, pairwise test was performed using SPSS statistics (IBM SPSS version 20.0 software, IBM Corp).

Species richness was estimated by employing Chao 1 and ACE methods, while quantitative species richness/evenness by nonparametric Shannon and Simpson's indices in Mothur package v.1.8.0 (Schloss et al., 2009). Each pyrosequencing read was taxonomically assigned by using a combination of BLASTN-based searches (Altschul et al., 1990) and pairwise similarity comparisons in EzTaxon database (O. S. Kim et al., 2012a). Relative abundance of each taxon was clustered by using Bionumerics version 6.0 software (Applied Maths, Sint Martens Latem, Belgium) while unweighted UniFrac Matrix (M. Hamady et al., 2010) was used to visualize bacterial community in an Principal Coordinates Analysis (PCoA) plot. An Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram was generated using Fast UniFrac analysis (M. Hamady et al., 2010). Canonical correspondence analysis (CCA) was used to visualize the pattern of soil bacterial community variation and distribution along the measured environmental variables.

6.4. Results

6.4.1. Physicochemical Characteristics of the Sampling Sites

Physicochemical parameters of the samples used in the study have been described in Table 25. While Tato Field thermal springs were bicarbonated in nature and have a higher bicarbonates (525-610 $mg.L^{-1}$) than sulfates (410-460 $mg.L^{-1}$), Tatta Pani hot springs were sulfate type considering bicarbonate level (133-159 $mg.L^{-1}$) was significantly lower than sulfates (545-684 $mg.L^{-1}$). Murtazaabad thermal springs were mixed type with high level of both sulfates and bicarbonates (710 – 940 $mg.L^{-1}$). The levels of sulfates and bicarbonates in Murtazaabad springs were also significantly higher than the other two sites.

Table 25 Physical and chemical parameters of sampling sites

Sample	Coordinates	Elevation (ft above Sea level	pH	EC µS cm^{-1}	Temp. (°C)	Ca^{2+}	Na^{+}	K^{+}	HCO_3^-	SiO_2	SO_4^{-2}	NH4	N2	CO (p.p.m.)
						Mg/L								
TF-H2-a	74°32'49.10"E, 35°28'30.40"N	3861	7.9	1119	85	2.5	200	25	540	91	425	200	8.4	20.3
TF-H2-b	74°32'46.10"E, 35°28'30.40"N	3841	8.2	1160	70	2.9	210	26	525	10	460	210	9.7	23.4
TF-H2-c	74°32'48.10"E, 35°28'30.40"N	3851	8.8	1569	90	3.5	300	20	610	14	410	300	9.6	25.3
TP-H3-a	33°36'42.90"N 73°56'48.34'E	2715	6.2	1060	75	0.1	150	4.9	145	45	690	250	13.8	42.3
TP-H3-b	33°36'43.12"N 73°56'49.10'E	2200	8.4	1032	60	0.1	165	2.5	133	58	684	265	13.2	40.0
TP-H3-c	33°36'42.12"N 73°56'49.34'E	2400	9.4	1550	90	0.2	170	4.6	159	56	545	270	11.3	42.4
MA-H4-a	74°36'19.76"E, 36°16'35.16"N	6968	7.5	1730	90	9	400	56	940	10	940	300	3.7	30.4
MA-H4-b	74°36'20.77"E, 36°16'30.34"N	6709	9.2	1742	95	5.3	420	60	710	6	910	420	2.3	29.8
MA-H4-c	74°36'19.77"E, 36°16'30.34"N	6809	6.7	1742	90	2.6	500	35	780	5	710	400	3.1	32.6

* EC, Electrical conductivity; Temp., Temperature

* Sample ID's were given according to location of hot water springs. 3 samples were collected for each site which were designated as site abbreviation followed by a, b and c for three sites respectively; TF-Tato Fields (This site also called as Tatta Pani Chilas in local language); TP = Tatta Pani (Kashmir); MA=Murtazaabad

Values are given as means of three readings

6.4.2. Microbial Diversity

A total of 70,836 quality-reads were observed for 10 samples after removal of low-quality and chimeric sequences (Table 26).

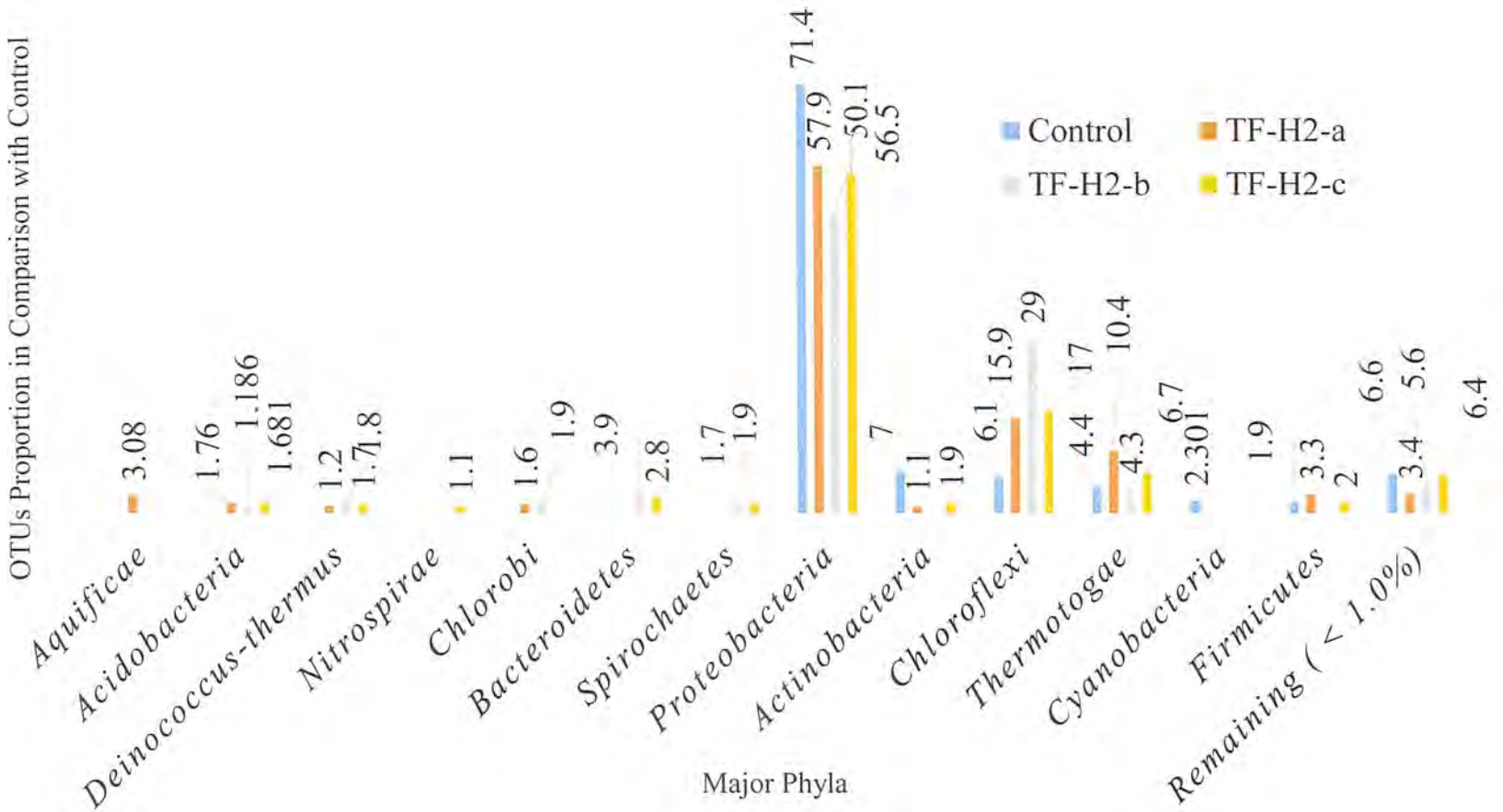
Table 26 Metadata for total number of reads and dropped reads of all samples

Samples*	Total Reads	Dropped Reads	Chimeric Reads
NCCP-H1	8273	3431	1191
TF-H2-a	7428	3068	1405
TF-H2-b	6485	2823	1049
TF-H2-c	5869	2919	808
TP-H3-a	7947	3393	1165
TP-H3-b	6819	2847	1415
TP-H3-c	7510	3268	1222
MA-H4-a	7161	3154	1152
MA-H4-b	6674	2665	1238
MA-H4-c	6670	2857	1148

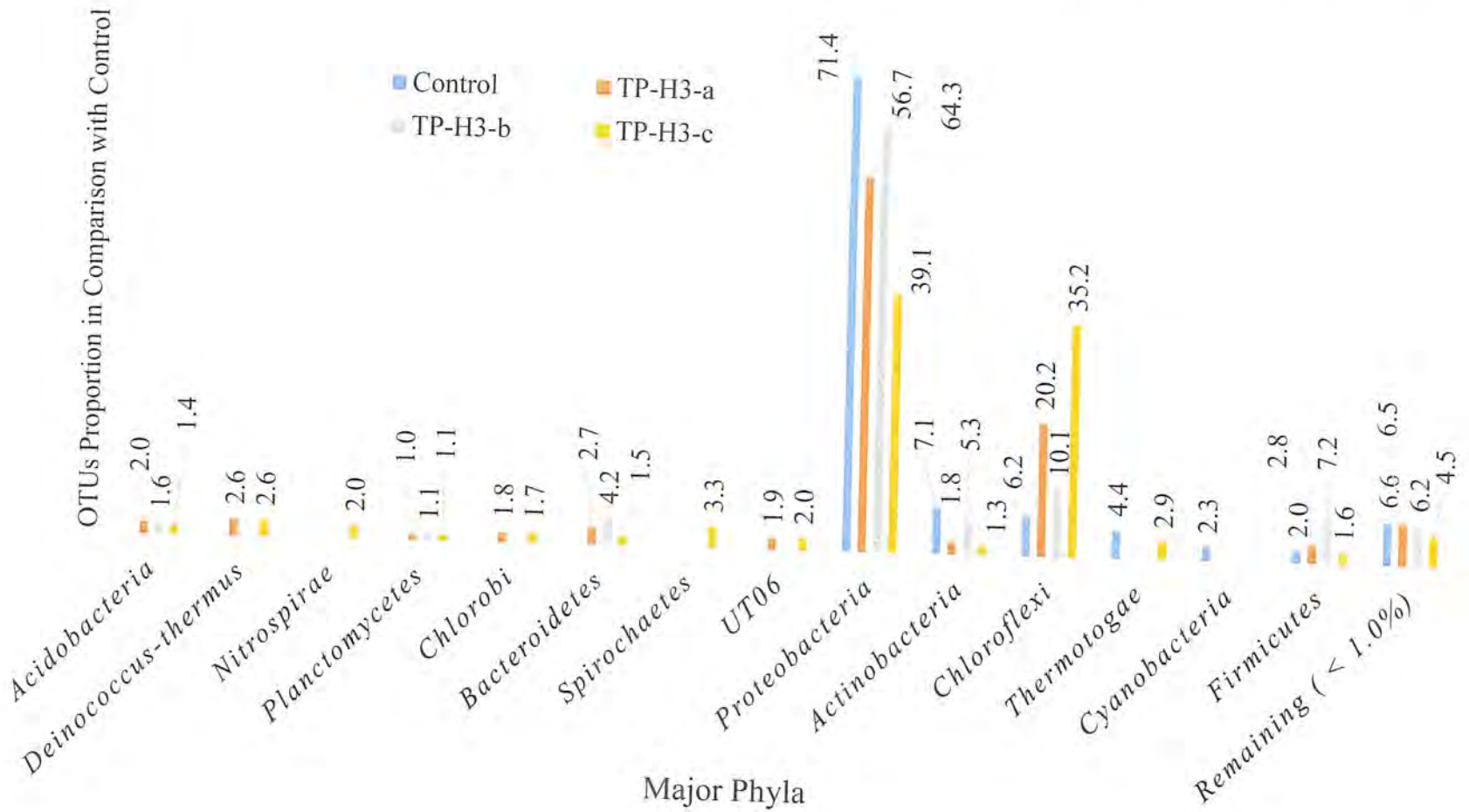
Forward Primer: GAGTTTGATCMTGGCTCAG
Reverse Primer: WTTACCGCGGCTGCTGG
****NCCP: National Culture Collection Pakistan; TF: Tato field; TP; Tatta pani; MA; Murtazaabad; H; Hot spring***

At 97 % OTU level, the quality reads can be distributed into 5,535 OTUs consisting of 972 microbial genera in 53 phyla (Figs. 35 and 36). Figure 37 is showing exclusive species present in a specific site and absent in control as well as in other sites.

a)


OTUs Proportion in Comparison with Control
Control
TF-H2-a
TF-H2-b
TF-H2-c
3.08
1.76
1.186
1.681
1.2
1.7
1.8
1.1
1.6
1.9
3.9
2.8
1.7
1.9
71.4
57.9
50.1
56.5
7
1.1
1.9
6.1
15.9
29
4.4
17
4.3
10.4
6.7
2.301
1.9
3.3
2
6.6
3.4
5.6
6.4
Aquificae
Acidobacteria
Deinococcus-thermus
Nitrospirae
Chlorobi
Bacteroidetes
Spirochaetes
Proteobacteria
Actinobacteria
Chloroflexi
Thermotogae
Cyanobacteria
Firmicutes
Remaining (< 1.0%)
Major Phyla

b)


OTUs Proportion in Comparison with Control
Control
TP-H3-a
TP-H3-b
TP-H3-c
2.0
1.6
1.4
2.6
2.6
2.0
1.0
1.1
1.1
1.8
1.7
2.7
4.2
1.5
3.3
1.9
2.0
71.4
56.7
64.3
39.1
7.1
1.8
5.3
1.3
6.2
20.2
10.1
35.2
4.4
2.9
2.3
2.8
2.0
7.2
1.6
6.6
6.5
6.2
4.5
Acidobacteria
Deinococcus-thermus
Nitrospirae
Planctomycetes
Chlorobi
Bacteroidetes
Spirochaetes
UT06
Proteobacteria
Actinobacteria
Chloroflexi
Thermotogae
Cyanobacteria
Firmicutes
Remaining (< 1.0%)
Major Phyla

c)

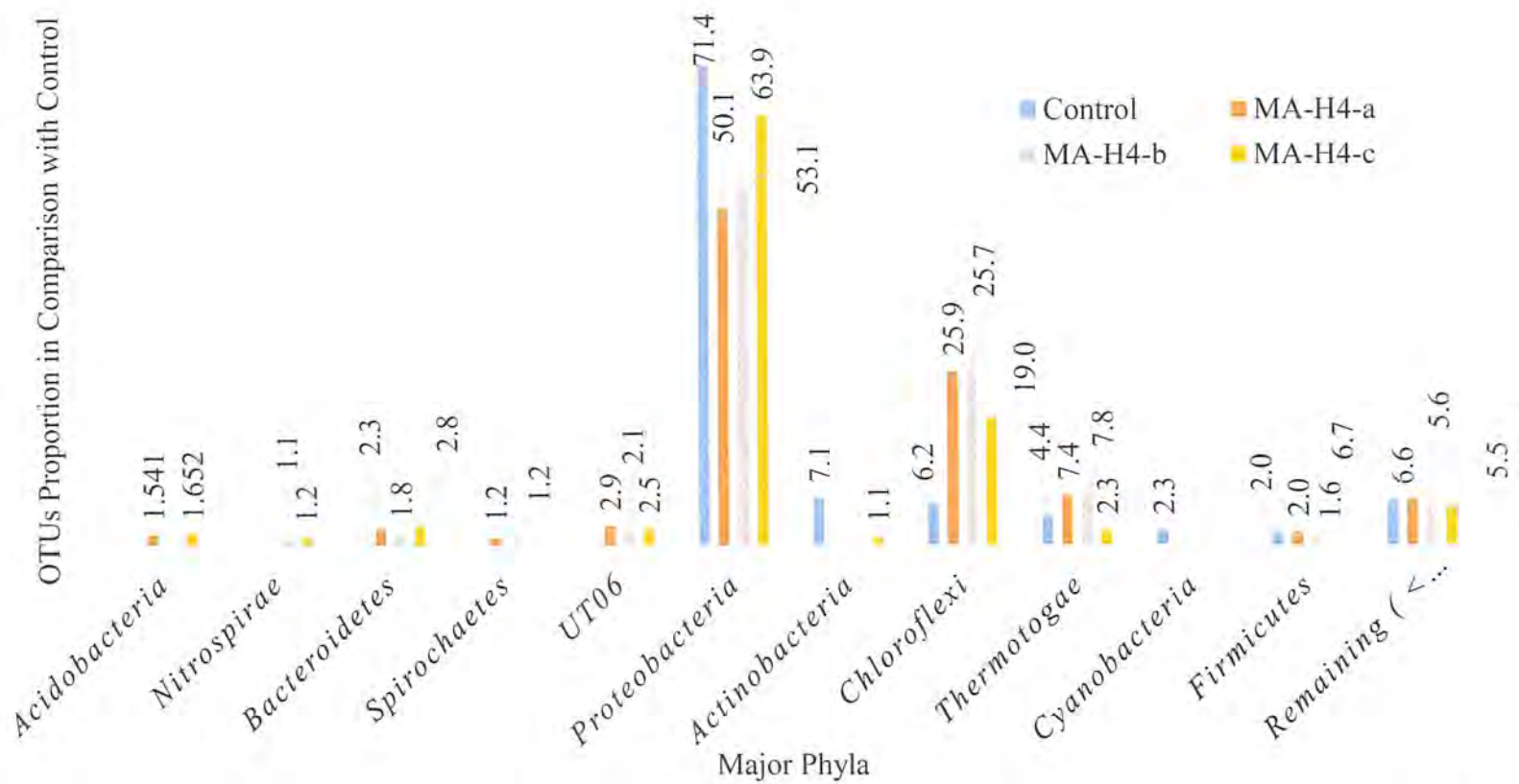


Figure 35. Proportion of OTUs present in sample sites in comparison with control species level at a) TF-H2-a, TF-H2-b, TF-H2-c and Control NCCP-H1; b) TP-H3-a, TP-H3-b, TP-H3-c and Control NCCP-H1; c) Ma-H4-a, Ma-H4-b, Ma-H4-c and Control NCCP-H1

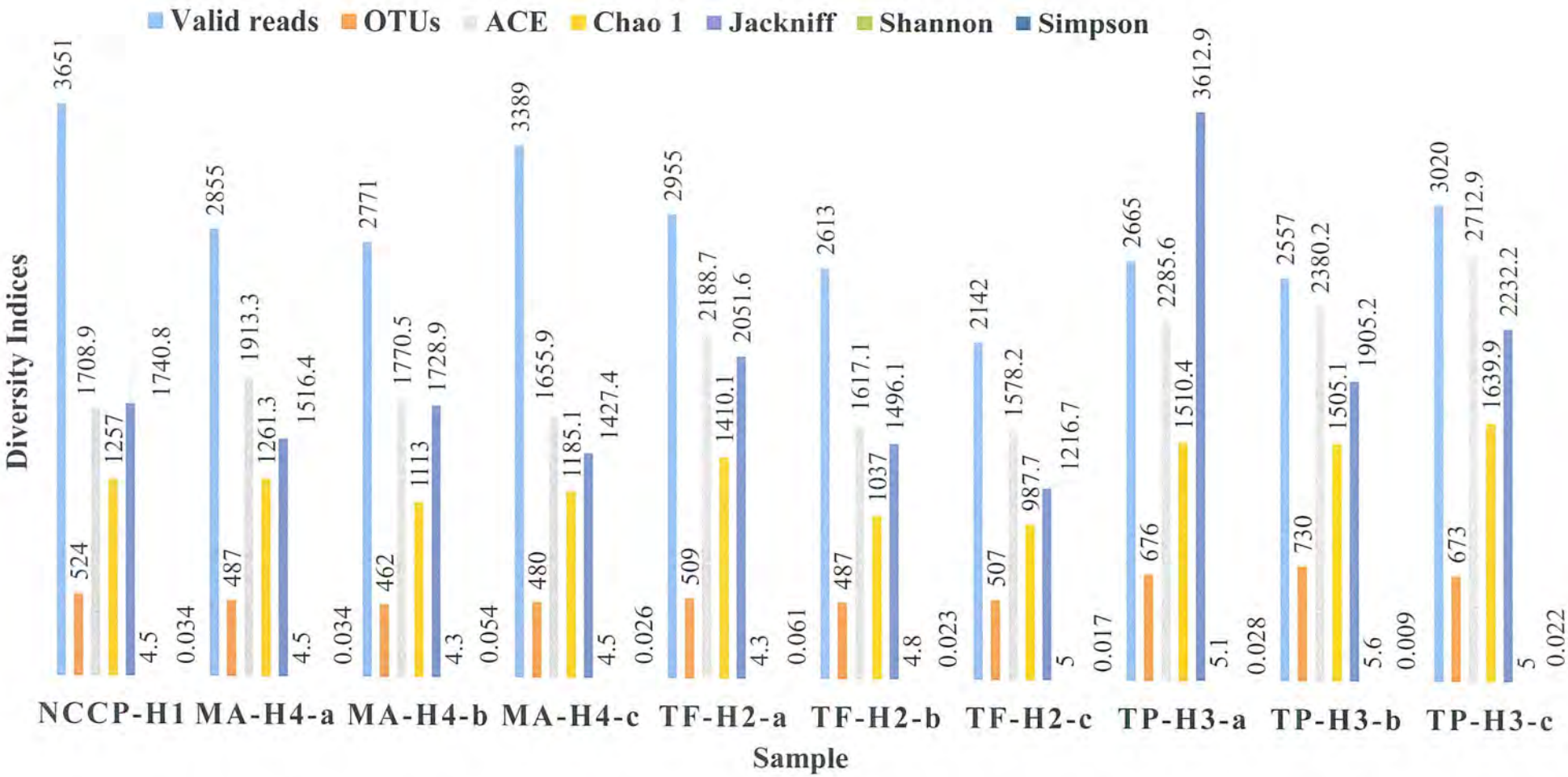
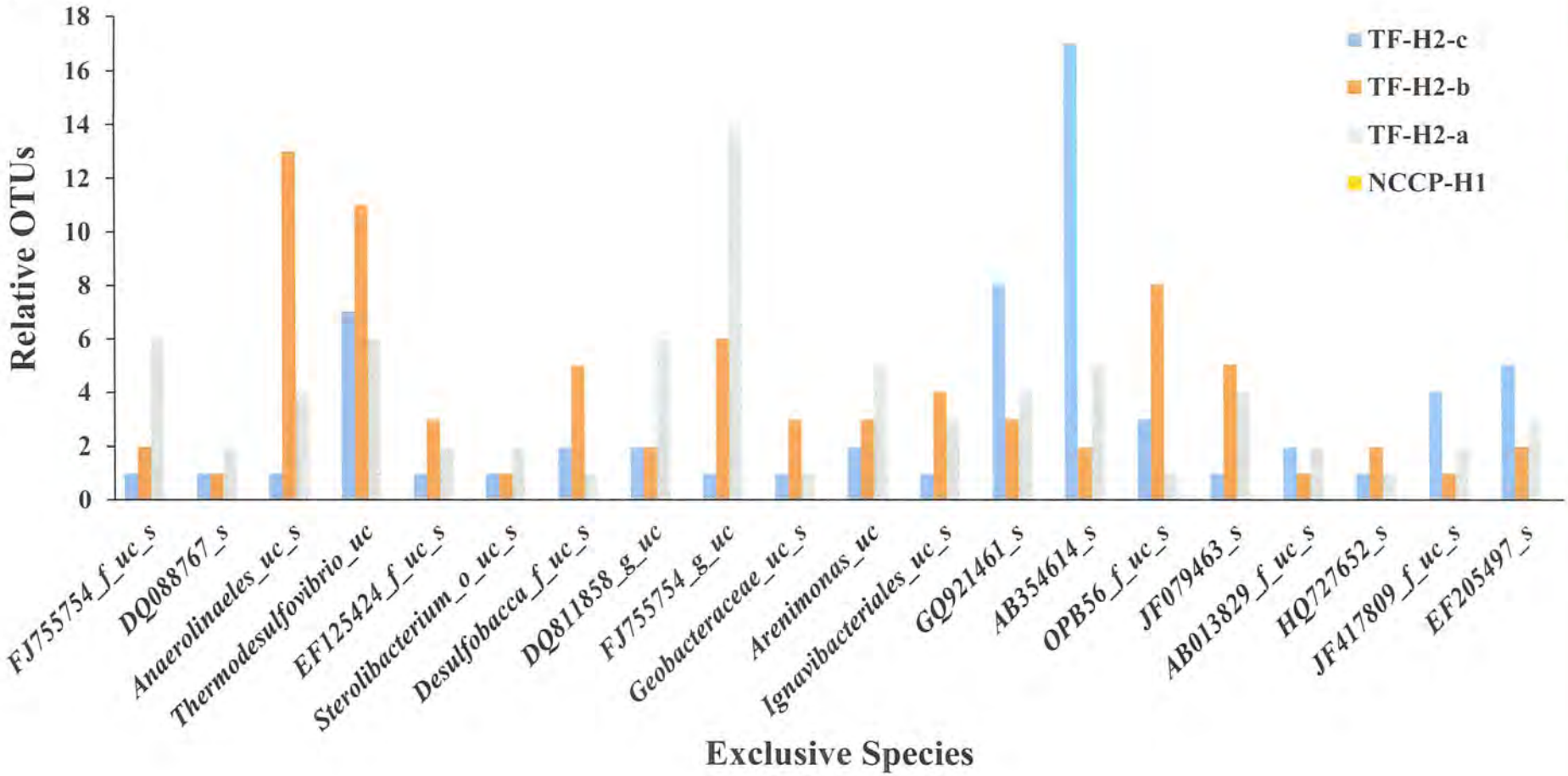
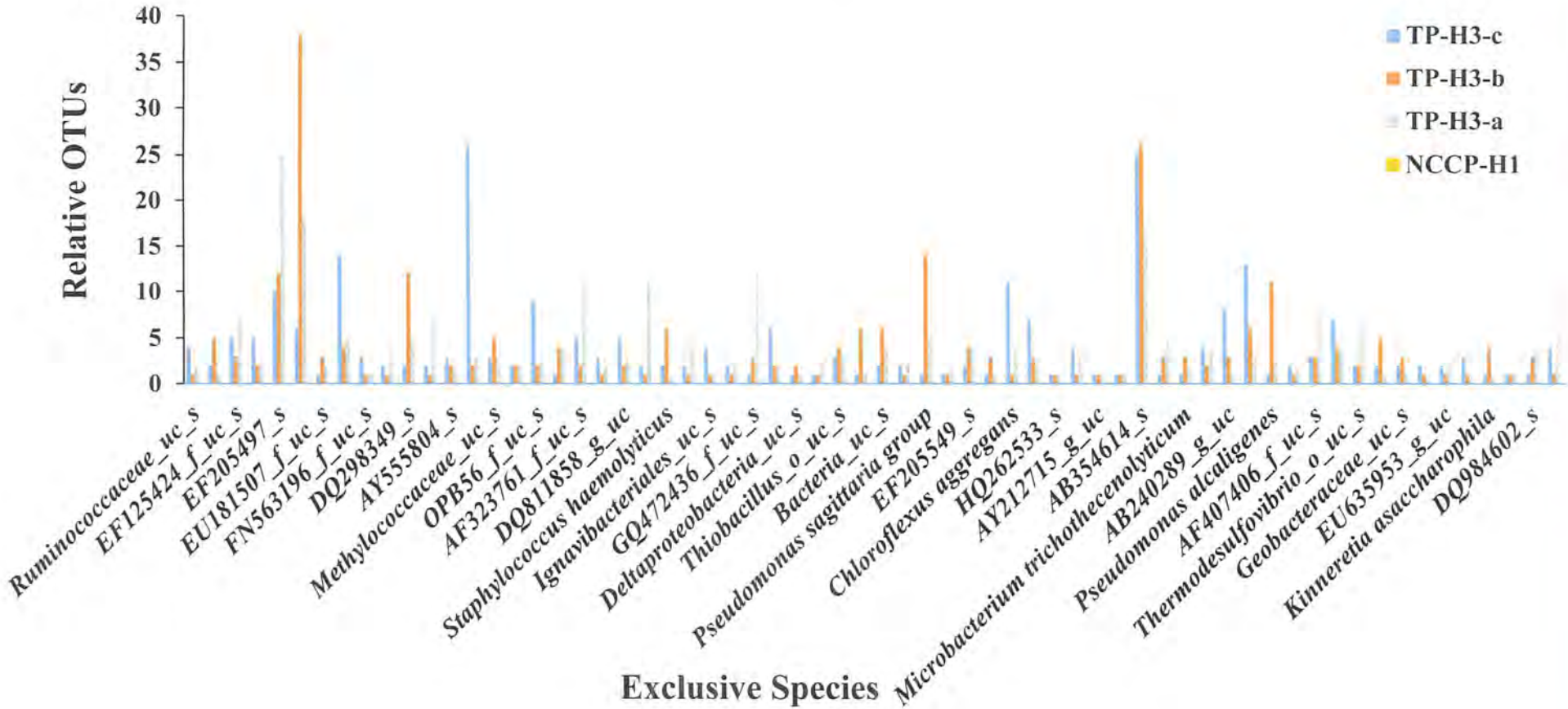


