1.0 INTRODUCTION

Compounds with oxygen, sulphur, phosphorus and nitrogen substitutions in the aromatic ring are called heterocyclic compounds. The environmental/natural sources of heterocyclic compounds include combustion (coal, biomass, refuse, diesel fuel, tobacco), commercial coal tar and its leachates, fossil fuels, wood-preserving wastes, municipal solid wastes, bleaching of paper pulp, volcanic activities and forest fires, cigarette smoke and automobile emission (Bressler and Fedorak, 2000; Hiraishi, 2003; Lobastova *et al*., 2004). Anthropogenic heterocyclic aromatic compounds are used as industrial solvents, dyes, explosives, pharmaceuticals and pesticides (Hiraishi, 2003).

Interest in the biodegradation of heterocyclic compounds is prompted by the ubiquitous distribution of these pollutants in diverse environment, their persistence and their toxic, mutagenic and carcinogenic properties (Jensen *et al*., 2003; Lobastova *et al*., 2004). The presence of nitrogen and sulphur containing heterocycles in crude oil is a serious environmental problem. Combustion of crude oil during the refining process results in their oxidation to NO_x and SO_x, which react with water in the air, forming acid rain (Kirimura *et al*., 1999).

Hydrocarbon contamination reduces biodiversity of the soil microbiota (Atlas *et al*., 1991) and imposes selective pressure that only favour limited number of fast-growing hydrocarbon degraders (Bundy *et al*., 2002). The use of culture-independent techniques premised on 16S rRNA analyses to study microbial diversity in hydrocarbon-contaminated soils allows quantitative assessment of soil microbial community, reveals the presence of viable but not yet culturable hydrocarbon degrading bacteria and eliminate cultivation bias as the DNA template used is extracted directly from the contaminated soils (Kubicek *et al*., 2003; Jain *et al*., 2005).

1.1 BACKGROUND OF STUDY

Petroleum crude oil, a major source of energy in the world is arguably the world's most compositionally complex organic mixture in terms of chemically distinct constituents. Petroleum crude oil production and operations such as oil exploration, exploitation, transportation, and distribution with its attendant oil spillage, seepages from oil tankers, release of effluents, and offshore drilling activities is adversely affecting the ecosystems (terrestrial and aquatic) especially in oil producing countries including Nigeria (Atlas, 1991; Cerniglia, 1992). This problem further exacerbate with indiscriminate disposal of spent and used oils at automobile and mechanic workshops, unprecedented use of generators and the release of exhaust into the environment, oil pipelines sabotage and vandalization by oil thieves and restive communities, particularly in the Niger Delta region of Nigeria.

Crude oil is a complex mixture consisting of aliphatics, alicyclics, aromatic and heteroaromatic compounds (Benedik *et al*., 1998). Nitrogen-containing compounds in crude oil are divided into 'non-basic' (indoles and pyrroles) and the 'basic' (pyridines and quinolines) molecules (Nojiri and Omori, 2007). The total nitrogen content in crude oil averages about 0.3% of which the non-basic compounds constitute about 70-75% (Benedik *et al*., 1998). Carbazole is a non-basic nitrogen heterocycle found in coal-tar creosote, fossil fuel and wood-preserving wastes and is recalcitrant, mutagenic and toxic (Nojiri and Omori, 2002). Their combustion in petroleum leads to the formation of nitrogen oxides, emission of which lead to acid rain (Kirimura *et al*., 1999).

Interest in the study of bacterial degradation of carbazole is spurred partly because of the ubiquitous nature of the pollutant and its mutagenic and toxic activities and also because carbazole is a structural analogue of dioxins and carbazole degrading enzymes can partly function as dioxin-degrading enzymes (Nojiri and Omori, 2007). The metabolic propensity of wide array of microorganisms to utilize hydrocarbons such as carbazole as source of carbon and energy necessitate the development of bioremediation techniques

where catabolic genes and enzymes of microorganisms are employed for biodegradation of hydrocarbon pollutants.

Three major degradation pathways have been reported for carbazole, lateral dioxygenation at carbon positions 3 and 4; monohydroxylation at carbon positions 1, 2 and 3; and angular dioxygenation at carbon positions 1 and 9a (Ouchiyama *et al*., 1993; Grifoll *et al*., 1995; Lobastova *et al*., 2004). In contrast to other two carbazole degradation pathways that lead to production of dead end metabolites and hydroxylated carbazole intermediates, angular dioxygenation lead to complete carbazole mineralization, however, very few bacteria genera with this ability have been reported (Nojiri and Omori, 2002; Nojiri and Omori, 2007).

In hydrocarbon-contaminated soils, microbial community structures are influenced by a number of factors such as soil type, concentration and bioavailability of the contaminants, nutrient contents, temperature, oxygen content and pH (Margesin and Schinner, 2001; Greer *et al*., 2010). Soil microbial diversity assessment using culture-dependent method is fast, cost effective and provides information on the active, heterotrophic component of the population. However, culturable cells represents <1% of the total microbial community in an environment (Hugenholtz, 2002). The use of culture independent method to decipher the microbial diversity of hydrocarbon-contaminated environments provides clues about the type of bacteria able to adapt to such habitats. It also reveal the presence of novel bacteria genera hitherto not reported to contribute to natural attenuation of hydrocarbon pollutants and capture viable but not culturable (VBNC) bacterial groups, which though not captured by cultural methods, play important roles in the decontamination process (Jain *et al*., 2005; Spiegelman *et al*., 2005; Rastogi and Sani, 2011).

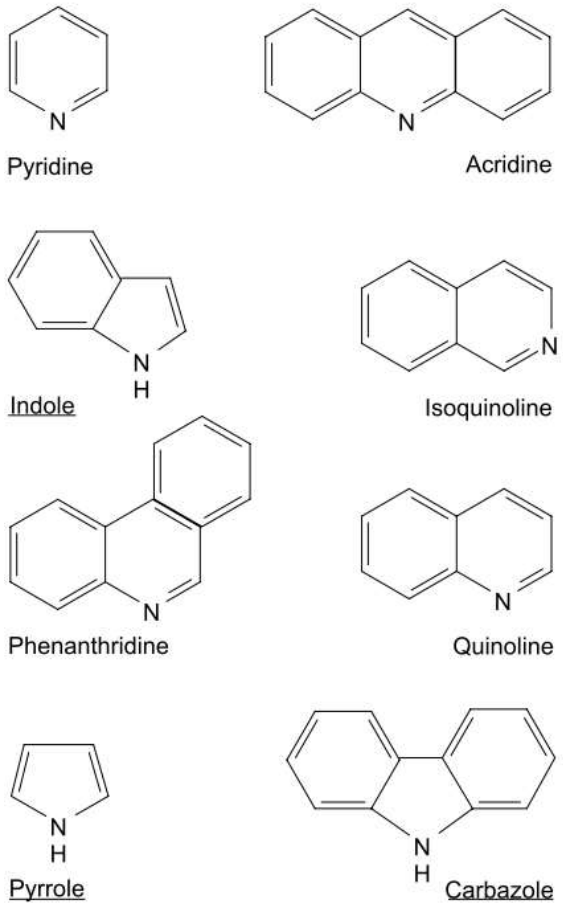


Figure 1.1: Common nitrogen heteroaromatic compounds found in fossil fuels. The nonbasic species are underlined (Benedik *et al*., 1998).

1.2 STATEMENT OF PROBLEM

Carbazole is a nitrogen heterocycle released into the environment with fossil fuels or their products such as creosote. It is recalcitrant, mutagenic and toxic (Nojiri and Omori, 2002). It is a model compound for the study of nitrogen-containing heterocyclic aromatic hydrocarbons. Hazardous carbazole derivatives such as N-methylcarbazole and 7-H-dibenzo[c,g]carbazole found in cigarette smoke and automobile emissions are genotoxic and carcinogenic and are categorized as "IARC Group 2B" carcinogens.

In spite of plethora of information on degraders of carbazole from temperate environment, there is virtually no report from African continent as a whole and Nigeria in

particular on the ability of autochthonous microorganisms to degrade carbazole. This is important as clean-up strategies are environment specific and local microorganisms with ability to degrade carbazole must be isolated and used as seed for bioremediation.

Owing to its relatively low mobility and weak sorption to soil and aquifer materials, there is ubiquitous distribution of carbazole in groundwater, aquatic sediments, seawater, industrial and urban air, subsoil region and bioaccumulation in marine organisms. This poses serious health and environmental concern due to the mutagenic and toxic properties of carbazole.

Carbazole degraders from different genera of bacteria have been isolated from diverse climes. Due to the physico-chemical properties of the Nigerian environment, which is quite different from the reported climes, it is imperative to isolate carbazole degraders that can cleave carbazole angularly resulting in its mineralization from our own environment and compare their metabolic potentials with carbazole degraders from temperate environments.

Soil contamination with petroleum hydrocarbons imposes significant changes to the structure and functions of the indigenous microflora and favours, over time, the dominance of fast hydrocarbon degraders that can utilize the pollutant as source of carbon and energy. Analysis of microbial diversity of these oil-impacted environments using culture-dependent method does not give a fair representation of the microbial richness of the site and could results in misleading assumptions and conclusions. However, culture-independent method premised on 16S rRNA analyses allows quantitative assessment of soil microbial community, reveals the presence of viable but non-culturable hydrocarbon degrading bacteria and overcome the limitation imposed by cultivation.

1.3 SIGNIFICANCE OF STUDY

Carbazole degraders have potential use for bioremediation of carbazole and dioxin-polluted sites. Because of the formation of carbazole through combustion of organic

materials in air and as a constituent of crude oil, pollution of the environment is a common occurrence. Thus, knowledge of its complete removal through microbial degradation is significant.

Carbazole is a structural analogue of dioxins and carbazole degrading enzymes can partly function as dioxin-degrading enzymes (Nojiri and Omori, 2007). Carbazole degraders obtained in this study can be employed to decontaminate and bioremediate environments polluted with carbazole and other dioxin-related compounds. This is significant due to ubiquitous nature of dioxins and their mutagenic and toxic activities.

The use of molecular techniques for detection and identification of novel degrading strains that cannot be captured using classical cultural methods is of paramount importance. Strains harboring novel degradative genes are identified and classified. This can increase the biodegradative gene pool for environmental restoration. Such genes can be purified and in liaison with Biochemist, Chemist and Biotechnologist can be seeded to polluted soil using appropriate bioremediation strategies.

1.4 AIM AND OBJECTIVES

General Objectives of Study

The aim of this study was to isolate and characterize carbazole-degrading bacterial strains from tropical hydrocarbon-contaminated soils; determine the extent of carbazole degradation by the isolates; and examine the bacterial diversity of one of the contaminated soils.

Specific Objectives of Study

1. To determine the physicochemical properties of selected hydrocarbon-contaminated soils.
2. To isolate and characterize carbazole-degrading bacteria from the hydrocarbon-contaminated soils.

3. To determine the degradative potential of the isolates on carbazole and identify key metabolite(s) produced by the isolates during carbazole biodegradation.
4. To determine the substrate specificity pattern of bacterial isolates.
5. To determine the degradative ability and efficiency of selected carbazole-degraders in soil microcosms.
6. To examine the bacterial diversity of a hydrocarbon-contaminated soil used in this study using clone library of ribosomal RNA sequences.

2.1 Carbazole: General Description

Carbazole ($C_{12}H_9N$, dibenzopyrrole diphenylenimine, CAS no. 86-74-8) is a non-basic tricyclic aromatic N-heteroatomic compound (Figure 2.1). It has a molecular weight of 167.21 g/mol, boiling and melting point of 355°C and 246°C (Lide, 2003), water solubility of 1.2 mg/l (Johansen *et al*., 1997), vapour pressure of 1 x 10^{-4} Pa (Peddinghaus *et al*., 2012), and octanol/water partition coefficient (log K_{ow}) of 3.72 (Blum *et al*., 2011). It is one of the π-excessive heterocycles (electron-rich rings) and is more recalcitrant than dibenzofuran but less than dibenzothiophene (Balaban *et al*., 2004). It is a white crystalline solid at ambient temperature. It sublimates, has a scent similar to creosote and exhibit strong fluorescence and long phosphorescence upon exposure to ultraviolet light (Collin and Höke, 1986). It is one of the major N-heterocyclic aromatic hydrocarbons in fossil fuels (coal, crude oil, oil derived from oil shales pyrolysis) and is also found in cigarette smoke and emitted from coal and wood combustion (Odabasi *et al*., 2006).

It is used as a chemical feedstock for the production of dyes, reagents, explosives, insecticides, lubricants and act as a colour inhibitor in detergents (Nojiri and Omori, 2007). It is also widely used as a model compound for the study of biodegradation of aromatic N-heterocyclic hydrocarbons (Xu *et al*., 2006). However, its release into the environment from diverse anthropogenic sources is of serious health and environmental concern, as carbazole is both mutagenic and toxic and classified as "benign tumorigen" (Smith and Hansch, 2000; Nojiri and Omori, 2007).

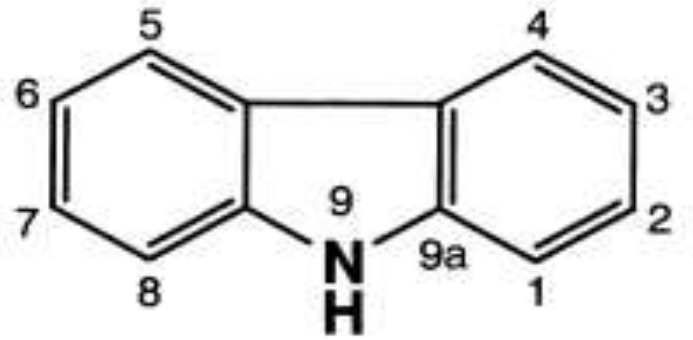


Figure 2.1: Molecular structure of carbazole (Nojiri *et al*., 2001a)

2.1.1 Solubility of carbazole

Heterocyclic aromatic compounds are known to exhibit higher polarity and water solubility due to substitution of one carbon atom by nitrogen, sulfur or oxygen (Meyer and Steinhart, 2000). These chemical properties lead to increase bioavailability and mobility as compared to the homologous polycyclic aromatic hydrocarbons resulting in various environmental influences of these compounds (Pearlman *et al*., 1984; Peddinghaus *et al*., 2012). Carbazole has an aqueous solubility at 25°C of 1.2 mg/l. It is less soluble than dibenzothiophene (1.5 mg/l) and dibenzofuran (4.8 mg/l) but more soluble than xanthene (1.0 mg/l) in spite of its higher molecular weight (Table 2.1). It is readily soluble in acetone and dimethyl sulfoxide, slightly soluble in ether and ethanol, and barely soluble in chloroform, acetic acid, carbon tetrachloride, and carbon disulfide (Collin and Höke, 1986).

Table 2.1: Properties of some Heterocyclic aromatic compounds

Group	Compound	Molecular Weight (g/mol)	Aqueous solubility at 25°C (mg/l)	Log K_{ow}
Nitrogen Heterocycles	Pyrrole	67.1	58,800	0.75
	Indole	117.0	1875	2.00
	Quinoline	129.2	6718	2.03
	Carbazole	167.2	1.2	3.72
	Acridine	179.2	46.6	3.48
	6-methylquinoline	143.2	631	2.57
Sulphur Heterocycles	Thiophene	84.1	3600	1.81
	1-Benzothiophene	134.2	130	3.12
	Dibenzothiophene	184.3	1.5	4.38
Oxygen Heterocycles	Benzofuran	118.1	678	2.67
	Dibenzofuran	168.2	4.8	4.12
	2-methylbenzofuran	132.2	160	3.22
	Xanthene	182.2	1.0	4.23

Source: Blum *et al*. (2011)

2.1.2 Aromaticity of carbazole

Aromaticity is a property of planar, cyclic, conjugated molecules that acts like unsaturated molecules and undergo substitution reaction rather than addition due to delocalization of electrons in the ring. It can also be considered a manifestation of cyclic delocalization and resonance (Balaban *et al*., 2004). The tendency to favour substitution rather than addition suggests that the parent unsaturated ring system has exceptional stability. Aromaticity cannot exist without conjugation (conjugation requires at least three overlapping *p* orbitals in the same plane). This is because aromatic molecules require

planarity and overlapping *p* orbitals so that electron can be delocalized for better quality. In the same vein, resonance exists because of electron delocalization and emerges in different patterns based on the structure and arrangement within a molecule. Resonance gives extra stability due to electron delocalization and can be conferred sometimes on a molecule due to cycling double bonds (Figure 2.2).

Aromaticity in a molecule is premised on possession of four specific qualities (Katritzky *et al*., 2010). These are (1) Stucture must be cyclic with conjugated Pi (π) bonds (2) each atom in the ring must have an unhybridized *p* orbital (3) all *p* orbitals must overlap continuously around the ring (planarity) and (4) 4n +2 π electrons (n is an integer: 0,1,2,3..) in cyclic conjugation are associated with each ring.

Aromatic heterocyclic compounds electronic structure is in agreement with Huckel's rule, which states that cyclic conjugated and planar systems having (4n + 2) π electrons are aromatic. The rings possess diamagnetic currents, react by substitution rather than addition, and bond orders and length tend to be intermediate between single and double (Balaban *et al*., 2004). Examples of these heterocycles are pyrrole, thiophene, and furan.

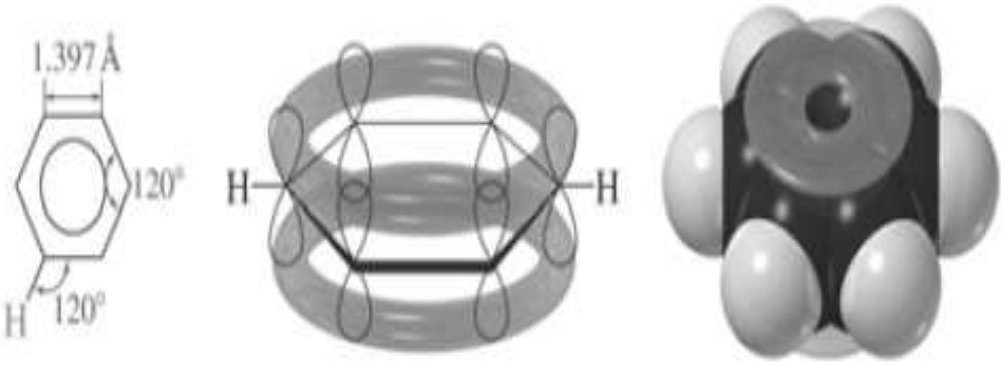
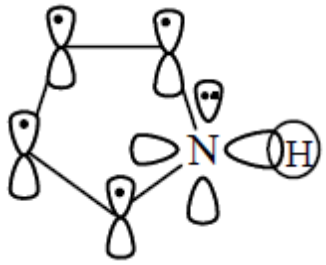


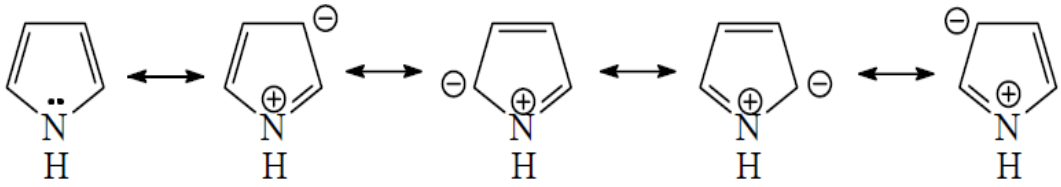
Figure 2.2: Resonance structure of benzene showing that the π electrons are evenly distributed around the atoms (delocalized) thus enhancing chemical stability (Wade Jr, 2006).

Carbazole (dibenzopyrrole) consist of two benzene rings fused together on either side of a pyrrole ring. Pyrrole is a five-membered ring in which the heteroatom has at least one pair of non-binding valence shell electrons. Hybridizing this heteroatom to a sp^2 state create a *p* orbital occupied by a pair of electrons and oriented parallel to the carbon *p*-orbitals resulting in a planar ring. The pi (π) system is occupied by six electrons. Four from two double bonds and two from the heteroatom. Hence, these five sp^2 hybridized atoms form planar six electrons delocalized π-cloud, which is responsible for the aromatic character of pyrrole (Figure 2.3a and Figure 2.3b).

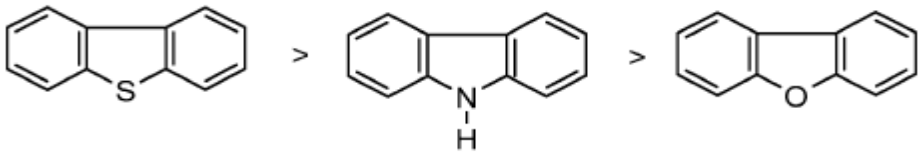
The resonance energies of pyrrole, thiophene, and furan are 5.3, 6.5 and 4.3 Kcal/mol which gives the order of aromaticity as thiophene > pyrrole > furan. In essence, carbazole is less aromatic than dibenzothiophene but more aromatic than dibenzofuran (Balaban *et al*., 2004) (Figure 2.3c).



(A)



(B)



I II III

(C)

Figure 2.3: Aromaticity of pyrrole ring and dibenzo- series. (A) Delocalized structure of pyrrole ring. (B) Resonance structure of pyrrole showing the donation of electrons by nitrogen to the ring and the electron richness of the ring system as six electrons is distributed over five atoms. (C) Aromaticity scales of dibenzothiophene (I), carbazole (II) and dibenzofuran (III) showing the order of aromaticity (Balaban *et al*., 2004).

2.2 Carbazole: Industrial and Medical Importance

Carbazoles are dominant as structural motifs in various synthetic materials and naturally occurring alkaloids. It exhibit material properties as optoelectronic materials, conducting polymers and synthetic dyes (Roy *et al*., 2012). Several dyes such as Hydron Blue^{TM}, Naphthol^{TM} dyes, anthraquinone vat dyes, styryl dyes, and dioxazine dyes are synthesized

from carbazole. Similarly, 1,3,6,8-tetranitro-carbazole (Nitrosan^{TM}) is used as an insecticide while reaction of carbazole with phenol and formaldehyde in the presence of acidic catalysts form Novalacs, which can be cured with hexamethylenetetramine to produce highly heat resistant polymers (Collin and Höke, 1986). Carbazole is also used to synthesize the monomer, N-vinylcarbazole, which can be polymerized to form polyvinyl carbazole (PVK) (Pearson and Stolka, 1981; Collin and Höke, 1986).

Naturally occurring carbazole are blessed with profound biological activities such as antitumor, psychotropic, anti-inflammatory, anti-histaminic, antibiotic and antioxidative activities (Figure 2.4) (Lobastova *et al*., 2004; Roy *et al*., 2012). The structural features of such carbazole-based natural products are the presence of nuclear hydroxyl groups (major structural feature), quinine functionality and prenyl groups (Roy *et al*., 2012). In pharmaceutical industry, hydroxylated carbazole derivatives are value-added substances exhibiting strong antioxidant activity and widely used in the treatment of encephalopathy, cardiopathy, hepatopathy and arteriosclerosis (Seto, 1991). Furthermore, carbazole moiety is considered as one of the pharmacophores in the cardiovascular pharmaceuticals carvedilol and carazolol, which are used in the treatment of hypertension, ischemic heart disease, and congestive heart failure (Roy *et al*., 2012).

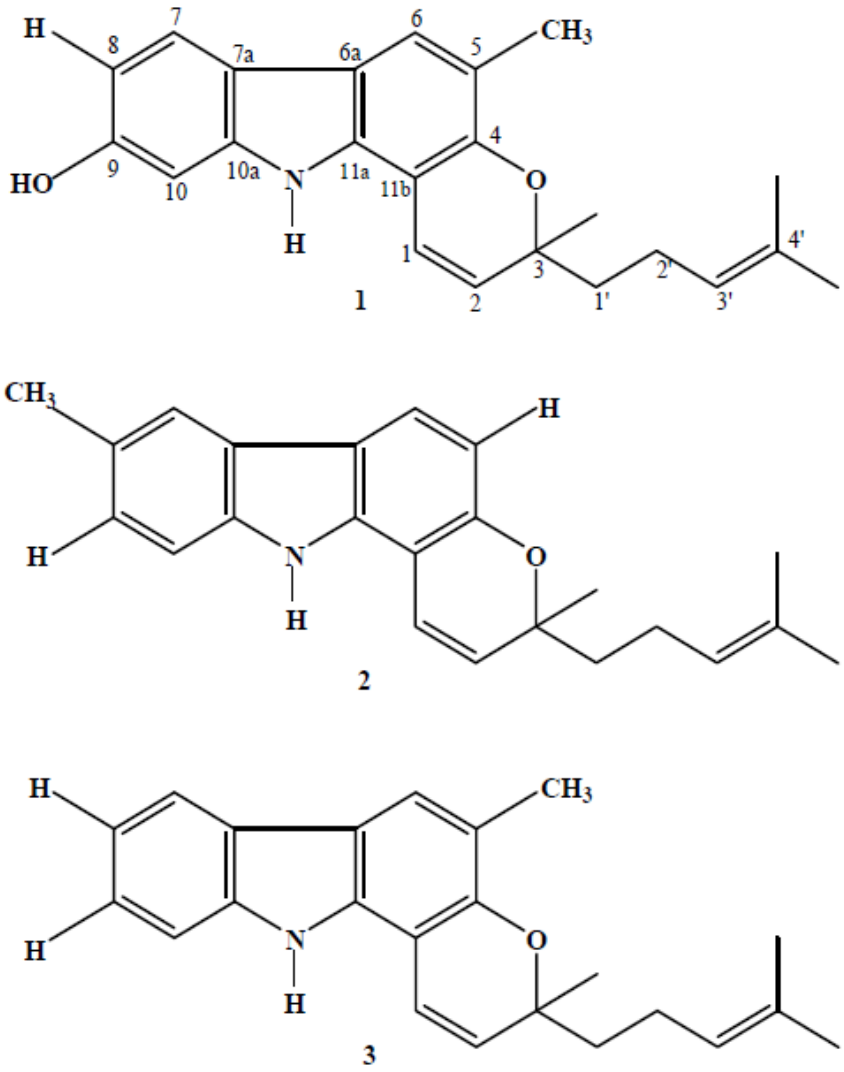


Figure 2.4: Chemical structures of some carbazole alkaloids (1) mahanine (2) mahanimbicine and (3) mahanimbine isolated from leaves of *Murraya koenigii* (Nagappan *et al*., 2011).

2.3 Carbazole in petroleum crude refining

In the petroleum industry, the removal of nitrogen heteroaromatics is important for three reasons. First, their combustion directly causes the formation of nitrogen oxides (NO_x), which contribute to acid rain and depletion of the ozone layer (Kirimura *et al*., 1999). Second, nitrogen-containing aromatic compounds presence can cause poisoning of refining catalysts, resulting in a decrease in yield (Girgis and Gates, 1991; Williams and

Chisti, 2001). Carbazole directly affects the refining process by its conversion into basic derivatives during cracking, which allows it to adsorb to the active sites of the cracking catalyst. It also serves as a potent direct inhibitor of hydrodesulfurization, a property that enables it to be included in the refining process to meet sulphur content criteria (Benedik *et al*., 1998; Nojiri and Omori, 2007). Finally, the presence of nitrogen heteroaromatics promotes corrosion of refining equipments thereby increasing the refining costs (Benedik *et al*., 1998).

2.4 Environmental fates of carbazole

2.4.1 Atmospheric fate

Carbazole is a semi-volatile organic compound (SOC) found in ambient air in gas phase and sorbed to aerosol (Odabasi *et al*., 1999). The fate, transport and removal of carbazole from the atmosphere by dry and wet deposition processes are strongly influenced by its gas-particle partitioning (Bidleman, 1988). The vapor pressure of carbazole (1×10^{-4} Pa) suggests that carbazole will exist in the vapor and particulate phases in the ambient atmosphere. Carbazole is released to the atmosphere in emissions from waste incineration, tobacco smoke, aluminum manufacturing, and rubber, petroleum, coal, and wood combustion (Smith *et al*., 1978; Jacobs and Billings, 1985; Pereira *et al*., 1987). Upon its release into the atmosphere, photochemically produced hydroxyl radicals (estimated half-life of 3 hrs) rapidly degrade vapor-phase carbazole. In the particulate phase, photodegradation of carbazole is dependent on the adsorbing substrate as substrates containing more than 5% carbon can stabilize photodegradation and permit long-range global transport of the pollutant (Behymer and Hates, 1988).

2.4.2 Terrestrial fate

Biodegradation by indigenous carbazole degraders in the soil is the dominant fate process for carbazole even though photolysis of carbazole in soil had been reported (Behymer and Hates, 1988; Grosser *et al*., 1991). However, adsorption of carbazole to environmental substrates will limit or prevent photolysis. The average K_{oc} (organic

carbon normalized partition coefficient) value of carbazole is 637 (Ainsworth *et al*., 1989), which suggest low mobility of carbazole in soil. Sorption of carbazole to soil is non-linear and highly correlated with organic content of soils (Bi *et al*., 2007).

2.4.3 Aquatic fate

In aquatic environment, biodegradation and photolysis are the dominant fate processes for carbazole. Half-lives of carbazole in a river, pond, eutrophic lake, and oligotrophic lake have been estimated as 0.5, 10, 10, and 3 hr respectively (Smith *et al*., 1978). Absence of carbazole degraders in the microbial community will foreclose biodegradation as a fate process while adsorption of carbazole to sediment will make photolysis unattainable (Pereira *et al*., 1987; Grosser *et al*., 1991). Volatilization is not a fate process in aquatic environment since carbazole is non-volatile in water (Meylan and Howard, 1991). Metabolism of carbazole to its N-methyl and N-acetyl derivatives by aquatic organisms has been reported. Furthermore, bioaccumulation and acute toxicity of NSO-heterocycles in aquatic organisms such as *Daphnia*, midge, and algae have been documented (Eisentraeger *et al*., 2008).

2.5 Toxicity of carbazole

Heterocyclic aromatic compounds are highly ubiquitous in the environment and are known to exhibit diverse ecotoxic effects such as acute toxicity, developmental and reproductive toxicity, cytotoxicity, photo-induced toxicity, mutagenicity, and carcinogenicity (Bundy *et al*., 2001; Barron *et al*., 2004; Robbiano *et al*., 2004; Brack *et al*., 2007; Eisentraeger *et al*., 2008).

Human exposure to carbazole occurs through tobacco smoking and inhalation of polluted air (IARC, 1983), while inhalation of vapours, dust, and dermal contact have been reported as possible routes of carbazole exposure to workers. There is no relevant epidemiological data to the carcinogenicity of carbazole to humans, though limited evidence in experimental animals for the carcinogenicity of carbazole have been reported (IARC, 1999).

In a study conducted on groups of 50 male and 50 female B6C3 F1 mice fed with different concentration of technical grade carbazole (96% purity) for 96 weeks, neoplastic lesions were found in the liver and fore stomach of the dead mice. The lesions were classified as neoplastic nodules and hepatocellular carcinomas. However, no such tumor was observed in the respective control groups (IARC, 1983).

Carbazole is mutagenic and toxic. Its toxicity to aquatic organisms is well documented (Eisentraeger *et al*., 2008; Peddinghaus *et al*., 2012). In a recent study on embryotoxic potential of NSO-heterocyclic compounds using groups of 3-month old zebrafish *Danio rerio*, carbazole displayed a very high embryotoxic potential with LC50 value of 1.07 mg/l, a value preceded only by acridine (0.7 mg/l) (Peddinghaus *et al*., 2012).

Although carbazole is not a human carcinogen, its hazardous derivatives such as N-methylcarbazole and 7-H-dibenzo (c,g) carbazole (and its derivatives) found in cigarette smoke and automobile emission are genotoxic and carcinogenic and have been categorized as "IARC Group 2B carcinogens" (Smith *et al*., 2000). 7H-dibenzo (c,g) carbazole (Figure 2.5) is a potent multi-site and multi-species carcinogen (Szafarz *et al*., 1988; Warshawsky *et al*., 1996) that induces tumour at the site of application and at distant sites, specifically in the liver (Renault *et al*., 1998).

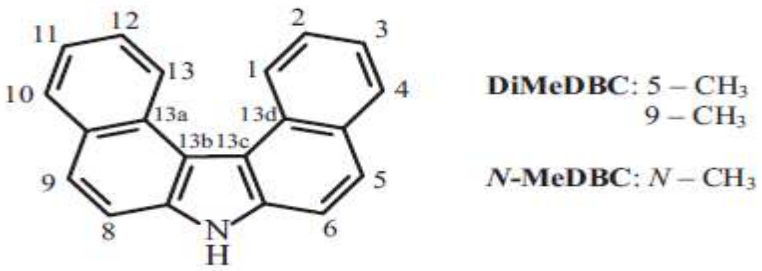


Figure 2.5: Chemical structure of 5,9-dimethyl dibenzo (c,g) carbazole (DBC) (Valovicova *et al*., 2012).

Synthetic methyl derivatives of 5,9-dimethyl dibenzo (c,g) carbazole, dimethyl-DBC and N-methyl-DBC exhibit specific attachment to liver and skin and together with the parent

compound (DBC) induces significant levels of DNA strand-breaks, micronuclei, and DNA adducts in immortalized human keratinocytes HaCat cells (Valovicova *et al*., 2012).

2.6 Biodegradation of heterocyclic aromatic compounds

Aerobic degradation of mono- and polycyclic aromatic hydrocarbons usually proceed via the upper (or peripheral) and lower (or ring cleavage) pathways (Diaz, 2004). The critical step in the upper pathway is the dearomatization of the benzene ring nucleus through mono- or dioxygenation, resulting in the introduction of two hydroxyl groups into the benzene ring forming *cis*-dihydrodiols (Mason and Cammack, 1992). Enzymes called dioxygenases catalyze the incorporation of both atoms of dioxygen into aromatic substrates and this type of dioxygenation is called **lateral dioxygenation** or **cis-dihydroxylation** (Nojiri and Omori, 2002). Cis-dihydrodiols are stereoselectively dehydrogenated by cis-dihydrodiol dehydrogenase, which re-aromatize the benzene ring nucleus to form a catechol moiety that undergo ring fission in *meta* or *ortho* fashion to produce intermediates of TCA cycle (Figure 2.6) (Nojiri *et al*., 2001b; Vaillancourt *et al*., 2006).

However, in contrast to lateral dioxygenation, investigation on the bacterial degradation of nitrogen, sulphur, and oxygen heteroaromatics such as dibenzofuran (DF), dibenzothiophene (DBT), carbazole (CAR), and dibenzo-*p*-dioxin (DD) revealed a new type of oxidative attack with high regioselectivity and specificity for the angular position (Nojiri and Omori, 2002). In this novel oxidative attack, the carbon atom bonded to the heteroatoms and the adjacent carbon in the aromatic ring is both oxidized (Figure 2.7) (Nojiri *et al*., 2001b). This reaction termed **angular dioxygenation** is catalyzed by the Rieske non-heme iron oxygenases called angular dioxygenases (Nojiri and Omori, 2002).

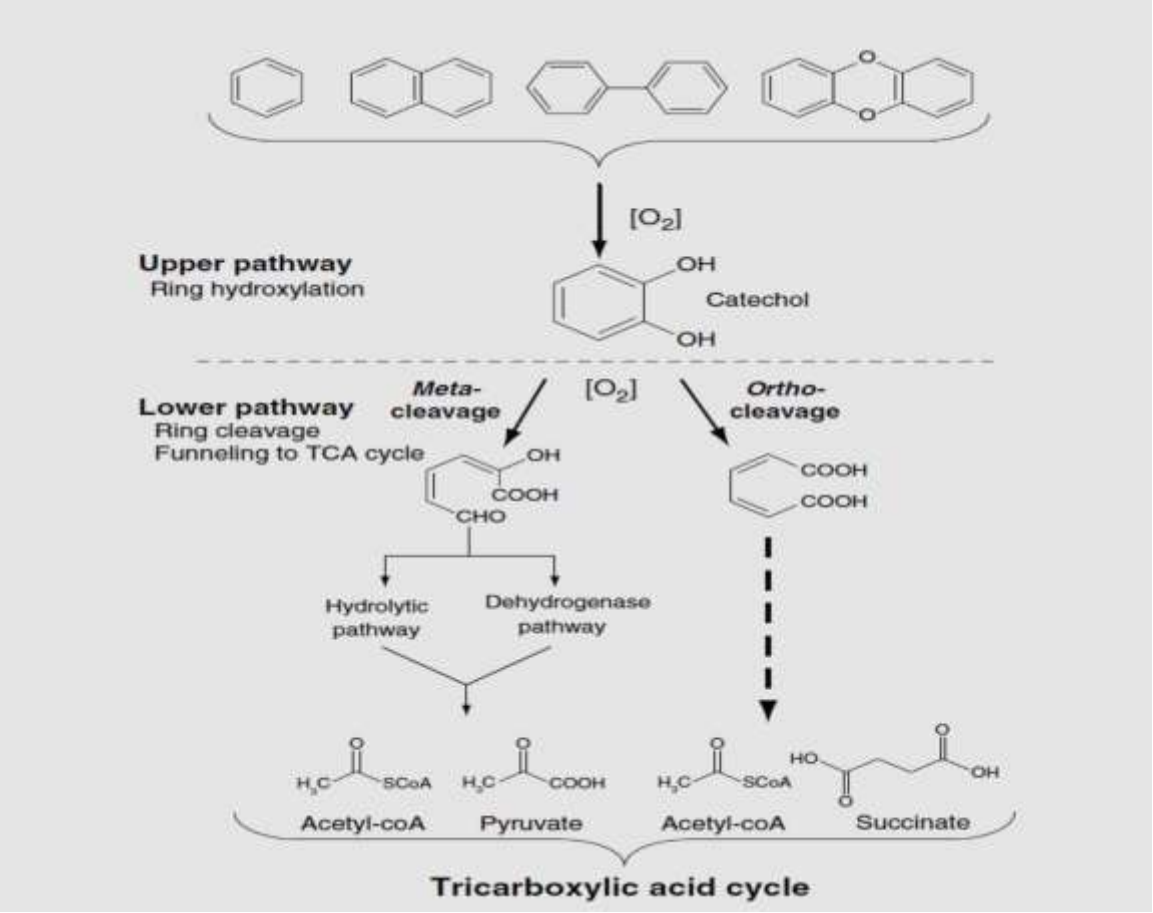


Figure 2.6: General schematic of aerobic aromatic degradation. The dashed line separate the peripheral (upper) pathway and the ring cleavage (lower) pathway (George and Hay, 2011).

Observations by several investigators indicate that angular dioxygenation is influenced by the electronegativity of the atom connecting the two aromatic rings. High electronegativities of connecting atoms (O and N) in dibenzofuran, dibenzo-*p*-dioxin and carbazole allow their use as substrates by angular dioxygenases (Bressler and Fedorak, 2000). However, low electronegativity of the connecting atom (S) in dibenzothiophene requires its oxidation to sulfone before it can be attacked by angular dioxygenases (Casellas *et al*., 1998; van Afferden *et al*., 1990, 1993).

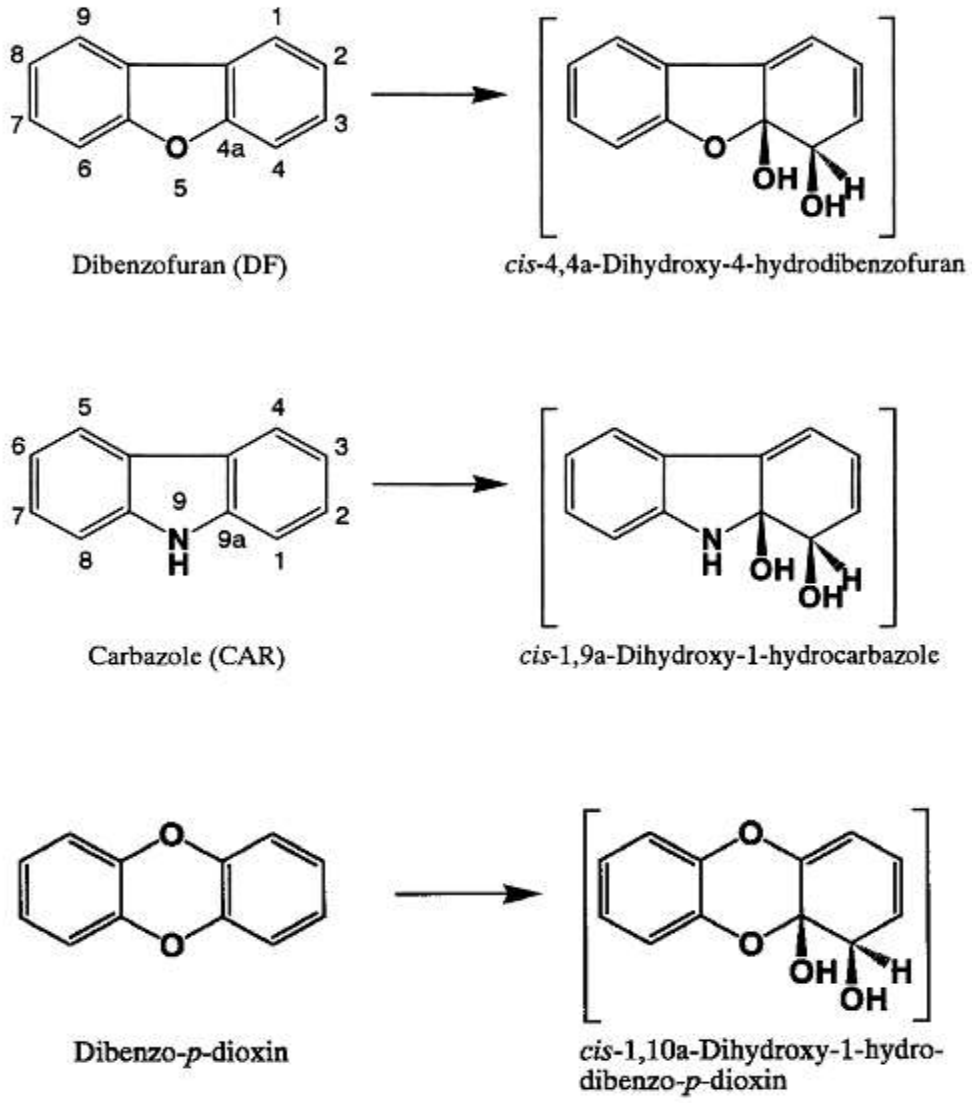


Figure 2.7: Angular dioxygenation of dibenzofuran, carbazole, and dibenzo-*p*-dioxin (Nojiri *et al*., 2001a; Nojiri and Omori, 2002).

Lateral dioxygenation of these aromatic heteroatoms have been reported with the initial hydroxylation of the aromatic ring occurring at the lateral positions 1,2 and 2,3 or 3,4 carbon atoms in DF, DD and CAR (Grifoll *et al*., 1995; Lobastova *et al*., 2004; Chang, 2008). Lateral dioxygenation of these aromatic heteroatoms is similar to the degradative pathways of naphthalene, biphenyl and other polycyclic aromatic hydrocarbons (PAHs), which produce yellow-coloured intermediate metabolites. However, this type of

dioxygenation usually results in incomplete bacterial degradation of these compounds and production of dead-end metabolites and monohydroxylated products (Cerniglia *et al*., 1979; Klecka and Gibson, 1980; Grifoll *et al*., 1995; Wittich, 1998; Lobastova *et al*., 2004).

2.6.1 Biodegradation of Dibenzofuran (DF) via angular dioxygenation

Dibenzofuran (DF), an O-heterocycle has been used as an insecticide, a component in heat-transfer oils, a carrier for dyeing and printing textile, and for synthesis of other compounds (Elvers *et al*., 1989; Xu *et al*., 2006). Sources of dibenzofuran in the environment include combustion (coal, biomass, refuse, diesel fuel, and tobacco), commercial coal tar and its leachate, incomplete combustion of propane, and photolysis of chlorinated biphenyl ether (Wittich, 1998; Bressler and Fedorak, 2000). Microorganisms that degrade DF are increasingly sought because of their potential ability to co-metabolize highly toxic polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Habe *et al*., 2001).

Initial step in the biodegradation of DF are categorized into two pathways- angular dioxygenation and lateral dioxygenation (Xu *et al*., 2006). The angular attack catalyzed by dibenzofuran 4,4a-dioxygenase (DFDO) occurs at carbon atom 4 and 4a position of DF. Incorporation of molecular oxygen into the angular position creates unstable hemiacetals that break up spontaneously to yield 2,3,2'-trihydroxybiphenyl (THBP). Subsequent ring (*meta*) cleavage of THBP catalyzed by 2,3,2'-THBP dioxygenase yield 2-hydroxy-6-oxo-6- (2-hydroxyphenyl)-2,4-hexadienoic acid (HOHPDA) (Xu *et al*., 2006). Hydrolysis of HOHPDA by HOHPDA hydrolases yield 2-oxo-4-pentenoate and salicylic acid. Salicylic acid is converted to catechol or gentisic acid, which are fed into the tricarboxylic acid (TCA) cycle (Figure 2.8) (Hiraishi, 2003).

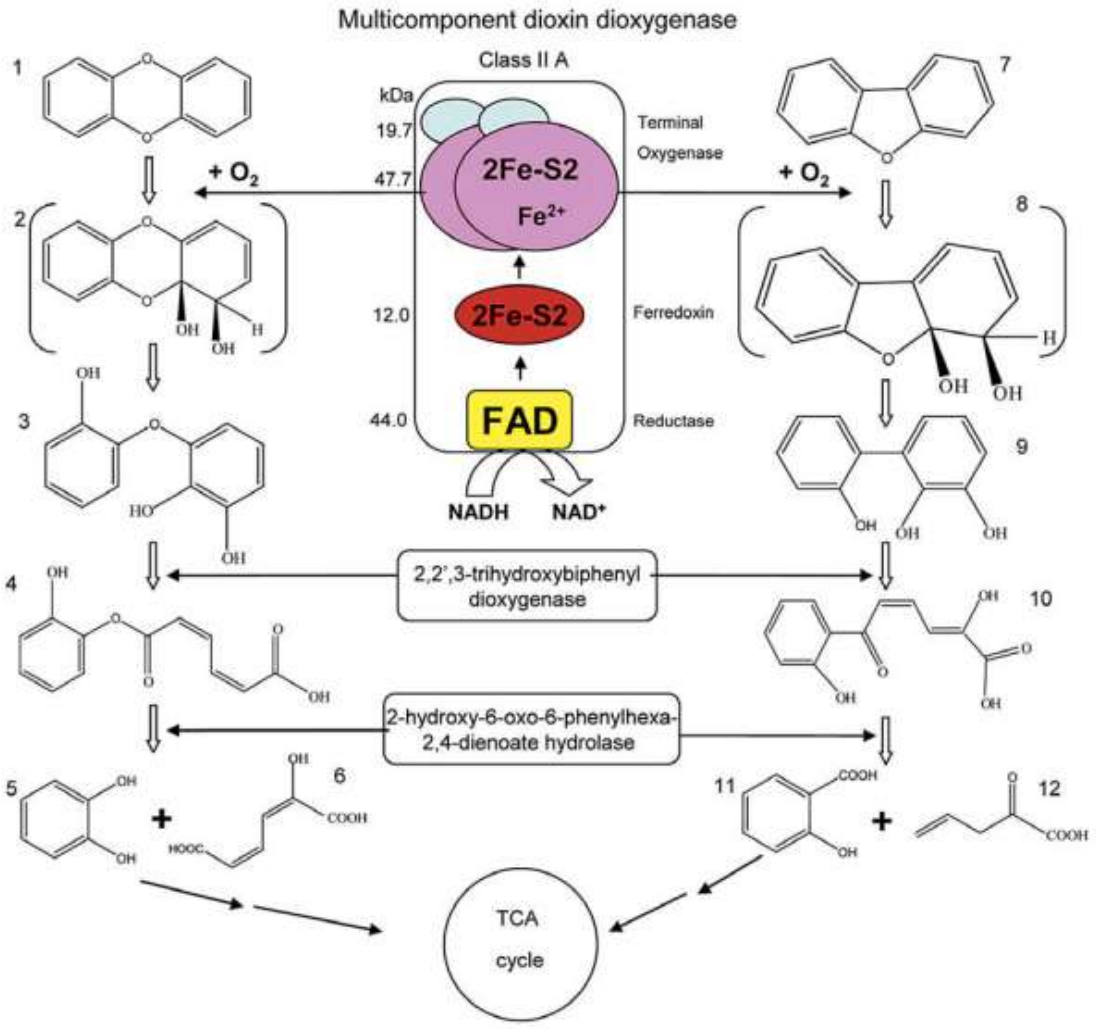


Figure 2.8: Biodegradative pathway of DD and DF via angular dioxygenation. (1) dibenzo-p-dioxin; (2) 4,4a-dihydro-dihydroxydibenzo-p-dioxin; (3) 2,2',3-trihydroxybiphenyl ether; (4) 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)hexa-2,4-dienoate; (5) catechol; (6) 2-hydroxy-muconate; (7) dibenzofuran; (8) 4,4a-dihydro-dihydroxydibenzofuran; (9) 2,2',3-trihyhroxybiphenyl; (10) 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoate; (11) salicylic acid; (12) 2-oxopent-4-enoate (Chang, 2008).

2.6.2 Biodegradation of Dibenzo-*p*-dioxin (DD) via angular dioxygenation

Dioxins are a group of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and coplanar polychlorinated biphenyls (PCBs) produced during incomplete combustion of chlorinated compounds, fossil fuels and municipal solid waste (Nojiri *et al*., 2001a; Hiraishi, 2003). Dioxins are also produced during industrial production of herbicides, insecticides, fungicides, and bleaching of paper pulp using chlorinated compounds (Halden and Dwyer, 1997, Hiraishi, 2003). Dioxins also encompasses low chlorinated and non-chlorinated DD and DBF as well as their structural analogues like diphenyl ethers (DE), carbazole (CAR), and Fluorene (Nojiri and Omori, 2002; Hiraishi *et al*., 2003). Dioxins are highly toxic, carcinogenic, ubiquitous and persistent in the environment with the half life of 2,3,7,8-Cl_4DD in soils and sediments as long as 1-10 years (Habe *et al*., 2001).

Initial angular dioxygenation for DD has been reported with the angular dioxygenase, dioxin 4,4a-dioxygenase (DDDO) attacking at the angular C4a and adjacent C4 position of DD (Armengaud *et al*., 1998). The resultant unstable hemiacetal intermediate is spontaneously converted to 2,2',3-trihydroxydiphenyl ether. Thus a single step oxygenation (angular dioxygenation) destroys the planar structure of dioxin from which the high toxicity of dioxin is derived (Nojiri and Omori, 2002; Takagi *et al*., 2002).

The dihydroxylated ring of the product (2,2',3-trihydroxydiphenyl ether) is *meta*-cleaved by an extradiol dioxygenase and subsequent hydrolysis of the *meta*-cleavage product yields catechol (Wittich *et al*., 1992). However, non identification of possible product of the hydrolysis of the meta-cleavage compound, 2-hydroxymuconic acid led to suggestion that only *meta*-cleavage enzyme, 2,3dihydroxybiphenyl 1,2-dioxygenase is needed for the production of catechol from 2,3-dihydroxydiphenyl ether (Pfeifer *et al*., 1993). Catechol formed from DD was mineralized via *meta* and/or *ortho* cleavage pathways (Figure 2.8) (Nojiri and Omori, 2002).

2.6.3 Biodegradation of dibenzothiophene (DBT)

Dibenzothiophene is a component of creosote, crude oils, and shale oils and often co-exist with other aromatic compounds in the environment (Seo *et al*., 2006). Dibenzothiophene and its derivatives are the major sulfur containing aromatic compounds in fossil fuels accounting for up to 70% of the sulfur content (Kertesz and Wirtek, 2001). The presence of these sulfur containing aromatic compounds in fossil fuels poses serious threats, as they are recalcitrant to conventional chemical desulfurization methods used in fossil fuel combustion (Shih *et al*., 1992). Combustion of sulfur containing fossil fuels generates sulfur dioxide (SO_2), which contribute to acid rain and air pollution (Xu *et al*., 2006).

Bacterial genera like *Gordonia*, *Arthrobacter*, *Rhodococcus*, *Mycobacterium* and *Pseudomonas* can degrade DBT through three independent reaction categories- sulfur oxidation, carbon-carbon cleavage (Kodama pathway, sulfoxidation and angular dioxygenation), and sulfur-specific cleavage (4S pathway) (Gupta *et al*., 2005; Santos *et al*., 2006; Seo *et al*., 2006; Xu *et al*., 2006). Due to economic importance of petroleum desulfurization, sulfur-specific cleavage where microorganisms specifically cleaved the carbon-sulfur bond in the DBT removing sulfur in the process while carbon and calorific values remain intact has been studied extensively (McFarland , 1999; Ohshiro and Izumi, 1999; Monticello, 2000; Gupta *et al*., 2005).

Angular dioxygenation of DBT was first reported by van Afferden *et al*. (1988) when a DBT degrader, *Brevibacterium* sp strain DO that grow on DBT as a sole source of sulfur, carbon and energy was isolated. Metabolic intermediates of DBT by strain DO as detected by co-chromatography, UV spectroscopy and GC-MS are DBT-sulfoxide, DBT-sulfone, and benzoic acid (van Afferden *et al*., 1990). In this pathway, angular dioxygenation of DBT only occurs after the formation of DBT-sulfone (Bressler and Fedorak, 2000). This pathway result in complete mineralization of DBT with the dihydroxy compound formed meta-cleaved and the ring fission product degraded to benzoic acid and sulfite, which then oxidizes to sulfate (Nojiri *et al*., 2001a; Figure 2.9).

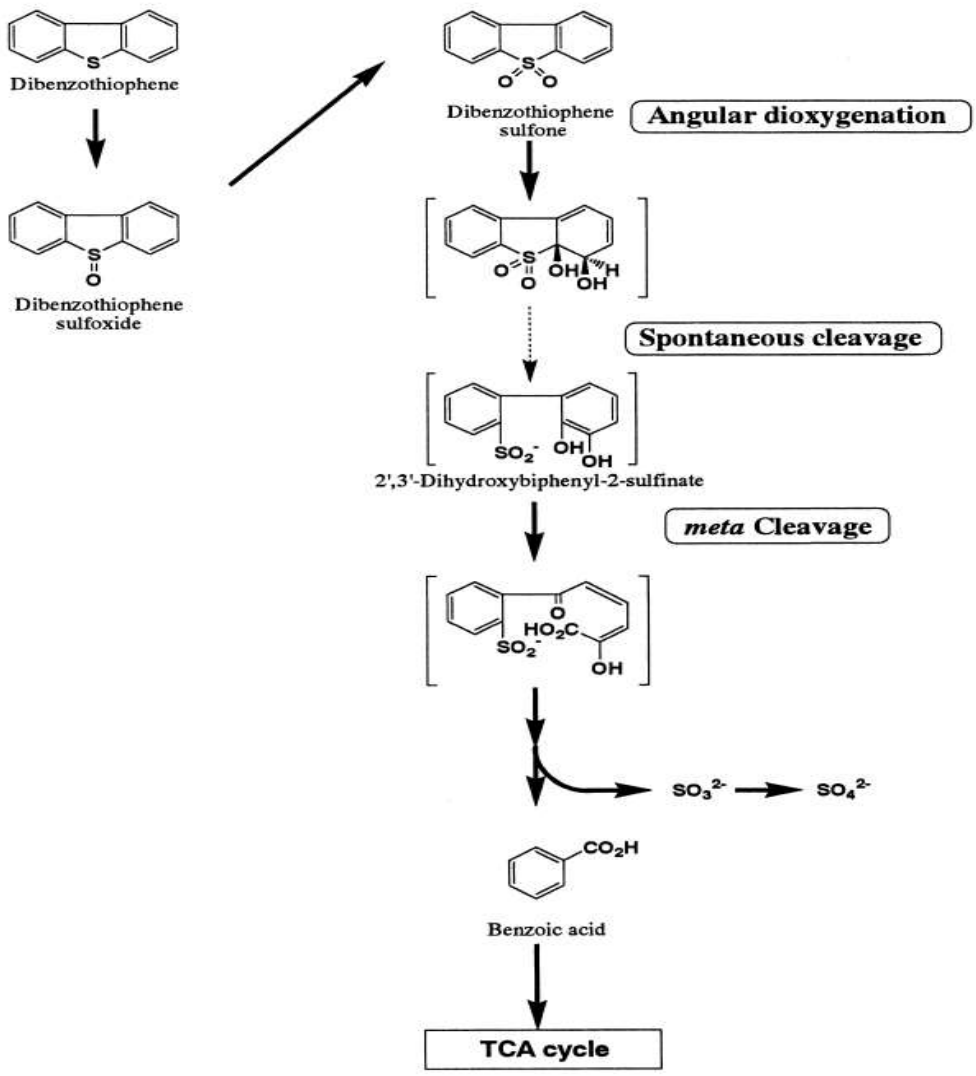


Figure 2.9: Biodegradative pathway of dibenzothiophene via angular dioxygenation (Nojiri *et al*., 2001a).

2.6.4 Bacterial degradation of carbazole

2.6.4.1 Diversity of carbazole degrading bacteria

Various carbazole-degrading bacteria have been isolated from diverse niches by enrichment cultural technique using carbazole as the sole source of nitrogen, carbon and energy or carbon, nitrogen and energy. Majority of carbazole degraders reported in literature are aerobic, Gram-negative bacteria with the exception of very few carbazole degraders such as *Nocardioides aromaticivorans* IC177 (Inoue *et al*., 2005) and

Gordonia sp. F.5.25.8 (Santos *et al*., 2006) that are aerobic, Gram-positive bacteria (Table 2.2). About 23 and 39% of carbazole degraders isolated from activated sludge, soil, and freshwater samples belong to the genera *Pseudomonas* and *Sphingomonas*, respectively (Nojiri and Omori, 2007). Recent research on carbazole degraders from marine environments using seawater-based enrichment culture has led to the isolation of novel carbazole degraders with unique carbazole degradative genes and enzymes different from those found in various carbazole degraders from soil, freshwater and activated sludge (Fuse *et al*., 2003; Maeda *et al*., 2009a, 2009b; Maeda *et al*., 2010).

Interest in the study of bacterial degradation of carbazole is spurred partly because of the ubiquitous nature of the pollutant and its mutagenic and toxic activities and also because carbazole is a structural analogue of dioxins and carbazole degrading enzymes can partly function as dioxin-degrading enzymes (Nojiri and Omori, 2007).

2.6.4.2 Degradation pathways of carbazole

Three major degradation pathways have been reported for carbazole. Lateral dioxygenation at carbon positions 3 and 4, monohydroxylation at carbon positions 1, 2, and 3 and angular dioxygenation at carbon positions 1, and 9a (Grifoll *et al*., 1995; Lobastova *et al*., 2004; Nojiri, 2012).

Table 2.2: Some Carbazole-Degrading Bacteria

Bacterial strain	**Medium[1]**	**Products[2]**	**References**
Ralstonia sp. RJGII.123	Carbon	Anthranilic acid	Grosser *et al*., 1991; Schneider *et al*., 2000
P. resinovorans CA10	Carbon, nitrogen	Anthranilic acid, catechol	Ouchiyama *et al*., 1993; Nojiri *et al*., 1999
P. resinovorans CA06	Carbon, nitrogen	Anthranilic acid, catechol	Ouchiyama *et al*., 1993

P. stutzeri ATCC31258	Carbon	Anthranilic acid	Hisatsuka and Sato, 1994
Pseudomonas sp. LD2	Carbon	Anthranilic acid	Gieg *et al*., 1996
Burkholderia sp. CB1	Carbon, nitrogen	Not detected	Shotbolt-Brown *et al*., 1996
Xanthomonas sp. CB2	Carbon, nitrogen	Not detected	Shotbolt-Brown *et al*., 1996
Sphingomonas sp. CB3	Carbon, nitrogen	Not detected	Shepherd and Lloyd-Jones, 1998
P. stutzeri OM1	Carbon, nitrogen	Anthranilic acid	Ouchiyama *et al*., 1998;
Sphingomonas sp. CDH-7	Carbon, nitrogen	Anthranilic acid	Kirimura *et al*., 1999
Sphingomonas sp. GTIN11	Nitrogen	Anthranilic acid	Kilbane II *et al*., 2002
Sphingomonas sp. KA1	Carbon	None	Habe *et al*., 2002
Pseudomonas rhodesiae KK1	Carbon	None	Yoon *et al*., 2002
Neptunomonas naphthovorans CAR-SF	Carbon	None	Fuse *et al*., 2003
Pseudomonas sp. XLDN4-9	Nitrogen	None	Li *et al*., 2004
Achromobacter sp. IC074	Carbon, nitrogen	None	Inoue *et al*., 2005
Stenotrophomonas sp. IC193	Carbon, nitrogen	None	Inoue *et al*., 2005
Janthinobacterium sp. J3	Carbon, nitrogen	None	Inoue *et al*., 2004
Pantoea sp. J14	Carbon, nitrogen	None	Inoue *et al*., 2004

[1]Carbon and nitrogen: carbazole was added to the isolation medium as the carbon, nitrogen and energy sources; carbon: carbazole was added to the medium as the carbon and energy source; nitrogen: carbazole was added as the nitrogen source.

[2]Major metabolic intermediate produced when the bacterium is grown on carbazole.

2.6.4.2.1 Lateral dioxygenation of carbazole

Grifoll *et al*. (1995) first suggested Lateral dioxygenation of carbazole by fluorene-degrading bacteria *Pseudomonas cepacia* F297 at C3 and C4 carbons yielding 4-(3'-hydroxy-2'-indoyl)-2-oxo-3-butenoic acid (Figure 2.10) as detected by GC-FID and GC-MS. However, strain F297 cannot utilize carbazole as source of carbon and energy (Grifoll *et al*., 1995).

2.6.4.2.2 Hydroxylation of carbazole

In contrast, Lobastova *et al*. (2004) were able to identify 1-, 2- and 3-hydroxycarbazoles as the bioconversion products following monohydroxylation of carbazole at position 1, 2, and 3 by *Aspergillus flavus* VKM F-1024 using TLC, GC, MS and ^{1}H NMR respectively. 3-hydroxycarbazole was detected as the major product while 1-hydroxy- and 2-hydroxycarbazoles are detected as minor products. Yamazoe *et al*. (2004), and Seo *et al*. (2006) also reported bioconversion of carbazole to hydroxycarbazoles.

Furthermore, bacterial dioxygenases such as naphthalene 1,2-dioxygenase from *Pseudomonas* sp. NCIB 9816-4 and biphenyl dioxygenase from *Beijerinckia* sp. B8/36 also catalyze the initial oxidation of carbazole to 3-hydroxycarbazole (Resnick *et al*., 1993). Hydroxylated carbazole derivatives have strong antioxidant activity and are value-added substances in pharmaceutical industry with diverse application in therapies for encepalopathy, cardiopathy, hepatopathy and arteriosclerosis (Lobastova *et al*., 2004).

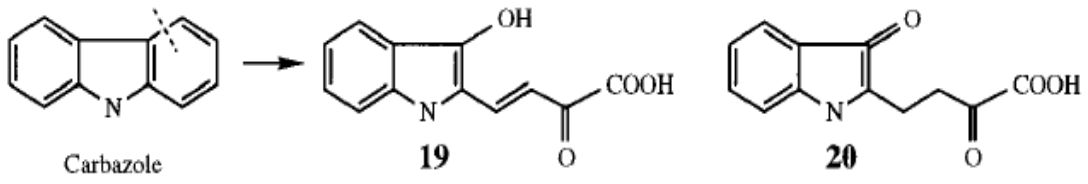


Figure 2.10: Lateral dioxygenation of carbazole at C3 and C4. The metabolites detected from the methylated acidic extract are 4-(3'-methoxy-2'-indolyl)-2-oxo-3-butenoic acid (Methylated, **19**) and 4-(3'-oxo-2'-indolinyl)-2-oxo-3-butenoic acid (Methylated, **20**) (Grifoll *et al*., 1995).

2.4.6.2.3 Angular dioxygenation of carbazole

Some carbazole degraders reported in literature degrade carbazole via angular dioxygenation, a novel type of oxidative attack that occurred at the ring-fused position and mediated by a multicomponent enzyme, carbazole 1,9a-dioxygenase (CARDO) with addictive preference for angular positions (Nojiri *et al*., 1999). In contrast to lateral dioxygenation and monohydroxylation, angular dioxygenation result in complete mineralization of carbazole with the resulting catechol converted to tricarboxylic acid (TCA) cycle intermediate (Nojiri and Omori, 2002).

Ouchiyama *et al*. (1993) isolated a carbazole-degrader, *Pseudomonas resinovorans* CA10, from activated sludge of a municipal wastewater treatment facility in Tokyo, Japan. The strain is capable of growth on carbazole as sole source of carbon, nitrogen and energy and accumulates anthranilic acid and catechol as catabolic intermediates of carbazole. It also grows on anthranilic acid as carbon and nitrogen source and accumulates catechol suggesting carbazole conversion to catechol via anthranilic acid (Ouchiyama *et al*., 1993). Furthermore, production of 2'-aminobiphenyl-2,3-diol and its meta-cleavage product 2-hydroxy-6-oxo-6-(2'-aminopheny)-hexa-2,4-dienoate (HOADA) from the culture medium of CA10 grown on carbazole was suggested. Based on these findings and its similarity with dibenzofuran degradation pathway, a carbazole degradation pathway was proposed (Figure 2.11). The pathway is divided into upper and lower pathway. The upper pathway encompasses the conversion of carbazole to catechol while the lower pathway involve catechol mineralization (Nojiri, 2012).

Carbazole is dioxygenated at angular (C9a) and adjacent (C1) carbon atoms to produce an unstable hemiaminal (1-hydro-1,9a-dihydroxycarbazole) which is spontaneously cleaved to form 2'-aminobiphenyl-2,3-diol. This metabolic intermediate is converted to anthranilic acid via meta-cleavage and subsequent hydrolysis. Anthranilic acid is converted to catechol by dioxygenation at the C1 and C2 positions followed by spontaneous deamination and decarboxylation reactions (Kobayashi and Hiyaishi, 1970). Formed catechol is converted to a tricarboxylic acid (TCA)-cycle intermediate via *ortho*-cleavage (as in *P. resinovorans* CA10) or *meta*-cleavage (as in *Pseudomonas stutzeri* strain OM1) pathways (Ouchiyama *et al*., 1993; Ouchiyama *et al*., 1998; Figure 2.11).

Anthranilic acid has been detected from the culture extracts of several carbazole degraders and is regarded as the main metabolite of carbazole angular dioxygenation (Ouchiyama *et al*., 1993; Gieg *et al*., 1996; Ouchiyama *et al*., 1998; Kirimura *et al*., 1999; Schneider *et al*., 2000; Kilbane II *et al*., 2002; Inoue *et al*., 2005). Anthranilic acid is a biotic compound and is formed by the degradation of tryptophan in several living organisms (Hayaishi and Stanier, 1951). It is an important intermediate in the metabolism of many N-heterocyclic compounds and plays important role in *Pseudomonas* quinolone signal, which is involved in quorum sensing in *Pseudomonas aeruginosa* cells (Calfee *et al*., 2001).

It is worthy to note however, that once angular dioxygenation and subsequent ring cleavage occurs for carbazole, the resulting 2'-aminobiphenyl-2,3-diol is degraded via the analogous biphenyl degradation pathways (Furukawa *et al*., 2004).

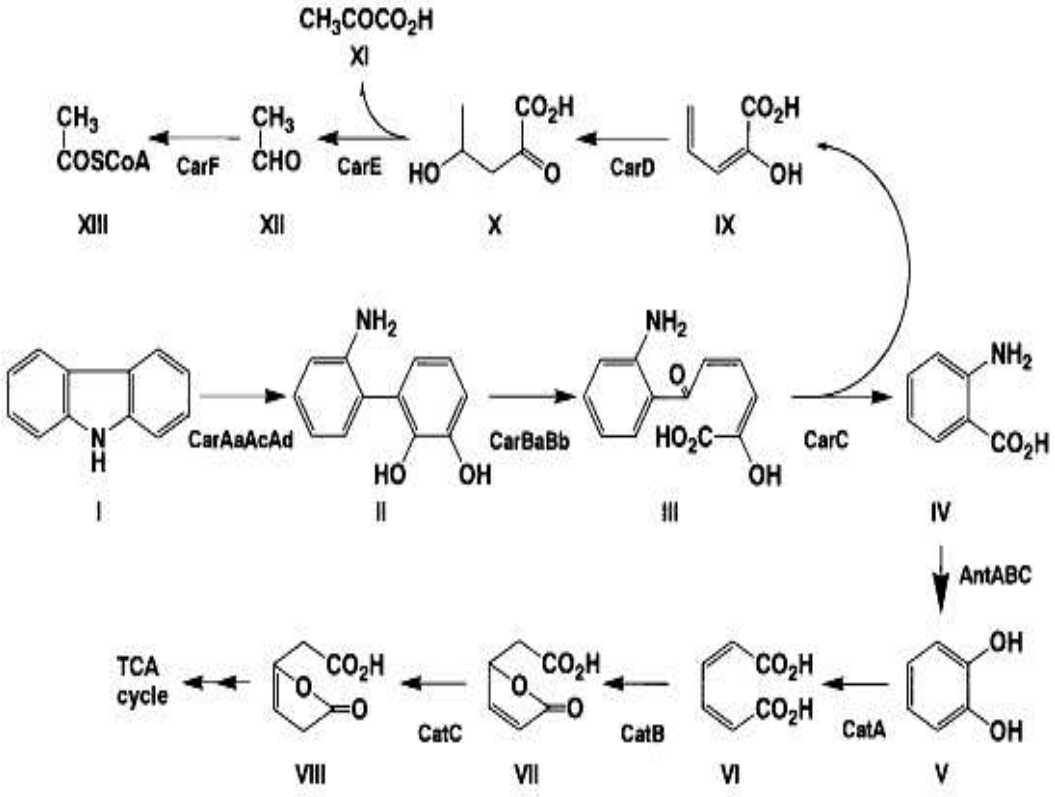


Figure 2.11: Carbazole degradation pathway in *P. resinovorans* CA10. Enzymes designations: CarAaAcAd, carbazole 1,9a-dioxygenase; CarBaBb, 2'-aminobiphenyl-2,3-diol 1,2-dioxygenase; CarC, 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoate

hydrolase; CarD, 2-hydroxypenta-2,4-dienoate hydratase; CarE, 4-hydroxy-2-oxovalerate aldolase; CarF, acetaldehyde dehydrogenase (acylating); AntABC, anthranilate 1,2-dioxygenase; CatA, catechol 1,2-dioxygenase; CatB, *cis,cis*-muconate lactonizing enzyme; CatC, muconolactone δ-isomerase. Compounds: I, CAR; II, 2'-aminobiphenyl-2,3-diol; III, 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoate; IV, anthranilic acid; V, catechol; VI, *cis,cis*-muconate; VII, muconolactone; VIII, β-ketoadipic acid enol-lactone; IX, 2-hydroxy-penta-2,4-dienoate; X, 4-hydroxy-2-oxovalerate; XI, pyruvate; XII, acetaldehyde; XIII, acetyl coenzyme A (Nojiri *et al*., 2001b).

2.7 Carbazole degradative genes

2.7.1 *Pseudomonas*-type *car* gene cluster

The CAR degradative genes of *P. resinovorans* CA10 have been extensively studied. Sato *et al*. (1997a, 1997b) first succeeded in cloning the genes involved in upper pathway of carbazole degradation from *P. resinovorans* CA10 genome by shotgun cloning using *meta*-cleavage activity. The resultant gene fragment contains seven degradative genes, one open reading frame (ORF) that encoded a putative protein or unknown function, and two partial possible genes. Functional analysis of the degradative genes shows two identical copies of *carAa*, *carAc*, and *carAd*, which encode terminal oxygenase, ferredoxin, and ferredoxin reductase components of carbazole 1,9a-dioxygenase (CARDO); *carBa* and *carBb*, which encode structural and catalytic subunits of the *meta*-cleavage enzyme (2'-aminobiphenyl-2,3-diol 1,2-dioxygenase); and *carC*, which encode the meta-cleavage compound (HOADA) hydrolase.

Gene walking around the car_{CA10} gene cluster revealed the entire gene structure (Figure 2.12). 2-hydroxypenta-2,4-dienoate (HPD degradative *carDFE* genes (meta-cleavage pathway genes) was found downstream of the *carAd* gene. In addition, *antABC* gene encoding anthranilate 1,2-dioxygenase (Figure 2.12a) was found in the 21-kb region upstream from *carAa* (Nojiri *et al*., 2001b). This anthranilate degradative gene cluster is a putative composite transposon flanked by two homologous insertion sequences ISPre1 and ISPre2. Furthermore, *antR* gene encoding a transcriptional regulator of the ant operon was found outside the putative composite transposon containing *antABC* (Figure 2.12a), which regulates the inducible expression of the *car* gene cluster (Urata *et al*., 2004). Tn5

mutagenesis was used to isolate the β-ketoadipate pathway (*ortho*-cleavage pathway) genes involved in catechol mineralization from strain CA10 genome (Kimura *et al*., 1996).

Carbazole-degrading bacteria from the genera *Pseudomonas*, *Burkholderia*, and *Janthinobacterium* have been reported that have nearly identical carbazole degradative genes with car_{CA10} (Figure 2.12a) and are designated *Pseudomonas*-type *car* gene cluster. Even though these carbazole-degraders are isolated from different origin, comparison of the gene organization and flanking regions of their *car* gene clusters suggests evolutionary diversity as reflected in differences in copy number of *car* gene cluster among carbazole degraders (Inoue *et al*., 2004). This phenomenon may arise because *car* gene clusters are sometimes borne on plasmids or transposons and/or flanked by IS (insertion sequence) elements (Inoue *et al*., 2004).

2.7.2 *Sphingomonas*-type *car* gene cluster

The genus *Sphingomonas* was found to possess a *car* gene cluster homologue (though relatively low homology, <60% identity) showing similarity in gene organization and phylogeny with the car_{CA10} gene cluster. Isolation of *car* gene clusters in sphingomonads, was first reported in *Sphingomonas* sp. GTIN11 (Kilbane II *et al*., 2002) and *Sphingomonas* sp. (reclassified as *Novosphingobium* sp) KA1 (Habe *et al*., 2002) and the $car_{KA1/GTIN11}$ gene cluster homologue have been reported to occur in various carbazole-degrading *Sphingomonas* and related strains (Inoue *et al*., 2004, 2005).

The *car* gene clusters isolated from these two *Sphingomonas* strain is different from car_{CA10} gene cluster in two ways. First, unlike the car_{CA10} gene cluster, it does not contain the NAD(P)H:ferredoxin oxidoreductase gene involved in the initial dioxygenase, but contain the genes for terminal oxygenase (*carAa*) and ferredoxin (*carAc*), the *meta*-cleavage enzyme (*carBaBb*), and HOADA hydrolase (*carC*) (Figure 2.12b). Second, though *Sphingomonas* CarAa exhibit significant homology with CA10 CarAa (>55% identity), its ferredoxin (CarAc) is neither related to $CarAc_{CA10}$ nor with other Rieske ferredoxins but shows similarity to the putidaredoxin-type ferredoxins. Because the

terminal oxygenase of strain KA1 ($CarAa_{KA1}$) can receive electrons from strain KA1 ferredoxin ($CarAc_{KA1}$) and catalyze angular dioxygenation of carbazole, it implies that ferredoxin selectivity differs between strain $CarAa_{CA10}$ and $CarAa_{KAI/GTIN11}$ (Inoue *et al*., 2004). Furthermore, two copies of car_{KA1} gene cluster (*car*-I_{KA1} and *car*-II_{KA1}) were found to be domiciled on a >250-kb circular plasmid pCAR3 in *Novosphingobium* sp. KA1 along with the presence of NAD(P)H:ferredoxin oxidoreductase genes (*fdrI* and *fdrII*) and a third putidaredoxin-type ferredoxin gene. These findings show clearly that the plasmid pCAR3 contains the complete set of genes responsible for carbazole mineralization in strain KA1 (Urata *et al*., 2006).

2.7.3 The *car* gene cluster in *Nocardioides aromaticivorans* IC177

Quite distinct *car* gene cluster different from the *Pseudomonas* and *Sphingomonas*-types was found in a Gram-positive bacterium *N. aromaticivorans* IC177 (Inoue *et al*., 2005, 2006). The car gene were clustered in the *carAaCBaBbAcAd* and *carDFE* gene clusters encoding the enzymes responsible for degradation of carbazole to anthranilate and 2-hydroxypenta-2,4-dienoate (HPD) (upper pathway) and HPD to pyruvate and acetylcoenzme A (lower pathway), respectively (Inoue *et al*., 2006).

However, the position of *carC* relative to *carBaBb* in strain IC177 is the opposite of that in *car* gene clusters of the *Pseudomonas* and *Sphingomonas*-types (Figure 2.12c) (Inoue *et al*., 2006). In the *car* gene operons in strain IC177, the genes overlap each other by 1 or 4 bp with *carDFE* genes closely linked and located upstream of the *carAaCBaBbAcAd* gene cluster. In addition, organization of carbazole catabolic operon in strain IC177 occurred in a more orderly fashion as functional units than those in Gram-negative strains, such as strains CA10, J3, GTIN11, and KA1 (Nojiri and Omori, 2007).

2.7.4 The *car* gene cluster in *Sphingomonas* sp. CB3

Interestingly, the *car* gene cluster of strain CB3 differs from those of the three above-mentioned types in terms of gene organization and phylogeny (Figure 2.12d) but showed

marked similarity with naphthalene and biphenyl degradative *bph* gene cluster (Shepherd and Lloyd-Jones, 1998). The *car* genes of strain CB3 are arranged in the order of *carAaAbAcAdBCD*, and the terminal oxygenase component of strain CB3 unlike those of other CAR degraders, which are composed of a single subunit, is composed of two subunits, CarAa and CarAb respectively (Shepherd and Lloyd-Jones, 1998). Although carbazole metabolic activity of the enzymes encoded in carbazole catabolic operon in CB3 has not been confirmed, its transcription was detected when carbazole was used as source of carbon by strain CB3 (Nojiri and Omori, 2007).

2.7.5 The *car* gene cluster in marine carbazole-degraders

Carbazole-degrading bacteria from different genera such as *Neptuniibacter*, *Erythrobacter*, *Marinobacter*, *Caulobacter*, *Hyphomonas*, *Lysobacter*, *Sphingosinicella*, *Kordiimonas*, and *Terrabacter* have been isolated from marine environment (Fuse *et al*., 2003; Inoue *et al*., 2005; Maeda *et al*., 2009a, 2009b). Southern hybridization analysis performed under strict conditions at 68°C (hybridization conditions for similarity of >90%) and 55°C (hybridization conditions for similarity >60%) using car_{CA10} and car_{KA1} gene cluster probes for 14 marine isolates showed that they lack *car* genes highly similar to car_{CA10} and car_{KA1}. This suggests that marine isolates are evolutionarily different from their terrestrial counterpart with unique *car* gene clusters and CARDO. Furthermore, hybridization analysis at 55°C showed that eight of the 14 marine isolates have novel *car* gene clusters that are highly different from the car_{CA10} and car_{KA1} genes.

Shotgun cloning experiments was performed to isolate the car gene clusters of three marine carbazole degraders, *Neptuniibacter* sp. strain CAR-SF, *Lysobacter* sp. strain OC7, and novel genus strain OC9, respectively.

