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Polymerase Chain Reaction-Based Restriction Fragment Length Polymorphism Analysis of the 16S-23S Ribosomal Genes Spacer Region in Sphingomonads

by

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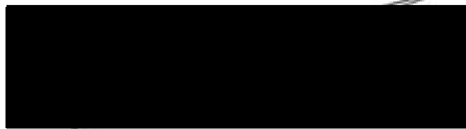

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ABSTRACT

Polymerase Chain Reaction-Based Restriction Fragment Length Polymorphism Analysis of the 16S-23S Ribosomal Genes Spacer Region in Sphingomonads

by Siba Riad Al-Medawar

The ability of sphingomonads present in drinking water to cause community and hospital acquired opportunistic infections has raised the need to establish rapid, reproducible, and feasible assays that could screen and identify sphingomonads to the genus, species and subspecies level. In this study, a total of 129 samples with yellow-orange-pigmented colonies were isolated from drinking water in Lebanon and were devided into 10 biotypes based on colony morphology. PCR-RFLP analysis of the 16S-23S ITS was done on 18 ATCC reference strains and 20 sphingomonas-specific 16S rRNA gene PCR positive isolates representing the ten biotypes to investigate the level of 16S-23S ITS (Intergenic Transcribed Spacer) region polymorphism and thus its ability to discriminate between the different sphingomonads. The first step was PCR amplification of the ITS, using two universal primers that target the constant regions flanking the ITS. This was followed by RFLP (restriction fragment length polymorphism) of the amplified ITS using 3 restriction endonucleases: *Hinf*I, *Alu*I, and *Bsp*143I., ITS size ranged between 400-1100 bp and was not variable enough to differentiate between *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis*. However, analysis of restriction products revealed differences in the number and size of bands obtained. Sixteen distinct banding patterns were recognized among the reference strains and twelve among the drinking water isolates. Each of the four genera had a unique and reproducible restriction pattern, but were also variable among species of the same genus, and strains of the same species. Several isolates had restriction patterns that were similar to those generated by the reference strains, and isolates having the same colony morphology generated the same restriction pattern. This study revealed that the ITS PCR-RFLP is a rapid technique that can be used to

generate molecular fingerprints of sphingomonads. To the extent of our knowledge, this study is the first comprehensive record of the different 16S-23S ITS sizes that can be found in the four major sphingomonad genera (*Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis*). Moreover, this is the first study that describes the use of 16S-23S ITS PCR-RFLP for subtyping the different sphingomonad species. However, employing a polyphasic approach, based on both biotyping and molecular fingerprinting, proved efficient in overcoming problems usually faced when attempting to identify organisms especially those of environmental origin.

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LIST OF ABBREVIATIONS

ARDRA: Amplified ribosomal DNA restriction analysis

ATCC: American Type Culture Collection

BT: Biotype

EPS: Exopolysaccharide

ERIC: enterobacterial repetitive intergenic consensus Polymerase Chain Reaction

GalAGSL: glycolipid α-galacturonosyl ceramide

GSL: Glycosphingolipid

HST: Hashwa Sima Tokajian

IL: Interleukine

IP: isoprenyl phosphate

IPP: isoprenyl phosphate precursor

ITS: Intergenic Transcribed Spacer

LPS: Lipopolysaccharide

MNC: Mononuclear cell

NKT: Natural Killer T-Lymphocyte

PCR: Polymerase Chain Reaction

PFGE: Pulsed-field gel electrophoresis

PMB: Polymyxin B

R: Reference strain

rDNA: ribosomal DNA

RFLP: Restriction Fragment Length polymorphism

rRNA: ribosomal RNA

TAE: Tris-acetate-EDTA

TBE: Tris-borate-EDTA

Tn5: Transposon 5

TNF: Tumour necrosis factor

tRNA: transfer RNA

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I dedicate this thesis to my beloved family

Chapter 1

INTRODUCTION

Most sphingomads are oligotrophic and are capable of surviving under low nutrient conditions such as soil and marine environments (Fegatella and Cavicchioli, 2000; Momma *et al.*, 1999), and are most commonly isolated from fresh water environments including water distribution systems, river water, mineral water, shower curtains, terrestrial subsurface sediments, sewage treatment plants (Tabata *et al.*, 1999; Fujii *et al.*, 2001; Lee *et al.*, 2001; Kelley *et al.*, 2004; Takeuchi *et al.*, 2001). Although most sphingomonads are environmental microorganisms, some strains have also been associated with nosocomial infections. Most of these nosocomial infections originate from contaminated medical devices (indwelling catheters, bronchofiberoscopes, and ventilators), solutions, and water (UV irradiated water used in surgery and dental unit water lines) (Yabuuchi *et al.*, 1990; Lemaitre *et al.*, 1996; Barbeau *et al.*, 1996). Contamination of these medical devices occurs when sphingomonads present in biofilms in water distribution systems recover from their dormant state upon transfer to a more hospitable environment (Mossel *et al.*, 2004).

In Lebanon, yellow-pigmented colonies are abundant in drinking water samples especially where an intermittent mode of supply is employed inducing frequent biofilm sloughing (Tokajian *et al.*, 2005; and Tokajian and Hashwa, 2004). This raised the need to establish rapid, reproducible, and feasible assays that could screen and identify sphingomonads to the genus, species and subspecies level.

Objectives:

- Develop an effective experimental approach that can be used to identify sphingomonads recovered from drinking water.
- Test the reliability of a polyphasic approach in which biotyping is combined with molecular fingerprinting.
- Determine the size of the ITS (Intergenic Transcribed Spacer) region in sphingmonads recovered from drinking water samples and ATCC reference strains through 16S-23S ITS specific PCR.
- RFLP (restriction fragment length polymorphism) of the amplified ITS using restriction endonucleases to determine the level of polymorphism in the ITS region in sphingomonads isolated from drinking water and compare them to ATCC reference strains.

Chapter 2

LITTERATURE REVIEW

2.1. Background:

The genus *Sphingomonas* (sphingosine containing monad) was first proposed by Yabuuchi *et al.* (1990), who had recocgnized that certain yellow pigmented, Gram-negative, rod-shaped bacteria, belonging to the alpha subclass of proteobacteria, had phenotypic and taxonomic properties that differentiated them from members of the genus that they have been assigned to. Comparative sequence analysis of the partial 16S rRNA of 16 strains showed low-homology with their type species. Moreover, cellular lipid and fatty acid analysis showed that these strains had a unique sphingolipid with acidic sugar such as glucoronic acid as a sole carbohydrate moiety, and 2-hydroxymyristic acid as a sole fatty acid component. In addition, these strains possessed ubiquinone 10 as the major respiratory quinone. According to these results Yabuuchi *et al.* (1990) proposed *Sphingomonas* genus with the type species *Sphingomonas paucimobilis*. Based on homology values (deoxyribonucleic acid, deoxyribonucleic acid hybridization, phenotypic characteristics, composition of cellular fatty acids, long chain bases, and total extractable lipids) three new species *S. parapaucimobilis*, *S. yanoikuyae*, *S. adhaesiva*, and one new combination, *S. capsulata* were also described. All members of the group were found to be motile with a single polar flagellum except for *S. capsulata*, which had a huge capsule and was, thus, nonmotile (Yabuuchi *et al.*, 1990). The yellow pigment of *S. paucimobilis* was identified as nostoxanthin, which serves as a

chemotaxonomic marker for nonphotosynthetic bacteria (Jenkins *et al.*, 1979).

Based on the complete 16S rDNA sequencing *Sphingomonas* was separated into four monophyletic clusters (Figure 1). These four clusters were assigned as four distinct genera including: *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* referred to collectively as sphingomonads (Takeuchi *et al.*, 2001). These genera were also separated based on certain morphological, physiological, biochemical, and genetic characteristics, which supported the phylogenetic classification. The Genus *Sphingomonas* had yellow or off-white colonies, with a G+C content of 62-68 mol%. The genus *Sphingobium* had yellow or whitish brown colonies with a G+C content of 62-67 mol%, while *Novosphingobium* had yellow or whitish brown colonies, and a G+C content of 62-67 mol%. Finally, *Sphingopyxis* had yellow or whitish brown colonies and a G+C content of 63-65 mol% (Takeuchi *et al.*, 2001).

2.2. Environmental and Medical Importance of Sphingomonads:

2.2.1. Rhizosphere:

Some sphingomonads live in close association with plants and are found in rhizosphere soil, or on plant roots. *Sphingomonas assdharolytica* and *Sphingomonas mali* were isolated from apple tree root, *Sphingomonas pruni* from peach tree roots, *Novosphingobium rosa from* rose roots, and *Sphingomonas roseiflava* isolated from ears of some Gramineae (Momma *et al.*, 1999; Takeuchi *et al.*, 1995; Yun *et al.*, 2000).

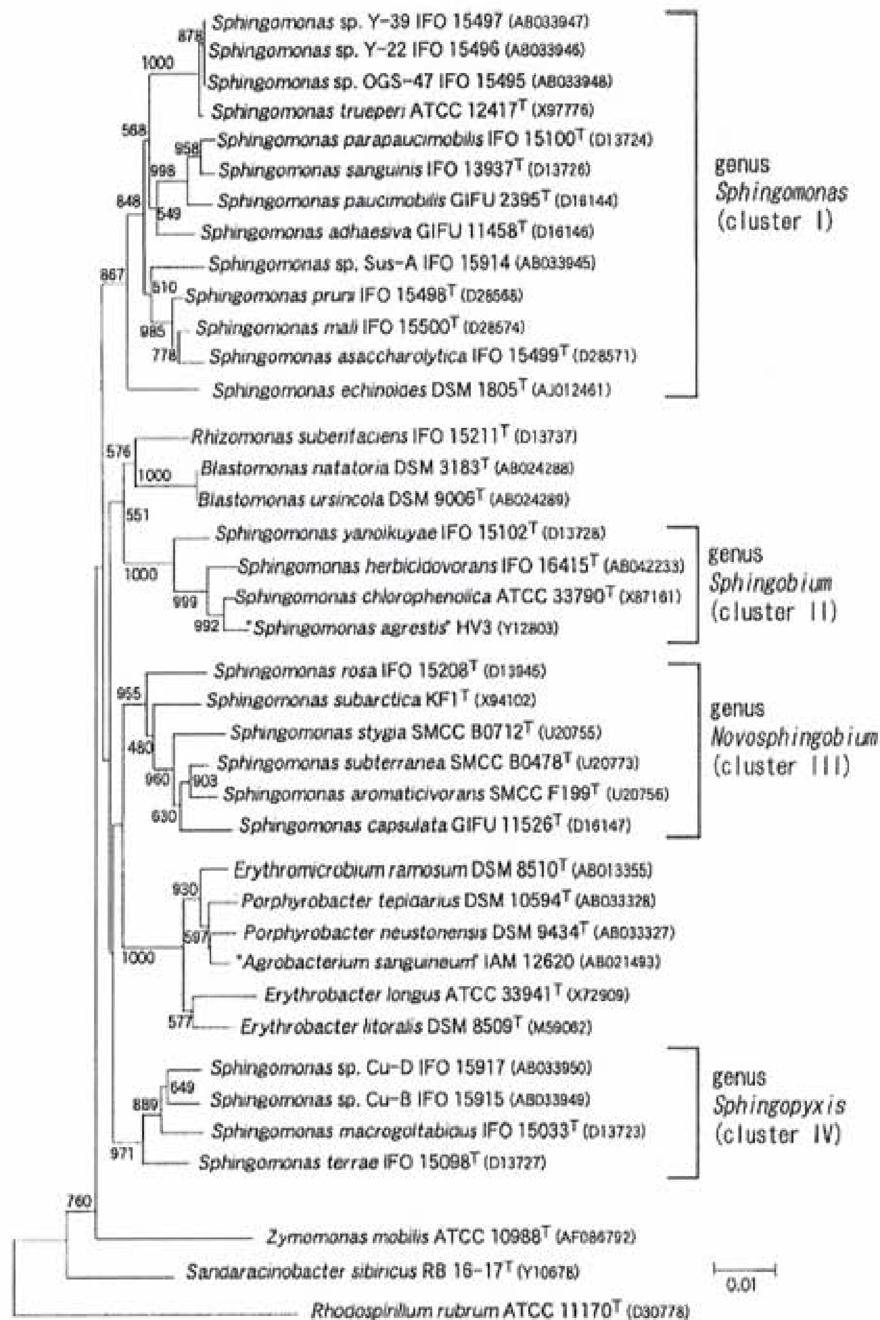

Figure 1: Phylogenetic tree based on complete 16S rDNA sequences separated the sphingomonas genus in to four monophyletic clusters *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* (Takeuchi *et al.*, 2001).

In most cases their association with plants is considered symbiotic-non-phytopathogenic, with some sphingomonads being recognized as antagonists for phytopathogens (plant pathogens) (Berg and Ballin, 1994; Kim *et al.*, 1998; Fukui *et al.*, 1999; Romanenko *et al.*, 2007). However, some sphingomonads could be phytopathogenic, as with *Sphingomonas melonis* that causes brown spots on yellow Spanish melon fruits and *Sphingomonas suberficiens,* which causes corkey root disease of lettuce (Buonario *et al.*, 2002; Van Bruggen *et al.*, 1990).

2.2.2. Oligotrophic environments:

Most sphingomads are oligotrophic and are capable of surviving under low nutrient conditions such as soil and marine environments, and are most commonly isolated from fresh water environments including water distribution systems, river water, mineral water, shower curtains, terrestrial subsurface sediments, sewage treatment plants (Fegatella and Cavicchioli, 2000; Momma *et al.*, 1999; Tabata *et al.*, 1999; Fujii *et al.*, 2001; Lee *et al.*, 2001; Kelley *et al.*, 2004; Takeuchi *et al.*, 2001). Sphingomonads are mesophiles growing in temperatures ranging from 20-40°C. Some strains although isolated from psychrotrophic temperatures, can still grow under mesophilic conditions (Eguchi *et al.*, 1996).