Figure 36. Chart determining the alpha diversity indices using CD-Hit method at 95 % confidence interval

a)


Relative OTUs
18
16
14
12
10
8
6
4
2
0
TF-H2-c
TF-H2-b
TF-H2-a
NCCP-H1
FJ755754_f_uc_S
DQ088767_S
Anaerolinaeles_uc_S
Thermodesulfovibrio_uc
EF125424_f_uc_S
Sterolibacterium_o_uc_S
Desulfobacca_f_uc_S
DQ811858_g_uc
FJ755754_g_uc
Geobacteraceae_uc_S
Arenimonas_uc
Ignavibacteriales_uc_S
GQ921461_S
AB354614_S
OPB56_f_uc_S
JF079463_S
AB013829_f_uc_S
HQ727652_S
JF417809_f_uc_S
EF205497_S
Exclusive Species

b)


Relative OTUs
40
35
30
25
20
15
10
5
0
TP-H3-c
TP-H3-b
TP-H3-a
NCCP-H1
Ruminococcaceae_uc_$
EF125424_f_uc_$
EF205497_$
EU181507_f_uc_$
FN563196_f_uc_$
DQ298349_$
AY555804_$
Methylococcaceae_uc_$
OPB56_f_uc_$
AF323761_f_uc_$
DQ811858_g_uc
Staphylococcus haemolyticus
Ignavibacteriales_uc_$
GQ472436_f_uc_$
Deltaproteobacteria_uc_$
Thiobacillus_o_uc_$
Bacteria_uc_$
Pseudomonas sagittaria group
EF205549_$
Chloroflexus aggregans
HQ262533_$
AY212715_g_uc
AB354614_$
Microbacterium trichothecenolyticum
AB240289_g_uc
Pseudomonas alcaligenes
AF407406_f_uc_$
Thermodesulfovibrio_o_uc_$
Geobacteraceae_uc_$
EU635953_g_uc
Kinneretia asaccharophila
DQ984602_$
Exclusive Species

c)

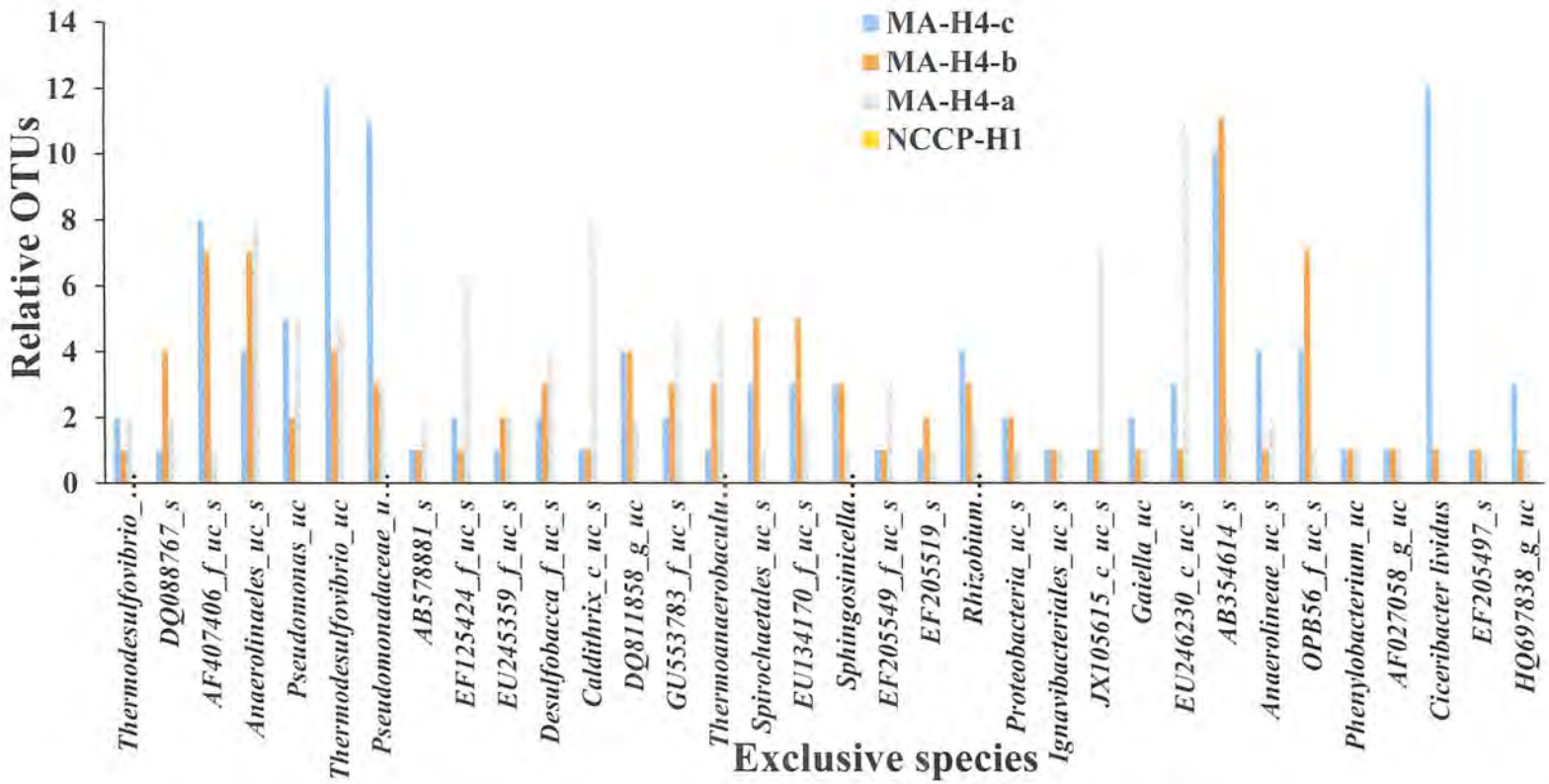


Figure 37. Taxon exclusive or XOR analysis for comparison of multiple communities to detect taxa present in selected sets of samples and absent in control by CD-Hit program and fast clustering of sequence data, a) Exclusive OTUs at species level present in TF-H2 sites and absent in control, b) Exclusive OTUs at species level present in TP-H3 sites and absent in control, c) Exclusive OTUs at species level present in MA-H4 sites and absent in control

Figure 38a

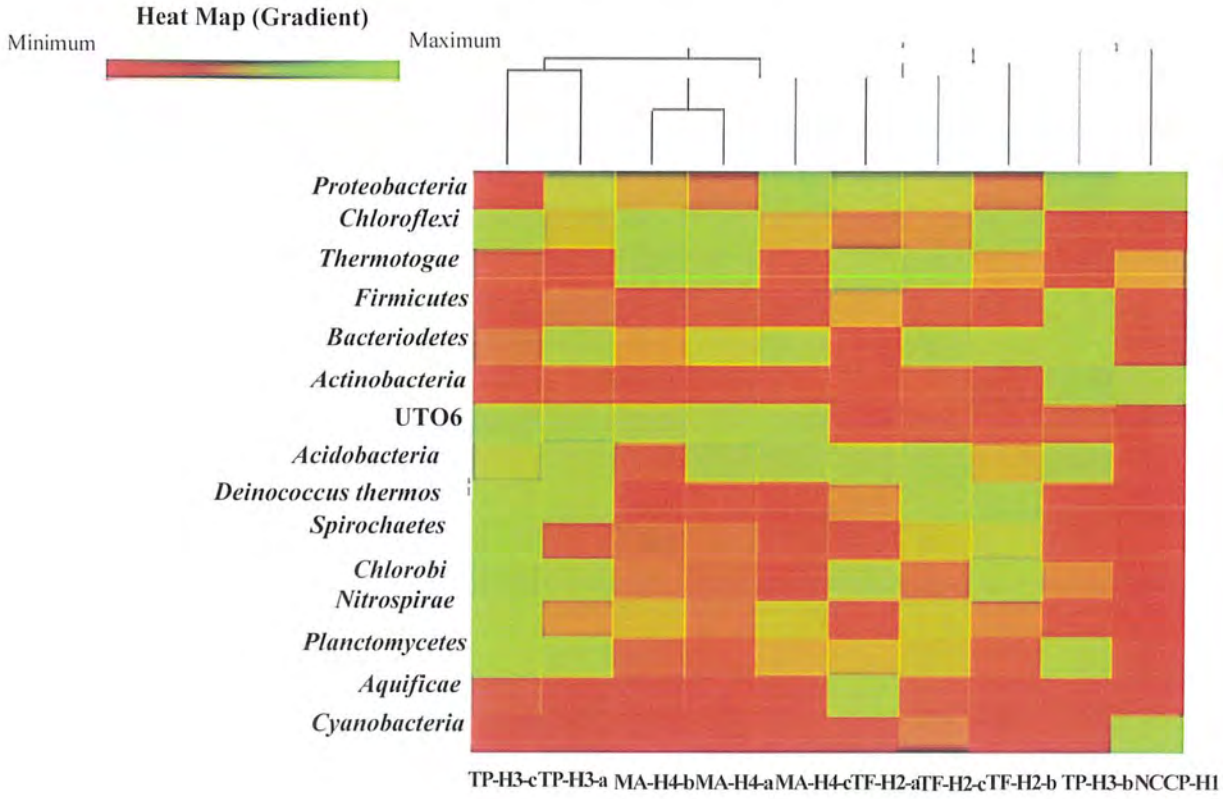

Heat Map (Gradient)
Minimum
Maximum
Proteobacteria
Chloroflexi
Thermotogae
Firmicutes
Bacteriodetes
Actinobacteria
UTO6
Acidobacteria
Deinococcus thermos
Spirochaetes
Chlorobi
Nitrospirae
Planctomycetes
Aquificae
Cyanobacteria
TP-H3-c
TP-H3-a
MA-H4-b
MA-H4-a
MA-H4-c
TF-H2-a
TF-H2-c
TF-H2-b
TP-H3-b
NCCP-H1

Figure 38(b)

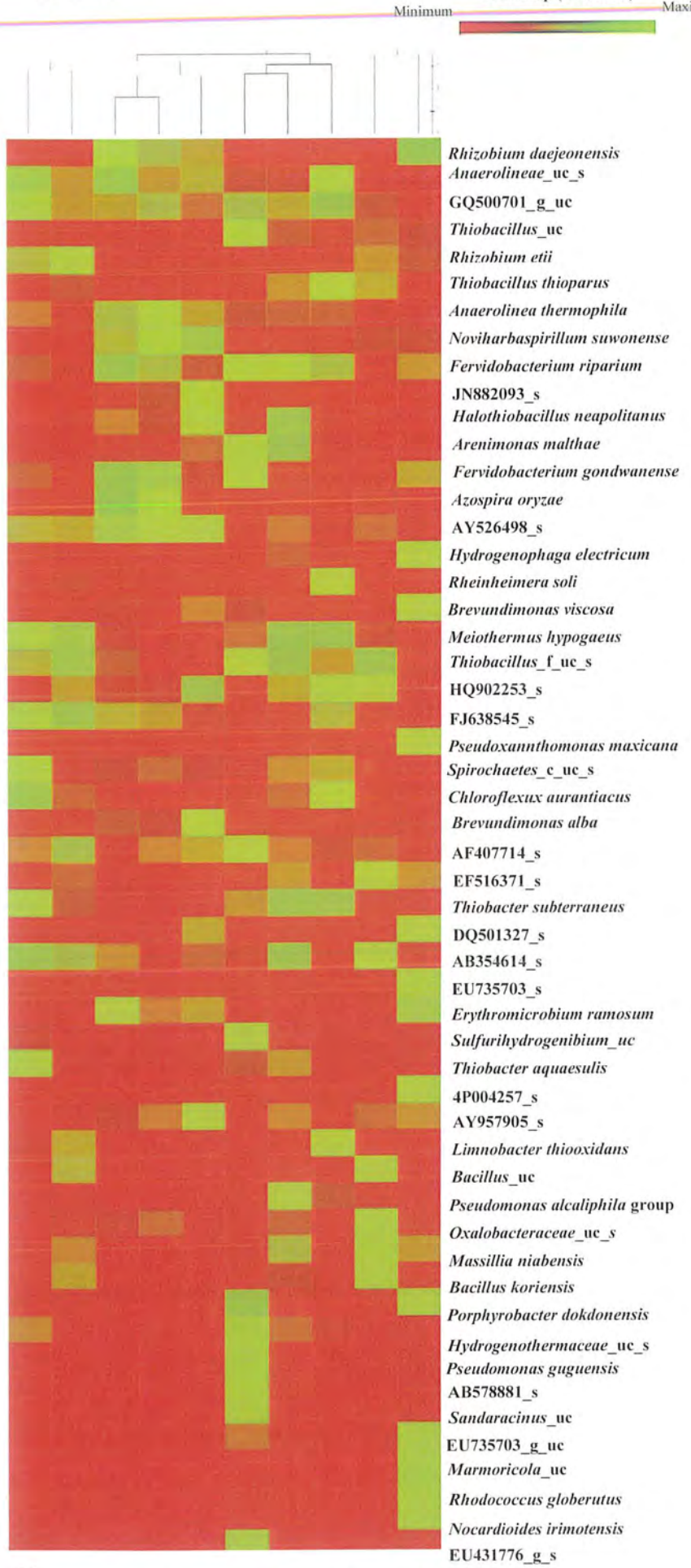

Heat Map (Gradient)
Minimum
Maximum
Rhizobium daejeonensis
Anaerolineae_uc_s
GQ500701_g_uc
Thiobacillus_uc
Rhizobium etii
Thiobacillus thioparus
Anaerolinea thermophila
Noviharbaspirillum suwonense
Fervidobacterium riparium
JN882093_s
Halothiobacillus neapolitanus
Arenimonas malthae
Fervidobacterium gondwanense
Azospira oryzae
AY526498_s
Hydrogenophaga electricum
Rheinheimera soli
Brevundimonas viscosa
Meiothermus hypogaeus
Thiobacillus_f_uc_s
HQ902253_s
FJ638545_s
Pseudoxannthomonas maxicana
Spirochaetes_c_uc_s
Chloroflexux aurantiacus
Brevundimonas alba
AF407714_s
EF516371_s
Thiobacter subterraneus
DQ501327_s
AB354614_s
EU735703_s
Erythromicrobium ramosum
Sulfurihydrogenibium_uc
Thiobacter aquaesulis
4P004257_s
AY957905_s
Limnobacter thiooxidans
Bacillus_uc
Pseudomonas alcaliphila group
Oxalobacteraceae_uc_s
Massillia niabensis
Bacillus koriensis
Porphyrobacter dokdonensis
Hydrogenothermaceae_uc_s
Pseudomonas guguensis
AB578881_s
Sandaracinus_uc
EU735703_g_uc
Marmoricola_uc
Rhodococcus globerutus
Nocardioides irimotensis
EU431776_g_s
NCCP-H1
TP-H3-b
TF-H2-b
TF-H2-c
TF-H2-a
MA-H4-c
MA-H4-a
MA-H4-b
TP-H3-a
TP-H3-c

Figure 38 Heat map indicating the clustering of OTUs retrieved from the hot spring samples. The heat map was constructed on the basis of Unifrac phylogenetic clustering indices. The color indicates the relative abundance of OTUs in the samples. The taxonomy of each OTU is provided at a) Phylum level and b) Species level.

Heat maps generated based on the composition of taxa at phylum level by using UniFrac beta-diversity phylogenetic clustering indices divided the samples into four major clusters (Fig. 38a).

Phylum *Proteobacteria* was most abundantly present in control sample NCCP-H1. Among the hot spring samples, the highest number of OTUs for phylum *Proteobacteria* was observed in TP-H3-b and the least in TP-H3-c. The sample TP-H3-b along with control NCCP-H1 formed a separate cluster far from samples TP-H3-a and TP-H3-c. This feature may be attributed to the fact that sample TP-H3-b has the lowest temperature (60 °C), among all other sampling sites, which might be a feasible temperature for growth of bacteria belonging to phylum *Proteobacteria* (Fig. 38a). Major species present among phylum *Proteobacteria* were found to be *Rhizobium etli* and *Rhizobium daejeonense* (Fig. 38b).

OTUs for phylum *Chloroflexi* were most dominant in TP-H3-c, MA-H4-a, MA-H4-b and TF-H2-b and lowest in control (Fig. 38a). At species level, OTUs for *Anaerolinaceae_uc_s* were present in TP-H3-c and TF-H2-b, and taxonomically unclassified species in MA-H4-a and MA-H4-b (Fig. 38b). For the sample TF-H2-b, 29 % of bacteria (759 species) were constituted by phylum *Chloroflexi*, of which 89 % belonged to class *Anaerolineae*. Remaining 11 % was comprised of unclassified OTUs. Among Tatta Pani hot spring sites, TP-H3-a at 75 °C yielded 538 OTUs for phylum *Chloroflexi*, of which 443 belonged to class *Anaerolineae*. Sample TP-H3-b at 60 °C with 259 *Chloroflexi* OTUs was the least among other sites. Sample TP-H3-c at 90 °C had 1062 OTUs for phylum *Chloroflexi*. The majority of these OTUs were comprised of class *Anaerolineae* with 934 species.

OTUs for phylum *Thermotogae* were most abundant in TF-H2-a and MA-H4-a and the least in TP-H3-a and TP-H3-b (Fig. 38a). In control, the proportion of phylum *Thermotogae* was low as compared to Tato Field and Murtazaabad but was higher than Tatta Pani (Fig. 38a). Phylum *Firmicutes* had least number of OTUs in MA-H4-c and most in TP-H3-b. Though it was present in very low proportion in control, the amount

was higher than in MA-H4-c and TF-H2-b. OTUs for phylum *Actinobacteria* were most abundant in TP-H3-b, but is comparatively lesser than the OTUs detected in NCCP-H1 (Fig. 38a). The phyla *Spirochaetes* and *Nitrospirae* were more abundant in TP-H3-c, while phylum *Aquificae* was predominantly detected in TF-H2-a. Murtazaabad hot springs had a minimum number of OTUs and diversity of the phylum *Deinococcus-thermus* as compared to Tato Field and Tatta Pani hot springs. Greater diversity of phylum *Deinococus-thermus* was observed in TF-H2-b with detection of OTUs belonging to *Meiothermus cerbereus, Thermus scotoductus, Thermus_uc, Thermus antranikianii* and *Deinococcus_uc* while least in TF-H2-a. OTUs for families *Thermaceae* and *Deinococcaceae* in MA-H4-c were significantly higher and more diverse than other sites of Murtazaabad and Tato fields. *Meiothermus granaticius* and *Thermus scotoductus* were unique to MA-H4-c among all the sites.

At species level, OTUs for 53 major species including 28 classified and 25 taxonomically unclassified species were shared among the different sites (Fig. 38b). OTUs of *Anaerolinaceae_uc_s* were shared by TF-H2-a, TF-H2-b, TP-H3-a, TP-H3-b, MA-H4-a and MA-H4-b. OTUs of *Thiobacillus thioparus* were present in TF-H2-b while those of *Thiobacillus_uc* were found abundantly in TF-H2-a. *Fervidobacterium riparium*'s OTUs were shared by MA-H4–a, MA-H4-b and the three samples of Tato Field springs. While OTUs belonging to *Arenimonas malthae* was present in only TF-H2-c and TF-H2-a, those of *Rheinheimera soli* and *Hydrogenophaga electricum* were found in TF-H2-b and TF-H2-c respectively. OTUs of *Halothiobacillus neapolitanus* were present in TF-H2-a and TF-H2-b. *Anaerolinea thermophila*, *Novihebaspirillum suwonense* and *Rhizobium daejeonense* were detected only in Murtazaabad springs while *Rhizobium etli* was restricted to Tatta Pani Springs. OTUs for *Halothiobacillus neapolitanus* was present in TF-H2-a and TF-H2-b.

6.4.3. Statistical Analysis of Microbial and Physicochemical Data

Since rarefaction curves of the total number of sequence reads against numbers of OTUs for all samples did not appear to reach an asymptote, more sampling would be necessary to cover an extensive taxonomic diversity (Fig. 39). Inter-variability among the samples from each site as determined by rarefaction curves between the average rarefaction values for each site yielded similar results. At phylum level, the significant differences within samples of the same sampling site as determined by the Tukey's HSD, LSD and Tamhane pairwise tests were relatively low. While Tato Field had significant values ranging from 0.35 to 0.98, these values ranged from 0.83 to 1.0 for Tatta Pani and Murtazaabad springs (Tables 27, 28 and 29).

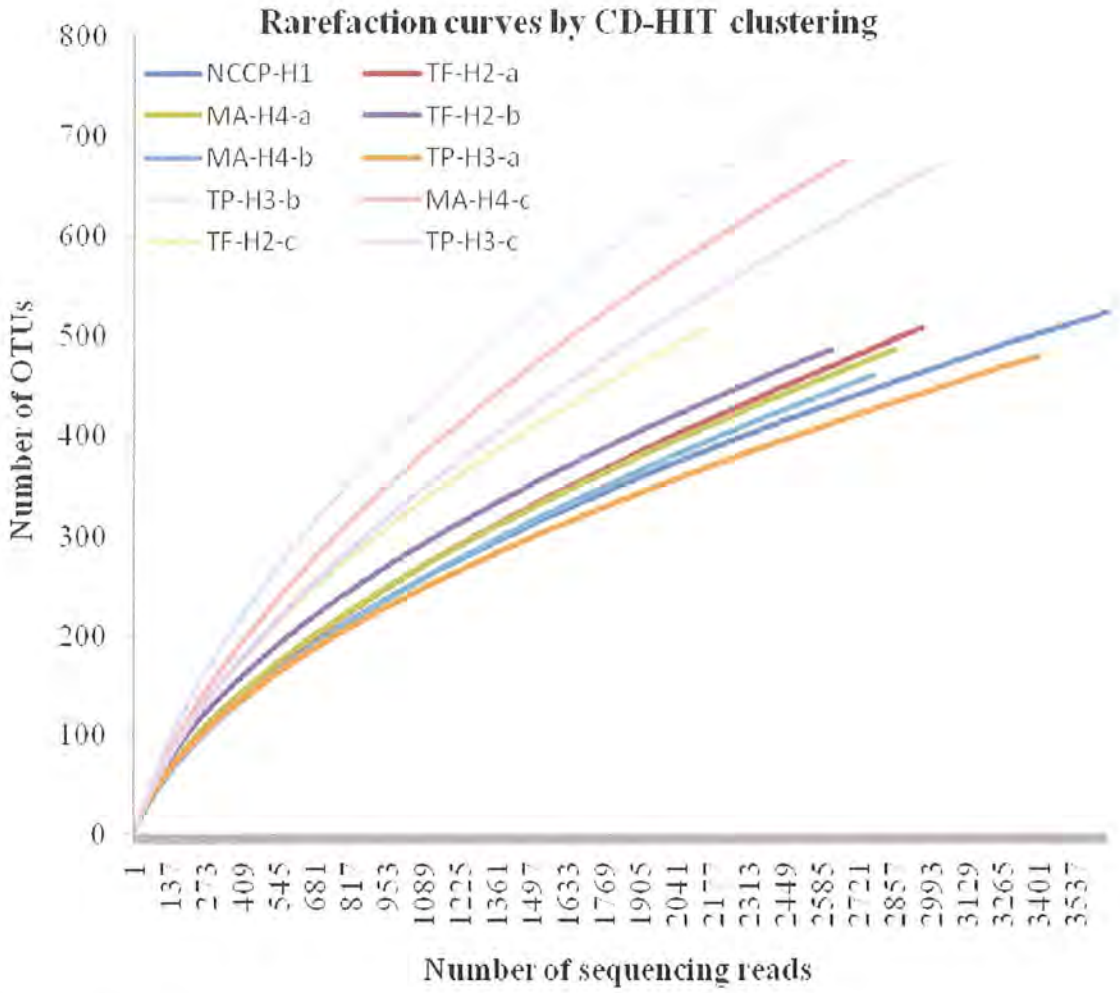


Figure 39. Rarefaction curves for total number of sequence reads against number of OTUs for all samples of sites NCCP-H1, TF-H2(a, b, c), TP-H3(a, b, c) and MA-H4(a, b, c) by CD-HIT clustering

Table 27 Statistical analysis for significant difference within samples of Tato Field-TF-H2 with Tukey HSD, LSD and Tamhane

Analysis	(I) coding	(J) coding	Mean Difference (I-J)	Significance	95% Confidence Interval	
					Lower Bound	Upper Bound
Tukey HSD	TF-H2-a	TF-H2-b	0.222	0.919	-1.10	1.55
		TF-H2-c	0.527	0.620	-0.80	1.85
	TF-H2-b	TF-H2-a	-0.222	0.919	-1.55	1.10
		TF-H2-c	0.305	0.852	-1.02	1.63
	TF-H2-c	TF-H2-a	-0.527	0.620	-1.85	0.80
		TF-H2-b	-0.305	0.852	-1.63	1.02
LSD	TF-H2-a	TF-H2-b	0.222	0.695	-0.89	1.33
		TF-H2-c	0.527	0.352	-0.58	1.63
	TF-H2-b	TF-H2-a	-0.222	0.695	-1.33	0.89
		TF-H2-c	0.305	0.589	-0.80	1.41
	TF-H2-c	TF-H2-a	-0.527	0.352	-1.63	0.58
		TF-H2-b	-0.305	0.589	-1.41	0.80
Tamhane	TF-H2-a	TF-H2-b	0.222	0.982	-1.35	1.79
		TF-H2-c	0.527	0.704	-0.77	1.83
	TF-H2-b	TF-H2-a	-0.222	0.982	-1.79	1.35
		TF-H2-c	0.305	0.894	-0.84	1.46
	TF-H2-c	TF-H2-a	-0.527	0.704	-1.83	0.77
		TF-H2-b	-0.305	0.894	-1.46	0.84

