博士論文

Isolation and Identification of Novel Species of Heavy-metals Tolerant Bacteria from Pakistan for Their Potential Usage in Bioremediation and in Agriculture

（パキスタン由来の重金属耐性を示す新種細菌の
単離･同定及びそのバイオレメディエーションや
農業への応用の可能性についての研究）

By

SAIRA ABBAS
サイラ　アバス

DEPARTMENT OF APPLIED BIOLOGICAL CHEMISTRY
GRADUATE SCHOOL OF AGRICULTURE AND LIFE SCIENCES
THE UNIVERSITY OF TOKYO
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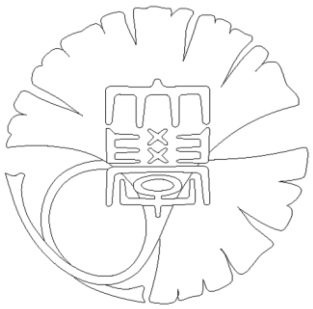

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サイラ　アバス

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By

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サイラ　アバス

A thesis submitted as a requirement
for the degree of

DOCTORATE OF PHILOSOPHY

IN

ENVIRONMENTAL MICROBIOLOGY AND BIOTECHNOLOGY

DEPARTMENT OF APPLIED BIOLOGICAL CHEMISTRY
GRADUATE SCHOOL OF AGRICULTURE AND LIFE SCIENCES
THE UNIVERSITY OF TOKYO
TOKYO-JAPAN
2016

Dedicated
to

My loving Mother

and

Father

Who always pray

To see the bud of

Their wishes

Bloom into a Flower

CERTIFICATE

"It is certified that the contents and form of the thesis entitled "***Isolation and Identification of Novel Species of Heavy-metals Tolerant Bacteria from Pakistan for Their Potential Usage in Bioremediation and in Agriculture***" submitted by **Ms. SAIRA ABBAS**, has been found satisfactory for the award of degree of the Doctorate of Philosophy under **JSPS Ronpaku Program**."

(Sign and Official seal)
The Dean,
Faculty of Agricultural and Life Sciences
The University of Tokyo
Tokyo, Japan

Committee:

CHAIRMAN:

Professor Dr. Toru Fujiwara
Research Supervisor from Japan
Laboratory of Plant Nutrition and Fertilizer
Department of Applied Biological Chemistry
The University of Tokyo, Japan

MEMBER:

Dr. Iftikhar Ahmed
Research Supervisor from Pakistan
Principal Scientific Officer / Director (IMCCP),
NARC, Islamabad, Pakistan

MEMBER:

Dr. Moriya Ohkuma
Head (Microbes Division), JCM,
Riken BioResource Centre, Tsukuba, Japan

MEMBER:

Professor Dr Masaharu Ishii
Laboratory of Applied Microbiology, Department of Biotechnology
The University of Tokyo, Japan

MEMBER:

Professor Dr. Keishi Senoo
Soil Science Laboratory
Department of Applied Biological Chemistry
The University of Tokyo, Japan

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(SAIRA ABBAS)

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List of Abbreviations

%	Percentage
µl	microliter
API	Analytical Profile Index
BLAST	Basic Local Alignment Search Tool
Cd	Cadmium
Cr	Chromium
Cu	Copper
DDBJ	DNA Data Bank of Japan
g/L	gram per liter
HCl	Hydrochloric acid
JCM	Japan Collection of Microorganisms
KCl	Potassium chloride
KCTC	Korean Collection for Type Cultures
m	meter
M	Molar
MEGA	Molecular Evolutionary Genetics Analysis
min	Minute
ml	Milliliter
Mn	Manganese
NaCl	Sodium chloride
NCCP	National Culture Collection of Pakistan
nm	Nanometer
nov	Novel
^{o}C	Degree Celsius
PCR	Polymerase chain reaction
pH	Power of hydrogen ion
rDNA	Ribosomal Deoxyribo Nucleic Acid
rRNA	Ribosomal Ribo Nucleic Acid
Sp.	Species
TE	Tris-EDTA ((Ethylenediamine Tetraacetic Acid)
TSA	Tryptic soya agar
TSB	Tryptic soya broth
w/v	Weight by Volume
Zn	Zink

CHAPTER I

EXECUTIVE SUMMARY

論文の内容の要旨

専攻　　応用生命化学専攻
氏名　　サイラ　アバス
指導教員名　藤原　徹

論文題目

Isolation and Identification of Novel Species of Heavy-metals Tolerant Bacteria from Pakistan for Their Potential Usage in Bioremediation and in Agriculture

（パキスタン由来の重金属耐性を示す新種細菌の単離･同定及びそのバイオレメディエーションや農業への応用の可能性についての研究）

Environmental degradation is a global phenomenon, but is significantly more deleterious in the developing countries, which house the largest population of human inhabitants within considerably smaller areas. With such a large populace, comes the demand of development in the agricultural sector and the need for economical growth and industrialization, which is often at the expense of the environment. Over the years, the rapid industrialization in the entire world introduced pollutants of heavy-metals in to the environment as a waste discharge or as a by-product of manufacturing process of human activity. Heavy metals are stable and persistent in the ecosystem because these cannot be degraded. Pakistan is also facing destructive environmental degradation as a result of heavy-metals pollutants.

Heavy metals are elements with high densities and belong to the transition group of the periodic table. Due to the low abundance in soil, these elements are called trace elements. In different ways, metals are involved in various aspects of metabolism, growth and differentiation. Metal resistant microorganisms may be helpful to other living organisms as they show the potential of bioremediation to toxic effects of heavy-metals pollutants and are also important in observing the causes, genetic transfer of microbial metal resistance and other processes.

This thesis reports isolation, identification and characterization of highly heavy metal tolerant bacteria, which may have potential usage in bioremediation of heavy-metals polluted soil/water system and in agriculture. Some strains isolated in these studies, to our knowledge, were the most tolerant strains against Cadmium (Cd) and Arsenic (As) compared to previous reports. Further, we report several novel species of bacteria that showed high tolerance against different metals. Some of the isolates demonstrated biosorption activity as well as plant growth promotion under heavy-metals contaminated environment.

More than 68 strains were isolated from the discharge of Industrial areas (Sialkot, Islamabad and Kasur). All bacterial strains were found to be highly tolerant against different heavy metals including Chromium (Cr), Copper (Cu), Cd, Lead (Pb), and As (Figure 1.1). Some strains tolerated concentration up to 3600 ppm for Cr, 3300 ppm for Cu, 3000 ppm for Cd, 2100 ppm for Pb and 3000 ppm for As. Among these highly tolerant isolates, maximum tolerance limit (MTL) was observed for NCCP-601, 602, 603, 647, 657, 660, 661, 621, 653 (against Cr 3600 ppm), NCCP-601, 602, 603 (against Cu 3300 ppm), NCCP-601, 602, 603 (against Cd 3000 ppm), and NCCP-644, 650 (against Pb 2100 ppm and As 3000 ppm). The isolates reported in these studies showed higher tolerance to Cu, Cd, Pb and As compared to previous reports.

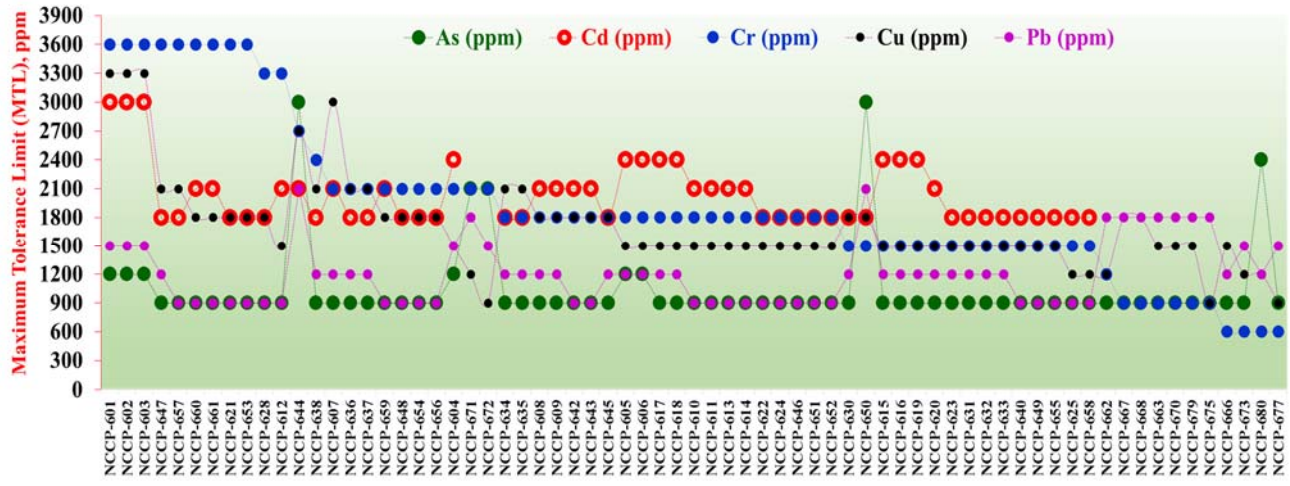

Figure 1.1. Maximum tolerance limit (MTL) of isolated bacterial strains against different heavy metals (Cr, Cu, Pb, Cd and As).

Those strains, which showed maximum tolerance, were further analyzed for biosorption ability. In this experiment, 23 strains (phylogenetically different) were grown on TSB containing 50 ppm of each heavy-metals (Cr, Cu, Cd, Pb, and As) separately up to OD_{600} >1.2 to stationary phase (48 hours of growth) to test for biosorption of respective metal. Maximum biosorption occurred for Pb followed by Cd and Cu, whereas biosorption of As and Cr was significantly lower by all the isolated strains. The isolates significantly differed in their biosorption capacity. Among these, at least four isolates, NCCP-614 (99 %), NCCP-605 (96 %), NCCP-655 (91 %) and NCCP-624 (88 %) showed maximum biosorption of Pb. Similarly, strains NCCP-614 also showed maximum biosorption capacity of 89 % for Cd.; however, maximum biosorption of copper was done by NCCP-625 (42 %) followed by NCCP-614 (38 %) and NCCP-647 (36 %). Our results indicated that these isolates can be used for bioremediation of soil/water system contaminated with heavy-metals (Pb, Cd and Cu).

Diverse bacterial community tolerant to heavy-metals was observed during this study. Identification based on comparative 16S rRNA gene sequence data demonstrated that these heavy metal tolerant strains belonged to nineteen different genera (Figure 1.2). The majority of the isolates were related to the genera *Bacillus*, *Pseudomonas* and *Staphylococcus.* Sequence similarity analysis showed that several isolates belonging to the genera, *Bacillus*, *Alcaligenes, Acinetobacter, Pseudomonas, Citrobacter* and *Bravibacterium,* had 97.9 to 100 % similarity of 16S rRNA gene sequence and thus, some of these strains were characterized taxonomically to delineate as novel species. However, other strains had the highest 16S rRNA gene sequence

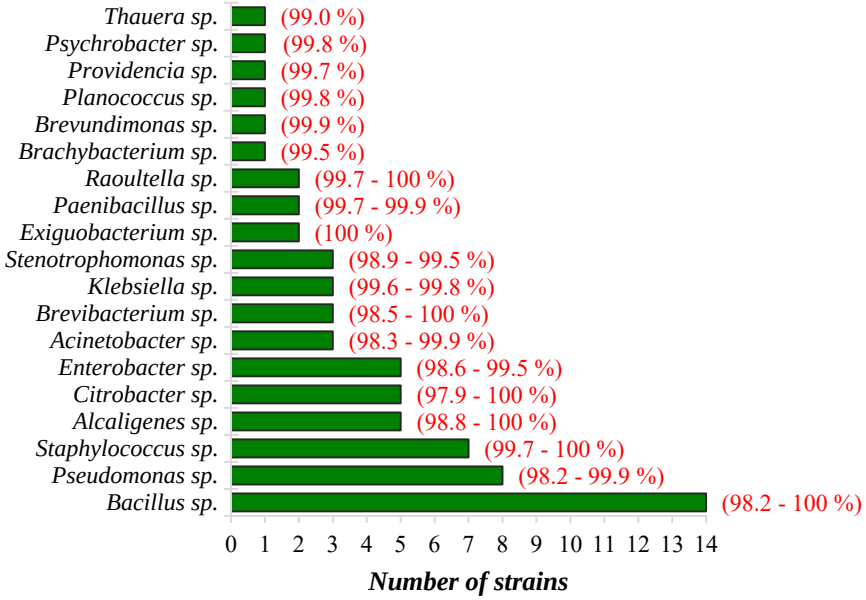

Figure 1.2. Biodiversity of isolated heavy-metals tolerant strains. The values in front of each bar show the percent similarity range of the strains with the known closely related species of the respective genera.

similarity (> 99%) with the closely related taxa in their respective clusters and therefore, were not included in the taxonomic characterization studies.

Based upon phylogenetic analyses, DNA-DNA homology, phenotypic and chemotaxonomic data, the isolated strains that belonged to the genera; *Acinetobacter, Alcaligenes* and *Bacillus* were characterized as novel species: *Acinetobacter pakistanensis* sp. nov. (Abbas et al. 2014), *Alcaligenes pakistanensis* sp. nov. (Abbas et al. 2015a), and *Bacillus malikii* sp. nov. (Abbas et al. 2015b). The detailed taxonomic characterization studies of these novel species are described in Chapters 5-7, respectively. Identification of these heavy-metals tolerant strains as new species will provide enormous information to understand the biochemistry of heavy-metals in living cells. These novel taxa would also be a source of new genes involved in metals transport / tolerance mechanisms.

To test the potential usage of the heavy-metals tolerant isolates in agriculture, 23 phylogenetically different bacterial strains were also analysed for screening of *nifH* and *acdS* gene(s). The strains with these genes are well established bioinoculants (biofertilizer) for PGPR activity and can be used in heavy-metals contaminated soils. Summary of the results of this study (Figure 1.3) showed that at least 13 isolates contained *nifH* gene in their genome, whereas at least 8 strains showed *acdS* gene. Some strains (NCCP-650, NCCP-611, NCCP-660, NCCP-635, NCCP-622, NCCP-614 and NCCP-605) were found to have both genes. These heavy-metals tolerant strains may have the potential for plant growth promotion and can be used as bioinoculants (biofertilizer) in agriculture. It is also worth mentioning here that strain NCCP-650, which contained both the genes, has been delineated as a novel species: *Alcaligenes pakistanensis* sp. nov. This strain also contained nitrite reductase, *nirK* gene, which has also its significance in bioremediation process.

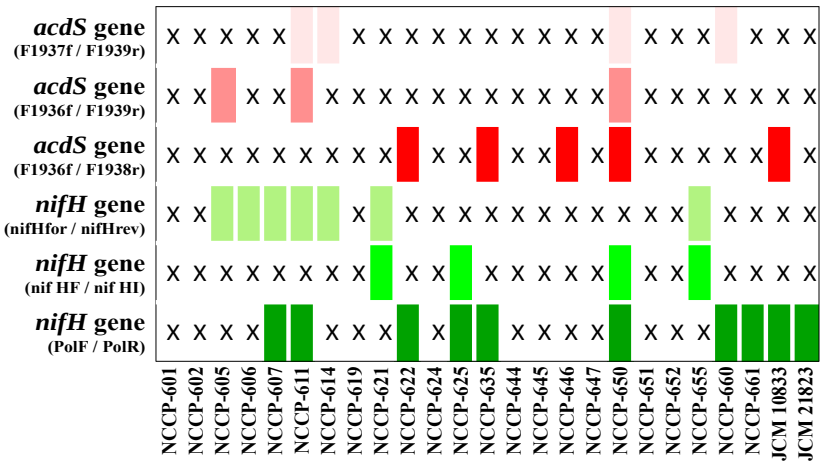

Figure 1.3. Summary of the amplification of *nifH* and *acdS* genes associated bands using different sets of primers.

Three bacterial strains (NCCP-650^T, 644^T and 602), which were candidate promising novel species, were further investigated for growth promotion activity for *Brassica napus* under axenic condition when irrigated with water containing 50 ppm of each metal separately. Strain NCCP-614 was also included in this study as it was efficient for biosorption of Pb, Cd and Cu. Strain JCM 10833 was used as positive control. Two of these strains (NCCP-650^T and 614) showed the presence of both *nifH* and *acdS* genes, whereas these genes were absent in NCCP-602 and 644^T. The results of this experiment showed (Figure 1.4) that all the strains tested in this study, played significant role in growth promotion of *Brassica* plants compared to control (no addition of strain or metal), when irrigated with water containing Pb or Cd. However application of water containing Cr or As salts significantly reduced plant growth. Among the strains, NCCP-650^T proved to be the best for increase in growth of *Brassica* plants under Pb, Cd and Cr stress, whereas NCCP-644 and NCCP-602 performed better under Cu and As stress, respectively. JCM 10833 did not improve growth of *Brassica* plants as a positive control under heavy-metals stress condition, probably due to the reason that this strain was sensitive to heavy-metals stresses. Strain NCCP-650^T belongs to a new species, *Alcaligenes pakistanensis*, and many strains of this genus are also reported as PGPR strains.

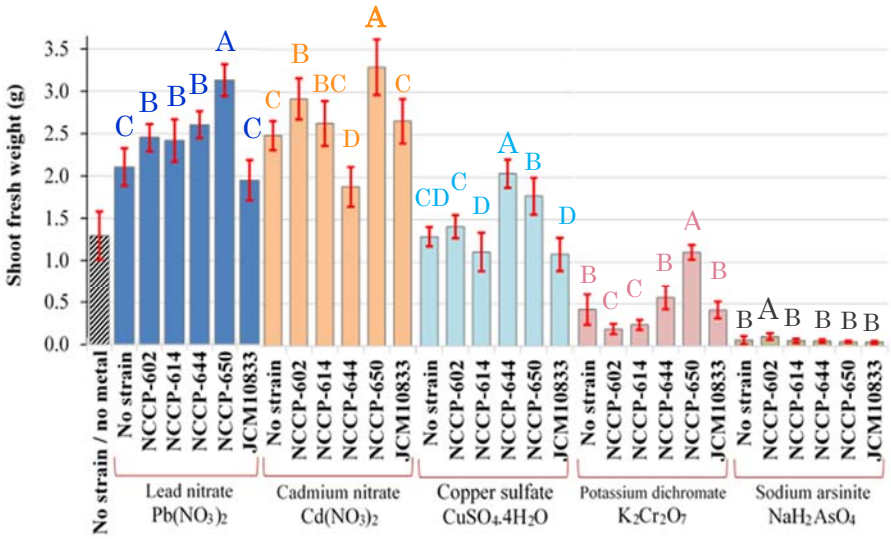

Figure 1.4. Effect of selected heavy-metals tolerant PGPR strains on growth promotion of *Brassica napus* irrigated with heavy-metal (50-ppm) containing water. Bars of same color under each metal stress, carrying the same alphabet letters are statistically non-significant (p 0.05)

Finally, the findings of heavy metal tolerance and essentiality for the novel species of heavy-metals tolerant bacteria provide a genetic resource to identify the gene(s) responsible for the mechanism of heavy metal tolerance in bacteria. The potential of these strains for bioremediation and their PGPR activity under stress condition would be important for agriculture.

CHAPTER II

INTRODUCTION

The primary function of ecosystem is to sustain life on earth. It provides vital needs as ecological services for all the living things located within that particular system. Another important aspect of ecosystem is its ability to clean, recycle and removal of pollutants and contaminants, existing in the water, soil or air. It is prominent that ecosystem should not be disturbed, or interfered, especially through human activity. If such activity is crucial then equilibrium has to be maintained within the ecosystem to sustain itself. Due to the active spread and development of industries, heavy metals, which are either used or produced as byproducts by several manufacturing, industrial, refining and mining processes, have become global persistent environmental pollutants. However, the demands of a rising human population in many regions, and its associated demand for development, industrialization and other related activities, most of the ecosystem are increasingly coming under threat and exploited by pollution, degradation and destruction.

In Pakistan, industrialization started in the 1950s but the rapid developmental process regarding factories and manufacturing process were not environmentally friendly. The wastes generated by the industries are directly discharge in to the air, water, and adjacent land without any prior treatment or detoxification. It is impossible to run the Pakistan towards development and independence, without process to confirm the necessary protection mechanism.

The term “heavy metals” is commonly used for those elements having high densities and belongs to the transition group of the periodic table. Due to the low abundance in soil these elements are called trace elements (De Vries et al. 2002). Heavy metals can be present in soils as free cations, as complexes (e.g. $CdCl^{-3}$, $ZnCl^{+2}$) with organic and inorganic ligands, and linked with soil colloids (Wang et al. 2010), they can also accumulate in biological systems finding their way into the food web via different mechanisms (Giller et al. 1998). Metals at low concentration are important components in all life processes, but high concentration of heavy metals are toxic to living system. Cadmium (Cd), lead (Pb), chromium (Cr), arsenic (As) and nickel (Ni) are known to be the most common heavy metals contaminating the environment worldwide.

In different ways, metal are involve in various aspects of metabolism, growth and differentiation (Beveridge and Doyle 1989). Metal resistant microorganisms may be helpful to other living organisms as they show the potential toxicity (Jansen et al. 1994) and also important in observing the causes, genetic transfer of microbial metal resistance and other process (De Rore et al. 1994). Industrial wastewaters, and mining process are main sources for heavy metal pollution, as they are responsible for removing of contaminated metals such as zinc (Zn), mercury (Hg), Cr, Cd, copper (Cu) and Pb in to the environment (Malik 2004; Soares et al. 2003). Due to the anthropogenic and natural processes heavy metal are gradually increasing in microbial habitats, consequently microbes have develop system to tolerate the presence of heavy metals by numerous process e.g. complexation, efflux, or removal of metal ions either by terminal electron acceptors in anaerobic respiration (White et al. 1995).

Some metals in the environment are called as bioavailable (soluble, non-soluble and mobile) and other are non-bioavailable (non-mobile, complexed, sorbed and precipitated). The bioavailable metal which is taken up by the environment are present in high concentration and toxic to biological systems (Roane and Kellogg 1996). Toxicity to an organism can be defined as the ability of a material or its natural potential to cause adverse effect in living systems and its effect depends upon the bioavailability of the toxic material (Rasmussen et al. 2000).

A number of health disorders have been reported due to toxicity of the heavy metals, for example contact dermatitis, periodontitis and stomatitis, pneumoconiosis, male infertility, mild renal dysfunction and the most prominent is resistance to antibiotics (Bruce and Hall 1995; Selden et al. 1995). Metals and metal-containing compounds continually influence to the human body. Trace elements are essential for normal functioning while some metals causes the toxic effects. There are some important factors prevailing the destructiveness of metals such as genetic susceptibility, duration of exposure and total concentration. The heavy metals show its toxicity, when administrated on a chronic basis (Shitova et al. 1992).

Heavy metals are non-degradable and thus persistent in the environment and acts as widespread pollutants. The heavy metals have potential applications in numerous expensive industries and later discharged into atmosphere as effluents. The presence of heavy metals in atmosphere and its surroundings can later microbial communities and disturb their activities (Zouboulis et al. 2004). Treatment with living organisms has been well-known method for dealing with a broad range of pollutants existing in the environment. Bioremediation is one of the environment friendly and inexpensive method than other physical-chemical methods.

It is important for the removal or conversion of hazardous compound in to harmless products. Through this technique appropriate microbes go through with number of physical and chemical reaction in the contaminated water, after the microbial metabolism the contaminated material are degraded and removed.

Bioremediation by microbes has now developed as an exceptional technique to such conventional chemical treatments (Brierley 1990). In all over the world, heavy metal pollution is a common environmental problem establishing a main risk for ecosystems and human health with expensive cleanup costs. In agriculture and industries, the input of heavy metals has led to the discharge and inadequate removal of massive amounts of heavy metals (Ansari and Malik 2007). Microbes interacts with small quantities of metals as metalloid distribution in the environment; on the other hand, the interaction with larger quantities are required for energy metabolism and leaving great impact on environment (Ehrlich 1997). However, due to strong ionic nature metals binds to many cellular ligands and relocate essential metals from their normal binding sites and rending the toxic. Many *Eubacteria* and *Archaea* are able to oxidize Fe (II), Mn (II), Co (III), AsO_2, Se or reduce Mn (IV), Fe (III) Co (II) AsO_2, SeO_3 on a large scale and conserve energy in these reactions (Xian 1989).

Certain microorganisms having 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase activity or those having potential for nitrogen fixation play very important role in agriculture. ACC deaminase is a multimeric enzyme with a molecular mass of about 35–42 kDa (Glick 2005), which has ability to break the part of ACC including the cyclopropane ring, in turns it form ammonia and α-ketobutyrate. Under stressed environment such as with heavy-metals contamination, bacteria having ACC deaminase can significantly promote the plant growth by regulating ethylene (Bhattacharyya and Jha 2012). Nitrogen fixation is the most important feature existing in the life forms of living organisms and is mostly administrated by nitrogen fixation (*nif*) genes harboring in microbes. The expression of *nif* genes is strictly monitored by environmental factors like ammonia and oxygen levels. These genes have specify α and β subunits mainly from molybdenum iron protein and later govern by different enzymes like dinitrogenase and dinitrogenase reductase and further encoded by *nifH* genes (Rubio and Ludden 2008). In many microorganisms, like *Azospirillum brasilense*, *Herbasipirillum seropedicae, Gluconacetobacter diazotrophicus* and *Klebsiella pneumoniae* there is only one *nifH* gene and the *nifH, D, K* genes are recorded as a single unit. However, some diazotrophs have multiple *nifH* genes. It is stated that *Clostridium pasteurianum* has six

nifH (*nifH1*) and *nifH*-like (*nifH2*, *nifH3*, *nifH4*, *nifH5* and *nifH6*) (Chen 2005; Johnson et al. 1993).

Biosorption is a process that uses cheap biomaterials to sequester metals from various aqueous solutions and the biomaterials used in this process are termed as biosorbents (Fomina and Gadd 2014). Biosorption process have been used to sequester precious, heavy, toxic and radioactive metal ions such as Ag, Au, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Pd, Pt, U, Th and Zn (Fomina and Gadd 2014). Biosorbents can be sourced from waste biomaterials generated from agriculture, food and pharmaceutical industries; this makes biosorption an economically alternative treatment method. Biomaterials containing acidic groups such as carboxyl, hydroxyl, and weak basic groups such as amides/amines are efficient in adsorbing metal ions (Volesky 1999). Heavy metals accumulation in the environmental systems is a serious concern for human and animal health. In soil system, heavy metals have deleterious effect on microbial communities at microscopic level, which in turns disturb the nutrient cycles in the soil environment. If the soil environment is disturbed due to irregularities in nutrient cycles, the overall long-term soil fertility is lost and sometime seriously threatened. The development of modern techniques in molecular biology can greatly improve soil and environmental systems that are threatened due to heavy metals stress.

In Pakistan, there is insufficient reported work of isolation of heavy metal tolerant bacteria having importance in agriculture, however, no work have done on the identification of these bacteria specially based upon the 16Sr RNA gene sequencing. Keeping in view, there is a need of work in this direction related with the identification and non-reported heavy metal tolerant bacteria that will be useful for bioremediation; thus, experiments were conduct to pursue the following objectives:

- To isolate, identify and characterizing the promising novel species of heavy-metal tolerant bacteria from Pakistani ecology using poly-phasic taxonomic approach.

- To study biosorption / bioremediation potential of isolated strains and their potential usage in agriculture under heavy-metals contaminated environments

CHAPTER III

REVIEW OF LITERATURE

3.1. Heavy metals in soils

Heavy metals represent environmental concerns, because of their widespread use and distribution, particularly their toxicity to human beings and the biosphere. However, these also include some elements that are vital for living organisms at relatively low concentrations (Alloway 1990). These elements are usually transition metals, which have high densities (>5 g cm^{-3}) when compared with other materials (Baird and Cann 2005) (Table 2.1). Heavy metals also include essential elements such as Iron (Fe) and Zinc (Zn) as well as toxic metals like lead (Pb), cadmium (Cd), and Mercury (Hg).

Table 3.1. Densities of some heavy metals and other substances (adapted from Baird and Cann (2005).

Substance	Density (g cm^{-3})
Mercury (Hg)	13.5
Lead (Pb)	11.3
Zinc (Zn)	9.16
Cadmium (Cd)	8.7
Arsenic (As)	5.8
Aluminium (Al)	2.7
Magnesium (Mg)	1.7
Water (H_2O)	1.0

Soil is an important resource that produces food and other raw materials for humans. However, soil is often a sink for wastes, including heavy metals (Park et al. 2011), from a variety of human activities (Han et al. 2001). In a risk based approach, soil can be regarded as contaminated if the bioavailable concentrations of heavy metals is high enough to cause harm directly or indirectly to humans, plants, animals, water quality, the wider ecosystem, buildings and or construction materials (DEFRA 2002; USEPA 2001). Some heavy metals are essential for living organisms at lower concentrations e.g. Zn, Mn, Cu, Co, and Cr and are also known as trace elements or micronutrients (Park et al. 2011; Vodyanitskii 2013).

The term toxic heavy metal includes those elements that are non-essential such as Pb, Cd, Hg, Ba, Ti, Sb, U and As (DEFRA 2002; Park et al. 2011). Heavy metals are emitted into the soil environment through anthropogenic and geogenic processes (El-Nahry and Doluschitz 2010). In nature, heavy metals commonly occur in soil parent materials (Dhillon and Dhillon 1991; Park et al. 2011) and the main natural source of heavy metals in soil environment is the weathering of parent material (Park et al. 2011; Tripathi et al. 2007). In this Chapter, a brief overview of heavy-metals, their interaction w.r.t. microorganisms and bioremediation/biosorption processes is presented.

3.1.1. Chromium

Chromium (Cr) is not essential element for plants (Taiz and Zeiger 1998). Chromium has economic importance in industrial use but also as major metal pollutant of the environment. Chromium compounds are used in industries for textile dyeing, wood preservation, pigmentation, chrome plating, tanning and manufacturing pulp and paper. The tanning industries specially produces large amount of Cr as a pollutant (Sharma and Adholeya 2011). The organic Cr from tanning industries discharged as sludge and effluent directly into the environment and imposing great environmental concern and human health as pollutant. The Cr salt is used to convert animals hide to leather and the waste water in directly discharged into the environment (Sharma and Adholeya 2011). Hexavalent Cr salts do not directly precipitate in the environment and bound to the different components of soil (Losi et al. 1994) and leached to the underground drinking sources and become hazard to animals and humans.

3.1.2. Cadmium

Cadmium (Cd) is a member of group II-B of the periodic table and is a relatively rare metal (Alloway 1990) which makes it uncommon in most “natural” soils and waters (Evangelou 1998). The average content of Cd in soil is less than 1 ppm, with the normal range 0.005-0.02 ppm in plants (Mulligan et al. 2001). It is very similar to zinc, undergoing joint geochemical processes, and its oxidation state is (like zinc) +2. Cadmium is rendered as a by-product of the mining and smelting of lead and zinc (Alloway 1990; Baird and Cann 2005; Evangelou 1998). The production of this metal has increased rapidly in the last few decades from 11000 t in 1960 to 19000 t in 1985 (Alloway 1990). This heavy metal is used in semiconductors, nickel-cadmium batteries, electroplating, PVC manufacturing, various alloys, pigments and control rods for nuclear reactors (Alloway 1990; Evangelou 1998). Soil and water contamination by Cd originates from the mining and smelting industries, atmospheric pollution, sewage sludge application and burning of fossil fuels (Alloway 1990; Evangelou 1998). Cadmium has no essential biological function and

is thus highly toxic to living organisms. Chronic exposure to cadmium in humans has several toxic effects, such as high blood pressure, kidney, lung, liver and testes damage (Alloway 1990; Baird and Cann 2005; Evangelou 1998). Cadmium is also associated with a disease called *Itai-Itai*, meaning "it hurts" in Japanese (Alloway 1990; Evangelou 1998).

3.1.3. Copper

Copper (Cu) is an essential plant micronutrient that is required for a number of enzymatic activities, particularly in nitrogen metabolism (Taiz and Zeiger 1998). Copper toxicity is due to the alteration of plasma membrane of cells, leading to leakage of potassium and other solutes. Due to similarities in valency and size between Cu, Mn and Fe, copper interferes with the accumulation of these other metals by competing for binding sites, thus resulting in deficiencies of these essential elements (Ebbs and Kochian 1997). Copper also effects the photosynthesis capacity of plants by replacing Fe in the phytosystem II reaction center. Energy transformation efficiency is thereby decreased, since Cu does not transfer energy as effectively as Fe (Ouzounidou and Ilias 2005). This interference at the photosystem II reaction center leads to reduced CO_2 assimilation and consequently reduction in plant transpiration rate, which in turn results in a lower growth potential (Ouzounidou and Ilias 2005).

3.1.4. Lead

Lead (Pb) induces a broad range of toxic effect to animals and living organisms. Lead toxicity impairs root elongation, seedling development, transpiration rate, seed germination, cell division, chlorophyll production, chloroplast development and overall plant growth (Gupta et al. 2010; Gupta et al. 2009; Krzesłowska et al. 2009; Maestri et al. 2010; Sharma and Dubey 2005). The severity of these effects depend upon Pb concentration, the stage of plant development, degree of exposure, intensity of plant stress and target plant organs studied. Plants use different mechanisms to detoxify metals including excretion, metal uptake, selective uptake, compartmentalization, and complex binding with different ligands (Gupta et al. 2010; Krzesłowska et al. 2009; Maestri et al. 2010). The response of plants to Pb exposure are often used as tool to access the environmental quality assessment. The examples includes growth of legumes on Pb contaminated soils (Alexander et al. 2006). *Brassica napus* (Zaier et al. 2010) and *Pelargonium* (Arshad et al. 2008) are considered as Pb hyper-accumulators, and have ability to extract large amount of Pb from contaminated soil without showing any morphological toxicity symptoms.

3.1.5. Arsenic

Arsenic (As) has a crustal average of 1.5-2 ppm (Shenker et al. 2001). The background

concentration range of As in soil is 1-40 mg/kg (Schallenberg et al. 2012) with an average As concentrations in soils of approximately 5 ppm (Shenker et al. 2001). In natural soils, high As concentrations are often related to sulfide deposits and their weathering to produce local mineralized forms (Shenker et al. 2001). Anthropogenic sources of As contamination in soil include sulfide mining, fossil fuels combustion, smelting, use of As containing pesticides and copper chrome arsenate used as a wood processing (Rahman et al. 2006; Shenker et al. 2001). In addition, groundwater has been found to be contaminated with As in more than 20 countries including the United States of America, China, India and Bangladesh (Rahman et al. 2006) and using this water to irrigate soils can create As contamination issues such as leaving risks of soil accumulation of the toxic element and possible exposure of As contamination to the food chain through plant uptake and animal consumption (Huq and Naidu 2004).

Arsenic is not essential for plants and appears not to be involved in specific metabolic reactions when supplied at low concentrations (Zhao et al. 2009). Naturally, As may be present in four oxidation states -3, 0, +3 and +5 (Hughes et al. 2011). The major forms of As in the soils are arsenate (As(V)) and arsenite (As(III)) (Shenker et al. 2001) with the latter having greater toxicity to most species including plants (Finnegan and Chen 2012). The symptoms of As toxicity in plants frequently include poor seed germination and reductions in root growth (Garg and Singla 2011). These effects may relate to rapid disruption of plasma membrane structure, including fluidization (Smith et al. 2010). At higher concentrations, As has been reported to interfere with metabolic processes and sometimes lead to plant death (Tripathi et al. 2007). Where plants survive to high As exposure, they may show retarded growth, severe chlorosis and nutrient deficiencies (Mascher et al. 2002), together with reduced photosynthetic oxygen evolution. Critical concentrations of As in shoot tissue range from approximately 21 to 325 $\mu g\ g^{-1}$ depending on the species and cultivar (Shaibur et al. 2008).

3.2. Bioavailability of heavy metals in soils

The bioavailable fraction of a heavy metal is defined as that proportion of the total pool of heavy metals in a soil which is extractable in a chemical regent and can potentially be absorbed by plants (Chojnacka et al. 2005; Fairbrother et al. 2007). Heavy metals generally have low solubility and so are mainly in forms that are unavailable to plant uptake (Violante et al. 2010). Thus, in natural undisturbed environments, heavy metals are rarely present in high enough bioavailable concentrations to cause significant toxicity to plants.

Heavy metal bioavailability in soil is related to the solubility of contaminant in that soil (Degryse et al. 2009; Hooda 2007). However, there is debate as to which fraction of heavy

metals in soil corresponds to the bioavailable pool (Hooda 2007). Bioavailability has been associated with heavy metal ion activity in the soil solution and the exchangeable heavy metal fraction (Hooda 2007; Violante et al. 2010). Nevertheless, there is yet no general consensus among researchers on how to measure bioavailability of heavy metals in soil. Heavy metal bioavailability, rather than total concentration, is important when accurately assessing the risk associated with soil contamination (Garg and Singla 2011; McLaughlin et al. 2000; Nolan et al. 2005; Oliver et al. 2004). Heavy metals that are present in soils occur in several fractions such as the soil solution, exchangeable, organically and colloidally bound, residual and within primary phase of minerals (McBride et al. 1997). Soil organisms and plants are not able to access the entire heavy metal pool in the soil. Among these heavy metal fractions, the most available heavy metals and potentially phytotoxic are present in soil solution and absorbed to inorganic soil constituents at ion exchange sites, with the other heavy metal fractions being not/less available for plant uptake (Peijnenburg and Jager 2003).

3.2.1. Microbial interactions

Rhizobia are bacteria that form symbiotic associations with legumes and are responsible for nitrogen fixation from the atmosphere into forms accessible by plant roots e.g. NH^{4+} ions (Sessitsch et al. 2002). Consequently, legumes absorb more cations than anions and, thus, acidify the surrounding rhizosphere (Gregory 2006). Nitrogen assimilation by rhizobia results in changes of pH and increases heavy metal solubility and bioavailability where the symbionts exist (Kopittke et al. 2007). Research was carried out in the rhizosphere of Ni accumulating plants and plants grown on serpentine soils (El-Aziz et al. 1991; Ma et al. 2009). It is reported that the rhizosphere bacteria increased plant availability of Ni by increasing Ni accumulation via the production of siderophores. The siderophores induced the dissolution of minerals carrying Ni and thus promoted shoot and root biomass indirectly (Ma et al. 2009; Wenzel et al. 2003).

Among other microbes in the rhizosphere are mycorrhiza which are mutualistic associations between certain soil fungi and the roots of most plant species (Bundrett et al. 1996). It was found that mycorrhiza assist in nutrient uptake at lower concentrations of metals (Arines et al. 1989). Also mycorrhiza are able to reduce metal uptake and in some cases increase plant metal tolerance under conditions of metal contamination (Weissenhorn et al. 1995). For instance, mycorrhizal *Trifolium pratense* (red clover) plants grown in acid soils with high Mn bioavailability had less Mn in the roots and the shoots than non-mycorrhizal plants (Arines et al. 1989). Also mycorrhizae are able to make plants more tolerant to metals when grown in soils containing high heavy metals concentrations (such as Zn, Cu, Mn, Ni,

Cr) (Denny and Ridge 1995; Leyval et al. 1997).

3.2.2. Heavy metal uptake and transport in plant and soil

Heavy metals are absorbed passively by plant roots via ion channels (Weis and Weis 2004). The mechanism for uptake is largely defined by the electrochemical gradients that apply to the transport of a specific nutrient (Reid 2001). A number of selective transport pathways for heavy metals into plants are now being discovered. For instance, iron bound to phytosiderophores can be transported by Yellow stripe1 (YS1) across the plasma membrane (Roberts et al. 2004). Yellow stripe1 gene synthesizes Fe(III)-phytosiderophore (Fe-III-PS) transporter in maize (*Zea mays*). Non-selective channels transport Ca^{2+} across the root membrane and it is now well understood that the uptake of other metals at normal soil solution concentrations occur via this pathway (Reid 2001). Competition between cations at the surface of the root shows that non-selective channels facilitate the absorption of essential and non-essential metals (Reid and Hayes 2003). As a result, under conditions of elevated heavy metals, competition for the transport sites leads to favoring the absorption of heavy metals over macronutrients, thus inducing or exacerbating deficiencies of nutrient cations (Palmer and Guerinot 2009; Reid and Hayes 2003).

Plants that have Fe deficiency are sometimes found to have higher Zn and Mn concentrations as well (Conte and Walker 2011). In Arabidopsis, root membrane protein IRT1, a general cation transporter, enhances Zn and Mn uptake (Vert et al. 2002). A number of transporters are responsible for Fe uptake from soil. This involves transporting from the roots to shoots, transporting to generative parts of plant, xylem unloading, mobilization when seed germination occurs and loading- unloading of Fe from vacuoles. The Fe transporters ZmYS1 and OsYSL15 are able to move additional metals other than Fe such as Cu, Zn and Ni (Conte and Walker 2011).

3.3. Bioremediation

Bioremediation, or biological remediation, is an eco-friendly and cost effective technology that involves the use of organisms such as plants and/or bacteria to remediate and stabilize contaminated sites (Anyasi and Atagana 2013; Sharma 2012). The technology involves biological agents such as plants and microorganisms to transform or degrade pollutants into less hazardous or nonhazardous substances (Park et al. 2011). Various organisms like fungi, algae, bacteria and plants have been reported to efficiently bioremediate

contaminants (Vidali 2001). The bioremediation technology offers a substitute pathway to more traditional techniques for the remediation of contaminated sites.

Bioremediation uses natural processes and relies upon organisms to change pollutants and environmental conditions to changes life functions to more adoptable manner (Zhuang et al. 2007). Their metabolic processes are proficient of using chemical pollutants as an efficient energy source, representing the contaminants to a harmless form by limiting their bioavailability or producing less toxic finished products (Zhuang et al. 2007). Bioremediation is an efficient way to degrade or mitigate hydrocarbons, organic compounds and solvents, herbicides, heavy metals, nitrogenous compounds and pesticides (Park et al. 2011).

Microorganisms used to perform the function of bioremediation are known as bioremediators. These bioremediators are grouped into two broad categories: aerobic and anaerobic. Aerobic microorganisms work in presence of oxygen and can degrade pesticides and hydrocarbons with many of these microbes use the pollutant as the source of energy (Sharma, 2012). Anaerobic microorganisms work in absence of oxygen and are less frequently used in comparison to aerobic ones (Sharma 2012).

3.3.1. *In-situ* bioremediation

Bioremediation involves two different strategies: *in-situ* and *ex-situ*. *In-situ* bioremediation is process of remediation without excavation of contaminated land (Khan et al. 2000). Often, it is applied to the breakdown of contaminants in saturated soils. It uses beneficial micro-organisms to degrade the chemicals in the contaminated environment and cost less than conventional remediation technologies (Kumar et al. 2011). *In-situ* bioremediation includes techniques like bioventing, biosparging and bioaugmentation (Sharma 2012).

Bioventing involves using a low flow of air to provide adequate oxygen for sustaining microbial activity (Lee et al. 2006). Bioventing is typically used to treat contaminants that are biodegradable under aerobic conditions. Bioventing accelerates natural processes as it provides a low flow of air, which augments the growth of microorganisms naturally present in soil (LEE and Swindoll 1993). Biosparging is injecting oxygen under pressure in to the saturated zone to transfer volatile (unstable) compounds to the unsaturated zone for biological breakdown by naturally occurring microorganisms (Sharma 2012). Biosparging is relatively cheap, easy to install and quickly distributes oxygen across the site to maximize microbial functioning (Muehlberger et al. 1997). Bioaugmentation involves naturally occurring microbial strains or genetically engineered variants to treat contaminated soil (Niu et al. 2009). Maintenance of this system is difficult as it requires monitoring to ensure the complete

degradation of the contaminants (Sharma 2012). Also optimizing the efficiency of the microorganisms in an uncontrolled external environment is difficult to achieve and assess (Bouwer and Zehnder 1993).

3.3.2. *Ex-situ* Bioremediation

Ex-situ bioremediation involves removing contaminated soils from the ground for treatment that can occur in another location either on or off the site (Khan et al. 2000). It is often considered to be less advantageous than *in-situ* remediation because the contamination is moved elsewhere and has the possibility to create significant risks in the excavation and transport of harmful material (Kumar et al. 2011). *Ex-situ* bioremediation includes techniques such as composting, land farming and biopiling (Kumar et al. 2011; Sharma 2012).

Composting is a controlled process by which organic materials are degraded by microorganisms under elevated temperature, resulting in the production of organic and/or inorganic by-products (Namkoong et al. 2002). Optimized compost temperatures are in between 55° to 65° C (Antizar-Ladislao et al. 2007). The volume of material often increases due to the addition of amendment agents after composting, which is a limitation of this technology (Sharma 2012). Land farming is a technique, where contaminated soil is taken and spread in a thin layer over a ground surface until the contaminants are degraded by aerobic microorganisms (Harmsen et al. 2007). Microorganisms are frequently added to the soil to achieve rapid degradation rate and mixing of soil is done at rapid rate in order to increase the contact area between the contaminants and microorganisms (Khan et al. 2004). Large areas of land are required for land farming, which is a limitation to the suitability of this technology (Khan et al. 2004). Biopiling is a technology, where excavated soils are piled and get mixed with microorganisms by using applied aeration. The piles should be covered to prevent overflow, evaporation and to advance solar heating (Filler et al. 2001). The contaminants are often condensed to carbon dioxide and water (Wu and Crapper 2009). Biopiling is similar to land farming but in the latter the soil is aerated artificially.