2.2.3. Exopolysaccharides and Biofilm Formation:

Some sphingomonads can exist in a sessile state under certain environmental conditions such as availability of carbon and nitrogen source and presence of oxygen. Sphingomonads that can exist in a sessile state are capable of forming aggregates (biofilm), which require

the synthesis of capsular exopolysaccharides that are similar in structure but not identical among the different sphingomonads and are collectively named as sphingans after the genus (Pollock, 1993). The specific structural variations within sphingans make them valuable for controlling the viscosity of aqueous solutions in many food and other industrial applications such as pharmeucetical industries (Fialho *et al.*, 1999). For example, GS-1, which is a sphingan formed by *Sphingomonas paucimobilis* strain GS-1, has a viscosity that is 5.5 fold greater than xanthan gum, produced by *Xanthomonas campestris*, and is stable over a pH range of 2-10. In addition, in the presence of NaCl, GS-1 has a gel strength 4 times that of agar and is able to withstand autoclaving (Ashtaputre and Shah, 1995). Like most biofilms, the architecture of the one developed by sphingomonads was shown to be mushroom shaped and formed of microcolonies (Venugopalan *et al.*, 2005). The three phases of biofilm formation, which are described by Davy and O'toole (2000) include: attachment, formation of microcolonies, and exopolysaccharides (EPS) production leading to mature biofilm formation. EPS production in sphingomonads is not needed during the attachment phase, rather it is required during the maturation phase, where it is used for development of the biofilm architecture (Venugopalan *et al.*, 2005).

The synthesis of sphingans and biofilm production are controlled by environmental factors as well as genetic determinants. Presence of nitrogen and carbon, salt concentration, temperature, pH, and the availability of oxygen are the major factors affecting the synthesis of sphingans and biofilm formation, which can be used to enhance sphingans production for industrial use. Sphingans synthesis requires the presence of certain enzymes and membrane transporters, which

are encoded by gene clusters that are homologous among the different sphingan producers (Vartak *et al.*, 1995).

2.2.4. Medical importance:

There has been an increasing awareness of the possible role of heterotrophic organisms that grow at ambient temperatures in causing opportunistic infections. Although most sphingomonads are environmental microorganisms, some strains have also been associated with nosocomial infections mainly through waterborne routes (Laskin and White, 1999). Sphingomonads can colonize different body sites without causing any disease symptoms. Infections with sphingomonads are rare in healthy immunocompetent people but can cause infections in immunocompromised patients that are not considered as serious as other Gram-negative bacteria (such as *Pseudomonas*) when treated with the proper antibiotics (Hsueh *et al.*, 1998). Lemaitre *et al.* (1996) reported the isolation of *S. paucimobilis* from tracheal secretions of 85 mechanically ventilated babies due to contaminated respiratory ventilators. Intrestingly, non of the babies developed pneumonia or sepsis. Hsueh *et al.* (1998) isolated *S. paucimobilis* from clinical specimens from six patients with nosocomial infections. Two of the six patients had intravascular catheter-related bacteremia and the other four patients had bacteremic biliary tract infection, urinary tract infection, ventilator-associated pneumonia, and wound infection. The same isolates were also recovered from blood and bile of one of the patients, which indicated that *S. paucimobilis* can be invasive in such patients.

Other Sphingomonads isolated from clinical specimens include: *S. adhesiva* from blood, *S. parapaucimobilis* from urine, vaginal swab, and sputum specimen, *S. sanguinis* from blood (Christakis *et al.*, 2004; Yabuuchi *et al.*, 1990). Most of these nosocomial infections originate from contaminated medical devices (indwelling catheters, bronchofiberoscopes, and ventilators), solutions, and water (UV irradiated water used in surgery and dental unit water lines) (Yabuuchi *et al.*, 1990; Lemaitre *et al.*, 1996; Barbeau *et al.*, 1996). Contamination of these medical devices occurs when sphingomonads present in biofilms in water distribution systems recover from their dormant state upon transfer to a more hospitable environment (Mossel *et al.*, 2004). Sphingomonads have also been associated with copper pipe degradation in water distribution system (Laskin and White, 1999).

2.3. Catabolism:

The wide distribution of sphingomonads in nature is probably due to their broad catabolic versatility. Sphingomonads can catabolize various sugars, polysaccharides, aromatic compounds, and other xenobiotics. The sugar that is assimilated by most sphingomonads is glucose (Yabuuchi *et al.*, 2002). Sphingomonads also produce polysaccharide lyases that degrade exopolysaccharides such as alginate (Hisano *et al.*, 1996).

2.3.1. Genetics and Genomics

Studying the genetics of sphingomonads is needed to understand sphingans biosynthetic pathways required for industrial applications, and explore the evolution of catabolic pathways for chemicals that can

be used in bioremediation (Pollock *et al.*, 1994; and Yamazaki *et al.*, 1996; Feng *et al.*, 1997).

Large plasmids found in sphingomonads contain aromatic catabolic properties and may play a significant role in the evolution and dissemination of many genes required for the metabolism of pesticides. Five plasmids required for metabolism of carbofuran were isolated from CF06. Loss of the plasmids induced by growth at 42°C or Tn5 (transposon 5) introduction resulted in the inability of the strain to grow on carbofuran as a sole source of carbon. When these plasmids were introduced into *Pseudomonas fluorescens* M480R, this organism was able to use carbofuran as a sole source of carbon for growth and energy (Feng *et al.*, 1997).

Uptil now the genome of few sphingomonads and their plasmids have been sequenced. The aromatic catabolic plasmid, pNL1, from *Novosphingobium aromaticivorans* F199 was the first conjugative plasmid that encodes pathways for the complete catabolism of aromatic organic compounds to be completely sequenced (Romine *et al.*, 1999). Almost half of the pNL1 plasmid carries genes that are probably involved in either transport or catabolism of aromatic compounds. The rest of the plasmid encodes for other functions such as plasmid maintenance, transfer, or replication (Figure 2) (Romine *et al.*, 1999).

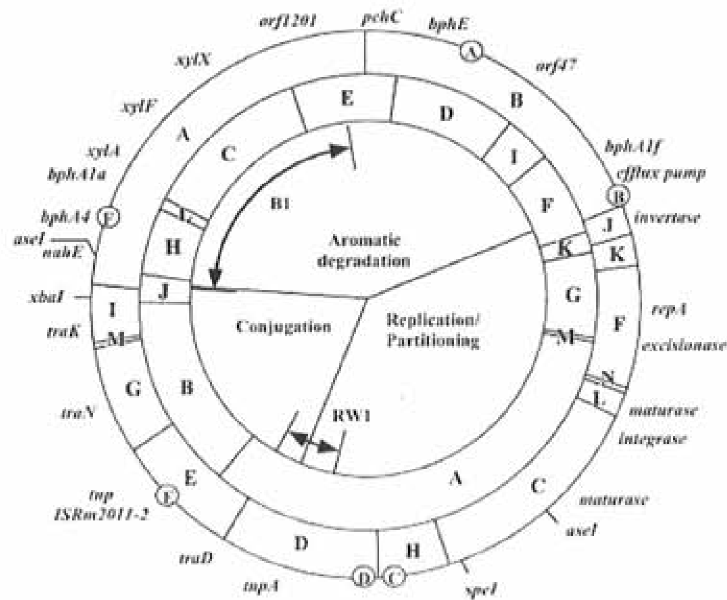

Figure 2: Physical map of pNL1. Bidirectional arrows show regions with homology to *S. yanoikuyae* B1 and *Sphingomonas* sp. strain RW1 (Romine *et al.*, 1999).

2.4. Membrane structure

The cell envelop of sphingomonads is made up of a cell membrane (consists of proteins, phospholipids, and respiratory quinones) and an outer membrane, which is mainly made up of glycosphingolipids (GSLs) in addition to some proteins and phospholipids (Figure 3) (Kawasaki *et al.*, 1994).

Two major types of GSLs have been isolated from *S. paucimobilis*, the monoglycosylated GSL-1 and the tetraglycosylated GSL-4A (Kawahara *et al.*, 1991). GSL1 is more abundant in the outer membrane, while GSL-4A affects the surface hydrophobicity and antigeicity (Figure 3) (Kawahara *et al.*, 1999).

The presence of GSLs and the absence of LPS in the outer membrane of sphingomonads is the most important characteristic that

distinguishes these bacteria from other Gram-negative bacteria (Kawahara *et al.,* 1991). Similar to LPS, the carbohydrate chain of GSLs is exposed to the outside of the cells (Kawasaki *et al.,* 1994). However, due to the shorter carbohydrate chain in GSL the cell surface of sphingomonads is more hydrophobic than that of other LPS containing Gram-negative bacteria. The high hydrophobicity explains the ability to metabolize aromatic compounds due to the influx of hydrophobic substances (Kawahara *et al.,* 1999).

GSLs have additionally many antigenic and structural functions. GSL-4A induces the release of TNF, IL-6, and IL-1 by human mononuclear cells (MNC) similar to LPS. However, GSL-1 does not induce their release but instead it inhibits GSL-4A-induced IL-1 and IL-6 release (Krizwon *et al.,* 1995). GSLs also affect the rate of incorporation and the gating behavior of porin molecules (Wiese et al., 1996), activate the alternative instead of the classical complement pathway (Wiese et al., 1996), and stimulate phagocytosis and phagosome-lysosome fusion by human polymorphonuclear leukocytes (PMN) (Miyazaki *et al.,* 1995).

S. paucimobilis glycolipid α-galacturonosyl ceramide (GalAGSL) was the first bacterial antigen described that could activate natural killer T (NKT) cells (Wu *et al.,* 2005). Previously, it was known that NKT cells play a role in autoimmunity and cancer (Hong *et al.,* 2001; Kawano *et al.,* 1998). The role of NKT cells in host defense against microbial infections was not clear (Wu *et al.,* 2006). The recent discovery of GalAGSL in sphingomonads as a NKT cell antigen, established the role of NKT cells in antimicrobial defense (Wu *et al.,* 2005). Thus, GalAGSL serves as an alternative to the LPS, which normally activate dendritic and other cell types, in the innate immune response (Wu *et al.,* 2006).

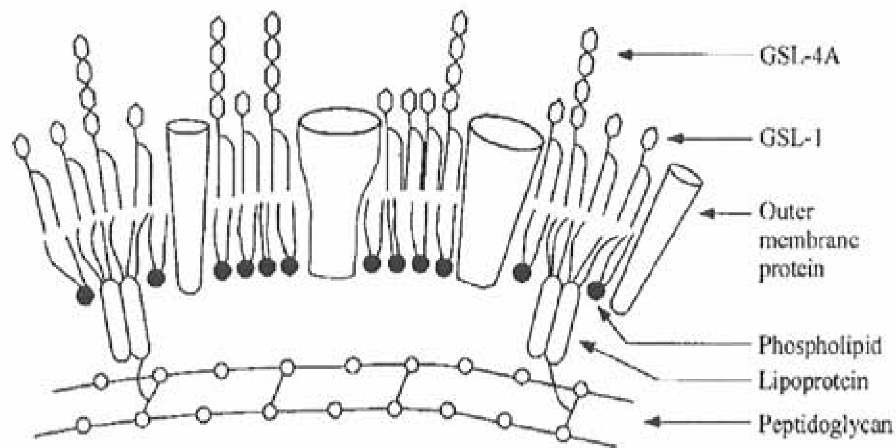

Figure 3: Schematic representation of membrane composition of *S. paucimobilis* (Kawahara *et al.*, 1999).

2.5. Antibiotic Susceptibility and treatment:

Some sphingomonads produce β-lactamases, and are thus resistant to β-lactam containing antibiotics. Moreover, the high membrane hydrophobicity of sphingomonads where the carbohydrate portion of GSL is much shorter and simpler than that of LPS, makes sphingomonads susceptible to hydrophobic antibiotics since it allows their influx (Smalley *et al.*, 1983). GSLs also cause the reduction in the surface charge density, and thus reduction of bound Polymyxin B (PMB) rendering sphingomonads resistant to PMB (Wiese *et al.*, 1996).

Treatment of infections caused by sphingomonads involves catheter removal in case of catheter related sepsis along with the proper antibiotic treatment. Usually imipenem alone or a third generation cephalosporin plus an aminoglycoside are adequate to treat such infections (Hseueh *et al.*, 1998).

2.5.1. Mechanism of Bacitracin Resistance:

The mechanism of resistance to bacitracin was studied by Pollock *et al.* (1994). Bacitracin binds to isoprenyl phosphate (IP) precursor (IPP) which is needed for essential synthesis of peptidoglycan and exopolysaccharides (Stone *et al.*, 1971). The reason why sphingomonads are able to survive bacitracin treatment was that IPP produced for exopolysacchride synthesis is in excess and will chelate bacitracin residues allowing other unbound IPP to support essential peptidoglycan synthesis (Pollock *et al.*, 1994).

2.6. Isolation and Identification:

The ability of sphingomonads to cause apportunistic infection urges the need to explore new methods for rapid isolation and identification. According to Yabuuchi *et al.* (1990), identification of sphingomonads is based on the isolation of Gram-negative, chemoheterotrophic, strictly aerobic, rods that produce yellow-pigmented colonies due to the carotenoid pigment nostoxanthin that is present in most species. Furthermore, the presence of 2-hydroxymyristic acid (14:0 2-OH) in sphingomonads as the major hydroxylated fatty acid instead of 3-hydroxy fatty acids, and sphingoglycolipids instead of lipopolysaccharide usually present in Gram-negative bacteria are all characteristic markers for the genus *Sphingomonas* (Yabuuchi *et al.*, 1990).

However, there is no one protocol that is designed for isolation and identification of sphingomonads. Different enrichment cultures and identification techniques have been used depending on the site of

isolation (drinking water, medical devices, clinical specimens, and chemically contaminated environments). If the sample is obtained from a contaminated site (sediment, soil, or water) and it's showing a certain biodegradation process then the isolation would be carried out by placing the inoculum from the site on an enrichment medium that contains the target contaminant as the sole source of carbon and energy together with mineral salts. The initial culture should be incubated for a long period of time followed by successive subculturing (Sorensen *et al.*, 2001). However, sphingomonads, recovered from drinking water, are oligotrophic and can be enriched on low nutrient conditions such as the R2A medium (Reasoner and Geldreich, 1985; Tokajian *et al.*, 2005).