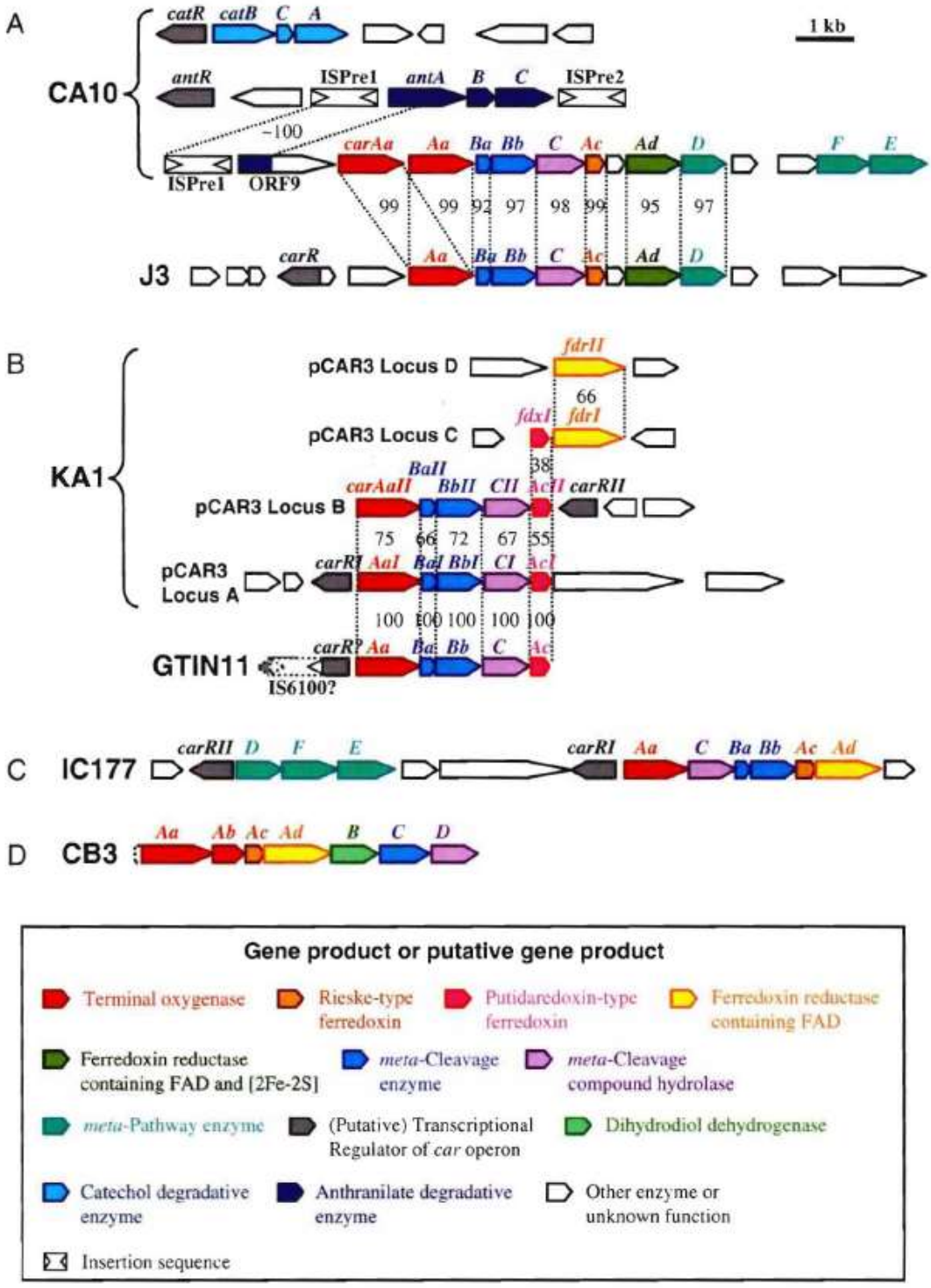


Figure 2.12: Genetic structure of the gene clusters involved in carbazole biodegradation by (a) *P. resinovorans* CA10 and *Janthinobacterium* sp. J3, (b) *Sphingomonas* (*Novosphingobium*) sp. KA1 and *Sphingomonas* sp. GTIN11, (c) *N. aromaticivorans* IC177, and (d) *Sphingomonas* sp. CB3 (Nojiri and Omori, 2007).

2.7.5.1 *car* gene cluster of *Neptuniibacter* sp. strain CAR-SF

The *car* gene cluster of strain CAR-SF are arranged in the order *carAaBaBbC*, resembling the order of arrangement of the *Pseudomonas* and *Sphingomonas*-type *car* gene clusters showing 48-77% similarity with car_{CA10} and car_{J3} genes and thus designated as a *Pseudomonas*-type *car* gene cluster (2.13a) (Nagashima *et al*., 2010). However, in comparison with the car_{CA10} and car_{J3} gene clusters, the $car_{CAR\text{-}SF}$ gene cluster lacks the ferredoxin *carAc* and ferredoxin reductase *carAd* genes, though a $carAc_{CA10}$-like gene was revealed by Southern hybridization analysis. This shows that unlike in car_{CA10} and related *Pseudomonas*-type *car* gene clusters, ferredoxin gene of CARDO was in a different location in CAR-SF strain and not in the $car_{CAR\text{-}SF}$ gene cluster (Nagashima *et al*., 2010).

2.7.5.2 *car* gene cluster of *Lysobacter* sp. strain OC7

The car gene cluster in strain OC7 are arranged in the order *carAaCBaBb*, with the position of *carC* and *carBaBb* inverted when compared to their positions in *Pseudomonas* and *Sphingomonas*-type *car* gene clusters. However, the genes arrangement followed the same order as in the *car* gene cluster of strain IC177 (Maeda *et al*., 2009b). The open reading frames (ORFs) containing the *car* gene cluster of strain OC7 share 39-52% similarity with *carAa*, *carC*, *carBa*, and *carBb* genes of strains CA10 and KA1, and showed no similarity with *car* genes of strain CB3, making the *car* genes of strain OC7 phylogenetically distinct from previously reported *car* gene products (Figure 2.12). Furthermore, southern hybridization analysis shows that only *Caulobacter* sp. strain OC6, a phylogenetically different genus (*α-proteobacteria*) hybridized with the car_{OC7} gene cluster (from strain OC7 belonging to *γ-proteobacteria* group) probe with more than 90% similarity (Maeda *et al*., 2009b). This finding is interesting as it reveals the evolutionary diversity of *car* gene clusters and importance of genetic exchange in its distribution across different phylogenetic groups.

The product of $carAa_{OC7}$ possessed consensus sequences of a Rieske-type [2Fe-2S] cluster and mononuclear heme iron (Maeda *et al*., 2009b). However, its ferredoxin and ferredoxin reductase genes are not located near the *car* gene cluster of strain OC7, as in

strain CAR-SF. In addition, as in CAR-SF, *E. coli* harbouring only *carAa*$_{OC7}$ was unable to convert CAR but *E. coli* cells harbouring pBOC77 (*carAa*$_{OC7}$*AcAd*$_{CA10}$) converted CAR to 2'-aminobiphenyl-2,3-diol. However, the transformation ratio of CAR by pBOC77 (*carAa*$_{OC7}$*AcAd*$_{CA10}$) was 32-36%, which is less than 99% recorded for *E. coli* cells harbouring pUCARA (*carAaAaAcORFcarAd*) (Sato *et al*., 1997a) or pSF6 (*carAa*$_{CAR\text{-}SF}$*AcAd*$_{CA10}$) used as positive controls, thus revealing weak electron-transfer efficiency of CarAa$_{OC7}$AcAd$_{CA10}$ and suggesting a different electron transfer components and RO class for CARDO$_{OC7}$ (Maeda *et al*., 2009b).

2.7.5.3 *car* gene cluster of novel genus strain OC9

The CARDO system and the arrangement of *car* gene cluster in strain OC9 present a new question in relation to evolution and diversity of *car* genes in bacteria. First, the recovered ORFs of strain OC9 share 35-65% homology with previously reported *car* genes (*carRAaCBaBb*). However, a ferredoxin-like gene (*carAc*) found immediately downstream of *carR* does not show homology with any of the reported ferredoxin component of CARDO as it possess a chloroplast-type ferredoxin (Maeda *et al*., 2010). This is a unique type of ferredoxin completely different from the Rieske and putidaredoxin-types reported for strains CA10, KA1, IC177, and CB3 CARDO systems (Sato *et al*., 1997a; Shepherd and Lloyd, 1998; Inoue *et al*., 2006; Urata *et al*., 2006). Second, the *car* gene cluster of strain OC9 were arranged in the order *carAcRAaCBaBb* with *carRAc* and *carAaBaBb* having opposite orientation, thus suggesting that the *carAc* and *carAa* genes transcribed within different transcription units (Figure 2.13b).

The product of *carAa*$_{OC9}$ possessed consensus sequences of a Rieske-type [2Fe-2S] cluster and mononuclear heme iron (Maeda *et al*., 2010). However, unlike *carAa*$_{CAR\text{-}SF}$ and *carAa*$_{OC7}$ that could not transform *E. coli* cells without CarAc, *E. coli* cells harbouring only *carAa*$_{OC9}$ in a resting cell reaction converted CAR to 2'-aminobiphenyl 2,3-diol, though the conversion ratio (12%) is low when compared to that of *E. coli* cells harbouring genes for both *carAa* and *carAc* (100%) respectively (Maeda *et al*., 2010).

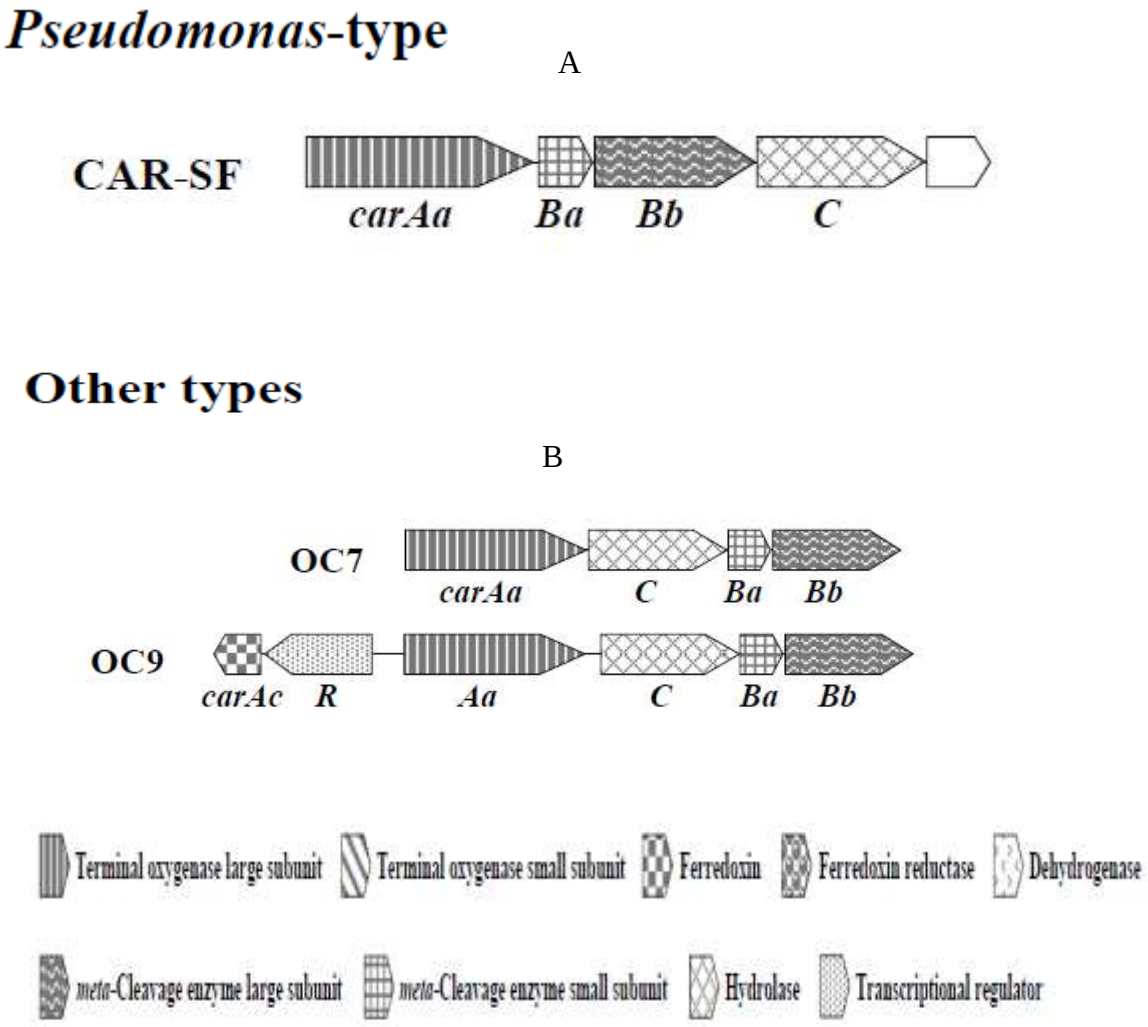


Figure 2.13: The genetic structures of *car* gene clusters in marine carbazole-degrading bacteria. (A) *Pseudomonas*-type *car* gene cluster of *Neptuniibacter* sp. strain CAR-SF. (B) other types of *car* gene clusters found in *Lysobacter* sp. strain OC7 and novel genus strain OC9 (Maeda *et al*., 2009b; Maeda *et al*., 2010; Nagashima *et al*., 2010).

2.8 The CARDO system in carbazole degraders and its substrate specificity

The extensively studied CARDO system in *Pseudomonas resinovorans* CA10 is a three-component dioxygenase system belonging to the Rieske nonheme iron oxygenase system (ROS) and consist of a terminal oxygenase and electron transport proteins (Sato *et al*., 1997a; Nam *et al*., 2002). The terminal oxygenase component of CARDO (CARDO-O) is a homotrimeric enzyme that contain one Rieske [2Fe-2S] cluster ($[2Fe\text{-}2S]_R$ and one

active-site iron (Fe^{2+}) in a single subunit (CarAa) (Nojiri and Omori, 2007). The electron transport proteins of CARDO, which mediate electron transport from NAD(P)H to CARDO-O, comprise ferredoxin (CARDO-F; a monomer of CarAc), which contains one $[2Fe\text{-}2S]_R$, and ferredoxin reductase (CARDO-R; a monomer of CarAd), which contains one FAD and one plant –type [2Fe-2S] cluster ($[2Fe\text{-}2S]_P$) (Sato *et al*., 1997a; Nam *et al*., 2002).

Phylogenetic analysis revealed a very low homology (<19% overall length –wise identity) of the amino acid sequence of CARDO with almost all known catalytic subunits of ROS terminal oxygenases (Figure 2.14) (Nojiri and Omori, 2007). In addition, CARDO-O consists of only catalyic α subunit with the α_3 configuration in contrast to typical class III ROSs whose terminal oxygenase components consist of both α and β subunits with the $\alpha_3\beta_3$ (or $\alpha_2\beta_2$) configuration (Nojiri and Omori, 2007). This homotrimeric structure is typical of class IA ROSs, whose terminal oxygenases have α_3 configurations (Ferraro *et al*., 2005).

CARDO catalyzes diverse oxygenation of aromatic compounds. Aside from angular dioxygenation, which is the most interesting feature of CARDO, biotransformation experiments with *E. coli* cells harbouring *carAa, carAc*, and *carAd* revealed the ability of CARDO to catalyze lateral dioxygenation and monooxygenation of aromatic substrates exhibiting broad substrate specificity (Figure 2.15) (Nojiri *et al*., 1999; Takagi *et al*., 2002). It was also observed that angular dioxygenation by CARDO occurs effectively at the angular position adjacent to an oxygen or nitrogen atom (due to high electronegativity of oxygen and nitrogen), but not a sulfur or carbon atom (Bressler and Fedorak, 2000; Nojiri and Omori, 2007).

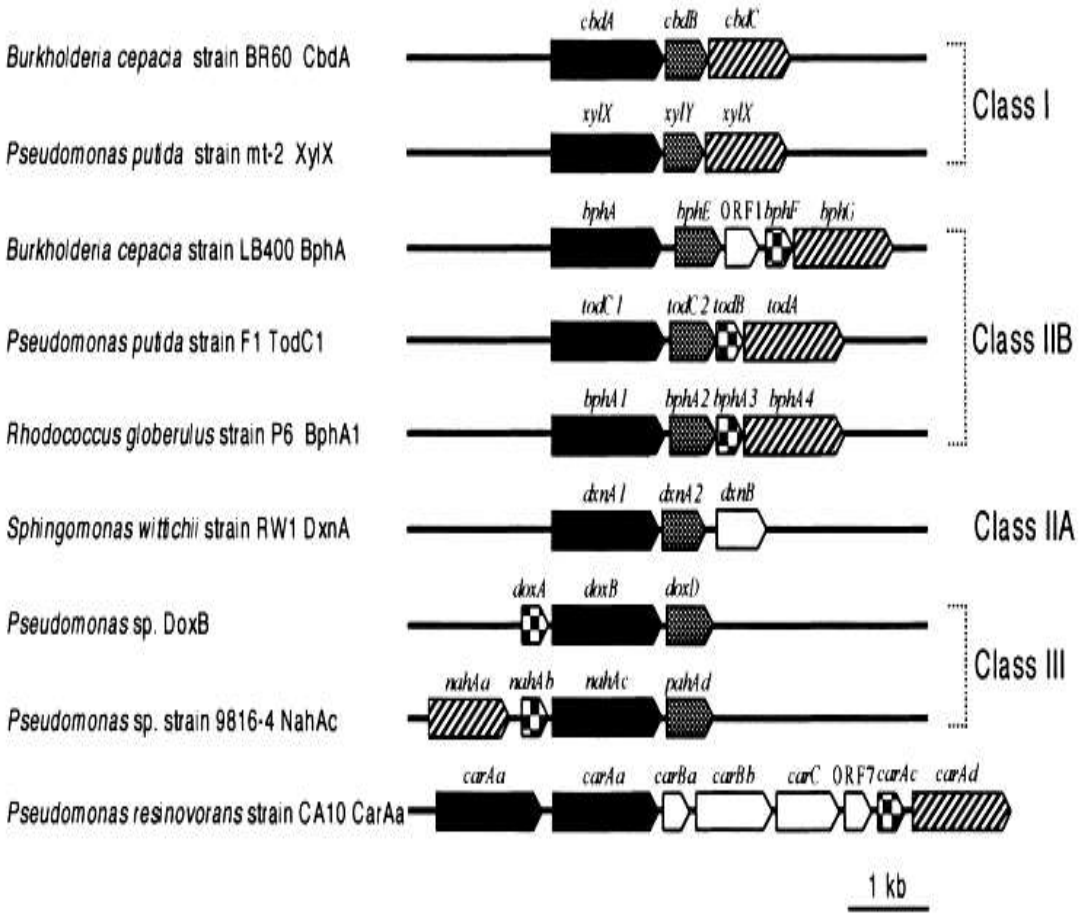


Figure 2.14: Organization of genes coding for different proteins of three component dioxygenase systems in representative strains of bacteria capable of degrading aromatic compounds (Hiraishi, 2003).

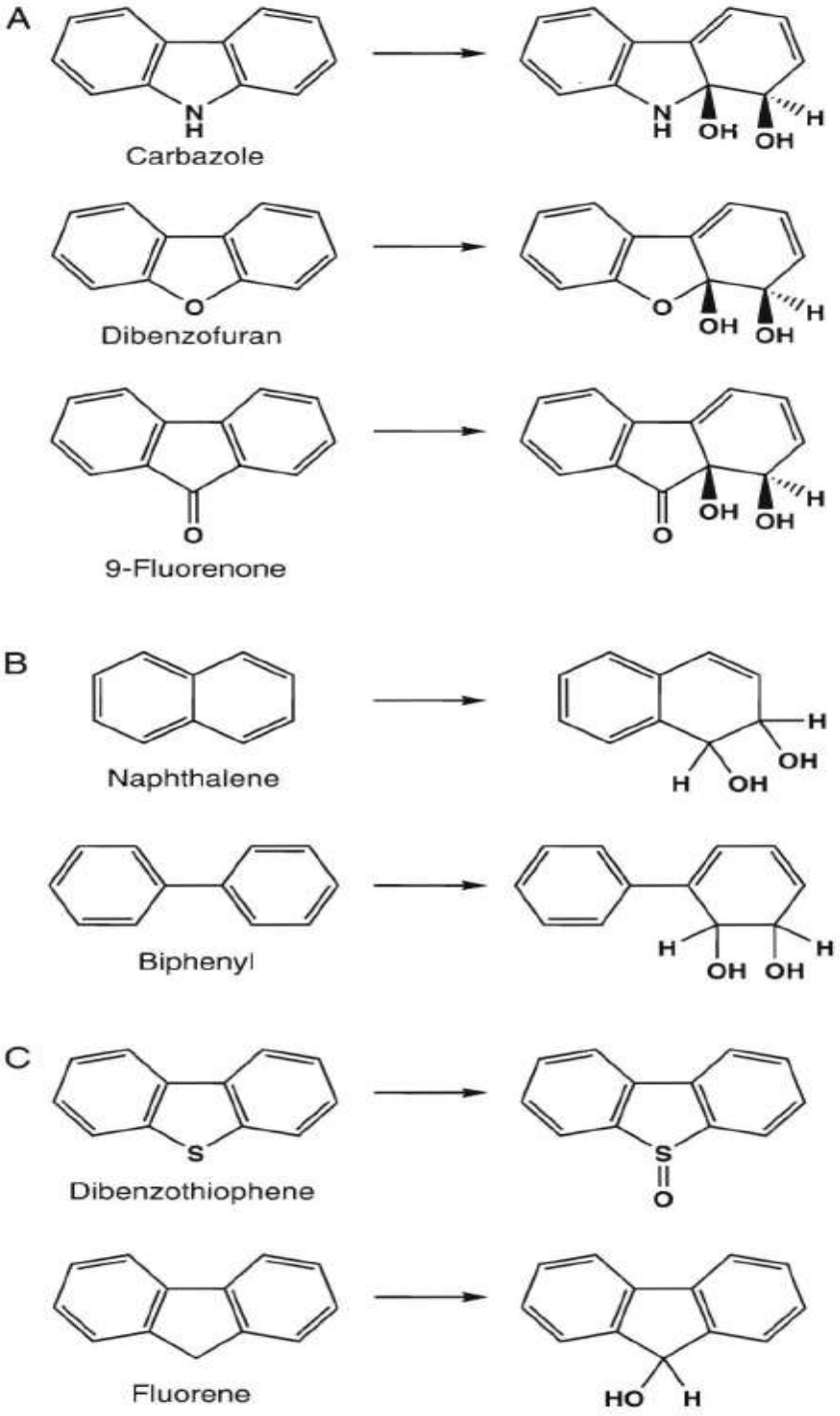


Figure 2.15: Diverse oxygenations catalyzed by CARDO. (a) Angular dioxygenation of carbazole, dibenzofuran and 9-fluorenone. (b) Lateral dioxygenation of naphthalene and biphenyl. (c) Monooxygenation of methylene carbon in fluorene and sulfoxidation of sulfide sulfur in dibenzothiophene (Nojiri *et al*., 1999; Takagi *et al*., 2002).

2.9 Factors affecting biodegradation of carbazole and related hydrocarbons in soil

Biodegradation is the breakdown of organic contaminants that occur due to microbial activity. It is a series of biological degradation steps or pathway catalyzed by specific enzymes that ultimately results in mineralization (oxidation of the parent compound to carbon dioxide and water) or transformation (partial oxidation of the parent compound). A number of parameters influence the survival and activity of microorganisms in any environment. It is therefore necessary to understand the factors limiting microbial degradation in soil in order to design appropriate technology needed to optimize the process of degradation.

2.9.1 Temperature

Temperature plays a very crucial role in biodegradation of hydrocarbons due to its direct effect on the chemistry of the pollutants and the physiology and diversity of microorganisms (Jain *et al*., 2011). Temperature affects the solubility of hydrocarbons (Foght *et al*., 1996) and although diverse ranges of temperature have been reported for hydrocarbon degradation, the rate of biodegradation generally decreases with decreasing temperature (Jain *et al*., 2011). Highest degradation rates in the soil environments generally occur in the range of 30-40°C (Bossert and Bartha, 1984). Carbazole biodegradation in soil generally occur at room temperature (28-30°C) (Habe *et al*., 2002; Widada *et al*., 2002). However, a soil bacterium *Pseudomonas* sp. strain C3211 isolated from a temperate climate degraded carbazole, dibenzothiophene and dibenzofuran at 10°C with acetone as co-substrate. In this strain, degradation was faster at 10°C than at 25°C thus, indicating that the strain is adapted to life at low temperatures (Jensen *et al*., 2003).

2.9.2 Nutrients

In a typical soil environment, essential nutrients are always limiting as microorganisms competed for it for growth and cellular activities. As a result, hydrocarbon degraders may not be present in sufficient number required for bioremediation of polluted sites (Vidali, 2001). Their growth and activity must be stimulated (biostimulation) by addition of nutrients and oxygen that serves as the basic building blocks of life needed by

autochthonous microorganisms to create necessary enzymes to break down the contaminants (Vidali, 2001). Nitrogen and phosphorus are necessary for cellular metabolism and can be found in low concentration in many soils. However, for effective biodegradation of pollutants, addition of fertilizers, pig dung, poultry droppings and other materials rich in nitrogen and phosphorus have been reported with relative successes (Atlas and Bartha, 1998; Okolo *et al*., 2005; Yakubu, 2007). Various concentration of carbon, nitrogen, phosphorus ratio has been suggested in literature. Vidali (2001) suggested C:N:P ratio of 100:10:1 for effective hydrocarbon degradation. Wang and Bartha (1990) found that effective remediation of hydrocarbon in soil required the addition of nitrogen and phosphorus to maintain a C:N ratio of 200:1 and a C:P ratio of 1000:1.

2.9.3 Chemical composition

Petroleum hydrocarbons can be divided into four classes: saturates, aromatics, asphaltenes and resins (pyridines, quinolines, carbazoles, sulfoxides, and arnides). Susceptibility of hydrocarbons to microbial attack varies based on their composition (Jain *et al*., 2011). Heterocyclic compounds in general are more difficult to degrade than their analogous mono- and polycyclic aromatics counterparts that contain only carbon. This is due to the higher electronegativity of nitrogen and oxygen atoms compared with the carbon atom (Bressler and Fedorak, 2000), which result in deactivation of the molecule toward electrophilic substitution. Heterocyclic compounds with five-membered rings and one heteroatom like carbazole are relatively biodegradable due to the involvement of non-bonding lone pair of heteroatom (in this case nitrogen) in aromatization, which make it more prone to attack by electrophilic agents and are hence more readily biologically hydroxylated (Balaban *et al*., 2004; Maier, 2009).

2.9.4 Solubility

A direct correlation has been reported between biodegradation of hydrocarbons in soil and their inherent water solubility as degrading bacteria in the unsaturated soil mainly occur in the interstitial water of soil (Jain *et al*., 2011). The number of aromatic rings influences hydrophobicity of aromatic compounds especially polycyclic aromatics

(PAHs) (Cerniglia, 1992). However, heterocyclic aromatic compounds exhibit higher polarity and water solubility due to substitution of one carbon atom by nitrogen, sulfur or oxygen (Meyer and Steinhart, 2000), which lead to their increased bioavailability and mobility as compared to homologous PAHs (Peddinghaus *et al*., 2012).

2.9.5 Bioavailability

Two steps are involved in biodegradation. First, is the uptake of the substrate by the cell, and second is the biodegradation of the substrate. However, for the pollutant to be taken up and metabolized it must be available in a water-soluble form, otherwise degradation of pollutant will be impeded if it has low water solubility or strongly sorbed to soil or sediments (Maier, 2000). Bioavailability is the amount of a substance that is physiochemically accessible to microorganisms (Jain *et al*., 2011).

Three modes of microbial uptake of liquid organic pollutants have been suggested. These include utilization of the solubilized organic compound, direct contact of cells with the organic compound through cell modification such as *fimbriae* (Rosenberg *et al*., 1982) or cell surface hydrophobicity that increase attachment of the cell to the organic compounds (Zhang and Miller, 1994), and direct contact with fine substrate droplets dispersed in aqueous phase. For solid phase organic pollutants, microorganisms can take up the substrate either by direct contact with the substrate or by utilization of solubilized substrate. The latter appeared to be the most important mode of microbial uptake to solid phase organics based on available evidence. This implies that low water solubility has a greater impact on degradation of solid-phase organics than on liquid-phase organics (Maier, 2009).

To overcome the challenges pose by hydrophobicity of organic contaminants and enhance uptake rate and biodegradation, some microorganisms produces biosurfactants, which serves two purposes. First, they increase the aqueous solubility of the contaminant through formation of micelles or vesicles that associate with the contaminant. Second, they facilitate attachment of cells to the contaminant by making the cell surface more hydrophobic thereby enhance its attachment to a separate oil phase (Herman *et al*., 1997).

Furthermore, introduction of external non-ionic surfactants and chemicals like Tween 20, polyoxyethylene, glycol monolaurate, dimethyl sulfoxide influences biodegradation rates of carbazole and enhance its dispersion and bioavailability (Ouchiyama *et al*., 1993; Hisatsuka and Sato, 1994).

Bioavailability of organic compound in soil can be affected by sorption of the compound by soil or sediment and diffusion of contaminants into soil matrix microsites that are inaccessible to bacteria due to pore size exclusion especially as the contaminant aged (Alexander, 1995). It can also be affected by incorporation of contaminant into soil organic matter catalyzed by oxidative enzymes present in the soil matrix, an irreversible process termed humification (Bollag, 1992).

2.9.6 Oxygen

Oxygen is a very important factor in biodegradation of organic compounds as it determines the bacterial pattern of dissimilatory and energy yielding process (Jain *et al*., 2011). Microbial degradation of aliphatic (Singer and Finnerty, 1984), cyclic (Perry, 1984), and aromatic (Cerniglia, 1997) hydrocarbons by bacteria and fungi required molecular oxygen as electron sink, which is used by microorganisms in the initial attack and in subsequent steps during the degradation process. Carbazole biodegradation is usually aerobic and to date, no single report exist detailing anaerobic biodegradation of carbazole.

2.9.7 Soil pH

Soil pH is an important parameter that is highly variable, ranging from 2.5 in mine spoils to 11.0 in alkaline deserts (Bossert and Bartha, 1984). Most bacteria favors a neutral pH of 7.0, however, fungi are more tolerant to acidic conditions (Jain *et al*., 2011). Highest degradation rates are generally observed at neutral pH. However, microorganism adapted to acidic environment with propensity for hydrocarbon degradation at a very acidic pH (pH 2-3) have been isolated from historically contaminated sites, though their diversity is low when compared with microorganisms that grow at neutral pH (Uyttebroek *et al*., 2007). Widada *et al*. (2002) reported that the proliferation and survival of marked strain CA10 cells in soil microcosms supplemented with 100 µg/kg of carbazole was influenced

by pH and organic matter. At soil pH of 6 with low organic matter, the number of marked CA10 rapidly decreased while at soil pH of 7.3 and 2.5% organic matter, a high cell density was maintained up to 21 days after inoculation and complete biodegradation of carbazole was shortened from 21 to 7 days.

2.9.8 Organic matter content

In soil, oxygen and organic matter concentrations decrease with depth. This explains the high microbial population and activity synonymous with surface soils. In contrast, microbial population in deep vadose zone and groundwater region are two orders of magnitude lower than the population in surface soil due to reduced organic matter content and oxygen (Maier, 2009). As such, surface soil has higher biodegradation rates than vadose zone and groundwater due to presence of nutrients and oxygen, which support aerobic biodegradation of contaminants by microorganisms. Thus, decreasing rate of biodegradation will persist with increase in depth.

2.9.9 Water activity

Activity of soil microorganisms are optimized when between 38 and 81% of soil pore space is saturated with water. Availability of water and oxygen are maximized in this range of water content. However, at higher water contents, oxygen replenishment is limited due to slow rate of oxygen diffusion through water, thereby limiting aerobic activity (Maier, 2009). Thus, the available water for microbial growth and metabolism may limit hydrocarbon biodegradation in soil (Leahy and Colwell, 1990). In a study of oil sludge degradation in soil, Dibble and Bartha (1979) reported optimal rates of biodegradation at 30 to 90% water saturation. Similarly, Grosser *et al*. (1991) while studying the mineralization of pyrene, benzo (a) pyrene and carbazole by indigenous soil microorganisms used 80% water holding capacity as optimum value that support effective biodegradation of the pollutants.

2.10 Bioremediation

Bioremediation is the exploitation of biodegradative activities of microorganisms to remove environmental pollutants and recalcitrant xenobiotics (Habe *et al*., 2001). Rapid

industrialization in agriculture, expansions in the chemical industry and the need to generate cheap form of energy to drive domestic and industrial activities have resulted in an ever-increasing reliance on anthropogenic organic chemicals with its attendant contamination of a significant number of soil environments (Reid *et al*., 2000).

Bioremediation offers a refreshing and environment-friendly alternative to conventional technologies that involve removal, alteration, or isolation of the pollutant. Such technology typically consists of excavation of contaminated soil followed by its incineration or containment. However, these technologies are expensive, not environment-friendly and in most cases do not destroy the pollutants but transfer them from one environment to the other (Crawford, 2002).

Bioremediation can be classified into three types. Biotransformation is the alteration of contaminant molecule into less or non-hazardous molecules; biodegradation is the catabolism of the contaminant usually by microorganisms into less complex compounds or inorganic molecule; and mineralization is the complete catabolism of the contaminant by microorganisms into inorganic constituents such as carbon dioxide or methane and water (Maier, 2009). Any of these three classes of bioremediation can occur *in situ* (at the site of contamination) or *ex situ* (contaminant taken out of the site of contamination and treated elsewhere).

The containment of the contaminant in *ex situ* bioremediation allows for their easy monitoring and maintenance of optimum conditions, which facilitate the bioremediation process. However, the removal of the contaminant is time-consuming, costly and potentially dangerous as it increases exposure of the contaminant to the workers and the public. The contaminant extraction strategies to be adopted in *ex situ* bioremediation will depend on the nature of the contaminant (whether it is liquid, gas or solid phase), its chemical properties and its toxicity (Andreoni and Gianfreda, 2007).

In contrast, *in situ* bioremediation allows biodegradation of contaminants without excavation or removal and applied biostimulation or bioaugmentation for decontamination of polluted soil. Biostimulation is the addition of supplementing carbon sources, nutrients, oxygen and other electron donors and acceptors to the contaminated

site in order stimulate the activity of indigenous or inoculated degrading strains (Andreoni and Gianfreda, 2007). Bioaugmentation is the introduction of strains with desired degradative capabilities against the target pollutants, either with or without nutrients, into the contaminated environment to augment the indigenous microbial population. Although *in situ* bioremediation is preferred because of, lower cost and reduced risk of exposure of the contaminant, it is very difficult to control condition and monitor progress because the bioremediation site is not contained (Crawford, 2002).

Soil microcosm studies in the laboratory allow manipulation of various environmental factors and growth conditions that could favor optimum activity of degrading microorganisms and facilitate effective biodegradation and bioremediation of polluted soils. Results obtained from such studies could be useful in designing novel bioremediation strategies that may be necessary in reclaiming polluted soil in field conditions.

However, it must be borne in mind that the purpose of soil bioremediation is not only to enhance timely degradation, transformation, remediation or detoxification of pollutants by biological means, but also to protect soil quality (Adriano *et al*., 1999). Soil quality is defined as the capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health (Doran and Parkin, 1994).

2.11 Soil Microbial Diversity

Soil is a very complex natural environment with respect to the size of microbial community and the diversity of species. One gram of forest soil contains an estimated 4 x 10^7 prokaryotic cells while one gram of cultivated soils and grasslands contained an estimated 2 x 10^9 prokaryotic cells respectively (Richter and Markewitz, 1995). Based on DNA-DNA reassociation kinetics, Torsvik *et al*. (1990) estimated that there are 4000 different bacterial "genomic units" in 1 g of soil. Similarly, DNA-DNA reassociation studies conducted on DNA isolated from different soil samples revealed that the number of distinct prokaryotic genomes per gram of soil range between 2,000 and 18,000

genomes (Torsvik *et al*., 1996, 1998, 2002). Extreme spatial heterogeneity of soil, its multiphase nature, and complex biological and chemical properties are believed to contribute immensely to the microbial diversity present in soil samples (Daniel, 2005).

2.11.1 Soil as a habitat for microorganisms

Soil is a highly complex, heterogeneous environment comprising mineral particles of different sizes, shapes and chemical characteristics; soil biota and organic compounds in various stage of decomposition. Soil microbiota including bacteria, fungi and protozoa is closely associated with soil particles, especially clay-organic matter complexes (Foster, 1988). Prokaryotes are the most abundant organisms in soil and constitute the largest component of soil biomass. The microhabitats for soil microorganisms include the interior (complex pore spaces between and inside the aggregates) and exterior surfaces of soil aggregates of varying sizes (<250 µm, 250 µm to 2 mm, and >2 mm) and compositions (Foster, 1988; van Elsas *et al*., 2002). Cellular metabolism and survival of soil microorganisms are strongly influenced by the availability of water and essential nutrients while cyclic perturbation in surface soil water content and other environmental factors causes fluctuation in the composition of soil microbial community (Daniel, 2005).

Prokaryotes plays a key role in the biogeochemical cycles of essential and trace elements and are thus heavily implicated in nutrients and energy exchanges within the soil. Their activities also has the potential to reflect past history of a given soil environment (Ranjard *et al*., 2000). It is therefore imperative to understand the interrelationships between bacteria and their environment by studying structural and functional diversity of bacterial communities in soil and their response to various natural and anthropogenic perturbations.

2.11.2 Methods of studying microbial diversity in soil

Soil microbial diversity comprises species diversity, genetic diversity and ecosystem biodiversity (Solbrig, 1991). Species diversity encompasses species richness (presence of many different species), the total number of species present, species evenness (equal abundance), and the distribution of species (Trevors, 1998; Ovreas, 2000). Typically, diversity studies include the relative diversities of communities across a gradient of

stress, disturbance or other biotic or abiotic differences (Hughes *et al*., 2001). In hydrocarbon-contaminated soils, microbial community structures are influenced by a number of factors such as soil type, concentration and bioavailability of the contaminants, nutrient contents, temperature, oxygen content and pH (Margesin and Schinner, 2001; Greer *et al*., 2010). Measurement of microbial diversity in soil employs two different methods, the biochemical-based method and the molecular-based method (Kirk *et al*., 2004).

2.11.2.1 Biochemical-based methods

The use of standard culture technique to study microbial diversity in soil involves isolation and characterization of microorganisms using commercially available growth media such as Luria-Bertani medium, nutrient agar, and tryptic soy agar (Kirk *et al*., 2004). Diversity using standard plate counts is assessed using selective plating and direct viable counts. These methods are fast, cost effective and provide information on the active, heterotrophic component of the population. However, difficulty in dislodging bacteria or spores from soil particles, growth medium selections, growth conditions, inability to culture large number of bacterial species, cultivation bias towards fast growing organisms and the potential for colony spreading or colony-colony inhibition are major drawbacks that influence the diversity of the microbial community (Dix and Webster, 1995; Trevors, 1998; Tabacchioni *et al*., 2000).

The consequence of using standard plate counts to study microbial diversity is that >99% of soil microorganisms that are viable but non-culturable (VBNC) will remain undetected even if they are metabolic active, abundant and contribute immensely to soil health and detoxification of anthropogenically perturbed soil. Molecular microbial surveys based on 16S rRNA conducted by Schloss and Handelsman (2004) revealed bacterial divisions such as BRC1, OP10, OP11, SC3, TM7, WS2, and WS3 that have no cultural representatives and are recognized only by their nucleotide sequences. This is in spite of the fact that these division-level clades, especially OP11 are highly diverse and widely distributed in different environments, thus exposing our limited knowledge on microbial diversity in soil (Rastogi and Sani, 2011).

In addition, it has been observed that soil microorganisms retrieved using conventional cultural techniques are rarely numerically abundant or functionally significant in soil and are considered as "weeds" of the microbial world constituting <1% of all microbial species (Hugenholtz, 2002). As an example, it is common knowledge that most of the bacterial species cultured from soil samples belong to one of the "big four" phyla- *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* due to their ease of cultivation under laboratory conditions. However, *Acidobacteria*, which constitute on average 20% of soil bacterial communities are rarely isolated because they are difficult to culture and are represented by few genera (Schloss and Handelsman, 2004).

Fatty acid methyl ester (FAME) analysis is a biochemical method that does not depend on cultural method to determine microbial biodiversity in soil. It is a method based essentially on the fact that fatty acids constitute a relatively constant proportion of the cell biomass (Kozdroj and van Elsas, 2001) and some signature fatty acids exist that can differentiate major taxonomic groups within a community. Thus, fatty acid groupings are used to provide information on the soil microbial community as a change in the fatty acid profile represents a change in microbial population (Ibekwe and Kennedy, 1998).

In FAME analysis, fatty acids are extracted directly from soil, methylated and analyzed by gas chromatography (Ibekwe and Kennedy, 1999). FAME analysis can be used to detect changes in the composition of bacterial community and follow signature fatty acids of different groups of microorganisms (Kirk *et al*., 2004). Though FAME analysis can be used to study microbial diversity, it is a poor method with many drawbacks. These include the influence of factors such as temperature and nutrition on cellular fatty acid composition, confounding of the FAME profiles by other organisms, and the impracticability of using individual fatty acids to represent specific species as individuals can have numerous fatty acids and the same fatty acids can occur in many species (Graham *et al*., 1995; Bossio *et al*., 1998).

Thus, for in-depth characterization of soil microbial communities, a technique that circumvents the need for cultivation and isolation is highly desired. The search for such technique led to the molecular approach for studying soil microbial diversity.

2.11.2.2 Molecular-based methods

Due to obvious limitations highlighted in the use of biochemical-based methods for studying soil microbial diversity, researchers have switched more often to molecular strategies to decipher qualitatively and quantitatively soil microbial diversity. These molecular based methods have been broadly classified into two major categories depending on their capability of revealing the microbial diversity structure and functions- the partial community analysis approaches and whole community analysis approaches (Rastogi and Sani, 2011).

2.11.2.2.1 Partial community analysis approach

This approach is premised on the polymerase chain reaction (PCR)-based methods where total RNA/DNA extracted from the soil sample is used as a template for the characterization of microorganisms. The PCR product generated reflects in principle a mixture of microbial gene signatures from all organisms present in the soil sample. In PCR amplification of extracted total DNA of soil samples, the target gene is the 16S rRNA. This is because these genes are large molecule (~1500 bp) with considerable genetic information, ubiquitous in all prokaryotes, structurally and functionally conserved, contain variable and highly conserved regions and do not engage in lateral gene transfer (Rosseló-Mora and Amann, 2001; Hugenholtz, 2002). Furthermore, the availability of a comprehensive sequence dataset for comparison in widely accessible databases makes 16S rRNA a “gold choice” in microbial ecology (Rosseló-Mora and Amann, 2001; Rastogi and Sani, 2011).

The amplified PCR products from environmental DNA are analyzed primarily by (1) clone library method, (2) genetic fingerprinting techniques such as amplified ribosomal DNA restriction analysis (ARDRA), ribosomal intergenic spacer analysis (RISA), denaturing gradient gel electrophoresis/temperature gradient gel electrophoresis (DGGE/TGGE), and terminal-restriction length polymorphism (T-RFLP), and (3) DNA microarrays or by a combination of these techniques. Through amplification and comparisons of PCR-amplified DNA sequences, these techniques have been used to

characterize microbial communities from contaminated environments (Malik *et al*., 2008).

Clone library method is the most widely used method to analyze PCR products amplified from environmental DNA. It involves cloning the PCR products and sequencing the individual gene fragments (DeSantis *et al*., 2007). The obtained sequences are then compared to known sequences in a database such as GenBank, Ribosomal Database Project (RDP) and Greengenes. Cloned sequences are assigned to phylum, class, order, family, subfamily, or species at sequence similarity cut-off values of 80, 85, 90, 92, 94, or 97% respectively (DeSantis *et al*., 2007). Although more expensive and time-consuming than community fingerprinting techniques, sequence analysis of clone libraries provides an unparallel level of phylogenetic resolution due to the long read lengths generated by Sanger sequencing technology (Leigh *et al*., 2010).

Genetic fingerprinting techniques produce community fingerprints generated from the physical separation of rRNA or DNA sequences on a gel (Muyzer, 1999) based on either sequence polymorphism or length polymorphism using PCR product amplified from environmental DNA. Genetic fingerprinting techniques are rapid and allow analysis of large number of samples simultaneously. Though it only produces a "snapshot" of microbial community as it only detect dominant organisms, produces complex bands, and exhibit weak phylogenetic resolution (Kirk *et al*., 2004; Nakatsu, 2007) , it is still widely used to demonstrate an effect on microbial communities or differences between microbial communities (Rastogi and Sani, 2011).

2.11.2.2.1.1 Clone library Analysis of 16S rRNA

Construction of clone libraries from PCR- amplified 16S rRNA genes obtained from soil total DNA extracts is the most widely used means of assessing soil microbial community composition and diversity. Although it is more expensive and time-consuming than community fingerprinting techniques such as terminal restriction fragment length polymorphism (T-RFLP) and DGGE, sequence analysis of clone libraries provides unparalleled level of phylogenetic resolution due to the long read lengths generated by Sanger sequencing technology (Leigh *et al*., 2010). The major steps involved in the

construction of clone libraries for microbial community analysis include soil sample collection, DNA extraction, PCR amplification of 16S rDNA, cloning of 16S rDNA PCR products, plasmid extraction/colony PCR and sequence analysis.

2.11.2.2.1.1.1 Soil Sample collection

Due to the heterogeneous nature of soil samples, detailed information on the chemical, physical and biotic factors such as particle size, soil type, water content, pH, temperature, and plant cover are useful for evaluation and comparison of the outcomes of soil-based studies (Daniel, 2005). The amount of soil sample needed usually depend on the microbial biomass present (Nakatsu, 2007). Commonly used kits for soil DNA extraction usually recommend 0.5 g, which yield sufficient amount of DNA for community analysis (Mumy and Findlay, 2004). Sampling is easier for surface soils compared with other soil compartments and due to soil heterogeneity, large quantities of soil are usually collected and homogenized to ensure that they are representative of that ecosystem, which must be confirmed by adequate replication (Daniel, 2005; Kang and Mills, 2006). Alteration of the composition of soil microbial community may ensue if soil is disturbed during sampling or transported and stored for a long time (Daniel, 2005).

2.12.2.2.1.1.2 DNA extraction

The essence of this step is to obtain high quality DNA from a mixed soil microbial community. DNA extraction from soil is particularly challenging because of soil heterogeneity, the extent of microbial diversity and the adherence of microorganisms to soil particles (Martin-Laurent *et al*., 2001). Compounding the problem of soil DNA extraction is the coextraction of humic substances, which interferes with restriction-enzyme digestion and PCR amplification, reduce cloning efficiency, transformation eficiency, and the specificity of DNA hybridization (Steffan *et al*., 1988; Tsai and Olsen, 1992; Tebbe and Vahjen, 1993). Though the basics of soil DNA extraction is fundamentally the same, however, protocols with many technical variations for extraction abound throughout the literature (Niemi *et al*., 2001; de Lipthay *et al*., 2004). There is no apparent consensus on a single method of soil DNA extraction and empirical testing is often required to identify a protocol that is effective for a particular soil sample. The

major factors influencing method choice are maximum cell lysis, minimal contamination from soil organic chemicals, release of DNA from soil and minimal shearing of DNA. other factors worthy of consideration include the amount of soil required, time needed to perform the extraction, required technical expertise, and cost (Nakatsu, 2007).

2.11.2.2.1.1.3 Polymerase chain reaction (PCR) amplification

Clone library analysis strongly relies on PCR amplification and as such, selection of appropriate primers and optimization of PCR conditions especially when DNA extracts from a new community are being examined or new PCR primer are being used are very critical for accurate characterization of soil microbial communities (Schmalenberger *et al*., 2001; Nakatsu 2007).

In most microbial community analysis, the small subunit, 16S rRNA has been used extensively as a phylogenetic marker as it contains both variable and conserved regions. Highly conserved regions act as alignment guides and are convenient sites for annealing of universal primers, while moderately and highly variable regions allow discrimination between groups and organisms (Head *et al*., 1998). The selected target genes are amplified by the polymerase chain reaction (PCR) from DNA extracts obtained from soil and the PCR products are sequenced.

In optimizing PCR conditions, variables such as primer-annealing temperature, concentration of the polymerase enzyme cofactor Mg^{2+}, and DNA template concentration are commonly optimized for successful amplification (Boleda *et al*., 1996; Ishii and Fukui, 2001). Chimeras formation, preferential amplification of selected targets, non-specific amplification of non-targets, and the production of single-stranded products are some of the PCR-generated errors that can be recognized by conducting replicate analyses and making comparisons of profiles under different PCR conditions (Nakatsu, 2007; Qiu *et al*., 2001; Thompson *et al*., 2002).

2.11.2.2.1.1.4 Cloning, Plasmid extraction/Colony PCR and Sequence Analysis

However, before sequencing the PCR products, the mixture of amplicons with different sequences must be separated from each other, and additional copies of each must be made, a process termed clone library construction.

Cloning involves the ligation of the PCR-amplified genes into plasmid vector using either "T-A" cloning or TOPO TA cloning system, followed by the transformation of competent cells with the recombinant vector. Colonies derived from individual transformed cells are then picked and regrown to produce larger quantities of plasmid for extraction (or colony PCR) followed by sequence analyses (Leigh *et al*., 2010). Obtained sequences may be compared to GenBank, Ribosomal Database Project (RDP-II), Greengenes and EMBL databases (Cole *et al*., 2005; DeSantis *et al*., 2006; Benson *et al*., 2007; Kulikova *et al*., 2007) to determine the taxonomic affiliations of the source organisms.

Various researchers have employed 16S rRNA clone library analysis to decipher the microbial diversity of hydrocarbon-contaminated soils. This is not surprising as this technique provides an unparallel level of phylogenetic resolution due to the long read lengths generated by Sanger sequencing technology (Leigh *et al*., 2010). This in effect allows detailed information to be generated about the structure and diversity of microbial communities in these oil-impacted ecosystems.

A study was conducted by Nogales *et al*. (2001) to analyze the bacterial community structure and diversity of a polychlorinated biphenyl-polluted soil using both rDNA and two rRNA clone libraries. The authors observed that nearly 29% of the cloned sequences in the rDNA library were identical to sequences in the rRNA libraries. In addition, a qualitative correspondence of clone frequency in the two types of libraries were observed with α- and β- *Proteobacteria* and *Acidobacterium* phyla dominating. However, the major difference in the two types of libraries as opined by the authors is the absence of clone representatives of the *Actinobacteria* phylum in the rDNA library.

Similarly, a combination of culture-independent and culturing method was used in another study to determine the impacts of hydrocarbon contamination on the diversity of

bacterial communities in coastal soil from Ross Island, Antarctica (Saul *et al*., 2005). The 16S rRNA clone library results indicates 76% and 6% of the 367 clones phylogenetically analyzed belongs to the phyla *Proteobacteria* and *Actinobacteria*. Similar results, albeit with higher percentage of *Actinobacteria* were obtained when culturing method was used with 65% and 26% of the 88 bacterial isolates belonging to the phyla *Proteobacteria* and *Actinobacteria*, respectively. However, the divisions, *Fibrobacter*/*Acidobacterium*, *Cytophaga*/*Flavobacterium*/*Bacteroides* (CFB), *Deinococcus*-*Thermus* and Low G+C Gram positives were inconspicuously absent from the hydrocarbon-contaminated soil (Saul *et al*., 2005).

Investigation on the diversity of the active microflora in a degrading soil remediation system for mineral oil hydrocarbon-contaminated soils was conducted by Popp et al. (2006) using small-subunit (SSU) rRNA analysis. The two clone libraries generated were dominated by γ-*Proteobacteria*, followed by α- and β-*Proteobacteria*. In addition, lower clone frequency of the phyla *Actinobacteria*, *Firmicutes*, *Bacteroidetes* and *Epsilonproteobacteria* were observed in the two clone libraries. Furthermore, novel bacteria genera such as *Zymomonas* and *Rhodoferax* were recovered from the mineral oil hydrocarbon-contaminated soil (Popp *et al*., 2006).

Similar findings highlighting the preponderance of the phylum *Proteobacteria* in hydrocarbon-contaminated soils using both culture-dependent and culture-independent methods have also been reported by other authors (Barragan *et al*., 2008; Liu *et al*., 2009; Zhang *et al*., 2012). This is in contrast to pristine soils where *Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, Gram-positives with low GC content, and *Alphaproteobacteria* are abundant (Popp *et al*., 2006; Barragan *et al*., 2008; Zhang *et al*., 2012).

Several reasons have been adduced to the predominance of *Proteobacteria* in hydrocarbon-contaminated soils. Many of the bacterial genera belonging to this phylum are efficient hydrocarbon degraders and the consequential reduction of biodiversity because of hydrocarbon contamination favors their propagation (Barragan *et al*., 2008; Greer *et al*., 2010). In addition, it has also been observed that in many diversity studies on hydrocarbon-contaminated soils where the phylum *Proteobacteria* is dominant, the class

γ- *Proteobacteria* seems preponderant (Saul *et al*., 2005; Popp *et al*., 2006; Liu *et al*., 2009). This is attributed to a concept called 'γ-shift' believed to occur under nutrient oversupply conditions (Amann *et al*., 1995) as exemplified in highly polluted, hydrocarbon-contaminated niches. This encourages the dominance of γ- *Proteobacteria* due to degradation of high levels of contaminants (Chao and Hsu, 2004; Gerdes *et al*., 2005).

Several authors have reported distribution and occurrence of other bacterial phyla in hydrocarbon-contaminated soils using 16S rRNA clone library. Though in relatively low numbers, bacterial phyla such as *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia*, *Chlorobi*, *Spirochaetes*, *Chloroflexi*, *Deinococcus-Thermus*, and candidate divisions (OP5, OP8, OP10, OP11, TM7, BRC1, and OD1) have been recovered from diverse contaminated soil environments (Nogales *et al*., 2001; Saul *et al*., 2005, Allen *et al*., 2007; Liu *et al*., 2009). In addition, a sizeable number of clones recovered from these hydrocarbon-impacted niches are regarded as 'Unclassified bacteria'. This implied that these clones are not affiliated to any known bacterial phyla and a new phylum may be required for their placement. Furthermore, in spite of their diversity and wide distribution in different environments, clones belonging to the 'candidate divisions' have no cultural representatives and are recognized only by their nucleotide sequences (Rastogi and Sani, 2011). Thus, 16S rRNA clone library offers better phylogenetic resolution than community fingerprinting tools and is the most widely used means of assessing microbial community composition and diversity.

3.1 MATERIALS

3.1.1 Study Sites

Samples for this study were obtained from three different sites (Figure 3.1).

3.1.1.1 Abandoned coal power plant soil (ACPP)

This site is located within the premises of Power Holding Company of Nigeria (PHCN) formerly National Electric Power Authority (NEPA) at Ijora-Olopa, Lagos. The coordinates of the site are latitude 6° 28' 1″ N and longitude 3° 22’ 47″ E. The soil has a long history of contamination due to the use of coal plant for electric power generation in the 1950s with concomitant disposal of used and spent oils. The polluted soil is dark in colour and often moistened with oils.

3.1.1.2 Mechanic workshop, Okokomaiko (MWO)

This site is an open ground located within a mechanic workshop at Tipper garage bus stop, Okokomaiko where engine parts are repaired and serviced. The coordinates of the site are latitude 6° 28' 23″ N and longitude 3° 11' 14″ E. The sampling point is a designated place where all used and spent oils within the workshop are dumped for years. The soil is dark-brown in colour, moistened with oil with an attendant irritating odour.

3.1.1.3 NEPA substation, UNILAG (NESU)

This site is located within the premises of NEPA substation, at the University of Lagos, Akoka. The coordinates of the site are latitude 6° 31' 18″ N and longitude 3° 23' 47″ E. The sampling point is also an open ground at the entrance of the workshop where used and spent oils are deposited as evident in the colour and odour of the soil.

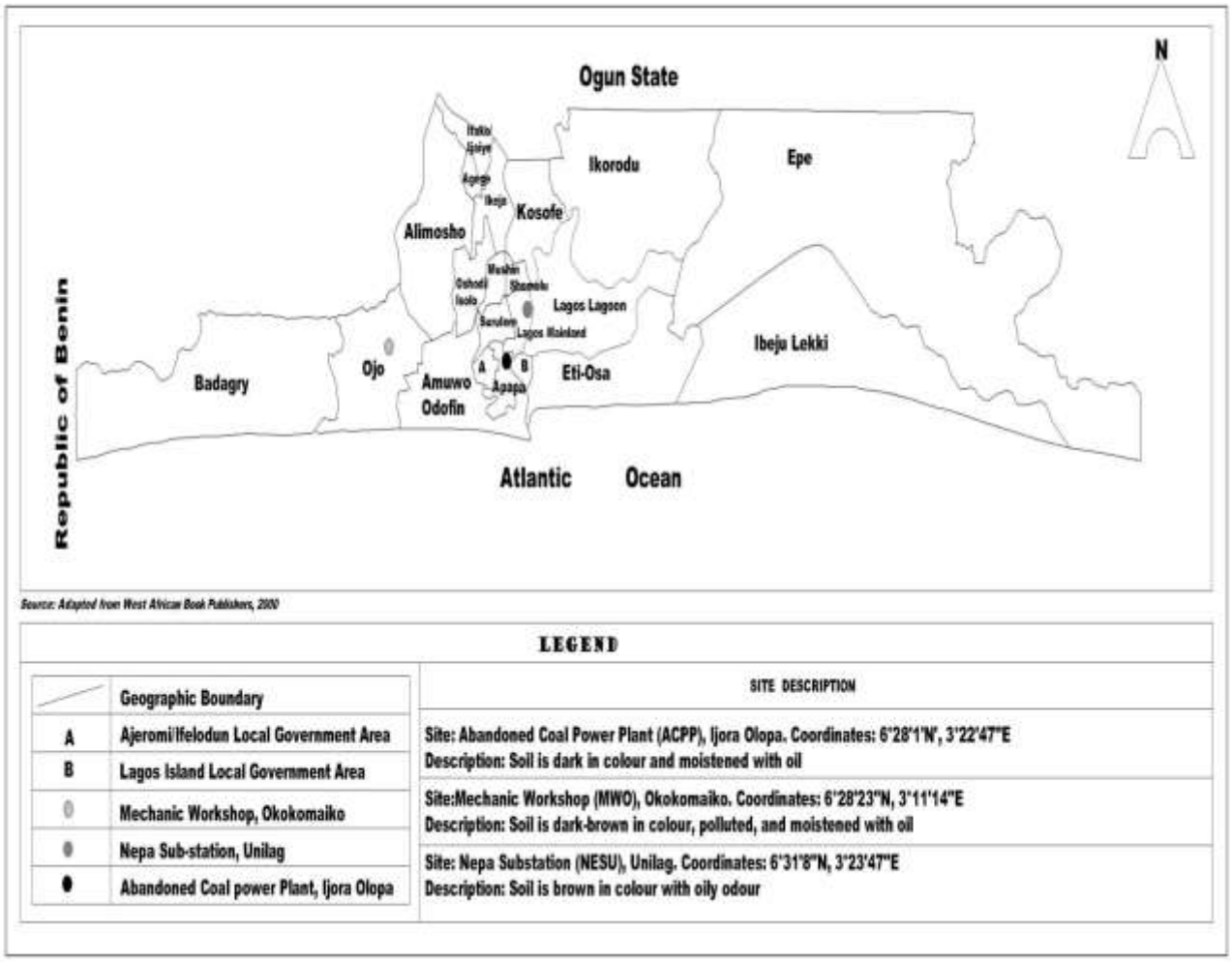


Figure 3.1: Map of Lagos State, Nigeria showing the sampling points used in this study. The location of ACPP (●), MWO (●) and NESU (●) were indicated on the map.

3.1.2 Sampling

Soil samples were collected at a depth of 10-12 cm using sterile hand trowel after removing the debris from the soil surface. Samples for physicochemical analysis were collected in clean black polythene bags, while samples for microbiological analysis were collected in sterile screw-capped bottles. Immediate analysis of the samples were carried out within 5 h of collection or stored at 4°C until treatment is feasible.

3.1.3 Chemicals Used for the Study

Ethyl acetate, dimethyl sulfoxide (DMSO), carbazole, anthranilic acid, pyrene, acenaphthene, dibenzothiophene, dibenzothiophene sulfone, ethyl carbazole, sodium sulfate, and other chemicals and reagent were of analytical grade from Kanto chemicals (Japan) and Sigma Aldrich. All other reagents and chemicals used for molecular biology work were of molecular grade and obtained from Sigma Aldrich and Kanto chemicals.

3.1.4 Sterilization and Aseptic Techniques

Materials used in this study were sterilized as follows:

3.1.4.1 Glassware

All glassware e.g. beakers, test tubes, conical flasks, and McCartney bottles were washed in detergent solution, rinsed with tap water and allowed to dry before placing them in the oven for sterilization. Dry heat sterilization with the oven was carried out at 170°C for at least 6 h.

3.1.4.2 Media

All media were sterilized by pouring them into conical flasks and test tubes, plugging the flasks and tubes with non-absorbent cotton wool and wrapping the wool and the neck of the flasks with aluminium foil. The media was sterilized in an autoclave at 121°C for 15 min.

3.1.4.3 Sugar Solutions

Due to the possibility of sugar decomposition at autoclaving temperature, sugar solutions were tyndallized for 30 min daily by moist heat steaming at 100°C for three days.

3.1.4.4 Physiological Saline

Appropriate volume (9 ml) of physiological saline (distilled water containing 0.85% NaCl) was dispensed in MacCartney bottles and sterilized at 121°C for 15 minutes.

3.1.4.5 Work Bench

Alcohol (70%) was used to wipe the working area and rid it of unwanted microorganisms. This is achieved by thorough wiping of the workbench with alcohol before and after each experiment.

3.1.4.6 Inoculating Loop

Inoculating loop was sterilized by flaming the platinum loop over a blue bunsen flame until red-hot and thereafter allowed to cool before use.

3.1.4.7 Glass Rod

Glass rod (hockey stick) was sterilized by first dipping it in absolute alcohol, then igniting it in a bunsen flame and thereafter allowed cooling before use.

3.1.4.8 Filter Papers

Filter papers were wrapped in aluminium foil paper and sterilized by autoclaving at 121°C for 15 min.

3.1.4.9 Serial Dilutions

McCartney bottles containing 9 ml of sterile physiological saline were used. Soil sample (10 g) was weighed and added into a conical flask containing 90 ml of sterile diluent (physiological saline), then mixed thoroughly and labelled as 10^{-1} dilution. Using a fresh sterile pipette tip, 1.0 ml of the 10^{-1} dilution was aseptically transferred to another bottle to obtain a 10^{-2} dilution. This exercise continued serially until 10^{-7} dilution was reached.

3.1.4.10 Culture Media

In this study, various types of solid and liquid media were used. They were generally sterilized by autoclaving at 121°C for 15 min except indicated otherwise.

3.1.4.10.1 Liquid Media

The Carbon-Free mineral medium (CFMM) described by Habe *et al*. (2002) and modified by the addition of 0.05 g of yeast extract was routinely used for enrichment.

The composition is presented in Appendix I. Part A of the medium was sterilized by autoclaving at 121°C for 15 min, while stock solution of Part B was prepared by dissolving appropriate concentration of the minerals in 1 ml distilled water and filter-sterilized using 0.22 µm pore size filter. Part A and B were then mixed thoroughly after sterilization. Other liquid media used include mineral salts medium described by Kästner *et al*. (1994), nutrient broth, Luria Bertani (LB) broth, peptone water, and methyl red Voges-Proskauer medium. The compositions of the liquid media used are listed in Appendix I.

3.1.4.10.2 Solid Media

Solid media used include nutrient agar, potato dextrose agar, nutrient gelatin, starch agar, starch casein nitrate agar for enumeration of actinomycetes, and Ashby's mannitol salt agar for enumeration of nitrogen fixers. Carbon-Free mineral medium was solidified by addition of 1.6% (w/v) bacteriological agar to the medium. The compositions of all solid media used are listed in Appendix I.

3.1.5 Reagents

Heat-stable reagents such as Tris-HCl, EDTA (ethylenediamenetetraacetic acid), Tris-base, glacial acetic acid, sodium acetate, and potassium acetate were sterilized by autoclaving at 121°C for 20 min. However, heat-labile reagents like glucose solution, stock ampicillin, stock isopropyl-β-D-thiogalactopyranoside (IPTG) were filter-sterilized using a 0.22 µm pore size filter and stored at -20°C.

3.1.6 Incubation

Incubation temperature of all inoculated media was at room temperature (27 ± 2°C), 37°C or as otherwise stated.

3.2 METHODOLOGY

3.2.1 Determination of Physico-Chemical Properties of Soil

3.2.1.1 Moisture Content

Soil samples of known weight (10 g) were put in crucibles (of known weight), and placed in an oven maintained at 105°C for at least 24 hrs or until the weights were constant. The samples were removed from the oven, allowed to cool, and weighed again. The losses in weights were taken as the weights of moisture for the respective samples and are expressed as percentages of soil samples.

3.2.1.2 Soil pH

The pH of soil sample was determined using the electrometric method. Known weight of soil sample (10 g) was mixed with 25 ml of sterile distilled water in a beaker. The soil slurry was stirred and allowed to stand for 1 h. After a second of stirring, the pH was measured in the supernatant with a pH meter (Model HI 99104, Hanna Instruments, Mauritius).

3.2.1.3 Soil Conductivity

Soil sample (5 g) was mixed with distilled water (100 ml) in a conical flask and shaken for 20 h or overnight. The solution was thereafter filtered. Conductivity cell (PW 9504 Philips) was calibrated with 0.01 M standard potassium chloride (KCl) solution and rinsed first with distilled water followed by twice rinsing with soil water suspension. The cell was dipped in the filtered solution and the conductivity in µs/cm was measured.

3.2.1.4 Water-Holding Capacity

Soil sample (150 g) was weighed and dried in an oven at 105 °C for 24 h or until the weight is constant. The oven-dried soil was soaked in water for 24 h. After 24 h, the water was decanted from the soil ensuring that no soil was decanted with the water. The soaked soil was thereafter weighed. The difference between the weights of soaked soil and oven-dried soil divided by the weight of oven-dried soil was taken as the water holding capacity of the soil sample and is expressed in percentages.

3.2.1.5 Grain Size Analysis

This test was performed to determine the percentage of different grain sizes contained within a soil. The mechanical or sieve analysis was performed to determine the distribution of the coarser, larger sized particles (> 75 µm), while the hydrometer was used to determine the distribution of the finer particles (< 75 µm).

3.2.1.5.1 Sieve Analysis

3.2.1.5.1.1 Wet Sieve Analysis

Oven-dried soil sample (100 g) was transferred into a dish. Sodium hexametaphosphate solution (100 ml) and water (200 ml) were added to cover soil mixture and allowed to stand for 30 min. The soil mixture was transferred onto a 75 µm-sized sieve and washed through the sieve with tap water until the filtrate is clear. The residue was carefully poured into a pan using back washing and allowed to sit for a short period until the top of the suspension becomes clear. Sufficient volume of the clear top water was removed, and the remaining soil-water suspension was placed in the oven for 24 h. The oven-dried residue was weighed after 24 h and used for dry sieve analysis.

3.2.1.5.1.2 Dry Sieve Analysis

The sieves were arranged in descending order with the larger sieve size (5 mm) at the top and the receiving pan placed beneath the smallest sieve (75 µm). The oven-dried residue was transferred into the topmost sieve and covered with lid. The nest of sieves was agitated by lateral and vertical motions accompanied by a jarring action for 10 min to ensure continuous movement of the soil over the sieve surface. Each sieve was then shaken separately over a clean tray until no more soil material passes. The soil material retained in the tray was returned to the next smaller sieve, which in turn was shaken. The material retained on each sieve was weighed and the value recorded.

3.2.1.5.2 Sedimentation by Hydrometer Method

The soil sample that has passed through the 75 µm sieve was mixed with 125 ml of sodium hexametaphosphate until the soil is thoroughly wet. The soil was allowed to soak

for at least 10 min. The soil slurry was transferred to a 1 L measuring cylinder and made up to 1 L mark with distilled water. Rubber bungs were inserted into the soil suspension and shaken vigorously end-over-end about 60 times in 2 min, and then immediately placed on the ground. The rubber bungs were removed and the hydrometer was immersed in the suspension to a depth slightly below its floating position and allowed to float freely. Hydrometer readings were taken at the top of the meniscus after periods of 0.5 min, 1 min, 2 min, and 4 min. The hydrometer was removed slowly, rinsed clean with distilled water and placed in the 1 L measuring cylinder (control blank) containing 125 ml of sodium hexametaphosphate, and 875 ml of distilled water. The top of the meniscus reading R_o of the blank was observed and recorded. The hydrometer was re-inserted in the soil suspension for readings after periods of 8 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h from the start of sedimentation. The hydrometer was inserted 15 s before each reading and carefully withdrawn after each reading to prevent disturbance of the suspension. The temperature of the suspension was observed and recorded once during the first 15 min and subsequently after every reading.

A graph of cumulative percentage (P) versus particle size diameter (D) was plotted and the amount of clay, silt and sand in the soil sample was interpolated and expressed in percentages.

3.2.1.6 Total Organic Carbon

The loss on ignition method (LOI) described by Chopra and Kanwar (1998) was used. Air-dried soil sample (10 g) was put in an empty, clean and dry porcelain dish (of known weight) and placed in a muffle furnace. The temperature of the furnace was gradually increased to 575°C for 8 h. The difference in weight before and after heating was taken as total organic carbon and is expressed as percentage of soil sample.

3.2.1.7 Total Hydrocarbon Content

This was determined using gravimetric method. Each soil sample (10 g) was acidified with concentrated sulphuric acid and extracted thrice with diethyl ether (25.9 ml). The acidic ether extract was dehydrated over anhydrous sodium sulphate and was evaporated under a hood in a water bath. The residue was allowed to cool in a desiccator and

weighed. Total hydrocarbon was then calculated by dividing the weight of the residue by the weight of sample and multiplied by 1000.

3.2.1.8 Total Nitrogen Content

Total nitrogen content of soil sample was determined using the Macro-Kjeldahl digestion method described by Black (1965). Soil sample (5 g) was measured into a clean, dry 500 ml Kjeldahl flask. Distilled water (20 ml) was added to the soil, shaken, and allowed to stand for 30 min. To increase and promote the rate of oxidation of organic matter during acid digestion, 1 tablet of mercury oxide catalyst and 100 g of K_2SO_4 were added to the mixture. Concentrated H_2SO_4 (30 ml) was thereafter added. Upon removal of all the water, the heat was increased until the digest became clear. The mixture was boiled for additional 5 h and the H_2SO_4condensed halfway up the neck of the flask. After allowing the flask to cool, 100 ml of water was added. Total nitrogen was thereafter determined colorimetrically.

3.2.1.9 Available Phosphorus

This is determined spectrophotometrically using the method described by Bray and Kurtz (1945). Soil sample (1 g) was air-dried, sieved (2 mm) and ground to 0.1-0.15 mm using a pebble mill. It was placed in a centrifuge tube and 7 ml of Bray No. 1 extracting solution (1 N NH4F and 0.5 N HCl) was added. The mixture was shaken vigorously for 1 min and centrifuged at 2000 rpm for 15 min. The supernatant (0.5 ml) was pipetted into a colorimeter tube and 2 ml of Reagent C solution (70 ml of Reagent A {ammonium molybdate, potassium antimonyltartarate, conc. H_2SO_4} and ascorbic acid in a 500 ml volume) was added. The transmittance was measured on the spectrophotometer at 880 nm and the amount of available phosphorus calculated and expressed as milligram per kilogram of soil (mg/kg).

3.2.1.10 Potassium Content

This is determined using flame photometry method. Ammonium acetate/acetate (50 ml) (38.55 g ammonium acetate dissolved in 29 ml of glacial acetic acid and diluted to 1 litre with distilled water) was added to 10 g of soil sample. The mixture was shaken for 30

minutes. It was allowed to stand for several minutes and the solution was filtered through a Whatman No. 30 filter paper. The potassium content was determined on a flame photometer and expressed in milligram per kilogram of soil.

3.2.1.11 Heavy Metals

Heavy metals content of soil sample was determined using atomic absorption spectrophotometer (Alpha 4, AAS) after sample digestion. Soil sample (5 g) was mixed with conc. Nitric acid (10 ml). The sample was heated until the brown fumes disappeared. It was allowed to cool and distilled water was added to adjust the volume to 50 ml. The filtrate was filtered off and analyzed using atomic absorption spectrophotometer. The filtrate was aspirated into a flame an atomized monochromatic light specific for each metal was passed through a monochromator for wavelength selection. It was measured with a photoelectric detector and results were recorded and expressed in milligram per kilogram.

3.2.2 Microbiological Analysis of Soil Samples

3.2.2.1 Total Heterotrophic Counts

Heterotrophic bacterial and fungal counts were enumerated by plating aliquots (100 µl) of appropriately diluted soil samples on nutrient agar and acidified potato dextrose agar containing streptomycin (1 mg/100 ml), respectively. Incubation was carried out aerobically at room temperature (27± 2°C) and counted after 24 h and 48 h for bacteria and fungi respectively.

3.2.2.2 Hydrocarbon-Utilizing Bacterial and Fungal Counts

Hydrocarbon-utilizing bacterial and fungal counts were estimated on mineral salts medium (MSM) described by Kästner *et al*. (1994). The medium contained per litre of distilled water Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NH_4Cl, 0.50 g; and $MgSO_4.7H_2O$, 0.20 g. The pH of the medium was adjusted to 7-7.2 for bacteria and 5.6 for fungi. Sterile trace elements solution (1 ml/l) described by Bauchop and Elsden (1960) was aseptically added

to the medium after sterilization. The MSM was also fortified with nystatin (50 µg/ml) and streptomycin (1 mg/100 ml) for bacterial and fungal estimations, respectively. Sterile crude oil served as the sole carbon and energy source and made available to the cultures through vapour-phase transfer (Raymond *et al*., 1976). The Petri dishes were taped round with masking tape to increase vapour pressure between them. Plates were counted after incubation at room temperature (27± 2°C) for 5-7 days.

3.2.2.3 Total counts for Nitrogen-Fixers and Actinomycetes

Nitrogen-fixing bacteria were estimated by plating aliquots of appropriately diluted soil samples on Ashby's mannitol agar. Enumeration of actinomycetes was achieved by plating aliquots of diluted soil samples on starch-casein nitrate agar as formulated by Kuster and Williams (1964). Plates were counted after incubation in the dark at room temperature (27± 2°C) for 7 days.

3.2.3 Isolation of Carbazole-Degrading Bacteria

3.2.3.1 Continuous Enrichment Method

Bacteria able to degrade carbazole were isolated on carbazole mineral salt medium. The Carbon-free mineral medium (CFMM) described by Habe *et al*. (2002) was used. The medium contains per litre of distilled water NH_4NO_3, 3.0 g; Na_2HPO_4, 2.2 g; KH_2PO_4, 0.8 g; $MgSO_4.7H_2O$, 0.1 g; $FeCl_3.6H_2O$, 0.05 g; and $CaCl_2.2H_2O$, 0.05 g. The medium was supplemented with yeast extract (0.05 g). The pH of the medium for bacteria was adjusted to 7.0 and nystatin included at 50 µg/ml. Contaminated soil sample (5 g) was added to 45 ml of CFMM medium containing 50 mg of carbazole per litre.

Enrichment was carried out by shaking for 4 to 5 weeks in the dark at room temperature until there was turbidity. For selective isolation of actinomycetes, contaminated soil was air-dried; 5 g was added to 45 ml of CFMM medium containing 50 mg of carbazole per litre and supplemented with 50 µg/ml and 20 µg/ml of nystatin and nalidixic acid respectively. After five transfers, carbazole degraders were isolated by plating out dilutions from the final flask on Luria Bertani (LB) agar. Several of the colonies that

appeared were further purified on LB agar. Ability to degrade carbazole was confirmed by inoculating pure isolates into fresh CFMM medium flasks containing carbazole (50 ppm) as sole carbon and energy source and observing for turbidity.

3.2.4 Maintenance of Isolates

Pure isolates were maintained in glycerol: LB medium (50:50) containing trace amount of carbazole. Colonies growing on LB medium were harvested with sterile wire loop, pooled and transferred to the medium. The mixture was shaken well to homogenize without foaming and kept in the freezer at -20^{o}C.

3.2.5 Identification and Characterization of Carbazole-Degrading Isolates

Pure cultures of carbazole-degrading isolates were identified on the basis of their colonial morphology, cellular morphology, biochemical characteristics according to the identification scheme of Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) and molecular techniques.

3.2.5.1 Colonial Morphology

Colonial characteristics of the isolates such as shape, colour, elevation and margins were observed on nutrient agar and Luria Bertani (LB) agar plates after incubation for 18-24 h at room temperature. Production of pigments was also noted.

3.2.5.2 Gram staining

Under aseptic conditions, smears of fresh culture of the isolates were made on clean slides. The smears were air-dried, heat-fixed and flooded with the primary stain, crystal violet for 1 min. Gram's iodine were added as a mordant and allowed to stay for 1 min. the smears were decolourized with 95% alcohol, which was added in drops until the drippings from the slide are colourless. The smears were counterstain with safranin for 20-30 seconds. They were air-dried and examine under oil immersion objective lens. Gram-positive organisms appeared purple while Gram-negative organisms appeared light red.

3.2.5.3 Biochemical Characteristics of Isolates

3.2.5.3.1 Motility Test

This test was carried out to know whether the isolates possess flagella, the organelle of motility, or not. Agar stab technique was used. A soft agar medium was prepared by adding bacteriological agar (1%) to SIM medium (Sulfide Indole Motility) and sterilized by autoclaving at 121°C for 15 min. The sterile medium was dispensed in test tubes and allowed to cool to form a gel. The gel was stab-inoculated with a colony of the isolate using a sterile straight wire. Inoculated test tubes were incubated at room temperature. Motility of the isolate is demonstrated when culture growth is not restricted to the line of inoculation.

3.2.5.3.2 Spore Staining

A thin smear was made from a 24-48 hr old culture of the test organisms and heat fixed on different slides. The heat-fixed smears were flooded with the primary stain, malachite green and heated over a beaker of boiling water for 5-6 min. The slides were replenished with more malachite green as it evaporates to prevent drying. The slides were subsequently rinsed with water for 30 seconds and counterstain with safranin for 60-90 seconds. The safranin was washed off and the slides air-dried and examined under oil immersion objective lens. Vegetative portion of the organism stained light red, while the spores stained green.

3.2.5.3.3 Catalase Test

This test is used to detect the presence of catalase enzyme, which catalyzes the destruction of hydrogen peroxide releasing oxygen in the process. A smear of fresh culture (18-24 h old) of the isolate was made on a clean slide. Few drops (3-5) of 3% solution of hydrogen peroxide (H_2O_2) were added using a dropper pipette. The appearance of gas bubbles due to release of oxygen gas indicate the presence of catalase enzyme.

3.2.5.3.4 Oxidase Test

This test is used to demonstrate the ability of microorganisms to produce cytochrome oxidase enzyme. Two to three drops of freshly prepared oxidase test reagent, tetramethyl-*p*-phenylenediaminedihydrochloride were added to a filter paper and placed on a clean Petri dish. Using a glass rod, the test organism is smeared on the filter paper. Oxidation of the test reagent results in a colour change from pink to purple to dark purple within 20-30 seconds.

3.2.5.3.5 Indole Production Test

This test was carried out to demonstrate the ability of some microorganisms to degrade the amino acid tryptophan using tryptophanase enzyme and producing indole as one of the products. The presence of indole is detectable by adding Kovac's reagent, which has among its component *p*-dimethylaminobenzaldehyde that forms a complex with indole yielding a cherry red colour. Colonies of isolates were inoculated into SIM agar, which contain tryptophan as substrate and incubated at room temperature for 48 h. After incubation, Kovac's reagent (0.5 ml) was added to the culture and shaken gently. Observation of a cherry red colour at the reagent layer indicates indole production.

3.2.5.3.6 Methyl-Red Test

This test is used to demonstrate the ability of some organisms to oxidize glucose with the production and stabilization of high concentration of acid end product. The isolates were inoculated into 10 ml of the Methyl-Red Voges-Proskauer (MR-VP) medium and incubated at room temperature for 3 days. One-third of each culture was transferred to a sterile empty tube for Voges-Proskauer (VP) test. Methyl red (5 drops) was added to two-third of the culture remaining in each tube. Observation of a red colour is indicative of a positive reaction, while a yellow colour is indicative of a negative reaction.

3.2.5.3.7 Voges-Proskauer Test

This test is used to show the ability of some organisms to produce non-acidic or neutral end products, such as acetylmethyl-carbinol, from the organic acid that result from glucose metabolism. Barritt's reagent consisting of a mixture of 5% alcoholic solution of α-naphthol (Solution A) and 40% potassium hydroxide (KOH, Solution B) is used to detect the presence of acetylmethyl-carbinol. Solution A (0.6 ml) and Solution B (0.2 ml) were added to the one-third aliquot from the methyl red test and shaken vigorously to aerate. Observation of a red colouration in the mixture within 20 minutes is indicative of a positive reaction.

3.2.5.3.8 Hydrogen Sulphide (H_2S) production

This test is used to demonstrate the ability of some microorganisms to produce hydrogen sulphide from decomposition of substrates, such as sulphur-containing amino acids like cysteine and methionine or organic sulphur compounds like thiosulphates, sulphites, or sulphates. Due to the colourless nature of hydrogen sulphide, it can only be detected when it reacts with a heavy metal salt incorporated into the medium thereby producing black metal sulphides. SIM medium consisting peptone and sodium thiosulphate as substrates and ferrous ammonium sulphate as H_2S indicator is used. Bacteriological agar (1.0%) was added to make the medium semisolid. Test organisms were stab-inoculated into the SIM agar and incubated at 35°C for 48 h. Observation of a black precipitate (FeS) along the line of stab-inoculation is indicative of the presence of H_2S.

3.2.5.3.9 Urease Test

This test detect the ability of some bacteria to produce urease enzyme, which attack the nitrogen and carbon bond in urea, producing ammonia, carbon dioxide and water as end products. Hydrolysis of urea produces ammonia, which accumulates in the medium creating alkaline condition that eventually led to pH increase and colour change of the pH indicator, phenol red. Sterile urea broth tubes were inoculated with the test organisms and incubated at 35°C for 48 h-72 h. Observation of a colour change from orange-red to deep pink or purplish red is indicative of a positive test for urea hydrolysis.

3.2.5.3.10 Sugar Fermentation

This test is used to detect the production of organic acids or organic acid and gas during sugar fermentation. One percent solutions of glucose, fructose, sucrose, galactose, lactose, xylose, arabinose, mannose, melibiose, raffinose, maltose, cellobiose, trehalose, rhamnose, mannitol, inositol, and sorbitol were used. Each fermentation broth tube contains peptone water (1%), phenol red (0.1%) as pH indicator, and an inverted Durham tube to trap gas produced by the test organisms during fermentation. The medium (10 ml) was inoculated with the test organisms and incubated at room temperature for 24-48 h, and observed every 8 h for colour change of the pH indicator. Observation of a colour change from red to yellow indicate production of organic acid, while visual observation of gas bubbles in the inverted Durham indicate production of gas from sugar fermentation.