3.3.3. Phytoremediation

Phytoremediation is an *in-situ* biomediation process that uses green plants and the microorganisms that are associated with them to extract, sequester, or detoxify pollutants. Plants have the capacity to accumulate, take up, eliminate or degrade solvents, crude oil, heavy metals and other harmful pollutants. Phytoremediation is a clean, cost-effective, environment-friendly technology, especially for treating large and diffused areas that are contaminated. There are many successful examples where phytoremediation has been

employed, and where it has been documented to work well for remediating contaminated industrial environments (Suárez et al. 2008). Depending on the method used and nature of the contaminant involved, phytoremediating areas where metals and other inorganic compounds exist, may utilize one of several techniques (Neumann et al. 1998) as follows:

(a) Phytoextraction: also known as phytoaccumulation, removes metals by taking ability of some plants to (hyper-)absorb and accumulate or translocate metals or/metalloids, by concentrating them within the biomass. The main usage of this type of remediation is to reduce the concentration of metals in contaminated soils so that they have better results in agriculture, horticulture, forestry and many more (Kumar et al. 1995).

(b) Phytoimmobilization, utilizes plants, mostly in combination with other soil additives for reducing contaminant transfer to other ecosystem components and also the food web and food chain. The "stabilized" inorganic or organic compound is normally incorporated into soil humus or into plant lignins. The main purpose for phytostabilization is not to degrade the metal but to stabilize them *in-situ*. This approach is particularly applicable when low-concentration, diffused, and vast areas of contamination are to be treated (Sharma 2012).

(c) Phytostimulation: plant roots promote the development of rhizospheric microorganisms that are proficient of degrading the contaminant, and the microbes utilize plant root exudates as a carbon source (Sharma 2012).

(d) Rhizovolatilization: employs metabolic capabilities of plants and the associated rhizosphere microorganisms to transform pollutants into instable (volatile) compounds that are released into the atmosphere with less harm. New plant growth promoting rhizobacteria (PGPR) strains, and heavy metals are absorbed by roots, are converted into less toxic forms before releasing into the atmosphere.

(e) Phytodegradation: In this technique plant use specific enzymes to degrade or mineralized different organic contaminants.

(f) Rhizofiltration: use terrestrial plants to precipitate, concentrate, and/or absorb pollutants in the aqueous system. Rhizofiltration is also used to partially treat industrial and agricultural runoff. Plants that can potentially accumulate large quantities of metals by natural methods have been identified, and are being studied for their use to remediate heavy metal contaminants (Dushenkov et al. 1995). These plants are called hyper-accumulators, and are found in areas having elevated metal concentrations in soil. These plants are relative slow growing and attains only small size. Depending upon the amount of metal at a particular site and the type of soil, even hyper-accumulating plants may

require 15–20 years to remediate a contaminated site. This time frame is usually too slow for practical application. Therefore, research undertaken to find such plants should emphasize species that are fast growing and accumulate greater amounts of biomass, in addition to their being tolerant to one or more heavy metals. Moreover, the success of phytoremediation depends on the metal in the soil being in the bioavailable fraction. Hence, it is also important that researchers study the bioavailability and uptake of target metals by hyper-accumulating plants. When research is dedicated to finding optimal hyper-accumulator plants, key study goals should include both (1) evaluating the impact of metal stress on beneficial rhizoshperic microbes and crops and (2) forecasting the application of bioremediation techniques that could be used to clean up heavy metals from the polluted soils

Approximately, 400 species of terrestrial plants have been recognized as hyper-accumulators of various heavy metals (Baker et al. 2000) and have potential applications as bioagents for phytoextraction of heavy metals. The efficiency of phytoaccumulation depends upon many factors like rate of heavy metal uptake and less phytotoxicity with enhanced production of biomass (Garbisu and Alkorta 2001). Some studies pointed that hyper-accumulators are not suitable for reducing phytotoxicity due to low biomass production in heavy metal contaminated soils and having relatively slow growth. These limitations can be enhanced using rhizobacteria as efficient plant growth promoting bioinoculants (Dushenkov et al. 1995). New potential mechanisms need to be explored that show efficient interaction of plants with microbiota (Tank and Saraf 2009).

3.3.4. Rhizoremediation

Rhizoremediation is a procession of remediation of soil by rhizobacteria i.e. bacteria that inhabit the rhizosphere of plants (Hong et al. 2011). The benefits of microbe-plant symbiosis within the plant rhizosphere can be combined into an effective remediating technology, which is a relatively new approach that has potentials to provide practical remediation outcomes (Khan et al. 2009). To tolerate heavy metal stress in contaminated soils, some microbes have developed certain mechanisms that can be applied to withstand the uptake of heavy metals (**Figure 3.1**). These mechanisms include, **(1) exclusion:** pumping heavy metal ions outside to the cell, here the metal ions are kept at bay and away from the target sites; **(2) extrusion:** where the metals are pushed out from the cell through chromosomal/plamid mediated events; **(3) accommodation:** where metals form complex with different cell components including metal binding proteins, that is gathering and sequestration of the metal ions inside the cell; **(4) biotransformation:** where the toxic metal

is reduced to a less toxic form by conversion; **(5) methylation and demethylation** and **(6) desorption/ adsorption** of heavy metals (Khan et al. 2009). These defense mechanisms enable tolerant microorganisms to function metabolically in heavy metal polluted environments.

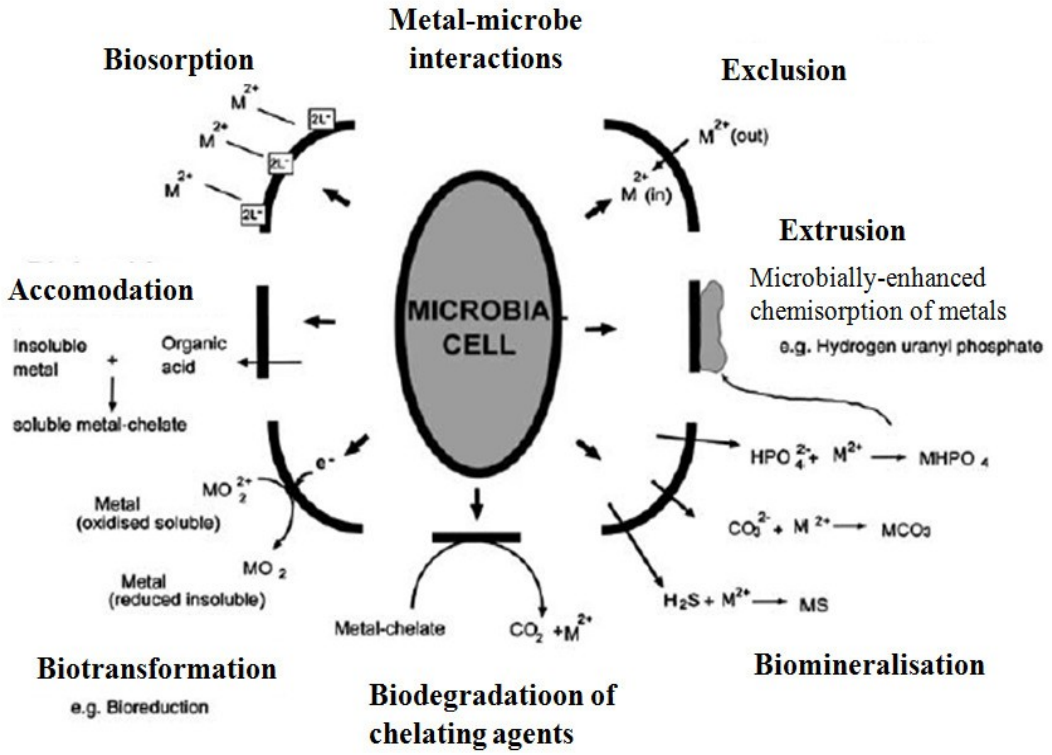

Figure 3.1. Metal tolerance mechanisms developed by soil microbes (Khan et al. 2009).

3.4. Plant Growth Promoting Rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) constitutes numerous species of soil bacteria that grow in the rhizosphere of plants and stimulate plant growth by various mechanisms (Hayat et al. 2010; Vessey 2003). These microorganisms interact with the plant roots and promote growth of plants in diverse soil conditions. The localization and colonization of rhizospheric bacteria in roots impact beneficial aspect to plants (Tank and Saraf 2009). PGPRs are used in some agricultural systems to improve crop yield and quality (Mehboob et al. 2011; Naveed et al. 2008). For example, the legume–rhizobium symbiosis turns atmospheric nitrogen (N) into forms that plants can use and is a vital part of the N cycle (Zahran 1999). As leguminous plants are important sources of protein for humans and the animal worldwide, the use of rhizobial inoculants for legumes ensure efficient N fixation and this process is occurring for almost 100 years (Smith 1998). Simultaneously inoculant markets were also developed in Myanmar (Thein and Hein 1997) and Thailand (Kongngoen

et al. 1997). In addition to the use in agricultural systems, there is also its potential for utilizing the properties of PGPR in other systems such as to use PGPR in the remediation and stabilization of contaminated land (Cardón et al. 2010). Some PGPR have also been shown to protect their host plant from pathogenic microorganisms (Kandavel and Sekar 2010) and toxic effects of heavy metals (Reichman and Parker 2007).

Numerous techniques convert the contaminants to less toxic form, less bioavailable products and even making contaminants to less mobile form but removing heavy metals from contaminated soil or environment is challenging and rather difficult. Studies showed that heavy metal cannot be degraded biologically, due to the fact that bioavailability and specificity of heavy metals to other ligands changes with the environmental factors specifically with Cu, Zn, Ni and Cr (Pilon-Smits 2005). Similarly, Cu, Zn, Ni and Cr promotes beneficial aspects in plant root system, but their high concentrations affect the total biomass of microbial communities (Giller et al. 1998), and thus, change microbial community structure (Gray and Smith 2005) or affect their activity (Römkens et al. 2002).

3.4.1. Use of heavy-metal tolerant bacteria as PGPR

Currently, there are many bacteria that have been reported to develop tolerance or resistance to elevated levels of heavy metals. This ability to grow and live in the presence of high concentrations of heavy-metals exists in many rhizospheric microorganisms. Tolerance of microbes to toxic effects of heavy-metals is the ability to cope with metal toxicity by means of intrinsic properties of the microorganisms, whereas resistance is the ability of microbes to detoxify heavy metals by being activated in direct response to the high concentrations of heavy-metal (Ledin 2000). Toxic forms of heavy metal should be either completely removed from the contaminated soil or transformed or immobilized in ways that render them safe. For persistence under heavy metal stressed environment, PGPR have developed a range of mechanisms by which they can transform, mobilize or immobilize heavy metals, thereby interpreting them inactive (Nies 1999). These mechanisms include **(1) exclusion** metal ions form there target sites, **(2) accumulating** metal ions to their compatible complexes either with ligands or with metal binding proteins like metalothinonins (Kao et al. 2006), **(3) extruding** metals from the cell through plasmid/chromosomal mediated events **(4) using methylation** and demethylation process and **(5) bio-transforming** the heavy metal to a less toxic form.

One or more of the above-mentioned mechanisms allow the microbes to function metabolically in metal-contaminated sites/soils. Interest in exploiting these bacterial properties to remediate heavy metal contaminated sites is growing, and results from their application are promising and challenging (Hallberg and Johnson 2005; Lloyd and Lovley 2001).

3.4.2. Synergistic interaction of PGPR and plants in heavy metal remediation

Many plant–microbe interactions have been investigated but most of the studies performed so far have mainly emphasized only plant–pathogen interactions. Recently, research on the ecology of microbes in rhizospheric soil was focused on the microbiological decontamination or detoxification of soil as affected by heavy metals. The important fact that PGPR promotes plant growth is well documented (Babalola 2010), and more recently, PGPR have been successfully used to reduce plant stress in metal contaminated soils. The microorganisms that are associated with roots establish a synergistic relationship with plant roots which enhances nutrient absorption and improves plant performance, as well as the quality of soils (Yang et al. 2009).

Bacteria interact with plant root system and promote growth in a variety of ways. Few bacteria are pathogenic in nature and affect or inhibit the plant growth. In PGPR systems, bacteria promotes the growth of plants without effecting the soil conditions (Glick et al. 2007). Some microbial communities have the ability to sequester heavy metals, and therefore may be useful for bioremediating of contaminated areas (Hallberg and Johnson 2005). During bioremediation process, PGPR improves the phytoextraction process either by altering the availability, solubility, transfer of nutrients and transport of heavy metals by reducing releasing chelates, converting to less toxic forms or altering soil pH (Ma et al. 2011). Considering the metabolites produced by PGPR, siderophores contributes a prominent role in heavy metal accumulation and mobilization (Rajkumar et al. 2012). Inoculating soil with *Pseudomonas aeruginosa* efficiently release the Pb and Cr into soil solution (Braud et al. 2009) and can realistically serve as a model system, because it is a well-known pathogen. Although no field success has yet been achieved by doing so, the concept of inoculating seeds/rhizospheric soils with selected metal mobilizing bacteria to improve phytoextraction in metal contaminated soils has merit and leave sufficient gap for future research.

3.4.3. Role of PGPR in heavy metal contaminated soil

The potential of using PGPR has shown promising results not only in laboratories but also in green house studies; however, the responses in field trails are variable (Bowen and Rovira 1999). PGPRs not only increase the growth of plants but also remediate contaminated soils in association with plants (Zhuang et al. 2007). The studies showed that PGPR play an active role in plants grown in heavy metal contaminated soils by improving plant growth and tolerance to heavy metals (Cardón et al. 2010; Dary et al. 2010; Koo and Cho 2009; Pishchik et al. 2009; Tank and Saraf 2009; Zaidi et al. 2006). The heavy metal tolerant PGPR *Bacillus subtilis* strain SJ-101 improved the growth of *Brassica juncea* in Ni contaminated soil (Tank and Saraf 2009; Zaidi et al. 2006).

Several rhizobial species are reported not only to tolerate heavy metal but also improve plant growth under exposure to excess heavy metals (Cardón et al. 2010). For example, *Bradyrhizobium* strain RM8 showed tolerance against Ni and Zn, *Rhizobium* sp. RL9 isolated from lentil nodules is effective against Zn, whereas *Rhizobium* sp. RP5 from pea nodules showed tolerance against Zn and Ni, beside these also produced substantial amounts of indole acetic acid (Wani et al. 2007; Wani et al. 2008a; Wani et al. 2008b). A variety of PGPR strains have been reported in literature that remediate heavy metal induced toxicity (**Table 3.2).**

Table 3.2. Examples of PGPR ability to tolerate a variety of heavy metals in plants

PGPR	Heavy metals	Plant	Reference
Rhizobacterium sp. D14	As	*Populus deltoids* LH05-17	Wang et al. (2011)
Rhizobium sp. RP5	Zn & Ni	Pea	Wani et al. (2008a)
Rhizobium sp. RL9	Zn	Lentil	Wani et al. (2008b)
Rhizobacteria	Cd	Wheat and barley	Shenker et al. (2001)
Pseudomonas sp. RJ10	Cd	*Brassica napus*	Sheng and Xia (2006)
Bacillus sp. RJ31	Cd	*Brassica napus*	Sheng and Xia (2006)
Pseudomonas putida KNP9	Pb & Cd	Mung bean	Tripathi et al. (2005)
Bradyrhizobium japonicum CB1809	As	Soybean	Reichman and Parker (2007)
Pseudomonas & Bacillus sp.	Cr	Mustard	Rajkumar et al. (2006)
Brevibacillus	Zn	*Trifolium repens*	Vivas et al. (2006)
Sinorhizobium Pb002	Pb	*Brassica juncea*	Di Gregorio et al. (2006)
Bradyrhizobium sp. (vigna) RM8	Ni	*Vigna radiate*	Wani et al. (2007)
Pseudomonas sp.	Ni	Chickpea	Tank and Saraf (2009)
Bacillus subtilis SJ- 101	Ni	Indian Mustard	Zaidi et al. (2006)

Several rhizobacterial strains help in modifying heavy metal induced plant toxicity (Shenker et al. 2001). PGPR strains, *Acinetobacter* and *Pseudomonads* improve uptake of Zn, Mg, K, Ca, P and Fe by crop plants (Esitken et al. 2006). Studies on certain rhizobacteria in heavy metal uptake indicated that *Pseudomonas* are able to produce siderophores and grow well in presence of heavy metals in chickpea plants grown in Ni contaminated soil (Tank and Saraf 2009).

Several rhizobacterial strains possess heavy metal reducing ability. For example, certain rhizobacteria are able to tolerate As accumulated by the silverback fern (*Pityrogramma calomelanos*) (Rahman et al. 2006). Rhizosphere microbes that were collected from roots of *P. calomelanos* significantly increased the biomass and As concentration of plants, suggesting that these rhizobacteria improved phytoextraction of As (Jankong et al. 2007). In another study, it was found that the fern *Pteris vittata* is an As hyper accumulator and inoculation of As reducing bacteria increased plant biomass by 53% and As uptake by 44% (Smedley and Kinniburgh 2002). Similar studies also showed that growth promoting effect of *Bradyrhizobium japonicum* CB1809 improved plant growth of soybean in As contaminated growing medium (Reichman and Parker 2007). It was however noted that, the plant uptake of As was not increased by inoculation with the *Bradyrhizobium* and thus the bacteria has significant potential for use *in situ* phytostabilization.

Recently, several strains of plant growth promoting rhizobacteria *Agrobacterium radiobacter*, *Azospirillum lipoferum* and *Arthrobacter mysorens* were isolated from barley plants grown in Cd and Pb-treated soil (Wang et al. 2011). The effect of these strains were evaluated by re-inoculating in barley plants grown in uncontaminated and contaminated soils. The results showed that inoculated barley plants had improved growth and uptake of nutrient elements compared to control plants when grown in soil contaminated with Cd and Pb (Wang et al. 2011). It was concluded from this study that accumulation of Cd and Pb in barley plants was reduced by the bacteria which accounted for increased growth of inoculated plants. In another study, Cr tolerant rhizobacteria were isolated from the rhizosphere of a Cr contaminated site. These bacteria were used to inoculate *Vigna radiata* in Cr contaminated soil and the inoculated plants were found to have an increase in biomass, root length and shoot length over non-inoculated plants grown in the same soil (Shenker et al. 2001).

3.5. Bacteria possessing ACC deaminase activity for stress alleviation

PGPRs support plant growth through several mechanisms including: increasing nutrient uptake efficiency by plants, producing plant growth hormones, and protecting host

plants from the pathogens (Díaz-Zorita and Fernández-Canigia 2009; Gholami et al. 2009; Hayat et al. 2010; Wu et al. 2005). Inoculating cereals with PGPRs can increase nutrient uptake, plant height, tissue nitrogen content, root length, leaf size and over all plant biomass (Ahmad et al. 2008; Bashan et al. 2004; Salantur et al. 2006). ACC deaminase positive (ACC+) bacteria are one group of PGPRs that degrade 1- aminocyclopropane-1-carboxylic acid (ACC), the precursor to ethylene. ACC+ bacteria have the ability to reduce ACC and ethylene levels between two-to-four fold, and thus, can improve plant growth under abiotic stress (e.g., drought, salinity, heavy metals).

ACC deaminase is a multimeric enzyme with a molecular mass of about 35–42 kDa (Glick 2005). It is a sulfhydryl enzyme that uses pyridoxal 5-phosphate as an important co-factor. It has been documented that D-cysteine and D-serine can be served as substrate for the ACC deminase enzyme, whereas L-alanine and L-serine can be competitive to ACC deaminase. ACC deaminase has ability to break the part of ACC including the cyclopropane ring; consequently it forms ammonia and α-ketobutyrate. The enzyme is mostly located inside the cytoplasm of bacterial cell and the plant system takes the ACC from the bacterial cell and catalyze the ACC by the enzyme.

The ACC deaminase enzyme has been found in different types of bacteria, such as Gram- positive bacteria, Gram-negative bacteria, endophytic bacteria and rhizobia (Glick 2005). Indole acetic acid (IAA) is produced by ACC+ bacteria, which stimulates plant production. The mechanism of reducing high levels of ethylene by ACC+ bacteria is outlined in **Figure 3.2**. The presence of PGPRs containing ACC-deaminase improved the root system of the plants as well as the number of nodules, and the nodule weight of chickpea plants (Shahzad et al. 2008), and these bacteria were able to adjust the ethylene and improve nutrient availability (Shahzad et al. 2008). Shaharoona et al. (2006) reported that ACC+ bacteria can decrease the effect of ethylene under both normal and stress conditions on plants because of their ACC-deaminase activity. The ACC+ bacteria increased the root elongation and the seedling length. It was also found that the production of ethylene by soybean roots was decreased by the inoculation of ACC+ containing *Bradyrhizobium japonicum*, which reduced the negative effect of ethylene on nodule formation.

ACC+ bacteria can improve plants tolerance to heavy metals, fungal phytopathogens, and flooding (Nie et al. 2002). Under drought stress, plants treated with bacterial strains showed greater fresh and dry weights compared to non-inoculated plants. ACC deaminase activity improved peanut plant root growth in their early stages of growth (Mayak et al. 2004). In the late stages of growth, PGPR activity of ACC+ bacteria helped

to increase plant biomass and yield through siderophores production, phosphorus solubilizaiton, and nitrogen fixation. As a result of these PGPR activities, there was more availability of nutrients and greater production of nodules. ACC+ bacteria were also reported to increase plant height and shoot N and P content (Dey et al. 2004), as well as increase the resistance of the plants to salinity through the reduction of salinity-induced ethylene biosynthesis (Nadeem et al. 2009).

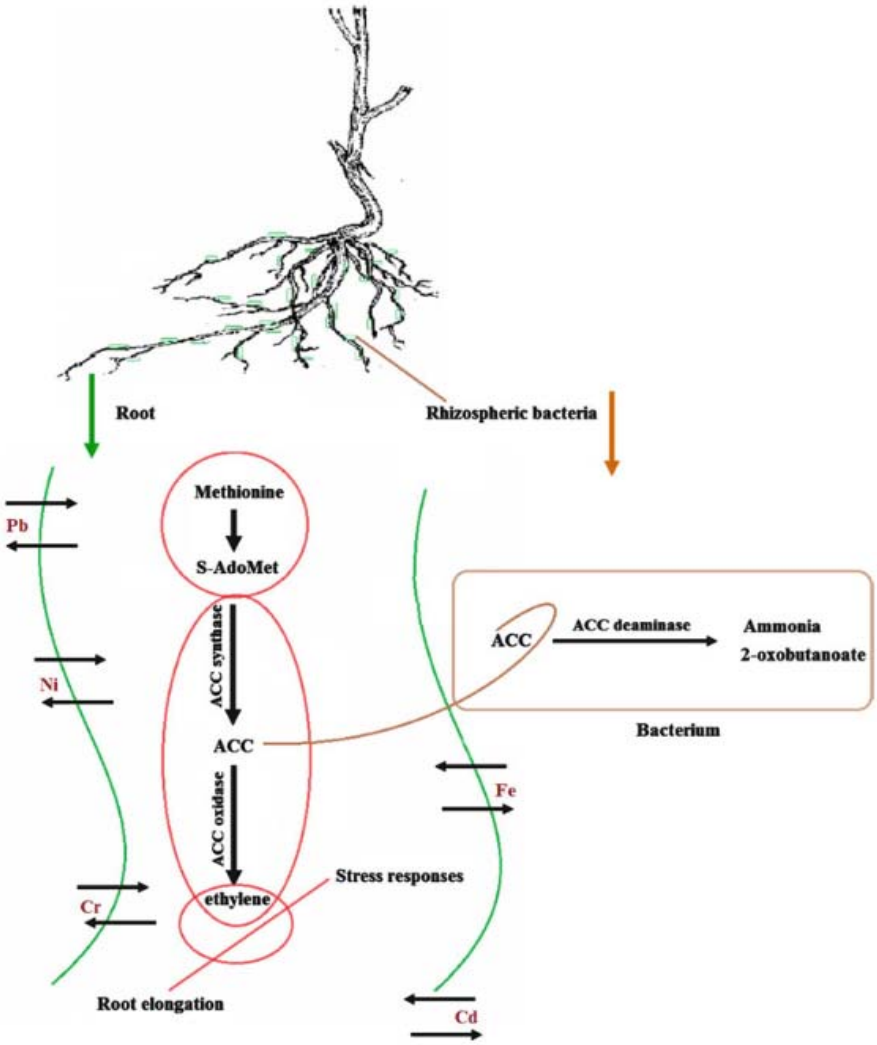

Figure 3.2.

Diagrammatic model showing the process for reducing ethylene levels in roots by using bacterial containing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Glick et al. 2007).

There are many strains of ACC+ bacteria that have been identified. A well known example is *Pseudomonas fluorescens*, which was reported to increase plant root and shoot elongation (Glick et al. 1994). *Pseudomonas putida* was also able to support the growth and germination of canola seeds under salinity stress by producing the enzyme ACC-deaminase (Jalili et al. 2009). Also, *Azospirillum brasilense* was found to be an ACC+ species that

improve shoot and root growth (Holguin and Glick 2001). Similarly, *Kluyvera ascorbata* SUD165 produced a siderophores and displayed ACC+ deaminase activity which reduce Ni uptake by canola seeds (Burd et al. 1998).

3.5.1. Hormones as stress releasing agents in plants

The capacity of phytohormone production is another process for plant growth promotion. Plant growth hormones consist of auxin, gibberellin, cytokinin and abscisic acid. Not only higher plants but also bacteria can synthesize these hormones. The naturally occurring auxin or notably indole-3-acetic acid (IAA) has broad physiological effects (Lambrecht et al. 2000), including the regulation of plant growth and development (Thimann 1935). Auxins have been implicated in cell enlargement, cell division, root initiation, root growth inhibition, apical dominance, phototropism, geotropism and also increased growth rate (Frankenberger Jr and Arshad 1995). Enormous auxins producing bacteria which found in 80% of rhizosphere are the strains that belong to genera: *Azobacter*, *Bacillus*, *Pseudomonas*, *Enterobacter*, *Azospirillum*, *Rhizobium*, and *Bradyrhizobium*. These bacteria secrete auxins as endogenous pool for plant growth.

Gibberellins are a group of endogenous phytohormones that enhance plant development processes including seed germination, stem elongation, and induction of physiological responses such as flowering and fruit setting (Sakamoto et al. 2004). These are also reported to be the mediators of certain environmental signals e.g. photoperiod and light quality. In 1950s, there was an application of gibberellins acid (GAs) from cultures of fungus *Gibberella fujikuroi* to restore dwarf mutants of maize and pea. To date, there have been 4 GAs (GA_1, GA_2, GA_3, and GA_4) identified in bacteria (MacMillan 2001) including *Acetobacter diazotrophicus*, *Azospirillum lipoferum*, *Azospirillum brasilense*, *Bacillus licheniformis*, *Bacillus pumilus*, *Herbospirillum seropedicae*, and *Rhizobium phaseoli* (Sakamoto et al. 2004).

Cytokinins are N_6 substituted aminopurines that act as endogenous plant growth regulators (PGR). These are organic constituents that influence developmental and physiological processes of plants at very low concentrations, less than 1 μM, and presented in plants as a free base form or a t-RNA constituent. Cytokinin plays an important role in controlling cell fate and cell division in plants. Besides this, cytokinin has diverse effect on axillary bud outgrowth, growth and development of intacted plants, leaf expansion, control

of nutrient metabolism, delay of senescence and accumulation of chlorophyll (Sakakibara 2006). Up to date, several cytokinin producing bacteria was identified and characterized in cultures such as *Agrobacterium* sp., *Pseudomonas* sp. (Akiyoshi et al. 1987), *Azotobacter*, *Azospirillum*, *Rhizobium*, *Bacillus*, and *Paenibacillus polymyxa* (Arshad and Frankenberger 1992; Nieto and Frankenberger 1989; Timmusk et al. 1999). Studies showed that exogenous supply of cytokinins could stimulate cell division and increase the level of endogenous cytokinins (Jacqmard et al. 1994). Arshad and Frankenberger (1992) also reported the influence of rhizophere microorganisms in plant growth and development. The plant growth promoting factor is a very complex phenomenon resulting from the combination of many mechanisms. Increasing of nutrient solubilization, nitrogen, phosphorus, and iron uptake are known to trigger plant growth. Nitrogen is one of the major nutrients for plant growth and it becomes an integral component of nucleic acids, biomolecules and proteins (Bøckman 1997). However, plants cannot directly take up nitrogen from the atmosphere nitrogen, and it must be converted into utilizing forms by three processes; i) conversion of atmospheric nitrogen into oxides of nitrogen in the atmosphere; ii) conversion of nitrogen to ammonia and iii) fixation of nitrogen as nitrogenase by microorganisms using a complex enzyme system. Thus, the most intensive application of plant associated nitrogen-fixation bacteria is used as biofertilizers for the alternative of inorganic nitrogen fertilizer (Vessey 2003). The variety of nitrogen fixing bacteria such as *Bacillus fusiformis* (Park et al. 2005), *Azospirillum brasilense* Sp-245, *Azospirillum lipoferum* CRT1, *Xanthobacte*r sp., *Enterobacter* sp., *Azotobacte*r sp., *Bacillus* sp., *Pseudomonas corrugate*, *Azotobacter chrococcum*, *Pseudomonas tolaasii*, *Sphingomonas trueperi*, *Pseudomonas fluorescens* and *Pseudomonas veronii*, have been isolated from various crop rhizospheres which increase plant nitrogen content and grain yield (Reis and Teixeira 2015).

3.6. Biosorption of heavy metals

Biosorption is the ability of certain types of microbial biomass to accumulate heavy metals from aqueous solutions. This phenomenon is also called as a biological ion exchange (Volesky 1999). A large number of micro-organisms belonging to various groups, including fungi, bacteria, algae and yeasts have been reported to bind with number of heavy metals (Volesky 1999). The leading mechanisms for biosorption includes **adsorption**, **bioprecipitation** and **ion exchange** (Volesky 1999). Recently, immobilization techniques

have been developed, but these techniques are expensive and complicated to use (Liu et al. 2003).

In recent years biosorption has been considered to be the safe and cost effective process for the removal of heavy metals from different solutions. The major advantage of biosorption includes **efficient removal of heavy metals** from different media in comparison to conventional methods. The effluents are in order of only few ppb of residual metals (Volesky 1999). The bioadsorption technology works over wide range of pH, temperatures and pressures. Similarly, this technology is derived from cheap raw materials and produces less chemical sludge and easy to dispose of. The most challenging task in bioadsorption technology is selection of biomass that is extremely available in large quantities and also inexpensive. Volesky and Holan (1995) identified several microbes, which have metal-binding capacities.

Numerous studies pointed out the capacity of activated sludge to accumulate heavy metals. This activated sludge mainly accumulate Cu and Zn from acid mine drainages (Utgikar et al. 2000). The pH stabilization is very important in order to achieve maximum efficiency of metals from activated sludge. Liu et al. (2003) used aerobic granules as biosorbent for the removal of Cd from industrial waste water. The uptake of Cd was in range of 43-566 mg g^{-1} depending upon the initial concentration of Cd^{+2} and biomass concentration.

Agricultural waste such as pecan, walnut, hazelnut, ground nut shells and peanuts in modified or natural form were also served as biosorbents (Demirbaş et al. 2002; Kurniawan et al. 2006; Shukla and Pai 2005). These agricultural wastes adsorbed Cu (II), Ni (II), Pb (II), Cd (II) and Zn (II). In some cases, thermal treatment in the presence of citric acid and phosphoric acid is needed to improve the efficiency of adsorption (Chamarthy et al. 2001). Coconut shells as biosorbent removed Cd from water with a concentration ranged between 20 to 1000 mg L^{-1}. The biosorbent showed a high biosorption capacity for Cd (II) with recovery of 285.7 mg g^{-1} of Cd (II) (Pino et al. 2006). Coir pith have capacity to biosorb Ni (II), Co (II) and Cr (III) from a mixture of ionic solutions. The system showed biosorption capacity of 15.9 mg g^{-1} for Ni (II), 11.6 mg g^{-1} for Cr (III) and 12.8 mg g^{-1} for Co (II) (Parab et al. 2006). Orange peel was verified as low-cost adsorbent for Ni(II) removal from

electroplating wastewater (Ajmal et al. 2000). The system showed a maximum biosorption of 96 % at 50 °C for initial concentration of 50 mg L^{-1} at pH 6. The possibility of potato peel waste for the removal of Ni(II) from aqueous solution has also been investigated (Devi Prasad and Abdullah 2009). Metal uptake increased from 0.07 to 0.20 mmol g^{-1} as concentration of Ni(II) increased from 20 to 120 mg L^{-1}. Other important wastes as biosorbents are listed in **Table 3.3**.

Table 3.3. Heavy metal removal from different agricultural waste biosorbents.

Metals	Adsorbents	References
Ni^{+2}	*Cassia fistula*	Hanif et al. (2007)
Pb^{+2}, Zn^{+2}, Fe^{+2}, Ni^{+2}	Waste tea leaves	Ahluwalia and Goyal (2005)
Cu^{+2}, Cd^{+2}, Ni^{+2},	Peat material	Sõukand et al. (2010)
Pb^{+2}, Hg^{+2}, Cd^{+2},	Rice husk	Krishnani et al. (2008)
Pb^{+2}	Maize	(Opeolu 2009)
Cr^{+2}, Pb^{+2}, Mn^{+2},	Fly ash	Gupta et al. (2003)
Cd^{+2}, Co^{+2}, Cr^{+3},	*Sargassum natans*	Volesky (1999)
Pb^{+2}	*Lemna minor*	Rahmani and Sternberg (1999)
Cr^{+3}, Cd^{+2}, Pb^{+2}	Saw dust	Hamadi et al. (2001)
Cd^{+2}	Cellulose xanthate	Tare et al. (1992)

Among the microorganisms, fungal biomass offers the advantage of having high percentage of cell wall material with excellent metal-binding capacities. Kogej and Pavko (2001) showed *Rhizopus nigricans* submerged culture as biosorbent for Pb (II) in aqueous solution. Say et al. (2001) studied the biosorption of Cu, Pb and Cd ions onto the dry fungal biomass of *Phanerochaete chryosporium.* The maximum uptake capacity of the biomass was 26.6, 85.9 and 27.8 mg g^{-1} for Cu, Pb and Cd, respectively. The increase in uptake was occurred with increasing pH from 2.0 to 6.0. Similarly, algal biomass also showed biosorption activity. Sahmurova et al. (2008) observed *Enteromorrpha compressa* as biosorbent for Zn (II) and Cd(II) removal from landfill leachate. The optimum conditions include pH of 4, contact time for 60 min and maintaining a temperature around 25 °C. Kaewsarn (2002) used calcium treated marine algae *Candina* sp. as a biosorbent for the uptake of Cu (II) from aqueous solutions. The maximum uptake capacity was 0.8 mmol g^{-1} at a solution pH of 5.0.

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CHAPTER IV

Isolation and molecular characterization of heavy metal tolerant bacteria for their potential usage in bioremediation and in agriculture

ABSTRACT

Soil pollution with heavy metals have adverse effect on microbial community and soil health. The present study was conducted to isolate heavy metal tolerant bacterial strains and to elucidate their potential usage for soil bioremediation and in agriculture. More than 68 heavy-metals tolerant strains, isolated from industrial discharge, were screened for the maximum tolerance limit. The results showed that some strains tolerated concentration up to 3600 ppm for Cr, 3300 ppm for Cu, 3000 ppm for Cd, 2100 ppm for Pb and 3000 ppm for As. Phylogenetically different strains (23) were further analyzed for biosorption of heavy-metals. Maximum biosorption occurred for Pb followed by Cd and Cu, whereas biosorption of As and Cr was lower by all the isolated strains. Identification based on comparative 16S rRNA gene sequence demonstrated that these heavy-metal tolerant strains belonged to 19 genera and majority of isolates were related to genera, *Bacillus* (21 %), *Pseudomonas* (12 %) and *Staphylococcus* (10 %). The results of molecular characterization for *nifH* and *acdS* gene(s) showed that at least 7 strains were found to contain both genes in their genome (15 isolates contained *nifH* gene, whereas at least 8 strains showed *acdS* gene). Four strains (NCCP-650^{T}, NCCP-644, NCCP-614, and NCCP-602) were further investigated for plant growth promotion activity in *Brassica napus* under axenic condition when irrigated with water containing 50 ppm of each metal separately. The results showed that all the strains tested in this study, played significant role in growth promotion of *Brassica* plants in comparison to control (no addition of strain or metal). Among the strains, NCCP-650^{T} proved to be the best for increase in growth of *Brassica* plants due to presence of both *nifH* and *acdS* genes. These heavy-metals tolerant strains may have the potential for plant growth promotion and can be used as bioinoculants (biofertilizer) in agriculture under heavy-metals contaminated soils. Our results also indicated that some of these isolates can be used for bioremediation of soil/water system contaminated with heavy-metals (Pb, Cd and Cu).

Keywords: Heavy metals, Bioremediation, tanneries discharge, Heavy-metals tolerant PGPR

INTRODUCTION

Heavy metals represent environmental concerns, because of their widespread use and distribution, particularly their toxicity to human beings and the biosphere. However, these also include some elements that are vital for living organisms at relatively low concentrations (Alloway 1990). These elements are usually transition metals, which have high densities (>5 g cm^{-3}) when compared with other materials (Baird and Cann 2005). Heavy metals include essential elements such as iron (Fe) and zinc (Zn) as well as toxic metals like lead (Pb), cadmium (Cd), and mercury (Hg). Soil is an important resource that produces food and other raw materials for humans. However, soil is often a sink for wastes, including heavy metals (Park et al. 2011). Phytoextraction is the potential and cost effective solution for bioremediation of heavy metal contaminated soils. Whereas, the conventional remediation technologies are less effective and sometime imposes detrimental effect to soil characteristics (Biswas et al. 2015; Wan et al. 2016).

Numerous plant species as hyperaccumulators grow in heavy metal contaminated soils, but these cannot be used in field of bioremediation due to their sluggish growth and low biomass (Shen and Liu 1998). Moreover, the heavy metals is occluded or adsorbed by iron-manganese oxides or complexes, organic matter, primary or secondary metabolites and by carbonates (Garbisu and Alkorta 2001). These metal complexes limit the bioavailability of heavy metals in soil and reduces the efficiency of phytoremediation (Chen et al. 2004; Sheng and Xia 2006). Currently, several bacteria have been reported to possess tolerance or resistance against toxic levels of heavy metals. This ability to grow and live in the presence of high concentrations exists in many rhizospheric microorganisms. Tolerance of microbes to toxic effects of heavy-metals is the ability to cope with metal toxicity by means of intrinsic properties of the microorganisms, whereas resistance is the ability of microbes to detoxify heavy metals by being activated in direct response to the high concentrations of heavy-metal (Ledin 2000).

Bacteria develop a variety of mechanisms for plant growth promotion in soils with high concentration of heavy metals; i.e. production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, production of phytohormones, siderophores, indole acetic acid and many others. Plant growth promoting rhizobia (PGPR) have shown promising results in laboratories as well as greenhouse studies; however, the responses in field trails are variable (Bowen and Rovira 1999). PGPRs not only increase the growth of plants but also remediate contaminated soils in association with plants (Zhuang et al. 2007). Studies have shown that

PGPR play an active role in plants grown in heavy metal contaminated soils by improving plant growth and tolerance to heavy metals (Cardón et al. 2010; Dary et al. 2010; Koo and Cho 2009; Tank and Saraf 2009).

Soil microbes have potential to affect the heavy metal availability and mobility to the plants. The rhizospheric bacteria can increase the uptake of nickel (Ni) in *Alyssum murale* and Cd in *Brassica napus* (Abou-Shanab et al. 2006; Sheng and Xia 2006). Similarly, the heavy metal tolerant PGPR *Bacillus subtilis* strain SJ-101 improved the growth of *Brassica juncea* in Ni contaminated soil (Tank and Saraf 2009). PGPR strains, *Acinetobacter* and *Pseudomonads* improved the mobility of few important metals by plants (Esitken et al. 2006). Arbuscular mycorrhizal fungi also stimulates the phytoextraction process by forming association between the natural and toxic heavy metals and improves the plant growth and total metal uptake (Wang et al. 2007).

Biosorption is the ability of certain types of microbial biomass to accumulate heavy metals from aqueous solutions. The leading mechanisms for biosorption includes adsorption, bio precipitation and ion exchange (Volesky 1999). Recently, immobilization techniques have been developed, but these techniques are expensive and complicated to use (Liu et al. 2003). Agricultural waste such as pecan, walnut, hazelnut, ground nut shells and peanuts in modified or natural form were also served as biosorbents (Demirbaş et al. 2002; Kurniawan et al. 2006; Shukla and Pai 2005). These agricultural wastes efficiently adsorbed Cu, Ni, Pb , Cd and Zn. Recently, Oves et al. (2013) studied the biosorbing potentials of *Bacillus thuringiensis* and showed that strain have potential to biosorb 94% Ni followed by Cu (91.8%) and Cd (87%).

The objectives of this study were to isolate and characterize heavy metal resistant bacteria from different industrial discharges in Pakistan. The bacterial isolates were thoroughly characterized based on physiological and molecular characteristics. Further, the biosorption of the isolated strains was conducted for Pb, Cd, Cu, Cr and As. Later, the potential biosorbant strains were selected for greenhouse experiment for accessing the growth promoting ability in *Brassica* plants.

MATERIALS AND METHODS

Sample collection and isolation of heavy-metals tolerant bacteria

The soil, sewage and/or water samples were collected in sterilized plastic bottles from the discharge of tanneries/textile industries areas of Sialkot, Kasur and Islamabad in Pakistan. Samples were brought to the laboratory and stored at 4 ºC until further usage. The effluent

samples were analyzed for heavy metal (Pb, Cd, Cu, Cr and As) by atomic absorption spectrophotometer (Perkin Elmer). The standard stock solutions (1000 mg L^{-1}) of the metals were procured from Sigma-Aldrich.

For isolation of bacterial strains, the samples were diluted in phosphate buffer saline (PBS) solution supplemented with incremental addition of heavy metals (100 ppm per day). The supernatant was streaked on agar plats of different media (tryptic soya agar (TSA), nutrient agar (NA), marine agar; Difco, USA) containing 300-600 ppm of heavy metals (Pb, Cd, Cu, Cr and As), separately and plats were incubated at 28 ºC. The heavy-metals were added using the salts; $Pb(NO_3)_2$, $Cd(NO_3)_2$, $CuSO_4$.$4H_2O$, $K_2Cr_2O_7$ and NaH_2AsO_4, respectively. The growth was observed after 24 to 72 h or till the appearance of bacterial colonies. The isolated colonies showing different morphology (in terms of shape, texture, color, margin and elevation) were further purified by sub-culturing method. The purified cultures of bacterial strains were maintained on agar plates and were also stored at -80 ºC in 35 % glycerol stock solution.

Characterization of isolated bacterial strains

The purified bacterial colonies were morphologically characterized for colony color, form, elevation, margin etc. Cell of the isolates were also analyzed for Gram's staining, morphology and motility using microscope (Olympus, CX31 equipped with Digital Camera 5A). The growth characteristics of bacterial strains were determined for pH range (5 to 10), temperature range (4 to 50 ºC) and tolerance to NaCl (0-30 %). Cells were grown in tryptic soya broth (Difco, USA) for pH range and growth was observed after 24 hr using spectrophotometer (IMPLEN, Germany) at OD600 nm wavelength, whereas temperature range was determined by growing cells on TSA and incubated at various temperature conditions (4 to 50 ºC). Tolerance to NaCl was determined by growing the cells on TSA containing various concentration of NaCl (0-30 %; with 1 % increment).

Screening of bacterial isolates for maximum tolerance limit of heavy metals

Maximum tolerance limit (MTL) is the concentration of respective heavy-metal in the medium, after which the tested isolate failed to grow. MTL for each heavy metal by the isolated bacterial strains was determined according to the method of Malik and Jaiswal (2000). For this experiment, media was supplemented with various heavy metals (Pb, Cd, Cu, Cr and

As) using salts as mentioned above, initially at concentration of 300 ppm with gradual increase by additional 300 ppm up to the MTL for the tested isolate. Each heavy-metal containing plate was subdivided into four equal sectors and the isolate was streaked separately in each quarter. The same method was carried out with control plates (plates without metal). Each sample was made in triplicate. Finally, the plates were incubated at 28˚C for 4-6 days to observe the growth of bacteria.