Table 28. Statistical analysis for significant difference within samples of Tatta Pani-TP-H3 sites with Tukey HSD, LSD and Tamhane

Analysis	(I) coding	(J) coding	Mean Difference (I-J)	Significance	95% Confidence Interval	
					Lower Bound	Upper Bound
Tukey HSD	TP-H3-a	TP-H3-b	2.038	0.999	-99.25	103.33
		TP-H3-c	-6.698	0.987	-107.99	94.59
	TP-H3-b	TP-H3-a	-2.038	0.999	-103.33	99.25
		TP-H3-c	-8.736	0.977	-110.03	92.55
	TP-H3-c	TP-H3-a	6.698	0.987	-94.59	107.99
		TP-H3-b	8.736	0.977	-92.55	110.03
LSD	TP-H3-a	TP-H3-b	2.038	0.962	-82.51	86.59
		TP-H3-c	-6.698	0.876	-91.25	77.85
	TP-H3-b	TP-H3-a	-2.038	0.962	-86.59	82.51
		TP-H3-c	-8.736	0.839	-93.29	75.82
	TP-H3-c	TP-H3-a	6.698	0.876	-77.85	91.25
		TP-H3-b	8.736	0.839	-75.82	93.29
Tamhane	TP-H3-a	TP-H3-b	2.038	1.000	-103.20	107.28
		TP-H3-c	-6.698	0.998	-108.57	95.18
	TP-H3-b	TP-H3-a	-2.038	1.000	-107.28	103.20
		TP-H3-c	-8.736	0.996	-113.24	95.77
	TP-H3-c	TP-H3-a	6.698	0.998	-95.18	108.57
		TP-H3-b	8.736	0.996	-95.77	113.24

Table 29. Statistical analysis for significant difference within samples of Murtazaabad MA-H4 with Tukey HSD, LSD and Tamhane

Analysis	(I) coding	(J) coding	Mean Difference (I-J)	Significance	95% Confidence Interval	
					Lower Bound	Upper Bound
Tukey HSD	MA-H4-a	MA-H4-b	1.642	0.999	-114.83	118.11
		MA-H4-c	-10.038	0.977	-126.51	106.43
	MA-H4-b	MA-H4-a	-1.642	0.999	-118.11	114.83
		MA-H4-c	-11.679	0.969	-128.15	104.79
	MA-H4-c	MA-H4-a	10.038	0.977	-106.43	126.51
		MA-H4-b	11.679	0.969	-104.79	128.15
LSD	MA-H4-a	MA-H4-b	1.642	0.973	-95.58	98.87
		MA-H4-c	-10.038	0.839	-107.26	87.19
	MA-H4-b	MA-H4-a	-1.642	0.973	-98.87	95.58
		MA-H4-c	-11.679	0.813	-108.90	85.55
	MA-H4-c	MA-H4-a	10.038	0.839	-87.19	107.26
		MA-H4-b	11.679	0.813	-85.55	108.90
Tamhane	MA-H4-a	MA-H4-b	1.642	1.000	-102.71	105.99
		MA-H4-c	-10.038	0.996	-136.23	116.15
	MA-H4-b	MA-H4-a	-1.642	1.000	-105.99	102.71
		MA-H4-c	-11.679	0.994	-138.55	115.19
	MA-H4-c	MA-H4-a	10.038	0.996	-116.15	136.23
		MA-H4-b	11.679	0.994	-115.19	138.55

Table 30 Pair wise comparison between studied sites at phylum level

(I) Tato Field, Tatta pani, Murtazaabad	(J) Tato Field, Tatta pani, Murtazaabad	Mean Difference (I-J)	Significance	95% Confidence Interval for Difference[a]	
				Lower Bound	Upper Bound
Tato Field	Tatta pani	-0.115	0.027	-0.755	0.525
	Murtazaabad	-0.281	0.010	-0.921	0.360
Tatta pani	Murtazaabad	-0.166	0.024	-0.806	0.474
	Tato Field	0.115	0.027	-0.525	0.755
Murtazaabad	Tato Field	0.281	0.010	-0.360	0.921
	Tatta pani	0.166	0.024	-0.474	0.806

*Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

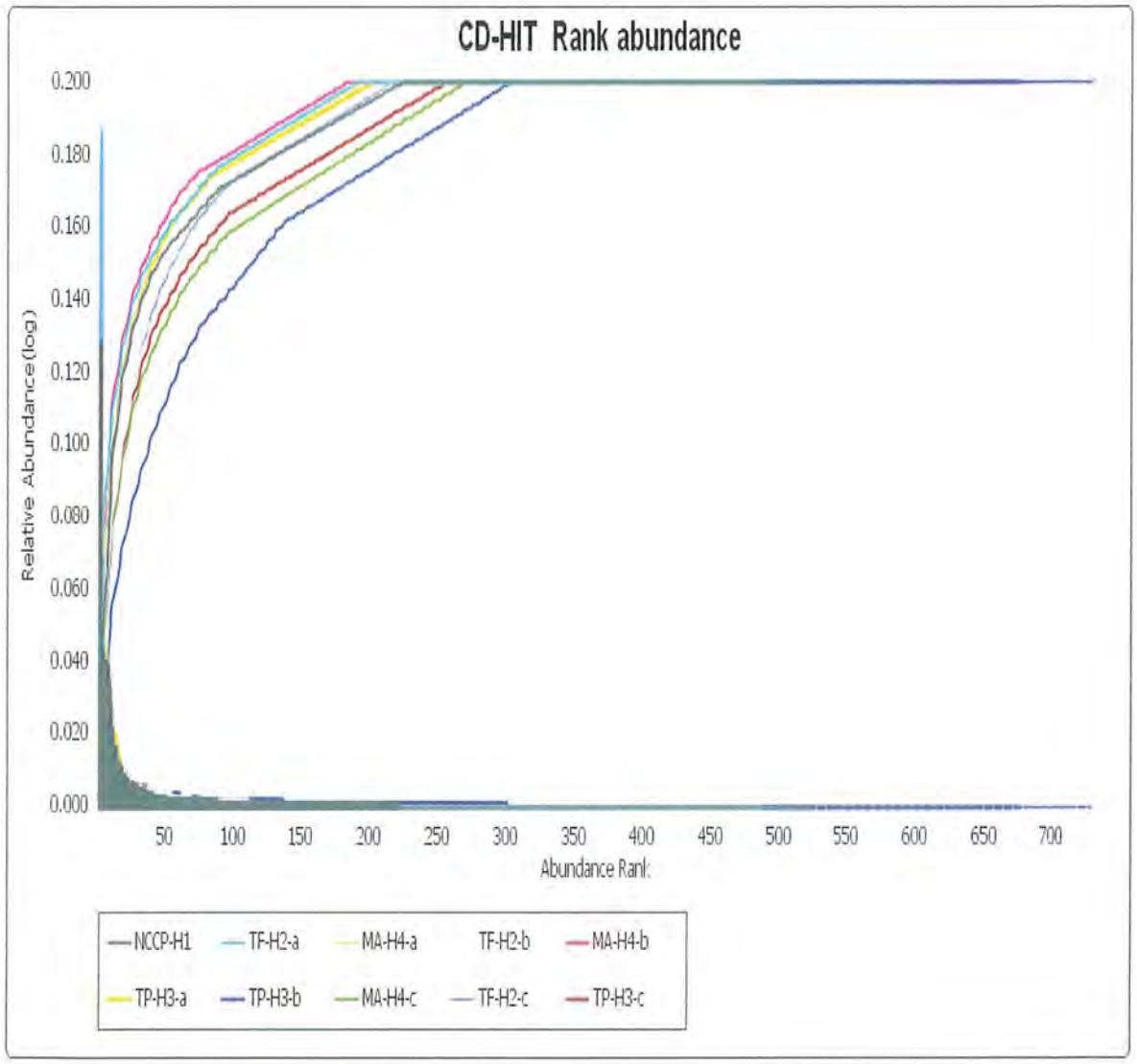


Figure 40. Relationship between Relative abundance and abundance rank between the control and hot water spring sites of TP-H3, TF-H2 and Ma-H4 by CD-HIT analysis

Among the Tatta Pani samples, both richness and abundance were highest in TP-H3-c. For the Murtazaabad hot spring samples, richness was highest in MA-H4-c but relative abundance was highest in MA-H4-b (Fig. 40\ If samples between different sites are compared at 95 % confidence level, the significance values were ranging between 0.010 and 0.027 (Table 30). Among samples from Tato Field, TF-H2-a showed highest relative abundance while richness was highest in sample TF-H2-c (Fig.. 40).

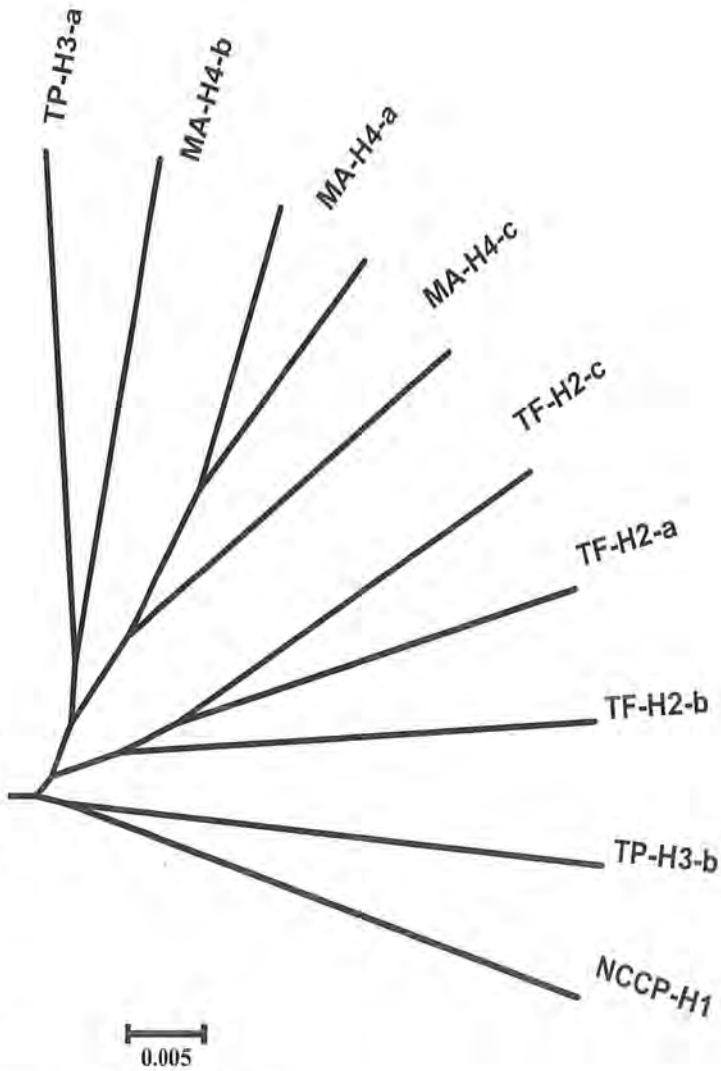


Figure 41. UPGMA dendrogram based on UniFrac distance matrix for comparison of microbial-community structures in the hot springs of Tato Field (TF-H2), Tatta Pani (TP-H3) and Murtazaabad (MA-H4).

UPGMA dendrogram generated based on UniFrac distance matrix (Fig. 41, Table 31) showed four major clades. In two of the clades, the three samples of Tato Field and Murtazaabad hot springs formed single closed independent clusters. However samples of Tatta Pani formed two clades relatively independent of each other, where TP-H3-c and TP-H3-a shared a branch while TP-H3-b clustered with NCCP-H1.

The PCoA plot based on the UniFrac distance matrix (Fig. 42) showed three non-overlapped clusters separating the three sites from each other with PC1 at 36.4 %, PC2 31.9 % and PC3 25.5 %. Slight rotation of axis did not have any effect on the cluster. Control NCCP-H1 formed a separate cluster and did not coincide with any of the three clusters.

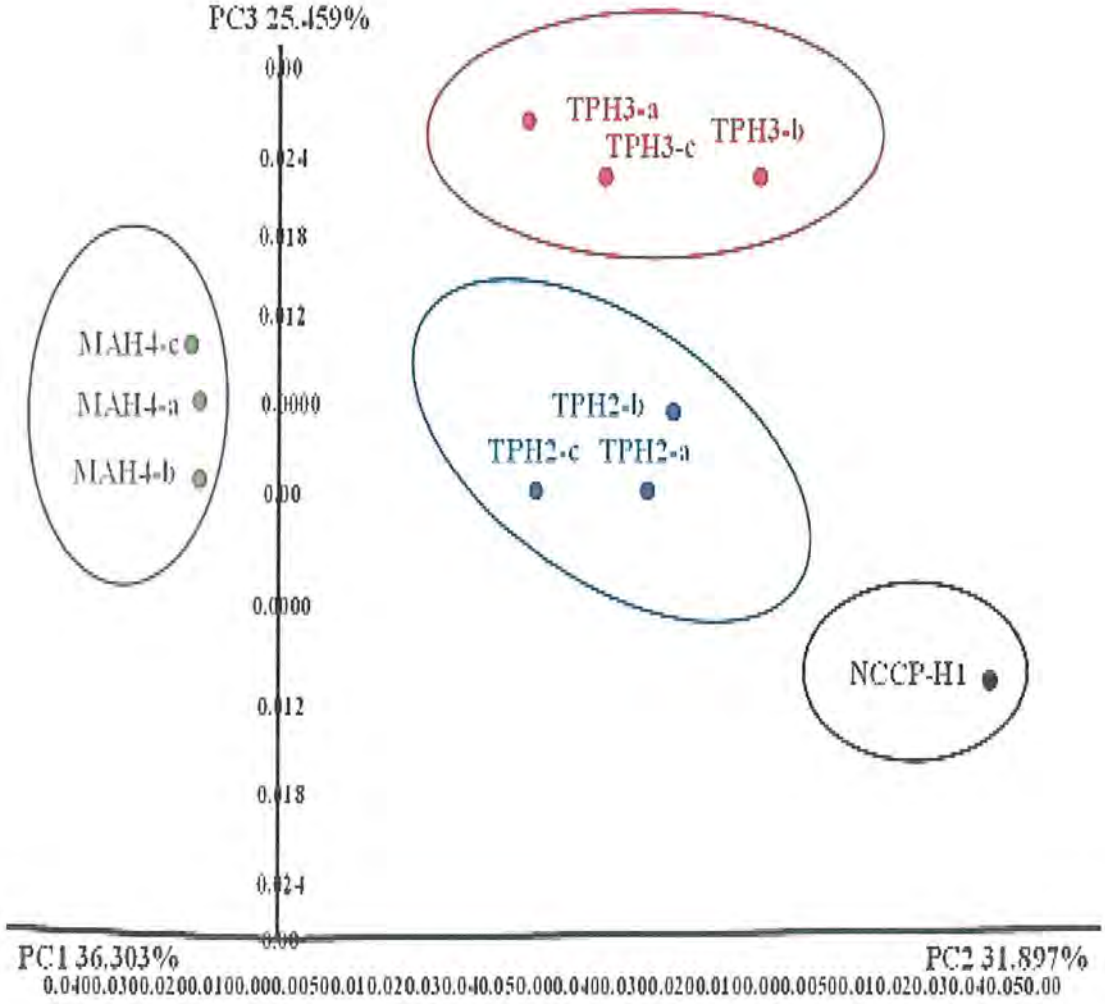


Figure 42. Principal coordinate analysis (PCoA) plot for the hot spring samples using UniFrac distance matrix. All OTUs were clustered into three clusters at the species level.

Two-way ANOVA was used to analyze the significance of physicochemical parameters on diversity of important taxa (Table 32), which showed significant values for seven phyla between different sampling sites (P values ranges from 0.001 for phylum *Deinococcus-Thermus* between TF-H2 and MA-H4 to 0.093 for phylum *Bacteroidetes* between TP-H3 and MA-H4).

Table 31 Values for Unifrac distance matrix

NCCP-H1	0									
TF-H2-a	0.082	0								
TF-H2-b	0.094	0.063	0							
TF-H2-c	0.077	0.054	0.060	0						
TP-H3-a	0.060	0.079	0.081	0.061	0					
TP-H3-b	0.070	0.0746	0.077	0.062	0.070	0				
TP-H3-c	0.084	0.083	0.065	0.072	0.074	0.081	0			
MA-H4-a	0.070	0.074	0.070	0.065	0.051	0.073	0.061	0		
MA-H4-b	0.065	0.075	0.072	0.066	0.054	0.077	0.062	0.033	0	
MA-H4-c	0.070	0.075	0.068	0.061	0.068	0.060	0.058	0.064	0.058	0
Sample	**NCCP-H1**	**TF-H2-a**	**TF-H2-b**	**TF-H2-c**	**TP-H3-a**	**TP-H3-b**	**TP-H3-c**	**MA-H4-a**	**MA-H4-b**	**MA-H4-c**

Table 32 Two-way ANOVA for relative sequence abundances in comparison with temperature

Taxa	TP-H3*TF-H2	TP-H3*MA-H4	TF-H2*MA-H4
Proteobacteria	0.003	0.006	0.005
Chloroflexi	0.043	0.015	0.042
Nitrospirae	0.054	0.03	0.03
Aquificeae	0.004	0.070	0.029
Bacteroidetes	0.03	0.092	0.030
Thermotogae	0.049	0.042	0.006
Deinococcus-thermus	0.008	0.005	0.001
Actinobacteria	0.004	0.050	0.037

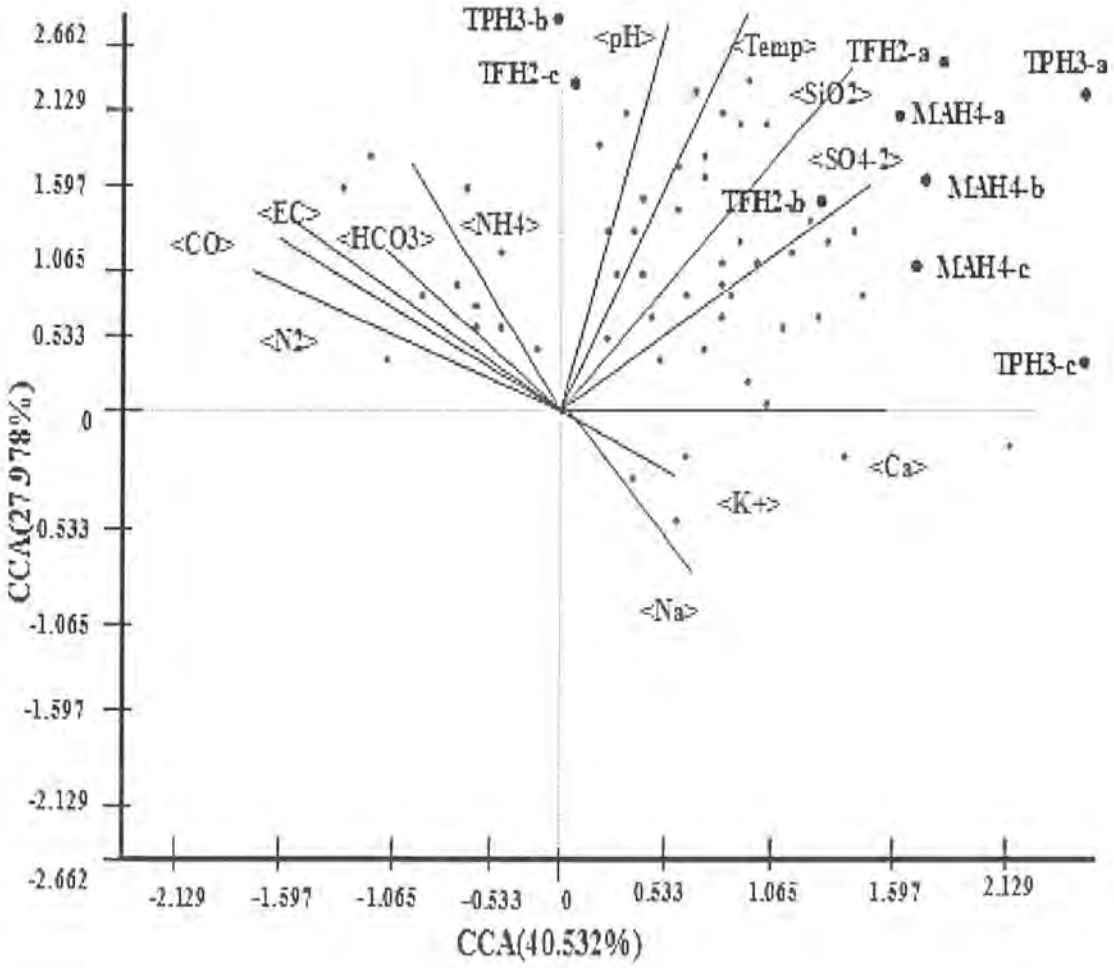


Figure 43. Canonical correspondence analysis (CCA) plot evaluating the effect of physicochemical parameters on bacterial community structures. Physicochemical factors included electrical conductivity, pH, temperature, and concentration of N_2, CO, HCO_3^-, NH_4^+, SiO_2, SO_4^{-2}, Ca^{+2}, K^+ and Na^+. Red dots represent the OTUs at phylum level, while the black dots represent the samples TF-H2-a, TF-H2-b, TF-H2-c, TP-H3-a, TP-H3-b, TP-H3-c, MA-H4-a, MA-H4-b and MA-H4-c.

Environmental data as shown in Table 25 were used to determine influence of physico-chemical parameters on bacterial diversity by Canonical Corresponding Analysis (CCA; Fig. 43). CCA1 showed 40.5 % variation along X- axis while CCA2 showed 28.0 % variation along Y-axis. Factors like temperature, pH, silica and sulfate contents were the driving factors determining bacterial distribution in specific sites

6.5. Discussion

6.5.1. Correlation between Microbial Diversity and Physicochemical Parameters

Higher species richness and abundance in sediments of Tatta Pani than in sediments of Tato Field and Murtazaabad (Figs 43, 44) may be due to the moderate temperature, high silicates and high sulfate contents of Tatta Pani Springs (Table 25). The dominant OTUs in moderately thermophilic environment comprised of phyla *Chloroflexi*, *Proteobacteria* and *Cyanobacteria* (Cox, Shock, & Havig, 2011; Otaki, Everroad, Matsuura, & Haruta, 2012). Along the Tatta Pani hot water spring situated in India, phylum *Proteobacteria* was reported as the dominant microorganisms (Mohanrao & Singh et al., 2016) . (Lau et al., 2009) and (Yim, Hongmei, Aitchison, & Pointing, 2006) also reported the influence of temperature (<70 °C) for the presence of *Cyanobacteria* and *Chloroflexi* in hot springs. In the present study, OTUs of the phyla *Proteobacteria* and *Chloroflexi* were found to be dominantly present in all the sampling sites, but not phylum *Cyanobacteria.* The reason may be attributed toward metabolic advantages of *Chroloflexi* over *Cyanobacteria* in hot spring sediments(Liu et al., 2011) (S. R. Miller, Strong, Jones, & Ungerer, 2009).

Thermophilic and hyperthermophilic bacteria had been predominantly isolated from streamers with temperature above 75 °C and mainly comprised of the phyla *Aquificeae, Deinococcus-Thermus. Thermodesulfobacteria* and *Thermotogae* and some members of the phyla *Proteobacteria* and *Firmicutes* (S. R. Miller et al., 2009) (Shang Wang et al., 2013) . Murtazaabad hot springs with relatively higher temperature (90-95 °C) favored the growth of thermophilic bacterial phylum *Thermotogae*. These domains were also detected in higher proportion in sites of Tata Field and Tatta Pani where average temperature is above 85 °C and least at TP-H3-b where temperature is found to be 60 °C. However OTUs of phyla *Aquificeae* and *Deinococcus-Thermus* were more dominant in sites ranging in temperature from 70 to 85 °C. At sites with low silica and high temperature, OTUs belonging to phylum *Chloroflexi* were dominant.

Kambura et al., (2016) believed that existence of phyla *Actinobacteria* and *Firmicutes* was an adaptation in low-nutrient conditions of the hot springs. In the present study, the maximum OTUs of both the phyla in TP-H3-b may be attributed to slightly alkaline pH (pH 8.4) and lowest temperature (60 °C). A much higher OTUs for most abundantly present order *Rhizobiales* in stagnant water of TP-H3-a (75 °C) and less in MA-H4-a (90 °C) may be related with long water residence

time (Dodsworth, Hungate, & Hedlund, 2011) . Deep water areas of the Murtazaabad hot water spring favored the sulfur-reducing bacteria.

6.5.2 Comparison with Previous Studies of Hot springs along the MKT

Previous studies of thermophilic bacteria in hot springs along the Main Karakoram Thrust have mostly focused on microbial cultivation studies (M. Kumar, Yadav, Tiwari, Prasanna, & Saxena, 2014) (Javed et al., 2012a) (Sharma, Vyas, & Pathania, 2013). The bacterial phylum *Firmicutes* have been detected as the dominant taxa in cultures, along with a small proportion of *Proteobacteria* and *Actinobacteria*. Our studies revealed the presence of *Firmicutes* and *Actinobacteria* in low temperature thermal springs and *Chloroflexi* and *Thermotogae* in high temperature springs. A comparison between the previous metagenomics-based study (Mohanrao & Singh et al., 2016) and this study revealed consistency in the identification of the dominant phylum *Proteobacteria* (Fig. 35). There is however discrepancy with regards to the proportion of other thermophilic taxa, especially phylum *Chloroflexi*.

6.5.3. Microbial Community Composition

The composition of microbial communities in thermal springs of Tato Field, Tatta Pani and Murtazaabad were largely dependent on the temperature, pH, residence time and physicochemical parameters. The correlation of the microbial community composition with the geochemistry of the thermal springs in the MKT can be better understood by a comparison of the dominant bacterial groups with relation to other geothermal systems around the world.

