การคัดกรองและลักษณะสมบัติของแบคทีเรียซึ่งออกซิไดซ์อาร์ซีไนต์ไปเป็นอาร์ซีเนตจากของเสียโรงงานฟอกหนังสัตว์และดินเกษตรกรรม

นาย กิจจา จิตรภิรมย์

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ปีการศึกษา 2552

SCREENING AND CHARACTERIZATION OF ARSENITE/ARSENATE OXIDIZING BACTERIA FROM TANNERY WASTES AND AGRICULTURAL SOILS

Mr. KITJA CHITPIROM

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Environmental Sciences
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Thesis Title	SCREENING AND CHARACTERIZATION OF ARSENITE/ARSENATE OXIDIZING BACTERIA FROM TANNERY WASTES AND AGRICULTURAL SOILS
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Field of Study	Environmental Sciences
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กิจจา จิตรภิรมย์: การคัดกรองและลักษณะสมบัติของแบคทีเรียซึ่งออกซิไดซ์อาร์ซีไนต์ไปเป็นอาร์ซีเนตจากของเสียโรงงานฟอกหนังสัตว์และดินเกษตรกรรม (SCREENING AND CHARACTERZATION OF ARSENITE/ARSENATE OXIDIZING BACTERIA FROM TANNERY WASTES AND AGRICULTURAL SOILS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.อัญชริดา อัครจรัลญา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.สมบูรณ์ ธนาศุภวัฒน์, 114 หน้า

แบคทีเรียทนต่ออาร์ซีนิกจำนวน 93 ไอโซเลตซึ่งแยกได้จากตัวอย่างของเสียโรงงานฟอกหนังสัตว์ 7 ตัวอย่าง ที่เก็บจาก จังหวัดสมุทรปราการ และดินเกษตรกรรม 9 ตัวอย่างที่เก็บจากจังหวัดนครปฐม และจังหวัดเพชรบุรี โดยวิธีอินริชเมนท์ (enrichment) ซึ่งเติมโซเดียมอาร์ซีไนต์ (sodium arsenite) ความเข้มข้น 1.3 มิลลิโมลาร์ แบคทีเรียในกลุ่มนี้มีค่าความทนต่ออาร์ซีนิก (MIC) 5-≥40 มิลลิโมลาร์อาซีไนต์ และ 200- ≥450 มิลลิโมลาร์อาร์ซีเนต และพบว่ามีแบคทีเรียจำนวน 27 ไอโซเลตเป็นแบคทีเรียที่มีความทนต่ออาร์ซีนิกสูง (MIC ≥ 40 มิลลิโมลาร์อาร์ซีไนต์ และ > 400 มิลลิโมลาร์อาร์ซีเนต) จากการศึกษาลักษณะทางสัณฐานวิทยา การเจริญ สรีรวิทยา และชีวเคมี ยูบิควิโนน (ubiquinone) และ ลำดับเบสของยีน 16S rRNA ทำให้สามารถจัดแบ่งแบคทีเรียเหล่านี้ออกเป็น 9 กลุ่ม ในสกุล *Klebsiella* คือ กลุ่มที่ 1 และ กลุ่มที่ 8 (9 ไอโซเลต) สกุล *Acinetobacter* คือกลุ่มที่ 2, 3 และกลุ่มที่ 7 (9 ไอโซเลต) สกุล *Pseudomonas* คือกลุ่มที่ 4 และกลุ่มที่ 6 (4 ไอโซเลต) สกุล *Comamonas* คือกลุ่มที่ 5 (4 ไอโซเลต) และ สกุล *Enterobacter* คือกลุ่มที่ 9 (1 ไอโซเลต)

จากการศึกษาพบว่ามีเพียง 1 ไอโซเลตเท่านั้น คือ *Comamonas* sp. A3-3 ในกลุ่มที่ 5 มีศักยภาพในการนำไปใช้บำบัดอาร์ซีนิกทางชีวภาพ เพราะสามารถออกซิไดซ์อาร์ซีไนต์ไปเป็นอาร์ซีเนต ผลการวิเคราะห์ชนิดของอาร์ซีนิกโดยใช้วิธี XANES spectroscopy ยืนยันว่าไอโซเลต A3-3 สามารถออกซิไดซ์อาร์ซีไนต์ได้จริง ประสิทธิภาพการออกซิไดซ์อาร์ซีไนต์ไปเป็นอาร์ซีเนตเท่ากับร้อยละ 65.3 เมื่อวิเคราะห์โดยวิธีดัดแปลงการไตเตรทกับไอโอดีน *Comamonas* sp. A3-3 แยกได้จากดินเกษตรกรรมสามารถเจริญได้ที่อุณหภูมิ 40°C ที่ความเข้มข้นของเกลือร้อยละ 4.5 (w/v) ในช่วง pH 5 ถึง 11 กรดไขมันหลักในเซลล์เป็น $C_{16:0}$, $C_{18:1}\,\omega 7c$ และ $C_{17:0\ CYCLO}$ ยูบิควิโนนหลักเป็น Q-8 ปริมาณ G+C ของ DNA เท่ากับ 69.6 โมลเปอร์เซ็นต์ ผลการวิเคราะห์ลำดับเบสของยีน 16S rRNA มีค่าความคล้ายคลึงกับ *Comamonas kerstersii* LMG 3475^T, *C. aquatica* LMG 2370^T และ *C. koreensis* YH12^T ร้อยละ 96.9 , 96.8 และ 96.4 ตามลำดับ แบคทีเรียสายพันธุ์นี้ (A3-3^T = NBRC 106524^T) มีความแตกต่างจาก *C. kerstersii* LMG 3475^T และ*Comamonas* สปีชีส์อื่นๆโดยอาศัยลักษณะทางฟีโนไทป์และตำแหน่งทางวิวัฒนาการ จึงจัดเป็นสปีชีส์ใหม่ในสกุล *Comamonas* และเสนอตั้งชื่อเป็น *Comamonas terra*

สาขาวิชา วิทยาศาสตร์สิ่งแวดล้อม ลายมือชื่อนิสิต.....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5087755020: MAJOR ENVIRONMENTOL SCIENCE
KEYWORDS: ARSENITE OXIDIZING BACTERIA/AESENIC RESISTANT BACTERIA/TENERY WASTES/AGICULTURAL SOILS

KITJA CHITPIROM: SCREENING AND CHARACTERZATION OF ARSENITE/ARSENATE OXIDIZING BACTERIA FROM TANNERY WASTES AND AGRICULTURAL SOILS: THESIS ADVISOR: ASSOC. PROF. ANCHARIDA AKARACHARANYA, THESIS CO-ADVISOR: ASSOC. PROF. SOMBOON TANASUPAWAT, Ph.D., 114 pp.

Ninety-three arsenic resistant bacteria were isolated from 7 tannery waste samples collected from Samutprakan province and 9 agricultural soil samples collected from Nakhonpathom and Petburi Provinces, Thailand by enrichment culture method using medium containing 1.3 mM sodium arsenite. Minimum inhibitory concentration (MIC) of these isolates ranged from 5-$\geq$40 mM arsenite and 200- $\geq$450 mM arsenate. Highly arsenic resistant bacteria (27 isolates) had minimum inhibitory concentrations (MICs) for arsenite and arsenate of $\geq$40 mM and >400 mM, respectively. On the basis of morphological, cultural, physiological and biochemical characteristics; ubiquinone system, and 16S rRNA gene sequence analyses, they were identified as following: nine isolates each of *Klebsiella* (Groups 1 and 8) and *Acinetobacter* (Groups 2, 3 and 7), four isolates each of *Pseudomonas* (Groups 4 and 6) and *Comamonas* (Group 5), and one isolate of *Enterobacter* (Group 9)**.**

Only one isolate in Group 5, *Comamonas* sp. A3-3, appeared to have potential for bioremediation due to a capability of oxidizing arsenite to arsenate. The arsenite oxidizing activity was confirmed by an arsenic speciation using a XANES spectroscopic method. An efficiency of the arsenite oxidizing activity determined by modified arsenite-iodine titration method was 65.3%. The arsenite oxidizing strain, *Comamonas* sp. A3-3, which isolated from agricultural soil grew at 40°C, in the presence of 4.5% (w/v) NaCl and at pH 5 to 11. Major cellular fatty acids were $C_{16:0}$, $C_{18:1}$ $\omega 7c$ and $C_{17:0\ CYCLO}$. Ubiquinone with eight isoprene unit (Q-8) was a predominant ubiquinone. DNA G+C content was 69.6 mol%. Phylogenetic analysis using 16S rRNA gene sequences showed that the strain A3-3 was closely related to *C. kerstersii* LMG 3475^T, *C. aquatica* LMG 2370^T and *C. koreensis* YH12^T with 96.9, 96.8 and 96.4% similarities, respectively. The strain A3-3 could be clearly distinguished from *C. kerstersii* LMG 3475^T and related *Comamonas* species based on its phenotypic characteristics and phylogenetic position. This strain (A3-3^T = NBRC 106524^T) could be a novel species in the genus *Comamonas*, for which the name *Comamonas terra* is proposed.

Field of Study : Environmental science Student's Signature .....

Academic Year : 2009 Advisor's Signature .....

Co-Advisor's Signature .....

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CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

As (III)	=	Arsenite or trivalent arsenic
As (V)	=	Arsenate or pentavalent arsenic
ATCC	=	American Type Culture Collection, Maryland, U.S.A.
ATP	=	Adenosine triphosphate
˚C	=	Degree celsius
mm	=	Millimeter
DMA	=	Dimethylarsinic acid
DNase	=	Deoxyribonuclease
EDTA	=	Disodiumethylenediaminetetraacetate
EMBL	=	European Molecular Biology Laboratory
EPA	=	Environmental Protection Agency
g	=	Gram or Gravity
GenBank	=	National Institute of Health genetic sequence database
h	=	Hour
HCl	=	Hydrochloric acid
HPLC	=	High performance liquid chromatograhphy
JCM	=	Japan Collection of Microorganisms
KCTC	=	Korean Collection for Type Cultures
kDa	=	Kilo dalton
KOH	=	Potassium hydroxide
l	=	Liter
LD_{50}	=	Lethal dose 50%
LMG	=	Laboratory for Microbiology, Gent University, Belgium
LUH	=	Collection L. Dijkshoorn, Leiden University Medical Center, Leiden, Netherlands
M	=	Molar
MEGA	=	Molecular Evolutionary Genetics Analysis
MeOH	=	Methanol
mg	=	Milligram
MIC	=	Minimum inhibitory concentration

min	=	Minute
ml	=	Milliliter
mM	=	Millimolar
MMA	=	Methylasonic acid
mm	=	Millimeter
NaCl	=	Sodium chloride
NaOH	=	Sodium hydroxide
NBRC	=	Biological Resource Center, National Institute of Technology and Evaluation (NITE), Japan
NCBI	=	National Center for Biotechnology Information
NCCB	=	Netherlands Culture Collection of Bacteria, Netherlands
nm	=	Nanometer
OD	=	Optica density
PCR	=	Polymerase chain reaction
ppb	=	part per billion
ppm	=	part per million
rRNA	=	Ribosomal ribonucleic acid
rpm	=	Round per minute
s	=	Second
SDS	=	Sodium dodesylsulfate
sp.	=	Species
TLC	=	Thin layer chromatography
UK	=	United Kingdom
US	=	United States
UV	=	Ultraviolet
v/v	=	volume / volume
v/w	=	volume / weight
XANES	=	X-ray absorption near-edge structure
µg	=	Microgram
µl	=	Microliter
ø	=	Diameter

CHAPTER I

INTRODUCTION

Arsenic is a member of group V in the periodic table of element, naturally occurs both as inorganic and organic compounds, and in four oxidation states (+5, +3, 0 and -3). Generally, inorganic arsenic compounds are more toxic than organic arsenic compounds, and arsenite is more broadly toxic than arsenate (NRC, 1999). Arsenic is usually found in conjunction with sulfur in minerals such as arsenopyrite (FeAsS), realgar (AsS), orpiment (As_2S_3) and enargite (Cu_3AsS_4) (Oremland and Stolz, 2005). The arsenic is spreaded out by both natural sources such as geological formation, geothermal and volcanic activities (ATSDR, 2000; Smedley and Kinniburgh, 2002) and anthropogenic sources such as mining, smelting, tanning and battery producing activities; the use of arsenical wood preservatives, the use of arsenical herbicides, insecticides, fungicides and fertilizers in agriculture (Walton *et al*., 2004; Khan *et al*., 2006). High arsenic concentration in environment may enter food-chain then causes harmful health effects to humans, animals and other organisms. (Olson *et al*., 1940; Walsh and Keeney, 1975; NRC, 1999; ATSDR, 2000; Pamela, 2000; DNREC, 2002; Oremland and Stolz, 2005). Chronic exposure to arsenic increases the risk of developing cancer (Eguchi *et al*., 1997).

Although, arsenic is generally toxic to life, it has been demonstrated that many bacteria are arsenic resistance and/or post arsenic metabolizing system (ATSDR, 2000; Smedley and Kinniburgh, 2002). Several mechanisms of bacterial arsenic resistance were proposed; 1) a strictly phosphate-specific transport system, which prevents uptake of arsenate, an analogous of phosphate, 2) an efflux system mediated by plasmid or chromosomally encoded *ars*-operon, 3) arsenic accumulation in cells (Takeuchi, *et al*., 2007). Resistance to arsenite and arsenate via the *ars* operon were reported in both Gram-positive and Gram-negative bacteria (Mukhopadhyay *et al*., 2002). The *ars* operon is usually consisted of a minimum of three co-transcribed genes, *arsR* (encoding of regulatory repressor), *arsB* (encoding of membrane arsenite permease pump) and *arsC* (encoding of intracellular arsenate reductase) in the order

of *arsRBC*. Bacteria posed the 3 elements of *ars* operon confers resistance to both arsenite and arsenate (Mateos *et al*., 2006). Bacterial oxidation of arsenite to arsenate is known as arsenic detoxification method because arsenate is less toxic, less soluble than arsenite. Most of bacteria use the *ars* operon system to aerobically oxidize arsenite. Recently, arsenite oxidase an initial electron donor in aerobically arsenite resistance, encoded from *aso* gene was reported by Silver and Phung (2005). Known arsenite oxidizing bacterial strains are distributed in more than 20 genera and have been isolated from various environments. They include both chemolithotrophs; *Acidicaldus* sp., *Acidithiobacillus* sp. (Brierley and Brierley, 2001; D'Imperio *et al*., 2007) and heterotrophs; *Agrobacterium* sp., *Alcaligenes* sp*., Burkholderia* sp., *Thiomonas* sp*., Acinetobacter* sp., *Pseudomonas* sp. (Santini *et al.,* 2000; Quémenéur *et al.*, 2008; Krumova *et al.*, 2008 ; Cai *et al*., 2009). An arsenate reduction via dissimilatory reduction that coupled growth with arsenate as terminal electron acceptor had been shown in either strictly anaerobic or facultative anaerobic bacteria (Niggemyer *et al*., 2001). In 2007 Takeuchi *et al*. reported that an arsenic resistant *Marinomonas communis* removed arsenic from culture medium amended with arsenate by accumulation in their cells.

Since bacterial arsenic detoxification method is low in cost and environmentally friendly (Clausen, 2000; Srinath *et al*., 2002; Tsuruta, 2004). Therefore, this study aims to screen for arsenite oxidizing bacteria which has high arsenic resistance to apply for arsenic bioremediation.

Research objectives

The main objectives of this present study are as followed:

1. Screening and characterization of arsenite oxidizing bacteria isolated from tannery wastes and agricultural soils.
2. Evaluation of arsenite oxidizing efficiency of the isolated bacteria.

CHAPTER II

LITERATURE REVIEW

2.1 Arsenic chemistry

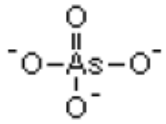
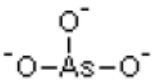
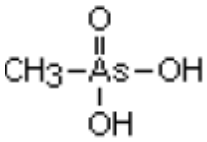
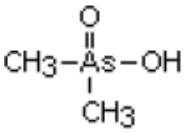
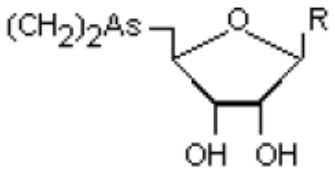
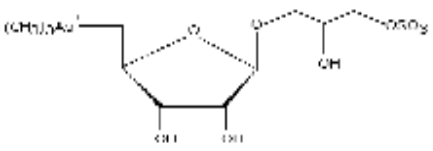
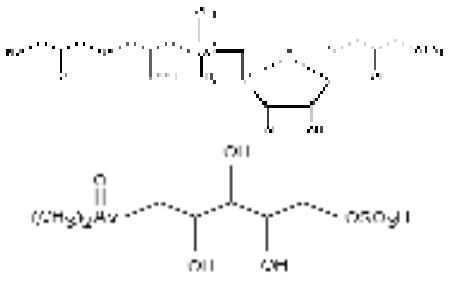
Arsenic has an atomic number of 33 and belongs to group V member in the periodic table of elements. It naturally occurs in four oxidation states (+5, +3, 0 and -3). The oxidized As(III) and reduced As(V) are the most widespread forms in nature. The arsenic is widely distributed in the earth's crust in trace quantities. It is usually found in conjunction with sulfur in minerals such as arsenopyrite (FeAsS), realgar (AsS), orpiment (As_2S_3) and enargite (Cu_3AsS_4) (Oremland and Stolz, 2005). A typical range for arsenic contamination in soils is 0.2 to 40 mg/kg with a median concentration of 6 mg/kg. (Huysmans and Frankenberger, 1990). However, the concentration may be higher in certain areas as a result of weathering and anthropogenic activities such as metal mining, smelting, fossil fuel combustion, battery producing activities, the use of arsenical wood preservatives, the use of arsenical herbicides, insecticides, fungicides and fertilizers in agriculture (ATSDR, 2000; Smedley and Kinniburgh, 2002; Walton *et al*., 2004; Khan *et al*., 2006). Arsenic generates multiple adverse human and animal health effects which generally inorganic arsenic compounds are more toxic than organic arsenic compounds, and arsenite is more broadly toxic than arsenate (NRC, 1999).

2.2 Sources and occurrance of arsenic in the environment

2.2.1 Natural sources

There are more than 200 mineral species of arsenic, about 60% are arsenates, 20% are sulfides and sulfosalts and the remaining 20% include arsenides, arsenites, oxides and elemental arsenic (Onishi, 1969). List of most common naturally occurring arsenic compounds and their molecular structures are shown in Table 2.1.

Table 2.1 Naturally occurring of arsenic compounds.

Names	Synonyms	Structures
arsenate		
arsenite		
methylarsonic acid	monomethylarsonic acid, MMA	
dimethylarsinic acid	cacodylic acid, DMA	
trimethylarsine oxide		$(CH_3)_3As{=}O$
tetramethylarsonium ion		$(CH_3)_4As^+$
arsenobetaine		$(CH_3)_3As^+CH_2COO^-$
arsenocholine		$(CH_3)As^+CH_2CH_2OH$
dimethylarsinoylribosides		
trialkylarsonioribosides		
dimethylarsinoylribitol sulfate		

Arsenopyrite (FeAsS) is the most common form of arsenic minerals. An ability of arsenic to bind to sulfur ligands means that it tends to be found associated with sulfide-bearing mineral deposits, either as separate As minerals or as a trace of a minor constituent of sulfide minerals. This leads to elevate arsenic level in soils in many mineralized areas where the concentrations of associated arsenic can range from a few milligrams to > 100 mg/kg.

In atmosphere, arsenic compounds are relatively volatile and consequently contribute significant fluxes. It has been estimated that the atmospheric flux of arsenic is about 73, 500 tons/year of which 60% is of natural origin such as volcanic activity and the rest is derived from anthropogenic sources (Chilvers and Peterson, 1987).

In water, concentration of arsenic in open ocean water is typically 1–2 µg/litre. Arsenic concentration in groundwater of geochemical origin were more than 1mg/l in Taiwan, West Bengal, India and Bangladesh (Chen *et al*., 1994; Chatterjee *et al.*, 1995; Das *et al*., 1995; Mandal *et a*l., 1996) . High level of arsenic concentration in drinking-water were also found in Chile (Borgono *et al*., 1977); North Mexico (Cebrian *et al*., 1983); and several areas of Argentina (Astolfi *et al.*, 1981; Nicolli *et al*., 1989; De Sastre *et al*., 1992).

2.2.2 Anthropogenic sources

Smelting of non-ferrous metals and production of energy from fossil fuel are two major industrial processes that lead to anthropogenic arsenic contaminations of air, water and soil. Other sources of contamination are manufacturing and usage of arsenical pesticides and wood preservatives.

Smelting activities generate largest arsenic anthropogenic input into atmosphere (Chilvers and Peterson, 1987).Tailing from metal-mining operations are significant source of contaminations surrounding topsoil and because of leaching.The contamination can lead to groundwater too. Levels as high as 2,000 and 500-9,300 mg/kg soil were reported in contaminated area near an old lead smelter (Temple *et al*., 1977) and in gold smelters, Canada, respectively (Hutchinson, 1887).

Industrial activity, Sadler *et al*. (1994) evaluated arsenic concentration in soil contaminated by tannery waste in Australia. Topsoils ranged from < 1 to 435 mg/kg, whilst the depth soils ranged from <1 to 1,010 mg/kg. Smedley and

Kinniburgh (2002) reported an arsenic concentration of up to 5,000 μg/l in groundwater associated with the former tinmining activity in Ron Phibun District, Thailand.

Agicultural activity, an As_2O_3 was widely used for cattle ticks (*Boophilus microplus*) control in Australia between 1900 and 1950 (Seddon 1951). From the 1960s there was a shift from inorganic arsenical herbicides (including lead and calcium arsenate and copper acetoarsenite) to inorganic and organic arsenical herbicides (arsenic acid, sodium arsenate, monomethylarsonic acid [MMA] and dimethylarsinic acid [DMA]). Annual historical applications of lead arsenate to orchards in USA ranged from 32 to 700 kg As/ha. Residue in orchard soils as high as 2500 mg/kg have been reported (Woolson, 1973). High levels of arsenic contamination in orchard areas ranged from 2,000 to 124,238 mg/kg soil have been reported by Walsh and Keeney (1975). Inaddition, arsenical animal feed additive have been widely used for controlling diseases, especially coccidiosis (Jackson and Bertsch, 2001). Total arsenical pesticides usage excluding wood preservatives, was estimated at $7–11 \times 10^3$ tons As/year.

2.3 Environmental distribution

Arsenic is primarily emitted into atmosphere by coal-fired power generation, smelting, burning vegetation and vulcanism. Microbial methylation and reduction also release arsenic into atmosphere; microorganisms form volatile methylated derivatives of arsenic under both aerobic and anaerobic conditions, and reduce arsenic compounds to release arsine gas (Cheng and Focht, 1979; Tamaki and Frankenberger, 1992). Arsenic is released into atmosphere primarily as As_2O_3 or, less frequently, as one of several volatile organic compounds. The arsenic released to air mainly exists in the form of particulate matter (Coles *et al*., 1979). These particles are dispersed by wind to a varying extent, depending on their size, and the particles are returned to earth by wet or dry deposition. Arsines that are released from microbial sources in soil or sediment undergo oxidation in the air, reconverting the arsenic to less volatile form that settle back to the ground (Wood, 1974; Parris and Brinckman, 1976).

Dissolved forms of arsenic in water column such as arsenate, arsenite, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) can interchange

oxidation state depending on Eh, pH and biological processes (Ferguson and Gavis, 1972). Methylation and demethylation reactions are also important transformation controlling the mobilization and subsequent distribution of arsenicals (Mok and Wai, 1994). Transport and partitioning of arsenic in water depends on chemical form of the arsenic and on interaction with other materials present. Arsenic compounds may be adsorbed from water on to clays, iron oxides, aluminium hydroxides, manganese compounds and organic materials (Callahan *et al*., 1979; Welch *et al*., 1988). The distribution and transport of arsenic in sediment is a complex process that depends on water quality, native biota and sediment type. There are a potential for arsenic release when there is fluctuation in Eh, pH, soluble arsenic concentration and sediment organic content (Abdelghani *et al*., 1981).

Arsenic flows into the estuary as arsenate and arsenite from river water and mine adits. There is oxidation of arsenite to arsenate, microbiological reduction of arsenate to arsenite and removal of arsenic by dilution with seawater and subsequent transport out of the estuary. Inorganic arsenic can be adsorbed on to charged particles of iron oxyhydroxides and manganese oxides and deposited as flocculated particles to sediment. There is subsequent release of dissolved arsenite and arsenate following the reduction and dissolution of the iron and manganese carrier phases in the anoxic sediments. Arsenate can be reduced, either microbially or chemically, to arsenite within the anoxic sediment, and arsenic (as arsenate or arsenite) can enter by sediment resuspension (Sanders, 1980; Knox *et al*., 1984). Inorganic arsenic was removed from the water column by phytoplankton and recycled during phytoplankton degradation and consumption (Millward *et al*., 1997)

Arsenic compound from weathered rock and soil may be transported by wind or water erosion and adsorbed by soils. However, rainwater or snowmelt may leach soluble forms into surface water or groundwater, and soil microorganisms may reduce a small amount to volatile forms (arsines). Under reducing conditions, arsenite dominates in soil (Haswell *et al*., 1985) but elemental arsenic and arsine can also be present (Walsh and Keeney, 1975). Arsenic would be present in well-drained soils as $H_2AsO_4^-$ if the soil was acidic or as $HAsO_4^{2-}$ if the soil was alkaline. Oxidation, reduction, adsorption, dissolution, precipitation and volatilization of arsenic reactions commonly occur in soil (Bhumbla and Keefer, 1994). In the porewater of aerobic

soils arsenate is the dominant arsenic species, with small quantities of arsenite and MMA in mineralized areas.