Identification of the bacterial strains

Strains were identified based on 16S rRNA gene sequence analysis following the method described earlier (Ahmed et al. 2007). For this purpose, template DNA was extracted from fresh cells of the strain by colony polymerase chain reaction (PCR) at 94 ºC for ten min. 16S rRNA gene was amplified in thermal cycler (Applied Biosystems, Veriti, USA) using Pre-mix Ex-Taq kit (Takara Cat # RR003A, Japan) with forward primer 9F (5'-GAG TTT GAT CCT GGC TCA G-3') and reverse primer 1510R (5'-GGC TAC CTT GTT ACG A-3') by following PCR conditions: 1 cycle of pre-denaturation at 94 ºC for 2 min, 30 cycles of denaturation at 94 ºC for 1 min, primers annealing at 50ºC for 1 min, and extension at 72 ºC for 1.30 min, and final extension at 72 ºC for 5 min. The amplified 16S rRNA gene was confirmed on agarose gel (0.8% (w/v)) and was purified using purification kit (Invitrogen, USA) according to the manufacturer's protocol. The purified 16S rRNA gene PCR product was sequenced using forward primer 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and reverse primer 1492R (5'-ACC TTG TTA CGA CTT-3') from Macrogen, Korea (http://dna.macrogen.com/eng/). BioEdit software was used for editing and to achieve assembled consensus sequence. The assembled sequence was subjected to BLAST search on Ez-Taxon Server and DNA Data Bank of Japan, (DDBJ) for identification of the strains and to find the percent sequence similarity with the closely related validly published species. The sequences of 16S rRNA gene of closely related validly published species were retrieved for phylogenetic analysis of the isolated strain. Alignment of sequences was carried out using Clustal W (version 1.6) (Thompson et al. 1997) and the phylogenetic analysis was performed to determine the evolutionary relationship of the strain with other validly published strains. Phylogenetic trees were constructed using three algorithms (data not shown): neighbor-joining (NJ), maximum likelihood (ML) and maximum-parsimony (MP) contained in MEGA-6 software package (Tamura et al. 2013).

Biosorption of heavy metals

To determine biosorption of heavy metals, 22 strains (phylogenetically different strains) were selected and were tested in this experiment in three independent replicates. *Escherichia coli* was used as a control. Bacterial cultures were grown in 5 mL TSB medium containing 50 ppm of each heavy metal (Pb, Cd, Cu, Cr and As) separately, with vigorous shaking at 30 °C incubation temperature. The growth was monitored after 48 h by measuring absorbance at 600 nm using a spectrophotometer (Hitachi, Japan). Cultures were harvested after 48 hr (when OD_{600} was >1.2 or growth reached at stationary phase) by centrifugation at 7500 *g* for 10 min and supernatant were collected in separate tubes and were analysed for heavy metals on Inductively Coupled Plasma-OES (Optima 8300, Perkin Elmer). Control samples of each metal (without bacterial strain) were used to determine the degree of removal of heavy metals from the solution in the test tube (Chen et al. 2006) using the following formula:

$$\frac{\text{CPS of sample}}{\text{CPS of control}} \times 100 = X, \quad \text{and, } \textit{Biosorption capacity} (\%) = 100 - X$$

Whereas Control sample is the medium containing 50 ppm of respective metal.

Percent decrease of metal content than control sample is the Biosorption capacity of the strain grown in sample. Finally the data of biosorption of heavy-metals was subject to statistical analysis using the GLM procedure in SAS and the differences between means were determined using Student-Neuman-Keuls (SNK) test.

Screening of bacterial strains for *nifH* and *acdS* genes

To test the potential usage of the heavy-metals tolerant isolates in agriculture, 23 phylogenetically different bacterial strains were also analysed for screening of *nifH* and *acdS* gene(s). The strains with these genes are well established bioinoculants (biofertilizer) for PGPR activity and can be used in heavy-metals contaminated soils. In this study, all the strains (23) used in biosorption experiment, were tested for the presence of *nifH* and *acdS* genes using different primer sets to detect the specific amplicon of respective gene. Two strains, JCM 10833 (*Bradirhizobium japonicum*) and JCM 21823 (*Rhizobium etli*) were also included as positive control. Genomic DNA was extracted from young bacterial cells (14-16 hrs) using

QIAamp DNA Mini Kit by following the instructions of manufacturer (Qiagen Cat # 51304, Germany).

Amplification of *acdS* gene was performed in 50 µL volume using Pre-mix Ex-Taq kit (Takara Cat # RR003A, Japan) with three sets of primers (Table 4.1) and 50-100 ng of genomic DNA as a template. The amplification was performed with the following PCR conditions: 1 cycle of pre-denaturation at 95 ºC for 5 min, 30 cycles of denaturation at 95 ºC for 30 sec, primers annealing at 50 ºC for 30 sec, and extension at 72 ºC for 30 sec, and final extension at 72 ºC for 7 min (Blaha et al. 2006). DNA band of expected amplicon size with each primer set was analyzed on 0.8 % agarose gel.

Amplification of *nifH* gene was performed using Pre-mix Ex-Taq kit (Takara Cat # RR003A, Japan) with three sets of primer (PolF/PolR, nifHF/ nifHI and nifHfor/ nifHrev; Table 4.1) and 50-100 ng of genomic DNA as a template. The amplification was performed using the PCR conditions as described previously for each set of primers (Supplementary Table 1, (Laguerre et al. 2001; Poly et al. 2001; Sarita et al. 2008). DNA band size of expected amplicons with each primer set was analyzed on 0.8 % agarose gel.

Evaluation of heavy-metal tolerant strains for growth promotion of *Brassica* plants

Three bacterial strains (NCCP-650^T, 644^T and 602), which were candidate promising novel species, were further investigated for growth promotion activity for *Brassica napus* under axenic condition when irrigated with water containing 50 ppm of each metal separately. Strain NCCP-614 was also included in this study as it was efficient for biosorption of Pb, Cd and Cu. Strain JCM 10833 was used as positive control. Two of these strains (NCCP-650^T and 614) showed the presence of both *nifH* and *acdS* genes, whereas these genes were absent in NCCP-602 and 644^T.

The greenhouse experiment was performed by growing seeds of *Brassica napus* in plastic pouches filled with soil and were watered with 50 ppm each of five (separately) heavy-metals Pb, Cu, Cr, Cd and As (using salts: $(Pb(NO_3)_2$, $CuSO_4.4H_2O$, $K_2Cr_2O_7$, $Cd(NO_3)_2$, NaH_2AsO_4, respectively) during the whole growth period (68 days). The experiment was conducted in complete random design (CRD) with three replications keeping the strains as

Table 4.1. Set of primers used in present study

Target genes	Primer pair for amplification	Sequence	Annealing temp. (°C)	Expected amplicon size of target gene (bp)	Reference
nifH	PolF (forward)	5' - TGC GAY CCS AAR GCB GAC TC - 3'	50 °C	360	(Poly et al. 2001)
	PolR (reverse)	5' - ATS GCC ATC ATY TCR CCG GA - 3'			
nifH	nifHF (forward)	5' -TACGGNAARGGSGGNATCGGCAA - 3'	57 °C	780	(Laguerre et al. 2001)
	nifHI (reverse)	5' – AGCATGTCYTCSAGYTCNTCCA - 3'			
nifH	nifHfor (forward)	5' - TAYGGNAARGGNGGHATYGGYATC - 3'	50 °C	420	(Sarita et al. 2008)
	nifHrev (reverse)	5' - ATRTTRTTNGCNGCRTAVABBGCCATCAT - 3'			
acdS	F1936 (forward)	5'- GH GAM GAC TGC AAY WSY GGC - 3'	50 °C	792	(Blaha et al. 2006)
	F1938 (reverse)	5'- AT CAT VCC VTG CAT BGA YTT - 3'			
acdS	F1936 (forward)	5'- GH GAM GAC TGC AAY WSY GGC - 3'	50 °C	558	
	F1939 (reverse)	5' - GA RGC RTC GAY VCC RAT CAC - 3'			
acdS	F1937 (forward)	5' - MGV AAG CTC GAA TAY MTB RT - 3'	50 °C	516	
	F1939 (reverse)	5' - GA RGC RTC GAY VCC RAT CAC - 3'			

more important factor than heavy-metals treatment. The plants were harvested after 68 days of growth period and plant growth parameters (shoot length, shoot dry weight) were recorded. Statistical analysis was performed using the GLM Procedure in SAS version 9.4 (SAS Institute Inc., Cary, USA). Least squares means were estimated for the main effects of strains and metals as well as their interaction effect. Standard error of the difference between means was estimated using the estimate statement in the model. Statistical analysis was performed using the GLM Procedure in SAS version 9.4 (SAS Institute Inc., Cary, USA). Least squares means were estimated for the main effects of metal and strain as well as their interaction effect. Standard error of the difference between means was estimated using the estimate statement in the model. The differences between means for length and shoot fresh weight were determined using Student-Neuman-Keuls (SNK) test in the GLM procedure of SAS.

RESULTS

Physico-chemical analysis of the effluent

Effluent samples were analyzed for different physiochemical parameters, including metal ion discharge, pH of the effluent samples and physical appearance. It was found that pH of samples was mostly alkaline, while most of these samples were highly colored with foul smelling. The effluent samples contained various heavy metals mostly Cr, Cu, Cd, Pb As and Ni (Table 4.2). The level of Cr and Pb in most of the samples were found above the permissible limits of 0.05 ppm and 0.01 ppm, respectively (CPCB 2008).

Table 4.2: Physicochemical analysis of effluent samples

Sample	pH	Electrical conductivity (dS m^{-1})	Cu (ppm)	Cr (ppm)	Pb (ppm)	Cd (ppm)	Ni (ppm)
Kohinoor drainage water	7.5	0.91	0.057	0.128	0.411	0.003	0.97
Kohinoor drain sludge	8.0	0.61	0.003	0.232	0.069	0.005	0.056
Kohinoor treatment plant water	8.2	0.68	ND	0.254	0.208	ND	0.148
Sector I-9 industrial water	8.5	0.76	0.031	0.171	0.169	ND	ND
Sector I-9 industrial sludge1	8.5	0.79	0.031	0.211	0.239	0.004	ND
Sector I-9 Industrial sledge2	7.5	0.63	0.050	0.149	0.099	ND	ND

The values mentioned are average of triplicate samples. ND, not detected.

Isolation and morphological characterization of bacterial strains

Initially 172 strains were isolated from the discharge of Industrial areas (Islamabad, Sialkot and Kasur) from Pakistan. The isolated strains were enriched with different heavy-metals and were differentiated on the basis of colony morphology. Later on, 68 strains were selected on the basis of colony morphology and the purified strains were designated as NCCP-601 to onward (Table 4.3). Most of the strains were round, lobed and filamentous in shape, having entire margins but some were having irregular margins. Colony color of most of strains was white and pale yellow but some strains were of peach and off-white in color. Morphologically different strains were further subjected to other experiments and stored in -80ºC refrigerator.

Table 4.3: Morphological characteristics of isolated heavy metal tolerant strains

Strain	Form	Margin	Surface	Elevation	Opacity	Color	NaCl tolerance (%) Range (Optimum)
NCCP-601	Round	Entire	Smooth	Convex	opaque	orange	0-18 (2)
NCCP-602	Round	Entire	Smooth	Convex	opaque	white	0-16 (2)
NCCP-603	Round	Entire	Smooth	Convex	opaque	white	0-4 (0)
NCCP-604	Round	Entire	Smooth	Convex	opaque	white	0-10 (1)
NCCP-605	Round	Entire	Smooth	Convex	opaque	white	0-8 (1)
NCCP-606	Round	Entire	Smooth	Convex	opaque	white	0-5 (1)
NCCP-607	Round	Entire	Smooth	Convex	opaque	white	0-7 (1)
NCCP-608	Round	Entire	Smooth	Convex	opaque	white	-
NCCP-609	Round	Entire	Smooth	Convex	opaque	white	0-8 (1)
NCCP-610	Round	Entire	Smooth	Convex	opaque	white	0-8 (1)
NCCP-611	Round	Entire	Smooth	Convex	opaque	white	0-7 (1)
NCCP-612	Round	Entire	Smooth	Convex	opaque	white	0-15 (2)
NCCP-613	Round	Entire	Smooth	Convex	opaque	white	0-8 (1)
NCCP-614	Round	Entire	Smooth	Convex	opaque	white	0-4 (0)
NCCP-615	Lobed	Irregular	Smooth	Convex	opaque	Pale yellow	0-7 (1)
NCCP-616	Round	Entire	Smooth	Convex	opaque	White	-
NCCP-617	Round	Entire	Smooth	Convex	opaque	White	-
NCCP-618	Round	Entire	Smooth	Convex	opaque	White	0-6 (1)
NCCP-619	Round	Entire	Smooth	Convex	opaque	Yellow	0-9 (1)
NCCP-620	Round	Entire	Smooth	Convex	opaque	White	0-10 (1)
NCCP-621	Round	Entire	Smooth	Convex	opaque	White	0-9 (1)
NCCP-622	Round	Entire	Smooth	Convex	opaque	White	0-6 (1)
NCCP-623	Round	Entire	Smooth	Convex	opaque	White	-

NCCP-624	Round	Entire	Smooth	Convex	opaque	White	0-4 (0)
NCCP-625	Filamentous	Irregular	Dull	Convex	opaque	White	0-14 (2)
NCCP-626	Round	Irregular	Smooth	Convex	opaque	Whitish yellow	-
NCCP-627	Round	Entire	Smooth	Convex	opaque	Peach	-
NCCP-628	Round	Entire	Smooth	Convex	opaque	White	0-15 (2)
NCCP-629	Round	Entire	Smooth	Convex	opaque	White	-
NCCP-630	Round	Irregular	Smooth	Flat	opaque	Yellow	0-4 (0)
NCCP-631	Round	Irregular	Smooth	Flat	opaque	White	-
NCCP-632	Round	Entire	Smooth	Convex	opaque	White	-
NCCP-633	Round	Entire	Smooth	Convex	opaque	White	-
NCCP-634	Round	Entire	Smooth	Convex	opaque	White	-
NCCP-635	Round	Entire	Smooth	Convex	opaque	White	0-5 (0)
NCCP-636	Round	Entire	Smooth	Convex	opaque	White	-
NCCP-637	Round	Entire	Smooth	Flat	opaque	White	-
NCCP-638	Round	Entire	Smooth	Flat	opaque	White	-
NCCP-639	Round	Entire	Smooth	Convex	opaque	White	-
NCCP-640	Round	Irregular	Smooth	Flat	opaque	White	-
NCCP-641	Round	Entire	Smooth	Flat	opaque	Light yellow	-
NCCP-642	Round	Entire	Smooth	Flat	opaque	white	-
NCCP-643	Round	Entire	Smooth	Flat	opaque	white	-
NCCP-644	Round	Irregular	Smooth	Convex	opaque	white	0-4 (0)
NCCP-645	Round	Entire	Smooth	Convex	opaque	white	0-5 (0)
NCCP-646	Round	Entire	Smooth	Convex	opaque	white	0-5 (1)
NCCP-647	Round	Entire	Smooth	Convex	opaque	white	0-8 (1)
NCCP-648	Round	Entire	Smooth	Convex	opaque	white	-
NCCP-649	Round	Entire	Smooth	Convex	opaque	White	-
NCCP-650	Round	Entire	Smooth	Convex	opaque	White	0-7 (1)
NCCP-651	Round	Entire	Smooth	Convex	opaque	White	0-8 (1)
NCCP-652	Round	Entire	Smooth	Convex	opaque	White	0-4 (0)
NCCP-653	Round	Entire	Smooth	Convex	opaque	White	-
NCCP-654	Round	Entire	Smooth	Convex	opaque	White	-
NCCP-655	Round	Entire	Smooth	Convex	opaque	White	0-8 (1)
NCCP-656	Round	Entire	Smooth	Flate	opaque	White	-
NCCP-657	Lobed	Irregular	Smooth	Flate	opaque	White	-
NCCP-658	Round	Entire	Smooth	Flate	opaque	Yellow	-
NCCP-659	Round	Entire	Smooth	Concave	opaque	White	-
NCCP-660	Round	Entire	Smooth	Flate	opaque	White	0-20 (2)
NCCP-661	Round	Entire	Smooth	Convex	opaque	Yellow	0-9 (1)
NCCP-662	Round	Entire	Smooth	Flate	opaque	White	0-10 (1)
NCCP-663	Round	Entire	Smooth	Convex	opaque	Yellow	0-9 (1)

NCCP-666	Round	Entire	Smooth	Flate	Transparent	White	0-9 (1)
NCCP-667	Round	Entire	Smooth	Convex	opaque	Yellow	0-7 (1)
NCCP-668	Round	Entire	Smooth	Convex	opaque	White	0-10 (1)
NCCP-670	Round	Entire	Smooth	Convex	opaque	White	0-4 (0)
NCCP-671	Round	Entire	Rough	Flate	opaque	White	0-20 (2)
NCCP-672	Irregular	Irregular	Smooth	Flate	opaque	White	0-20 (2)
NCCP-673	Round	Entire	Smooth	Flate	Transparent	White	0-12 (1)
NCCP-675	Round	Entire	Smooth	Convex	opaque	White	0-7 (1)
NCCP-677	Round	Entire	Smooth	Convex	opaque	White	0-4 (0)
NCCP-679	Round	Entire	Smooth	Convex	opaque	White	0-7 (1)
NCCP-680	Round	Entire	Smooth	Convex	opaque	White	0-3 (0)

Maximum tolerance limit (MTL) of isolated bacterial strains for heavy-metals and NaCl

All the isolated bacterial strains were found to be tolerant against different heavy metals including Cr, Cu, Cd, Pb and As (Figure 4.1). The results demonstrated that some of the isolated strains tolerated 3600 ppm for Cr, 3300 ppm for Cu, 3000 ppm for Cd, 1500 ppm for Pb and 1200 ppm for As. Among these highly tolerant isolates, maximum tolerance limit (MTL) was observed for NCCP-601, 602, 603, 647, 657, 660, 661, 621, 653 (against Cr 3600 ppm), NCCP-601, 602, 603 (against Cu 3300 ppm), NCCP-601, 602, 603 (against Cd 3000 ppm), and NCCP-644, 650 (against Pb 2100 ppm and As 3000 ppm). The isolates reported in these studies showed higher tolerance to Cu, Cd, Pb and As compared to previous reports (Haq et al. 1999; Shakoori et al. 2002; Shakoori and Muneer 2002; Shakoori and Qureshi 2000; Shakoori et al. 2010). Most of the bacterial isolates were also found to grow over a wide range of NaCl concentration ranging from 0 to 20% (Table 4.3).

Biosorption of heavy metals

Based on MTL, the isolated strains were further tested for possible usage in biosorption of heavy metals from 50 mL of TSB containing 50 ppm of respective heavy-metal. All isolated bacterial strains showed significant reduction in heavy metal concentration in TSB medium. Maximum biosorption occurred for Pb followed by Cd and Cu. However, there was no significant reduction in concentrations of Cr and As by any of the isolated strains (Figure 4.2). The isolates significantly differed in their biosorption capacity for Pb, Cd and Cu (Figure 4.2a, b & c), but there was no significant difference for As and Cr (Figure 4.2d & e). Among these, three isolates, NCCP-614 (99 %), NCCP-605 (96 %) and NCCP-655 (91 %) showed maximum biosorption of Pb (Figure 4.2a). Similarly, strains NCCP-614 and NCCP-655 also showed maximum biosorption capacity of 89 % and 59 % for Cd, respectively (Figure 4.2b); however, maximum biosorption of Cu was done by NCCP-625 (42 %) followed by NCCP-619 (38 %)

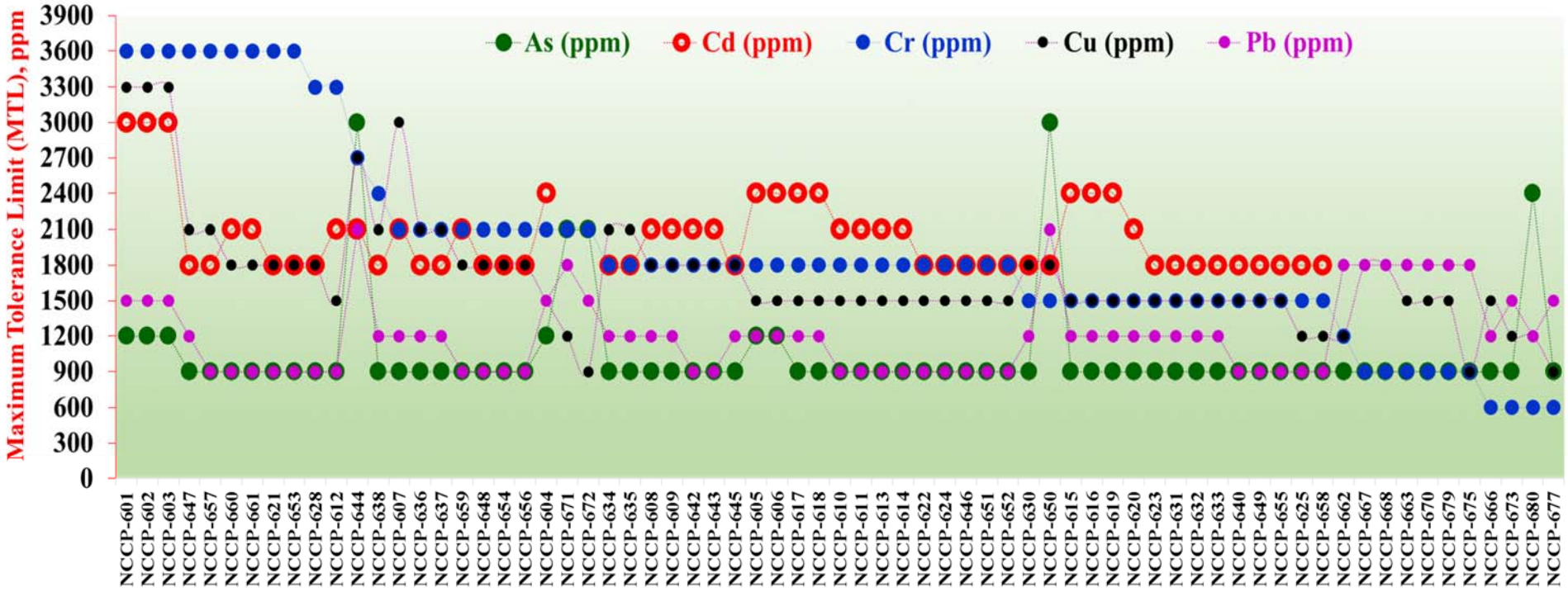

Figure 4.1. Maximum tolerance limit (MTL) of isolated bacterial strains against different heavy metals (Cr, Cu, Pb, Cd and As)

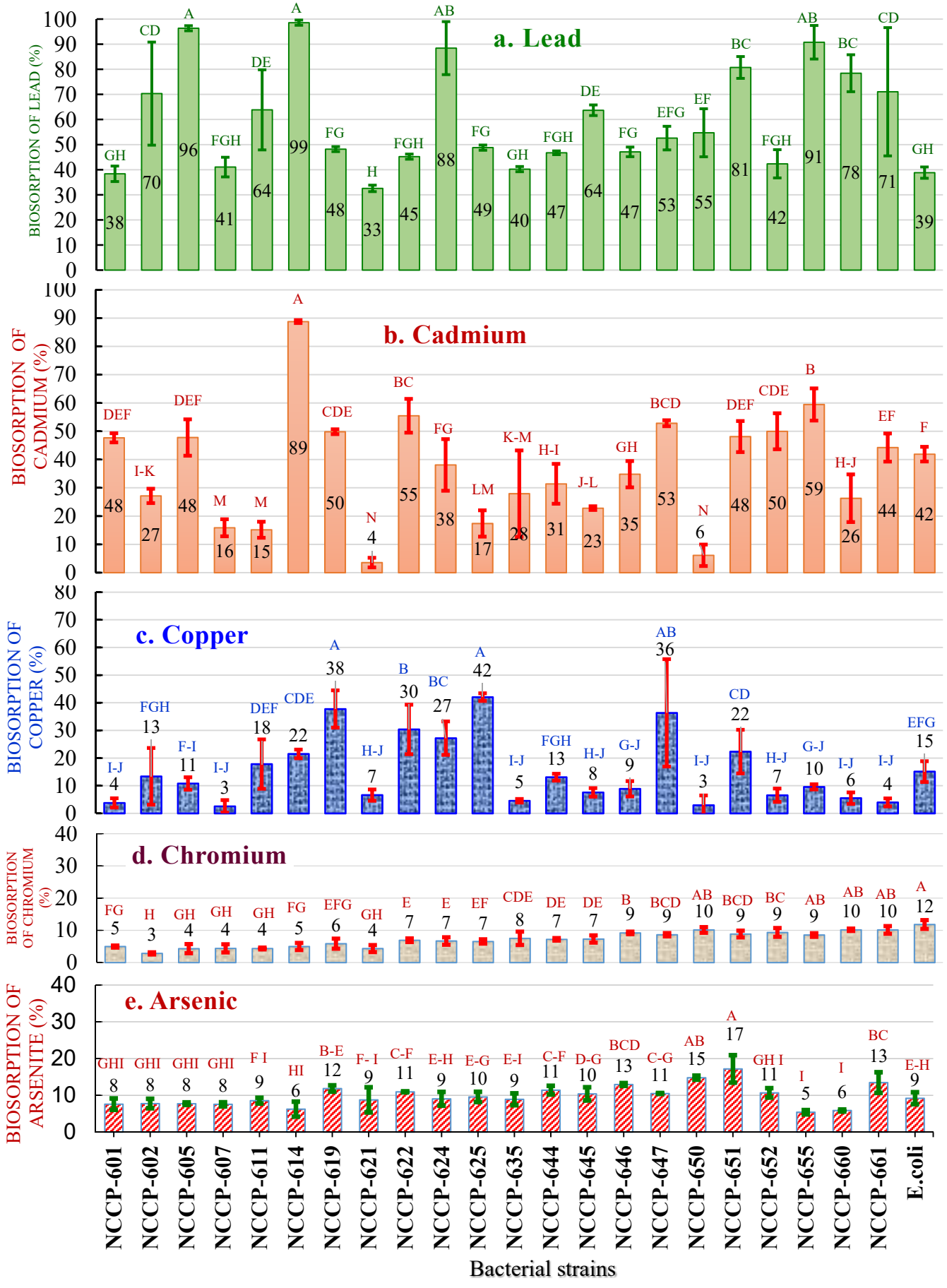

Figure 4.2. Biosorption capacity (%) of heavy-metals tolerant strains. (a) lead, (b) cadmium, (c) copper, (d) chromium and (e) arsenic. Bars carrying the same alphabet letter in each metal are statistically not significant (*p = 0.05*)

and NCCP-647 (36 %) (Figure 4.2c). The bacterial isolates showed almost no significant biosorption with Cr and As (Figure 4.2d and e). Our results indicated that these isolates can be used for bioremediation of soil/water system contaminated with heavy-metals (Pb, Cd and Cu).

Identification of the bacterial strains

It is accepted that culturable microorganisms from any given sample taken from an environment represent only a small portion of the total population that actually present. In our study, more than 68 bacterial strains were identified taxonomically on the basis of 16S rRNA gene sequence (Table 4.4), which were isolated from effluent samples and found to be highly tolerant to heavy-metals. Identification based on comparative 16S rRNA gene sequence data demonstrated that a diverse bacterial community was observed (Figure 3a & 3b). The isolated population belonged to three phyla, including *Proteobacteria* (56%), *Firmicutes* (38%) and *Actinobacteria* (6%) (Figure 4.3a). These heavy metal tolerant strains are related to nineteen different genera (Figure 4.3a & b). The dominant number of strains belonged to *Bacillus* (21 %) *Pseudomonas* (12 %) and *Staphylococcus* (10 %).

The value in front of each genus (Figure 4.3b) show the percent sequence similarity of 16S rRNA gene with the closely related species in their respective genera. This sequence similarity of the isolates was 97.9 to 100 % with the closely related species of genera, *Bacillus*, *Alcaligenes, Acinetobacter, Pseudomonas, Citrobacter* and *Bravibacterium,* (Figure 4.3b; Table 4.4), which indicate that some of these strains can be characterized taxonomically to delineate as novel species. Keeping in view of these results, along with phylogenetic analyses, DNA-DNA homology, phenotypic and chemotaxonomic data, three isolated strains were characterized as novel species: *Acinetobacter pakistanensis* sp. nov. (Abbas et al. 2014), *Alcaligenes pakistanensis* sp. nov. (Abbas et al. 2015a), and *Bacillus malikii* sp. nov. (Abbas et al. 2015b). However, other strains had high similarity (> 99%) of 16S rRNA gene sequence with the closely related taxa in their respective clusters and therefore, were not included in the taxonomic characterization studies.

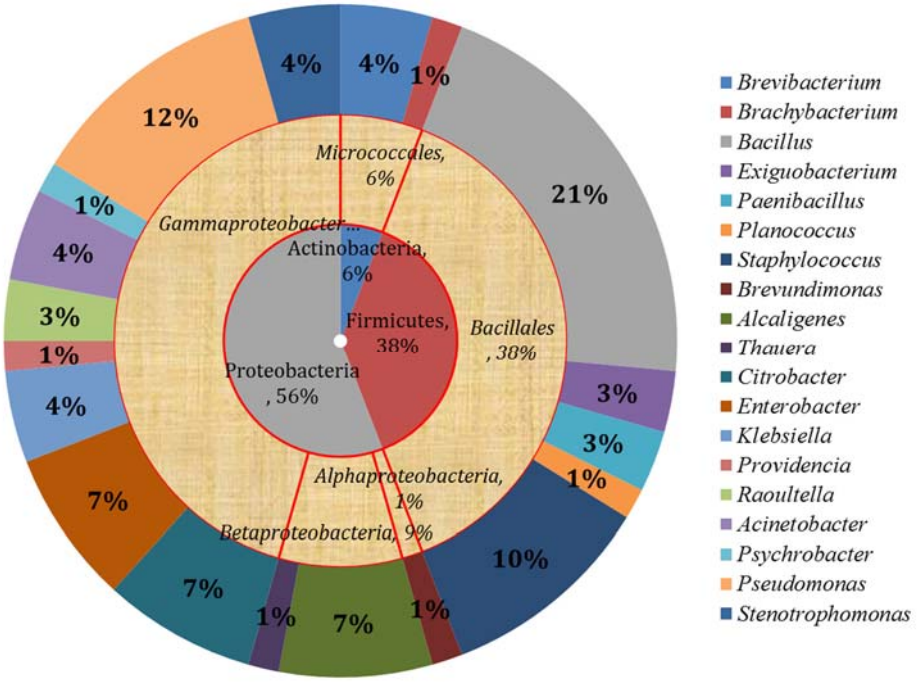

Figure 4.3 (a). Biodiversity pie chart of isolated heavy-metals tolerant strains. Outer ring shows the percentage of isolated population in a particular genus (see legend).

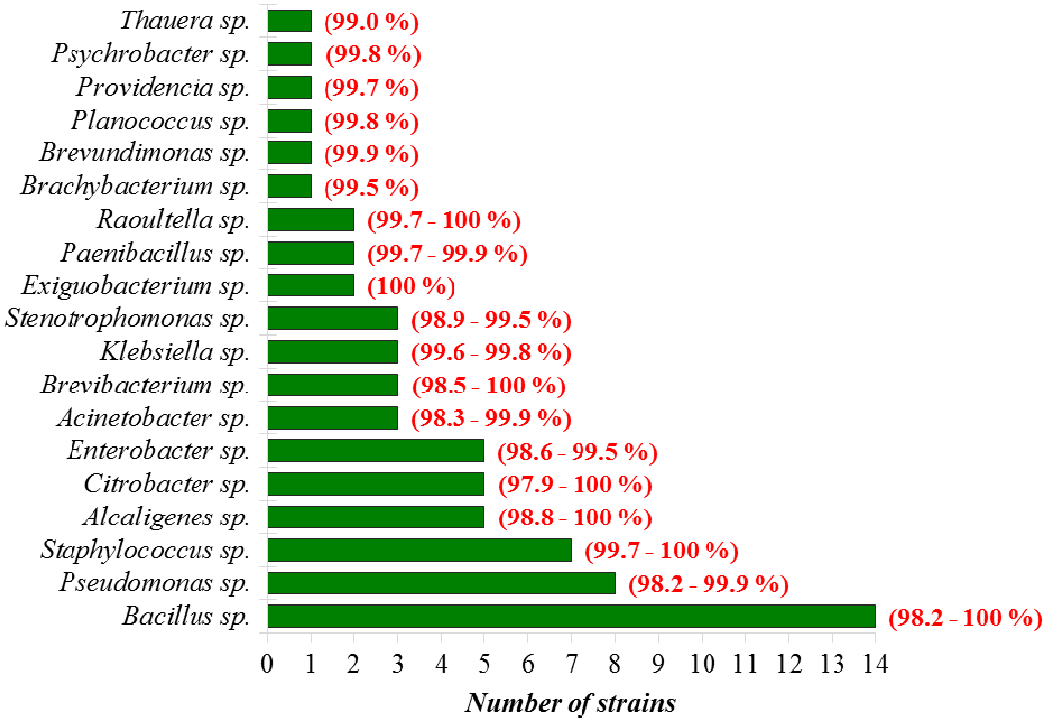

Figure 4.3 (b). Biodiversity isolated heavy-metals tolerant strains. The values in front of each bar show the percent similarity range of the strains with the known closely related species of the respective genera.

Table 4.4. Identification of isolated Heavy metal tolerant strains based on 16S rRNA gene sequence and their accession numbers published in DNA database.

Strain ID	Number of nucleotides of 16S rRNA gene	Accession number of 16S rRNA gene	Closely related validly published taxa	Similarity %age of 16S rRNA gene sequence with closely related species	Cover age	No. of closely related species having >97% (>98%) similarity of 16S rRNA gene sequence
NCCP-601	1416	AB920786	*Staphylococcus xylosus* (D83374)	99.93	96.0	37 (21)
NCCP-602	1432	AB920787	*Brevibacterium ammoniilyticum* (JF937067)	98.56	97.0	12 (4)
NCCP-603	1393	AB920788	*Acinetobacter guillouiae* (APOS01000028)	98.63	95.4	(2)
NCCP-604	1417	AB920789	*Providencia vermicola* (AM040495)	99.72	96.9	10 (9)
NCCP-605	1393	AB920790	*Citrobacter youngae* (AJ564736)	100.00	95.2	62 (29)
NCCP-606	1393	AB920791	*Enterobacter aerogenes* (CP002824)	99.43	95.3	62 (31)
NCCP-607	1427	AB920792	*Enterobacter aerogenes* (CP0028240)	99.02	96.6	57 (20)
NCCP-608	1092	AB920793	*Enterobacter aerogenes* (CP002824)	99.45	74.6	63 (30)
NCCP-609	1092	AB920794	*Citrobacter youngae* (AJ564736)	100.00	74.5	59 (28)
NCCP-610	832	AB920795	*Citrobacter youngae* (AJ564736)	99.77	56.8	42 (8)
NCCP-611	1432	AB920796	*Raoultella ornithinolytica* (AJ251467)	99.72	98.0	53 (12)
NCCP-612	984	AB920797	*Staphylococcus equorum* subsp. *equorum* (AB009939)	100.00	66.7	22 (12)
NCCP-613	1415	AB920798	*Citrobacter youngae* (AJ564736)	97.94	95.2	1
NCCP-614	874	AB920799	*Stenotrophomonas rhizophila* (AJ293463)	98.97	59.6	30 (2)
NCCP-615	961	AB920800	*Alcaligenes aquatilis* (AJ937889)	99.90	65.8	(4)
NCCP-616	1368	AB920801	*Alcaligenes aquatilis* (AJ937889)	100.00	94.2	(4)
NCCP-617	1356	AB920802	*Enterobacter aerogenes* (CP002824)	98.60	93.0	40 (9)
NCCP-618	1376	AB920803	*Brevibacterium frigoritolerans* (AM747813)	99.93	93.5	7 (4)
NCCP-619	1241	AB920804	*Bacillus persicus* (HQ433471)	99.52	66.2	27 (1)
NCCP-620	1087	AB920805	*Klebsiella pneumoniae subsp. rhinoscleromatis* (ACZD01000038)	99.63	74.4	47 (20)
NCCP-621	852	AB920806	*Exiguobacterium indicum* (AJ846291)	100.00	57.4	8 (5)
NCCP-622	1399	AB920807	*Brevibacterium frigoritolerans* (AM747813)	100.00	95.0	7 (4)

NCCP-623	815	AB920808	*Klebsiella pneumoniae* subsp. *pneumoniae* (AJJI01000018)	99.75	55.7	60 (24)
NCCP-624	970	AB920809	*Pseudomonas veronii* (AF064460)	99.59	66.4	49 (32)
NCCP-628	1132	AB920810	*Staphylococcus equorum subsp. equorum* (AB009939)	100.00	76.8	24 (12)
NCCP-630	1023	AB920811	*Pseudomonas aeruginosa* (Z76651)	99.90	70.1	(2)
NCCP-631	861	AB920812	*Klebsiella pneumoniae* subsp. *rhinoscleromatis* (ACZD01000038)	99.77	58.8	58 (28)
NCCP-632	946	AB920813	*Pseudomonas aeruginosa* (Z76651)	99.89	64.8	3 (2)
NCCP-633	1049	AB920814	*Pseudomonas aeruginosa* (Z76651)	99.81	72.0	(2)
NCCP-634	1133	AB920815	*Staphylococcus equorum* subsp. *equorum* (AB009939)	99.82	76.8	20 (11)
NCCP-635	1099	AB920816	*Paenibacillus motobuensis* (AY741810)	99.72	74.5	(1)
NCCP-636	1155	AB920817	*Planococcus rifietoensis* (AJ493659)	99.83	78.4	19 (10)
NCCP-637	1266	AB920818	*Bacillus flexus* (AB021185)	99.37	86.3	4 (4)
NCCP-638	965	AB920819	*Bacillus flexus* (AB021185)	100.00	65.4	4 (4)
NCCP-640	976	AB920820	*Pseudomonas aeruginosa* (Z76651)	99.90	67.0	2 (2)
NCCP-642	993	AB920821	*Bacillus anthracis* (AB190217)	100.00	67.4	12 (10)
NCCP-643	950	AB920822	*Bacillus anthracis* (AB190217)	100.00	64.5	12 (10)
NCCP-644	1413	AB916465	*Acinetobacter kyonggiensis* (FJ527818)	98.33	96.7	10 (1)
NCCP-645	966	AB920823	*Pseudomonas fragi* (AF094733)	99.38	66.3	50 (22)
NCCP-646	840	AB920824	*Pseudomonas azotoformans* (D84009)	98.20	57.5	9 (1)
NCCP-647	1124	AB920825	*Staphylococcus equorum* subsp. *equorum* (AB009939)	99.73	75.9	18 (10)
NCCP-648	1390	AB920826	*Psychrobacter faecalis* (AJ421528)	99.78	95.1	27 (9)
NCCP-649	532	AB920827	*Stenotrophomonas rhizophila* (AJ293463)	99.25	36.2	4 (1)
NCCP-650	1412	AB920828	*Alcaligenes faecalis* subsp. *parafaecalis* (AJ242986)	98.78	96.5	4 (3)
NCCP-651	1403	AB920829	*Bacillus cereus* (AE016877)	100.00	95.1	12 (11)
NCCP-652	846	AB920830	*Thauera mechernichensis* (Y17590)	99.05	58.0	6 (6)
NCCP-653	1134	AB920831	*Bacillus aerophilus* (AJ831844)	99.47	76.6	5 (5)

NCCP-654	1118	AB920832	*Pseudomonas extremaustralis* (AHIP01000073)	99.46	76.3	48 (30)
NCCP-655	1119	AB920833	*Stenotrophomonas rhizophila* (AJ293463)	99.46	76.2	6 (2)
NCCP-656	1091	AB920834	*Bacillus safensis* (AF234854)	99.54	74.1	5 (5)
NCCP-657	1401	AB920835	*Bacillus subtilis* subsp. *inaquosorum* (AMXN01000021)	99.36	95.1	15 (12)
NCCP-658	1407	AB920836	*Bacillus gibsonii* (X76446)	99.64	95.2	3 (3)
NCCP-659	1400	AB920837	*Bacillus safensis* (AF234854)	99.93	95.0	10 (5)
NCCP-660	881	AB920838	*Staphylococcus haemolyticus* (L37600)	100.00	59.7	38 (16)
NCCP-661	1092	AB920839	*Brachybacterium nesterenkovii* (X91033)	99.45	75.6	6 (2)
NCCP-662	1492	AB968093	*Bacillus niabensis* (AY998119)	98.17	100	3 (2)
NCCP-663	858	AB968094	*Alcaligenes faecalis subsp. faecalis* (D88008)	99.18	58.9	4 (4)
NCCP-666	1494	AB968095	*Bacillus cohnii* (X76437)	99.86	100	4 (2)
NCCP-667	1479	AB968096	*Alcaligenes aquatilis* (JX986974)	99.32	100	4 (3)
NCCP-668	829	AB968097	*Citrobacter amalonaticus* (FR870441)	100.00	56.6	24 (10)
NCCP-670	811	AB968098	*Brevundimonas mediterranea* (AJ227801)	99.88	58.6	8 (5)
NCCP-671	882	AB968099	*Bacillus sonorensis* (AYTN01000016)	99.21	59.9	15 (10)
NCCP-672	789	AB968100	*Staphylococcus xylosus* (D83374)	99.87	53.5	23 (12)
NCCP-673	892	AB968101	*Exiguobacterium mexicanum* (AM072764)	100.00	60.1	8 (6)
NCCP-675	868	AB968102	*Raoultella ornithinolytica* (AJ251467)	100.00	59.4	55 (21)
NCCP-677	1492	AB968103	*Paenibacillus motobuensis* (AY741810)	99.86	100	1 (1)
NCCP-679	828	AB968104	*Enterobacter aerogenes* (CP002824)	99.28	56.7	55 (33)
NCCP-680	1501	AB968105	*Acinetobacter bouvetii* (APQD01000004)	99.93	100	12 (5)

Screening of *nifH* and *acdS* genes of isolated bacterial strains

To test the potential usage of isolated heavy-metals tolerant isolates in agriculture, phylogenetically different isolates were also analysed for screening of *nifH* gene using different primer sets like PolF/PolR, nifHF/nifHI and nifHfor/nifHrev (Table 4.1). *Rhizobium etli* JCM 21823^T and *Bradyrhizobium japnicum* JCM 10833^T were used as positive control. The *nifH* gene was amplified by these primers and amplicons of approximately 360, 420 and 780 bp, previously reported to be associated with presence of *nifH* gene, were identified (Table 4.5). The results depicted that *nifH* gene was amplified in at least 15 isolates with one or two primer sets (Figure 4). Similarly, the isolates were also analyzed for presence of *acdS* gene in their genome using three sets of primers like F1936f/F1938r, F1936f/F1939r and F1937f/F1939r (Supplementary Table 1). The *acdS* gene was amplified in at least 8 strains by these primers with an approximate size of 792, 558 and 516 bp, respectively. The careful analysis of results showed that at least 7 strains (NCCP-650, NCCP-611, NCCP-660, NCCP-635, NCCP-622, NCCP-614 and NCCP-605) were found to have both *nifH* and *acdS* genes (Figure 4). It is also worth mentioning here that strain NCCP-650^T, which contained both the genes, has been delineated as a novel species: *Alcaligenes pakistanensis* sp. nov. and this strain also contained nitrite reductase, *nirK* gene (Abbas et al. 2015a), which has its significance in bioremediation process. These heavy-metals tolerant strains may have the potential for plant growth promotion and can be used as bioinoculants (biofertilizer) in agriculture.

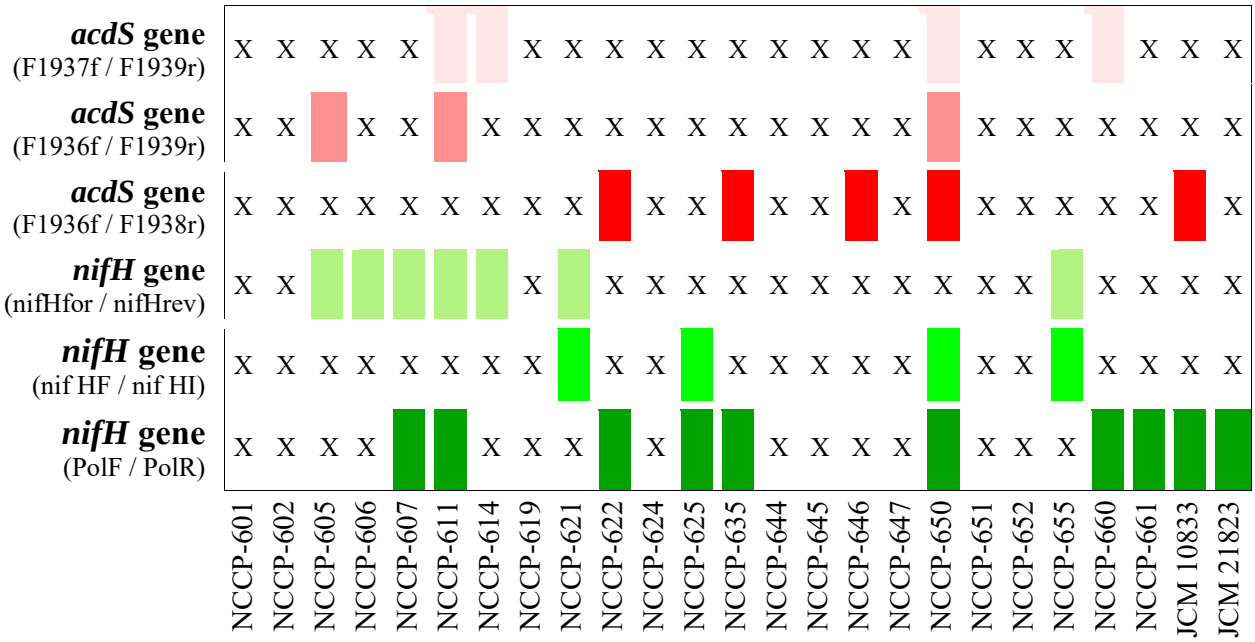

Figure 4.4. Summary of amplified PCR products with different markers of *nifH* and *acdS* genes. Green color denotes the amplification of *nifH* gene and red color denotes amplification of *acdS* gene, whereas X indicates that no amplification occur.