3.2.5.3.11 Starch Hydrolysis

This test is used to demonstrate the ability of some organisms to hydrolyze starch to dextrins, maltose and glucose using α-amylase enzyme. Sterile starch agar plates were inoculated with the test organisms and incubated at room temperature for 24-48 h. After incubation, the plates were flooded with dilute iodine solution. Observation of clear zones around the colonies is indicated of starch hydrolysis, while changing of the medium colour from straw to blue-black indicate a negative result.

3.2.5.3.12 Gelatin Liquefaction

This test is used to demonstrate the ability of some organisms to hydrolyze gelatin to amino acid using gelatinase enzyme. Sterile nutrient gelatin deep tubes were stab-inoculated with the test organisms and incubated at room temperature for 48 h or longer depending on the bacterial species. After incubation (35°C), the tubes were kept in the refrigerator at 4°C for 30 min. inability of the medium to resolidify indicate the production of gelatinase enzyme.

3.2.5.3.13 Nitrate Reduction

This test is used to demonstrate the ability of some organisms to reduce nitrates to nitrites or molecular nitrogen using nitrate reductase enzyme. Sterile nutrient broth tubes supplemented with 0.1% potassium nitrate (KNO_3) were inoculated with the test organisms and incubated at room temperature for 24-48 h. After incubation, 0.5 ml of Solution A (sulfanilic acid) and 0.5 ml of Solution B (α-naphthylamine) were added to each of the culture tubes and mix. Observation of a distinct cherry red colour indicates a positive test. To ascertain whether or not nitrates were reduced beyond nitrite stage, several grains of zinc powder were added to tubes showing negative result and shaken gently. Observation of a red colour within 5-10 min confirms that nitrates were not reduced. However, if there is no colour change after the addition of zinc powder, it indicates that nitrates in the medium were reduced beyond nitrite to ammonia or nitrogen gas i.e. a positive result.

3.2.5.4 Molecular Characterization of Isolates

Genotypic identification of the isolates was achieved on the basis of 16S rRNA gene analysis. This analysis was carried out at the Biotechnology Research Center, University of Tokyo, Japan.

3.2.5.4.1 DNA Isolation

Total DNA extraction from six bacterial isolates was performed as described by Ausubel *et al*. (1990) with slight modification. Pure bacterial strains was inoculated into a 5 ml carbon free minimal medium (CFMM) amended with 0.3 mM of carbazole dissolved in dimethyl sulfoxide and incubated on a rotary shaker (300 rpm) at 30°C for 48 hours. Bacterial culture (2 ml) was poured into Eppendorf tubes and centrifuged at 13,000 rpm for 2 minutes at room temperature. The supernatant was discarded and the cell pellet resuspended in 567 µL Tris-EDTA buffer pH 8 (TE buffer; 1 M Tris-HCl, 0.5 M EDTA) by vortexing. Three microlitres of proteinase K solution (10 mg/mL in sterile distilled water) and 30 µL of 10% sodium dodecyl sulphate (SDS) were added, mixed thoroughly

using AS ONE tube rotator for 5-10 minutes and incubated at 37°C for 60 minutes. One hundred microlitres of 5 M NaCl was added to the mixture and gently mixed using the rotator for 5-10 minutes. Thereafter, 80 µL of pre-warmed (65°C) Cetyltrimethyl ammonium Bromide/ sodium chloride solution (CTAB/NaCl solution) was added to the mixture, mixed properly using the rotator and incubated at 65°C for 10 minutes in a TAITEC, water bath shaker. The DNA is first purified by the addition of 800 µL of chloroform to the mixture, gently mixed using the rotator for 10 minutes and centrifuged at 13,000 for 15 minutes at room temperature. The resulting supernatant was transferred to a new sterile Eppendorf tubes (2 mL) and 500-700 µL of phenol: chloroform (25:25) was added to further purify the DNA. The organic/aqueous fractions that results was mixed using the rotator for 10 minutes and centrifuged at 13,000 rpm for 15 minutes at room temperature. The aqueous fraction containing the DNA was transferred to new sterile Eppendorf tubes (1.5 mL) and the DNA precipitated with the addition of equal volume of isopropanol. The solution was mixed properly using the rotator for 30-40 minutes and the precipitate (DNA) collected by centrifugation at 15,000 rpm for 10 minutes at room temperature. The resulting supernatant was removed and the precipitate washed by the addition of 400 µL of 70% ethanol and centrifuged at 15,000 rpm for 5 minutes at room temperature. After the removal of the supernatant, the DNA pellet was dried in A-3S Aspirator (Eyela Tokyo Rikakikai Co.) and eluted in TE buffer containing DNA free RNase A solution (10 mg/mL in glycerol stock), followed by incubation at 37℃ for 60 minutes. The total DNA obtained was stored at 4℃ until being used.

3.2.5.4.2 Nucleic acid Analysis

The concentration and the purity of the genomic DNA obtained are analyzed using PC-controlled Beckman Coulter DU 800 UV-Vis. Spectrophotometer. Sterile distilled MilliQ water (49 µL) is mixed with I µL of genomic DNA eluted in TE buffer (with RNase A) in 1.5 mL Eppendorf tubes and the entire volume (50 µL) was dispensed in a one-sample 50 µL-microcell inside the spectrophotometer. Sterile distilled MilliQ water (50 µL) was used as a blank. The sample identity and the dilution factor used was thereafter fed into DU 800 system and application software (Version 3.0, Build 52.0.102) and prompted for analysis.

3.2.5.4.3 Amplification of 16S rRNA Genes of Bacterial Isolates

The universal primers 27f (5′-AGAGTTTGATC{A/C}TGGCTCAG-3′) corresponding to the position 8-27 (*E.coli* SSU rRNA, GenBank accession J01695) and 1378r (5′-CGGTGTGTACAAGGCCCGGGAACG-3′) corresponding to the position 1378-1401 (Heuer *et al*., 1997) respectively, were used to amplify 16S rRNA genes from the bacterial isolates using the polymerase chain reaction (PCR). The PCR amplification reaction mixture contains 20 pmol of the forward and the reverse primers, 10 µL of Ex *Taq* buffer (Mg^{2+} plus) (Takara, Otsu, Japan), 2.5 mM of each deoxyribonucleotide triphosphate (dNTP), 2.5 U (0.5 µL) of Ex *Taq* polymerase (Takara) and 1.0 µL of the purified genomic DNA in a total volume of 100 µL per bacterial isolate. The PCR reaction mix (without Ex Taq Polymerase and Genomic DNA) was prepared in a 1.5 mL Eppendorf tube, vortexed and centrifuged, after which Ex Taq polymerase was added, tapped gently and spin down for effective mixing using Nano Spin NS-060 (Nippon Gene). The mixture (99 µL) was thereafter dispensed in PCR tubes with 1 µL of purified genomic DNA of each bacterial isolate added to each tube. The reaction mix (20 µL) was dispensed in a PCR tube and 1 µL of Sterile distilled MilliQ water was added to serve as negative control. The PCR tubes was tapped gently and spin down after which it is placed in the PCR machine for amplification. Polymerase chain reaction was carried out in a GeneAmp PCR system (PCR Thermal Cycler, PERSONAL, Takara). The thermocycling conditions consisted of an initial denaturing step at 95°C for 3 min, 30 amplification cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, maximal ramp rates throughout, with the final step at 72°C extended to 7 min before cooling to 4℃. The PCR products (about 1.5 kb) were resolved on agarose gel electrophoresis to confirm that the products of the expected sizes were obtained.

3.2.5.4.4 Agarose Gel Electrophoresis

Following the nucleic acid analysis to determine the purity and concentration of the 16S PCR product, agarose gel electrophoresis was carried out to characterize the sizes of the DNA fragments after PCR amplification. One percent (w/v) agarose gel (Agarose ME, Nacalai tesque, Kyoto, Japan) in 1 x Tris-acetate/EDTA buffer (TAE buffer) was prepared by heating in a microwave oven until agarose was completely melted. The

melted agarose gel was cooled to about 50°C and poured in gel chamber with comb. DNA sample was mixed with 0.1 volume of gel-loading buffer containing bromophenol blue and loaded into slot of agarose gel. Submerged gel electrophoresis was carried out at 100 V for 30-35 min in minigel electrophoresis chamber with 1 x TAE as running buffer (Mupid-2x Submarine Electrophoresis System, Tokyo Co. Ltd., Japan). Gel was stained with ethidium bromide (0.5 µg/mL in distilled water) for 15 min and washed with distilled water for 5 min. DNA bands was visualized under UV-light transilluminator (Nippon Gene, Japan). OneSTEP Marker 6 (λ/*Sty* I digest; fragment sizes in kb, 19.33, 7.74, 6.22, 4.26, 3.47, 2.69, 1.88, 1.49, 0.93, 0.42, and 0.07; Nippon Gene) was used as a standard DNA marker.

3.2.5.4.5 Recovering of 16S PCR Amplicons from Agarose Gel

PCR products were extracted from agarose gel and purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) according to manufacturer's instructions. The area of the gel containing the DNA fragment was excised using a clean blade. The gel slice containing the excised DNA was placed in a 1.5 mL Eppendorf tube. Ten microlitres of membrane binding solution was added per 10 mg of gel slice, vortexed and incubated at 50-65ºC until the gel slice is completely dissolved. The dissolved gel mixture was transferred using a sterile pipette to a mini-column assembly (provided) and incubated at room temperature for I minute followed by centrifugation at 16,000 rpm for 1 min. The flow-through was discarded. Seven hundred microlitres of membrane wash solution with ethanol (provided) was added to the mini-column assembly and centrifuged at 16,000 rpm for 1 min. The flow-through was discarded. This step was repeated with 500 µL of membrane wash solution with ethanol followed by centrifugation at 16,000 rpm for 5 min. The mini-column was removed from the collection tube and transferred to a clean 1.5 mL Eppendorf tube. Fifty microlitres of nuclease-free water was added to the mini-column, incubated at room temperature for I min. followed by centrifugation at 16,000 rpm for 1 min. The mini-column was discarded and the obtained DNA (now in Eppendorf tubes) is stored at 4ºC or -20ºC until being used.

3.2.5.4.6 Ligation of DNA fragment into plasmid vector

PCR products of partial 16S rRNA gene were ligated into pT7Blue(R) plasmid vector (2887 bp in length, Ap^r, lacZ, Novagen, USA). DNA inserts and plasmid vector were mixed together with the molar ratio 2:1 and finally mixed with equal volume of Ligation high contained in DNA Ligation kit (Toyobo). The reaction mixture was incubated at 16°C for 12 hrs or overnight after which it was used for transformation of competent *Escherichia coli*.

3.2.5.4.7 Transformation of *E. coli* with Recombinant Plasmid

Standard method (Sambrook *et al*., 1989) was used to transform competent *E. coli* DH5α (*F- ϕ80d lacZΔM15 Δ(lacZYA-argF)U169 endA1recA1 hsdR17 (rk-mk-) deoR thi-1 supE44 λ gyrA96 relA1*) (Toyobo) with recombinant pT7Blue(R) plasmid. Ligated products (5-15 µL) were mixed with 100 µL of DH5α and the tubes were kept on ice for 20 min. The mixture was thereafter subjected to heat shock at 42°C for 90 sec and immediately returned back to ice for 1 min. Five hundred microlitres of LB medium was added to the mixture tubes and incubated at 37°C for 45 min. A 50-200 µL of the mixture was spread onto LB agar plates amended with ampicillin, X-gal and IPTG. The plates were incubated at 37°C for 13-14 hours. The resultant transformants (white colonies) were picked and inoculated onto 5 mL LB broth amended with 100 µg/mL of ampicillin to populate the clones. The tubes were incubated at 37°C on a rotary shaker for 8-12 hours and thereafter centrifuged at 5,000 rpm for 2 minutes to pellet the *E. coli* DH5α recombinant cells.

3.2.5.4.8 Plasmid Extraction and Digestion with Restriction Enzymes

Plasmids were extracted by rapid alkaline extraction method. After centrifugation of DH5α cells containing plasmid, the pelleted cells were resuspended in 100 µL Tris-EDTA/glucose buffer pH 8 (TEG buffer) and vortexed for 10-20 seconds. Two hundred microlitres of freshly prepared lysis solution consisting of sodium hydroxide-sodium dodecyl sulphate solution (10x SDS: 1N NaOH: water; 1:2:7) were added to the tubes, mixed by inversion 3-6 times and incubated on ice for 5 minutes. One hundred and fifty microlitres of 5 M potassium acetate (KOAc) solution was added, mixed by inversion 3-

6 times, incubated on ice for 10 minutes and centrifuged at 12,000 rpm for 10 minutes at 4°C. Aqueous phase was transferred to a new 1.5 mL Eppendorf tube. Four hundred and fifty microlitres of phenol: chloroform solution (25:25) was added to the aqueous phase, vortexed for 1 minute and centrifuged at 12,000 rpm for 5-10 minutes at room temperature. The resultant aqueous phase was also transferred to a new 1.5 mL Eppendorf tube. Plasmid was precipitated with 450 µL of isopropanol, vortexed for few seconds and collected by centrifugation at 12,000 rpm for 5 minutes at room temperature. The supernatant was removed with sterile pipette followed by addition of 400 µL of 70% ethanol. The tube was inverted once and centrifuged at 12,000 rpm for 5 minutes at room temperature. The supernatant was carefully removed to avoid removal of plasmid with it. The tubes were dried in an aspirator for 5 minutes and the resultant plasmid DNA is eluted in 50-100 µL TE buffer/RNase solution depending on the concentration of the pelleted plasmid DNA. The eluted DNA was incubated at 37°C for more than 1 hour after which it is stored at -20°C until it is needed. The extracted plasmid was double-digested with EcoRI and PstI restriction enzymes (Takara Bio, Japan) following manufacturer's instructions. Agarose gel electrophoresis with 1% agarose and OneSTEP Marker 6 prepared as described previously was used to characterize the sizes of DNA fragments after digestion with restriction endonuclease enzymes.

3.2.5.4.9 Nucleotide Sequencing and Sequence Analysis

DNA cycle sequencing reaction mixture was prepared by using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The pre-reaction mixes prepared in a 96-Well reaction plate include 1 µL of DNA template, 2 µL of each primer, 8.0 µL Terminator Ready Reaction Mix and distilled water in a total volume of 20 µL. Four different primers were used. T7 promoter primer (5′-TAATACGACTCACTATAGGG-3′) (Sigma Genosys, Japan) and U-19mer primer (5′-GTTTTCCCAGTCACGACGT-3′) (Invitrogen) which anneal with the upstream or downstream region of the insert were used. Additionally, a forward and a reverse primer f2L′ (5′-CCAGCAGCCGCGGTAATAC-3′) and r2L′ (5′-GACTACCAGGGTATCTAATC-3′) (Sigma Genosys, Japan) which anneal with the inside of the insert were also used. DNA cycle sequencing was performed with Gene

Amp PCR system 9600 (Perkin Elmer Cetus, CT, USA). PCR temperature regimen consist of initial denaturation at 96°C for 1 minute, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes and then hold at 4°C.

Nucleotide sequence determination was carried out by the chain termination method using ABI PRISM 3730xl DNA Sequencer (Applied Biosystems, UK) according to the manufacturer's instructions. The 16S rRNA sequences obtained were compared with the sequences deposited at the GenBank databases using the BLAST algorithm. Phylogenetic tree was constructed by the neighbor-joining (NJ) method using CLC Sequence Viewer 6.5.2 and bootstrapped with 500 repetitions.

3.2.6 Substrate Specificity

Biodegradation ability of carbazole degraders on different hydrocarbon substrates was evaluated in CFMM containing the respective hydrocarbons as sole carbon and energy source at concentration of 100 ppm. Stock solution of the substrates was prepared by dissolving 1 g of respective hydrocarbon substrates in 10 ml dimethyl sulfoxide (DMSO) and filter-sterilized the solution using hydrophobic filter (Advantec, JP020AN). Sterile CFMM (5 ml) was prepared in test tubes and 5 µl of stock solution of the respective hydrocarbons was added. Carbazole degraders were added at 1% (v/v). Incubation was carried out at room temperature in the dark for 14 days. Degradation was monitored by evaluation of cell increase and visual observation for turbidity. The hydrocarbons tested include naphthalene, fluorene, acenaphthene, pyrene, anthranilic acid, dibenzothiophene, dibenzothiophene-sulfone, dibenzofuran, 3,3'-dimethoxybenzidine, and *p*-hydroxybenzoic acid. Liquid hydrocarbons like crude oil and engine oil were autoclaved and added separately to the sterile CFMM at 0.1% (v/v).

3.2.7 Biodegradation Studies

3.2.7.1 Evaluation of Carbazole Biodegradation

Carbazole degradation was carried out by inoculating replicate 250-ml flasks containing 50 ml of CFMM medium already supplemented with carbazole as a sole carbon, nitrogen and energy source at concentration of 50 ppm. Flasks were inoculated with 0.5 ml of CFMM-washed 18 h LB-grown cells and subsequently incubated with shaking for 30 days at room temperature. Culture flasks prepared as stated above but inoculated with heat-killed cells were used as controls. Degradation was monitored by withdrawing a flask from the lot at intervals of 3 days and plating out aliquots of appropriate dilutions onto nutrient agar for total viable counts (TVC). Residual carbazole was quantified by Gas Chromatographic (GC) analysis.

3.2.7.2 Analytical Studies

3.2.7.2.1 Extraction of Residual Carbazole for Gas Chromatography

Residual carbazole was extracted twice by adding 10 ml of hexane to broth culture (20 ml) in flask and shaken vigorously for 30 minutes using a mechanical shaker. After removing the aqueous phase with separating funnel, the solvent was allowed to vent off in a pre-heated oven overnight to about 1 ml to concentrate the analyte (carbazole).The residual carbazole concentration was determined by injecting 1 µl of the resultant solution for gas chromatographic analysis.

3.2.7.2.2 Gas Chromatographic Analysis

Residual carbazole was determined by Gas Chromatography equipped with Flame ionization detector (GC/FID). A standard carbazole (1 µl) was first injected into the GC/FID to obtain a standard chromatogram, which give a standard peak area for standard carbazole. This was carried out to identify the run time and retention time for carbazole prior to injection of the sample analyte. Afterwards, hexane extract (1 µl) was injected into GC/FID. The column SE-30 was 60 m long. The carrier gas was nitrogen. The injector and detector temperature were maintained at 220 °C and 250 °C, respectively.

The column was programmed at an initial oven temperature of 70 °C for 2 min, then ramped at 10°C/min to 200 °C and held for 5 min.

3.2.7.2.3 Detection of Metabolites of Carbazole Degradation

Analyses for the detection of metabolites of carbazole biodegradation from ethyl acetate extracts of growing cells and resting cells of isolated carbazole degraders was carried out using gas chromatography-mass spectrometry (GC-MS). The analysis was carried out at the Biotechnology Research Center, University of Tokyo, Japan.

3.2.7.2.3.1 Metabolites Detection from Growing Cells

Growing cultures (5 ml) of the isolates were centrifuged (13,000 rpm, 2 min) to remove the residual substrate and the cells. The supernatants were twice extracted with ethyl acetate (4,000 rpm, 10 min) after acidification to pH 2 with 1N HCl. The ethyl acetate layer was dried with anhydrous sodium sulfate and concentrated by a rotary evaporator under reduced pressure at 20°C. The concentrated ethyl acetate extracts were derivatized with methylation with PTAH, m-(trifluoromethyl)-phenyltrimethylammonium hydroxide (TMTFTH). GC/MS analysis was performed on a JEOL JMS-K9 Ultra Quad GC/MS (JEOL Ltd., Tokyo, Japan) interfaced with an Agilent Technology 6890N Network GC system equipped with a splitless injector. A capillary column InertCap$^{(R)}$ (5% phenyl-95% methylpolysilarylene; I.D. 0.25 mm, length 15 m, film thickness 0.25 µm) (GL Sciences Inc. Japan) was used as the analytical column. Each sample (1 µl) was injected into the column at 80°C in the splitless mode. After 2 minutes at 80°C, the column temperature was increased to 280°C at 16°C min^{-1}. The head pressure of the helium carrier gas was 65 kPa.

3.2.7.2.3.2 Metabolites Detection from Resting Cells

Bacterial cells were cultivated in 200 ml of CFMM/carbazole medium at 30°C for 2-3 days. The cells were harvested by centrifugation (5g, 4°C) for 15 minutes and washed twice with CFMM buffer. The resultant cells were distributed in 5 ml aliquot into test tubes and 50 µL of a 50 ppm stock solution of carbazole in dimethyl sulfoxide (DMSO)

were added to each tube of resting cells. The reaction mixture was incubated at 30°C on a rotary shaker (300 rpm) for 1, 2, 3, 24, and 48 hours. The metabolites were extracted as described above and each extract analysed by GC-MS.

3.2.7.2.3.3 HPLC Analysis of Anthranilic Acid Metabolites

High-performance liquid chromatography (HPLC) was performed to detect anthranilic acid metabolites from anthranilic acid-grown cultures of strains SL1, SL4, and SL6, respectively. Anthranilic acid (AN; 50 ppm) was supplied as sole sources of carbon and energy and cultures were incubated in the dark at room temperature for 14 days. HPLC analysis was done using Shimadzu Model LC-2010 HT (Kyoto, Japan) equipped with a variable wavelength photodiode array detector and fitted with uBondapak C18 column Model WAT 025875 (250 mm length; 4.6 mm ID; 5 µm thickness; WATERS Scientific). Acetonitrile-extracts (5 µl) from growing cells culture were analyzed using acetonitrile and water (60:40 v/v) mobile phase, at a flow rate of 2 ml min^{-1}. Column temperature was set at 30°C. Major products were monitored at an absorbance of 254 nm and identified with reference to retention times of standards used.

3.2.7.2.3.4 Catechol Dioxygenase Assay

Two mililitres of strains SL1, SL4 and SL6 cells were harvested by centrifugation at the late logarithmic phase from CFMM medium containing carbazole and were suspended in 1 ml CFMM. Cells were lysed by the addition of 20 µl toluene and after vigorous mixing, unbroken cells and cell debris were removed by centrifugation at 16,000 x g for 30 sec. The clear supernatants were immediately used for the assay or placed on ice for not more than 10 min. Activity assays were performed using GENESYS 10S UV-Vis spectrophotometer (Thermo Scientific, USA). The reaction as initiated by the addition of 100 µl catechol solution (100 µM) to a reaction reaction mixture in a 1-cm light path quartz cuvette containing 800 µl phosphate buffer and 100 µl of crude lysate. The blank cuvette contained the same amount of enzyme in the same buffer with the exception of catechol. Activities of catechol 1,2-dioxygenase and 2,3-dioxygenase were monitored at 260 and 375 nm, respectively.

3.2.8 Degradation of Carbazole in Soil Microcosm

Degradation of carbazole in soil microcosm was carried using a method modified from Kästner *et al*. (1998). Nine different set up were made as follows

I. Sterilized soil +carbazole

II. Sterilized soil + strain SL1 + carbazole

III. Sterilized soil + strain SL4 + carbazole

IV. Sterilized soil + strain SL6 + carbazole

V. Sterilized soil + SL1 + SL4 + SL6 + carbazole

VI. Native soil + carbazole (NSC)

VII. Native soil + SL1+ carbazole (NSC1)

VIII. Native soil + SL4 + carbazole (NSC4)

IX. Native soil + SL6 + carbazole (NSC6)

3.2.8.1 Soil Sample

Soil samples were obtained from uncontaminated farmland site at a vegetable garden, opposite Lagos State University, Ojo. Each experimental set up consisted of a 300 ml metal cup containing 100 g of soil. The soil was sterilized by autoclaving for 20 minutes followed by 24 h incubation at room temperature for three consecutive times. Native soil is the soil used without sterilization. Soil physicochemical parameters were determined as described in section 3.4. Carbazole was applied to the soil at a concentration of 100 mg/kg or 10 mg/100 g.

3.2.8.2 Spiking method

Carbazole (10 mg) was spiked on 100 g of soil by first dissolving it in 1 ml of dichloromethane in 250 ml conical flask and allowing the solvent to vent off. Water (10 ml) was then added to the flask and brought to boiling to detach the carbazole from the

flask. The carbazole suspension in water was then poured on the soil in the metal cup and mixed thoroughly.

3.2.8.3 Inocula Preparation for Soil Inoculation

Inocula were incubated in 250 ml flasks at room temperature in CFMM (Habe *et al*., 2002) with carbazole (100 ppm). Cells were harvested at the logarithmic phase of growth by centrifugation. The cell pellets were then washed twice with decreasing concentration of CFMM and twice with sterile distilled water. Inoculation was carried out after resuspension of cell pellets in 2.5 ml of sterile distilled water. Total viable bacteria and residual carbazole were determined from soil samples at day 0 and day 30.

3.2.8.4 Analytical Method

Residual carbazole was determined by Gas Chromatography equipped with Flame ionization detector (GC/FID). Soil sample (10 g) was weighed into a glass amber bottle and dichloromethane (10 ml) was added to the soil and shaken vigorously for 30 minutes using a mechanical shaker. The sample was thereafter filtered into a glass beaker and the filtrate was vented off to about 1 ml to concentrate the analyte (carbazole). The concentrate was then dispensed in a vial and stored at 4 °C prior to analysis.

A standard carbazole (1 µl) was first injected into the GC/FID to obtain a standard chromatogram, which give a standard peak area for standard carbazole. This is carried out to identify the run time and retention time for carbazole prior to injection of the sample analyte. Afterwards, sample analyte (1 µl) was injected into GC/FID. The column OV-3 is 60 m long. The carrier gas was nitrogen. The injector and detector temperature were maintained at 220 °C and 250 °C, respectively. The column was programmed at an initial oven temperature of 70 °C for 2 min, then ramped at 10°C/min to 200 °C and held for 5 min.

3.2.9 Bacterial Diversity Studies

Metagenomic study of polluted soil sample from MWO study site was carried out at the Biotechnology Research Center, University of Tokyo, Bunkyo-Ku, Tokyo, Japan.

3.2.9.1 Preparation of Soil Sample

Polluted soil sample from MWO study site was used. This site was chosen among the three sampling sites used in this study because of its unusually high hydrocarbon content (157 g/kg) and presence of various heavy metals. Soil sample was passed through a 2-mm mesh size sieve. The sieved soil was thoroughly mixed in a large plastic bag to avoid variability among the results of replicate soil samples.

3.2.9.2 DNA Extraction from Polluted Soil

DNA extraction was done using FASTDNA® Spin kit for soil (MP Bio, Japan) according to manufacturer's instruction. Soil sample (500 mg, wet weight) was added to a Lysing Matrix E tube followed by addition of sodium phosphate buffer (978 µl) and MT buffer (122 µl) respectively. The tubes are secured in FastPrep instrument (MP Bio), homogenized for 40 seconds at a speed setting of 6.0, and centrifuged at 14,000 x g for 15 min to pellet debris. The resulting supernatant was transferred to a clean 2.0 ml microcentrifuge tube and 250 µl PPS (Protein Precipitation Solution) was added and mixed by shaking the tubes by hand ten times. The tubes were centrifuged at 14,000 x g for 5 min to pellet precipitate and supernatant was transferred to a clean 15 ml tube to allow better mixing and DNA binding. Binding Matrix Solution (1 ml), which was resuspended before use was added to the supernatant and the tube was placed on a rotator for 2 min to allow binding of DNA. The tubes were thereafter placed in a tube rack for 3 min to allow settling of Binding Matrix. Five hundred microlitres of the resulting supernatant was removed and discarded, and the Binding Matrix was resuspended in the remaining supernatant. Approximately 600 µl of the resulting mixture was transferred to a SPIN™ Filter and centrifuged at 14,000 x g for 1 min. the flow-through in the collection tube was emptied. The remaining mixture was added to the SPIN™ Filter, centrifuged at 14,000 x g for 1 min, and the flow-through in the collection tube emptied. Five hundred microlitres of prepared SEWS-M (prepared by adding 100 ml of 100%

ethanol to 12 ml of concentrated SEWS-M wash solution) was added to the SPIN™ Filter and centrifuged at 14,000 x g for 1 min. Flow-through in the collection tube was emptied and the SPIN™ Filter was replaced. Further centrifugation at 14,000 x g for 2 min was done to dry the matrix of residual SEWS-M wash solution. The SPIN™ Filter was removed, placed in a new collection tube and air-dried (with the cap open) for 5 min at room temperature. Binding Matrix (above the spin filter) was gently resuspended in 50 µl DES (DNase/pyrogen free water) by brief vortexing of the SPIN™ Filter and incubated at 55°C for 5 min in a heat block to increase purified DNA yield. Finally, centrifugation at 14,000 x g for 1 min was done to elute the DNA into the collection tube and the SPIN™ Filter was discarded. The eluted DNA was stored at -20°C for extended period or 4°C until use. Agarose gel electrophoresis of the extracted total DNA was performed as described in Section 3.10.1.4 using 5-10 µl of the eluted DNA solution, 0.9 % agarose and Marker 6 (λ/*Sty* I digest; Nippon gene).

3.2.9.3 Clone Library Analysis

Clone library analysis was used to decipher the microbial community diversity of MWO polluted soil. The steps involved in 16S rRNA clone library construction are amplification of 16S rDNA with *Ex Taq* (TaKaRa) from total DNA extracted from the polluted soil, cloning of the 16S rDNA PCR products with TOPO® TA Cloning® kit for sequencing (Invitrogen), performance of colony PCR with *Ex Taq* (TaKaRa), and determination of the identity of each cloned sequence.

3.2.9.3.1 Amplification of 16S rDNA from total DNA extracted from soil

Bacteria specific primers $27F_{MOD}$ (5′-AGRGTTTGATCMTGGCTCAG-3′) and $1492R_{MOD}$ (5′-TACGGYTACCTTGTTAYGACTT-3′) (Vergin *et al*., 1998) were used to amplify 16S rDNA gene using genomic DNA extracted from MWO polluted soil as template. Amplification of 16S rDNA gene in the extracted DNA was done using *Ex Taq* polymerase (TaKaRa). The PCR reaction mixture consist of 40 µl of 10x *Ex Taq* buffer, 32 µl of dNTPs mixture (2.5 mM each), 2 µl each of the forward and the reverse primers (100 µM each), 4 ul of *Ex Taq* polymerase (TaKaRa), 4 µl of DNA template (113 ng/ µl), and 316 µl of distilled water in a total reaction volume of 400 µl. PCR reaction mix

containing 4 µl distilled water instead of DNA template was used as a negative control. The thermocycling condition consists of an initial denaturation step at 94°C for 3 minutes followed by 15 amplification cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes and a final extension at 72°C for 5 minutes before cooling to 4°C.

3.2.9.3.2 Detection and Purification of PCR Product

Agarose gel electrophoresis, as described in Section 3.7.4.4, was performed to confirm that the PCR product of the expected size has been obtained (approximately 1400 bp). Agarose gel (1.0 %) was prepared. Electrophoresis was performed using 1-2 µl of the PCR product and OneStep Marker 6 (Nippon gene). The PCR product was purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) according to manufacturer's instructions as described in Section 3.2.5.4.5

3.2.9.3.3 Cloning of 16S rDNA PCR Product

Cloning of the 16S rDNA PCR product was carried out using TOPO® TA Cloning® kit for sequencing (Invitrogen), a cloning system that does not require the use of blue/white screening method for identification of recombinants.

Principle

TOPO® TA Cloning® kit contains pCR™ 4-TOPO plasmid vector that allows direct recombinants selection by disruption of the lethal *E. coli* gene, *ccdB* (Bernard *et al*., 1994) fused to the C-terminus of the Lac*Z*α fragment. Ligation of a PCR product into the vector disrupts the expression of the *LacZα-ccdB* gene fusion in the vector thus permitting growth of only positive recombinants upon transformation in OneShot DH5α™-T1^R competent cells (kit provided). Cells containing non-recombinant vector are killed upon plating.

3.2.9.3.4 Ligation of the PCR Product into Plasmid Vector

The TOPO cloning reaction mixture (Table 3.1) were mixed gently and incubated at room temperature (22°C to 23°C) for 5-30 min. It is thereafter placed on ice. During the incubation, one vial of OneShot DH5α™-T1^R competent *E. coli* cells was thawed on ice

for each transformation. In addition, a vial of SOC medium (kit provided) was warmed to room temperature and selective LB agar plates containing 50 µg/ml of kanamycin were pre-warmed to 37°C.

Table 3.1: Ligation Reaction Mixture

Reagent	**Volume (µl)[a]**
Insert (PCR product to be cloned)	0.5-4.0 (10-100 ng total DNA)
Salt solution (kit provided)	1
Sterile water	(for final volume of 5 µl with insert and salt)
TOPO® vector	1
Final volume	6

[a]The ratio of insert to vector is 3:1 to maximize diversity recovery. One microlitre of TOPO vector contains 3.9 fmol (10 ng) DNA. For a ~1500 bp PCR product, 10-12 fmol (10-12 ng) was used.

3.2.9.3.5 Transformation of competent cells with Recombinant Vector

Two microlitres of the TOPO cloning reaction mixture was added into a vial of chemically-competent DH5α™-T1^R *E. coli* cells and mixed gently by stirring or flicking tube. It is then incubated on ice for 5-30 min. The cells were heat-shocked in a water bath at 42°C for 30 s without shaking and immediately transferred to ice. Room temperature SOC medium (250 µl) was added to the tube and the tube was capped tightly and shaken horizontally (200 rpm) at 37°C for 1 hour. Each transformation mix (10-50 µl) were spread on pre-warmed selective plates (LB agar plates containing 50 µg/ml of kanamycin) and incubated overnight at 37°C.

3.2.9.3.6 Colony PCR

Colony PCR was performed to identify the clones that have taken up the recombinant plasmid vector harboring the 16S rDNA genes. T7 promoter primer (5′-TAATACGACTCACTATAGGG-3′) and M13 reverse primer (5′-GGAAACAGCTATGACCATG-3′) were used. Each transformed colony (from section 3.2.9.3.5) on the selective LB agar plates was picked with a sterilized toothpick and suspended in 96-well plates (5 plates) containing 25 µl of PCR reaction solution in each well. The PCR reaction solution consists of 2.5 µl of 10x *Ex Taq* buffer (TaKaRa), 2 µl

of dNTPs mixture (2.5 mM each), 0.5 µM each of forward and reverse primers, 0.125 µl of *Ex Taq* (5U/µl) polymerase (TaKaRa), template from 1 colony and distilled water to bring the total volume to 25 µl. The thermocycling condition consists of an initial denaturation step at 98°C for 2 minutes followed by 30 amplification cycles of 98°C for 10 s, 55°C for 30 s and 72°C for 1.5 minutes and a final extension at 72°C for 5 minutes before cooling to 4°C.

3.2.9.3.7 Detection and Purification of PCR Products

Agarose gel electrophoresis was performed to confirm that the PCR products of the expected size have been obtained. PCR products from randomly selected 24 wells from each of the five 96-well plates were analyzed using 1% agarose gel at 200 V for 30 min. OneStep Marker 6 (λ/sty I digest; Nippongene) was used. The PCR products were enzymatically purified using Shrimp alkaline phosphatase and exonuclease I (TaKaRa) in order to remove unused dNTPs and primers.

3.2.9.3.8 Sequencing

Sanger sequencing was performed on all five 96-well plates (480 clones). Sequencing reaction was conducted using BigDye® Terminator v3.1 Sequencing kit (Life Technologies) using 1400R sequencing primer (ACGGGCGGTGTGTAC). The sequencer used was Applied Biosystems 3730xl DNA Analyzer (Life Technologies).

3.2.9.3.9 Sequence Data Analysis

Bases below 20 (Phred quality score) were trimmed, and all the sequence data were converted to reverse complement. In the sequence data analysis, sequence reads that are less than 200 bp were excluded. Chimera check of the sequenced clones was conducted using DECIPHER V1.0.4 (Wright *et al*., 2012). Sequence alignment was carried out using INFERNAL aligner tool in the RDP Pipeline (Nawrocki and Eddy, 2007). Taxonomic affiliations of each of the sequenced clones were determine using RDP Classifier v2.5 (Wang *et al*., 2007) in the RDP-II database, which classify sequences into Bergey's hierarchical taxonomy with confidence level for each taxonomic level provided. Hierarchical cluster analysis (Complete linkage clustering) was perfomed using RDP

Pipeline and the resulting clusters were used to generate representative sequences from each cluster (OTU). Phylogenetic trees were constructed with the representative sequences (97% sequence identity) using neighbor-joining algorithm within the program MEGA 5.1 (The Biodesign Institute) and bootstrapped with 100 repetitions. Alpha-diversity indices such as Shannon-Weiner index, Chao1, Simpson's inverse, Fisher's Alpha, evenness and rarefaction curve for the clone library were computed using the RDP Pipeline and EstimateS v9.1.0 (Colwell, 2013). Rarefaction curve was plotted where X-axis represent the number of clones (sequences) and Y-axis represent the number of OTU. Good's coverage formula [1-(n/N)] x 100 (where n is the number of single clone OTU and N is the total number of sequences for the analysed sample) (Good, 1953) was used to evaluate the MWO library coverage

3.2.9.3.10 Nucleotide Sequence Accession Numbers

The sequence data of the partial 16S rRNA gene of all the 437 clones reported in this study have been deposited in the GenBank nucleotide sequence database under the accession numbers **KF916697** - **KF917133**.

3.2.10 Statistical analysis

Mean generation times (ΔT_d) and specific growth rates (μ) of the isolates on carbazole was calculated using non-linear regression of growth curves for the period when growth rates were maximal using Prism version 5.0 (Graphpad software, San Diego, CA, USA).

4.1 Physico-chemical Properties of Study Sites

The physico-chemical properties of the soil samples from Abandoned Coal Power Plant (ACPP), Mechanic Workshop, Okokomaiko (MWO) and NEPA Substation, UNILAG (NESU) are shown in Tables 4.1. The results obtained for thevariables determined in each site showed remarkable differences, which reflect their degree of pollution.

Moisture content was highest at ACPP site (11.1%) and lowest at MWO site (6.85%). The pH of the three sampling sites was weakly acidic with a pH value of 5.4 at ACPP site and 6.1 at MWO site respectively.

There is a marked difference in the quantity of available nutrients in the polluted soil samples with conductivity values of 318 µs/cm, 159.4 µs/cm, and 67.4 µs/cm recorded for MWO, NESU, and ACPP sampling sites, respectively.

The total organic carbon (TOC) content of the polluted soils indicated variations for carbon stored in the soil organic matter (SOM). The TOC content of the soils is generally less than 4% with TOC values of 3.1%, 1.93%, and 1.01% for ACPP, MWO, and NESU sites, respectively.

The total hydrocarbon content (THC) of the soil samples showed distinct variations. MWO site has a staggering THC value of 157 x 10^3 mg/kg, followed by ACPP site with a THC value of 133 x 10^2 mg/kg. NESU site was the least polluted with a value of 216 mg/kg of soil.

The concentration of macronutrients such as nitrogen, phosphorus, and potassium in the polluted soils varied widely. ACPP site has a high concentration of phosphorus and potassium values (363.4 mg/kg, 18.4 mg/kg) when compared to the concentrations obtained from MWO (1.34 mg/kg, 2.10 mg/kg) and NESU (0.19 mg/kg, 0.28 mg/kg) sites respectively. However, nitrogen content of the three study sites is less than 1%.

Heavy metals such as lead, iron, zinc, manganese, nickel, copper, and cadmium were detected in reasonable concentrations from the three polluted sampling sites. ACPP site has the highest lead (Pb) concentration (4.7 mg/kg) when compared to the concentration obtained from MWO (0.11 mg/kg) and NESU (0.06 mg/kg) sites respectively. Cadmium

(1.12 mg/kg) and copper (5.10 mg/kg) were only detected at NESU site while higher concentration of zinc (3.31 mg/kg) and nickel (4.34 mg/kg) were recorded at MWO site.

Table 4.1: Physico-chemical Properties of the Soil samples

Parameters	**Study sites**		
	MWO	**NESU**	**ACPP**
pH	6.10	5.80	5.40
Moisture (%)	6.85	7.89	11.1
Conductivity (µs/cm)	318	159.4	67.4
Total organic carbon (%)	1.93	1.01	3.1
Total hydrocarbon content (mg/kg)	157×10^3	216	134×10^2
Potassium (mg/kg)	2.10	0.28	18.4
Phosphorus (mg/kg)	1.34	0.19	363.4
Nitrogen (%)	0.10	0.05	0.18
Sodium (mg/kg)	ND	ND	542
Chloride (mg/kg)	ND	ND	68.0
Iron (mg/kg)	2.27	28.83	ND
Manganese (mg/kg)	1.83	3.24	ND
Zinc (mg/kg)	3.31	0.47	ND
Lead (mg/kg)	0.11	0.06	4.70
Nickel (mg/kg)	4.34	3.42	ND
Cadmium (mg/kg)	ND	1.12	ND
Copper (mg/kg)	ND	5.10	ND

N.D: Not detected; MWO: Mechanic workshop, Okokomaiko; ACPP: Abandoned coal power plant soil, Ijora-Olopa; NESU: NEPA substation, UNILAG.

4.2 Microbiological Properties of Study Sites

The microbiological properties of the three study sites are shown in Table 4.2. The highest population density for total heterotrophic bacteria was obtained from NESU (8.40 x 10^9cfu/g) and the lowest from ACPP (6.18 x 10^7cfu/g). However, for total heterotrophic fungi, the highest population density was obtained from MWO (8.20 x 10^7cfu/g) while the lowest was obtained from ACPP (6.09 x 10^7cfu/g). The highest population of hydrocarbon degrading bacteria (6.72 x 10^6cfu/g) and fungi (5.4 x 10^5cfu/g) as well as the highest population of actinomycetes (4.6 x 10^5cfu/g) were obtained from MWO. The ACPP study site had the highest population of nitrogen fixers (6.2 x 10^5cfu/g).

The percentage of hydrocarbon-degrading bacteria and fungi in the total heterotrophic bacteria and fungi communities at MWO site is 0.91% and 0.65%, respectively. At ACPP site, hydrocarbon-degrading bacteria and fungi communities constitute 0.79% and 0.62% of the heterotrophic bacteria and fungi communities while at NESU site, the percentages were 0.0045% and 0.43% of the heterotrophic bacteria and fungi communities.

Table 4.2: Microbiological Characteristics of the Soil samples

STUDY SITES	**THB (cfu/g)**	**THF (cfu/g)**	**HUB (cfu/g)**	**HUF (cfu/g)**	**TNF (cfu/g)**	**TA (cfu/g)**
MWO	7.4×10^8	8.2×10^7	6.7×10^6	5.4×10^5	2.8×10^4	4.6×10^5
ACPP	6.2×10^7	6.1×10^7	4.9×10^5	3.8×10^5	6.2×10^5	3.2×10^5
NESU	8.4×10^9	6.3×10^7	3.8×10^5	2.7×10^5	4.3×10^4	2.8×10^5

Key: THB- Total heterotrophic bacteria THF- Total heterotrophic fungi

HUB- Hydrocarbon utilizing bacteria HUF- Hydrocarbon utilizing fungi

TNF- Total Nitrogen Fixers TA- Total Actinomycetes

4.3 Isolation and Characterization of Carbazole Degraders

Six bacterial isolates belonging to four genera namely *Pseudomonas*, *Stenotrophomonas*, *Achromobacter*, and *Microbacterium* were obtained by continuous enrichment method from two soil sampling sites, MWO and ACPP. Carbazole degraders were not isolated from NESU sampling site.

4.3.1 Strains SL1, SL2 and SL3

Strains **SL1**, **SL2** and **SL3** were isolated from ACPP study site. These strains are aerobic, motile, Gram-negative rods that are oxidase and catalase positive and urease negative. They failed to ferment most of the sugars tested with exception of glucose, xylose, galactose and mannose. They were positive for nitrate reduction and showed negative reaction to H_2S and indole production. Colonies on LB agar appeared circular, round and smooth with no pigmentation. Thus, the three isolates were putatively identified as *Achromobacter* species.

Multiple sequence alignments of the cloned 16S rDNA partial fragments of the three isolates (SL1, 1383 bp; SL2, 1383 bp; SL3, 1383 bp; Figure 4.1 - 4.3; Plate 4.1 - 4.2) with the nucleotide sequences in the NCBI databases revealed homology with the members of the genus *Achromobacter* exhibiting strong relationship with 99% homologies. Alignment of the nucleotide sequences of the three strains produces 99% homology thus, affirming the results of the homology search.

The nucleotide sequence of the three strains were deposited in the DDBJ, EMBL, and GenBank databases and assigned the name *Achromobacter* sp. with accession numbers **AB646575.2**, **AB646576.2** and **AB646577.2** for strains SL1, SL2 and SL3 respectively. The phylogenetic tree (Figure 4.7) showed closest relationship between these three strains and *Achromobacter* species.

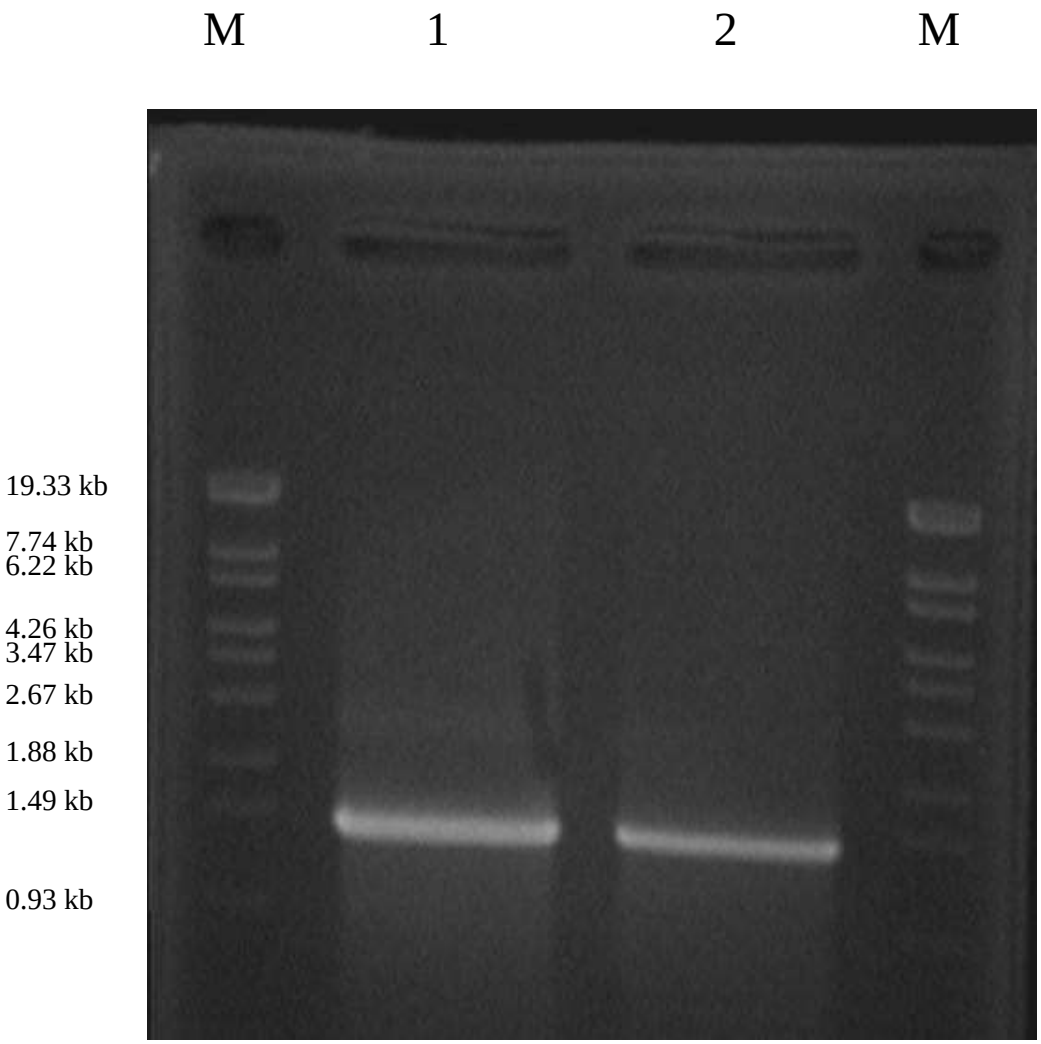


Plate 4.1: Electrophoretogram showing the bands of 16S rDNA amplicons. Agarose (1%) was used. Lanes are indicated as –M, OneSTEP Marker 6 (λ/Sty I digest); Lane 1: PCR amplicon of SL2 16S rDNA gene; Lane 2: PCR amplicon of SL6 16S rDNA gene.

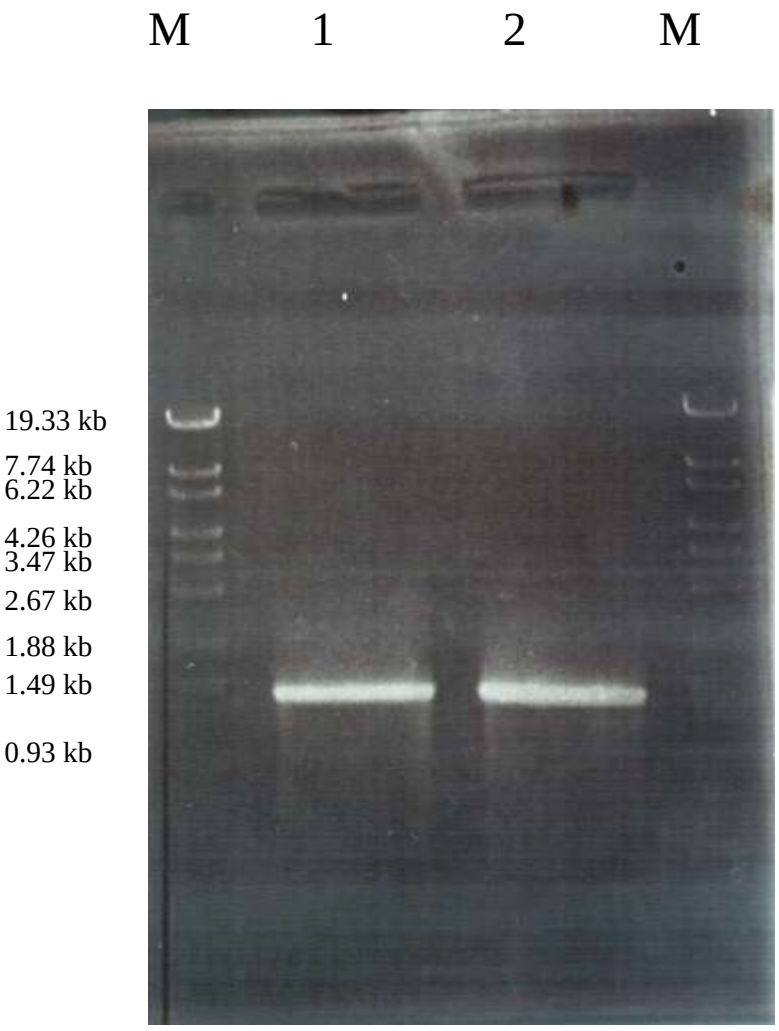


Plate 4.2: Electrophoretogram showing the bands of 16S rDNA amplicons. Agarose (1%) was used. Lanes are indicated as –M, OneSTEP Marker 6 (λ/Sty I digest); Lane 1: PCR amplicon of SL1 16S rDNA gene; Lane 2: PCR amplicon of SL3 16S rDNA gene.

>AB646575.2
AGAGTTTGATCCTGGCTCAGATTGAACGCTAGCGGGATGCCTTGCACATGCAAGTCGAAC
GGCAGCACGGACTTCGGTCTGGTGGCGAGTGGCGAACGGGTGAGTAATGTATCGGAACGT
GCCCAGTAGCGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTACGGGG
GAAAGCAGGGGATCTTCGGACCTTGCACTATTGGAGCGGCCGATATCGGATTAGCTAGTT
GGTGGGGTAACGGCCTACCAAGGCGACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACA
ATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAG
CACTTTTGGCAGGAAAGAAACGTCGCGGGCTAATACCTCGCGAAACTGACGGTACCTGCA
GAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCC
AGAGCTTAACTTTGGAACTGCATTTTTAACTACCGGGCTAGAGTGTGTCAGAGGGAGGTG
GAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCA
GCCTCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGCCTTCGGGCCTTGGT
AGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAA
AGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGA
AAAACCTTACCTACCCTTGACATGTCTGGAATGCCGAAGAGATTTGGCAGTGCTCGCAAG
AGAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGT
AGGGCTTCACACGTCATACAATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCT
AATCCCAGAAACCCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGG
AATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCG
//

Figure 4.1: Nucleotide sequence (1383 bp) of *Achromobacter* sp. strain SL1 (**AB646575.2**)

>AB646576.2
AGAGTTTGATCATGGCTCAGATTGAACGCTAGCGGGATGCCTTACACATGCAAGTCGAAC
GGCAGCACGGACTTCGGTCTGGTGGCGAGTGGCGAACGGGTGAGTAATGTATCGGAACGT
GCCCAGTAGCGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTACGGGG
GAAAGCAGGGGATCTTCGGACCTTGCACTATTGGAGCGGCCGATATCGGATTAGCTAGTT
GGTGGGGTAACGGCCTACCAAGGCGACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACA
ATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAG
CACTTTTGGCAGGAAAGAAACGTCGCGGGCTAATACCCCGCGAAACTGACGGTACCTGCA
GAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCC
AGAGCTTAACTTTGGAACTGCATTTTTAACTACCGGGCTAGAGTGTGTCAGAGGGAGGTG
GAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCA
GCCTCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGTCTTCGGACCTTGGT
AGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAA
AGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGA
AAAACCTTACCTACCCTTGACATGTCTGGAATGCCGAAGAGATTTGGCAGTGCTCGCAAG
AGAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGT
AGGGCTTCACACGTCATACAATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCT
AATCCCAGAAACCCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGG
AATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCG
//

Figure 4.2: Nucleotide sequence (1383 bp) of *Achromobacter* sp. strain SL2 (**AB646576.2**)

>AB646577.2
AGAGTTTGATCCTGGCTCAGATTGAACGCTAGCGGGATGCCTTACACATGCAAGTCGAAC
GGCAGCACGGACTTCGGTCTGGTGGCGAGTGGCGAACGGGTGAGTAATGTATCGGAACGT
GCCCAGTAGCGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTACGGGG
GAAAGCAGGGGATCTTCGGACCTTGCACTATTGGAGCGGCCGATATCGGATTAGCTAGTT
GGTGGGGTAACGGCCTACCAAGGCGACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACA
ATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAG
CACTTTTGGCAGGAAAGAAACGTCGCGGGCTAATACCCCGCGAAACTGACGGTACCTGCA
GAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCC
AGAGCTTAACTTTGGAACTGCATTTTTAACTACCGGGCTAGAGTGTGTCAGAGGGAGGTG
GAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCA
GCCTCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGCCTTCGGGCCTTGGT
AGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAA
AGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGA
AAAACCTTACCTACCCTTGACATGTCTGGAATGCCGAAGAGATTTGGCAGTGCTCGCAAG
AGAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGT
AGGGCTTCACACGTCATACAATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCT
AATCCCAGAAACCCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGG
AATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCG
//

Figure 4.3: Nucleotide sequence (1383 bp) of *Achromobacter* sp. strain SL3 (**AB646577.2**)

4.3.2 Strain SL4

Strain **SL4** was isolated from MWO study site. The strain was Gram negative, aerobic, non-sporulating, motile rod. Colonies on LB agar are smooth, circular and muddy white in appearance. The strain was positive for oxidase, catalase and nitrate reductase but negative for indole, methyl red/Voges-Proskauer, gelatinase, urease and amylase. Colonies utilized glucose, fructose, arabinose and galactose but failed to ferment xylose, lactose and raffinose.

Multiple sequence alignments of the cloned 16S rDNA partial fragment of strain SL4 (1389 bp; Figure 4.4; Plate 4.3) with the nucleotide sequences in the NCBI databases revealed homology with the members of the genus *Pseudomonas,* placing this bacterium within the *Pseudomonas* clade producing 99% homology with reference sequences.

The nucleotide sequence of strain SL4 was deposited in the DDBJ, EMBL, and GenBank databases and assigned the name *Pseudomonas* sp. strain SL4 with the accession number **AB646578.2**. The phylogenetic tree (Figure 4.7) showed closest relationship between *Pseudomonas* sp. strain SL4, and reference sequences (*Pseudomonas* spp) retrieved from NCBI GenBank, based on 16S rRNA gene nucleotide sequences.

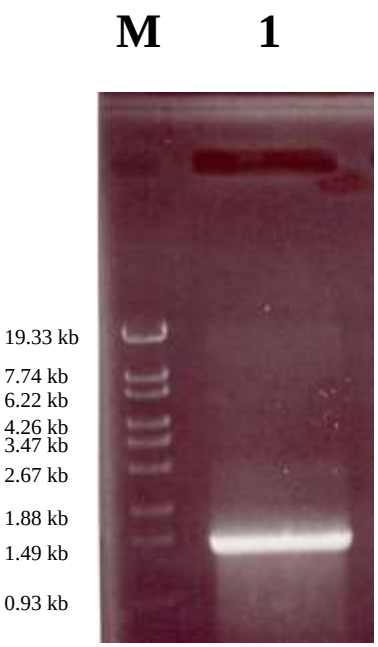


Plate 4.3: Electrophoretogram showing the bands of 16S rDNAamplicons. Agarose (1%) was used. Lanes are indicated as –M, OneSTEP Marker 6 (λ/Sty I digest); Lane 1: PCR amplicon of SL4 16S rDNA gene.

>AB646578.2
AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
GGATGAAGGGAGCTTGCTCCCGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCT
GCCTGGTAGTGGGGGACAACGTTCCGAAAGGAGCGCTAATACCGCATACGTCCTACGGGG
GAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTA
GGTGGGGTAATGGCTCACCTAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCA
CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG
CACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACA
GAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAGATGGATGTGAAATCCC
CGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGTACGGTAGAGGGTGGTG
GAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG
ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAG
TGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAATTCA
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
AAGAACCTTACCTGGCCTTGACATGTCCGGAATCCTGCAGAGATGCGGGAGTGCCTTCGG
GAATCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTAAGGTGGGCACTCTAA
GGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
ACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGT
GGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGA
AGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTG
TACACACCG
//

Figure 4.4: Nucleotide sequence (1389 bp) of *Pseudomonas* sp. strain SL4 (**AB646578.2**)

4.3.4 Strain B_A

Strain B_A was isolated from ACPP study site. The isolate is Gram-negative, aerobic, motile, non-spore forming, rod-shaped bacteria. Colonies of strain B_A on LB agar were circular, smooth, glossy, and convex with a pale yellow appearance. Biochemical tests indicated that strain B_A was positive for oxidase, catalase, and gelatinase and urease negative. It exhibited negative activities for nitrate reduction as well as H_2S and indole production. Most of the sugars tested such as glucose, arabinose, mannitol, mannose, inositol, rhamnose, sucrose, sorbitol, melobiose, maltose and inositol supported the growth of strain B_A. It was thus putatively identified as a *Xanthomonas* sp.

Furthermore, the molecular identification of strain B_A based on 16S rDNA homology of a partial 1397 bp sequence (Figure 4.5; Plate 4.4) with the sequences in NCBI database confirmed strain B_A as *Stenotrophomonas maltophilia*. Strain B_A exhibited 99% homology with *Stenotrophomonas maltophilia* strains. The nucleotide sequence of strain B_A was deposited and registered in the DDBJ, EMBL, and GenBank nucleotide sequence databases and was assigned the name *Stenotrophomonas maltophilia* strain B_A with the accession number **AB646574**. The phylogenetic tree (Figure 4.7) showed closest relationship between *Stenotrophomonas maltophilia* strain B_A, and reference sequences (*Stenotrophomonas maltophilia* strains) retrieved from NCBI GenBank, based on 16S rRNA gene nucleotide sequences.

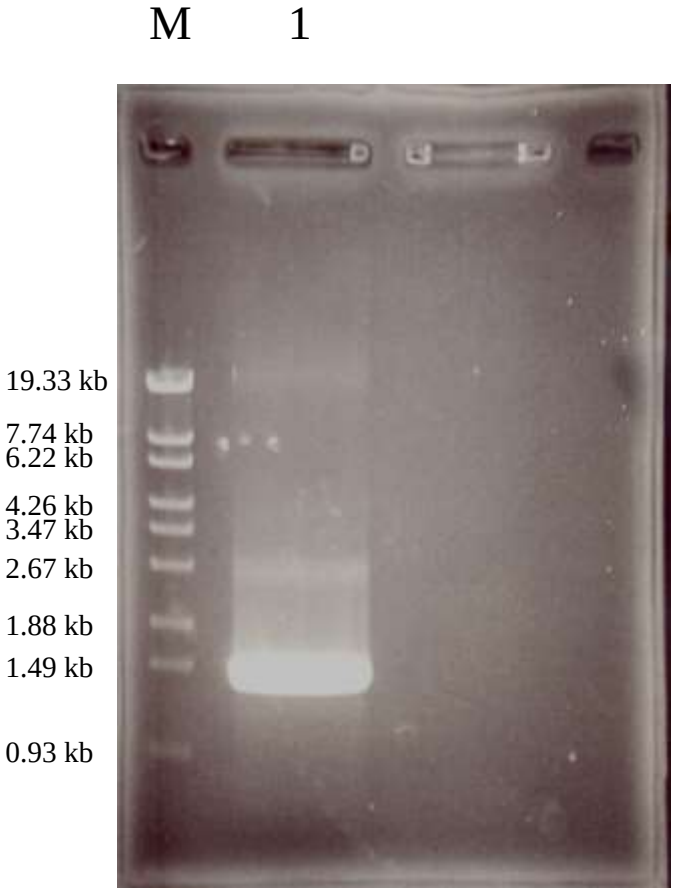


Plate 4.4: Electrophoretogram showing the band of 16S rDNA amplicon.Agarose (1%) was used. Lanes are indicated as –M, OneSTEP Marker 6 (λ/Sty I digest); Lane 1: PCR amplicon of B_A 16S rDNA gene.

>AB646574
AGAGTTTGATCATGGCTCAGAGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGGCAG
CACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTT
TTTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGG
GGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGC
CCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGG
TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGTCA
TACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGG
CTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCG
CGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGT
TTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAAT
GTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCA
TGGCGAAGGCAGCTACCTGGACCAACATTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGC
AGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCT
TACCTGGCCTTGACATGTCGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAACACA
GGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGG
AGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTACTACAAT
GGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATCCCAGAAACCCTATCTCAGTCCGG
ATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGC
GGTGAATACGTTCCCGGGCCTTGTACACACCG

Figure 4.5: Nucleotide sequence (1397 bp) of *S. maltophilia* strain B_A (**AB646574**)

4.3.3 Strain SL6

Strains **SL6** was isolated from MWO site. They are obligately aerobic, Gram positive, non-spore forming, irregular rods occurring singly or in clusters. On LB agar, SL6 was circular, smooth, translucent, yellow-pigmented, opaque, low-convex, moist colonies with entire margins. Strain SL6 colonies are catalase positive but negative for oxidase, methyl red, Voges-Proskauer, indole, gelatinase and H_2S production. It was positive for starch hydrolysis and is unable to utilize all the sugars tested with exception of mannitol, and salicin. The strain was thus putatively identified as *Corynebacterium* species.

However, comparison of the 16S rDNA partial fragment of strain SL6 (1374 bp; Figure 4.6; Plate 4.1) with the nucleotide sequences in the NCBI databases indicates significant alignments of the strain with *Microbacterium* species. Strain SL6 exhibited 99% homology with *Microbacterium esteraromaticum* strains. The nucleotide sequences of strain SL6 was deposited in the DDBJ, EMBL and GenBank databases and was assigned the name *Microbacterium esteraromaticum* strain SL6, with the accession number **AB646579.2**. The phylogenetic tree (Figure 4.7) showed closest relationship between *Microbacterium esteraromaticum* strain SL6, and reference sequences (*Microbacterium esteraromaticum* strains) retrieved from NCBI GenBank, based on 16S rRNA gene nucleotide sequences.

>AB646579.2
AGAGTTTGATCATGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAAC
GATGAAGCCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGCAACC
TGCCCCTGACTCTGGGATAAGCGCTGGAAACGGCGTCTAATACTGGATATGTCCCGTCAC
CGCATGGTGTGCGGGTGGAAAGATTTTTCGGTTGGGGATGGGCTCGCGGCCTATCAGCTT
GTTGGTGAGGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
ACAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTA
AACCTCTTTTAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAAAAGCACCGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGAATTATTGGGCG
TAAAGAGCTCGTAGGCGGTCTGTCGCGTCTGCTGTGAAATCCCGAGGCTCAACCTCGGGC
TTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCG
GTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAAC
TGACGCTGAGGAGCGAAAGGGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCC
CGTAAACGTTGGGAACTAGTTGTGGGGTCCTTTCCACGGATTCCGTGACGCAGCTAACGC
ATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGG
ACCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAG
GCTTGACATACACGAGAACGGGCCAGAAATGGTCAACTCTTTGGACACTCGTGGACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGGATACTGCCGGGGTC
AACTCGGAGAAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCAC
GCATGCTACAATGGCCGGTACAATGGGCTGCGATACCGTAAGGTGGAGCGAATCCCAAAA
AGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGT
AATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
//

Figure 4.6: Nucleotide sequence (1374 bp) of *M. esteraromaticum* strain SL6 (**AB646579.2**)

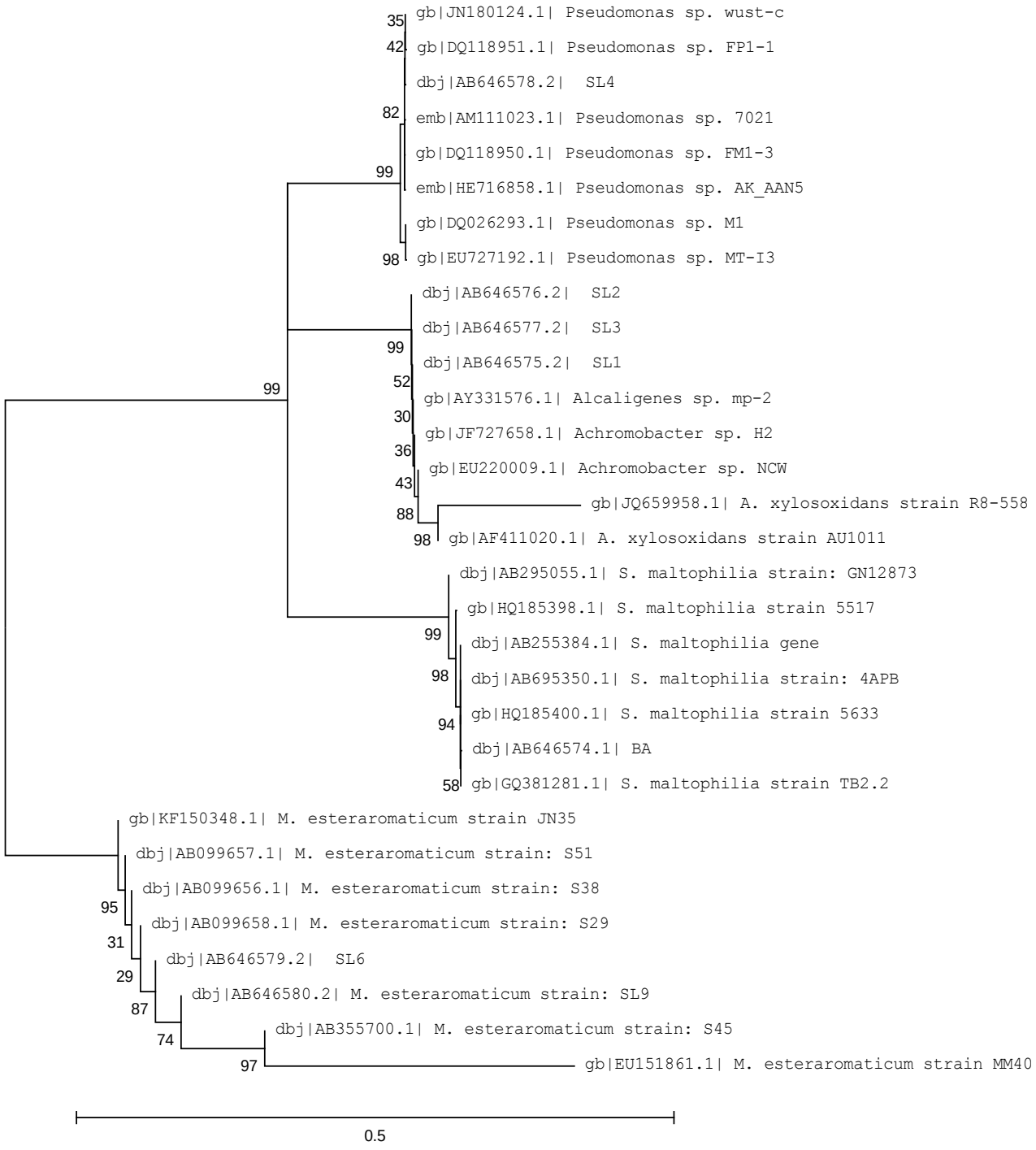


Figure 4.7: Phylogenetic tree resulting from neighbor joining analysis of 16S rRNA showing the phylogenetic positions of carbazole-degrading strains SL1, SL2, SL3, SL4, SL6 and B_A and related species of the genus *Achromobacter*, *Pseudomonas*, *Microbacterium and Stenotrophomonas* retrieved from NCBI GenBank. Accession number of each microorganism used in the analysis is shown before the species name.

4.4 Substrate Specificity of Isolates

The substrate utilization patterns of the isolates are shown in Table 4.3. All the isolates failed to grow on naphthalene and dibenzofuran. Strain SL1 grew luxuriantly on anthranilic acid, dibenzothiophene- sulfone, carbazole and crude oil utilizing them as sole source of carbon and energy. It weakly utilized acenaphthene, 3,3′-dimethoxybenzidine, N-ethyl carbazole,*p*-hydroxybenzoic acid and engine oil, while it recorded poor growth on fluorene, pyrene and dibenzothiophene.

Strain SL4 showed luxuriant growth on dibenzothiophene-sulfone, 3,3'-dimethoxybenzidine, carbazole, and crude oil utilizing them as sole source of carbon and energy. It exhibited weak utilization of acenaphthene, anthranilic acid, and engine oil, with poor growth on pyrene, *p*-hydroxybenzoic acid, and N-ethyl carbazole, while it failed to utilize fluorene and dibenzothiophene.

Strain SL6 showed luxuriant growth on crude oil and carbazole and failed to utilize 3,3'-dimethoxybenzidine. The isolate weakly utilized fluorene, pyrene, dibenzothiophene-sulfone, N-ethyl carbazole, and anthranilic acid, while it showed poor utilization of acenaphthene, dibenzothiophene, *p*-hydroxybenzoic acid, and engine oil.

Strain B_A was unable to utilize *p*-hydroxybenzoic acid and show luxuriant growth on carbazole. It exhibited weak utilization of acenaphthene, dibenzothiophene-sulfone, and crude oil, while it shows poor utilization of fluorene, pyrene, anthranilic acid, dibenzothiophene, 3,3'-dimethoxybenzidine, engine oil and N-methyl carbazole.

Table 4.3: Substrate Specificity of Carbazole-Degrading Isolates

Substrate	**Isolates**			
	SL1	**SL4**	**SL6**	**B_A**
Naphthalene	-	-	-	-
Fluorene	+	-	++	+
Acenaphthene	++	++	+	++
Pyrene	+	+	+	+
Carbazole	+++	+++	+++	+++
Dibenzofuran	-	-	-	-
Dibenzothiophene	+	-	+	-
Dibenzothiophene-sulfone	+++	+++	++	++
3,3'-dimethoxybenzidine	++	+++	-	+
N-ethyl carbazole	++	+	++	+
Anthranilic acid	+++	+++	++	+
p-hydroxybenzoic acid	++	+	+	-
Crude oil	+++	+++	+++	++
Engine oil	++	++	+	+

+++ Luxuriant growth (>10^6 cfu/ml after 5 days of incubation); ++ Weak growth (>10^6 cfu/ml after 1 week of incubation); + Poor growth (>10^6 cfu/ml after 2 weeks of incubation); - No growth (<10^6 cfu/ml after 2 weeks of incubation).

4.5 Biodegradation Studies

4.5.1 Time Course of Growth of Isolates on Carbazole

The growth profiles of four carbazole-degrading strains out of the six strains characterized were monitored on carbazole. The four strains (SL1, SL4, SL6, and B_A) were selected based on their higher degradative ability on carbazole.

The growth profile of strain SL1 on carbazole after 30 days of incubation showed an initial slow growth followed by an exponential increase from an initial population of 9.3 x 10^6 cfu/ml to 7.4 x 10^9cfu/ml in 12 days. The population thereafter dropped gradually. The isolate exhibited specific growth rate and mean generation time of 0.0229 h^{-1} and 30 h, respectively (Figure 4.8, Table 4.4). From the initial carbazole concentration recovered from the heat-killed control (Figure 4.9), strain SL1 reduced the initial concentration to 11.60 mg/L after 30 days of incubation at room temperature constituting 81.3% carbazole degradation with degradation rate and rate of degradation of 0.113% h^{-1} and 0.057 mg l^{-1} h^{-1}, respectively. Figure 4.10 shows the GC-FID chromatogram of n-hexane extract of residual carbazole from strain SL1 culture after 30 days of incubation.

Strain SL4 displayed an initial slow growth on carbazole followed by a steady exponential increase in the population from an initial population of 9.2 x 10^6 cfu/ml to 8.1 x 10^9 cfu/ml in 12 days. Thereafter, the population density slowly declined. The isolate exhibited specific growth rate and mean generation time of 0.0238 h^{-1} and 29.0 h, respectively (Figure 4.11, Table 4.4). Strain SL4 exhibited the highest carbazole biodegradation ability by reducing the initial concentration to 9.34 mg/L after 30 days of incubation at room temperature constituting 85% degradation. The degradation rate and rate of degradation were 0.118% h^{-1} and 0.062 mg l^{-1} h^{-1}, respectively. Figure 4.12 shows the GC-FID chromatogram of n-hexane extract of residual carbazole from strain SL4.

After an initial slow growth, strain SL6 population increased exponentially from an initial population of 9.1 x 10^6 cfu/ml to 5.3 x 10^9 cfu/ml in 18 days. The population thereafter declined gradually. The isolate exhibited specific growth rate and mean generation time of 0.0125 h^{-1} and 55.4 h, respectively (Figure 4.13, Table 4.4). Strain SL6 reduced the initial carbazole concentration to 22.03 mg/L after 30 days of incubation at room

temperature constituting 64.4% of carbazole degradation with degradation rate and rate of degradation of 0.089% h^{-1} and 0.036 mg l^{-1} h^{-1}, respectively. Figure 4.14 shows the GC-FID chromatogram of n-hexane extract of residual carbazole from strain SL6 after 30 days of incubation.

Strain B_A also exhibited an initial slow growth on carbazole followed by an exponential increase from initial population density of 9.2 x 10^6cfu/ml to 7.4 x 10^9cfu/ml in 12 days. The population thereafter steadily declined. The isolate however exhibited specific growth rate and mean generation time of 0.0233 h^{-1} and 29.5 h, respectively (Figure 4.15, Table 4.4). Strain B_A reduced the initial concentration to 14.59 mg/L after 30 days of incubation at room temperature constituting 76.4% carbazole degradation respectively with degradation rate and rate of degradation of 0.106% h^{-1} and 0.050 mg l^{-1} h^{-1}, respectively. Figure 4.16 shows the GC-FID chromatogram of n-hexane extract of residual carbazole from strain B_A after 30 days of incubation.

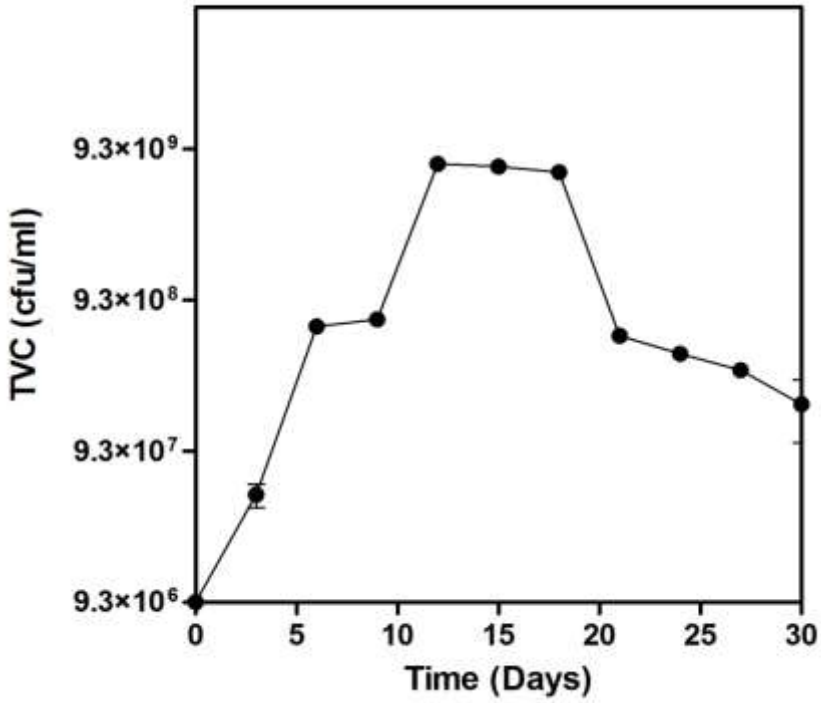


Figure 4.8: Population dynamics of *Achromobacter* sp. strain SL1 on carbazole after 30 days of incubation at room temperature. Carbazole was supplied at a concentration of 50 ppm. Data points represent the mean of three replicate flasks. Error bars represent standard deviation.

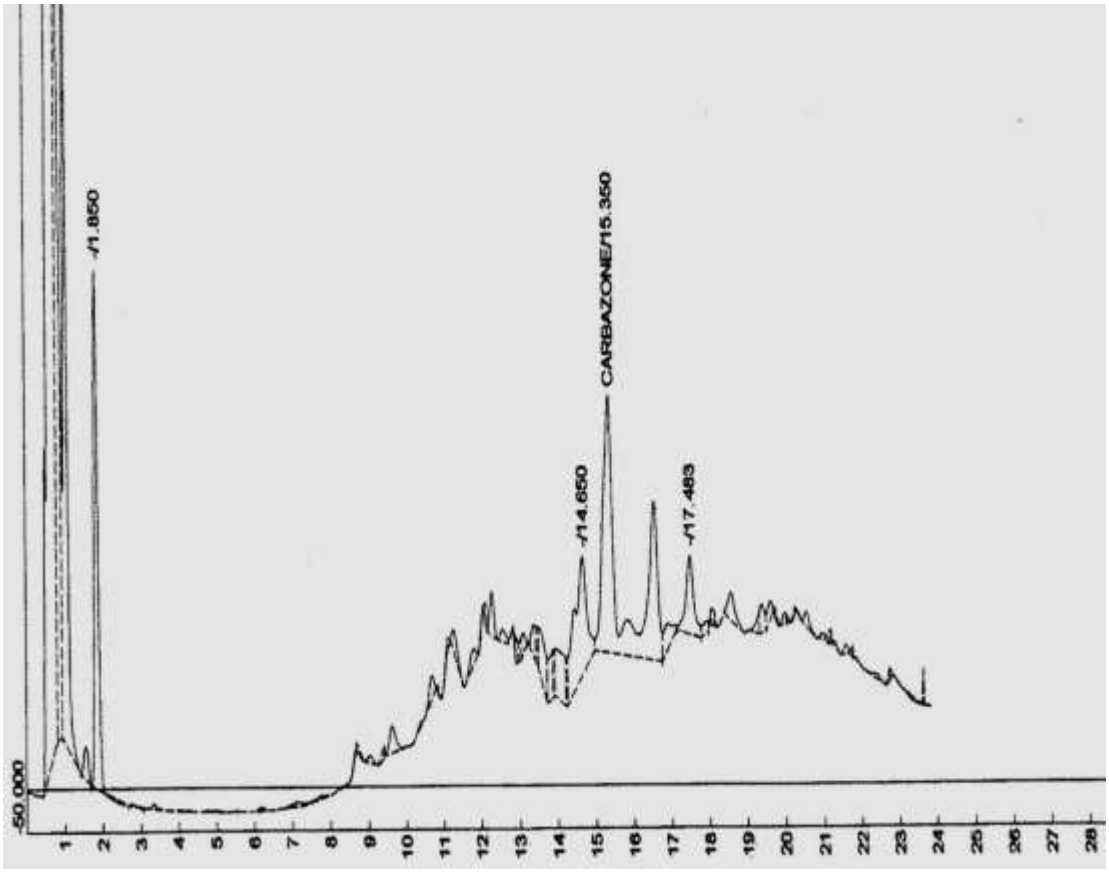


Figure 4.9: Gas chromatographic traces of n-hexane extract of recovered carbazole from control flask (containing heat-killed cells).