***Proteobacteria*:** Phylum *Proteobacteria* has been previously observed to be dominant in many geothermal environments (Mohanrao & Singh et al.,2016) (Deng, Cui, Hernández, & Dumont, 2014) (Lau et al., 2009) (Tekere, Lötter, Olivier, Jonker, & Venter, 2011) but this observation is not consistent with hot springs in Yellowstone National Park and Tengchong (Carrine E Blank et al., 2002) (Hou et al., 2013) (Hedlund et al., 2015) . In our study *Proteobacteria* was detected as the dominant bacteria and it constituted 54.6 % of the total OTUs. Among them, many genera in class *Gammaprotobacteria* and *Alphaproteobacteria* of types I and II methanotrophs (C. E. Sharp, Martínez-Lorenzo, Brady, Grasby, & Dunfield, 2014) (Dedysh et al., 2002) are common in this study. In Tato Field springs, type II methanotrophs belonging to genera *Hyphomicrobium, Methyloligella* and *Methylobacterium* were detected. Methane-oxidizing *Betaproteobacteria* of the genus *Methylopila* were present in TF-H2-a while

in TF-H2-b and TF-H2-c, genus *Methyloversatilis* was dominant. The detection of type I methane oxidizers belonging to genus *Methylothermus* of the family *Methylothermaceae* at all sites in our study were in congruence with the findings of thermotolerant methanotrophic bacteria *Methylothermus thermalis* and *M. subterraneus* from a hot spring and hot subsurface thermal aquifers in Japan (Tsubota, Eshinimaev, Khmelenina, & Trotsenko, 2005). The dominant methanotrophs identified in TP-H3-a were *Methylothermaceae_uc* and *Methylobacterium rhodesianum*, and in TP-H3-b was unclassified species belonging to genus *Methyligella* of type II methanotrophs. However, no classified methanotroph of the class *Betaproteobacteria* was detected in the Tatta Pani springs. Unclassified OTUs of order *Methylococcales_uc* were found in majority of the thermal springs of this study except in TP-H3-a and TP-H3-b. In Murtazaabad thermal springs MA-H4-a and MA-H4-b, type II methanotrophs belonging to genera *Methylobacteriaceae_uc, Methyloversatilis_uc* and various unclassified species were detected, but not type I methanotrophs. In MA-H4-c, family *Methylococcaceae*, which is usually present in effluent of thermal springs (Merkel, Podosokorskaya, Sokolova, & Bonch-Osmolovskaya, 2016) was detected

Sulfate-reducing bacteria (SRB) of the class *Deltaproteobacteria* are a major constituents in geothermal related environments such as the alkaline and saline oxbow lake which serve as short term reservoir for geothermal water (Borsodi et al., 2016) . This group is commonly found in the present study, and constituted 12.2 %, 10.1 % and 7.3 % of phylum *Proteobacteria* in three sites of Tato Field springs. Despite low OTUs among the Tato Field sites, the diversity of SRB was more in TF-H2-c . Among Tatta Pani sites, 140, 79 and 217 OTUs in TP-H3-a, TP-H3-b and TP-H3-c respectively, were affiliated with SRB species of *Deltaproteobacteria*. The distributions of these OTUs may be attributed to temperature and oxygen content of the sites. The samples in TP-H3-b were collected from surface water and therefore had low temperature and higher oxygen content than the relatively deeper TP-H3-c. pH was another factor that had an effect on the activity and thereby the distribution of SRB. TP-H3-a was slightly acidic and other 2 sites alkaline. Among the Tatta Pani sites, the genera *Desulfatirhabdium* and *Desulfomicrobium apsheronum* sp. were unique to TP-H3-a. In Murtazaabad thermal springs, SRB of the class *Deltaproteobacteria* were evenly distributed among the three sites.

Chloroflexi: OTUs for *Anaerolinea thermophila* was the most dominant group among phylum *Chloroflexi* in our study. Other prominent OTUs of classified species belonged to class *Anaerolineae* which included *Bellilinea caldifistulae, Thermanaerothrix daxensis*, *Chloroflexus aurantiacus* and *Chloroflexus aggregans*. The species *Chloroflexus aurantiacus* has also been reported in Zodletone thermal spring (M. S. Elshahed et al., 2003). Castenholz (Alexander & Imhoff, 2006) reported hidden mats of photoheterotrophic *Chloroflexi* with cyanobacterial mats in Hunter's hot water spring. OTUs for an unclassified family in halotolerant class *Dehalococcoidetes* of group II *Chloroflexi* were present in the thermal springs of Tatta Pani and Murtazaabad. The diversity of phylum *Chloroflexi* was found to be directly influenced by temperature. This is exemplified by the presence of highest OTUs for phylum *Chloroflexi* in Murtazaabad hot springs which had the highest temperature among the study sites. *Chloroflexus* relatives of neutral to alkaline hot springs viz. *Chloroflexus aurantiacus* and *Chloroflexus aggregans* as reported by (Nübel, Bateson, Madigan, Kühl, & Ward, 2001), were also detected in all sites of the present study. Our study also detected members of *Chloroflexi* in shared habitats with unclassified OTUs of *Cytophaga, Veruucomicrobia*, *Chlorobium, Firmicutes* and *Planctomycetes*, which is in congruence with the findings of Elshahed et al. (M. S. Elshahed et al., 2003). In another study, dual mode of life present in thermal springs was revealed with both aerobic and anaerobic lifestyle of the community members in a single hot water spring (Tripathy, Padhi, Mohanty, Samanta, & Maiti, 2016)]. It can be confirmed by the presence of most prominent phylum *Proteobacteria* and significant number of phylum *Choroflexi* in the current study.

***Thermotogae*:** In the present study, *Thermotogae* was the third most dominant phylum after *Proteobacteria* and *Chloroflexi*. The detection of phylum *Thermotogae* may be attributed to the high temperature of hot springs (>85 °C) in TF-H2-a and Murtazaabad. Similar, cultures of genus *Fervidobacterium* of the order *Thermotogales* have been isolated from Mae Fang National Park, Thailand (Podosokorskaya et al., 2011) and Changbai Mountain, China (J. Cai et al., 2007) where the average temperature of the sampling sites is above 80 °C.

Aquificae: OTUs belonging to the phylum *Aquifaceae* were predominantly detected in TF-H2-a (91 OTUs), with the most dominant species being *Sulfurihydrogenibium_uc* (59 OTUs). S. Wang et al. (2013) reported that *Aquificae*

occurred at high temperature springs and their distribution was directly proportional to temperature and silica contents. In the current study, no clear relationship could be established between the distribution of *Aquificae* and temperature. For instance, OTUs of phylum *Aquificae* in Murtazaabad hot springs (>90 °C) were significantly less than Tato Field thermal springs (70-90 °C), while no *Aquificae* was being detected in Tatta Pani springs (60-90 °C). Rather than temperature, the higher level of *Aquificales* in Tato Fields could be attributed to the level of silica. Similar findings had been reported for the distribution of *Aquificales* in Yellowstone National Parks (C. E. Blank, S. L. Cady, & N. R. Pace, 2002; Spear et al., 2005b).

6.5.4. Physiological Functions

It will be difficult to infer physiological functions from the sequence data. However certain tentative physiological roles can be determined based on the taxonomic affiliations of the metagenome. Studies from sulfur-rich geothermal springs revealed that methane produced in anoxic water bodies are oxidized by a consortium of methanotrophs and SRB (Mostafa S Elshahed et al., 2003) (Tripathy et al., 2016) (Delgado-Serrano et al., 2014) . As expected, *Proteobacteria* of the type I and II methanotrophs and SRB are a major constituent in the study sites, and likely involved in the mineral recycling under the low oxygen conditions of hot springs, which in turn help in energy production. In acidic hot springs, this metabolism of energy recycling may be initiated by methane oxidizing phylum *Verrucomicrobia* (Islam, Jensen, Reigstad, Larsen, & Birkeland, 2008) (C. Sharp, Stott, & Dunfield, 2012), as indicated by its presence in slightly acidic sites TP-H3-a (pH 6.2) and MA-H4-c (pH 6.7).

Phylum *Clostridia* which are known to be obligatory dependent on methanogens or on the presence of an external electron acceptor (J. Hugenholtz & Ljungdahl, 1990) was observed in TF-H2-a. Few OTUs of the thermophilic, anaerobic, Mn(IV)- and Fe(III)-reducing *Carboxydocella* of the species *Carboxydocella_uc* and *Carboxydocella manganica* were detected in MA-H4-a and MA-H4-b. These strains can assimilate CO chemolithoautotrophically under aerobic conditions and used CO dehydrogenases under anaerobic conditions (Sokolova et al., 2002) . Few OTUs of genus *Ammoniphilus* were present in the three sites of Tatta Pani. They were obligatory oxalotrophic and haloalkalitolerant bacteria and required a high concentration of ammonium ions and pH of 6.8-9.5 (Tables 25). In MA-H4-a, unclassified species belonged to genera *Anaerosporobacter* and *Nitratireductor* were detected. This finding

could be in lineal with oxidation of NO_2 to nitrate by *Nitrospira* at high temperature (Erko Stackebrandt, 2014) and subsequent reduction of nitrate to nitrous oxide or complete oxidation to N_2 by members of the order *Thermales*, *Aquificales* and *Bacillales* (Nakagawa & Fukui, 2002) (Mishima et al., 2009).

6.6. Conclusion

The geothermal fields in Tato Field, Tatta Pani and Murtazaabad were highly diverse in their environmental conditions and encompassed sites ranging in temperature from 60 to 95 °C and in pH from 6.2 to 9.4. Distinct bacterial lineages and unclassified bacteria were observed at all taxonomic levels in the hot springs of MKT located in Pakistan. These phyla include *Proteobacteria, Chloroflexi, Thermotogae, Bacteriodetes, Actinobacteria, Deinococcus-thermus, Nitrospirae, Acidobacteria* and others well reported unclassified thermal spring phyla such as UT06, OP11, BRC1, OD1, OP8, OP1, OP3, OP9, OMAN and NKB19. The composition of the microbial communities in these geothermal sites were however dependent on the environmental parameters such as temperature, pH and physicochemical conditions. Apart from the dominant group, the presence of many unclassified bacteria gives an implication of a complex physiological process in nature (Kamruzzaman et al., 2010) (Sogin et al., 2006) , but could not determine from the metagenomics data of the present study. This information could however be supplemented if comparative studies were done along with cultivable bacteria associated with these thermal springs (Selim et al., 2016) (Sahoo et al., 2016) .

CHAPTER 7

Conclusion

Of various extreme environments for the growth of microorganisms, high temperature tolerant organisms have been presented as biotechnologically important extremophiles. It is obvious that we have only scratched the surface in revealing a new vista this regime. Several species of bacteria were examined from these special sites and high temperature tolerant species were identified and characterized in systematic taxonomy. Based upon our taxonomic data, three novel temperature tolerant species; *Nocardioides pakistanensis* sp. nov., *Microvirga pakistanensis* sp. nov. (Amin et al., 2016 and b) and *Streptomyces caldifontis* sp. nov., (Amin et al 2017). Three Actinobacterial strains have been isolated from hot water spring soils from Tatta Pani hot water spring, Kotli Kashmir and Bacterial strain was isolated from Cholistan desert soil, Bahawalpur Pakistan. These novel species were not as such thermophilic but high temperature tolerant because they survived in hot water springs where temperature remained above 65°C whole year and also reaches up to 900°C. One salt tolerant strain, *Zafaria solitudinis* gen. nov. sp. nov. that was isolated from Tatta pani hot water spring soil from Kotli Kashmir has been proposed in a novel genus NCCP-1664^T gen. nov. (Amin et al. 2016). This novel strain was high salt tolerant and can grow efficiently up to 16% (w/v) NaCl concentration. It was initially proosed to be new species of Arthrobacter genus but lateron Busse et al 2016 described whole new classification between Arthrobacter genus according to peptidoglycan type, quinone type and polar lipids profile. NCCP-1664^T contained polar lipids, which was different from already described groups and new genera between Arthrobacter genus, Phylogenetic position by using all algorithms and these chemotaxonomic features became the basis for proposal of new genus.

In addition to diversity studies in lab 454 pyrosequencing approach was used to get complete diagram of our selected sites for presence of bacteria. Various distinct lineages and unclassified bacteria were observed at all taxonomic levels. Distinct phyla which seemed to outcompete were *Proteobacteria, Chloroflexi, Thermotoga, Bacteriodetes, Deinococcus thermus, Nitrospirae, Acidobacteria* and others well reported thermal spring phyla were UT06, OP11, BRC1, OD1, OP8, OP1, OP3, OP9,

OMAN, NKB19. Environmental properties like pH, temperature and sulfur influenced the community structure at most that was depicted by presence of sulfur and nitrate reducing bacteria, but the influence of other factors on microbial community assemblage like anaerobic stress in deep water; methane, ammonia and presence of planktonic material were supported by presence of *Chloroflexi, Anaerobiales*. Dominance of *Chloroflexus* and low number of order *Aquificales* were also studied by Skirnisdottir, et al. at upper temperature of 88 to 90°C that matched our results. High temperature and low oxygen site of Tatta pani spring (TP-H3-c) had largest OTUs for sulfur bacteria. Deltaproteobacteria purple sulfur bacteria were most dominant in sulfur rich (Tatta pani hot water spring) TP-H3 sites.

Presence of Cyanobacteria at (Tato Field hot water spring) TF-H2-a and (Tato Field hot water spring) TF-H2-b effected number and diversity of purple sulfur bacteria even at high temperature. Highest numbers of OTUs for purple sulfur bacteria 217 were present in (Tatta Pani hot water spring samples) TP-H3-c which was sulfur rich site and second highest number was observed in (Tato Field hot water spring) TF-H2 where sulfur level was much lower but phylum Cyanobacteria population was present to support their growth. Another unique phylum UT06 was present with rich diversity in (Tata Pani hot water spring) TP-H3 and (Murtazaabad hot water spring) MA-H4 and low in Tato field samples. Its diversity and number were evenly high in all samples of MA-H4 but in TP-H3 its distribution was not even.

Similar physiochemical properties of hot water springs located at far distances and varying geographical locations were responsible for linked microbial community. Presence of closely related microbial species in neighbouring hot water springs indicated that movement of water and soil was also responsible for designing microbial community structure in adjacent environments. These geothermal sites should be considered to explore natural biogeochemical cycles and role of specific microorganisms in energizing these cycles to exploit these potentials in near future. In these hot water springs enhanced conditions of high temperature, alkaline pH, methanogenesis all met automatically and genes for methanogenesis were switched in methanogens. Study suggested that methanotrophy in these thermal sites was not restricted to only one type of methanotroph, members of type I methanotroph, aerobic methanotroph, beta proteobacteria methanotroph and type II methanotroph all collectively were responsible for methane cycle in thermal systems.

For Cholistan desert pyrosequencing study we found that both alpha and beta diversity measures are much higher than previous evaluations for desert soil. Presence of specific OTUs were linked with the location of samples e.g., Hydrogenophaga electricum and Anaerolinae thermophila were profusely present in NCCP-D1-b as compared to NCCP-D1-a, and Arenimonas malthae, Rhizobium daejeonensis and Anaerolinae thermophile were substantial in number in NCCP-D3-b as compared to NCCP-D3-a. Phylu Proteobacteria was most dominant in all samples by overall bacterial diversity and specially Actinobacterial diversity in rhizospheric samples was significantly higher as compared to non rhizospheric samples.

Mutualistic and symbiotic relationships between different culturable bacteria were already being studied between many bacteria but how nonculturable bacteria determines the presence and dominance of classified and culturable bacteria has not been studied yet and various unexplored metabolic aspects of microbiota of these hot water springs are to be studied yet.

PROPOSED FUTURE STUDIES

Future studies should be focused to unravel all ignored geochemically important sites specially to fill gap of limited culturing techniques and substrates so far known to study these non-cultured bacteria. The taxonomic results obtained in this study provide information to the ecological studies for exploration in this regime and thus discovers new whole area for further research in the subject of 'Extremophiles'. Although we have tried our best to give a clear picture of bacterial distribution of two sites but we would like to propose few future studies as extension of present study

1. Controlled experiments would help identify which factors influencing species distribution at most.
2. Similarly rhizosphere versus non rhizospaheric soil organisms can be observed in relation to rhizospere specific carbon compounds.
3. Metabolic and genome wide studies can help researchers to extend this research for narrowing down habitat in genomically interconnected populations.
4. Discrete, particular bacterial lineages can be found in contrasting soil types.
5. In case if genomic interconnections are low, variations in the core and accessory genomes can be found to solve distribution of distinct biogeographical patterns. For example, (N. Kumar et al., 2015) (Boucher et al., 2011) (Porter, Chang, Conow, Dunham, & Friesen, 2017)reported that core genomes of *Rhizobium leguminosarum* and *Vibrio cholerae* have high similarity but the accessory genome is much varied. The accessory genome is principal pool of microbial adaptive variation because it can be vanished or gained via horizontal transfer (Polz, Alm, & Hanage, 2013) (Porter et al., 2017) .
6. Specific classes of bacteria can be studied along with their biogeochemical cycles i;e; methane cycle related microorganisms, anaerobes, nitrogen cycle related bacteria, sulfate reducing bacteria and archea.
7. Novel enzymes can be extracted from novel genomes by metagenomics studies and whole genome analysis. (Pasternak et al., 2013) stated that that impact of bacterial diversity and their abundance on nature can be explained only by using full-genome proteomic comparisons..

REFERENCES

Ahmad, M., Akram, W., Hussain, S. D., Sajjad, M. I., & Zafar, M. S. (2001). Origin and subsurface history of geothermal water of Murtazabad area, Pakistan-an isotopic evidence. *Appl Radiat Isot.* 55(5), 731-736.

Ahmad, M., Tasneem, M., Rafique, M., Akram, W., Iqbal, N., Rafiq, M., & Fazil, M. (2005). *Identification of recharge sources and dating of groundwater using isotope and CFC techniques.* Paper presented at the Proceedings of the first international conference on environmentally sustainable development v. 1-3.

Ahmad, S., Ali, A., Ullah, I., Naz, N., Ali, A., & Ali, N. (2013). Bacteriological and biochemical evaluation of the spring's water of district Buner Khyber Pakhtunkhwa Pakistan. *Int J Adv Res Technol.* 2, 452-460.

Ahn, J. H., Lim, J. M., Kim, S. J., Song, J., Kwon, S. W., & Weon, H. Y. (2014). *Nocardioides paucivorans* sp. nov. isolated from soil. *J Microbiol.* 52(12), 990-994. doi: 10.1007/s12275-014-4587-x

Ahsan Mustaqeem, S., Qureshi, S. R., & Waqar, A. Exergetic and Energetic Analysis of Power Generation from Geothermal Resources in Pakistan.

Al-Dhabi, N. A., Esmail, G. A., Duraipandiyan, V., Arasu, M. V., & Salem-Bekhit, M. M. (2016). Isolation, identification and screening of antimicrobial thermophilic *Streptomyces* sp. Al-Dhabi-1 isolated from Tharban hot spring, Saudi Arabia. *Extremophiles.* 20(1), 79-90.

Alexander, B., & Imhoff, J. F. (2006). Communities of green sulfur bacteria in marine and saline habitats analyzed by gene sequences of 16S rRNA and Fenna-Matthews-Olson protein. *Int Microbiol.* 9(4), 259-266.

Alou, M. T., Rathored, J., Lagier, J.-C., Khelaifia, S., Labas, N., Sokhna, C., Dubourg, G. (2016). *Massilibacterium senegalense* gen. nov., sp. nov., a new bacterial genus isolated from the human gut. *New microbes new infect (NMNI).* 10, 101-111.

Altenburger, P., Kämpfer, P., Schumann, P., Steiner, R., Lubitz, W., & Busse, H.-J. (2002). *Citricoccus muralis* gen. nov., sp. nov., a novel actinobacterium isolated from a medieval wall painting. *Int J Syst Evol Microbiol.* 52(6), 2095-2100.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol.* 215(3), 403-410. doi: 10.1016/s0022-2836(05)80360-2.

Amin, A., Ahmed, I., Habib, N., Abbas, S., Hasan, F., Xiao, M., Li, W.-J. (2016). *Microvirga pakistanensis* sp. nov., a novel bacterium isolated from desert soil of Cholistan, Pakistan. *Arch. microbiol.* 1-7.

An, S., Couteau, C., Luo, F., Neveu, J., & DuBow, M. S. (2013). Bacterial diversity of surface sand samples from the Gobi and Taklamaken deserts. *Microb Ecol.* 66(4), 850-860. doi: 10.1007/s00248-013-0276-2.

Andrew, D. R., Fitak, R. R., Munguia-Vega, A., Racolta, A., Martinson, V. G., & Dontsova, K. (2012). Abiotic factors shape microbial diversity in Sonoran Desert soils. *Appl Environ Microbiol.* 78(21), 7527-7537. doi: 10.1128/aem.01459-12.

Angel, R., Soares, M. I., Ungar, E. D., & Gillor, O. (2010). Biogeography of soil archaea and bacteria along a steep precipitation gradient. *Isme j.* 4(4), 553-563. doi: 10.1038/ismej.2009.136.

Angers, D. A., & Caron, J. (1998). Plant-Induced Changes in Soil Structure: Processes and Feedbacks. *Biogeochemistry.* 42(1/2), 55-72.

APHA. (1985). *Standard Methods for the Examination of Water and Wastewater* (6th ed.). Washington, DC: American Public Health Association, USA.

Ardley, J. K., Parker, M. A., De Meyer, S. E., Trengove, R. D., O'Hara, G. W., Reeve, W. G., Howieson, J. G. (2012). *Microvirga lupini* sp. nov., *Microvirga lotononidis* sp. nov. and *Microvirga zambiensis* sp. nov. are *alphaproteobacterial* root-nodule bacteria that specifically nodulate and fix nitrogen with geographically and taxonomically separate legume hosts. *Int J Syst Evol Microbiol.* 62(Pt 11), 2579-2588. doi: 10.1099/ijs.0.035097-0.

Arnórsdóttir, J., Sigtryggsdóttir, Á. R., Thorbjarnardóttir, S. H., & Kristjánsson, M. M. (2009). Effect of proline substitutions on stability and kinetic properties of a cold adapted subtilase. *J. biochem.* 145(3), 325-329.

Bakht, M. S. (2000). *An over view of geothermal resources of Pakistan.* Paper presented at the Proc World Geotherm Congr.

Bakht, M. S. (2000). *An overview of geothermal resources of Pakistan.* Paper presented at the Proceedings World Geothermal Congress.

Barns, S. M., Fundyga, R. E., Jeffries, M. W., & Pace, N. R. (1994). Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proceedings of the National Academy of Sciences.* 91(5), 1609-1613.

Bates, S. T., Berg-Lyons, D., Caporaso, J. G., Walters, W. A., Knight, R., & Fierer, N. (2011). Examining the global distribution of dominant archaeal populations in soil. *Isme j.* 5(5), 908-917. doi: 10.1038/ismej.2010.171.

Beijerinck, M. (1913). Jaarboek van de Koninklijke Akademie v. *Wetenschoppen. Muller, Amsterdam, The Netherlands.* 43(2).

Berelson, W. M., Corsetti, F. A., Pepe-Ranney, C., Hammond, D. E., Beaumont, W., & Spear, J. R. (2011). Hot spring siliceous stromatolites from Yellowstone National Park: assessing growth rate and laminae formation. *Geobiology, 9*(5), 411-424. doi: 10.1111/j.1472-4669.2011.00288.x.

Berg, G., & Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol Ecol.* 68(1), 1-13. doi: 10.1111/j.1574-6941.2009.00654.x.

Bernardet, J.-F., Nakagawa, Y., & Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol.* 52(3), 1049-1070.

Beye, M., Bakour, S., Traore, S., Raoult, D., & Fournier, P.-E. (2016). "*Lascolabacillus massiliensis*": a new species isolated from the human gut. *New microbes new infect.* 11, 91-92.

Bezsudnova, E. Y., Boyko, K. M., Polyakov, K. M., Dorovatovskiy, P. V., Stekhanova, T. N., Gumerov, V. M., Popov, V. O. (2012). Structural insight into the molecular basis of polyextremophilicity of short-chain alcohol dehydrogenase from the hyperthermophilic archaeon *Thermococcus sibiricus*. *Biochimie.* 94(12), 2628-2638.

Blank, C. E., Cady, S. L., & Pace, N. R. (2002). Microbial composition of near-boiling silica-depositing thermal springs throughout Yellowstone National Park. *Appl Environ Microbiol.* 68(10), 5123-5135.

Blank, C. E., Cady, S. L., & Pace, N. R. (2002). Microbial composition of near-boiling silica-depositing thermal springs throughout Yellowstone National Park. *Appl Environ Microbiol.* 68(10), 5123-5135.

Borsodi, A. K., Szirányi, B., Krett, G., Márialigeti, K., Janurik, E., & Pekár, F. (2016). Changes in the water quality and bacterial community composition of an alkaline and saline

oxbow lake used for temporary reservoir of geothermal waters. *Environ Sci Pollut.* 1-13.

Boucher, Y., Cordero, O. X., Takemura, A., Hunt, D. E., Schliep, K., Bapteste, E., Polz, M. F. (2011). Local mobile gene pools rapidly cross species boundaries to create endemicity within global *Vibrio cholerae* populations. *MBio.* 2(2), e00335-00310.

Bouizgarne, B., & Aouamar, A. A. B. (2014). Diversity of plant associated actinobacteria *Bacterial Diversity in Sustainable Agriculture.* (pp. 41-99): Springer.

Bravakos, P., Kotoulas, G., Skaraki, K., Pantazidou, A., & Economou-Amilli, A. (2016). A polyphasic taxonomic approach in isolated strains of *Cyanobacteria* from thermal springs of Greece. *Mol phylogenet evol.* 98, 147-160.

Briggs, B. R., Brodie, E. L., Tom, L. M., Dong, H., Jiang, H., Huang, Q., Huang, L. (2014). Seasonal patterns in microbial communities inhabiting the hot springs of Tengchong, Yunnan Province, China. *Environ Microbiol.* 16(6), 1579-1591.

Brock, T. D. (2012). *Thermophilic microorganisms and life at high temperatures*: Springer Science & Business Media.

Brock, T. D., & Freeze, H. (1969). *Thermus aquaticus* gen. n. and sp. n., a nonsporulating extreme thermophile. *J bacteriol.* 98(1), 289-297.

Buée, M., De Boer, W., Martin, F., van Overbeek, L., & Jurkevitch, E. (2009). The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant Soil.* 321(1), 189-212. doi: 10.1007/s11104-009-9991-3.

Burgess, E. A. (2009). Geomicrobiological description of two contemporary hydrothermal pools in Uzon Caldera, Kamchatka, Russia, as models for sulfur biogeochemistry: uga.

Burgess, E. A., Wagner, I. D., & Wiegel, J. (2007). Thermal Environments and Biodiversity *Physiology and Biochemistry of Extremophiles*: American Society of Microbiology.

Busarakam, K., Brown, R., Bull, A. T., Tan, G. Y. A., Zucchi, T. D., da Silva, L. J., Goodfellow, M. (2016). Classification of thermophilic actinobacteria isolated from arid desert soils, including the description of *Amycolatopsis deserti* sp. nov. *A Van Leeuw.* 109(2), 319-334.

Busse, H.-J. (2016). Review of the taxonomy of the genus *Arthrobacter*, emendation of the genus *Arthrobacter* sensu lato, proposal to reclassify selected species of the genus *Arthrobacter* in the novel genera *Glutamicibacter* gen. nov., *Paeniglutamicibacter* gen. nov., *Pseudoglutamicibacter* gen. nov., *Paenarthrobacter* gen. nov. and

Pseudarthrobacter gen. nov., and emended description of *Arthrobacter roseus*. *Int J Syst Evol Microbiol.* 66(1), 9-37.

Byrer, D. E., Rainey, F. A., & Wiegel, J. (2000). Novel strains of *Moorella thermoacetica* form unusually heat-resistant spores. *Arch. microbiol.* 174(5), 334-339.

Cai, J., Wang, Y., Liu, D., Zeng, Y., Xue, Y., Ma, Y., & Feng, Y. (2007). *Fervidobacterium changbaicum* sp. nov., a novel thermophilic anaerobic bacterium isolated from a hot spring of the Changbai Mountains, China. *Int J Syst Evol Microbiol.* 57(Pt 10), 2333-2336. doi: 10.1099/ijs.0.64758-0.

Cai, L., Ye, L., Tong, A. H., Lok, S., & Zhang, T. (2013). Biased diversity metrics revealed by bacterial 16S pyrotags derived from different primer sets. *PloS one.* 8(1), e53649. doi: 10.1371/journal.pone.0053649.

Campbell, B. J., Polson, S. W., Hanson, T. E., Mack, M. C., & Schuur, E. A. (2010). The effect of nutrient deposition on bacterial communities in Arctic tundra soil. *Environ Microbiol.* 12(7), 1842-1854. doi: 10.1111/j.1462-2920.2010.02189.x.