Table 4.5: Results of *nifH* and *acdS* genes amplification with different sets of primers.

Name of Strain	*nif*H gene (PolF / PolR)	*nif*H gene (nifHF / nifHI)	*nif*H gene (nifHfor / nifHrev)	*acdS* gene (F1936f / F1938r)	*acdS* gene (F1936 / F1939r)	*acdS* gene (F1937f / F1939r)
NCCP-601	–	–w	–w	–w	–w	–w
NCCP-602	–w	–w	–w	–w	–w	–w
NCCP-605	–w	–w	+	–w	+m	–w
NCCP-606	–w	ND	+	–w	–w	–w
NCCP-607	+m	ND	+m	–w	–w	–w
NCCP-611	+m	–w	+	–w	+m	+m
NCCP-614	–w	–w	+m	–w	–w	+m
NCCP-621	–w	+m	+	–w	–w	–w
NCCP-622	+	–w	–w	+m	–w	–w
NCCP-624	–w	–w	–	–w	–w	–w
NCCP-625	+	+m	–w	–w	–w	–w
NCCP-635	+m	ND	–w	+m	–w	–w
NCCP-644	–w	–w	–w	–w	–w	–w
NCCP-645	–w	–w	–	–	–w	–w
NCCP-646	–w	ND	–w	+m	–w	–w
NCCP-647	–w	–w	–	–w	–w	–w
NCCP-650	+m	+m	–	+m	+m	+m
NCCP-651	–	–w	–	–w	–w	–w
NCCP-652	–w	–w	–w	–w	–w	–w
NCCP-655	ND	+m	+	–w	–w	–
NCCP-660	+	ND	ND	–	–w	+m
NCCP-661	+m	–w	–	–	–w	–w
JCM 10833	+	–w	–w	+m	–w	–w
JCM 21823	+	–w	–w	–w	–w	–w

ND, not determined; +, PCR product of the expected size; -, no PCR product; -w, no expected PCR products, only products of unexpected and nonspecific size; +m, expected PCR products plus other products of unexpected and nonspecific size.

Evaluation of heavy-metal tolerant strains for growth promotion of *Brassica* plants

The selected heavy-metal tolerant strains were tested for growth promotion in *Brassica napus* irrigated with water containing 50 ppm of each heavy metal separately under axenic condition. The data showed that the effects of strains, heavy metals and the interaction of the two were significant on shoot fresh weight and shoot lengths. Different heavy metals have significant effect on plant growth such as lead nitrate, cadmium nitrate and copper sulfate have positive effect whereas sodium arsenate and potassium dichromate have negative effect on plant growth. The reason could be due to the presence of nitrate in the salts of lead nitrate and

cadmium nitrate. The results also showed (Figure 4.5) that all the strains used in this study, played significant role in increasing shoot fresh weight and shoot length of *Brassica* plants compared to control (no addition of strain or metal), when irrigated with water containing 50 ppm of Pb or Cd (Figure 4.5a & 4.5b). However, application of water containing Cr or As salts significantly reduced plant growth and no positive response was observed in plants irrigated with water containing 50 ppm of As, because shoot length and shoot fresh weight was quite negligible in comparison to other heavy metals treatment. Among the strains, NCCP-650^{T} proved to be

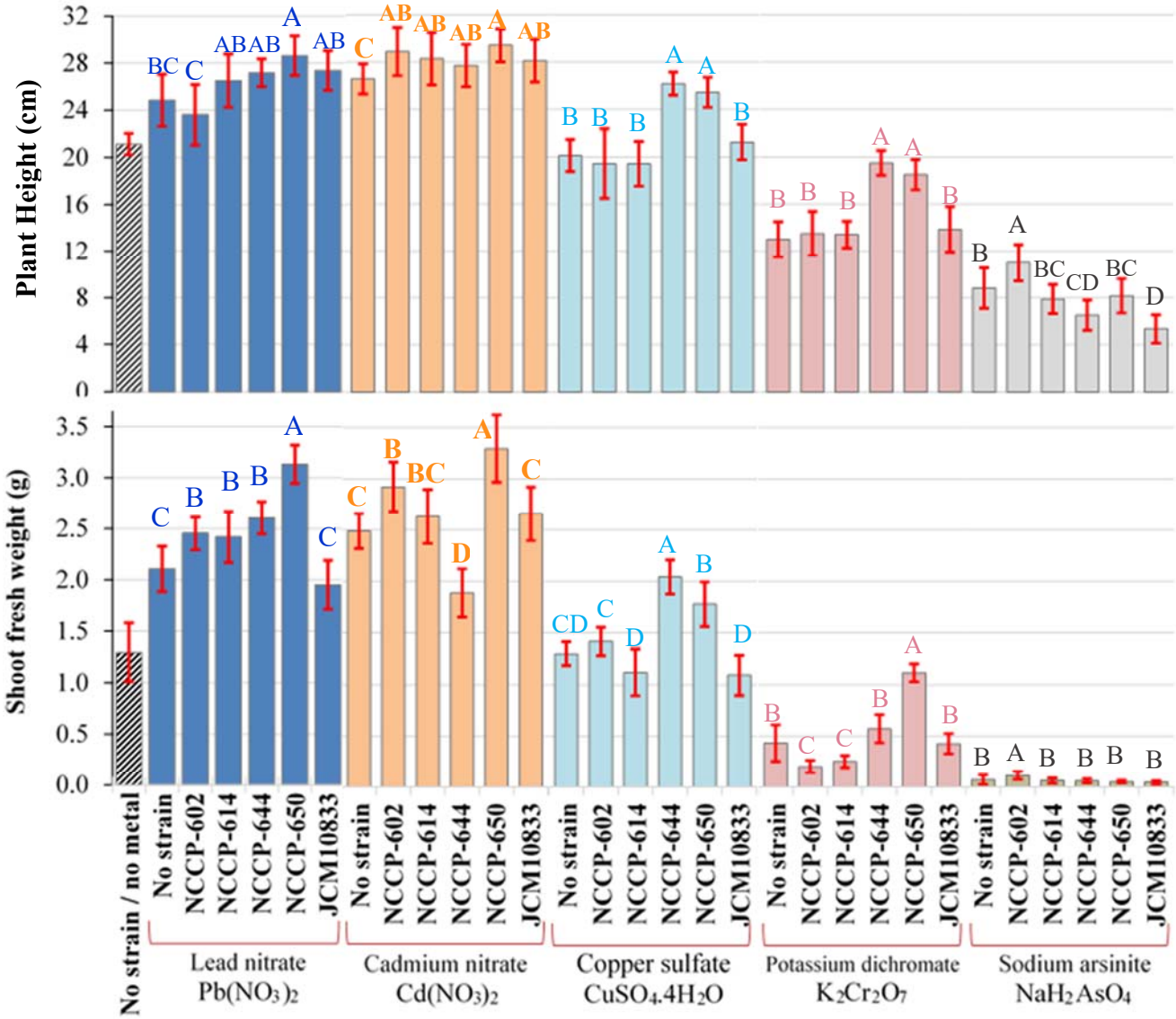

Figure 4.5 (a). Effect of selected heavy-metals tolerant PGPR strains on growth promotion of *Bassica napus* irrigated with heavy-metal (50 ppm) containing water. Bar s of same color in each parameter carrying the same alphabet letters are statistically non-significant (*p* 0.05).

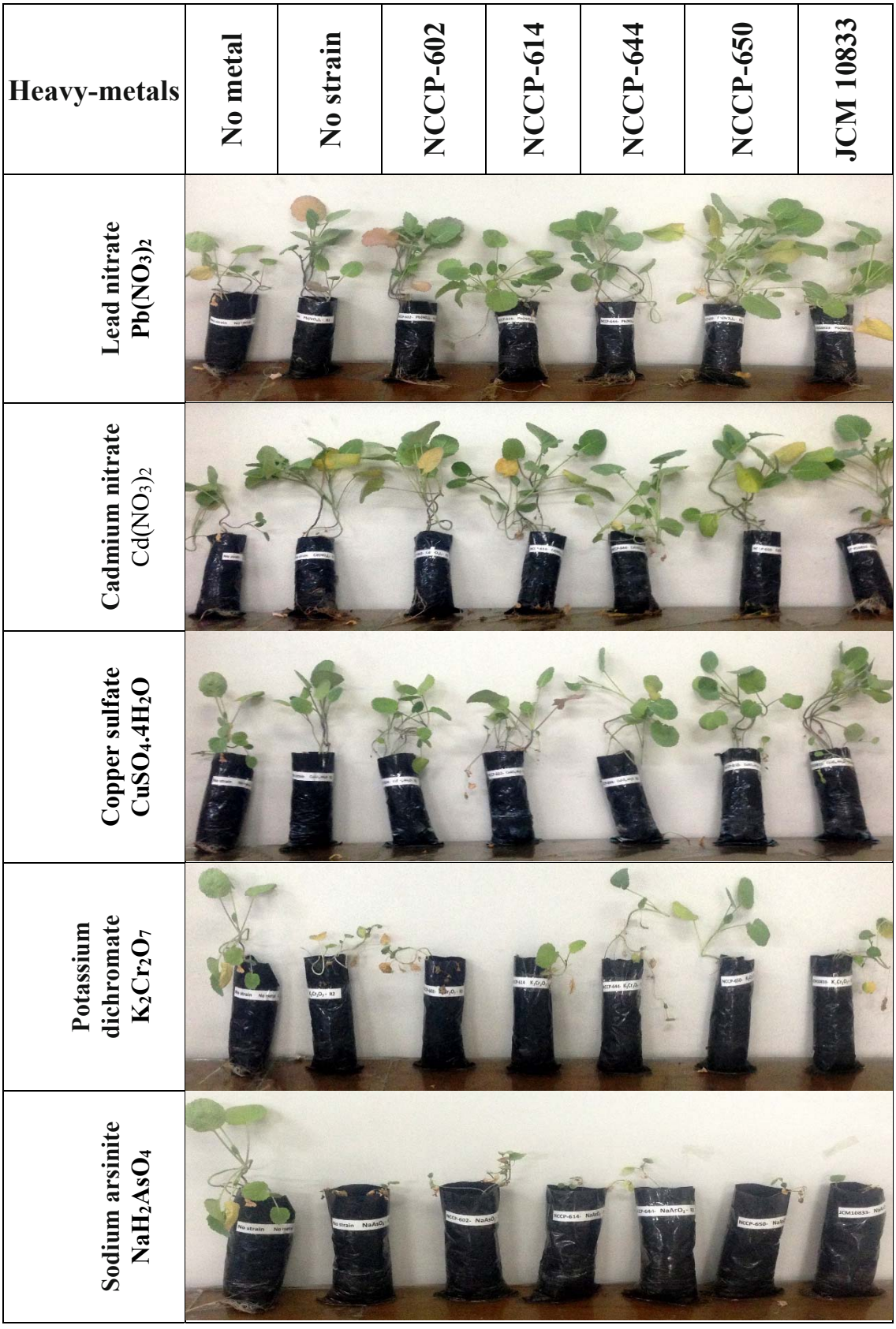

Figure 4.5 (b). Picture showing the effect of selected heavy-metals tolerant PGPR strains on growth promotion of *Bassica napus* irrigated with heavy-metal (50 ppm) containing water.

the best for increase in growth of *Brassica* plants under Pb, Cd and Cr stresses, whereas NCCP-602 played a positive role under As stress. Strain NCCP-650^T belongs to a new species, *Alcaligenes pakistanensis* (Abbas et al. 2015a) and many strains of this genus are also reported as having PGPR activity (Duca et al. 2014). Strain JCM 10833 (*Bradyrhizobium japonicum*) did not show significant role in plant growth improvement as a positive control under heavy metal stress condition. The reason could be that this strain is sensitive to heavy-metals stress and cannot survive. This is in agreement with the recent studies using this strain under heavy-metals stress.

DISCUSSION

Biodiversity of heavy metal resistant bacterial communities from industrial discharge were examined using standard techniques. The initial chemical analysis pointed slight high pHs with ample amount of heavy metal pollution. The discharge sample values exceeded the threshold values described by various environmental protecting agencies. Nevertheless, the polluted samples contain various bacterial communities that have tendency to biosorb various heavy metals. The primary characterization shows different bacterial communities with various shapes and colonies. The composition of waste help bacterial cells to cope with and adapt to external conditions (Mitchell and Kogure 2006). In our study, we found different bacterial communities according to the composition of industrial wastes. The analysis indicated eighty different strains on the basis of phenotypic characterization. However, the phenotypic characterization cannot be used for direct comparison of different strains (Fritze 2002).

Molecular characterization was performed with 16S rRNA sequencing that shows the identity of each strain. In our study, we observed dominant genera as *Bacillus* followed by *Pseudomonas*, *Staphylococcus*, *Alcaligenes*, *Citrobacter* and *Enterobacter*. Among the isolated population, at least three strains have been found as a candidate novel species, which were characterized to propose new names: *Alcaligenes pakistanensis* NCCP-650^T (Abbas et al. 2015a), *Bacillus malikii* NCCP-662^T (Abbas et al. 2015b) and *Acinetobacter pakistanensis* NCCP-644^T (Abbas et al. 2014). Bestawy et al. (2013) identified *Enterobacter*, *Stenotrophomonas, Providencia, Comamonas, Delftia and Ochrobactrum* as dominant genera from activated industrial effluent sludge in Egypt. Gram positive and Gram negative bacteria's have a very strong anionic cell wall. This anionic cell wall allows bacteria to bind

the metal for nucleation (Kelly et al. 2004). The isolated strains showed good MTL against Cr, Cu, Cd, Pb and As. The tolerance limit of bacteria against a particular metal gradually increased over time and bacterial generations produced after a certain time show good resistance against metal concentration and can be used in decontamination purposes (Kelly et al. 2003). The MTL follows the order of Cr > Cu > Cd > Pb > As, however the MTL values always varies in relation to strain type and evaluation parameters during study.

The heavy metal resistant bacteria can play important role in bioremediation of contaminated soil/water environment by showing their resistance to heavy metal stresses and also by reducing their toxicity in contaminated soil surroundings (Filali et al. 2000). In recent years, biosorption has been considered to be a safe and cost effective process for the removal of heavy metals from different solutions. The major advantage of biosorption includes efficient removal of heavy metals from different mediums in comparison to conventional methods. The effluents are in order of only few ppb of residual metals (Volesky 1999). The genetic makeup and long term exposure of bacterial strains to these heavy metals might develop resistance against potential toxicity and adverse effects (Lim and Aris 2014). Previous studies pointed that *Bacillus* sp. JDM-2-1 could tolerate Cr^{+6} (4800 µg/mL) and *S. capitis* could tolerate Cr^{+4} (2800 µg/mL). Similarly, these strains were able to resist Cd^{+2} (50 µg/mL), Cu^{+2} (200 µg/mL), Pb^{+2} (800 µg/mL), Hg^{+2} (50 µg/mL) and Ni^{+2} (4000 µg/mL) (Zahoor and Rehman 2009). In another study, *Pseudomonas aeruginosa* could tolerate Pb^{+2} (650 µg/mL), Cu^{+2} (200 µg/mL), Cd^{+2} (50 µg/mL), Zn^{+2} (50 µg/mL), Ni^{+2} (550 µg/mL) and Cr^{+6} (100 µg/mL) (Rehman et al. 2008). These bacterial isolates have been reported previously to exhibit high resistance to heavy metals by Roane et al. (2001).

The heavy metal tolerance of the populations isolated in this study may have been acquired by adaptation, a genetically altered tolerance, or to a shift in species composition, where organisms already tolerant became more competitive (Li et al. 2006). Previous studies have mentioned the peculiar characteristics of some members of this genus for antibiotic resistance and Cd reduction (Chien et al. 2007). Our results regarding Cd reduction with *Stenotrophomonas* sp. was well supported with previous study of Chien et al. (2007) indicated that *Stenotrophomonas* sp. isolated from heavy metal contaminated soil showed a much higher tolerance to heavy metals than those obtained from culture collections (Chien et

al. 2007). Previous study reported that *Staphylococcus* sp. have capacity to biosorb Cu^{+2} but its efficiency is directly proportional to concentration of Cu in the medium (Andreazza et al. 2011; Stanley and Ogden 2003).

Heavy metal resistant bacteria belonging to different genera such as *Pseudomonas, Mycobacterium*, *Agrobacterium, Arthrobacter*, *Achromobacter*, *Sphingomonas and Microbacterium* have been found to potentially promote plant growth and reduce stress symptoms in plants (Abou-Shanab et al. 2007; Jiang et al. 2008; Ma et al. 2009). Some rhizobacteria have ability to reduce the toxicity of heavy metals, resulting in the stimulation of plant growth. In our study, the selected strains promoted the growth of *Brassica* in heavy metal contaminated soil. The results are much promising for Pb, Cd, Cr and Cu. These results are in agreement with the previous study of Belimov et al. (2005). They isolated and characterized Cd-tolerant bacteria associated with the roots of the metal accumulating plant *Brassica juncea* L. Czern. grown in heavy metal contaminated soils, making the selection of PGPR strains which might be useful to increase plant biomass production under unfavorable environmental conditions. He et al. (2009) characterized the Cd-resistant bacteria and investigated their plant growth promotion potential. They observed that Cd and Pb uptakes in Cd-hyperaccumulator tomato plants grown in heavy metal contaminated soil, improved the efficiency of phytoremediation of Cd-contaminated soils. On the contrary, the isolated strains did not promote the growth of plants in As contaminated soil, since As is considered as very toxic to plants and microorganisms. The reduced growth might be due to low production of siderophores, which in turn restricted the movement of As in the soil. Production of siderophores are necessary for mobilization of As in the soil together with iron ions, which rendered As more soluble and bioavailable to plants (Drewniak et al. 2008; Wang et al. 2011).

Conclusions

Limited research work has been reported on identification of novel species of bacteria from Pakistani ecology. There is also very less number of reports on biosorption of heavy metals by heavy-metals tolerant bacterial strains from Pakistan. The strains isolated in our studies were found to be highly tolerant to Cd, Cu, Pb and As. Some strains were found to be more tolerant to Cd, Pb, Cu and As compared to previous reports. Our results indicated that two isolates (NCCP-614 and NCCP-655) resulted more than 90 % and 59 % biosorption of

Pb and Cd, respectively, whereas another three isolates (NCCP-625, NCCP-619 and NCCP-647) did more than 36 % biosorption of Cu. These isolates can be used for bioremediation of soil/water system contaminated with Pb, Cd and Cu. Phylogenetic identification of these heavy-metals tolerant strains based on 16S rRNA gene sequence data showed that at least three strains belonged to novel species, which can be characterized by polyphasic taxonomy. Molecular characterization based on *nifH* and *acdS* genes indicated that at least 7 strains contained both of these genes and these strains have the potential for plant growth promotion and can be used as bioinoculants (biofertilizer) in agriculture. Among these, strain NCCP-650^T, which contained both the genes, was found to significantly increase growth of *Brassica napus* in greenhouse experiment, when irrigated with water contaminated with heavy-metals. This strain also reported to contain nitrite reductase, *nirK* gene, which has its significance in bioremediation process too. These novel taxa would also be a source of new genes involved in metals transport / tolerance mechanisms. The potential of these strains for bioremediation and their PGPR activity under stress condition would be important for agriculture.

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CHAPTER V

Heavy metal-tolerant and psychrotolerant bacterium *Acinetobacter pakistanensis* sp. nov. isolated from a textile dyeing wastewater treatment pond

ABSTRACT

A Gram-stain negative, cocci to short rod, non-motile, strictly aerobic, heavy metal-tolerant and psychrotolerant bacterium, designated NCCP-644^T, was isolated from a textile dyeing wastewater treatment pond. The optimum (and ranges of) temperature and pH for growth of strain NCCP-644^T was 20-25°C (3-37°C) and 7–8 (6-10), respectively. The strain can tolerate 0-3 % NaCl (w/v) and heavy metals (Cr 2700 ppm, As 3000 ppm, Pb 2100 ppm and Cu 2700 ppm) in tryptic soya agar medium. The sequences of the 16S rRNA gene and three housekeeping genes, *gyrB, rpoB* and *atpD*, of strain NCCP-644^T showed the highest similarity (98.3, 91.9, 95.3 and 96.6 %, respectively) with the strain "*Acinetobacter kyonggiensis*" KSL5401-037^T. Phylogenetic analyses from the sequence of the16S rRNA, *gyrB* and *rpoB* genes also revealed the affiliation of NCCP-644^T with members of the genus *Acinetobacter*. The chemotaxonomic data [major quinones as Q-9; major cellular fatty acids as summed feature 3 (iso-$C_{15:0}$ 2OH/$C_{16:1}$ ω7*c*) followed by $C_{18:1}$ ω9c, $C_{16:0}$, and $C_{12:0}$ 3-OH; major polar lipids as diphosphatidyl glycerol, phosphatidyl serine, phosphatidyl monomethyl ethanol, phosphatidyl inositol, phosphatidyl ethanolamine, phosphatidyl glycerol and one unknown phospholipid] also support the affiliation of strain NCCP-644^T with the genus *Acinetobacter*. The level of DNA–DNA relatedness between strain NCCP-644^T and the closely related type strains of the genus Acinetobacter was < 54.7 %. Based on the phylogenetic analyses, DNA–DNA hybridisation and biochemical characteristics, strain NCCP-644^T differed from the validly named *Acinetobacter* species and thus, it represents a new species. The name, *Acinetobacter pakistanensis* sp. nov.is proposed for the type strain NCCP-644^T (= LMG 28467^T = KCTC 42081^T = JCM 18977^T).

Keywords:

Heavy metal tolerant, psychrotolerant, textile dyeing wastewater, *Acinetobacter pakistanensis*

INTRODUCTION

Brisou and Prévot (1954) proposed the genus *Acinetobacter* with the type species *A. calcoaceticus* in the family *Moraxellaceae*, and it comprises thirty three validly named species (Euzéby 2014). Furthermore, there are seven other species, "*A. marinus*", "*A. seohaensis*" (Yoon et al. 2007), "*A. septicus*" (Kilic et al. 2008), "*A. antiviralis*" (Lee et al. 2009), "*A. kyonggiensis*" (Lee and Lee 2010), "*A. oleivorans*" (Kang et al. 2011) and "*A. oryzae*" (Chaudhary et al. 2012), which have been published but yet not validated. Recently, three new species, *A. guangdongensis, A. bohemicus* and *A. apis* have been published (Feng et al. 2014; Kim et al. 2014; Krizova et al. 2014) but these are also still not validated names. Although several members of the genus *Acinetobacter* were isolated from clinical specimens and received; therefore, much higher interest, a majority of the members isolated from diverse environmental habitats, such as activated sludge, wetlands, forest soil, seawater, dump sites, wastewater, cotton, cankar bark, floral nectar and wild rice (Álvarez-Pérez et al. 2013; Carr et al. 2003; Choi et al. 2013; Lee and Lee 2010; Li et al. 2014a; Li et al. 2014b; Li et al. 2013; Malhotra et al. 2012; Nishimura et al. 1988; Vaz-Moreira et al. 2011; Yoon et al. 2007). This genus consists of aerobic, Gram-negative coccobacillary rods, non-motile and non-fermentative bacteria. Many of these species can survive in a wide range of environmental conditions for a long period. Several of these organisms can grow over a wide temperature range on typical culture medium. Recently, *Acinetobacter harbinensis* has been reported to grow at temperatures as low as 2ºC (Li et al. 2014a). Several other *Acinetobacter* strains also identified from frozen food, therefore, these species attract particular interest.

Several microorganisms tolerate toxic concentrations of heavy metals (Affan et al. 2009; Tripathi et al. 2010; Tripathi et al. 2011; Zahoor and Rehman 2009). These heavy metal-tolerant bacteria offer an opportunity to exploit in the bioremediation of contaminated soil/water systems. During our studies of microbial diversity for heavy metal-tolerant bacteria, strain NCCP-644^{T} was isolated from a textile dyeing wastewater sample collected from a wastewater treatment pond. The objective of this study is to delineate the taxonomic position of the strain by phenotypic, chemotaxonomic and phylogenetic characterization. Based on the

results, it is concluded that strain NCCP-644^T represents a new species of the genus *Acinetobacter*.

MATERIALS AND METHODS

Isolation and growth of the strains

Strain NCCP-644^T was isolated from a wastewater sample collected from the treatment pond of textile dyeing wastewater plant of Kohinoor mills, Islamabad, Pakistan. The strain was recovered on tryptic soy agar (TSA, Difco) supplemented with different concentrations of heavy metals, including Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2} using the dilution plate method. The purified strain was maintained on agar medium and stored in glycerol (35 %, w/v) at –80ºC and used for the phenotypic and phylogenetic characterization experiments. Based on the sequence similarity of the 16S rRNA gene, the type strains of the closely related taxa "*Acinetobacter kyonggiensis*" JCM 17071^T, *A. harbinensis* KCTC 32411^T, *A. bouvetii* JCM 18991^T, *A. beijerinckii* JCM 18990^T, and *A. johnsonii* JCM 20194^T and the type species of the genus *A. calcoaceticus* JCM 6842^T were used as reference strains for these characterization experiments under the same laboratory conditions. The characterization experiments were performed at 25ºC unless otherwise mentioned.

Heavy metals tolerance

To determine the tolerance of the novel strain to toxic concentrations of heavy metals, the isolated strain NCCP-644^T and the reference strains were grown on TSA supplemented separately with different concentrations of the heavy metals Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2} for five to seven days. The concentration of the heavy metals Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2} in the agar media ranged from 300-3000 ppm and were prepared using the salts $K_2Cr_2O_7$, Na_2HAsO_4, $Pb(NO_3)_2$ and $CuSO_4.5H_2O$, respectively.

Morphology and phenotypic characterization

Colony morphology of strain NCCP-644^T was observed on well-isolated colonies, grown on TSA for 2 days. A phase-contrast microscope (Nikon Optiphot-2, Japan) was used to examine the cells of strain NCCP-644^T grown on TSA for 24-48 h. Gram staining was

performed using a commercial kit (bio-Me´rieux, France) according to manufacturer's instructions. The motility of strain NCCP-644^T was determined with M medium (bioMérieux, France) and microscopy. The oxygen dependence was determined on TSA by incubating the cells in an anaerobic chamber (Mitsubishi Gas Chemicals Co., Inc.) for 10 days.

The optimum and range of pH for growth was determined in tryptic soy broth (TSB; Difco) by adjusting the pH to a range of 4.0 to 10.0 at an increment of 1 pH unit, and the OD_{600} was monitored using a spectrophotometer (Beckman Coulter Model DU730, USA). The pH values were adjusted with HCl or Na_2CO_3 and buffers (Sorokin 2005) and were verified after autoclaving. The temperature range for growth was determined on TSA (pH 7.0) by incubating the cells at different temperatures (3, 5, 10, 15, 20, 25, 30, 33, 37, 40, 45 and 50ºC) for 6 days. Growth in various NaCl concentrations was investigated in mTGE agar medium (Difco), which contains (per litre) beef extract (6 g), tryptone (10 g), dextrose (2 g), and agar (15 g), supplemented with various concentrations of NaCl (0–10 %; w/v), with the pH adjusted to pH 7.0 and incubation for 6 days.

The physiological and biochemical characteristics were determined using the API 20E, API 20NE and API 50CH galleries (bioMérieux, France). Because negative reactions were primarily obtained with the API 50CH and API 20E for the utilization of various carbon sources, we also analyzed an extended array of biochemical features of the strains using the API Rapid 32 ID system (bioMérieux, France). The catalase and oxidase activities were determined using the API Color Catalase and API Oxidase Reagent (bioMérieux, France). Antibiotic resistance was assessed with an ATB-VET strip (bioMérieux, France), and the enzyme activities were determined with an API ZYM strip (bioMérieux, France). The API suspension medium was used to inoculate the strips. All commercial kits were used according to the manufacturers' protocols, except for the API Rapid 32 ID, for which the strains were grown aerobically on TSA at 25ºC rather than anaerobic growth on blood agar media.

Amplification, sequencing and phylogenetic analysis of 16S rRNA and housekeeping genes, *gyrase subunit B (gyrB), RNA polymerase beta subunit (rpoB), translation initiation factor IF-2 (infB)* and *ATP synthase beta subunit (atpD)*

A nearly complete 16S rRNA gene was amplified as previously described (Ahmed et al. 2007). The housekeeping genes *gyrB, atpD* and *infB* were amplified using the primers and PCR conditions described previously (Brady et al. 2008), whereas the *rpoB* gene was amplified and sequenced **(Table 5.1)** according to the method of La Scola et al. (2006). The purified PCR product was sequenced at Macrogen, Korea (http://dna.macrogen. com/eng) using universal primers for the 16S rRNA gene and internal primers for the housekeeping genes as described above (Brady et al. 2008; La Scola et al. 2006). The sequences were assembled using the BioEdit software to obtain a consensus sequence of the genes, which were then submitted to the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/).

Table 5.1. Amplification and sequencing primers for *gyrB, rpoB, atpD* and *infB* genes

S. No.	Target genes	Primer pair for amplification	Sequence	Annealing temp (°C)	Expected amplicon size of target gene
1.	*gyrB*	gyrB 01-F (forward) gyrB 02-R (reverse)	5'- TAARTTYGAYGAYAACTCYTAYAAAGT - 3' 5'- CMCCYTCCACCARGTAMAGTT - 3'	55 °C	742 bp
2.	*atpD*	atpD 01-F (forward) atpD 02-R (reverse)	5' – RTAATYGGMGCSGTRGTNGAYGT - 3' 5' - TCATCCGCMGGWACRTAWAYNGCCTG - 3'	55 °C	657 bp
3.	*infB*	infB 01-F (forward) infB 02-R (reverse)	5' – ATYATGGGHCAYGTHGAYCA - 3' 5' – ACKGAGTARTAACGCAGATCCA - 3'	55 °C	615 bp
4.	*rpoB*	rpoB CM7-F rpoB CM31b-R	5'- AACCAGTTCCGCGTTGGCCTG - 3' 5'- CCTGAACAACACGCTCGGA - 3'	55 °C	
5.	*rpoB*	Ac696F (forward) Ac1093R (reverse)	5'- TAYCGYAAAGAYTTGAAAGAAG - 3' 5'- CMACACCYTTGTTMCCRTGA - 3'	48 °C	350 bp
6.	*rpoB*	Ac1055F (forward) Ac1598R (reverse)	5'- GTGATAARATGGCBGGTCGT 3263 - 3' 5'- CGBGCRTGCATYTTGTCRT - 3'	48 °C	450 bp
7.	*rpoB*	Ac696F (forward) Ac1598R (reverse)	5'- TAYCGYAAAGAYTTGAAAGAAG - 3' 5'- CGBGCRTGCATYTTGTCRT - 3'	48 °C	700 bp

The strain was identified using the sequence of the 16S rRNA gene on the Ez-Taxon Server (http://eztaxon-e.ezbiocloud.net) and a BLAST search of the housekeeping genes on the DDBJ/NCBI servers. The 16S rRNA gene sequences of closely related validly published type strains were retrieved from the EzTaxon Server database and phylogenetic trees were constructed as described previously (Ahmed et al. 2014) using three algorithms, the maximum parsimony (MP), neighbor-joining (NJ) and maximum likelihood (ML) methods. The sequence similarities of the housekeeping genes *gyrB, rpoB* and *atpD* were estimated with the closely related available sequences using the Kimura 2-parameter model. The phylogenetic trees were constructed using nucleotide sequences of the *gyrB* and *rpoB* genes with the sequences of related species of the genus *Acinetobacter*. The stability of the relationship was assessed using bootstrap analysis with 1,000 re-samplings for the tree topology.

DNA base composition, DNA–DNA hybridization

For the DNA G+C content analysis and DNA–DNA hybridization, the genomic DNA of strain NCCP-644^T and the reference strains were isolated using a Qiagen Genomic-tip 500/G following the manufacturer's protocol, with a minor modification in which the RNase T1 was used in addition to the RNase A. The DNA–DNA hybridization of strain NCCP-644^T and the reference strains "*A. kyonggiensis*" JCM 17071^T, *A. harbinensis* KCTC 32411^T, *A. bouvetii* JCM 18991^T, *A. beijerinckii* JCM 18990^T, and *A. johnsonii* JCM 20194^T was performed at 45 ºC with photobiotin-labelled DNA and microplates as described by Ezaki et al. (1989). A Fluoroskan Ascent Plate Reader (Thermo Lab Systems, USA) was used for the fluorescence measurements.

To determine the DNA G+C contents, the genomic DNA was digested with P1 nuclease and alkaline phosphatase. The DNA G+C contents were analyzed on an HPLC (model UFLC, Shimadzu, Japan) at 270 nm using solvent $NH_4H_2PO_4$ (0.02 M) - CH_3CN (v/v 20:1) with a Cosmosil 5C18 column (4.6 by 150 mm; Nacalai Tesque; reversed phase silica gel; C18).

Chemotaxonomic analyses

For whole-cell fatty acid analysis, strain NCCP-644^T and the reference strains were grown on TSA for 24 h. The cellular fatty acid methyl esters were prepared (Sasser 1990) and analysed on a GC (6890; Hewlett Packard) according to the standard protocol of the Sherlock Microbial Identification System (MIDI Sherlock version 4.5, MIDI database TSBA40 4.10). The respiratory quinones and polar lipids of strain NCCP-644^T and the closely related reference strain "*A. kyonggiensis*" JCM 17071^T were analyzed from 150–200 mg lyophilized cells grown in TSB for 24 h as described by Minnikin et al. (1984). Isoprenoid quinones were purified by TLC and then analyzed by HPLC. The polar lipids were examined by two-dimensional TLC, using HPTCL plates (10 × 10 cm) Silica gel 60 (Merck), as described by Kudo (2001).

RESULTS AND DISCUSSION

Strain NCCP-644^T formed off-white colonies, which are moist and raised with entire margins. The colonies are 1–2 mm in size after 1–2 days when grown on TSA (Difco) medium at 25 °C. Old colonies (10 days) may become bigger (3-4 mm). Cells of the strain are Gram-stain negative, non-motile, strictly aerobic and cocci to short rod (coccobacillus), which occur primarily in pairs and sometimes in triplet form. The optimum growth of cells was at pH 7.0-8.0 (range of 6.0–10.0). Strain NCCP-644^T is sensitive to NaCl and can tolerate only up to 0–3 % NaCl (w/v) (optimum without NaCl) in TSB (Difco) medium. The strain can grow at 3–37 ºC (optimum 20–25 ºC). No growth was observed at 40 ºC and there was a slight growth at 37 ºC after many days. Strain NCCP-644^T exhibited many phenotypic features that were similar to that of closely related taxa "*A. kyonggiensis*" JCM 17071^T, *A. harbinensis* KCTC 32411^T, *A. bouvetii* JCM 18991^T, *A. beijerinckii* JCM 18990^T, *A. johnsonii* JCM 20194^T and *A. calcoaceticus* JCM 6842^T; but many biochemical and physiological characteristics also differentiated these species **(Table 5.2)**. Of these, the most notable differentiating

Table 5.2. Differentiating phenotypic and biochemical characteristics of strain NCCP-644^T and the type strains of closely related *Acinetobacter* species

All strains were negative for the production of acid from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, Methyl-*β*D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, amygdalin, arbutin, esculin, salicin, D-celiobiose, D-maltose, D-lactose, D-melibiose, D-saccharose (sucrose), D-trehalose, inulin, D-melezitose, D-raffinose, amidon (starch), glycogen, xylitol, gentiobiose, D-furanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate.

All strains were positive for catalase but negative for oxidase, *β*-galactosidase (2-nitrophenyl-*β*D galacto pyranoside), arginine dihydrolase, lysine and ornithine decarboxylases, urease, tryptophan deaminase, indole production, H_2S production, and hydrolysis of esculin and gelatin. No oxidation/fermentation of D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin, and L-arabinose was observed. All strains were positive for the assimilation of malate but negative for glucose, arabinose, mannose, mannitol, maltose and potassium gluconate.

High enzyme activity was observed in all strains for leucine arylamidase, esterase lipase (C 8), valine arylamidase, esterase (C 4), arginine arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase, whereas no enzyme activity was observed for trypsin, α-chymotrypsin, α- galactosidase, *β*-galactosidase, *β*-glucoronidase, α-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase, α-fucosidase, α-galactosidase, ß-galactosidase, ß-galactosidase, A-glucosidase, ß-glucosidase, A-arabinosidase, ß-glucuronidase, glutamic acid decarboxylase, α-fucosidase and pyroglutamic acid arylamidase.

All strains were resistant to the antibiotics (μg mL^{-1}) penicillin (0.25), cephalothin (8), cefoperazon (4), lincomycin (2), pristinamycin (2), tylosin (2) and metronidazol (4) but sensitive to the antibiotics streptomycin (8), spectinomycin (64), kanamicin (8), gentamicin (4), apramycin (16), doxycyclin (4), colistin (4), cotrimoxazol (2/38), flumequin (4), oxolinic acid (2) and enrofloxacin (0.5).

+++, Very strongly positive; ++, strongly positive; +, Positive; w+, weakly positive; –, negative; nd, no found. R, resistant to the antibiotic; wR, weakly resistant; S, sensitive.

	NCCP-644^T	"*A. kyonggiensis*" JCM 17071^T	*A. harbinensis* KCTC32411^T	*A. bouvetii* JCM 18991^T	*A. beijerinckii* JCM 18990^T	*A. johnsonii* JCM 20194^T	*A. calcoaceticus* JCM 6842^T
Growth at:							
Temperature (ºC) range, (optimum)	3-37 (20-25)	5-30 (20-25)	2-35 (8-20) †	5-37 (20-30)	5-40 (25-33)	10-33 (20-30)	5-37 (20-30)
pH range (optimum)	6-10 (7-8)	6-9 (7-8)	6-8.5 (7.2) †	6-9 (6-8)	6-10 (7-8)	nd	nd
NaCl range (%), (optimum)	0-3 (0-1)	0-3 (0)	0-4 (0)†	0-3 (0-2)	0-4 (0-2)	0-4 (1-2)	0-2 (0-1)
Tolerance to heavy metals:							
Chromium (ppm)	2700	1200	nd	600	1800	300	300
Arsenic (ppm)	3000	2400	3000	3000	3000	900	1200
Lead (ppm)	2100	900	900	1500	1500	300	900
Copper (ppm)	2700	1200	900	1200	2100	300	300
Utilisation of citrate	–	–	–	–	+	–	–
Nitrate reduction to N_2	+	+	–	+	+	+	+
Voges-Proskauer reaction	+	–	+	+	+	+	+
Acid from N-acetyl glucosamine	–	+	–	–	–	–	–
Assimilation of:							
Capric acid	+	–	+	+	+	+	–
Adipic acid	–	–	–	–	–	–	+
Malate	+	+	–	+	+	+	+
Trisodium citrate	–	–	–	w+	+	+	–
Phenyl acetic acid	–	–	–	–	–	–	w+
Enzyme activity **(API-Zym and Rapid 32 ID)**							
Alkaline phosphatase	+++	++	–	++	w+	w+	+++
Lipase (C 14)	+	+	w+	++	+++	++	w+
Cysteine arylamidase	+	+	w+	+	+	+	w+
Acid phosphatase	+	w+	+	++	++	+	+++

Napthol-As-BI-phosphohydrolase	+++	–	w+	+	++	+	++
Proline arylamidase	+	+	+	–	+	+	+
Glutamyl glutamic acid arylamidase	+	–	–	–	+	–	+
***Resistance to* (μg mL^{-1}) (API-ATB Vet)**							
Amoxycilin (4)	S	R	S	wR	R	R	R
Amox-clav. acid ((4/2)	S	wR	S	wR	wR	R	R
Oxacillin (2)	R	R	R	R	R	R	S
Chloramphenicol (8)	S	S	S	R	R	S	R
Tetracycline (4)	S	S	S	wR	R	S	S
Erythromycin (1)	wR	S	R	R	R	wR	S
Sulphamethizole (100)	S	S	wR	S	S	R	S
Nitrofurantoin (25)	R	R	R	R	R	R	S
Fusidic acid (2)	R	R	R	R	R	R	S
Rifamcin (4)	S	S	wR	S	R	S	S
G+C content, mol %	40.6	41.2–42.1[†]	45.5[†]	43.8[†]	42.0[†]	44.0–45.0[†]	39.0-42.0[†]

#

All data are from this study unless otherwise noted.

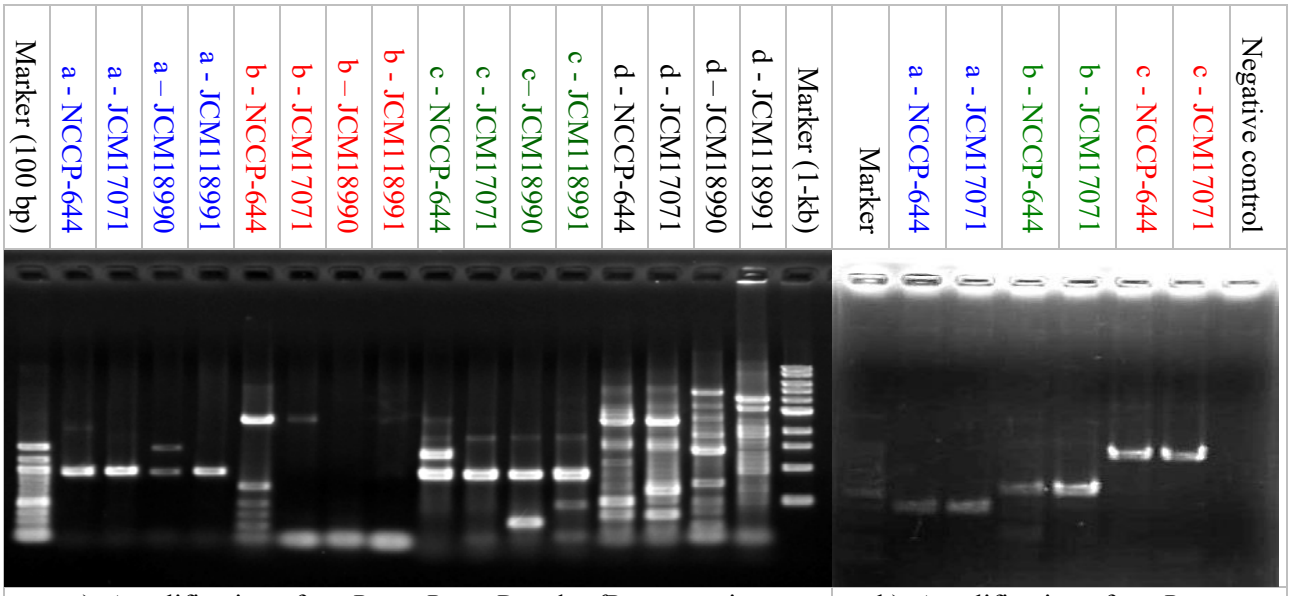
[†] Data from previous studies (Bouvet and Grimont 1986; Lee and Lee 2010; Li et al. 2014a; Nishimura et al. 1987).

characteristics compared to the most closely related species, "*A. kyonggiensis*" JCM 17071^T, are growth of NCCP-644^T at 3 °C, positive reactions for Voges-Proskauer and assimilation of capric acid. Strain NCCP-644^T also differed from the reference strains for tolerance against toxic concentrations of heavy metals (Table 5.2). Strain NCCP-644^T showed growth at 3 °C, which is unusual for other species of the genus *Acinetobacter,* except *A. harbinensis.*

The isolated novel strain NCCP-644^T showed tolerance to toxic concentrations of heavy metals, including Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}. It grew well in TSA medium containing Cr 2700 ppm, As 3000 ppm, Pb 2100 ppm and Cu 2700 ppm. By comparison, the closely related reference strains were also tested for tolerance to heavy metals under similar conditions. It was found that the toxic effects of Cr^{+2}, Pb^{+2} and Cu^{+2} significantly differentiated the NCCP-644^T from the reference species (Table 5.2). However, the majority of the strains in this study tolerated high concentrations of As^{+2}, except "*A. kyonggiensis*" JCM 17071^T and *A. calcoaceticus* JCM 6842^T. By comparison with the previously reported heavy metal-tolerant bacteria (Affan et al. 2009; Tripathi et al. 2010; Tripathi et al. 2011; Zahoor and Rehman 2009), strain NCCP-644^T is considered highly tolerant to toxic concentrations of heavy metals.

Genotypic characterization

Specific amplicons of *gypB* and *atpD* genes were successfully amplified using the primers mentioned in Table 5.1.; however, *rpoB* and *infB* genes could not have been amplified (**Figure 5.1 a**) and only non-specific bands appeared in *infB* gene amplicons. The *rpoB* gene was thus amplified using primers specific to the genus *Acinetobacter* (**Figure 5.1 b**). Comparative sequence analyses of the 16S rRNA, *gyrB, rpoB* and *atpD* genes confirmed the inter species relatedness of strain NCCP-644^T with the closely related type strain "*A. kyonggiensis*" JCM 17071^T and the other described *Acinetobacter* species **(Table 5.3)**. The sequence (1413 nucleotides) of 16S rRNA gene of strain NCCP-644^T was compared with the sequences of closely related type strains on the Ez-Taxon Server



a). Amplification of *gyrB, rpoB, atpD* and *infB* genes using
a. *gyrB* primers No. 1 (amplicon size: 742-bp)
b. *rpoB* primers No. 2 (amplicon size: 558-bp)
c. *atpD* primers No. 3 (amplicon size: 657-bp)
d. *infB* primers No. 4 (amplicon size: 615-bp)
using primer pairs as mentioned in Table 5.1.

b). Amplification of *rpoB* gene using specific primers for the genus *Acinetobacter*:
a. primers No. 5 (amplicon size: 350 bp)
b. primers No. 6 (amplicon size: 450 bp)
c. primers No. 7 (amplicon size: 700 bp)
using primer pairs as mentioned in Table 5.1.