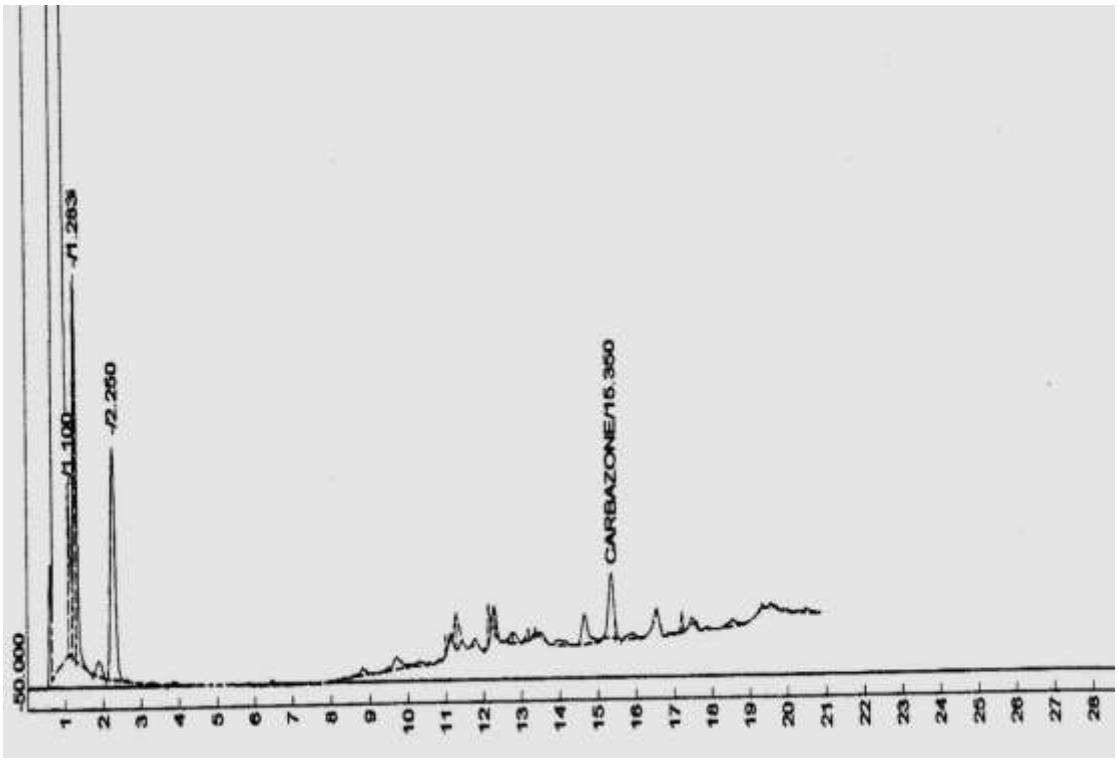


Figure 4.10: Gas chromatographic traces of n-hexane extract of recovered carbazole from the culture flask of *Achromobacter* sp. strain SL1 after 30 days of incubation.

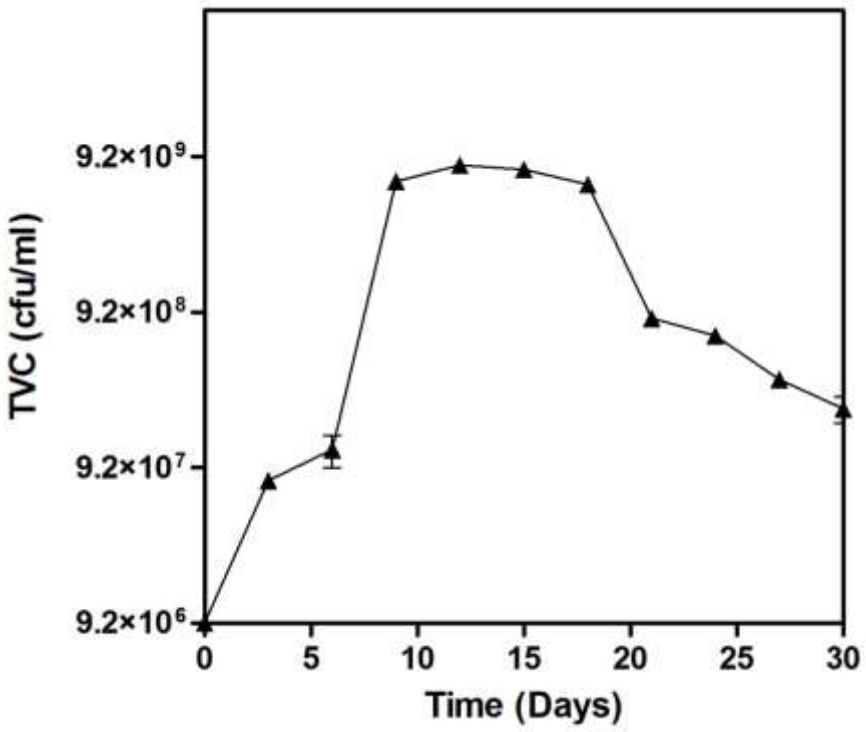


Figure 4.11: Population dynamics of *Pseudomonas* sp. strain SL4 on carbazole after 30 days of incubation at room temperature. Carbazole was supplied at a concentration of 50 ppm. Data points represent the mean of three replicate flasks. Error bars represent standard deviation.

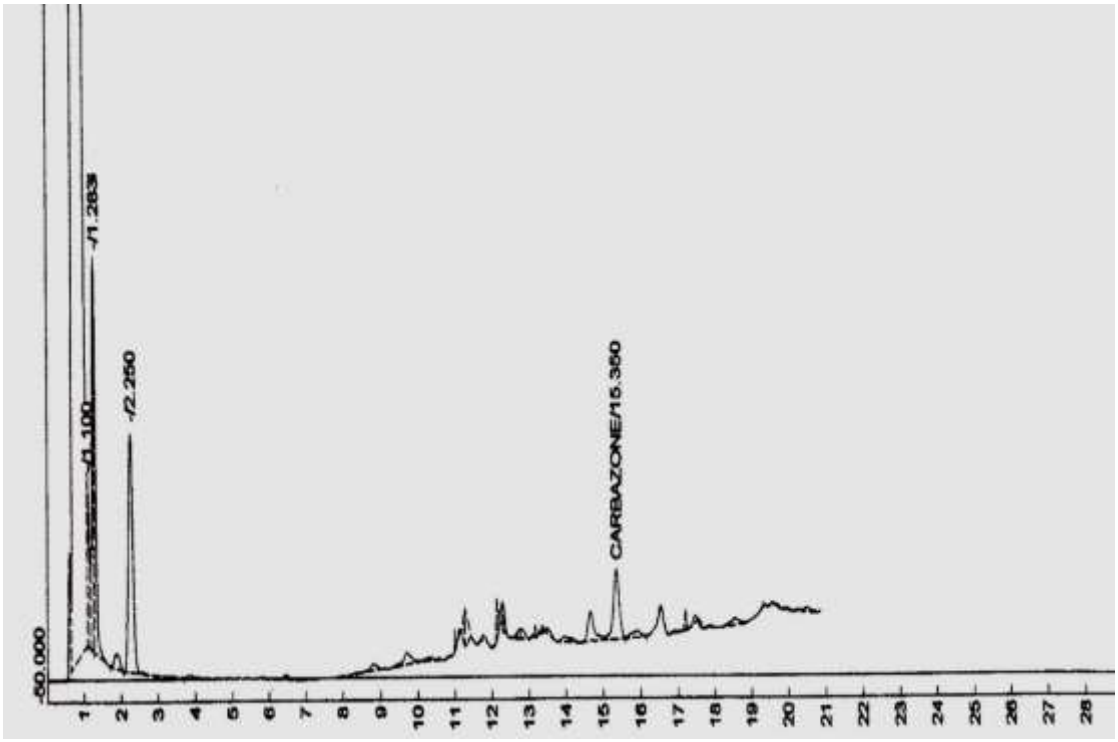


Figure 4.12: Gas chromatographic traces of n-hexane extract of recovered carbazole from the culture flask of *Pseudomonas* sp. strain SL4 after 30 days of incubation.

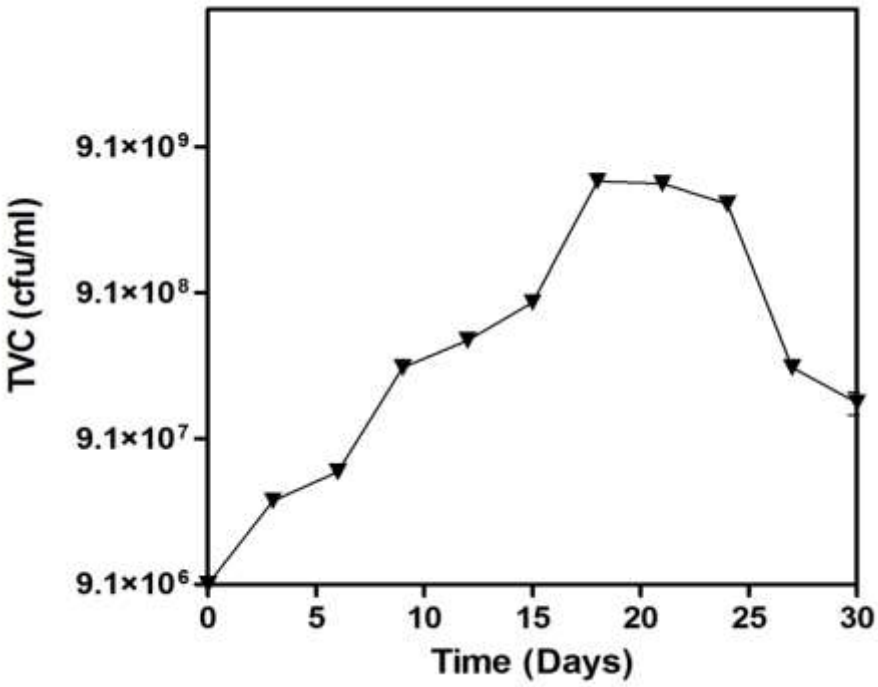


Figure 4.13: Population dynamics of *Microbacterium esteraromaticum* strain SL6 on carbazole after 30 days of incubation at room temperature. Carbazole was supplied at a concentration of 50 mg/L. Data points represent the mean of three replicate flasks. Error bars represent standard deviation.

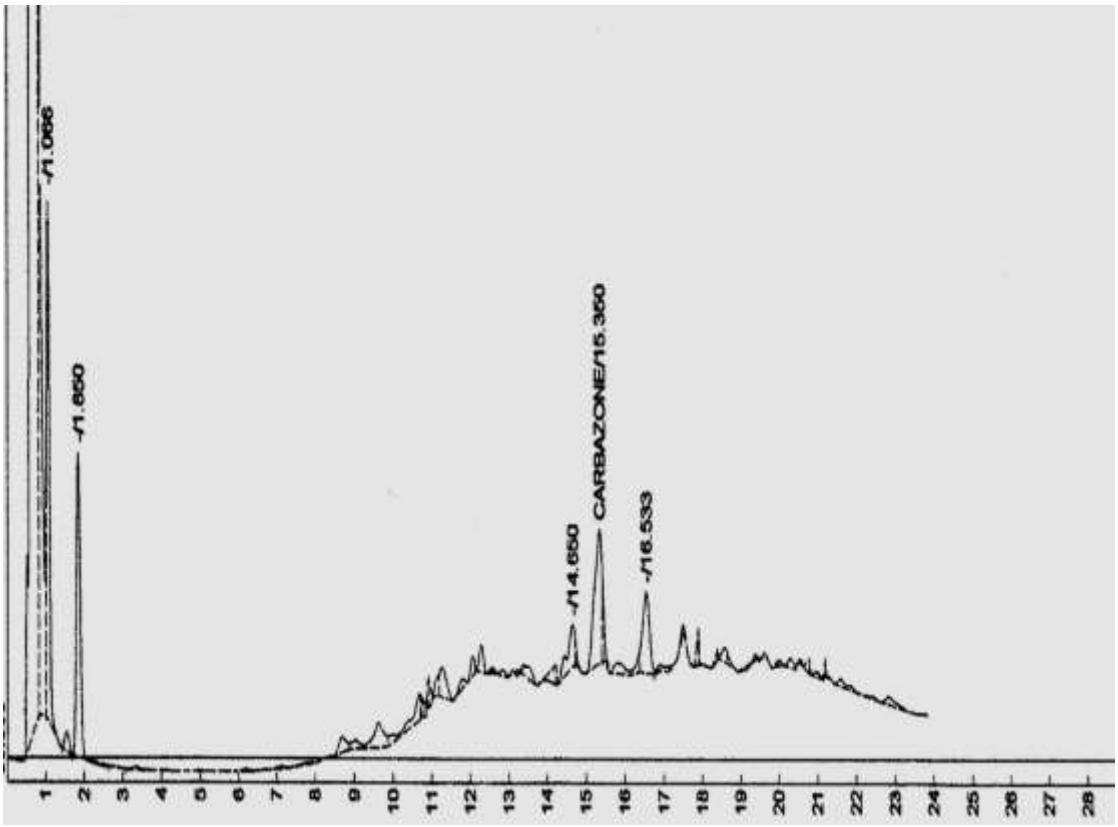


Figure 4.14: Gas chromatographic traces of n-hexane extract of recovered carbazole from culture flask of *Microbacterium esteraromaticum* strain SL6 after 30 days of incubation.

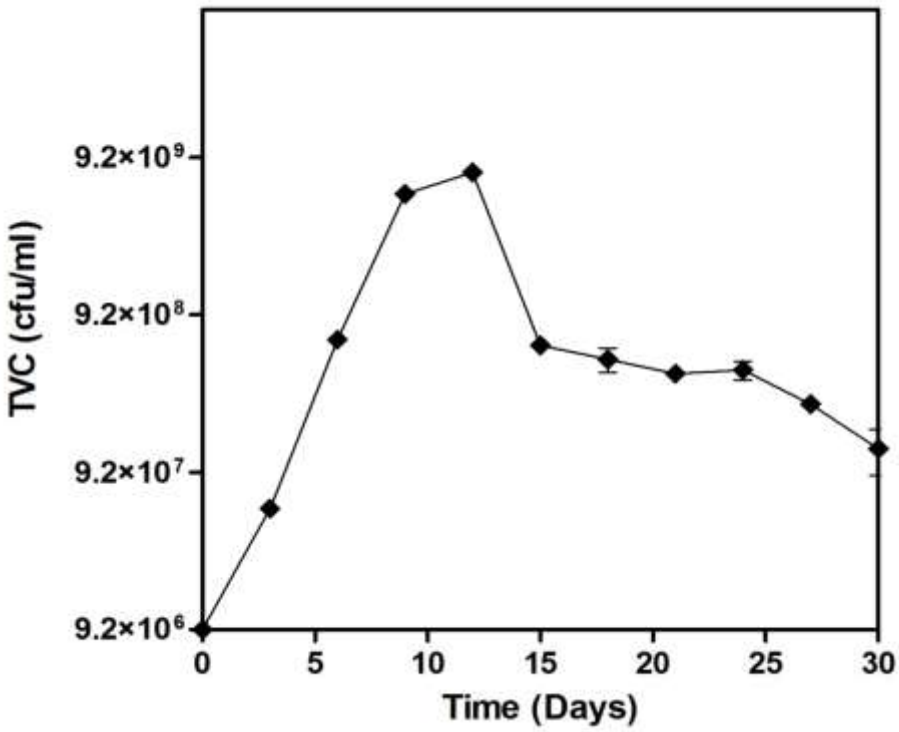


Figure 4.15: Population dynamics of *Stenotrophomonas maltophilia* strain B_A on carbazole after 30 days of incubation at room temperature. Carbazole was supplied at a concentration of 50 ppm. Data points represent the mean of three replicates flasks. Error bars represent standard deviation.

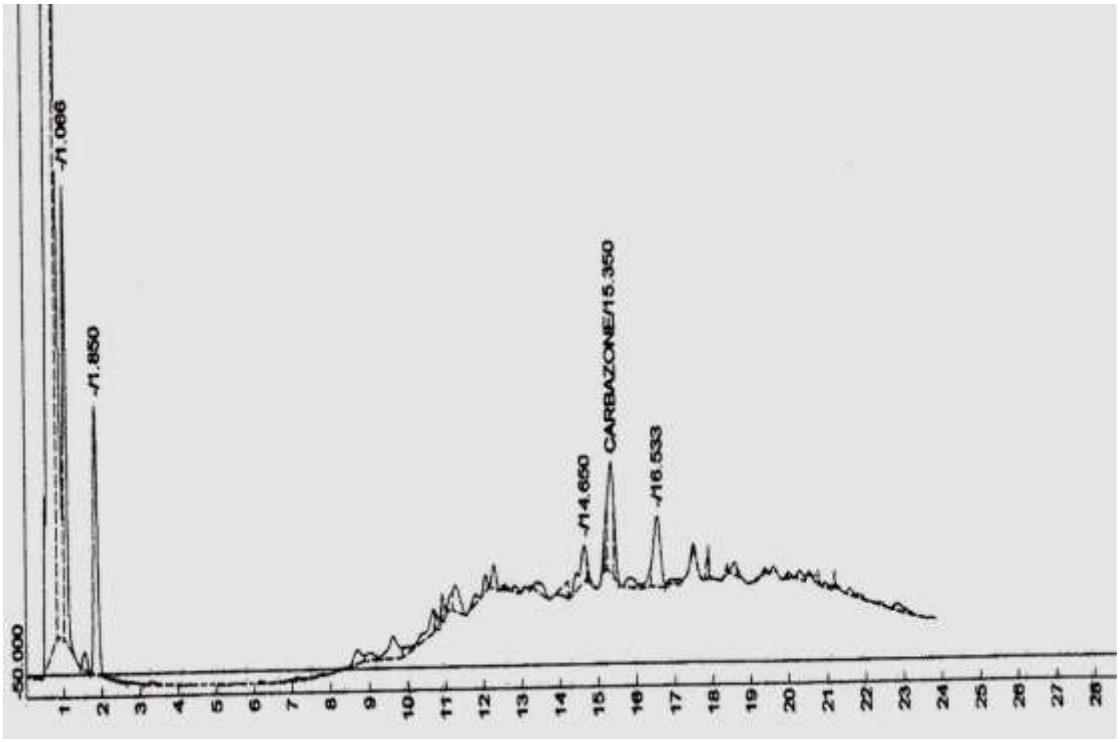


Figure 4.16: Gas chromatographic traces of n-hexane extract of recovered carbazole from the culture flask of *Stenotrophomonas maltophilia* strain B_A after 30 days of incubation.

Table 4.4: Growth Kinetics of the Isolates on Carbazole

Isolates	**Specific growth rate,μ (h^{-1})**	**Mean generation time, ΔT_d (h)**	**% degradation***	**Degradation rate (%/h)**	**Rate of degradation (mg l^{-1} h^{-1})**
Achromobacter sp. strain SL1	0.0229	30.0	81.3	0.113	0.057
Pseudomonas sp. strain SL4	0.0238	29.0	85.0	0.118	0.062
M. esteraromaticum strain SL6	0.0125	55.4	64.4	0.089	0.036
S. maltophilia strain B_A	0.0233	29.5	76.4	0.106	0.050

*Percent degradation values represent the net decrease (FID area counts) calculated with reference to the amount recovered from heat-killed control flasks.

4.5.2 Detection of Metabolite(s) of Carbazole Biodegradation

Gas chromatography-mass spectrometry was used to detect metabolites of carbazole biodegradation from the four strains. GC-MS analysis of ethyl acetate extracts of the growing and resting cells of three out of the four strains showed anthranilic acid as the only metabolite detected. However, HPLC analysis of acetonitrile extracts of culture of anthranilic acid-grown cells of strains SL1, SL4, and SL6 showed detection of catechol and small traces of *cis,cis* muconic acid (in SL1 only). Anthranilic acid was detected neither in ethyl acetate extract of growing cells nor resting cells of strain B_A.

4.5.2.1 *Achromobacter* sp. Strain SL1

The GC-MS chromatogram and mass spectra data of anthranilic acid detected from both the growing and the resting cells of strain SL1 grown on 50 ppm of carbazole are shown in Figure 4.17-4.21.

GC-MS analysis of the ethyl acetate extracts of the resting cells of strain SL1 after 1 h, 2 h, 3 h, and 24 h of incubation indicate the detection of anthranilic acid methylated at the COOH group with retention time of 5.21 min from the extract with concentrations that initially accumulated for the first two hours. The concentration however started to decrease as evident in peak reduction after 3 h and 24 h. The depletion of the accumulated anthranilic acid after 2 h suggest further metabolism of the accumulated anthranilic acid to catechol which can be metabolized further to the intermediate of the TCA cycle.

GC-MS analysis of the ethyl acetate extract of the growing cells of strain SL1 after 5 days of incubation also resulted in the detection of transient amounts of anthranilic acid methylated at both the COOH and the NH_2 groups with retention time of 5.30 minutes. Detection of anthranilic acid in trace amount from the ethyl acetate extract of the growing cells is a clear indication that this metabolite is not a dead-end metabolite and it is being metabolized further by the isolate.

HPLC analysis of acetonitrile extract of growing cells of strain SL1 in 50 ppm of anthranilic acid after 14 days of incubation showed the detection of catechol as major

metabolite and small amount of *cis,cis* muconic acid at retention times 1.3 min and 8.97 min, respectively (Figure 4.22). The metabolites were identified with reference to retention times of standards (Anthranilic acid; Catechol; Figure 4.22).

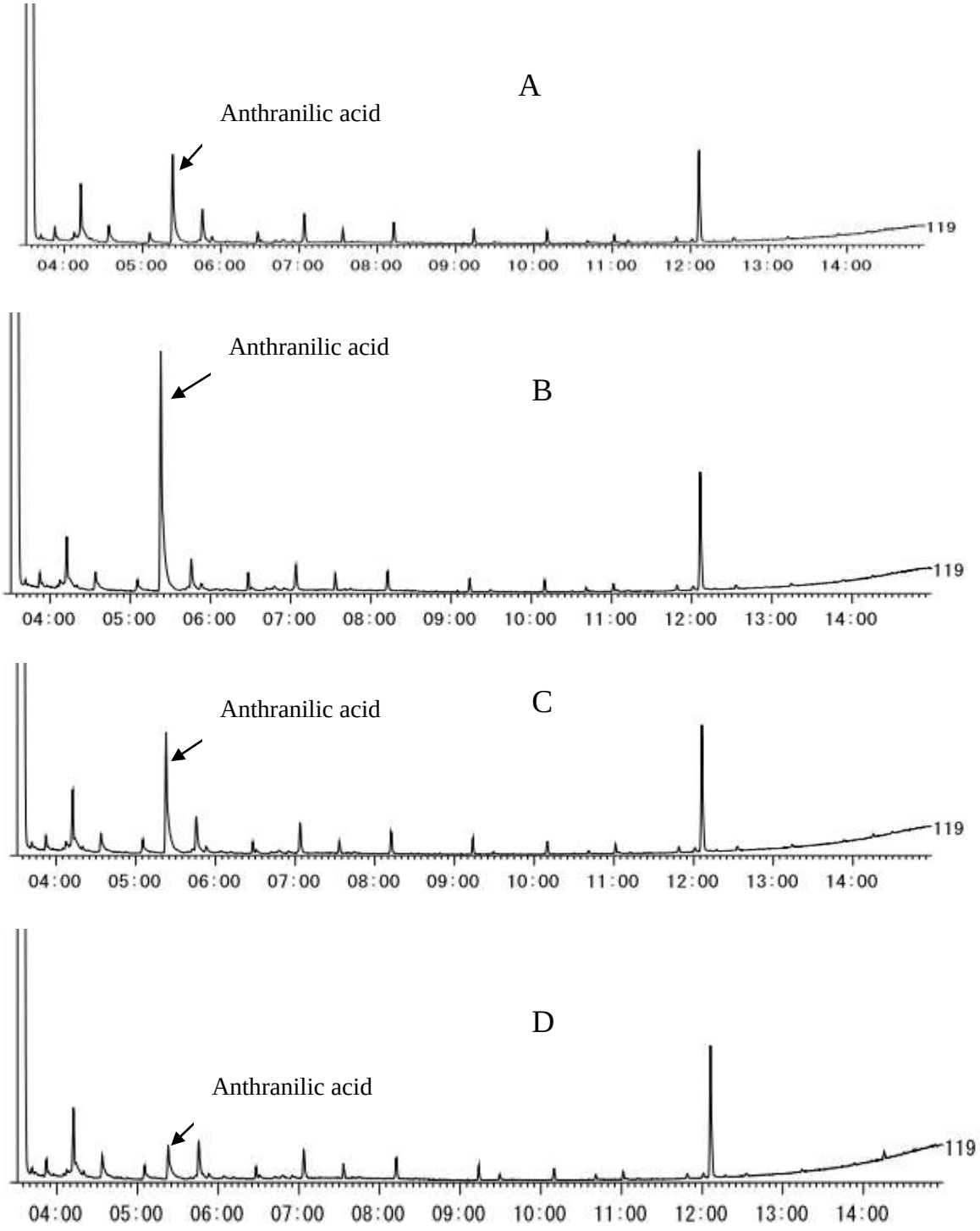


Figure 4.17: GC-MS chromatograms showing the peaks of anthranilic acid (methylated) recovered from resting cell culture ethyl acetate extracts of strain SL1 grown on carbazole (50 ppm) as carbon and energy source after (A) 1 h, (B) 2 h, (C)3 h and (D) 24 h of incubation at 30°C and 300 rpm.

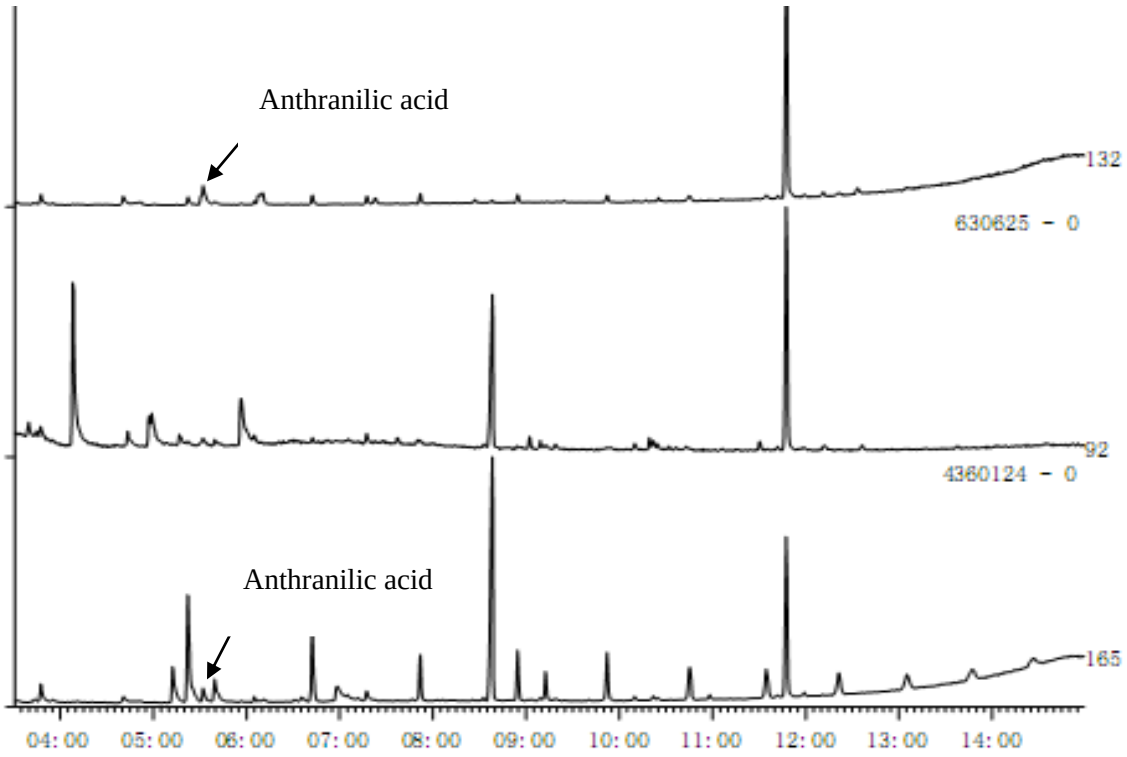


Figure 4.18: GC-MS chromatograms showing the peaks of anthranilic acid (N-methyl, methyl ester) recovered from growing cell culture ethyl acetate extracts of strain SL1 grown on carbazole (50 ppm) as carbon and energy source after 5 days of incubation at 30°C and 300 rpm.

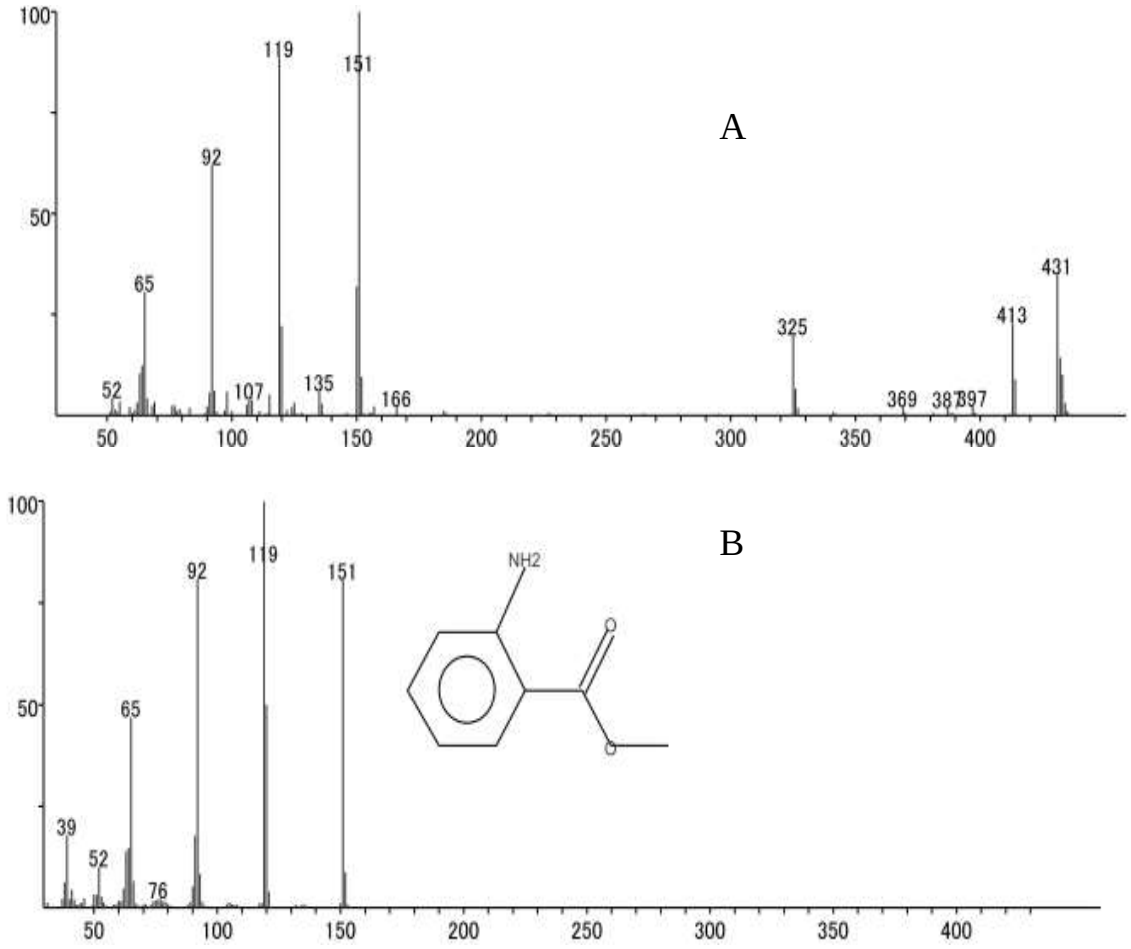


Figure 4.19: GC-MS mass spectra data for (A) methylated anthranilic acid recovered from ethyl acetate extract of resting cells culture of strain SL1 grown on carbazole (50 ppm) and (B) standard anthranilic acid (methylated).

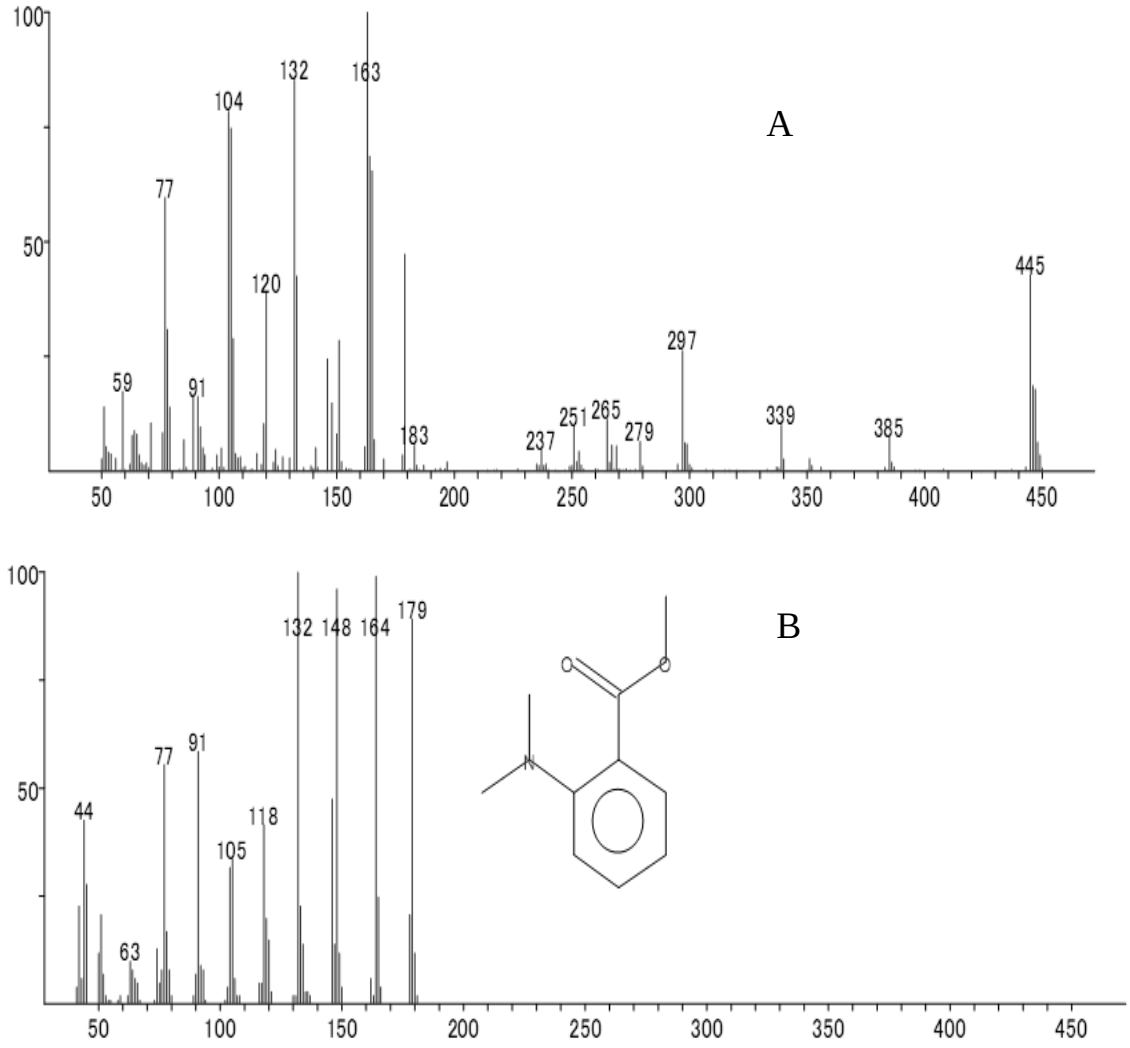


Figure 4.20: GC-MS mass spectra data for (A) anthranilic acid (N,N-dimethyl, methyl ester) recovered from ethyl acetate extract of growing cells culture of strain SL1 grown on carbazole (50 ppm) for 5 days and incubated at 30°C and 300 rpm and (B) standard anthranilic acid (N,N-dimethyl, methyl ester).

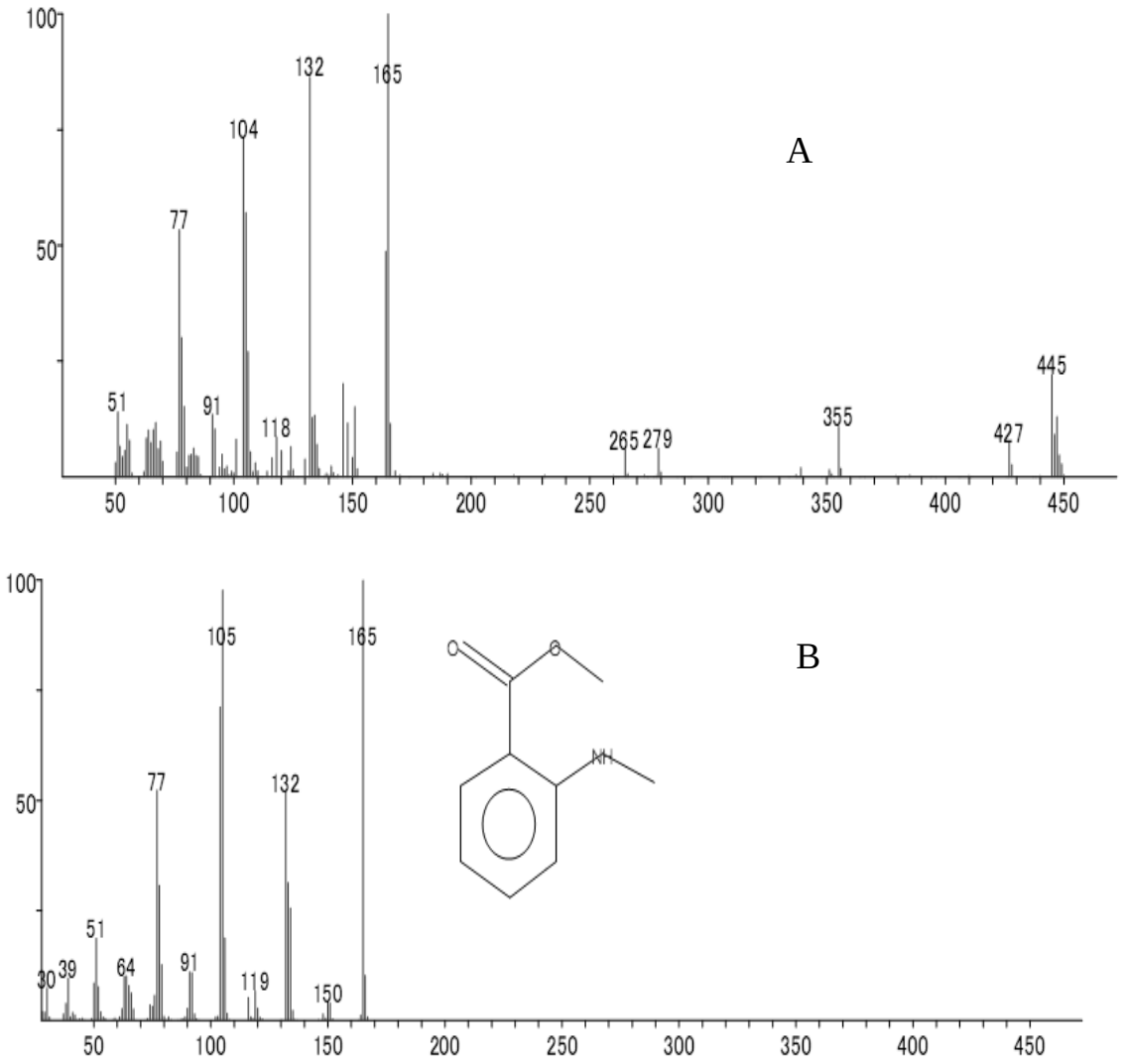


Figure 4.21: GC-MS mass spectra data for (A) anthranilic acid (N-methyl, methyl ester) recovered from ethyl acetate extract of growing cells culture of strain SL1 grown on carbazole (50 ppm) for 5 days and incubated at 30°C and 300 rpm and (B) standard anthranilic acid (N-methyl, methyl ester).

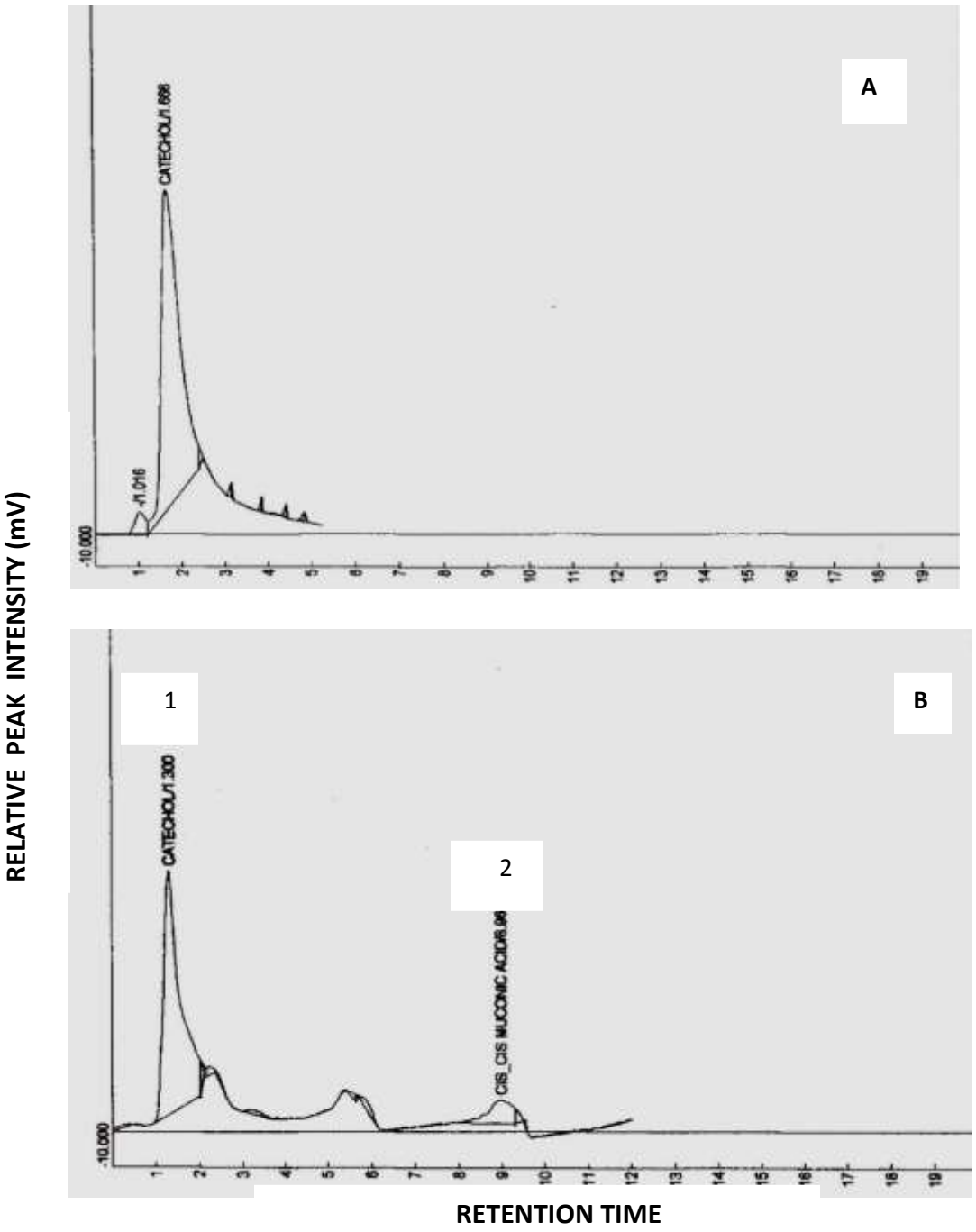


Figure 4.22: HPLC chromatograms of acetonitrile extracts of AN (anthranilic acid) cultures (50 ml CFMM with 0.3 mM AN) of strains SL1. Catechol (CAT) standard (A), acetonitrile extract of AN culture of strain SL1 (B). Peaks 1 (CAT), 3 (*cis cis* muconic acid) were identified by comparing the retention times with those of authentic CAT and AN.

4.5.2.2 *Pseudomonas* sp. Strain SL4

The GC-MS chromatogram and mass spectra data of anthranilic acid detected from both the growing and the resting cells of strain SL4 grown on 50 ppm of carbazole are shown in Figure 4.23-4.26.

GC-MS analysis of the ethyl acetate extracts of the resting cells of strain SL4 after 1 h, 2 h, 3 h, and 24 h of incubation indicate the detection of anthranilic acid methylated at the COOH group with retention time of 5.20 minutes. Anthranilic acid was consistently detected throughout the time points (i.e. 1 h, 2 h, 3 h, and 24 h) used.

GC-MS analysis of the ethyl acetate extract of the growing cells of strain SL4 after 5 days of incubation however resulted in the detection of transient amounts of anthranilic acid methylated at both the COOH and the NH_2 groups with retention time of 5.30 minutes. Detection of anthranilic acid in trace amount from the ethyl acetate extract of the growing cells is a clear indication that this metabolite is not a dead-end metabolite and it is being metabolized further by the isolate.

HPLC analysis of acetonitrile extract of growing cells of strain SL4 in 50 ppm of anthranilic acid after 14 days of incubation showed the detection of catechol as major metabolite and undegraded anthranilic acid at retention times 1.85 min and 1.17 min, respectively (Figure 4.27). This indicates that not all the anthranilic acid was degraded by the isolates after 14 days of incubation.

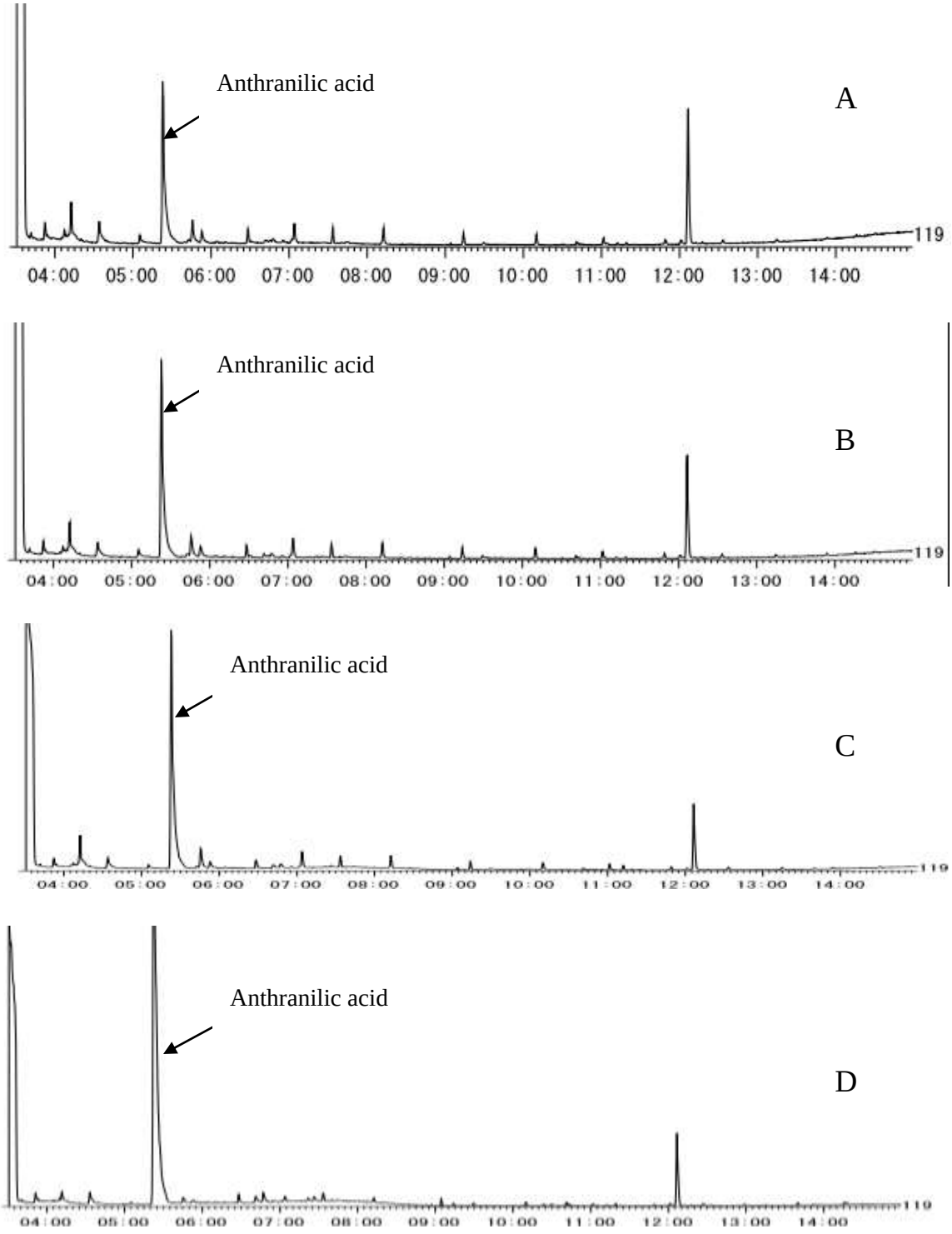


Figure 4.23: GC-MS chromatograms showing the peaks of anthranilic acid (methylated) from ethyl acetate extract of resting cell culture of strain SL4 grown on carbazole (50ppm) as carbon and energy source after (A) 1 hr, (B) 2 hr, (C) 3h and (d) 24hr of incubation at 30°C and 300 rpm.

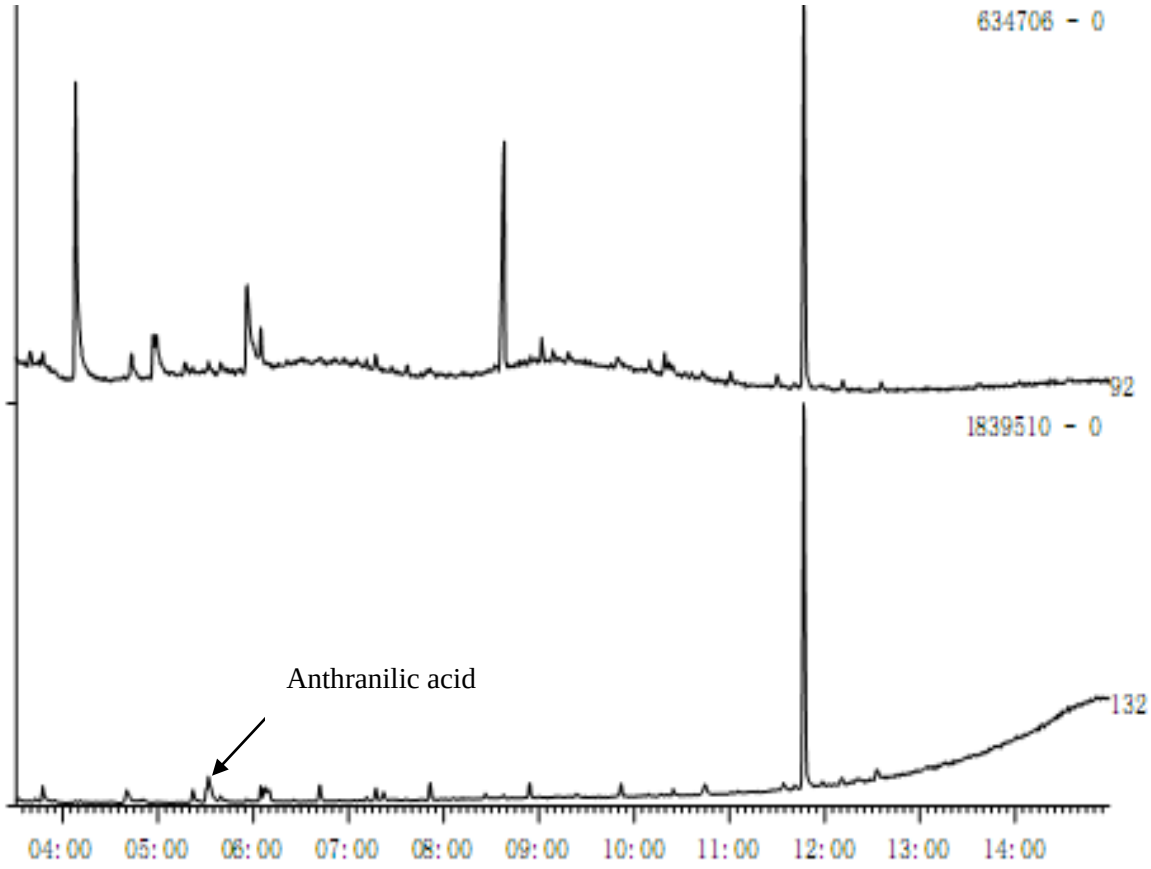


Figure 4.24: GC-MS chromatograms showing the peaks of anthranilic acid (N,N-dimethyl, methyl ester) recovered from ethyl acetate extract of growing cell culture of strain SL4 grown on carbazole (50 ppm) as carbon and energy source after 5 days of incubation at 30°C and 300 rpm.

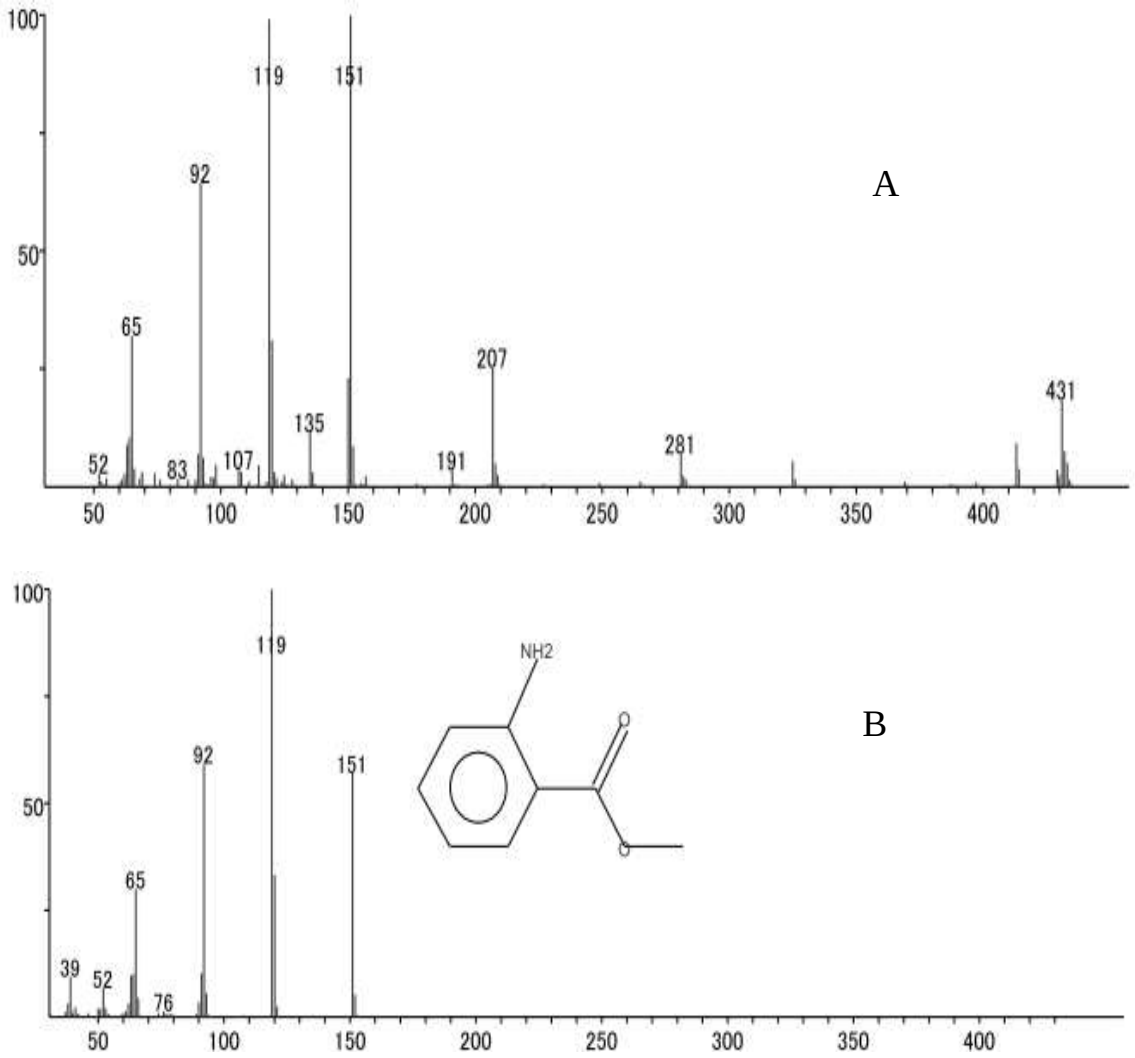


Figure 4.25: GC-MS mass spectra data for (A) methylated anthranilic acid recovered from ethyl acetate extract of resting cell culture of strain SL4 grown on carbazole (50 ppm) and (B) standard anthranilic acid (methylated).

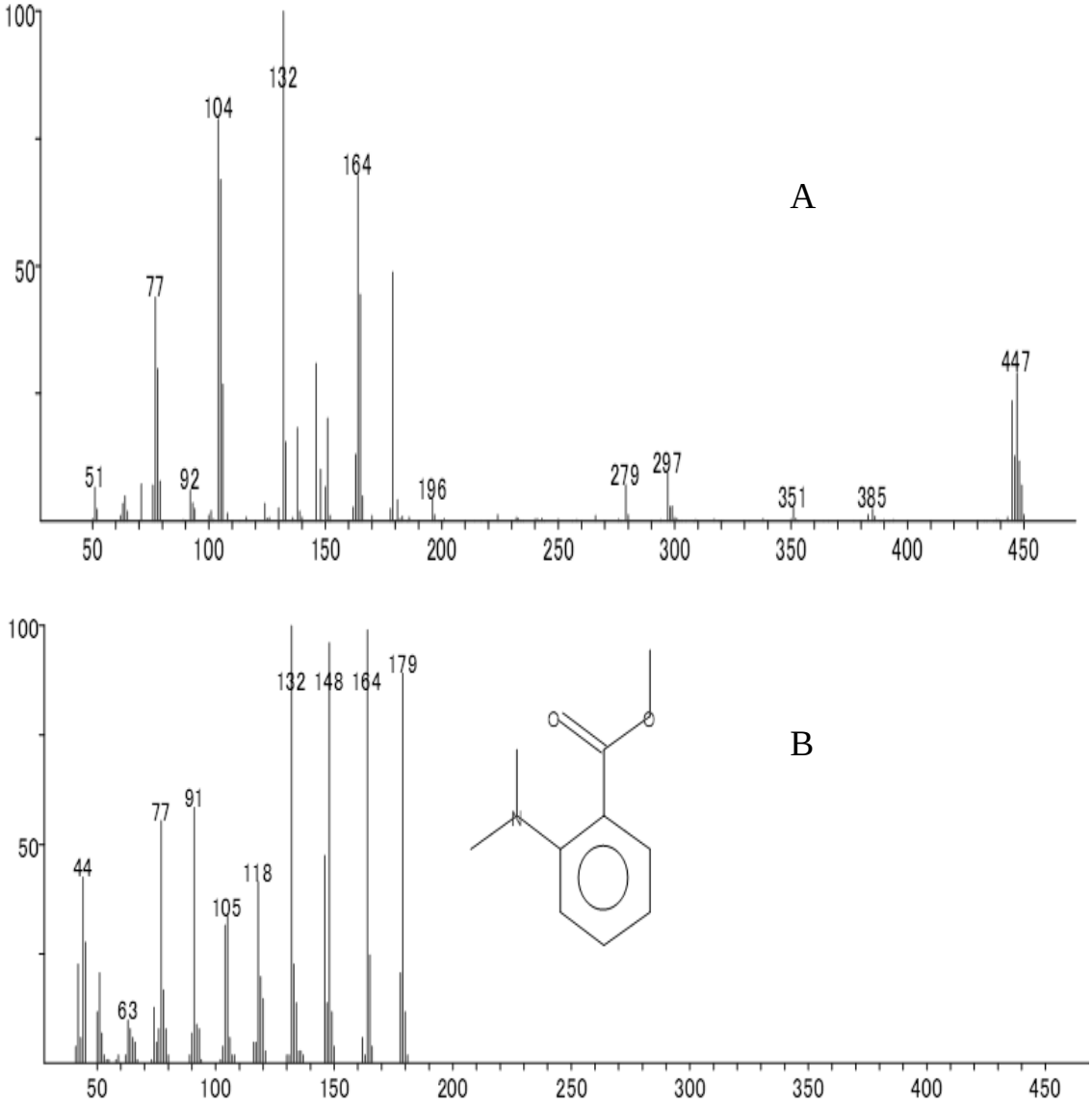


Figure 4.26: GC-MS mass spectra data for (A) anthranilic acid (N,N-dimethyl, methyl ester) recovered from growing cell culture extract of strain SL4 grown on carbazole (50 ppm) for 5 days and incubated at 30°C and 300 rpm and (B) standard anthranilic acid (N,N-dimethyl, methyl ester).

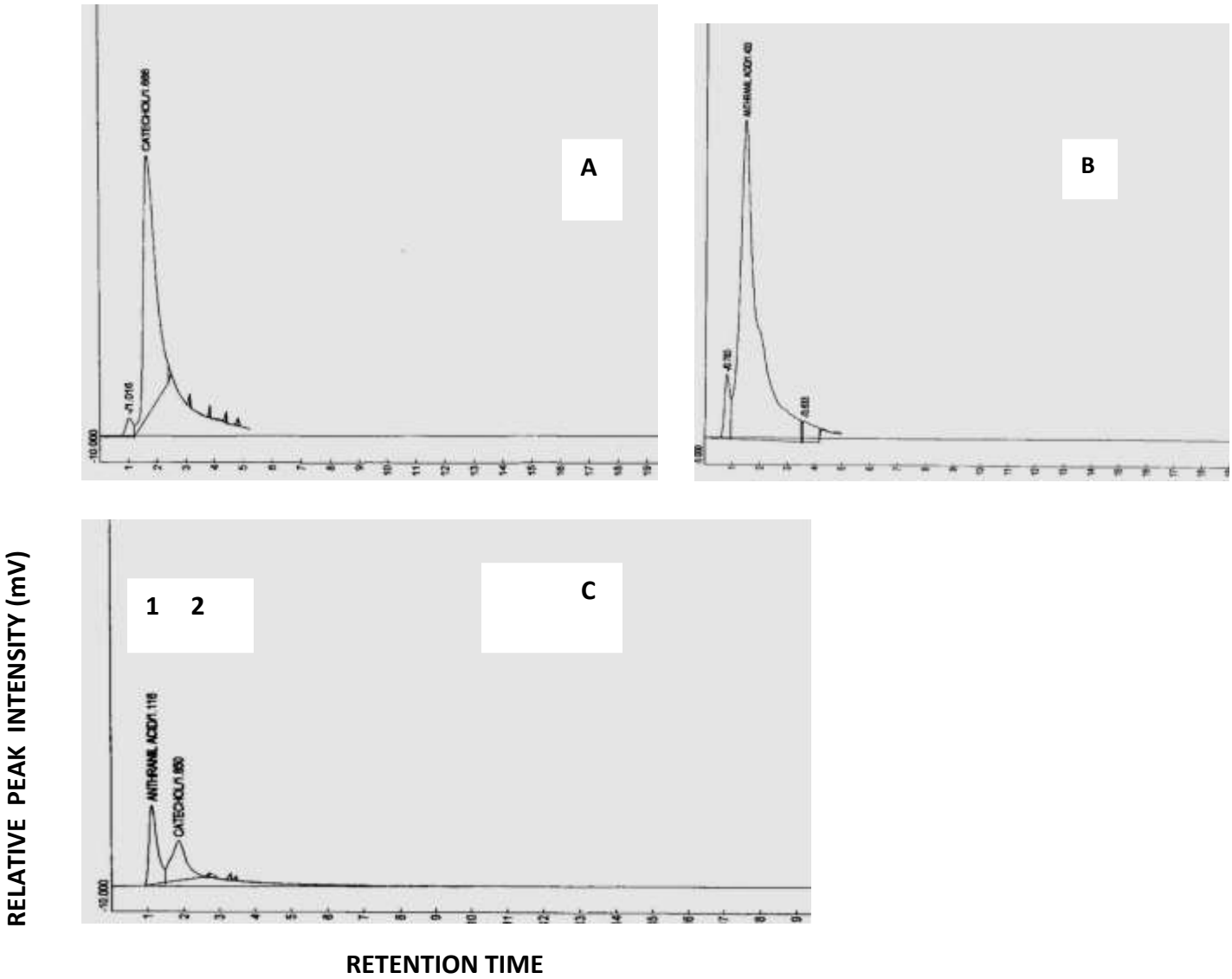


Figure 4.27: HPLC chromatograms of acetonitrile extract of AN (anthranilic acid) cultures (50 ml CFMM with 0.3 mM AN) of strains SL4. Standards used are CAT (catechol) (A) and AN (B) while (C) is acetonitrile extract of AN culture of strain SL4. Peaks 1 (AN) and 2 (CAT) were identified by comparing the retention times with those of authentic CAT and AN.

4.5.2.3 *Microbacterium esteraromaticum* strain SL6

The GC-MS chromatogram and mass spectra data of anthranilic acid detected from both the growing and the resting cells of strain SL6 grown on 50 ppm of carbazoleare shown in Figure 4.28-4.31.

GC-MS analysis of the ethyl acetate extracts of the resting cells of strain SL6 after 1 h, 2 h, 3 h, and 24 h of incubation indicate transient detection of anthranilic acid methylated at the COOH group with retention time of 5.20 minutes. Anthranilic acid concentration briefly accumulate after 1 h of incubation, followed by its depletion throughout the remaining time points (i.e. 2 h, 3 h, and 24 h) used. This indicates that the metabolite is metabolized further by the isolate thus making its accumulation impossible.

GC-MS analysis of the ethyl acetate extract of the growing cells of strain SL6 after 5 days of incubation also resulted in the detection of minute amounts of anthranilic acid methylated at both the COOH and the NH_2 groups with retention time of 5.30 minutes. Detection of anthranilic acid in trace amount from the ethyl acetate extract of the growing cells is a clear indication that this metabolite is not a dead-end metabolite, and it is being metabolized further by the isolate.

HPLC analysis of acetonitrile extract of growing cells of strain SL6 in 50 ppm of anthranilic acid after 14 days of incubation showed the detection of catechol as major metabolite and undegraded anthranilic acid at retention times 1.85 min and 1.17 min, respectively (Figure 4.32). The detection of anthranilic acid in the extract after 14 days of incubation indicates that not all the anthranilic acid was degraded within 14 days.

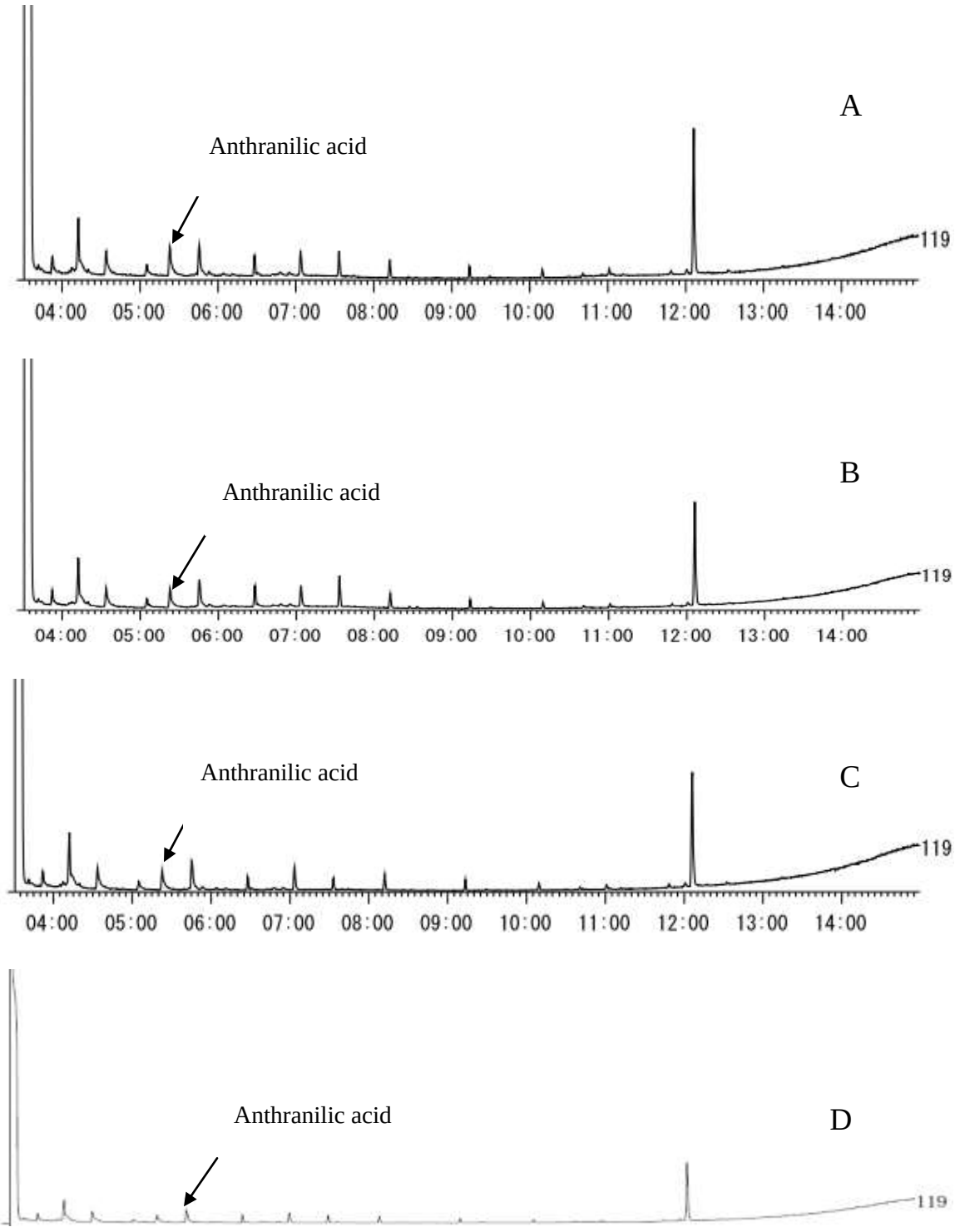


Figure 4.28: GC-MS chromatograms showing the peaks of anthranilic acid (methylated) recovered from ethyl acetate extract of resting cell culture of strain SL6 grown on carbazole (50 ppm) as carbon and energy source after (A) 1 hr, (B) 2 h, (C) 3 h and (D) 24 h of incubation at 30°C and 300 rpm.

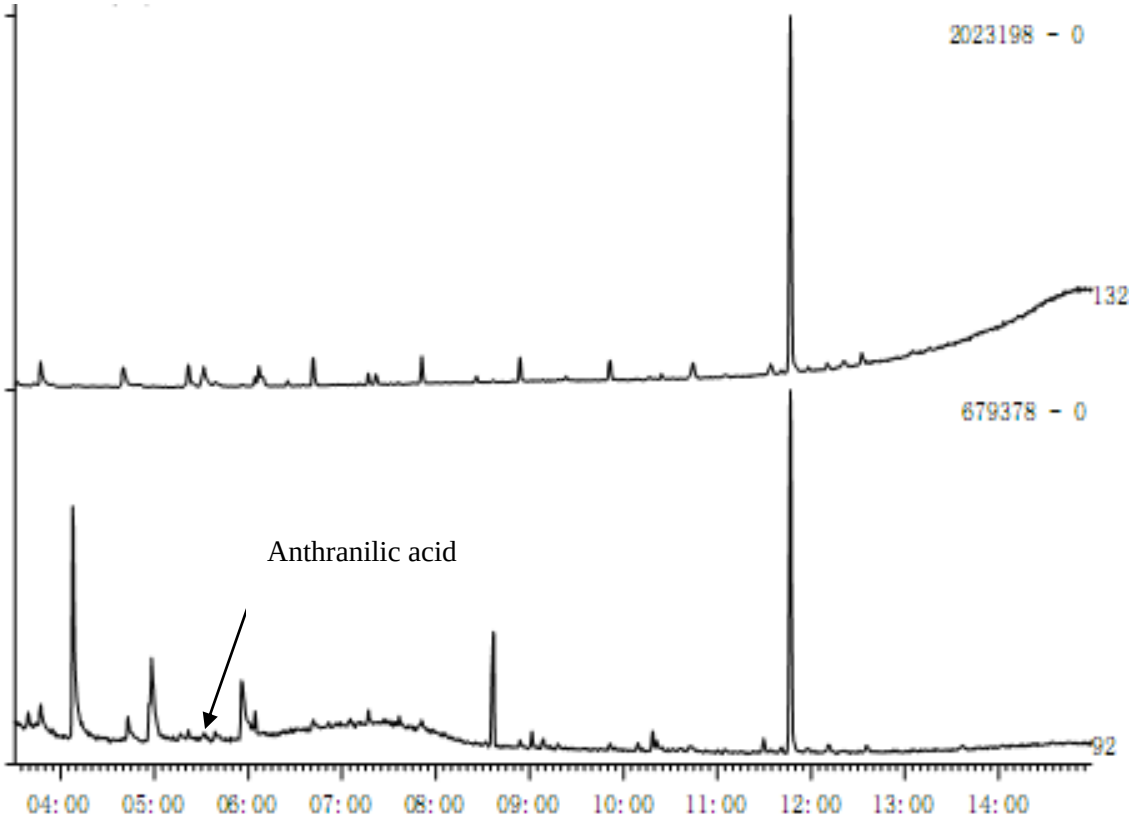


Figure 4.29: GC-MS chromatograms showing the peaks of anthranilic acid (N-methyl, methyl ester) recovered from ethyl acetate extract of growing cell culture of strain SL6 grown on carbazole (50 ppm) as carbon and energy source after 5 days of incubation at 30°C and 300 rpm.

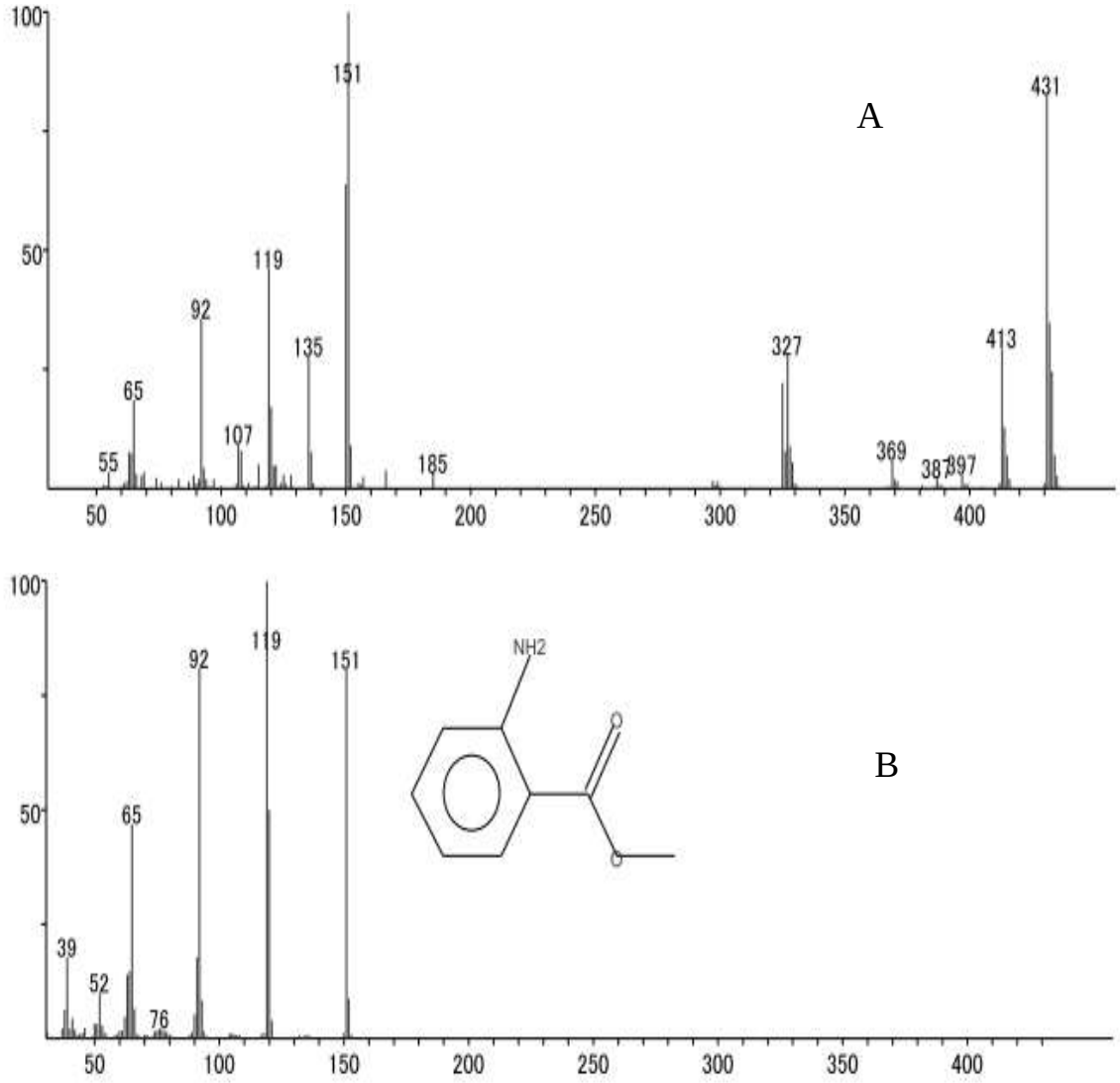


Figure 4.30: GC-MS mass spectra data for (A) methylated anthranilic acid recovered from ethyl acetate extract of resting cell culture of strain SL6 grown on carbazole (50 ppm) and (B) standard anthranilic acid (methylated).

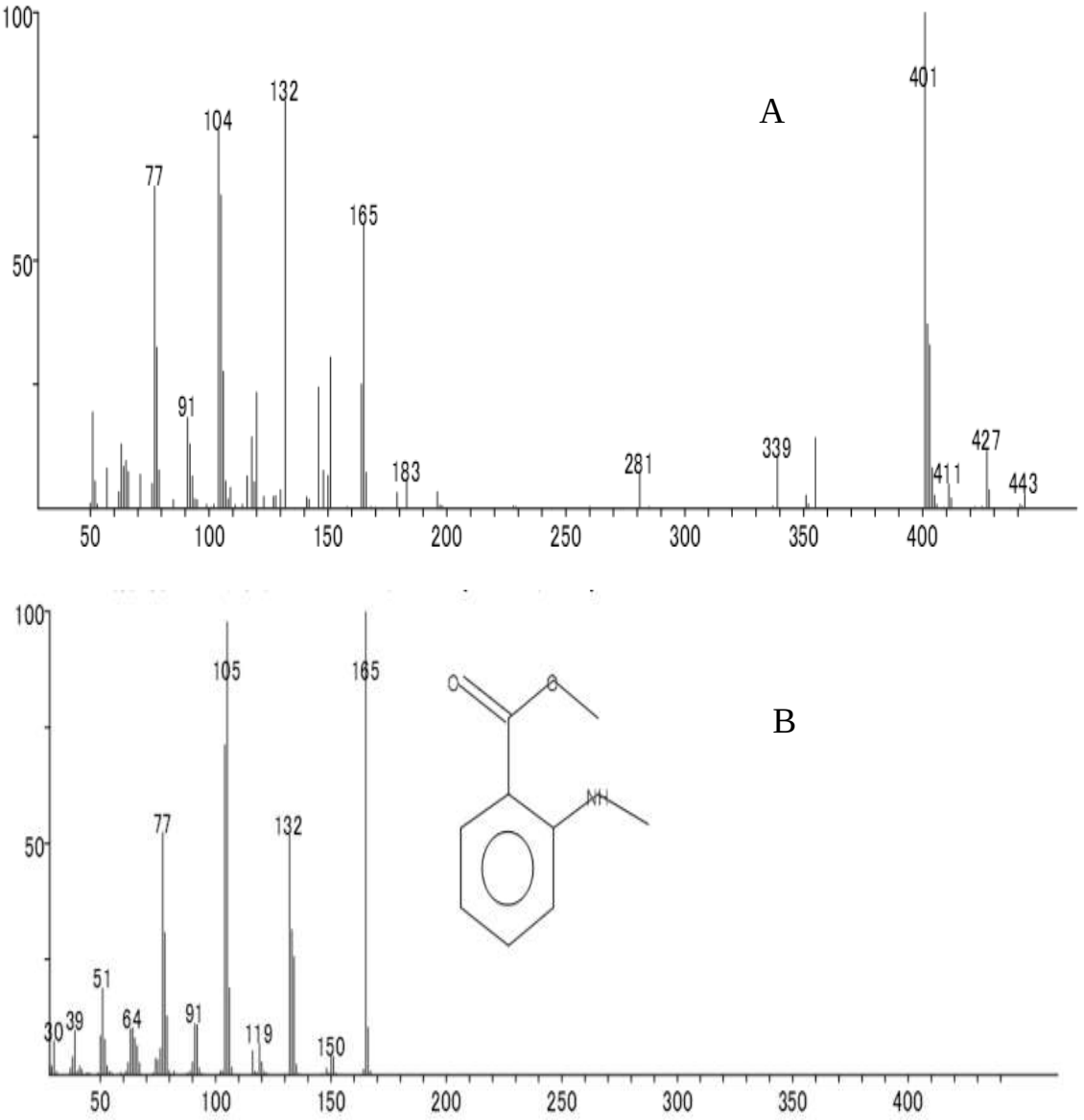


Figure 4.31: GC-MS mass spectra data for (A) anthranilic acid (N-methyl, methyl ester) recovered from ethyl acetate extract of growing cells culture of strain SL6 grown on carbazole (50 ppm) for 5 days and incubated at 30°C and 300 rpm and (B) standard anthranilic acid (N-methyl, methyl ester).