Biomethylation and bioreduction are probably the most important environmental transformation of the elements, since they can produce organometallic species that are sufficiently stable to be mobile in air and water. However, the biomethylated forms of arsenic are subject to oxidation and bacterial demethylation back to inorganic forms. Three major modes of biotransformation of arsenic species have been found to occur in the environment:1) redox transformation between arsenite and arsenate, 2) the reduction and methylation of arsenic, and 3) the biosynthesis of organoarsenic compounds (Andreae, 1983). However, the arsenic concentration in environment may enter food-chain then causes harmful health effects to humans, animals and other organisms. (Olson *et al*., 1940; Walsh and Keeney, 1975; NRC, 1999; ATSDR, 2000; Pamela, 2000; DNREC, 2002; Oremland and Stolz, 2005).

2.4 Arsenic toxicity

Generally, inorganic arsenic compounds are more toxic than organic arsenic compounds, and arsenite or trivalent inorganic is more broadly toxic than arsenate or pentavalent inorganic arsenic (NRC, 1999). Intramuscular LD_{50} of sodium arsenite and sodium arsenate, administered to young mouse were 8 and 21 mg/kg body weight, repectively (Bencko *et al*. 1978).Oral LD_{50} of As_2O_3, a trivalent arsenical, administered to young mouse, ranges from 26 to 39 mg/kg body weight.The symptoms observed from As_2O_3 intoxication include convulsion, retching and haemorrhaging in the intestinal tract (Kaise *et al*., 1985; Harrison *et al*., 1958).

The toxicity of arsenite is due to the formation of strong bond with functional group, such as the thiol of cysteine residue and the imidazolium nitrogen of histidine residue of cellular protein. The activity of enzymes or receptors is due in part to the functional groups on amino acids such as sulfhydryl group on cysteine or coenzyme such as lipoic acid, which has vicinal thiol groups. Thus, if arsenite binds to a critical thiol or dithiol, the enzyme may be inhibited (Aposhian, 1989). Arsenite inhibits pyruvate dehydrogenase (Peters, 1955; Szinicz and Forth, 1988), a lipoic acid

dependent enzyme involved in gluconeogenesis. The acute toxicity of inorganic arsenic may result in part from inhibition of gluconeogenesis and ultimately depletion of carbohydrate from the organism (Reichl *et al*., 1988; Szinicz and Forth, 1988). However, binding of arsenite to protein at non-essential sites may be detoxication mechanisms (Aposhian, 1989). Arsenite inhibits the binding of steroids to the glucocorticoid receptor, but not other steroid receptors (Lopez *et al*., 1990; Simons *et al.*, 1990). The glucocorticoid receptor has vicinal thiols that are involved with steroid binding (Simons *et al*., 1990).

In the case of arsenate, pentavalent inorganic arsenic; a mechanisms of toxicity, is its reduction to a trivalent form, such as arsenite. Arsenite is more toxic than arsenate, as evidenced by the lower amount of it needed to elicit a toxic response. Another potential mechanisms is the result of the mimetic effect of arsenate and phosphate, which affects global cell metabolism (Tseng, 2004; Joris and Simon, 2006). Arsenate can replace phosphate in the sodium pump and anion exchange transport system (Kenney and Kaplan, 1988).

Arsenic has effects on widely different organ systems in the body. It has produced serious effects in humans after both oral and inhalation exposures, it has many end-points, and the exposure is widespread all over the world.

Short –term effects, ingestion of large doses of arsenic may lead to acute symptom within 30–60 min. Acute gastrointestinal syndrome is the most common presentation of acute arsenic poisoning. This syndrome starts with a metallic or garlic like taste associated with dry mouth, burning lips and dysphagia. Violent vomiting may ensue and may eventually lead to haematemesis. Gastrointestinal symptom, which are caused by paralysis of capillary control in the intestinal tract, may lead to a decrease in blood volume, lowered blood pressure and electrolyte imbalance. Thus, after the initial gastrointestinal problems, multi-organ failure may occur, including renal failure, respiratory failure, failure of vital cardiovascular and brain functions, and death. Survivors of the acute toxicity often develop bone marrow suppression (anaemia and leukopenia), haemolysis, hepatomegaly, melanosis and polyneuropathy resulting from damage to the peripheral nervous system. Polyneuropathy is usually more severe in the sensory nerves, but may also affect the motor neurones (ATSDR, 2000).

Long-term effects, Arsenic is a human carcinogen such as lung and skin cancer observed in a sheep-dip factory manufacturing sodium arsenite (Hill and Faning, 1948). Several further case series also reported unexpectedly high lung cancer mortality in different occupational exposure situations (Osburn, 1957; Roth, 1958; Latarjet *et a*l., 1964; Lee and Fraumeni, 1969). Chronic skin effects of arsenic, including pigmentation changes, hyperkeratosis and skin cancer, from drinking water, were reported as early as the 19th century (Hutchinson, 1887; Dubreuilh, 1910). A large number of case series on arsenical skin cancer after exposure via drinking-water were published from Argentina, Chile, Mexico and Taiwan in the early 1900s (Zaldivar, 1974). An endemic peripheral vascular disease (PVD), known as wu chiao ping or blackfoot disease (BFD), leading to progressive gangrene of the legs, has been known in Taiwan since the 1920s. It has increased in prevalence since the 1950s, and has been subject of intense investigation since the late 1950s (Wu *et al*., 1961; Chen and Wu, 1962).

2.5 Arsenic analytical methods

Many chemical analysis techniques are improved for detection of arsenical compounds in samples, all methods had disadvantage or limitation. Colorimetric methods have been used for determination of arsenic but these methods are semi quantitative and lack sensitivity. Gutzeit's test (Vogel, 1954) is based on generation of arsine from arsenic compounds by an addition of zinc granule to concentrated sulfuric acid. The arsine is detected by means of a strip of filter paper moistened with silver nitrate or mercuric chloride. The arsine reacts with silver nitrate to give a grey spot, and with mercuric chloride to give a yellow to reddish-brown spot. Sensitivity of the test is about 1 µg. Modification of this method using mercuric bromide is found in a test kit currently being used in Bangladesh for groundwater testing which has a limit of detection of 50–100 µg/l under field conditions.George *et al*. (1973) reported that sensitivity of arsenic measurement by the colorimetric method in poultry and swine tissues using silver diethyldithiocarba- mate (AgDDTC) as testing reagent, was 0.1 mg/kg. Dhar *et al.* (1997) reported a detection limit of 0.04 mg/litre with 95% confidence limit using the AgDDTC in chloroform with hexamethylenetetramine.

Atomic absorption spectrometry (AAS) offers a possibility of selectivity and sensitivity. Popular methods for generating atoms for AAS are flame and electrothermally heated graphite furnaces. the atomic absorption spectrometric methods are known as flame AAS (FAAS), electrothermal AAS (ETAAS) and hydride generation AAS (HGAAS). Although they are suitable for total arsenic determination after appropriate digestion. FAAS is relatively less sensitive for the arsenic determination than ETAAS and HGAAS. The technique is routinely used to speciate a limited number of compounds. These methods are known to be interfered by some factors (Boampong *et al*., 1988; Welz *et al.*, 1990; Julshamn *et al.*, 1996).

Inductively coupled plasma-mass spectrometry (ICP-MS) is a very high sensitivity method for arsenic determination (sub-nanogram to sub-picogram). It is susceptible to isobaric interferences arising from plasma sample. Hydrochloric acid and perchloric acid are not desirable for sample preparation, because chloride ions generated in the plasma can combine with argon gas to form argon chloride (ArCl). This has the same mass as arsenic (mass, 75) which can lead to error if not corrected. Therefore, whenever possible, only nitric acid should be used in sample preparation. Careful sample preparation is as important as final measurement, and special care should be taken to avoid contamination and loss by volatilization, adsorption and precipitation. However, the ICP-MS method coupled with HPLC or HGAAS or micellar liquid chromatography (MLC) were used for detection and speciation of arsenite, arsenate, MMA and DMA in the samples (Heitkemper *et al.* 1989; Hakala and Pyy, 1992; Shum *et* al., 1992; Larsen *et al*., 1993; Ding *et al*., 1995; Magnuson *et al.*, 1996; Le and Ma, 1997; Thomas *et al.*, 1997).

Particle-induced X-ray emission spectrometry (PIXES) is a technique which sample (target) bombarded by charged particles emits characteristic X-rays of the elements present. The PIXES is a multi-elemental technique with a detection limit of approximately 0.1 μg As/g. It has an advantage of using small amount of samples (1 mg or less) and being a non-destructive technique. Application of the PIXES in an environmental field has mostly focused on atmospheric particulate material (aerosol samples) (Maenhaut, 1987). Castilla *et al*. (1993) described an arsenite and arsenate determination using X-ray fluorescence (XRF) spectroscopy in water with a detection limit of 3.1 ng/g. Huffman *et al*. (1994) described the use of X-ray absorption fine

structure (XAFS) spectroscopy to provide arsenic speciation information in coal and combustion ashes at concentrations of 10–100 mg/kg. Although there are a variety of methods to determine concentration and oxidation state of arsenic in coal and ash, there have been few attempts to determine mineral forms of arsenic. X-ray absorption near-edge structure (XANES) spectroscopy which give detail about an electronic structure and local arrangement of atoms around absorbing atom seem to be a powerful and promising tool for determination of local structure of various chemical samples. Further attempts are being made to bring XANES analysis to a more quantitative level such as arsenic species application (Mihelic *et al*., 2004). Arčon *et al.* (2005) detected an arsenic molecular information and identified the most abundant modes of arsenic bonding from soil in UK by using XANES method. The results show arsenic is predominantly in pentavalent form, tetrahedrally bound to oxygen atoms in the first coordination sphere.

2.6 Standard and regulation for inorganic arsenic exposure

The Occupational Safety and Health Administration (OSHA) set a permissible exposure limit (PEL) of arsenic for occupational exposure at 10 micrograms of inorganic arsenic per cubic meter of air (10 $\mu g/m^3$), averaged over any 8-hour period for a 40-hour workweek and the recommended exposure limit (REL) set by the National Institute for Occupational Safety and Health (NIOSH), is 2 $\mu g/m^3$ for a 15 min ceiling, based on classification of arsenic as a potential human carcinogen. In 1986, EPA promulgated the National Emissions Standards for Hazardous Air Pollutants for three stationary source categories known to emit inorganic arsenic: primary copper smelters, glass-manufacturing plants and arsenic plants. However, there is no ambient air standard for arsenic (La Dou, 1997; ATSDR, 2000).

The EPA Office of Drinking Water has set a maximum contaminant level (MCL) for arsenic in drinking water of 10 ppb. The World Health Organization (WHO) recommends a provisional drinking water guideline of 10 ppb (US.EPA, 2001; US.EPA, 2002).

The US. Food and Drug Administration (FDA) have established tolerance levels for arsenic in by-products of animals treated with veterinary drugs. These permissible levels range from 0.5 ppm in eggs and uncooked edible tissues of

chickens and turkeys to 2 ppm in certain uncooked edible by-products of swine (American Medical Association, 1986).

In 1989, EPA began to phase out household, ant poisons containing sodium arsenate, because of the danger of ingestion by small children. The EPA Office of Pesticide Programs has restricted the use of inorganic arsenic to pressure-treated wood. It has also cancelled all registered uses of inorganic arsenic for nonwood preservative purposes (Table 2.2).

Table 2.2 Standards and regulations for human health (ATSDR, 2006).

Agencies	Focus	Level	Comments
American Conference of Governmental Industrial Hygienists	Air: workplace	10 μg/m^3	Advisory; TLV/TWA
National Institute for Occupational Safety and Health	Air: workplace	2 μg/m^3	Advisory; 15-minute ceiling limit
Occupational Safety and Health Administration	Air: workplace	10 μg/m^3	Regulation; PEL over 8-hour day
U.S. Environmental Protection Agency	Air: environment	Not applicable	Not applicable
	Water	10 ppb	Regulation; maximum contaminant level in drinking water
Food and Drug Administration	Food	0.5-2 ppm	Regulation; applies to animals treated with veterinary drugs

2.7 Arsenic treatment methods

In general, toxic chemicals were removed and treated by the following methods; chemical precipitation, membrane filtration, ion-exchange or adsorption. Coagulation and filtration are the precipitative process. This process can be optimized to remove dissolved inorganic As(V) from water. The mechanisms involve adsorption and co-precipitation of As(V) to an aluminum or ferric hydroxide. As(III) is not effectively removed because of its overall neutral charge under natural pH. Because

As(III) removal is more difficult than those of As(V), therefore, pre-oxidation of arsenite to arsenate is typically necessary. The efficiency and economics of the system are contingent upon several factors, including type and dosage of coagulant, mixing intensity, and pH. Optimized coagulation-filtration system is capable of achieving over 90% removal of As(V). Both aluminum and iron coagulants can remove arsenic, but the iron coagulant (ferric chloride or ferric sulfate) is more effective. Lime softening is also a precipitative process used to remove arsenic. Addition of lime results in pH increase to above 10.5, then magnesium hydroxide is formed. In this pH range, magnesium hydroxide and As(V) is removed by co-precipitation. Enhanced lime softening treatment processes was currently used for arsenic removal (Rubel, 1984; US.EPA, 2004; US.EPA, 2005).

Reverse osmosis (RO) and membrane separation technologies are attractive arsenic treatment processes for small water systems. They can address numerous water quality problems while maintaining simplicity and ease of operation. RO is a pressure-driven membrane separation process capable of removing arsenic from water by means of particle size, dielectric characteristics, and hydrophilicity/ hydro-phobicity. RO also effectively removes other constituents from water, including organic carbons, salts, dissolved minerals, and color. This treatment process is relatively insensitive to pH, although pH adjustment may be required to protect the membrane from fouling (US.EPA, 2004; US.EPA, 2005).

Activated alumina (AA) is an adsorption process that uses porous, granular material with ion exchange properties. In drinking water treatment, packed-bed AA adsorption is commonly used for removal of natural organic matter and fluoride. The removal of As(V) by adsorption can be accomplished by continuously passing water under pressure through one or more beds. AA media can either be regenerated or disposed and replaced with fresh media. The efficiency and economics of the system are contingent upon several factors: pre-oxidation of As (III) to As(V), constituent interference with the adsorption process, and the need for pH adjustment to <6.5 (Amy *et al*., 2004; US.EPA, 2004; US.EPA, 2005).

Ion exchange is a physical-chemical ion exchange process in which ions are exchanged between a solution phase and solid resin phase. For arsenic removal, an anion resin in the chloride form is used to remove As(V). Anion exchange resins also

remove other anions such as sulfate, nitrate, and uranium. Because As(III) occurs in water below pH 9 with no ionic charge, As(III) is not removed by the anion exchange process. When the resin eventually becomes saturated with arsenate and other anions such as nitrate and sulfate, the resin must be regenerated. In the regeneration step, a sodium chloride brine solution is passed through the spent resin where the adsorbed arsenate and other anions are replaced with chloride ions. Because of high concentrations of arsenic in the spent brine, it likely will be classified as a hazardous waste. The advantages of the anion exchange technology for arsenic removal is simplicity of operation, long resin life, ease of regeneration, and lack of pH impact on the exchange capacity (Clifford, 1999; Jain and Loeppert, 2000; US.EPA. 2000). However, those methods have disadvantages such as high cost and a generation of secondary contaminants (Table 2.3). So that, bioremediation is one of choice for arsenic treatment methods. Many bacteria are arsenic resistance and/or post arsenic metabolizing system (ATSDR, 2000; Smedley and Kinniburgh, 2002). Bacterial oxidation of arsenite to arsenate is known as arsenic detoxification. Bacterial arsenic detoxification is of increasing interest for bioremediation due to low in cost and environmentally friendly (Clausen, 2000; Srinath *et al*., 2002; Tsuruta, 2004).

Table 2.3 Advantages and disadvantages of currently arsenic treatment methods (Ahmed *et al*., 2000).

Methods	Advantages	Disadvantages
Co-precipitation:	No monitoring of a break through is required. Relatively low cost simple chemicals. Low capital costs.	Serious short and long term problems with toxic sludge. Multiple chemicals requirement Operation requires training and discipline.
Alum coagulation	Durable powder chemicals normally available	Pre-oxidation may be required
Iron coagulation	More efficient than alum on weigh basis	Medium removal of As (III)
Lime softening	Most common chemicals	Re-adjustment of pH is required.
Membrane techniques:	Low space requirement. Capable of removal of other contaminants, if any.	High costs. High tech operation and maintenance. Toxic waste-water. Re-adjustment water quality is required.
Reverse Osmosis		Membrane does not withstand oxidizing agents.
Sorption techniques:	No daily sludge problem.	Requires monitoring of break through or filter use. Requires periodical regeneration or medium shift.
Activated alumina	Relatively well known and commercially available.	Re-adjustment of pH is required.
Iron coated sand	Expected to be cheap. No regeneration is required.	Yet to be standardized. Toxic solid waste.
Ion exchange resin	Well defined medium and hence capacity.	High cost medium. High tech operation and maintenance. Regeneration creates a sludge problem.

2.8 Arsenic resistant bacteria

2.8.1 Biodiversity of arsenic resistant bacteria

Arsenic resistant bacteria were found in various environments. *Aeromonas* sp. was frequently found in association with fish. *Bacillus arsenicus*, *B. indicus* were isolated from a siderite concretion and an aquifer, respectively. *B. arseniciselenatic* was isolated from an anoxic mud; sediment; of an alkaline, hypersaline and arsenic rich water in California. The *Bacillus* sp. was survived in the unfavorable condition because of the presence of spore (Anderson and Cook, 2004; Blum *et al*., 1998; Suresh *et al*., 2004). High arsenic resistant *Pseudomonas aeruginosa* was isolated from marine environment (De Vincente *et al*., 1990). *Acinetobacter*, *Flavobacterium*, *Pseudomonas*, *Sinorhizobium* and *Sphingomanas* were isolated from arsenic contaminated soils in Thailand, which the *Sphingomanas* was found to be an arsenite-oxidizing bacteria (Saowapar Kinegam *et al*., 2008).

Cai *et al*. (2009) reported that MIC range for arsenite of bacteria isolates from arsenic-contaminated soils in China was 2 mM to 34 mM. Low arsenite MIC (3 mM) isolates were belong to *Bacillus* sp. and *Exiguobacterium* sp., which high arsenite MIC ($\geq$20 mM) isolates were belong to *Acinetobacter* sp, *Arthrobacter* sp., *Comamonas* spp., *Rhodococcus* sp., *Stenotrophomonas* sp., *Agrobacterium* sp. and *Pseudomonas* sp. Pepi *et al.*, (2007) found that *Bacillus fusiformis*, *B. thuringiensis*, *Pseudomonas* sp. and *Aeromonas molluscorum* were high resistant to both arsenite (16.68 mM) and arsenate (133.47 mM).

Known arsenite oxidizing bacteria are distributed in more than 20 genera and have been isolated from various environments. They include both chemolithotrophs; *Acidicaldus* sp., *Acidithiobacillus* sp. (Brierley and Brierley, 2001; D'Imperio *et al*., 2007) and heterotrophs; *Agrobacterium sp., Alcaligenes* sp*., Burkholderia sp., Thiomonas* sp*., Acinetobacter* sp., *Pseudomonas* sp. (Santini *et al.,* 2000; Krumova *et al.*, 2008; Quéméneur *et al.*, 2008; Cai *et al*., 2009). Indeed, high arsenic resistance without a redox based metabolisms of an arsenite is noted, for example, Cai *et al*. (2009) isolated a highly arsenic resistant *Comamonas* sp.from an arsenic-contaminated soil in Chaina but the *Comamonas* could not oxidize arsenite to arsenate. Another arsenic resistant and arsenite oxidizing bacteria are shown in Table 2.4.

Table 2.4 Arsenic resistant and arsenite oxidizing bacteria.

Genera	Levels of arsenic resistance		Ability of arsenite oxidizing	References
	As(III) (mM)	As(V) (mM)		
Gram-positive bacteria				
Low G+C (*Firmicutes*)				
Bacillus	1.75-16.85	40-320	Absent	1,4, 8
Sporosarsina	14	160	ND	1
Planococcus	14	>640	ND	1
Exiguobacterium	3	ND	Absent	4
High G+C (*Actinobacteria*)				
Tsukamurella	1.75	160	ND	1
Zimmermannella	14	640	ND	1
Pseudocavibacter	3.5	640	ND	1
Plantibacter	1.75	80	ND	1
Microbacterium	3-56	640	ND	1,4
Arthrobacter	1.75-20	160-320	Absent	1,3
Kocuria	7	640	ND	1
Micrococcus	3.5-7	370-640	ND	1
Streptomyces	1.75	40	ND	1
Rhodocuccus	12-20	320	Absent	1, 3,4
Corynebacterium	>12	>400	ND	7
Arthobacter	3.5	160	ND	1
Gram-negative bacteria				
Alphaproteobacteria				
Agrobacterium	8-25	ND	**Present**	3,4,6
Sinorhizobiun	1.75	160	ND	1
Phyllobacterium	3.5	320	ND	1
Aminobacter	3.5	160	ND	1
Paracoccus	3.5	160	ND	1
Ensifer	1.75	160	ND	1
Azoarcus	6	>10	**Present**	3

Table 2.4 (Cont.) Arsenic resistant and arsenite oxidizing bacteria.

Genera	Levels of arsenic resistance		Ability of arsenite oxidizing	References
	As(III) (mM)	As(V) (mM)		
Gram-negative bacteria				
Betaproteobacteria				
Acidovorax	10	ND	Absent	4
Comamonas	7-26	ND	Absent	4
Achromobacter	13	ND	**Present**	4
Variovorax	3.5-7	160-320	ND	1
Janthinobacterium	4-6.65	ND	Absent	4
Gammaproteobacteria				
Pseudomonas	1.75-23	11-640	**Present**	1, 3, 5
Acinetobacter	3.5-23	40-320	Absent	1,2,3,4
Stenotrophomonas	3.5-21	320	Absent	1,4
Escherichia	6.65	ND	ND	2
Enterobacter	6.65	ND	ND	2
Klebsiella	6.65-9	ND	Absent	2,4
Yersinia	6.65	ND	ND	2
Serratia	6.65	ND	ND	2
Xanthomonas	6.65	ND	ND	2
Aeromonas	9-16.68	80-133.47	Absent	4,8

1, reported by Achour *et al*., 2007; 2, reported by Saltikov and Olson, 2002; 3, Krumova *et al*., 2008; 4, Cai *et al*., 2009; 5, Turner, 1954; 6, Salmassi *et al*., 2002 ; 7, Mateos *et al*., 2006; 8, Pepi *et al*., 2007 ; ND, not detected.

2.8.2 Mechanisms of arsenic resistance

Bacteria have developed different strategies to transform arsenic including arsenic methylation, arsenite oxidation, cytoplasmic arsenate reduction and respiratory arsenate reduction, although these processes may increase arsenic toxicity or contribute toward detoxification (McBride and Wolfe, 1971; Mukhopadhyay *et al*., 2002; Tseng, 2004; Silver and Phung, 2005, Joris and Simon, 2006).

Mechanisms have been reported to make bacteria resist to arsenic are minimizing of arsenate uptake through a specific phosphate uptake system, reducing of arsenate to arsenite then conjugate with glutathione or other thaioles, binding of arsenite with glyceroporin membrane protein (Peters, 1955; Szinicz and Forth, 1988), accumulating of arsenate in thir cells (Takeuchi *et al*. 2007). Craig (1989) found that fungi could convert arsenic to methylarsonic acid and dimethylarsinic acid.

Enzymes which responsible for an arsenic resistant mechanisms mainly are encoded from a cytoplasmic *ars*-operon (Niggemyer *et al*., 2001). This operon, encoding of *ars* genes which widely distributed in bacteria. It can be found on plasmid (e.g *Staphylococcus* plasmids pI258 and pSX267) or chromosome (e.g *Escherichia coli*, *Pseudomonas aeruginosa* and other enterobacteria) (Cai *et al*., 1998; Diorio *et al*., 1995; Gotz *et al*., 1983). They most commonly consist of either three (a*rsRBC*) or five (*arsRDABC*) genes arranged in a single transcriptional unit. The *ArsB*, an integral membrane protein that pumps arsenite out of cell, is often associated with an ATPase subunit (*ArsA*). *ArsC* is an arsenate reductase that converts arsenate to arsenite prior to efflux. *ArsR* is a trans-acting repressor involved in basal regulation of the *ars* operon, while *ArsD* is a second repressor controlling an upper level of *ars* genes expression. Both Gram-positive and Gram-negative bacteria were reported to both arsenite and arsenate resistant by the *ars*-operon (Mukhopadhyay *et al*., 2002). Achour *et al*. (2007) studied diversity of arsenite transporter genes. The results showed that 70.7% of all isolates contained a gene related to *arsB* or *ACR3*. More than 30 various *ars* genes were found. Some of isolates carried both *arsB* and *ACR3*-like genes. Phylogenetic analysis of protein sequences deduced from an amplicons indicated a prevalence of arsB in Firmicutes and Gammaproteobacteria, while ACR3(1) and ACR3(2) were mostly present in *Actinobacteria* and *Alphaproteobacteria*, respectively. An analysis at the National Center for bioinformatics “Cluster of Orthologus Genes (COG)” site finds 47 presumed ArsC protein sequences and 29 presumed ArsB sequences at NCBI web site (http://www.ncbi.nlm.nih.gov/cgi-bin/COG/palox?seq=ArsC and http://www.ncbi. nlm. nih.gov/cgi-bin/COG/palox?seq=ArsB).