Currently, there are three approaches for identification and classification of bacteria. The first approach is based on the use of standard characteristics including morphological, physiological and biochemical features. The second is based on genotypic and molecular methods. Finally, a combination of the two approaches constitutes what is known as the polyphasic approach, which is the most common choice for the identification and reclassification of microorganisms (Prakash *et al.*, 2007).

2.6.1. Biochemical Approaches for Identification of sphingomonads:

Identification of sphingomonads based on their biochemical profiles is achieved through many commercially available kits including Biolog (Biolog, Inc., Hayward, California), API (bioMerieux, Marcy-L'Etoile, France), and Biotype 100 (biomerieux). The metabolic pattern (metabolic fingerprint) from 95 biochemical tests can be generated using the Biolog system. The bacteria are suspended in a special

inoculating fluid. Tetrazolium violet, which is present in all the wells, is used as an indicator redox dye forming a purple color upon substrate oxidation. After 24 hours of incubation, wells are keyed into Biolog's computer system, which cross-references the pattern to an extensive library of species. Sorensen *et al.* (2001) used the Biolog system for identification of *Sphingomonas* sp. (SRS2), and Yang *et al.* (2005) tested the ability *S. chlorophenolica*, to utilize (oxidize) various carbon sources. In addition, Pollock (1993) added bacterial isolates secreting gellan-related polysaccharides as *Sphingomonas* based on metabolic fingerprints generated using the Biolog system, fatty acid profiles, and pigment spectroscopy. On the other hand, the API 20NE and Biotype 100 were used by Yabuuchi *et al.* (2002) for biochemical characterization of different sphingomonads. Biolog and all the biochemical identification schemes mentioned earlier have many limitations, as they may yield results that are usually not reproducible, ambiguous and misleading. A metabolic pattern could be obtained in many instances with no species ID because of the lack of similar metabolic patterns in the database; the data base being limited to clinical rather than environmental isolates (Amy *et al.*, 1992; Tokajian and Hashwa, 2004; Tokajian *et al.*, 2005).

2.6.4. Molecular Approaches for Identification of Sphingomonads:

Various molecular approaches have been applied for the identification of sphingomonads to the species and subspecies level. ERIC-PCR (enterobacterial repetitive intergenic consensus Polymerase Chain Reaction), and BOX-PCR (BOX element PCR) were used by Busse *et al.* (2003), and Buonaurio *et al.* (2001) to determine relatedness among newly isolated sphingomonas species. Pulsed-field gel electrophoresis

(PFGE) was used by Eguchi *et al.* (2001) to determine the genome size of strain AFO1 that was isolated from the north pacific and was compared to the banding pattern of that demonstrated by *S. alaskensis* RB2256.

Another DNA-based typing method is Ribotyping. The genes coding for ribosomal RNAs in prokaryotes are arranged in an operon in the following order 5'-16S-23S-5S-3' which is separated by two spacer regions known as ITS (Intergenic Transcribed Spacer) (Condon *et al.*, 1995). Each species can contain from one to eleven ribosomal operons depending on its growth rate (Garcia-Martinez *et al.*, 1996).

The most commonly used components in ribotyping are 16S rRNA gene and the 16S-23S ITS. Yabuuchi *et al.* (1990) used partial 16S rRNA sequencing for the phylogenetic analysis of sphingomonads, while the complete 16S rRNA sequence was used to describe the four main genera: *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis*. Moreover, a PCR based assay using sphingomonas specific primers within the 16S rRNA gene was first suggested by Leys *et al.* (2004). However, this PCR, although very specific, could not identify sphingomonads to the species or sub-species level. Amplified ribosomal DNA restriction analysis (ARDRA) which includes: PCR amplification of 16S rRNA gene followed by restriction digestion and electrophoresis, was also used for PCR-based fingerprinting (Han *et al.*, 2003). However, different 16S genes cannot be easily separated based on the size, which is nearly constant, and it is not often divergent enough to yield good separation between species of the same genus limiting its use (Linton *et al.*, 1994a; Linton *et al.*, 1994b; Rainey *et al.*, 1996; Normand *et al.*, 1996).

This can be solved by targeting the ITS. The size and sequence of the ITS, especially between 16S and 23S rDNA, can be extremely variable

between species, strains, and even among different operons within the same cell (intercisronic) (Condon *et al.*, 1995). This variability is mainly because most of the ITS is made of non-coding sequences which puts it under less evolutionary pressure and makes it more prone to undergo insertion deletion events and thus more genetically variable than the other rRNA coding regions incluing 23S and 16S rRNA genes. Moreover, the size of ITS depends largely on the number of functional units present within the ITS such as tRNAs which ranges from none to two per spacer. Other functional units include: sequences for the recognition of enzymes such as the ribonuclease III, and antiterminator sequences such as *boxA* (Colleran *et al.*, 1991; Gürtler and Stanisich, 1996; Normand *et al.*, 1996; Bram *et al.*, 1980; Harvey *et al.*, 1988; and Berg *et al.*, 1989) (Figure 4). Although the ITS doesn't code for a final product, it has an important function in processing pre-RNAs due to the presence of these functional units, which are conserved among closely related species because they are under more selective pressure than the other non-coding sequences (Garcia-Martinez *et al.*, 1996).

The alternation of conserved and variable zones in the ITS constitutes a good target for designing PCR primers and other molecular probes (Garcia-Martinez *et al.*, 1999). The amplification of the ITS using primers targeting the constant regions flanking it can yield a characteristic banding pattern made up of one/several band(s) which have the same or different size depending on the number of ribosomal operons and number of tRNAs per spacer in this cell (Condon *et al.*, 1995). In addition, PCR amplification of the ITS can be combined with sequencing and/or restriction digestion for identification purposes. For example, Ferrera *et al.* (2006) used ITS sequencing for phylogenetic analysis, while Matar *et al.* (1993) used a panel of five

restriction enzymes including *AluI*, *HaeIII*, *TaqI*, *HinfI*, and *MseI*, to digest the ITS and generate fingerprints for ribotyping different *Rochalimaea* species.

So far only one study used the ITS to study genetic similarity among sphingomonads isolated from soil (Johnsen *et al.*, 2002). However, the ITS amplification was only a small part of the study and there was no mention of the different sizes or sequences of the ITS PCR product. Nowadays, many databases for the ITS sequence and size among different classes of microorganisms exist (Garcia-Martinez *et al.*, 2001). However, there is no available database for the ITS sequences and sizes among the different sphingomonads.

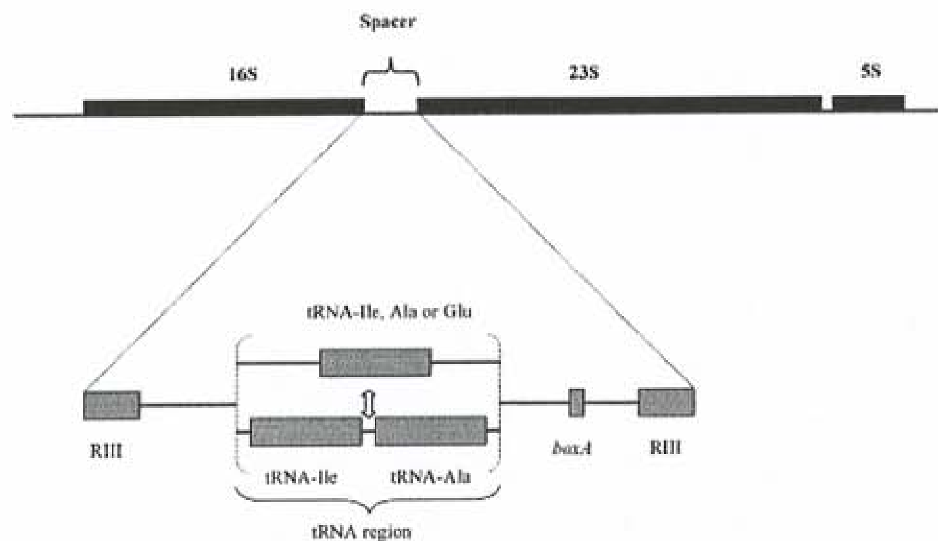

Figure 4: Schematic representation of The ITS showing it's position within the rRNA genes and three functional region including tRNA genes and sequences for the recognition of enzymes such as the ribonuclease III, and *boxA* (Garcia-Martinez *et al.*, 1999).

Chapter 3

MATERIALS AND METHODS

3.1. Bacterial strains

The study was conducted using all forms and derivatives of yellow pigmented colonies isolated both from an intermittent drinking water distribution network (Tokajian *et al.*, 2005), and Polyethylene and cast iron household storage tanks in Lebanon over a period of two years (Tokajian and Hashwa, 2004). The isolates were designated as HST-1 through 129.

3.2. Reference strains

Reference strains used in this study were obtained from the ATCC (Table 1). Growth conditions (growth media, incubation time, and incubation temperature) used were according to ATCC recommendations.

3.3. Isolation and purification of yellow pigmented colonies

Yellow pigmented colonies were isolated and purified on R2A agar (Oxoid) (Reasoner and Geldreich, 1985). Plates were incubated at 28°C for 7 days. Pure colonies were kept at -20 °C on glycerol. A total of 113 derivatives of yellow pigmented colonies, out of around 129, representing all the different morphological entities within this population were chosen and used for biotyping, 16S rRNA gene based studies, and finger printing.

Table 1. ATCC reference strains.

ATCC #	Species
33790	*Sphingobium chlorophenolicum*
53874	*Sphingobium chlorophenolicum*
49356	*Sphingomonas suberifaciens*
51230	*Sphingobium yanoikuyae*
51231	*Sphingomonas parapaucimobilis*
51380	*Sphingopyxis macrogoltabida*
51382	*Sphingomonas sanguinis*
51838	*Sphingomonas pruni*
51839	*Sphingomonas asaccharolytica*
51840	*Sphingomonas mali*
700279	*Novosphingobium subterraneum*
700280	*Novosphingobium stygium*
BAA-1092	*Sphingomonas paucimobilis*
51381	*Sphingomonas terrae*
51837	*Sphingomonas rosa*
1466	*Novosphingobium capsulatum*
51229	*Sphingomonas adhaesiva*
35951	*Blastomonas natatoria*

3.4. Biolog and Metabolic fingerprinting

The chosen colonies were identified using the Biolog (Biolog, Inc., Hayward, California) microbial identification system. The metabolic profile of each organism using the Biolog microplates was compared automatically, by using the MicroLog software, with the MicroLog GN database (release 4.01A). Biolog identifications were reported if the similarity index of the genus or species was 0.5 or greater at 24 h of incubation. A phenogram was generated using the UPGMA algorithm (CLC bio A/S, Denmark).

3.5. DNA Extraction

DNA extraction was done using the NucleoSpin DNA extraction kit (Macherey-Nagel, Germany), according to the manufacturer's instructions.

3.6. PCR amplification

All PCR assays were performed on PerkinElmer GeneAmp 9700 (PerkinElmer, Wellesly, Massachusetts).

3.6.1. 16S rRNA Gene Amplification

16S rRNA gene amplification was used as positive PCR control ensuring the integrity of all sample DNA used for 16S rRNA gene based studies, and finger printing. The 16S rDNA gene was amplified using the gene sequence specific primers: forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (TiBMolBiol, Germany) (Lane *et al.*, 1985). The amplification reaction contained 1.5 µl DNA extract, 1U AmpliTaq Gold polymearse (Applied Biosystems, USA), 2.5 mM $MgCl_2$, 1X PCR buffer, 0.4 mM of each deoxynucleoside triphosphate (dNTP), and 0.25 µM of the forward and reverse primers in a final volume of 20 µl. The cycles used were as follows: initial Denaturation at 95°C for 2 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 53 °C for 30 sec and elongation step at 72°C for 2 min. The expected PCR amplicon was around 1500 bp long and was visualized by ethidium bromide staining on 1% agarose gel using 1x TAE buffer.

3.6.2. Sphingomonas-specific 16S rRNA gene based PCR assay

The Sphingomonas-specific modified primer set consisting of the forward primer Sphingo 108f (5'-GCGTAACGCGTGGGAATCTG-3') and the reverse primer Sphingo 420r (5'-TTACAACCCTAAGGCCTTC-3') (Leys *et al.*, 2004). The PCR mixture contained 2 μl DNA, 1 U of AmpliTaq Gold *polymerase* (Applied Biosystems, USA), 20 pmol of the forward and reverse primers, 10 nmol of each deoxynucleoside triphosphate (dNTP), 2.5 mM $MgCl_2$ and 1X PCR buffer in a final volume of 50 μl (Leys *et al.*, 2004). PCR amplification was comprised of the following 3 steps: heating at 95 °C for 5 min; 50 cycles of denaturation at 95°C for 5 sec, annealing at 62 °C for 10 sec and extension at 74 °C for 30 sec, and a final extension at 74 °C for 2 min. The expected PCR amplicon was around 320 bp long and was visualized by ethidium bromide staining on 2.5% agarose gel using 1x TAE buffer.

3.7. Molecular Fingerprinting

3.7.1. ITS PCR amplification

The ITS region was amplified using the modified forward primer 1492F (5'-AAGTCGTAACAAGGTAACC-3') targeting the end of 16S rRNA gene, and the reverse primer 115R (5'-GGGTTBCCCCATTCRG-3') that targeted the 23S rRNA gene (Garcia- Martinez *et al.*, 1999).

The PCR reaction mixture contained 1-10 ng DNA template, 1 U of AmpliTaq Gold *polymerase* (Applied Biosystems, USA), 200 mM of

each dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer, and 0.4 μM of each primer in a final volume of 50 μl. PCR amplification was comprised of the following 3 steps: initial cycle of 94 °C for 5 min, 30 cycles of denaturation at 94 °C, for 30 sec, annealing at 48 °C for 30 sec, and extention at 72 °C for 1 min and a final extension for 5 min at 72 °C. The PCR product was visualized by ethidium bromide staining on a 1.5% agarose gel using 1x TBE buffer.