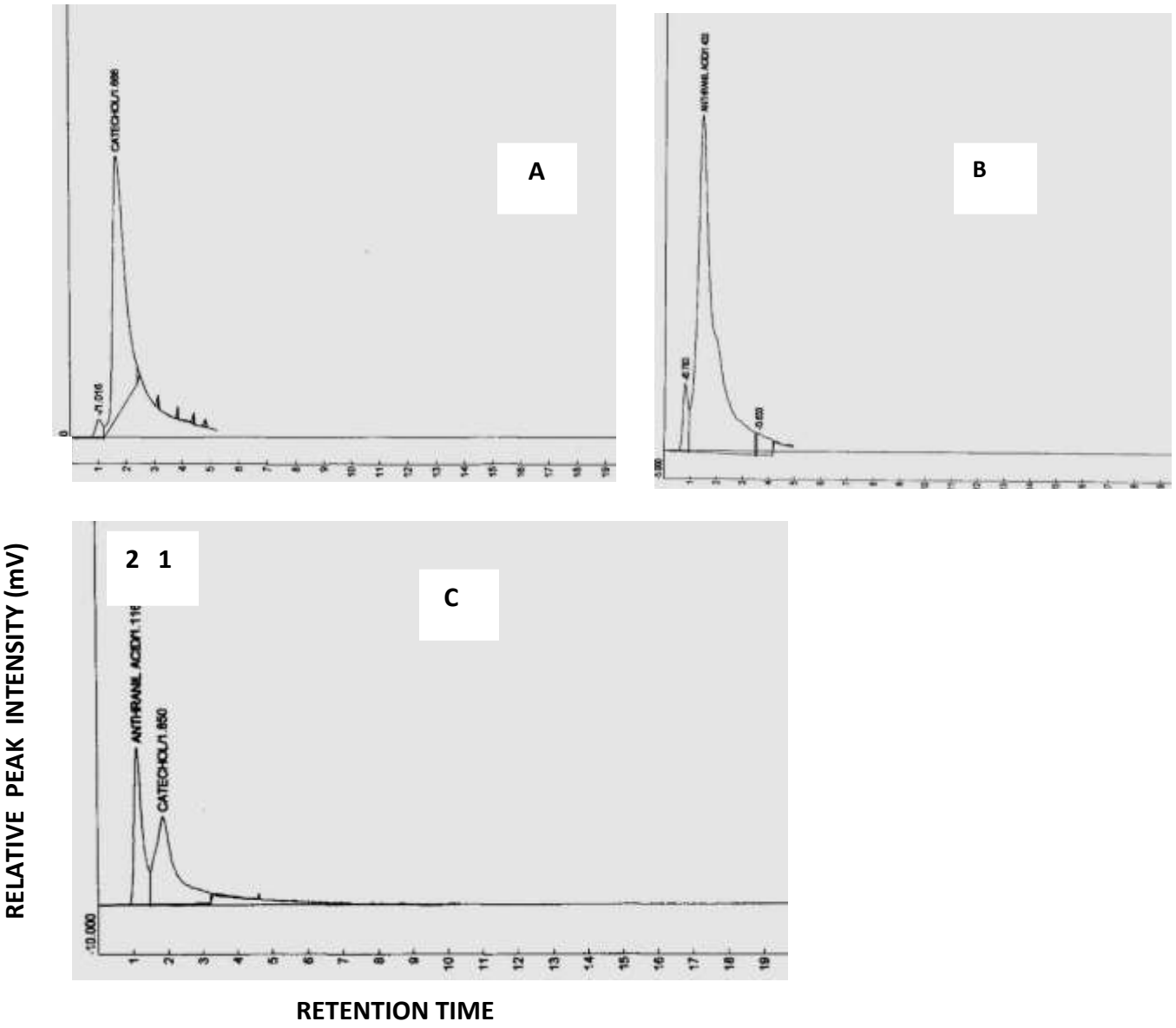


Figure 4.32: HPLC chromatograms of acetonitrile extract of AN (anthranilic acid) cultures (50 ml CFMM with 0.3 mM AN) of strains SL6. Standards used are CAT (catechol) (A) and AN (B) while (C) is acetonitrile extract of AN culture of strain SL6 Peaks 1 (CAT) and 2 (AN) were identified by comparing the retention times with those of authentic CAT and AN.

4.5.2.4 Catechol dioxygenation by Carbazole-degrading isolates

Three of the four carbazole-degrading strains (*Achromobacter* sp. strain SL1, *Pseudomonas* sp. strain SL4 and *M. esteraromaticum* strain SL6) also degrade catechol via the *ortho* pathway as reflected in increase activity (increase in absorbance spectra values) at 260 nm when monitored using UV-Vis spectrophotometer. The increase activity at 260 nm indicate the formation of *cis,cis*-muconate via catechol 1,2-dioxygenase activity for the three strains (Figure 4.33a). At absorbance value of 375 nm, there is consistent decrease in absorbance spectra values, which indicate that the three carbazole-degrading strains lack ability to degrade catechol using the *meta* pathway (Figure 4.33b).

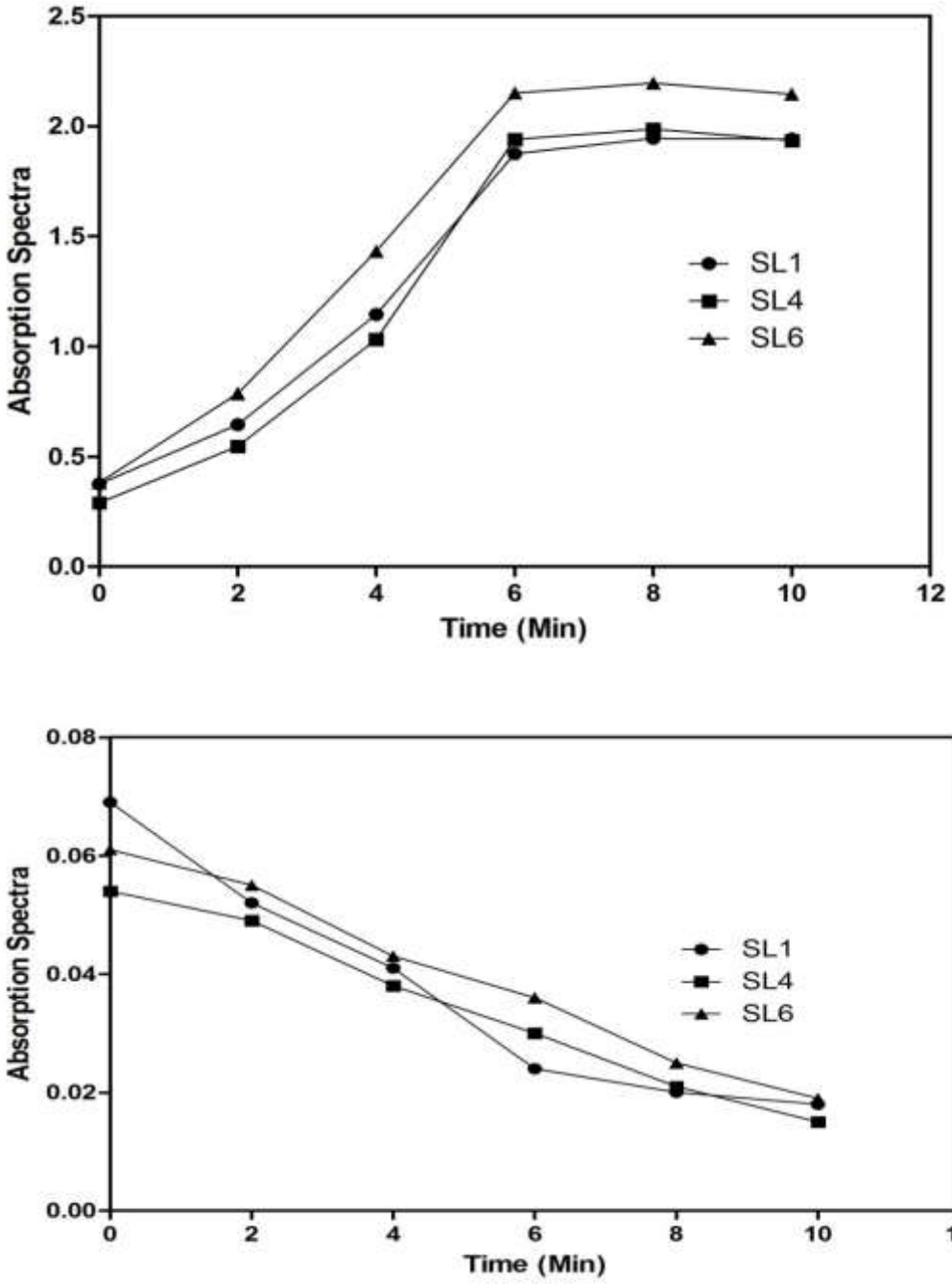


Figure 4.33: Enzymatic transformation of catechol to cis, cis muconic acid by lysate of carbazole-grown cells. The reaction was started in a sample cuvette containing 100 ml of cell lysate in 800 ml of phosphate buffer, pH 7.5, by the addition of 100 mM catechol. Optical absorption spectra were recorded at periodic intervals of 0, 2, 4, 6, 8, and 10 min. Increase in absorption spectra at 260 nm indicate conversion of catechol to *cis, cis* muconic acid by the three isolates (A). Consistent decreases in absorption spectra were observed at 375 nm (B).

4.5.3 Carbazole Biodegradation in Soil Microcosm

4.5.3.1 Physico-Chemical Properties of Soil used in Microcosm Study

The physico-chemical properties of the agricultural soil sample used in microcosm study are shown in Table 4.5. The pH of the soil was weakly acidic and close to neutral (6.6) while the moisture content was 9.36%. Grain size determination shows that the soil is dark grey clayey silty sand with sand content of 89% and silt and clay content of 11%. The water holding capacity of the soil was 40% while the values for total organic carbon, total hydrocarbon, total nitrogen, available phosphorus and potassium were 1.24%, 769.23 mg/kg, 0.06%, 3.53 mg/kg and 2.52 mg/kg respectively.

Table 4.5: Physico-Chemical Properties of Soil used in Microcosm Study

Parameters	**Value**
Moisture (%)	9.36
pH	6.6
Total organic carbon (%)	1.24
Total hydrocarbon content (mg/kg)	769.23
Total Nitrogen (%)	0.06
Available Phosphorus (mg/kg)	3.53
Potassium (mg/kg)	2.52
Sand (%)	89
Clay and Silt (%)	11
Water Holding Capacity (%)	40

4.5.3.2 Population Dynamics of Isolates and Degradation of Carbazole in Soil

The population dynamics of inoculated organisms in sterilized and native (unsterilized) soil microcosm are shown in Table 4.6. In microcosm study with sterilized soils, strain SL4 has the highest survival rate in the soil as the initial population density (1.2 x 10^7cfu/g) increased to 1.8 x 10^7cfu/g after 30 days in soil. Strain SL1 appeared to have the least population density among the three isolates with a population density of 1.9 x 10^6cfu/g after 30 days in soil, a value slightly lower than the initial population at the beginning of the experiment. The combination of the three isolates on carbazole shows a modest increase in population density from an initial population density of 1.7 x 10^7 to a final population of 2.1 x 10^7cfu/g. In native soil, there was a decrease in population density in all the four set-ups when compared to the initial populations. The autochthonous bacterial population in the soil had the lowest population density after 30 days of incubation with 100-ppm carbazole.

The chromatograms of dichloromethane extracts of residual carbazole in sterile soil microcosm at day 0 and day 30 as detected by gas chromatography are shown in Figure 4.34-4.37. From the initial carbazole concentration of 100 mg/kg introduced into the sterile soil, 88.12 mg/kg of carbazole was recovered after 30 days of incubation.

In sterilized soil inoculated with strain SL1, out of the initial carbazole concentration of 100 mg/kg introduced into the soil at day 0, 33.04 mg/kg was recovered after 30 days of incubation constituting 33.04% of recovered carbazole, which represent 66.96% carbazole removal (Figure 4.34; Table 4.7).

In sterilized soil inoculated with strain SL4, the initial carbazole concentration of 100 mg/kg was reduced to 17.85 mg/kg after 30 days of incubation constituting 17.85% of recovered carbazole, which represent 82.15% carbazole removal (Figure 4.35; Table 4.7).

Strain SL6 degraded the initial carbazole concentration of 100 mg/kg at day 0 to 31.46 mg/kg after 30 days in soil constituting 31.46% recovered carbazole, which represent 68.54% carbazole removal (Figure 4.36; Table 4.7).

The combination of the three isolates on carbazole as inocula reduced the initial carbazole concentration of 100 mg/kg at day 0 to 12.87 mg/kg after 30 days constituting 12.87% of recovered carbazole, which represent 87.13% carbazole removal (Figure 4.37; Table 4.7).

In microcosm study with native (unsterilized) soil, The chromatograms showing dichloromethane extracts of residual carbazole at day 0 and day 30 as detected by gas chromatography are shown in Figure 4.38-4.41. From the initial carbazole concentration of 100 mg/kg introduced into the native soil (NSC), 80.81 mg/kg of carbazole was recovered after 30 days of incubation constituting 80.81% of recovered carbazole, which represent 19.19% carbazole removal by indigenous bacteria in the soil (Figure 4.38, Table 4.7).

In native soil inoculated with strain SL1 (NSC1), out of the initial carbazole concentration of 100 mg/kg introduced into the soil at day 0, 8.36 mg/kg was recovered after 30 days of incubation constituting 8.36% of recovered carbazole, which represent 91.64% carbazole removal (Figure 4.39, Table 4.7).

In native soil inoculated with strain SL4 (NSC4), out of the initial carbazole concentration of 100 mg/kg introduced into the soil at day 0, 12.71 mg/kg was recovered after 30 days of incubation constituting 12.71% of recovered carbazole, which represent 87.29% carbazole removal (Figure 4.40, Table 4.7).

In native soil inoculated with strain SL6 (NSC6), out of the initial carbazole concentration of 100 mg/kg introduced into the soil at day 0, 10.87 mg/kg was recovered after 30 days of incubation constituting 10.87% of recovered carbazole, which represent 89.13% carbazole removal (Figure 4.41, Table 4.7).

Table 4.6: Bacterial Population Density During Soil Microcosm Study

Isolate Code	Time (Days) 0	30
SSC	0	0
SSC1	1.3×10^7	1.9×10^6 cfu/g
SSC4	1.2×10^7	1.8×10^7 cfu/g
SSC6	1.4×10^7	1.6×10^7 cfu/g
SSC146	1.7×10^7	2.1×10^7 cfu/g
NSC	1.5×10^6	1.2×10^4 cfu/g
NSC1	8.6×10^8	6.4×10^7 cfu/g
NSC4	9.8×10^7	1.7×10^7 cfu/g
NSC6	1.1×10^8	3.4×10^7 cfu/g

Key:

SSC= Sterilized soil + carbazole

SSC1= Sterilized soil + carbazole + strain SL1

SSC4= Sterilized soil + carbazole + strain SL4

SSC6= Sterilized soil + carbazole + strain SL6

SSC146= Sterilized soil + carbazole + strains SLI, SL4, SL6.

NSC= Native soil + carbazole

NSC1=Native soil + carbazole + strain SL1

NSC4= Native soil + carbazole + strain SL4

NSC6= Native soil + carbazole + strain SL6

Table 4.7: Carbazole Degradation Rates of Isolates in Soil Microcosm

Isolate code	Initial carbazole conc. (Day 0; mg/kg)	Final carbazole conc. (Day 30; mg/kg)	% recovered carbazole after 30 days	% carbazole removed after 30 days
SSC	100	88.12	88.12	11.88
SSC1	100	33.04	33.04	66.96
SSC4	100	17.85	17.85	82.15
SSC6	100	31.46	31.46	68.54
SSC146	100	12.87	12.87	87.13
NSC	100	80.81	80.81	19.19
NSC1	100	8.36	8.36	91.64
NSC4	100	12.71	12.71	87.29
NSC6	100	10.87	10.87	89.13

Key:

SSC= Sterilized soil + carbazole

SSC1= Sterilized soil + carbazole + strain SL1

SSC4= Sterilized soil + carbazole + strain SL4

SSC6= Sterilized soil + carbazole + strain SL6

SSC146= Sterilized soil + carbazole + strains SLI, SL4, SL6

NSC= Native soil + carbazole

NSC1=Native soil + carbazole + strain SL1

NSC4= Native soil + carbazole + strain SL4

NSC6= Native soil + carbazole + strain SL6

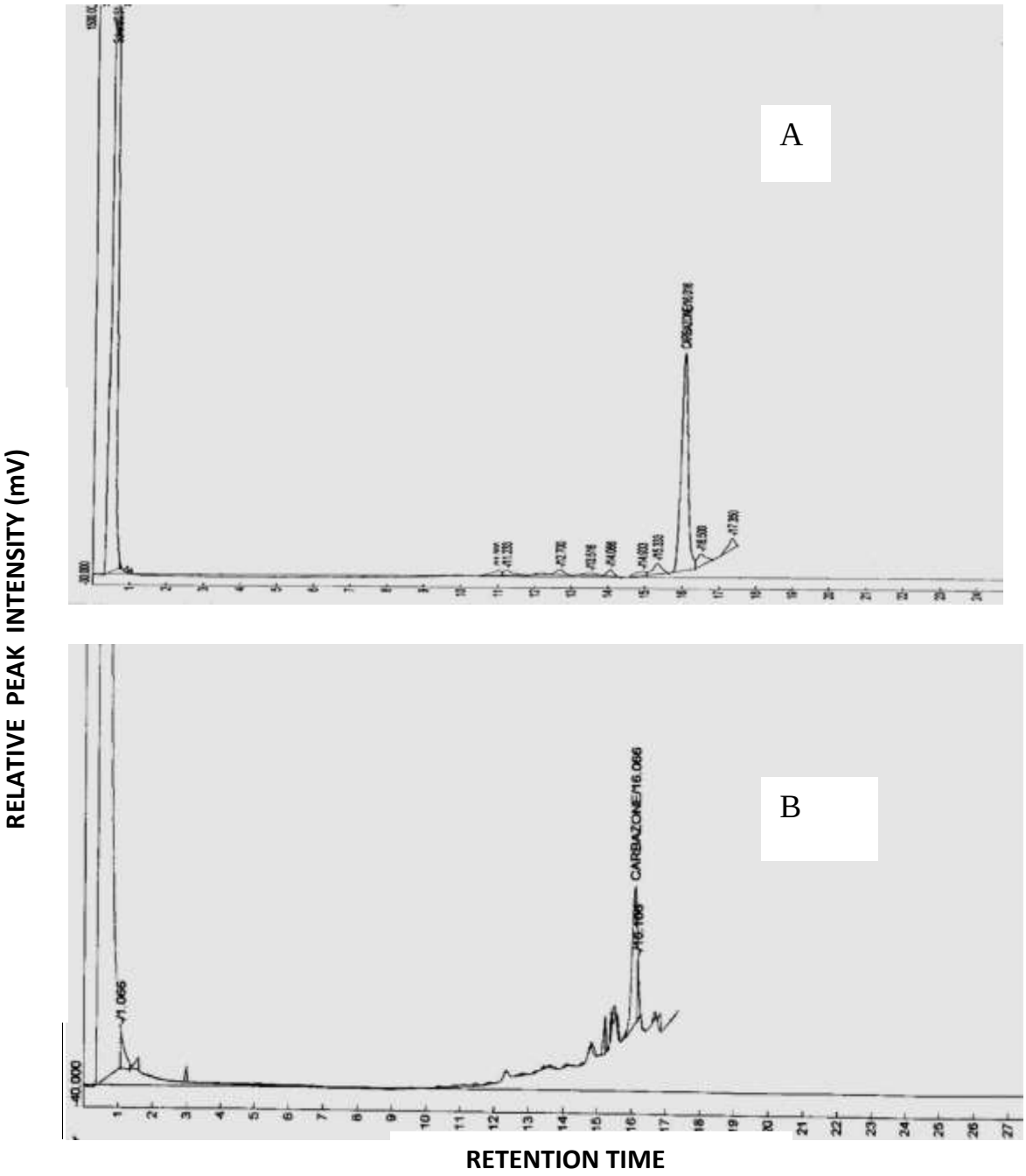


Figure 4.34: GC-FID chromatogram of dichloromethane extract of residual carbazole from *Achromobacter* sp. strain SL1-inoculated sterilized carbazole spiked soil at day 0 (A) and after 30 days of incubation (B).

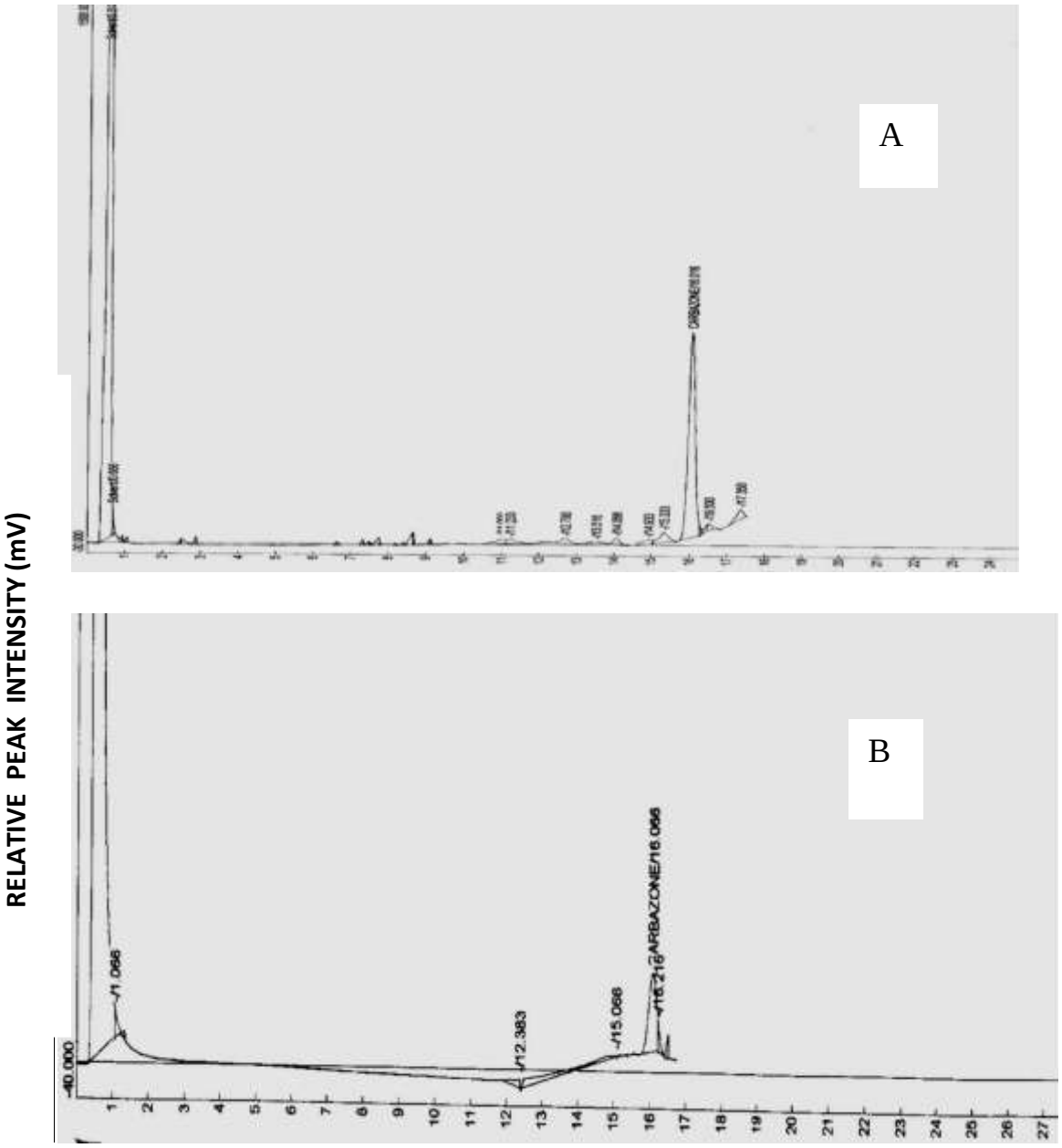


Figure 4.35: GC-FID chromatogram of dichloromethane extract of residual carbazole from *Pseudomonas* sp. strain SL4-inoculated sterilized carbazole spiked soil at day 0 (A) and after 30 days of incubation (B).

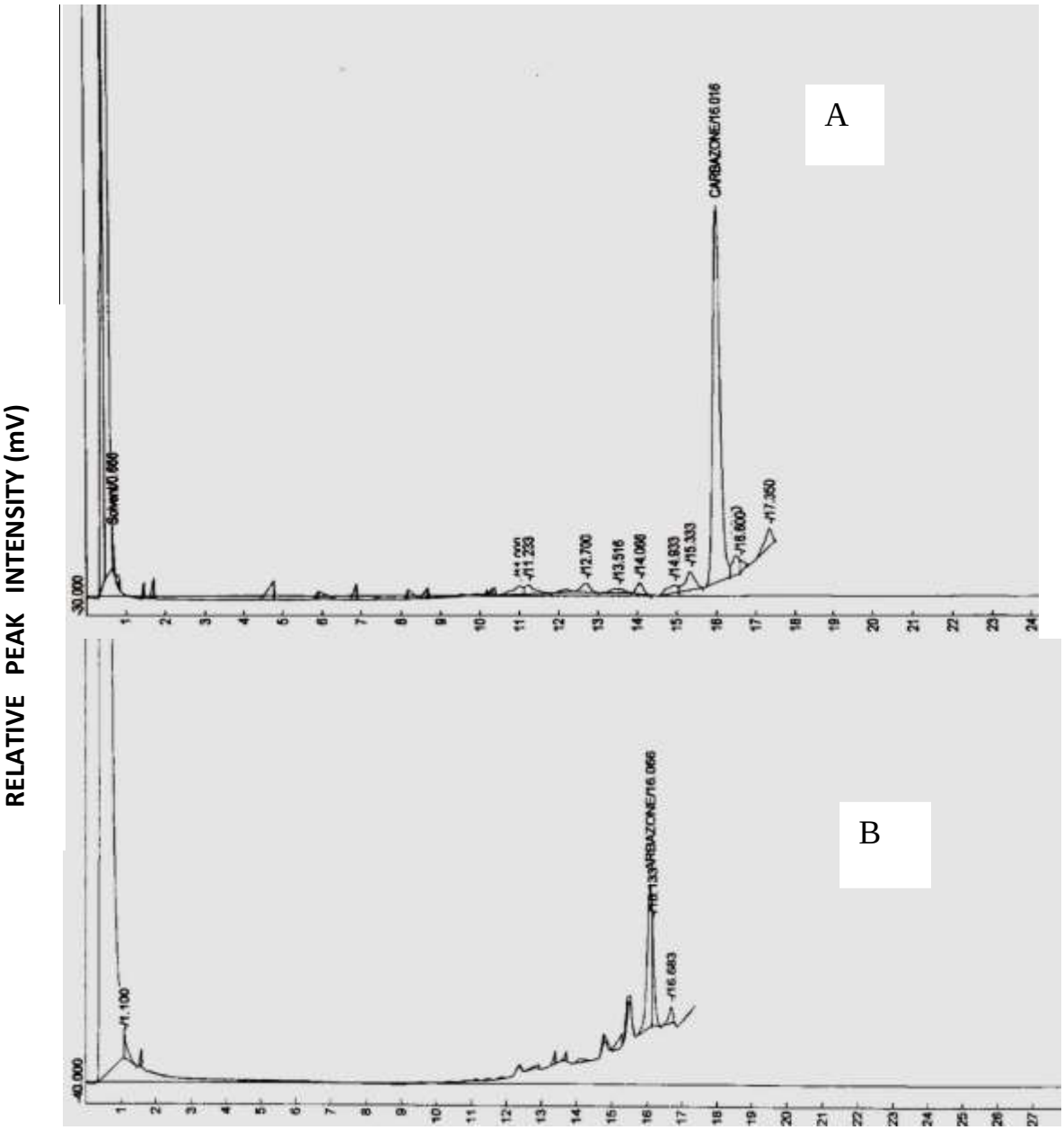


Figure 4.36: GC-FID chromatogram of dichloromethane extract of residual carbazole from *Microbacterium esteraromaticum* strain SL6-inoculated sterilized carbazole spiked soil at day 0 (A) and after 30 days of incubation (B).

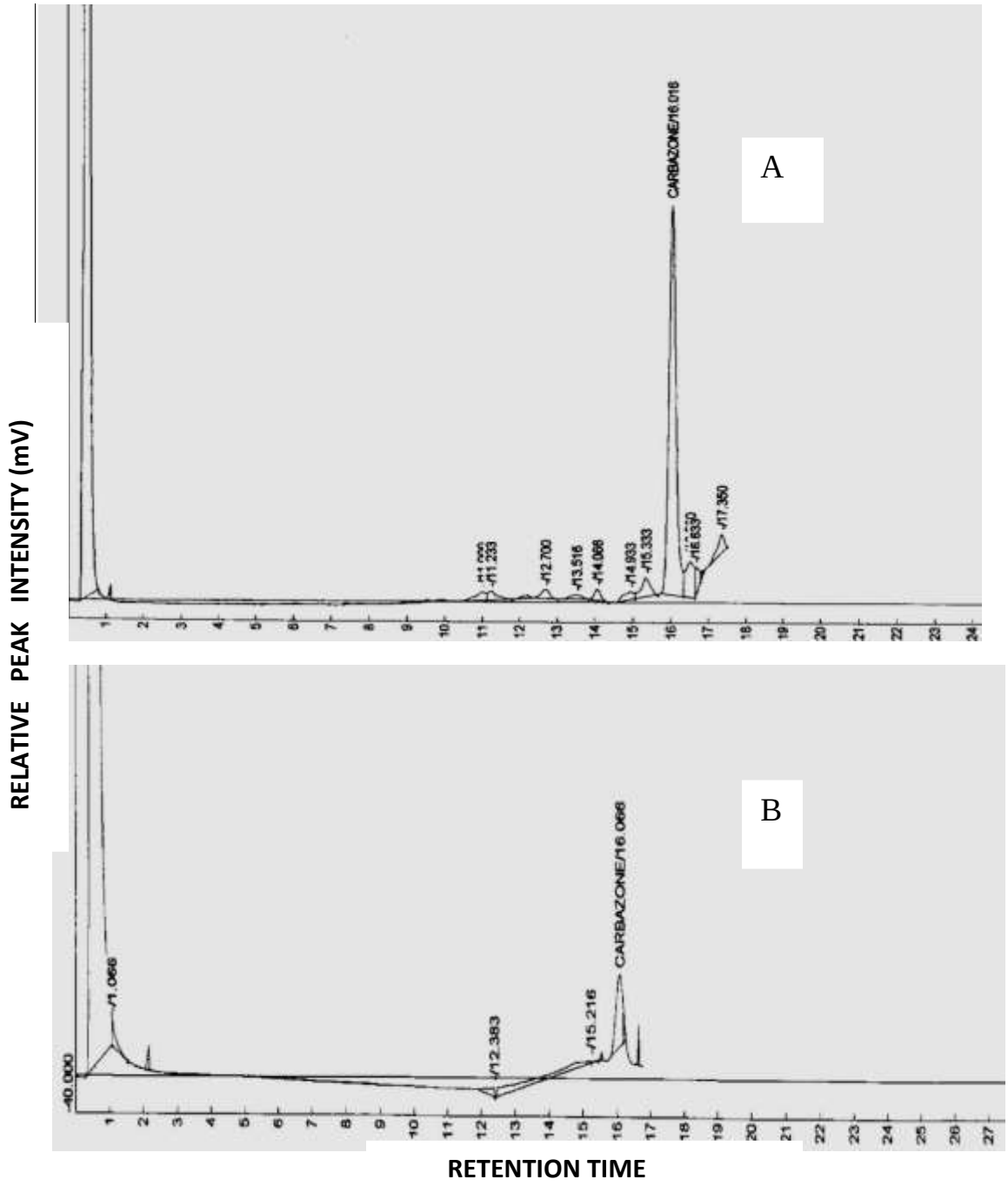


Figure 4.37: GC-FID chromatogram of dichloromethane extract of residual carbazole from strains SL1, SL4 and SL6 (SSC146)-inoculated sterilized carbazole spiked soil at day 0 (A) and after 30 days of incubation (B).

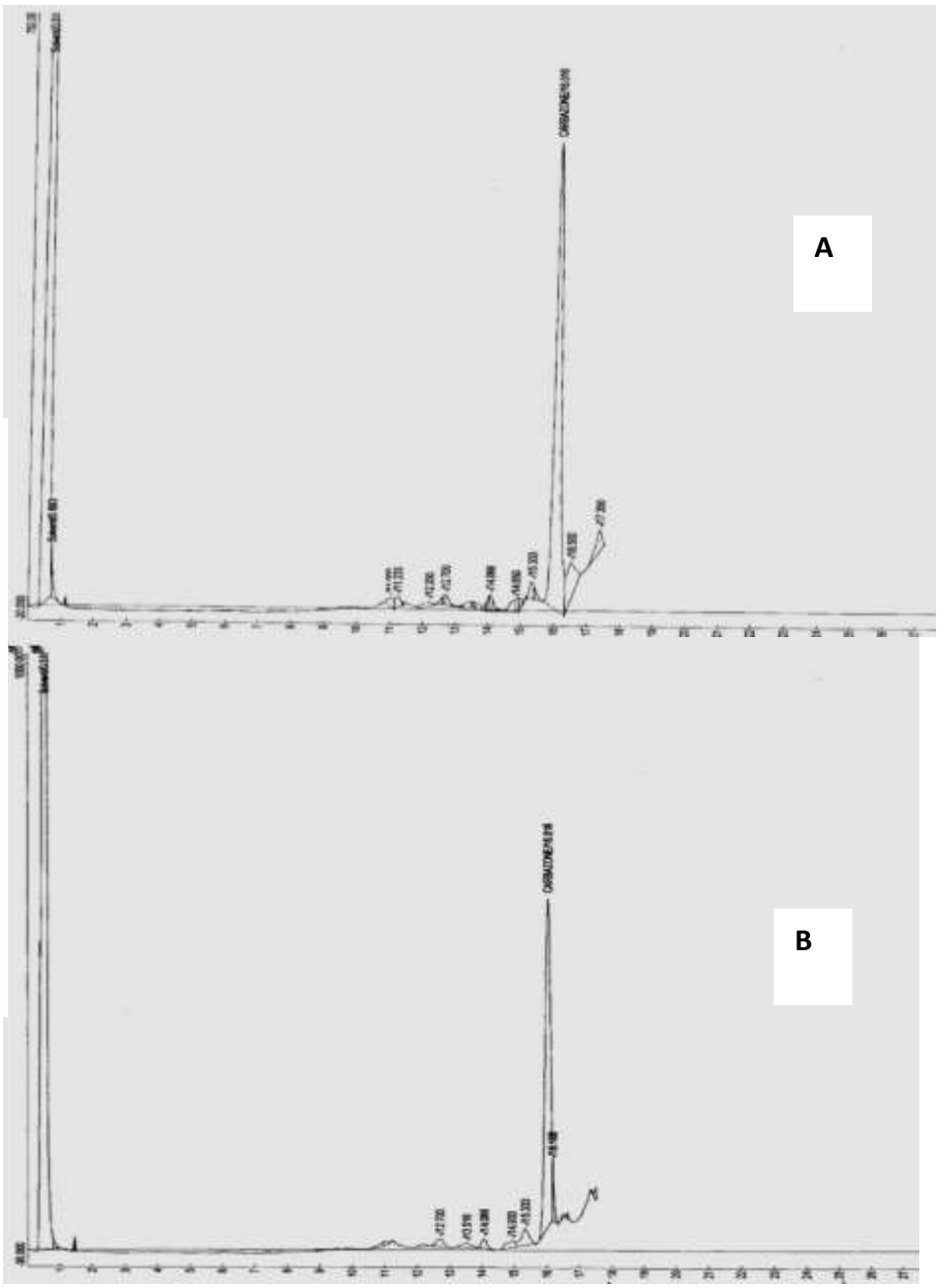


Figure 4.38: GC-FID chromatogram of dichloromethane extract of residual carbazole extracted from native soil carbazole spiked soil (NSC) at day 0 (A) and after 30 days of incubation (B).

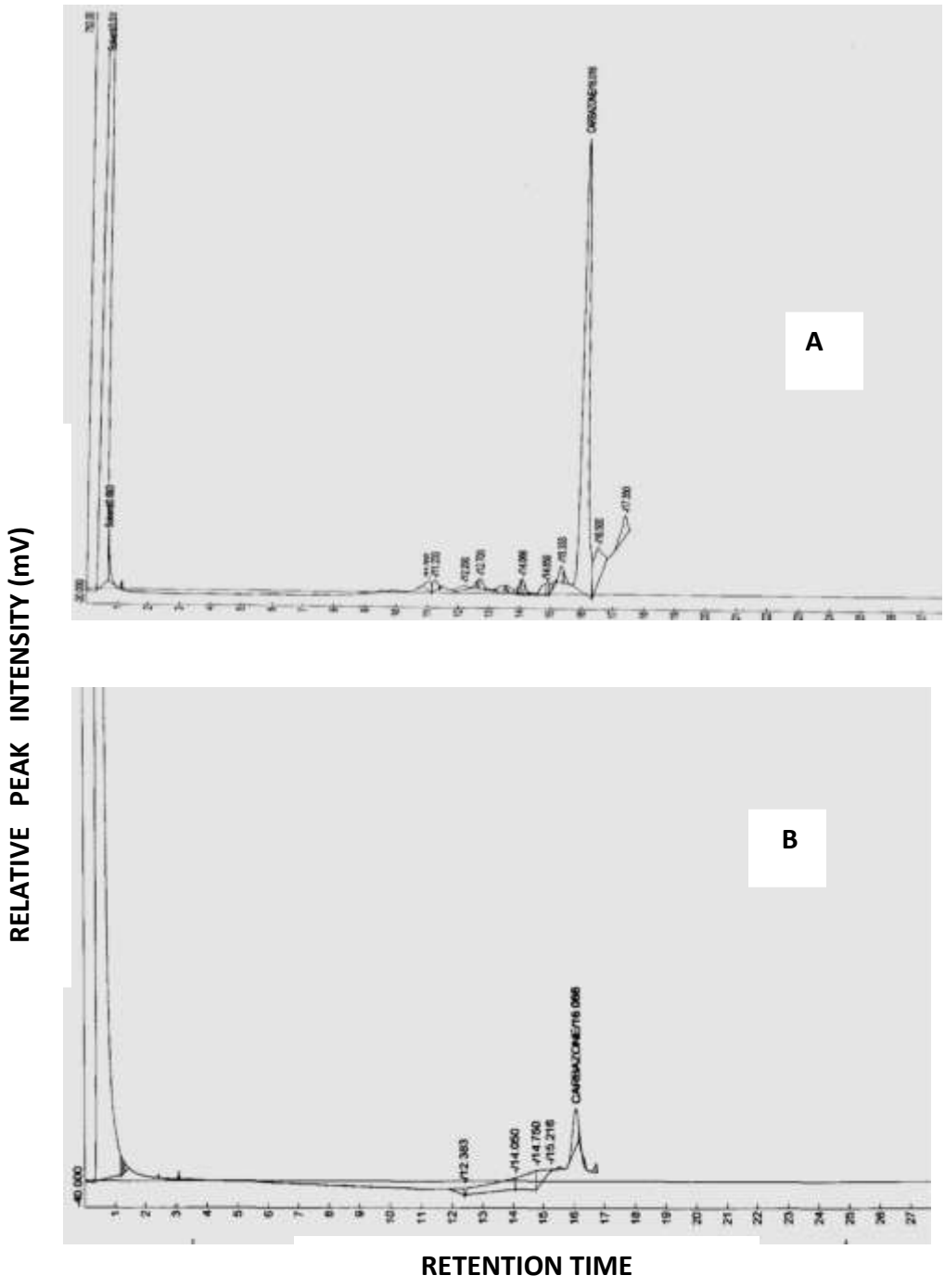


Figure 4.39: GC-FID chromatogram of dichloromethane extract of residual carbazole from strain SL1-inoculated native soil carbazole spiked soil (NSC1) at day 0 (A) and after 30 days of incubation (B).

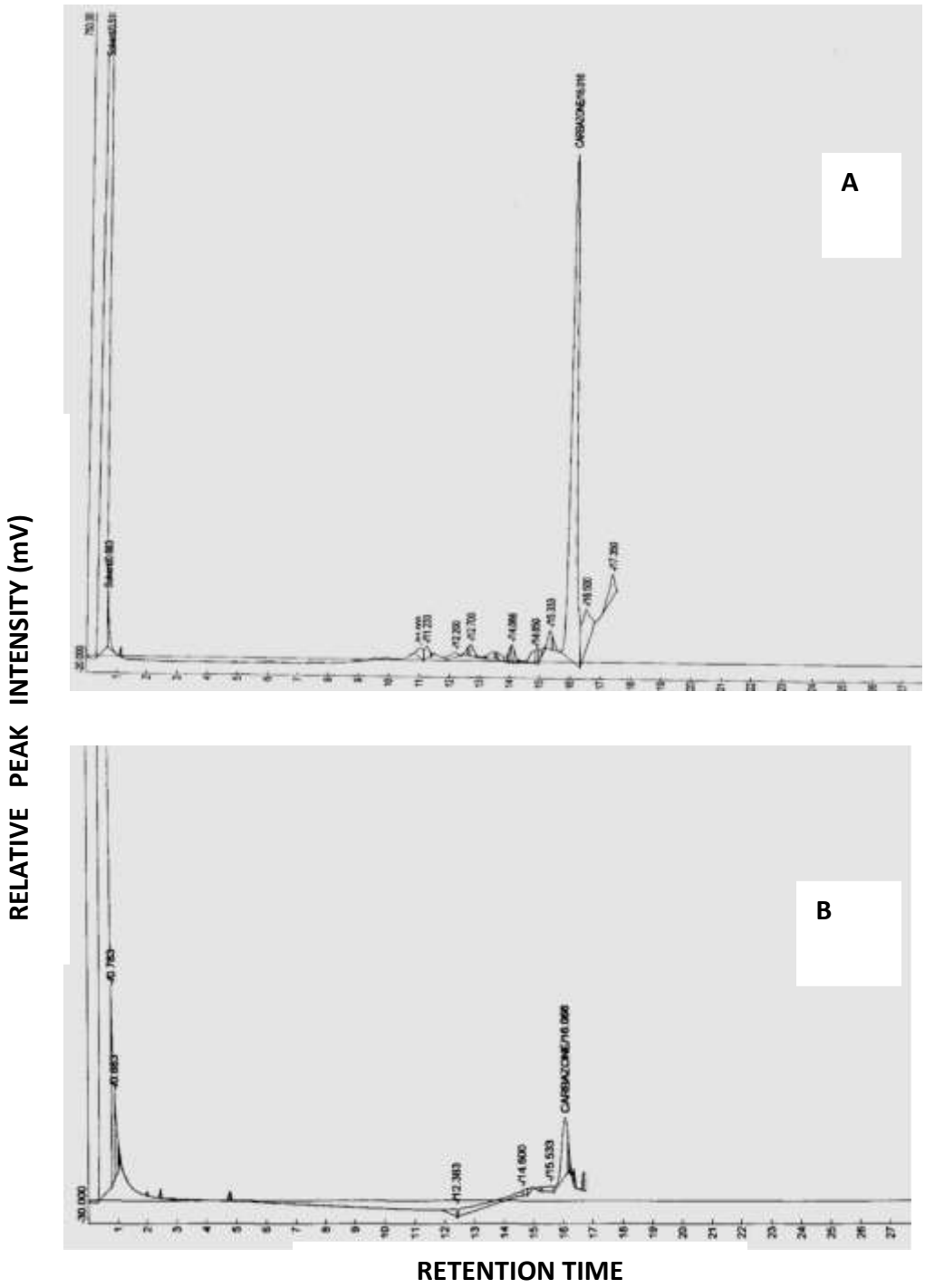


Figure 4.40: GC-FID chromatogram of dichloromethane extract of residual carbazole from strain SL4-inoculated native soil carbazole spiked soil (NSC4) at day 0 (A) and after 30 days of incubation (B).

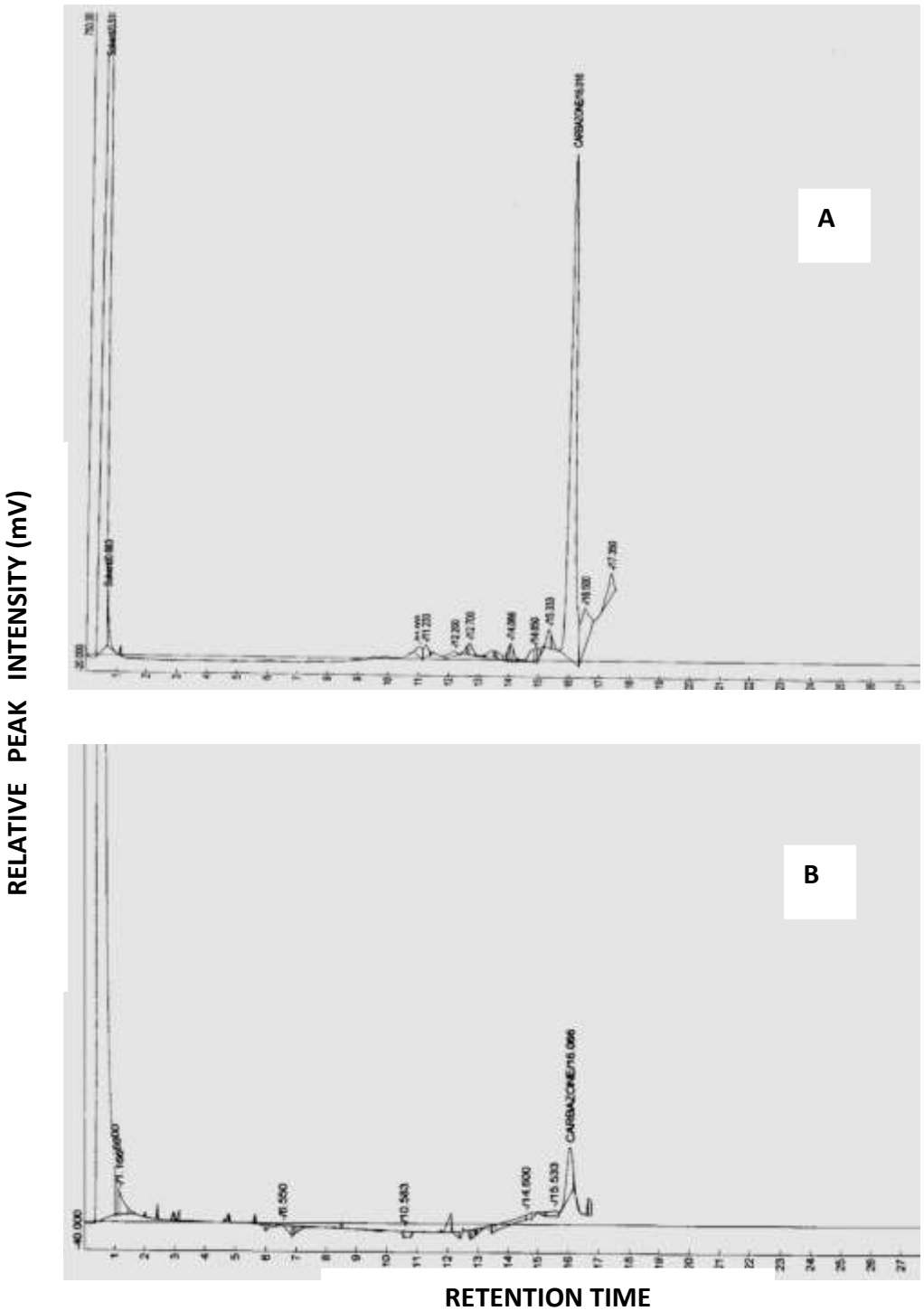


Figure 4.41: GC-FID chromatogram of dichloromethane extract of residual carbazole from strain SL6-inoculated native soil carbazole spiked soil (NSC6) at day 0 (A) and after 30 days of incubation (B).

4.6 Bacterial Diversity Studies

4.6.1 Isolation of Total DNA from MWO Polluted soil

The use of FastDNA® SPIN Kit for Soil (MP Bio) for DNA extracted from hydrocarbon-polluted soil MWO led to the isolation of a 19.33-kb bacterial total DNA resolved using 0.9% agarose gel as shown in Plate 4.5.

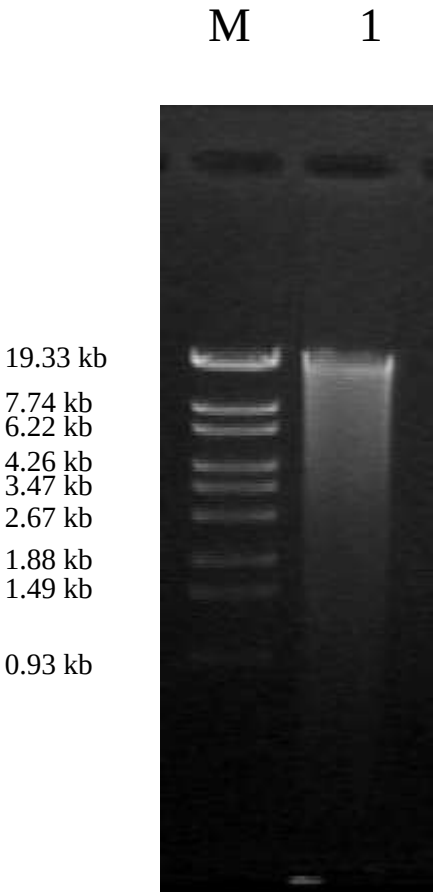


Plate 4.5: Electrophoretogram showing the band of bacterial total DNA extracted from MWO polluted soil using FastDNA® SPIN Kit for Soil (MP Bio). Agarose (0.9%) was used. Lanes are indicated as –M, OneSTEP Marker 6 (λ/Sty I digest); Lane 1: Bacterial total DNA.

4.6.2 Analysis of 16S rDNA Clone library of MWO polluted soil

4.6.2.1 Amplification of 16S rDNA Gene

Amplification of 16S rDNA gene using bacterial specific primer set $27F_{MOD}$/$1492R_{MOD}$ and TaKaRa *Ex Taq* from total DNA extracted from MWO polluted soil led to the detection of approximately 1.4 kb PCR product (Plate 4.6).

4.6.2.2 Amplification of 16S rDNA Gene from Transformed Colonies using Colony PCR

The use of colony PCR with sequencing primers T7 promoter/M13 reverses primers to amplify 16S rDNA gene in each of the transformed colonies as shown in Figure 4.35 led to the detection of PCR products of approximately 1.6 kb as resolved on 1% agarose gel and OneStep Marker 6 (λ/Sty I digest) (Plate 4.7).

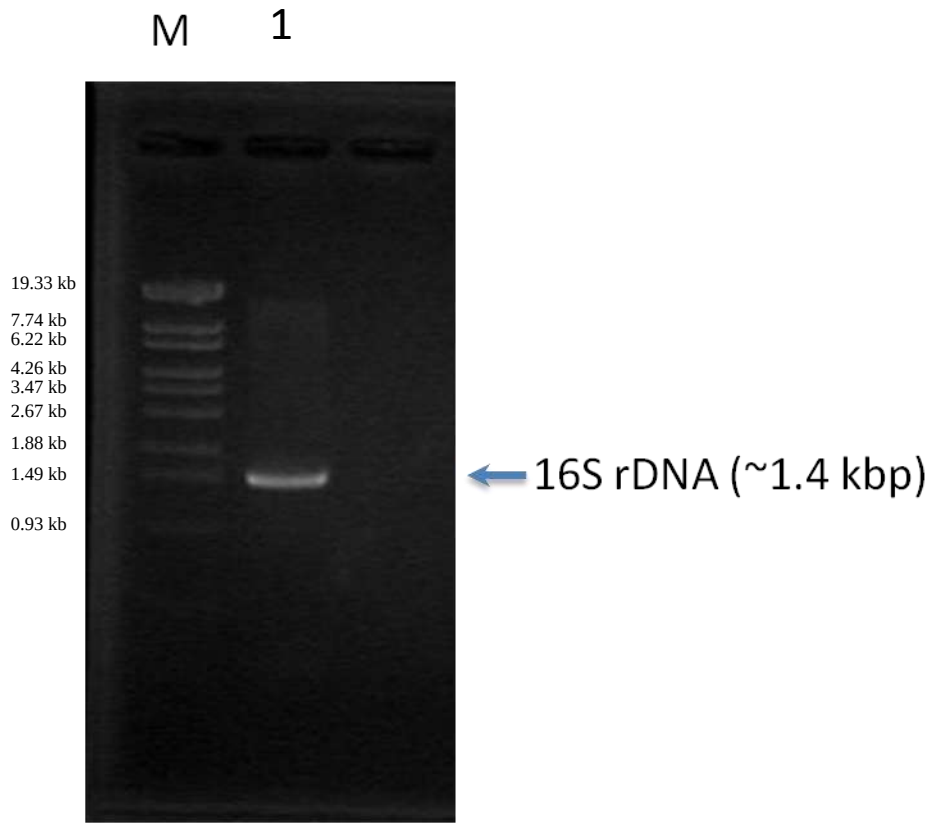


Plate 4.6: Electrophoretogram showing the band of 16S rDNA amplicon. Agarose (1%) was used. Lanes are indicated as –M, OneStep Marker 6 (λ/Sty I digest); Lane 1: PCR product of 16S rDNA gene from bacterial total DNA extracted from MWO polluted soil using $27F_{MOD}/1492R_{MOD}$ bacterial specific primers.

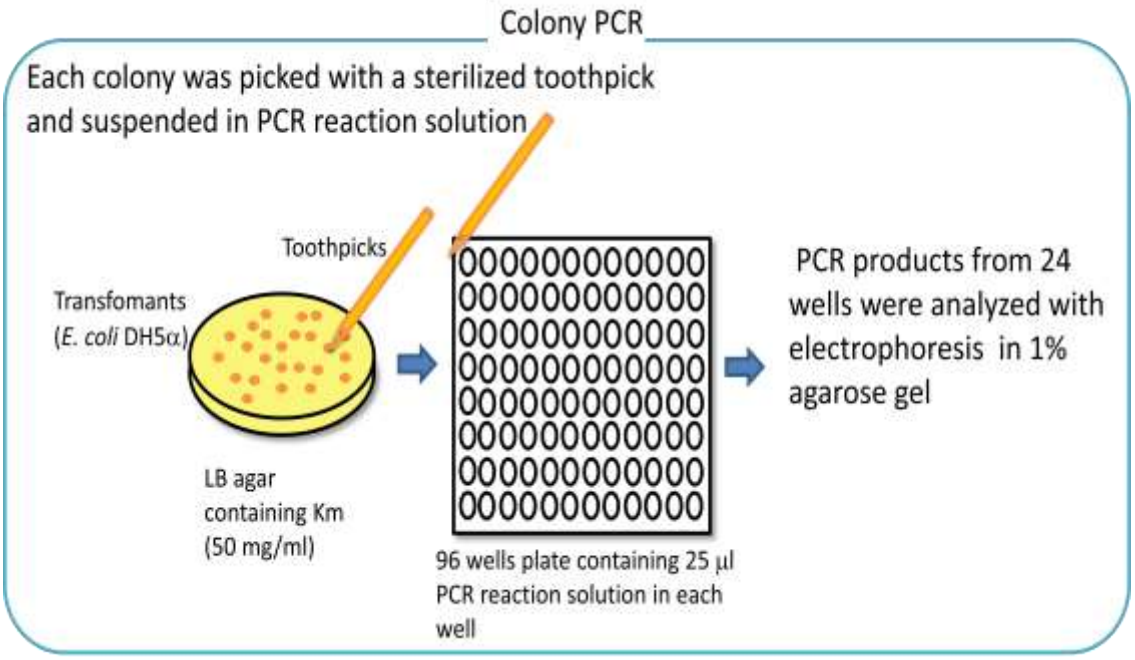


Figure 4.42: Colony PCR of transformed clones

M----------I---------- M----------I----------

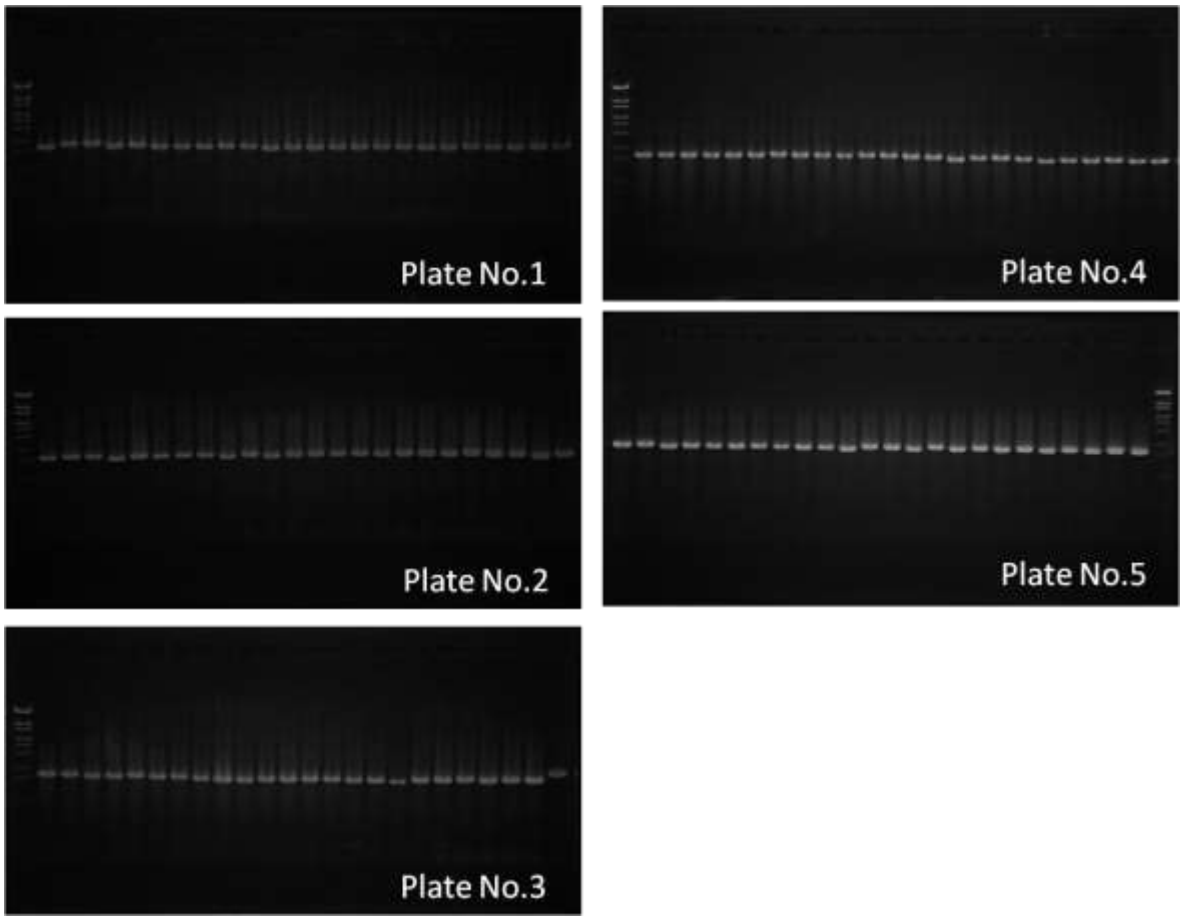


Plate 4.7: Electrophoretogram showing the band of 16S rDNA amplicons after colony PCR of each of the transformed colonies. Agarose (1%) was used at 200 V for 30 min. Twenty-four wells from each of the five 96-well plates were randomly sampled and the 16S rDNA genes in each of the colony in the wells amplified and resolved on agarose gel to confirm the presence of the inserts. Lanes are indicated as –M, OneStep Marker 6 (λ/Sty I digest); -I-: PCR products of 16S rDNA gene from transformed colonies.

4.6.3 Phylogenetic analysis of MWO Polluted Soil Clone Library

A total of 480 clones were sequenced using 1400R sequencing primer. Out of these, six clones have no sequence data, seven clones have less than 200 bp nucleotide sequences, eighteen clones were regarded as chimera sequences while twelve clones failed the sequence similarity check with the GenBank/EMBL/DDBJ database. Four hundred and thirty-seven clones cutting across thirteen phylogenetic groups (Phyla) were identified using the RDP Classifier in the RDP-II database. Out of 437 clones analyzed phylogenetically, 228 clones (52.2%) belong to the phylum *Proteobacteria*, 54 clones (12.36%) belongs to the phylum *Bacteroidetes*, 41 clones (9.38%) belong to the phylum *Chloroflexi*, and 22 clones (5%) belongs to the phylum *Acidobacteria*. In addition, 15 clones (3.43%) belong to the phylum *Firmicutes*, 11 clones (2.52%) is ascribed to *Verrucomicrobia*, 34 clones (7.78%) belong to TM7, 6 clones (1.37%) belong to the phylum *Actinobacteria*, and 4 clones (0.92%) belongs to the phylum *Planctomycetes*. Furthermore, three clones (0.69%) belong to the phylum *Chlorobi*, two clones (0.46%) was ascribed to *Spirochaetes* while one clone each (0.23%) belong to the phyla *Chlamydiae* and OD1. Fourteen clones (6.18%) were regarded as unclassified bacteria (Figure 4.43). The nucleotide sequences of the entire 437 clones in MWO library is displayed in Appendix IV.

4.6.3.1 Proteobacteria

The phylum *Proteobacteria* constitutes more than 50% of the total number of clones in MWO clone library. Out of the 228 clones phylogenetically ascribed to this phylum, the class *Gammaproteobacteria* contributes 111 clones (48.7%) thus indicating the dominance of this class in the phylum. Furthermore, within the class *Gammaproteobacteria*, unclassified *Gammaproteobacteria* clones dominates contributing 81 out of the 111 clones ascribed to this class. In addition, hydrocarbonoclastic bacteria genera belonging to *Gammaproteobacteria* class such as *Acinetobacter*, *Lysobacter* and *Pseudoxanthomonas* are recovered from the hydrocarbon-polluted soil thus indicating their contribution to natural attenuation of hydrocarbon pollutants in the soil.

Two classes, *Alphaproteobacteria* and *Betaproteobacteria* contributed 37 and 42 clones to the phylum constituting 16.2% and 18.4%, respectively. Thirteen uncultured genera (19 clones) were recovered for the class *Alphaproteobacteria*. The remaining clones in this class (18 clones) belong to unclassified groups. In addition, well-known xenobiotic degraders like *Sphingomonas*, *Novosphingobium*, and *Erythrobacter* were also recovered from the polluted soil. In the clone library, 11 uncultured genera (23 clones) were recovered for the class *Betaproteobacteria*. The remaining 19 clones in this class belong to unclassified groups. The class *Deltaproteobacteria* contributed 38 clones (16.7%) to the phylum out of which 19 are uncultured genera. The remaining nineteen clones are affiliated to the unclassified groups.

A clone (SL-0113), which cannot be assigned to any class in the phylum *Proteobacteria*, is believed to be a novel clone, and was thus identified as unclassified *Proteobacteria*.

4.6.3.2 Bacteroidetes

In the clone library, three classes of the phylum *Bacteroidetes* were identified. They are *Sphingobacteria*, *Bacteroidetes*_incertae_sedis and *Flavobacteria*. Fifty-four clones were ascribed to the phylum *Bacteroidetes*. Out of these, 29 clones (53.7%) belong to the class *Sphingobacteria* while the remaining 46.3% was shared between *Bacteroidetes*_incertae_sedis and *Flavobacteria*. Seven uncultured genera (21 clones) were ascribed to *Sphingobacteria*. The remaining eight clones belong to unclassified groups in the class. The class *Bacteroidetes*_incertae_sedis has two representative uncultured genera *Ohtaekwangia* sp. and *Proxilibacter* sp. contributing 20 clones (37%) to the phylum. The class *Flavobacteria* is represented with five clones (9.26%) consisting of three uncultured genera, *Crocinitomix*, *Flavobacterium* and *Owenweeksia* species, respectively.

4.6.3.3 Chloroflexi (Green Non-Sulfur Bacteria)

Three classes were identified as belonging to this phylum. They are *Anaerolineae*, *Caldilineae*, and *Dehalococcoidetes*. Forty-one clones in the clone library were ascribed to this phylum. Out of these, 36 clones (87.8%) belong to the class *Anaerolineae*, two clones belong to the class *Caldilineae* and one clone belongs to the class

Dehalococcoidetes. In the class *Anaerolineae*, 17 out of the thirty-six clones belong to the uncultured genus *Bellilinea* while the uncultured genera *Anaerolinea* and *Longilinea* contributed five clones to the class. The remaining 14 clones in the class belong to unclassified *Anaerolineaceae*. The uncultured genera *Caldilinea* (two clones) and *Dehalogenimonas* (one clone) are the only representatives of the class *Caldilineae* and *Dehalococcoidetes*.

Two clones cannot be ascribed to any class in the phylum *Chloroflexi*. It was thus identified as unclassified *Chloroflexi* in the RDP-II database.

4.6.3.4 Acidobacteria

In MWO polluted soil clone library, the phylum *Acidobacteria* constitute 5% of the library as only 22 clones could be ascribed to this phylum. Subdivisions Gp3, Gp4, Gp6, and Gp7 are identified in this clone library. Subdivision Gp4 contributes 12 clones to the phylum while subdivisions Gp3, which has an uncultured genus *Bryobacter* sp. contributed five clones to the phylum. In addition, subdivisions Gp6 and Gp7 contributed three and two clones to the library, respectively.

4.6.3.5 Firmicutes

In this clone library, the phylum *Firmicutes* constitute 3.43% (15 clones) of the library with three classes, *Clostridia, Negativicutes* and *Bacilli* identified. The class *Clostridia* (10 clones) is represented in this clone library by four uncultured genera (*Tissierella*, *Mahella*, *Saccharofermentans* and *Sedimentibacter*) and four unclassified groups. The class *Bacilli* was represented by one uncultured genus, *Bacillus* sp. while unclassified_*Veillonellaceae* was the only representative of the class *Negativicutes*.

However, three clones cannot be placed in any class of the phylum and was identified in the RDP database as unclassified *Firmicutes*.

4.6.3.6 Actinobacteria

The phylum *Actinobacteria* constitute a meager 1.37% of the MWO clone library contributing only six clones to the library. The representatives of this phylum in the

library consisted of four uncultured genera. The uncultured genera are *Gordonia*, *Cellulomonas*, *Aciditerrimonas* and *Mycobacterium*, respectively.

4.6.3.7 Verrucomicrobia

The phylum *Verrucomicrobia* constitute 2.52% of the clone library contributing 11 clones to the MWO clone library. It consists of two classes, *Verrucomicrobiae* and *Opitutae* and one subdivision, subdivision3. Subdivision3 was represented with six clones; *Verrucomicrobiae* was represented with the uncultured genus *Prosthecobacter* sp. (two clones), while *Opitutae* was represented with the uncultured genus *Opitutus* sp. (two clones). One clone cannot be ascribed to any of the existing classes and was identified as unclassified_*Verrucomicrobia*.

4.6.3.8 Planctomycetes and Chlorobi

The phylum *Planctomycetes* contributed four clones to the MWO polluted soil clone library, which amount to 0.92% of the library. The only class associated with the phylum *Planctomycetes* in the library is *Planctomycetacia* represented by an uncultured genus, *Planctomyces* sp. (three clones) and one clone of unclassified *Planctoycetaceae*.

Similarly, the phylum *Chlorobi*, which contributed three clones (0.69%) to the library, has only one class, *Ignavibacteria*, which was represented by an uncultured genus, *Ignavibacterium* sp. (three clones).

4.6.3.9 Spirochaetes and Chlamydiae

The phyla *Spirochaetes* and *Chlamydiae* contributed three clones to the MWO polluted soil clone library. The phylum *Spirochaetes* contributed two clones to the library amounting to 0.46%. Two uncultured genera, *Turneriella* sp. and *Treponema* sp. represent the phylum in the clone library. The phylum *Chlamydiae* contributed one clone to the library amounting to 0.23%. The uncultured genus, *Parachlamydia* is the only representative of the phylum *Chlamydiae* in the library.

4.6.3.10 TM7 and OD1

The phyla TM7 and OD1 collectively contributed 35 clones (8%) to the MWO polluted soil clone library. Thirty-four clones (7.8%) belonging to the uncultured genus, TM7_genera_incertae_sedis represented TM7. In addition, OD1 (one clone) was represented by only one uncultured genus, OD1-genera_incertae-sedis.

4.6.3.11 Unclassified Bacteria

Fourteen in the entire clone library do not show any affiliation to the existing phyla or candidate divisions in the RDP-II and NCBI databases and thus cannot be placed under any phylum. The clones were thus phylogenetically identified as unclassified_ Bacteria.

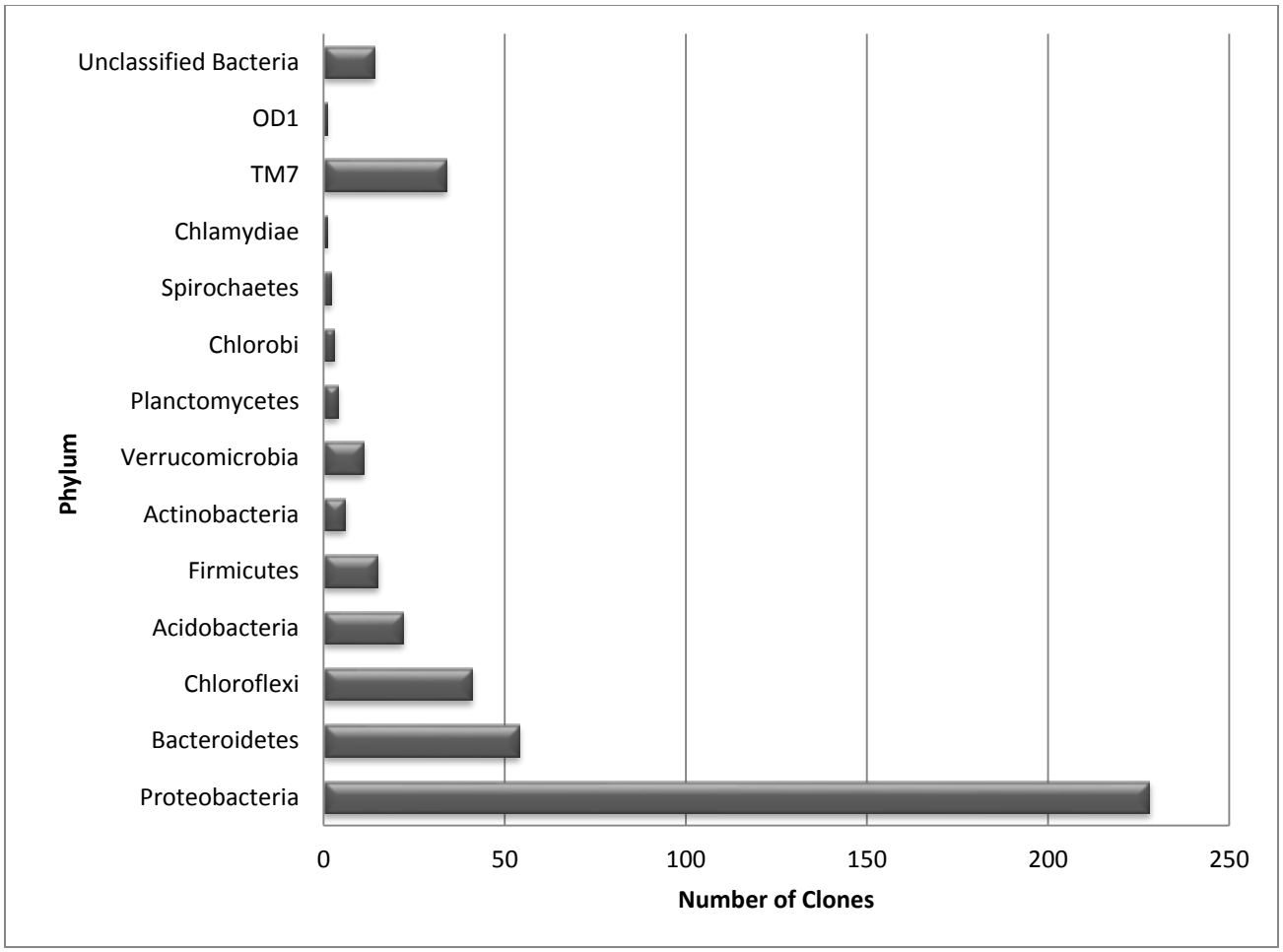


Figure 4.43: Frequency bar chart of the clones found in the clone library of MWO polluted soil. The x-axis shows the number of clones found in each phylum and unclassified bacteria, while the y-axis shows the major existing or possibly novel phylogenetic groups (phylum) found in the clone library. The phylum *Proteobacteria* has the highest number of clones in the clone library (228 clones; 52.2%) while the phyla *Chlamydiae* and OD1 have the least number of clones in the library (1 clone each; 0.23% each).

To discern the evolutionary relationship between members of the same phylum/ class/division as well as relationship with members from different groups in the MWO polluted soil clone library, phylogenetic trees were constructed using the nucleotide sequences of the representative sequences (Figure 4.44-4.53).

Based on the evolutionary distance values considered as the cut off values for bacterial phylum, family/class, genus and species delineations (80, 90, 95, and 97%, respectively), it was observed that in the MWO clone library, 318 OTUs revealed relationship at the species level. At genus and family/class levels, 281 and 189 OTUs were recovered as shown in the rarefaction curve (Figure 4.54). In addition, the clone library coverage indicates 42, 52 and 77% of the clone library is covered at the species, genus and family/class levels, respectively.

Furthermore, all the diversity indices used in this study showed consistent decrease as the taxonomic hierarchy increases. For instance, Shannon index (H′) decreases from 5.59 (species delineation) to 4.82 (family/class delineation). Chao1 decreases from 1125.7 (species delineation) to 291.89 (family/class delineation). Similar decreases were observed in other diversity indices (Table 4.8).

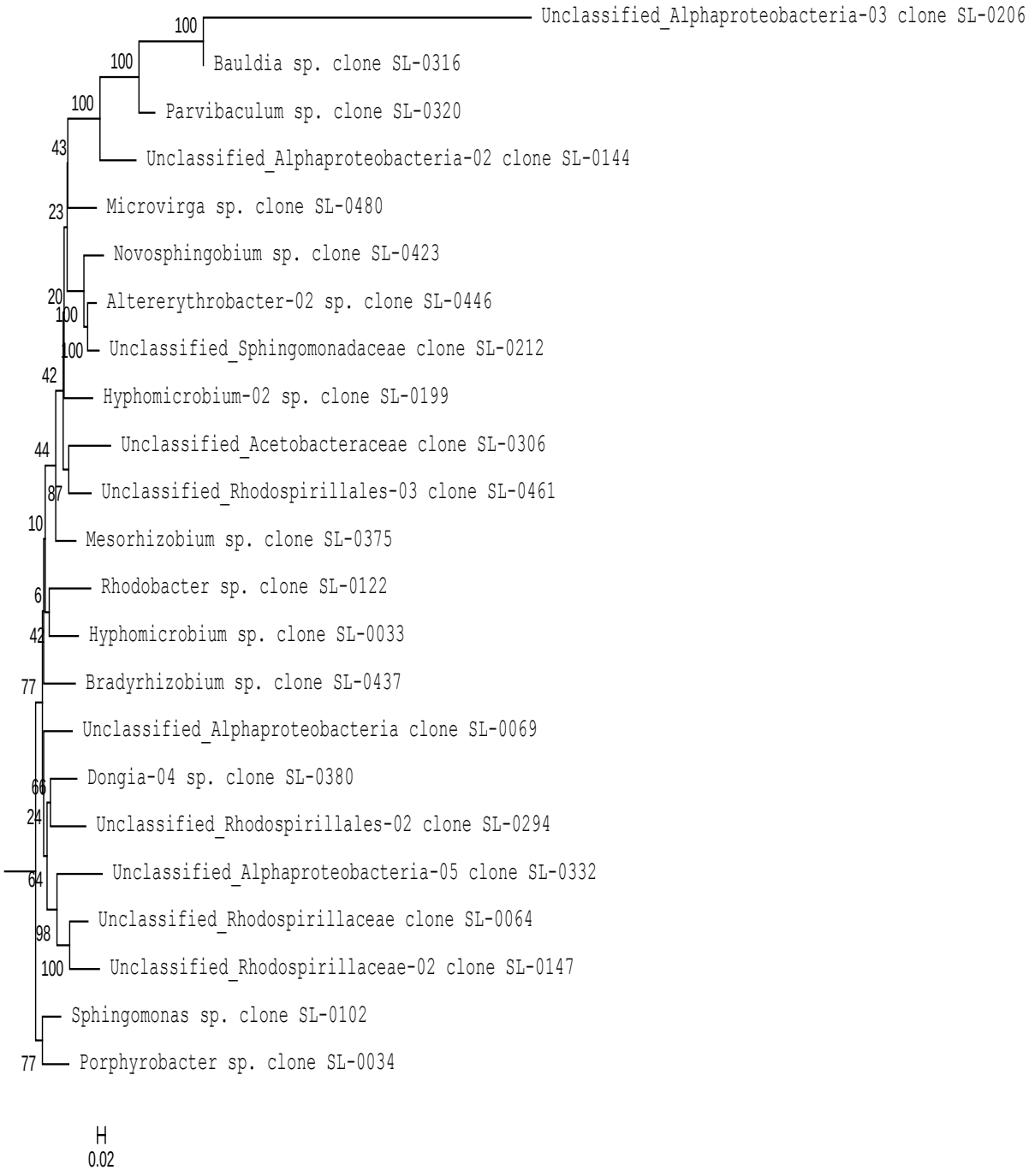


Figure 4.44: 16S rRNA gene-based phylogenetic tree based on the neighbor joining method showing phylogenetic relationship of representative bacterial clones belonging to the class *Alphaproteobacteria* from MWO polluted soil clone library. Bootstrap values of 100 repetitions were used. The scale bar represents the number of changes per nucleotide position.

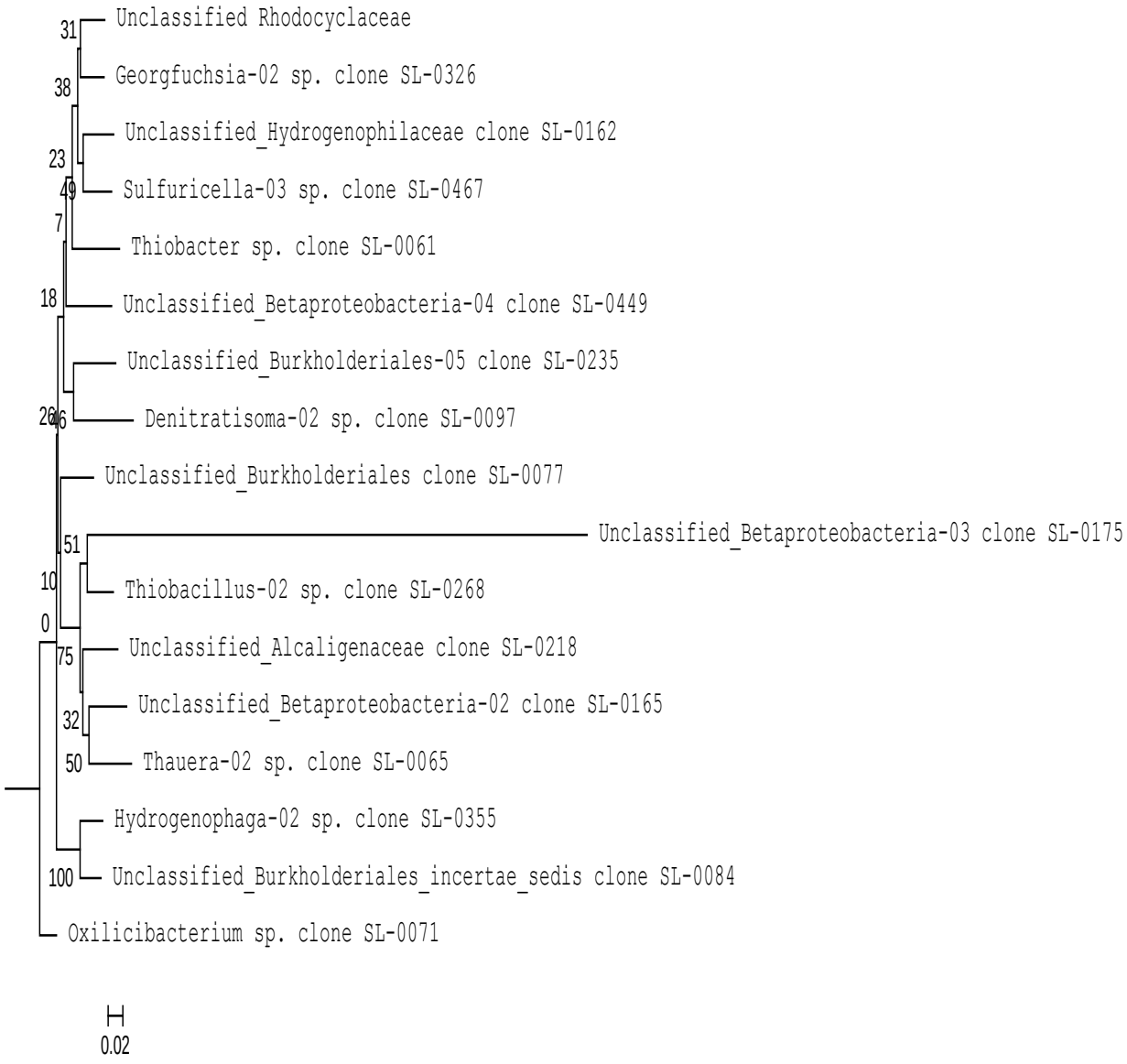


Figure 4.45: 16S rRNA gene-based tree based on the neighbor joining method showing phylogenetic relationship of representative bacterial clones belonging to the class *Betaproteobacteria* from MWO polluted soil clone library. Bootstrap values of 100 repetitions were used. The scale bar represents the number of changes per nucleotide position.