Bacterial oxidation of arsenite to arsenate which in many cases are considered primarily a detoxification metabolism since arsenate is much less toxic

than arsenite. Arsenite oxidation has been identified in various bacteria including *Pseudomonas* (Turner, 1954), *Alcaligenes* (Osborne and Ehrlich, 1976), *Thiomonas* (Bruneel *et al*., 2003), *Herminiimonas* (Weeger *et al*., 1999), *Agrobacterium* (Salmassi *et al*., 2002) and *Thermus* (Gihring and Banfield, 2001; Gihring *et al.,* 2001). Some of these bacteria were able to use arsenite as sole electron donor and grew as lithotrophs. An arsenite-oxidizing heterotropic bacteria have not been shown to gain energy through arsenite oxidation and probably use arsenite oxidation as a detoxification mechanisms (Cai *et al*., 2009).

Arsenite oxidation was catalyzed by a periplasmic arsenite oxidase. This enzyme contains two subunits encoded by genes *aoxA*/*aroB*/*asoB* (small Rieske 2Fe-2S subunit) and *aoxB*/*aroA*/*asoA* (large Mo-pterin subunit), respectively. The *aoxAB* operon was putative arsenite oxidase genes in various microorganisms such as *Thermus thermophilus*, *Chloroflexus aurantiacus* and *Aeropyrum pernix* but not for a facultative autotropic arsenite-oxidizing bacterium *Alkalilimnicola ehrlichei* (Oremland *et al*., 2002). First arsenic-related genes (*asoA* and *asoB*) were found in *Alcaligenes faecalis* (Schmidt and Shaw, 2001; Mukhopadhyay *et al*., 2002; Santini and Vanden Hoven, 2004; Silver and Phung, 2005) Genes for arsenite oxidase region of *A*. *faecalis* are shown in Figure 2.1. Recently *aoxB*-like sequences have been widely found in different arsenic contaminated soil and water systems (Inskeep *et al*., 2007).

Cai *et al.* (2009) found that bacteria which contained both arsenite oxidase gene (*aoxB*) and arsenite transporter gene (*ACR3* or *arsB*) displayed a higher average arsenite resistance level than those possessing an arsenite transporter gene only.

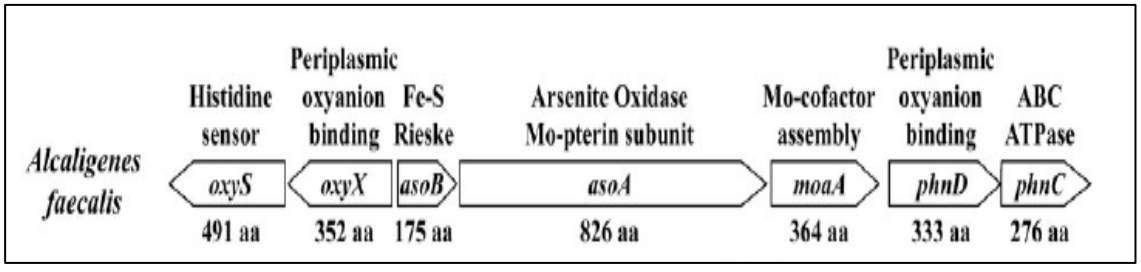


Figure 2.1 Genes in arsenite oxidase region of *A*. *faecalis* (NCBI accession number AY297781; http://www.uic.edu/depts/mcmi/faculty/silver.html).

2.9 Enzymatic reactions in arsenite oxidation

Oxidation of arsenite represents a potential detoxification process that allows microorganisms to tolerate higer level of arsenite. The enzymatic reactions were first studied in *Alcaligenes faccalis* (Anderson *et al*., 1992). The arsenite oxidase enzyme is located on outer surface of inner membrance and exhibits arsenite oxidation activity in presence of azurin or cytochrome *c* as electron accepter. Its structure shows two subunits, a larger 88-kDa polypeptide containing Mo-pterin and HiPIP 3Fe-4S center, and a smaller 14-kDa subunit with Rieske 2Fe-2S center (Anderson *et al*., 2001; Ellis *et al*., 2001). Arsenite oxidase is closest in sequence to subfamilies nitrate reductase and formate dehydrogenas, but their amino acids are only 20% identical in each pairing (Ellis *et al*., 2001).

After arsenite ($AsO_3H^-_2$) binds to large subunit of the enzyme, free 2 electrons transfer to Mo, then oxidizes arsenite to arsenate and reduces Mo(VI) to Mo(IV). After that, cells release arsenite (AsO_4H^{2-}), 2 electrons transfered from Mo(IV) to 3Fe-4S center and regenerated Mo(IV) reaction center. Two electrons are transfered from 3Fe-4S center in large subunit to Rieske 2Fe-2S center of small subunit. Finally, electrons are transferred to an oxygen respiratory chain, either through azurin or cytochrome *c* (Anderson *et al*., 2001) (Figure 2.2.). Useful energy is not generated by this respiratory process and bacterial cells cannot grow with arsenite as a sole energy source. This indicated that the arsenite-oxidation function was to detoxify arsenite (Ehrlich., 2001).

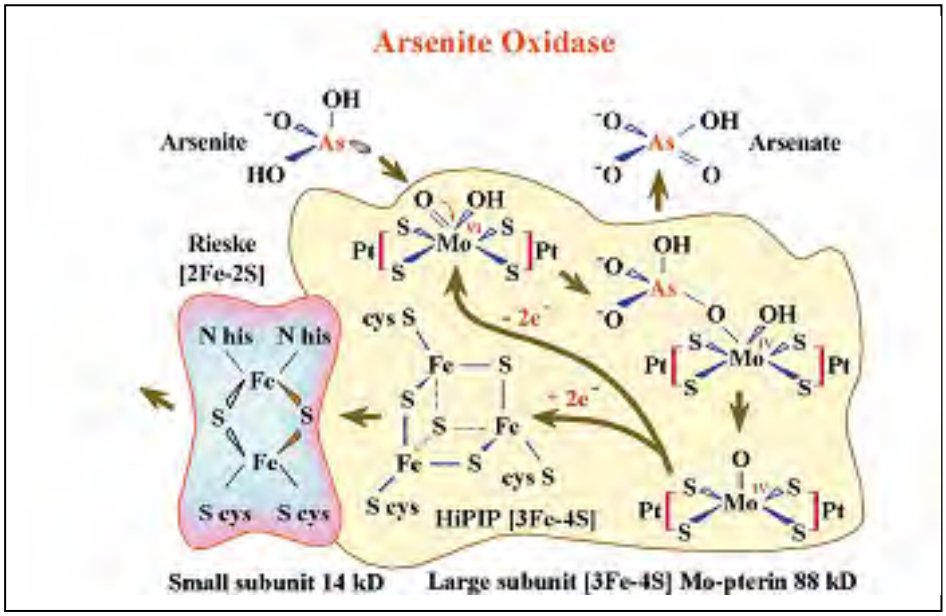


Figure 2.2 The structure and proposed reaction of arsenite oxidase enzyme (Ehrlich, 2001; Mukhopadhyay, *et al*., 2002).

CHAPTER III

EXPERIMENTAL

3.1 Sample collection and isolation of arsenic resistant bacteria

Due to high levels of arsenic contamination in tanning industry area and agricultural soils were reported (Sadler *et al*., 1994; Walsh and Keeney, 1975). Tannery wastes (untreated water, treated water, sledges) and waste contaminated soils from tanning industry (Samutprakan province) and agricultural soils from mix plantations, flower plantation, orchards and farms in Nakhonpathom and Petburi Provinces, Thailand, where there were an argicutural activity for longer than 20 years, (estimate arsenic contaminated areas) were collected for arsenic determination by ICP-MS technique and arsenic resistant bacteria isolation.

The arsenic resistant bacteria were isolated from the samples by inoculation of either 0.5 g soil or 0.5 ml water sample in 10 ml of chemically defined liquid medium (CDM), pH 7.0, which consisted of sodium citrate 0.5 g/l, casamino acids 0.05 g/l, $NaNH_4HPO_4$ 1.5 g/l, KH_2PO_4 1 g/l, $MgSO_4.7\ H_2O$ 0.4 g/l, Vitamin I solution (pyridoxal HCl 0.25%, thiamine 1%, calcium pantothenate 1%, riboflavin 1%, niacin 1%, *p*-aminobenzoate 0.5%, pyridoxine HCl 2%, vitamin B12 0.002%) 10 ml, and Vitamin II solution (folic acid 0.0125%, biotin 0.00025%) 2 ml/l, supplemented with 1.3 mM final concentration of sodium arsenite ($NaAsO_2$; Merck, Germany) and incubated at 30°C with shaking (200 rpm) in the dark for 48 h. The cultures were subcultured twice, by transferring at 1% (v/v) to the same fresh medium containing the 1.3 mM $NaAsO_2$ and incubating under the same conditions. The resulting arsenic resistant cultures were purified by streak plate method on CDM agar medium. The purified cultures were kept at 4°C on R2A medium (yeast extract 0.5 g/l, proteose peptone 0.5 g/l, casamino acids 0.5 g/l, dextrose 0.5 g/l, soluble starch 0.5 g/l, sodium pyruvate 0.3 g/l, KH_2PO_4 0.3 g/l, $MgSO_4.\ 7\ H_2O$ 0.05 g/l, agar 1.5 g/l), pH 7.0.

3.2 Minimum inhibitory concentration (MIC)

MIC was determined by modified method of Muller *et al.* (2003). The single colony of arsenic resistant bacteria isolated was inoculated into TYEG broth (tryptone 1 g/l, yeast extract 3 g/l and glucose 1 g/l; pH 7.0) supplemented with final concentration of either sodium arsenite (5 - 40 mM) or sodium arsenate ($Na_2HAsO_4.7H_2O$; Fluka, Spain) (5 - 450 mM) 4 ml in test tube (ø 1.5 x 15 cm) and incubated at 30°C with shaking (200 rpm) in the dark for 48 h. Bacterial growth was monitored from turbidity (OD 660 nm) of the inoculated media. MIC was defined as the lowest concentration of arsenite or arsenate added which completely inhibited growth (Muller *et al.*, 2003). Triplicate measurements were conducted for each isolates.

3.3 Arsenite oxidation and arsenate reduction

The arsenic resistant bacteria isolated were screened for arsenite oxidizing and arsenate reducing activities by a modified microplate method of Diliana *et al.* (2004) and by the method of Lett *et al.* (2001). For the microplate method, 12 ml of a 48 h bacterial culture, grown in CDM medium, was centrifuged at 4°C, 5,500 x g for 15 min, and cells pellets were collected, washed twice with sterile deionized water and then suspended in 1.2 ml deionized water. A 20 µl aliquot of the bacterial suspension was mixed with 80 µl of 0.2 M Tris HCl buffer pH 7.4 containing sodium arsenite or sodium arsenate at a final concentration of 2.7 and 1.3 mM, respectively. The cell suspensions were incubated at 30°C in the dark for 72 h, then silver nitrate solution was added to a final concentration of 0.2 M. A change from bright yellow colour of sodium arsenite to a brownish colour of sodium arsenate indicated a likely arsenite oxidizing activity, whilst on the arsenate plates a change from the brownish to a bright yellow colour indicated arsenate reduction. For the method described by Lett *et al.* (2001), 48 h colonies grown on CDM agar containing 2.7 mM sodium arsenite were flooded with 0.1 M silver nitrate. Colonies surrounded by a brownish zone indicated arsenite oxidizing activity.

3.4 Removal of arsenic from culture broth

Bacteria were cultivated in TYEG medium at 30°C with shaking (200 rpm) for 24 h. The overnight cultures were transferred at 2.5 % (v/v) into 20 ml of TYEG medium supplemented with 1.3 mM sodium arsenate (100 mg/l arsenate) in a 250 ml flask and incubated at 30°C with shaking. Bacterial growth was monitored by measuring the optical density at the wavelength of 660 nm (OD 660 nm). Stationary phase cultures were centrifuged at 4°C (23,200 x g) for 20 min. The resulting supernatants were analyzed for arsenic content and an arsenic concentration in culture broth with and without inoculation was compared. Triplicate measurements were conducted for each isolates.

3.5 Confirmation of arsenite oxidizing activity and efficiency of arsenite oxidizing activity

3.5.1 XANES spectroscopy method

Single colony of the resistant bacteria (grown on TYEG agar) was inoculated into 5 ml of TYEG broth supplemented with 1.33 mM sodium arsenite (0.1 g/l) in test tube (ø 1.5 x 15 cm) and incubated at 30°C (200 rpm) in dark 24 h, then transferred at 1% (v/v) to the same medium and incubated at the same condition for 72 h. The culture was centrifuged at 4°C (23,200 x g) for 20 min and the resulting supernatant was determined of arsenite oxidizing efficiency by XANES spectroscopy (Hormes *et al*., 2006); an aliquot of approximately 1 ml was pipetted on a arsenic-free filter paper and directly measured. 'Thickness' of the sample was optimized in order to avoid possible self-absorption effects. Medium without inoculation, incubated at 30°C with shaking for 72 h was used as control. The arsenic forms in the culture media were compared with analytical resenic referance compounds (Appendic D).

3.5.2 Modified method of arsenite-iodine titration

The arsenic resistant bacteria were cultivated in TYEG medium at 30°C with shaking (200 rpm) for 24 h. The overnight culture was transferred at 1 % (v/v) into 40 ml of TYEG medium supplemented with 5.0 mM sodium arsenite (0.376 g/l) in a 250 ml flask and incubated at 30°C with shaking in dark, 24 h. The bacterial culture was centrifuged at 4°C (23,200 x g) for 20 min. Residual arsenite in the supernatant was determined. First, 0.5 ml of reagent A and 0.5 ml of reagent B were

added into a test tube, then titration with the culture supernatant. Residual arsenite in the culture broth with and without inoculation was compared. Triplicate measurements were conducted for each isolates. Residual arsenite in the culture supernatant was calculated by using an equation of arsenite–iodine titration strandard curve (Appendix D).

3.6 Arsenite oxidase and arsenite transporter genes

The *aoxB* gene encoding the catalytic subunit of the arsenic oxidase enzyme which found from arsenite oxidizing bacteria, seems to be a valuable molecular marker for investigating its ecology and the potential of arsenite oxidation in the environment. A recent study described primers targeting the first quarter of the *aoxB* gene to detect its presence and expression in the environment and suggested that the gene is widely distributed among the Bacteria and also is widespread in soil-water systems containing arsenic (Inskeep *et al*., 2007). Genes encoding the arsenite detoxification (*ars* genes) are widely distributed in bacteria and archaea and can be found on plasmids or chromosomes. They most commonly consist of either three (*ars*RBC) or five (*ars*RDABC) genes arranged in a single transcriptional unit. Recently, different studies have focused on the detection of *ars* genes in environmental samples, to correlate their presence with the arsenic resistance phenotype and/or arsenic-transforming capacities of bacterial isolates (Niggemyer *et al*., 2001; Mukhopadhyay *et al*., 2002). Depending on the purpose, 3 primer set (*aoxB*, *aroB* and *arsB*) were chosen as the target genes.

Three primer pairs were using for detection of arsenite oxidase and arsenite transporter genes, primer set 1; *aoxB*M*1*-2F (5'-CCACTTCTGCATCGTGGGNTG YGGN TA-3′) bind at nucleotide positions 66 to 92 in *H. arsenicoxydans aoxB* open reading frame) and *aoxBM3*-2R (5′-TGTCGTTGCCCCAGATGADNCCYTTYTC-3′) positions 1150 to 1177) were designed by Que´me´neur *et al.* (2009), primer set 2; *aroB*-1F (5'-GTSGGBTGYGGMTAYCABGYCTA-3') bind at nucleotide positions 85 to 107 in *Rhizobium* sp. str.NT26 and *aroB*-1R (5'-TTGTASGCBGGNCGR TTRTGRAT-3') positions 592 to 614 as described by Inskeep *et al*. (2007) and primer set 3; *darsB*-1F (5′-GGTGTGGAACATCGTCTGGAAYGCNAC-3′) *darsB*-1R (5′-

CAGGCCGTACACCACCAGRTACATNCC-3′) were designed by Achour *et al.* (2007). Degenerate nucleotide sites are indicated by standard ambiguity codes as follows: N =A, C, G, or T; R =A or G; V=A, C, or G and Y =C or T; B = G, T or C; M = A or C, S = G or C; V = A, C, or G.

All PCR reaction mixtures contained approximately 50 ng DNA template, 1xPCR buffer, 0.2 mM of each deoxyribonucleoside triphosphates, 0.2 mM of each primer and 1 U *Taq* DNA polymerase (Eppendorf) in 25 µl volume. Amplifications were performed in a Master cycler gradient (Mastercycler® geadient; Eppendorf).

Cycling condition for set 1 primers consisted of 35 cycles, annealing temperature of 52°C, 1 min 10 s elongation time (Que´me´neur *et al*., 2009).

Optimized PCR condition for set 2 primers were 95°C for 4 min followed by 9 cycles of 95°C for 45 s, 50°C (decreased by 0.5°C after each cycle) for 45 s, 72°C for 50 s, followed by 25 cycles of 95°C for 45 s, 46°C for 45 s and 72°C for 50 s and a finalextension of 72°C for 5 min (Inskeep *et al*., 2007).

Cycling conditions for set 3 primer consisted of 5 min of denaturation at 94°C followed by 35 cycles of 45 s of denaturation at 94°C, 45 s of annealing at 57°C to 52°C with a 0.5°C decrement per cycle during the first 10 cycles and 30 s of primer extension at 72°C. This was followed by a final extension reaction at 72°C for 7 min (Achour *et al*., 2007).

The PCR amplified products were analyzed by running 5 µl of the PCR product on a 1% agarose gel in Tris-acetate EDTA buffer which stained in an ethidium bromide solution (0.5 mg/ml) and examined under UV-transilluminator to visualize the amplified arsenite oxidizing and arsenite transporter genes band.

3.7 Identification methods

Morphological, cultural, physiological, and biochemical characteristics of the isolates were determined using the methods described by Barrow and Feltham (1993), Leifson (1963), Sirilak Namwong (2005) along with several supplementary tests.

3.7.1 Phenotypic characteristics

3.7.1.1 Cell morphology and cultural characteristics

The arsenic resistant bacteria isolated were cultivated on TYEG agar medium at 30°C for 24 h and cell morphology, spore formation and pigmentation were recorded. Colonial appearances were examined after incubated for 1-2 days.

3.7.1.1.1 Gram staining: Thin smear of bacterial colony was prepared on a clean slide. Slide was fixed by passing through flame. The smear was convered with Gram crystal violet for 30 sec, then rinsed with water. Next, the smear was convered with Gram iodine for 30 sec, then rinsed with water. Decolourized with ethanol 95% and washed with water, then the smear was counter stained for 30 s by Gram safranin. Blotted slide dried and examined under oil immersion (1,000x).

3.7.1.1.2 Flagella staining: Standard microscope slide, pre cleaned by the manufacturer was used. The slide was flamed briefly, then draw a thick line with a wax pencil across its width so that the stain was confined to two-thirds of surface. To this area, 3 drops of sterile distilled water was added. An inoculating needle was touched to the top of an isolated colony from the TYEG agar slant medium and gently mixed in the water. There was no visible opalescence. The suspension was spreaded over the staining area and then tapped off onto a disinfectant soaked gauze sponge. The slide was air-dried on a level surface. Staining by method of Forbes (1981), the slide was stained by 1 ml of stain for 1 min at room temperature, washed in tap water, counterstained with Hucker modification of Gram crystal violet for 1 min, then washed, blotted dry, and examined under oil immersion by starting near to the wax line drawn.

3.7.1.2 Physiological and biochemical characteristics

3.7.1.2.1 Oxidase: Each colony was streaked on TYEG agar and incubated at 37°C for 1 day. A few drops of 1% tetramethyl-p-phenylenediamine were dropped on sterile filter paper disc. The test culture was then smeared across the moist paper disc with sterile loop. The apperance of dark-purple colour on paper within 30 sec denoted a positive reaction.

3.7.1.2.2 Catalase: A small amount of pure culture was transferred from agar onto slide. Hydrogen peroxide (H_2O_2), 3% (v/v) was immediately dropped onto the portion of colony on slide. The evolution of gas bubbles indicated a positive test.

3.7.1.2.3 Growth at different temperatures: Bacterial cells were inoculated on TYEG agar medium and incubated at 4°C, 30°C, 37°C, 40°C, 50°C and 60°C. Growth examination was performed after 5 days.

3.7.1.2.4 Growth in different NaCl concentration: Bacterial cells were inoculated into TYEG medium containing 3% and 5% (w/v) NaCl and incubated at 30°C for 5 days, then the growth was examined.

3.7.1.2.5 Growth at different pH: Bacterial cells were inoculated into TYEG broth, pH 5.0, 6.0, 8.0 and 9.0, then incubated at 30°C for 5 days.

3.7.1.2.6 Glucose oxidation/fermentation: Bacterial cells were inoculated into OF medium supplemented with 1% glucose in 2 test tubes (overlay or not overlay with mineral oil) and incubated at 30°C for 5 days. Color of an indicator changed to yellow indicated a positive test.

3.7.1.2.7 Aesculin hydrolysis: Bacterial cells were inoculated into aesculin broth and incubated at 30°C for 5 days. Black color formation indicated a positive test.

3.7.1.2.8 Gelatin hydrolysis: Bacterial cells were inoculated onto gelatin medium and incubated at 30°C for 5 days then the cultures were kept at 4°C for 15-20 min. The inoculated gelatin medium did not solidify indicated a positive test.

3.7.1.2.9 Starch hydrolysis: Bacterial cultures were streaked onto 10% (w/v) starch agar medium and incubated at 30°C for 5 days, then flooded with Lugol's iodine solution. Clear colorless zone surrounded the colony indicated starch hydrolysis.

3.7.1.2.10 Tween 80 hydrolysis: Bacterial cultures were streaked onto TYEG agar medium containing 1.0% (w/v) Tween 80 and incubated at 30°C for 5 days. Clear zone surrounded the colony indicated Tween 80 hydrolysis.

3.7.1.2.11 Deoxyribonuclease (DNase) activity: Bacterial cultures were streaked onto DNase test agar medium and incubated at 30°C for 5 days then flooded with 1 N HCl. Clear zone surrounded the colony indicated a positive test.

3.7.1.2.12 Indole: Bacterial cells were inoculated into peptone water and incubated at 30°C for 5 days. The culture broth was then mixed with Kovac's reagent. Red color developed indicated a positive test.

3.7.1.2.13 Nitrate reduction: Bacterial cells were inoculated into nitrate broth and incubated at 30^{o}C for 5 days, then one drop each of Solution A and Solution B of nitrate reduction test reagents were added. Red color developed within 5 minutes indicated a positive test. Negative test was confirmed by addition of zinc dust into cultured broth to develop red colour of nitrite.

3.7.1.2.14 Simmon citrate: Bacterial culture were streaked onto citrate agar slant and incubated at 30 ^{o}C for 5 days. Blue color formation indicated a positive test.

3.7.1.2.15 Utilization of carbohydrate: Utilization of various carbohydrates as sole carbon and energy sources was tested in a mineral basal medium (MB) supplemented with 0.2% (w/v) test substrate as described by Stanier *et al.* (1966). Growth was determined spectrophotometrically after 5 days of cultivation.

3.7.1.2.16 Hydrogen sulfide production: Bacterial cultures were streaked onto Triple Sugar Iron agar (TSI) slant and incubated at 30^{o}C for 5 days. Black color formation indicated a positive test.

3.7.1.2.17 Urease activity: Bacterial cultures were streaked onto urea agar slant containing urea 2% (w/v). Color change of phenol red indicator to pink a positive test.

3.7.1.2.18 Methyl red and Voges-Proskauer: Bacterial cells were inoculated into MR-VP broth and incubated at 30^{o}C for 5 days. For MR test, 5 ml aliquot of the culture was mixed with 5 drops of methyl red reagent. Red color formation on surface of the culture indicated positive test. For VP test, 0.6 ml of alpha-naphthol solution and 0.2 ml of potassium hydroxide solution (Barritt reagent). An appearance of pink to red color indicated positive test (presence of acetyl methylcarbinol).

3.7.1.2.19 API 20E API 20NE and API 50CH: Capability to assimilate some substrates was tested by using API 20E (for the identification of enteric bacteria) API 20NE (for the identification of non-fastidious and non-enteric bacteria) and API 50CH (for determination of carbohydrates metabolism) strip as described by manufacturer (bioMérieux, France).

3.7.1.3 Chemotaxonomic characteristics

3.7.1.3.1 Cellular fatty acids: Cellular fatty acids are located in cell membrance as a component of polar lipids and glycolipids. Fatty acids of Gram negative bacteria generally are lipopolysaccharides constituent. Although bacteria have various kinds of fatty acids in their cells, the fatty acids are produced through highly regulated synthetic pathways (Lennarz, 1966). Cellular fatty acid analysis was performed as following; Dried cells (40 mg) were transferred into screw-cap tube containing 1 ml of saponification reagent, shaken well, heated to 100°C for 30 min, then cooled down to room temperature in water. After that methylation reagent was added, mixed and heated to 80°C for 10 min, cooled rapidly in ice water. Then, added extraction solvent, transferred upper layer to new tube, added base wash reagent, mixed for 5 min and added saturated sodium chloride solution for purification. The upper layer of the fatty acid suspension obtained was transferred to a new vial for automated GC analysis. (Minnikin *et al*., 1984; Sasser, 1990).