Figure 5.1. Gel pics of amplification of *gyrB, rpoB, atpD* and *infB* genes by PCR

database (http://www.ezbiocloud.net/eztaxon). The highest sequence similarity (98.3 %) of 16S rRNA gene of strain NCCP-644^T was observed with "*A. kyonggiensis*" KSL 5401-034^T (Gen Bank accession no. FJ527818), followed by 97.7 % with *A. harbinensis* KCTC 32411^T (KC843488) and 97.6 % with *A. bouvetii* DSM 14964^T (APQD01000004), whereas the similarity values were 94.3 % (*A. towneri* DSM 14962^T) to 97.5 % (*A. johnsonii* CIP 64.6^T) with the other related members in the genus *Acinetobacter*. The phylogenetic analyses (**Figure 5.2**) showed that strain NCCP-644^T clustered with "*A. kyonggiensis*" KSL 5401-034^T (FJ527818) at high (98 %) bootstrap support. The node of this cluster also appeared with the same species at high bootstrap values, 91 and 88 %, when the phylogenetic trees were reconstructed using the MP and ML algorithms, respectively. This suggested a strong coherence of strain NCCP-644^T with "*A. kyonggiensis*" KSL 5401-034^T (**Figures 5.3 & 5.4**).

The sequence similarity values of the housekeeping genes *gyrB, rpoB* and *atpD* of strain NCCP-644^T were also highest with "*A. kyonggiensis*" JCM 17071^T (92.3, 95.3 and

Table 5.3. Analysis of housekeeping genes *Acinetobacter pakistanensis* NCCP-644^T in comparison with the reference species and their accession numbers published in DNA database (in this study)

Strain Name	Name of housekeeping gene	Number of nucleotides housekeeping gene	Accession number of housekeeping gene	Closely related validly published taxa as per respective gene sequence similarity	Similarity %age of housekeeping gene sequence with closely related species
***Acinetobacter pakistanensis* NCCP-644^T**	***gyrB***	**876**	**AB924048**	***A. kyonggiensis* (AB924049)**	**92.3**
***Acinetobacter pakistanensis* NCCP-644^T**	***rpoB***	**873**	**AB938199**	***A. kyonggiensis* (AB938200)**	**95.3**
***Acinetobacter pakistanensis* NCCP-644^T**	***atpD***	**756**	**AB924051**	***A. kyonggiensis* (AB924052)**	**96.6**
A. kyonggiensis JCM 17071^T	*gyrB*	899	AB924049	*A. pakistanensis* NCCP-644^T (AB924048)	92.0
A. kyonggiensis JCM 17071^T	*rpoB*	887	AB938200	*A. pakistanensis* NCCP-644^T (AB938199)	95.3
A. kyonggiensis JCM 17071^T	*atpD*	744	AB924052	*A. pakistanensis* NCCP-644^T (AB924051)	96.6
A. beijerinckii JCM 18990^T	*atpD*	751	AB924053	*A. oleivorans* (CP002080)	96.0
A. bouvetii JCM 18991^T	*gyrB*	948	AB924050	*A. nosocomialis* (JX523706)	99.0
A. bouvetii JCM 18991^T	*atpD*	727	AB924054	*A. baumannii* (CP006768)	86.0

96.6 %, respectively); however, significantly low similarity values for the *gyrB* gene analysis (82.7 % for *A. harbinensis* KCTC 32411^T to 71.1 % for *A. Bouvetii* CCUG 50766^T) and *rpoB* gene analysis (85.3 % for *A. gerneri* NIPH 2282^T to 76.7 % for *A. soli* CCUG 59023^T) were observed with all other species of the genus. The *atpD* gene of strain NCCP-644^T also showed low sequence similarities (90.3 % with *A. bouvetii* JCM 18991^T and 89.5 % with *A. johnsonii* JCM 20194^T). The NJ phylogenetic trees based on the *gyrB* and *rpoB* genes sequence analyses with the type strains of the *Acinetobacter* species also showed strong coherence of strain NCCP-644^T with "*A. kyonggiensis*" JCM 17071^T with a high bootstrap value (100 %, **Figures 5.4 & 5.5**). This was also confirmed when a phylogenetic analyses was performed on the deduced amino acid sequences of the *gyrB* and *rpoB* genes. Based on some characteristics, the strain NCCP-644^T is closely related

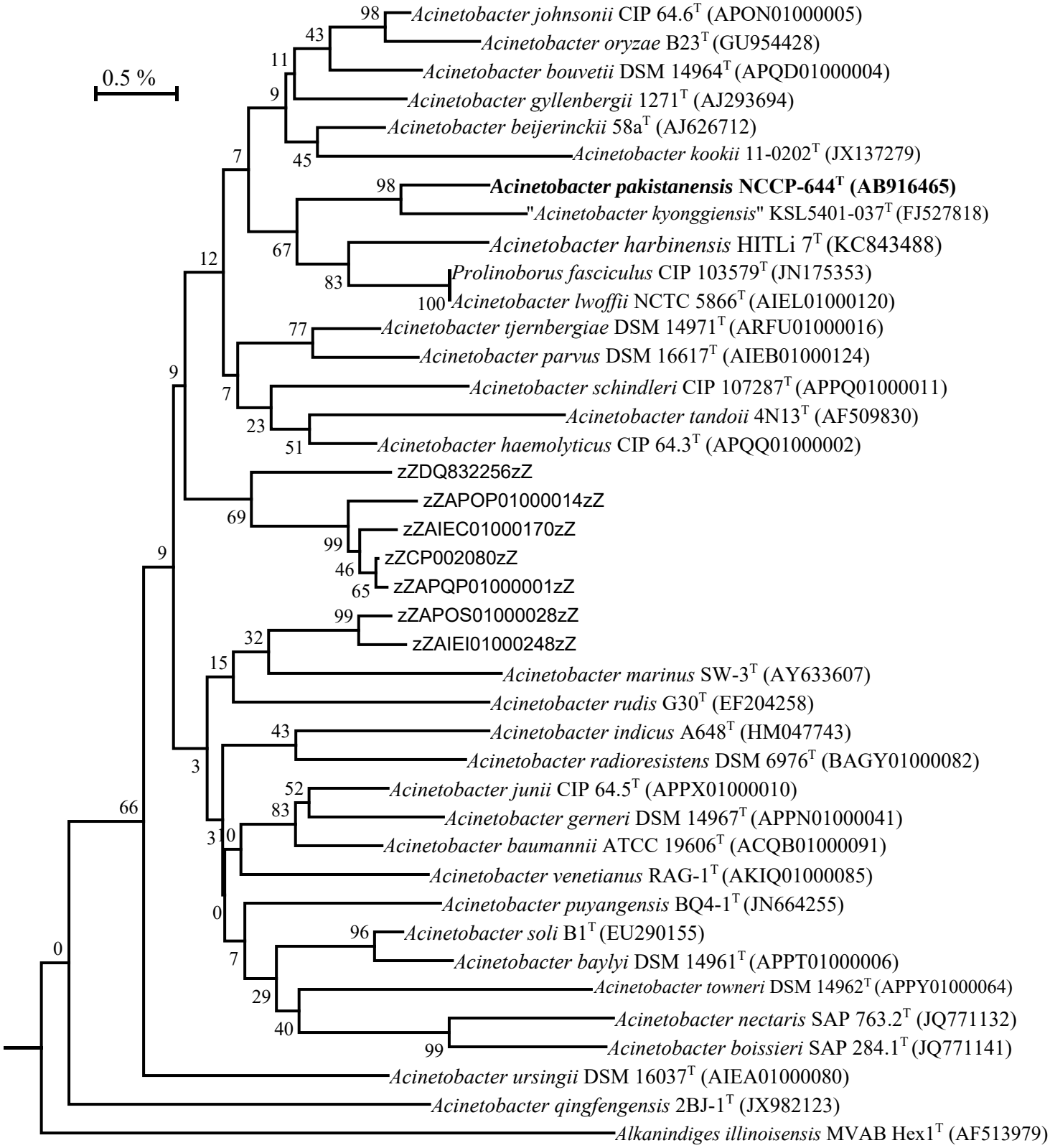


Figure 5.2.

Neighbour-joining phylogenetic tree constructed from sequences of the 16S rRNA gene showing the inter-relationship of strain NCCP-644^T with the type strains of genus *Acinetobacter*, which is based on a comparison of 1232 nucleotides and is rooted using *Alkanindiges illinoisensis* MVAB Hex1^T (AF513979) as an out-group. The bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The accession number of each type strain is shown in the parentheses.

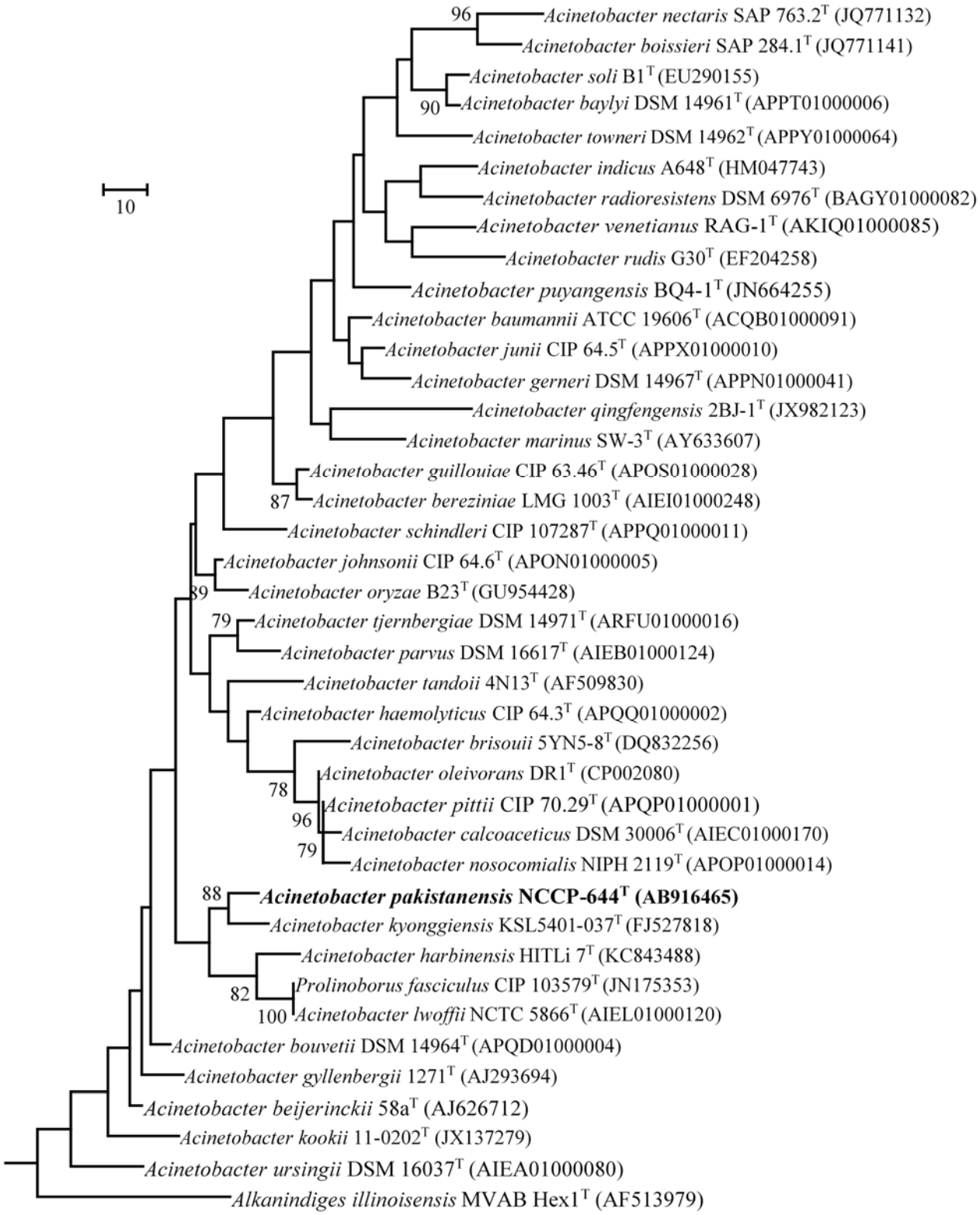


Figure 5.3.

Maximum parsimony phylogenetic tree inferred from the sequences of the 16S rRNA gene showing the inter-relationship of strain NCCP-644^T with the type strains of genus *Acinetobacter*, which is based on a comparison of 1232 nucleotides and is rooted using *Alkanindiges illinoisensis* MVAB Hex1^T (AF513979) as an out-group. The bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The accession number of each type strain is shown in the parentheses.

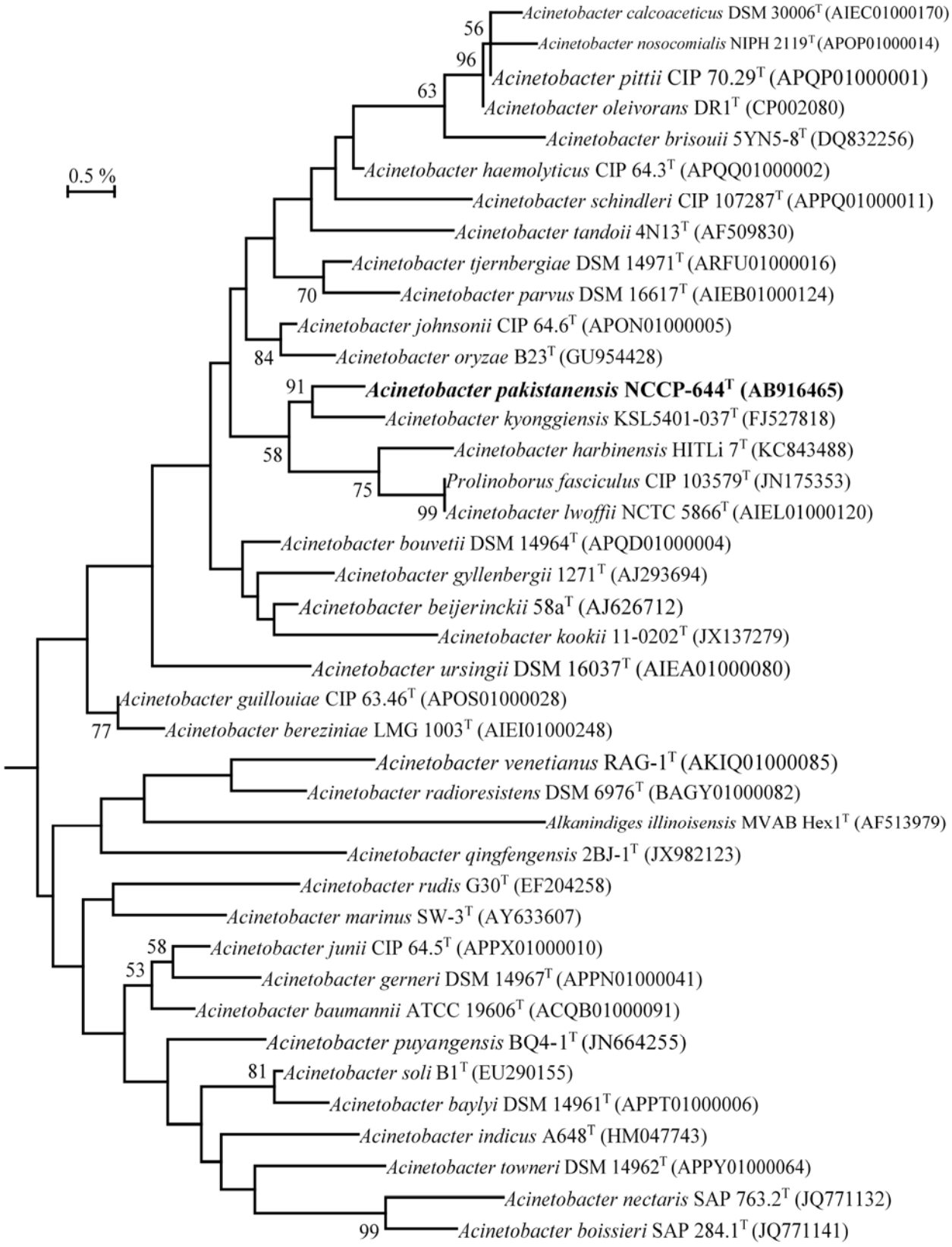


Figure 5.4.

Maximum-likelihood phylogenetic tree inferred from the sequences of 16S rRNA gene showing the inter-relationship of strain NCCP-644T with the type strains of genus *Acinetobacter*, which is based on a comparison of 1232 nucleotides. The bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The accession number of each type strain is shown in the parentheses.

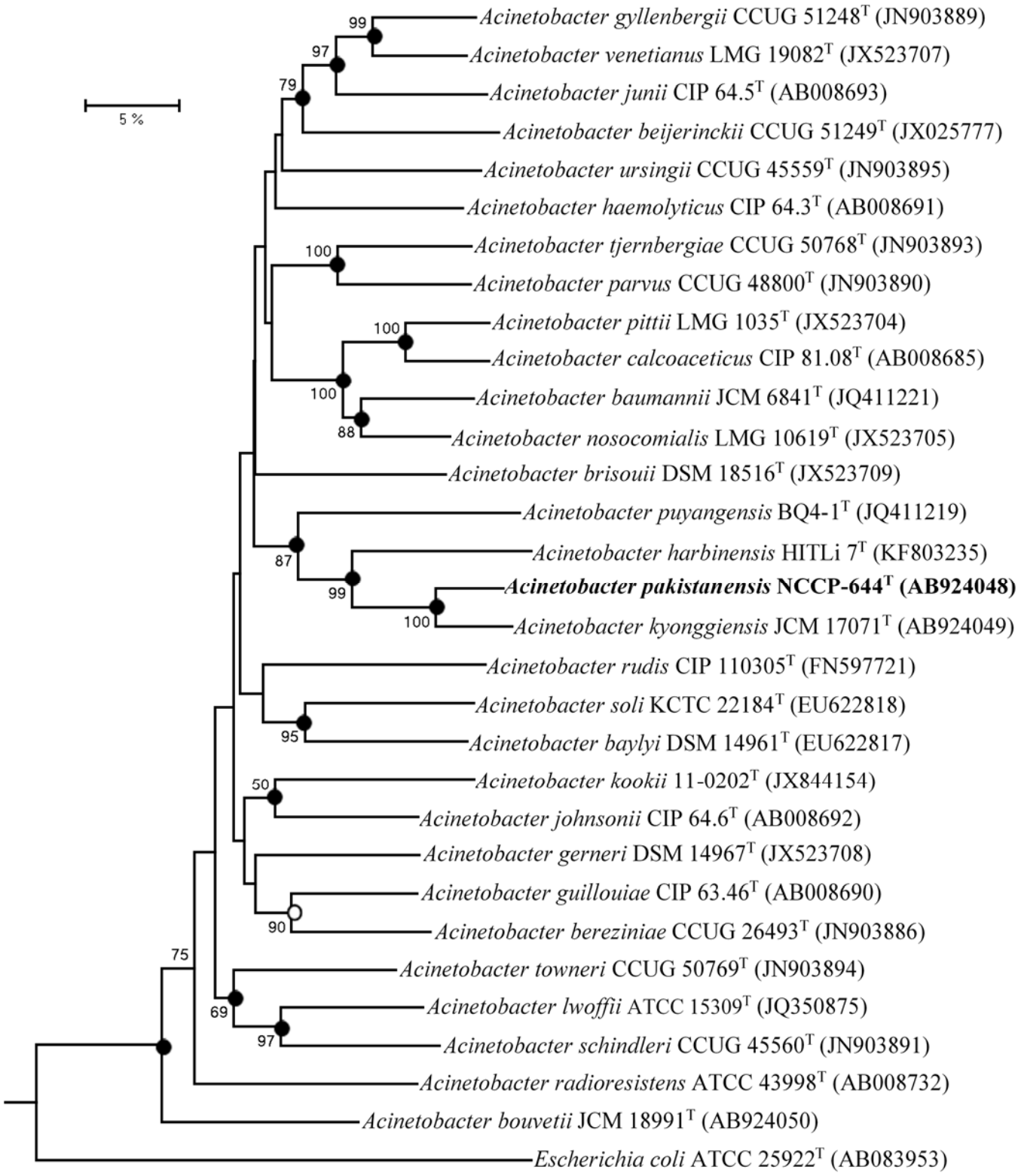


Figure 5.5.

Neighbour-joining phylogenetic tree constructed from sequences of the *gyrB* gene showing the inter-relationship of strain NCCP-644^T with the closely related type strains of genus *Acinetobacter*, which is based on a comparison of 794 nucleotides and is rooted using *Escherichia coli* ATCC 25922^T (AB083953) as an out-group. The bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The nodes indicated by empty circles were recovered by at least two algorithms, whereas the nodes with solid circles were recovered by three algorithms (NJ, MP and ML). The length of the bar is the 5 % sequence divergence. The accession number of each type strain is shown in the parentheses.

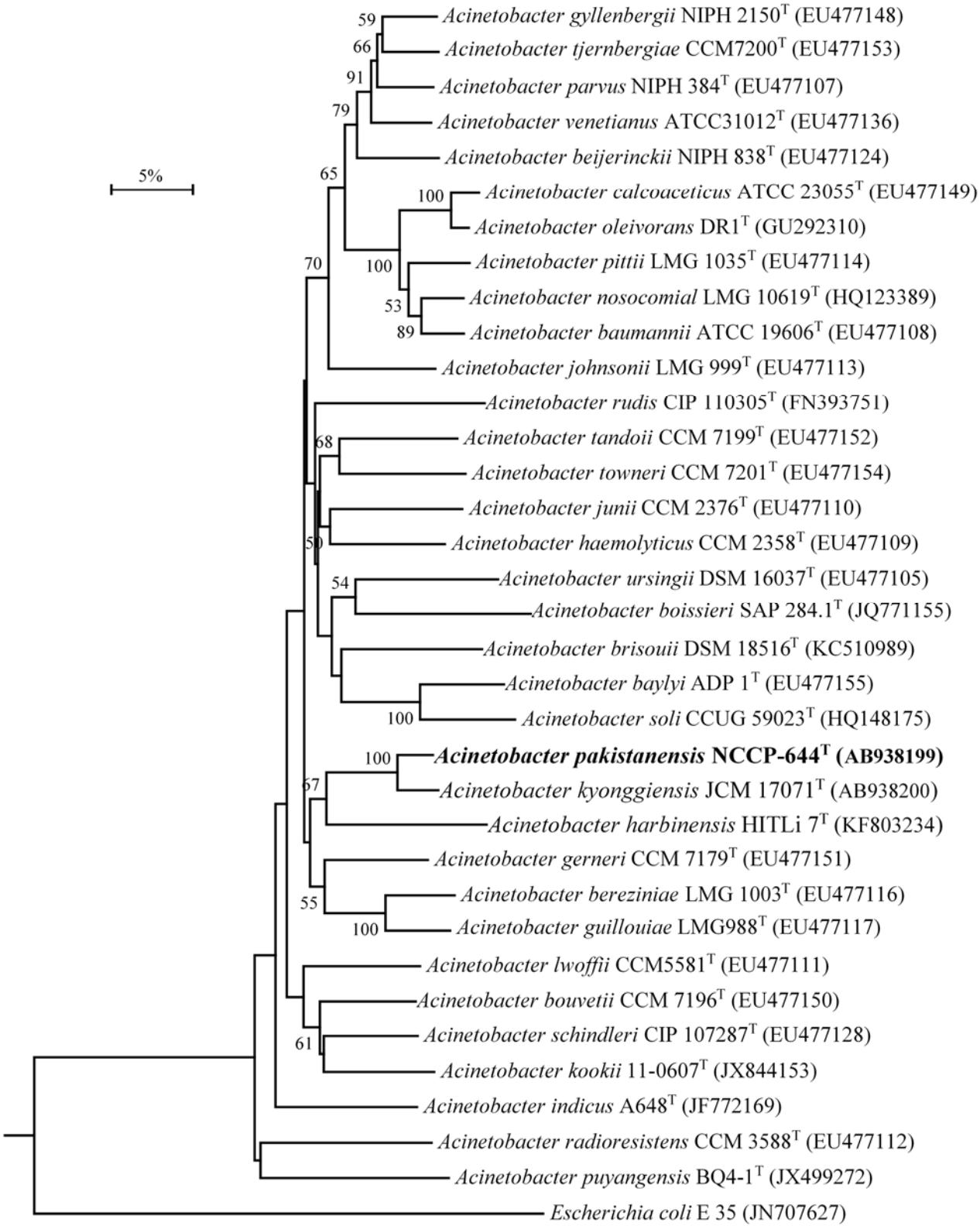


Figure 5.6.

Neighbour-joining phylogenetic tree constructed from sequences of the *rpoB* gene showing inter-relationship of strain NCCP-644^T with the closely related type strains of genus *Acinetobacter*, which is based on a comparison of 842 nucleotides and is rooted using *Escherichia coli* E35 (JN707627) as an out-group. The bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The length of the bar is the 5 % sequence divergence. The accession number of each type strain is shown in the parentheses.

to "*A. kyonggiensis*" JCM 17071^T, However, many other data presented here also show some differences with these type strains of the genus *Acinetobacter* (Table 5.2). In summary, our strain NCCP-644^T is closely related to "*A. kyonggiensis*" and *A. Harbinensis* based on the analyses of the 16S rRNA, *rpoB* and *gyrB* gene sequences; however, it is distinct from all other recognized species of the genus *Acinetobacter*. Although the 16S rRNA gene sequence of our strain NCCP-644^T exhibited greater than 97 % similarity with several species, such as "*A. kyonggiensis*", *A. harbinensis, A. bouvetii, A. johnsonii*, *A. beijerinckii, A. haemolyticus, A. tjernbergiae* and *A. tandoi* of the genus *Acinetobacter*, the findings of the three housekeeping genes sequences for *gyrB, rpoB* and *atpD* indicated that our strain NCCP-644^T is distinct from recognized species of the genus *Acinetobacter*. Previously, it was suggested that any strain with less than 95 % sequence similarity of *rpoB* gene with the validly named species might belong to a new species in the genus *Acinetobacter* (Gundi et al. 2009; La Scola et al. 2006; Narciso-da-Rocha et al. 2013). Additionally, it was also proposed that a genetic distance of 0.041 for the *gyrB* gene sequences corresponds to a 70 % DNA relatedness value, which is the upper limit to delineate any novel species in bacteria (Yamamoto et al. 1999).

In this study, our strain NCCP-644^T showed sequence similarity of the *gyrB* and *rpoB* genes with "*A. kyonggiensis*" JCM 17071^T (95.3 and 92.3 %, respectively), with lower values observed for the other type strains of the recognized species of the genus *Acinetobacter*. Therefore, the multilocus sequence analysis based on the three housekeeping genes supports the hypothesis that our strain NCCP-644^T is a novel species. To confirm this hypothesis, DNA– DNA hybridization analysis was also performed. It was found that the DNA–DNA reassociation between strain NCCP-644^T and the reference strains "*A. kyonggiensis*" JCM 17071^T, *A. harbinensis* KCTC 32411^T, *A. johnsonii* JCM 20194^T, *A. beijerinckii* JCM 18990^T and *A. bouvetii* JCM 18991^T was maximum 54.7 % **(Table 5.4)**, which are less than the 70 % threshold that is required to delineate any strain to a new species (Wayne et al. 1987).

Table 5.4. DNA-DNA relatedness between strain (NCCP-644^T) of *Acinetobacter pakistanensis* sp. nov. with the type strains of their most closely related species.

Strains	NCCP-644^T = *Acinetobacter pakistanensis* sp. nov.
"Acinetobacter kyonggiensis" JCM 17071^T	54.7
Acinetobacter harbinensis KCTC 32411^T	19.6
Acinetobacter johnsonii JCM 20194^T	20.6
Acinetobacter beijerinckii JCM 18990 ^{T}	12.3
Acinetobacter bouvetii JCM 18991^T	10.3

The G+C content of genomic DNA of strain NCCP-644^T was 40.6 mol% as determined by HPLC **(Figure 5.7)** using a procedure previously described (Hayat et al. 2013). These results are in consistent with results previously reported in the members of the genus *Acinetobacter* (Bouvet and Grimont 1986). However, the DNA G+C content of *A. johnsonii* JCM 20194^T was higher at 44.0-45.0 mol% (Bouvet and Grimont 1986). This discrepancy may be a result of the different methodologies. In this study, the DNA G+C content was detected by HPLC, as opposed to detection with the spectrophotometrically denaturation method, followed by Bouvet and Grimont (1986).

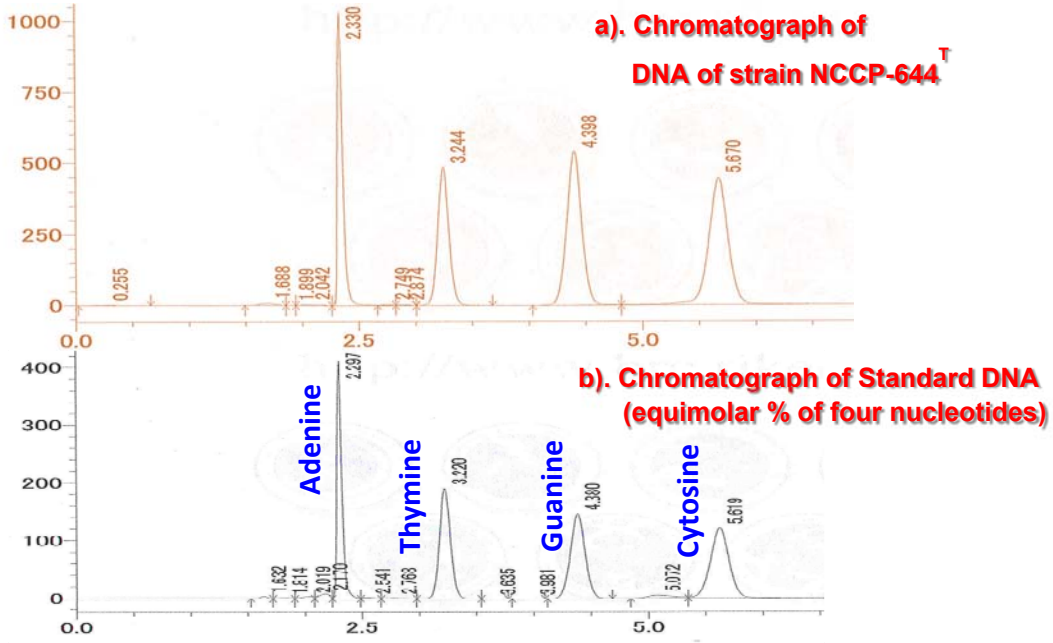


Figure 5.7. Chromatograph of DNA G+C Content analysis of (a) *Acinetobacter pakistanensis* NCCP-644^T in comparison with the (b) DNA standard on HPLC.

Chemotaxonomic analysis

The cellular fatty acid profile of strain NCCP-644^T comprised predominantly of summed feature 3 (iso-$C_{15:0}$ 2OH / $C_{16:1}$ ω7*c*; 37.2 ± 3.4 %), followed by $C_{18:1}$ ω9*c* (16.8 ± 2.0 %), $C_{16:0}$ (12.5 ± 0.9 %), $C_{12:0}$ 3-OH (9.6 ± 1.8 %), $C_{12:0}$ (7.6 ± 0.7 %), $C_{10:0}$ (6.6 ± 0.8 %) and other minor components **(Table 5.5)**. The major components of this profile are similar to those present in other members of the genus, although significant variation in the values of these components clearly differentiates our strain from the closely related reference strains. However, a significant amount of $C_{12:0}$ 2-OH (3.3 ± 2.4 %) is present in *A. calcoaceticus* JCM 6842^T, but only traces of this component are detected in NCCP-644^T and/or a small amount is present in the other reference strains (Table 5.5). Strain NCCP-644^T contained ubiquinone Q-9 as a major component (78 %), whereas Q-8 was a minor component (12 %), and traces of Q-10 were also detected **(Figure 5.8)**. The closely related type strain of the reference species "*A. kyonggiensis*" JCM 17071^T also contained a similar quinone system (Q-9, 83 %; Q-8, 10 % and traces of Q-10). These results are consistent with previous reports for the members of *Acinetobacter* (Vaz-Moreira et al. 2011); however, our results for "*A. kyonggiensis*" JCM 17071^T differed from those reported previously by Lee and Lee (2010), who described the presence of only Q-8 as the major quinone system in "*A. kyonggiensis*" JCM 17071^T.

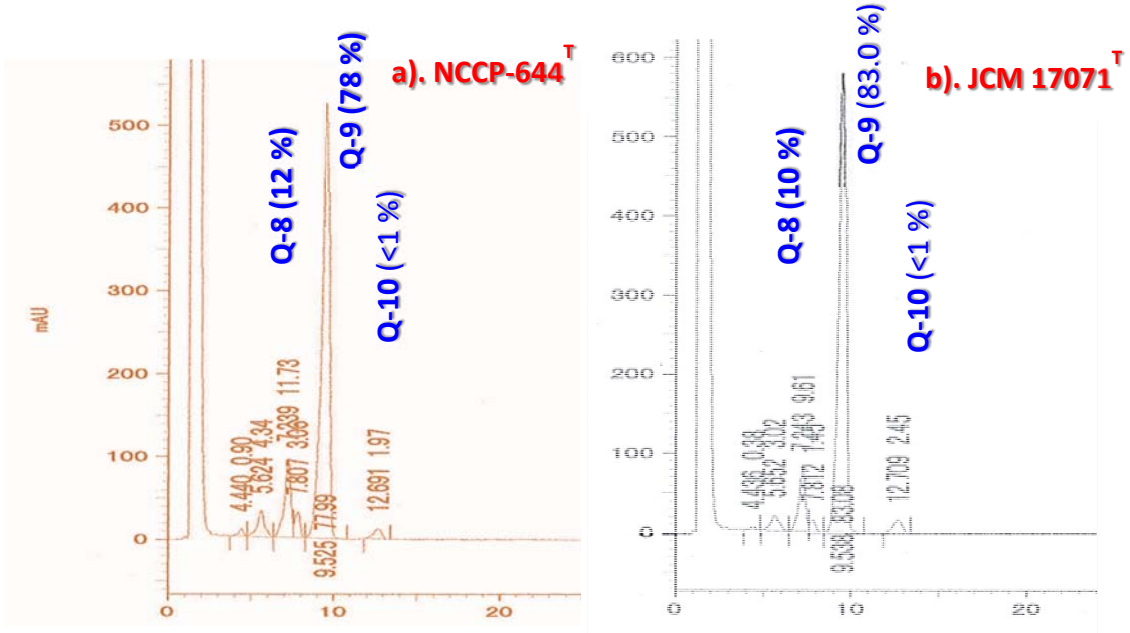


Figure 5.8. Chromatograph of menaquinone analysis of (a) *Acinetobacter pakistanensis* NCCP-644^T in comparison with (b) the reference species JCM 17071^T

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Table 5.5. Cellular fatty acid profiles (%) of strain NCCP-644^T compared to the reference type strains of the genus *Acinetobacter*.

Characteristics	NCCP-644^T	"*A. kyonggiensis*" JCM 17071^T	*A. harbinensis* KCTC 32411^T	*A. bouvetii* JCM 18991^T	*A. beijerinckii* JCM 18990 ^{T}	*A. johnsonii* JCM 20194 ^{T}	*A. calcoaceticus* JCM 6842^T
$C_{10:0}$	**6.60 ±0.82**	**4.31 ±0.25**	**1.91**	**2.61 ±0.11**	**1.28 ±0.55**	**0.28 ±0.01**	**0.23 ±0.08**
$C_{12:0}$	**7.57 ±0.66**	**3.32 ±0.18**	**5.1**	**8.91 ±0.20**	**6.96 ±2.32**	**10.00 ±3.38**	**10.16 ±1.77**
$C_{14:0}$	0.56 ±0.02	1.14 ±0.02	0.5	1.05 ±0.11	0.78 ±0.32	0.93 ±0.21	0.88 ±0.02
$C_{16:0}$	**12.53 ±0.93**	**16.38 ±3.37**	**11.93**	**17.40 ±2.44**	**14.52 ±0.10**	**14.12 ±0.08**	**11.60 ±4.18**
$C_{18:0}$	0.38 ±0.01	1.73 ±0.67	1.98	0.54 ±0.13	0.68 ±0.07	0.56 ±0.16	1.29 ±0.09
$C_{10:0}$ 2-OH	nd	nd	nd	nd	2.09 ±0.59	nd	0.03 ± 0
$C_{12:0}$ 2-OH	0.27 ±0.08	0.18 ±0.02	0.18	0.21 ±0.04	2.90 ±1.12	1.67 ±0.75	3.27 ±2.40
$C_{12:0}$ 3-OH	**9.56 ±1.79**	**5.39 ±0.57**	**4.63**	**6.84 ±0.44**	**9.12 ±1.37**	**6.79 ±1.37**	**6.09 ±2.40**
$C_{16:0}$ N alcohol	0.71 ±0.60	2.69 ±2.67	nd	0.07 ±0.05	0.19 ±0.09	1.01 ±0.57	1.41 ±1.39
$C_{16:1}$ ω9*c*	0.68 ±0.34	1.12 ±0.64	0.81	nd	0.76 ±0.04	0.48 ±0.17	0.37 ± 0
$C_{17:1}$ ω8*c*	0.76 ±0.40	0.08 ±0.01	0.04	0.15 ±0.04	1.51 ±0.03	0.18 ±0.04	1.21 ±0.26
$C_{18:1}$ ω7*c*	2.43 ±0.59	3.00 ±0.52	2.48	2.53 ±1.19	0.49 ±0.10	3.66 ±1.54	1.72 ±1.12
$C_{18:1}$ ω9*c*	**16.76 ±2.01**	**26.71 ±3.46**	**37.05**	**13.52 ±2.70**	**24.95 ±7.84**	**23.22 ±8.90**	**14.15 ±4.14**
$C_{18:3}$ ω6*c* (6, 9, 12)	0.27 ±0.24	0.92 ±1.10	nd	nd	0.23 ±0.05	0.51 ±0.28	1.41 ±1.41
Summed features 2*	0.22 ±0.04	0.12 ±0.02	0.07	0.16 ±0.01	0.33 ±0.06	0.68 ±0.11	2.59 ±0.12
Summed features 3*	**37.20 ±3.43**	**31.53 ±2.40**	**32.66**	**45.27 ±1.90**	**30.08 ±0.03**	**34.91 ±3.92**	**34.44 ±6.66**

*Summed feature 2 comprises one or more of iso-$C_{16:1}$ I / $C_{14:0}$ 3OH, and summed feature 3 comprises one or more of $C_{16:1}$ ω7c / iso-$C_{15:0}$ 2OH, which could not be separated by the MIDI system.

All data were obtained in this study. The data are the mean of two values except for *A. harbinensis* KCTC 32411^T. The values are the percentages of total fatty acid detected.

nd, not detected

The cellular fatty acid component values were deleted if present at less than 1% in all species and/or absent in some species.

Polar lipids profile of strain NCCP-644^T is similar (Figure 5.9) to "*A. kyonggiensis*" JCM 17071^T, which comprised of diphosphatidyl glycerol, phosphatidyl monomethyl ethanol, phosphatidyl glycerol, phosphatidyl serine, phosphatidyl inositol and one unknown phospholipid. However, the presence of phosphatidyl ethanolamine differentiates our strain NCCP-644^T from "*A. kyonggiensis*" JCM 17071^T, in which it is absent **(Figure 5.9)**.

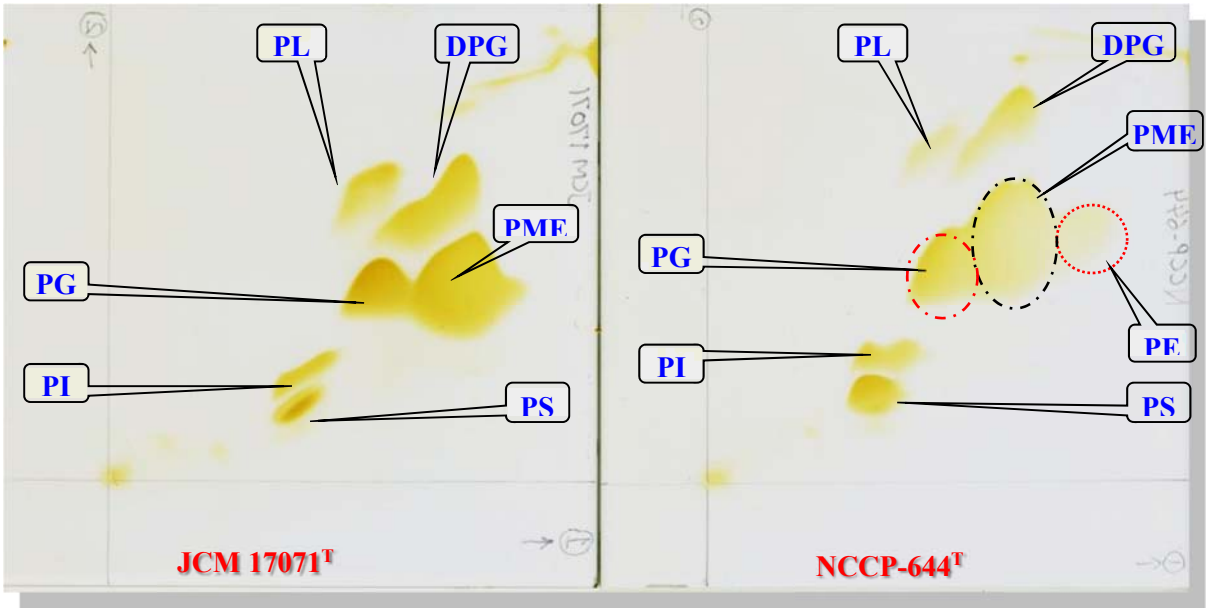

Figure 5.9.

Polar lipid profiles of strain NCCP-644^T compared to the closely related strain "*Acinetobacter kyonggiensis*" JCM 17071^T (reference strain).

DPG (diphosphatidylglycerol), PG (phosphatidylglycerol), PE (phosphatidylethanolamine), PME (phosphatidyl monomethylethanol), PI (phosphatidylinositol), PS (phosphatidylserine) and PL (one phospholipid of unknown structure).

Based on the physiological and biochemical characteristics, chemotaxonomic data and phylogenetic analyses of the 16S rRNA and *gyrB* genes along with genotypic (DNA-DNA relatedness) data presented in this paper, the isolated strain NCCP-644^T is concluded to be a new species in the genus *Acinetobacter* with the proposed name, *A. pakistanensis* sp. nov., the type strain NCCP-644^T and its description is provided below.

Description of *Acinetobacter pakistanensis* sp. nov.

Acinetobacter pakistanensis (pa.kis.tan.en′sis. N.L. masc. adj. *pakistanensis* from Pakistan, where the organism was isolated).

The cells are Gram-stain negative, cocci to short rod (coccobacillus), occur primarily in pairs and sometimes triplet form, which are non-motile and strictly aerobic. The colonies are moist, raised, off-white in colour with entire margins and are 1–2 mm in size after 1–2 days when grown on TSA (Difco) medium at 25°C. The cells grow at 3–37°C (optimum at 20–25°C), at pH ranges of 6.0–10.0 (optimum at pH 7–8) and in 0–3 % NaCl (w/v) (optimum without NaCl) in TSB (Difco) medium. Positive for Voges-Proskauer reaction, nitrate reduction to N2 and catalase but negative for oxidase, arginine dihydrolase, *β*-galactosidase (2-nitrophenyl-*β*D-galactopyranoside), lysine- and ornithine-decarboxylases, tryptophan deaminase, H2S production, citrate utilization and indole production. In addition, esculin, urea and gelatin are not hydrolyzed by this strain. There is no oxidation/fermentation of D-melibiose, L-arabinose, amygdalin, D-sucrose, D-mannitol, L-rhamnose, D-glucose, D-sorbitol and inositol. The strain can assimilate malate and capric acid but not glucose, mannose, mannitol, arabinose, maltose, adipic acid trisodium citrate or potassium gluconate, phenyl acetic acid. The strain is negative for acid production from D-mannitol, D-galactose, inulin, D-sorbitol, L-xylose, L-rhamnose, D-mannose, methyl-*β*D-xylopyranoside, *N*-acetyl glucosamine, D-trehalose, arbutin, D-glucose, D-fucose, inositol, erythritol, D-lyxose, D-adonitol, D-melibiose, L-fucose, methyl-α D-mannopyranoside, D-melezitose, D-xylose, D-maltose, dulcitol, amygdalin, D-tagatose, D-cellobiose, D-ribose, potassium gluconate, methyl-αD-glucopyranoside, D-raffinose, xylitol, L-arabinose, D-fructose, D-arabitol, glycerol, gentiobiose, esculin, D-lactose, salicin, D-saccharose (sucrose), L-sorbose, potassium 2-ketogluconate, amidon (starch), D-arabinose, glycogen, D-furanose, L-arabitol and potassium 5-ketogluconate (API-50CH, bioMérieux, France). The strain has high enzyme activity for leucine arylamidase, napthol-As-BI-phosphohydrolase, lipase (C 14), leucyl glycine arylamidase, arginine arylamidase, esterase lipase (C 8), proline arylamidase, alkaline phosphatase, cysteine arylamidase, glycine arylamidase, tyrosine arylamidase, alanine

arylamidase, valine arylamidase, esterase (C 4), phenylalanine arylamidase, histidine arylamidase, serine arylamidase, esterase (C 4), glutamyl glutamic acid arylamidase, leucine arylamidase and acid phosphatase. However, the strain has no enzyme activity for α-arabinosidase, α-fucosidase, α-chymotrypsin, glutamic acid decarboxylase, α-glucosidase, α-mannosidase, α- galactosidase, α-fucosidase, *β*-glucosidase, *β*-glucuronidase, trypsin, *N*-acetyl-*β*-glucosaminidase, *β*-galactosidase, α- & *β*-galactosidase, α- & *β*-glucosidase, *β*-glucuronidase and pyroglutamic acid arylamidase (API-Zym and API Rapid 32 ID, bioMérieux, France). Major polar lipids are phosphatidyl monomethyl ethanol (PME), phosphatidyl inositol (PI) diphosphatidyl glycerol (DPG), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl glycerol (PG) and one unknown phospholipid. Major cellular fatty acids are summed feature 3 (iso-$C_{15:0}$ 2-OH / $C_{16:1}$ ω7*c* as defined by MIDI), followed by $C_{18:1}$ ω9*c*, $C_{16:0}$, $C_{12:0}$ 3-OH, $C_{12:0}$ and $C_{10:0}$. The major component of quinone system detected as Q-9 and minor component as Q-8. The genomic DNA G+C content of the type strain is 40.6 mol%.