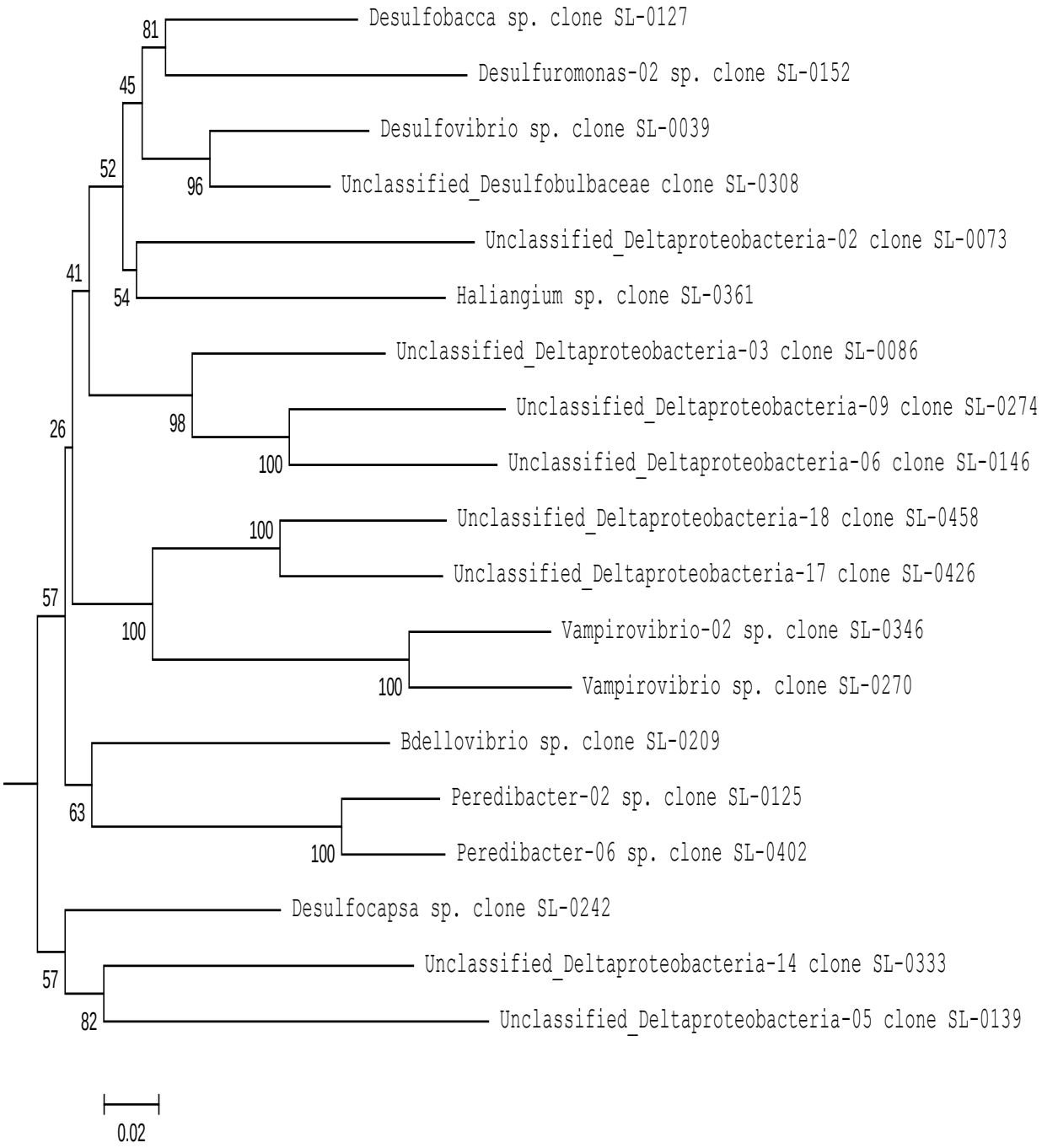


Figure 4.46: 16S rRNA gene-based phylogenetic tree based on the neighbor joining method showing phylogenetic relationship of representative bacterial clones belonging to the class *Deltaproteobacteria* from MWO polluted soil clone library. Bootstrap values of 100 repetitions were used. The scale bar represents the number of changes per nucleotide position.

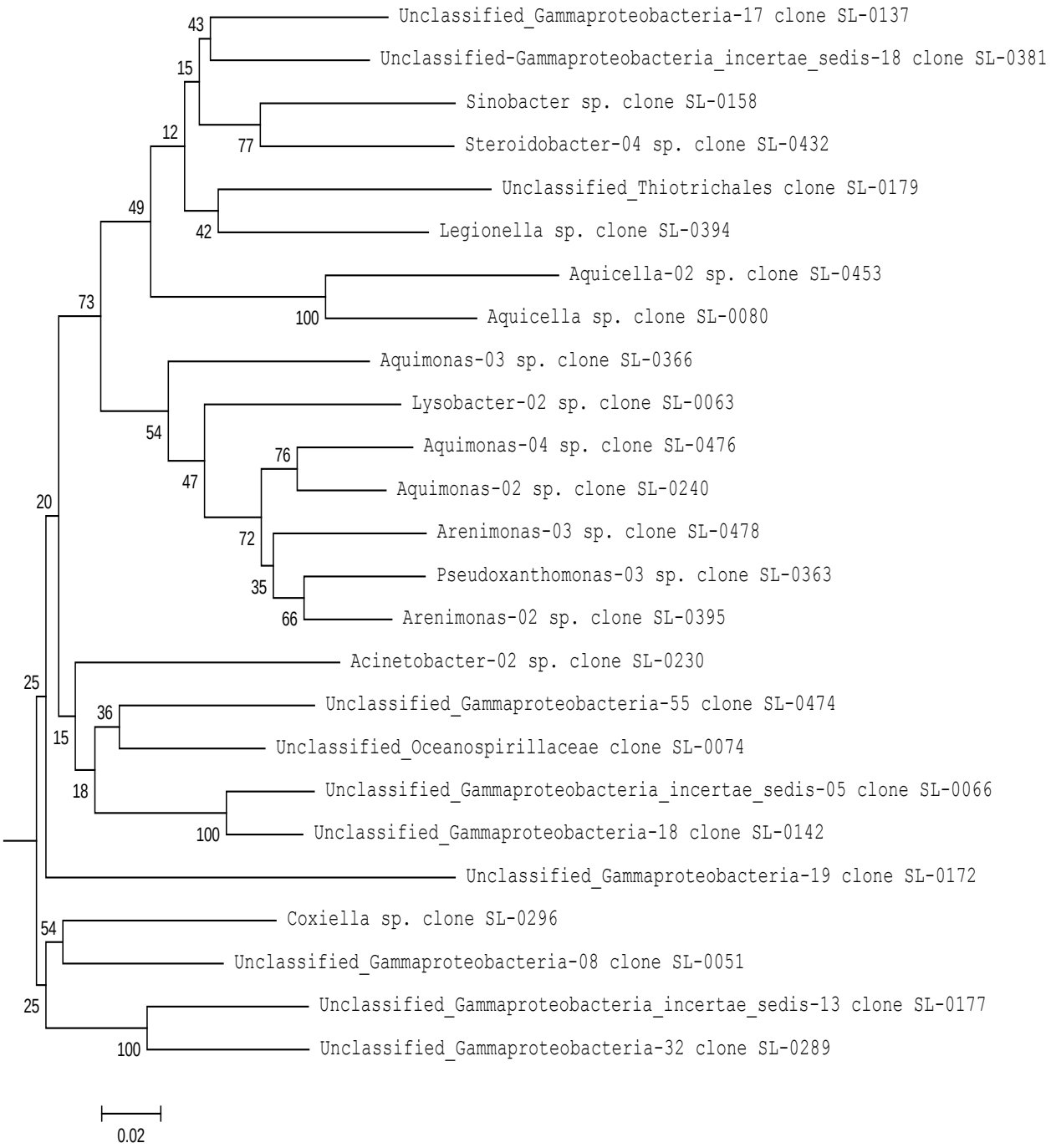


Figure 4.47: 16S rRNA gene-based phylogenetic tree based on the neighbor joining method showing phylogenetic relationship of representative bacterial clones belonging to the class *Gammaproteobacteria* from MWO polluted soil clone library. Bootstrap values of 100 repetitions were used. The scale bar represents the number of changes per nucleotide position.

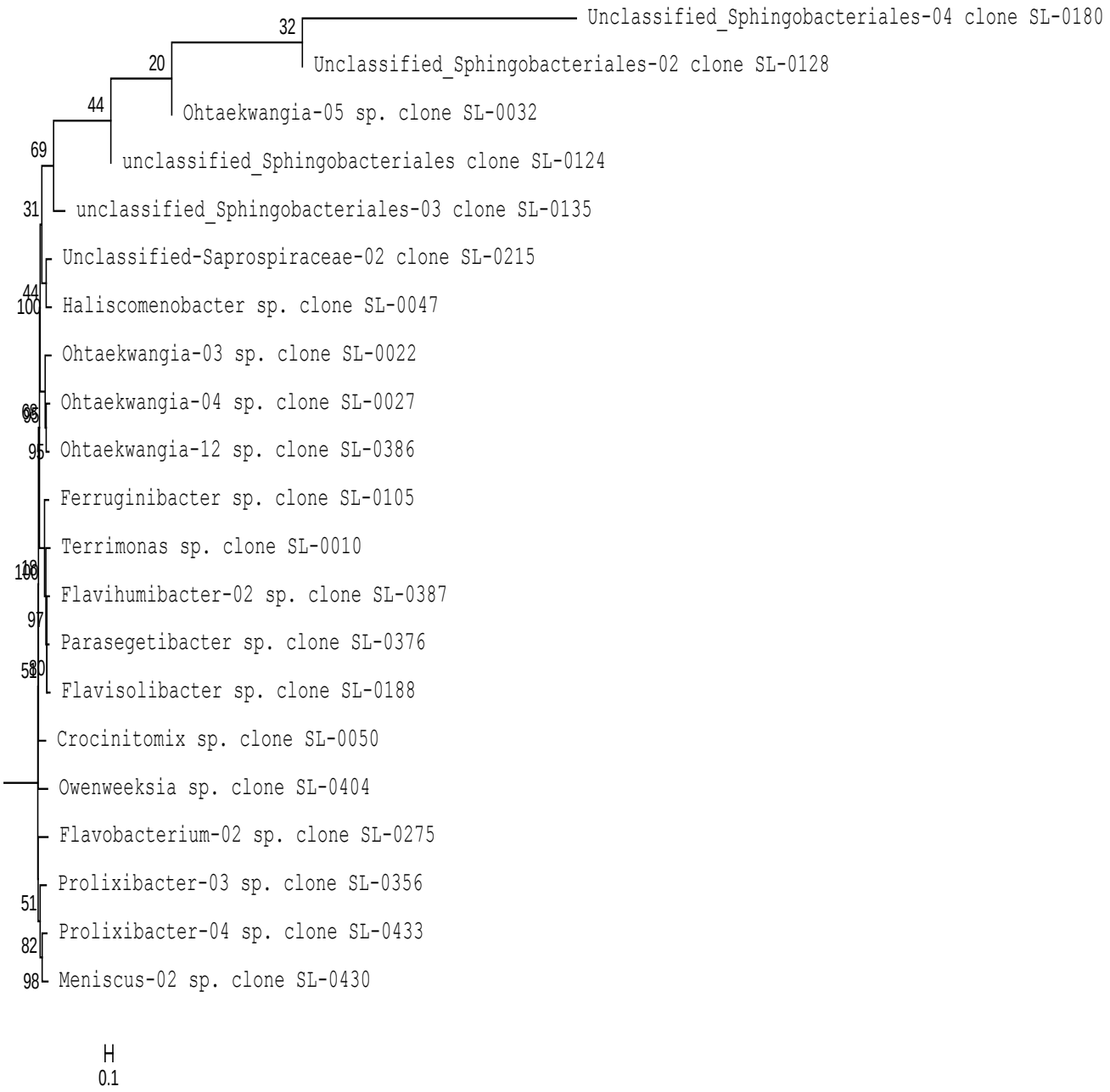


Figure 4.48: 16S rRNA gene-based phylogenetic tree based on the neighbor joining method showing phylogenetic relationship of representative bacterial clones belonging to the phylum *Bacteroidetes* from MWO polluted soil clone library. Bootstrap values of 100 repetitions were used. The scale bar represents the number of changes per nucleotide position.

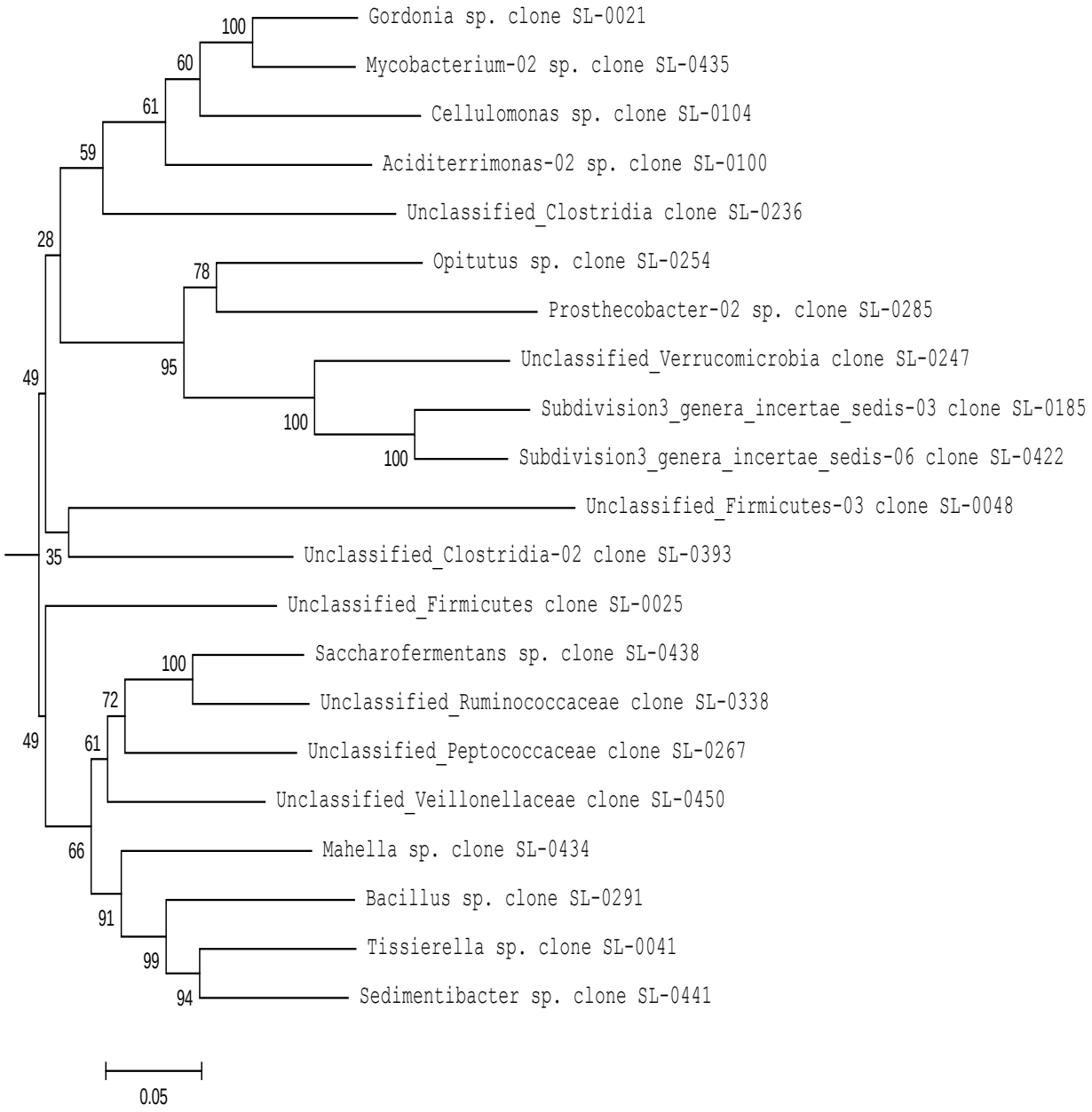


Figure 4.49: 16S rRNA gene-based phylogenetic tree based on the neighbor joining method showing phylogenetic relationship of representative bacterial clones belonging to the phyla *Firmicutes*, *Actinobacteria*, and *Verrucomicrobia* from MWO polluted soil clone library. Bootstrap values of 100 repetitions were used. The scale bar represents the number of changes per nucleotide position.

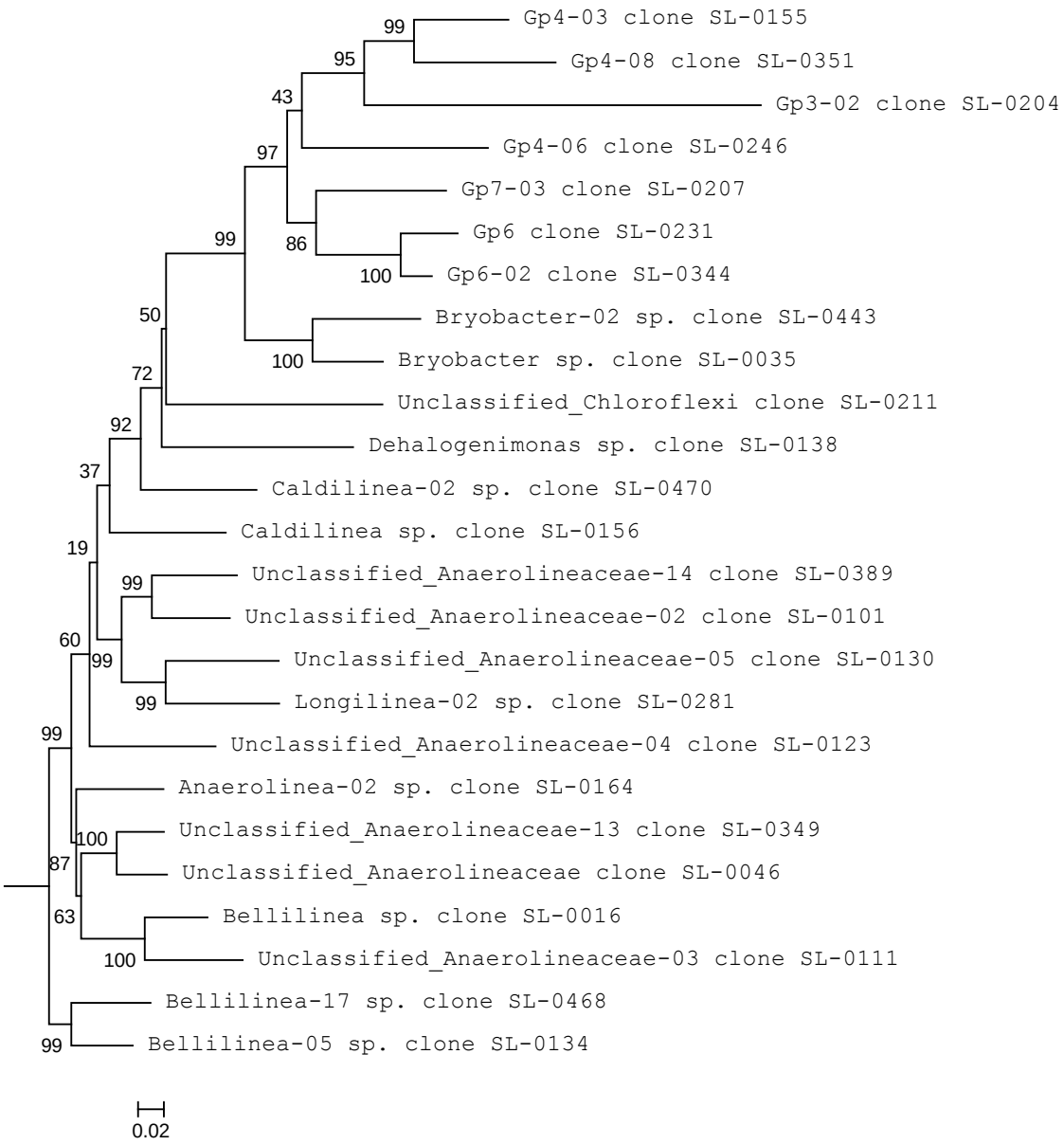


Figure 4.50: 16S rRNA gene-based phylogenetic tree based on the neighbor joining method showing phylogenetic relationship of representative bacterial clones belonging to the phyla *Chloroflexi* and *Acidobacteria* from MWO polluted soil clone library. Bootstrap values of 100 repetitions were used. The scale bar represents the number of changes per nucleotide position.

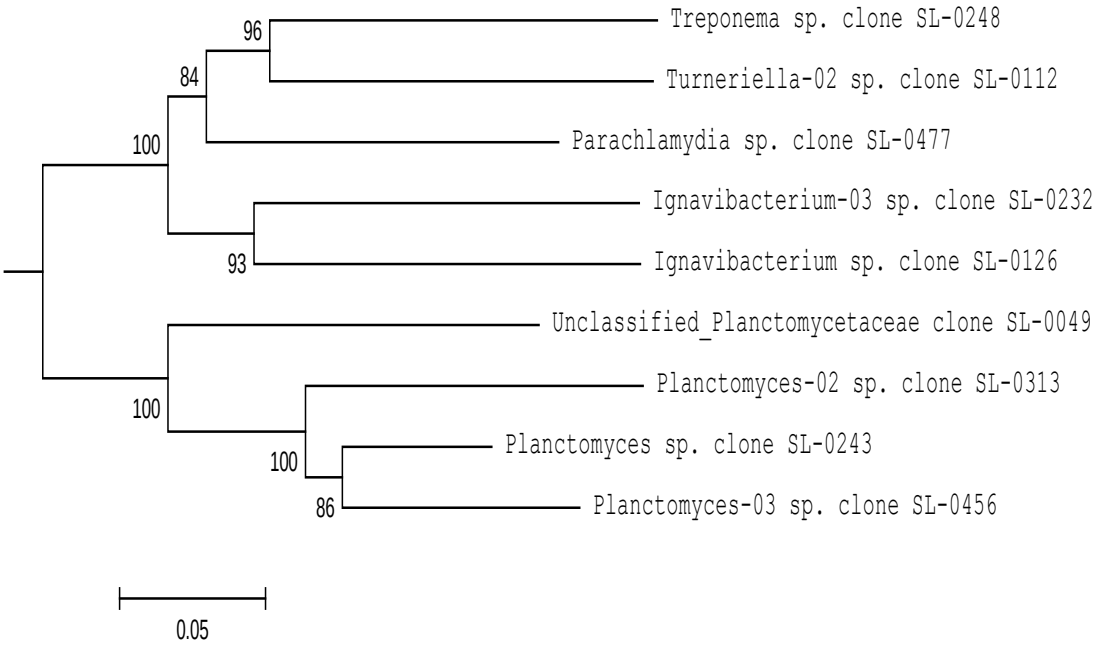


Figure 4.51: 16S rRNA gene-based phylogenetic tree based on the neighbor joining method showing phylogenetic relationship of representative bacterial clones belonging to the phyla *Spirochaetes, Chlamydiae, Chlorobi* and *Planctomycetes* from MWO polluted soil clone library. Bootstrap values of 100 repetitions were used. The scale bar represents the number of changes per nucleotide position.

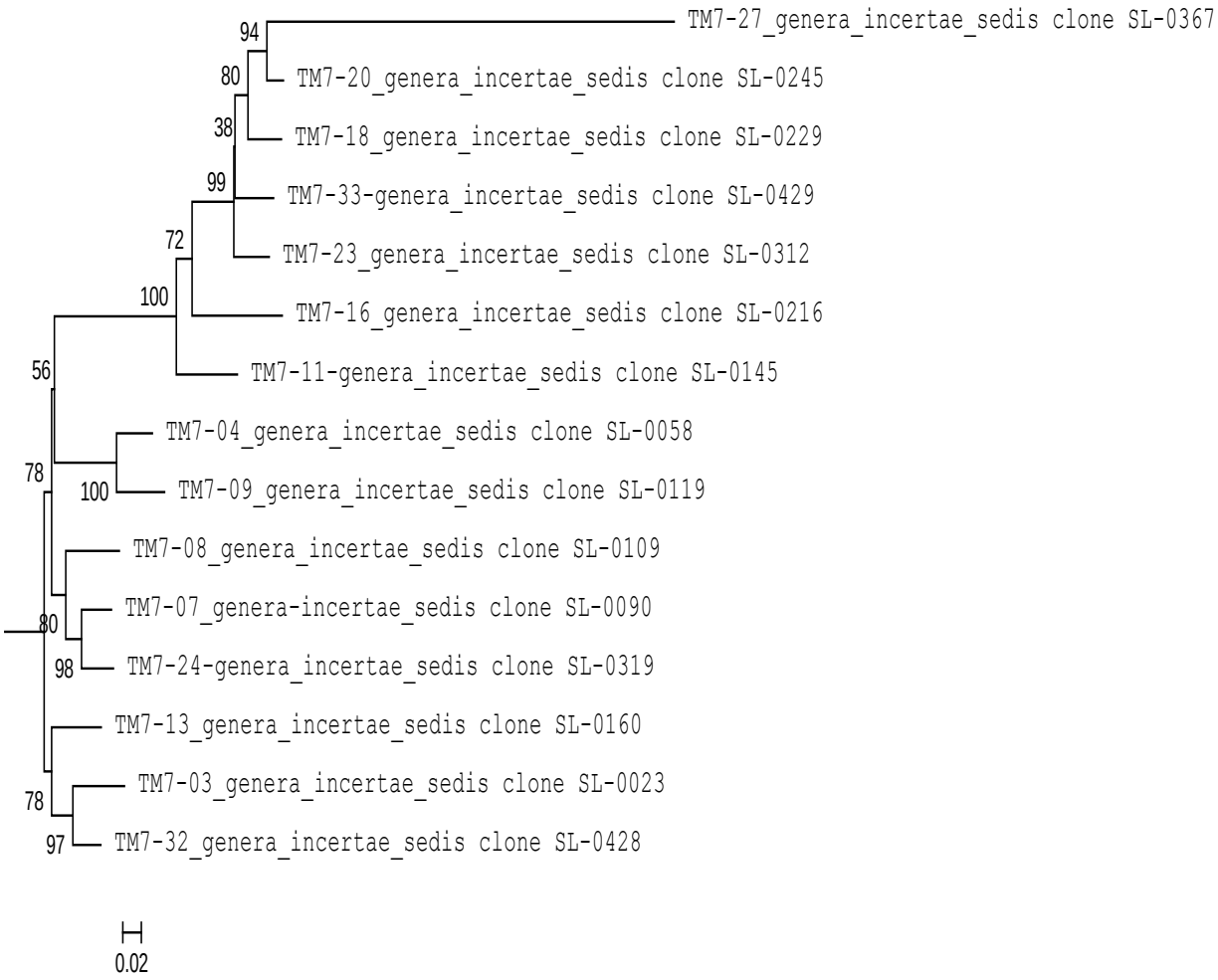


Figure 4.52: 16S rRNA gene-based phylogenetic tree based on the neighbor joining method showing phylogenetic relationship of representative bacterial clones belonging to the phylum TM7 from MWO polluted soil clone library. Bootstrap values of 100 repetitions were used. The scale bar represents the number of changes per nucleotide position.

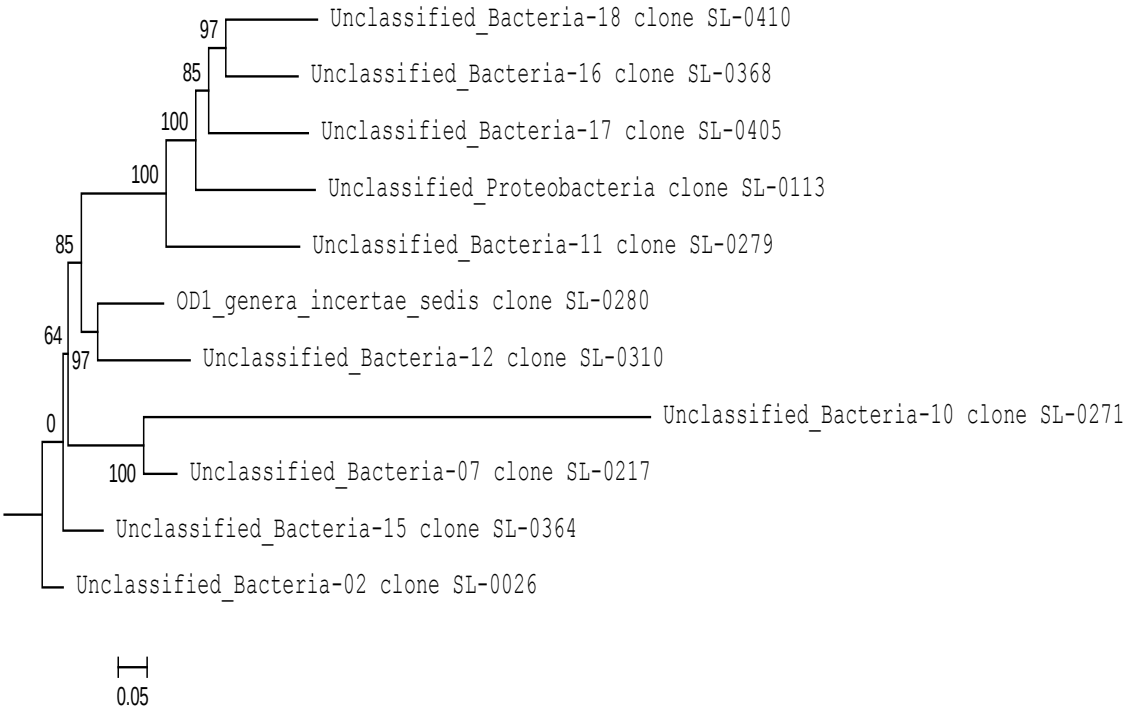


Figure 4.53: 16S rRNA gene-based phylogenetic tree based on the neighbor joining method showing phylogenetic relationship of representative bacterial clones belonging to "Unclassified Bacteria", Unclassified_*Proteobacteria* and Phylum OD1 from MWO polluted soil clone library. Bootstrap values of 100 repetitions were used. The scale bar represents the number of changes per nucleotide position.

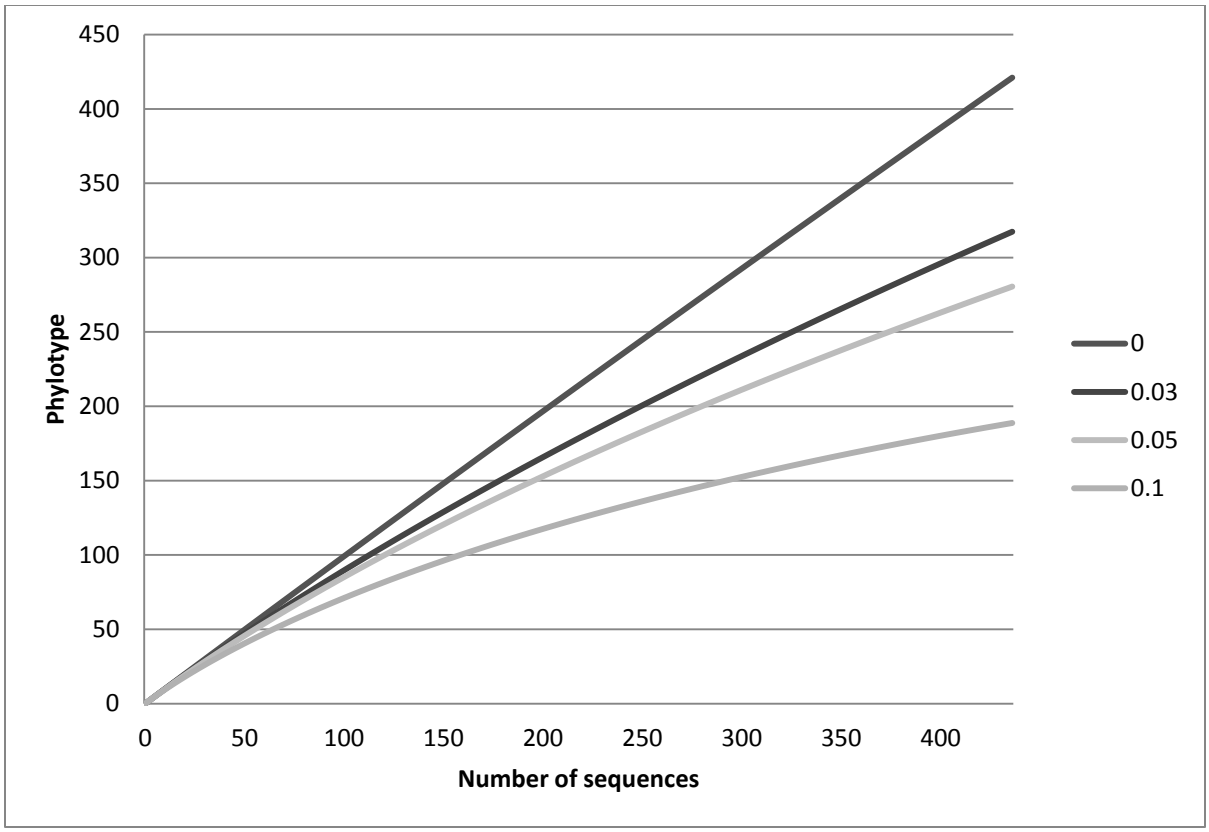


Figure 4.54: Rarefaction curve of number of unique sequences recovered vs. number of clones sequenced for MWO clone library. The phylotypes (OTUs) are 422, 318, 281 and 189 at evolutionary cut-off distances of 0.00 (100%), 0.03 (97%), 0.05 (95%) and 0.1 (90%), respectively.

Table 4.8: Diversity indices of Bacterial community in MWO library

Diversity Indices	**Cut-off distances**		
	97% (0.03)	**95% (0.05)**	**90% (0.10)**
Phylotypes	318 (288, 348)[a)]	281 (254, 308)	189 (173, 205)
Coverage (%)	42	52	77
Shannon index (H′)	5.59	5.40	4.82
Simpson's index (1/D)	203	153	69
Fisher's Alpha	525	340	126
Chao1	1126	837	292
Evenness (E)	0.97	0.96	0.92

[a)] The values in parentheses represent the 95% confidence intervals

DISCUSSION

The use of autochthonous microorganisms inhabiting hydrocarbon-polluted niches for biodegradation and bioremediation has been widely accepted as a formidable approach due to avalanche of successes recorded by researchers (Jain *et al*., 2005; Andreoni and Gianfreda, 2007). The mechanisms of adaptation employed by these organisms include synthesis of inducible enzymes, mutation such as single nucleotide change or DNA rearrangement that results in degradation of the pollutant and acquisition of genetic information from closely related or phylogenetically distinct population within the hydrocarbon-impacted community through horizontal gene transfer (Top and Springael, 2003). Bioremediation technologies help natural attenuation process works faster by either inoculation into soils of strains with desired degradative capabilities (bioaugmentation) or addition of appropriate nutrients (biostimulation) to stimulate the activity of autochthonous degrading strains (Andreoni and Gianfreda, 2007).

In a typical contaminated soil, inorganic nutrients especially the macronutrients is always limiting or lacking resulting in slow pollutant degradation even in the presence of carbon and energy required for growth (Giordani *et al*., 1998; Vidali, 2001). For instance, the concentration of nitrogen and phosphorus at NESU and MWO site are very low, which may be due to their high demand by microorganisms for sugar phosphorylation, synthesis of amino acids, nucleic acids, nucleotides and other cellular processes (Andrew and Jackson, 1996). In essence, amendment of these polluted soils with nitrogen and phosphorus is necessary to enhance biodegradation of organic pollutants.

Activity of soil microorganisms are optimized when between 38 and 81% of soil pore space is saturated with water. Availability of water and oxygen are maximized in this range of water content. Thus, the amount of available water for microbial growth and metabolism may limit hydrocarbon biodegradation in soil (Leahy and Colwell, 1990). The observed low moisture content of the soils used in this study (6.85-11.1%) could be ascribed to a hydrocarbon-mediated reduction in the water holding capacity of the soils (Dibble and Bartha, 1979). The weakly acidic pH (5.40-6.10) observed at the hydrocarbon-contaminated sites used in this study could be attributed to hydrocarbon

inputs. Earlier report indicates that environments, which receive hydrocarbon inputs, tend to be more acidic and very poor in nutrient content (Chikere and Okpokwasili, 2002).

Soil is a mixture of minerals, salts and organic materials and the overall chemical richness of any environment is a reflection of its conductivity values. A high value of electrical conductivity indicates the presence of appreciable amount of ions in the soil samples (Mushtaq and Khan, 2010). Highest conductivity value was obtained from MWO site (318 µs/cm) followed by NESU site (159.4 µs/cm). This is not surprising considering the degree of pollution of MWO as indicated by unusually high total hydrocarbon content and presence of various heavy metals. The acidic nature of the soils used in the study favour bioaccumulation and biomagnifications of heavy metals (Parth *et al*., 2011). In addition, a direct positive correlation between heavy metals presence and increase in soil electrical conductivity has been established in previous studies (Anju and Banerjee, 2011).

Heavy metals are ubiquitous and persistent environmental pollutants that are introduced into the environment through anthropogenic activities and other sources of industrial wastes. The presence of heavy metals such as iron, lead, cadmium and nickel at the sampling sites used in this study indicate gross pollution as heavy oils and spent oils rich in heavy metals are indiscriminately disposed at some of these sites. Heavy metals in low concentrations are micronutrients, which play indispensable roles in cell growth and metabolic functions. However, at high concentrations, heavy metals induce oxidative stress, interfere with protein folding, and function (Nies, 1999). Bacteria to counteract heavy metals stress have devised various resistance mechanisms. These include formation and sequestration of heavy metals in complexes, reduction of a metal to a less toxic species, and direct efflux of a metal out of the cell (Nucifora *et al*., 1989; Nies and Silver, 1995; Outten *et al*., 2000). Isolation of carbazole degraders from MWO and ACPP sampling sites in spite of the high heavy metals presence especially at MWO may be due to the possibility of the degraders harbouring genes for heavy metals resistance and the high hydrocarbon and organic carbon contents of the two sites. However, the relatively low hydrocarbon and organic carbon contents coupled with the presence of heavy metals

at the NESU sampling site, UNILAG may be responsible for the inability to isolate carbazole degraders from this site.

Hydrocarbon contamination imposes selective pressure that only favor limited number of fast-growing hydrocarbon degraders, which are enriched in the (typically oligotrophic) soil environment (Bundy *et al*., 2002). In the present study, a direct correlation was established between the total hydrocarbon content and population of hydrocarbon-utilizing bacteria obtained from the three sampling sites. Site MWO has the highest total hydrocarbon content and the population of hydrocarbon-utilizing bacteria obtained from this site is significantly higher than those from ACPP and NESU are. In addition, the proportion of hydrocarbon utilizers relative to the total heterotrophs observed in this study was generally less than 1%. Though this value is higher than previously reported values for tropical hydrocarbon-contaminated environments (Adebusoye *et al*., 2007), it nonetheless indicates that only a small fraction of the population actually participated in the decommissioning of organic pollutants.

Microbially mediated processes dominate the removal mechanism of carbazole and other hydrocarbon pollutants in soil. This is because photochemical reactions are not possible in the soil subsurface. Successful isolation of degraders of anthropogenic compounds require previous exposure to the compounds, which result in evolution of adapted microflora that have acquired the necessary degradative genes and capable of transforming and mineralizing the compounds after a long period of exposure (Wackett and Hershberger, 2001). Carbazole degraders are isolated from polluted environments through classical continuous enrichment method. Majority of carbazole degraders reported in literature are aerobic, Gram-negative bacteria with the exception of very few carbazole degraders such as *Nocardioides aromaticivorans* IC177 (Inoue *et al*., 2005) and *Gordonia* sp. F.5.25.8 (Santos *et al*., 2006) that are aerobic, Gram-positive bacteria. About 23 and 39% of carbazole degraders isolated from activated sludge, soil, and freshwater samples belong to the genera *Pseudomonas and Sphingomonas*, respectively (Nojiri and Omori, 2007). Carbazole degraders isolated in this study are members of the genera *Achromobacter*, *Pseudomonas*, *Microbacterium*, and *Stenotrophomonas*, respectively. Aside from *Pseudomonas* species, reports on carbazole degradation by other

isolated bacteria genera in this study are very few or non-existent (Inoue *et al*., 2005; Farajzadeh and Karbalaei-Heidari, 2012).

The substrate spectrum analysis of the carbazole degraders isolated in this study on various hydrocarbon substrates revealed different utilization patterns. This may be attributed to the varied composition of the substrates and the diverse nature of hydrocarbon products present at the site from which the isolates were recovered (Leahy and Colwell, 1990). All the isolates failed to grow on naphthalene and dibenzofuran and none of the isolates grows luxuriantly on all the polycyclic aromatic hydrocarbons tested. In addition, shared specificity for dibenzothiophene-sulfone (DBT-S), carbazole, N-ethyl carbazole, anthranilic acid, crude oil and engine oil by all the isolates were also observed. These findings corroborate earlier reports that carbazole degraders have limited substrate specificity for growth (Grosser *et al*., 1991; Kimura and Omori, 1995). For example, two carbazole degrading *Sphingomonas* strains, CB3 and CDH7 were reported to lack specificity for fluorene, naphthalene, dibenzothiophene, dibenzofuran, biphenyl and phenanthrene (Shotbolt-Brown *et al*., 1996; Kirimura *et al*., 1999). It is noteworthy that the best studied carbazole degrader, *Pseudomonas resinovorans* strain CA10, lacks specificity for dibenzothiophene-sulfone (Takagi *et al*., 2002). However, carbazole-degrading strains isolated in this study grew luxuriantly on DBT-S indicating possibly the acquisition of novel degradative genes because of long exposure to various hydrocarbon products by the isolates. Specificity for DBT-S has also been reported for carbazole degrading actinomycetes, *Nocardioides aromaticivorans* strain IC177 (Inoue *et al*., 2005). Furthermore, the degradation of 3,3-dimethoxybenzidine (3,3 DMB) by strains SL4 and SL1 is good news as 3,3 DMB is a congener of the known human bladder carcinogen, benzidine and is classified by the International Agency for Research on Cancer as Group 2B carcinogens.

The luxuriant growth of the isolates observed on crude oil as compared to sparse growth on engine oil may be attributed to two factors. First, crude oil, a complex mixture of different chemical composition may favorably support growth of microorganisms better than refined petroleum product such as engine oil due to diverse nutrient options available in crude oil as source of carbon and energy (Salam *et al*., 2011). Second, at the

ACPP and MWO sites where these isolates were recovered, different types of oil products may have been used for lubrication and fuelling of the coal plant coupled with indiscriminate disposal of spent engine oil and diesel at MWO site. These pollutants inevitably found their way into the soil along with product of coal combustion thereby resulting in adaptation of autochthonous organisms to the pollutants due to selective pressure and acquisition of degradative abilities (Wackett and Hershberger, 2001).

The genus *Achromobacter* is widely distributed in nature. They are nutritionally versatile with propensities for degradation of anthropogenic compounds such as hydrocarbons, polycyclic aromatic hydrocarbons, heterocyclic aromatic hydrocarbons, and polychlorinated biphenyls (Hong *et al*., 2008; Ilori *et al*., 2008; Eixarch and Constanti, 2010; Kaczorek *et al*., 2013). In this study, *Achromobacter* sp. strain SL1 isolated from ACPP site, which exhibited specific growth rate and doubling time of 0.0229 h^{-1} and 30.0 h degrades 81.3% of 50 ppm (0.3 mM) carbazole within 30 days with a rate of degradation of 0.057 mg l^{-1} h^{-1}. Reports on carbazole degradation by *Achromobacter* spp. are scanty and only two reports existed globally, which detailed carbazole biodegradation by *Achromobacter* species. Inoue *et al*. (2005) isolated *Achromobacter* sp. strain IC074 that degrade carbazole and harbor carbazole degradative genes *carR*, *carAa*, and *carAc* highly homologous to *Novosphingobium* sp. strain KA1. Similarly, Farajzadeh and Karbalaei-Heidari (2012) reported the isolation of an *Achromobacter* sp. strain CAR1389, which degraded 90% of 6 mM carbazole within 7 days.

The genus *Pseudomonas* encompasses arguably the most diverse and ecologically significant group of bacteria due to their remarkable degree of physiological and genetic adaptability. *Pseudomonas* is reputed to posses broad substrate affinity not only for different classes of hydrocarbons such as alicyclics, heterocyclics and aromatics (Vankateswaran *et al*., 1995; Nojiri *et al*., 1999; Obayori *et al*., 2008) but also for a plethora of xenobiotic compounds (Amund and Adebiyi, 1991; Habe *et al*., 2001; Wackett and Hershberger, 2001). Catabolic versatility of this genus is predicated on the presence of mobile genetic elements harboring degradative genes, which further suggest that these genes are recruited from preexisting catabolic pathways through horizontal gene transfer. *Pseudomonas* strain SL4 isolated in this study, which exhibited specific

growth rate and doubling time of 0.0238 h^{-1} and 29 h degraded 85% of 0.3 mM carbazole within 30 days with a rate of degradation of 0.062 mg l^{-1} h^{-1}. This value is lower than 98% carbazole degradation in 56 h reported for *Pseudomonas* sp. XLDN4-9 (Li *et al*., 2006) but higher than 12% carbazole degradation in 10 days reported for *Pseudomonas rhodesiae* KK1 (Yoon *et al*., 2002), respectively.

The genus *Stenotrophomonas* are known to exhibit diverse degradative abilities on hydrocarbons, aromatic hydrocarbons, polycyclic aromatic hydrocarbons, heterocyclics and xenobiotics such as pesticides and herbicides as well as antibiotics (Juhasz *et al*., 2002; Inoue *et al*., 2005; Guzik *et al*., 2009; Nayak *et al*., 2009; Verma *et al*., 2011; Guo *et al*., 2013). In particular, *S. maltophilia* strains have shown extensive catabolic versatility on high and low molecular weight PAHs such as phenanthrene, benzo(a)pyrene, dibenzo(a,h)anthracene and coronene (Juhasz *et al*., 1996; 2000; 2002; Guo *et al*., 2013). Globally, only one report on carbazole degradation by *Stenotrophomonas* species has been reported (Inoue *et al*., 2005). However, in this study *Stenotrophomonas maltophilia* strain B_A isolated from ACPP site degraded 76.4% of 0.3 mM carbazole within 30 days with rate of degradation of 0.05 mg l^{-1} h^{-1}. On carbazole, this strain also exhibited specific growth rate and doubling time of 0.0233 h^{-1} and 29.5 h, respectively. Although involvement of a *Stenotrophomonas* sp in carbazole degradation have been reported, nevertheless, globally, this is the first report highlighting carbazole degradative potential of a *Stenotrophomonas maltophilia* strain.

The phylum *Actinobacteria* encompasses bacteria genera such as *Mycobacterium*, *Rhodococcus*, and *Gordonia* with unrivalled capability to degrade recalcitrant pollutants due to their metabolic versatility, genetic plasticity and ability to survive in harsh environments (Larkin *et al*., 2005; Mutnuri *et al*., 2005; Kanaly and Harayama, 2010). Information on biodegradative abilities of *Microbacterium* spp is relatively new as the genus is a known human opportunistic pathogen. However, two crude oil degrading *Microbacterium* spp. Identified as *M. oleivorans* and *M. hydrocarbonoxydans* were isolated by Schippers *et al*. (2005) from oil storage cavern 126 and oil contaminated soil in Germany. Similarly, Manickam *et al*. (2006) reported a hexachlorocyclohexane-degrading *Microbacterium* sp. ITRCI capable of degrading four major isomers of the

toxic compound. Furthermore, four bioemulsifier-producing *Microbacterium* strains were isolated from oil-contaminated mangrove with heavy metals removal abilities (Aniszweski *et al*., 2010). In this study, *Microbacterium esteraromaticum* strain SL6 isolated from MWO site degraded 64.4% of 0.3 mM carbazole within 30 days with a rate of degradation of 0.036 mg l^{-1} h^{-1}. The growth kinetics of the isolate on carbazole indicated a specific growth rate and doubling time of 0.0125 h^{-1} and 55.4 h, respectively. Although the degradation rate is lower than 80% in 30 days and 40% in 10 days reported for *Nocardioides aromaticivorans* strain IC177 and *Gordonia* sp. strain F.5.25.8 (Inoue *et al*., 2005; Santos *et al*., 2006), it was however higher than 57% reported for *Arthrobacter* sp. P1-1 (Seo *et al*., 2006). Nevertheless, it is noteworthy that globally this is the first report detailing carbazole degradation potential of a *Microbacterium* sp.

Angular dioxygenation, the initial dioxygenation of carbazole is a distinct reaction mediated by carbazole 1,9a dioxygenase (CARDO), which exhibit high regioselectivity and addictive preference for the angular position as hydroxylation occurs at the ring-fused position (Nojiri and Omori, 2007). Angular dioxygenation result in complete mineralization of carbazole with the resulting intermediate, anthranilic acid converted to catechol, which is degraded via *ortho* or *meta* pathways to tricarboxylic acid (TCA) cycle intermediate (Nojiri and Omori, 2002). In this study, three of the four carbazole-degrading bacterial strains, SL1, SL4 and SL6 cleaved carbazole angularly and methylated derivative of the COOH group and both the COOH and NH_2 groups of anthranilic acid were detected in the resting and growing cells cultures similar to those found from other carbazole-degrading strains that cleaved carbazole angularly. These results indicated that the degradation pathway for carbazole to anthranilic acid in these strains was similar to that of the most extensively studied carbazole degrader *Pseudomonas resinovorans* strain CA10 (Ouchiyama *et al*., 1993). Furthermore, Anthranilic acid has been detected from the culture extracts of several carbazole degraders and is regarded as the main metabolite of carbazole angular dioxygenation (Ouchiyama *et al*., 1993; Gieg *et al*., 1996; Ouchiyama *et al*., 1998; Kirimura *et al*., 1999; Schneider *et al*., 2000; Kilbane II *et al*., 2002; Inoue *et al*., 2005). Degradation of carbazole to anthranilic acid is important because anthranilic acid is an easily degradable

and harmless substrate, and various organisms for the tryptophan biosynthesis pathway (Gibson and Pittard, 1968; Maeda *et al*., 2009b) assimilate it.

To discern the fate of anthranilic acid produced by the isolates during growth on carbazole, anthranilic acid was used as the only source of carbon and energy for the isolates and production of catechol was monitored using HPLC at absorbance of 254 nm. Catechol was detected in the three isolates with small amount of *cis cis* muconic acid in strain SL1. In addition, with exception of strains SL1, residual anthranilic acid were detected by HPLC in the culture extracts of strains SL4 and SL6. These results indicate the possibility that anthranilic acid was converted to catechol by anthranilate 1,2-dioxygenase and the catechol formed mineralized by the β-ketoadipate pathway via *ortho* cleavage by strains SL1, SL4 and SL6 as confimed by catechol dioxygenase assay using UV-vis spectrophotometry. Detection of catechol from anthranilic acid has been reported in previous studies on carbazole degraders with angular dioxygenation ability. Ouchiyama *et al*. (1993) detected anthranilic acid and catechol (when anthranilic acid was used as carbon source) from culture extracts of strain *P. resinovorans* strain CA10 using HPLC and GC-MS and regarded anthranilic acid and catechol as the main metabolites of carbazole biodegradation. Furthermore, aside from catechol, small traces of cis cis muconic acid was detected by HPLC when anthranilic acid was used as carbon source for strain CA10 (Ouchiyama *et al*., 1993).

Soil microcosm studies in the laboratory allow manipulation of various environmental factors and growth conditions that could favor optimum activity of degrading microorganisms and facilitate effective biodegradation and bioremediation of polluted soils. Results obtained from such studies could be useful in designing novel bioremediation strategies that may be necessary in reclaiming polluted soil in field conditions. Bioaugmentation (seeding) is the introduction of strains with desired degradative capabilities against the target pollutants, either with or without nutrients, into the contaminated environment to augment the indigenous microbial population (Andreoni and Gianfreda, 2007). In this study, *Achromobacter* sp. strain SL1, *Pseudomonas* sp. strain SL4 and *M. esteraromaticum* strain SL6 were still detectable after 30 days of inoculation in both sterilized soil and native soil at very high concentrations ($>10^6$ cfu/g).

Such survival may have resulted in the ability of the seeded strains to compete favorably with the autochthonous organisms in the soil. In addition, the percentage of carbazole removed in native soil (unsterilized soil) in the presence of each of the three strains is higher (NSC1 91.64%, NSC4 87.29%, NSC6 89.13%) than the percentage of carbazole removed by the three strains in sterilized soil (SSC1 66.96%, SSC4 82.16%, SSC6 68.54%). This increase may be due to positive collaboration between the autochthonous and seeded bacterial strains, which resulted in higher carbazole degradation rates. Moreover, agricultural practices such as pesticides and herbicides application in agricultural farms may have resulted in acquisition of some catabolic genes by the autochthonous bacteria, which perhaps allowed them relative ability to degrade carbazole (19.19%).

In addition, it was observed that the percentage of carbazole removed by a consortium of the three strains (SSC146) in the sterilized soil is higher (87.13%) than the percentage of carbazole removed by the individual strain (SSC1 66.96%, SSC4 82.16%, SSC6 68.54%). This finding further buttressed the assertion that mixed bacterial culture degrade better than pure strains. Aside from their ability to compete favorably with the autochthonous organisms in the soil, the strongest factor for the survival of the seeded strains may be their ability to detect and utilized the introduced carbazole as source of carbon and energy as reflected in the reduction of this N-heterocyclic compound over a period of 30 days.

The use of culture independent method to decipher the microbial diversity of hydrocarbon-contaminated environments provides clues about the type of bacteria able to adapt to such habitats and reveal the presence of novel bacteria genera hitherto not reported to contribute to natural attenuation of hydrocarbon pollutants. It also captures viable but not culturable (VBNC) bacterial groups, which though not captured by cultural methods, plays important role in the decontamination process.

In this study, bacterial community composition in MWO hydrocarbon-contaminated soil sample was characterized by cloning of 16S rDNA amplified from total DNA extracted from the soil. The clone library analysis revealed the predominance of *Proteobacteria* (52.2%) with representatives from α-, β-, γ- and δ-classes, respectively. These results

correlates with previous findings that soils with greater hydrocarbon contamination have higher percentages of *Proteobacteria* (Saul *et al*., 2005; Barragán *et al*., 2008; Vivas *et al*., 2008). It also buttress the observation that post-contamination with hydrocarbons mostly results in a shift in bacterial community composition towards an enrichment of *Proteobacteria* (Labbé *et al*., 2007; Zhang *et al*., 2012).

In this study, the class *γ-Proteobacteria* dominated *Proteobacteria* phylum contributing 111 clones (48.7%). This is not surprising, as the dominance of this class has been reported to occur under nutrient oversupply conditions (Amman *et al*., 1995). This phenomenon termed γ-shift, which usually result from degradation of high levels of contaminants is exemplified at MWO site with a high amount of total hydrocarbon (570 g/kg). the presence of very high total hydrocarbon content at the sampling site provide a high nutrient supply for hydrocarbon degraders, which might have resulted in γ-shift (Popp *et al*., 2006) thus allowing *γ-Proteobacteria* to dominates.

Hydrocarbonoclastic bacteria such as *Pseudoxanthomonas, Acinetobacter* and *Lysobacter*, belonging to the class *γ-Proteobacteria* were also recovered from MWO site. The prevalence of *Acinetobacter* clones obtained in this study may be due to the nature of oils disposed at this site, which are mostly engine oils and diesel. This correlates with previous findings that *Acinetobacter* concentration in soil increase within a few days of contamination with diesel (Gallego *et al*., 2001; Chao and Hsu, 2004). In addition, *Acinetobacter* clones obtained in this study shows 100% similarity with all *Acinetobacter* strains retrieved from the NCBI database, majority of which are hydrocarbon degraders. *Pseudoxanthomonas* clones obtained in this study shares 100% similarity with uncultured *Pseudoxanthomonas* (Accession numbers, FM209103, FM209104, FM209176) recovered from hydrocarbon-contaminated soils. Among the two clones of *Arenimonas* obtained from MWO site, one clone (SL-0222) shares 100% similarity with *Arenimonas malthae* strain CC-JY-1 (DQ239766) isolated from diesel oil-contaminated soil sample in Taiwan (Young *et al*., 2007), thus indicating possible role of this strain in hydrocarbon contaminated soils.

The class *α-Proteobacteria* and *β-Proteobacteria* in this study contributed 37 and 42 clones to the *Proteobacteria* phylum. Members of the sphingomonad consisting of the

genera *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* belonging to *α-Proteobacteria* are well-known aromatic, polycyclic aromatic, and heteroaromatic hydrocarbon degraders and their preponderance in hydrocarbon-contaminated environment is well established (Gibson *et al*., 1973; Fredrickson *et al*., 1995; Pinyakong *et al*., 2000; Takeuchi *et al*., 2001; Habe *et al*., 2002; Pinyakong *et al*., 2003). In this study, uncultured clones of *Sphingomonas* and *Novosphingobium*, were recovered from MWO polluted soil. *Sphingomonas* clone SL-0102 shares 99% similarity with uncultured *Sphingomonas* sp. 2F12 (HM438630) isolated from anthracene contaminated soil. Similarly, *Novosphingobium* clone SL-0423 shares 97% similarity with *N. aromaticivorans* strains FM1 (AB331237) and SMCC B0522 (U20774) isolated from deep-terrestrial-subsurface sediments (Balkwill *et al*., 1997) and river water (Inoue *et al*., 2008), respectively. The presence of these strains at MWO site may not be unconnected to the fact that used and spent engine oil and diesel disposed at this site are rich in polycyclic aromatic hydrocarbons (PAHs), which may serve as source of carbon and energy to these strains thereby facilitate their proliferation. Other uncultured *α-Proteobacteria* genera recovered from MWO site with possible role are *Hyphomicrobium* sp. clone SL-0033, a prosthecate bacterium that shares 96% similarity with uncultured *Hyphomicrobium* sp. AMNF5 (AM934754) isolated from a pilot-scale bioremediation process of aliphatic hydrocarbon-contaminated soil (Militon *et al*., 2010). Also worth mentioning is the recovery of *Parvibaculum* sp. clone SL-0452, which shares 98% similarity with complete genome of *P. lavamentivorans* DS-1 (CP000774) that degrade linear alkylbenzenesulfonate (LAS) and 16 other anionic and non-ionic surfactants (Schleheck *et al*., 2011). It is noteworthy that though *Parvibaculum* spp. are frequently detected in habitats with hydrocarbon degradation using culture independent techniques (Sanchez *et al*., 2005; Paixao *et al*., 2010), this is the first report to the best of my knowledge highlighting possible role of *P. lavamentivorans* in natural attenuation of hydrocarbon pollutants in a polluted soil in the entire African continent.

The class *β-Proteobacteria* are chemoautotrophic bacteria that utilized inorganic compounds, which they fix into the production of all necessary organic compounds. Uncultured genera belonging to this class such as *Acidovorax*, *Hydrogenophaga*, *Thauera*, and *Thiobacillus* are soil microorganisms and have been implicated in

degradation of PAHs, autotrophic oxidation of sulfur compounds and degradation of aromatic hydrocarbons under denitrifying conditions (Eriksson *et al*., 2003; Kelly *et al*., 2005).

The class *δ-Proteobacteria* contributed 39 clones (16.3%) to the phylum *Proteobacteria* and are dominated by anaerobic sulfate-reducing bacteria (SRB), methane oxidizers and iron-reducing bacteria such as *Desulfovibrio*, *Desulfobacca*, *Desulforhabdus*, *Desulfocapsa* and *Desulfuromonas,* respectively. They used organic acid such as acetate produced by syntrophic organisms as electron donor for sulfate or Fe (III) reduction. In this study, Uncultured *Desulfobacca* clone SL-0127 shared 95% similarity with uncultured *Desulfobacca* sp. ZZ-L3E11 (EF613372) recovered in a benzene-contaminated aquifer under sulfate-reducing condition. These results is not surprising as hydrocarbon metabolism in anoxic conditions has been reported for several species of denitrifying, iron reducing and sulfate reducing bacteria (Heider *et al*., 1999; Spormann and Widdel, 2000). In particular, SRB were known to utilize varieties of organic compounds as energy and carbon source ranging from monocarboxylic and dicarboxylic acids (Rabus *et al*., 2006), amino acids, methylated sulfur, nitrogen compounds, polyaromatic hydrocarbons, aromatic hydrocarbons and saturated hydrocarbons (Tang *et al*., 2009).

Although 228 clones are ascribed to the phylum *Proteobacteria* in this study, about 60% of these clones cannot be assigned to known genera. This correlates with previous findings, which observed that only 19 to 36% of the proteobacterial sequences could be assigned to a known genus (Janssen, 2006), thus, indicating that many proteobacterial groups remain undescribed and unnamed.

The phyla *Actinobacteria* (high G+C content) and *Firmicutes* (low G+C content), the Gram-positive representatives in the studied soil contributed less number of clones to the MWO library (1.37% *Actinobacteria*, 3.43% *Firmicutes*) compared to other phyla. Several reasons could be adduced. First, *Actinobacteria*, which are dominant in pristine soil (Saul *et al*., 2005) are known to grow slowly and may be outcompeted by *Proteobacteria* that are fast growers and preponderant in hydrocarbon contaminated soils. Second, DNA extraction from Gram-positive bacteria may not have been as effective as

Gram-negative bacteria because the cell wall structure of the former is difficult to lyse (Burgmann *et al*., 2001). Moreover, previous investigators have also reported low abundances of Gram-positive bacteria in bacterial clone libraries constructed from extracted total DNA from hydrocarbon-contaminated soils (Popp *et al*., 2006; Liu *et al*., 2009; Zhang *et al*., 2012).

Actinobacteria phylum encompasses bacteria genera with unrivalled capability to degrade recalcitrant pollutants due to their metabolic versatility, genetic plasticity and ability to survive in harsh environments (Larkin *et al*., 2005; Mutnuri *et al*., 2005; Kanaly and Harayama, 2010). In this study, *Actinobacteria* contribute a paltry six clones with representative uncultured genera such as *Cellulomonas*, *Gordonia*, *Aciditerrimonas* and *Mycobacterium*, respectively. The uncultured genera shares significant relationship with hydrocarbon-degrading strains recovered from NCBI database. *Cellulomonas* sp. clone SL-0104 shares 99% homology with *Cellulomonas* sp. T26 (HQ702749) isolated from coalmine soil; *Gordonia* sp. clone SL-0021 shares 100% homology with *Gordonia* sp. PCSB4 (HM449700) isolated from petroleum-contaminated soil while *Mycobacterium* sp. clone SL-0435 shares 99% homology with the complete genome of *Mycobacterium* sp. JLS (CP000580) isolated from creosote-contaminated soil. In the phylum *Firmicutes*, the class *Bacilli* is represented by only one uncultured genera *Bacillus* sp. clone SL-0291, which shares 98% homology with *Bacillus firmus* N12-3 (HM030743) isolated from production water of oil reservoir.

The class *Sphingobacteria* is the dominant group in *Bacteroidetes* phylum recovered from MWO polluted soil with 29 clones. Previous findings have also established their dominance within the phylum as their diverse oxygen requirements (aerobes, anaerobes, and facultative anaerobes) favor their proliferation at different depth of soil matrices (Janssen, 2006). The class *Flavobacteria* was represented by uncultured genera *Flavobacterium*, *Crocinitomix* and *Owenweeksia*. Members of this genus are chemoorganotrophic and have been reported to increase in the mid-phase of bioremediation of petroleum-contaminated soil (Kaplan and Kitts, 2004; Popp *et al*., 2006).

The phyla *Acidobacteria*, *Chloroflexi*, *Planctomycetes*, *Chlorobi*, *Spirochaetes*, *Chlamydiae*, *Verrucomicrobia* and *Deinococcus-Thermus* encompass indigenous soil bacteria of MWO polluted soil with diverse physiological properties. Significant proportions of representative of these phyla have been recovered from pristine soils (Saul *et al*., 2005; Brons *et al*., 2008). However, in hydrocarbon-contaminated soil, the representatives of these phyla were few or largely absent. Saul et al. (2005) observed that *Cytophaga/Flavobacterium/Bacteroidetes* (CFB) group, *Fibrobacter/Acidobacterium* goup, and *Deinococcus-Thermus* were largely absent from hydrocarbon-contaminated soils around Scott Base, Antarctica. The low representation of these phyla in MWO clone library may not be unconnected to hydrocarbon contamination at this site, which negatively affect these phyla and reduce their population.

Previous studies have indicated the diversity and abundance of candidate divisions TM7, OD1, BRC1, and OP11 in pristine and contaminated soils (Allen *et al*., 2007; Brons *et al*., 2008). Molecular microbial surveys based on 16S rRNA conducted by Schloss and Handelsman (2004) recovered bacterial divisions such as BRC1, OP10, OP11, SC3, TM7, WS2, and WS3 that have no cultural representatives and are recognized only by their nucleotide sequences. In this study, candidate divisions TM7 and OD1 were recovered from MWO polluted soil. This correlates with similar findings by Allen *et al*. (2007) indicating the recovery of these candidate divisions from libraries representing petroleum-contaminated zones.

Microbial diversity, which constitutes an extraordinary reservoir of life in the biosphere (Jain *et al*., 2005) is composed of species richness and evenness. In essence, highest diversity is observed in communities with many different species present (richness) in relatively equal abundance (evenness). The determination of abundances of sequence types in a diversity study is useful in predicting abundances of microorganisms in the studied environment (Dojka *et al*., 1998). Shannon-Weiner diversity index (H') of bacterial clone library in this study was 5.59 (97%; species delineation). This is higher than 4.41 reported by Nogales *et al*. (2001) for bacterial DNA clone library constructed from polychlorinated biphenyl polluted soil. It is equally higher than 3.93, 3.78 and 3.30 reported by Popp *et al*. (2006) and Zhang *et al*. (2012) from hydrocarbon-contaminated

soils. It is however lower than 6.49-9.54 reported by Sutton *et al*. (2013) for diesel-contaminated soil.

Clone library analysis used in this study revealed the preponderance of a number of bacterial phylotypes that shared high similarities with strains from NCBI database previously isolated from hydrocarbon-contaminated environments or described to be involved in hydrocarbon biodegradation. This implies that MWO polluted site harbors a large number of bacteria well suited to hydrocarbon contamination and potentially contributing to natural attenuation of hydrocarbon pollutants. The recovery of novel bacterial genera and the preponderance of members of *Proteobacteria* phylum in the polluted soil gives credence to these assertions.

6.1 SUMMARY OF FINDINGS

Total hydrocarbon contents (values ranging from 216-1570679 mg/kg), moisture content (6.85-11.1%) and total organic carbon (1.01-3.10%) were determined for the three hydrocarbon-contaminated soil samples. Heavy metals such as lead (0.11-4.70 mg/kg), nickel (3.42-4.34 mg/kg) and cadmium (1.12 mg/kg) as well as physiological microbial groups such as THB (6.2×10^7-8.4×10^9), HUB (3.8×10^5-6.7×10^8), THF (6.1×10^7-8.2×10^7) and HUF (2.7×10^5-5.4×10^5) were also recovered from the soils.

Six carbazole-degrading bacterial strains were isolated from two of the hydrocarbon-contaminated soils and identified phenotypically and genotypically as *Achromobacter* sp. Strain SL1, *Achromobacter* sp. Strain SL2, *Achromobacter* sp. Strain SL3, *Pseudomonas* sp. Strain SL4, *Microbacterium esteraromaticum* strain SL6, and *Stenotrophomonas maltophilia* strain B_A. The sequences were deposited at GenBank and assigned accession numbers AB646575.2, AB646576.2, AB646577.2, AB646578.2, AB646579.2 and AB646574 respectively.

Pseudomonas sp. strain SL4 was the best carbazole utilizer among the isolates degrading 85% of initial carbazole concentration within 30 days. It was closely followed by *Achromobacter* sp. strain SL1 (81.3%). The least carbazole degrader among the isolates was *M. esteraromaticum* with 64.4% degradation of carbazole.

Anthranilic acid, the central metabolite of angular dioxygenation of carbazole and catechol, was detected in the growing cells and resting cells extracts of strains SL1, SL4 and SL6 using GC-MS and HPLC. The retention time of methylated anthranilic acid was 5.25 min, with a mass fragmentation pattern of 119 (M^+, 100), 151 (90), 92 (73), 65 (41). The three strains degrade catechol via the *ortho* pathway producing *cis cis* muconic acid as established by UV-Vis spectroscopy.

The bacterial strains were able to utilize polycyclic aromatic hydrocarbons like acenaphthene, fluorene (with exception of strain SL4), and pyrene. They also utilized heterocyclic aromatic compounds like dibenzothiophene (with exception of strains SL4 and B_A), dibenzothiophene-sulfone, 3,3'-dimethoxybenzidine and anthranilic acid. However, none of the strains utilized napththalene and dibenzofuran.

In soil microcosm study, the level of carbazole removed by each of the three bacterial isolates was within a range 66.96-82.16%. The combination of the three bacterial isolates led to the removal of 87.13% of initial concentration of carbazole. In native soil (unsterilized soil), carbazole removal rate was within 19.19-91.64%, respectively after 30 days of incubation.

Four hundred and thirty-seven clones were retrieved from Mechanic Workshop, Okokomaiko (MWO) hydrocarbon-contaminated soil using clone library analysis of 16S rRNA. Each of the clones was sequenced and identified to genus level using the Ribosomal Database Project (RDP-II) and the NCBI. The 437 clones cut across 13 bacterial phyla. They are *Proteobacteria, Bacteroidetes, Chloroflexi, Acidobacteria, Firmicutes, Actinobacteria, Verrucomicrobia, Planctomycetes, Chlorobi, Spirochaetes, Chlamydiae,* TM7 and OD1. Fourteen clones, which did not affiliate with the existing bacteria phyla, were designated by RDP-II and NCBI as 'Unclassified Bacteria'. The sequences were deposited in GenBank under the accession number **KF916697 - KF917133**.

6.2 CONCLUSION

Carbazole degrading bacteria with ability for angular dioxygenation and mineralization were isolated from hydrocarbon-contaminated tropical soils. The isolates could also be used as seed for bioremediation of soils polluted with carbazole and other hydrocarbons. In addition, bacterial diversity study revealed that novel hydrocarbon-degrading bacterial strains abound in tropical hydrocarbon-contaminated soil.

CONTRIBUTIONS TO KNOWLEDGE

1. Globally, this is the first report detailing carbazole degradation potential of a *Microbacterium* sp. Furthermore, the organism (along with two other strains) was found to degrade carbazole by angular dioxygenation, a rare occurrence among carbazole degraders.

2. A total of four hundred and thirty-seven bacterial clones cutting across thirteen different bacterial phyla (*Proteobacteria, Bacteroidetes, Chloroflexi, Acidobacteria, Firmicutes, Actinobacteria, Verrucomicrobia, Planctomycetes, Chlorobi, Spirochaetes, Chlamydiae*, TM7 and OD1) were identified as contributing to natural attenuation of hydrocarbon-pollutants in Mechanic Workshop, Okokomaiko (MWO) soil. Some of these strains represent unculturable bacterial strains that were previously not known to be associated with hydrocarbon-polluted soil and the entire 437 sequences had been deposited in GenBank under the accession number **KF916697 - KF917133**.

3. Detection of anthranilic acid, catechol and *ortho*-cleavage of catechol to *cis cis* muconic acid by *Achromobacter* sp. strain SL1, *Pseudomonas* sp. strain SL4 and *Microbacterium esteraromaticum* strain SL6 established complete mineralization of carbazole to intermediate of tricarboxylic acid cycle. To the best of my knowledge, this is the first report of bacterial isolates from African environment with ability of complete mineralization of carbazole.

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APPENDIX I

MEDIA AND REAGENTS

Luria Bertani medium (LB)

Tryptone/peptone bacteriological	10 g
Yeast extract	5 g
Deionized water	800 mL

Adjust pH to 7.0 with 5 N NaOH. The volume was adjusted to 1 L with deionized water. For solid medium, 1.6% agar was added. The medium was sterilized by autoclaving at 15 psi for 20 minutes.

Carbon free mineral medium (CFMM, pH 7) described by Habe et al. (2002)

Part A

NH_4NO_3	3.0 g/L
Na_2HPO_4	2.2 g/L
KH_2PO_4	0.8 g/L
Deionized water	1 L

Part A was sterilized by autoclaving at 15 psi for 20 minutes.

Part B

$MgSO_4.7H_2O$	0.1 g
$FeCl_3.6H_2O$	0.05 g
$CaCl_2.2H_2O$	0.05 g

Part B was prepared as stock solution by dissolving each chemical in 1 mL distilled water and sterilized by filtering through a 0.22 µm pore size filter. After sterilization, A and B was mixed thoroughly.

Starch Casein Nitrate Agar (for actinomycetes)

Soluble starch	10.0 g
Casein (vitamin-free)	0.3 g
KNO_3	2.0 g
K_2HPO_4	2.0 g
NaCl	2.0 g
$CaCO_3$	0.02 g
$MgSO_4.7H_2O$	0.05 g
$FeSO_4.7H_2O$	0.01 g
Agar	18 g
Nystatin, Cycloheximide	50 µg/ml

Adjust pH to 7.0-7.2 with 5 N NaOH. The volume was adjusted to 1 L with deionized water. The medium was sterilized by autoclaving at 15 psi for 20 minutes.

Ashby's Mannitol Agar (Nitrogen-free medium)

KH_2PO_4	0.2 g
$MgSO_4$	0.2 g
NaCl	0.2 g
$CaSO_4$	0.1 g
$CaCO_3$	5.0 g
Mannitol	10.0 g
Agar	20.0 g

Adjust pH to 7.0 with 5 N NaOH. The volume was adjusted to 1 L with deionized water. The medium was sterilized by autoclaving at 15 psi for 20 minutes.

Nutrient Broth	**g/Litre**
Beef extract	1.0 g
Peptone	5.0 g
NaCl	5.0 g
Yeast extract	2.0 g
Distilled water	1000 ml

Peptone water	
Peptone	10.0 g
NaCl	5.0 g
Distilled water	1000 ml
pH	7.2

Nutrient agar	
Beef extract	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.4

Nutrient gelatin	
Beef extract	3.0 g
Peptone	5.0 g
Gelatin	120.0 g
Distilled water	1000 ml

Gelatin was added to water and allowed to stand for 30 minutes before heating to further dissolve it. The pH was adjusted to 7.0 and sterilized by autoclaving at 15 psi for 20 minutes. The gelatin was thereafter dispensed in sterile bottles and allowed to set.

Urea Medium

Peptone	1 g
NaCl	5 g
KH_2PO_4	2 g
Agar	20 g
Distilled water	1000 ml
pH	6.8

Starch Agar

Soluble starch	10 g
Nutrient agar	1000 ml

Potato Dextrose Agar

Potato extract	4 g
Dextrose	20 g
Agar	15 g
Distilled water	1000 ml
pH	5.6

SIM Medium

Ammonium ferric citrate	0.2 g
Casein peptone	20.0 g
Meat peptone	6.6 g

Sodium thiosulfate	0.2 g
Agar	3.0 g
Distilled water	1000 ml

Reagents

Kovac's reagent

4-dimethylaminobenzaldehyde	5 g
n-butanol	75 ml
concentrated HCl	25 ml

Lugol's Iodine

Iodine	5 g
Potassium iodide	10 g
Distilled water	100 g

Tetramethyl-*p*-phenylenediamine solution

Tetramethyl-*p*-phenylenediamine	1 g
Distilled water	100 ml

Tris-EDTA buffer (TE buffer, pH 8)

1 M Tris-HCl, pH 8	1 mL
0.5 M EDTA pH 8	0.2 mL

Distilled water was added to 100 mL and the solution sterilized at 15 psi for 20 minutes.

Tris-EDTA/glucose buffer (TEG buffer, pH 8)

Part A

1 M Tris-HCl pH 8	2.5 mL
0.5 M EDTA pH 8	2.0 mL

Part B

1 M glucose solution	5 mL

Part A was sterilized by autoclaving at 15 psi for 20 minutes. Part B was sterilized by filtering through a 0.22 µm pore size filter. After sterilization, A and B are mixed thoroughly.

50x Tris-acetate/EDTA buffer (50x TAE buffer, pH 8)

Tris-base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA pH 8	100 mL

Tris-base (242 g) was weighed and dissolved in 750 mL distilled water. Glacial acetic acid (57.1 mL) and 100 mL of EDTA (pH 8) were carefully added and the stock solution is adjusted to a final volume of 1L. Working solution of TAE buffer (1x TAE) was prepared by diluting the stock solution 50x in distilled water.

10x Tris-borate/EDTA buffer (10x TBE buffer)

Tris-base	108 g
Boric acid	55 g
EDTA	7.5 g

Tris-base, boric acid and EDTA were dissolved in 800 mL of distilled water. The buffer was diluted to 1L with distilled water and placed in a hot water bath to dissolve the undissolved white clumps.

10% sodium dodecyl sulphate (10% SDS)

SDS	10 g
Distilled water	70 mL

The solution was dissolved at 70°C using a water bath and adjusted to a final volume of 100 mL.

Sodium hydroxide/sodium dodecyl sulphate solution (NaoH-SDS solution)

1 M NaCl: 10% SDS: distilled water = 2:1:7

Cetyltrimethyl ammonium bromide/sodium chloride solution (CTAB/NaCl solution)

(10% CTAB in 0.7 M NaCl)

NaCl	4.1 g
Distilled water	80 mL
CTAB	10 g

The three components were added with stirring while heating at 65°C in a water bath. The solution was adjusted with distilled water to a final volume of 100 mL.

5 M NaCl

NaCl	292 g
Distilled water	880 mL

The solution was adjusted with distilled water to a final volume of 1L.

3 M sodium acetate

$CH_3COONa.3H_2O$	4.081 g

Distilled water 6 mL

The two components were mixed and the pH adjusted to 5.2 with glacial acetic acid. The solution was brought to a final volume of 10 mL and sterilized by autoclaving at 15 psi for 20 minutes.

5 M potassium acetate (KOAc)

CH_3COOK 29.4 g

Glacial acetic acid 11.5 mL

The solution was brought to a final volume of 100 mL with distilled water and sterilized by autoclaving at 15 psi for 20 minutes.

Stock ampicillin (Ap; 0.1 g/mL)

Ampicillin 0.1 g

Distilled water 1 mL

The solution was filter-sterilized using a 0.22 µm pore size filter. It is stored at -20°C.

Stock Isopropyl-β-D-thiogalactopyranoside (IPTG, 1 M)

IPTG 238 mg

Distilled water 1 mL

The solution was filter-sterilized using a 0.22 µm pore size filter. It is stored at -20°C

Stock 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal, 2% w/v)

X-Gal 20 mg

Dimethylformamide 1 mL

The tube containing the solution was wrapped in aluminium foil to prevent damage by light. It is stored at -20°C.