3.7.1.3.2 Quinone analysis: Isoprenoid quinones are important in respiratory electron transport system. Various kinds of quinones are found in bacterial cells and quinones type is used to classify bacterial. A modified method of Komagata and Suzuki (1987) was performed: Dried cells (100-500 mg) were extracted with chloroform: MeOH (2:1) for a few hours, then filtered and dried under rotary evaporator. The dried sample was dissolved in small amount of acetone and applied onto a silica gel TLC (Merck no.1.05744, E. Merck, Darmstadt, FRG). The applied TLC was then developed with petroleum ether-diethyl ether system (85: 15, v/v) and menaquinone band was visualized by UV (254 nm) radiation. The menaquinone band was scraped and dissolved in HPLC-grade methanol. The suspension was filtered and dried under N_2 gas. The menaquinone sample was analyzed by HPLC using methanol-isopropanol (4:1) eluent and μ-BondapakC_{18} column (Water Associates, Milford, Mass, USA).

3.7.2 Genotypic charecteristics

3.7.2.1 DNA base composition: DNA was extracted by method of Saito and Miura (1963). Log phase cells grown on TYEG or R2A agar medium at 30^{o}C for 24 h were harvested by scraping and suspended in 10 ml of saline-EDTA buffer pH 8.0. Bacterial cell lysis was induced by addition of lysozyme at final concentration of

20 mg/ml in 0.1 M Tris buffer pH 9.0 containing 10% (w/v) sodium dodecyl sulfate (SDS) and incubation at 55°C for 10 min. The cell suspension became turbid to opalescent then viscous indicated cell lysis. Cellular protein was denatured and extracted with 4 ml of phenol-chloroform (1:1 v/v) for 30 sec, then centrifuged at room temperature, 12,000 rpm (9,200 x g) for 10 min. DNA was precipitated by adding cold 95% (v/v) ethanol into the supernatant. The DNA precipitate was spooled with a glass rod, rinsed with 70% then 95% (v/v) ethanol, air dried, then dissolved in 5 ml of 0.1 x SSC. RNase A solution (0.3 ml) was added into the DNA solution, incubated at 37°C for 20 min, then extracted by 2 ml of phenol-chloroform. After centrifugation at room temperature, 12,000 rpm (9,200 x g) for 10 min, the upper layer was transferred to new tube. DNA was precipitated by adding cold 95% (v/v) ethanol and spooled with a glass rod then rinsed with 70% then 95% (v/v) ethanol. The DNA precipitate was air dried and dissolved in 5 ml of 0.1 x SSC. Purity and quantity of DNA were determined from ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}) as described by Marmur and Doty (1962).

DNA base composition was analyzed by the method of Tamaoka and Komagata (1984) which DNA was hydrolysed to nucleosides by nuclease P1 (EC 3.1.3.30) and alkaline phosphatase (EC 3.1.3.1). DNA sample (0.5-1.0 g of DNA/l of distilled water; OD_{260} = 10-20) was boiled in boiling water bath for 5 min and immediately cooled in ice water. Ten microliter of the DNA solution was mixed with 10 µl of nuclease P1 solution, incubated at 50°C for 1 h, then 10 µl of alkaline phosphatase solution was added and kept at 37°C for 1 hour. DNA base composition of the DNA hydrolysate was analyzed by HPLC using conditions as shown in Table 3.1.

Table 3.1 Conditions for high-performance liquid chromatography.

Detector wave length	270 nm
Column	Nakarai Cosmosil packed column $5C_{18}$ (150X4.6 mm)
Column temperature	Room temperature
Eluent	0.2 M $NH_4H_2PO_4$-acetonitrile (20:1, v/v)
Flow rate	1ml / min

3.7.2.2 16S rRNA gene sequence and phylogenetic analysis

3.7.2.2.1 16S rRNA gene sequence analysis: The PCR was performed in a total volume of 50 µl containing 1 µl of DNA sample, 0.25 µl of *Taq* DNA polymerase, 5 µl of 10x polymerase buffer, 4 µl of dNTP mixture, 2.5 µl of 10 µM forward primer (20F; 5'-AGAGTTTGATCCTGGCTC-3') and reverse primer (1495R; 5'-CTACGGCTACCTTGTTA-3') and 34.75 µl of Milliq water. A Master cycler gradient (Mastercycler® geadient; Eppendorf) was used with a temperature profile of 5 min at 94°C followed by 30 cycles of 30 s at 94°C (denaturing of DNA), 30 s at 60°C (primer annealimg), and 2 min at 72°C (polymerization) and a final extension for 5 min at 72°C. The PCR amplified products were analyzed by running 5 µl of the PCR product on a 1% agarose gel in Tris-acetate EDTA buffer. Agarose gel was stained in an ethidium bromide solution (0.5 mg/ml) and examined under UV-transilluminator (UVP Inc.) to visualize the amplified 16S rRNA gene band.

The 16S rRNA gene fragments were amplified with 357R; 5'-CTGCTGCCTCCCGTAG-3', 802R; 5'-TACCAGGGTATCTAATCCC-3', 1100R; 5'-AGGGTTGCGCTCGTTG-3' and 1541R; 5'-AAGGAGGTGATCCAGCC-3' primers as described by Somboon Tanasupawat *et al*. (2004). The amplified 16S rRNA was used as templates for sequencing with big dye terminator sequencing Kit (Perkin Elmer) and annalyzed by ABI377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for each sample was performed in a DNA Thermal Cycler (Gene Amp® PCR System 2400; Perkin Elmer) with a temperature profile of 30 s at 96°C followed by 25 cycles of 10 s at 96°C (denaturing of DNA), 5 s at 50°C (primer annealing), and 4 min at 60°C (polymerization). Sequencing for each sample was carried out in both forward and reverse directions.

3.7.2.2.2 Phylogenetic analysis: Homology search was performed by using the standard BLAST sequence similarity searching program version 2.2.1 from the web server http://www.ncbi.nlm.nih.gov/BLAST/__against previously reported sequences at GenBank/EMBL/DDBJ databases. The sequence was multiply aligned with selected sequences obtained from the three main databases by using the CLUS-TAL W version 1.81. The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by

using the neighbor-joining (Saitou and Nei, 1987) and maximum parsimony methods in the MEGA program version 2.1 (Kumar *et al.*, 2001). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1,000 resamplings. Values of sequence similarity among the closest strains were calculated manually after pairwise alignments obtained using CLUSTAL X program (Thompson *et al.*, 1997). Gaps and ambiguous nucleotides were eliminated from the calculations.

3.7.2.3 DNA-DNA hybridization: DNA-DNA hybridization was determined by using microplate hybridization technique as described by Ezaki *et al*. (1989) for noval species confirmation ($\geq$ 98% of 16S rRNA gene sequencing similarity to bacterial type strains): Photobiotinylation of DNA was performed by mixing 10 μg/ml of photobiotin with an equal volume of denatured purified DNA solution of a type strain (10 μg of DNA/ ml), and then irradiated by sunlamp (500 W) for 25 min. After irradiation, free photobiotin was removed by n-butanol extraction. The biotanylated DNA was used for hybridization immediately. A 100 μl of heat denatured purified DNA solution of unknown or type strains (10 μg of DNA/1ml of phosphate buffered saline containing 0.1 M $MgCl_2$) was incubated at 37^{o}C for 2 h in microdilution plates (Nunc Corp., Denmark).

After 2 h, 200 μl of a prehybridization solution (20xSSC, 5% [v/v] Denhardt solution, 50% [v/v] formamide containing 10 μg/ml of denatured salmon sperm DNA) was added to microdilution plates and further incubated for 1 h. Then, the prehybridization solution was discarded, replaced by 100 μl of hybridization mixture (20xSSC, 5% [v/v] Denhardt solution, 3% [w/v] dextrane sulfate, 50% [v/v] formamide, 10 μg/ml of denatured salmon sperm DNA) containing 10 μg of the biotinytlated DNA and covered with aluminium foil. The microplate was incubated overnight (15 h) at 40-52^{o}C. After hybridization, each microplate wells was washed three times by 200 μl of 0.2xSSC buffer. A 100 μl of streptavidin peroxidase conjugate solution (Boehringer, Germany) was added and the microplate was incubated at 37^{o}C for 30 min. After incubation, each microplate wells was washed three times by phosphate buffered, then added 100 μl of substrate, 3.3',5.5'–tetramethyl benzidine–H_2O_2 solution (Wako, Japan), into each well. The microplate

was again incubated at 37^{o}C for 10 min. The reaction was stopped by addition of 2 M H_2SO_4 and color intensity was measured by Microplate Reader (Bio Rad, Model 3350, USA) at wavelength of 450 nm. The homology values for the DNA-DNA hybridization were calculated (Somboon Tanasupawat *et al*., 2000). Definition of same species generally includes strains with approximately 70% or greater of DNA-DNA relatedness (Wayne *et al*., 1987).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Sample collection and isolation of arsenic resistant bacteria

Arsenic concentration of the seven tannery wastes ranged from 0.05 to 10.03 mg/kg which were lower than the reported of Sadler *et al*. (1994). The nine agricultural soil samples contained 4.11 - 4.35 mg/kg arsenic which were lower than median arsenic contaminated soils reported by Woolson (1973) or Walsh and Keeney (1975). The typical range for arsenic contamination in soils is 0.2 to 40 mg/kg with a median concentration of 6 mg/kg (Huysmans and Frankenberger, 1990). Maximum limit of contamination soils with arsenic were not seted, but EPA has set a maximum contaminant level (MCL) for arsenic in drinking water of 10 ppb (US.EPA, 2001), based on classification of arsenic as a potential human carcinogen (La Dou, 1997; ATSDR, 2000).

From the seven tannery wastes, 42 bacterial isolates (45.16%) grew in the presence of 1.3 mM sodium arsenite were isolated. The nine agricultural soils, 51 isolates (54.84%) gerw in the presence of 1.3 mM sodium arsenite were isolated (Table 4.1).

Table 4.1 Source of samples and isolate number.

Source of samples	Isolate number	No. of isolates	Percentage of isolates (%)
Tannery wastes		**42**	**45.16**
Untreated water (2 samples)	W1-1, W1-2, W1-3, W1-4 W1-5, W1-6, W8-1, W8-2 W8-3, W8-6, W9-1, W9-2 W9-3, W9-4, W9-5, W9-6	16	17.20
Treated water (1 samples)	W3-5, W10-1, W10-2, W10-3 W10-4, W10-5, W10-6	7	7.53
Sledge (3 samples)	SL4-1, SL4-2, SL4-3, LS4-4 SL4-5, SL4-6, SL5-1, SL5-2 SL5-4, SL5-5 SL6-1, LS6-2 SL6-3, SL6-5, SL6-6	15	16.13
Waste contaminated soil (1 sample)	SE7-2, SE7-3, SE7-4, SE7-5	4	4.30

Table 4.1 (Cont.) Source of samples and isolate number.

Sources of samples	Isolate number	No. of isolates	Percentage of isolates (%)
Agricultural soils		**51**	**54.84**
Farm (2 samples)	F1-1, F1-2, F1-3, F1-4 F1-5, F1-6, F2-1, F2-2 F2-3, F2-4 F2-5, F2-6	12	12.90
Orchard (3 samples)	A3-1, A3-2, A3-3, A3-4 A3-5, A3-6, A4-1, A4-3 A4-4, A4-5 A4-6, A7-1 A7-2, A7-3, A7-4, A7-5 A7-6	17	18.28
Mix plantation (2 samples)	A5-2, A5-3, A5-4, A5-5 A5-6, A6-1, A6-2, A6-4 A6-5, A6-6	10	10.76
Flower plantation (2 samples)	A8-1, A8-2, A8-3, A8-4 A8-5, A8-6, A9-1, A9-2 A9-3, A9-4 A9-5, A9-6	12	12.90

4.2 Minimum inhibitory concentration (MIC)

The frequency distribution (as discrete categories) of their minimum inhibitory concentration (MIC) levels for arsenate and arsenite are summarized separately in Figure 4.1, whilst the combined MICs for both arsenate and arsenite are shown as a scatter plot in Figure 4.2.

The 42 arsenic-resistant isolates enriched from the tannery wastes had MICs ranged from 5 to ≥40 mM and 200 to ≥450 mM for arsenite and arsenate, respectively. Sixteen (17.20%) from the 42 isolates had MIC of arsenite and arsenate ≥40 mM and ≥400 mM, respectively, whilst four isolates (4.30%) had MIC of arsenate >450 mM (Figure 4.1A, 4.1B). The 51 arsenic-resistant isolates enriched from the agricultural soils had MICs ranged from 5 to ≥40 mM and 200 to>450 mM for arsenite and arsenate, respectively. However, distribution profile for arsenite MIC of the isolates from agricultural soils was lower than those from tannery wastes, whilst no significant difference was noted for arsenate MIC between the two habitats. Nevertheless, eleven isolates (11.83%) from the agricultural soil had MIC ≥40 mM and ≥400 mM for arsenite and arsenate, respectively, whilst four isolates (4.30%) had MIC for arsenate > 450 mM (Figure 4.1C, 4.1D). The 16 bacterial isolates from the

tannery wastes and 11 bacterial isolates from the agricultural soil samples which had both MIC of arsenite > 40 mM and MIC of arsenate ≥400 mM (Figure 4.2) were selected for further characterization and identification.

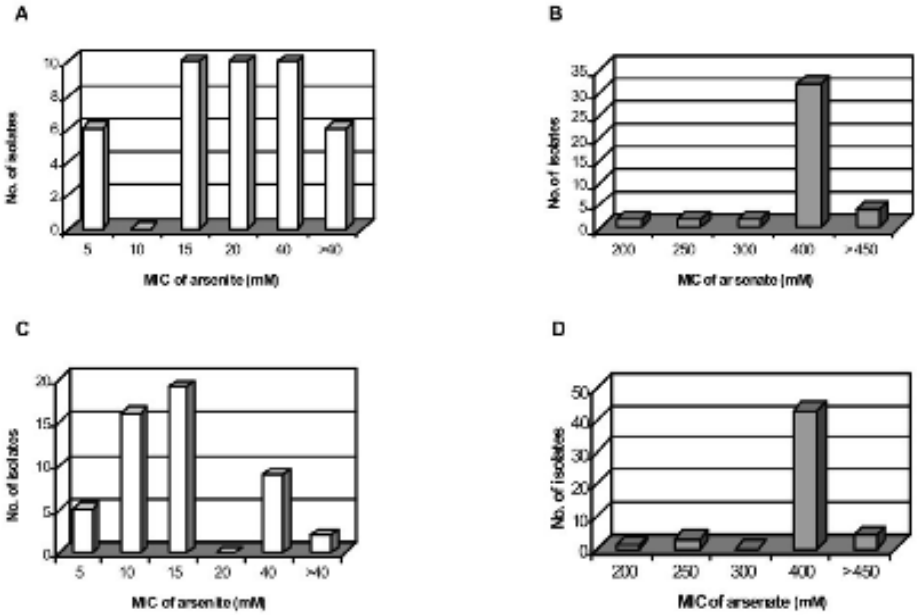


Figure 4.1 Frequency distribution of minimum inhibitory concentrations (MIC) for arsenite (A, C) and arsenate (B, D) of the arsenic resistant bacteria isolated from tannery wastes (A, B) and agricultural soils (C, D). For each isolate at least triplicate separate MIC evaluations were performed.

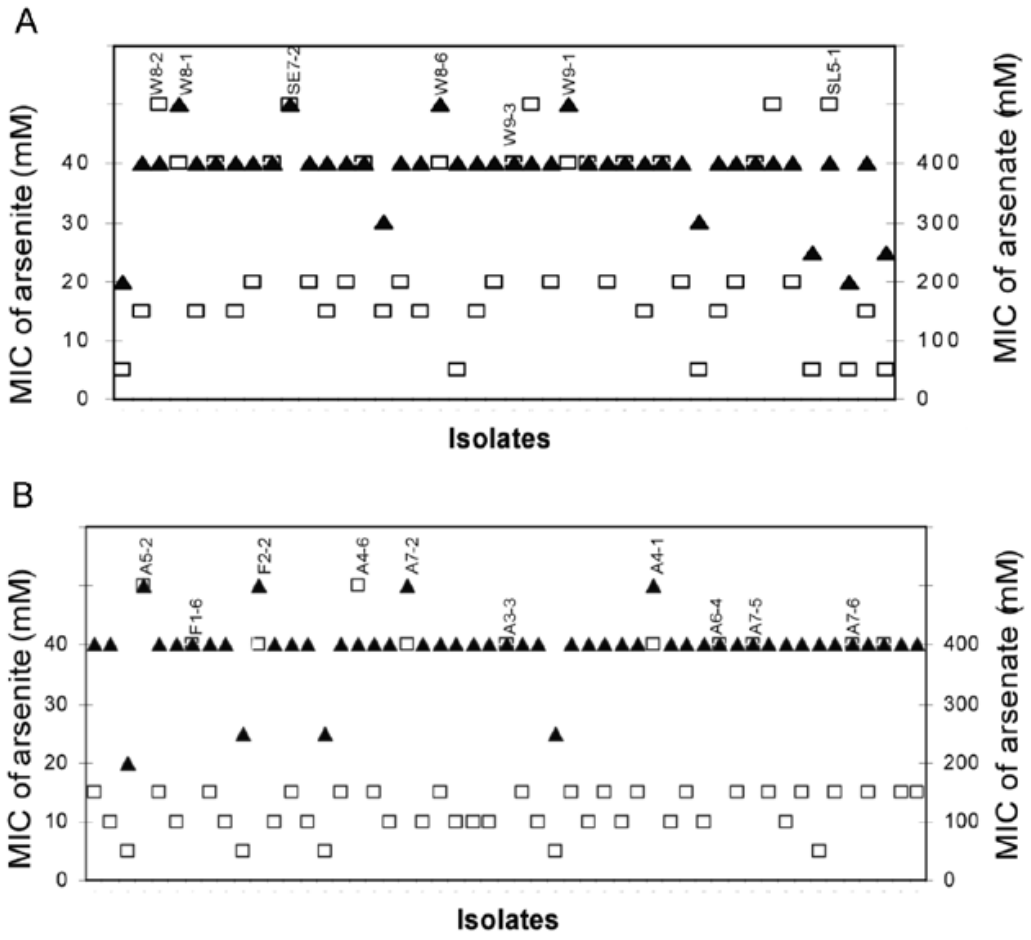


Figure 4.2 Combined arsenite and arsenate resistance levels of the selected arsenic resistant bacterial isolates. The 16 selected isolates from tannery water (A), the 11 selected isolates from agricultural soil (B). □: MIC of arsenite, ▲: MIC of arsenate.

4.3 Arsenite oxidation and arsenate reduction

Among all of the isolates which had MICs for arsenite and arsenate ≥ 40 mM and ≥ 400 mM, respectively, from the tannery wastes and agricultural soils, there only one isolate, *Comamonas* sp. A3-3 isolated from agricultural soil, oxidized arsenite to arsenate (Appendix E). No isolate reduced arsenate to arsenite under the tested condition. Known arsenite oxidizing bacterial strains are distributed in more than 20 genera and have been isolated from various environments. They include both chemolithotrophs; *Acidicaldus* sp., *Acidithiobacillus* sp. (Brierley and Brierley, 2001; D'Imperio *et al*., 2007) and heterotrophs; *Agrobacterium sp., Alcaligenes* sp.,

Burkholderia sp.*, Thiomonas* sp.*, Acinetobacter* sp., *Pseudomonas* sp. (Santini *et al.,* 2000; Krumova *et al.*, 2008; Quemeneur *et al.*, 2008; Cai *et al*., 2009). Cai *et al*. (2009) isolated a highly arsenic resistant *Comamonas* sp. from arsenic-contaminated soil in China but it could not oxidize arsenite to arsenate.

4.4 Removal of arsenic from culture broth

Some microorganisms take up arsenate via phosphate transporters and then reduce the arsenate internally to arsenite which is then either extruded from cell or sequestered in intracellular components, either as free arsenite or as conjugates with glutathione or other thiols (Mateos *et al.*, 2006). Therefore, the representative arsenic resistant strains, A4-6, A5-2, A6-4 and W8-2, selected according to their high arsenite (MIC $\geq$40 mM) and arsenate (MIC $\geq$400 mM) resistance, and their differences in colony morphology, were grown in TYEG medium containing 1.3 mM sodium arsenate. As shown in Figure 4.3, the presence of arsenate at 1.3 mM in growth media had no effect on growth of three isolates A4-6, A5-2 and W8-2, but the growth of isolate, A6-4, was retarded within the first 6 h. After the cultures reached stationary growth phase, cells were separated by centrifugation and the supernatants were analyzed for arsenic concentration by using ICP-MS. The arsenic concentration in the culture supernatants was found to be the same as those in fresh medium without inoculation (Figure 4.4). The result indicated none of the four isolates tested took up an arsenate. Thus, reduction of arsenate to arsenite and sequestering in cells is not likely to be a mechanism of their resistance. Trend of higher total arsenic in the culture supernatants than in the fresh medium due to carry over of arsenic in bacterial inoculum. Takeuchi *et al*. (2007) reported that an arsenic resistant *Marinomonas communis* was capable of removing arsenic from culture medium amended with arsenate by accumulation in their cells. Another possible of arsenic resistant mechanism, microorganism could convert inorganic arsenic to organic arsenic form (Knowles and Benson, 1983; Craig, 1989; Cevantes *et al.,* 1994) or some bacteria had an integral membrane protein that pumps arsenite out of cell (Mukhopadhyay *et al*., 2002). The ICP-MS could not detect the change of arsenic species in culture medium for confirmation of those arsenic resistant mechanisms.

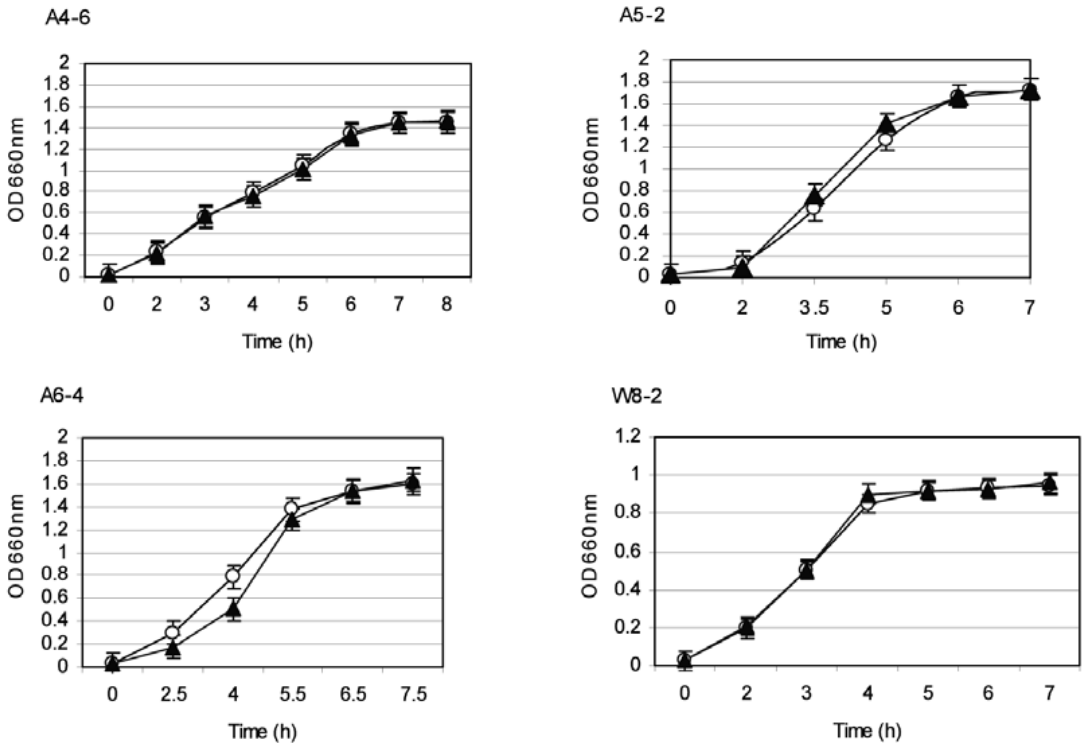


Figure 4.3 Growth of isolates A4-6, A5-2, A6-4 and W8-2 in the absence (O) and in the presence (▲) of 1.3 mM sodium arsenate. Data are shown as mean ± 1 SD and are derived from triplicate independent experiments.

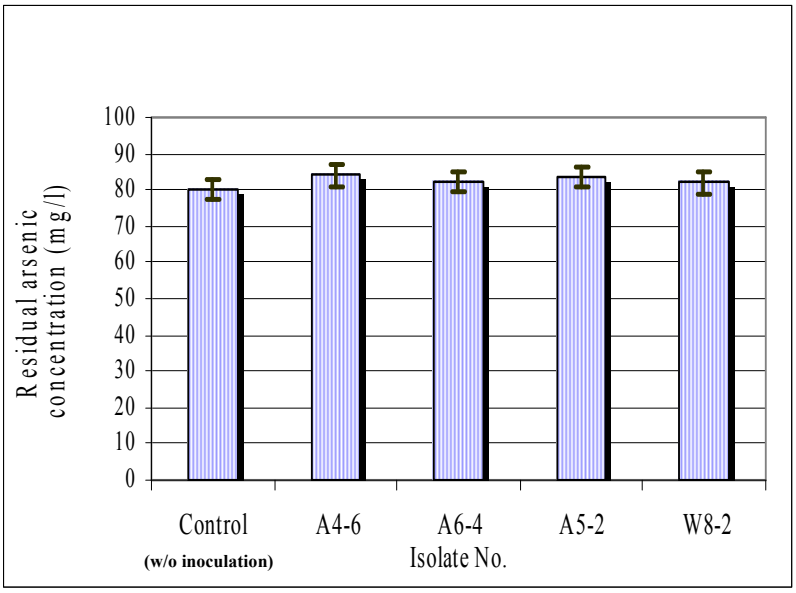


Figure 4.4 Arsenic concentration of cultured broth after cultivation of the representative strains.