3.7.2. ITS Restriction digestion

Amplicon DNA was digested without any purification with the following enzymes, *Hinf*I, *Alu*I, and *Bsp*143I (Fermentas). 0.5 μL of each enzyme was added to 25 μl of the ITS PCR product (concentration around 0.25 μg/μl) along with 2.5 μl 10x tango buffer for 16 hours at 37 °C (Fermentas). The restriction reaction was stopped at 65° C for 20 mins (Fermentas). The digested DNA was then visualized by ethidium bromide staining on a 3% agarose gel using 1x TBE buffer.

Chapter 4

RESULTS

4.1. Isolation and purification of yellow pigmented colonies

Ten biotypes, based on colonial morphology, were recovered from water storage tanks, and water distribution networks in Lebanon over a period of two years (Figure 5d and Figure 5e). The biotypes (BT) were as follows: BT1: whitish yellow , BT2: light translucent yellow, BT3: light opaque yellow, BT4: large yellow mucoid, BT5: small yellow mucoid, BT6: translucent orange, BT7: fuzzy orange, BT8: yellowish orange , BT9: dark yellow , BT10: brown. BT9 was the most predominant biotype, while the least predominant were BT2 and BT3 (Figure 6). Reference strains were designated as R1 to R17. R1 (*S. paucimobilis*) and R14 (*N. capsulatum*) had large yellow mucoid colonies. R4 (*S. yanoikuyae*) had whitish yellow colonies. R16 (*S. rosa*) had fuzzy orange colonies. R2 (*S. macroglotabida*), R5 (*N. subterraneum*), R10 (*S. chlorophenolicum* 1: ATCC 53874), R12 (*S. asaccharolytica*), R7 (*S. pruni*), and R9 (*Sphingomonas mali*) had light yellow colonies. R6 (*N. stygium*) had light opaque yellow colonies; and R13 (*S. adhaesiva*), R3 (*S. sanguinis*), R8 (*S.pararpaucimobilis*), R15 (*Sphingomonas terrae*), R17 (*Sphingomonas suberifaciens*) had dark yellow colonies, R11 (*S. chlorophenolicum* 2: ATCC 33790) had bright yellow colonies, and R18 (*B. natatoria*) had brown colonies (Figure 5 and Figure 5c).

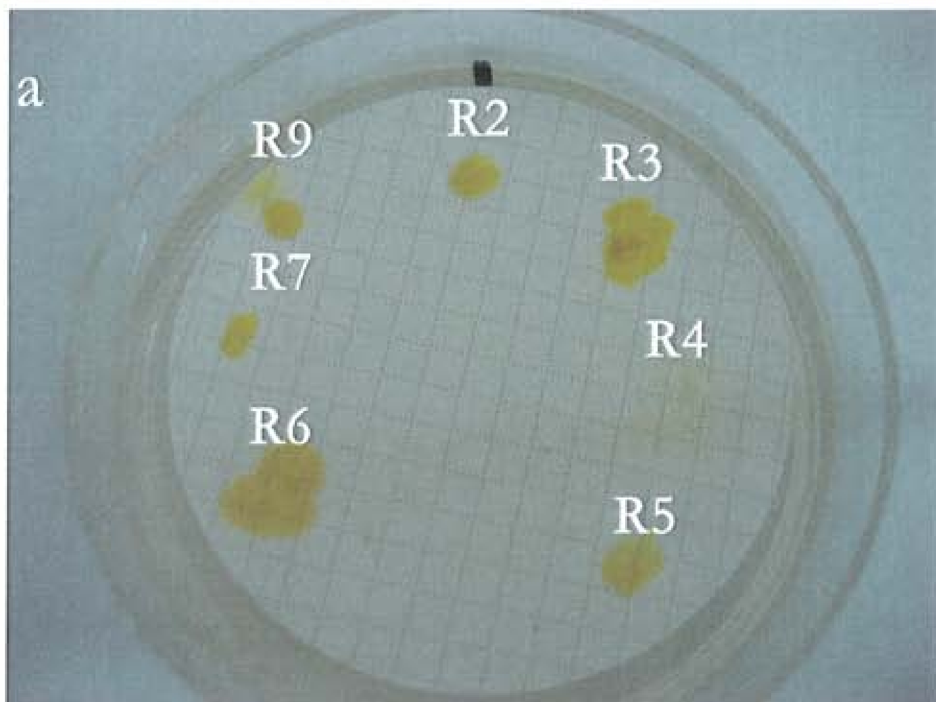

Figure 5a: Colonies representing the following ATCC reference strains: R2 (*S. macroglotabida*), R3 (*S. sanguinis*), R4 (*S. yanoikuyae*), R5 (*N. subterraneum*), R6 (*N. stygium*), R7 (*S. pruni*), and R9 (*Sphingomonas mali*).

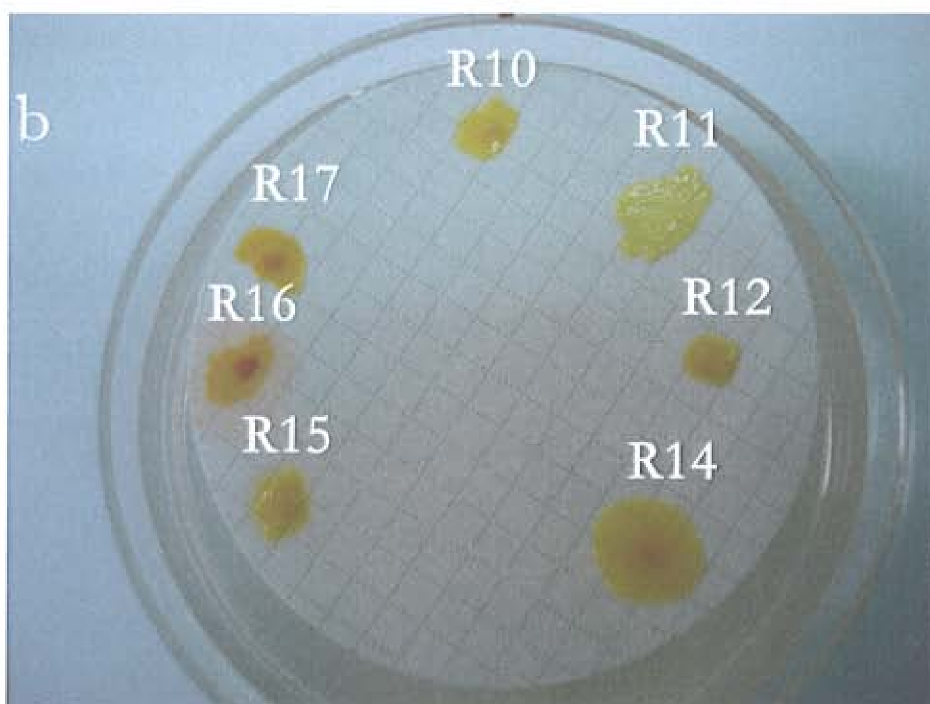

Figure 5b: Colonies representing the following ATCC reference strains: R10 (*S. choromphenolicum* 1: ATCC 53874), R11 (*S. choromphenolicum* 2: ATCC 33790), R12 (*S. asaccharolytica*), R14 (*N. capsulatum*), R15 (*Sphingomonas terrae*), R16 (*S. rosa*), and R17 (*Sphingomonas suberifaciens*).

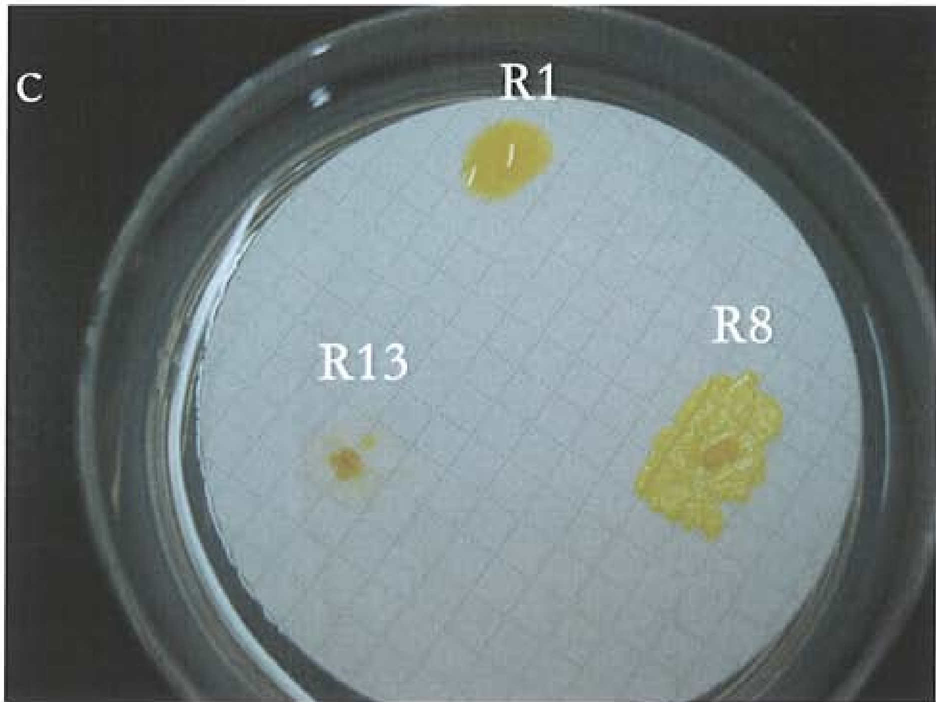

Figure 5c: Colonies representing the following ATCC reference strains: R1 (*S. paucimobilis*), R8 (*S.pararpaucimobilis*), and R13 (*S. adhaesiva*).

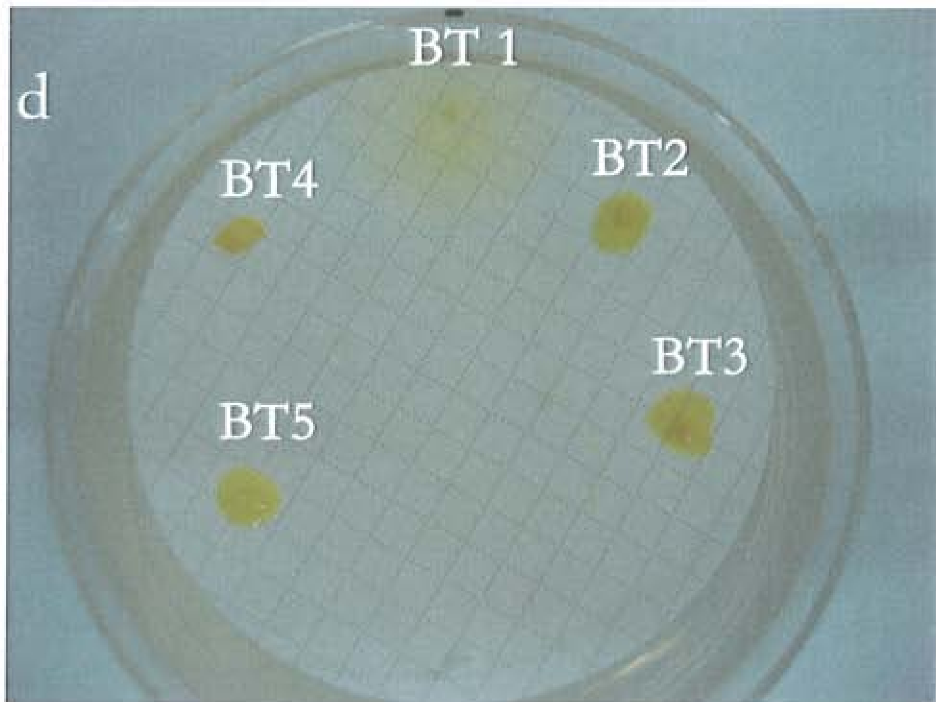

Figure 5d: Different forms and derivatives of yellow pigmented colonies, representing biotypes 1-5, recovered water storage tanks and the water distribution networks in Lebanon over a period of two years. BT1: whitish yellow, BT2: light translucent yellow, BT3: light opaque yellow, BT4: large yellow mucoid, BT5: small yellow mucoid.

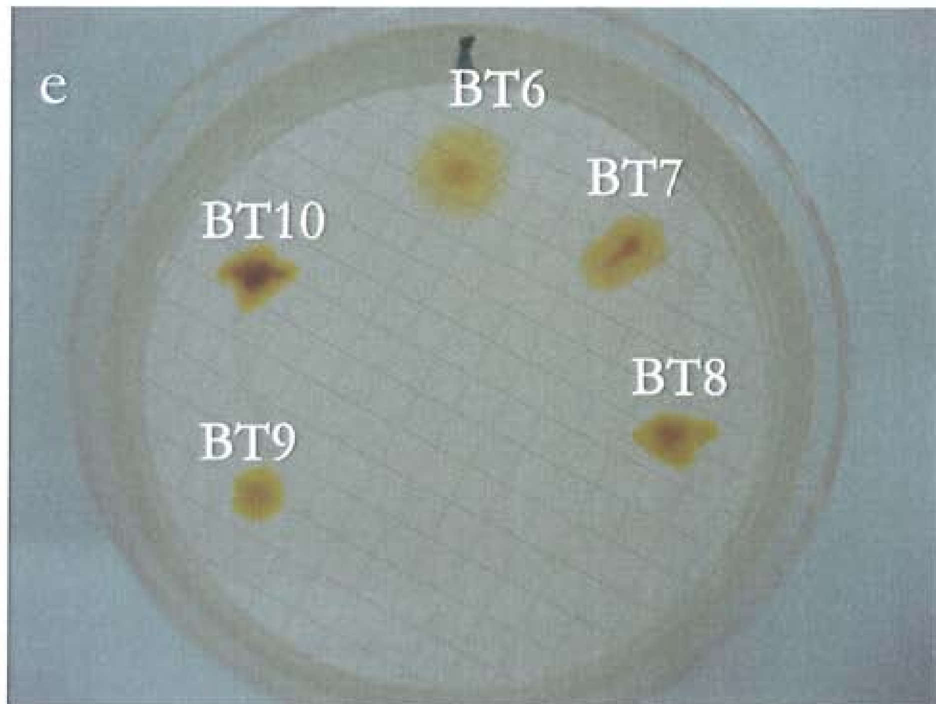

Figure 5e: Different forms and derivatives of yellow pigmented colonies, representing biotypes 6-10, recovered from water storage tanks and the water distribution networks in Lebanon over a period of two years. BT6: translucent orange, BT7: fuzzy orange, BT8: yellowish orange , BT9: dark yellow , BT10: brown.