1x TAE Running Buffer

Reagent	Amount
50x TAE buffer	140 ml
dH_2O	6,860 ml
Total volume	7,000 ml

2x Gel Loading Dye

Reagent	Amount	Final concentration
2% bromothymol blue	0.25 ml	0.05%
2% xylene cyanol	0.25 ml	0.05%
100% glycerol	7.0 ml	70%
dH_2O	2.5 ml	
Total volume	10.0 ml	

Store at room temperature

APPENDIX II

TABLES

Table 1: Time course of growth of SL1 on carbazole

Time (Days)	TVC (cfu/ml)	
0	9.2×10^6	9.4×10^6
3	4.2×10^7	5.4×10^7
6	6.0×10^8	6.4×10^8
9	7.0×10^8	6.8×10^8
12	7.6×10^9	7.2×10^9
15	7.0×10^9	7.2×10^9
18	6.6×10^9	6.4×10^9
21	5.3×10^8	5.5×10^8
24	4.3×10^8	3.9×10^8
27	3.0×10^8	3.4×10^8
30	2.5×10^8	1.3×10^8

Table 2: Time course of growth of SL4 on carbazole

Time (Days)	TVC (cfu/ml)	
0	9.4×10^6	9.0×10^6
3	7.7×10^7	7.5×10^7
6	1.0×10^8	1.4×10^8
9	6.5×10^9	6.3×10^9
12	7.5×10^9	8.7×10^9
15	7.8×10^9	7.4×10^9
18	6.0×10^9	6.2×10^9
21	7.9×10^8	8.9×10^8
24	6.8×10^8	6.2×10^8
27	3.6×10^8	3.2×10^8
30	2.5×10^8	1.9×10^8

Table 3: Time course of growth of SL6 on carbazole

Time (Days)	TVC (cfu/ml)	
0	9.0×10^6	9.2×10^6
3	3.5×10^7	3.3×10^7
6	5.6×10^7	5.2×10^7
9	2.6×10^8	3.0×10^8
12	4.4×10^8	4.2×10^8
15	8.0×10^8	7.6×10^8
18	5.1×10^9	5.5×10^9
21	5.2×10^9	5.0×10^9
24	3.9×10^9	3.5×10^9
27	2.7×10^8	2.9×10^8
30	1.8×10^8	1.4×10^8

Table 4: Time course of growth of B_A on carbazole

Time (Days)	TVC (cfu/ml)	
0	9.2×10^6	9.2×10^6
3	5.6×10^7	5.2×10^7
6	6.8×10^8	6.0×10^8
9	5.7×10^9	5.1×10^9
12	7.6×10^9	7.2×10^9
15	6.0×10^8	5.8×10^8
18	5.4×10^8	4.2×10^8
21	3.8×10^8	4.0×10^8
24	3.7×10^8	4.5×10^8
27	2.5×10^8	2.5×10^8
30	1.6×10^8	1.0×10^8

APPENDIX III

MASS SPECTRA

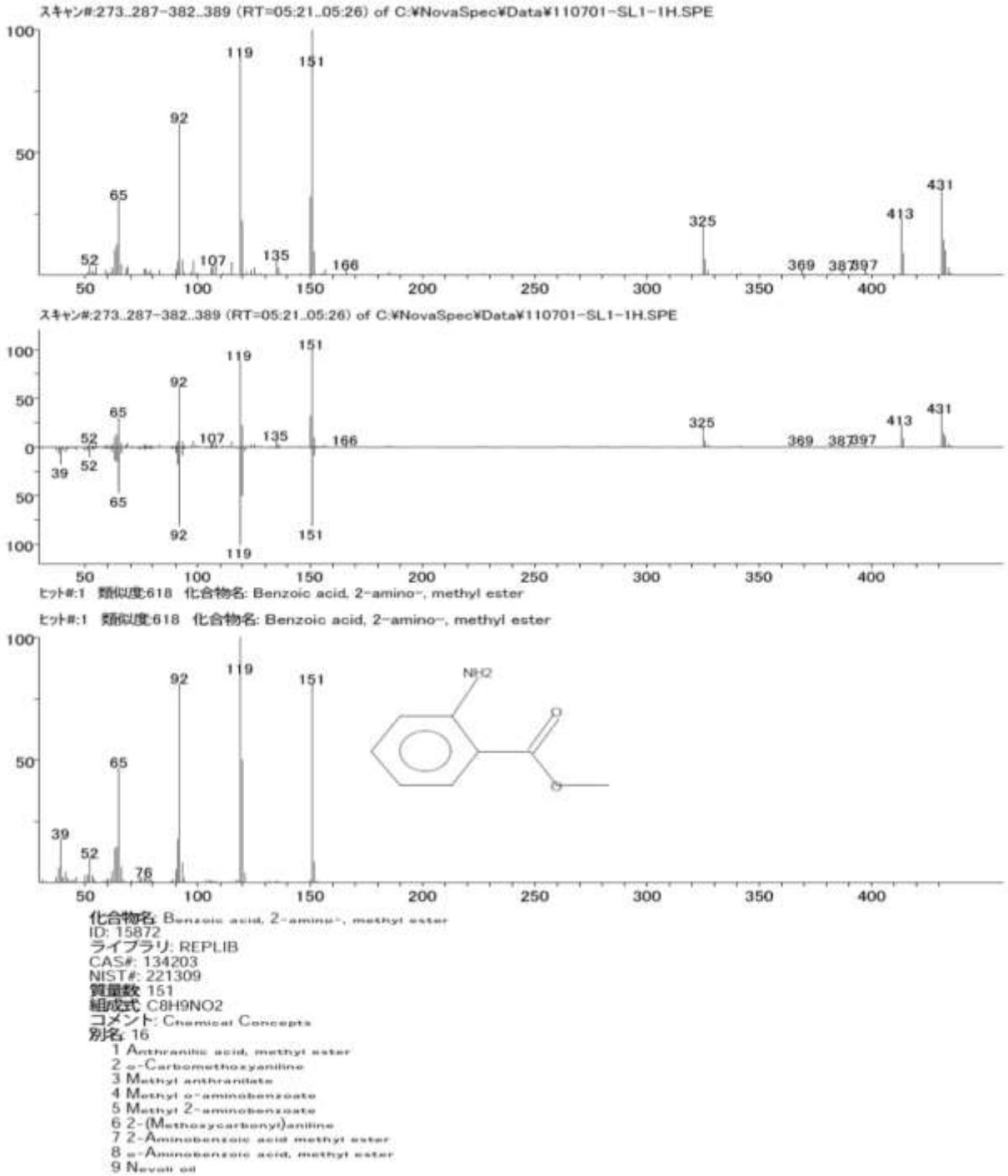

Figure 1a: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL1 after 1 hour of incubation.

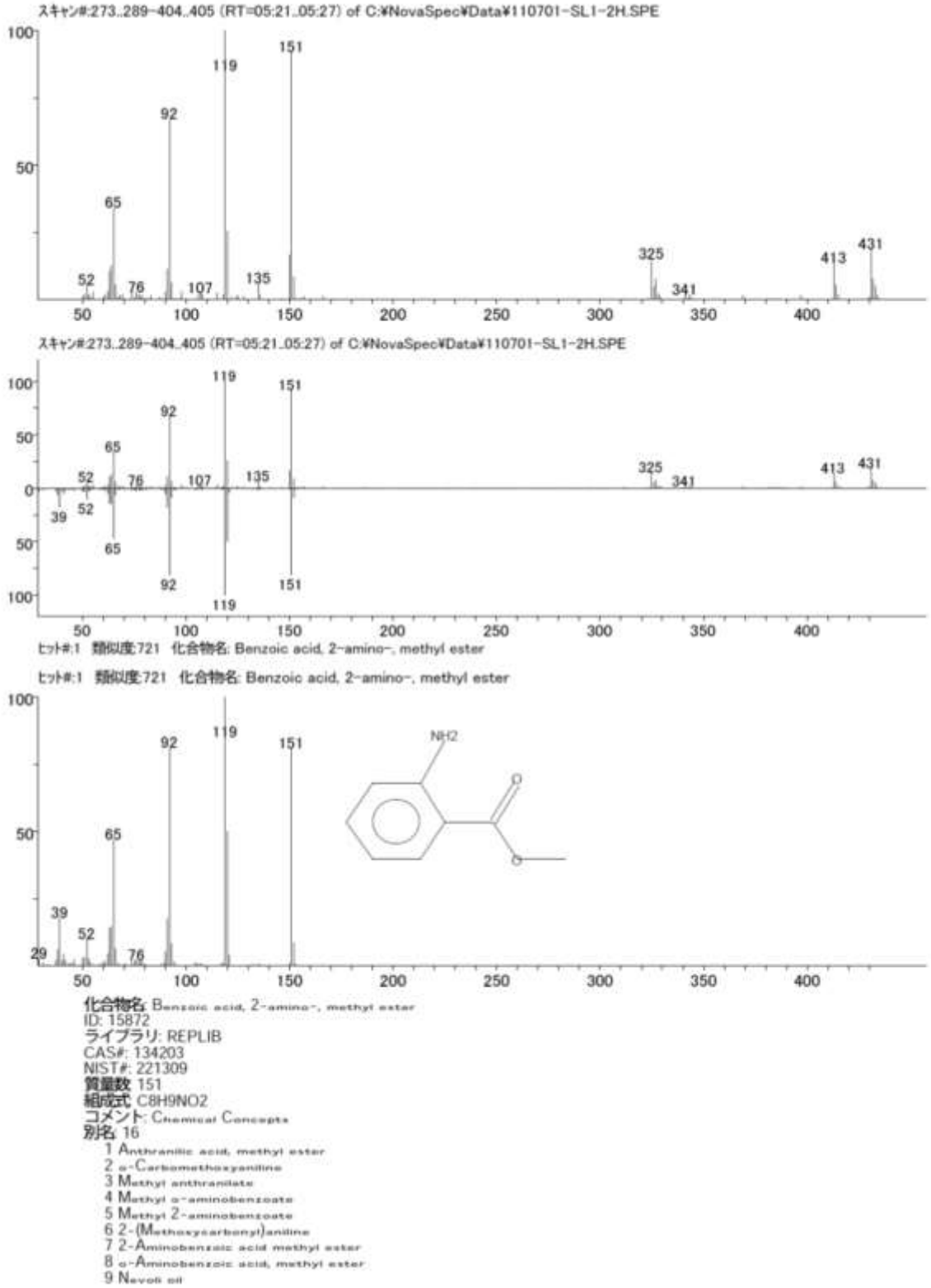

Figure 1b: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL1 after 2 hours of incubation.

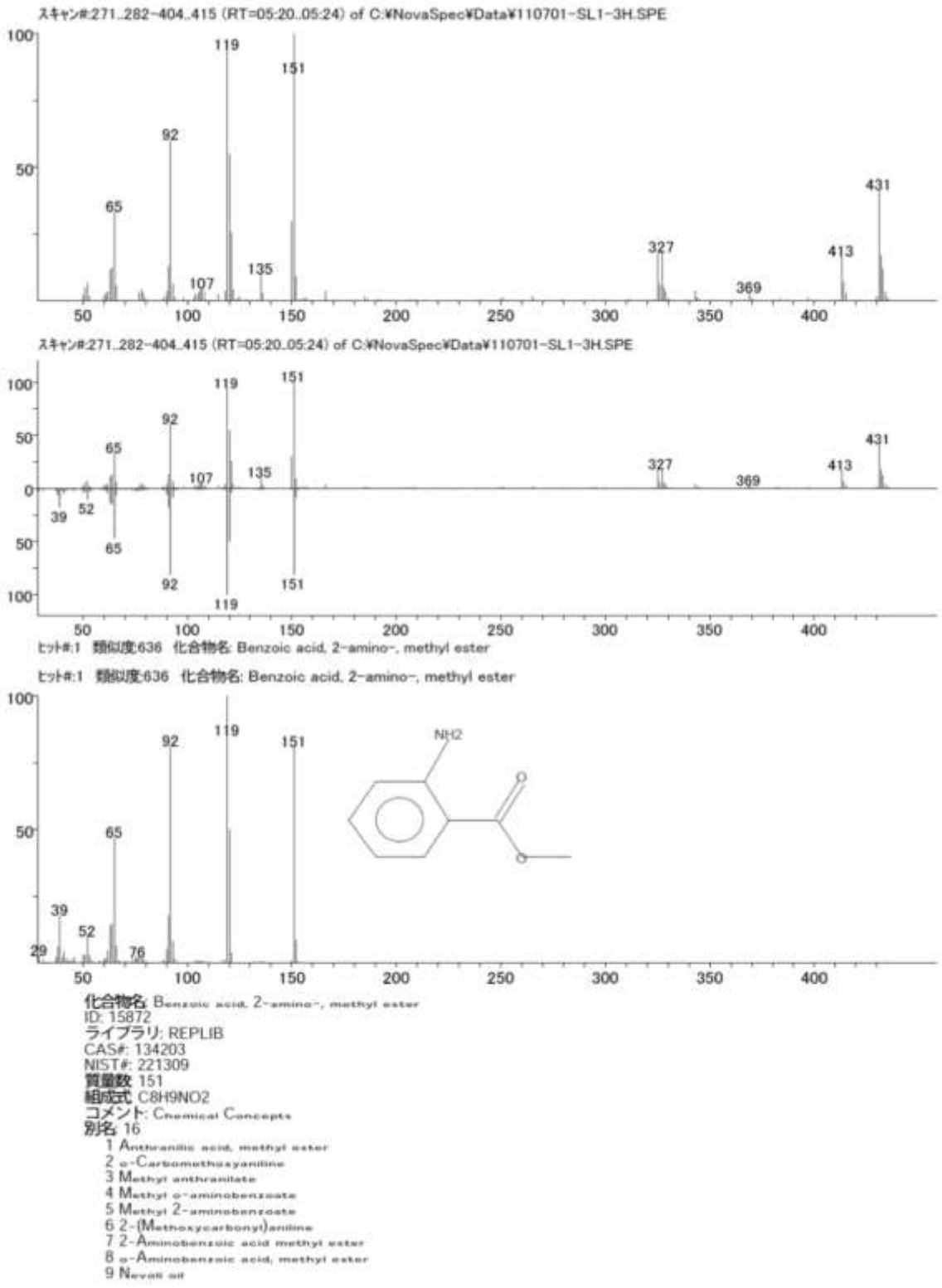

Figure 1c: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL1 after 3 hours of incubation.

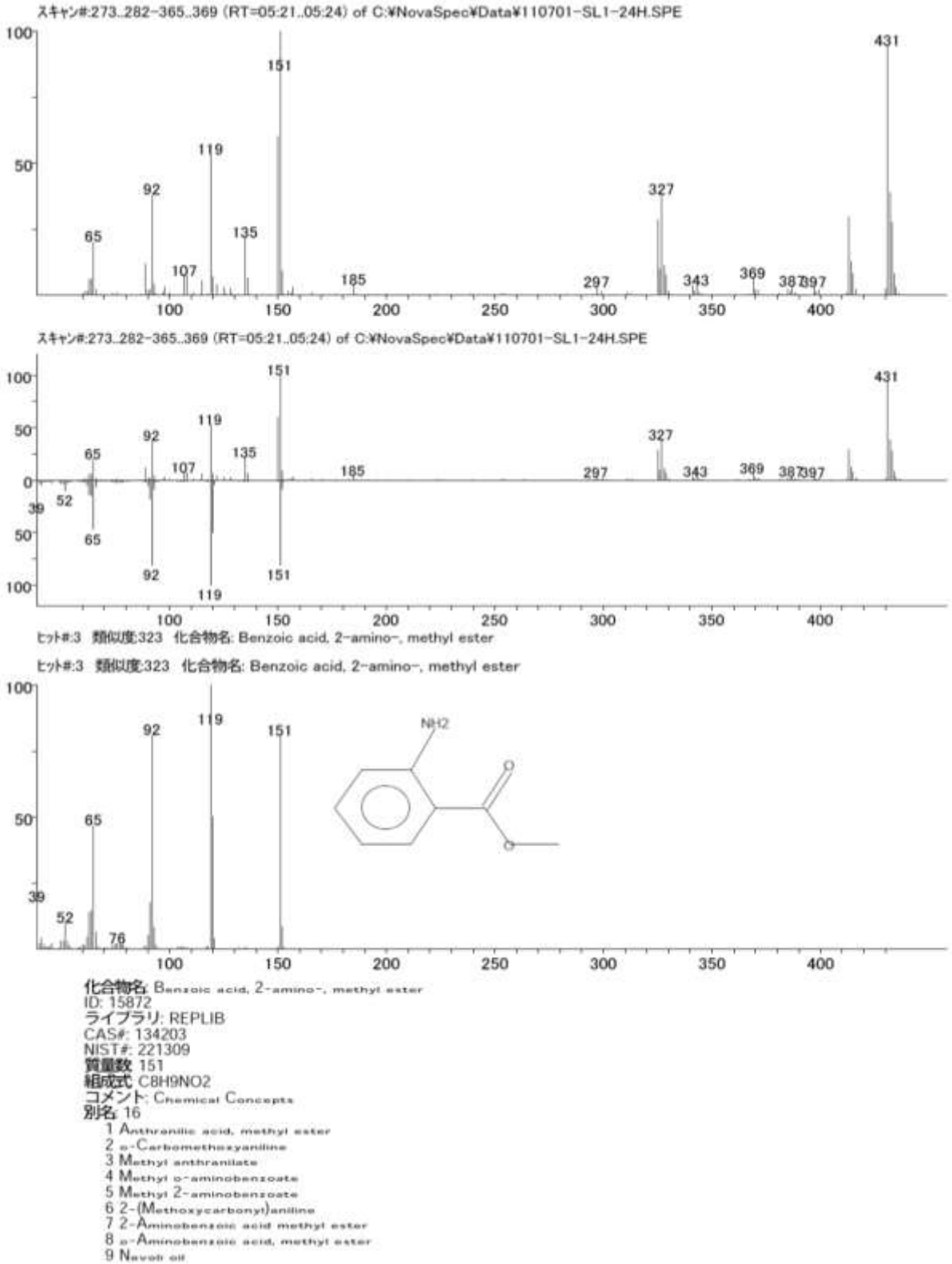

Figure 1d: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL1 after 24 hours of incubation.

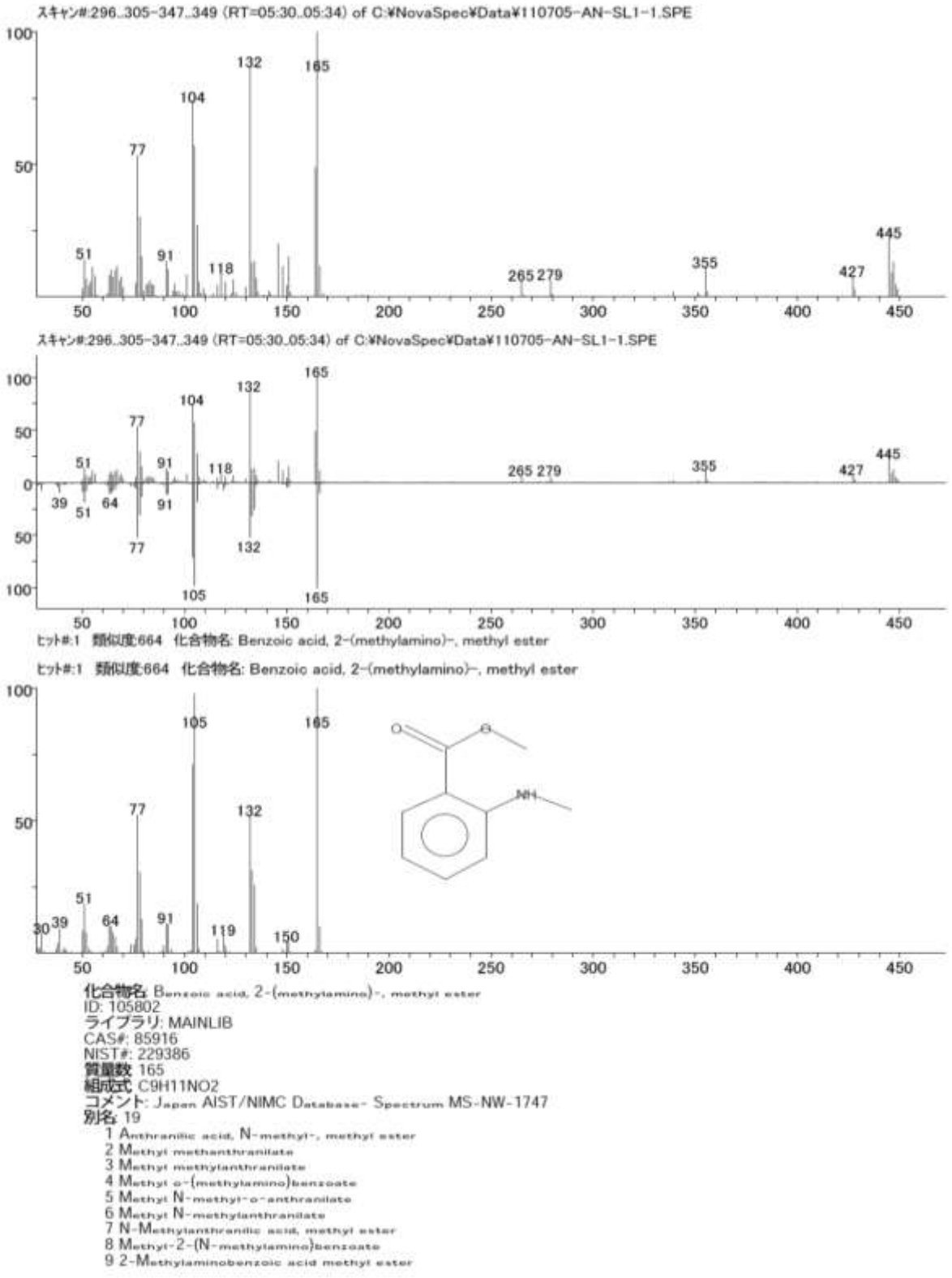

Figure 2: GC-MS mass spectra methylated AN (anthranilic acid) from ethyl acetate extract of growing cells of strain SL1 after 5 days of incubation.

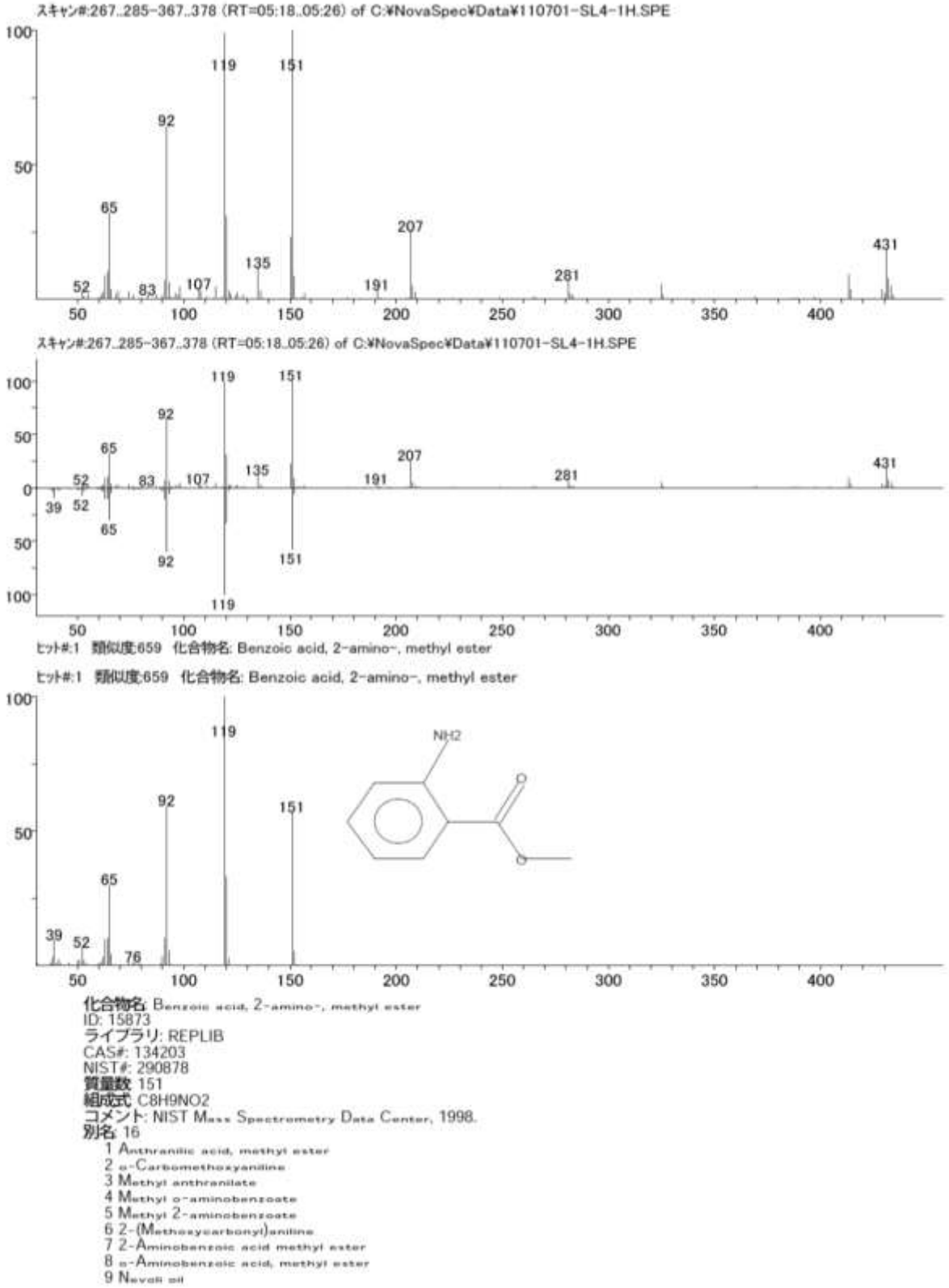

Figure 3a: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL4 after 1 hour of incubation.

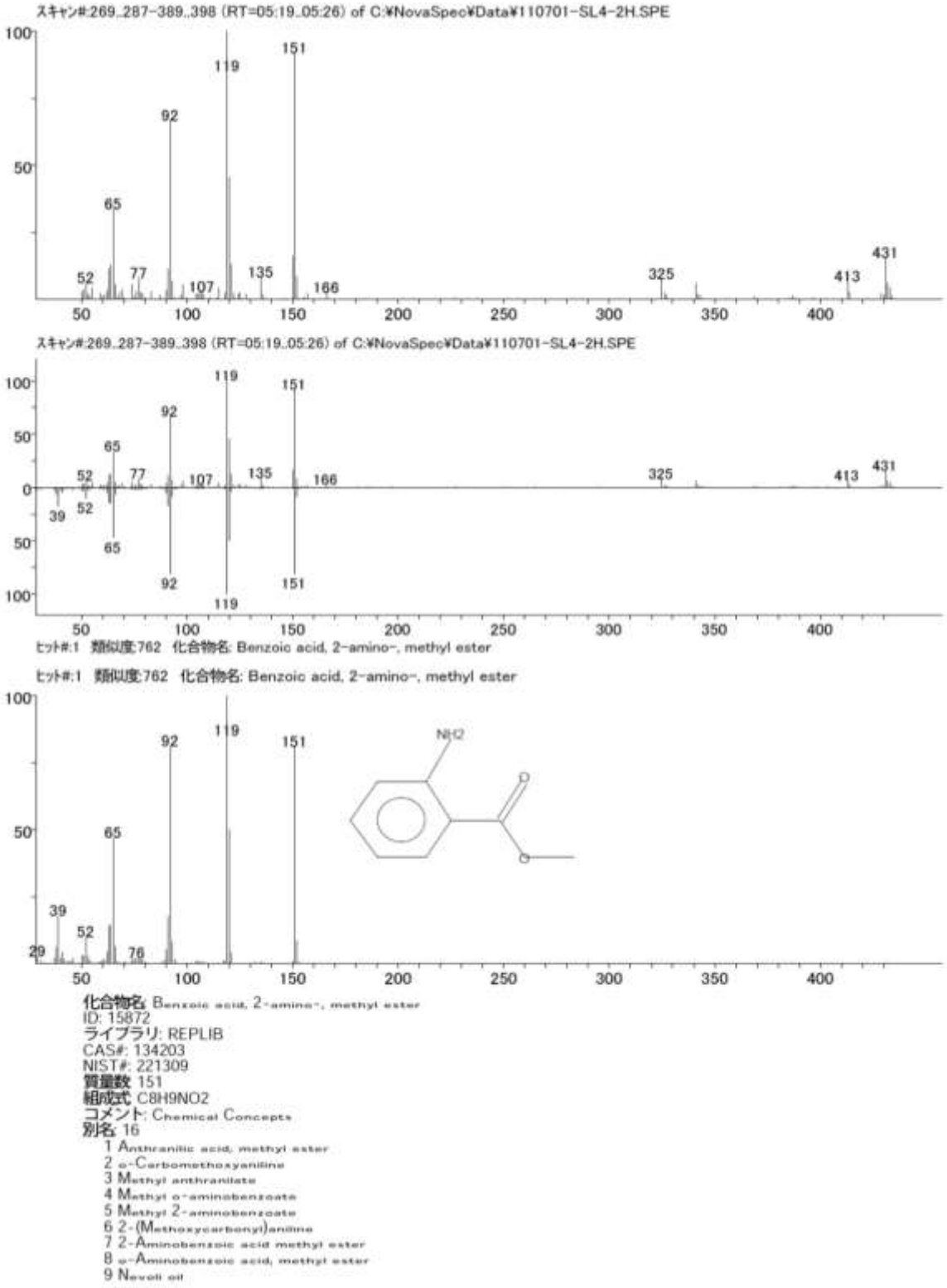

Figure 3b: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL4 after 2 hours of incubation.

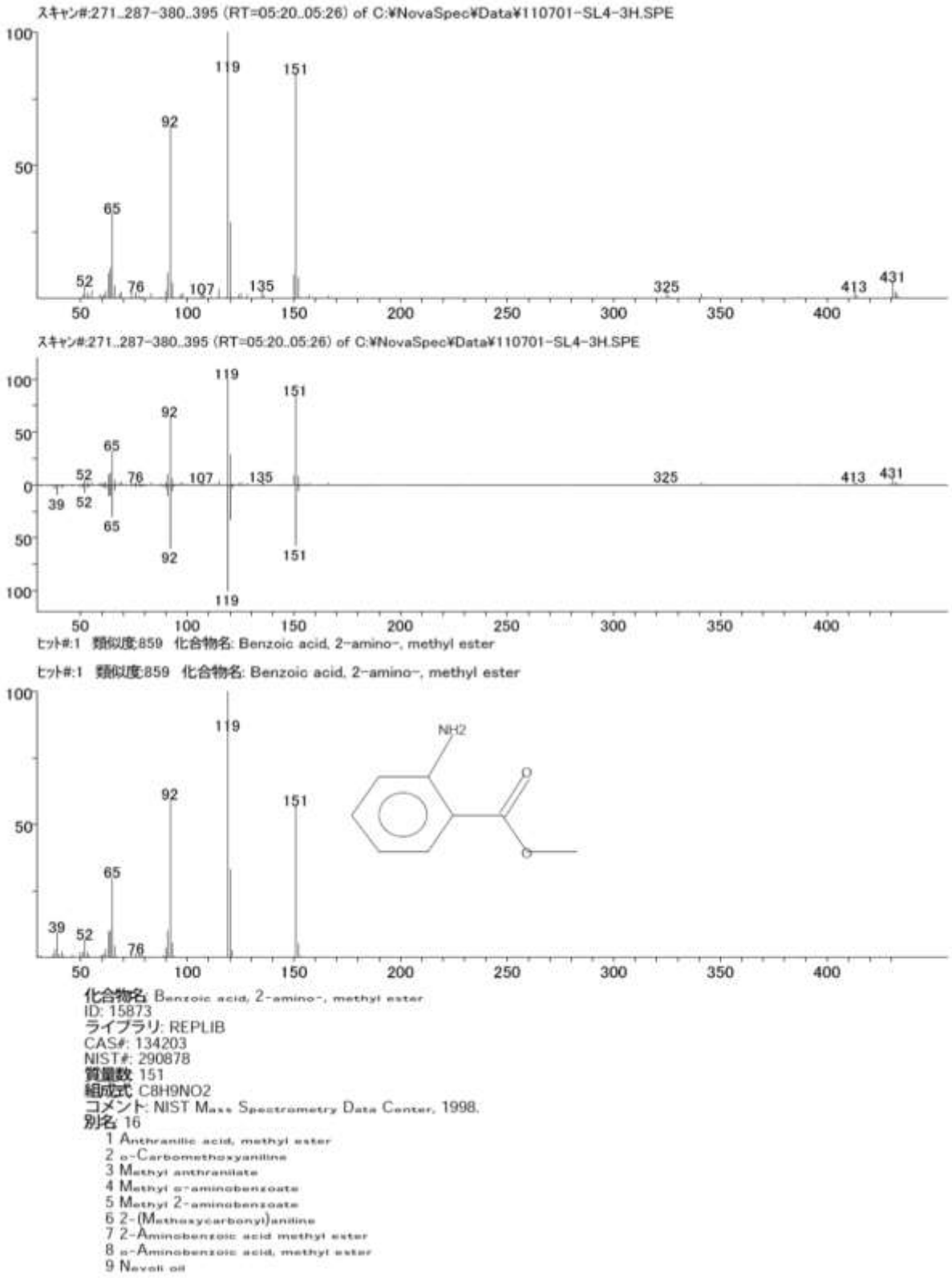

Figure 3c: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL4 after 3 hours of incubation.

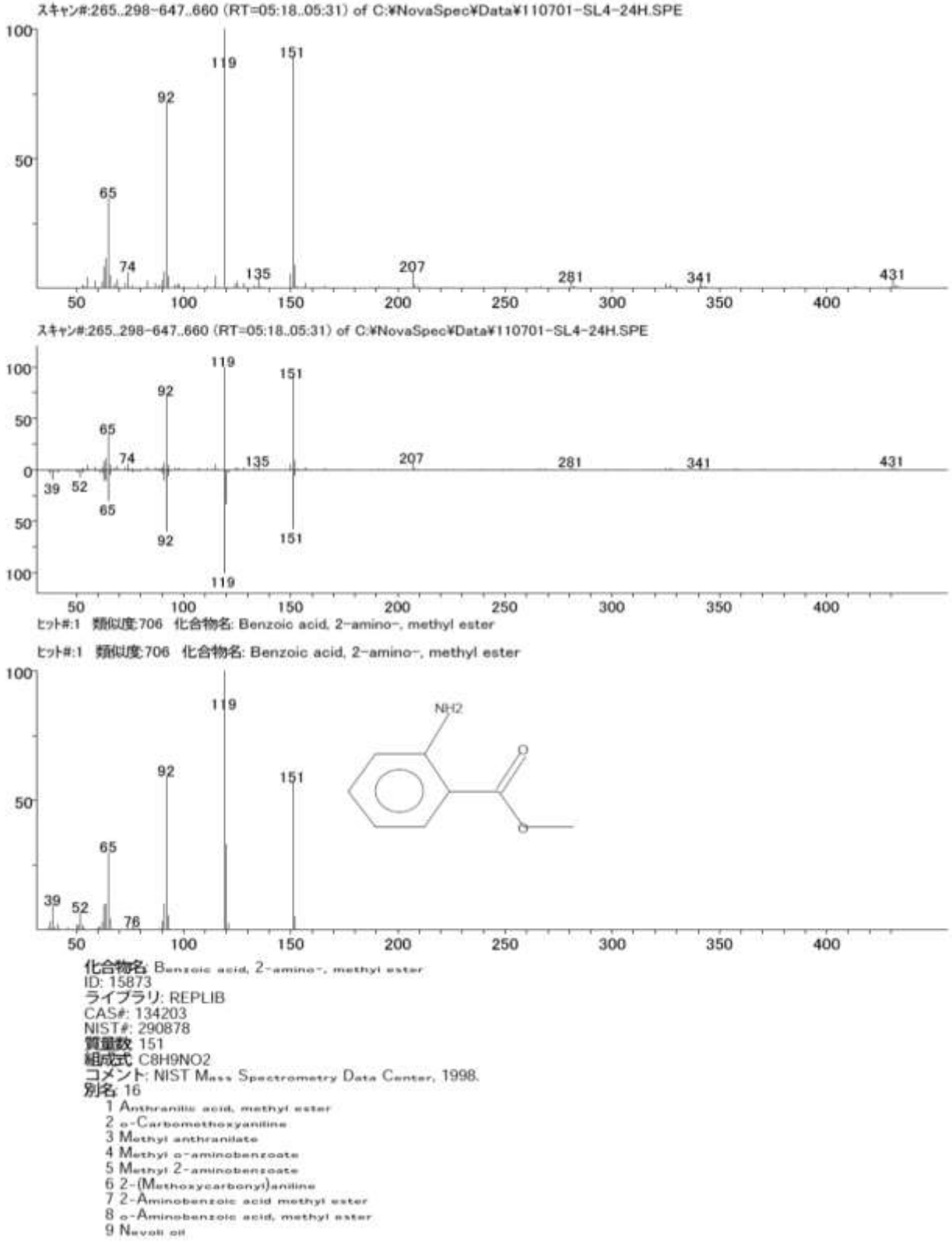

Figure 3d: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL4 after 24 hours of incubation.

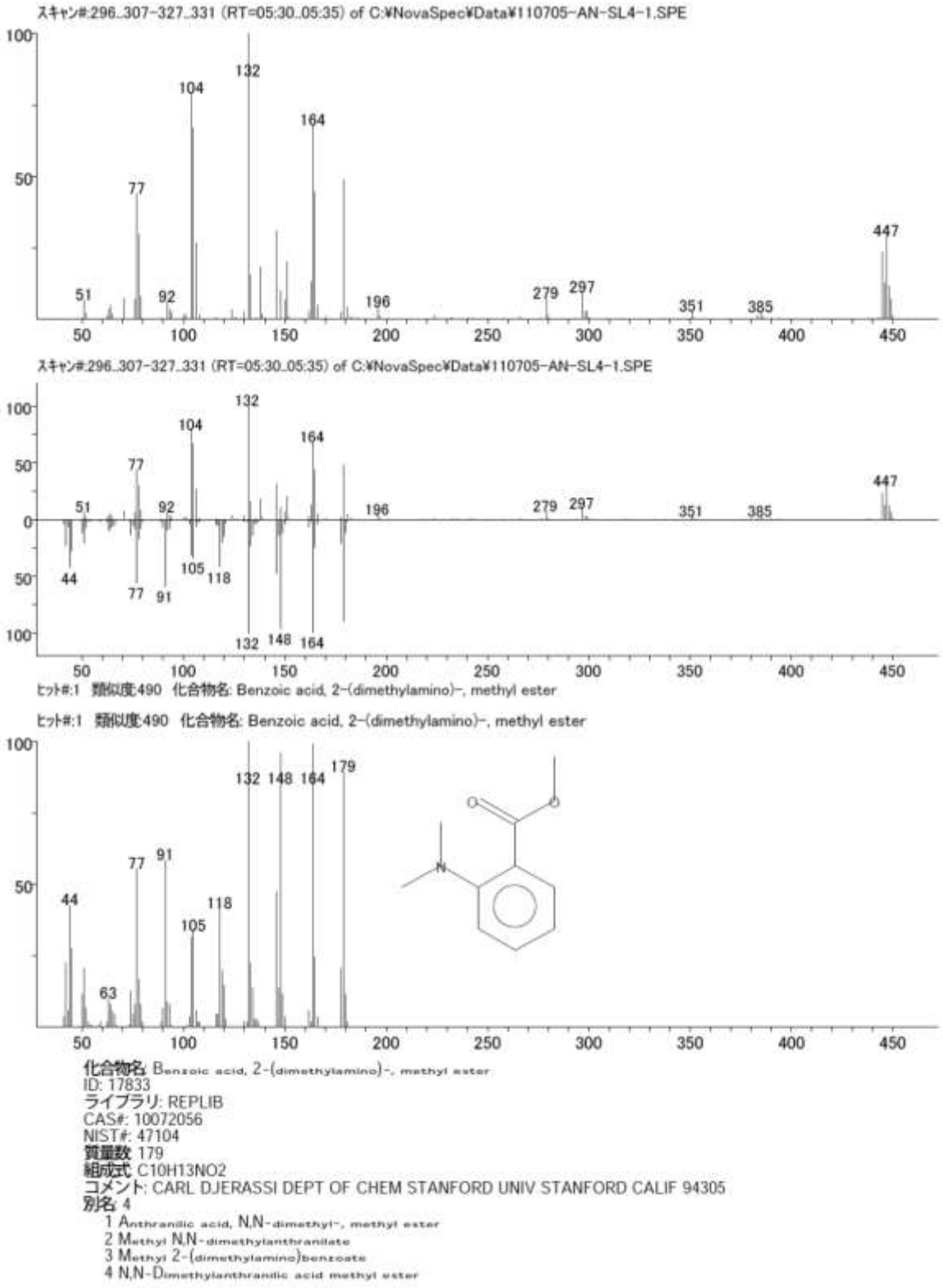

Figure 4: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of growing cells of strain SL4 after 5 days of incubation.

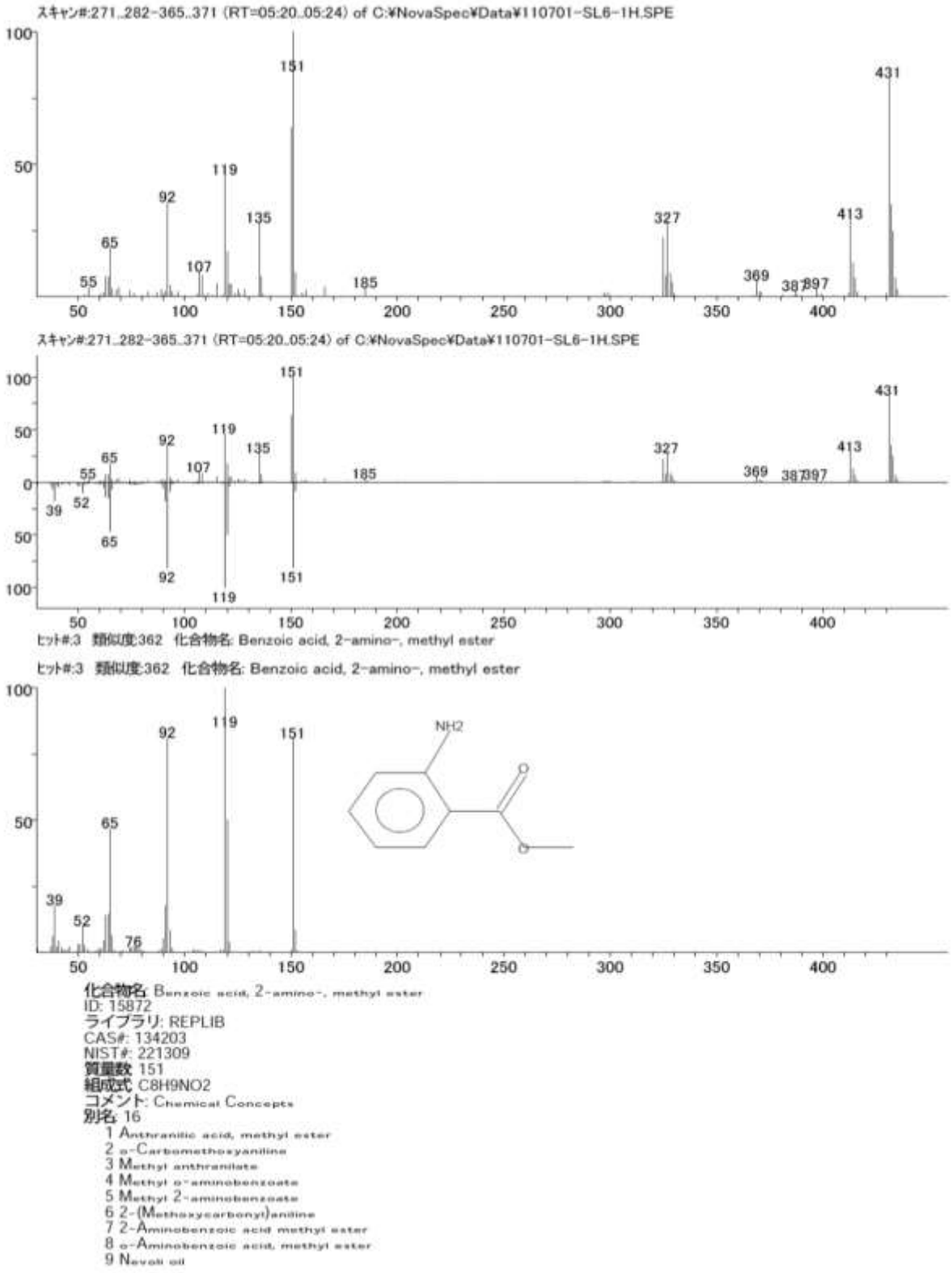

Figure 5a: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL6 after 1 hour of incubation.

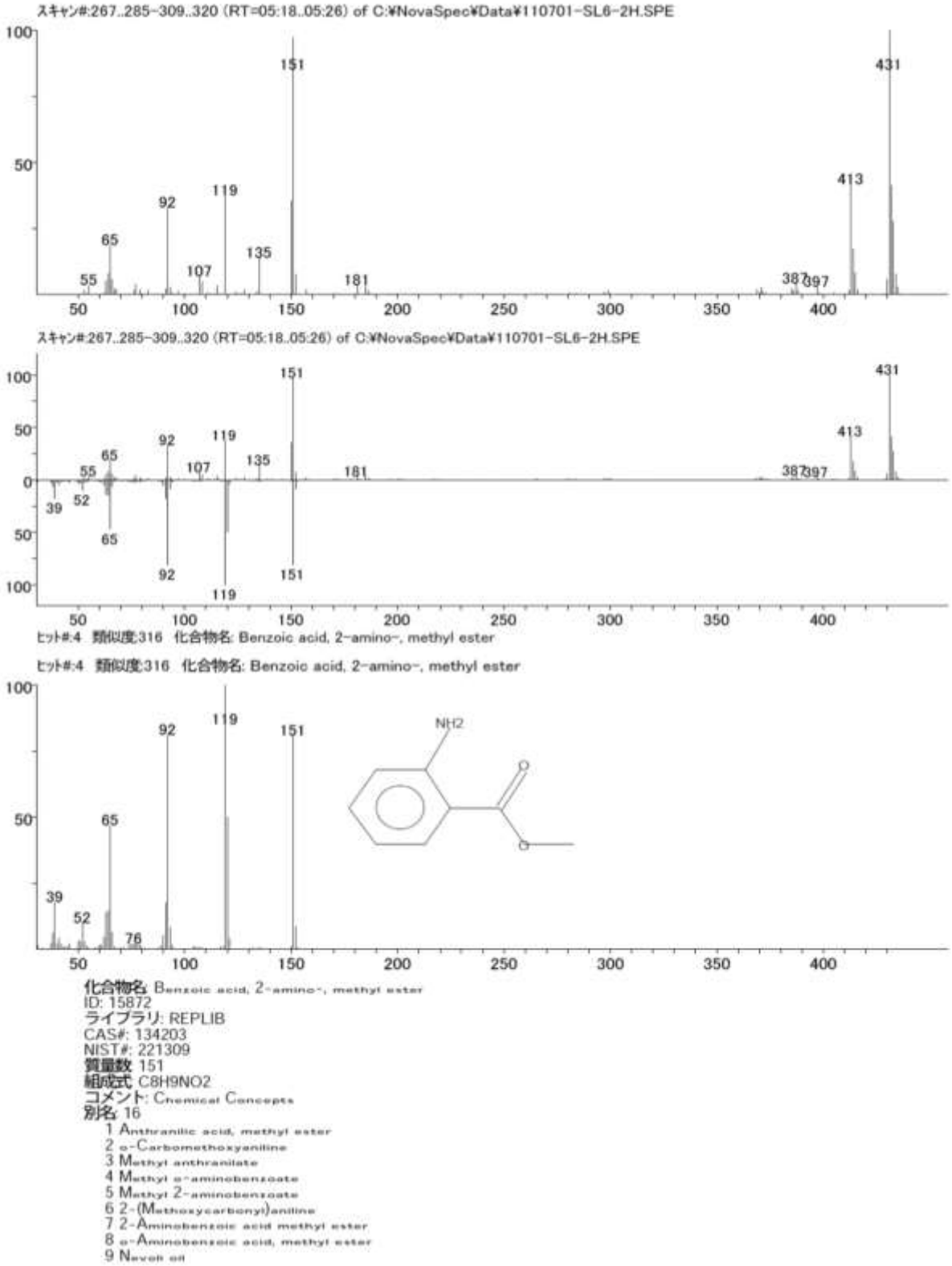

Figure 5b: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL6 after 2 hours of incubation.

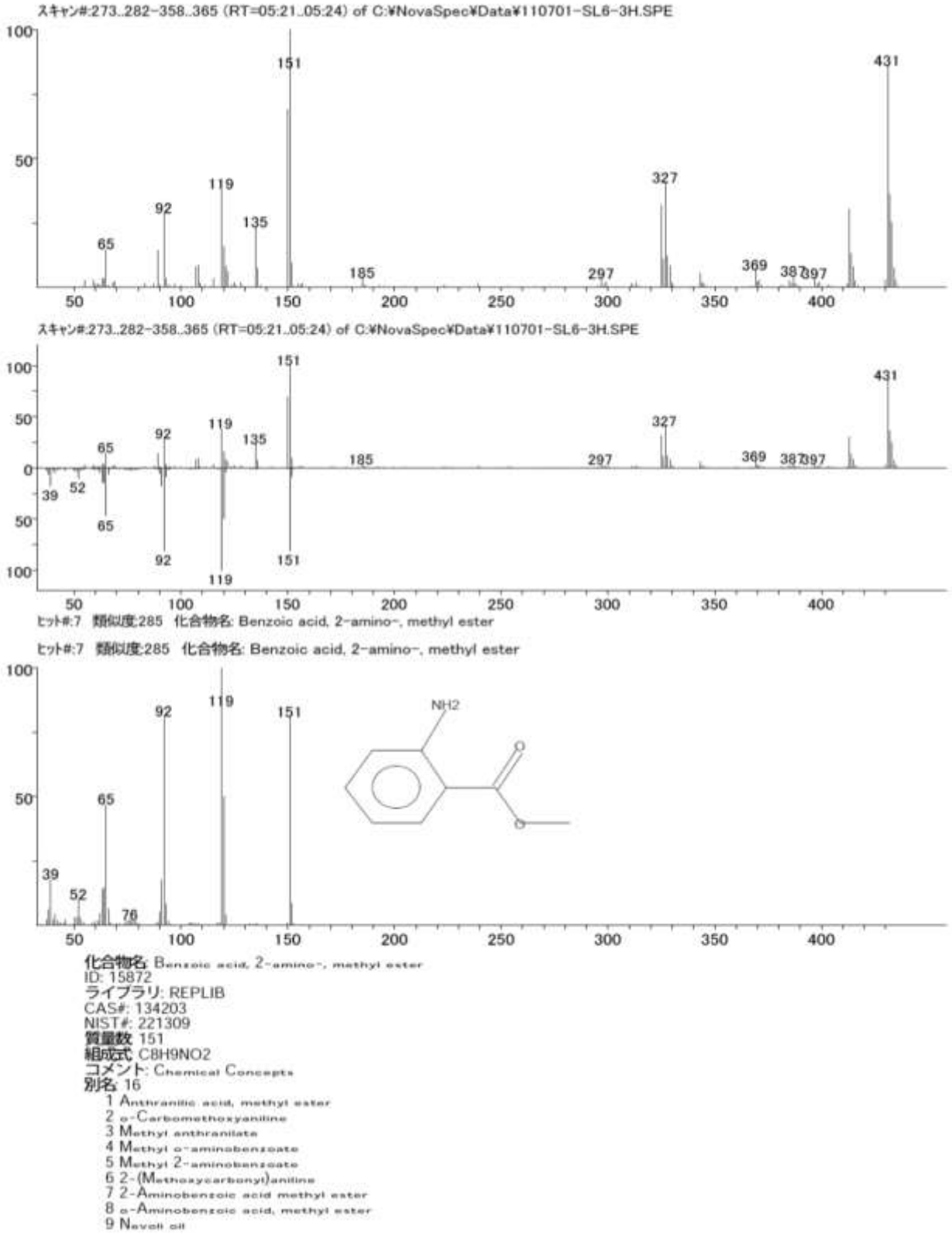

Figure 5c: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL6 after 3 hours of incubation.

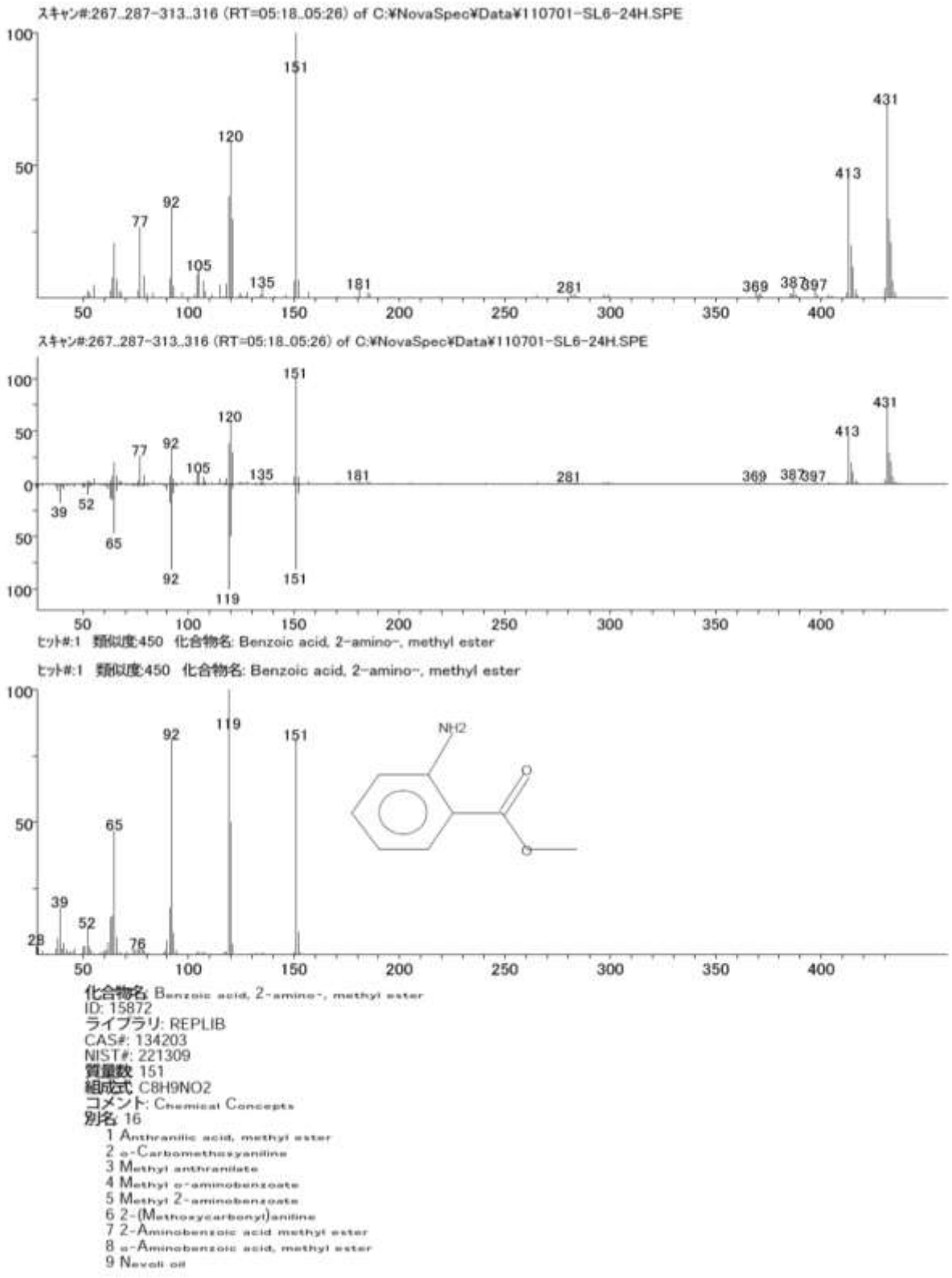

Figure 5d: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of resting cells of strain SL6 after 24 hours of incubation.

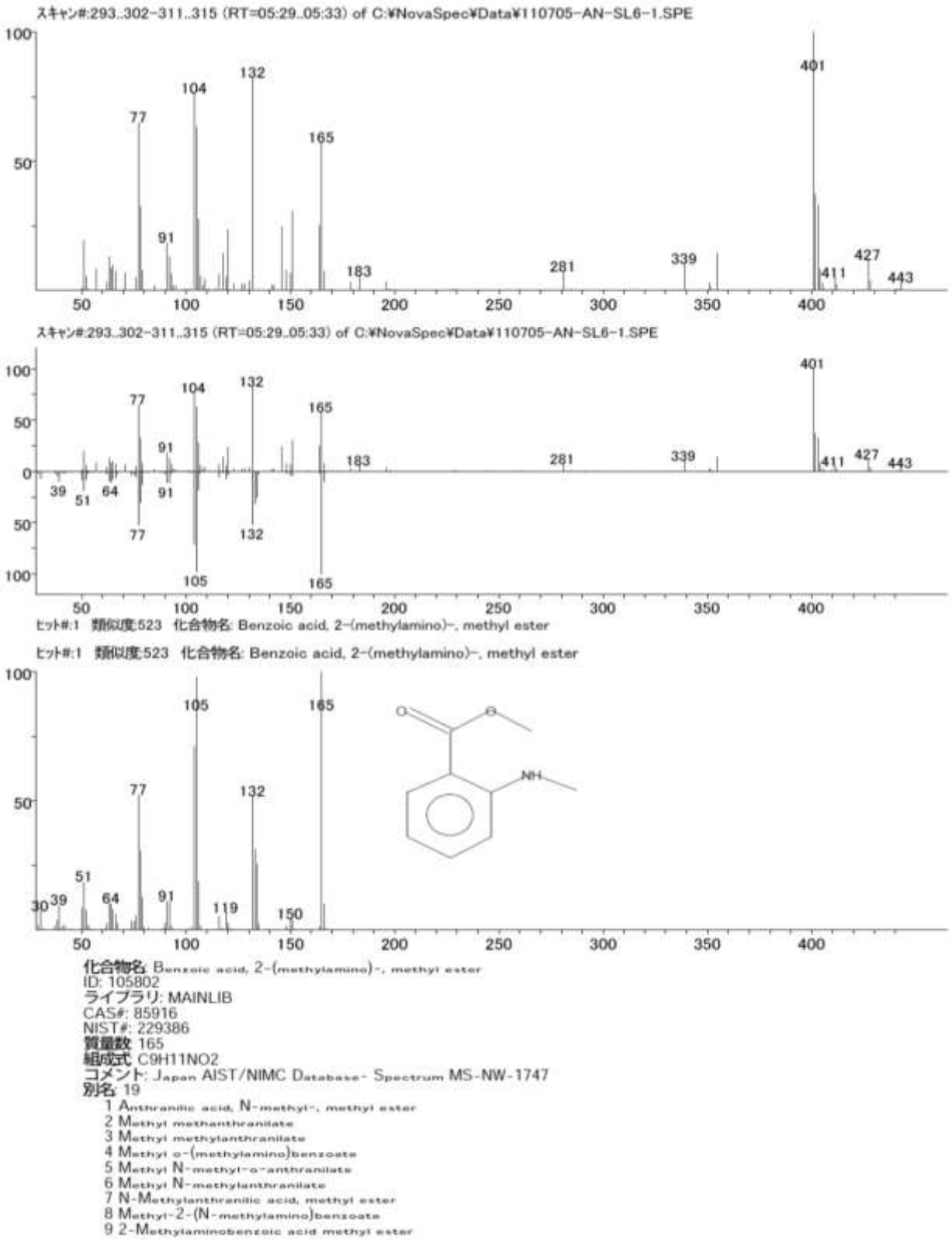

Figure 6: GC-MS Mass spectra of methylated AN (anthranilic acid) from ethyl acetate extract of growing cells of strain SL6 after 5 days of incubation.

APPENDIX IV

MWO CLONE LIBRARY

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>Unclassified_Gammaproteobacteria-03 clone SL-0015**(KF916710)**
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>Bellilinea sp. clone SL-0016**(KF916711)**
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>Hydrogenophaga sp. clone SL-0018**(KF916712)**
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>Unclassified_Deltaproteobacteria clone SL-0019**(KF916713)**
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>Bellilinea-02 sp. clone SL-0020**(KF916714)**
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>Gordonia sp. clone SL-0021**(KF916715)**
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>Ohtaekwangia-03 sp. clone SL-0022**(KF916716)**
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>TM7-03_genera_incertae_sedis clone SL-0023**(KF916717)**
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>Unclassified_Gammaproteobacteria-04 clone SL-0024**(KF916718)**
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>Unclassified_Firmicutes clone SL-0025**(KF916719)**
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>Unclassified_Bacteria-02 clone SL-0026**(KF916720)**
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>Ohtaekwangia-04 sp. clone SL-0027**(KF916721)**
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>Unclassified_Firmicutes-02 clone SL-0028**(KF916722)**
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>Peredibacter sp. clone SL-0029**(KF916723)**
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>Unclassified_Gammaproteobacteria-05 clone SL-0030**(KF916724)**
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>Aciditerrimonas sp. clone SL-0031**(KF916725)**
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>Ohtaekwangia-05 sp. clone SL-0032**(KF916726)**
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>Hyphomicrobium sp. clone SL-0033**(KF916727)**
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>Porphyrobacter sp. clone SL-0034**(KF916728)**
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>Bryobacter sp. clone SL-0035**(KF916729)**
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>Flavihumibacter sp. clone SL-0036**(KF916730)**
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>Bellilinea-03 sp. clone SL-0038**(KF916731)**
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>Desulfovibrio sp. clone SL-0039**(KF916732)**
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>unclassified_Gammaproteobacteria_incertae_sedis-03 clone SL-
0040**(KF916733)**
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>Tissierella sp. clone SL-0041**(KF916734)**
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>Unclassified_Betaproteobacteria clone SL-0042**(KF916735)**
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>Unclassified_Gammaproteobacteria-06 clone SL-0043**(KF916736)**
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>Unclassified_Bacteria-04 clone SL-0044**(KF916737)**
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>Unclassified_Gammaproteobacteria-07 clone SL-0045**(KF916738)**
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>Unclassified_Anaerolineaceae clone SL-0046**(KF916739)**
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>Haliscomenobacter sp. clone SL-0047**(KF916740)**
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>Unclassified_Firmicutes-03 clone SL-0048**(KF916741)**
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>Unclassified_Planctomycetaceae clone SL-0049**(KF916742)**
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>Crocinitomix sp. clone SL-0050**(KF916743)**
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>Unclassified_Gammaproteobacteria-08 clone SL-0051**(KF916744)**
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>Unclassified-Saprospiraceae clone SL-0052**(KF916745)**
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>Gp4 clone SL-0053**(KF916746)**
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>Unclassified_Gammaproteobacteria-09 clone SL-0055**(KF916747)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-04 clone SL-0056**(KF916748)**
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>Unclassified_Rhodocyclaceae sp. clone SL-0057**(KF916749)**
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>TM7-04_genera_incertae_sedis clone SL-0058**(KF916750)**
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>Haliscomenobacter-02 sp. clone SL-0059**(KF916751)**
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>Thauera sp. clone SL-0060**(KF916752)**
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>Thiobacter sp. clone SL-0061**(KF916753)**
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>Unclassified_Gammaproteobacteria-10 clone SL-0062**(KF916754)**
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>Lysobacter-02 sp. clone SL-0063**(KF916755)**
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>Unclassified_Rhodospirillaceae clone SL-0064**(KF916756)**
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>Thauera-02 sp. clone SL-0065**(KF916757)**
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0066**(KF916758)**
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>TM7-05_genera_incertae_sedis clone SL-0067**(KF916759)**
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>TM7-06_genera_incertae_sedis clone SL-0068**(KF916760)**
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>Unclassified_Deltaproteobacteria-02 clone SL-0073**(KF916765)**
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>Unclassified_Oceanospirillaceae clone SL-0074**(KF916766)**
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>Erythrobacter sp. clone SL-0075**(KF916767)**
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>Unclassified_Gammaproteobacteria-13 clone SL-0076**(KF916768)**
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>Unclassified_Burkholderiales clone SL-0077**(KF916769)**
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>Ohtaekwangia-06 sp. clone SL-0078**(KF916770)**
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>Pseudoxanthomonas sp. clone SL-0079**(KF916771)**
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>Aquicella sp. clone SL-0080**(KF916772)**
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>Subdivision3_genera_incertae_sedis clone SL-0082**(KF916773)**
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>Unclassified_Burkholderiales-02 clone SL-0083**(KF916774)**
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>Unclassified_Burkholderiales_incertae_sedis clone SL-0084**(KF916775)**
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>Unclassified_Deltaproteobacteria-03 clone SL-0086**(KF916776)**
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>Subdivision3_genera_incertae_sedis-02 clone SL-0087**(KF916777)**
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>Bellilinea-04 sp. clone SL-0089**(KF916778)**
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>TM7-07_genera-incertae_sedis clone SL-0090**(KF916779)**
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>Unclassified_ Rhodocyclaceae-02 clone SL-0091**(KF916780)**
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>Tissierella-02 sp. clone SL-0092**(KF916781)**
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>Sulfuricella sp. clone SL-0093**(KF916782)**
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>Unclassified_Oceanospirillaceae-02 clone SL-0095**(KF916783)**
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>Unclassified_Gammaproteobacteria-14 clone SL-0096**(KF916784)**
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>Denitratisoma-02 clone SL-0097**(KF916785)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-06 clone SL-0098**(KF916786)**
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>Aciditerrimonas-02 sp. clone SL-0100**(KF916787)**
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>Unclassified_Anaerolineaceae-02 clone SL-0101**(KF916788)**
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CGGA
>Sphingomonas sp. clone SL-0102**(KF916789)**
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>Cellulomonas sp. clone SL-0104**(KF916790)**
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>Ferruginibacter sp. clone SL-0105**(KF916791)**
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>Flavobacterium sp. clone SL-0106**(KF916792)**
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CTCC
>TM7-08_genera_incertae_sedis clone SL-0109**(KF916793)**
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>Unclassified_Anaerolineaceae-03 clone SL-0111**(KF916794)**
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>Turneriella-02 sp. clone SL-0112**(KF916795)**
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>Unclassified_Proteobacteria clone SL-0113**(KF916796)**
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>Unclassified_Deltaproteobacteria-04 clone SL-0114**(KF916797)**
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>Ohtaekwangia-07 sp. clone SL-0115**(KF916798)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-07 clone SL-
0116**(KF916799)**
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>Gp7 clone SL-0117**(KF916800)**
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>Unclassified_Gammaproteobacteria-15 clone SL-0118**(KF916801)**
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>TM7-09_genera_incertae_sedis clone SL-0119**(KF916802)**
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>Acinetobacter sp. clone SL-0120**(KF916803)**
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>TM7-10_genera_incertae_sedis clone SL-0121**(KF916804)**
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>Rhodobacter sp. clone SL-0122**(KF916805)**
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>Unclassified_Anaerolineaceae-04 clone SL-0123**(KF916806)**
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>unclassified_Sphingobacteriales clone SL-0124**(KF916807)**
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GGGCAGCTACTGCGCGAGCAGATGCCAATCTCAAAAACCGTCCTCAGTTC
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>Peredibacter-02 sp. clone SL-0125**(KF916808)**
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TCG
>Ignavibacterium sp. clone SL-0126**(KF916809)**
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>Desulfobacca sp. clone SL-0127**(KF916810)**
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>Unclassified_Sphingobacteriales-02 clone SL-0128**(KF916811)**
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>Unclassified_Anaerolineaceae-05 clone SL-0130**(KF916812)**
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>Unclassified_Gammaproteobacteria-16 clone SL-0131**(KF916813)**
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AGTC
>Anaerolinea sp. clone SL-0132**(KF916814)**
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>Bellilinea-05 sp. clone SL-0134**(KF916815)**
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>unclassified_Sphingobacteriales-03 clone SL-0135**(KF916816)**
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>Bellilinea-06 sp. clone SL-0136**(KF916817)**

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>Unclassified_Gammaproteobacteria-17 clone SL-0137**(KF916818)**
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>Dehalogenimonas sp. clone SL-0138**(KF916819)**
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>Unclassified_Deltaproteobacteria-05 clone SL-0139**(KF916820)**
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>Gp4-02 clone SL-0140**(KF916821)**
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>Gp7-02 clone SL-0141**(KF916822)**
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>Unclassified_Gammaproteobacteria-18 clone SL-0142**(KF916823)**
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CGACTCC
>Peredibacter-03 sp. clone SL-0143**(KF916824)**
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>Unclassified_Alphaproteobacteria-02 clone SL-0144**(KF916825)**
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>TM7-11-genera_incertae_sedis clone SL-0145**(KF916826)**
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>Unclassified_Deltaproteobacteria-06 clone SL-0146**(KF916827)**
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>Unclassified_Rhodospirillaceae-02 clone SL-0147**(KF916828)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-08 clone SL-
0148**(KF916829)**
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CGCCAACCCGCGAGGGGGAGCTAATCTCACAAAGCCGTTCGTAGTCCGGA
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>Terrimonas-02 sp. clone SL-0149**(KF916830)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-09 clone SL-
0150**(KF916831)**
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>Haliscomenobacter-03 sp. clone SL-0151**(KF916832)**
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>Desulfuromonas-02 sp. clone SL-0152**(KF916833)**
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>Bellilinea-07 sp. clone SL-0153**(KF916834)**
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>Unclassified_Anaerolineaceae-06 clone SL-0154**(KF916835)**
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>Gp4-03 clone SL-0155**(KF916836)**
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>Caldilinea sp. clone SL-0156**(KF916837)**
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>Unclassified_Anaerolineaceae-07 clone SL-0157**(KF916838)**
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>Sinobacter sp. clone SL-0158**(KF916839)**
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>TM7-12_genera_incertae_sedis clone SL-0159**(KF916840)**
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>TM7-13_genera_incertae_sedis clone SL-0160**(KF916841)**
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>Unclassified_Burkholderiales-03 clone SL-0161**(KF916842)**
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>Unclassified_Hydrogenophilaceae clone SL-0162**(KF916843)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-10 clone SL-0163**(KF916844)**
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>Anaerolinea-02 sp. clone SL-0164**(KF916845)**
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>Unclassified_Betaproteobacteria-02 clone SL-0165**(KF916846)**
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>Steroidobacter sp. clone SL-0167**(KF916847)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-11 clone SL-
0168**(KF916848)**

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>Unclassified_Anaerolineaceae-08 clone SL-0169**(KF916849)**
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>Ferruginibacter-02 sp. clone SL-0170**(KF916850)**
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TCG
>Dongia sp. clone SL-0173**(KF916853)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-12 clone SL-
0174**(KF916854)**
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>Dongia-02 sp. clone SL-0176**(KF916856)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-13 clone SL-
0177**(KF916857)**
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>Unclassified_Thiotrichales clone SL-0179**(KF916859)**
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>Unclassified_Sphingobacteriales-04 clone SL-0180**(KF916860)**
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>Unclassified_Gammaproteobacteria-20 clone SL-0182**(KF916861)**
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>Unclassified_Gammaproteobacteria-21 clone SL-0183**(KF916862)**
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>Subdivision3_genera_incertae_sedis-03 clone SL-0185**(KF916863)**
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GAAG
>Ohtaekwangia-08 sp. clone SL-0186**(KF916864)**
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>Prosthecobacter sp. clone SL-0187**(KF916865)**
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CCAGTACAATATGAACCGAGACCGCGAGGTGGAGGAAATCAATAAAACTG
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>Flavisolibacter sp. clone SL-0188**(KF916866)**
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>Subdivision3_genera_incertae_sedis-04 clone SL-0189**(KF916867)**
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>Subdivision3_genera_incertae_sedis-05 clone SL-0190**(KF916868)**
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>Fangia sp. clone SL-0191**(KF916869)**
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>Altererythrobacter sp. clone SL-0192**(KF916870)**
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>Unclassified_Gammaproteobacteria-22 clone SL-0193**(KF916871)**
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>TM7-14_genera_incertae_sedis clone SL-0194**(KF916872)**
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>TM7-15_genera-incertae_sedis clone SL-0195**(KF916873)**
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>Unclassified_Gammaproteobacteria-23 clone SL-0196**(KF916874)**

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>Gp4-04 clone SL-0197**(KF916875)**

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>Ohtaekwangia-09 sp. clone SL-0198**(KF916876)**

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>Hyphomicrobium-02 sp. clone SL-0199**(KF916877)**

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AGAGGTGAGTGGAATTCCTAGTGTAGAGGTGAAATTCGTAGATATTAGGA
AGAACACCAGTGGCGAAGGCGGCTCACTGGTCCGGTACTGACGCTGAGGT
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGGATGCTAGCCGTCGGCAAGCTTGCTTGTCGGTGGCGCAGCT
AACGCATTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
ACGCAACGCGAAGAACCTTACCAGCCCTTGACATGTCCGGACGGTTTCCA
GAGATGGATTCCTCCTAGCAATAGGTCGGAACACAGGTGCTGCATGGCTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTCGCCATTAGTTGCCATCATTCAGTTGGGCACTCTAGTGGGACTGCC
GGTGATAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTT
ACGGGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGCAGCCAC
TCAGCGATGAGGAGCTAATCCCAAAAAGCCGTCTCAGTTCGGATTGAGCT
CTGCAACTCGAGCTC

>Aquimonas sp. clone SL-0200**(KF916878)**

ATGACGGTACCGTAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGC
GGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGT
GCGTAGGCGGTTGGCTAAGTCAGATGTGAAAGCCCTGGGCTCAACCTGGG
AATGGCATTTGAAACTGGCTGGCTAGAGTGCGGTAGAGGATGGCGGAATT
CCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAACATCCGTGGCGA
AGGCGGCCATCTGGACCAGCACTGACGCTGAGGCACGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTG
GATGTTGGGCTCAACTCGGAGCTCAGTGTCGAAGCTAACGCGTTAAGTTC
GCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGCAGA
ACCTTACCTGGTCTTGACATGTCGCGAACCCTGCAGAGATGCGGGGGTGC
CTTCGGGAACGCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTG
CCAGCACGTTATGGTGGGAACTCTAAGGAGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTAC
ACACGTACTACAATGGTCGGTACAGAGGGTTGCGAGACCGCGAGGTGGAG
CCAATCCCAGAAAACCGATCCCAGTCCGGATTGGAGTCTGCAACTCGACT
CC

>Unclassified_Bacteria-06 clone SL-0201**(KF916879)**

GCGTTGTTCGGGATTACTGGGCGTAAAGCGCGCGCAGGCGGGTCCTGTAA
GTCGGAAGTGAAATTTCACGGCTCAACCGTGAAGCTGCTTCTGATACTGC
GGATCTGGAGATCGGTAGAGGTCGGTAGAATTACAGGTGTAGCGGTGGAA
TGCGTAGATATCTGTAAGAATACCCGTGGCGAAGGCGGCCGACTGGGCCG
AATCTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGGGCACTAGGTGCCGGGGGGAGCGAC
CCCTTCGGTGCCGCAGCTAACGCGATAAGTGCCCCGCCTGGGGAGTACGG
CCGCGAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGGTTTGAC
ATGCAGATGAAAGCTTCTGGAAACAGGGGCCCTTCTTCGGAACATTTGCA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTGCCCCGTGTTACTAACAGGTAAAGCTGA
GGACTCTCGGGGGACTGCCGGCGTCAAGCCGGAGGAAGGTGGGGACGACG
TCAAGTCATCATGGCCCTTACGCCTAGGGCGACACACGTGCTACAATGGC
CAGGACAGAGGGCTGCGAAGCGGCAACGTGGAGCGAATCCCAGAAACCTG
GTCCAAGTTCGGATTGTGGGCTGAAACTCGCCCACAGAAGCCGGAATCG

>Longilinea sp. clone SL-0203**(KF916880)**

GCTAACTACGTGCCAGCAGCCGCGGTAAAACGTAGGAGGCGAGCGTTATC
CGGATTTACTGGGTGTAAAGCGCGTGCAGGCGGACGGGGAAGTGGTGCGT
GAAAGCGCCCGGCTCAACCGGGCGAGGCCGTGCCAAACTGCCCGGCTGGA
GGCAGGTAGAGGCGTGTGGAATTCCGGGTGTAGTGGTGAAATGCGTAGAG
ATCCGGAGGAACACCAGTGGCGAAGGCGACACGCTGGGCCTGACCTGACG

CTCAGACGCGAAAGCATGGGGAGCGAACGGGATTAGAAACCCCGGTAGTC
CATGCTGTAAACGATGTCGACTAGGTGTGGGGGTGTAACAGCCTCTGTGC
CGCAGCCAACGTGATAAGTCGACCACCTGGGGACTACGGCCGCAAGGTTA
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCGTGTGGTT
TAATTCGAGGCTACACGAAGAACCTTACCTGGGCTTGACATCACGGTGGT
AGCGACCCGAGAGGGGAGCGACCTTCGGGAGCCGTGACAGGTGCCGCATG
GCTGTCGTCAGCTCGTGTCGTGAGATGTCCGGTTAAGTCCGGTAACGAGC
GCAACCCTCGCCGTCAGTTATAGGTTGTCTGACGGGACTGCCCGTTGAAC
GCGGGAGGAAGGTGGGGATGACGTCAAGTCAGCATGGCCCTTATGTCCAG
GGCTACACACACGCTACAATGGCCGGTACAATGGGTCGCCAACCCGCGAG
GGGGAGCCAATCCACCAAAGCCGGTCTCAGTTCGGATTGCAGGCTGTAAC
CCGCCTGCATGAAGTCGGA
>Gp3-02 clone SL-0204**(KF916881)**
AAGTCTGGTGTGAAATCTCCCGGCTTAACTGGGAGGGTGCGCCGGAAACT
GGGTTGCTGGAGTGTGGGAGAGGCAAGCGGAATTCCTGGTGTAGCGGTGA
AATGCGTAGATATCAGGAGGAACACCTGCGGTGTAGACGGATTGCTGGAC
CATGACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCCTAAACGATGCATACTTGGTGTGGGCAGTTCAT
TCTGTCTGTGCCGGAGCTAACGCGTTAAGTATGCCGCCTGGGGAGTACGG
TCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGGGCTCGAA
CGGCTGCAGACACCTTCTGGAAACAGAGGGATTCCCGCAAGGGACTGTAG
TCGAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCAACCCGCAAGGGGC
ACTCTCAGGAGACCGCCAGCGATAAGTTGGAGGAAGGTGGGGATGACGTC
AAGTCATCATGGCCTTTATGTCCAGGGCTACACACGTGCTACAATGGGCG
GTACAAAC
>Bellilinea-08 sp. clone SL-0205**(KF916882)**
GCTAACTACGTGCCAGCAGCCGCGGTAACACGTAGGAGACTAGCGTTATT
CGGATTTACTGGGCGTAAAGCGCGTGCAGGCGGTTCGGTAAGTTGGATGT
GAAAGCTCCCGGCTTAACTGGGAGAGGTCGTTCAATACTGTCGAACTTGA
GAGTGGTAGAGGGAGGTGGAATTCCGGGTGTAGTGGTAAAATGCGTAGAT
ATCCGGAAGAACACCAGTGGCGAAAGCGGCCTCCTGGACCATTTCTGACG
CTCAGACGCGAAAGCTAGGGTAGCGAACGGGATTAGAGACCCCGGTAGTC
CTAGCCGTAAACGATGTAGACTTGGCGTTGGAGGGGTTAAATCCTTCAGT
GCCGAAGCTAACGCGATAAGTCTACCGCCTGGGGACTACGGCCGCAAGGT
TAAAACTCAAAGGAATTGACGGGGCCCCGCACAAGCAGCGGAGCGTGTGG
TTTAATTCGATGATACACGAAGAACCTTACCAGGGTTTGACATGCTGGTA
GTAGGGATCCGAAAGGTGACCGACCCCTCGGGGAGCCAGCACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTCGGTTAAGTCCGCTAAC
GAGCGCAACCCTTGCTGTGTGTTACATGTGTCACACGGGACTGCCGGTCT
TAAACCGGAGGAAGGTGGGGATGATGTCAAGTCCGCATGGCCTTTATATC
CTGGGCTACACACACGCTACAATGGCCGGTACAATAGGTTGCGAAGCCGC
AAGGCGGAGCCAATCCTCAAAGCTGGCCTCAGTTCAGATTGCAGGCTGCA
ACCCGCCTGC
>Unclassified_Alphaproteobacteria-03 clone SL-0206**(KF916883)**
GCGGTGGAGCATGTTCTTTAATTCGAAGCAACGCGAAGAACCTTACCTAC
GCTTGTATCCTGATCGCGACTTTCAGAGATGAGAGTCTTCAGTTCGGCTG
GATCAGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTATGTTGCCATCAGG
TAATGCTGGGCACTCATACGAGACTGCCGGTGATAAGCCGGAGGAAGGCG
GGGACGACGTCAAGTCATCATGGCCCTTACGCGTAGGGCTAGAAACGTGC
TACAATGGCAATGACAGTGGGCAGCAACACGGCAACGTGAAGCTAATCTC
CAAAAGTTGTCTCAGTTCAGATTGTCCTCTGCAACTCGAGGGCATGAAGC
TGGAATCG
>Gp7-03 clone SL-0207**(KF916884)**
GTGCCAGCAGCCGCGGTAATACAGAGGGGGCAAGCGTTATTCGGAATTAT