Strain NCCP-644^T (= LMG 28467^T = KCTC 42081^T = JCM 18977^T) is the type strain isolated from a sample of textile dyeing wastewater collected from a treatment pond at Kohinoor mills, Islamabad, Pakistan.

The GenBank/EMBL/DDBJ accession numbers for strain NCCP-644^T (= JCM 18977^T = KCTC 42081^T) are AB916465 (16S rRNA gene), AB924048 (*gyrB* gene), AB938199 (*rpoB* gene) and AB924051 (*atpD* gene); for strain JCM 17071^T, these are AB924049 (*gyrB* gene), AB938200 (*rpoB* gene) and AB924052 (*atpD* gene); for strain JCM18990T, it is AB924053 (*atpD* gene); and for strain JCM 18991^T, the accession numbers are AB924050 (*gyrB* gene) and AB924054 (*atpD* gene).

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CHAPTER VI

A heavy metal tolerant novel bacterium, *Alcaligenes pakistanensis* sp. nov., isolated from industrial effluent in Pakistan

ABSTRACT

Two strains, NCCP-650^T and NCCP-667, were isolated from industrial effluent and their taxonomic positions were investigated using a polyphasic taxonomic approach. The strains were found to be Gram-stain negative, strictly aerobic, motile short rods, which are tolerant to heavy-metals (Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}). Cells were observed to grow at a temperature range of 10–37 °C (optimal 25–33 °C), pH range of 5.5–10.0 (optimal 6.5–7.5) and can tolerate 0–7 % NaCl (w/v) (optimum 0–1 %) in tryptic soya agar medium. Sequencing of the 16S rRNA gene and two housekeeping genes, *gyrB* and *nirK*, of the isolated strains revealed that both strains belong to the *Betaproteobacteria* showing highest sequence similarities with members of the genus *Alcaligenes*. The chemotaxonomic data [major quinones as Q-8; predominant cellular fatty acids as summed features 3 ($C_{16:1}$ ω7c/iso-$C_{15:0}$ 2-OH) and $C_{16:0}$ followed by Summed features 2 (iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH), $C_{17:0}$ cyclo and $C_{18:1}$ ω7*c*; major polar lipids as diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and one unidentified aminolipid] also supported the affiliation of the isolated strains with the genus *Alcaligenes*. DNA–DNA hybridizations between the two strains and with closely related type strains of species of the genus *Alcaligenes* confirmed that both isolates belong to a single novel species within the genus *Alcaligenes*. On the basis of phylogenetic analyses, physiological, biochemical characteristics and DNA–DNA hybridization, the isolated strains can be differentiated from established *Alcaligenes* species and thus, represent a novel species, for which the name *Alcaligenes pakistanensis* sp. nov. is proposed with the type strain NCCP-650^T (=LMG 28368^T = KCTC42083^T = JCM 30216^T).

Keywords:
Heavy metals tolerance, Industrial effluent, *Alcaligenes pakistanensis, nirK* gene, *gyrB* gene

INTRODUCTION

The genus *Alcaligenes* was proposed in 1919 (Castellani and Chambers 1919) and placed in the family *Alcaligenaceae* (De Ley et al. 1986) with the type species, *Alcaligenes faecalis*. So far, the genus *Alcaligenes* contains only two species *A. aquatilis* and *A. faecalis*, and the latter has been split into three sub species *A. faecalis* subsp. *faecalis*, *A. faecalis* subsp. *parafaecalis* and *A. faecalis* subsp. *phenolicus* (Euzéby 1997). Though the members of this genus were isolated from soil, sediment, bioprocess residues and water (Rehfuss and Urban 2005; Schroll et al. 2001; Van Trappen et al. 2005), some strains have also been reported to be isolated from clinical specimens (Busse and Auling 2005) and thus attract particular interest. This genus comprises of aerobic, motile, Gram-negative non-fermentative coccobacillary rods, which form non-pigmented colonies on nutrient agar. Some organisms were reported to have potential roles in bioremediation due to the presence of genes encoding for copper containing nitrite reductase and phenol hydroxylase (Rehfuss and Urban 2005). Several microorganisms are reported to tolerate toxic concentrations of heavy metals (Abbas et al. 2014; Affan et al. 2009; Tripathi et al. 2010; Tripathi et al. 2011; Zahoor and Rehman 2009). These heavy metal tolerant bacteria offer an opportunity to exploit their role in bioremediation of environments contaminated with heavy-metals. During our studies of microbial diversity for heavy-metal tolerant bacteria, strains NCCP-650^T and NCCP-667 were isolated on tryptic soy agar (TSA, BD,USA) by a dilution plate method from industrial effluent samples. The aim of this study was to delineate the taxonomic position of the isolated strains by polyphasic taxonomic characterization. On the basis of results obtained, the strains NCCP-650^T and NCCP-667 are considered to represent a novel species of the genus *Alcaligenes*.

MATERIALS AND METHODS

Isolation and growth of the strains

Strain NCCP-650^T was isolated from industrial effluent (water and sludge sample) collected from industrial wastewater discharge channel of an industrial area (lat/lon = ‘‘33.66 N 73.05 E’’), Islamabad, Pakistan, whereas strain NCCP-667 was isolated from tanneries

effluent (water and sludge sample) collected from a leather factory (lat/lon = ‘‘31.10 N 74.45 E’’), Kasur, Pakistan. The strains were recovered on TSA supplemented with different concentrations of heavy-metals (Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}) by a dilution plate method. The purified strains were maintained on agar medium as well as stored in glycerol (35 %, w/v) at –80 ºC, and subjected to polyphasic taxonomic characterization experiments. Type strains of closely related taxa, *Alcaligenes aquatilis* LMG 22996^T, *A. faecalis* subsp. *parafaecalis* DSM 13975^T, *A. faecalis* subsp. *faecalis* JCM 20522^T and *A. faecalis* subsp. *phenolicus* DSM 16503^T were used as reference strains in the majority of experiments under the same laboratory conditions. The characterization experiments were performed at 30 ºC unless otherwise mentioned.

Heavy metals tolerance

To demonstrate the tolerance of isolated strains to toxic concentrations of heavy metals, the strains NCCP-650^T, NCCP-667 and the reference strains were grown on TSA supplemented separately with different concentrations of heavy-metals (Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}) for 5–7 days. The concentration of heavy-metals (Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}) in agar media was in the range of 300–3000 ppm (in an incremental addition of 300 ppm), which were prepared using the salts: $K_2Cr_2O_7$, Na_2HAsO_4, $Pb(NO_3)_2$ and $CuSO_4.5H_2O$, respectively.

Morphology and phenotypic characterization

Colony morphology of the isolated strains was observed on isolated colonies grown on TSA for 2 days. A phase-contrast microscope (Nikon Optiphot-2, Japan) was used to examine cells of the isolated strains grown on TSA for 24–48 h and further detailed by scanning electron microscope. For the electron microscopic analysis, cells were fixed in a 2.5 % (v/v) paraformaldehyde–glutaraldehyde mixture buffered with 0.1 M phosphate (pH 7.2) for 2 h, dehydrated in graded ethanol, substituted by isoamyl acetate and after drying at critical point sputter-coated with gold (SC502, Polaron) and observed using a scanning electron microscope (FEI Quanta 250 FEG). Gram staining was performed using commercial color (Gram-staining) kit (Cat. 55542, bioMe´rieux, France) according to manufacturer’s

instructions. The motility of strains was determined with M medium (bioMe´rieux, France) in addition to microscopy. Relation to oxygen was determined on TSA by incubation in an anaerobic chamber (Mitsubishi Gas Chemicals Co., Inc.) for 10 days.

The optimum and range of pH for growth was determined in tryptic soy broth (TSB; BD, USA) by adjusting to a range of pH 4.0–10.5 (at increment of 0.5 pH unit) and OD_{600} was monitored using a spectrophotometer (Beckman Coulter Model DU730, USA). The pH values adjusted by using buffers, HCl or Na_2CO_3 (Sorokin 2005) and were verified after autoclaving. The temperature range for growth was determined on TSA (pH 7.0) by incubating at different temperatures (3, 5, 10, 15, 20, 25, 30, 33, 37, 40, 45, 50 ºC) for 6 days. Growth at various NaCl concentrations was investigated in TGE (pH 7.0), which contains (per litre): beef extract (6 g), tryptone (10 g), dextrose (2 g); agar (15 g) and supplemented with various concentration of NaCl (0–10 %; w/v), at adjusted pH 7.0, and incubated for 6 days. Physiological and biochemical characteristics were determined using API 20E (Cat. 20100), API 20NE (Cat. 20050) and API 50CH (Cat. 50300) galleries (bioMe´rieux, France). Since the strains showed mainly negative reactions for utilization of various carbon sources with API 20E, API 50CH and API 20NE, thus an extended array of metabolic features of the strains was analyzed using the Biolog GN2 characterization system (Biolog Inc. USA). Biolog tests were performed by growing the strain NCCP-650^T along with reference strains on Biolog Universal Growth (BUG) agar medium according to the instructions of the manufacturer. Catalase and oxidase activities were determined by using API Color Catalase (Cat. 55561) and API Oxidase (Cat. 55635) reagents (bioMe´rieux, France). Resistance to antibiotics was assessed with an ATB-VET (Cat. 14289) strip (bioMe´rieux, France) and enzyme activities were determined with an API ZYM (Cat. 25200) strip (bioMe´rieux, France). API suspension medium was used to inoculate the strips. All commercial kits were used according to the manufacturers' protocols.

Amplification, sequencing and phylogenetic analyses of 16S rRNA and housekeeping genes, *gyrase subunit B* (*gyrB*) and *nitrite reductase* (*nirK*) genes

Nearly complete 16S rRNA gene sequence of the isolated strains was amplified and sequenced as previously described (Roohi et al. 2014); whereas, for housekeeping genes: *gyrB, rpoB, atpD* and *infB* genes were tried to amplify using the primers and PCR conditions described earlier by Brady et al. (2008) as mentioned previously in Table 5.1. With these primer sets, only *gyrB* gene (specific band) could be amplified successfully, whereas non-specific bands produced with primers sets of *rpoB, atpD* and *infB* genes (Figure 6.5). To demonstrate the presence of genes coding for copper containing nitrite reductase (*nirK*) and the phenol hydroxylase in the isolated strains in comparison with the reference species, PCR was performed using the primers (mentioned in **Table 6.1**) and PCR conditions as described previously by Rehfuss and Urban (2005), Futamata et al. (2001) and Braker et al. (1998). The specific band of *nirK* gene was successfully amplified but non-specified bands were appeared with amplicons of phenol hydroxylase gene (Figure 6.6). All the purified PCR products were sequenced using the same primers on an ABI DNA analyzer. The sequences obtained were assembled using BioEdit software to get consensus sequence of the genes and submitted to DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). The DDBJ/EMBL/GenBank accession numbers for strains NCCP-650^T (=LMG 28368^T = KCTC 42083^T = JCM 30216^T), NCCP-667, LMG 22996^T, DSM 13975^T, JCM 20522^T and DSM 16503^T are LC001699–LC001704 (*gyrB* gene) and AB983284–AB983289 (*nirK* gene), respectively; whereas the 16S rRNA gene accession numbers for strains NCCP-650^T and NCCP-667 are AB920828 and AB968096, respectively.

Table 6.1. Amplification and sequencing primers for phenol hydroxylase and nitrite reductase *(nirK) genes*

Target genes	Primer pair for amplification	Sequence	Annealing temp (°C)	Expected amplicon size of target gene	Reference
Phenol hydroxylase	PHE2f (forward)	5'- (CCBTTCATGTCSGGHGC) - 3'	56 °C	700 bp	Futamata et al. (2001)
	PHE r (reverse)	5'- (ATYTGRTGCACMGGCARCC) - 3'			
Nitrite reductase	nirK1f (forward)	5'- (ATCATGGTSCTGCCGCG) - 3'	56 °C	470 bp	Braker et al. (1998)
	nirK5r (reverse)	5'- (GCCTCGATCAGRTTGTGGTT) - 3'			

The strain was identified using the sequence of 16S rRNA gene on Ez-Taxon Server (http://eztaxon-e.ezbiocloud.net) and BLAST search at the DDBJ/NCBI servers. The 16S rRNA gene sequences of closely related validly named type strains were retrieved from the database of the EzTaxon Server and phylogenetic trees were constructed as described previously (Ahmed et al. 2014) using three algorithms: neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods. The stability of the relationship was assessed with bootstrap analysis, by performing 1000 re-sampling for the tree topology. The sequence similarities of *gyrB* and *nirK* genes of isolated strains were compared with the sequences of reference strains using the Kimura 2-parameter model contained in MEGA 6 software package.

DNA base composition, DNA–DNA hybridization

For DNA G+C content analysis and DNA–DNA hybridization, genomic DNA of strains NCCP-650^T and NCCP-667 and the reference strains were isolated using Qiagen Genomic-tip 500/G following the manufacturer's protocol, with a minor modification in which RNase T_1 was used in addition to RNase A. To confirm that strains NCCP-650^T and NCCP-667 belong to the same species, DNA–DNA hybridization was performed at 45 ºC with photobiotin-labelled DNA and microplates as described by Ezaki et al. (1989), using a Fluoroskan Ascent Plate Reader (Thermo Lab Systems, USA) for fluorescence measurements. To establish a separate identity of the isolated strains NCCP-650^T and NCCP-667 as a new species, DNA–DNA hybridization between strain NCCP-650^T and with the reference strains, *Alcaligenes aquatilis* LMG 22996^T, *A. faecalis* subsp. *parafaecalis* DSM 13975^T, *A. faecalis* subsp. *faecalis* JCM 20522^T and *A. faecalis* subsp. *phenolicus* DSM 16503^T, were performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were converted to percentage DNA–DNA relatedness values.

To determine DNA G+C contents, the genomic DNA was digested with P1 nuclease and alkaline phosphatase. The DNA G+C contents were analyzed by HPLC (model UFLC,

Shimadzu, Japan) at 270 nm using solvent $NH_4H_2PO_4$ (0.02 M) – CH_3CN (v/v 20:1) with Cosmosil 5C18 column (4.6 by 150 mm; Nacalai Tesque, Japan; reversed phase silica gel; C18).

Chemotaxonomic analyses

For cellular fatty acids analysis, the isolates and the reference strains were grown on TSA for 24 h. The cellular fatty acid methyl esters were prepared (Sasser 1990) and were analyzed by GC (6890; Hewlett Packard) according to the standard protocol of the Sherlock Microbial Identification System (MIDI Sherlock version 4.5, MIDI database TSBA40 4.10). Respiratory quinone and polar lipids of strain NCCP-650^T and the closely related reference strains were extracted and analyzed from 100 mg lyophilized cells grown in PYE (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2) for 24 h as described by Tindall (1990a); Tindall (1990b) and Altenburgera et al. (1996). Polyamines were extracted and analyzed from biomass grown in PYE medium as described by Busse and Auling (1988). For HPLC analysis, conditions were applied reported by Busse et al. (1997). HPLC equipment applied for analyses of quinones and polyamines was reported by Stolz et al. (2007). Strain NCCP-650^T along with the reference species were analysed for whole cell sugars using lyophilized cells as described by Staneck and Roberts (1974) with the modification that sugars were identified on HPLC instead of TLC (Mikami and Ishida 1983).

RESULTS AND DISCUSSION

The isolated strains, NCCP-650^T and NCCP-667, formed off-white colonies, which were circular, low-convex with smooth surface and older colonies spread with irregular margins on TSA medium. Cells of the strains were Gram-stain negative, aerobic, motile short rods, mostly occurring in pairs, sometimes in single and rarely in quadrant form (**Figure 6.1**). Cells grew at pH range of 5.5–10.0 (optimal at pH 6.5–7.5) and showed no growth at pH 5.0 or 10.5. The strains tolerated 0–7 % NaCl (w/v) (optimum 0–1 %) in TSB medium. The growth of the strains was observed at temperature range of 10–37 °C (optimal growth at 25–33 °C); no growth was observed at 40 °C after 6 days, which differentiated strain NCCP-650^T from all the reference species that exhibited growth at 40–42 °C with optimum growth at 33–

37 °C (Rehfuss and Urban 2005).

Characterization by API 50CHB, API 20E and API 20NE kits produced mostly negative results (except positive results for acetoin production, citrate utilization and assimilation of capric acid, malate, trisodium citrate and phenyl acetic acid) and were similar to those of the closely related reference species; however, several physiological (mainly obtained in Biolog GN2 characterization) and some biochemical characteristics also differentiated the novel strains from the reference species (detailed results are provided in **Table 6.2** and in the species description). Both the isolates were overall similar in the phenotypic features but some variable results were also obtained in Biolog tests and API ATB-Vet for analysis of resistance to antibiotics (Table 6.2).

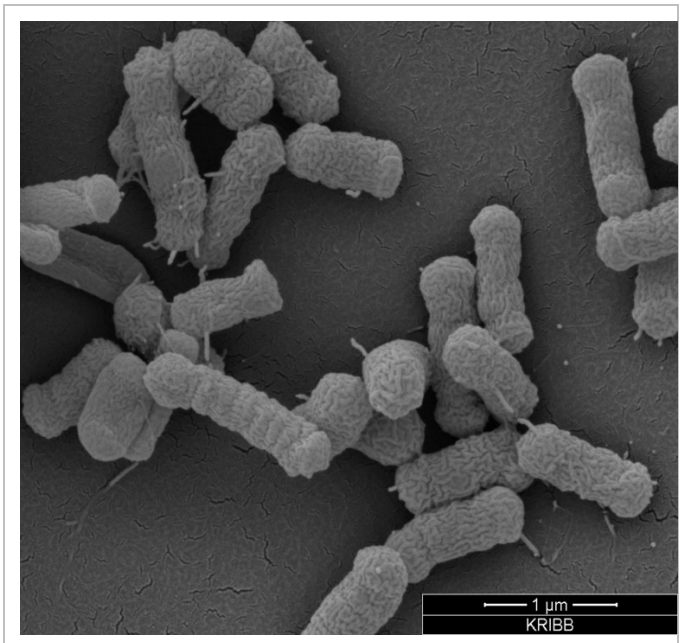

Figure 6.1. Scanning electron micrograph of cells of *Alcaligenes pakistanensis* NCCP-650^T grown on tryptic soy agar medium at 30°C for 48 hr.

The two isolates, NCCP-650^T and NCCP-667 showed tolerance to toxic concentrations of heavy metals, including Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}. They grew well on TSA medium containing Cr 1500 ppm, As 3000 ppm, Pb 2100 ppm and Cu 1800 ppm. In comparison, the closely related reference strains were also tested for tolerance to heavy-metals under similar conditions. It was found that the isolated strains differed for tolerance to Cr^{+2} and Pb^{+2} from the closely related reference species (Table 6.2), however, no difference was observed for tolerance to Cu^{+2}, As^{+2} and boron when compared with the reference species, suggesting that the members of genus *Alcaligenes* are mostly tolerant to heavy metals. Compared with the previously reported heavy-metal tolerant bacteria (Abbas et al. 2014; Affan et al. 2009; Tripathi et al. 2010; Tripathi et al. 2011; Zahoor and Rehman 2009), the strains NCCP-650^T and NCCP-667 can be considered as highly tolerant to toxic concentrations of heavy-metals.

Table 6.2. Characteristics that differentiate novel strain NCCP-650^T from the type strains of closely related species of the genus *Alcaligenes*

+++, Very strongly positive; ++, strongly positive; +, Positive; w+, weakly positive; –, negative; v, variable results between the strains; R, resistant to the antibiotic; wR, weakly resistant; S, sensitive. Rha (rhamnose); Rib (ribose); Glu (glucose); Man (mannose); Gal (galactose). All data are from this study unless otherwise mentioned.

Characteristics	**NCCP-650^T / NCCP-667**	***A. aquatilis* LMG 22996^T**	***A. faecalis* subsp. *parafaecalis* DSM 13975^T**	***A. faecalis* subsp. *faecalis* JCM 20522 ^{T}**	***A. faecalis* subsp. *phenolicus* DSM 16503^T**
Growth at Temperature (ºC) range (optimum)	10-37 (25-33)	10-40 (25-33)	10-40 (33-37)	10-40 (33-37)	10-40 (33-37)
Tolerance to heavy-metal:					
Chromium (ppm)	1500	900	900	900	900
Lead (ppm)	2100	1800	1800	1800	1800
Tryptophane deaminase	–	+	–	+	–
Oxidation/reduction of substrate: (Biolog)					
Dextrin	–	–	–	–	+
Glycogen	–	w	–	w	+
Tween 40	v	–	–	+	+
Tween 80	v	+	–	+	+
D-fructose	v	–	–	–	–
L-fucose	v	–	–	–	–
D-galactose	v	–	–	–	–
Maltose	v	–	–	–	w
D-mannitol	v	–	–	–	w
D-mannose	v	–	–	–	+
γ-hydroxy butyric acid	+	–	+	+	+
α-keto butyric acid	–	+	–	+	+
Succinamic acid	+	+	–	w	+
L-alanyl-glycine	–	–	–	–	+
L-asparagine	+	–	–	+	+
L-aspartic acid	v	+	–	+	+
Glycyl-L-aspartic acid	–	–	–	–	+
Glycyl-L-glutamic acid	–	–	–	–	+
L-histidine	w	–	–	+	+

Hydroxy-L-proline	–	–	–	–	+
L-pyroglutamic acid	–	+	+	w	+
D-serine	+	–	–	–	+
L-serine	w	–	–	–	+
L-threonine	+	+	–	w	+
D,L-carnitine	–	–	–	–	+
γ-amino butyric acid	–	–	–	–	+
Urocanic acid	+	–	–	–	+
Phenylethylamine	+	+	+	+	–
D,L-α-glycerol phosphate	–	–	–	–	+
Glucose-1-phosphate	–	–	–	–	+
Glucose-6-phosphate	–	–	–	–	+
***Enzyme activity* (API-Zym)**					
Alkaline phosphatase	++	–	+++	+	+++
Esterase (C 4)	+	w+	++	++	++
Esterase lipase (C 8)	+	w+	w+	+	++
***Resistance to* (μg mL^{-1}):**					
Amoxycilin (4)	v	R	R	R	R
Amox-clav.acid ((4/2)	S	R	R	R	R
Cefoperazon (4)	S	S	R	S	R
Streptomycin (8)	R	S	R	R	R
Kanamicin (8)	S	S	R	S	R
Gentamicin (4)	S	S	R	S	S
Apramycin (16)	v	R	R	S	R
Chloramphenicol (8)	S	R	R	R	R
Sulfamethizol (100)	S	S	S	S	wR
Flumequin (4)	S	S	R	S	R
Oxolinic acid (2)	S	S	S	S	R
Whole cell sugars (molar ratio, %)	Rib (80) Man (15) Glu (5)	Rib (95) Glu (5)	Rib (96) Glu (4)	Rib (100)	Rib (73) Gal (13) Man (10) Glu (4)
G+C content, mol %	55.5	56[†]	56[†]	56–59[†]	54.8[†]

[†] data from previous studies (Rehfuss and Urban 2005; Schroll et al. 2001; Van Trappen et al. 2005) .

Phylogenetic analyses, DNA–DNA hybridization and DNA base composition

Comparative sequence analyses of the 16S rRNA, *gyrB* and *nirK* genes were used to confirm the inter species relatedness of isolated strains NCCP-650^T and NCCP-667 with type strains of closely related reference species. Sequence comparison of the 16S rRNA genes of strains NCCP-650^T and NCCP-667 were carried out using Ez-Taxon Server database (http://www.ezbiocloud.net/). The 16S rRNA gene sequence of strain NCCP-650^T showed the highest similarity (98.79 %) with *A. aquatilis* LMG 22996^T (GenBank accession no. JX986976); the similarity values with other established species of the genus *Alcaligenes* were 98.76–98.22 %. The 16S rRNA gene sequence similarity between the isolated strains was 99.22 %. A neighbor-joining phylogenetic tree constructed based on a comparison of 1321 aligned nucleotides (without gaps and ambiguous nucleotides) showed that both isolates formed a coherent unit at a high bootstrap value (95 %) within the clade comprising species of genus *Alcaligenes* (**Figure 6.2**). The nodes of this clade also appeared with the same species at high bootstrap values, when phylogenetic trees were constructed using maximum-likelihood and maximum parsimony algorithms (**Figures 6.3 & 6.4**), suggesting a strong affiliation of the isolated strains NCCP-650^T and NCCP-667 with the established species of the genus *Alcaligenes*.

The sequence similarity values of housekeeping genes *gyrB* and *nirK* of the isolates NCCP-650^T and NCCP-667 were 100 % with each other, respectively; however, there were notably low similarity values (89.1–85.1 and 92.4–89.7 %, respectively; Tables 6.3 & 6.4) with the species of genus *Alcaligenes*. The analysis based on deduced amino acid sequences of the *gyrB* gene of the isolates showed 95.2–89.7 % similarity with the reference species (the highest similarity with *A. faecalis* subsp. *parafaecalis* DSM 13975^T).

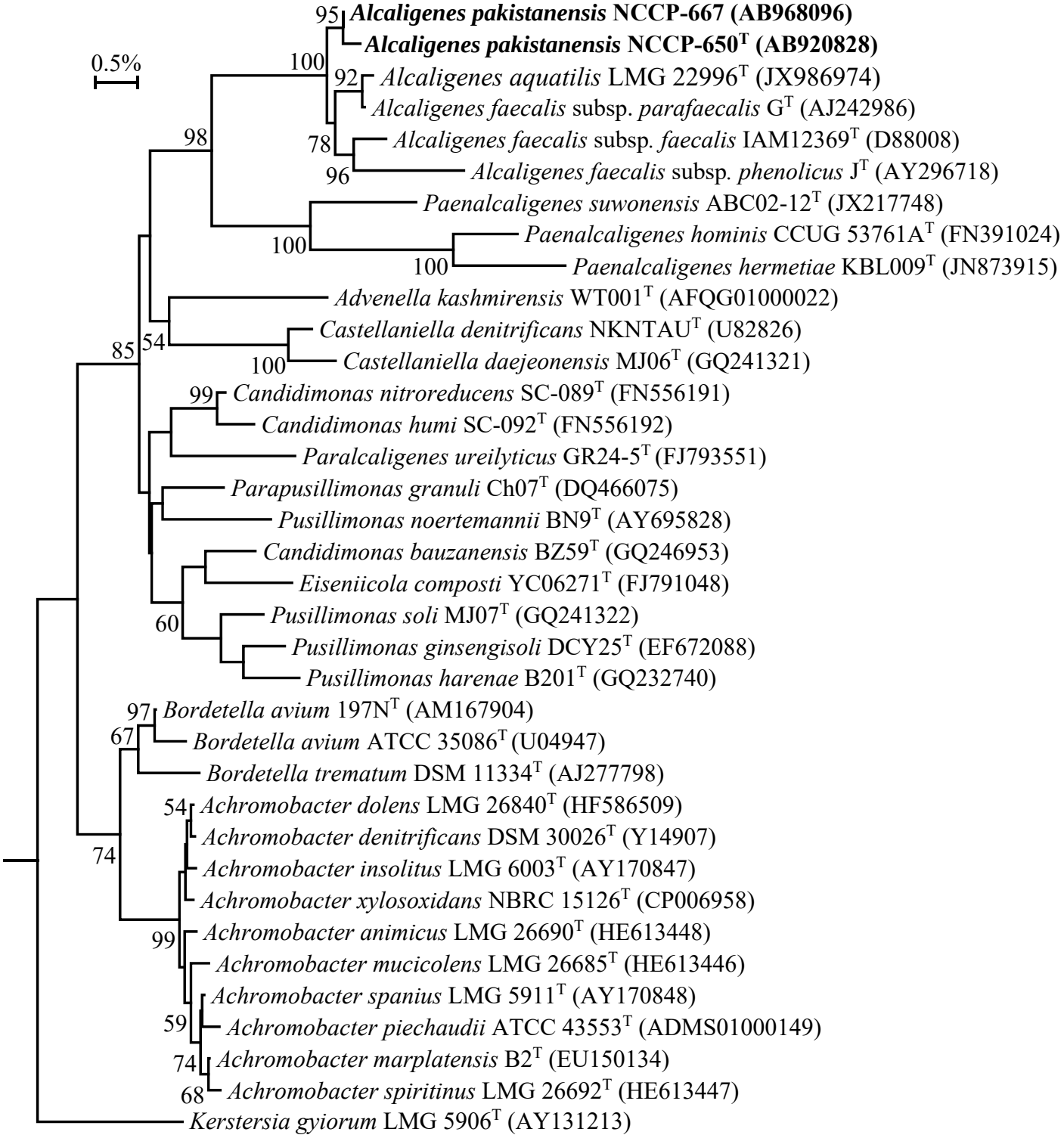

Figure 6.2.

Neighbour-joining phylogenetic tree inferred from 16S rRNA gene sequence showing inter-relationship of strain NCCP-650^T with the closely related species of the genus *Alcaligenes* and other related genera. The tree was generated using the MEGA 6.0 software package (Tamura et al. 2013) based on a comparison of 1321 nucleotides and was rooted by using *Kerstersia gyiorum* LMG 5906^T (AY131213) as an out-group. Bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. Bar, 0.5 % sequence divergence. The accession number of each type strain is shown in parentheses.

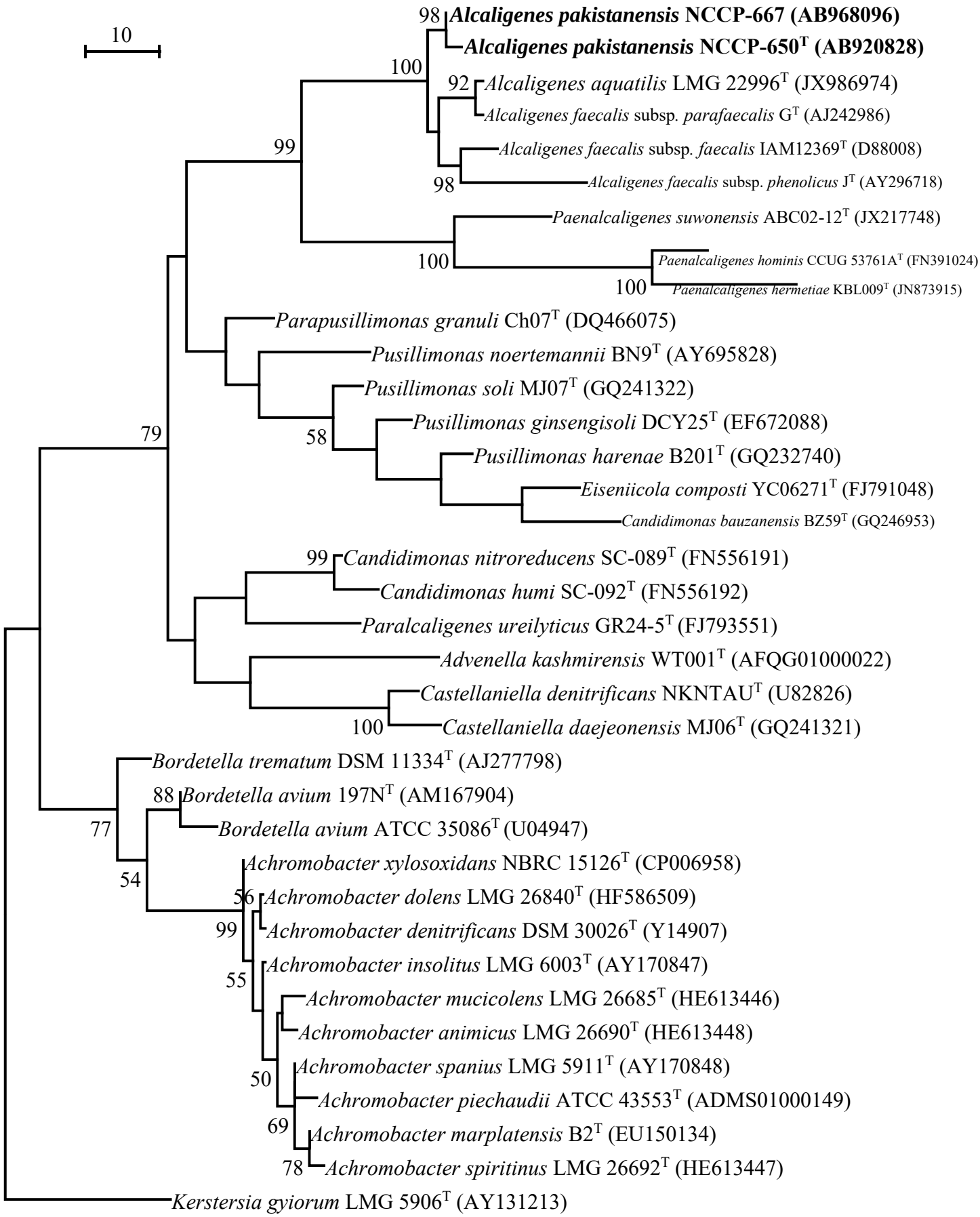

Figure 6.3. Maximum-parsimony phylogenetic tree inferred from 16S rRNA gene sequence showing inter-relationship of strain NCCP-650^T with the closely related species of genus *Alcaligenes* and other related genera.

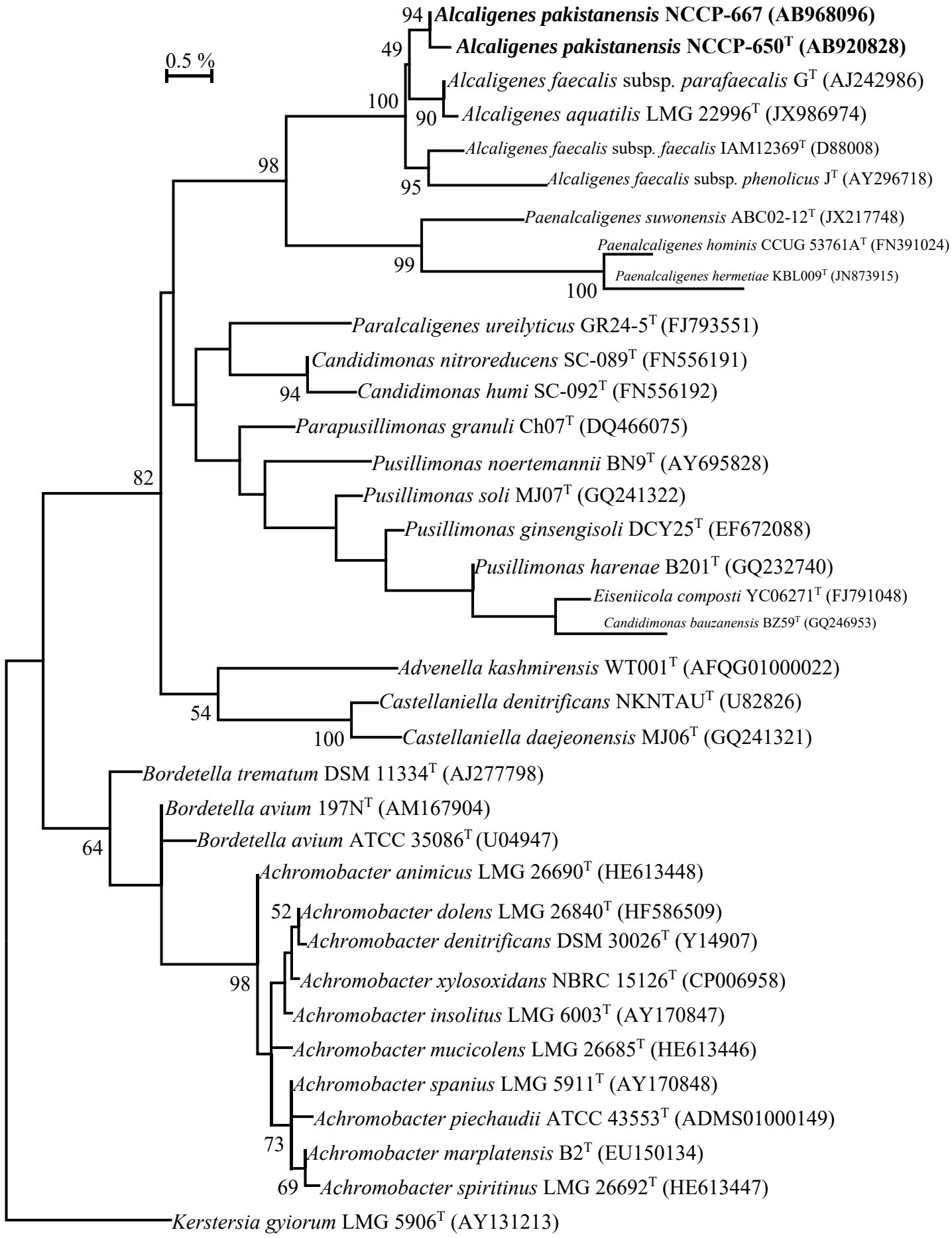

Figure 6.4. Maximum-likelyhood phylogenetic tree inferred from 16S rRNA gene sequence showing inter-relationship of strain NCCP-650T with the closely related species of genus *Alcaligenes* and other related genera.

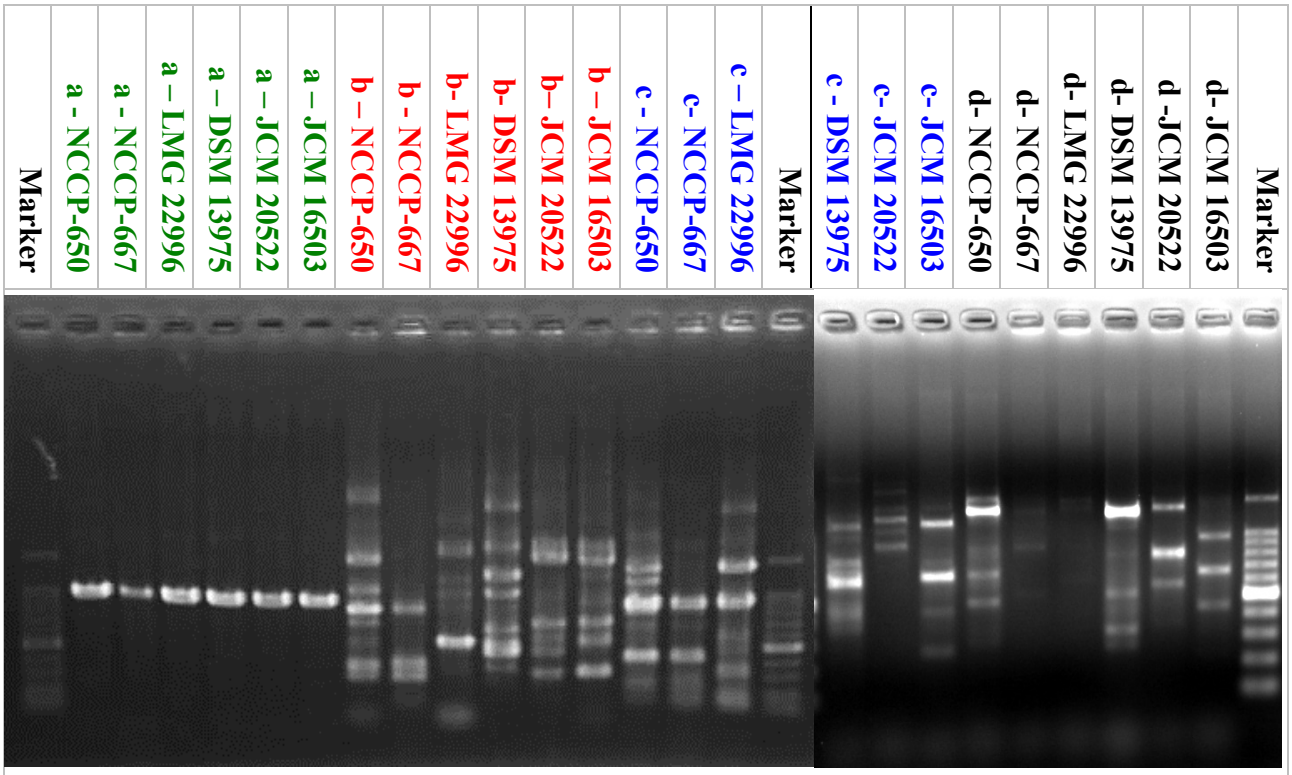

Figure 6.5. Gel pics of amplification of *gyrB, rpoB, atpD* and *infB* genes by PCR using primer pairs as mentioned in Table 5.1:
a. *gyrB* primers No. 1 (amplicon size: 742-bp)
b. *rpoB* primers No. 2 (amplicon size: 558-bp)
c. *atpD* primers No. 3 (amplicon size: 657-bp)
d. *infB* primers No. 4 (amplicon size: 615-bp)

Table 6.3. Sequence similarity index (%) of *gyrB* gene for NCCP-650^T and NCCP-667 with closely related reference strains and their accession numbers for *gyrB* gene published in DNA database (in this study).

S. No.	Strains	1	2	3	4	5	6	Accession Numbers
1	*Alcaligenes pakistanensis* NCCP-650^T	100						LC001699
2	*Alcaligenes pakistanensis* NCCP-667	100	100					LC001700
3	*Alcaligenes faecalis* subsp. *parafaecalis* DSM 13975^T	86.5	86.5	100				LC001702
4	*Alcaligenes faecalis* subsp. *phenolicus* DSM 16503^T	87.2	87.2	90.5	100			LC001704
5	*Alcaligenes faecalis* subsp. *faecalis* JCM 20522^T	89.1	89.1	90.4	95.3	100		LC001703
6	*Alcaligenes aquatilis* LMG 22996^T	85.1	85.1	92.7	90.0	90.6	100	LC001701

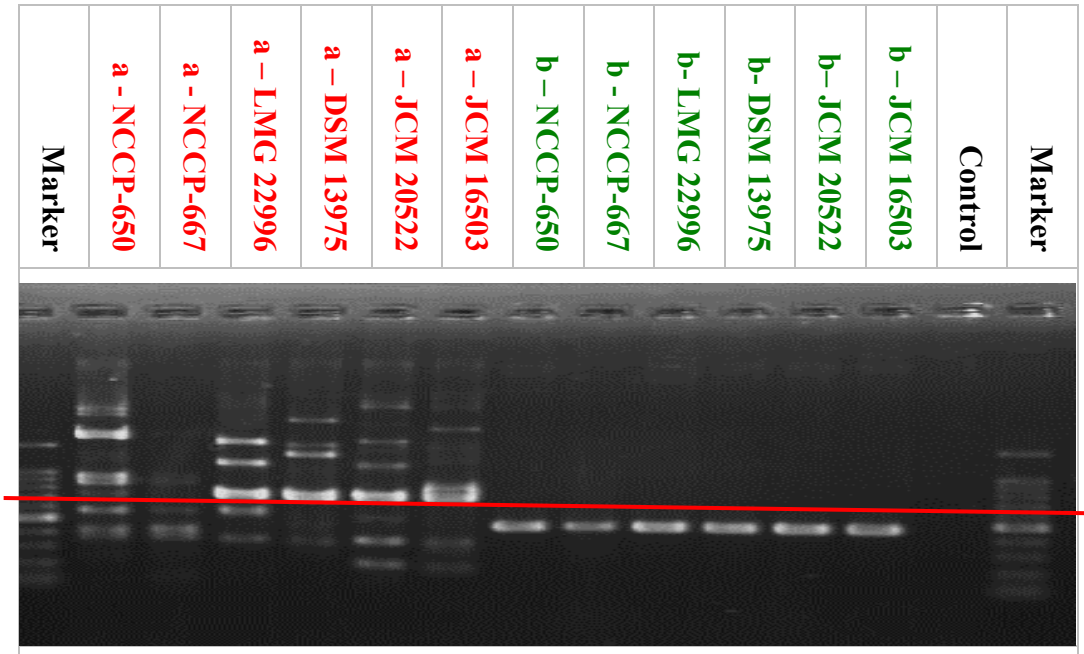

Figure 6.6. Gel pics of amplification of phenol hydroxylase and *nirK* genes by PCR using primers as mentioned in Table 6.1
a. ***Phenol hydroxylase gene*** primers No. 1 (amplicon size: 700-bp)
b. ***nirk* gene** primers No. 2 (amplicon size: 470-bp)

Table 6.4. Sequence similarity index (%) of *nirK* gene for NCCP-650^T and NCCP-667 with closely related reference strains and their accession numbers for *gyrB* gene published in DNA database (in this study).