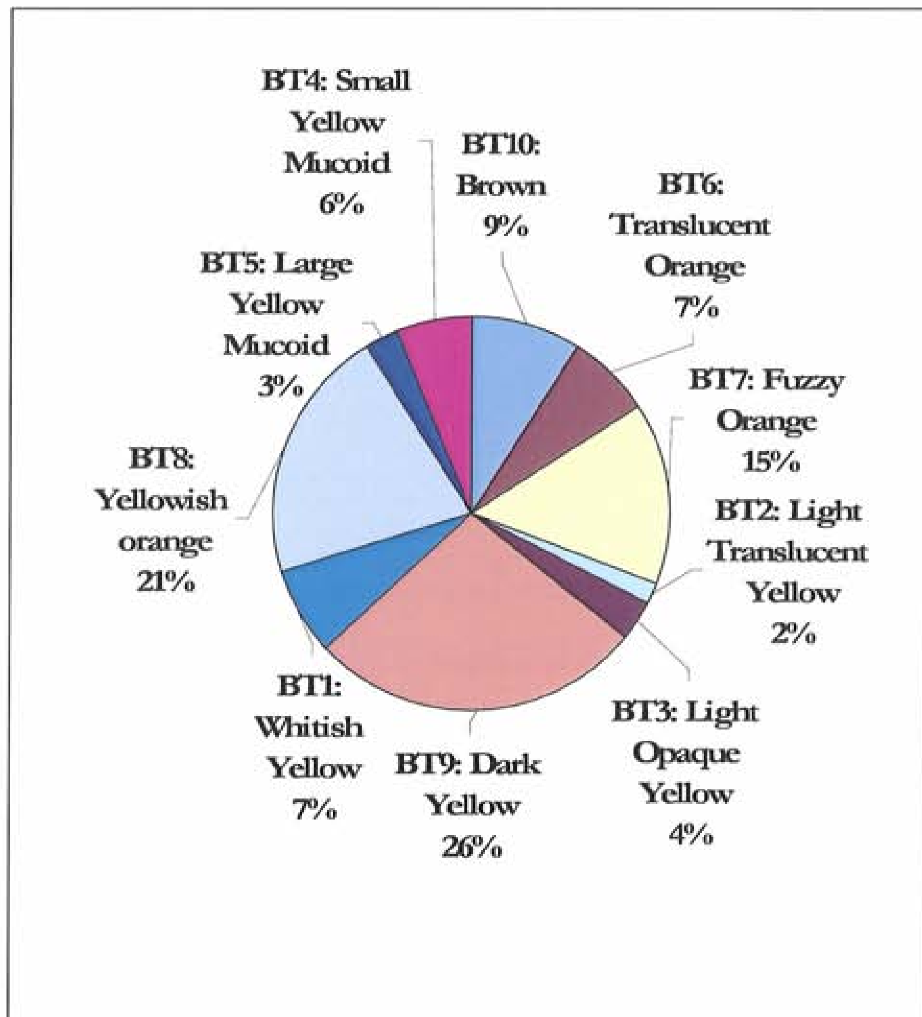

Figure 6: Distribution of sphingomonads isolated from drinking water samples collected from water storage tanks and the water distribution networks in Lebanon among the different colony types.

4.2. Biolog

The metabolic profiles of all isolates, recovered from water storage tanks, and water distribution networks in Lebanon over a period of two years that showed at least one band in sphingomonas-specific 16S rRNA based PCR assay were identified using the Biolog system. Seventeen out of 113 sphingomonas-specific 16S rRNA PCR positive isolates (*ANNEX I*) and seven of the ATCC reference strains (*S. paucimobilis*, *S. macroglotabida*, *S. sanguinis*, *S. yanoikuyae*, *S. pararpaucimobilis*, *N. capsulatum*, and *S. terrae*) were identified as sphingomonad using the Biolog system. On the other hand, positive results at the genus level was also obtained with ATCC reference strains: *S. suberifaciens*, *S. chloromphenolicum* 1, *S. mali*, *N. stygium*, and *S. pruni*. The remaining including: *S. chloromphenolicum* 2, *S. asaccharolytica*, *N. subterraneum*, *S. rosa*, and *S. adhaesiva*, were either not identified as a sphingomonad or gave repeatedly no ID.

4.3. Metabolic fingerprinting:

Using the metabolic profiles of these isolates along with those of ATCC reference strains, a phenogram was generated using the UPGMA algorithm (CLC bio A/S, Denmark). Two phenograms were generated: one using the metabolic profile of all seventeen samples that where identified as sphingomonads using the Biolog system along with the metabolic profiles of the reference strains (Figure 8). The second phenogram was generated using representatives of the different biotypes along with the reference strains (Figure 9).

As Figure 8 shows HST-69 (BT1, and identified as *S. sanguinis* using the biolog system) was clustered with *S. chloromphenolicum* 1. *S. rosa* was

clustered with both HST-66 (BT2 and identified as *S. paucimobilis* using the Biolog system) and HST-51 (BT9 and identified as *S. yanoikuyae* using the Biolog system). HST-73 (BT6) and HST-80 (BT8), were closely associated and clustered with HST-105 (BT6) and HST-50 (BT6) and were all identified as *S.paucimobilis* B using the Biolog system. HST-43 (BT9 and identified as *S. yanoikuyae* using the Biolog system), HST-44 (BT2 and identified as *S. yanoikuyae* using the Biolog system), HST-16 (BT9 and identified as *S. sanguinis* using the Biolog system), HST-128 (BT2 and identified as *S. sanguinis* using the Biolog system), and HST-62 (BT1 and identified as *S. paucimobilis* A using the Biolog system) all clustered together. HST-17 (BT9), and HST-129 (BT8), were closely associated and were both identified as *S. macroglotabida* using the Biolog system.

In the second phenogram (Figure 9) HST-121 and HST-119, which belonged to BT5, clustered together. Isolates HST-105 and HST-73, which belonged to BT6, clustered together and appeared to be associated with the two of the reference strains *N. subterraneum* and *S. adhaesiva*. HST-99 and HST-100, which belonged to BT7, clustered together and appeared to be associated with the reference strain *S. macroglotabida*. HST-59 and HST-60, which belonged to BT1, clustered together. HST-122, which belonged to BT3, appeared to be closely associated with *S. asaccharolytica*. ATCC strains *S. yanoikuyae* and *S. sanguinis* were closely associated, and the same was true for *S. suberifaciens* and *S. terrae*. HST-70, which belonged to BT4, appeared to be associated with ATCC strain *S. macroglotabida*. However, HST-76, which also belonged to BT4, clustered with HST-53 (BT9). HST-52 and HST-54 which belonged to BT8 were not closely associated. HST-52 seemed to be more closely associated with HST-114 (BT 10). HST-128 and HST-66 belonged to BT 2, but didn't cluster together.

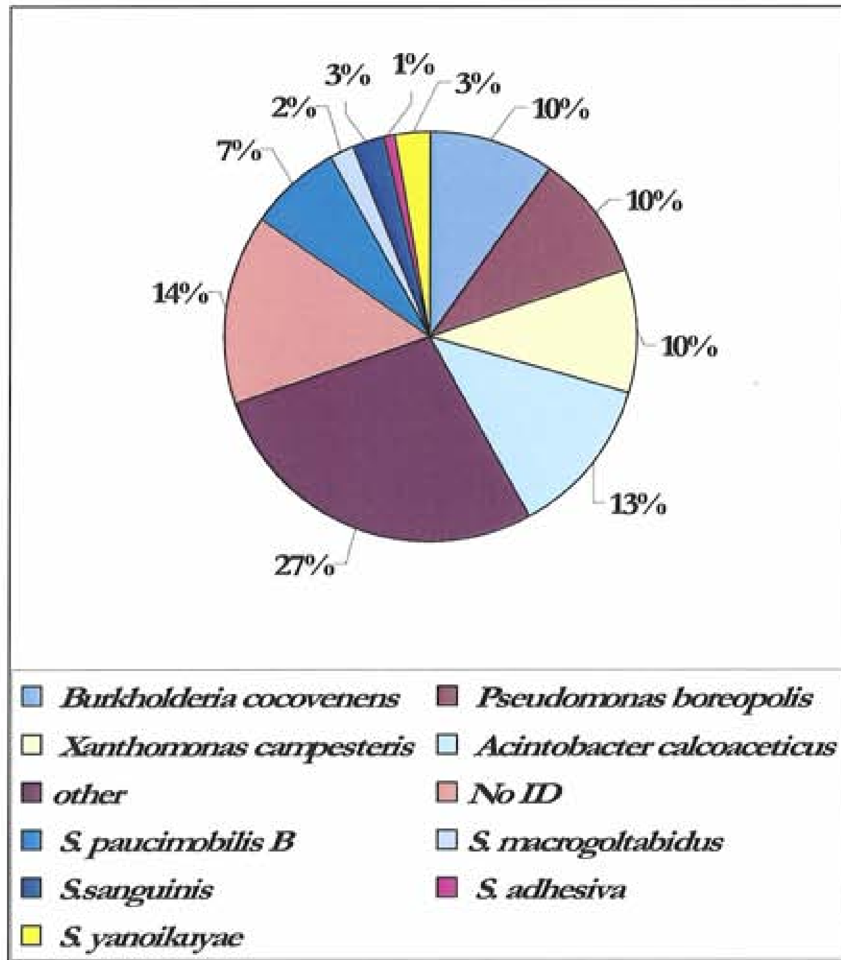

Figure 7: Distribution of the different isolates, recovered from water storage tanks and distribution networks in Lebanon over a period of two years, among the different species as identified by Biolog.

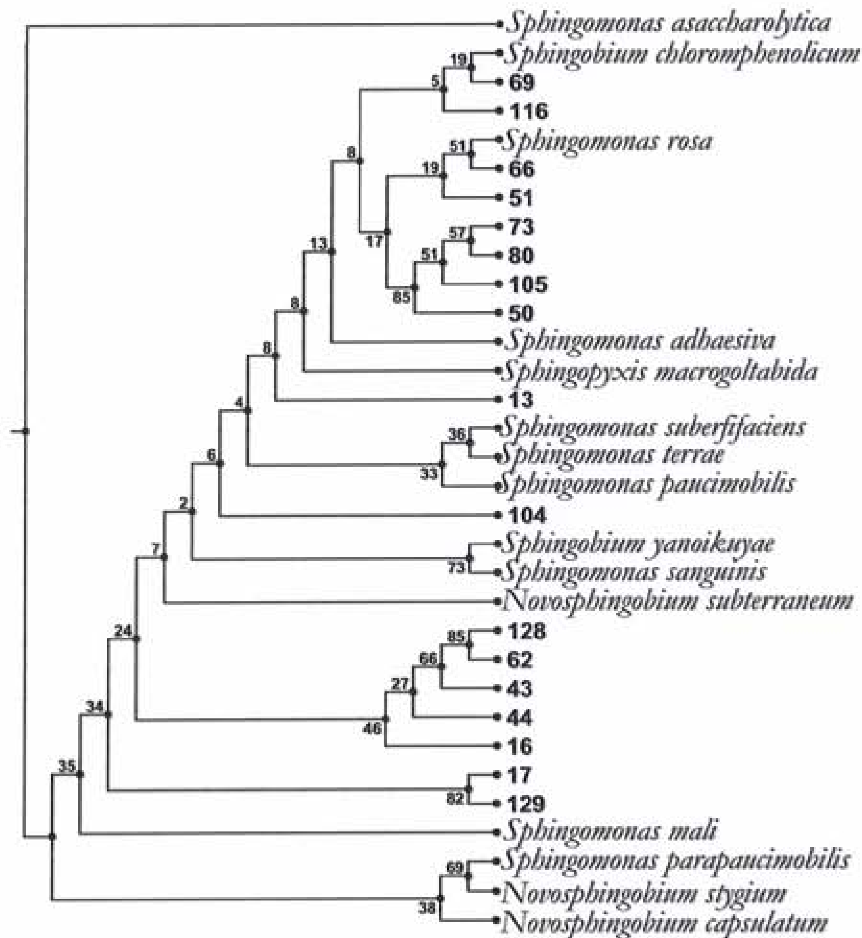

Figure 8: Phenogram generated with the metabolic profile of all 17 samples that where identified as sphingomonads using the Biolog system along with the metabolic profiles of the reference strains using the UPGMA algorithm (CLC bio A/S, Denmark). HST-69: *S. sanguinis*, HST-116: *S. yanoikuyae*, HST-66: *S. paucimobilis* B, HST-51: *S. yanoikuyae*, HST-73: *S. paucimobilis* B, HST-80: *S. paucimobilis* B, HST-105: *S. paucimobilis* B, HST-50: *S. paucimobilis* B, HST-13: *S. paucimobilis* B, HST-104: *S. adhaesiva* , HST-128: *S. sanguinis*, HST-62: *S. paucimobilis* B, HST-43: *S. paucimobilis* B, HST-44: *S. yanoikuyae*, HST-16: *S. sanguinis*, HST-17: *S. macrogoltabida*, HST-129: *S. macrogoltabida*.