4.5 Confirmation of arsenite oxidizing activity and efficiency of arsenite oxidizing activity

4.5.1 XANES spectroscopy method

XANES spectroscopy was used to determine the valence of excited arsenic atoms in liquid media directly. There was a distinct shift in the absorption K-edge in A3-3 inoculated broth represented a change in oxidation state from sodium arsenite (11,871.2 $\pm$ 0.5 eV) to arsenic(V) oxide (11,876.0 $\pm$ 0.5 eV). Comparison to arsenic standards (Appendix D), indicated arsenite oxidizing capability of the isolate A3-3 (Figure 4.5). Presently, no publication of arsenic speciation in bacteria studied by using XANES spectroscopy method. Arsenic speciation in the leaves of Chinese Brake Fern (*Pteris vittata* L.) which has been discovered recently to be an extremely effective hyper-accumulator of arsenic, showed 94 % aqueous arsenite ion (AsO_3^-), 6 % As_2S_2 and other As(III) compounds, such as sodium salts and oxides have distinctly smaller (Webb *et al*., 2003).

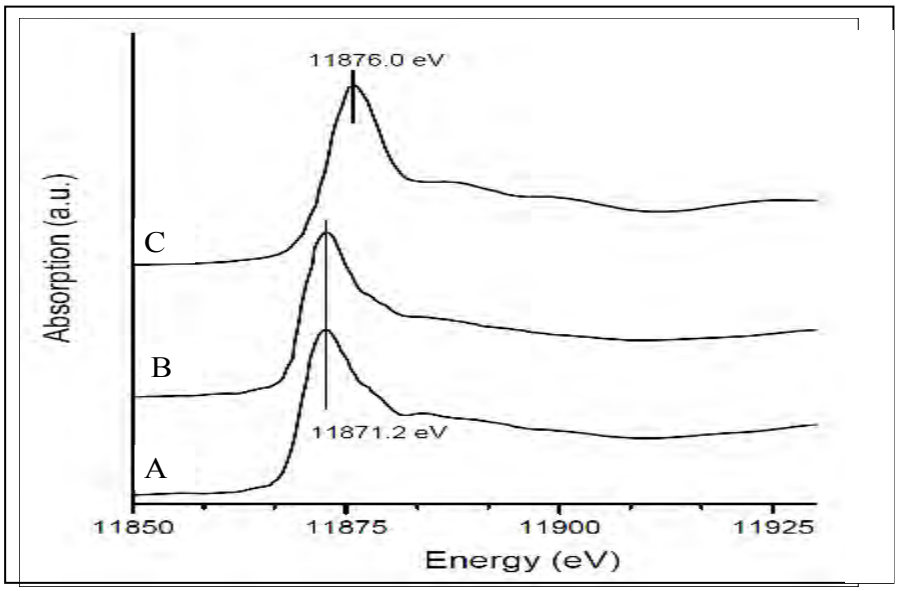


Figure 4.5 Arsenic K-edge XANES spectra analysis; freshly prepared bacterial culture medium (A), medium without inoculation (B) and after cultivation of A3-3 (C). Spectra shown here, are the average of two scans. The averaged spectrum was smoothed using the FFT-smoothing routine (5 point-setting) of the origin program.

4.5.2 Modified method of arsenite-iodine titration

Residual arsenite in culture broth with and without inoculation was analysed and compared. Arsenite concentration of the inoculated broth decreased from 4.9 mM to 1.7 mM (Figure 4.6). So, efficiency of arsenite oxidizing activity of A3-3 isolate determination by this method is 65.3% after 24 h incubation. Bacterial oxidation occurred at a rate of 0.0022 mM min^{-1} during growth phases. Valenzuela *et al*., (2009) reported nine bacterial isolates (*Pseudomonas*) were able to oxidize more than 95% of low arsenite concentration (0.5 mM) present in the culture medium, after long time incubation (7 days). Rehman *et al.* (2010) reported *P. lubricans* could oxidize As(III) which determined in crude extracts of bacterial cells were 42% (42 µg mg^{-1} of protein),78% (78 µg mg^{-1} of protein) and 95% (95 µg mg^{-1} of protein) from 200 µM arsenite present in the medium after 24, 48 and 72 h of incubation at optimal conditions, respectively.

The arsenite oxidizing ability of *Comamonas* A3-3 indicates its potential application in biological treatment of waste waters contaminated with arsenic. Similar report by Lièvremont *et al.* (2003) who demonstrated a new insight to the arsenic removal process, based on the use of arsenic-oxidizing bacteria for the conversion of arsenite into arsenate. Another type of treatment is based on the production of H_2S from sulfate-reducing bacteria, which consequently leads to the precipitation of arsenic under arsenic sulfides (Groudeva *et al*., 2001). Most of works for the biological removal of arsenic are based on the filtration of water trough sands or another support on which is possible to form a biofilm from arsenic oxidizing bacteria. Katsoyanidis and Zouboulis (2004) proposed the use of biological iron oxidation for the removal of arsenic. Biosorption has been demonstrated to be a useful alternative to conventional treatment systems for the removal of metals from the dilute aqueous solution such as, Loukidou *et al.* (2003) proposed employment of fungal, non- living biomass of *P. chrysogenum* as arsenate biosorptive agent.

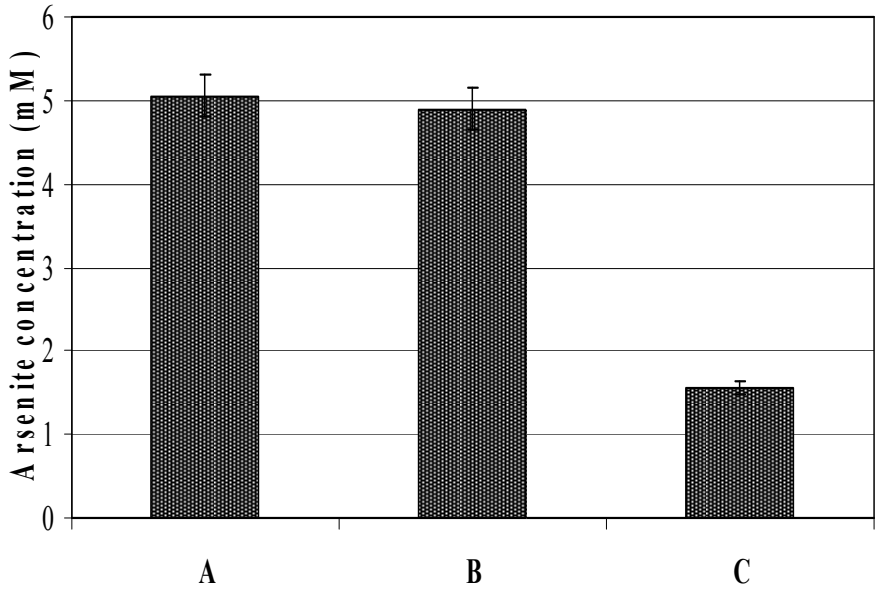


Figure 4.6 Residual arsenite concentrations in culture supernatant; freshly prepared bacterial culture medium (A), medium without inoculation cultivation (B) and after cultivation of A3-3 (C).

4.6 Arsenite oxidase and arsenite transporter genes

The three primer sets were tested on arsenite oxidizing A3-3 isolate for detection of arsenite oxidase gene and arsenite transporter gene. No genes amplification was obtained from this A3-3 isolate. The results suggest that the arsenite oxidase gene in this A3-3 isolate differ from the other described arsenite oxidase. Similar results were reported by Rhine *et al*. (2007) and Valenzuela *et al*., (2009) did not amplify any genes for arsenite oxidase, despite showing arsenite oxidizing activity under aerobically condition. Gram-positive and Gram-negative bacteria were resised to both arsenite and arsenate by *ars*-operon and *aoxAB* operon (arsenite oxidase genes). Genes in those operons were found more diversity such as > 30 various of *ars* genes from microorganism (Schmidt and Shaw, 2001; Mukhopadhyay *et al*., 2002; Santini and Vanden Hoven, 2004; Silver and Phung, 2005). Recently *aoxB*-like sequences have been widely found in different arsenic contaminated soil and water systems (Inskeep *et al*., 2007).

4.7 Bacterial identification

The twenty-seven high arsenic resistant bacterial isolates were divided into nine groups based on their morphological, cultural, physiological and biological characteristics. Groups 1 to 4 were isolated from tannery waste samples and groups 5 to 9 were isolated from agricultural soil samples. They were all Gram negative, and rod-shaped or short rod in morphology. Across the nine groups, colony morphology on TYEG agar medium was found to be quite diverse. So they are described within each group below. Most of them showed positive reaction for catalase, MR reaction, citrate utilization and growth in 3% (w/v) NaCl, but were negative for hydrolysis of gelatin, indole, could not grow at both 4 and 50°C and did not produce hydrogen sulfide (data not shown). The more variable characteristics in physiological and biochemical properties of each group are summarized in Table 4.2, except the principal ubiquinones of each representative strains which are shown separately in Table 4.3.

Group 1 contained eight isolates, W8-1, W8-6, W9-1, W9-4, W9-5, W9-6, WS1-2 and SL4-2, which had circular, convex, entire, mucoid and white colour colonies of 2.7 - 4.5 mm diameter. The representative isolate (randomly selected), W8-1, showed 98.9% 16S rRNA gene sequence similarity to *Klebsiella pneumoniae* subsp. ozaenae ATCC 11296^T, Figure 4.7 (Drancourt *et al*., 2001). Along with isolate W9-1 it contained Q8 as a major ubiquinone (Table 4.3), and had MICs for arsenite and arsenate of 40 and 450 mM, respectively.

Group 2 contained six isolates, W8-2, W9-2, W9-3, W10-1, W10-2 and W10-3, which had circular, raised, entire and white colour colonies of 0.5 - 2.0 mm diameter. The representative isolates (randomly selected), W8-2 and W9-3, showed 97.1 and 95.8% 16S rRNA gene sequence similarity to *Acinetobacter schindleri* LUH 5832^T, Figure 4.8 (Nemec *et al.*, 2001), respectively and contained Q9 as a major ubiquinone. The MICs of arsenite and arsenate were $\geq$40 and $\geq$400 mM, respectively.

Group 3 contained one isolate, SL5-1, which had circular, raised, entire and creamy-white colonies of 1.0 - 2.0 mm diameter, showed 16S rRNA gene sequence similarity of 96.4% to, and were phylogenetically placed within, *Acinetobacter calcoaceticus* NCCB 22016^T, Figure 4.8 (Nemec *et al.*, 2001) . Q9 was a major ubiquinone, and MICs for arsenite and arsenate were $\geq$40 and $\geq$400 mM, respectively.

Group 4 also contained one isolate, SE7-2, which had irregular, flat, undulate and white-yellowish colonies of 2.0 - 3.0 mm diameter, showed 16S rRNA gene sequence similarity of 94.3% to *Pseudomonas alcaligenes* ATCC 14909^T (Baïda *et al.,* 2002) . Q9 was a major ubiquinone, and MIC for arsenite and arsenate were ≥40 and ≥400 mM, respectively.

Group 5 contained four isolates, F1-4, F1-6, A3-3 and A7-5, which had circular, convex or raised, entire and white colonies of 1.0 - 2.5 mm diameter. Three representative isolates, F1-6, A3-3 and A7-5, showed 98.5, 96.8 and 98.4% 16S rRNA gene sequence similarity to *Comamonas testosteroni* ATCC 11996^T, *C. kerstersii* LMG 3475^T and *C. testosteroni* ATCC 11996^T, Figure 4.10 (Tamaoka *et al.*, 1987; Wauters *et al.*, 2003) respectively. Q9 was a major ubiquinone and MICs for arsenite and arsenate were 40 and 400 mM, respectively.

Group 6 contained three isolates, A6-4, F2-2 and A7-2, which had circular, flat, entire and white colonies of 0.5 - 1.5 mm diameter, and showed 97.7, 97.7 and 98.3% 16S rRNA gene sequence similarity to *Pseudomonas plecoglossicida* FPC 951^T, Figure 4.9 (Nishimori *et al.,* 2000) ,respectively. The F2-2 and A6-4 isolates contained Q9 as a major ubiquinone and had MICs for arsenite and arsenate of ≥40 and ≥400 mM, respectively.

Group 7 contained two isolates, A4-6 and A7-6, which had circular, raised, entire and white colour colonies of 1.0 - 2.5 mm diameter. They showed 99.5% 16S rRNA gene sequence similarity to *Acinetobacter calcoaceticus* NCCB 22016^T, Figure 4.8 (Nemec *et al.*, 2001), contained Q9 as a major ubiquinone. Their MICs for arsenite and arsenate were ≥40 and ≥400 mM, respectively.

Group 8 contained one isolate, A4-1, which had irregular, raised, undulate, mucoid and white colonies of 1.5 - 5.0 mm diameter, 96.6% 16S rRNA gene sequence similarity to *Klebsiella pneumoniae* subsp. *ozaenae* ATCC 11296^T , Figure 4.7 (Drancourt, 2001). Q8 was a major ubiquinone and MICs for arsenite and arsenate were 40 and >450 mM, respectively.

Finally, group 9 contained one isolate, A5-2, which had circular, umbonate, entire and white colonies of 1.0 - 2.5 mm diameter. It showed 98.5% 16S rRNA gene sequence similarity to *Enterobacter ludwigii* EN-119^T, Figure 4.7 (Hoffmann *et al*.,

2005). Q8 was a major ubiquinone and MICs for arsenite and arsenate were ≥40 and >450 mM, respectively.

Summary of phylogenetic relationships between all strains; W8-2, W9-3, SL5-1, A4-6, A7-6, A7-2, A6-4, F2-2, A4-1, W8-1, A5-2, A7-5, F1-6, A3 -3 and their reference species was shown in Figure 4.11.

Table 4.2 Differential characteristics of the grouped arsenic resistant isolates.

	Tannery wastes				**Agricultural soils**				
Group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Number of isolates	8	6	1	1	4	3	2	1	1
Cell shape	R	SR	SR	R	R	R	SR	R	R
Glucose O/F	+/+	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+
Growth at 40 ºC	+	-	+	-	-	- (+1)	+(-1)	+	+
Growth in 5%NaCl	+	-	w	-	-	- (+1)	-	+	+
Oxidase	-	-	-	+	+	+	-	-	-
VP reaction	w	-	-	-	-	-	-	w	-
Nitrate reduction	+	-	-	+	+(-1)	-(+1)	-	+	+
Urease activity	+(-1)	-	-	-	-	+(-1)	+(-1)	+	+
Hydrolysis of Tween 80	- (+1)	+	+	-	-	-	-	-	-
Aesculin	+	-	-	-	-	-	-	+	+
Starch	-	-	-	+	-	-	-	-	-
Utilization of Carbohydrates									
Mannitol	+	-	-	+	-	-	-	+	+
D-Xylose	+	-	+	-	-	-	+	+	+
L-Arabinose	+	-	+	-	-	-	+	+	+
D-Malonate	+(-1)	+	+	+	-	+(-1)	-	+	+
L-Citrulline	+	+	+	-	-	-	-	+	+
Suspected genera	*Kb*	*Ac*	*Ac*	*Ps*	*Co*	*Ps*	*Ac*	*Kb*	*En*

R, rods; SR, short rods; +, positive; w, weakly positive; –, negative

Kb, *Klebsiell*a; *Ac*, *Acinetobacter*; *Ps*, *Pseudomonas*; *Co*, *Comamonas*; *En*, *Enterobacter*.

Table 4.3 Frequency of different ubiquinone types found in representative strains of each arsenic resistant isolates.

Group[1]	**Isolate no.**	**Ubiquinone (%)**[2]			
		Q7	Q8	Q9	Q10
1	W8-1	6.1	**90.7**	3.2	0
1	W9-1	5.1	**75.7**	14.0	5.2
2	W8-2	0	12.3	**87.7**	0
2	W9-3	0	11.8	**88.2**	0
3	SL5-1	2.2	7.0	**90.8**	0
4	SE7-2	3.9	0.2	**95.9**	0
5	F1-6	3.0	**88.1**	8.9	0
5	A3-3	1.7	**93.4**	4.9	0
5	A7-5	2.2	**94.7**	3.1	0
6	F2-2	8.0	3.3	**88.7**	0
6	A6-4	4.8	1.0	**94.2**	0
7	A4-6	7.8	7.0	**85.2**	0
7	A7-6	8.8	1.8	**81.8**	7.6
8	A4-1	9.6	**83.4**	0	7.0
9	A5-2	14.7	**85.3**	0	0

[1]Goups are those defined by morphology, as in the results text.

[2]Ubiquinone types are shown as percentage of total ubiquinone in bacterial membrane, with the most frequent type being shown in bold type.

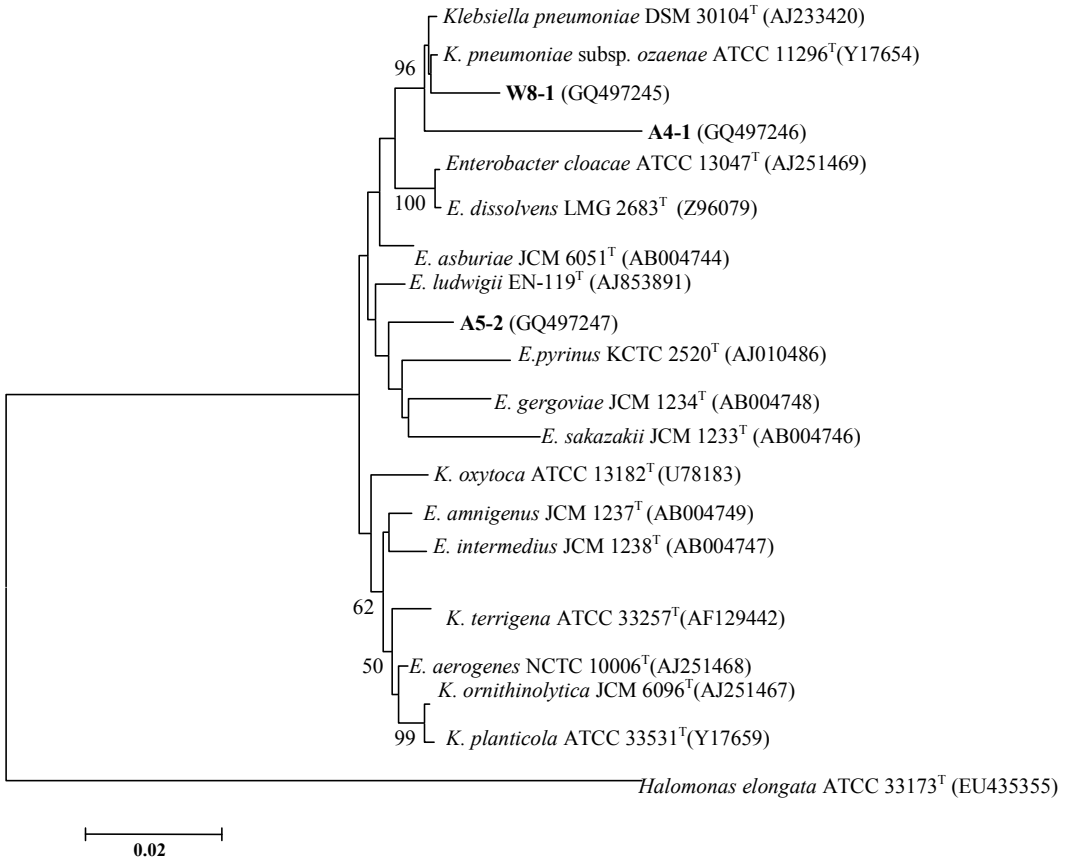


Figure 4.7 Neighbor-joining tree of 16S rRNA gene sequences showing phylogenetic relationship between strain W8-1, A4-1, A5-2 and selected type strains of *Klebsiella , Enterobacter* and *Halomonas*. Based on 1,000 replications, bootstrap percentages above 50% are shown. Bar, 0.02 substitutions per nucleotide position. GenBank accession codes are shown after each isolate name.

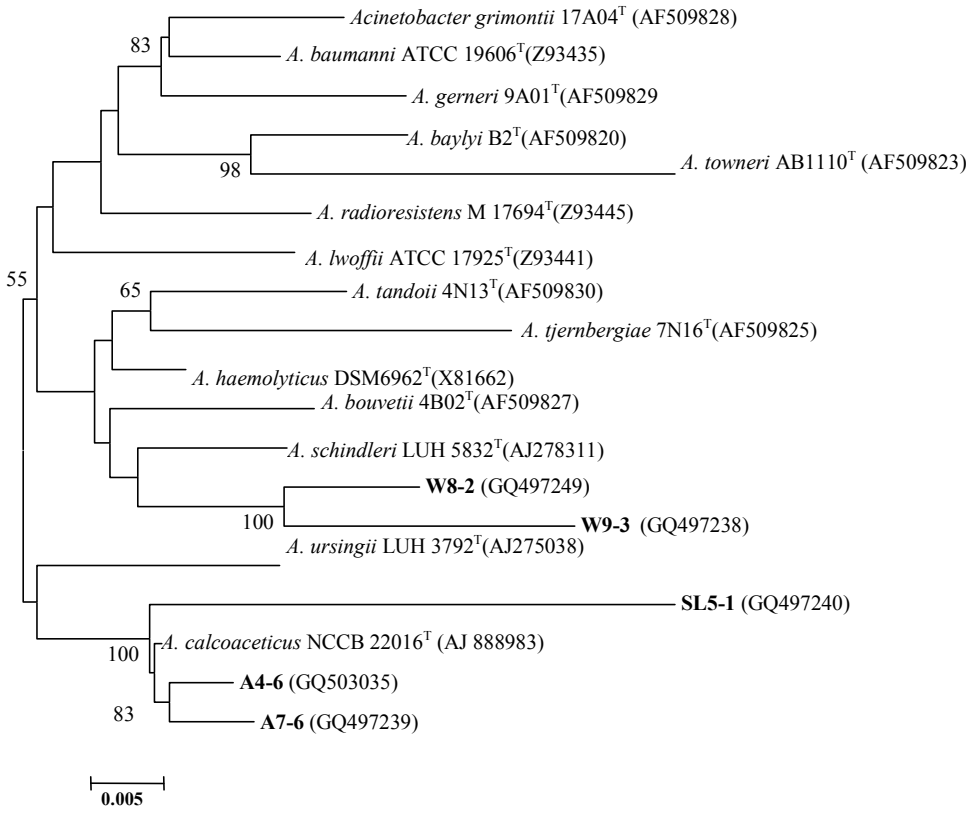


Figure 4.8 Neighbor-joining tree of 16S rRNA gene sequences showing phylogenetic relationship between strain W8-2, W9-3, SL5-1, A4-6, A7-6 and selected type strains of *Acinetobacter*. Based on 1,000 replications, bootstrap percentages above 50% are shown. Bar, 0.005 substitutions per nucleotide position. GenBank accession codes are shown after each isolate name.

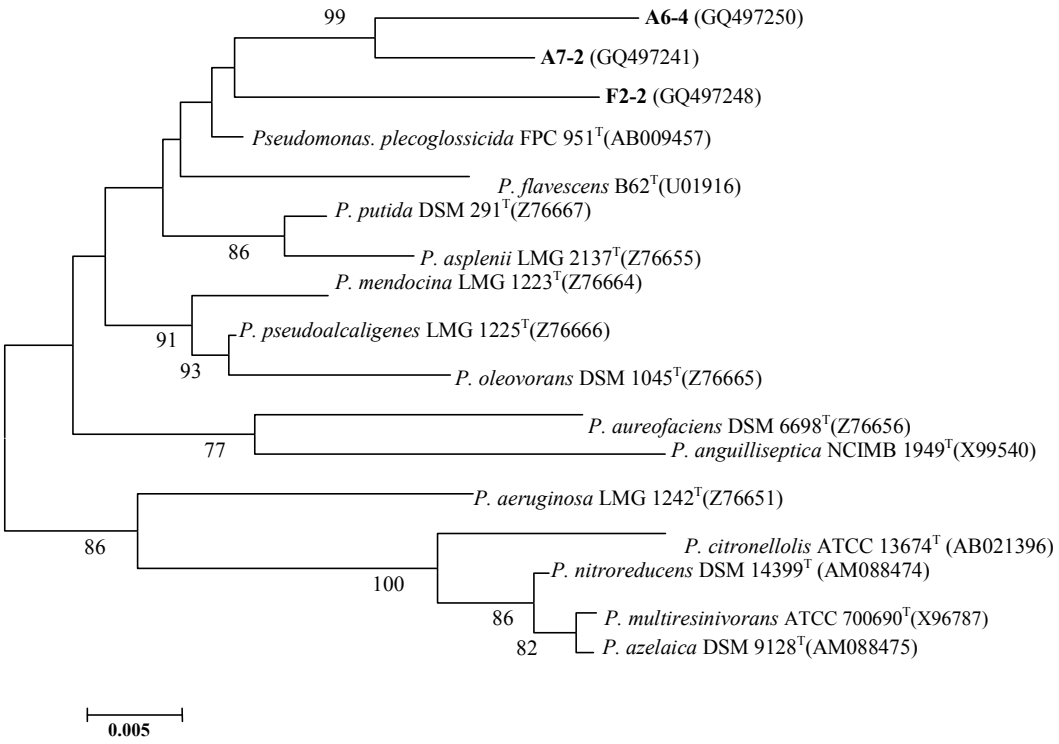


Figure 4.9 Neighbor-joining tree of 16S rRNA gene sequences showing phylogenetic relationship between strain A6-4, A7-2, F2-2 and selected type strains of *Pseudomanas*. Based on 1,000 replications, bootstrap percenttages above 50% are shown. Bar, 0.005 substitutions per nucleotide position. GenBank accession codes are shown after each isolate name.