Carreto, L., Moore, E., Nobre, M. F., Wait, R., Riley, P. W., Sharp, R. J., & DA Costa, M. S. (1996). *Rubrobacter xylanophilus* sp. nov., a new thermophilic species isolated from a thermally polluted effluent. *Int J Syst Evol Microbiol.* 46(2), 460-465.

Cary, S. C., McDonald, I. R., Barrett, J. E., & Cowan, D. A. (2010). On the rocks: the microbiology of Antarctic Dry Valley soils. *Nat Rev Microbiol.* 8(2), 129-138. doi: 10.1038/nrmicro2281.

Cavanaugh, C. M., Gardiner, S. L., Jones, M. L., Jannasch, H. W., & Waterbury, J. B. (1981). Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila Jones*: possible chemoautotrophic symbionts. *Science.* 213(4505), 340-342.

Cavicchioli, R. (2002). Extremophiles and the search for extraterrestrial life. Int J *Astrobiol.* 2(3), 281-292.

Cayol, J.-L., Ducerf, S., Patel, B., Garcia, J.-L., Thomas, P., & Ollivier, B. (2000). *Thermohalobacter berrensis* gen. nov., sp. nov., a thermophilic, strictly halophilic bacterium from a solar saltern. *Int J Syst Evol Microbiol.* 50(2), 559-564.

Chang, M. S., Gachal, G. S., Qadri, A. H., & Sheikh, M. Y. (2013). Ecological impacts on the status of Marsh Crocodiles in Manghopir Karachi. *I J A R.* 1(1), 42-46.

Chen, M., Xiao, X., Wang, P., Zeng, X., & Wang, F. (2005). *Arthrobacter ardleyensis* sp. nov., isolated from Antarctic lake sediment and deep-sea sediment. *Arch Microbiol.* 183(4), 301-305. doi: 10.1007/s00203-005-0772-y.

Chen, Y. G., Tang, S. K., Zhang, Y. Q., Li, Z. Y., Yi, L. B., Wang, Y. X., Cui, X. L. (2009). *Arthrobacter halodurans* sp. nov., a new halotolerant bacterium isolated from sea water. *A Van Leeuw.* 96(1), 63-70. doi: 10.1007/s10482-009-9336-5.

Cheng, C., Li, Y.-Q., Asem, M. D., Lu, C.-Y., Shi, X.-H., Chu, X., Li, W.-J. (2016). *Streptomyces xinjiangensis* sp. nov., an actinomycete isolated from Lop Nur region. *Arch Microbiol.* 1-7 (in press).

Chevaldonné, P., Fisher, C., Childress, J., Desbruyères, D., Jollivet, D., Zal, F., & Toulmond, A. (2000). Thermotolerance and the'Pompeii worms'. *Marine Ecology Progress Series.* 208, 293-295.

Christensen, H., Angen, O., Mutters, R., Olsen, J. E., & Bisgaard, M. (2000a). DNA-DNA hybridization determined in micro-wells using covalent attachment of DNA. *Int J Syst Evol Microbiol.* 50(3), 1095-1102.

Christensen, H., Angen, O., Mutters, R., Olsen, J. E., & Bisgaard, M. (2000b). DNA-DNA hybridization determined in micro-wells using covalent attachment of DNA. *Int J Syst Evolut Microbiol.* 50(3), 1095-1102.

Chu, H., Fierer, N., Lauber, C. L., Caporaso, J. G., Knight, R., & Grogan, P. (2010). Soil bacterial diversity in the Arctic is not fundamentally different from that found in other biomes. *Environ Microbiol.* 12(11), 2998-3006. doi: 10.1111/j.1462-2920.2010.02277.x.

Collins, M., Dorsch, M., & Stackebrandt, E. (1989). Transfer of *Pimelobacter tumescens* to *Terrabacter* gen. nov. as *Terrabacter tumescens* comb. nov. and of *Pimelobacter jensenii* to *Nocardioides* as *Nocardioides jensenii* comb. nov. *Int J Syst Evol Microbiol.* 39(1), 1-6.

Collins, M., & Jones, D. (1980). Lipids in the classification and identification of coryneform bacteria containing peptidoglycans based on 2, 4-diaminobutyric acid. *J Appl Bacteriol.* 48(3), 459-470. doi: 10.1111/j.1365-2672.1980.tb01036.x.

Collins, M. D., Pirouz, T., Goodfellow, M., & Minnikin, D. E. (1977). Distribution of menaquinones in *Actinomycetes* and *Corynebacteria*. *J Gen Microbiol.* 100(2), 221-230. doi: 10.1099/00221287-100-2-221.

Conn, H. J., & Dimmick, I. (1947). Soil bacteria similar in morphology to *Mycobacterium* and *Corynebacterium*. *J Bacteriol.* 54(3), 291-303.

Connon, S. A., Lester, E. D., Shafaat, H. S., Obenhuber, D. C., & Ponce, A. (2007). Bacterial diversity in hyperarid Atacama Desert soils. *J Geophys Res Biogeosci.* 112(G4), n/a-n/a. doi: 10.1029/2006JG000311.

Corliss, J. B., Dymond, J., Gordon, L. I., & Edmond, J. M. (1979). on the Galapagos Rift. *Science.* 203, 16.

Cowan, S. T., & Steel, K. J. (1974). Manual for the identification of medical bacteria. London: Cambridge University Press.

Cox, A., Shock, E. L., & Havig, J. R. (2011). The transition to microbial photosynthesis in hot spring ecosystems. *Chem. Geol.* 280(3), 344-351.

Cybulski, L. E., Ballering, J., Moussatova, A., Inda, M. E., Vazquez, D. B., Wassenaar, T. A., Killian, J. A. (2015). Activation of the bacterial thermosensor DesK involves a serine zipper dimerization motif that is modulated by bilayer thickness. *Proceedings of the National Academy of Sciences.* 112(20), 6353-6358.

Dastager, S. G., Lee, J. C., Ju, Y. J., Park, D. J., & Kim, C. J. (2008a). *Nocardioides koreensis* sp. nov., *Nocardioides bigeumensis* sp. nov. and *Nocardioides agariphilus* sp. nov., isolated from soil from Bigeum Island, Korea. *Int J Syst Evol Microbiol.* 58(10), 2292-2296. doi: 10.1099/ijs.0.65566-0.

Dastager, S. G., Lee, J. C., Ju, Y. J., Park, D. J., & Kim, C. J. (2008b). *Nocardioides tritolerans* sp. nov., Isolated from soil in Bigeum Island, Korea. *J Microbiol Biotechnol.* 18(7), 1203-1206.

Dastager, S. G., Lee, J. C., Ju, Y. J., Park, D. J., & Kim, C. J. (2009). *Nocardioides sediminis* sp. nov., isolated from a sediment sample. *Int J Syst Evol Microbiol.* 59(2), 280-284. doi: 10.1099/ijs.0.002162-0.

Dastager, S. G., Lee, J. C., Pandey, A., & Kim, C. J. (2010). *Nocardioides mesophilus* sp. nov., isolated from soil. *Int J Syst Evol Microbiol.* 60(10), 2288-2292. doi: 10.1099/ijs.0.019059-0.

Davidova, I. A., Wawrik, B., Callaghan, A. V., Duncan, K., Marks, C. R., & Suflita, J. M. (2016). *Dethiosulfatarculus sandiegensis* gen. nov., sp. nov., isolated from a methanogenic paraffin-degrading enrichment culture and emended description of the family *Desulfarculaceae. Int J Syst Evol Microbiol.* 66(3), 1242-1248.

Dedysh, S. N., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A., Semrau, J. D., Liesack, W., & Tiedje, J. M. (2002). *Methylocapsa acidiphila* gen. nov., sp. nov., a novel methane-

oxidizing and dinitrogen-fixing acidophilic bacterium from Sphagnum bog. *Int J Syst Evol Microbiol.* 52(1), 251-261.

Delaye, L., Becerra, A., & Lazcano, A. (2005). The last common ancestor: What's in a name? *Origins of Life and Evolution of Biospheres.* 35(6), 537-554.

Delgado-Serrano, L., López, G., Bohorquez, L. C., Bustos, J. R., Rubiano, C., Osorio-Forero, C., Zambrano, M. M. (2014). Neotropical Andes hot springs harbor diverse and distinct planktonic microbial communities. *FEMS microbiol ecol.* 89(1), 56-66.

Deng, Y., Cui, X., Hernández, M., & Dumont, M. G. (2014). Microbial diversity in hummock and hollow soils of three wetlands on the Qinghai-Tibetan Plateau revealed by 16S rRNA pyrosequencing. *PloS one.* 9(7), e103115.

Ding, L., Hirose, T., & Yokota, A. (2009). Four novel *Arthrobacter* species isolated from filtration substrate. *Int J Syst Evol Microbiol.* 59(4), 856-862. doi: 10.1099/ijs.0.65301-0.

Dodsworth, J. A., Hungate, B. A., & Hedlund, B. P. (2011). Ammonia oxidation, denitrification and dissimilatory nitrate reduction to ammonium in two US Great Basin hot springs with abundant ammonia-oxidizing archaea. *Environ Microbiol, 13*(8), 2371-2386.

Donahoe-Christiansen, J., D'Imperio, S., Jackson, C. R., Inskeep, W. P., & McDermott, T. R. (2004). Arsenite-oxidizing Hydrogenobaculum strain isolated from an acid-sulfate-chloride geothermal spring in Yellowstone National Park. *Appl Environ Microbiol.* 70(3), 1865-1868.

Dong, Y., Sanford, R. A., Boyanov, M. I., Kemner, K. M., Flynn, T. M., O'Loughlin, E. J., Fouke, B. W. (2016). *Tepidibacillus decaturensis* sp. nov.: a microaerophilic, moderately thermophilic iron-reducing bacterium isolated from a depth of 1.7 km in the Illinois Basin, USA. *Int J Syst Evol Microbiol.*

Dubourg, G., Lagier, J.-C., Robert, C., Armstrong, N., Couderc, C., Fournier, P.-E., & Raoult, D. (2016). *Risungbinella massiliensis* sp. nov., a new member of *Thermoactinomycetaceae* isolated from human gut. *A Van Leeuw.* 109(6), 773-784.

Dwivedi, V., Sangwan, N., Nigam, A., Garg, N., Niharika, N., Khurana, P., Lal, R. (2012). Draft genome sequence of *Thermus* sp. strain RL, isolated from a hot water spring located atop the Himalayan ranges at Manikaran, India. *J Bacteriol.* 194(13), 3534. doi: 10.1128/jb.00604-12.

Dworkin, J. P., Lazcano, A., & Miller, S. L. (2002). The roads to and from the RNA world. *J. theor. Biol.* 222(1), 127-134.

Elshahed, M. S., Senko, J. M., Najar, F. Z., Kenton, S. M., Roe, B. A., Dewers, T. A., Krumholz, L. R. (2003). Bacterial diversity and sulfur cycling in a mesophilic sulfide-rich spring. *Appl Environ Microbiol.* 69(9), 5609-5621.

Elshahed, M. S., Senko, J. M., Najar, F. Z., Kenton, S. M., Roe, B. A., Dewers, T. A., Krumholz, L. R. (2003). Bacterial diversity and sulfur cycling in a mesophilic sulfide-rich spring. *Appl Environ Microbiol.* 69(9), 5609-5621.

Engle, M., Li, Y., Woese, C., & Wiegel, J. (1995). Isolation and Characterization of a Novel Alkalitolerant Thermophile, *Anaerobranca horikoshii* gen. nov., sp. nov. *Int J Syst Evol Microbiol.* 45(3), 454-461.

Evtushenko, L. I., Krausova, V. I., & Yoon, J.-H. (2015). *Nocardioides Bergey's Manual of Systematics of Archaea and Bacteria*: John Wiley & Sons, Ltd.

Ezaki, T., Hashimoto, Y., & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol.* 39(3), 224-229. doi: 10.1099/00207713-39-3-224.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol.* 17(6), 368-376. doi: 10.1007/BF01734359.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evol.* 39(4), 783-791. doi: 10.2307/2408678.

Fierer, N., & Jackson, R. B. (2006). The diversity and biogeography of soil bacterial communities. *Proc Natl Acad Sci U S A.* 103(3), 626-631. doi: 10.1073/pnas.0507535103.

Fierer, N., & Lennon, J. T. (2011). The generation and maintenance of diversity in microbial communities. *Am J Bot.* 98(3), 439-448. doi: 10.3732/ajb.1000498.

Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool.* 20(4), 406-416. doi: 10.2307/2412116.

Forterre, P., Brochier, C., & Philippe, H. (2002). Evolution of the Archaea. *Theoretical population biology.* 61(4), 409-422.

Fuchs, T., Huber, H., Teiner, K., Burggraf, S., & Stetter, K. (1996). *Metallosphaera prunae*, sp. nov., a novel metal-mobilizing, thermoacidophilic Archaeum, isolated from a uranium mine in Germany. *Syst applmicrobiol.* 18(4), 560-566.

Funke, G., Hutson, R. A., Bernard, K. A., Pfyffer, G. E., Wauters, G., & Collins, M. D. (1996). Isolation of Arthrobacter spp. from clinical specimens and description of *Arthrobacter cumminsii* sp. nov. and *Arthrobacter woluwensis* sp. nov. *J clin microbiol.* 34(10), 2356-2363.

Ganzert, L., Bajerski, F., Mangelsdorf, K., Lipski, A., & Wagner, D. (2011). *Arthrobacter livingstonensis* sp. nov. and *Arthrobacter cryotolerans* sp. nov., salt-tolerant and psychrotolerant species from Antarctic soil. *Int J Syst Evol Microbiol.* 61(Pt 4), 979-984. doi: 10.1099/ijs.0.021022-0.

Gao, B., & Gupta, R. S. (2012). Phylogenetic framework and molecular signatures for the main clades of the phylum *Actinobacteria*. *Microbiol Mol Biol Rev.* 76(1), 66-112.

Garcia-Pichel, F., Johnson, S. L., Youngkin, D., & Belnap, J. (2003). Small-scale vertical distribution of bacterial biomass and diversity in biological soil crusts from arid lands in the Colorado plateau. *Microb Ecol.* 46(3), 312-321. doi: 10.1007/s00248-003-1004-0.

Gerday, C., & Glansdorff, N. (2007). *Physiology and biochemistry of extremophiles*: ASM Press.

Giovannelli, D., Chung, M., Staley, J., Starovoytov, V., Le Bris, N., & Vetriani, C. (2016). *Sulfurovum riftiae* sp. nov., a mesophilic, thiosulfate-oxidizing, nitrate-reducing chemolithoautotrophic Epsilonproteobacterium isolated from the tube of the deep-sea hydrothermal vent polychaete, Riftia pachyptila. *Int J Syst Evol Microbiol.*

Goodfellow, M., Kumar, Y., Labeda, D. P., & Sembiring, L. (2007). The *Streptomyces violaceusniger* clade: a home for streptomycetes with rugose ornamented spores. *A Van Leeuw.* 92(2), 173-199.

Goris, J., Suzuki, K.-i., Vos, P. D., Nakase, T., & Kersters, K. (1998). Evaluation of a microplate DNA-DNA hybridization method compared with the initial renaturation method. *Can J Microbiol.* 44(12), 1148-1153. doi: 10.1139/w98-118.

Goudjal, Y., Toumatia, O., Sabaou, N., Barakate, M., Mathieu, F., & Zitouni, A. (2013). Endophytic actinomycetes from spontaneous plants of Algerian Sahara: indole-3-acetic acid production and tomato plants growth promoting activity. *World J Microbiol Biotechnol.* 29(10), 1821-1829. doi: 10.1007/s11274-013-1344-y.

Grassia, G. S., McLean, K. M., Glénat, P., Bauld, J., & Sheehy, A. J. (1996). A systematic survey for thermophilic fermentative bacteria and archaea in high temperature petroleum reservoirs. *FEMS microbiol ecol.* 21(1), 47-58.

Greenwood, J., & Pickett, M. (1980). Transfer of *Haemophilus vaginalis* Gardner and Dukes to a New Genus, *Gardnerella: G. vaginalis* (Gardner and Dukes) comb. nov. *Int J Syst Evol Microbiol.* 30(1), 170-178.

Gregersen, T. (1978). Rapid method for distinction of Gram-negative from Gram-positive bacteria. *Eur J Appl Microbiol Biotechnol.* 5(2), 123-127.

Gulen, B., Petrov, A. S., Okafor, C. D., Vander Wood, D., O'Neill, E. B., Hud, N. V., & Williams, L. D. (2016). Ribosomal small subunit domains radiate from a central core. *Sci. rep.* 6.

Gupta, P., Reddy, G. S., Delille, D., & Shivaji, S. (2004). *Arthrobacter gangotriensis* sp. nov. and *Arthrobacter kerguelensis* sp. nov. from Antarctica. *Int J Syst Evol Microbiol.* 54(6), 2375-2378. doi: 10.1099/ijs.0.63110-0.

Hamady, M., Lozupone, C., & Knight, R. (2010). Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. *Isme j.* 4(1), 17-27. doi: 10.1038/ismej.2009.97.

Hamady, M., Walker, J. J., Harris, J. K., Gold, N. J., & Knight, R. (2008). Error-correcting barcoded primers allow hundreds of samples to be pyrosequenced in multiplex. *Nature methods.* 5(3), 235-237. doi: 10.1038/nmeth.1184.

Hanada, S., Takaichi, S., Matsuura, K., & Nakamura, K. (2002). *Roseiflexus castenholzii* gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium that lacks chlorosomes. *Int J Syst Evol Microbiol.* 52(1), 187-193.

Hasegawa, T., Takizawa, M., & Tanida, S. (1983). A rapid analysis for chemical grouping of aerobic *Actinomycetes*. *J Gen Appl Microbiol.* 29(4), 319-322. doi: 10.2323/jgam.29.319.

Hedlund, B. P., Reysenbach, A.-L., Huang, L., Ong, J. C., Liu, Z., Dodsworth, J. A., Liu, Y. (2015). Isolation of diverse members of the *Aquificales* from geothermal springs in Tengchong, China. *Front microbiol.* 6, 157.

Herman, R. P., Provencio, K. R., Herrera-Matos, J., & Torrez, R. J. (1995). Resource islands predict the distribution of heterotrophic bacteria in chihuahuan desert soils. *Appl Environ Microbiol.* 61(5), 1816-1821.

Hou, W., Wang, S., Dong, H., Jiang, H., Briggs, B. R., Peacock, J. P., Zhi, X. (2013). A comprehensive census of microbial diversity in hot springs of Tengchong, Yunnan Province China using 16S rRNA gene pyrosequencing. *PloS one.* 8(1), e53350.

Huang, Q., Dong, C. Z., Dong, R. M., Jiang, H., Wang, S., Wang, G., Li, X. (2011). Archaeal and bacterial diversity in hot springs on the Tibetan Plateau, China. *Extremophiles.* 15(5), 549-563.

Huber, R., Rossnagel, P., Woese, C., Rachel, R., Langworthy, T. A., & Stetter, K. O. (1996). Formation of ammonium from nitrate during chemolithoautotrophic growth of the extremely thermophilic bacterium *Ammonifex degensii* gen. nov. sp. nov. *Appl Syst microbial.* 19(1), 40-49.

Hug, L. A., Baker, B. J., Anantharaman, K., Brown, C. T., Probst, A. J., Castelle, C. J., Ise, K. (2016). A new view of the tree of life. *Nat. Microbiol.* 1, 16048.

Hugenholtz, J., & Ljungdahl, L. G. (1990). Metabolism and energy generation in homoacetogenic clostridia. *FEMS Microbiol Rev.* 7(3-4), 383-389.

Hugenholtz, P. (2002). Exploring prokaryotic diversity in the genomic era. *Genome biol.* 3(2), 1.

Hugenholtz, P., Pitulle, C., Hershberger, K. L., & Pace, N. R. (1998). Novel division level bacterial diversity in a Yellowstone hot spring. *J. bacteriol.* 180(2), 366-376.

Hur, M., Kim, Y., Song, H. R., Kim, J. M., Choi, Y. I., & Yi, H. (2011). Effect of genetically modified poplars on soil microbial communities during the phytoremediation of waste mine tailings. *Appl Environ Microbiol.* 77(21), 7611-7619. doi: 10.1128/aem.06102-11.

Ibrahim, M. H., Lebbe, L., Willems, A., & Steinbüchel, A. (2016). *Chelatococcus thermostellatus* sp. nov., a new thermophile for bioplastic synthesis: comparative phylogenetic and physiological study. *AMB Express.* 6(1), 1-9.

Inan, K., Ozer, A., Guler, H. I., Belduz, A. O., & Canakci, S. (2016). *Brevibacillus gelatini* sp. nov., isolated from a hot spring. *Int J Syst Evol Microbiol.* 66(2), 712-718.

Irlinger, F., Bimet, F., Delettre, J., Lefevre, M., & Grimont, P. A. (2005). *Arthrobacter bergerei* sp. nov. and *Arthrobacter arilaitensis* sp. nov., novel coryneform species isolated from the surfaces of cheeses. *Int J Syst Evol Microbiol.* 55(Pt 1), 457-462. doi: 10.1099/ijs.0.63125-0.

Islam, T., Jensen, S., Reigstad, L. J., Larsen, Ø., & Birkeland, N.-K. (2008). Methane oxidation at 55 C and pH 2 by a thermoacidophilic bacterium belonging to the *Verrucomicrobia* phylum. *Proceedings of the National Academy of Sciences.* 105(1), 300-304.

Islas, S., Velasco, A. M., Becerra, A., Delaye, L., & Lazcano, A. (2003). Hyperthermophily and the origin and earliest evolution of life. *Int microbiol.* 6(2), 87-94.

Itoh, T., Onishi, M., Kato, S., Iino, T., Sakamoto, M., Kudo, T., Ohkuma, M. (2016). *Athalassotoga saccharophila* gen. nov., sp. nov., isolated from an acidic terrestrial hot spring, and proposal of *Mesoaciditogales* ord. nov. and *Mesoaciditogaceae* fam. nov. in the phylum *Thermotogae*. *Int J Syst Evol Microbiol.* 66(2), 1045-1051.

Jahangir, T., Khuhawar, M., Leghari, S., & Leghari, A. (2001). Physico-chemical and biological study of Mangho Pir euthermal springs Karachi, Sindh, Pakistan. *Online J. Biol. Sci.* 1, 636-639.

Javed, M., Zahoor, S., Sabar, H., Haq, I., & Babar, M. (2012a). Thermophilic bacteria from the hot springs of gilgit (Pakistan). *J. Anim. Plant Sci.*(22), 83-87.

Javed, M., Zahoor, S., Sabar, H., Haq, I., & Babar, M. (2012b). Thermophilic bacteria from the hot springs of Gilgit (Pakistan). *J Ani Plant Sci.* 22(1), 83-87.

Jiang, Z., Li, P., Jiang, D., Dai, X., Zhang, R., Wang, Y., & Wang, Y. (2016). Microbial Community Structure and Arsenic Biogeochemistry in an Acid Vapor-Formed Spring in Tengchong Geothermal Area, China. *PloS one.* 11(1), e0146331.

Jolivet, E., Corre, E., L'Haridon, S., Forterre, P., & Prieur, D. (2004). *Thermococcus marinus* sp. nov. and *Thermococcus radiotolerans* sp. nov., two hyperthermophilic archaea from deep-sea hydrothermal vents that resist ionizing radiation. *Extremophiles.* 8(3), 219-227.

Jolivet, E., L'Haridon, S., Corre, E., Forterre, P., & Prieur, D. (2003). *Thermococcus gammatolerans* sp. nov., a hyperthermophilic archaeon from a deep-sea hydrothermal vent that resists ionizing radiation. *Int J Syst Evol Microbiol.* 53(3), 847-851.

Jones, S. E., & Lennon, J. T. (2010). Dormancy contributes to the maintenance of microbial diversity. *Proc Natl Acad Sci U S A.* 107(13), 5881-5886. doi: 10.1073/pnas.0912765107.

Joyce, G. F. (2002). The antiquity of RNA-based evolution. *Nature.* 418(6894), 214-221.

Kageyama, A., Morisaki, K., Omura, S., & Takahashi, Y. (2008). *Arthrobacter oryzae* sp. nov. and *Arthrobacter humicola* sp. nov. *Int J Syst Evol Microbiol.* 58(1), 53-56. doi: 10.1099/ijs.0.64875-0.

Kambura, A. K., Mwirichia, R. K., Kasili, R. W., Karanja, E. N., Makonde, H. M., & Boga, H. I. (2016). Bacteria and Archaea diversity within the hot springs of Lake Magadi and Little Magadi in Kenya. *BMC microbiology.* 16(1), 1.

Kämpfer, P. (2012). Genus I. *Streptomyces* Waksman and Henrici 1943, 339 emend. Witt and Stackebrandt 1990, 370 emend. Wellington, Stackebrandt, Sanders, Wolstrup and Jorgensen 1992, 159. *Bergey's manual of systematic bacteriology.* 5, 1455-1767.

Kamruzzaman, M., Udden, S. N., Cameron, D. E., Calderwood, S. B., Nair, G. B., Mekalanos, J. J., & Faruque, S. M. (2010). Quorum-regulated biofilms enhance the development of conditionally viable, environmental *Vibrio cholerae*. *Proceedings of the National Academy of Sciences.* 107(4), 1588-1593.

Kanso, S., & Patel, B. K. (2003). *Microvirga subterranea* gen. nov., sp. nov., a moderate thermophile from a deep subsurface Australian thermal aquifer. *Int J Syst Evol Microbiol.* 53(2), 401-406. doi: 10.1099/ijs.0.02348-0.

Kashefi, K., & Lovley, D. R. (2003). Extending the upper temperature limit for life. *Science.* 301(5635), 934-934.

Kelley, D. S., Baross, J. A., & Delaney, J. R. (2002). Volcanoes, fluids, and life at mid-ocean ridge spreading centers. *Annu. Rev Earth Pla.* 30(1), 385-491.

Kelley, D. S., Karson, J. A., Früh-Green, G. L., Yoerger, D. R., Shank, T. M., Butterfield, D. A., Proskurowski, G. (2005). A serpentinite-hosted ecosystem: the Lost City hydrothermal field. *Science.* 307(5714), 1428-1434.

Khan, K., Khan, I., Leghari, A., & Khan, M. (1987). Geology along the Karakoram Highway from Hasan Abdal to Khunjerab Pass. *Geological Survey of Pakistan, Quetta*, 39-43.

Kim, H. M., Choi, D. H., Hwang, C. Y., & Cho, B. C. (2008). *Nocardioides salarius* sp. nov., isolated from seawater enriched with zooplankton. *Int J Syst Evol Microbiol.* 58(9), 2056-2064. doi: 10.1099/ijs.0.65569-0.

Kim, K. K., Lee, K. C., Oh, H. M., Kim, M. J., Eom, M. K., & Lee, J. S. (2008). *Arthrobacter defluvii* sp. nov., 4-chlorophenol-degrading bacteria isolated from sewage. *Int J Syst Evol Microbiol.* 58(8), 1916-1921. doi: 10.1099/ijs.0.65550-0.

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., Chun, J. (2012a). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol.* 62, 716-721. doi: 10.1099/Ijs.0.038075-0.

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., Chun, J. (2012b). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol.* 62(Pt 3), 716-721. doi: 10.1099/ijs.0.038075-0.