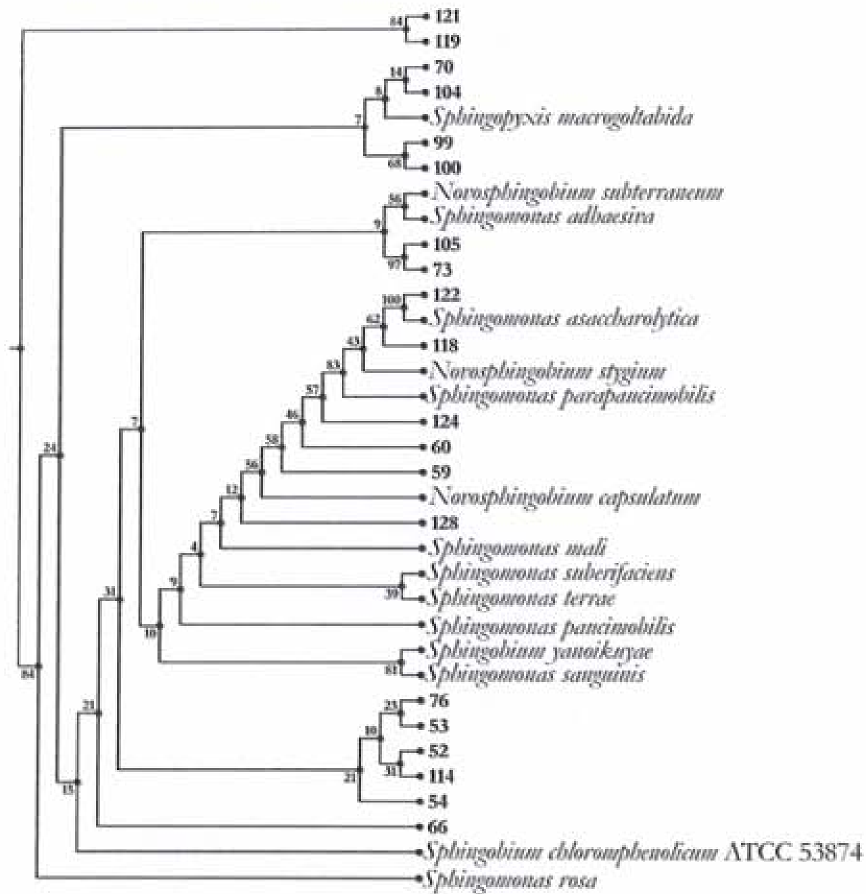

Figure 9: Phenogram generated with the metabolic profile of twenty samples representing the different biotypes along with the metabolic profiles of the reference strains using the UPGMA algorithm (CLC bio A/S, Denmark). Samples representing BT1 included: HST-59 and HST-60, BT2: HST-66 and HST-128, BT3: HST-104 and HST-122, BT4: HST-119 and HST-121, BT5: HST-70 and HST-76, BT6: HST-105 and HST-73, BT7: HST-99 and HST-100, BT8: HST-52 and HST-54, BT9: HST-53 and HST-129, BT10: HST-114 and HST-118.

4.4. PCR amplification

4.4.1 16S rDNA amplification

The quality of the extracted DNA from all sphingomonads was evaluated by the amplification of the 16S rDNA (Figure 10). All samples showed a clear band at 1500 bp.

4.4.2. Sphingomonas-specific 16S rRNA PCR

PCR assay based on the use of sphingomonad specific primer set was studied using agarose gel electrophoresis. All reference strains and tested isolates gave one clear band with a size of around 320 bp and an additional one having a size of 225 bp. None, however, was detected neither in the reagent control nor in the case of *E. coli* (Figure 11a, b, c, d, e). Out of 129 isolates with yellow-pigmented colonies, sixteen were negative for sphingomonas-specific 16S rRNA gene PCR (HST-29, HST-39, HST-42, HST-61, HST-81, HST-82, HST-83, HST-84, 85, HST-86, HST-87, HST-88, HST-89, HST-106, HST-117, HST-123).

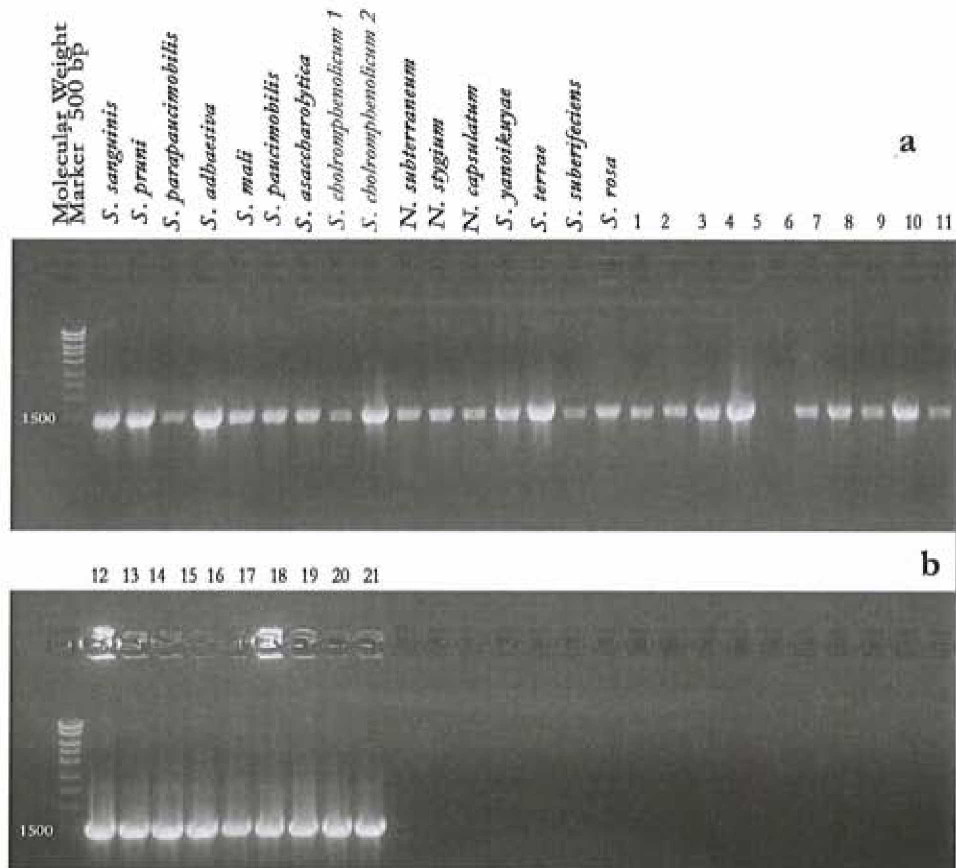

Figure 10: Amplification of 16S rRNA gene as a positive PCR control. **(a)**: well 1: Molecular weight marker 500 bp, well 2-18: ATCC reference strains, well 19-29: HST-1 -11. **(b)**: well 1: Molecular weight marker 500 bp, well 2-9 includes HST-1 -21. They all yielded a band at the expected position (1500bp).

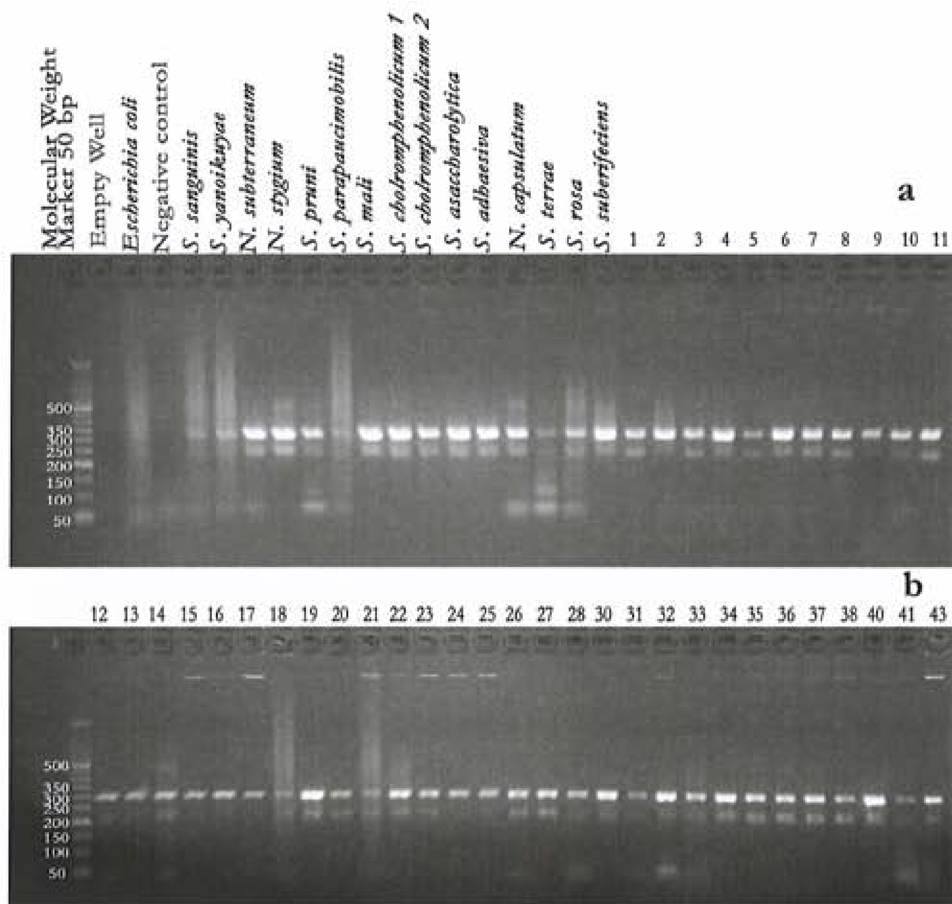

Figure 11: Amplification of sphingomonas-specific 16S rRNA gene. DNA samples were analyzed on a 2.5% agarose gel. **(a)**: Well 1: 50 bp Molecular weight marker VIII (Roche), well 2: Empty, well 3: *Escherichia coli* Gram-negative organisms used as a negative PCR control, well 4: negative reagent control, wells 5-19: reference strains. well 20-30: HST1-11. **(b)**: Well 1: 50 bp Molecular weight marker VIII (Roche), wells 2-30: HST-12 -43.

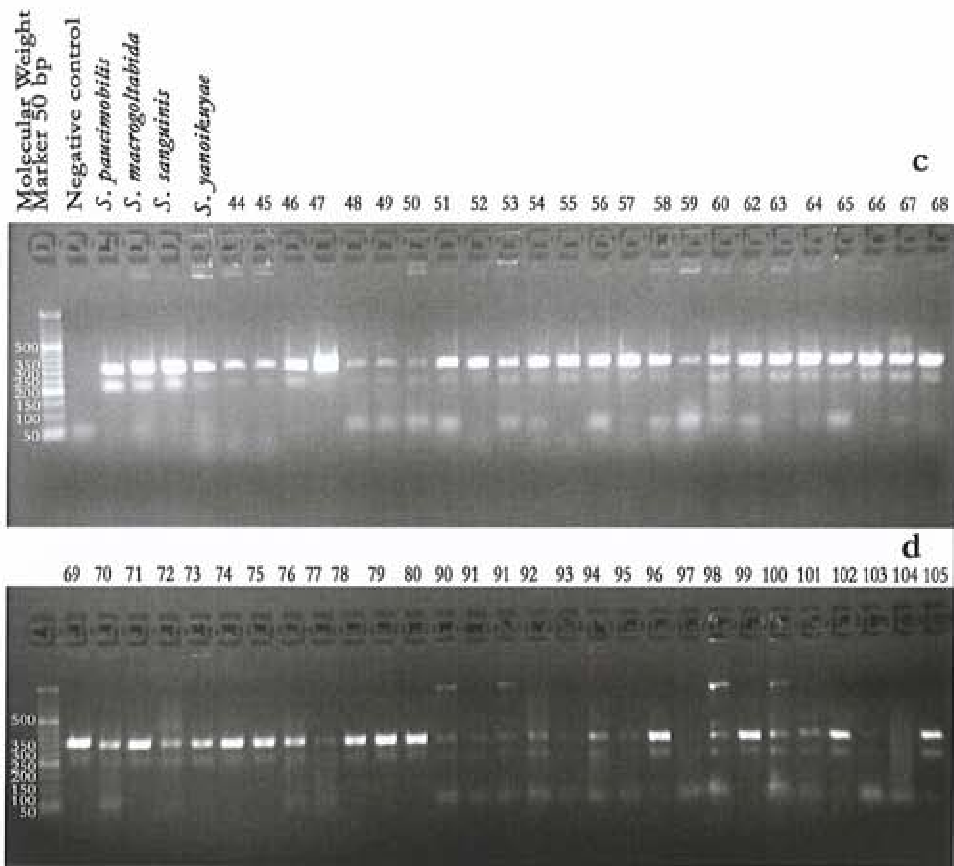

Figure 11: Amplification of sphingomonas-specific 16S rRNA gene. DNA samples were analyzed on a 2.5% agarose gel. **(c)**: Well 1: 50 bp Molecular weight marker VIII (Roche), well 2: negative reagent control, wells 3-6: ATCC reference strains, wells 7-30: HST-44 -68. **(d)**: Well 1: 50 bp Molecular weight marker VIII (Roche), wells 2-30: HST-69 -105.

Figure 11: Amplification of sphingomonas-specific 16S rRNA gene. DNA samples were analyzed on a 2.5% agarose gel. **(e)**: Well 1: 50 bp Molecular weight marker VIII (Roche), wells 2- 21: HST-108- 129.