S. No.	Strains	1	2	3	4	5	6	Accession Numbers
1	*Alcaligenes pakistanensis* NCCP-650^T	100						AB983284
2	*Alcaligenes pakistanensis* NCCP-667	100	100					AB983285
3	*Alcaligenes faecalis* subsp. *parafaecalis* DSM 13975^T	89.7	89.7	100				AB983286
4	*Alcaligenes faecalis* subsp. *phenolicus* DSM 16503^T	92.7	92.7	90.0	100			AB983287
5	*Alcaligenes faecalis* subsp. *faecalis* JCM 20522^T	90.4	90.4	90.9	92.0	100		AB983288
6	*Alcaligenes aquatilis* LMG 22996^T	92.4	92.4	92.5	92.5	94.9	100	AB983289

A single PCR product of the *nirK* gene (expected size of *470 bp) was amplified, but no amplicon (expected size *700 bp) of phenol hydroxylase (LmPH) gene was visualized on an ethidium bromide gel for the isolated strains (Figure 6.6). The similarities of deduced amino acids for *nirK* gene of the isolates were 99.10–97.26 % with *A. faecalis*, but low similarity (95.39 %) was observed with *A. aquatilis* LMG 22995^T. Both the

isolates showed 100 % similarity of deduced amino acids sequences of *nirK* and *gyrB* genes, suggesting that these belong to the same species.

In summary, the analyses of 16S rRNA, *gyrB* and *nirK* gene sequences showed that the strains NCCP-650^T and NCCP-667 are closely related to the members of genus *Alcaligenes*; however, based on physiological and biochemical features (Table 6.2), they are also distinct from all the recognized species of this genus. Although 16S rRNA gene sequences of the isolates exhibited greater than 97 % similarity with the members of genus *Alcaligenes*, the findings of the low sequence similarity of housekeeping gene sequences for *gyrB* and *nirK* supported the hypothesis that these strains belong to a novel species. To confirm this hypothesis, DNA–DNA hybridization analysis was also performed. It was found that the DNA–DNA relatedness between both the isolated strains NCCP-650^T and NCCP-667 was 93.6 (±3.1) %, confirming that these strains belong to the same species. However, DNA–DNA hybridization values betweenNCCP-650^T and the reference strains, *Alcaligenes faecalis* subsp. *parafaecalis* DSM 13975^T, *A. faecalis* subsp. *phenolicus* DSM 16503^T, *A. faecalis* subsp. *faecalis* JCM 20522^T and *A. aquatilis* LMG 22996^T were determined to be 39.8 (±4.1), 34.7 (±4.7), 31.6 (±5.5) and 10.8 (±3.2) %, respectively (Table 6.5). These values are clearly below the threshold value of 70 %, demonstrating that the two isolates are representatives of a novel species (Wayne et al. 1987). The DNA G+C content of strain NCCP-650^T was 55.5 mol% as determined by HPLC (Figure 6.7). These results are consistent with members of the genus as previously reported (Rehfuss and Urban 2005; Van Trappen et al. 2005), which support affiliation of the isolates to the genus *Alcaligenes*.

Table 6.5. DNA-DNA relatedness (%) between strain NCCP-650^T of *Alcaligenes pakistanensis* sp. nov. with the type strains of their most closely related species.

Strains	**NCCP-650^T =** *Alcaligenes pakistanensis* sp. nov.
Alcaligenes aquatilis LMG 22996^T	10.8 ± 3.2
Alcaligenes faecalis subsp. *faecalis* JCM 20522 ^{T}	31.6 ± 5.5
Alcaligenes faecalis subsp. *phenolicus* DSM 16503^T	34.7 ± 4.7
Alcaligenes faecalis subsp. *parafaecalis* DSM 13975^T	39.8 ± 4.1

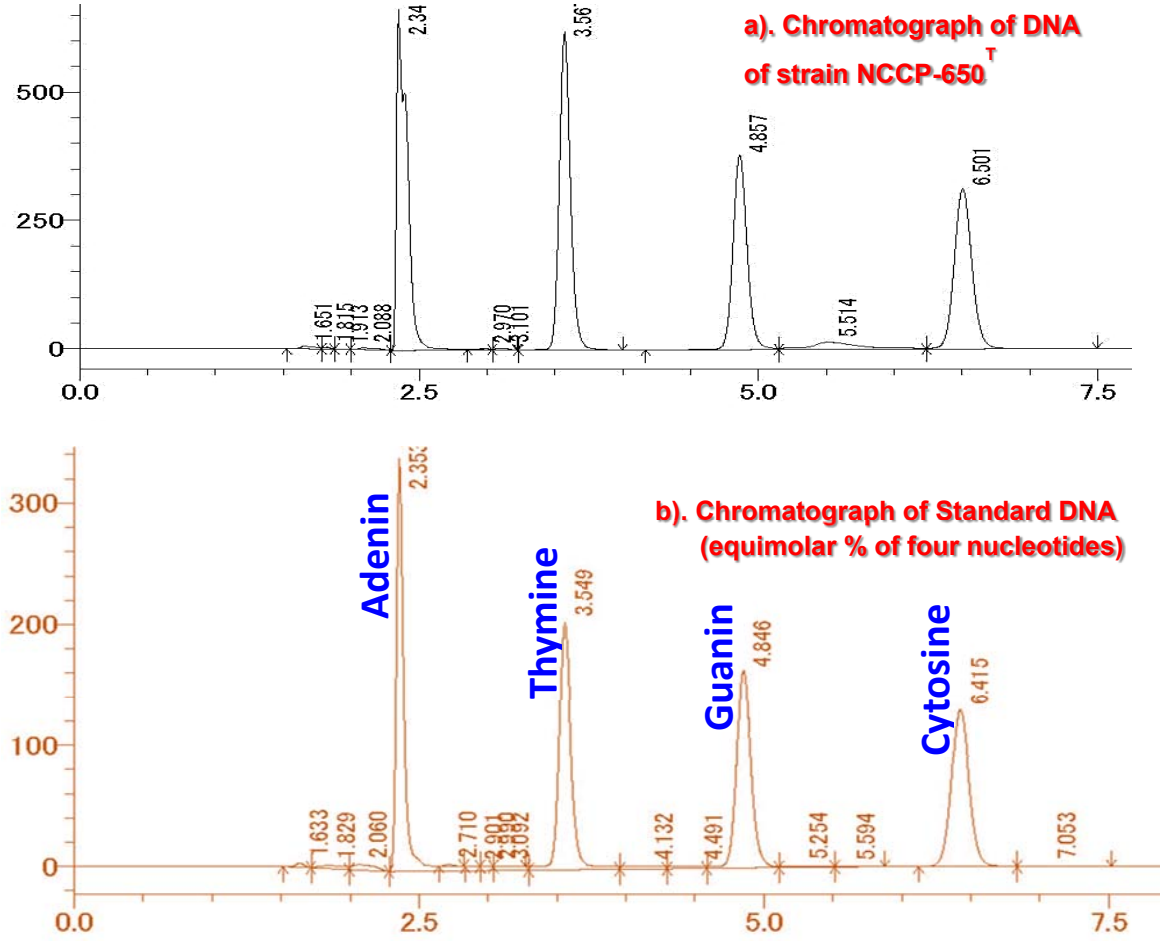

Figure 6.7. Chromatograph of DNA G+C contents analysis of (a) *Alcaligenes pakistanensis* NCCP-650^T in comparison with the (b) DNA standard on HPLC.

Chemotaxonomic analysis

The cellular fatty acid profile of the isolated strains consisted predominantly of summed features 3 ($C_{16:1}$ ω7*c*/iso-$C_{15:0}$ 2-OH as defined by MIDI) and $C_{16:0}$ followed by Summed features 2 (iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH), $C_{17:0}$ Cyclo, $C_{18:1}$ ω7c,$C_{12:0}$ 2-OH, $C_{14:0}$ and $C_{10:0}$ and other minor components (**Table 6.6**). Major components of this profile are similar to those found in other members of the genus, albeit some variation in values of these components clearly differentiated our strains from the closely related reference species of the genus. The presence of summed features 3, $C_{16:0}$, summed features 2 and $C_{17:0}$ Cyclo as major components has been observed in members of the *Alcaligenaceae* (Coenye et al. 2003; Vandamme et al. 1996; Vandamme et al. 1995).

Table 6.6. Cellular fatty acid profiles (%) of strains NCCP-650^T and type strains of reference species of the genus *Alcaligenes*

Characteristics	NCCP-650^T / NCCP-667	*A. aquatilis* LMG 22996^T	*A. faecalis* subsp. *parafaecalis* DSM 13975^T	*A. faecalis* subsp. *faecalis* JCM 20522 ^{T}	*A. faecalis* subsp. *phenolicus* DSM 16503^T
$C_{10:0}$	1.1±0.1	2.1±0.2	2.1±0.2	1.9±0.3	0.1±0
$C_{12:0}$	0.4±0	1.0±0.2	0.8±0.1	1.7±0.3	4.0±0.2
$C_{14:0}$	1.4±0.1	0.6±0.1	2.3±0.1	0.9±0	1.1±0.1
$C_{16:0}$	**32.4±1.0**	**29.6±1.5**	**30.6±2.2**	**29.9±2.9**	**31.5±0.9**
$C_{17:0}$	0.2±0.1	0.5±0	0.1±0	0.4±0	0.3±0
$C_{18:0}$	0.6±0.2	0.7±0.1	0.4±0.1	0.8±0.3	0.5±0
$C_{12:0}$ 2-OH	**2.0±0.1**	**2.1±0.2**	**2.5±0.3**	**2.6±0.4**	**2.7±0.1**
$C_{16:0}$ 3-OH	0.4±0	0.4±0	0.5±0.1	0.4±0	0.3±0
$C_{17:0}$ Cyclo	**8.9±1.6**	**8.2±3.2**	**9.0±0.2**	**8.0±2.7**	**12.3±1.0**
$C_{18:1}$ ω7*c*	**7.6±1.3**	**11.0±1.8**	**4.5±0.6**	**8.1±1.7**	**4.8±0.6**
Summed features 2*	**9.4±0.7**	**10.3±1.3**	**11.4±1.6**	**12.1±2.1**	**11.7±1.1**
Summed features 3*	**33.8±2.7**	**31.5±2.1**	**34.6±0.9**	**31.3±1.1**	**29.1±1.0**

*Summed feature 2 comprised one or more of iso-$C_{16:1}$ I / $C_{14:0}$ 3-OH, and Summed feature 3 comprised one or more of $C_{16:1}$ ω7*c* / iso-$C_{15:0}$ 2-OH, which could not have been separated by MIDI system.

All data are obtained in this study. Values (average of two readings of each strain from two independent experiments and their standard deviation) are percentages of total fatty acid detected.

Those values of cellular fatty acid components were deleted if present less than 1% in all the species and/or absent in some species.

Ubiquinone Q-8 was observed in strain NCCP-650^T as the major component of respiratory lipoquinone (**Figure 6.8**). Ubiquinone Q-8 was also detected in all the reference species. Previously, the type strain of the reference species, *A. faecalis* subsp. *parafaecalis* DSM 13975^T was reported to contain ubiquinone Q-8 system (Schroll et al. 2001). Our results are in agreement with those reported in *A. faecalis* subsp. *parafaecalis* DSM 13975^T and also conforms the reports that the predominant presence of Q-8 is a common trait of members of *Betaproteobacteria* including members of the *Alcaligenaceae* such as *A. faecalis* subsp. *faecalis* IAM 12586^T and *A. faecalis* subsp. *parafaecalis* DSM 13975^T (Schroll et al. 2001; Yokota et al. 1992).

The polar lipid profile of strainNCCP-650^T showed high similarity with those of the reference species (**Figure 6.9; Table 6.7**). The polar lipids of strain NCCP-650^T were predominantly identified to be diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and unidentified aminolipid (AL1). Furthermore, moderate to minor amounts of phosphatidylserine (PS), another unidentified aminolipid (AL2) and six unidentified polar lipids (L1–6) without an amino residue, a phosphate residue or sugar moiety were also observed. Though the presence of polar lipid L5 distinguished NCCP-650^T from all reference species and the presence of aminolipid AL2 from all reference species except *A. faecalis* subsp. *parafaecalis* DSM 13975^T (Figure 6.9; Table 6.7) not too much significance should be given to this observation because the amounts detected of these two lipids were rather low. Also the presence of several unidentified polar lipids in the reference species but absent in NCCP-650^T were too low to be considered as a robust distinguishing trait.

The polyamines pattern was composed of putrescine [45.9 µmol (g dry weight)$^{-1}$], spermidine [2.2 µmol (g dry weight)$^{-1}$] and traces of cadaverine and spermine [\0.1 µmol (g dry weight)$^{-1}$]. The absence of any detectable 2-hydroxyputresence is very rarely observed among *Betaproteobacteria* but the close relative of NCCP-650^T, *A. faecalis* subsp. *parafaecalis* DSM 13975^T was also reported to lack 2-hydroxyputresence (Schroll et al. 2001), whereas the type species of A. faecalis subsp. faecalis was shown to contain this polyamine though in relatively low amounts (Busse and Auling 1988). However, the absence of this polyamine in both NCCP-650^T and *A. faecalis* subsp. *parafaecalis* reflects the close relatedness between the two (**Figure 6.2**).

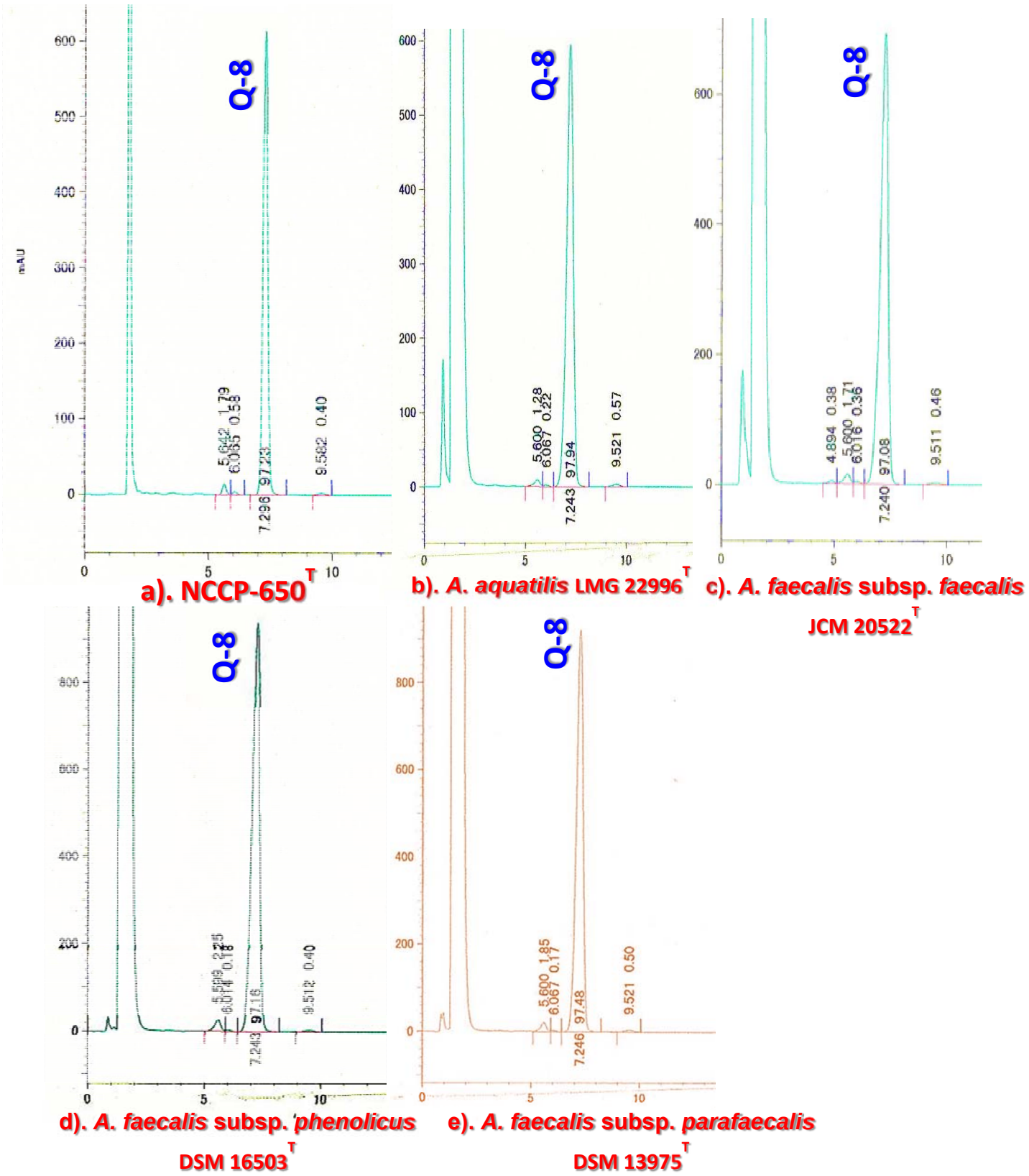

Figure 6.8. Chromatographs of menaquinone analyses of (a) *Alcaligenes pakistanensis* sp. nov. NCCP-650^T in comparison with analyses of the reference strains (b) *A. aquatilis* LMG 22996^T c). *A. faecalis* subsp. *faecalis* JCM 20522^T d). *A. faecalis* subsp. *phenolicus* DSM 16503^T and e) *A. faecalis* subsp. *parafaecalis* DSM 13975^T

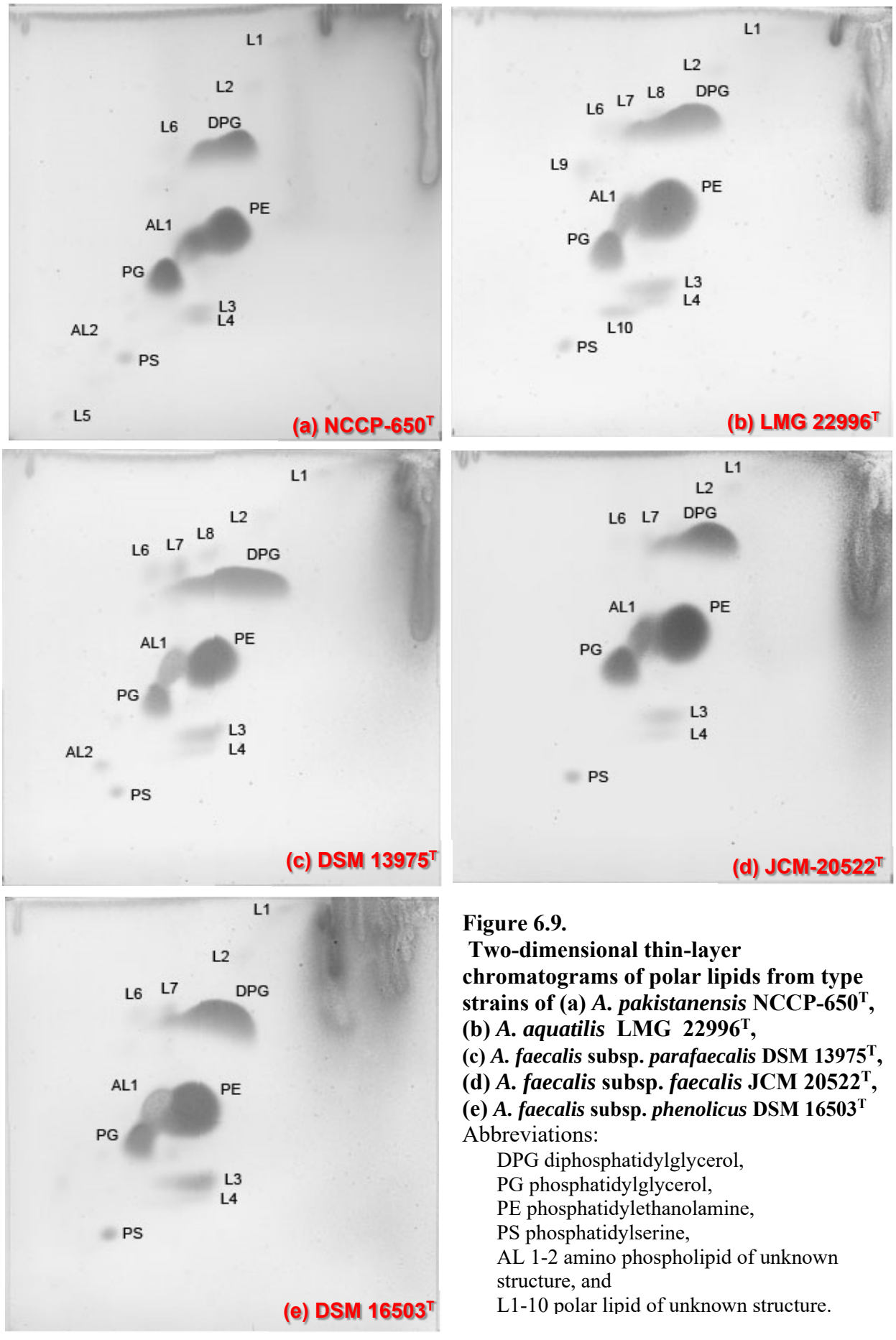

Figure 6.9.
Two-dimensional thin-layer chromatograms of polar lipids from type strains of (a) *A. pakistanensis* NCCP-650^T, (b) *A. aquatilis* LMG 22996^T, (c) *A. faecalis* subsp. *parafaecalis* DSM 13975^T, (d) *A. faecalis* subsp. *faecalis* JCM 20522^T, (e) *A. faecalis* subsp. *phenolicus* DSM 16503^T
Abbreviations:
DPG diphosphatidylglycerol,
PG phosphatidylglycerol,
PE phosphatidylethanolamine,
PS phosphatidylserine,
AL 1-2 amino phospholipid of unknown structure, and
L1-10 polar lipid of unknown structure.

Table 6.7. Comparison of polar lipids of type strains of (a) *Alcaligenes pakistanensis* NCCP-650^T, (b) *A. aquatilis* LMG 22996^T, (c) *A. faecalis* subsp. *parafaecalis* DSM 13975^T, (d) *A. faecalis* subsp. *faecalis* JCM 20522^T, (e) *A. faecalis* subsp. *phenolicus* DSM 16503^T

A. pakistanensis NCCP-650^T	*A. aquatilis* LMG 22996^T	*A. faecalis* subsp. *parafaecalis* DSM 13975^T	*A. faecalis* subsp. *faecalis* JCM 20522 ^{T}	*A. faecalis* subsp. *phenolicus* DSM 16503T
DPG	DPG	DPG	DPG	DPG
PG	PG	PG	PG	PG
PE	PE	PE	PE	PE
PS	PS	PS	PS	PS
AL1	AL1	AL1	AL1	AL1
AL2		AL2		
L1	L1	L1	L1	L1
L2	L2	L2	L2	L2
L3	L3	L3	L3	L3
L4	L4	L4	L4	L4
L5				
L6	L6	L6	L6	L6
	L7	L7	L7	L7
	L8	L8		
	L9			
	L10			

Abbreviations: DPG diphosphatidylglycerol, PG phosphatidylglycerol, PE phosphatidylethanolamine, PS phosphatidylserine, AL 1-2 amino phospholipid of unknown structure, and L1-10 polar lipid of unknown structure.

Ribose was found to be the major whole cell sugar in strain NCCP-650^T and the reference species, whereas mannose (15 %), and glucose (5 %) were detected as minor components in strain NCCP-650^T. The comparison of molar ratio of sugars in all the strains is presented in Table 6.2. Mannose was absent in *A. aquatilis* LMG 22996^T, *A. faecalis* subsp. *parafaecalis* DSM 13975^T and *A. faecalis* subsp. *faecalis* JCM 20522^T, whereas a significant amount (13 %) of galactose was detected in *A. faecalis* subsp. *phenolicus* DSM 16503^T (Table 6.2). Our data demonstrate that the genus *Alcaligenes* is heterogeneous in terms of minor whole cell sugars.

On the basis of phenotypic, genotypic, chemotaxonomic data and phylogenetic analyses, both the isolated strains NCCP-650^T and NCCP-667 belong to a single novel species of the genus *Alcaligenes*, for which the name *Alcaligenes pakistanensis* sp. nov., is proposed with the type strain NCCP-650^T and its description is given below:

Description of *Alcaligenes pakistanensis* sp. nov.

*Alcaligenes pakistanensis (*pa.kis.tan.en'sis. N.L. masc. adj. *pakistanensis* from Pakistan, where the organism was isolated).

Cells are Gram-stain negative, strictly aerobic, motile and short rods, mostly occur in pairs, sometimes in single and rarely occur in quadrant form. Colonies are off-white in color,

circular, low-convex with smooth surface; older colonies spread with irregular margins on TSA medium. Cells grow at temperature range of 10−37 °C (optimal growth at 25−33 °C), pH range of 5.5–10.0 (optimal at pH 6.5–7.5) and in 0–7 % NaCl (w/v) (optimum 0–1 %) in TSB medium. Tolerant to heavy-metals (i.e. Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}). Possesses the copper containing nitrite reductase *nirK* gene. Catalase and oxidase activities are positive. Positive for Voges–Proskauer reaction and citrate utilization but negative for nitrate reduction to N_2, indole production, lysine & ornithine decarboxylases, arginine dihydrolase, tryptophane deaminase, *β*-galactosidase (2-nitrophenyl-*β*-D-galactopyranoside) and H_2S production. Gelatin, urea and esculin are not hydrolyzed. Can assimilate capric acid, trisodiumcitrate, phenyl acetic acid and malate but not glucose, mannose, mannitol, arabinose, potassium gluconate, adipic acid and maltose. No oxidation/fermentation of D-glucose, D-sorbitol, amygdalin, L-arabinose, inositol, L-rhamnose, D-sucrose, D-melibiose, and D-mannitol. No acid is produced from substrates in the API-50CH system (bioMérieux, France). Strong enzyme activity observed for acid phosphatase, leucine arylamidase, alkaline phosphatase, esterase (C 4), esterase lipase (C 8), valine arylamidase, naphthol-As-BI-phosphohydrolase, but weak enzyme activity for lipase (C 14), cystine arylamidase, trypsin, α-chymotrypsin, whereas no enzyme activity for α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-glucosidase, *β*-glucosidase, *N*-acetyl- *β*-glucosaminidase, α-mannosidase, α-fucosidase (API-Zym, bioMérieux, France). The following compounds are used as sole carbon sources as determined by Biolog GN plates: methyl pyruvate, *cis*-aconitic acid, citric acid, formic acid, α-hydroxy butyric acid, *β*-hydroxy butyric acid, γ-hydroxy butyric acid, *p*-hydroxy phenyl acetic acid, D,L-lactic acid, malonic acid, propionic acid, succinic acid, bromo succinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-asparagine, L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, D-serine, L-serine, L-threonine, urocanic acid, phenylethylamine, mono-methyl-succinate (weak), acetic acid (weak), whereas variable results for Tween 40, Tween 80, L-arabinose, D-arabitol, D-fructose, L-fucose, D-galactose, maltose, D-mannitol, D-mannose, L-alanyl-glycine, L-aspartic acid, but the following substrates are not used as carbon source: dextrin, glycogen, D-cellobiose, L-erythritol, α-D-lactose, lactulose, α-keto butyric acid, α-keto glutaric acid, α-keto valeric acid,

quinic acid, D-saccharic acid, sebacic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, hydroxy-L-proline, L-pyroglutamic acid, D,L-carnitine, γ-aminobutyric acid, inosine, putrescine, D,L-α-glycerol phosphate, glucose-1-phosphate, glucose-6-phosphate. Major polar lipids are diphosphatidyl glycerol, phosphatidyl glycerol, phosphatidylethanolamine, and an unidentified amino-lipid (AL1). Moderate to minor amounts of phosphatidylserine, one unidentified amino-lipid (AL2) and six unidentified polar lipids (L1–6). The polyamine pattern contains the major compound putrescine and moderate amounts of spermidine. 2-hydroxy putrescine is absent. Predominant cellular fatty acids are summed features 3 ($C_{16:1}$ ω7*c*/iso-$C_{15:0}$ 2-OH as defined by MIDI) and $C_{16:0}$ followed by Summed features 2 (iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH), $C_{17:0}$ Cyclo, $C_{18:1}$ ω7*c*, $C_{12:0}$ 2-OH, $C_{14:0}$ and $C_{10:0}$. The major quinone is ubiquinone Q-8. The DNA G+C content of the type strain is 55.5 mol%.

Strain NCCP-650^T (=LMG 28368^T = KCTC 42083^T = JCM 30216^T) is the type strain, isolated from an industrial effluent (water and sludge) sample collected from Industrial waste water discharge channel of Sector I-9 Industrial area, Islamabad, Pakistan.

The DDBJ/EMBL/GenBank accession numbers for the type strain NCCP-650^T are AB920828 (16S rRNA gene), LC001699 (*gyrB* gene) and AB983284 (*nirK* gene).

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CHAPTER VII

A heavy metal tolerant novel bacterium, *Bacillus malikii* sp. nov., isolated from tannery effluent wastewater

ABSTRACT

The taxonomic position of a Gram-stain positive and heavy metal tolerant bacterium, designated strain NCCP-662^T, was investigated by polyphasic characterisation. Cells of strain NCCP-662^T were observed to be rod to filamentous shaped, motile and strictly aerobic, and to grow at 10–50 °C (optimum 30–37 °C) and at pH range of 6–10 (optimum pH 7–8). The strain was found to be able to tolerate 0–12 % NaCl (w/v) and heavy metals (Cr 1200 ppm, Pb 1800 ppm and Cu 1200 ppm) in tryptic soya agar medium. The phylogenetic analysis based on the 16S rRNA gene sequence of strain NCCP-662^T showed that it belongs to the genus *Bacillus* and showed high sequence similarity (98.2 and 98.0 %, respectively) with the type strains of *Bacillus niabensis* 4T19^T and *Bacillus halosaccharovorans* E33^T. The chemotaxonomic data showed that the major quinone is MK-7; the predominant cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{14:0}$, iso-$C_{16:0}$ and $C_{16:0}$ and iso-$C_{15:0}$; the major polar lipids are diphosphatidylglycerol, phosphatidylglycerol along with several unidentified glycolipids, phospholipids and polar lipids. The DNA G+C content was determined to be 36.9 mol%. These data also support the affiliation of strain NCCP-662^T with the genus *Bacillus*. The level of DNA–DNA relatedness between strain NCCP-662^T and *B. niabensis* JCM 16399^T was 20.5 ± 0.5 %. On the basis of physiological and biochemical characteristics, phylogenetic analyses and DNA–DNA hybridization data, strain NCCP-662^T can be clearly differentiated from the validly named *Bacillus* species and thus represents a new species, for which the name *Bacillus malikii* sp. nov. is proposed with the type strain NCCP-662^T (= LMG 28369^T = DSM 29005^T = JCM 30192^T).

Keywords:

Heavy metal tolerant, *Bacillus malikii*, Tannery effluent

INTRODUCTION

The genus *Bacillus* was first described by Cohn (1872) and contains Gram-stain positive endospore forming aerobic or facultative anaerobic rod-shaped bacteria, which have been reported in many environments including various soils, sediments, aquatic and terrestrial habitats and even in clinical samples (Logan and Vos 2009). With the advancement of systematic analyses, many of these bacilli have been reclassified into new genera. Ash et al. (1991) reorganised the taxonomy of genus *Bacillus* into 5 distinct phylogenetic groups based on 16S ribosomal RNA sequences. The polyphasic approach has further led to the reclassification of *Bacillus* species into several new genera such as *Alicyclobacillus* (Wisotzkey et al. 1992), *Alkalibacillus* (Jeon et al. 2005), *Alteribacillus* (Didari et al. 2012), *Anaerobacillus* (Zavarzina et al. 2009), *Aneurinibacillus, Brevibacillus* (Shida et al. 1996), *Bhargavaea* (Verma et al. 2012), *Fictibacillus* (Glaeser et al. 2013), *Geobacillus* (Nazina et al. 2001), *Gracilibacillus* and *Salibacillus* (Wainø et al. 1999), *Hydrogenibacillus* (Kämpfer et al. 2013), *Kyrpidia* (Klenk et al. 2011), *Lysinibacillus* (Ahmed et al. 2007b), *Jeotgalibacillus* (Yoon et al. 2001b), *Paenibacillus* (Shida et al. 1997), *Psychrobacillus* (Krishnamurthi et al. 2010), *Rummeliibacillus* (Vaishampayan et al. 2009), *Solibacillus* (Krishnamurthi et al. 2009), *Pullulanibacillus* and *Sporolactobacillus* (Hatayama et al. 2006), *Sporosarcina* (Yoon et al. 2001a), *Ureibacillus* (Fortina et al. 2001), *Viridibacillus* (Albert et al. 2007) and *Virgibacillus* (Heyndrickx et al. 1998).

Currently the genus *Bacillus* is comprised of more than 200 validly named species (http://www.bacterio.net/bacillus.html). The member of this genus can survive in a wide range of environmental conditions for long periods and endospores play a crucial role in their survival due to resistance to extreme environments (Nicholson et al. 2000).

Several microorganisms are reported to tolerate toxic concentration of heavy metals (Abbas et al. 2015; Abbas et al. 2014; Tripathi et al. 2010; Tripathi et al. 2011; Zahoor and Rehman 2009). Many strains of *Bacillus* species have been found to adsorb toxic metal ions and thus may have roles in the bioremediation of contaminated soil/ water systems (Hafez et

al. 2002; Nourbakhsh et al. 2002; Stolz and Oremland 1999). During our studies for the isolation of heavy metal tolerant bacteria, strain NCCP-662^T was isolated from a tannery effluent sample. This study was carried out to delineate the taxonomic position of the new bacterium by physiological, biochemical, genotypic and phylogenetic characterisation. On the basis of data from these comparative studies, strain NCCP-662^T is concluded to represent a novel species of the genus *Bacillus*, for which the name *Bacillus malikii* sp. nov. is proposed.

MATERIALS AND METHODS

Isolation and growth of the strains

Strain NCCP-662^T was isolated from tannery effluent (water and sludge sample), collected from Leather Pak Road, Younas Nagar, Kasur, Pakistan (31.10 N 74.45 E). The strain was recovered aerobically on tryptic soy agar (TSA, Difco) supplemented with different concentrations of heavy metals (Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}) by a dilution plate method. The purified strain was maintained on agar medium, as well as stored in glycerol (35 %, w/v) at – 80 °C, and subjected to polyphasic characterisation experiments to meet the minimum standards for describing novel taxa of aerobic endospore forming bacteria (Logan et al. 2009). Different agar media (Difco, USA) including nutrient agar (NA), marine agar 2216 (MA), tryptic soya agar (TSA), R2A agar, brain heart infusion (BHI), YEM and HM (Ventosa et al. 1982) were tested to investigate the media supporting growth of the isolated strain. Type strains of closely related taxa, *Bacillus niabensis* JCM 16399^T and *Bacillus halosaccharovorans* DSM 25387^T, were used as reference strains in the majority of the characterisation experiments, cultured under the same laboratory conditions. The characterisation experiments were performed at 30 °C unless otherwise mentioned.

Heavy metals tolerance

The tolerance of the novel strain NCCP-662^T and the reference strains to toxic concentrations of heavy metals was investigated by growing them on TSA supplemented separately with different concentrations of heavy metals (Cr^{+2}, Pb^{+2} and Cu^{+2}) for five to seven days. The concentration of heavy metals (Cr^{+2}, Pb^{+2} and Cu^{+2}) in the agar media was

kept in range of 300–3000 ppm, which were prepared using the salts $K_2Cr_2O_7$, $Pb(NO_3)_2$ and $CuSO_4.5H_2O$, respectively.

Morphological, physiological and biochemical characterization

Colony morphology of strain NCCP-662^T was observed following growth on MA or NA1 (NA supplemented with 1 % NaCl) for 2 days. A phase contrast microscope (Nikon Optiphot-2, Japan) was used to examine cells of strain NCCP-662^T grown on MA or NA1 for 24–48 h. For detailed cell morphology, cells grown on MA for 24 h were observed using a scanning electron microscope (FEI Quanta 250 FEG) following the protocol described previously (Abbas et al. 2015). Gram staining was performed using a commercial kit (bioMérieux, France) following the manufacturers' instructions. The motility of strain NCCP-662^T was determined in M medium (bioMérieux, France) in addition to assessment by microscopy. Anaerobic growth was determined on MA or NA1 by incubation in an anaerobic chamber (Mitsubishi Gas Chemicals Co., Inc.) for 10 days.

The optimum and range of pH for growth were determined in MA medium (Difco) adjusted to a range of pH 5.0–10.5. The pH values were adjusted by using buffers, HCl or Na_2CO_3 (Sorokin 2005) and pH of agar plates was verified using litmus paper (with precision of 0.2–0.5 pH unit). The temperature range for growth was determined on MA (pH 7.0) by incubating at different temperatures (3, 5, 10, 15, 20, 25, 30, 33, 37, 40, 45 and 50 °C) for 6 days. Growth at various NaCl concentrations was investigated in TGE (pH 7.0), which contained (per litre): beef extract (6 g), tryptone (10 g), dextrose (2 g) and agar (15 g) supplemented with various concentrations of NaCl (0–15 %; w/v), at pH 7.0 and incubated for 6 days.

Physiological and biochemical characteristics were determined using API 20E, API 20NE and API 50CH galleries (bioMérieux, France). Enzyme activities were determined with an API ZYM strip (bioMérieux, France). API saline medium was used to inoculate the strips. Catalase and oxidase activities were determined by using API Color Catalase and API Oxidase Reagent (bioMérieux, France). All commercial kits were used according to the

manufacturers' protocols using cells of the strains grown on MA for 24–36 h. Biolog testing was performed according to the instructions of the manufacturer by growing the strains on Biolog Universal Growth (BUG) agar medium supplemented with 1.5 % NaCl.

Amplification, sequencing of 16S rRNA gene and the phylogenetic analysis

The nearly complete 16S rRNA gene was amplified as previously described (Ahmed et al. 2007a). The purified PCR product was sequenced with six universal primers (27F, 520R, 530F, 907R, 1110F and 1492R) for 16S rRNA genes using the commercial services of Macrogen, Korea (http://dna.macrogen.com/eng). The sequences obtained were assembled using BioEdit software to obtain a consensus sequence of the genes and submitted to the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). The strain was identified using the sequence of the 16S rRNA gene at the Ez-Taxon Server (http://eztaxon-e.ezbiocloud.net) and by BLAST search on the DDBJ/NCBI servers for type strains. The 16S rRNA gene sequences of closely related type strains with validly published names were retrieved from the database of the EzTaxon Server and phylogenetic trees were constructed as described previously (Ahmed et al. 2014b) using three algorithms: maximum parsimony, neighbour joining and maximum likelihood methods. The stability of the relationship was assessed with bootstrap analysis, by performing 1000 re-sampling for the tree topology.

DNA base composition, DNA–DNA hybridization

For DNA G+C content analysis and DNA–DNA hybridization, genomic DNA of strain NCCP-662^T and the reference strains were isolated as described previously (Ahmed et al. 2014a). DNA–DNA hybridization between strain NCCP-662^T and the reference strains, *B. niabensis* JCM 16399^T, *B. halosaccharovorans* DSM 25387^T and *Bacillus herbersteinensis* DSM 16534^T, was carried out at 45 °C using photobiotin-labelled DNA as described by Ezaki et al. (1989). The experiment was performed in microplates (NUNC Immuno module Maxisorp F16 Black Cat # 4-75515) with five replications for each sample and fluorescence was measured using a Fluoroskan Ascent Plate Reader (Thermo Labsystems, USA).

DNA G+C contents were determined by digesting genomic DNA with P_1 nuclease and alkaline phosphatase, followed by analysis using HPLC (model UFLC, Shimadzu, Kyoto, Japan) at 270 nm with solvent $NH_4H_2PO_4$ (0.02 M) -CH_3CN (v/v 20:1) and a Cosmosil 5C18 column (4.6 × 150 mm; Nacalai Tesque; reversed phase silica gel; C18).

Chemotaxonomic analyses

Biomass of strain NCCP-662^T was obtained by cultivation on MA for 48 h for analysis of respiratory quinone, polar lipids and peptidoglycan. To extract isoprenoid quinones, about 100–150 mg lyophilized cells were used and quinones were purified on TLC as described by Minnikin et al. (1984) and then analysed using HPLC (Shimadzu, Kyoto, Japan) with a Cosmosil column (4.6 x 150 mm; Nacalai Tesque; reversed phase silica gel; 5C18) using methanol:2-propanol (2:1) as mobile phase; peaks of quinones were detected at UV 270 nm wave length. Polar lipids were examined by two-dimensional TLC, using HPTLC plates (10 × 10 cm) Silica gel 60 (Merck), as described by Kudo (2001). For analysis of diagnostic amino acids in the cell wall peptidoglycan, 10 mg lyophilized cells were hydrolysed (6 N HCl, 100 °C, 18 h). The whole cell hydrolysate was analysed by thin-layer chromatography on a HPTLC Cellulose (10 × 10 cm) plate (1.05787.001 Merck, Germany) using the solvent system methanol—distilled water—6 N HCl—pyridine (80:26:4:10). A standard solution of diaminopimelic acid (0.01 M, mixture of LL-, DD- and *meso*-A2pm) was also run on the TLC plate in parallel for identification of the product.

For whole cell fatty acid analysis, strain NCCP-662^T and the reference strains were grown on MA medium for 24 h. The cellular fatty acid methyl esters were prepared as described by Sasser (1990) and analysed using a gas chromatograph (model 6890; Hewlett Packard) according to the standard protocol of the Sherlock Microbial Identification System (MIDI Sherlock version 4.5, MIDI database TSBA40 4.10).

RESULTS

Strain NCCP-662^T was observed to form tiny whitish colonies, which were smooth with shiny surfaces, slightly convex and round with entire margins, and were slightly sticky

in texture on MA or NA1 medium after 24–36 h at 30 °C. Cells of the strain were observed to be motile, strictly aerobic, Gram-stain positive and spore-forming long rods or filaments that mostly occur in pairs or long chains (end to end) and occasionally singly (**Figure 7.1**). We observed growth of the strain at 10–50 °C, with optimum growth at 30–37 °C after 3 days, whereas the closely related reference strains could not survive at 10 °C. Cells of strain NCCP-662^T were found to grow in the presence of 0–12 % NaCl (w/v, optimum 1–3 %) and at pH 6.0–10.0 (optimum growth at pH 7–8) but no growth was observed at pH 5.0. In comparison to other media tested, optimal growth was observed using MA and NA media after 24–36 h at 30 °C.

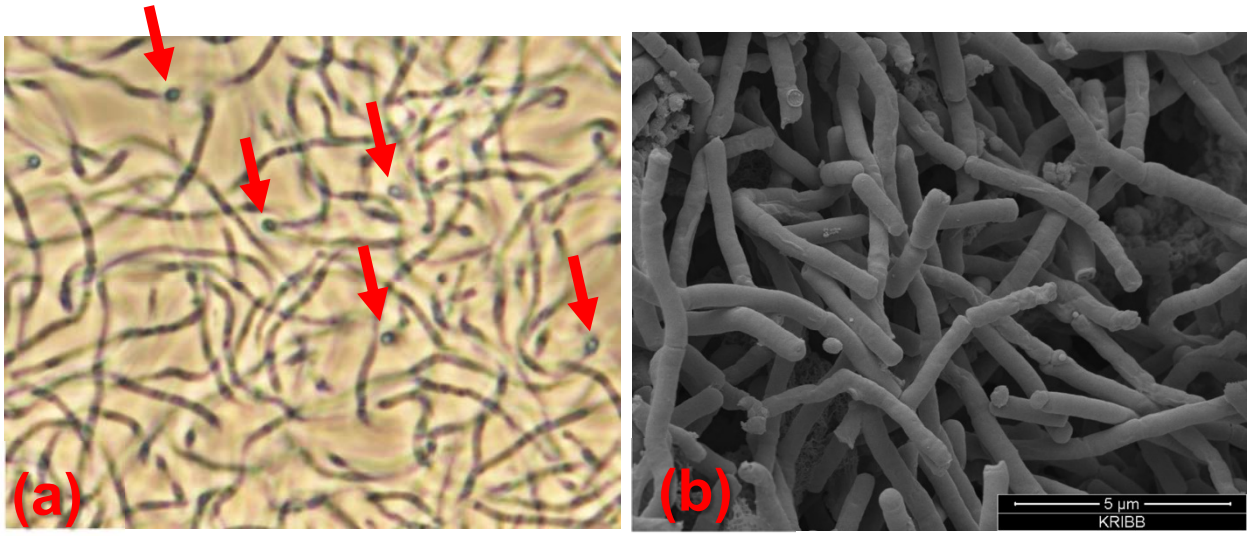

Figure 7.1.