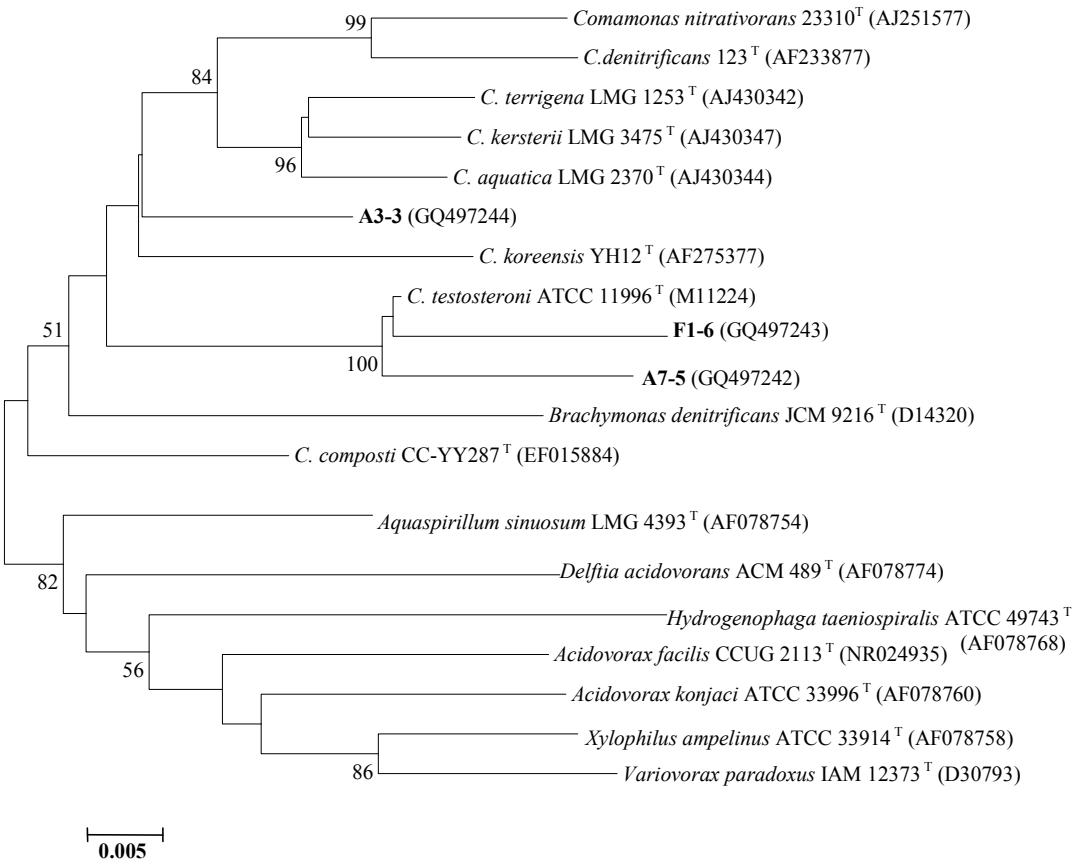


Figure 4.10 Neighbor-joining tree of 16S rRNA gene sequences showing phylogenetic relationship between strain F1-6, A7-5, A3-3 and selected type strains of *Comamonas*, *Xylophilus*, *Variovorax*, *Acidovorax*, *Acidovorax*, *Hydrogenophaga, Brachymonas, Delftia* and *Aquaspirillum*. Based on 1,000 replications, bootstrap percentages above 50% are shown. Bar, 0.005 substitutions per nucleotide position. GenBank accession codes are shown after each isolate name.

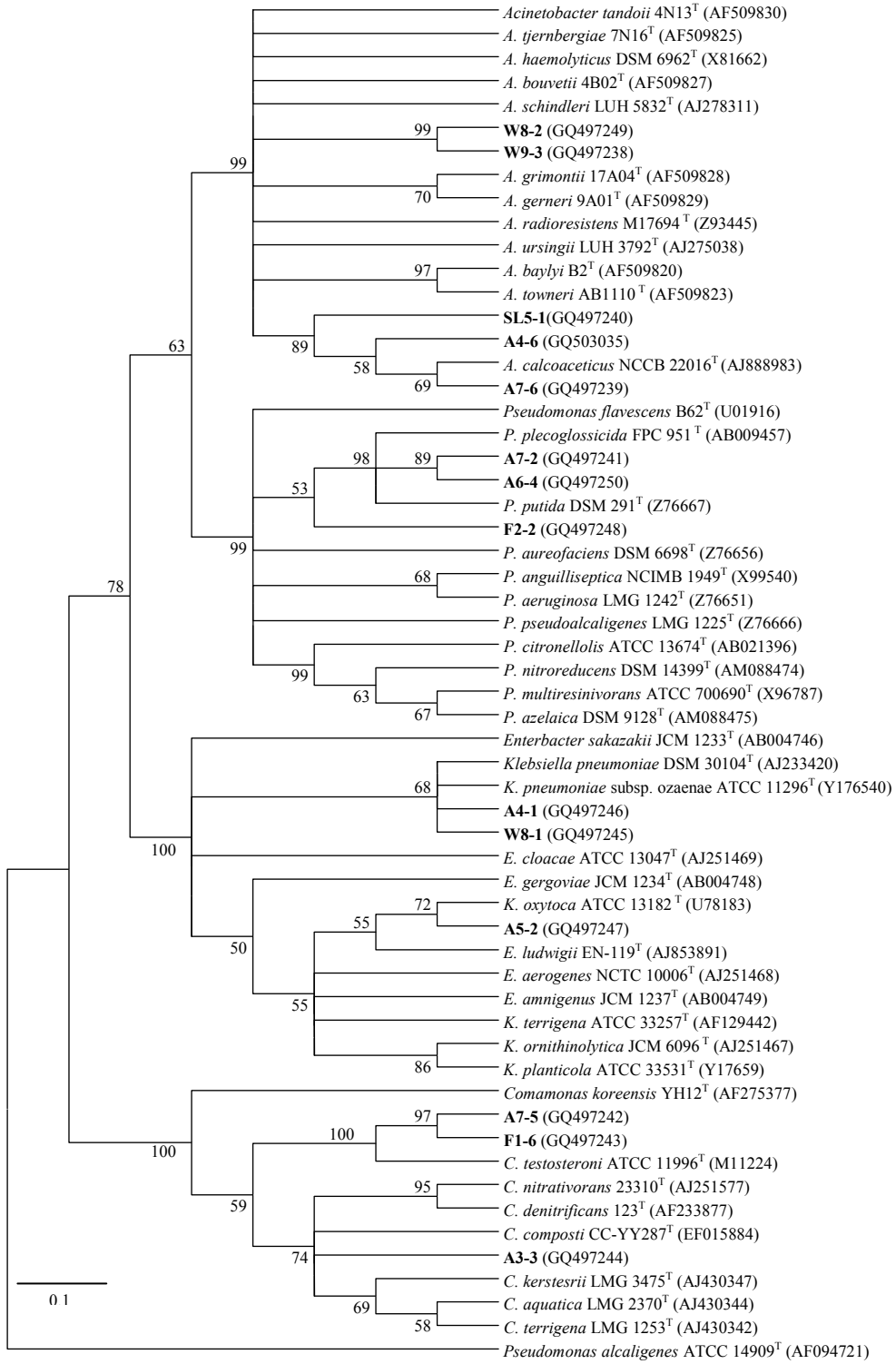


Figure 4.11 Neighbor-joining based phylogenetic trees of 16S rRNA gene sequences showing phylogenetic relationship between strains W8-2, W9-3, SL5-1, A4-6, A7-6, A7-2, A6-4, F2-2, A4-1, W8-1, A5-2, A7-5, F1-6, A3-3 and their reference species. Based on 1000 replications, bootstrap percenttages above 50% are shown. Bar, 0.1 substitutions per nucleotide position.

4.8 Arsenite oxidizing bacteria, *Comamonas* sp A3-3 and their relative isolates

Effect of arsenate on growth of the isolate A3-3 was tested. The isolate A3-3 was cultivated in TYEG medium at 30°C with shaking (200 rpm) for 24 h. The overnight culture was transferred at 2.5 % (v/v) into 20 ml of TYEG medium supplemented with 1.3 mM sodium arsenate (100 mg/l) in 250 ml flask and incubated at 30°C with shaking. Growth of the isolate A3-3 was monitored by monitoring of an optical density at 660 nm (OD 660 nm) and compared with those of isolate F1-6 and *Comamonas testosteroni* KCTC 2990 ^{T}. As shown in Figure 4.12, arsenate at 1.3 mM had no effect on growth of the isolate A3-3, F1-6 and *C. testosteroni* KCTC 2990 ^{T}.

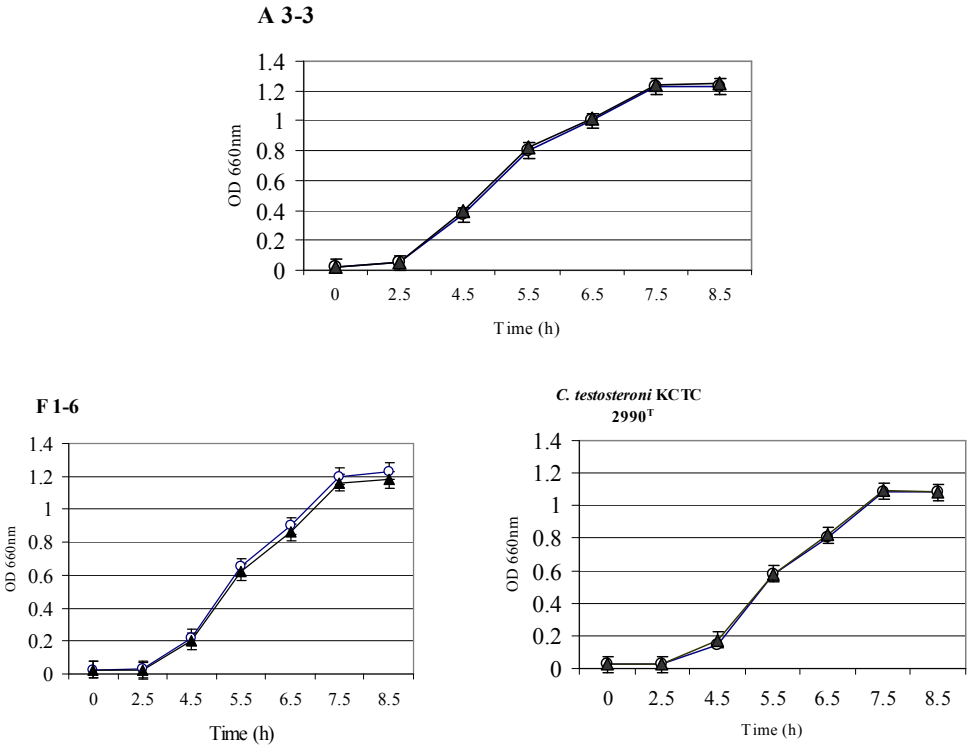


Figure 4.12 Growth comparison of isolate A3-3, F1-6 and *C. testosteroni* KCTC 2990 ^{T} in the absence (O) and in the presence (▲) of 1.3 mM sodium arsenate. Data are shown as the mean $\pm$ 1 SD and are derived from triplicate independent repeats.

The isolate A3-3 was Gram-negative rods with polar flagella; colony was circular, white, raised elevation, entire margin, 1-2.5 mm diameter, no pigmentation. Oxidase, catalase, utilization of simmon citrate, urease, VP, hydrolysis of esculin ferric citrate, oxidation of glucose were positive. Positive reaction for assimilation of potassium gluconate, adipic acid and malic acid by using API 20E, API 20NE and API 50CH systems. It grew at 40°C in the presence of 4.5% (w/v) NaCl and at pH 5 to11 but it could not grow at 4°C and 50°C in the presence of 5% (w/v) NaCl and at pH 4. Hydrolysis of TDA, lysine, arginine, gelatin, starch, Tween 80; indole, nitrate reduction; H_2S production, oxidation of glycerol were negative. Assimilation of carbohydrates determined were negative for rhamnose, sucrose, melibiose, amygdalin, arabinose, erythritol, D–arabinose, L–arabinose, D–ribose, D–xylose, L–xylose, D-adonitol, Methyl-βD-xylopyranoside, D–galactose, D–glucose, D–fructose, D–manose, L–sorbose, L–rhamnose, dulcitol, inositol, D–mannitol, D–sorbitol, Methyl-αD-mannopyranoside, Methyl-αD-glucopyranoside, N–acetylglucosamine, amygdalin arbutin, salicin, D–cellobiose, D–maltose, D–lactose (bovine origin), D–melibiose, D–saccharose (sucrose), D–trehalose,inulin, D–melezitose, D–raffinose, Amidon (starch), glycogen, xylitol, gentiobiose, D–turanose, D–lyxose, D–tagatose, D–fucose, L–fucose, D–arabitol, L–arabitol, Potassium 2-ketogluconate potassium 5-ketogluconat, caparic acid, trisodium citrate and phenyl acetic acid from API 20E, API 20NE and API 50CH determination. Differential characteristic and DNA G+C content of the isolate A3-3 and related *Comamonas* species were showed in Table 4.4

Ubiquinones Q8 (93.41%) was found to be major quinine and major cellular fatty acids are $C_{16:0}$, $C_{18:1}$ $\omega 7c$ and $C_{17:0\ CYCLO}$ but those of *Comamonas kerstersii* LMG 3475^T were $C_{16:0}$, $C_{18:1}$ $\omega 9c$, $C_{10:0}$ 3-OH, $C_{12:0}$ and $C_{14:0}$ (Wauters *et al*., 2003; Chou *et al*., 2007). The profiles of cellular fatty acids of the islolate A3-3 isolate and its related *Comamonas* species were shown in Table 4.5.

Phylogenetic analysis using 16S rRNA gene sequence of the isolate A3-3 showed lowly percentages simirarity (< 98%); 96.9 % similar to those of *C. kerstersii* LMG 3475 ^{T} (Wauters *et al*., 2003), 96.8% similar to *C. aquatica* LMG 2370^T (Wauters *et al*., 2003), 96.4% similar to *C. koreensis* YH12^T (Chang *et al*., 2002), 96.3% similarity to *C. testosteroni* ATCC 11996 ^{T} (Tamaoka *et al*.,1987), 96.2% similarity to *C. composti* CC-YY287^T (Young *et al*.,2008), 96.1% similarity to

C. terrigena LMG 1253^{T}(Wauters *et al*., 2003), 95.7% similarity to *C. nitrativorans* 23310^{T}(Etchebehere *et al*., 2001), 95.5% similarity to *C. denitrificans* 123^{T} (Gumaelius *et al*., 2001). A 16S rRNA gene sequence of the isolate A3-3 were 95% and 94.6% similarity to those of the isolate A7-5 and F1-6, respectively (Table 4.6). The position of the isolate A3-3 in phylogenetic tree was shown in Figure 4.9. The isolates F1-6 and A7-5 had 98.1% 16S rRNA sequences similarity to *C. testosteroni* ATCC 11996 ^{T} but had DNA-DNA hybridization 37.7 and 13.6% DNA-DNA hybridization to *C. testosteroni* KCTC 2990^{T}, respectively (Table 4.7). Therefore, the 3 arsenic resistant isolate, A3-3, A7-5 and F1-6 represent novel species of genus *Comamonas*, for which the name *C. terra* (NBRC106524^{T}), *C. soli* (NBRC106525^{T}) and *C. thailandensis* (NBRC106526^{T}), respectively were proposed. The bacterial isolates will be registered and preserved by National Institute of Technology and Evaluation (NITE), Biological Resource Center (NBRC), Japan. The GenBank accession number of A7-5, F1-6 and A3-3 are GQ497242, GQ497243 and GQ497244, respectively.

Table 4.4 Differential characteristic of isolate A3-3 and its related *Comamonas* species.

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Cell shape	R	R	S	R	R	C	R	R
Flagella	P	P	P	P	P	A	P	P
Growth at 37 °C	+	-	+	+	+	+	+	+
Growth at 40 °C	+	-	-	+	-	w	-	-
Growth in pH 4	-	-	-	-	-	-	-	-
Growth in pH 5	+	+	+	-	+	+	+	-
[1,2]Urease	+	-	-	-	-	-	-	-
[2]Nitrate reduction	-	+	+	+	+	+	+	+
Tween 80 hydrolysis	-	-	-	+	+	+	+	-
Citrate utilization	+	+	+	+	+	-	+	-

Table 4.4 (Cont.) Differential characteristic of isolate A3-3 and its related *Comamonas* species.

Characteristics	1	2	3	4	5	6	7	8
[2,3]**Assimilation of :**								
Amygdalin	-	-	w	-	-	-	-	-
Arbutin	-	-	+	-	-	-	+	-
Erythritol	-	-	-	-	-	-	-	+
Glucose	-	-	-	-	-	+	+	-
Glycogen	-	-	-	-	-	-	-	+
Inositol	-	-	-	-	-	+	-	-
Mannitol	-	-	-	-	-	+	-	-
Sucrose	-	-	w	-	-	-	-	-
Tagatose	-	-	-	-	-	+	-	-
Capric acid	-	-	-	-	-	-	+	-
Aipic acid	-	-	-	+	+	+	+	+
Trisodium citrate	-	-	-	-	-	+	w	-
Potassium gluconate	+	+	+	-	+	+	+	+
[4]G+C content (mol %)	69.6	62.7	64.5	61.0	64.0	66.0	62.5	64.0

+, positive; w, weakly positive; –, negative; R, rods; S, short rods; C, curved rods; P, polar flagella; A, absent.

[1]data from API 20E; [2]data from API 20NE; [3]data from API 50 CH.

[4]data for G+C content for reference strains were obtained from Chou *et al*. (2007), Chang *et al*. (2002), De Vos *et al*., (1985), and Tomaoka *et al* (1987).

Strains: 1, A3-3; 2, F1-6; 3, A7-5; 4, *Comamonas kerstersii* LMG 3475^T; 5, *C. aquatica* LMG 2370^T; 6, *C. koreensis* KCTC 12005^T; 7, *C. testosteroni* KCTC 2990 ^{T}; 8, *C. terrigena* KCTC 2989 ^{T}.

Table 4.5 Cellular fatty acid composition of isolate and their related *Comamonas* sp**.**

Fatty acids	1	2	3	4	5	6	7	8
Saturated straight-chain								
C $_{12:0}$	<0.5	3.29	3.23	2.6	3.0	2.3	2.4	2.8
C $_{14:0}$	<0.5	<0.5	<0.5	2.9	3.9	1.0	1.0	3.3
C $_{15:0}$	ND	ND	ND	<0.5	<0.5	9.4	1.0	3.7
C $_{16:0}$	**24.02**	**28.17**	**30.74**	**23.4**	**25.2**	**29.9**	**30.4**	**27.5**
C $_{17:0}$	<0.5	ND	<0.5	0.6	<0.5	2.6	0.8	1.5
Unsaturated straight-chain								
C $_{18:1\ \omega7c}$	**15.02**	**19.16**	**18.78**	ND	ND	ND	ND	ND
Saturated branched-chain								
C $_{17:0\ CYCLO}$	**25.94**	**27.63**	**22.43**	0.7	<0.5	**12.3**	3.8	2.4
Unsaturated branched-chain								
C $_{19:0\ CYCLO\ \omega8c}$	7.54	2.65	0.71	ND	ND	ND	ND	ND
Hydroxylated fatty acids								
C $_{10:0\ 3\text{-}OH}$	6.01	4.91	4.29	4.5	5.0	3.5	4.8	5.3
C $_{16:0\ 2\text{-}OH}$	6.34	3.41	3.23	<0.5	<0.5	2.2	2.0	<0.5
C $_{16:1\ 2\text{-}OH}$	1.40	1.64	1.72	<0.5	<0.5	<0.5	0.6	<0.5
[1]Summed feature 3	7.38	7.19	**12.93**	**28.2**	**42.4**	**26.1**	**33.1**	**38.4**
C $_{18:1}$/ C $_{18:1\ \omega7c}$	ND	ND	ND	**36.1**	**19.0**	9.6	**17.9**	**14.9**

[1]Summed feature 3 contains C $_{16:1\ \omega7c}$/ C $_{15:0\ iso\ 2\text{-}OH}$; ND, Not detected.

Strains: 1, A3-3; 2, F1-6; 3, A7-5 ; 4, *Comamonas kerstersii* LMG 3475^T(Chou *et al*., 2007); 5, *C. aquatica* LMG 2370^T(Chou *et al*., 2007); 6, *C. koreensis* DSM 18232^T(Chang *et al*., 2002); 7, *C. testosteroni* KCTC 2990^T (Tomaoka *et al*, 1987 ; Chang *et al*., 2002); 8*, C. terrigena* KCTC 2989^T (Chang *et al*., 2002).

Major fatty acids are shown in bold type.

Table 4.6 16S rRNA gene sequencing similarities with comparison to *Comamonas* type strains.

Type strains	Similarity (%)
Comamonas kerstersii LMG 3475^T	96.9
C. aquatica LMG 2370^T	96.8
C. koreensis YH12^T	96.4
C. testosteroni ATCC 11996^T	96.3
C. composti CC-YY287^T	96.2
C. terrigena LMG 1253^T	96.1
C. nitrativorans 23310^T	95.7
C. denitrificans 123^T	95.5
A7-5 NBRC106525^T	95.0
F1-6 NBRC106526^T	94.6

Table 4.7 DNA-DNA relatedness of *Comamonas testosteroni* KCTC 2990^T, isolate A7-5 and F1-6.

Strain	DNA-DNA hybridization (%)	
	***C. testosteroni* KCTC 2990^T**	**F1-6**
***C. testosteroni* KCTC 2990^T**	100	37.7
F1-6	41.6	100
A7-5	13.6	35.1

CHAPTER V

CONCLUSION

Highly arsenic resistant bacteria (27 isolates), which had a minimum inhibitory concentrations (MICs) for arsenite and arsenate of $\geq$40 mM and >400 mM, respectively, were isolated from tannery wastes and agricultural soils collected in Central Thailand. On the basis of the morphological, cultural, physiological and biochemical characteristics; principal ubiquinone type and 16S rRNA gene sequence analyses, they were identified as following: nine isolates each of *Klebsiella* (Groups 1 and 8) and *Acinetobacter* (Groups 2, 3 and 7), four isolates each of *Pseudomonas* (Groups 4 and 6) and *Comamonas* (Group 5), and one isolate of *Enterobacter* (Group 9)**.** Among these 27 isolates, only one isolate, *Comamonas* A3-3, appeared to have bioremediation potential due to capability of oxidizing arsenite to arsenate, as determined by silver nitrate staining method (Diliana *et al*., 2004) and arsenite agar plate (Lett *et al.*, 2001). XANES spectroscopic method was used for analysis of arsenic speciation presented in the culture broth after growing *Comamonas* A3-3 in arsenite containing medium. The XANES results confirmed arsenite oxidizing capability of *Comamonas* A3-3. The arsenite oxidizing efficiency was found to be 65.3% as determined by modified arsenite-iodine titration method. Arsenite oxidizing ability shown by *Comamonas* A3-3 indicates its potential application in biological treatment of wastewaters contaminated with arsenic.

The arsenite oxidizing isolate A3-3, was isolated from agricultural soil and was identified as *Comamonas* by polyphasic study. It had 16S rRNA sequences 96.9% similarity to *Comamonas kerstersii* LMG 3475^T. *Comamonas* A3-3 could be clearly distinguished from *Comamonas kerstersii* LMG 3475^T and other related *Comamonas* species based on its physiological and biochemical characteristics as well as its phylogenetic position. Therefore, the *Comamonas* A3-3 represented a novel species of genus *Comamonas*, for which the name *Comamonas terra* sp. nov is proposed. The type strain is A3-3 (NBRC106524^T).

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APPENDICES

APPENDIX A

CULTURE MEDIA AND REAGENT FOR IDENTIFICATION

All media were dispensed and steriled in autoclave for 15 min at 15 pounds pressure (121ºC) except for an acid from carbon sources test which was sterilized at 10 pounds for (110ºC) 10 min. Sodium arsenite ($NaAsO_2$; Merck, Germany) and sodium arsenate ($Na_2HAsO_4.7H_2O$; Fluka, Spain) were filter steriled.