4.4.3. Molecular Fingerprinting

4.4.3.1. ITS PCR amplification

The ITS region was amplified using the forward 1492F and the reverse primers 115R in all the ATCC reference strains and in all isolates that showed at least one band in sphingomonas-specific 16S rRNA gene based PCR assay. A highly variable PCR product of a size ranging between 400 and 1100 bp was obtained (*ANNEX* I, Figures 12 and 13). The majority of the isolates gave one intense and highly reproducible fragment, which was designated as primary product. Almost 50% of the isolates gave an intense band at 900 bp (Figure 12). Some of the isolates gave additional 2-3 bands, which appeared weaker and more variable and were designated as secondary products (Figure 13). When two isolates from each biotype were compared (based on the patterns generated with the primary products only) for size variability, most of the isolates belonging to the same biotype gave the same size (Figure 14b and Table 3). Moreover, each biotype had a characteristic ITS size except for some similarity between biotypes 8, 9, and 10 (primary 1000 bp band) and biotypes 2 and 6 (primary 900 bp band). On the other hand, the reference strains did not show much size variability, to allow easy differentiation among the four genera: *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* (Figure 14a and Table2). *Sphingobium chlorophenolicum* 1, *Sphingobium chlorophenolicum* 2, *Novosphingobium stygium*, and *Sphingomonas pruni* all gave 900 bp primary products. If the secondary products are taken in to consideration, then differentiation could be possible among some of the ATCC strains used (Figure 14a and Table 2). *S. chorophenolicum* 1

gave 2 additional secondary products at positions 500 and 700 bp, unlike *S. choromphenolicum* 2.

Table 2: ITS amplification patterns of ATCC strains.

ATCC strain	ITS size (bp)
S. paucimobilis	**350***, 500
S. macrogoltabida	400, **800**
S. sanguinis	**1000**
S. yanoikuyae	**400**, 500, 600
N. subterraneum	500, 700, **900**
N. stygium	500, 700, **900**
S. pruni	**900**
S. parapaucimobilis	**900**
S. mali	**900**
S. chloromphenicum 1	500, **900**, 700
S. chloromphenicum 2	**900**
S. assacharolytica	**900**
S. adhaesiva	**400**, 500, 700
N. capsulatum	500 700, 900, **1000**
S. terrae	**800**
S. rosa	**400**, 500, 700
S. suberficience	**1000**
B. natatoria	**900**

* Bold font: primary product.

Table 3: ITS amplification patterns of Biotypes.

Biotypes	HST Strains	ITS size (bp)
BT 1	HST-59	400,**700***, 900
	HST-60	400,**700**, 900
BT2	HST-66	500, 700, **900**
	HST-128	500, 700, **900**
BT3	HST-104	500, **900**
	HST-107	900, **1000**
BT4	HST-70	**900**
	HST-76	500, 700, **1000**
BT5	HST-119	400, **600**
	HST-121	400, **600**
BT6	HST-73	500, **900**
	HST-105	500, **900**
BT7	HST-96	**400**, 500, 600
	HST-99	**400**, 500, 600
BT8	HST-52	500, **1000**
	HST-54	**1000**
BT9	HST-124	500, **1000**
	HST-53	500, **1000**
BT10	HST-114	**1000**
	HST-118	**1000**

* Bold font: primary product.

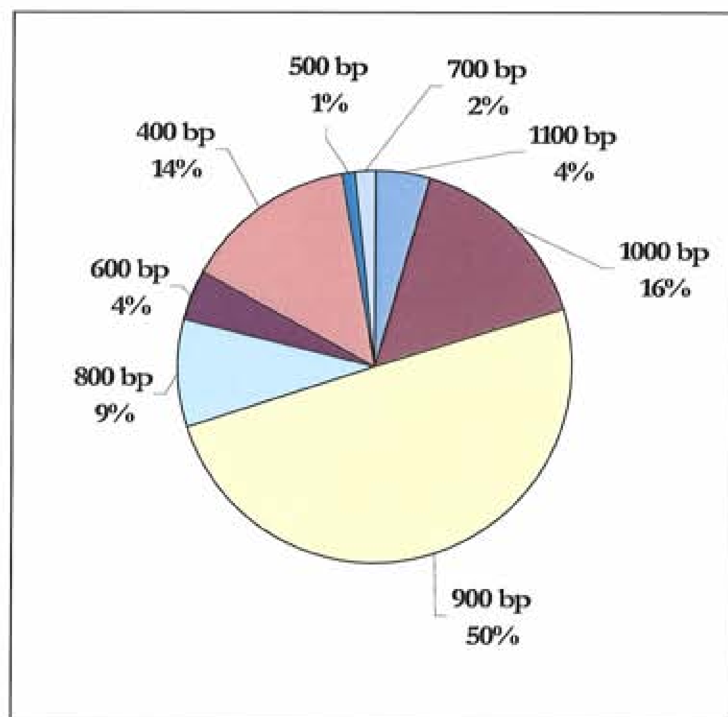

Figure 12: ITS size distribution among sphingomonads isolates.

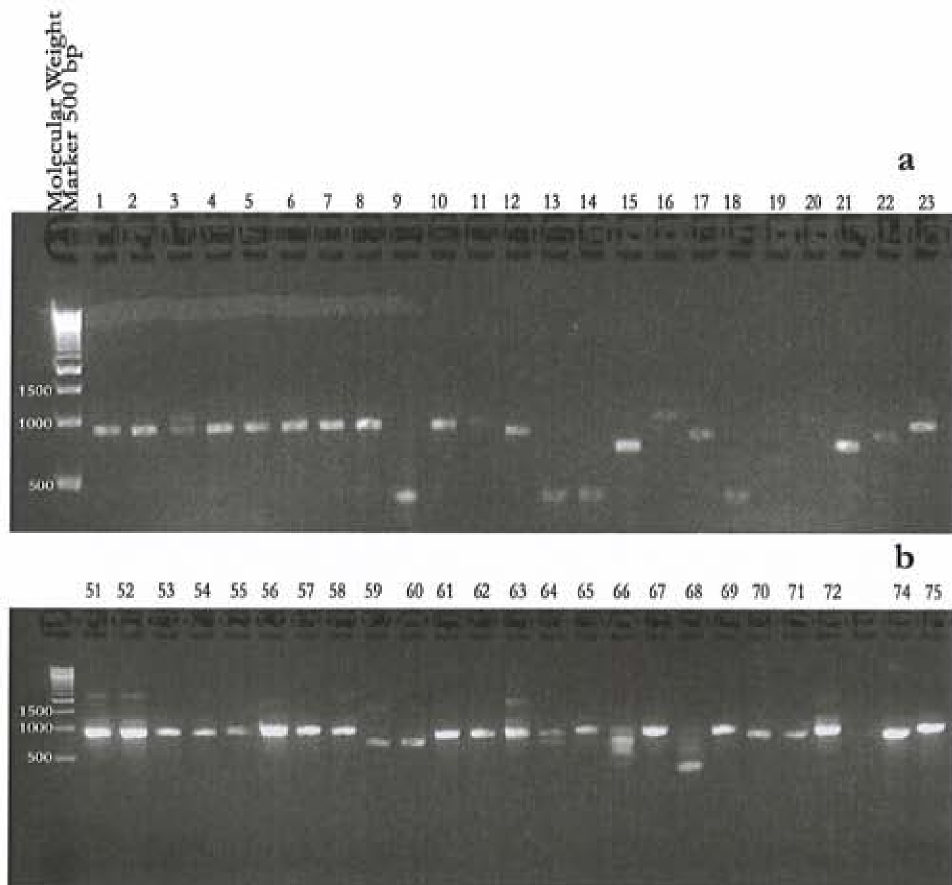

Figure 13: PCR-mediated amplification of the ITS. DNA samples were analyzed on a 1.5% agarose gel. **(a)**: Well 1: 500 bp Molecular weight marker (Roche), wells 2-30: HST-1 -23. **(b)**: Well 1: 500 bp Molecular weight marker (Roche), wells 2-30 51-75: HST-51 -75.

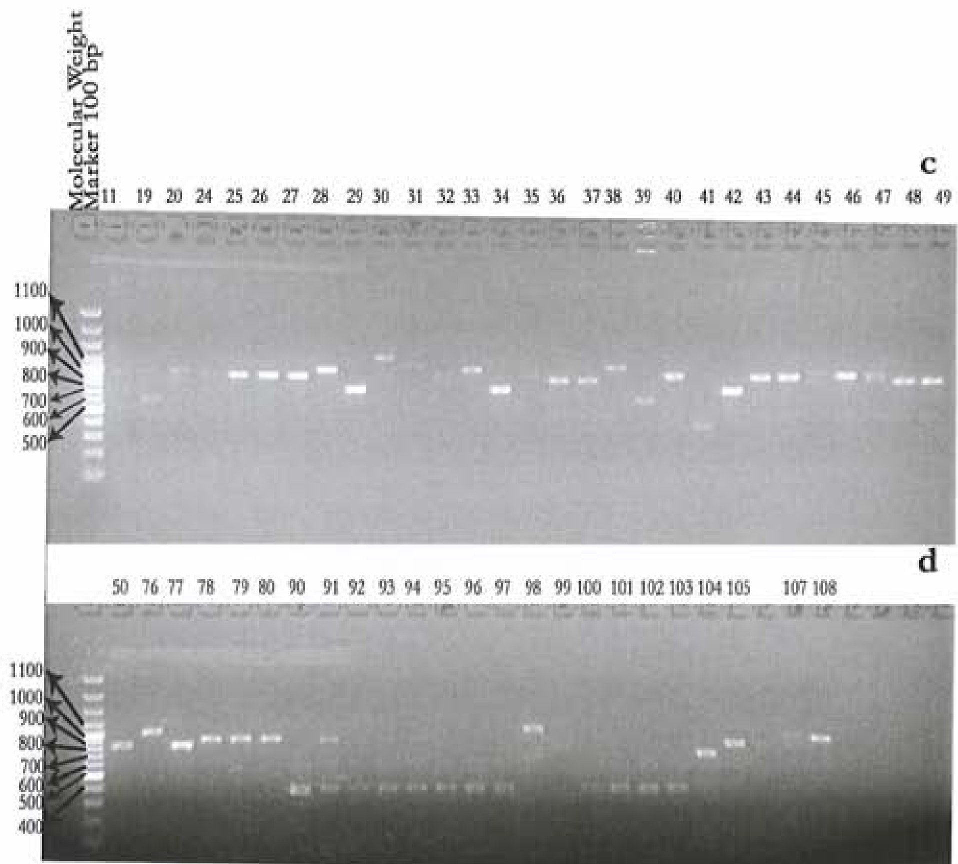

Figure 13: PCR-mediated amplification of the ITS. DNA samples were analyzed on a 1.5% agarose gel. **(c)**: Well 1: 100 bp Molecular weight marker (Roche). Wells 2-30: HST-11, HST-19, HST-20, and HST-24 - 49. **(d)**: Well 1: 100 bp Molecular weight marker (Roche), wells 2-26: HST-50 and HST-76 -108.

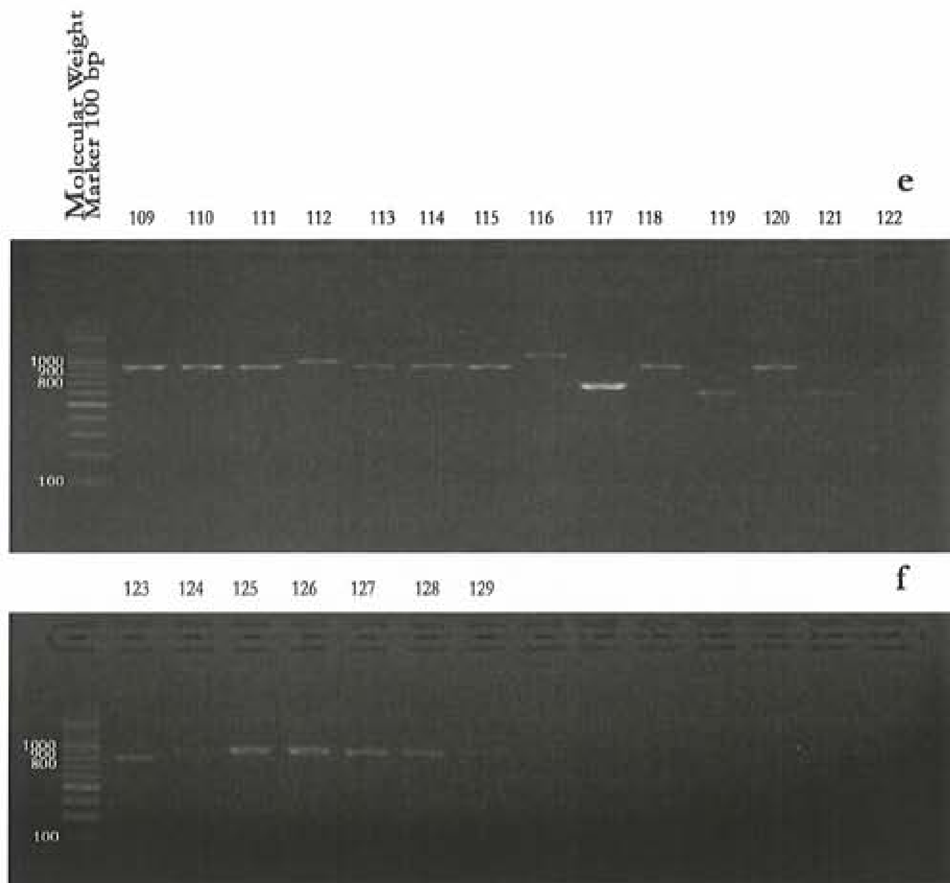

Figure 13: PCR-mediated amplification of the ITS. DNA samples were analyzed on a 1.5% agarose gel. **(e)**: Well 1: 100 bp Molecular weight marker (Roche), wells 2-15: HST-109 -122. **(f)**: Well 1: 100 bp Molecular weight marker (Roche), wells 2-8: HST-123 -129.