Kimura, H., Sugihara, M., Yamamoto, H., Patel, B. K., Kato, K., & Hanada, S. (2005). Microbial community in a geothermal aquifer associated with the subsurface of the Great Artesian Basin, Australia. *Extremophiles.* 9(5), 407-414.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol.* 16(2), 111-120.

Koeck, D. E., Hahnke, S., & Zverlov, V. V. (2016). *Herbinix luporum* sp. nov., a thermophilic cellulose-degrading bacterium isolated from a thermophilic biogas reactor. *Int J Syst Evol Microbiol.*

Kojima, H., Umezawa, K., & Fukui, M. (2016). *Caldimicrobium thiodismutans* sp. nov., a sulfur-disproportionating bacterium isolated from a hot spring, and emended description of the genus *Caldimicrobium*. *Int J Syst Evol Microbiol.* 66(4), 1828-1831.

Korn-Wendisch, F., Rainey, F., Kroppenstedt, R., Kempf, A., Majazza, A., Kutzner, H., & Stackebrandt, E. (1995). *Thermocrispum* gen. nov., a New Genus of the Order *Actinomycetales*, and Description of *Thermocrispum municipale* sp. nov. and *Thermocrispum agreste* sp. nov. *Int J Syst Evol Microbiol.* 45(1), 67-77.

Kovacs, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature.* 178(4535), 703.

Krishnan, R., Menon, R. R., Tanaka, N., Busse, H.-J., Krishnamurthi, S., & Rameshkumar, N. (2016). *Arthrobacter pokkalii* sp nov, a novel plant associated actinobacterium with plant beneficial Properties, isolated from saline tolerant Pokkali rice, Kerala, India. *PloS one.* 11(3), e0150322. doi: 10.1371/journal.pone.0150322.

Kroppenstedt, R. M. (1982). Separation of bacterial menaquinones by HPLC using reverse phase (RP18) and a silver loaded ion exchanger as stationary phases. *J Liq Chromat, 5*(12), 2359-2367. doi: 10.1080/01483918208067640

Kubo, K., Knittel, K., Amann, R., Fukui, M., & Matsuura, K. (2011). Sulfur-metabolizing bacterial populations in microbial mats of the Nakabusa hot spring, Japan. *Syst Appl Microbiol.* 34(4), 293-302. doi: 10.1016/j.syapm.2010.12.002.

Kubota, M., Kawahara, K., Sekiya, K., Uchida, T., Hattori, Y., Futamata, H., & Hiraishi, A. (2005). *Nocardioides aromaticivorans* sp. nov., a dibenzofuran-degrading bacterium isolated from dioxin-polluted environments. *Syst Appl Microbiol.* 28(2), 165-174. doi: 10.1016/j.syapm.2004.10.002.

Kumar, M., Yadav, A. N., Tiwari, R., Prasanna, R., & Saxena, A. K. (2014). Deciphering the diversity of culturable thermotolerant bacteria from Manikaran hot springs. *Ann Microbiol.* 64(2), 741-751.

Kumar, N., Lad, G., Giuntini, E., Kaye, M. E., Udomwong, P., Shamsani, N. J., Bailly, X. (2015). Bacterial genospecies that are not ecologically coherent: population genomics of *Rhizobium leguminosarum*. *Open biol.* 5(1), 140133.

Kumar, Y., & Goodfellow, M. (2010). Reclassification of *Streptomyces hygroscopicus* strains as *Streptomyces aldersoniae* sp. nov., *Streptomyces angustmyceticus* sp. nov., comb. nov., *Streptomyces ascomycinicus* sp. nov., *Streptomyces decoyicus* sp. nov., comb. nov., *Streptomyces milbemycinicus* sp. nov. and *Streptomyces wellingtoniae* sp. nov. *Int J Syst Evol Microbiol.* 60(4), 769-775.

Kuske, C. R., Ticknor, L. O., Miller, M. E., Dunbar, J. M., Davis, J. A., Barns, S. M., & Belnap, J. (2002). Comparison of soil bacterial communities in rhizospheres of three plant species and the interspaces in an arid grassland. *Appl Environ Microbiol.* 68(4), 1854-1863.

Labeda, D. P. (2016). Taxonomic evaluation of putative *Streptomyces scabiei* strains held in the ARS Culture Collection (NRRL) using multi-locus sequence analysis. *A Van Leeuw.*1-8 (in press).

Lau, M. C., Aitchison, J. C., & Pointing, S. B. (2009). Bacterial community composition in thermophilic microbial mats from five hot springs in central Tibet. *Extremophiles.* 13(1), 139-149.

Lauber, C. L., Hamady, M., Knight, R., & Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl Environ Microbiol.* 75(15), 5111-5120. doi: 10.1128/aem.00335-09.

Lazcano, A., & Bada, J. L. (2003). The 1953 Stanley L. Miller Experiment: Fifty Years of Prebiotic Organic Chemistry. *Origins Life Evol. B.* 33(3), 235-242. doi: 10.1023/a:1024807125069.

Lechevalier, M. P., & Lechevalier, H. (1970). Chemical composition as a criterion in the classification of aerobic *Actinomycetes*. *Int J Syst Bacteriol.* 20(4), 435-443.

Lee, D. W., Hyun, C. G., & Lee, S. D. (2007). *Nocardioides marinisabuli* sp. nov., a novel actinobacterium isolated from beach sand. *Int J Syst Evol Microbiol.* 57(12), 2960-2963. doi: 10.1099/ijs.0.65127-0.

Lee, H.-J., & Whang, K.-S. (2016). *Streptomyces rhizosphaerihabitans* sp. nov., and *Streptomyces adustus* sp. nov., isolated from bamboo forest soil. *Int J Syst Evol Microbiol.* (in press). doi: doi:10.1099/ijsem.0.001236.

Lee, S. D., & Lee, D. W. (2014). *Nocardioides rubroscoriae* sp. nov., isolated from volcanic ash. *A Van Leeuw.* 105(6), 1017-1023. doi: 10.1007/s10482-014-0161-0.

Lee, S. H., Jang, I., Chae, N., Choi, T., & Kang, H. (2013). Organic layer serves as a hotspot of microbial activity and abundance in Arctic tundra soils. *Microb Ecol.* 65(2), 405-414. doi: 10.1007/s00248-012-0125-8

Lee, Y.-E., Jain, M. K., Lee, C., & Zeikus, J. G. (1993). Taxonomic distinction of saccharolytic thermophilic anaerobes: description of *Thermoanaerobacterium xylanolyticum* gen. nov., sp. nov., and *Thermoanaerobacterium saccharolyticum* gen. nov., sp. nov.; reclassification of *Thermoanaerobium brockii*, *Clostridium thermosulfurogenes*, and *Clostridium thermohydrosulfuricum* E100-69 as *Thermoanaerobacter brockii* comb. nov., *Thermoanaerobacterium thermosulfurigenes* comb. nov., and *Thermoanaerobacter thermohydrosulfuricus* comb. nov., respectively; and transfer of *Clostridium thermohydrosulfuricum* 39E to *Thermoanaerobacter ethanolicus*. *Int J Syst Evol Microbiol.* 43(1), 41-51.

Leis, B., Angelov, A., Mientus, M., Li, H., Pham, V. T., Lauinger, B., Santos, H. (2015). Identification of novel esterase-active enzymes from hot environments by use of the host bacterium *Thermus thermophilus*. *Front microbiol.* 6, 275.

Levy, M., & Miller, S. L. (1998). The stability of the RNA bases: implications for the origin of life. *Proceedings of the National Academy of Sciences.* 95(14), 7933-7938.

Li, C., Jin, P., Liu, C., Ma, Z., Zhao, J., Li, J., Xiang, W. (2016). *Streptomyces bryophytorum* sp. nov., an endophytic actinomycete isolated from moss (Bryophyta). *A Van Leeuw.*

Li, J., Peng, X., Zhang, L., Jiang, L., & Chen, S. (2016). Linking Microbial Community Structure to S, N and Fe Biogeochemical Cycling in the Hot Springs at the Tengchong Geothermal Fields, Southwest China. *Geomicrobiol J.* 33(2), 135-150.

Li, W., & Godzik, A. (2006). Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics.* 22(13), 1658-1659. doi: 10.1093/bioinformatics/btl158

Li, W. J., Xu, P., Schumann, P., Zhang, Y. Q., Pukall, R., Xu, L. H., Jiang, C. L. (2007). *Georgenia ruanii* sp. nov., a novel actinobacterium isolated from forest soil in Yunnan

(China), and emended description of the genus *Georgenia*. *Int J Syst Evol Microbiol.* 57(7), 1424-1428. doi: 10.1099/ijs.0.64749-0.

Lin, S. Y., Wen, C. Z., Hameed, A., Liu, Y. C., Hsu, Y. H., Shen, F. T., Young, C. C. (2015). *Nocardioides echinoideorum* sp. nov., isolated from sea urchins (*Tripneustes gratilla*). *Int J Syst Evol Microbiol.* 65(6), 1953-1958. doi: 10.1099/ijs.0.000206.

Liu, B., Liu, G.-H., Cetin, S., Schumann, P., Pan, Z.-Z., & Chen, Q.-Q. (2016). *Bacillus gobiensis* sp. nov., isolated from a soil sample. *Int J Syst Evol Microbiol.* 66(1), 379-384.

Liu, L., Salam, N., Jiao, J.-Y., Jiang, H.-C., Zhou, E.-M., Yin, Y.-R., Li, W.-J. (2016). Diversity of Culturable Thermophilic Actinobacteria in Hot Springs in Tengchong, China and Studies of their Biosynthetic Gene Profiles. *Microb Ecol.* 1-13.

Liu, Z., Klatt, C. G., Wood, J. M., Rusch, D. B., Ludwig, M., Wittekindt, N., Bryant, D. A. (2011). Metatranscriptomic analyses of chlorophototrophs of a hot-spring microbial mat. *The ISME journal.* 5(8), 1279-1290.

Loveland-Curtze, J., Sheridan, P. P., Gutshall, K. R., & Brenchley, J. E. (1999). Biochemical and phylogenetic analyses of psychrophilic isolates belonging to the *Arthrobacter* subgroup and description of *Arthrobacter psychrolactophilus*, sp. nov. *Arch Microbiol.* 171(6), 355-363.

Lu, Y., Murase, J., Watanabe, A., Sugimoto, A., & Kimura, M. (2004). Linking microbial community dynamics to rhizosphere carbon flow in a wetland rice soil. *FEMS Microbiol Ecol.* 48(2), 179-186. doi: 10.1016/j.femsec.2004.01.004.

Ludwig, W., Euzéby, J., Schumann, P., Busse, H.-J., Trujillo, M. E., Kämpfer, P., & Whitman, W. B. (2012). Road map of the phylum Actinobacteria *Bergey's manual® of systematic bacteriology* (pp. 1-28): Springer.

Luo, G., Wang, W., & Angelidaki, I. (2013). Anaerobic digestion for simultaneous sewage sludge treatment and CO biomethanation: process performance and microbial ecology. *Environ sci technol.* 47(18), 10685-10693.

Luo, X., Sun, Y., Xie, S., Wan, C., & Zhang, L. (2016). *Streptomyces indoligenes* sp. nov., isolated from rhizosphere soil of Populus euphratica in Xinjiang, China. *Int J Syst Evol Microbiol*,.(in press).

Mages, I. S., Frodl, R., Bernard, K. A., & Funke, G. (2008). Identities of *Arthrobacter* spp. and *Arthrobacter*-like bacteria encountered in human clinical specimens. *J Clinic Microbiol.* 46(9), 2980-2986. doi: 10.1128/JCM.00658-08.

Magurran, A. E. (2013). *Measuring biological diversity*: John Wiley & Sons.

Mannisto, M. K., Kurhela, E., Tiirola, M., & Haggblom, M. M. (2013). Acidobacteria dominate the active bacterial communities of Arctic tundra with widely divergent winter-time snow accumulation and soil temperatures. *FEMS Microbiol Ecol.* 84(1), 47-59. doi: 10.1111/1574-6941.12035.

Margesin, R., Schumann, P., Sproer, C., & Gounot, A. M. (2004). *Arthrobacter psychrophenolicus* sp. nov., isolated from an alpine ice cave. *Int J Syst Evol Microbiol.* 54(6), 2067-2072. doi: 10.1099/ijs.0.63124-0.

Marmur, J. (1963). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *Method Enzymol.* 6, 726-738. doi: Doi 10.1016/0076-6879(63)06240-6.

Marschner, P., Yang, C. H., Lieberei, R., & Crowley, D. E. (2001). Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biol Biochem.* 33(11), 1437-1445. doi: http://dx.doi.org/10.1016/S0038-0717(01)00052-9.

Marteinsson, V. T., Birrien, J.-L., Reysenbach, A.-L., Vernet, M., Marie, D., Gambacorta, A., Prieur, D. (1999). *Thermococcus barophilus* sp. nov., a new barophilic and hyperthermophilic archaeon isolated under high hydrostatic pressure from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol.* 49(2), 351-359.

McCann, C. M., Wade, M. J., Gray, N. D., Roberts, J. A., Hubert, C. R., & Graham, D. W. (2016). Microbial Communities in a High Arctic Polar Desert Landscape. *Front Microbiol.* 7, 419. doi: 10.3389/fmicb.2016.00419.

McLean, E. (1982). *Soil pH and lime requirement.* Madison, WI, USA: American Society of Agronomy, Soil Science Society of America.

Merkel, A., Podosokorskaya, O., Sokolova, T., & Bonch-Osmolovskaya, E. (2016). Diversity of methanogenic archaea from the 2012 terrestrial hot spring (Valley of Geysers, Kamchatka). *Microbiology.* 85(3), 342-349.

Mesbah, M., Premachandran, U., & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic-acid by high-performance liquid-chromatography. *Int J Syst Bacteriol.* 39(2), 159-167.

Mesbah, N. M., & Wiegel, J. (2005). Halophilic thermophiles: a novel group of extremophiles. *Microbial diversity: current perspectives and potential applications. IK Publishing House, New Delhi.* 91-118.

Miller, S. L., & Bada, J. L. (1988). Submarine hot springs and the origin of life.

Miller, S. R., Strong, A. L., Jones, K. L., & Ungerer, M. C. (2009). Bar-coded pyrosequencing reveals shared bacterial community properties along the temperature gradients of two alkaline hot springs in Yellowstone National Park. *Appl Environ Microbiol.* 75(13), 4565-4572.

Minnikin, D., Collins, M., & Goodfellow, M. (1979). Fatty acid and polar lipid composition in the classification of *Cellulomonas, Oerskovia* and related taxa. *J Appl Bacteriol.* 47(1), 87-95. doi: 10.1111/j.1365-2672.1979.tb01172.x.

Mirete, S., Morgante, V., & González-Pastor, J. E. (2016). Functional metagenomics of extreme environments. *Curr Opin Biotech.* 38, 143-149.

Mishima, M., Iwata, K., Nara, K., Matsui, T., Shigeno, T., & Omori, T. (2009). Cultivation characteristics of denitrification by thermophilic *Geobacillus* sp. strain TDN01. *J gen appl microbiol.* 55(2), 81-86.

Mohanrao, M. M., & Singh, D. P. (2016). Deciphering the microbial diversity of tattapani hot water spring using metagenomic approach.

Nagy, M. L., Perez, A., & Garcia-Pichel, F. (2005). The prokaryotic diversity of biological soil crusts in the Sonoran Desert (Organ Pipe Cactus National Monument, AZ). *FEMS Microbiol Ecol.* 54(2), 233-245. doi: 10.1016/j.femsec.2005.03.011.

Nakagawa, T., & Fukui, M. (2002). Phylogenetic characterization of microbial mats and streamers from a Japanese alkaline hot spring with a thermal gradient. *The J gen appl microbiol.* 48(4), 211-222.

Navarro-Gonzalez, R., Rainey, F. A., Molina, P., Bagaley, D. R., Hollen, B. J., de la Rosa, J., McKay, C. P. (2003). Mars-like soils in the Atacama Desert, Chile, and the dry limit of microbial life. *Science.* 302(5647), 1018-1021. doi: 10.1126/science.1089143.

Neilson, J. W., Quade, J., Ortiz, M., Nelson, W. M., Legatzki, A., Tian, F., Maier, R. M. (2012). Life at the hyperarid margin: novel bacterial diversity in arid soils of the Atacama Desert, Chile. *Extremophiles.* 16(3), 553-566. doi: 10.1007/s00792-012-0454-z.

Neufeld, J. D., & Mohn, W. W. (2005). Unexpectedly high bacterial diversity in arctic tundra relative to boreal forest soils, revealed by serial analysis of ribosomal sequence tags. *Appl Environ Microbiol.* 71(10), 5710-5718. doi: 10.1128/aem.71.10.5710-5718.2005.

Nickson, R., McArthur, J., Shrestha, B., Kyaw-Myint, T., & Lowry, D. (2005). Arsenic and other drinking water quality issues, Muzaffargarh District, Pakistan. *Appl Geochem.* 20(1), 55-68.

Nobu, M. K., Dodsworth, J. A., Murugapiran, S. K., Rinke, C., Gies, E. A., Webster, G., Sass, H. (2016). Phylogeny and physiology of candidate phylum 'Atribacteria'(OP9/JS1) inferred from cultivation-independent genomics. *The ISME journal.* 10(2), 273-286.

Norris, T. B., Wraith, J. M., Castenholz, R. W., & McDermott, T. R. (2002). Soil microbial community structure across a thermal gradient following a geothermal heating event. *Appl Environ Microbiol.* 68(12), 6300-6309.

Nübel, U., Bateson, M. M., Madigan, M. T., Kühl, M., & Ward, D. M. (2001). Diversity and Distribution in Hypersaline Microbial Mats of Bacteria Related to *Chloroflexus* spp. *Appl Environ Microbiol.* 67(9), 4365-4371.

Nübel, U., Bateson, M. M., Vandieken, V., Wieland, A., Kühl, M., & Ward, D. M. (2002). Microscopic examination of distribution and phenotypic properties of phylogenetically diverse *Chloroflexaceae*-related bacteria in hot spring microbial mats. *Appl Environ Microbiol.* 68(9), 4593-4603.

O'Donnell, A. G., Falconer, C., Goodfellow, M., Ward, A. C., & Williams, E. (1993). Biosystematics and diversity amongst novel carboxydotrophic actinomycetes. *A Van Leeuw.* 64(3-4), 325-340.

O'Donnell, A. G., Goodfellow, M., & Minnikin, D. E. (1982). Lipids in the classification of *Nocardioides*: Reclassification of *Arthrobacter simplex* (Jensen) lochhead in the genus *Nocardioides* (Prauser) emend. O'Donnell et al. as *Nocardioides simplex* comb. nov. *Arch Microbiol.* 133(4), 323-329. doi: 10.1007/BF00521299

Orphan, V., Goffredi, S. K., Delong, E., & Boles, J. (2003). Geochemical influence on diversity and microbial processes in high temperature oil reservoirs. *Geomicrobiol. J.* 20(4), 295-311.

Orsi, W. D., Smith, J. M., Liu, S., Liu, Z., Sakamoto, C. M., Wilken, S., Worden, A. Z. (2016). Diverse, uncultivated bacteria and archaea underlying the cycling of dissolved protein in the ocean. *The ISME journal.*

Orsi, W. D., Smith, J. M., Liu, S., Liu, Z., Sakamoto, C. M., Wilken, S., Santoro, A. E. (2016). Diverse, uncultivated bacteria and archaea underlying the cycling of dissolved protein in the ocean. *Isme j.* 10(9), 2158-2173. doi: 10.1038/ismej.2016.20.

Osman, J. R., Zelicourt, A. d., Bisseling, T., Geurts, R., Hirt, H., & DuBow, M. S. (2016). *Bacterial Rhizosphere Biodiversity from Several Pioneer Desert Sand Plants Near Jizan, Saudi Arabia.* Paper presented at the The Open Conference Proceedings Journal.

Otaki, H., Everroad, R. C., Matsuura, K., & Haruta, S. (2012). Production and consumption of hydrogen in hot spring microbial mats dominated by a filamentous anoxygenic photosynthetic bacterium. *Microbes Environ.* 27(3), 293-299.

Pace, N. R. (1991). Origin of life-facing up to the physical setting. *Cell.* 65(4), 531-533.

Pace, N. R. (1997). A molecular view of microbial diversity and the biosphere. *Science.* 276(5313), 734-740.

Pang, J., & Allemann, R. K. (2007). Molecular dynamics simulation of thermal unfolding of *Thermatoga maritima* DHFR. *Phys. Chem. Chem. Phys.* 9(6), 711-718.

Pasternak, Z., Pietrokovski, S., Rotem, O., Gophna, U., Lurie-Weinberger, M. N., & Jurkevitch, E. (2013). By their genes ye shall know them: genomic signatures of predatory bacteria. *The ISME journal.* 7(4), 756-769.

Paul, S., Cortez, Y., Vera, N., Villena, G. K., & Gutiérrez-Correa, M. (2016). Metagenomic analysis of microbial community of an Amazonian geothermal spring in Peru. *Genomics Data.* 9, 63-66.

Pearson, A., Huang, Z., Ingalls, A., Romanek, C., Wiegel, J., Freeman, K., Zhang, C. (2004). Nonmarine crenarchaeol in Nevada hot springs. *Appl Environ Microbiol.* 70(9), 5229-5237.

Pedersen, K. (2000). Exploration of deep intraterrestrial microbial life: current perspectives. *FEMS microbiol lett.* 185(1), 9-16.

Pérez-Rodríguez, I., Rawls, M., Coykendall, D. K., & Foustoukos, D. I. (2016). Deferrisoma palaeochoriense sp. nov., a thermophilic, iron (III)-reducing bacterium from a shallow-water hydrothermal vent in the Mediterranean Sea. *Int J Syst Evol Microbiol.* 66(2), 830-836.

Petrov, A. S., Gulen, B., Norris, A. M., Kovacs, N. A., Bernier, C. R., Lanier, K. A., Hud, N. V. (2015). History of the ribosome and the origin of translation. *Proceedings of the National Academy of Sciences.* 112(50), 15396-15401.

Philippot, L., Raaijmakers, J. M., Lemanceau, P., & van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat Rev Microbiol.* 11(11), 789-799. doi: 10.1038/nrmicro3109.

Phillips, H. C. (1951). Characterization of the soil globiforme bacteria.

Phillips, R. W., Wiegel, J., Berry, C. J., Fliermans, C., Peacock, A. D., White, D. C., & Shimkets, L. J. (2002). *Kineococcus radiotolerans* sp. nov., a radiation-resistant, gram-positive bacterium. *Int J Syst Evol Microbiol.* 52(3), 933-938.

Phongsopitanun, W., Kudo, T., Ohkuma, M., Pittayakhajonwut, P., suwanborirux, k., & Tanasupawat, S. (2016). *Streptomyces verrucosisporus* sp. nov., isolated from marine sediments. *Int J Syst Evol Microbiol.* (in press). doi: doi:10.1099/ijsem.0.001240

Pitcher, D., Saunders, N., & Owen, R. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol.* 8(4), 151-156. doi: 10.1111/j.1472-765X.1989.tb00262.x.

Pledger, R. J., Crump, B. C., & Baross, J. A. (1994). A barophilic response by two hyperthermophilic, hydrothermal vent Archaea: an upward shift in the optimal temperature and acceleration of growth rate at supra-optimal temperatures by elevated pressure. *FEMS microbiol ecol.* 14(3), 233-241.

Podosokorskaya, O. A., Merkel, A. Y., Kolganova, T. V., Chernyh, N. A., Miroshnichenko, M. L., Bonch-Osmolovskaya, E. A., & Kublanov, I. V. (2011). *Fervidobacterium riparium* sp. nov., a thermophilic anaerobic cellulolytic bacterium isolated from a hot spring. *Int J Syst Evol Microbiol.* 61(Pt 11), 2697-2701. doi: 10.1099/ijs.0.026070-0.

Polz, M. F., Alm, E. J., & Hanage, W. P. (2013). Horizontal gene transfer and the evolution of bacterial and archaeal population structure. *Trends Genet.* 29(3), 170-175.

Porter, S. S., Chang, P. L., Conow, C. A., Dunham, J. P., & Friesen, M. L. (2017). Association mapping reveals novel serpentine adaptation gene clusters in a population of symbiotic Mesorhizobium. *The ISME journal.* 11(1), 248-262.

Powell, C. M. (1979). A speculative tectonic history of Pakistan and surroundings: some constraints from the Indian Ocean. *Geodynamics of Pakistan.* 13, 5-24.

Prashar, P., Kapoor, N., & Sachdeva, S. (2014). Rhizosphere: its structure, bacterial diversity and significance. *Rev Environ Sci Bio.* 13(1), 63-77.

Prauser, H. (1976). *Nocardioides*, a new genus of the order *Actinomycetales*. *Int J Syst Bacteriol.* 26(1), 58-65.

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2--approximately maximum-likelihood trees for large alignments. *PloS one.* 5(3), e9490. doi: 10.1371/journal.pone.0009490.

Puente, M. E., Li, C. Y., & Bashan, Y. (2009). Rock-degrading endophytic bacteria in cacti. *Environ Exp Bot.* 66(3), 389-401.

Qin, S., Li, W. J., Dastager, S. G., & Hozzein, W. N. (2016). Editorial: Actinobacteria in Special and Extreme Habitats: Diversity, Function Roles, and Environmental Adaptations. *Front Microbiol.* 7, 1415. doi: 10.3389/fmicb.2016.01415.

Qin, S., Yuan, B., Zhang, Y. J., Bian, G. K., Tamura, T., Sun, B. Z., Jiang, J. H. (2012). *Nocardioides panzhihuaensis* sp. nov., a novel endophytic actinomycete isolated from medicinal plant *Jatropha curcas* L. *A Van Leeuw.* 102(2), 353-360. doi: 10.1007/s10482-012-9745-8.

Radl, V., Simoes-Araujo, J. L., Leite, J., Passos, S. R., Martins, L. M., Xavier, G. R., Zilli, J. E. (2014). *Microvirga vignae* sp. nov., a root nodule symbiotic bacterium isolated from cowpea grown in semi-arid Brazil. *Int J Syst Evol Microbiol.* 64(Pt 3), 725-730. doi: 10.1099/ijs.0.053082-0

Rao, S., Chan, Y., Bugler-Lacap, D. C., Bhatnagar, A., Bhatnagar, M., & Pointing, S. B. (2016). Microbial Diversity in Soil, Sand Dune and Rock Substrates of the Thar Monsoon Desert, India. *Ind J Microbiol.* 56(1), 35-45. doi: 10.1007/s12088-015-0549-1.

Reth, S., Reichstein, M., & Falge, E. (2005). The effect of soil water content, soil temperature, soil pH-value and the root mass on soil CO 2 efflux–a modified model. *Plant and Soil.* 268(1), 21-33.

Reysenbach, A.-L., Longnecker, K., & Kirshtein, J. (2000). Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. *Appl Environ Microbiol.* 66(9), 3798-3806.

Reysenbach, A.-L., & Shock, E. (2002). Merging genomes with geochemistry in hydrothermal ecosystems. *Science.* 296(5570), 1077-1082.

Rhoades, J. (1982a). *Cation exchange capacity*. Madison, WI, USA: American Society of Agronomy, Soil Science Society of America.

Rhoades, J. (1982b). *Soluble salts*. Madison, WI, USA: American Society of Agronomy, Soil Science Society of America.