TGGGCGTAAAGGGCGCGTAGGCGGCTTTTCAAGTGGCGGGTGAAATCCCT
CGGCTTAACCGGGGAACTGCCTGCCAGACTGGATTGCTTGAGGCCGGGAG
AGGTGAGTGGAATTCCCAGTGTAGCGGTGAAATGCGTAGATATTGGGAGG
AACACCAGTGGCGAAGGCGGCTCACTGGACCGGTACTGACGCTGAGGCGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGAGTGCTTGGTGTAGCGGGTATCGACCCCTGCTGTGCCGAAGTC
AACACATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
AAGCAACGCGCAGAACCTTACCTGGGTTTGAACTGCACAGGAAAGTCTCA
GAGATGAGATCCCCTCTTCGGAGGTCTGTGTAGAGGTGCTGCATGGCTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTGTTCACAGTTACTAACGCGTAATGGCGAGAACTCTGTGGAGACTGC
CGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCTT
TATGTCCAGGGCTACACACGTGCTACAATGGACGGTACAGAGAGTCGCGA
GACCGCGAGGTGGAGCTAATCTCAAAAAGCCGTTCTCAGTTCGGATTGCA
CTCTGCAACTCGAGTGCATGAAG
>Unclassified_Gammaproteobacteria-24 clone SL-0208**(KF916885)**
GGAGTGACGTTACCCACAGAATAAGCACCGGCAAACTCCGTGCCAGCAGC
CGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAG
CGCACGTAGGTGGTTCGTTAAGTCGGATGTGAAATCCCTGGGCTCAACCT
GGGAGCTGCATTCGATACTGGCGGACTTGAGTACGAGAGAGGGGGGTGGA
ATTCCAGGTGTAGCGGTGAAATGCGTAGATATCTGGAGGAACACCAGTGG
CGAAGGCGGCCCCCTGGCTCGATACTGACACTGAGGTGCGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA
CTAGCCGTTGGGGAACTTGATTTCCCAGTGGCGTAGCTAACGCGCTAAGT
CGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACG
GGGACCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGACGCAACGCGAA
GAACCTTACCTGCTCTTGACATCCTGCGAACTTTCCAGAGATGGAAGGGT
GCCTTCGGGAGCGCAGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGT
TGCCAGCATTTAGTTGGGGACTCTAGGGAGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGCAGGGCTAC
ACACGTACTACAATGGCCGGTACAAAGGGTTGCGAAAGCGCGAGCTGGAG
CCAATCCCAAAAAGCTGGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT
CCATGAAGTCGGAATCG
>Bdellovibrio sp. clone SL-0209**(KF916886)**
AAGAAAGGATCGGCTAACTTCGTGCCAGCAGCCGCGGTAAGACGAGGGAT
CCAAGCGTTGTTCGGAATCATTGGGCGTAAAGCGGGTGTAGACGGCTTTG
TAAGTCAGGTGTGAAAGCCCAGGGCTCAACCCTGGAAGTGCATTTGATAC
TGCGAAGCTTGAGTGTGGGAGAGGCTAGTAGAATTCCTGGTGTAGTGGTG
AAATACGTAGATATCAGGAGGAATACCGGTGGCGAAGGCGGCTAGCTGGC
CCAACACTGACGTTGAGACCCGAAAGCGTGGGGATCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCATAAACGATGGATACTTGTTGTTGGAGGTATT
GACCCCTTCAGTGACGAAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTA
CGGTCGCAAGATTAAAACTCAAAGAAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTAGGCTT
GACATGTACTGGAAGAGTGGCAGAAATGTCCTCGCCCGCAAGGGTCGGTA
CACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCCTGCCTTTAGTTGCCAGCATTTAGTTGG
GCACTCTAGAGGGACTGCCGACGTTAAGTCGGAGGAAGGTGGGGATGACG
TCAAGTCCTCATGGCCCTTATGTCTAGGGCTACACACGTGCTACAATGGG
GCGTACAGACGGATGCATAACCGCGAGGTGAAGCCAATCCTACAAAACGC
CTCTAAGTTCAGATTGCAGTCTGCAACTCGACTGCATGAAG
>Sulfuricella-02 sp. clone SL-0210**(KF916887)**
CGGTTGCGGCTAATACCCGCGACTAATGACGGTACCTGCAGAAGAAGCAC
CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTCGTAAGTCAGAT

GTGAAAGCCCCGGGCTTAACCTGGGAACTGCGTTTGAAACTGCGAGGCTA
GAGTGTGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAG
AGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGCTAACACTGA
CGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCCCTAAACGATGTCAACTAGTTGTTGGTGGAGAAATCCATTAGT
AACGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGT
TAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGATTATGTGG
ATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGA
ACTTGCCAGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCT
GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTGCCATTAATTGCCATCATTCAGTTGGGCACTTTAAT
GGGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTC
ATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAG
GGCAGCCAACCCGCGAGGGGGAGCCAATCCCAGAAAGCCGATCGTAGTCC
GGATTGGAGTCTGCAACTCGACTCCATGAAGTC

>Unclassified_Chloroflexi clone SL-0211**(KF916888)**

GCGTTGTCCGGAATTACTGGGCGTAAAGAGCGCGCAGGCGGCACTTTAAG
TAGGGCGTGAAATCTCTCGGCTTAACTGAGAGGGGTCGTTCTAAACTGGA
GAGCTAACGAGGGCAGGAGAGGAAAGTGGAATTCCCGGTGTAGTGGTGAA
ATGCGTAGATATCGGGAGGAACACCTGTGGCGAAGGCGACTTTCTGGCCT
GTTCCTGACGCTGAGGCGCGAAGGCTAGGGGAGCGAACGGGATTAGATAC
CCCGGTAGTCCTAGCAGTAAACGATGGATACTAGGTGTTGGTGGTATTGA
CCCCACCAGTGCCGGAGCTAACGCATTAAGTATCCCGCCTGGGGAGTACG
GCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCG
GAGCGTGTGGTTTAATTCGACGCAACGCGCAGAACCTTACCAGGACTTGA
CATGCTTCTGACAGAGGCGGAAACGTCTTCTCTCTTCGGAGCAGATGCAC
AGATGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTCGTCGCTAGTTGAATTCTCTAGCGAGACTG
CCGGTAGAAAACCGGAGGAAGGTGGGGATGACGTCAAGTCAGCATGGCTC
TTACGTCCTGGGCTACACACACGCTACAATGGCCGGTACAATGGGCTGCC
AAGGGGCGACCCGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGC
AGGCTGCAACCCGCCTGC

>Unclassified_Sphingomonadaceae clone SL-0212**(KF916889)**

CCGTGCCAGCAGCCGCGGTAATACGGAGGGAGCTAGCGTTGTTCGGAATT
ACTGGGCGTAAAGCGTGCGTAGGCGGCTATTCAAGTCAGAGGTGAAAGCC
TGGAGCTCAACTCCAGAACTGCCTTTGAAACTAGATAGCTAGAATCTTGG
AGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGA
AGAACACCAGTGGCGAAGGCGACTCACTGGACAAGTATTGACGCTGAGGT
ACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGATAACTAGCTGTCCGGGCTCTCAGAGCTTGGGTGGCGCAGC
TAACGCATTAAGTTATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC
AAAGGAATTGACGGGGGCCTGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGCAGAACCTTACCAGCGTTTGACATCCTTGTCGCGGATAG
TGGAGACACTTTCCTTCAGTTCGGCTGGACAAGTGACAGGTGCTGCATGG
CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTCGTCCTTAGTTGCCATCATTCAGTTGGGCACTCTAAGGAAACT
GCCGGTGATAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCC
CTTACACGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGCAGC
AACCCTGCGAGGGGTAGCTAATCTCCAAAAACCGTCTCAGTTCGGATTGT
TCTCTGCAACTCGAGAGCATGAAGGCGGAATCG

>Unclassified_Gammaproteobacteria-25 clone SL-0213**(KF916890)**

GGAGCCCGTGACGCTACCCACAGAAGAAGCACCGGCAAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGT
AAAGCGCGCGTAGGCGGTTTGGTAAGCTGGATGTGAAATCCCCGGGCTCA
ACCTGGGAACTGCATCCAGAACTGCCAAGCTAGAGTATGGTAGAGGGTAG
TGGAATTTCCGGTGTAGCGGTGAAATGCGTAGATATCGGAAGGAACACCA
GTGGCGAAGGCGGCTACCTGGACCAATACTGACGCTGAGGTGCGAAAGCG

TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
TCAACTAGCCGTTGGGCTCCTTGAGGGTCTAGTGGCGCAGCTAACGCGAT
AAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACG
CGAAGAACCTTACCAGGCCTTGACATCCTGCGAACTTTCTAGAGATAGAT
TGGTGCCTTCGGGAGCGCAGTGACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCT
TAGTTGCCAGCACTTCGGGTGGGAACTCTAAGGAGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGGCCTGG
GCTACACACGTGCTACAATGGTCGGTACAGAGGGTCGCGAAGCCGCGAGG
TGGAGCTAATCCCAGAAAACCGGTCGTAGTCCGGATCGGAGTCTGCAACT
CGACTCCGTGAAG
>Ignavibacterium-02 sp. clone SL-0214**(KF916891)**
GAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGG
GCAAGCGTTGTCCGGATTTACTGGGTGTAAAGGGCGCGCAGGCGGAATAT
CAAGTCAGAGGTGAAATCCTACAGCTTAACTGTAGAACTGCCTTTGATAC
TGTTATTCTTGAGTTCGGGAGAGAGAGACGGAATTCCAGGTGTAGTGGTG
AAATACGTAGATATCTGGAAGAACACCAGTTGCGAAGGCGGTCTCTTGGT
CCGATACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCTGTAAACGATGAATACTAGGTGTTGGGTTTTTA
ACTCAGTGCCGCAGCTAACGCATTAAGTATTCCACCTGGGAAGTACGATC
GCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAG
CATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTAGGCTTGAAAG
GCAAGTGACAGGGTATGAAAGTACCCCTCCAGCAATGGCACTTGTACAGG
TGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCCTACTATTAGTTGCCACCAGGTTATGCTGAGCAC
TCTAATAGGACTGCCTACGCAAGTAGTGAGGAAGGTGGGGATGACGTCAA
GTCCGCATGGCCCTTACGCCTAGGGCCACACACGTGCTACAATGGATGTT
ACAATGGGTAGCTAAACCGCAAGGTGGAGCCAATCCTCCAAAGGCATCCT
CAGTTCGGATTGGAGTCTGAAACTCGACTCCATGAAGTTGGAATT
>Unclassified-Saprospiraceae-02 clone SL-0215**(KF916892)**
CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATCCGGAATT
ACTGGGTTTAAAGGGTGCGTAGGCGGCTGTGTAAGTCAGGAGTGAAAGTT
TGCGGCTTAACCGTAAAATTGCTTTTGATACTGCACGGCTAGAATCAGGA
TGAGGTCAGCGGAATGTGGCATGTAGCGGTGAAATGCATAGATATGCCAT
AGAACACCAATTGCGAAGGCAGCTGGCTAGACCTGCATTGACGCTGAGGC
ACGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCC
TAAACGATGCTTACTCGACGTATGGCGCTAGTCGTCGTGCGTCCAAGGGA
AACCGTTAAGTAAGCCACCTGGGGAGTACGACCGCAAGGTTGAAACTCAA
AGGAATTGACGGGGGTCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
TGATACGCGAGGAACCTTACCTGGGCTAGAATGCGCGTGACCGGTCGTGA
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AGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCT
GTCCTTAGTTGCCAGCTCATCGCAAGATGAAGGAACTCTAAGGAGACTGC
CGGCGTAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCTT
TATGCCCAGGGCGACACACGTGCTACAATGGCCGGTACAACGGGTTGCCA
AACCGCGAGGTGGAGCCAATCCCATAAAGCCGGTCTCAGTTCGGATCGGA
GTCTGAAACCCGACTCCGTGAAG
>TM7-16_genera_incertae_sedis clone SL-0216**(KF916893)**
GCAAATAGTGAAATCTGGTGGCTCAACCATCAACCCATTATTTGAACTGG
ATTGCTCGAGAGCGAGAGAGGTCACTGGAATTCCTTGTGTAGGAGTGAAA
TCCGTAGATATAAGGAGGAACACCAATGGCGTAGGCAGGTGACTGGCTCG
TTTCTGACACTGAGGCACGAAAGCGTGGGGAGCGAACGGGATTAGATACC
CCGGTAGTCCACGCCGTAAACGATGGATGCTAGCTGTTAGGAGTATCGAC
CCTTCTAGTAGCGAAGCTAACGCGTTAAGCATCCCGCCTGGGGAGTACGG
TCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGTGGTGG
AGCGTGTTCTTTAATTCGATGATAAGCGAAGAACCTTACCAGGGCTTGAC

ATCCTTGGAATTTCTCCGAAAGGAGAGAGTACTTTATTGGACCAAGTGAC
AGGTGTTGCATGGCCGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGT
CCTTCAACGAGCGCAACCCTTATGTTTAGTTGAATTTTTCTAAACAGACT
GCCTCGGTAACGGGGAGGAAGGAGGGGATGATGTCAGGTCATTATTACCC
TTACGTCCTGGGCTAGAAACGCGCTACAATGGCCGGTACAAAGGGCAGCC
AACCCGCGAGGGGGAGCAAATCCCATCAAAACCGGTCCCAGTTCGGATTG
CAGGCTGAAACTCGCCTGC
>Unclassified_Bacteria-07 clone SL-0217**(KF916894)**
TGGGCGTAAAGGGTGTGTAGGTGGTGTGATTAGTCGGATGTAAAATCCTG
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GGGTGTACGGAACTCAAGGTGTAGGGGTGAAATCCGTTGATATCTTGGGG
AACACCAAAAGCGAAGGCAGTGCACTGGCACTTTCCTGACACTGAAACAC
GAAAGCGTGGGTAGCGAATCGGATTAGATACCCGAGTAGTCCACGCCCTA
AACGCTGTCTGCTAGCTATGAGGAGTATCGACCCTCTTCGTGGCGTAGGT
AACCCGTTAAGCAGACCGCCTGGGTAGTACGAGCGCAAGCTTAAAACTCA
AAGGAATAGACGGGGGCTCGCACAAGCGGTGGATCATGGGGCTTAATTCG
TCACTAAGCGAGGAACCTTACCGAGGCTAGAAATCCTACTGCACGCTCCC
TGAAAGGGGAGAAGCCTTCGAGGGTGTAGGACAGGTGATGCATGGCCGTC
GTCAGTTCGTGGCTTGAGCTGTTCCCTTAAGTGGGGAAACGAACGCAACC
CTCGTTGCCTGTTACAAGTGTCAGGCGAGACTGCTCCCTCACGGGAGAGG
AAGGTGAGGATGACGCCAGGTCAGCATGTCCCTCGATGCCTCGGGCTGCA
CCCGTGATACAATGGGTAGTACAACGAGACGCAATGTGGTAACACGGAGC
AAATCTTTATAAAACTATCCTCAATTCGGATTGAGGTCTGCAACTCGACC
TCATGAAGTCGGAATCG
>Unclassified_Alcaligenaceae clone SL-0218**(KF916895)**
ACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATT
ACTGGGCGTAAAGCGTGCGCAGGCGGCTTTGTAAGACGGATGTGAAAGCC
CCGGGCTCAACCTGGGAAGTGCATTCGTGACTGCAAGGCTAGAGTGTGTC
AGAGGGAGGTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGAGATGCGGA
GGAACACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGCTCAGGC
ACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCC
TAAACGATGTCAACTAGTTGTCGGGGATTCATTTCCTTGGTAACGCAGCT
AACGCGTGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCG
ATGCAACGCGAAAAACCTTACCTACGCTTGACATGTCAGGAACCCTGAAG
AGATTTAGGGGTGCCCGAAAGGGAACCTGAACACAGGTGCTGCATGGCTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATCATTAGTTGCTACGCAAGGGCACTCTAATGAGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGCG
TAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGGTTGCCAACCCGC
GAGGGGGAGCCAATCCCATAAAGCCGATCGTAGTCCGGATTGCAGTCTGC
AACTCGACTGC
>Steroidobacter-02 sp. clone SL-0219**(KF916896)**
GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTAC
TGGGCGTAAAGCGCACGTAGGCGGGAAATTAAGTCGGATGTGAAATCCCC
GGGCTTAACCTGGGAACTGCATCCGATACTGGTTGCCTAGAGTATGGAAG
AGGGAAGCGGAATTCCAGGTGTAGCGGTGAAATGCGTAGATATCTGGAGG
AACATCAGTGGCGAAGGCGGCTTCCTGGTCCAATACTGACGCTGAGGTGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTA
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ACGCGTTAAGTTCTCCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAA
AGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
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>Terrimonas-03 sp. clone SL-0220**(KF916897)**
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>Unclassified_Anaerolineaceae-10 clone SL-0221**(KF916898)**
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>Arenimonas sp. clone SL-0222**(KF916899)**
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>Unclassified_Burkholderiales-04 clone SL-0223**(KF916900)**
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>Unclassified_Gammaproteobacteria-26 clone SL-0224**(KF916901)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-14 clone SL-
0226**(KF916902)**
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>Bellilinea-09 sp. clone SL-0227**(KF916903)**
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>TM7-17_genera_incertae_sedis clone SL-0228**(KF916904)**
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>TM7-18_genera_incertae_sedis clone SL-0229**(KF916905)**
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>Acinetobacter-02 sp. clone SL-0230**(KF916906)**
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>Gp6 clone SL-0231**(KF916907)**
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>Ignavibacterium-03 sp. clone SL-0232**(KF916908)**
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>Acidovorax sp. clone SL-0233**(KF916909)**
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>TM7-19_genera_incertae_sedis clone SL-0234**(KF916910)**
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>Unclassified_Burkholderiales-05 clone SL-0235**(KF916911)**
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>Unclassified_Alphaproteobacteria-04 clone SL-0237**(KF916913)**
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>Thiobacillus sp. clone SL-0238**(KF916914)**
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>Acinetobacter-03 sp. clone SL-0239**(KF916915)**
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GGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTC
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CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCG
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>Aquimonas-02 sp. clone SL-0240**(KF916916)**
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CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATT
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>Desulfocapsa sp. clone SL-0242**(KF916917)**
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GACTCCATGAAGTGGAATCG
>Planctomyces sp. clone SL-0243**(KF916918)**
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TGCAAGGCTTGAGTGAGACAGGGGTGTGTGGAACTTCTAGTGGAGCGGTG
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CGACGTCAAGTCATCATGGCCTTTATGTCTAGGGCTGCACACGTGCTACA
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AGCGCCGCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGG
AATCG
>Gp4-05 clone SL-0244**(KF916919)**
ACCCAGCAACGCCGCGTGAAGGATGAAGTATTTCGGTATGTAAACTTCGA
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CGAGCTTAACTCGGAACGGCCAGCTGATACTGCAGTGCTAGAGTGCAGAA
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TATGTTCAGGGCTACACACGTGCTACAATGGAAGGTACAAAACGTCGCAA
TCCCGCAAGGGGGAGCTAATCGCGAAAACCTTCCTCAGTTCGGATTGGAG
TCTGCAACTCGACTCCATGAAG
>TM7-20_genera_incertae_sedis clone SL-0245**(KF916920)**
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GGAATCG
>Gp4-06 clone SL-0246**(KF916921)**
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CCGCGGTAATACGTAGGGAGCAAGCGTTGTTCGGATTTACTGGGCGTAAA
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CGGAGGAAGGTGGGGATGATGTCAAGTCATCATGGCCTTTATGTTCAGGG
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ACTCCATGAAG
>Unclassified_Verrucomicrobia clone SL-0247**(KF916922)**
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CAGGAGGAACACCGGTGGCGAAGGCGGCTTCCTGGAAGGTTCCTGACTCT
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CGCGAGGTGGAGCCAATCCCAAAAACCGGGCCCAGTTCAGATTGCAGGCT
GCAACTCGCCTGCATGAAGCC
>Treponema sp. clone SL-0248**(KF916923)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-15 clone SL-
0249**(KF916924)**
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CGCGAGGGGGAGCTAATCTCACAAAGCCGTTCGTAGTCCGGATTGCAGTC

TGCAACTCGACTGCATGAAGTCGGAATCG
>TM7-21_genera_incertae_sedis clone SL-0250**(KF916925)**
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>Unclassified_Deltaproteobacteria-08 clone SL-0251**(KF916926)**
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AGAGGAGAGTGGAATTCCAGGTGTAGAGGTGAAATTCGTAGATATCTGGA
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TGAAAGCCGGAGTTCTCGCAAGAGACGCGGAGACAGGTGTTGCATGCCTG
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TCTGCAACTCGACTCCATGAAG
>Unclassified_Gammaproteobacteria-27 clone SL-0252**(KF916927)**
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CCATGAAGTCGGAATCG

>Bellilinea-10 sp. clone SL-0253**(KF916928)**
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CGGGGCTACACACACGCTACAATGGGCAGTACAATGGGTCGCTAAACCGC
GAGGTGGAGCCAATCCCCCAAAGCTGTCCTCAGTTCAGATTGCAGGCTGC
AACCCGCCTGCATGAAGCCGGA
>Opitutus sp. clone SL-0254**(KF916929)**
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>Unclassified_Gammaproteobacteria-28 clone SL-0255**(KF916930)**
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>Unclassified_Anaerolineaceae-11 clone SL-0256**(KF916931)**
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TGGGGAGCGAACGGGATTAGAAACCCCGGTAGTCCATGCCGTAAACGATG
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GCAAAGCCGGTCTCAGTTCGGATTGCAGGCTGCAACCCGCCTGCATGAAG
TCGGAG
>Peredibacter-04 sp. clone SL-0257**(KF916932)**
GTCCTTATGGCTAATATCCATAAGGGGTGATGGTACCAAAGGAATAAGCA
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>Unclassified_Gammaproteobacteria_incertae_sedis-16 clone SL-
0259**(KF916933)**
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>Unclassified_Rhodospirillales clone SL-0260**(KF916934)**
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>Unclassified_Bacteria-08 clone SL-0261**(KF916935)**
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>Peredibacter-05 sp. clone SL-0262**(KF916936)**
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>Methylohalomonas sp. clone SL-0263**(KF916937)**
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>Unclassified_Gammaproteobacteria-29 clone SL-0265**(KF916938)**
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>Unclassified_Gammaproteobacteria-30 clone SL-0266**(KF916939)**
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>Unclassified_Peptococcaceae 2 clone SL-0267**(KF916940)**
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>Thiobacillus-02 sp. clone SL-0268**(KF916941)**
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>Haliscomenobacter-04 sp. clone SL-0269**(KF916942)**
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>Vampirovibrio sp. clone SL-0270**(KF916943)**
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>Unclassified_Bacteria-10 clone SL-0271**(KF916944)**
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>Gp4-07 clone SL-0272**(KF916945)**
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>Desulfocapsa-02 sp. clone SL-0273**(KF916946)**
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>Unclassified_Deltaproteobacteria-09 clone SL-0274**(KF916947)**
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>Flavobacterium-02 sp. clone SL-0275**(KF916948)**
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>Unclassified_Gammaproteobacteria-31 clone SL-0276**(KF916949)**
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>Unclassified_Bacteria-11 clone SL-0279**(KF916950)**
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CCATGAAGTCGGAA
>OD1_genera_incertae_sedis clone SL—0280**(KF916951)**
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CCTCAGAAGCCGGAATCG
>Longilinea-02 sp. clone SL-0281**(KF916952)**
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AAGCGTGGGGAGCGAACGGGATTAGAGACCCCGGTAGTCCACGCCGTAAA
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CTGGTCGTAGTTACACGTGTCTACGACGACTGCCCGGGAGAACCGGGAGG
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>Unclassified_Oceanospirillales clone SL-0282**(KF916953)**
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CCGGGCTCAACCTGGGAGCTGCATTCGATACTGGCAGACTTGAATACGGT
AGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGA
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GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGTCGACTAGCCGTTGGGGAACTTGATTTCCCAGTGGCGTAGC
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GAGATGGATTGGTGCCTTCGGGAGCGCAGAGACAGGTGCTGCATGGCTGT
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GTGACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTA
CGAGCAGGGCTACACACGTACTACAATGGCCGGTACAAAGGGTTGCGAAA
GCGCGAGCTGGAGCCAATCCCAAAAAGCTGGTCGTAGTCCGGATTGGAGT
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>Unclassified_Sphingobacteriales-05 clone SL-0283**(KF916954)**
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AGCGAAAGCATTAAGTTATCCACCTGGGGAGTACGACCGCAAGGTTGAAA
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>Meniscus sp. clone SL-0284**(KF916955)**
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GTTGGATTCG
>Prosthecobacter-02 sp. clone SL-0285**(KF916956)**
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CCAGTACAATGTGAACCGAGACCGCGAGGTGGAGGAAATCAATAAAACTG
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>Unclassified_Sphingobacteriales-06 clone SL-0286**(KF916957)**
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A
>Unclassified_Deltaproteobacteria-10 clone SL-0287**(KF916958)**
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AACCGCCCTCAGTTCGGATCGCAGTCTGCAACTCGACTGC
>Unclassified_Gammaproteobacteria-32 clone SL-0289**(KF916959)**
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>Unclassified_Burkholderiales-06 clone SL-0290**(KF916960)**
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ACTCGACTGC
>Bacillus sp. clone SL-0291**(KF916961)**
AATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTAC
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GCCGCGAGGTGAAGCGAATCCCATAAAACCATTCTCAGTTCGGATTGTAG
GCTGCAACTCGCCTAC
>Acinetobacter-04 sp. clone SL-0292**(KF916962)**
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>Unclassified_Deltaproteobacteria-11 clone SL-0293**(KF916963)**
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CGCCGTAAACGATGGGCACTAGGTGTCGGGGGTATCCACTCCCTCGGTGC
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>Unclassified_Rhodospirillales-02 clone SL-0294**(KF916964)**
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>TM7-22_genera_incertae_sedis clone SL-0295**(KF916965)**
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CGCGCTACAATGGCCGGTACAAAGGGCAGCCAACCCGCGAGGGGGAGCAA
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ATGAAGCCGGAATC
>Coxiella sp. clone SL-0296**(KF916966)**
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GGAGTCTGCAACTCGACTCCATGAAGTCGGAATCG
>Desulfopila sp. clone SL-0297**(KF916967)**
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TTCGCGAGAAGGAGCCAATCCCATAAAGTATGTCTCAGTCCGGATTGGAG
TCTGCAACTCGACTCCATGAAG
>Bellilinea-11 sp. clone SL-0298**(KF916968)**
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AACCCGCCTGCATGAAGCCGGA
>Unclassified_Gammaproteobacteria-33 clone SL-0299**(KF916969)**
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AGTCGGAATCG
>Ferruginibacter-03 sp. clone SL-0300**(KF916970)**
GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATCCGGATTTAC
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>Pseudoxanthomonas-02 sp. clone SL-0301**(KF916971)**
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>Unclassified_Gammaproteobacteria-34 clone SL-0302**(KF916972)**
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>Unclassified_Gammaproteobacteria-35 clone SL-0303**(KF916973)**
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>Proxilibacter sp. clone SL-0304**(KF916974)**
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>Acinetobacter-05 sp. clone SL-0305**(KF916975)**
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>Unclassified_Acetobacteraceae clone SL-0306**(KF916976)**
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>Terrimonas-04 sp. clone SL-0307**(KF916977)**
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>Unclassified_Desulfobulbaceae clone SL-0308**(KF916978)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-17 clone SL-0309**(KF916979)**
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>Unclassified_Bacteria-12 clone SL-0310**(KF916980)**
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>Flavobacterium-03 sp. clone SL-0311**(KF916981)**
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C
>TM7-23_genera_incertae_sedis clone SL-0312**(KF916982)**
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>Planctomyces-02 sp. clone SL-0313**(KF916983)**
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>Georgfuchsia sp. clone SL-0314**(KF916984)**
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>Bauldia sp. clone SL-0316**(KF916985)**
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>Unclassified_Burkholderiales-07 clone SL-0317**(KF916986)**
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>Unclassified_Gammaproteobacteria-36 clone SL-0318**(KF916987)**
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>TM7-24-genera_incertae_sedis clone SL-0319**(KF916988)**

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>Parvibaculum sp. clone SL-0320**(KF916989)**
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>Unclassified_Gammaproteobacteria-38 clone SL-0323**(KF916992)**
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>Bellilinea-12 sp. clone SL-0325**(KF916994)**
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>Georgfuchsia-02 sp. clone SL-0326**(KF916995)**
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>Prolixibacter-02 clone SL-0327**(KF916996)**
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>Unclassified_Deltaproteobacteria-13 clone SL-0328**(KF916997)**
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>TM7-25_genera_incertae_sedis clone SL-0330**(KF916999)**
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>Unclassified_Gammaproteobacteria-39 clone SL-0331**(KF917000)**
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>Bellilinea-13 sp. clone SL-0334**(KF917003)**
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>Desulforhabdus sp. clone SL-0336**(KF917004)**
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>Bellilinea-14 sp. clone SL-0337**(KF917005)**
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>Unclassified_Ruminococcaceae clone SL-0338**(KF917006)**
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AATCG
>Ohtaekwangia-10 sp. clone SL-0341**(KF917008)**
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>Unclassified_Gammaproteobacteria-41 clone SL-0342**(KF917009)**
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>Unclassified_Bacteria-14 clone SL-0343**(KF917010)**
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>Gp6-02 clone SL-0344**(KF917011)**
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>Vampirovibrio-02 sp. clone SL-0346**(KF917012)**
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>Unclassified_Syntrophomonadaceae clone SL-0347**(KF917013)**

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>Georgfuchsia-03 sp. clone SL-0348**(KF917014)**
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>Unclassified_Anaerolineaceae-13 clone SL-0349**(KF917015)**
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GC
>Ohtaekwangia-11 sp. clone SL-0350**(KF917016)**

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>Gp4-08 clone SL-0351**(KF917017)**
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>Unclassified_Chromatiales clone SL-0352**(KF917018)**
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>Unclassified_Alphaproteobacteria-06 clone SL-0353**(KF917019)**
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>Thauera-03 sp. clone SL-0354**(KF917020)**
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>Hydrogenophaga-02 sp. clone SL-0355**(KF917021)**
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>Prolixibacter-03 sp. clone SL-0356**(KF917022)**
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>Derxia sp. clone SL-0357**(KF917023)**
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>Unclassified_Chloroflexi-02 clone SL-0358**(KF917024)**
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>Unclassified_Alphaproteobacteria-07 clone SL-0359**(KF917025)**
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>Gp4-09 clone SL-0360**(KF917026)**

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>Haliangium sp. clone SL-0361**(KF917027)**
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>TM7-26_genera-incertae_sedis clone SL-0362**(KF917028)**
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>Pseudoxanthomonas-03 sp. clone SL-0363**(KF917029)**
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>Unclassified_Bacteria-15 clone SL-0364**(KF917030)**
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>Unclassified_Gammaproteobacteria-42 clone SL-0365**(KF917031)**
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>Aquimonas-03 sp. clone SL-0366**(KF917032)**
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CTCC
>TM7-27_genera_incertae_sedis clone SL-0367**(KF917033)**
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>Unclassified_Bacteria-16 clone SL-0368**(KF917034)**
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>Dongia-03 sp. clone SL-0369**(KF917035)**
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>Unclassified_Hahellaceae clone SL-0370**(KF917036)**
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>Unclassified_Burkholderiales-08 clone SL-0371**(KF917037)**
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>Unclassified_Gammaproteobacteria-43 clone SL-0372**(KF917038)**
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>Unclassified_Gammaproteobacteria-44 clone SL-0373**(KF917039)**
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>Unclassified_Gammaproteobacteria-45 clone SL-0374**(KF917040)**
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>Mesorhizobium sp. clone SL-0375**(KF917041)**
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>Parasegetibacter sp. clone SL-0376**(KF917042)**
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>Bellilinea-15 sp. clone SL-0377**(KF917043)**
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>Unclassified_Deltaproteobacteria-15 clone SL-0378**(KF917044)**
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>Dongia-04 sp. clone SL-0380**(KF917045)**
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>Unclassified-Gammaproteobacteria_incertae_sedis-18 clone SL-
0381**(KF917046)**
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>Mycobacterium sp. clone SL-0382**(KF917047)**
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>Steroidobacter-03 sp. clone SL-0383**(KF917048)**
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>Ohtaekwangia-12 sp. clone SL-0386**(KF917049)**
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>Flavihumibacter-02 sp. clone SL-0387**(KF917050)**
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>Terrimonas-05 sp. clone SL-0388**(KF917051)**
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>Unclassified_Gammaproteobacteria-46 clone SL-0390**(KF917053)**
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>Gp4-10 clone SL-0392**(KF917054)**
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>Unclassified_Clostridia-02 clone SL-0393**(KF917055)**
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>Legionella sp. clone SL-0394**(KF917056)**
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>Arenimonas-02 sp. clone SL-0395**(KF917057)**
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>TM7-28_genera_incertae_sedis clone SL-0396**(KF917058)**
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>Unclassified_Gammaproteobacteria-47 clone SL-0398**(KF917059)**
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>Anaerolinea-03 sp. clone SL-0399**(KF917060)**
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>Peredibacter-06 sp. clone SL-0402**(KF917062)**
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>Owenweeksia sp. clone SL-0404**(KF917064)**
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>Unclassified_Bacteria-17 clone SL-0405**(KF917065)**
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GGGCTGAAACTCGCCCACAGAAGCCGGAATCG
>Hahella sp. clone SL-0406**(KF917066)**
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>TM7-29_genera_incertae_sedis clone SL-0407**(KF917067)**
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>Gp4-11 clone SL-0408**(KF917068)**
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>Bellilinea-16 sp. clone SL-0409**(KF917069)**
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GAGTGGTAGAGGGAGGTGGAATTCCGGGTGTAGTGGTAAAATGCGTAGAT
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TAAACCGGAGGAAGGTGGGGATGATGTCAAGTCCGCATGGCCTTTATATC
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ACCCGCCTGC
>Unclassified_Bacteria-18 clone SL-0410**(KF917070)**
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GTGATACAATGGTCGGTACAGTGGGCAAAGCCGCGAGGCTAAGGTAATCC
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GGGAATCG
>Ohtaekwangia-13 sp. clone SL-0412**(KF917071)**
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ATGAAG
>TM7-30_genera_incertae_sedis clone SL-0413**(KF917072)**
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>Unclassified_Deltaproteobacteria-16 clone SL-0414**(KF917073)**
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>Unclassified_Alphaproteobacteria-08 clone SL-0415**(KF917074)**
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>TM7-31_genera_incertae_sedis clone SL-0416**(KF917075)**
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>Unclassified_Gammaproteobacteria-48 clone SL-0417**(KF917076)**
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>Methylobacillus sp. clone SL-0419**(KF917077)**
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>Unclassified_Gammaproteobacteria-49 clone SL-0420**(KF917078)**
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>Unclassified_Gammaproteobacteria-50 clone SL-0421**(KF917079)**
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>Subdivision3_genera_incertae_sedis-06 clone SL-0422**(KF917080)**
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>Novosphingobium sp. clone SL-0423**(KF917081)**
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>Unclassified_Gammaproteobacteria-51 clone SL-0425**(KF917082)**
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GAAGTCGGAATCG
>Unclassified_Deltaproteobacteria-17 clone SL-0426**(KF917083)**
CCGTGCCAGCAGCCGCGGTAAGACGGAGGGTGCAAGCGTTGCTCGGAATC
ATTGGGCGTAAAGGGTGCGTAGGCGGTTTCTTAAGTCTGGCGTGAAAGCC
CAGGGCCCAGCCTTGGAAGGGCGCTAGAAACTGAGGAGCTTGAGTGCCGG
AGGGGAGAGTGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGA
GGAATACCGGTGGCGAAAGCGACTCTCTGGACGGTAACTGACGCTGAGGC
ACGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTG
TAAACGATGGACACTAGGTGTCGGGGGTATCCACTCCCTCGGTGCCGCCG
CTAACGCATTAAGTGTCCCGCCTGGGAAGTACGGCCGCAAGGTTAAAACT
CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTCAATT
CGATGCAACGCGAAAAACCTTACCTGGGTTTGACACCTGGCGAATTTTTC
CGAAAGGAAAAAGTGCCCGTAAGGGAGCGCCAAGACAGGTGCTGCATGGC
TGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTTATCCCTAGTTGCCCCCAGGTGAAGCTGTGGCACTCTAAGGAGA
CTGCCCGTGTTAAGCGGGAGGAAGGTGGGGACGACGTCAAGTCATCATGG

CCTTTATATCCAGGGCTACACACGTGCTACAATGGGTGGTACAGAGAGTT
GCGAAGTCGTGAGATGAAGCTAATCTCAAAAAGCCATCCTCAGTTCGGAT
CGAAGTCTGCAACTCGACTTC
>Unclassified_Gammaproteobacteria-52 clone SL-0427**(KF917084)**
GCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTG
GGCGTAAAGCGCACGTAGGTGGTTCGTTAAGTCGGATGTGAAATCCCTGG
GCTCAACCTGGGAGCTGCATTCGATACTGGCGGACTCGAGTACGAGAGAG
GGGGGTGGAATTCCAGGTGTAGCGGTGAAATGCGTAGATATCTGGAGGAA
CACCAGTGGCGAAGGCGGCCCCCTGGCTCGATACTGACACTGAGGTGCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGTCGACTAGCCGTTGGGGAACTTGATTTCCCAGTGGCGTAGCTAAC
GCGCTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAT
GAATTGACGGGGACCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGACG
CAACGCGAAGAACCTTACCTGCTCTTGACATCCTGCGAACTTTCCAGAGA
TGGAAGGGTGCCTTCGGGAGCGCAGAGACAGGTGCTGCATGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCCTTAGTTGCCAGCATTTAGTTGGGGACTCTAGGGAGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCTCTTACGAG
CAGGGCTACACACGTACTACAATGGCCGGTACAAAGGGTTGCGAAAGCGC
GAGCTGGAGCCAATCCCAAAAAGCTGGTCGTAGTCCGGATTGGAGTCTGC
AACTCGACTCCATGAAGTCGGAATCG
>TM7-32_genera_incertae_sedis clone SL-0428**(KF917085)**
GTGACGAATATGACGGTAGCAGACGAATAAGGATCGGCTAACTACGTGCC
AGCAGCCGCGGTCATACGTAGGATCCGAGCGTTATCCGGATTTACTGGGC
GTAAAGAGTTGCGTAGGTGGCAAAGTAAGTTAGTAGTGAAAGCGTGTGGC
TCAACCATACTCACATTACTAAAACTGCTTAGCTTGAAGATGAGAGAGGT
CACTGGAATTCCTTGTGTAGGAGTGAAATCCGTAGATATAAGGAGGAACA
CCGATGGCGTAGGCAGGTGACTGGCTCATTCTTGACACTAAGGCACGAAA
GCGTGGGGAGCAAACGGGATTAGATACCCCGGTAGTCCACGCCGTAAACG
ATGGATGCTAGCTGTTAGGAGTATCGACCCTCCTAGTAGCGCAGCTAACG
CGTTAAGCATCCCGCCTGTGGAGTACGGTCGCAAGACTAAAACATAAAGG
AATTGACGGGGACCCGCACAAGCGGTGGAGCGTGTTGTTTAATTCGATGG
TAAGCGAAGAACCTTACCCAGGCTTGACATCCTGCTAATCACTCCGAAAG
GAGAGAGTGCCTTCGGGCCGCAGTGACAGGTGTTGCATGGCCGTCGTCAG
CTCGTGTCGTGAGATGTTTGGTTAAGTCCATCAACGAGCGCAACCCTTAT
AGTTAGTTGTATTTCTCTAGCTAGACTGCCCTGGCAACAGGGAGGAAGGG
GGGGATGATGTCAGGTCAGTATTACCCTTACGCCTGGGGCTACAAACACG
CTACAATGGCCGGTACAAAGGGCTGCCAACCCGCGAGGGGGAGCAAATCC
CATCAAAGCCGGTCTCAGTTCGGATAGCAGGCTGAAACTCGCCTGCTGAA
GCTGGAATCG
>TM7-33-genera_incertae_sedis clone SL-0429**(KF917086)**
GAGTTGCGTAGGCGGTCGGTAAAGCGAATAGTGAAACCTGGTGGCTCAAC
CATTCAGACTATTATTCGAACTCACCGACTCGAGAGTAGCAGAGGTAACT
GGAATTTCTTGTGTAGGAGTGAAATCCGTAGATATAAGAAGGAACACCGA
TGGCGTAGGCAGGTTACTGGGCTATTTCTGACGCTAAGGCACGAAAGCGT
GGGGAGCGAACCGGATTAGATACCCGGGTAGTCCACGCCGTAAACGATGG
ATACTAGCTGTTGGAGGTATCGACCCCTTCAGTAGCGAAGCTAACGCGTT
AAGTATCCCGCCTGTGGAGTACGGCCGCAAGGCTAAAACATAAAGGAATT
GACGGGGACCCGCACAAGCGGTGGATCGTGTTCTTTAATTCGATGATAAA
CGAAGAACCTTACCAGGGCTTGACATCCTAAGAAGGCTTCCGAAAGGAAA
CTGTGCCGTAAGGAACTTAGTGACAGGTGATGCATGGCCGTCGTCAGCTC
GTGTCGTGAGATGTTTGGTTAAGTCCATCAACGAGCGCAACCCTTGTGTC
TAGTTGTATTTTTCTAGACAGACTGCCCCGGTAACGGGGAGGAAGGAGGG
GATGATGTCAGGTCAGTATTTCCCTTACGTCCTGGGCTAGAAACACGATA
CAATGGCTGGTACAATGCGCCGCGAAGCCGCGAGGTGAAGCAAATCGCAC
CAAAGCCAGTCCCAGTTCGGATTGCAGGCTGAAACTCGCCTGCATGAAGT
CGGAATCG

>Meniscus-02 sp. clone SL-0430**(KF917087)**
GTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGATTTAT
TGGGTTTAAAGGGTGCGCAGGTGGTTGAATAAGTCAGTGGTGAAAGTCTG
CCGCTTAACGGTAGGATTGCCATTGATACTGTTTAACTTGAGTTTAGGTG
AGGTAGGCGGAATGTGTAGTGTAGCGGTGAAATGCATAGATATTACACAG
AACACCGATTGCGAAGGCAGCTTACTAATCTACAACTGACACTGAGGCAC
GAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTA
AACGATGATCACTCGCTGTTTGCGATATACAGTAAGCGGCTAAGCGAAAG
CGATAAGTGATCCACCTGGGGAGTACGATCGCAAGGTTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGAGGAACATGTGGTTTAATTCGATGA
TACGCGAGGAACCTTACCTGGGCTTAAATGGGGAGTGACAGCTGGCGAAA
GTTGGTTTTCTTCGGACACTCTTCAAGGTGCTGCATGGTTGTCGTCAGCT
CGTGCCGTGAGGTGTCGGGTTAAGTCCCATAACGAGCGCAACCCTTACTG
TTAGTTGCCAGCGGGTCAAGCCGGGAACTCTAACGGGACTGCCACCGTAA
GGTGAGAGGAAGGTGGGGATGACGTCAAATCAGCACGGCCCTTATGTCCA
GGGCTACACACGTGTTACAATGGCCGGTACAAAGGGCAGCTACACCGCGA
GGTGATGCTAATCTCGAAAGCCGGTCTCAGTTCGGATCGAAGTCTGCAAC
CCGACTTC
>Georgfuchsia-04 sp. clone SL-0431**(KF917088)**
ACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATT
ACTGGGCGTAAAGCGTGCGCAGGCGGCGACATAAGACAGATGTGAAATCC
CCGGGCTCAACCTGGGAACTGCGTTTGTGACTGTGTTGCTCGAGTGTGGC
AGAGGGGGGTGGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGA
GGAACACCGATGGCGAAGGCAGCCCCCTGGGTTAACACTGACGCTCATGC
ACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCC
TAAACGATGCCAACTAGGTGTTGGGGAAGGAGACTTCCTTAGTGCCGTAG
CTAACGCGTGAAGTTGGCCGCCTGGGGAGTACGGTCGCAAGATTAAAACT
CAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATT
CGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCC
AGAGATGGCTTGGTGCTCGAAAGAGAACCTGGACACAGGTGCTGCATGGC
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTTGTCATTAGTTGCCACCATTCAGTTGGGCACTCTAATGAGACTG
CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC
TTATGGGTAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGGTTGCC
AACCCGCGAGGGGGAGCCAATCCCACAAAGCCGATCGTAGTCCGGATTGG
AGTCTGCAACTCGACTCCATGAAGTCGGAATCG
>Steroidobacter-04 sp. clone SL-0432**(KF917089)**
GTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGAATTAC
TGGGCGTAAAGCGCACGTAGTCGGCTTTGCAGGTCGGGTGTGAAATCCCC
GGGCTCAACCTGGGAACTGCATTCGAGACTGCATTGCTAGAGTATGGGAG
AGGGAAGCGGAATTTCTGGTGTAGCAGTGAAATGCGTAGATATCAGAAGG
AACATCAGTGGCGAAGGCGGCTTCCTGGACCAATACTGACGATCAGGTGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTA
AACGATGAGAACTGGATGTCGGGAGGGTTTGCCTTCCGGTGTCGTAGCTA
ACGCGTTAAGTTCTCCGCCTGGGAAGTACGGCCGCAAGGTTGAAACTCAA
AGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
TGCAACGCGAAGAACCTTACCTGGTCTTGACATCCCAGGAATCCTGCAGA
GATGCGGGAGTGCCTTCGGGAACCTGGAGACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTGCCCTTAGTTGCCATCATTCAGTTGGGAACTCTAAGGGGACCGCCGGT
GACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTATG
ACCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAACCC
GCGAGGGGGAGCCAATCCCAAAAAGCCGATCGTAGTCCGGATTGCAGTCT
GCAACTCGACTGC
>Prolixibacter-04 sp. clone SL-0433**(KF917090)**
CCGTGCCAGCAGCCGCGGTAATACGGAGGATGCGAGCGTTATCCGGATTT
ATTGGGTTTAAAGGGTGCGTAGGCGGAAAAATAAGTCAGTGGTGAAAACC

TTCAGCTTAACTGGAGACTTGCCATTGATACTGTTATTCTTGAGTGTGGT
TAAGGTAGGCGGAATGTGTAATGTAGCGGTGAAATGCTTAGATATTACAC
AGAACACCGATTGCGAAGGCAGCTTACTGAGCCATAACTGACGCTGATGC
ACGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGATCACTCGCTGTTGGCGATACACAGTCAGCGGCTAAGCAAA
AGCATTAAGTGATCCACCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGAGGAACATGTGGTTTAATTCGAT
GATACGCGAGGAACCTTACCTGGGCTTAAATGTATAGTGCATTTCACCGA
AAAGTGAATTTCCTTCGGGACTCTATGCAAGGTGCTGCATGGTTGTCGTC
AGCTCGTGCCGTGAGGTGTCGGGTTAAGTCCCATAACGAGCGCAACCCTT
ATCGTTAGTTGCCAGCGGGTAATGCCGGGAACTCTAGCGAAACTGCCGGT
GTAAACCGAGAGGAAGGTGGGGATGACGTCAAATCAGCACGGCCCTTATG
TCCAGGGCTACACACGTGTTACAATGGCCGGTACAGAGGGCAGCTACCTG
ATGACAGGGTGCGAATCTCGAAAGCCGGTCTCAGTTCGGATCGGAGTCTG
CAACTCGACTCC

>Mahella sp. clone SL-0434**(KF917091)**

CGACGGTACCATCAGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGGGGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGGGCG
TGTAGGCGGCAGACTAGGTCAGATGTGAAACACCAGGGCTCAACCGTGGT
ATTGCATTTGAAACCGGTTTGATTGAGTGCAGGAGAGGAAAGCGGAATTC
CTAGTGTAGCGGTGAAATGCGTAGATATTAGGAAGAACACCGGTGGCGAA
GGCGGCTTTCTGGACTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGC
AAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGGATACTAG
GTGTGGGAGGTATCGACCCCTTCCGTGCCGCAGTTAACACAATAAGTATC
CCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCAGCGGAGCGTGTGGTTTAATTCGAAGCTACGCGAAGAA
CCTTACCAGGTCTTGACATCCCCTGAAGTATGCAGAAATGCATACGTCCT
ATCAGAAATGATAAGACAGAGAGACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTC
TTAGTTGCCAGCACGTAGCGGTGGGCACTCTAAGCGAGACTGCCGTGGAT
GACACGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACC
TGGGCTACACACGCGCTACAATGGCCGCCACAGAGAGAAGCGAAGCGGCA
GCGCAGAGCCAATCCCAGAAAAGCGGTCCCAGTTCGGATTGTGGGCTGCA
ACCCGCCCGCATGAAGTGGA

>Mycobacterium-02 sp. clone SL-0435**(KF917092)**

AGAAGAAGGACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
TCCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTG
TCGCGTTGTTCGTGAAAACCGGGGGCTTAACCCTCGGCGTGCGGGCGATA
CGGGCAGACTTGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGT
GGAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGG
GCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTGGGTTTCCT
TCCTTGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAG
TACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGGGGCCCGCACAAGC
GGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGT
TTGACATGCGCAGGACGCCGGTAGAGATATCGGTTCCCTTGTGGCCTGTG
TGCAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGTCTCATGTTGCCAGCACGTGATGGT
GGGGACTCGTGAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATG
GCCGGTACAAAGGGCTGCGATGCCGTGAGGTGGAGCGAATCCTTTCAAAG
CCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGGAAGTCGGAGT
CG

>Gp3-03 clone SL-0436**(KF917093)**

ACGTGCCAGCAGCCGCGGTAATACGTAGGCAGCGAGCGTTGTTCGGAGTT
ACTGGGCGTAAAGCGTGCGTAGGCGGTGGTCCAAGTCTGGTGTGAAATCT
CCCGGCTTAACTGGGAGGGTGCGCCGGAAACTGGGCCGCTGGAGTGTGGG

AGAGGTAAGCGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGA
GGAACACCTGTGGTGTAGACAGCTTACTGGACCATGACTGACGCTGAGGC
ACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCC
TAAACGATGCATACTTGGTGTGGGCAGTTCAGTCTGTCCGTGCCGGAGCT
AACGCGTTAAGTATGCCGCCTGGGGAGTACGGTCGCAAGGCTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
ACGCAACGCGAAGAACCTTACCTGGGCTCGAACGGCTGTGGACCGTCTCT
GGAAACAGAGGCTTTCCCGCAAGGGACTGCAGTCGAGGTGCTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGCCCTGTGTTGCTAAGCCGAAAGGCTGCACTCTCAGGGGACCGC
CAGCGATAAGCTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCTT
TATGTCCAGGGCTACACACGTGCTACAATGGGCGGTACAGAGCGTAGCAA
ACCCGCGAGGGGGAGCAAATCGCAAAAAACCGTTCTCAGTTCGGATTGCA
GGCTGCAACCCGCCTGCATGAAG

>Bradyrhizobium sp. clone SL-0437**(KF917094)**
GTGCGGGAAGATAATGACGGTACCGCAAGAATAAGCCCCGGCTAACTTCG
TGCCAGCGGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACT
GGGCGTAAAGGGTGCGTAGGCGGGTCTTTAAGTCAGGGGTGAAATCCTGG
AGCTCAACTCCAGAACTGCCTTTGATACTGAAGATCTTGAGTTCGGGAGA
GGTGAGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGA
ACACCAGTGGCGAAGGCGGCTCACTGGCCCGATACTGACGCTGAGGCACG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGAATGCCAGCCGTTAGTGGGTTTACTCACTAGTGGCGCAGCTAAC
GCTTTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACG
CAACGCGCAGAACCTTACCAGCCCTTGACATCCCGGTCGCGGACTCCAGA
GACGGAGTTCTTCAGTTCGGCTGGACCGGAGACAGGTGCTGCATGGCTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCCCGTCCTTAGTTGCTACCATTCAGTTGAGCACTCTAAGGAGACTGCCG
GTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTT
ACGGGCTGGGCTACACACGTGCTACAATGGCGGTGACAATGGGATGCTAA
GGGGTGACCCTTCGCAAATCTCAAAAAGCCGTCTCAGTTCGGATTGGGCT
CTGCAACTCGAGCCCATGAAGTTGGAATCG

>Saccharofermentans sp. clone SL-0438**(KF917095)**
ACGTGCCAGCTGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTT
ACTGGGTGTAAAGGGCGTGTAGGCGGGTTTGCAAGTCGGATGTGAAATTC
CCAGGCTTAACTTGGGCGGGTCATCCGAAACTGCAGATCTTGAGTACTGG
AGAGGATAGTGGAATTCCTAGTGTAGCGGTAAAATGCGTAGATATTAGGA
GGAACACCAGTGGCGAAGGCGGCTATCTGGACAGTAACTGACGCTGAGGC
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAATACTAGGTGTAGGGGGTATCGACCCCCCCTGTGCCGCAG
CTAACGCAATAAGTATTCCACCTGGGGAGTACGGCCGCAAGGTTGAAACT
CAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGTATGTGGTTTAATT
CGACGCAACGCGAAGAACCTTACCAGGGCTTGACATCCTCTGACGACGGA
AGAGATTTCGTTTTCCCTTCGGGGACAGAGAGACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCCTATTGCCAGTTGCCAGCAAGAAAGATGGGCACTCTGGCGAGACTGC
CGTTGACAAAACGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCT
TATGTCCTGGGCTACACACGTACTACAATGGCAACAACAGAGGGCAGCCA
TGCCGCGAGGCAGAGCGAATCCCAAAATGTTGTCTCAGTTCAGATTGCAG
GCTGCAACTCGCCTGC

>Flavihumibacter-03 sp. clone SL-0439**(KF917096)**
GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATCCGGATTCAC
TGGGTTTAAAGGGTGCGTAGGCGGGTATGTAAGTCCGTGGTGAAATCTCC
GAGCTTAACTCGGAAACTGCCGTGGGTACTGCGTATCTTGAATGTTGTGG
AGGTGAGCGGAATATGTCATGTAGCGGTGAAATGCTTAGATATGACATAG
AACACCAATTGCGAAGGCAGCTCACTACACAAATATTGACGCTGAGGCAC

GAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTA
AACGATGATTACTCGACATACGCGATACACAGTGTGTGTCTGAGCGAAAG
CATTAAGTAATCCACCTGGGAAGTACGACCGCAAGGTTGAAACTCAAAGG
AATTGACGGGGGTCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGA
TACGCGAGGAACCTTACCTGGGCTAGAATGCTGGGAGACCGTGGGTGAAA
GCTCACTTTGTAGCAATACACTGCCAGTAAGGTGCTGCATGGCTGTCGTC
AGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCT
ATCAGTAGTTGCCAACAGGTCAAGCTGGGAACTCTACTGAAACTGCCGTC
GTAAGACGTGAGGAAGGAGGGGATGATGTCAAGTCATCATGGCCTTTATG
CCCAGGGCTACACACGTGCTACAATGGGGCGTACAAAGGGCTGCCACTTA
GTGATAAGGAGCGAATCCCAAAAAACGCCTCTCAGTTCGAATCGGAGTCT
GCAACTCGACTCC

>Unclassified_Gammaproteobacteria-53 clone SL-0440**(KF917097)**
AAGTCGGATGTGAAATCCCTGGGCTTAACCTGGGAACTGCATCCGATACT
GGTTGCCTAGAGTATGGAAGAGGGAAGCGGAATTCCAGGTGTAGCGGCGA
AATGCGTAGATATCTGGAGGAACATCAGTGGCGAAGGCGGCTTCCTGGTC
CAATACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCCTAAACGATGAGAACTAGTTGTTGGAAGGGTCT
GCCTTTCAGTGACGCAGCTAACGCGTTAAGTTCTCCGCCTGGGGAGTACG
GCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGCCCTTGA
CATGCCAGGAATCCCGCAGAGATGTGGGAGTGCCTTCGGGAGCCTGGACA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTGTCTCTAGTTACTAACAGTTAGGCTGAG
CACTCTAGAGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGT
CAAGTCATCATGGCCCTTATGGGCAGGGCTACACACGTGCTACAATGGAC
GGTACAGAGGGTCGCCAACCCGCGAGGGGGA

>Sedimentibacter sp. clone SL-0441**(KF917098)**
GACGGTAGCCAAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGGGGCGAGCGTTGTCCGGATTTACTGGGCGTAAAGGGTGA
GTAGGCGGTAATATGTGTCAGATGTAAAAGGCTAAAGCTTAACCATAGTT
AGCATTTGAAACTGTATTACTTGAGTGCAGGAGAGGTAAGTGGAATTCCT
AGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCAGTGGCGAAGG
CGACTTACTGGACTGTAACTGACGCTGAGTCACGAAAGCGTGGGTAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAGTGCTAGGT
GTTGGGTAGCGATACTCAGTGCCGAAGTAAACACAATAAGCACTCCGCCT
GGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCCGC
ACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAAGGCTTGACATCCCCTTGACCGGCACAGAGATGTGCCCTCTCCTTCGG
GAGCAAGGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCATTAGTTGCCAGC
ATTTAAGGTGGGCACTCTAATGAGACTGCCGATGATAAATCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTACACACGT
GCTACAATGGTCGGTACAAAGGGCAGCGAAGGAGCGATCTGGAGCAAATC
CCAATAAACCGATCTCAGTTCGGATTGTAGGCTGCAACTCGCCTAC

>Unclassified_Bacteria-19 clone SL-0442**(KF917099)**
AGGGGCGTAAACACCTTTTATGAGGAGAAAGTTTATTGATGTTACTCCAT
GAATAAGGGGCTCCCAATTCTGTGCCAGCAGGAGCGGTAATACAGAAGCC
CCAAGCGTTACCCGGATTTACTGGGCGTAAAGGATGCGTAGGCGGTTATA
TTAGTCAGGTGTTAAATCCTGGGGCTCAACCTCAGGCTCGCATTTGAAAC
GGTATAACTAGAAGGAATGAGAGGTGAACAGAACTCACGGTGTAGGGGTG
AAATCCGTTGATATCGTGGGGAATACCAAATGCGAAGGCAGTTCACTGGC
ATTTTCTTGACGCTGAGGCATGAAAGCGTGGGTAGCGAACGGGATTAGAT
ACCCCGGTAGTCCACGCCCTAAACGCTGTCTGCTAGCTATGAGGAGAATC
GACCCTCCTCGTGGCGTAGGTAACCCGATAAGCAGACCGCCTGGGTAGTA
CGAGCGCAAGCTTAAAACTCAAAGGAATAGACGGGGGCTCGCACAAGCGG
TGGAGCGCGTGGCTTAATTCGTCGCTAAGCGAAAAACCTTACCGAGGCTA

GATATCCTACTGCACGACCTGGGAAACCAGGTAAGCTTTCGAAGGTGTAG
GACAGGTGATGCATGGCCGTCGTCAGTTCGTGGCTTGAGCTGTTCCCTTA
AGTGGGGAAACGAACGCAACCCTCGTTGCCTGTTACAAGTGTCAGGCGAG
ACTGCTCCCTCACGGGAGAGGAAGGTGAGGATGACGCCAGGTCAGCATGT
CCCTTGATGCCTCGGGCTGCACACACGCTACAATGGGGTGCACAACGGGA
CGCAATATCGTAAGATGGAGCAAATCCTTATAAAACACCCCCCAGTTCGG
ATTGTGGGCTGCAACTCGCCCAC
>Bryobacter-02 sp. clone SL-0443**(KF917100)**
TAAAGGTCTTTCGACGGGGAAAATAATGATGGTACCCGTATAAGAAGGAG
CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGCTCCCAGCGTTG
TTCGGAATTACTGGGCGTAAAGCGAGTGTAGGCGGTCCATCAAGTTGGTT
GTGAAATCTCCTGGCTCAACTGGGAGGGTGCGACCAAAACTGATGGACTA
GAGTCTGGGAGAGGAGAGTGGAATTCCTGGTGTAGCGGTGAAATGCGTAG
ATATCAGGAGGAACACCGGTGGTGAAGACGGCTCTCTGGACCGGTACTGA
CGCTGAGACTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCCCTAAACGATGCGTACTTGGTGTAGGCTCTTCACTGAGTCTGT
GCCGGAGTTAACACGTTAAGTACGCCGCCTGGGGAGTACGGTCGCAAGGC
TGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGACGCAACGCGAAGAACCTTACCTAGGCTTGAACTGCAGTGG
CCGTTCTTAGAAATAGGAATTTCCCTTCGGGGACTGCTGTAGAGGTGCTG
CACGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTCGTCCTGTGTTACCAAAAATTGGGACTCGCAGGAGACC
GCCAGCGACAAGCTGGAGGAAGGTGGGGACGACGTCAAGTCATCGTGGCC
TTTATGTCTAGGGCTACACACGTGCTACAATGGGCGGTACAACGGGTTGC
GAAGTCGCAAGGCGGAGCTAATCCCTAAAAGCCGTCCTCAGTTCGGATTG
CAGGCTGCAACTCGCCTGCATGAAG
>Ohtaekwangia-14 sp. clone SL-0444**(KF917101)**
GCGTTGTCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGCCCTGTAAG
TCAGTGGTGAAATATTTCAGCTTAACTGAGAGGGTGCCATTGATACTGCA
GGGCTTGAGTACAGATGAGGTAGGCGGAATTGACGGTGTAGCGGTGAAAT
GCATAGATATCGTCAAGAACACCGATAGCGAAGGCAGCTTACCAAGCTGT
AACTGACGCTGAGGCACGAAAGTGTGGGGATCAAACAGGATTAGATACCC
TGGTAGTCCACACTGTAAACGTTGATGACTCGATGTTGGCGATACACAGC
CAGCGTCCAAGAGCAATCGTTAAGTCATCCACCTGGGGAGTACGCCGGCA
ACGGTGAAACTCAAAGGAATTGACGGGGGTCCGCACAAGCGGTGGAGCAT
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>Ohtaekwangia-15 sp. clone SL-0445**(KF917102)**
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>Altererythrobacter-02 sp. clone SL-0446**(KF917103)**
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>TM7-34_genera_incertae_sedis clone SL-0448**(KF917104)**
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>Unclassified_Betaproteobacteria-04 clone SL-0449**(KF917105)**
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CTGC
>Unclassified_Veillonellaceae clone SL-0450**(KF917106)**
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>Opitutus-02 sp. clone SL-0451**(KF917107)**
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>Parvibaculum-02 sp. clone SL-0452**(KF917108)**
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>Aquicella-02 sp. clone SL-0453**(KF917109)**
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>Unclassified_Betaproteobacteria-05 clone SL-0454**(KF917110)**
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>Steroidobacter-05 clone SL-0455**(KF917111)**
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>Planctomyces-03 sp. clone SL-0456**(KF917112)**
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GGAATCG
>Georgfuchsia-05 sp. clone SL-0457**(KF917113)**
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TGC
>Unclassified_Deltaproteobacteria-18 clone SL-0458**(KF917114)**
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>Gp4-12 clone SL-0459**(KF917115)**
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>Unclassified_Gammaproteobacteria_incertae_sedis-19 clone SL-0460**(KF917116)**
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>Unclassified_Rhodospirillales-03 clone SL-0461**(KF917117)**
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>Georgfuchsia-06 sp. clone SL-0462**(KF917118)**
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>Ohtaekwangia-16 sp. clone SL-0464**(KF917119)**
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>Unclassified_Sphingomonadaceae-04 clone SL-0466**(KF917120)**
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GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGATAACTAGCTGTCCGGGCTCTCAGAGCTTGGGTGGCGCAGCTA
ACGCATTAAGTTATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAA
AGGAATTGACGGGGGCCTGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
AGCAACGCGCAGAACCTTACCAGCGTTTGACATCCTTGTCGCGGATAGTG
GAGACACTTTCCTTCAGTTCGGCTGGACAAGTGACAGGTGCTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTCGTCCTTAGTTGCCATCATTCAGTTGGGCACTCTAAGGAAACTGC
CGGTGATAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCT
TACACGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGCAGCAA
CCCTGCGAGGGGTAGCTAATCTCCAAAAACCGTCTCAGTTCGGATTGTTC
TCTGCAACTCGAGAGCATGAAGGCGGAATCG
>Sulfuricella-03 sp. clone SL-0467**(KF917121)**
CAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTT
CGTAAGTCAGATGTGAAAGCCCCGGGCTTAACCTGGGAACTGCGTTTGAA
ACTGCGAGGCTAGAGTGTGGCAGAGGGGGGTAGAATTCCACGTGTAGCAG
TGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTG
GGCTAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAG
ACACCCTGGTAGTCCACGCCCTAAACGATGTCAACCAGTTGTTGGTGGAG
AAATCCATTAGTAACGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTA
CGGCCGCAAGGTTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG
TGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTT
GACATGCCAGGAACTTGCCAGAGATGGCTTGGTGCCCGAAAGGGAACCTG
GACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTGCCATTAATTGCCATCATTCAGTT
GGGCACTTTAATGGGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATG

GTCGGTACAGAGGGCAGCCAACCCGCGAGGGGGAGCCAATCCCAGAAAGC
CGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAAT
CG
>Bellilinea-17 sp. clone SL-0468**(KF917122)**
GCTAACTACGTGCCAGCAGCCGCGGTAAAACGTAGGAGGCAAGCGTTATC
CGGATTCACTGGGCGTAAAGCGCGTGCAGGCGGTTCGGTAAGTTGGGCGT
GAAATCTCCCGGCTCAACTGGGAGAGGTCGTTCAATACTACCGGGCTTGA
GAGCAGAAGAGGAAAGTGGAATTCCCGGTGTAGTGGTGAAATGCGTAGAT
ATCGGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTTTCTGACG
CTCAGACGCGACAGCTAGGGTAGTAAACGGGATTAGAGACCCCGGTAATC
CTAGCCGTAAACGATGTAAACTTGGCGTCGGTGGCTTAAACTCCATCGGT
GCCGCAGCCAACGCGATAAGTTTACCGCCTGGGGACTACGGCCGCAAGGT
TAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCGTGTGG
TTTAATTCGATGCTACACGAAGAACCTTACCCGGGTTTGACATGCAAGTG
GTAGTGATCTGAAAGGTGAACGACCCGCAAGGGAGCTTGCACAGGTGTTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTCGGTTAAGTCCGCTAAC
GAGCGCAACCCTCGCCACGTGTTACATGTGTCACGTGGGACCGCCGGTAT
CAAGCCGGGGGAAGGTGGGGATGACGTCAAGTCCGCATGGCCTTTATGTC
CGGGGCTACACACACGCTACAATGGGCAGTACAATGGGTCGCTAAACCGC
GAGGTGGAGCCAATCCCCCAAAGCTGTCCTCAGTTCAGATTGCAGGCTGC
AACCCGCCTGC
>Caldilinea-02 sp. clone SL-0470**(KF917123)**
GCTAACTACGTGCCAGCAGCCGCGGTAAAACGTAGGAGGCGAGCGTTATC
CGGATTTACTGGGCGTAAAGCGCGCGCAGGCGGTGCGGTGAGTCGGACGT
GAAAGCTCCTGGCTTAACTGGGAGAGGCCGTTCGATACTGCCACACTTGA
GGTTGGGAGAGGGGTGCGGAATTCCCGGTGTAGTGGTGGAACGCGTAGAG
ATCGGGAGGAACACCCGTGGCGAAGGCGGCACCCTGGCCCACACCTGACG
CTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACCATGTCAACTAGGTGTCGGCAGTGTTACACTGGCGGCG
CCGGAGCTAACGCGTTAAGTTGACCGCCTGGGGACTACGGCCGCAAGGCT
AAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCGTGTGGT
TTAATTCGATGCAACACGAAGAACCTTACCTGGGTTTGACATGACCGTAG
TAGTGAAGCGAAAGCGGAACGAGCCTTCGGGCAGCGGCCACAGGTGTTGC
ATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCCTGTTGCCAGTTATACGTGTCTGGCGAGACTGCCGGTATC
AAGCCGGAGGAAGGTGGGGATGACGTCAAGTCAGCATGACCTTTATATCC
AGGGCTACACACACGCTACAATGACCGGCACAATGCGTCGCCAAGCCGCG
AGGCGGAGCTAATCGCACCAAAACCGGTCTCAGTTCGGATTGGAGGCTGC
AACCCGCCTCCATGAAGCTGGA
>Unclassified_Gammaproteobacteria-54 clone SL-0471**(KF917124)**
GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGATTTAC
TGGGCGTAAAGCGCACGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCC
GGGCTCAACCTGGGAGCGGCATTCAATACTGGCAAACTGGAGTACGAGAG
AGGGGGGTGGAATTCCAGGTGTAGCGGTGAAATGCGTAGATATCTGGAGG
AACACCGGTGGCGAAGGCGGCCCCCTGGCTCGATACTGACACTGAGGTGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGTCGACTAGCCGTTGGGAAACTTGCTTTCTCAGTGGCGTAGCTA
ACGCGCTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAA
ATGAATTGACGGGGACCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGA
CGCAACGCGAAGAACCTTACCTGCTCTTGACATGTCGAGAACTTTCCAGA
GATGGATGGGTGCCTTCGGGAACTCGAACACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTGTCCTTAGTTGCCAGCATTTAGTTGGGGACTCTAGGGAGACTGCCGGT
GACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACG
AGCAGGGCTACACACGTACTACAATGGCCGGTACAAAGGGTTGCGAAAGC
GCGAGCTGGAGCCAATCCCAAAAAGCCGGTCGTAGTCCGGATTGGAGTCT
GCAACTCGACTCCATGAAGTCGGAATCG

>Bdellovibrio-02 sp. clone SL-0472**(KF917125)**
AAATTATGATGGTACCCTGTAAGAAAGGATCGGCTAACTTCGTGCCAGCA
GCCGCGGTAAGACGAGGGATCCCAGCGTTGTTCGGAATCATTGGGCGTAA
AGCGGGTGTAGACGGCTTTGTAAGTCAGGTGTGAAAGCCCAGGGCTCAAC
CCTGGAAGTGCATTTGATACTGCGAAGCTTGAGTGTGGGAGAGGCTAGTA
GAATTCCTGGTGTAGTGGTGAAATACGTAGATATCAGGAGGAATACCGGT
GGCGAAGGCGGCTAGCTGGCCCAACACTGACGTTGAGGCCCGAAAGCGTG
GGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCATAAACGATGGA
TACTTGTTGTTGGAGGTATTGACCCCTTCAGTGACGAAGCTAACGCGTTA
AGTATCCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGAAATTG
ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGC
GAAGAACCTTACCTAGGCTTGACATGTACTGGAAGAGTGGCAGAAATGTC
CTCGCCCGCAAGGGTCGGTACACAGGCGCTGCATGGCTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGCCTTT
AGTTGCCAGCATTTAGTTGGGCACTCTAGAGGGACCGCCGACGTTAAGTC
GGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCTAGGGC
TACACACGTGCTACAATGGGGCGTACAGACGGATGCATAGCCGCGAGGTG
AAGCCAATCCTACAAAACGCCTCTAAGTTCAGATTGCAGTCTGCAACTCG
ACTGCATGAAG
>Unclassified_Gammaproteobacteria_incertae_sedis-20 clone SL-
0473**(KF917126)**
ACCGAAAGGCTTTGACGTTACTTGCAGAAAAAGCACCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGG
GCGTAAAGCGCGCGTAGGCGGCTTGTTAAGTCGGATGTGAAATCCCCGAG
CTCAACTTGGGAACTGCATTCGATACTGACTCGCTAGAGTGTGGTAGAGG
GAAGTGGAATTCCAGGTGTAGCGGTGAAATGCGTAGATATCTGGGGGAAC
ATCAGTGGCGAAGGCGGCTTCCTGGACCAACACTGACGCTGAGGCGCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTCAACTAGCCGTAGGAAGCATCTGGCTTTTTGTGGCGCAGCTAACG
CGATAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAATG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGC
AACGCGAAAAACCTTACCTGCCCTTGACATGTCAGGAATCCTTCAGAGAT
GAGGGAGTGCCTTCGGGAGCCTGAACACAGGTGCTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTG
TCCTTAGTTGCCAGCGGTTCGGCCGGGAACTCTAAGGAGACTGCCGGTGA
TAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTATGGG
CAGGGCTACACACGTGCTACAATGGCCGGTACAGAAGGTTGCCAACCCGC
GAGGGGGAGCTAATCCTGTAAAGCCGGTCGTAGTCCGGATCGCAGTCTGC
AACTCGACTGC
>Unclassified_Gammaproteobacteria-55 clone SL-0474**(KF917127)**
CAGAATAAGCACCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGGAGG
GTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGTAGGTGGTTC
GTTAAGTCGGATGTGAAATCCCTGGGCTCAACCTGGGAGCTGCATTCGAT
ACTGGCGGACTCGAGTACGAGAGAGGGGGGTGGAATTCCAGGTGTAGCGG
TGAAATGCGTAGATATCTGGAGGAACACCAGTGGCGAAGGCGGCCCCCTG
GCTCGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGGAAC
TTGATTTCCCAGTGGCGTAGCTAACGCGCTAAGTCGACCGCCTGGGGAGT
ACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGACCCGCACAAGCG
GTGGAGTATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGCTCT
TGACATCCTGCGAACTTTCCAGAGATGGAAGGGTGCCTTCGGGAGCGCAG
AGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCATTTAGTTG
GGGACTCTAGGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGAC
GTCAAGTCATCATGGCTCTTACGAGCAGGGCTACACACGTACTACAATGG
CCGGTACAAAGGGTTGCGAAAGCGCGAGCTGGAGCCAATCCCAAAAAGCT
GGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATC

G
>Ferruginibacter-04 sp. clone SL-0475**(KF917128)**
GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATCCGGATTCAC
TGGGTTTAAAGGGTGCGTAGGTGGATTAGTAAGTCTGTGGTGAAATCTCC
GTGCTTAACTCGGAAACTGCCGTGGATACTATTAGTCTTGAATCTCCTGG
AGGTAAGCGGAATATGTCATGTAGCGGTGAAATGCTTAGATATGACATAG
AACACCAATTGCGAAGGCAGCTTACTACGGGAGCATTGACACTGAGGCAC
GAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGTCCTA
AACGATGATTACTCGACATACGCGATACACTGTGTGTGTCTGAGCGAAAG
CATTAAGTAATCCACCTGGGAAGTACGATCGCAAGATTGAAACTCAAAGG
AATTGACGGGGGTCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGA
TACGCGAGGAACCTTACCTGGGCTAGAATGCGGTCTGACCGCCTGTGAAA
GCAGGTTTTGTAGCAATACACAGATCGTAAGGTGCTGCATGGCTGTCGTC
AGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCT
ATCACTAGTTGCCATCAGGTAATGCTGGGAACTCTAGTGAAACTGCCGTC
GTAAGACGCGAGGAAGGAGGGGATGATGTCAAGTCATCATGGCCTTTATG
CCCAGGGCTACACACGTGCTACAATGGCGAGTACAAAGGGCAGCTACCTG
GTAACAGGATGCTAATCTCAAAAAACTCGTCTCAGTTCGAATTGGGGTCT
GCAACTCGACCTCATGAAGCTGGAATCG
>Aquimonas-04 sp. clone SL-0476**(KF917129)**
GTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTAC
TGGGCGTAAAGCGTGCGTAGATGGTGCGTTAAGTCGGATGTGAAAGCCCC
GGGCTCAACCTGGGAACTGCATCCGATACTGGCGGACTAGAGTGTGATAG
AGGATGGCGGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGG
AACATCCGTGGCGAAGGCGGCCATCTGGATCAACACTGACGTTGAGGCAC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTA
AACGATGTCGACTGGATGTTGGGTGCAACTTGGCACTCAGTATCGAAGCT
AACGCGTTAAGTCGACCGCCTGGGGAGTACGCGCGCAAGCGTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCG
ATGCAACGCGCAGAACCTTACCTGGTCTTGACATGTCGAGAACCCTGCGG
AGACGTGGGGGTGCCTTCGGGAACTCGAACACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTGTCCTTAGTTGCCAGCACTTCGGGTGGGAACTCTAAGGAGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTA
CGACCAGGGCTACACACGTACTACAATGGTCGGTACAGAGGGTTGCAATA
CCGCGAGGTGGAGCCAATCCCAGAAAACCGATCCCAGTCCGGATTGGAGT
CTGCAACTCGACTCCATGAAGTCGGAATCG
>Parachlamydia sp. clone SL-0477**(KF917130)**
GGCAAATTTGAGTGTACTTGGTAAAGAAGCACCGGCTAACTCCGTGCCAG
CAGCTGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTATTGGGCGT
AAAGGGCGCGTAGACGGGAAGGCAAGTCAGATGTTAAAGCCCGGGGCTTA
ACCCCGGAAAAGCATTTGAAACTGCCTTTCTTGAGGATAGACGGAGAAAA
CGGAATTCCACAAGTAGCGGTGAAATGCGTAGATATGTGGAAGAACACCG
GTGGCGAAGGCGGTTTTCTAGTTTATTCCTGACGTTGAGGCGCGAAAGCT
AGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCTAGCTGTAAACGATG
TATACTTGGTGTAGCCGGAATCAACCCTGGCTGTGCCGTAGCTAACGTGT
TAAGTATACCGCCTGGGGAGTACGCTCGCAAGGGTGAAACTCAAAAGAAT
TGACGGGGACCCGCACAAGCAGTGGAGCATGTGGTTTAATTCGATGCAAC
GCGAAGAACCTTACCTGGGCTTGACATGCATTGGACCGCTCTAGAAATAG
GGCTTCCCTTCGGGGCCGGTGCACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCAC
TAGTTGCCAACACGTAATGGTGGGAACTCTAGTGAGACTGCCTGGGTTAA
CCAGGAGGAAGGTGAGGATGACGTCAAGTCCGCATGGCCCTTATGTCCAG
GGCTACACACGTGCTACAATGGACGGTACAGAAGGCAGCTTAGCCGTGAG
GTAAAGCAAATCCTAGAAAGCCGTTCCCAGTTCGGATTGTAGTCTGCAAC
TCGACTACATGAAG

>Arenimonas-03 sp. clone SL-0478**(KF917131)**
GTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTAC
TGGGCGTAAAGCGTGCGTAGGTGGTTCGTTAAGTCAGATGTGAAAGCCCC
GGGCTCAACCTGGGAATTGCATTTGATACTGGCGGGCTAGAGTGCGGTAG
AGGAGAGTGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAGG
AACATCAGTTGCGAAGGCGGCTCTCTGGACCAGCACTGACACTGAGGCAC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTA
AACGATGCGAACTGGACGTTGGGAGCAATCAGGCTCTCAGTGTCGAAGCT
AACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCG
ATGCAACGCGCAGAACCTTACCTGGCCTTGACATCCACGGAAGCCCTGAG
AGATCGGGGTGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTGTCCTTAGTTGCCAGCGCGTAATGGCGGGAACTCTAAGGAGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
ACGGCCAGGGCTACACACGTACTACAATGGTGGGGACAGAGGGTCGCCAA
GGCGCGAGCCGGAGCCAATCCCAGAAACCCCATCCTAGTCCGGATCGGAG
TCTGCAACTCGACTCC
>Ferruginibacter-05 sp. clone SL-0479**(KF917132)**
TACTTTTAGTCTTGAATATCCTGGAGGTGAGCGGAATATGTCATGTAGCG
GTGAAATGCTTAGATATGACATAGAACACCAATTGCGAAGGCAGCTCACT
ACGGGATCATTGACACTGAGGCACGAAAGCGTGGGGATCAAACAGGATTA
GATACCCTGGTAGTCCACGCCCTAAACGATGGATACTCGACATACGCGAT
ATACTGTGTGTGTCTGAGCGAAAGCATTAAGTATCCCACCTGGGAAGTAC
GATCGCAAGATTGAAACTCAAAGGAATTGACGGGGGTCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCTGGGCTAG
AATGCGGTCTGACCGCCTGTGAAAGCAGGTTTTGTAGCAATACACAGATC
GTAAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCCTATCACTAGTTGCCATCAGGTAATGCT
GGGAACTCTAGTGAAACTGCCGCCGTAAGGCGTGAGGAAGGAGGGGATGA
TGTCAAGTCATCATGGCCTTTATGCCCAGGGCTACACACGTGCTACAATG
GCGAGTACAAAGGGCAGCTACCTGGTAACAGGATGCTAATCTCAAAAAAC
TCGTCTCAGTTCGAATTGGGGTCTGCAACTCGACCCCATGAAGCTGGAAT
CG
>Microvirga sp. clone SL-0480**(KF917133)**
GCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATCACTG
GGCGTAAAGGGCGCGTAGGCGGCTTTGTAAGTCGGGGGTGAAAGCCTGTG
GCTCAACCACAGAATTGCCTTCGATACTGCATGGCTTGAGACCGGAAGAG
GTAAGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAA
CACCAGTGGCGAAGGCGGCTTACTGGTCCGGTTCTGACGCTGAGGCGCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGAATGCCAGCCGTTGGCGAGCTTGCTCGTCAGTGGCGCAGCTAACG
CTTTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC
AACGCGCAGAACCTTACCAGCCTTTGACATGTCCCGTATGAGGAGTGGAG
ACACACCTCTTCAGTTCGGCTGGCGGGAACACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTCGCCCCTAGTTGCCATCATTGGGTTGGGCACTCTAGGGGGACTGCCGG
TGATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTA
CGGGCTGGGCTACACACGTGCTACAATGGCGGTGACAAAGGGCAGCGAAC
CCGCGAGGGGGAGCTAATCCTTAAAAGCCGTCTCAGTTCAGATTGCACTC
TGCAACTCGAGTGCATGAAGGCGGAATCG

Figure 1: The MWO clone library. Nucleotide sequences of the entire 437 clones in the library analyzed phylogenetically using the RDP-II database. Sequence similarity check was conducted for the entire sequences using the GenBank/EMBL/DDBJ database. Numbers in bold parentheses are accession numbers.