Cell morphology of *Bacillus malikii* NCCP-662^T grown at 30ºC for 48 hr. (a) Vegetative cells and endospores (indicated by arrows) as viewed under phase-contrast microscopy, (b) The cells of NCCP-662^T observed under scanning electron microscopy.

Strain NCCP-662^T exhibits many phenotypic features that are similar to those of closely related reference strains; however, it also differs from these species in certain physiological and biochemical characteristics. The detailed physiological and biochemical characteristics of strain NCCP-662^T are given in the species description and **Table 7.1**. Among these, the most notable differentiating characteristics in comparison with the closely related species, *B. niabensis* JCM 16399^T, are growth of strain NCCP-662^T at 10 °C, positive reactions for oxidase, Voges-Proskauer reaction, hydrolysis of esculin but no hydrolysis of gelatin.

Table 7.1. Characteristics that differentiate novel strain NCCP-662^T from the type strains of closely related species of the genus *Bacillus.*

All the strains are positive for catalase and *β*-galactosidase (2-nitrophenyl-*β*D galactopyranoside) but negative for indole production, arginine dihydrolase, tryptophan deaminase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production and urease (API 20E, bioMérieux, France). No anaerobic fermentation of D-glucose. There was no assimilation of D-glucose, D-maltose, capric acid, adipic acid, trisodium citrate and phenyl acetic acid in all the strains (API 20NE, bioMérieux, France).

All strains are positive for production of acid from esculin, L-arabinose, D-xylose, methyl-*β*D-xylopyranoside, D-glucose, D-fructose, D-mannose, methyl-αD-glucopyranoside, amygdalin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose (sucrose), D-trehalose, amidon (starch), glycogen, gentiobiose and negative for production of acid from erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, L-sorbose, dulcitol, xylitol, D-lyxose, D-Tagatose, D-fucose, L-fucose, L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate ((API 50CH, bioMérieux, France).

All the strains are positive for oxidation/reduction of the following substrates: dextrin, amygdalin, D-cellobiose, α-D-glucose, maltose**,** maltoriose, D-mannose**,** D-melibiose, D-ribose**,** sucrose, pyruvic acid and glycerol and negative for D-gluconic acid, 3-methyl-D-glucose and xylitol (Biolog, USA).

Strong enzyme activity is observed in all the strains for β-glucosidase and moderately positive for esterase lipase (C 8) and esterase (C 4), whereas no enzyme activity is present for leucine arylamidase, acid phosphatase, lipase (C 14), valine arylamidase, cystine arylamidase, trypsin, naphthol-As-BI-phosphohydrolase, α-galactosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase and α-fucosidase ((API Zym, bioMérieux, France).

	NCCP-662^T	***B. niabensis* JCM 16399^T**	***B. halosaccharovorans* DSM 25387^T**
Pigment	Whitish	Yellowish white	Creamy
Spore shape and position	Spore in non-swollen sporangium at sub-terminal position	Swollen† sporangium	Swollen sporangium†
Temperature (ºC) range, (optimum)	10-50 (30-37)	20-50 (30)	15-45 (33-40)
Tolerance to heavy-metals:			
Chromium (ppm)	1200	900	900
Lead (ppm)	1800	900	900
Copper (ppm)	1200	600	600
Oxidase	+	–	+
Voges–Proskauer test	+	–	w+

Nitrate reduction	–	+	–
Hydrolysis of:			
Esculin	+	–	+
Gelatin	–	w+	+
Assimilation of:			
L-arabinose	–	+	+
D-mannose	–	–	+
D-mannitol	–	–	w+
N-acetyl glucosamine	–	–	w+
Potassium gluconate	–	+	+
Malate	–	–	+
Acid production (aerobically) from:			
Glycerol	–	+	+
D-galactose	–	+	+
L-rhamnose	–	+	+
Inositol	–	+	+
D-mannitol	–	+	+
D-sorbitol	–	+	–
Methyl α-D-mannopyranoside	–	–	+
N-acetyl glucosamine	–	+	+
Arbutin	+	+	–
Inulin	–	+	+
D-melezitose	–	–	+
D-raffinose	–	+	+
D-turanose	–	+	+
D-arabitol	–	–	+
***Oxidation/reduction of*:**			
α-, *β*-cyclo dextrin	–	+	–
Glycogen	–	+	–
N-acetyl-D-glucosamine	–	–	+
N-acetyl-β-D-mannosamine	–	–	+
L-arabinose	–	+	+
D-arabitol	–	–	+
Arbutin	–	+	+
D-fructose	–	+	+
D-galactose	–	+	+
Gentiobiose	–	w	w
m-inositol	–	+	+
α-D-lactose	–	+	w
Lactulose	–	+	+
D-mannitol	–	+	+
D-melezitose	–	w	+
β-methyl-D-galactoside	–	+	–

α-methyl-D-glucoside	–	+	+
β-methyl-D-glucoside	–	+	+
Palatinose	–	+	+
D-psicose	–	+	+
D-raffinose	–	+	w
Salicin	–	+	+
D-sorbitol	–	+	+
Stachyose	–	+	+
D-trehalose	–	+	+
Turanose	–	+	+
D-xylose	–	+	+
Acetic acid	–	–	+
α-keto valeric acid	–	w	+
L-malic acid	–	–	+
Pyruvic acid methyl ester	+	–	–
Succinic acid mono-methyl ester	+	+	–
D-alanine	–	–	+
Adenosine	–	–	+
2'-deoxy adenosine	–	–	+
Inosine	–	–	+
Thymidine	–	–	+
Uridine	–	–	+
***Enzyme activity* (API-Zym)**			
Alkaline phosphatase	+	+++	+++
α –chymotrypsin	+	–	+
β-galactosidase	–	+	–
β-glucuronidase	–	–	+
α- glucosidase	+++	+++	–
G+C content, mol %	36.9	37.7–40.9†	42.6†

+++, Very strongly positive; ++, strongly positive; +, Positive; w+, weakly positive; –, negative;
All data are from this study unless otherwise mentioned.
† data from previous studies (Kwon et al. 2007; Mehrshad et al. 2013)

Strain NCCP-662^T shows no anaerobic fermentation of D-glucose. There was no assimilation of D-glucose, D-maltose, capric acid, adipic acid, trisodium citrate, L-arabinose, D-mannose, D-mannitol, *N*-acetyl glucosamine, potassium gluconate, malate and phenyl acetic acid (API 20NE, bioMérieux, France). Acid is produced aerobically from esculin, arbutin, L-arabinose, D-xylose, methyl-*β*D-xylopyranoside, D-glucose, D-fructose, D-mannose, methyl-*α*D-glucopyranoside, amygdalin, salicin, D-cellobiose, D-maltose, D-

lactose, D-melibiose, D-saccharose (sucrose), D-trehalose, amidon (starch), glycogen, gentiobiose but no production of acid from glycerol, D-galactose, L-rhamnose, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, *N*-acetyl glucosamine, erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, L-sorbose, dulcitol, xylitol, D-lyxose, D-tagatose, inulin, D-melezitose, D-raffinose, D-turanose, D-arabitol, D-, L-fucose, L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate (in API-50CH tests). Positive for oxidation/reduction of the following substrates: dextrin, amygdalin, D-cellobiose, *α*-D-glucose, maltose, maltoriose, D-mannose, pyruvic acid methyl ester, succinic acid mono-methyl ester, D-melibiose, D-ribose, sucrose, pyruvic acid and glycerol; and negative for the following substrates: *α* -, *β*-cyclo dextrin, glycogen, *N*-acetyl-D-glucosamine, *N*-acetyl-*β*-D-mannosamine, L-arabinose, D-arabitol, arbutin, D-fructose, D-galactose, gentiobiose, m-inositol, *α*-D-lactose, lactulose, D-mannitol, D-melezitose, *β*-methyl-D-galactoside, *α*-methyl-D-glucoside, *β*-methyl-D-glucoside, palatinose, D-psicose, D-raffinose, salicin, D-sorbitol, D-alanine, adenosine, 2'-deoxy adenosine, inosine, thymidine, uridine, stachyose, D-trehalose, turanose, D-xylose, acetic acid, *α*-keto valeric acid, L-malic acid, D-gluconic acid, 3-methyl-D-glucose and xylitol (in the Biolog tests). Strong enzyme activity is observed for *α*-glucosidase, *β*-glucosidase and moderately positive activity for esterase lipase (C 8), alkaline phosphatase, a-chymotrypsin and esterase (C 4), whereas the isolate has no enzyme activity for *β*-galactosidase, *β*-glucuronidase, leucine arylamidase, acid phosphatase, lipase (C 14), valine arylamidase, cystine arylamidase, trypsin, naphthol-As-BI-phosphohydrolase, *α*-galactosidase, *N*-acetyl- *β*-glucosaminidase, *α*-mannosidase and *α*-fucosidase (API Zym, bioMérieux, France).

Strain NCCP-662^T also differs from the reference strains with respect to the tolerance against typically toxic concentrations of heavy metals (Table 7.1). Strain NCCP-662^T was found to grow well in NA/TSA media containing (separately) Cr 1200 ppm, Pb 1800 ppm or Cu 1200 ppm. The closely related reference strains cannot survive at these concentrations of the heavy metals. When compared with the previously reported heavy metal tolerant bacteria, strain NCCP-662^T can be considered as less tolerant to toxic concentration of heavy metals than *Acinetobacter pakistanensis* (Abbas et al. 2014) but its tolerance level is still high compared to many other bacteria (Affan et al. 2009; Tripathi et al. 2010).

Phylogenetic analysis, DNA–DNA hybridization and DNA base composition

The comparison of the almost complete 16S rRNA gene sequence (1492 nucleotides; DDBJ/EMBL/GenBank accession number AB968093) of strain NCCP-662^T showed high sequence similarity (98.2 %) with *B. niabensis* 4T19^T (GenBank accession no. AY998119), 98.0 % with *B. halosaccharovorans* E33^T (HQ433447) and less than 98 % with other members of the genus *Bacillus*. The phylogenetic analyses revealed that strain NCCP-662^T clustered with a clade comprising of *B. niabensis* and *B. halosaccharovorans* as close neighbours (**Figure 7.2**). This affiliation of the isolate with *B. niabensis* and *B. halosaccharovorans* was also confirmed by reconstructing phylogenetic trees using maximum parsimony and neighbour-joining algorithms (**Figures 7.3 & 7.4**), suggesting that strain NCCP-662^T coherently clusters with *B. niabensis* and *B. halosaccharovorans* although clear bootstrap support was found only in neighbour joining tree. Strain NCCP-662^T is closely related to *B. niabensis* and *B. halosaccharovorans* in many other characteristics, although the data presented here also demonstrated some differences from these closely related reference strains (Table 7.1), which suggests that strainNCCP-662^T represents a novel species of the genus *Bacillus*.

The DNA–DNA relatedness between strain NCCP-662^T and the reference strains *B. niabensis* JCM 16399^T, *B. halosaccharovorans* DSM 25387^T and *B. herbersteinensis* DSM 16534^T was determined as 20.5 ± 0.5 %, 14.4 ± 2.3 % and 12.2 ± 3.7 %, respectively. These values are less than the 70 % threshold needed to assign the strain to a novel species (Wayne et al. 1987). Strain NCCP-662^T was found to have 36.9 mol% G+C content of the genomic DNA as determined by HPLC (**Figure 7.5**). This value is consistent with the placement of strain within the genus *Bacillus* and is similar to the range of G+C content reported for the closely related reference strain *B. niabensis* JCM 16399^T but lower than that of *B. halosaccharovorans* DSM 25387^T (Table 7.1). DNA G+C contents of members of the genus *Bacillus* are reported to have a wide heterogeneity, ranging from 32 to 66 % (Logan and Vos 2009).

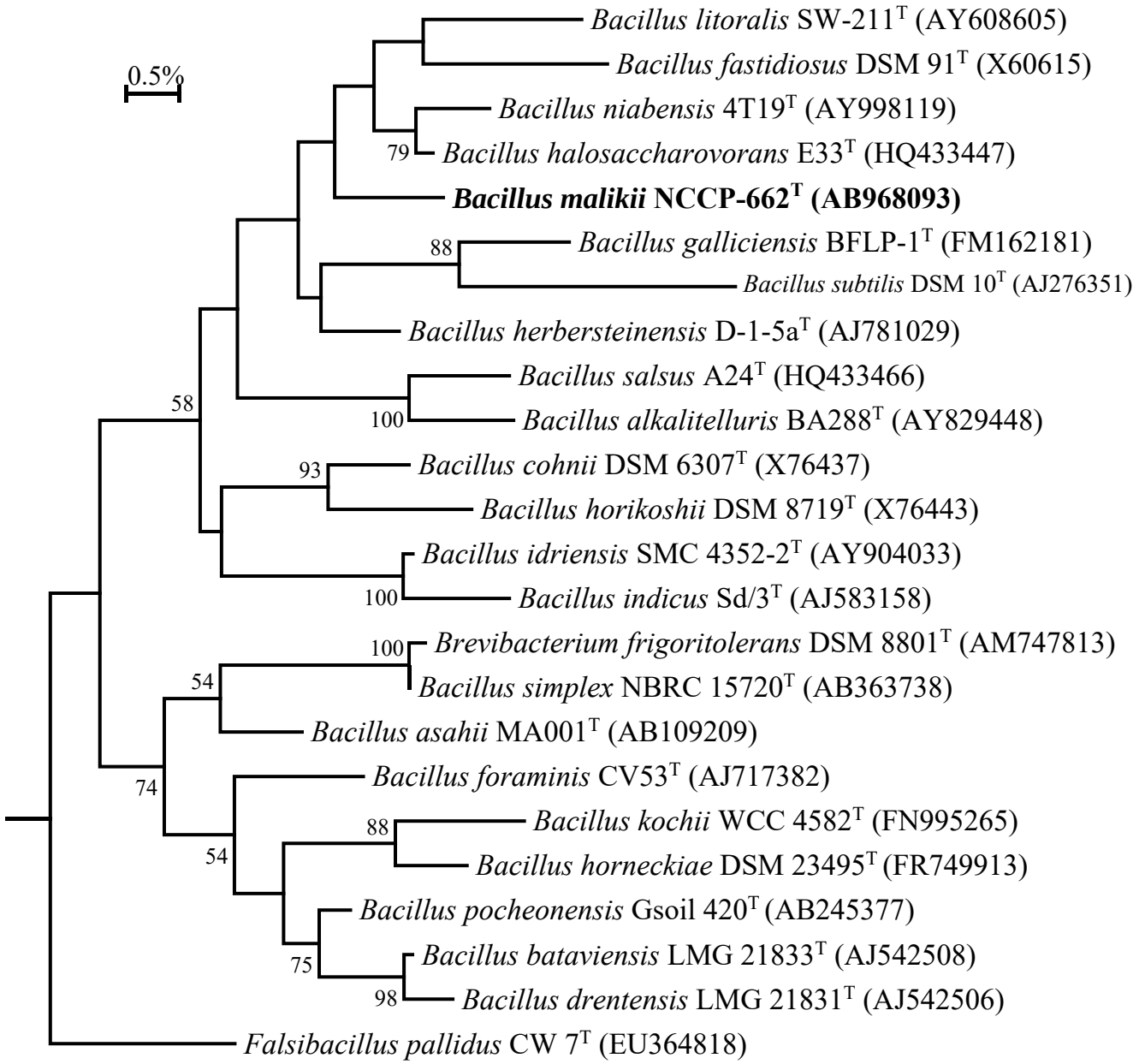

Figure 7.2.

Maximum-likelihood phylogenetic tree inferred from 16S rRNA gene sequences showing inter-relationship of strain NCCP-662^T with type strains of the genus *Bacillus*. Data with gaps and ambiguous nucleotides were removed from the alignment for the construction of the tree, which was generated using MEGA 6.0 software package (Tamura et al. 2013) based on a comparison of 1307 nucleotides, and was rooted by using *Falsibacillus pallidus* CW 7^T (EU364818) as an out-group. Bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The accession number of each type strain sequence is shown in parentheses.

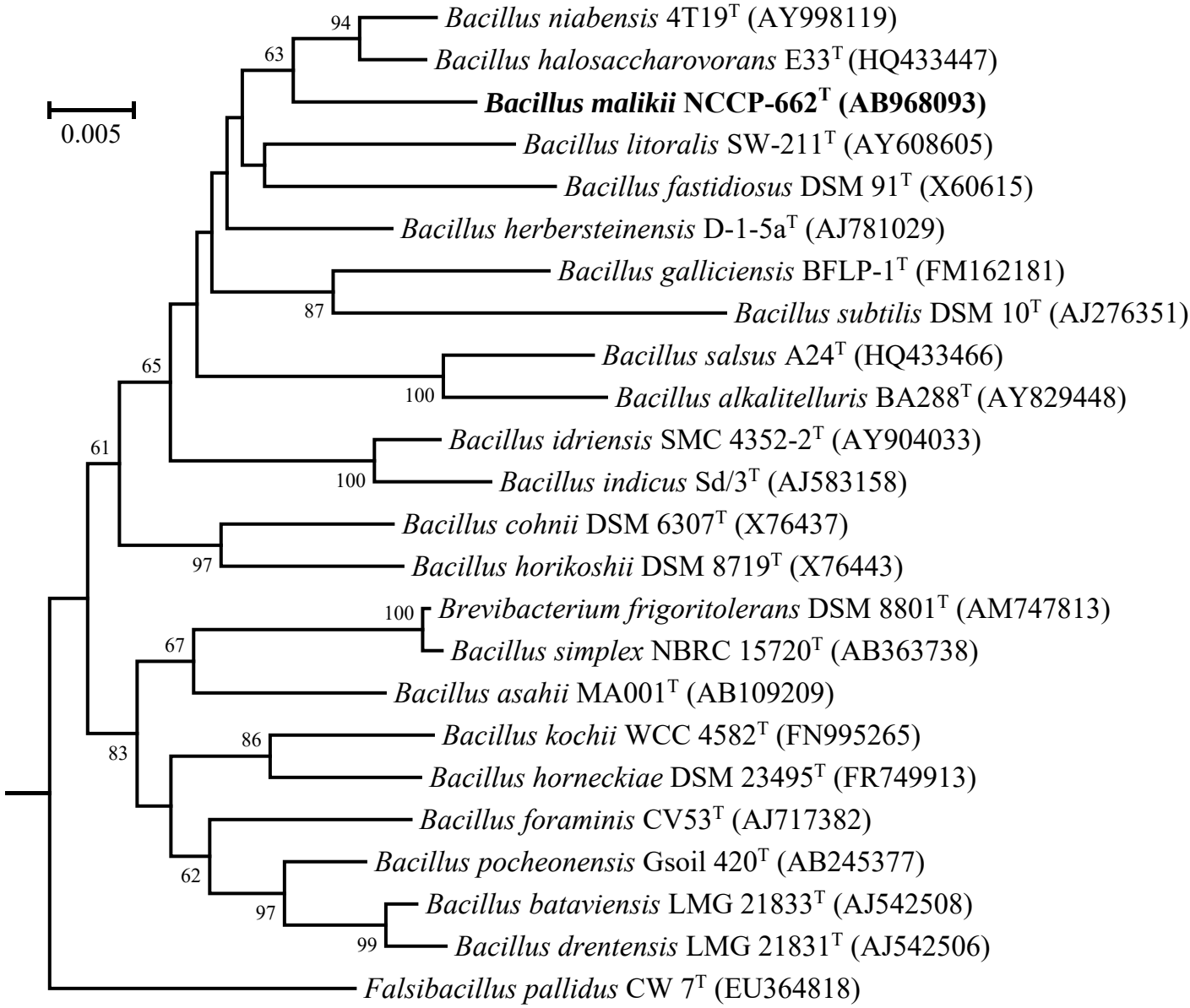

Figure 7.3.

Neighbour-joining phylogenetic tree inferred from 16S rRNA gene sequences showing inter-relationship of strain NCCP-662^T with type strains of the genus *Bacillus*. Data with gaps and ambiguous nucleotides were removed from the alignment for the construction of tree, which was generated using MEGA 6.0 software package (Tamura et al. 2013) based on a comparison of 1307 nucleotides, and was rooted by using *Falsibacillus pallidus* CW 7^T (EU364818) as an out-group. Bootstrap values (only >60% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The accession number of each type strain sequence is shown in parentheses.

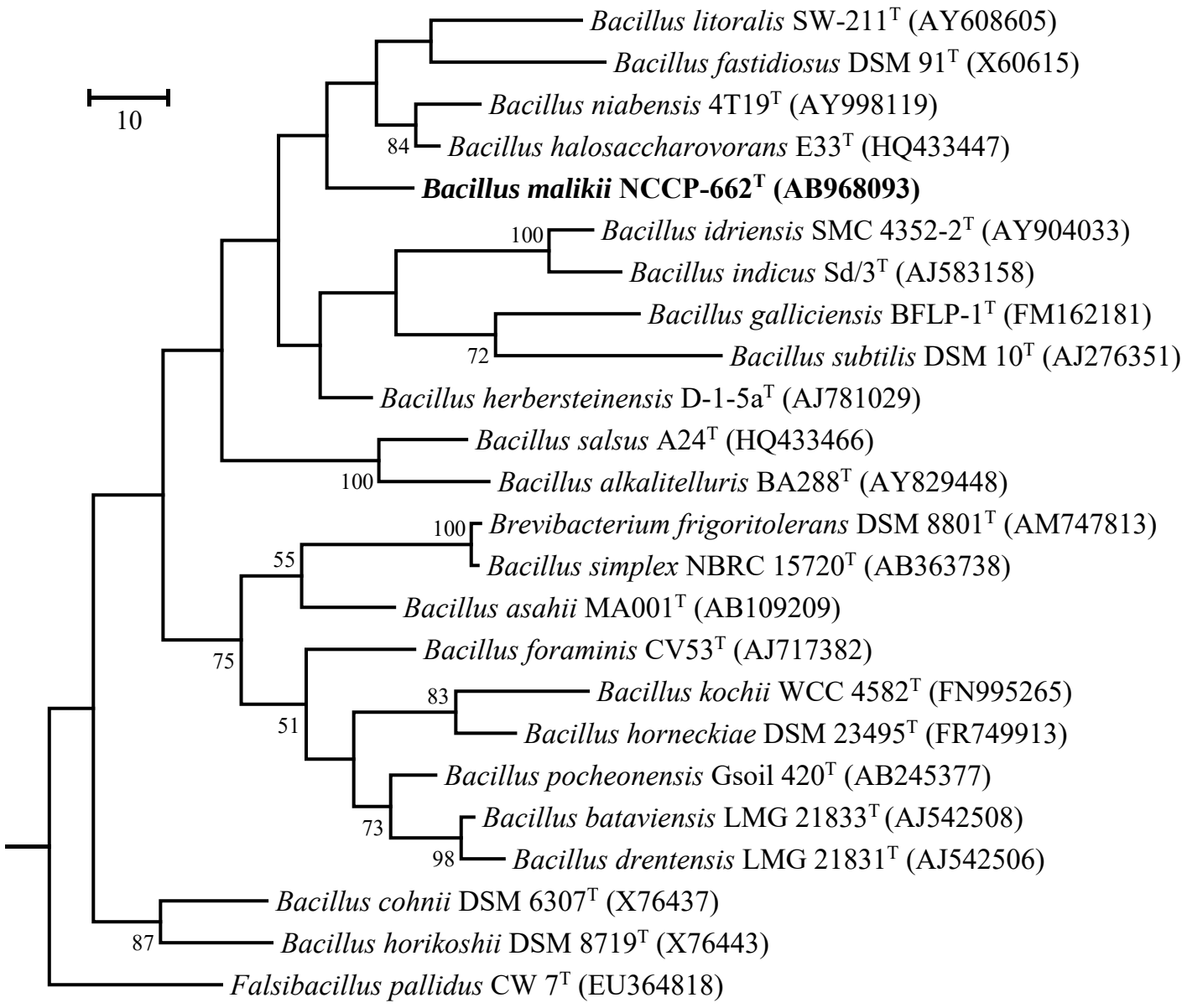

Figure 7.4.

Maximum-parsimony phylogenetic tree inferred from 16S rRNA gene sequences showing inter-relationship of strain NCCP-662^T with type strains of the genus *Bacillus*. Data with gaps and ambiguous nucleotides were removed from the alignment for the construction of tree, which was generated using MEGA 6.0 software package (Tamura et al. 2013) based on a comparison of 1307 nucleotides, and was rooted by using *Falsibacillus pallidus* CW 7^T (EU364818) as an out-group. Bootstrap values (only >60% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The accession number of each type strain sequence is shown in parentheses.

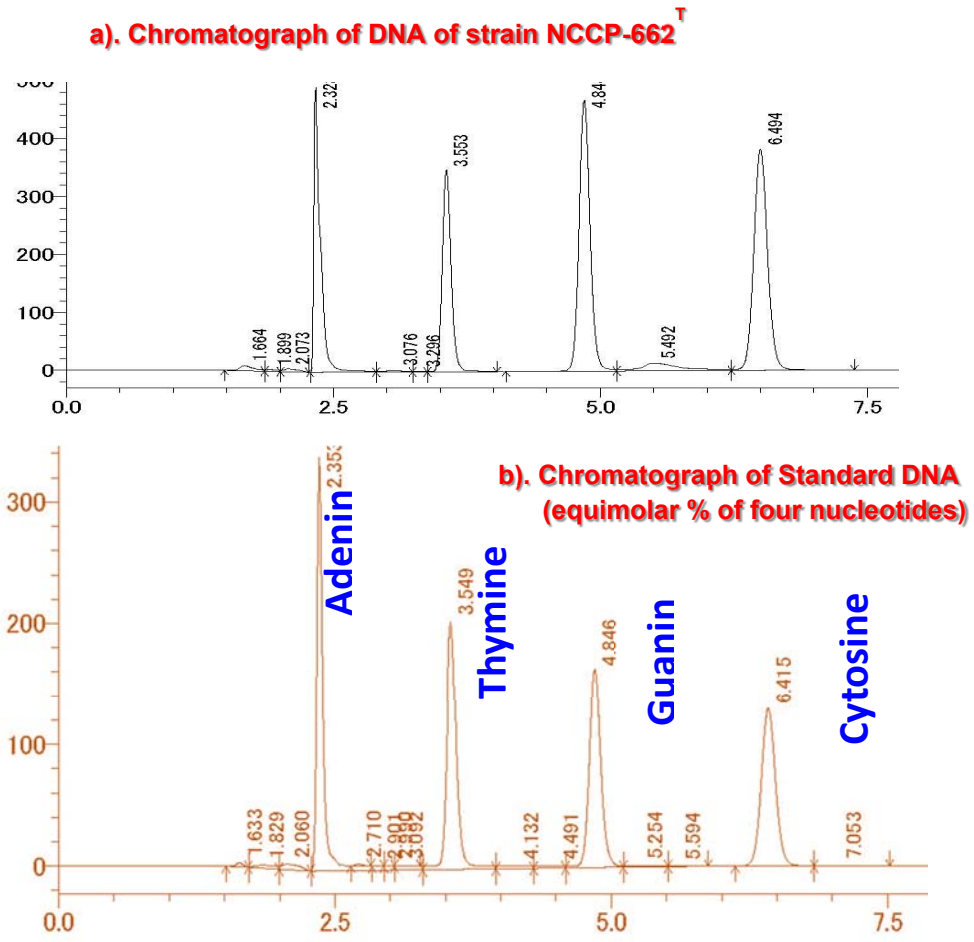

Figure 7.5.

Chromatograph of DNA G+C Content analysis of (a) *Bacillus malikii* NCCP-662^T in comparison with the (b) DNA standard on HPLC.

Chemotaxonomic analysis

The cellular fatty acid profile of strain NCCP-662^T was found to consist predominantly of anteiso-$C_{15:0}$, iso-$C_{14:0}$, iso-$C_{16:0}$ and $C_{16:0}$, followed by iso-$C_{15:0}$ and $C_{16:1}$ ω11*c*. Minor amounts (less than 5 % but more than 1 %) of $C_{15:0}$, anteiso-$C_{17:0}$, $C_{16:1}$ ω7*c* alcohol, $C_{14:0}$, $C_{17:0}$ and iso-$C_{17:0}$ were also detected (**Table 7.2**). The major components of this profile are similar to those present in closely related reference strains, although variation in amounts of some components clearly differentiated the novel strain from these and other members of the genus *Bacillus*. Strain NCCP-662^T was found to contain higher amounts of iso-$C_{14:0}$, $C_{16:0}$ and $C_{16:1}$ ω11*c* (7.2 %) compared to the reference strains (Table 7.2).

Table 7.2. Cellular fatty acid profiles (%) of strain NCCP-662^T in comparison with the type strains of reference species of the genus *Bacillus.*

Characteristics	NCCP-662^T	*B. niabensis* JCM 16399^T	*B. halosaccharovorans* DSM 25387^T
$C_{14:0}$	1.4	0.1	0.4
$C_{15:0}$	3.7	0.7	1.0
$C_{16:0}$	10.4	4.7	2.5
$C_{17:0}$	1.4	0.3	0.3
iso-$C_{14:0}$	16.2	4.3	4.8
iso-$C_{15:0}$	8.3	5.1	13.9
iso-$C_{16:0}$	12.2	9.8	4.9
iso-$C_{17:0}$	1.2	1.4	1.3
anteiso-$C_{15:0}$	30.4	54.9	57.0
anteiso-$C_{17:0}$	2.8	14.2	6.8
$C_{16:1}$ ω7*c* alcohol	2.6	1.0	1.5
$C_{16:1}$ ω11*c*	7.2	1.4	2.0
Summed features 4*	0.2	0.5	1.5

*Summed feature 4 comprised one or more of iso-$C_{17:1}$ I / anteiso-$C_{17:1}$ B, which could not have been separated by MIDI system.

All the data are obtained in this study. Values are percentages of total fatty acid detected.

Those values of cellular fatty acid components were deleted if present less than 1% in all the species and/or absent in some species.

The cell wall peptidoglycan contained *meso*-diaminopimelic acid as a diagnostic amino acid (**Figure 7.6**), which is the characteristic of members of the genus *Bacillus*. The respiratory quinone system of strain NCCP-662^T was found to contain MK-7 (83 %) as a major component, whilst MK-6 (11 %) and MK-8 (6 %) were also detected as minor components (**Figure 7.7**). The presence of anteiso-$C_{15:0}$ as a major fatty acids, *meso*-diaminopimelic acid in the peptidoglycan and MK-7 as the predominant respiratory quinone of strain NCCP-662^T are typical characteristics of members of the genus *Bacillus* (Bagheri et al. 2012; Kämpfer et al. 2006; Mehrshad et al. 2013; Roohi et al. 2014).

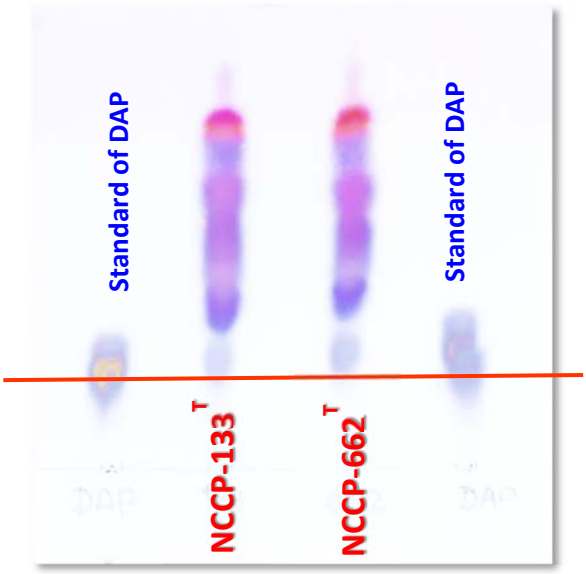

Figure 7.6

Chromatograph of diaminopimelic Acid (*m*-DAP) in cell wall peptidoglycan of *Bacillus malikii* sp. nov. NCCP-662^T in comparison with a reference strain: *Bacillus boraciitolerans* NCCP-133^T

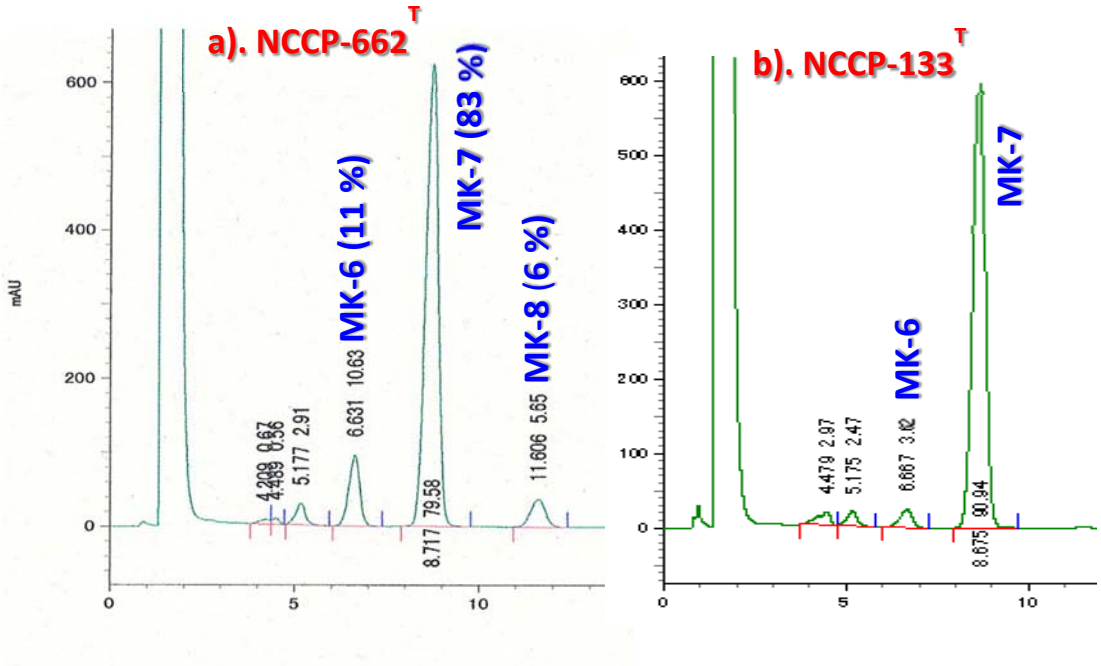

Figure 7.7

Chromatograph of menaquinones analysis of a) *Bacillus malikii* sp. nov. NCCP-662^T in comparison with a reference strain of the genus, b) *Bacillus boraciitolerans* NCCP-133^T

The polar lipids profile of strain NCCP-662^T were found to be comprised of predominantly diphosphatidylglycerol and phosphatidylglycerol, along with several unidentified glycolipids (GL1-6), phospholipids (PL1-2) and polar lipids (L1-2) (**Figure 7.8**). This polar lipids profile is very similar to that of the closely related species *Bacillus halosaccharovorans* (Mehrshad et al. 2013) and *B. herbersteinensis* (Wieser et al. 2005) by lacking phosphatidylethanolamine and having two glycolipids (GL5-6) with similar chromatographic motility. The presence of diphosphatidylglycerol, phosphatidylglycerol and two glycolipids (GL5–GL6) in the profile of strain NCCP-662^T are also consistent with the profile of the type species of the genus, *B. subtilis* DSM 10^T (Kämpfer et al. 2006). However, the profile of NCCP-662^T differs from that of *B. subtilis* in the absence of phosphatidylethanolamine and an amino phospholipid, as well as the additional occurrence of several unidentified glycolipids (GL1-4), phospholipids (PL1-2) and polar lipids (L1-2). Although the presence of glycolipids GL1-4 distinguished NCCP-662^T from the reference species (Figure 7.8), not too much significance should be given to this observation because the amounts of these glycolipids detected were rather low.

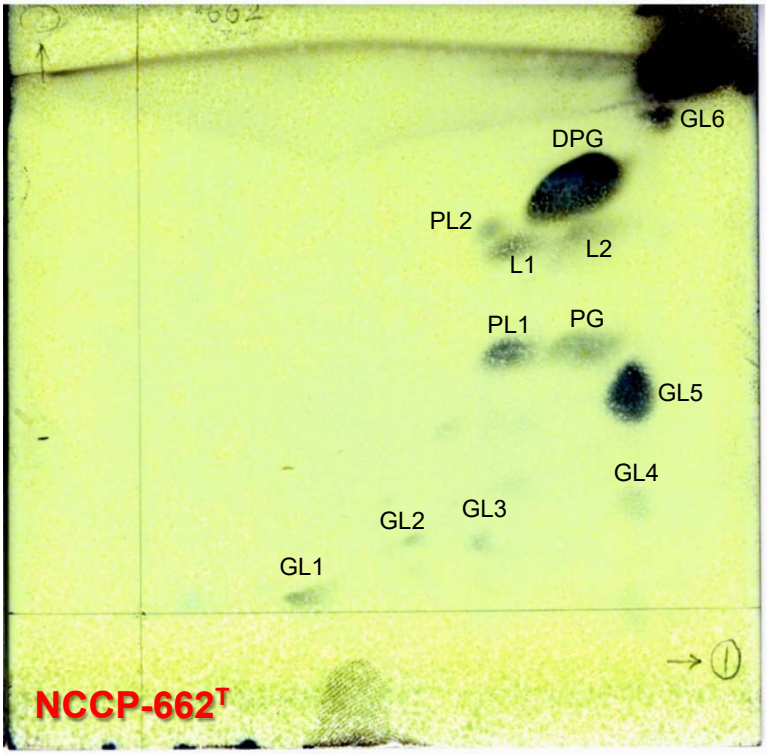

Figure 7.8.

Polar lipids profile of strain NCCP-662^T.

DPG (Diphosphatidylglycerol),
PG (Phosphatidylglycerol),
GL1-6 (Glycolipids of unknown structure),
PL1-2 (Phospholipids of unknown structure) and
L1-2 (Polar lipids of unknown structure).

The phenotypic, physiological and phylogenetic analyses, as well as evidence of DNA–DNA relatedness and chemotaxonomic data, clearly distinguish the isolated strain NCCP-662^T from other closely related validly named members of the genus *Bacillus* and thus

the strain is considered to represent a novel species of the genus, for which the name *Bacillus malikii* sp. nov. is proposed with the type strain NCCP-662^T and its description is given here:

Description of *Bacillus malikii* sp. nov.

Bacillus malikii (ma.li'ki.i N.L. masc. gen. n. *malikii* after the name of Dr. Kausar Abdullah Malik, a distinguished professor of microbiology and biotechnology in Pakistan).

Cells are Gram-stain positive, strictly aerobic, motile and spore-forming long rods or filaments that mostly occur in pairs or long chains. The colonies are small (2–3 mm in diameter) and round with entire margins, smooth and shiny surfaces, slightly convex and whitish in colour, which are slightly sticky in texture on nutrient agar, tryptic soy agar and marine agar media. Cells grow at 10–50 °C (optimum growth at 30–37 °C), at pH ranges of 6.0–10.0 (optimal at pH 7–8) and in 0–12 % NaCl (w/v, optimum 1–3 %). Tolerant to heavy metals (Cr, As, Pb and Cu). Catalase, oxidase, *β*-galactosidase (2-nitrophenyl- *β*D galactopyranoside) and Voges–Proskauer reaction are positive but negative for indole production, arginine dihydrolase, tryptophan deaminase, lysine- and ornithine-decarboxylases, citrate utilisation, H_2S production, urease and nitrate reduction. Esculin is hydrolysed but not gelatin. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol along with several unidentified glycolipids, phospholipids and polar lipids. The predominant cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{14:0}$, iso-$C_{16:0}$ and $C_{16:0}$, followed by iso-$C_{15:0}$, $C_{16:1}$ ω11*c*, $C_{15:0}$, anteiso-$C_{17:0}$ and $C_{16:1}$ ω7*c* alcohol. The major respiratory quinone is menaquinone MK-7, with minor amounts of MK-6 and MK-8. The DNA G+C content of the type strain is 36.9 mol%.

The type strain NCCP-662^T (=LMG 28369^T = DSM 29005^T = JCM 30192^T) was isolated from a tannery effluent sample collected from the treatment pond of a leather factory. The DDBJ/EMBL/GenBank accession number for 16S rRNA gene sequence of strain NCCP-662^T is AB968093.

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CHAPTER VIII

GENERAL CONCLUSION AND FUTURE PROSPECTS

Environmental pollution by heavy-metal contaminated industrial effluents significantly deleterious in the developing countries. The main threats to human health from heavy metals are associated with exposure to Pb, Cd, Hg, Cu, Ni and As. Conventional technologies for removing heavy metals, including ion exchange, reverse osmosis, evaporative recovery, and chemical precipitation is often inefficient and very expensive. Therefore, new methods for the removal of metals from effluent or their reduction to very low concentrations must be developed. In this regard, microbe-based technologies can serve as alternatives to conventional methods for pollution removal of heavy-metals from the environment. These heavy metal tolerant bacteria can be useful for bioremediation of soil/water contaminated heavy metals. Limited research work has been reported on bioremediation of heavy metals by heavy-metals tolerant bacterial strains in Pakistan.

In the present studies, more than 68 strains of bacteria were isolated from the discharge of Industrial areas (Sialkot, Islamabad and Kasur), which were found to tolerate toxic concentrations of heavy metals including Cr, Cu, Cd, Pb, and As. Some strains tolerated a concentration up to 3600 ppm for Cr, 3300 ppm for Cu, 3000 ppm for Cd, 2100 ppm for Pb and 3000 ppm for As. Among these highly tolerant isolates, maximum tolerance limit (MTL) was observed, NCCP-601, 602, 603, 647, 657, 660, 661, 621, 653 against Cr (3600 ppm), NCCP-601, 602, 603 against Cu (3300 ppm), NCCP-601, 602, 603 against Cd (3000 ppm), NCCP-644, NCCP-650 against Pb (2100 ppm) and As (3000 ppm). The isolates reported in these studies showed the highest tolerance to Cd and As compared to previous reports.

Our studies on biosorption of heavy-metals indicated that two isolates, NCCP-614

(99 %), NCCP-605 (96 %), NCCP-655 (91 %) and NCCP-624 (88 %) showed maximum biosorption of Pb. Similarly, strains NCCP-614 also showed maximum biosorption capacity of 89 % for Cd.; however, maximum biosorption of copper was done by NCCP-625 (42 %) followed by NCCP-614 (38 %) and NCCP-647 (36 %). These isolates can be used for bioremediation of soil/water system contaminated with Pb, Cd and Cu.

Identification based on comparative 16S rRNA gene sequence data demonstrated that these heavy metal tolerant strains belonged to nineteen different genera. The majority of the strains belonged to *Bacillus*, *Pseudomonas* and *Staphylococcus*. Sequence similarity analysis showed that though most of the isolates had high 16S rRNA gene sequence similarity (> 99%) with the closely related taxa in their respective clusters; however, some strains belonging to the genera, *Bacillus*, *Alcaligenes*, *Acinetobacter*, *Pseudomonas*, *Citrobacter* and *Bravibacterium*, had 97.9 to 100 % similarity of 16S rRNA gene sequence and thus, these strains were characterized taxonomically to delineate as novel species based upon phylogenetic analyses, DNA-DNA homology, phenotypic and chemotaxonomic data. The strains delineated as novel species were named as: ***Acinetobacter pakistanensis*** sp. nov., ***Alcaligenes pakistanensis*** sp. nov., and ***Bacillus malikii*** sp. nov.

The isolated heavy-metals tolerant strains were also characterized at molecular level based on *nifH* and *acdS* genes. The results indicated that at least 7 strains contained both of these genes and these strains have the potential for plant growth promotion and can be used as bioinoculants (biofertilizer) in agriculture. Among these, strain NCCP-650, which contained both the genes, was found to significantly increase growth of *Brassica napus* in greenhouse experiment, when irrigated with water containing heavy-metals. This strain NCCP-650^{T} also found to contain nitrite reductase, *nirK* gene, which has its significance in bioremediation process too. This strain is a good candidate PGPR and thus, can be used in agriculture on heavy metals contaminated soils.

The findings of heavy metal tolerant novel species provide a genetic resource, which would also be a source of new genes involved in metals transport / tolerance mechanisms as well as to identify the gene(s) responsible for the mechanism of heavy metal tolerance in

bacteria because of its small genome size.. Such gene(s) may be useful in high metal soils for plant growth promotion and bioremediation. The potential of these strains for bioremediation and their PGPR activity under stress condition would be important for agriculture.

Future prospects / recommendations

Based upon the finding of the present research, further investigation can be carried out to formulate an agricultural technology for farmer's field or bioremediation of industrial effluents using these strains through the following course of action:

- Mechanisms of heavy metal tolerance in these strains having efficient biosorption capacity should be studied using molecular and physiological approaches.
- Bioreactor studies should be planned at industrial level using these strains having efficient biosorption capacity as pilot project to demonstrate heavy metal removal abilities from tannery/industrial effluents.
- Multiple metal tolerant microbes can be better utilized for decontamination of soil and water polluted areas at large scale.
- Bioassays should be studied for the confirmation of activities of nitrogenase and ACC-deaminase enzymes for the strains possessing *nifH* and *acdS* genes in their genome.
- Plant growth promoting strains may be used for agriculture as a bioinoculants in heavy metal contaminated soil/water systems. However, field experiments should be conducted for specific crops before large scale distribution of bioinoculants.

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