Roesch, L. F., Fulthorpe, R. R., Riva, A., Casella, G., Hadwin, A. K., Kent, A. D., Triplett, E. W. (2007). Pyrosequencing enumerates and contrasts soil microbial diversity. *Isme j.* 1(4), 283-290. doi: 10.1038/ismej.2007.53

Roh, S. W., Sung, Y., Nam, Y. D., Chang, H. W., Kim, K. H., Yoon, J. H., Bae, J. W. (2008). *Arthrobacter soli* sp. nov., a novel bacterium isolated from wastewater reservoir sediment. *J Microbiol.* 46(1), 40-44. doi: 10.1007/s12275-007-0239-8.

Rong, X., & Huang, Y. (2010). Taxonomic evaluation of the *Streptomyces griseus* clade using multilocus sequence analysis and DNA–DNA hybridization, with proposal to combine 29 species and three subspecies as 11 genomic species. *Int J Syst Evol Microbiol.* 60(3), 696-703.

Russell, N., Harrisson, P., Johnston, I., Jaenicke, R., Zuber, M., Franks, F., & Wynn-Williams, D. (1990). Cold adaptation of microorganisms [and discussion]. *Philos Trans R Soc Lond B: Biol Sci.* 326(1237), 595-611.

Sahoo, R. K., Gaur, M. K., Das, A., Singh, A., Kumar, M., & Subudhi, E. (2016). Comparative analysis of 16S rRNA gene Illumina sequence for microbial community structure in diverse unexplored hot springs of Odisha, India. *Geomicrobiol J.* 00-00.

Saitou, N., & Nei, M. (1987). The neighbor-joining method - a new method for reconstructing phylogenetic trees. *Mol Biol Evol.* 4(4), 406-425.

Salama, F., EL-GHANI, M. A., & El-Tayeh, N. (2013). Vegetation and soil relationships in the inland wadi ecosystem of central Eastern Desert, Egypt. *Turk J Bot.* 37(3), 489-498.

Salem, R. B., Abbassi, M. S., Cayol, J.-l., Bourouis, A., Mahrouki, S., Fardeau, M.-L., & Belhadj, O. (2016). Thermophilic *Bacillus licheniformis* RBS 5 isolated from hot Tunisian spring co-producing alkaline and thermostable [alpha]-amylase and protease enzymes. *J microb biotech food sci.* 5(6), 557.

Sarker, M. R., Akhtar, S., Torres, J. A., & Paredes-Sabja, D. (2015). High hydrostatic pressure-induced inactivation of bacterial spores. *Critical reviews in microbiology.* 41(1), 18-26.

Sasser, M. (1990). Identification of bacteria by gas chromatography of cellular fatty acids. *MIDI Technical Note 101.*

Schippers-Lammertse, A. F., Muijsers, A., & Klatser-Oedekerk, K. B. (1963). *Arthrobacter polychromogenes* nov. spec., its pigments, and a bacteriophage of this species. *A Van Leeuw.* 29(1), 1-15.

Schippers, A., Schumann, P., & Sproer, C. (2005). *Nocardioides oleivorans* sp. nov., a novel crude-oil-degrading bacterium. *Int J Syst Evol Microbiol.* 55(4), 1501-1504. doi: 10.1099/ijs.0.63500-0.

Schleifer, K. H., & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev.* 36(4), 407-477.

Schlesinger, W. H., Raikes, J. A., Hartley, A. E., & Cross, A. F. (1996). Erratum: On the Spatial Pattern of Soil Nutrients in Desert Ecosystems. *Ecology.* 77(4), 1270-1270.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol.* 75(23), 7537-7541. doi: 10.1128/aem.01541-09.

Selim, S., Hassan, S., Hagagy, N., Kraková, L., Grivalský, T., & Pangallo, D. (2016). Assessment of Microbial Diversity in Saudi Springs by Culture-Dependent and Culture-Independent Methods. *Geomicrobiol J.* 1-11.

Semêdo, L., Gomes, R., Linhares, A., Duarte, G., Nascimento, R., Rosado, A., Alviano, C. (2004). *Streptomyces drozdowiczii* sp. nov., a novel cellulolytic streptomycete from soil in Brazil. *Int J Syst Evol Microbiol.* 54(4), 1323-1328.

Semêdo, L. T. A. S., Gomes, R. C., Bon, E. P. S., Soares, R. M. A., Linhares, L. F., & Coelho, R. R. R. (2000). Endocellulase and exocellulase activities of two *Streptomyces* strains isolated from a forest soil. *Appl Biochem Biotechnol.* 84(1), 267-276. doi: 10.1385/abab:84-86:1-9:267.

Shah, A. A., Nawaz, A., Kanwal, L., Hasan, F., Khan, S., & Badshah, M. (2015). Degradation of poly (ε-caprolactone) by a thermophilic bacterium Ralstonia sp. strain MRL-TL isolated from hot spring. *Int Biodeterior Biodegradation.* 98, 35-42.

Sharma, N., Vyas, G., & Pathania, S. (2013). Culturable diversity of thermophilic microorganisms found in hot springs of Northern Himalayas and to explore their potential for production of industrially important enzymes. *Scholars Acad. J. Biosci.* 1, 165-178.

Sharp, C., Stott, M., & Dunfield, P. (2012). Detection of autotrophic verrucomicrobial methanotrophs in a geothermal environment using stable isotope probing. *Front microbiol.* 3, 303.

Sharp, C. E., Martínez-Lorenzo, A., Brady, A. L., Grasby, S. E., & Dunfield, P. F. (2014). Methanotrophic bacteria in warm geothermal spring sediments identified using stable-isotope probing. *FEMS microbiol ecol.* 90(1), 92-102.

Shin, K.-S., Shin, Y. K., Yoon, J.-H., & Park, Y.-H. (2001). *Candida thermophila* sp. nov., a novel thermophilic yeast isolated from soil. *Int J Syst Evol Microbiol.* 51(6), 2167-2170.

Shirling, E., & Gottlieb, D. (1966). Method for characterization of *Streptomyces* species. *Int J Syst Bacteriol.* 16(3), 313-340. doi: 10.1099/00207713-16-3-313.

Shuja, T. A. (1986). Geothermal areas in Pakistan. *Geothermics.* 15(5-6), 719-723.

Sievert, S. M., Kuever, J., & Muyzer, G. (2000). Identification of 16S ribosomal DNA-defined bacterial populations at a shallow submarine hydrothermal vent near Milos Island (Greece). *Appl Environ Microbiol.* 66(7), 3102-3109.

Skerman, V. B. D., McGowan, V., & Sneath, P. H. A. (1980). Approved lists of bacterial names. *Int J Syst Evol Microbiol.* 30(1), 225-420.

Skirnisdottir, S., Hreggvidsson, G. O., Hjorleifsdottir, S., Marteinsson, V. T., Petursdottir, S. K., Holst, O., & Kristjansson, J. K. (2000). Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats. *Appl Environ Microbiol.* 66(7), 2835-2841.

Slobodkina, G. B., Baslerov, R. V., Novikov, A. A., Viryasov, M. B., Bonch-Osmolovskaya, E. A., & Slobodkin, A. I. (2016). *Inmirania thermothiophila* gen. nov., sp. nov., a thermophilic, facultatively autotrophic, sulfur-oxidizing gammaproteobacterium isolated from a shallow-sea hydrothermal vent. *Int J Syst Evol Microbiol.* 66(2), 701-706.

Slobodkina, G. B., Panteleeva, A. N., Beskorovaynaya, D. A., Bonch-Osmolovskaya, E. A., & Slobodkin, A. I. (2016). *Thermostilla marina* gen. nov., sp. nov., a thermophilic, facultatively anaerobic planctomycete isolated from a shallow submarine hydrothermal vent. *Int J Syst Evol Microbiol.* 66(2), 633-638.

Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., Herndl, G. J. (2006). Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences.* 103(32), 12115-12120.

Sokolova, T., Kostrikina, N., Chernyh, N., Tourova, T., Kolganova, T., & Bonch-Osmolovskaya, E. (2002). *Carboxydocella thermautotrophica* gen. nov., sp. nov., a novel anaerobic, CO-utilizing thermophile from a Kamchatkan hot spring. *Int J Syst Evol Microbiol.* 52(6), 1961-1967.

Spear, J. R., Walker, J. J., & Pace, N. R. (2005a). Hydrogen and primary productivity: inference of biogeochemistry from phylogeny in a geothermal ecosystem. *Geothermal Biology and Geochemistry in Yellowstone National Park.* 113-128.

Spear, J. R., Walker, J. J., & Pace, N. R. (2005b). Hydrogen and primary productivity: inference of biogeochemistry from phylogeny in a geothermal ecosystem. In W. Inskeep & T. McDermott (Eds.), *Geothermal biology and geochemistry in Yellowstone National Park Bozeman.* MT (pp. 113-128). Thermal Biology Institute, Montana State University.

Srinivasan, S., Joo, E. S., Kim, E. B., Jeon, S. H., Jung, H.-Y., & Kim, M. K. (2016). *Altererythrobacter terrae* sp. nov., isolated from mountain soil. A Van Leeuw J Microb. 109(3), 397-404.

Sripreechasak, P., Tamura, T., Shibata, C., Suwanborirux, K., & Tanasupawat, S. (2016). *Streptomyces andamanensis* sp. nov., isolated from soil. *Int J Syst Evol Microbiol.* 66(5), 2030-2034. doi: doi:10.1099/ijsem.0.000987.

Stackebrandt, E. (2014). The family *Gracilibacteraceae* and transfer of the genus *Lutispora* into *Gracilibacteraceae The Prokaryotes* (pp. 149-151): Springer.

Stackebrandt, E., Fowler, V. J., Fiedler, F., & Seiler, H. (1983). Taxonomic Studies on *Arthrobacter nicotianae* and Related Taxa: Description of *Arthrobacter uratoxydans* sp. nov. and *Arthrobacter sulfureus* sp. nov. and Reclassification of *Brevibacterium protophormiae* as *Arthrobacter protophormiae* comb. nov. *Syst appl microbiol.* 4(4), 470-486.

Stackebrandt, E., & Goebel, B. M. (1994). Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Evol Microbiol.* 44(4), 846-849. doi: 10.1099/00207713-44-4-846.

Stackebrandt, E., Koch, C., Gvozdiak, O., & Schumann, P. (1995). Taxonomic Dissection of the Genus *Micrococcus: Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus Cohn 1872* gen. emend. *Int J Syst Evol Microbiol.* 45(4), 682-692.

Stefanova, K., Tomova, I., Tomova, A., Radchenkova, N., Atanassov, I., & Kambourova, M. (2016). Archaeal and bacterial diversity in two hot springs from geothermal regions in Bulgaria as demostrated by 16S rRNA and GH-57 genes. *Internat. microbiol.* 18(4), 217-223.

Stumm, W., Morgan, J. J., & Drever, J. I. (1996). Aquatic Chemistry. *J Environ Qual, 25*(5), 1162.

Summit, M., Scott, B., Nielson, K., Mathur, E., & Baross, J. (1998). Pressure enhances thermal stability of DNA polymerase from three thermophilic organisms. *Extremophiles.* 2(3), 339-345.

Sun, L., Toyonaga, M., Ohashi, A., Matsuura, N., Tourlousse, D. M., Meng, X.-Y., Yamaguchi, T. (2016). Isolation and characterization of *Flexilinea flocculi* gen. nov., sp. nov., a filamentous, anaerobic bacterium belonging to the class Anaerolineae in the phylum *Chloroflexi*. *Int J Syst Evol Microbiol.* 66(2), 988-996.

Sun, L., Toyonaga, M., Ohashi, A., Tourlousse, D. M., Matsuura, N., Meng, X.-Y.,Yamaguchi, T. (2016). *Lentimicrobium saccharophilum* gen. nov., sp. nov., a strictly anaerobic

bacterium representing a new family in the phylum *Bacteroidetes*, and proposal of *Lentimicrobiaceae* fam. nov. *Int J Syst Evol Microbiol.*

Szewzyk, U., Szewzyk, R., & Stenström, T. (1994). Thermophilic, anaerobic bacteria isolated from a deep borehole in granite in Sweden. *Proceedings of the National Academy of Sciences.* 91(5), 1810-1813.

Takahata, Y., Nishijima, M., Hoaki, T., & Maruyama, T. (2000). Distribution and physiological characteristics of hyperthermophiles in the Kubiki oil reservoir in Niigata, Japan. *Appl Environ Microbiol.* 66(1), 73-79.

Takeda, M., Suzuki, I., & Koizumi, J. (2004). *Balneomonas flocculans* gen. nov., sp. nov., a new cellulose-producing member of the alpha-2 subclass of *Proteobacteria*. *Syst Appl Microbiol.* 27(2), 139-145. doi: 10.1078/072320204322881745.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA 6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol.* 30(12), 2725-2729. doi: 10.1093/molbev/mst197.

Tang, S.-K., Wang, Y., Chen, Y., Lou, K., Cao, L.-L., Xu, L.-H., & Li, W.-J. (2009). *Zhihengliuella alba* sp. nov., and emended description of the genus *Zhihengliuella*. *Int J Syst Evol Microbiol.* 59(8), 2025-2032.

Tang, S. K., Wang, Y., Chen, Y., Lou, K., Cao, L. L., Xu, L. H., & Li, W. J. (2009). *Zhihengliuella alba* sp. nov., and emended description of the genus *Zhihengliuella*. *Int J Syst Evol Microbiol.* 59(8), 2025-2031. doi: 10.1099/ijs.0.007344-0.

Tekere, M., Lötter, A., Olivier, J., Jonker, N., & Venter, S. (2011). Metagenomic analysis of bacterial diversity of Siloam hot water spring, Limpopo, South Africa. *Afr J Biotechnol.* 10(78), 18005-18012.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25(24), 4876-4882. doi: 10.1093/nar/25.24.4876.

Tindall, J. B., Sikorski, J., Simbert, A. R., & Krieg, R. N. (2007). Phenotypic characterization and the principles of comparative systematics. In C. A. Reddy, T. J. Beveridge, J. A. Breznak, G. A. Marzluf, T. M. S. chmidt & L. R. Snyder (Eds.), *Methods for General and Molecular Microbiology* (3rd ed., pp. 330-393). Washington, DC: American Society for Microbiology.

Todaka, N., Shuja, T., Jamiluddin, S., Khan, N., Pasha, M., & Iqbal, M. (1988). A preliminary study for geothermal development project in Pakistan. *Geological Survey of Pakistan.* 4-47.

Tripathi, C., Mahato, N., Singh, A., Kamra, K., Korpole, S., & Lal, R. (2016). *Lampropedia cohaerens* sp. nov., a biofilm-forming bacterium isolated from microbial mats of a hot water spring, and emended description of the genus *Lampropedia*. *Int J Syst Evol Microbiol.* 66(3), 1156-1162.

Tripathy, S., Padhi, S. K., Mohanty, S., Samanta, M., & Maiti, N. K. (2016). Analysis of the metatranscriptome of microbial communities of an alkaline hot sulfur spring revealed different gene encoding pathway enzymes associated with energy metabolism. *Extremophiles.* 1-12.

Trujillo, M. E., Riesco, R., Benito, P., & Carro, L. (2015). Endophytic Actinobacteria and the Interaction of Micromonospora and Nitrogen Fixing Plants. *Front Microbiol.* 6, 1341. doi: 10.3389/fmicb.2015.01341.

Tsubota, J., Eshinimaev, B., Khmelenina, V. N., & Trotsenko, Y. A. (2005). *Methylothermus thermalis* gen. nov., sp. nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan. *Int J Syst Evol Microbiol.* 55(Pt 5), 1877-1884. doi: 10.1099/ijs.0.63691-0.

Tuo, L., Dong, Y. P., Habden, X., Liu, J. M., Guo, L., Liu, X. F., Sun, C. H. (2015). *Nocardioides deserti* sp. nov., an actinobacterium isolated from desert soil. *Int J Syst Evol Microbiol.* 65(5), 1604-1610. doi: 10.1099/ijs.0.000147.

Tvrzova, L., Schumann, P., Sproer, C., Sedlacek, I., Verbarg, S., Kroppenstedt, R. M., & Pacova, Z. (2005). Polyphasic taxonomic study of strain CCM 2783 resulting in the description of *Arthrobacter stackebrandtii* sp. nov. *Int J Syst Evol Microbiol.* 55(Pt 2), 805-808. doi: 10.1099/ijs.0.63428-0.

Unsworth, L. D., van der Oost, J., & Koutsopoulos, S. (2007). Hyperthermophilic enzymes–stability, activity and implementation strategies for high temperature applications. *Febs Journal.* 274(16), 4044-4056. doi: 10.1111/j.1742-4658.2007.05954.x.

van Elsas, J. D., Chiurazzi, M., Mallon, C. A., Elhottova, D., Kristufek, V., & Salles, J. F. (2012). Microbial diversity determines the invasion of soil by a bacterial pathogen. *Proc Natl Acad Sci U S A.* 109(4), 1159-1164. doi: 10.1073/pnas.1109326109.

Ventosa, A., Nieto, J. J., & Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. Microbiol Mol Biol Rev. 62(2), 504-544.

Verma, M., Lal, D., Kaur, J., Saxena, A., Kaur, J., Anand, S., & Lal, R. (2013). Phylogenetic analyses of phylum *Actinobacteria* based on whole genome sequences. *Res Microbiol.* 164(7), 718-728.

Virk, H., Sharma, A., & Sharma, N. (2002). Radon and helium monitoring in some thermal springs of North India and Bhutan. *Curr Sci.* 82(12), 1423-1424.

Wallenstein, M. D., McMahon, S. K., & Schimel, J. P. (2009). Seasonal variation in enzyme activities and temperature sensitivities in Arctic tundra soils. *Global Change Biol.* 15(7), 1631-1639.

Wang, S., Hou, W., Dong, H., Jiang, H., Huang, L., Wu, G., Zhang, L. (2013). Control of temperature on microbial community structure in hot springs of the Tibetan Plateau. *PloS one.* 8(5), e62901. doi: 10.1371/journal.pone.0062901.

Ward, D. M., Ferris, M. J., Nold, S. C., & Bateson, M. M. (1998). A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. Microbiol Mol Biol Rev. 62(4), 1353-1370.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Truper, H. G. (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol.* 37(4), 463-464. doi: 10.1099/00207713-37-4-463.

Weon, H. Y., Kwon, S. W., Son, J. A., Jo, E. H., Kim, S. J., Kim, Y. S., Ka, J. O. (2010). Description of *Microvirga aerophila* sp. nov. and *Microvirga aerilata* sp. nov., isolated from air, reclassification of *Balneimonas flocculans* Takeda et al. 2004 as *Microvirga flocculans* comb. nov. and emended description of the genus *Microvirga*. *Int J Syst Evol Microbiol.* 60(Pt 11), 2596-2600. doi: 10.1099/ijs.0.018770-0.

Westerberg, K., Elvang, A. M., Stackebrandt, E., & Jansson, J. K. (2000). *Arthrobacter chlorophenolicus* sp. nov., a new species capable of degrading high concentrations of 4-chlorophenol. *Int J Syst Evol Microbiol.* 50 (6), 2083-2092. doi: 10.1099/00207713-50-6-2083.

Wetherill, G. W. (1990). Formation of the Earth. Annu. Rev. Earth Pla. 18, 205-256.

Whitaker, R. J., Grogan, D. W., & Taylor, J. W. (2003). Geographic barriers isolate endemic populations of hyperthermophilic archaea. *Science.* 301(5635), 976-978.

White, D. E., Brannock, W., & Murata, K. (1956). Silica in hot-spring waters. Geochim. Cosmochim. Acta. 10(1-2), 27-59.

Wiegel, J. (1998a). Anaerobic alkalithermophiles, a novel group of extremophiles. *Extremophiles.* 2(3), 257-267.

Wiegel, J. (1998b). Lateral gene exchange, an evolutionary mechanism for extending the upper or lower temperature limits for growth of an microorganism? A hypothesis. *Thermophiles: The keys to molecular evolution and the origin of life.* 175-185.

Wiegel, J., Braun, M., & Gottschalk, G. (1981). *Clostridium thermoautotrophicum* species novum, a thermophile producing acetate from molecular hydrogen and carbon dioxide. *Curr. Microbiol.* 5(4), 255-260.

Wiegel, J., Hanel, J., & Aygen, K. (2003). Chemolithoautotrophic thermophilic iron (III)-reducer *Biochemistry and Physiology of Anaerobic Bacteria* (pp. 235-251): Springer.

Wieser, M., Denner, E. B., Kämpfer, P., Schumann, P., Tindall, B., Steiner, U., Patel, B. (2002). Emended descriptions of the genus *Micrococcus, Micrococcus luteus* (Cohn 1872) and *Micrococcus lylae* (Kloos et al. 1974). *Int J Syst Evol Microbiol.* 52(2), 629-637.

Wilde, S. A., Valley, J. W., Peck, W. H., & Graham, C. M. (2001). Evidence from detrital zircons for the existence of continental crust and oceans on the Earth 4.4 Gyr ago. *Nature.* 409(6817), 175-178.

Williams, S. (1989). Genus: *Streptomyces* (Waksman and Henrici 1943) *Bergey's Manual of Systematics in Bacteriology* (Vol. 4, pp. 2452-2492).

Williams, S., & Davies, F. (1965). Use of antibiotics for selective isolation and enumeration of actinomycetes in soil. *Microbiol.* 38(2), 251-261.

Wilson, E. O. (1992). The diversity of life. *Allen lane.*

Xu, P., Li, W.-J., Tang, S.-K., Zhang, Y.-Q., Chen, G.-Z., Chen, H.-H., Jiang, C.-L. (2005). *Naxibacter alkalitolerans* gen. nov., sp. nov., a novel member of the family '*Oxalobacteraceae*' isolated from China. *Int J Syst Evol Microbiol.* 55(3), 1149-1153.

Xu, P., Li, W. J., Tang, S. K., Zhang, Y. Q., Chen, G. Z., Chen, H. H., Jiang, C. L. (2005). *Naxibacter alkalitolerans* gen. nov., sp. nov., a novel member of the family '*Oxalobacteraceae*' isolated from China. *Int J Syst Evol Microbiol.* 55(3), 1149-1153. doi: 10.1099/ijs.0.63407-0

Yamamura, H., Ohkubo, S. Y., Nakagawa, Y., Ishida, Y., Hamada, M., Otoguro, M., Hayakawa, M. (2011). *Nocardioides iriomotensis* sp. nov., an actinobacterium isolated from forest soil. *Int J Syst Evol Microbiol.* 61(9), 2205-2209. doi: 10.1099/ijs.0.025080-0.

Yassin, A., Spröer, C., Siering, C., Hupfer, H., & Schumann, P. (2011). *Arthrobacter equi* sp. nov., isolated from veterinary clinical material. *Int J Syst Evol Microbiol.* 61(9), 2089-2094.

Yim, L. C., Hongmei, J., Aitchison, J. C., & Pointing, S. B. (2006). Highly diverse community structure in a remote central Tibetan geothermal spring does not display monotonic variation to thermal stress. *FEMS microbiol ecol.* 57(1), 80-91.

Yoon, J. H., Cho, Y. G., Lee, S. T., Suzuki, K., Nakase, T., & Park, Y. H. (1999). *Nocardioides nitrophenolicus* sp. nov., a *p*-nitrophenol-degrading bacterium. *Int J Syst Bacteriol.* 49(2), 675-680. doi: 10.1099/00207713-49-2-675.

Yoon, J. H., Kim, I. G., Kang, K. H., Oh, T. K., & Park, Y. H. (2004). *Nocardioides aquiterrae* sp. nov., isolated from groundwater in Korea. *Int J Syst Evol Microbiol.* 54(1), 71-75. doi: 10.1099/ijs.0.02585-0.

Yoon, J. H., Lee, J. K., Jung, S. Y., Kim, J. A., Kim, H. K., & Oh, T. K. (2006). *Nocardioides kongjuensis* sp. nov., an *N*-acylhomoserine lactone-degrading bacterium. *Int J Syst Evol Microbiol.* 56(8), 1783-1787. doi: 10.1099/ijs.0.64120-0.

Yoon, J. H., Park, S., Kang, S. J., Lee, J. S., Lee, K. C., & Oh, T. K. (2010). *Nocardioides daedukensis* sp. nov., a halotolerant bacterium isolated from soil. *Int J Syst Evol Microbiol.* 60(6), 1334-1338. doi: 10.1099/ijs.0.015354-0.

Yoon, Y., Lee, H., Lee, S., Kim, S., & Choi, K.-H. (2015). Membrane fluidity-related adaptive response mechanisms of foodborne bacterial pathogens under environmental stresses. *Int Food Res. J.* 72, 25-36.

Zaburannyi, N., Rabyk, M., Ostash, B., Fedorenko, V., & Luzhetskyy, A. (2014). Insights into naturally minimised *Streptomyces albus* J1074 genome. *BMC Genomics.* 15(1), 1-11. doi: 10.1186/1471-2164-15-97.

Zaigham, N. A., Nayyar, Z. A., & Hisamuddin, N. (2009). Review of geothermal energy resources in Pakistan. Renew. Sustainable Energy Rev. 13(1), 223-232.

Zhang, D.-C., Schumann, P., Liu, H.-C., Xin, Y.-H., Zhou, Y.-G., Schinner, F., & Margesin, R. (2010). *Arthrobacter alpinus* sp. nov., a psychrophilic bacterium isolated from alpine soil. *Int J Syst Evol Microbiol.* 60(9), 2149-2153.

Zhang, D. C., Schumann, P., Redzic, M., Zhou, Y. G., Liu, H. C., Schinner, F., & Margesin, R. (2012). *Nocardioides alpinus* sp. nov., a psychrophilic actinomycete isolated from alpine glacier cryoconite. *Int J Syst Evol Microbiol.* 62(2), 445-450. doi: 10.1099/ijs.0.031047-0.

Zhang, J., Ma, Y., & Yu, H. (2012). *Arthrobacter cupressi* sp. nov., an actinomycete isolated from the rhizosphere soil of Cupressus sempervirens. *Int J Syst Evol Microbiol.* 62(11), 2731-2736. doi: 10.1099/ijs.0.036889-0.

Zhang, J., Song, F., Xin, Y. H., Zhang, J., & Fang, C. (2009). *Microvirga guangxiensis* sp. nov., a novel alphaproteobacterium from soil, and emended description of the genus *Microvirga*. *Int J Syst Evol Microbiol.* 59(8), 1997-2001. doi: 10.1099/ijs.0.007997-0.

Zhang, R., & Wienhold, B. J. (2002). The effect of soil moisture on mineral nitrogen, soil electrical conductivity, and pH. Nutr Cycl Agroecosys. 63(2-3), 251-254.

Zhao, W.-D., Romanek, C. S., Mills, G., Wiegel, J., & Zhang, C.-L. (2005). Geochemistry and microbiology of hot springs in Kamchatka, Russia. *高校地質學報.* 11(2), 217-223.

Zhou, Y., Wei, W., Wang, X., & Lai, R. (2009). Proposal of *Sinomonas flava* gen. nov., sp. nov., and description of *Sinomonas atrocyanea* comb. nov. to accommodate *Arthrobacter atrocyaneus*. *Int J Syst Evol Microbiol.* 59(2), 259-263.

Zillig, W., Stetter, K. O., Wunderl, S., Schulz, W., Priess, H., & Scholz, I. (1980). The *Sulfolobus-"Caldariella"* group: taxonomy on the basis of the structure of DNA-dependent RNA polymerases. *Arch microbiol.* 125(3), 259-269.