1. Chemically Defined liquid Medium (CDM)

Sodium citrate	0.5	g
Casamino acid	0.05	g
$NaNH_4HPO_4$	3	g
KH_2PO_4	1	g
$MgSO_4.7\ H_2O$	0.4	g
Agar (for agar plate)	20	g
Distilled water	1	l
Adjust pH to 7.0, autoclaved and mixed with Vit I and II solution before pour plate		
Vitamin I solution	10	ml
(pyridoxal HCl 0.25%, thiamine 1%, calcium pantothenate 1%, riboflavin 1%, niacin 1%, *p*-aminobenzoate 0.5%, pyridoxine HCl 2%, vitamin B12 0.002%)		
Vitamin II solution	2	ml
(folic acid 0.0125%, biotin 0.00025%)		

2. R2A medium

Proteose peptone	0.5	g
Yeast extract	0.5	g
Casamino acids	0.5	g
Tris buffer	0.5	g

Dextrose	0.5	g
Soluble starch	0.5	g
Sodium pyruvate	0.3	g
KH_2PO_4	0.3	g
$MgSO_4$. 7 H_2O	0.05	g
Agar	1.5	g
Distilled water	1	l
Dissolved and adjusted pH to 7.0		

3. TYEG broth

Tryptone	1	g
Yeast extract	3	g
Glucose	1	g
Distilled water	1	l
Dissolved and adjusted pH to 7.0		

4. Mineral basal medium for utilization test (MB)

Utilization of various compounds (0.2 g/l) as sole carbon and energy sources were tested in a mineral liquid medium containing:

NH_4Cl	0.5	g
Na_2HPO_4.12 H_20	3.58	g
$CaCl_2$	0.002	g
KH_2PO_4	0.98	g
$MgSO_4$. $7H_20$	0.03	g
KCl	0.75	g
$FeSO_4.7H_20$	0.0006	g
Distilled water	1	l
Dissolved and adjusted pH to 7.0		

5. Aesculin broth

Aesculin	1	g
Ferric citrate	0.5	g
NaCl	100	g
Peptone water	1	l
Dissolved and adjusted pH to 7.4		

6. Gelatin agar

TYEG medium	100	ml
Gelatin	15	g
Dissolved and adjusted pH to 7.2		

7. Starch agar

TYEG agar medium	100	ml
Soluble starch	0.1	g
Dissolved and adjusted pH to 7.2		

8. Tween 80 agar medium

TYEG agar medium	100	ml
Tween 80	1	ml
$CaCl_2$	0.2	ml
Dissolved and adjusted pH to 7.2		

9. Deoxyribonuclease (DNase) media

Difco™DNase test agar	42	g
Distilled water	1	l
Adjusted pH to 7.3 ± 0.2 and heated to boiling to dissolve completely		

10. Peptone water (for indole test)

Peptone	1	g
Distilled water	1	l
Adjusted pH to 7.2		

11. Nitrate broth

Difco™Nitrate broth base	10	g
Distilled water	1	l
Dissolved and adjusted pH to 7.2		

12. MR-VP medium

Difco™MR-VP broth base	10	g
Distilled water	1	l
Dissolved and adjusted pH to 7.2		

13. Urea medium

BBL™ Urea Agar Base (steriled by filtration)	29	g
Agar	15	g
Distilled water	1	l
Dissolved and adjusted pH to 6.8 $\pm$ 0.2		

14. Simmon's citrate medium

BBL™ Simmons Citrate Agar	24.2	g
Distilled water	1	l
Dissolved and adjusted pH to 7.2		

15. OF medium

Difco™ OF Basal Medium	9.4	g
Distilled water	1	l
Carbohydrate	1 0	g
Dissolved and adjusted pH to 7.2		

16. Triple Sugar Iron Agar (TSI medium) for H_2S production

Difco™ Triple Sugar Iron Agar	65	g
Distilled water	1	l
Dissolved and adjusted pH to 7.2		

17. Gram Stain

Crystal Violet: Dissolved 2 g of crystal violet in 20 ml of 95% ethanol. Mix this solution with 80 ml of 1% Ammonium Oxalate solution. Standed for 24 h and filtered.

Gram Iodine: Dissolved 1 g of Iodine and 3 g of Potassium iodide in 300 ml distilled water. Stored in an amber bottle.

Decolorizer: 95% Ethyl Alcohol

Safranin: Dissolved 2.5 g of safranin in 10 ml of 95% ethanol, mixed with 100 ml of distilled water

18. Flagella staining

Basic fuchisin	0.5	g
Tannic acid	0.2	g
Aluminium sulfate	0.5	g

Dissolved the chemical compounds with 2.0 ml of 95% ethanol, 0.5 ml of glycerol, and 7.5 ml of tris(hydroxymethyl)aminomethane(tris)buffer.

19. Kovacs'reagent

ρ-dimethylaminobenzaldehyde	5	g
Amyl alcohol	75	g
Conc. HCl	25	ml

Dissolved the aldehyde in the alcohol by gently warming in a water bath (about 50-55ºC). Cooled and then added the acid with care. Protected from light and stored at 4°C.

20. Nitrate test reagent

Solution A: 0.33% sulphanilic acid in 5 N- acetic acid, dissolved by gentle heating.

Solution B: 0.6% dimethyl-α-napthylaminein 5 N-acetic acid, dissolved by gentle heating.

21. MR test reagent

methyl red solution: Dissolved 0.1 g of methyl red in 300.0 ml of 95% ethyl alcohol and adjusted final volumn to 500 ml by adding distilled water.

22. VP test reagent

Barritt reagent (A): Dissolved 5% alpha-naphthol solution in absolute ethanol.

Barritt reagent (B): Dissolved 40% potassium hydroxide (KOH) in distilled water.

APPENDIX B

REAGENTS FOR CHEMOTAXONOMIC CHARACTERIZATION

1. Cellular fatty acid analysis

1.1 Reagent 1 (Saponification reagent)

Sodium hydroxide	15	g
MeOH (HPLC grade)	50	ml
Mili-Q water	50	ml

Dissolved NaOH pellets in Mili-Q water then added MeOH

1.2 Reagent 2 (Methylation reagent)

6 N HCl	65	ml
MeOH (HPLC grade)	55	ml

Adjusted pH to below 1.5

1.3 Reagent 3 (Extraction solvent)

n-Hexane (HPLC grade or n-Hexane 1000)	50	ml
Methyl-tert-Butyl Ether (HPLC grade)	50	ml

1.4 Reagent 4 (Base wash reagent)

Sodium hydroxide	1.2	g
Milli-Q water	100	ml

Dissolved NaOH in water

1.5 Reagent 5

Saturated sodium chloride

2. Quinone analysis

2.1 Extraction of quinine: Chloroform : methanol (2:1, v/v)

2.2 Reverse plate thin-layer chromatography: Acetone : acetonitrile (80:20, v/v) for ubiquinone

2.3 HPLC: Elution sovent

Methanol : isopropyl ether (4:1, v/v)

APPENDIX C

REAGENTS FOR DNA EXTRACTION AND PURIFICATION, DNA BASE COMPOSITION, DNA-DNA HYBRIDIZATION AND 16S rRNA SEQUENCING

1. DNA extraction and DNA base composition

1.1 Saline –EDTA (0.15 M NaCl + 0.1 M EDTA)

NaCl	8.76	g
EDTA	37.22	g

NaCl and EDTA were dissolved in 1 l ultra pure water and adjusted the pH 8.0 by adding 5 N HCl and steriled by autoclaving at 121ºC, 15 pounds/inch pressure,for 15 min.

1.2 10% (W/V) SDS

Sodium dodecyl sulphate	10	g
Distilled water	90	ml

Dissolved and made up to 100 ml with distilled water.

1.3 Phenol: Chloroform (1:1, v/v)

Crystalline phenol was liquidified in water bath at 65°C and mixed with chloroform in the ratio of 1:1 (v/v). The solution was stored in a light tight bottle.

1.4 20 x SSC (20 x standard saline citrate)

NaCl	17.5	g
Sodium citrate	8.8	g
Distilled water	1	l

Dissolved and adjusted pH to 7.0 then steriled by autoclaveing at 121ºC, 15 pounds / inch 2 pressure, for 15 min. Note: To prepare 0.1× SSC and 0.2× SSC, the 20× SSC were diluted at 200 and 100 times, respectively before used.

1.5 RNase A solution

RNase A	20	mg
0.15 M NaCl	10	ml

Dissolved 20 mg of RNase A in 10 ml of 0.15 M NaCl and heated at 95°C for 5-10 min. Kept at -20°C.

1.6 0.1 M Tris-HCl (pH 7.5)

Tris (hydroxymethyl)aminomethane	1.21	g
Distilled water	90	ml

Dissolved and adjusted pH to 7.5 by adding 0.1 N HCl. Made up to volumn 100 ml by distilled water.

1.7 RNase T1 solution

RNase T1	80	ul
0.1 M Tris-HCl (pH 7.5)	10	ml

Mixed 80 µl of RNase T1 in 10 ml of 0.1 M Tris-HCl (pH 7.5) and heated at 95°C for 5 min. Kept at -20°C.

1.8 40 mM CH3COONa + 12 mM ZnSO4 (pH 5.3)

CH_3COONa	3.28	g
$ZnSO_4$	1.94	g
Distilled water	90	ml

Dissolved and adjusted pH to 5.3 by adding 0.1 N HCl or 0.1 N NaOH. Made up volumn to 100 ml by distilled water.

1.9 Nuclease P1 solution

Nuclease P1	0.1	mg
40 mM CH_3COONa + 12 mM $ZnSO_4$ (pH 5.3)	1	ml

Dissolved and stored at 4°C.

1.10 Alkaline phosphatase solution

Alkaline phosphatase	2.4	units
0.1 M Tris-HCl (pH 8.1)	1	ml

2. DNA-DNA hybridization

2.1 Phosphate-buffer saline (PBS)

NaCl	8.00	g
KCl	0.20	g
KH_2PO_4	0.12	g
Na_2HPO_4(anhydrous)	0.91	g
Distilled water	1	l

Dissolved then steriled by autoclaving at 121ºC, 15 pounds/inch 2 pressure, for 15 min.

2.2 20 x SSC (20 x standard saline citrate)

NaCl	17.5	g
Sodium citrate	8.8	g
Distilled water	1	l

Dissolved and adjusted pH to 7.0 then steriled by autoclaving at 121ºC 15 pounds / inch 2 pressure, for 15 min.

2.3 100 x Denhardt solution

Bovine serum albmin (Fraction V)	2	g
Polyvinylpyrrolidone	2	g
Ficoll400	2	ml

Dissolved in 100 ml ultra pure water and stored at 4ºC until used.

2.4 Salmon sperm

Salmon sperm DNA	10	mg/ml

Dissolved in 10 mM Tris + EDTA buffer pH 7.6 volume 1 ml, boiled for 10 min and then immediately cooled in ice water. Sonicated salmon sperm DNA solution for 3 min and stored at 4ºC until use.

2.5 Prehybridization solution

100x Denhardt solution	2	ml
10 mg/ml Salmon sperm DNA	1	ml
20x SSC	10	ml
Formamide	50	ml
Distilled water	34	ml

Dissolved a ingredients in ultra pure water and steriled by autoclaving at 121ºC,15 pounds / inch 2 pressure for 15 min and kept at 4ºC.

2.6 Hybridization solution

Prehybridization	100	ml
Dextran sulfate	5	g

Dissolved dextran sulfate in Prehybridization solution and kept at 4ºC.

2.7 Solution 1

Bovine serum albumin (Fraction V)	0.25	g
Triton X – 100	50	µl
PBS	50	ml

Mixed all of the ingredients and kept at 4ºC

2.8 Solution 2

Strepavidin–POD conjugate	1	µl
Solution1	4	ml

Dissolved Strepavidin-POD conjugate in solution 1 before used (freshly prepared).

2.9 Solution 3

3,3',5,5' Tetramenthylbenzidine (TMB)		
(10 mg/ml in DMFO)	100	ml
0.3% H_2O_2	100	ml
0.1 M citric acid + 0.2 M Na_2HPO_4 buffer pH 6.2		
in 10% DMFO	5	ml

Mixed all of the ingredients before use (freshly prepared).

3. 16S rRNA gene sequence analysis

3.1 Primers for 16S rRNA amplification and sequencing

3.1.1 Forward primer

20F; 5'-AGAGTTTGATCCTGGCTC-3'

3.1.2 Reverse primers

357R; 5'-CTGCTGCCTCCCGTAG-3'

802R; 5'-TACCAGGGT ATCTAATCCC-3'

1100R; 5'-AGGGTTGCGCTCGTTG-3'

1495R; 5'-CTACGGCTACCTTGTTA-3'

1541R; 5'-AAGGA GGTGATCCAGCC-3'

4. Arsenite oxidase and arsenite tranporter genes detection

4.1 Primer for arsenite oxidase genes

*aoxB*M*1*-2F; 5'-CCACTTCTGCATCGTGGGNTG YGGN TA-3′

*aoxB*M*3*-2R; 5′-TGTCGTTGCCCCAGATGADNCCYTTYTC-3′

aroB-1F; 5'-GTSGGBTGYGGMTAYCABGYCTA-3'

aroB-1R; 5'-TTGTASGCBGGNCGR TTRTGRAT-3'

4.2 Primer for arsenite tranporter genes

darsB-1F; 5′-GGTGTGGAACATCGTCTGGAAYGCNAC-3′

darsB-1R; 5′-CAG GCCGTACACCACCAGRTACATNCC-3′

APPENDIX D

STANDARD ASSAY METHODS

I. Modified method of arsenite-iodine titration

1. Determination of arsenite in culture medium broth

Arsenite was determined by modified method of standardization of arsenite-iodine titration (Sherren, 2001) as described by chemical equations:

$$Na_3AsO_3 + I_2 + H_2O \leftrightarrow Na_3AsO_4 + 2I^- + 2H^+ \quad (1)$$

$$I_2 + \text{starch} \rightarrow \text{Blue colour} \quad (2)$$

2. Reagents

A: 2.5 % starch solution

B: Gram stain iodine solution

C: 50 mM sodium arsenite solution

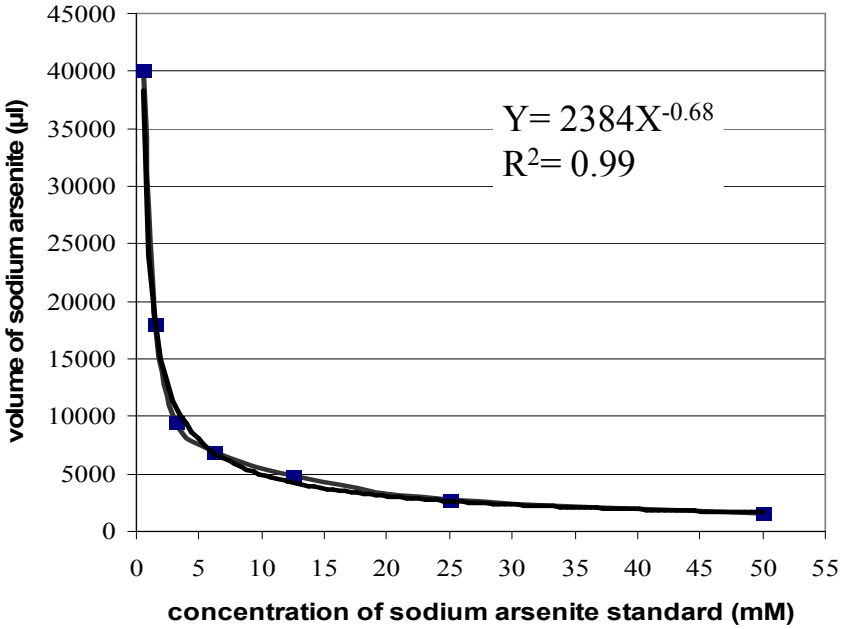
D: 2xTYEG medium, pH 7.0

3. Procedure and preparation of arsenite standard curve

3.1. Diluted 50 mM sodium arsenite solution to 25, 12.5, 6.25, 3.1, 1.5 and 0.5 mM with 2xTYEG medium, pH 7.0, for using as standard sodium arsenite solution.

3.2. Pipetted 0.5 ml reagent B into test tube, add 0.5 ml of Reagent A into the test tube and vortexed immediately.

3.3 Each tube (3.2) were titrated with standard sodium arsenite solution (3.1).Untill the solutions reached an end point, blue color change to colorless (5 replicate measurements). Recorded the volume of sodium arsenite used. An arsenite-iodine titration standard curve was shown in figure below.



II. XANES spectroscopy method

1. Measurements and experimental

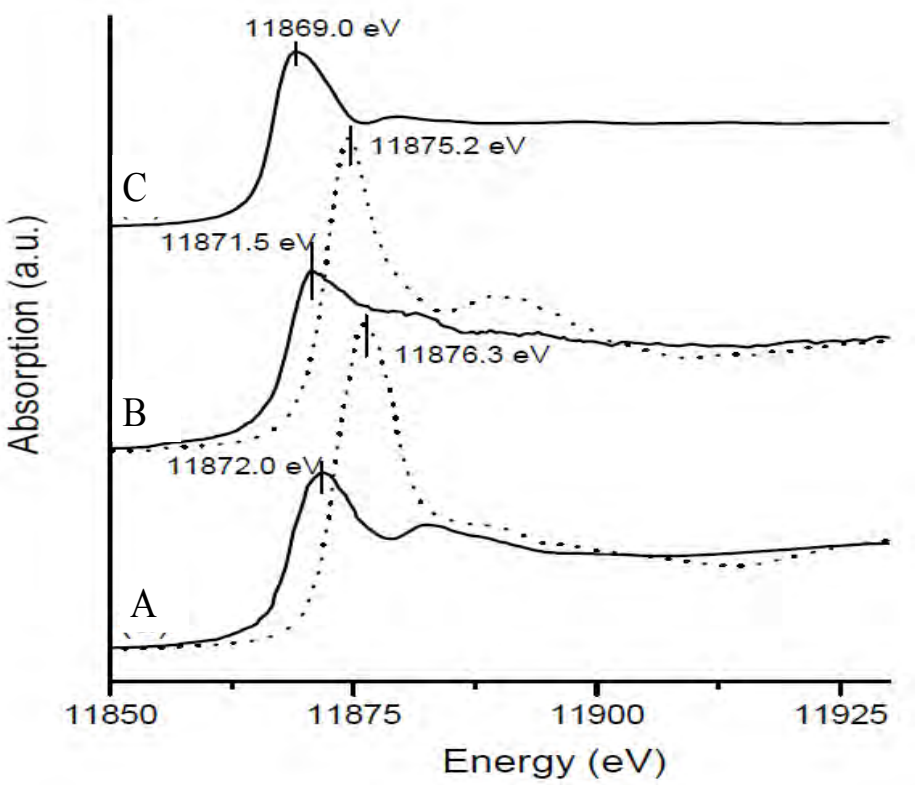
XANES spectra were recorded at the Double Crystal Monochromator beamline of the Center for Advanced Microstructures & Devices (CAMD), Louisiana State University, Baton Rouge, LA (Hormes *et al*., 2006). CAMD's storage ring was operated at energy of 1.3 GeV with electron currents between 200 and 80 mA. The monochromatic flux rate per second on the sample was about 5 x 10^8 photons (at 200 mA). The synchrotron radiation was monochromatized by a modified Lemonnier type double crystal X-ray monochromator equipped with Ge (422) crystals (Lemonnier *et al*., 1978). Measurements of reference compounds were performed twice in transmission mode with ionisation chambers (300 mbar argon pressure inside), measuring the beam intensities in front of and behind the sample. Measurements of samples (bacterial media) were performed twice in fluorescence mode using a Vortex^{TM} silicon drift fluorescence detector (SII Nano Technology Inc., USA) (sample chamber filled with 300 mbar argon); three independent samples of each bacterial medium was measured. Measurements of the bacterial media were performed according to Franz *et al*. (2007). For energy calibration of the spectra, the

spectrum of elemental arsenic was used as a "secondary standard" setting the first maximum of the derivative of the spectrum to energy of 11,867.00 eV. According to the step width, this value is reproducible to ±0.5 eV. Spectra were scanned with step widths of 2 eV in the pre-edge region between 11,800-11,855 eV, 0.5 eV between 11,855-11,900 eV, the main region of interest, and 1 eV between 11,900-12,050 eV with an integration time of 1 s per point. Using the ORIGIN program (ORIGIN Lab Corporation, Northampton, MA 01060, USA), a linear background determined in the pre-edge region was subtracted from the raw data to correct the spectra from contributions of higher shells and from supporting materials. Spectra were normalized at 11,930 eV. Fluorescence excitation spectra of the bacterial samples have been smoothed using the FFT-smoothing routine (5 point-setting) which is part of the ORIGIN program.

2. Analysis of reference arsenic compounds

Elemental arsenic, sodium arsenite, sodium arsenate, arsenic(III)oxide and arsenic(V)oxide were used as reference compounds. These compounds were of reagent grade, purchased from Sigma-Aldrich (Munich, Germany) and Alfa Aesar (Karlsruhe, Germany) respectively, used as received. The reference compounds were ground into fine powder, placed homogeneously on an arsenic-free, self-adhesive kapton® film (type 7010, CWC Klebetechnik, Frankenthal, Germany) and measured directly.

The Arsenic K-edge XANES spectra of the reference compounds were shown in figure below.



Analysis of reference arsenic compounds using XANES spectroscopy method: A, arsenic(III)oxide (solid line) and arsenic(V)oxide (dotted line); B, sodium arsenite (solid line), sodium arsenate (dotted line); C, elemental arsenic, a.u.; arbitrary units.

APPENDIX E

16S rRNA SEQUENCES OF REPRESENTATIVE STRAINS FIGURES OF 16S rRNA GENE ON AGAROSE GEL AND FIGURES OF ARSENITE OXIDIZING TEST

I. 16S rRNA gene sequences of representative strains

1. *Acinetobacter* sp.W8-2 (GQ497249, 1491 bp)

TCCAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACAT
GCAAGTCGAGCGGAGCGAGGGTGCTTGCACCTTAGCTTAGCGGCGGACGG
GTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATTCCGAAA
GGAATGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGG
ACCTTGCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTA
AAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGGACAATGGGCGGAAGCCTGATCCCAGCCATGCCGCGTGTGTG
AAGAAGACCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACCTC
AGATTAATACTCAAGGATAGTGGACGTTACTCGCAGAATAAGCACCGGCT
AACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGG
ATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTTTTAAGTCGGATGTGAA
ATCCCCGAGCTTAACCTGGGAATTGCATTCGATACTGGGAAGCTAGAGTA
TGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATC
TGGAGGAATACCGATGGCGAAGGCAGCCATCTGCGCCTAATACTGACCCT
GAGGTACGGAAAGCATCCCCCCCCCGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGG
CTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTC
GCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGGAGG
AAGCACGTGGGTTTAAATTCGATGCAACCCCGAAGAACCTTACCTGGTCT
TGACATACAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTG

ATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTACGTGAGATGTCCCTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTATTTGCCAGCACTTCGG
GTGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGA
CGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACA
ATGGTCGGTACAAAGGGTTGCTACCTCGCGAGAGGATGCTAATCTCAAAA
AGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGG
AATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGC
CTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGTAG
CTAGCCTAACTGCAAAGAGGGCGGTACCACGGTTACCCGTCT

2. *Acinetobacter* sp. W9-3 (GQ497238, 1499 bp)

TAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGC
AAGTCGAGCGGAGGGAGGGTGCTTGCTCCTTAGCTTAGCGGCGGACGGGT
GAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATTCCGAAAGG
GATGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGAC
CTTGCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAA
GGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGGACAATGGGCGGAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTGGAT
TTATATTATTTAGCAATAGTGGACGTTACTCGCAGAAATAATGCTACCGGC
TAATCTCTGTGCCAGGCAGCCCGCGGGTAATATACATGAGGGTGTGAGAG
CGTTTAATCCGGATTTTACTGGGCGTAAAAGCGTGCGTAGGCGGCTTTTTA
AGTCGCACTGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTG
GGAAGCTAGAGTATGGGAGAGGATGGTAGAACTCCCAGGTGTAGCGGCG
AAATGCGTATGAGATACTGGAGGAATACCGATGGCGAAGGCAGCCATCTG
GCCCTTAAATACTGACGCTGACGTACGAAAGCATGGGGAGCAAACAGGA
CTTAGATTACCCCGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGG
GGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGG
GGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCA
CAAGCGGGGGGAGAAATGTGGTTTAATTCGATGCAAACGCAGAAAGAAC
CTTAACCTGGTCTTGACATACCAGAGAACTTTCCAGAGATGGATTGGTGCC

TTCGGGAACTCTGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTAGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTATTTGCC
AGCACTTCGGGTGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGA
AGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACA
CGTGCTACAATGGTCGGTACAAAGGGTTGCTACCTCGCGAGAGGGTGCTA
ATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCA
TGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACG
TTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACC
AGAAGTAGCTAGCCTAACTGCAAAGAGGGCGGTACCACGGTTACCG

3. *Acinetobacter* sp. SL5-1 (GQ497240, 1539 bp)

TAAAAGGTTGGGGGTTGGGGGGAAAAAATGCATGGTAAATTTTTAATTTT
TTTTATGCAGAGTTTAATCATGGCTAAGATTGAACAGCGGCGACACTCTTA
ACACTAATTAAATCGTGTCCACAAGAGCTAAATTTACTACTAATCTCTTAG
CGTGGAGAGGGTAACTAATACTTAAGAATCACAATGTTGTTGGGGAGAAA
ATTTCAAAAGGAAATCTTAAATACCCATACTTCTTACGGGAGAAAGAAGG
AGATTTTTGGACCCTTGCTTAATAGAAGGACATGATTAGTTCGATTAGCTA
GTTCGGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCCGAGAG
GATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGGACAATGGGTGCAAGCCTGATCCAGCCATGCC
GCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGA
GGCTACTTTAGATAATACCTAGAGATAGTGGACGTTACTCGCAGAATAAG
CACCGGCTAACTCTGTGCCCAGCAGCCGCGGTAATACAGAGGGTGCAAGC
GTTAATCGGATTTACTGGGCGTAAAGCGCGCGTAGGCGGCTAATTAAGTC
AAATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTA
GCTAGAGTGTGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGC
GTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAACA
CTGACGCTGAGGTGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCATGCCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGG
CTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACCGGT
CGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGAC

ATAGTAAGAACTTTCCAGAGATGGATCGGTGCCCTCGGGAACTTACATAC
AGGTGCTGCACTGGCTGCCGTAAGCCCGTGTGCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTTTCCTTATTTGCCAGCGAGTAATGTCGG
GAACTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGAC
GTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGG
TCGGTACAAAGGGTTGCTACCTAGCGATAGGATGCTAATCTCAAAAAGCC
GATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATC
GCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGTAGCTAG
CCTAACTGCAAAGAGGGCGGTACCACGGTGGGCCGGAG