Zucchi, T. D., Kim, B.-Y., Kshetrimayum, J. D., Weon, H.-Y., Kwon, S.-W., & Goodfellow, M. (2012). *Streptomyces brevispora* sp. nov. and *Streptomyces laculatispora* sp. nov., actinomycetes isolated from soil. *Int J Syst Evol Microbiol.* 62(3), 478-483.

LIST OF PUBLICATIONS

Research Publications Published:
Following sources and a report submitted to Saudia University have been excluded for final similarity check with Turnitin.

- **Amin, Arshia**., Ahmed, I., Habib, N., Abbas, S., Xiao, M., Hozzein, W. N., & Li, W. J. (2016). *Nocardioides pakistanensis* sp. nov., isolated from a hot water spring of Tatta Pani in Pakistan. *Antonie van Leeuwenhoek*, 1-9. **IF=1.944**
- **Amin, Arshia**, Iftikhar Ahmed, Neeli Habib, Saira Abbas, Fariha Hasan, Min Xiao, Wael N. Hozzein, and Wen-Jun Li. *Microvirga pakistanensis* sp. nov., a novel bacterium isolated from desert soil of Cholistan, Pakistan." *Archives of Microbiology* (2016): 1-7. **IF=1.760**
- **Arshia Amin**, Iftikhar Ahmed, Nauman Khalid, Ghenijan Osman, Inam Ullah Khan, Min Xiao and Wen-Jun Li. *Streptomyces caldifontis* sp. nov., isolated from a hot water spring of Tatta Pani, Kotli, Pakistan. Antonie van Leeuwenhoek (2017) 110: 77. doi:10.1007/s10482-016-0778-2. **IF=1.944**
- **Arshia Amin**, Iftikhar Ahmed, Nimaichand Salam, Byung-Yong Kim, Dharmesh Singh, Xiao-Yang Zhi, Min Xiao, Wen-Jun Li. Diversity and Distribution of Thermophilic Bacteria in Hot Springs of Pakistan. Diversity and Distribution of Thermophilic Bacteria in Hot Springs of Pakistan. DOI: 10.1007/s00248-017-0930-1. MECO-D-16-00262.3. **IF=3.232**
- Inam Ullah Khan; Firasat Hussain; Ye Tian; Neeli Habib; Wen-Dong Xian; Zhao Jiang; **Arshia Amin**; Chang-Guo Yuan; En-Min Zhou; Xiao-Yang Zhi; Wen-Jun Li. *Tibeticola sediminis* gen. nov., sp. nov., a thermophilic bacterium isolated from hot spring. Ms. No. IJSEM-D-16-00941R3. **IF=2.782**

Research Publications submitted:

- **Arshia Amin,** Iftikhar Ahmed , Salam Nimaichand, Nauman Khalid, Peter Schumann, Neeli Habib, Firasat Hussain, Inam Ullah Khan, Javed Iqbal Dasti, Wen-Jun Li. Proposal of *Zafaria solitudinis* gen. nov. sp. nov., a moderately thermotolerant and halotolerant Actinobacteria isolated from Cholistan desert soil of Pakistan and *Alloarthrobacter* gen. nov. and transfer of *Arthrobacter ginkgonis* to *Alloarthrobacter ginkgonis* comb. nov., *Arthrobacter halodurans* to *Alloarthrobacter halodurans* comb. nov., *Arthrobacter deserti* to *Alloarthrobacter deserti* comb. nov. and *Arthrobacter rhombi* to *Alloarthrobacter rhombi* comb. nov. IJSEM.

- **Arshia Amin,** Iftikhar Ahmed, Nimaichand Salam, Nauman Khalid, Peijun Zuo, Min Xiao, Wael N. Hozzein, Wen-Jun Li. Bacterial Community Structure in the Cholistan Desert, Pakistan: Comparative Analysis between Rhizospheric and Non-Rhizospheric Soil Samples. Frontiers in Microbiology. Received on: 24 Dec 2016. Manuscript ID:250793

- Inam Ullah Khan; Neeli Habib; Firasat Hussain; Wen-Dong Xian; **Arshia Amin;** En-Min En-Min Zhou; Iftikhar Ahmed; Xiao-Yang Zhi; Wen-Jun Li. *Thermus caldifontis* sp. nov., a thermophilic bacterium isolated from a hot spring. Ms. No. IJSEM-D-16-01065R1
- Neeli Habib; Inam Ullah Khan; Firasat Hussain; Wen-Dong Xian; **Arshia Amin;** En-Min Zhou; Iftikhar Ahmed; Xiao-Yang Zhi; Wen-Jun Li. *Crenalkalicoccus tepidum* sp. nov., a moderately thermophilic bacterium isolated from a hot spring. International Journal of Systematic and Evolutionary Microbiology. Ms. No. IJSEM-D-17-00092
- *Nocardioides thalensis* sp. nov., isolated from a desert Inam Ullah Inam Ullah Khan; Firasat Hussain; Neeli Habib; Min Xiao; Iftikhar Ahmed; **Arshia Amin**; Xiao-Yang Zhi; Wen-Jun Li, Ms. No. IJSEM-D-17-00161
- Industrially important 249 bacterial Strains have been isolated, sequenced and submitted in gene bank, accession numbers are from LC065128, NCCP-1100 to LC065376, NCCP1664
- Raw pyrosequencing reads were submitted to the Sequence Read Archive (SRA), NCBI
  - under accession numbers SAMN03284242- SAMN03284258
  - http://www.ncbi.nlm.nih.gov/biosample?LinkName=bioproject_biosample_all&from_uid=271634
- New bacterial species have been submitted in following international culture collection centers and certificates were received for validation of new species of bacteria (KCTC) (Korean Collection for Type Cultures), JCM (Japan Collection of Microorganisms), CGMCC(China General Microbiological Culture Collection), DSMZ(Leibniz-Institute,
- Deutsche Sammlung Von Mikroorganismen und Zellkulturen GmbH)
- House keeping genes, *rec A, rpo B, gyr B* and *dna K* were also amplified and submitted under following accession numbers at DDBJ.
- 30 industrialy important strains among isolated strains have been sent to Korean company for Whole Genome Sequencing for future studies at molecular level.

Arch Microbiol (2016) 198:933–939
DOI 10.1007/s00203-016-1251-3

ORIGINAL PAPER

Microvirga pakistanensis sp. nov., a novel bacterium isolated from desert soil of Cholistan, Pakistan

Arshia Amin[1,2,3] · Iftikhar Ahmed[2,4] · Neeli Habib[1] · Saira Abbas[2,4] · Fariha Hasan[3] · Min Xiao[5] · Wael N. Hozzein[7] · Wen-Jun Li[1,5,6]

Received: 7 March 2016 / Revised: 27 May 2016 / Accepted: 31 May 2016 / Published online: 11 June 2016
© Springer-Verlag Berlin Heidelberg 2016

Abstract A Gram-negative, non-spore-forming, non-pigmented, strictly aerobic and non-motile short rod bacterium, designated NCCP-1258^T, was isolated from Cholistan desert soil, Bahawalpur, Pakistan. Growth of strain NCCP-1258^T was observed at pH range 6.5–9.5 (optimum 7.5–8.5) and temperature range 20–45 °C (optimum 40 °C), and it tolerated 0–2 % NaCl (optimum 0.5 %, w/v). Phylogenetic analysis based on 16S rRNA gene sequence comparison revealed that strain NCCP-1258^T belongs to genus *Microvirga* and is most closely related to *Microvirga lotononidis* (98.0 %), *Microvirga vignae* (97.4 %), *Microvirga lupini* (97.2 %), *Microvirga zambiensis* (97.2 %) and *Microvirga flocculans* (97.1 %). Analysis of the concatenated sequences of four housekeeping gene loci (*dnaK*, *gyrB*, *recA* and *rpoB*) also confirmed the placement of strain NCCP-1258^T within the genus *Microvirga*. DNA–DNA relatedness values of NCCP-1258^T with above-mentioned type strains were less than 42 %. The DNA G+C content of strain NCCP-1258^T was 64.3 mol%. Chemotaxonomic data (predominant menaquinone system was Q-10; major fatty acids were $C_{16:0}$, $C_{18:1}$ ω7*c* and $C_{19:0}$ cyclo ω8*c*; the polar lipid profile contained diphosphatidylglycerol, phosphatidylcholine, phosphatidyl dimethyl ethanolamine and phosphatidyl ethanolamine) also supported the affiliation of strain NCCP-1258^T to the genus *Microvirga*. On the basis of physiological and biochemical characteristics, phylogenetic analyses and DNA–DNA relatedness, strain NCCP-1258^T can be distinguished from the closely related taxa and thus represents a novel species of the genus *Microvirga*, for which the name *Microvirga pakistanensis* sp. nov. is proposed with the type strain NCCP-1258^T (=CGMCC 1.15074^T = KCTC 42496^T).

Communicated by Erko Stackebrandt.

Electronic supplementary material The online version of this article (doi:10.1007/s00203-016-1251-3) contains supplementary material, which is available to authorized users.

✉ Iftikhar Ahmed
iftikharnarc@hotmail.com

✉ Wen-Jun Li
liwenjun3@mail.sysu.edu.cn

1 Yunnan Institute of Microbiology, Yunnan University, Kunming, People's Republic of China

2 Institute of Microbial Culture Collection of Pakistan (IMCCP), National Agricultural Research Centre (NARC), Park Road, Islamabad 45500, Pakistan

3 Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan

4 PARC Institute of Advance Studies in Agriculture (PIASA), National Agricultural Research Centre (NARC), Park Road, Islamabad 45500, Pakistan

5 State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, People's Republic of China

6 Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Ürümqi 830011, People's Republic of China

7 Bioproducts Research Chair (BRC), College of Science, King Saud University, Riyadh 11451, Kingdom of Saudi Arabia

Antonie van Leeuwenhoek
DOI 10.1007/s10482-016-0778-2

ORIGINAL PAPER

Streptomyces caldifontis sp. nov., isolated from a hot water spring of Tatta Pani, Kotli, Pakistan

Arshia Amin · Iftikhar Ahmed · Nauman Khalid · Ghenijan Osman · Inam Ullah Khan · Min Xiao · Wen-Jun Li

Received: 23 June 2016 / Accepted: 29 September 2016
© Springer International Publishing Switzerland 2016

Abstract A Gram-staining positive, non-motile, rod-shaped, catalase positive and oxidase negative bacterium, designated NCCP-1331^T, was isolated from a hot water spring soil collected from Tatta Pani, Kotli, Azad Jammu and Kashmir, Pakistan. The isolate grew at a temperature range of 18-40 °C (optimum 30 °C), pH 6.0–9.0 (optimum 7.0) and with 0–6 % NaCl (optimum 2 % NaCl (w/v)). The phylogenetic analysis based on 16S rRNA gene sequence revealed that strain NCCP-1331^T belonged to the genus *Streptomyces* and is closely related to *Streptomyces brevispora* BK160^T with 97.9 % nucleotide similarity, followed by *Streptomyces drosdowiczii* NRRL B-24297^T with 97.8 % nucleotide similarity. The DNA–DNA relatedness values of strain NCCP-1331^T with *S. brevispora* KACC 21093^T and *S. drosdowiczii* CBMAI 0498^T were 42.7 and 34.7 %, respectively. LL-DAP was detected as diagnostic amino acid along with alanine, glycine, leucine and glutamic acid. The isolate contained MK-9(H_8) as the predominant menaquinone. Major polar lipids detected in NCCP-1331^T were phosphatidylethanolamine, phosphatidylinositol and

Electronic supplementary material The online version of this article (doi:10.1007/s10482-016-0778-2) contains supplementary material, which is available to authorized users.

A. Amin · M. Xiao · W.-J. Li (✉)
State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, People's Republic of China
e-mail: liwenjun3@mail.sysu.edu.cn

A. Amin · I. Ahmed (✉)
Institute of Microbial Culture Collection of Pakistan (IMCCP), National Agricultural Research Centre (NARC), Park Road, Islamabad 45500, Pakistan
e-mail: iftikharnarc@hotmail.com

A. Amin
Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan

A. Amin · G. Osman · I. U. Khan · W.-J. Li
Yunnan Institute of Microbiology, Yunnan University, Kunming, People's Republic of China

N. Khalid
School of Food and Agricultural Sciences, University of Management and Technology, Lahore 54000, Pakistan

N. Khalid
Centre for Chemistry and Biotechnology, School of Life and Environmental Sciences, Deakin University, Waurn Ponds, Victoria 3216, Australia

W.-J. Li
Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Ürümqi 830011, People's Republic of China

Published online: 11 October 2016

Microb Ecol
DOI 10.1007/s00248-017-0930-1

ENVIRONMENTAL MICROBIOLOGY

Diversity and Distribution of Thermophilic Bacteria in Hot Springs of Pakistan

Arshia Amin[1,2,3] · Iftikhar Ahmed[2] · Nimaichand Salam[4] · Byung-Yong Kim[5] · Dharmesh Singh[6] · Xiao-Yang Zhi[1] · Min Xiao[4] · Wen-Jun Li[1,4,7]

Received: 31 May 2016 / Accepted: 2 January 2017
© Springer Science+Business Media New York 2017

Abstract Chilas and Hunza areas, located in the Main Mantle Thrust and Main Karakoram Thrust of the Himalayas, host a range of geochemically diverse hot springs. This Himalayan geothermal region encompassed hot springs ranging in temperature from 60 to 95 °C, in pH from 6.2 to 9.4, and in mineralogy from bicarbonates (Tato Field), sulfates (Tatta Pani) to mixed type (Murtazaabad). Microbial community structures in these geothermal springs remained largely unexplored to date. In this study, we report a comprehensive, culture-independent survey of microbial communities in nine samples from these geothermal fields by employing a barcoded pyrosequencing technique. The bacterial phyla *Proteobacteria* and *Chloroflexi* were dominant in all samples from Tato Field, Tatta Pani, and Murtazaabad. The community structures however depended on temperature, pH, and physicochemical parameters of the geothermal sites. The Murtazaabad hot springs with relatively higher temperature (90–95 °C) favored the growth of phylum *Thermotogae*, whereas the Tatta Pani thermal spring site TP-H3-b (60 °C) favored the phylum *Proteobacteria*. At sites with low silica and high temperature, OTUs belonging to phylum *Chloroflexi* were dominant. Deep water areas of the Murtazaabad hot springs favored the sulfur-reducing bacteria. About 40% of the total OTUs obtained from these samples were unclassified or uncharacterized, suggesting the presence of many undiscovered and unexplored microbiota. This study has provided novel insights into the nature of ecological interactions among important taxa in these communities, which in turn will help in determining future study courses in these sites.

Keywords Main Karakoram Thrust · Hot springs · Pyrosequencing · 16S rRNA gene · Thermophilic bacterial diversity

Electronic supplementary material The online version of this article (doi:10.1007/s00248-017-0930-1) contains supplementary material, which is available to authorized users.

✉ Iftikhar Ahmed
iftikharnarc@hotmail.com

✉ Wen-Jun Li
liwenjun3@mail.sysu.edu.cn

[1] Key Laboratory of Microbial Diversity in Southwest China, Ministry of Education, Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, People's Republic of China

[2] Institute of Microbial Culture Collection of Pakistan (IMCCP), National Agricultural Research Centre (NARC), Islamabad 45500, Pakistan

[3] Department of Microbiology, Quaid-e-Azam University, Islamabad 45320, Pakistan

[4] State Key Laboratory of Biocontrol and Guandong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, People's Republic of China

[5] Chun Lab Inc., Seoul National University, Seoul 151-742, Republic of South Korea

[6] Environmental Genomics Division, National Environmental Engineering Research Institute (CSIR-NEERI), Nagpur 440024, India

[7] Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Ürümqi 830011, People's Republic of China

Published online: 19 January 2017

Antonie van Leeuwenhoek (2016) 109:1101–1109
DOI 10.1007/s10482-016-0711-8

ORIGINAL PAPER

Nocardioides pakistanensis sp. nov., isolated from a hot water spring of Tatta Pani in Pakistan

Arshia Amin · Iftikhar Ahmed · Neeli Habib · Saira Abbas · Min Xiao · Wael N. Hozzein · Wen-Jun Li

Received: 17 February 2016 / Accepted: 7 May 2016 / Published online: 11 May 2016
© Springer International Publishing Switzerland 2016

Abstract A Gram-staining positive, non-spore forming, non-pigmented and non-motile bacterium, designated as NCCP-1340^T, was isolated from a hot water spring, Tatta Pani, Pakistan. Cells of strain NCCP-1340^T were observed to be aerobic, rod shaped, catalase and urease positive but H_2S production and oxidase negative. Growth was observed at pH 6.0–8.0 (optimum pH 7.0) and at 20–40 °C (optimum 37 °C). The strain could tolerate 0–8 % NaCl (optimum 2 %, w/v). Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain NCCP-1340^T belongs to the genus *Nocardioides* and is closely related to *Nocardioides iriomotensis* JCM 17985^T (96.8 %), *Nocardioides daedukensis* KCTC 19601^T (96.6 %), *Nocardioides jensenii* KCTC 9134^T (96.1 %) and *Nocardioides daejeonensis* KCTC 19772^T (96.1 %). The DNA–DNA relatedness values of strain NCCP-1340^T with *N. iriomotensis* JCM 17985^T, *N. daedukensis* KCTC 19601^T and *N. jensenii* KCTC 9134^T were found to be less than 53 %. The DNA G+C content of strain NCCP-1340^T was determined to be 71.8 mol %. The affiliation of strain NCCP-1340^T to the genus *Nocardioides* was further supported by chemotaxonomic data which showed the presence of MK-8(H_4) as major menaquinone system;

Electronic supplementary material The online version of this article (doi:10.1007/s10482-016-0711-8) contains supplementary material, which is available to authorized users.

A. Amin · N. Habib · W.-J. Li (✉)
Yunnan Institute of Microbiology, Yunnan University, Kunming, People's Republic of China
e-mail: liwenjun3@mail.sysu.edu.cn

A. Amin · I. Ahmed (✉) · S. Abbas
Institute of Microbial Culture Collection of Pakistan (IMCCP), National Agricultural Research Centre (NARC), Park Road, Islamabad 45500, Pakistan
e-mail: iftikharnarc@hotmail.com

A. Amin
Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan

I. Ahmed · S. Abbas
PARC Institute of Advance Studies in Agriculture (PIASA), National Agricultural Research Centre (NARC), Park Road, Islamabad 45500, Pakistan

M. Xiao · W.-J. Li
State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, People's Republic of China

W. N. Hozzein
Bioproducts Research Chair (BRC) College of Science, King Saud University, Riyadh, Saudi Arabia

Korean Collection for Type Cultures (KCTC)
Korea Research Institute of Bioscience & Biotechnology (KRIBB)
125 Gwahak-ro, Yuseong-gu, Daejeon, 305-806, Korea
Tel: +82 42 860-4602, FAX: +82 42 860-4625

Certificate of Deposit

Ref.: Date of issue: MAR 12, 2015

Taxonomic designation	:	***Arthrobacter* sp.**
Accession number	:	**KCTC 39549**
Depositor(s)	:	Wenjun Li
Strain code by the depositor(s)	:	**NCCP-1664**

The above culture has been successfully deposited to KCTC, and is now available to the scientific research community, domestic or international.
KCTC is a member of the World Data Center for Microorganisms (WDCM), and maintains collections of microorganisms for research purposes. Our main functions include preservation and distribution of microbial resources for the academic research communities, domestic or international.
The distribution of any KCTC culture is subjected to the terms of KCTC regulations for the furnishing of biological resources.

K.S. Bae

Curator of the Actinobacterial collection
Kyung Sook Bae Ph.D.

Telephone: +82-42-860-4610
Fax: +82-42-860-4625
E-mail: ksbae@kribb.re.kr
Web: http://www.kctc.re.kr

中国微生物菌种保藏管理委员会普通微生物中心
China General Microbiological Culture Collection Center (CGMCC)

Address: Institute of Microbiology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101, China
Telephone: 86-10-64807355 Fax: 86-10-64807288 E-mail: cgmcc@sun.im.ac.cn Homepage: www.cgmcc.net

受理通知书

NOTIFICATION OF RECEIPT

CGMCC 1.15092

1. Name and address of the depositor or agent

   李文均 Wen-Jun LI
   Yunnan Institute of Microbiology, Yunnan University
   Kunming, Yunnan 650091, P.R. China

2. Strain reference given by depositor

   NCCP-1664

3. Deposited microorganisms appended

   ☐ Scientific description

   ■ Proposed taxonomic name

   Arthrobacter sp.

4. The deposited microorganism has been received and numbered as CGMCC 1.15092 on January, 2015. The strain has been checked for viability in the CGMCC and is stored using one of the standard methods used in the CGMCC.

5. This strain is available in the public accessible section of the CGMCC. It will be included in the published and online catalogue after publication of this number by the authors.

Signature of Head of CGMCC Yu-Guang ZHOU

Date March 12, 2015

Korean Collection for Type Cultures (KCTC), Microbial Resource Center
Korea Research Institute of Bioscience and Biotechnology (KRIBB)
125 Gwahak-ro, Yuseong-gu, Daejeon, 305-806, Korea
Tel: +82-42-860-4602, FAX: +82-42-860-4625

Certificate of Deposit

Date: February 11, 2015

The following information is confidential and acts only to confirm that a strain has been deposited to the KCTC.

***Microvirgae* sp. NCCP-1258** has been deposited in the general collection of microorganisms in the KCTC under the number **KCTC 42496.**

This strain is now readily available to the international scientific research community. Access to the strain is not restricted and it shall be supplied to anyone upon request.

Song-Gun Kim

Song-Gun Kim, Ph.D.
Curator responsible for the strain
Korean Collection for Type Cultures (KCTC)
Microbial Resource Center
Korea Research Institute of Bioscience and Biotechnology (KRIBB)
125 Gwahak-ro, Yuseong-gu, Daejeon, 305-806, Korea

Korean Collection for Type Cultures (KCTC)
Korea Research Institute of Bioscience & Biotechnology (KRIBB)
125 Gwahak-ro, Yuseong-gu, Daejeon, 305-806, Korea
Tel: +82 42 860-4602, FAX: +82 42 860-4625

Certificate of Deposit

Ref.: Date of issue: MAR 12, 2015

Taxonomic designation	:	***Streptomyces* sp.**
Accession number	:	**KCTC 39537**
Depositor(s)	:	Wenjun Li
Strain code by the depositor(s)	:	**NCCP1331**

The above culture has been successfully deposited to KCTC, and is now available to the scientific research community, domestic or international.
KCTC is a member of the World Data Center for Microorganisms (WDCM), and maintains collections of microorganisms for research purposes. Our main functions include preservation and distribution of microbial resources for the academic research communities, domestic or international.
The distribution of any KCTC culture is subjected to the terms of KCTC regulations for the furnishing of biological resources.

K S. Bae

Curator of the Actinobacterial collection
Kyung Sook Bae Ph.D.

Telephone: +82-42-860-4610
Fax: +82-42-860-4625
E-mail: ksbae@kribb.re.kr
Web: http://www.kctc.re.kr

Certificate of Deposit

Date of issue: April 25,2015

The following information is confidential and serves only to confirm that a strain has been deposited in the CPCC.

Strain *Streptomyces sp.* NCCP-1331^{T}
has been deposited in the CPCC under the number
CPCC 204147

This strain has been checked for viability and is stored using one of the standard methods used in the CPCC. It is now available to the international scientific community without any restriction upon request.

Liyan Yu

China Phamaceutical Culture Collection
Institute of Medicinal Biotechnology
Chinese Academy of Medical Sciences
Peking Union Medical College
No. 1, Beijing Tiantan Xili , Dongcheng District
100050 Beijing P. R. China.
Tel: +86-10-63187118, FAX: +86-10-63187118

Microbe Div
3-1-1 Koyada

No. 15020
29 Jul 20

This is to c
of Microor
by the autl

Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
Inhoffenstraße 7B, 38124 Braunschweig, Germany

DSMZ

Confirmation of the availability of a strain for the purpose of valid publication of a new name according to the Bacteriological Code

The following information is confidential and serves only to allow the International Journal of Systematic and Evolutionary Microbiology to confirm that a strain has been deposited and will be available from the DSMZ in accordance with the Rules of the Bacteriological Code (1990 revision) as revised by the ICSP at the plenary sessions in Sydney and Paris.

Strain Nocardioides sp. NCCP-1340 has been deposited in the DSMZ under the number

DSM 29942

This strain is available in the publicly accessible section of the DSMZ and restrictions have not been placed on access to information concerning the presence of this strain in the DSMZ. It will be included in published and online catalogues after publication of this number by the authors.

This strain has been checked for viability in the DSMZ and is stored using one of the standard methods used in the DSMZ. The depositor of this strain has also carried out a "depositor's check" and confirmed the identity of the strain held under this DSM number.

!! The DSMZ is not responsible for differences between the properties of the strain deposited in the DSMZ and properties given in the literature/databases !!

It is the sole responsibility of the depositor to ensure that type strains deposited in the DSMZ conform to the requirements of the appropriate Rules governing prokaryotes nomenclature and the deposition of type strains (Rules 18a, 27, & 30 of the ICNB/ICNP, including changes made at plenary sessions of the JC/ICSP).

Dr. R. Pukall, Curator Gram-positive Bacteria

Moriya Ohku
Head of the
The RIKEN

*NOTICE: Tl
the Internati
registered o
not reissue*

Geschäftsführer/
Managing Director:
Prof. Dr. Jörg Overmann
Aufsichtsratsvorsitzender/Head of
Supervisory Board: MR Dr. Axel Kollatschny

Braunschweigische Landessparkasse
Kto.-Nr./Account: 2 039 220
BLZ/Bank Code: 250 500 00
IBAN DE22 2505 0000 0002 0392 20
SWIFT (BIC) NOLADE 2 H

Handelsregister/
Commercial Register:
Amtsgericht Braunschweig
HRB 2570
Steuer-Nr. 13/200/24030

JAPAN COLLECTION OF MICROORGANISMS

Microbe Division, RIKEN BioResource Center
3-1-1 Koyadai, Tsukuba, Ibaraki, 305-0074, Japan

Phone : +81 29 836 9556
Fax : +81 29 836 9561
E-mail : inquiry@jcm.riken.jp

No. 150203
28 Oct 2015

CERTIFICATE OF DEPOSITION AND AVAILABILITY OF A MICROORGANISM

This is to certify that the following microorganism has been deposited into the Japan Collection of Microorganisms (JCM) and will be available to the public after publication by the author(s) of the scientific paper that describes this taxon, subject to the agreement by the user(s) to the terms and conditions of use of the strain:

JCM 30630 *Nocardioides* sp.
<- W.-J. Li; Yunnan Inst. of Microbiol., Yunnan Univ., China; NCCP-1340

Terms and conditions:

- In publishing the research results to be obtained by use of the BIOLOGICAL RESOURCE, an acknowledgment to the DEPOSITOR is requested.

M Ohkuma

Moriya Ohkuma, Ph.D.
Head of the Microbe Division (JCM)
The RIKEN BioResource Center

NOTICE: *This document is valid only for the proposal of a new scientific name with the above-mentioned strain in the International Journal of Systematic and Evolutionary Microbiology (IJSEM). The scientific name is based on the registered one as of the issue date, and any later update of the name, does not invalidate this document. JCM does not reissue this certificate in principle, unless the MTA (for deposition) in connection with the strain is renewed.*

国立研究開発法人 理化学研究所 バイオリソースセンター 微生物材料開発室
〒305-0074 茨城県つくば市高野台3丁目1番地1