4. *Acinetobacter* sp. A4-6 (GQ503035, 1480 bp)

TGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGG
AGAGAGGTAGCTTGCTACTGATCTTAGCGGCGGACGGGTGAGTAATGCTT
AGGAATCTGCCTATTAGTGGGGGACCAACATTTCGAAAGGAATGCTAATA
CCGCATACGTCCTTACGGGAGAAAGCAGGGGATCTTCGGACCCTTGCGCT
AATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACC
AAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA
CAATGGGTGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGCCCTT
ATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGTTAATACCT
AGAGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCA
GCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGATTTACTGGGCG
TAAAGCGCGCGTAGGCGGCTAATTAAGTCAAATGTGAAATCCCCGAGCTT
AACTTGGGAATTGCATTCGATACTGGTTAGCTAGAGTGTGGGAGAGGATG
GTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC
GATGGCGAAGGCAGCCATCTGGCCTAACACTGACGCTGAGGTGCGAAAGC
ATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGAT
GTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGAT
AAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACG
CGAAGAACCTTACCTGGCCTTGACATAGTAAGAACTTTCCAGAGATGGAT

TGGTGCCTTCGGGAACTTACATAACAGGTGCTGCATGGCTGTTCGTGAACT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCT
TATTTGCCAGCGAGTAATGTCGGGAACTTTAAGGATACTGCCAGTGACAA
ACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGGCCAG
GGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCTACCTAGCGAT
AGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAAC
TCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGT
TTGTTGCACCAGAAGTAGCTAGCCTAACTGCAAAGAGGGCGGTTACCCGG
TGTGGCCGATGACTGGTTGGGAACGTAA

5. *Acinetobacter* sp. A7-6 (GQ497239, 1484 bp)

TAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGC
AAGTCGAGCGGAGAGAGGTAGCTTGCTACTGATCTTAGCGGCGGACGGGT
GAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATTTCGAAAGG
AATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGAC
CTTGCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAA
GGCCTACCAAGGCGACGATCTGTAACGGGTCTGAGAGGAAGGTCCGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGT
TAATACCTAGAGATAGTGGACGTTACTCGCAGAAATAAGCACCCGGCTAA
ACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGGTGCAAGCGTTAATCGG
ATTTACTGGGCGTAAAGCGCGCGTAGGCGGCTAATTAAGTCAAATGTGAA
ATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTAGCTAGAGTGT
GGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCT
GGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAACACTGACGCTGA
GGTGCGAAAGCATGGGGAGCCAAACAGGGATTAGATACCCTGGTAAGTTC
CATGCCCGTAAAACGAATGTCTACTTAGCCGTTGGGGCCTTTGAAGCCTTT
AGTTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCA
AGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGGTTTAATTCGATGCAACGCGAAGAACCTTACCCTGGCCTTGACATA

GTAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTTACATACAGG
TGCTGCATGGCTGTCGTCAGCTCGTGTCAGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTTTCCTTATTTGCCAGCGAGTAATGTCGGGAACT
TTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAA
GTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGT
ACAAAGGGTTGCTACCTAGCGATAGGATGCTAATCTCAAAAAGCCGATCG
TAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAG
TAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACAC
ACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGTAGCTAGCCTAA
CTGCAAAGAGGGCGGTACCACGGTGGACCCGA

6. *Pseudomonas* sp. A7-2 (GQ497241, 1063 bp)

TTAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATG
CAAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGG
TGAGTAATGCCTAGGAATCTGCCCTGGTAGTGGGGGACAACGTTTCGAAA
GGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGG
GCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTA
ATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAATC
ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGGACAATGGGTAAAGCCTGAATCCCAGCCATGCCGCGTGTGTG
AAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGCCAGTA
AGTTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAAC
TCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAAT
TACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGC
CCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTACGG
TAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGA
AGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCA
GCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAAC
TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT
TCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTC

CAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGCT
GTGTGGATCCGTGT

7. *Pseudomonas* sp. A6-4 (GQ497250, 1089 bp)

GTTAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACAT
GCAAGTCGAGCGGAATAGACCGGGGAGCCTTGCCTCCCCTTGATTCAAGC
GGCCGGAACGGGGTGAGTAATGCCCTAGGGAATTCTGCCCTGGGTAGTGG
GGGACAACGTTTCGAAAGGAACGCTTAATACCGCATACGTCCTAACGGGA
GAAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAAGATGAGCCTAGGTTC
GGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCGTAA
CTGGGTCTGAGAGGATGATGAGAACACACTGGAACTGAGACACGGTCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGTAAATGG
GATCCCCAGCCATGCCGCGTGTTGTGAAGAAGGTCTTCGGATTGTAAAGC
ACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGT
TACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATA
CAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGAGCGTAG
GTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGC
ATCCAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGT
GTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG
ACCACCTGGACTGATACTGACAATGAGGTGCGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCG
TTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCC
TGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTT
ACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCG
GGAACTCTGACACAGGTGCTGCATGGCTGTTTTGAATCGTGT

8. *Pseudomonas* sp. F2-2 (GQ497248, 1459 bp)

CGCCTGCAGAGTTTGATTCCTGGCTCAGACTGAACGCTGGCGGCACCCCT
AACACATGCAAGTCGAGCGGATGACGGGACCTTGCTCCTTGATTCAGCGG
CGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGT
TCCGAAAGGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGG

ATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGG
TGAGGTAAAGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATG
ATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATACCGCGT
GTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGCG
CAGTAAGTTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCG
GCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGT
GAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAG
AGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGA
TATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGAATACTG
ACACTGAGGTTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAG
TGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGG
TTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG
GTTTAATTCGAAGCAACGCGAAGAACCTTTCACCAGGCCTTGACATGCAA
AAGAACTTTTCCAGAGATGGATTGGTGCCTTTCGGAAACTCTGACACCAG
GGTGCTGCAATGGCTGTCGTCCAGCTCGTGTCGTGAGATGTTGGGGTTAA
GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTCATGGTGG
GCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGG
TCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACC
GATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATC
GCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAG
TCTAACCTTC

9. *Klebsiella* sp. A4-1 (GQ497246, 1596 bp)

CCGGGGTGGGACCTAAAAGATAATAGGTTTCCGGGATAAAGGGCGCAAG
CGGTTCGGGTTGAAACGGAGGGGTTTCGTGGCACACAGCCCAGGCTTAGA
GTATGAATGTCACTAAAGCGTAAATGATTGATACGCTGAGCGGCAGAGCC
TATGACAAATGCAACTCGATTGCGGTAGCACAGGGAGAAAAGGCTTGCTA

TCAGGTGATCCGAGTAGGCGGCAGGGTGGGTAAGTAGAGCGCACTGAGG
GGAGGTTCCAGGGAGGAAACGCGGAAACTCTAAATATCCCGCATAATGTC
GCAAGATCAAAGTGGGGCGACCGATTGGCCTCATGCCATCAGATGTGCCC
GAACGGAATGAGCTAGTAAGTGGGGTAACGAGCTCACCTAGGCGACGAT
CCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGT
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCA
AGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGAGTTGTAA
AGCACTTTCAGCGGGGAGGAAGGCGTTAAGGTTAATAACCTTGGCGATTG
ACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT
AATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACG
CAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAAC
TGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCA
GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAG
GCGGCCCCCTGGACAAAGACTGACGCTCAGATGCGAAAGCGTGGGGAGC
AAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTT
GAGAGGTTGTGCCCTTGAAGCGTGGCTTCCGGAGCTAACGCGTTAAATCG
ACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGA
ACCTTACCTGGTCTTGACATCCACAGAACTTACCAGAGATGGATTGGTGCC
TTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTG
AACACTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGC
CAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAG
GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACA
CACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAG
CGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT
CCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTG
CAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTGTGAT
C

10. *Klebsiella* sp. W8-1 (GQ497245, 1526 bp)

GTTAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACAT
GCAAGTCGAGCGGGTAAGCACAGAGAGCTTGCTTCTTCGGGTGAACGAAC
CGGCGGGACGGGGTGAGTAAATGTCTGGGAAAACCTGCCTGATGGAGGG
GGATAACTTACTGAAAAACGGGTAGCTAATACCGCATAACGTCGCAAGAC
CAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGA
TTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGG
TCTGAGAGGATGACCGAACCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATG
CAAGCCATGCCGCGTGTGTGAAGAAGGCCTTTCGGGTTGTAAAGCACTTT
CAGCGGGGGAGGAAGGCGTTAAAGGTTAATAACCTTGGCGATTGACGTTA
CCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACG
GAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGC
GGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCAT
TCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGT
AGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGC
CCCCTGGACAAAGACTGACGCAATCAGGTGCGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAAACGAATGTTCGGATTT
GGGAGGTTGTGCCCCTTGAGGCGGTGGGCTTTCCGGAGCTAACGCGTTAA
AATCGACCGCCTGGGGAGTACGGCCGCAAAGGTTAAAACTCCAAATGAAT
TGACGGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAA
CGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGG
ATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTGCAA
CTCCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAT
CCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGAT
AAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACC
AGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCG
AGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCA
ACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTA
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGA
GTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCA
CTTTGTGATTCATGACTGGGGTGAATCGTA

11. *Enterobacter* sp. A5-2 (GQ497247, 1492 bp)

TAGTCTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGC
AAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGG
ACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG
GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACC
TTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTG
GGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAC
CAAGGCCACCACTGGAACCTGAGGACACCGGTCCAAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATG
CCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTTCAGCGGGGA
GGAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGA
AGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA
GCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAG
TCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGC
AGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAAT
GCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAA
AGACTGACGCTCAGGTGCGAAAGGCGTGCCCGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCT
TGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTA
CGGCCGCAAGGTTAAAACTCAAATGAAATTGACCGGGGGCCCCGCACAA
AGCCGGGGGAGGCATGGGGTTTTAATTCGATGCAACGCGAAGAACCTTTA
CCTACTTCTTGACATCCCAGAGAAACTTAGCAGAGATGCTTTGGGGCCTTC
GGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGA
AATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAG
CGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAA
GGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACAC
GTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGG
ACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCAT
GAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAA
AGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTACCACTTGGATAC

12. *Comamonas* sp. A7-5 (GQ497242, 1491 bp)

TAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCA
AGTCGAACGGTAACAGCCACTTCGGATGCTGACGAGTGGCGAACGGGTGA
GTAATACATCGGAACGTGCCTAGTAGTGGGGGATAACTACTCGAAAGAGT
AGCTAATACCGCATGAGATCTACGGATGAAAGCAGGGGACCTTCGGGCCT
TGTGCTACTAGAGCGGCTGATGGCAGATTAGGTAGTTGGTGGGATAAAGG
CTTACCAAGCCTGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACA
CTAAAACTGAGTACAACGGCCCAGACCTTCCTACGGGAGGCAGCAGTGGG
GAATTTTAGGACAATGGGCGCAAACGCCTGATACAGTCCAATGCGACGTC
GGGTGCCTAGGAGGAAGAGCCCTCGGGTTGTAAACTGCTTCTGTACGGAC
ACGAAAAGCTCTGGGGCTAATATCCCCGGGTCATGACGGTACCGTAAGAA
TAAGCACCGGCTAAACTACGTGCCCAGCAGCCGCGGTAAATACGTAGGGG
TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTT
GTAAGACAGTGGTGAAATCCCCGGGCTCAACCTGGGAACTGCCATTGTGA
CTGCAAGGCTAGAGTGCGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGT
GAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTG
GGCCCTGCACTGACGCCTCATGCACGAAAACGTGGGGAATCAAACAAGG
ATTTAGATACCCTGGTTAGTTCCACGCCCTAAACGGATGTCAAACTGGTTT
GTTGGGTTCTTAACTAACTCAGTAACGAAAGCTAACGCGTGAAGTTGACC
GCCCTGGGGAGTACGGCCGCAAAGGTTGAAACTCAAAGGATTTGACGGG
GACCCGCACAAGCGGTGGATGATGTGGTTTAATTTCGATGCAACGCGAAA
AAACCTTACCCACCTTTGACATGGCAGGAACTTACCAGAGATGGTTTGGT
GCTCGAAAGAGAACCTGCACACAGGTGCTGCATGGCTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCATTA
GTTGCTACATTCAGTTGAGCACTCTAATGGGACTGCCGGTGACAAACCGG
AGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATAGGTGGGGCTA
CACACGTCATACAATGGCTGGTACAAAGGGTTGCCAACCCGCGAGGGGGA
GCTAATCCCATAAAGCCAGTCGTAGTCCGGATCGCAGTCTGCAACTCGAC
TGCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGTCACGGTGAA
TACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTC
TCGCCAGAAGTAGGTAGCCTAACCGCAAGGAGGGCGCTACCA

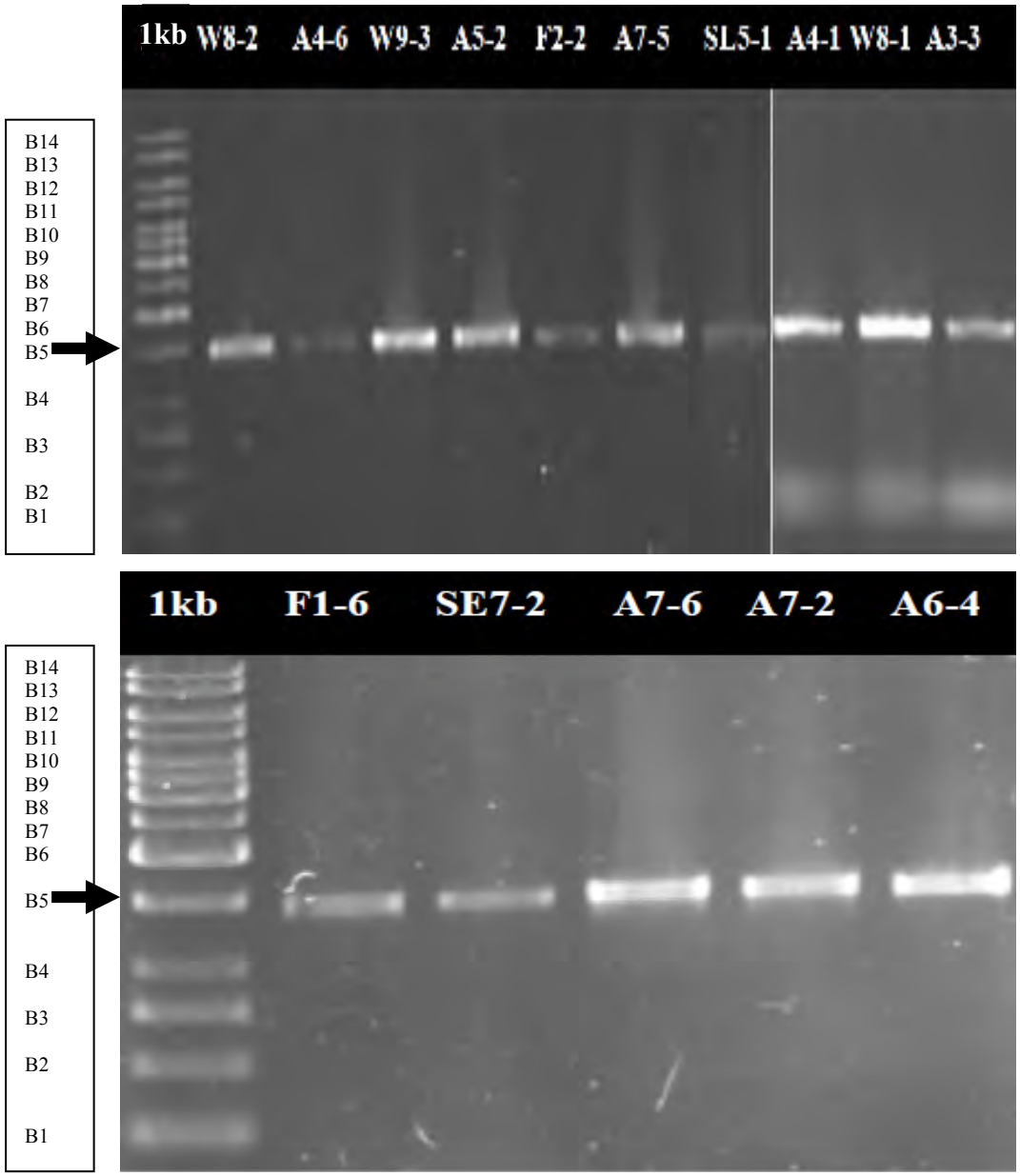
13. *Comamonas* sp. F1-6 (GQ497243, 1491 bp)

TAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCA
AGTCGAACGGTAACAGGTCTTCGGATGCTGACGAGTGGCGAACGGGTGAG
TAATACATCGGAACGTGCCTAGTAGTGGGGGATAACTACTCGAAAGAGTA
GCTAATACCGCATGAGATCTACGGATGAAAGCAGGGGACCTTCGGGCCTT
GTGCTACTAAGAAGCGCCTGATGGCCAGATTAGGGTAGGTGGTGGGGTAA
AAGGCTTTAACCAAGCCTGCGAATCTGTAAGCTGGTCCTGAAGAGGAACG
ACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCG
TGCAGGATGAAACCCCTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAA
GCCTGGGGCTAATATCCCCGGGTCATGACGGTACCGTAAGAATAAGCACC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAA
TCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGTGG
TGAAATCCCCGGGCTCAACCTGGGAACTGCCATTGTGACTGCAAGGCTAG
AGTGCGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAG
ATAAGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGCAGCT
GAGCGGCTGCATGGCACCCGAAAAAGACGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTGGTTGTTGGGTCTTA
ACTGACTCAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTAC
GGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGG
TGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCCACCTTTG
ACATGGCAGGAACTTACCAGAGATGGTTAGGTGCTCGAAAGAGAACCTGC
ACACAGGGTGCAGCATGGCTGTCGTCAGCTCGAGTCCGTGAGATGTTGGG
TTAAGTCCCCCAACGAGCGCCACCCTTGCCATTAGTTGCTACATTCAGTTG
AGCACTCTAATGGGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCCTCATGGCCCTTATAGGTGGGGCTACACACGTCATACAATG
GCTGGTACAAAGGGTTGCCAACCCGCGAGGGGGAGCTAATCCCATAAAGC
CAGTCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAAT
CGCTAGTAATCGTGGATCAGAATGTCACGGTGAATACGTTCCCGGGTCTT
GTACACACCGCCCGTCACACCATGGGAGCGGGTCTCGCCAGAAGTAGGTA
GCCTAACCGCAAGGAAGGCGCTACCACCGGCCCGGGTCCCAGC

14. *Comamonas* sp. A3-3 (GQ497244, 1487 bp)

TTAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGC
AAGTCGAACGGTAACAGCACTTTCGGTGGCTGACGAGTGGCGAAACGGGT
GAGTAATACATCGGAACGTGCCTAGTAGTGGGGGATAACTACTCGAAAGA
GTAGCTAATACCCCATGAGATCTAAGGATGAAAGCAGGGGACCTTCGGGC
CTTGCGCTACTAGAGCGGCTGATGGCAGATTAGGTAGTTGGTGGGATAAA
AGCTTACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATTTTGGACAATGGGCGCAAGCCTGATCCCAGCAATGCCGCGTGCAGGAT
GAAGGCCTTTCGGGTTGTAAACTGCTTTTGTACGGAAACGAAAAGCCCTC
TTCTAATACAGGAGGGTCATGACGGTACCGTAAGAATAAGCACCGGCTAA
CTACGTGCCAGCAGCCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGAGGTGAAA
TCCCCGGGCTCAACCTGGGAACTGCCTTTGTGACTGCAAGGCTAGAGTAC
GGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGC
GGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGTACTGACACTCA
TGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCCTAAACGATGTCAACTGGTTGTTGGGTCTTAATTGACTCAGTAACGAAG
CTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACT
CAAAGGAATTGACGGGGACCCGCCACAAGCGGTGGATGATGTGGTTTAAT
TCGATGCAACGCGAAAAACCTTACCCACCTTTGACATGGCAGGAATCCTT
TAGAGATAGAGGAGTGCTCGAAAGAGAACCTGCACACAGGTGCTGCATG
GCTGTCGTGCAAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCTTGCCATTAGTTGCTACGAAAGGGCACTCTAATGGGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCT
TATAGGTGGGGCTACACACGTCATACAATGGCTGGTACAAAGGGTTGCCA
ACCCGCGAGGGGGAGCTAATCCCATAAAGCCAGTCGTAGTCCGGATCGCA
GTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAG
AATGTCACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACAC
CATGGGAGCGGGTCTCGCCAGAAGTAGGTAGCCTAACCGCAAGGAGGGC
GCTTACCACGGCGGGGTTCGTGACTGGGGTGATCGTAA

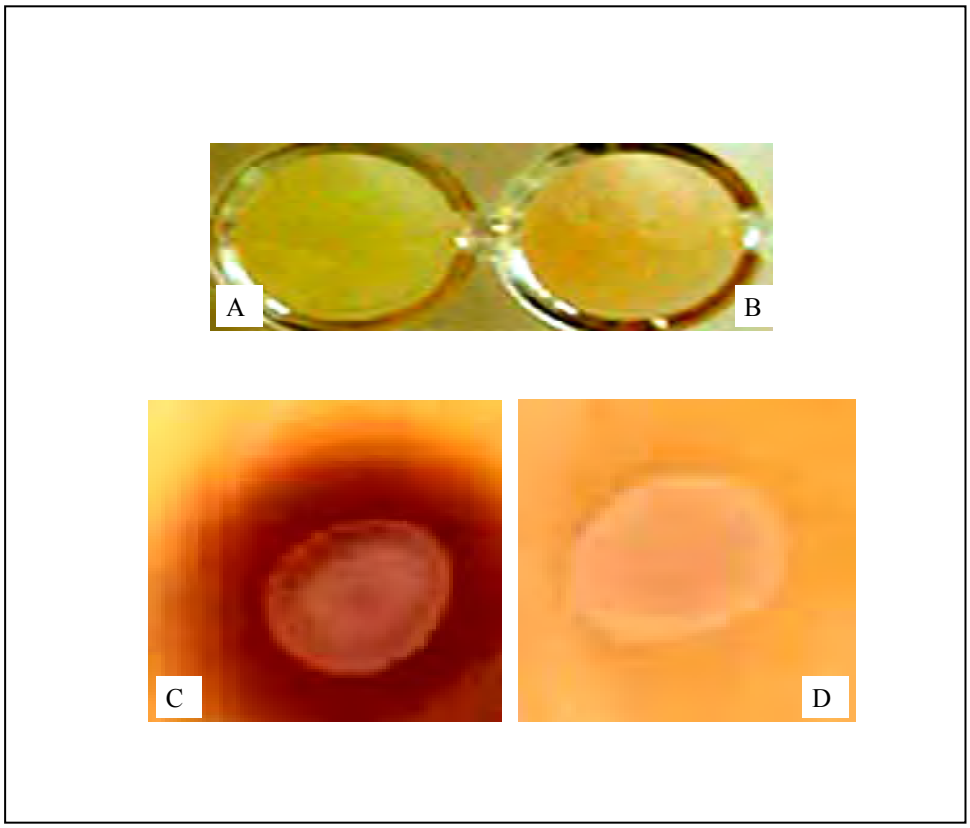
II. 16S rRNA gene on 1% agarose gel before purification by gel extraction method



Condition: 1% agarose gel electrophoresis

1KB DNA Ladder**:** B1(250bp), B2(500bp), B3(750bp), B4(1,000bp), **B5(1,500bp),** B6(2,000bp), B7(2,500bp), B8(3,000bp), B9(3,500bp), B10(4,000bp), B11(5,000bp), B12(6,000), B13(8,000bp) B14(10,000bp)

III. FIGURES OF ARSENITE OXIDIZING TEST



Microplate screening method (Diliana *et al*., 2004): Change of bright yellow colour of sodium arsenite (A) to brownish colour of sodium arsenate (B) after incubation and addition of silver nitrate indicated arsenite oxidation. Arsenite plate method (Lett *et al.*, 2001) after flooding with siver nitrate: Colony surrounded by a brownish zone indicated a likely arsenite oxidizing activity (C), colony without brownish zone indicated non arsenite oxidizing bacteria (D).

BIOGRAPHY

Family Name: Chitpirom **First Name: KITJA**

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Education Attainment

Year	Academic degree	Educational institute
1992/1996	B.Sc. (Agricultural), Animal Science	Prince of Songkla University THAILAND
2001/2006	B.P.H. (Occupational health and Safety)	Sukhothaithammathirat Open University THAILAND
2004/2005	Dip. in Medical Microbiology	Institute for Medical Research (IMR) MALAYSIA
2002/2004	M.S. (Sustainable Land Use and Natural Resource Management)	Kasetsart University THAILAND

Publications and Proceeding:

1. **Chitpirom K**., Akaracharanya A., Tanasupawat S., Leepipatpiboon N. and K. Kyoung-Woong. 2009. Isolation and Characterization of Arsenic Resistant Bacteria from Tannery Wastes and Agricultural Soils in Thailand. *Ann. Microbiol.* 59: 649-656.
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8. **Chitpirom K**., Charubhun N., Pitiyont B., Mungkung N.and Saksoog P.2006. Land Use Impacts on the Water Quality in the Upper Lam Phra Ploeng watersheds. *Proceeding of The 38th APACPH Conference 2006 & 2nd International Public Health Conference* Bangkok,Thailand,3-6 Dec.2006.