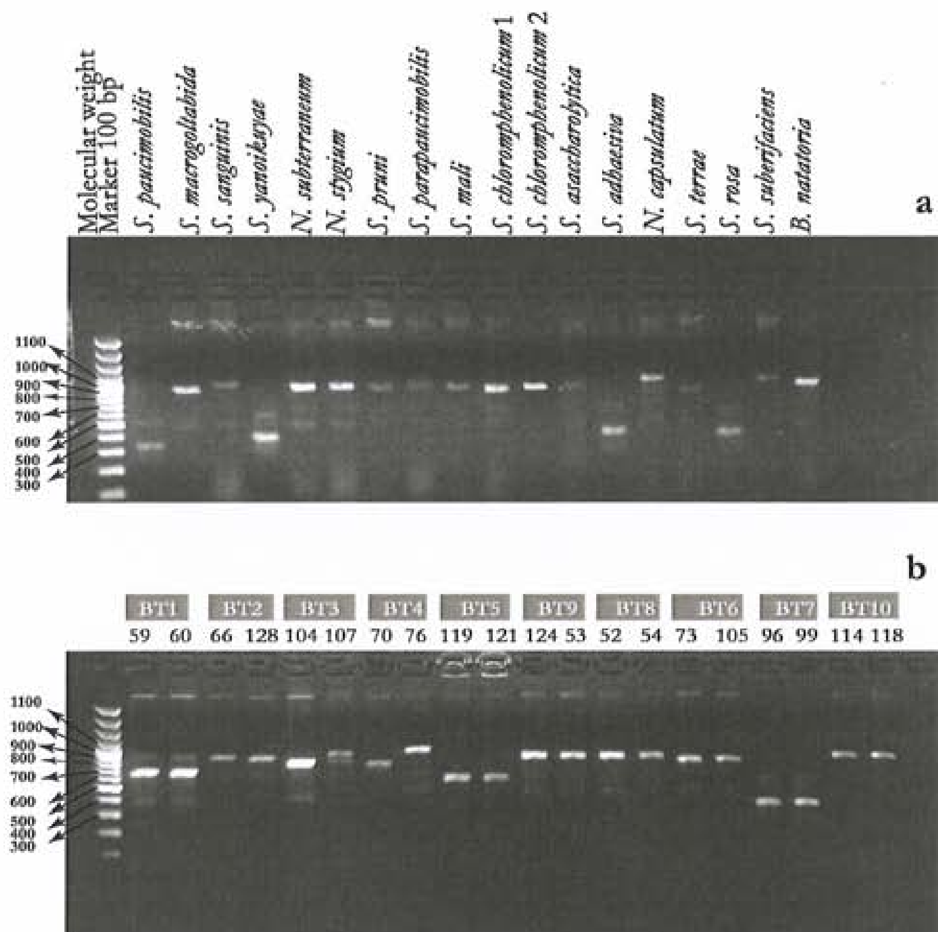

Figure 14: PCR-mediated amplification of the ITS. DNA samples were analyzed on a 1.5% agarose gel. **(a)**: Well 1: 100 bp Molecular weight marker (Roche). The rest of the wells contain ITS PCR product of the ATCC reference strains. **(b)**: Well 1: 100 bp Molecular weight marker (Roche). The rest of the wells contain ITS PCR product of 2 samples from each biotype.

4.4.3.2. ITS Restriction digestion:

ITS PCR product was digested using *Hinf*I, *Alu*I, and *Bsp*143I. Because of the lack of a comprehensive database for the different ITS sequences to study the various restriction sites in sphingomonads, enzymes were chosen from a panel of seven restriction enzymes (*Sma*I, *Hind*III, *Eco*RI, *Xho*I, *Hinf*I, *Alu*I, and *Bsp*143I). Out of those only *Hinf*I, *Alu*I, and *Bsp*143I were able to cut the amplified ITS of two chosen reference strains (*S. pararpaucimobilis* and *N. capsulatum*) and two isolates (HST-113 and HST-115) and were accordingly used in this study for all other reference strains and unknown isolates. Electrophoresis and fragment size analysis of restriction products revealed extensive variability in the number and size of bands obtained. This led to the recognition of sixteen distinct banding patterns among the reference strains (Table 4 and Figure 15a). All the reference strains gave different band patterns except for *N. subterraneum* and *N. stygium*, which also had the same ITS size (900 bp). Moreover, twelve distinct banding patterns were recognized among isolates representing the different biotypes (Figure 15). Each BT gave distinctive band patterns, and isolates belonging to the same biotype gave identical band patterns (Figure 15b). However, different band patterns appeared within the same BT such as HST-104 and HST-107 (BT 3), HST-70 and HST-76 (BT 4), and HST-124 and HST-53 (BT 9). In addition, some of the isolates had band patterns similar to those generated by the ATCC reference strains (Table 4). For example, BT10 gave a pattern similar to pattern 17, while patterns generated by HST-107 (BT3), BT2, and BT4 did not correlate with any of the patterns generated by the ATCC reference strains.

Table 4: ITS Restriction Patterns of ATCC reference strains.

Patterns	Number and sizes of bands (bp)	Corresponding ATCC Strains	Isolates with similar band patterns
1	25, 60, 100, 125, 150, 200	*S. paucimobilis*	HST-53(BT9), BT8
2	25, 50, 75, 90, 125, 100, 200	*S. macrogoltabida*	HST-124 (BT9)
3	50, 60, 80, 100, 150, 175, 250, 550, 750	*S. sanguinis*	NM*
4	50, 75, 120, 125, 175, 200, 300	*S. yanoikuyae*	BT6
5	50 60, 75, 90, 100, 150, 175, 180, 200	*N. stygium* *N. subterraneum*	BT5
6	50, 60, 75, 100, 175, 190, 350, 400, 500, 550	*S. pruni*	NM
7	150, 200, 400	*S. pararpaucimobilis*	NM
8	50, 60, 75, 100, 350	*S. mali*	NM
9	50, 75, 100, 150 ,175	*S. chloromphenolicum* 1	BT1
10	50, 90, 100, 175	*S. chloromphenolicum* 2	NM
11	50, 60, 75, 100, 350, 400, 500, 550,	*S. asaccharolytica*	NM
12	50, 60, 175, 200, 250	*S. adhaesiva*	NM
13	50, 75, 100, 120, 125, 150, 175, 200	*N. capsulatum*	HST-104 (BT3)
14	50, 100, 175	*S. terrae*	NM
15	25, 75, 150, 175, 200	*S. rosa*	BT7
16	50, 60, 75, 275, 300	*S. suberifaciens*	NM
17	25, 50, 60, 75, 175, 200, 225, 250, 300	*B. natatoria*	BT10

*NM: No match among the isolates.

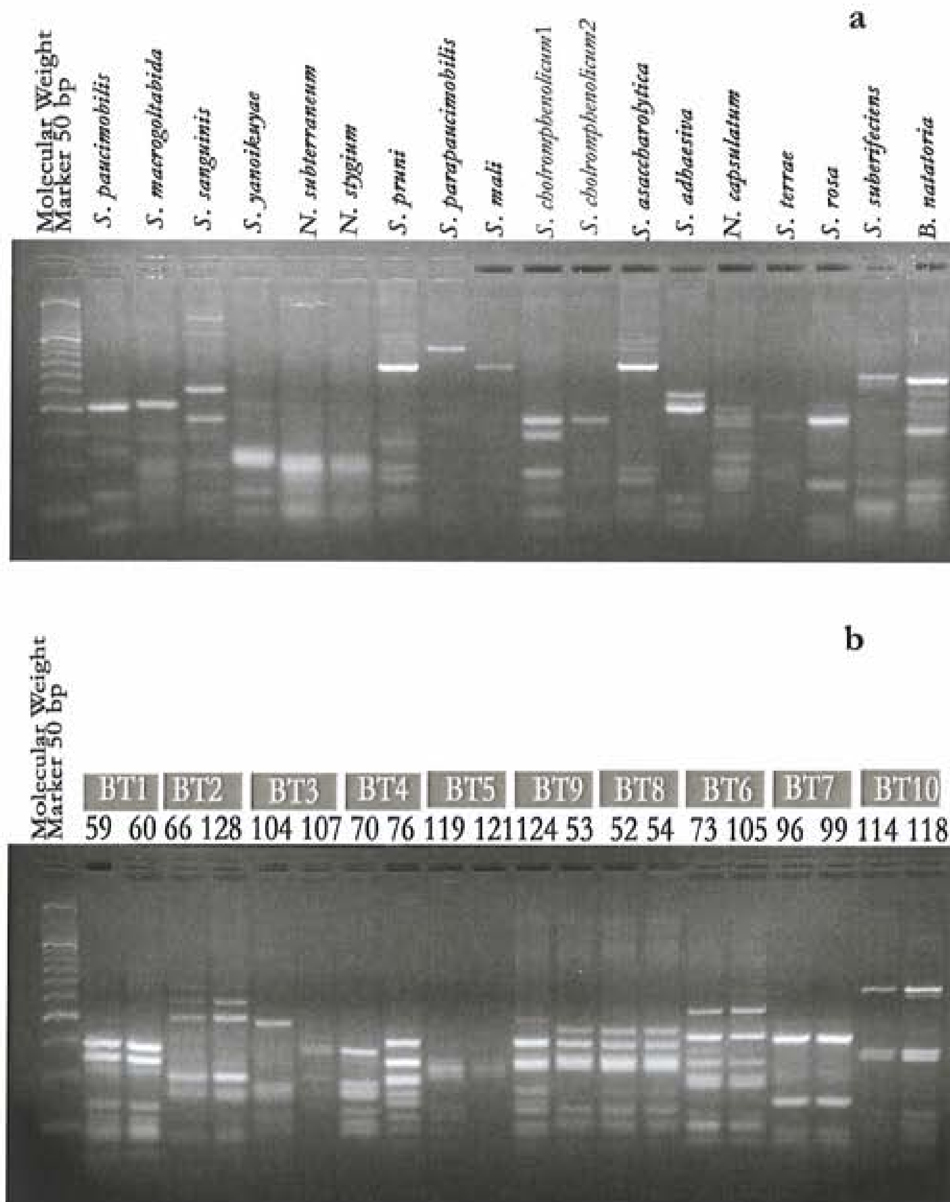

Figure 15: Electrophoresis picture of Restriction Digestion product of ATCC reference strains and biotypes representatives visualized by ethidium bromide staining on a 3% agarose gel using 1x TBE buffer. **(a)**: Well # 1: 50 bp Molecular weight marker (Roche). The rest of the wells contain ITS restriction product of the ATCC reference strains. **(b)**: Well # 1: 50 bp Molecular weight marker (Roche). The rest of the wells contain ITS restriction product of 2 samples from each biotype.

Chapter 5

DISCUSSION

The ability of sphingomonads present in drinking water to cause community and hospital acquired opportunistic infections has raised the need to establish rapid and feasible assays that could screen and identify sphingomonads to the genus, species and subspecies level. Previously, different forms of yellow-orange-pigmented colonies were isolated both from an intermittent drinking water distribution network (Tokajian *et al.*, 2005), and Polyethylene and cast iron household storage tanks in Lebanon (Tokajian and Hashwa, 2004).

A total of 129 samples with yellow-orange-pigmented colonies have been screened with sphingomonas-specific 16S rRNA PCR assay. Molecular and biochemical fingerprints where also compared using Biolog for biochemical fingerprinting, and ITS PCR-RFLP for molecular fingerprinting. Previously Leys *et al.* (2004) described the use of a culture-independent PCR-based detection method using the primers Sphingo 108f and Sphingo 420r which target the sphingomonas 16S rRNA gene to asses sphingomonas diversity in PAH-contaminated soils. The PCR resulted in 352 bp long product when analyzed on DGGE (Denaturing Gradient Gel Electrophoresis). In this study, a modified primer set was used to screen for sphingomonads amongst colonies growing on R2A agar. However the PCR resulted in 2 bands (320 bp and 225 bp) in 113 samples out of 129 samples collected from drinking water networks as well as storage tanks and in all the ATCC reference strains used in this study. Thus the 16S rRNA gene based PCR assay described in this study is rapid, feasible,

and reproducible method that can be easily employed to screen and confirm isolates belonging to sphingomonads. Nevertheless, this assay although specific it's not enough to differentiate closely related sphingomonads. Thus, it should be combined with other biochemical and/or molecular assays to identify and relate the different sphingomonad species.

The 16S rRNA gene based PCR assay described in this study was combined with Biolog (Biolog, Inc., Hayward, California) microbial identification system for metabolic fingerprinting and phylogenetic studies. Previously, Sorensen *et al.* (2001) used the Biolog system for identification of *Sphingomonas* sp. (SRS2), and Yang *et al.* (2005) tested the ability *S. chlorophenolica*, to utilize (oxidize) various carbon sources. In addition, Pollock (1993) added bacterial isolates secreting gellan-related polysaccharides as *Sphingomonas* based on metabolic fingerprints generated using the Biolog system. On the other hand, the API 20NE and Biotype 100 were used by Yabuuchi *et al.* (2002) for biochemical characterization of different sphingomonads. Biolog and all the biochemical identification schemes mentioned earlier have many limitations, as they may yield results that are usually not reproducible, ambiguous and misleading. A metabolic pattern could be obtained in many instances with no species ID because of the lack of similar metabolic patterns in the data base; the data base being limited to clinical rather than environmental isolates (Amy *et al.*, 1992; Tokajian and Hashwa, 2004; Tokajian *et al.*, 2005). In this study many of the ATCC strains such as *S. suberifaciens*, *S. chloromphenolicum*, *S. mali*, *N. stygium*, and *S. pruni* were correctly identified to the genus but not species level, while *S. choromphenolicum* , *S. asaccharolytica*, *N. subterraneum*, *S. rosa*, and *S. adhaesiva*, gave either an ID other than *Sphingomonas* or had repeatedly no ID. It's noteworthy that these strains, except for *S.*

adhaesiva, are not present in the Biolog database, while the ones available (*S. paucimobilis*, *S. macrogoltabida*, *S. sanguinis*, *S. yanoikuyae*, *S. pararpaucimobilis*, *N. capsulatum*, and *S. terrae*) were correctly identified. Out of 113 isolates confirmed as sphingomonads using the 16S rRNA gene based PCR assay only 17 were identified as sphingomonads using the Biolog system, with the same species giving variable metabolic profiles. For example, not all the samples identified as *S. paucimobilis* by the Biolog system had the same colony morphology and metabolic profile (*ANNEX I*). The opposite was also true some samples that had the same colony morphology and/or metabolic profile gave different IDs (*ANNEX I*).

The metabolic patterns of samples were keyed along with the metabolic patterns of ATCC reference strains in to a phenogram, generated using the UPGMA algorithm (CLC bio A/S, Denmark). Many of the isolates belonging to the same colony morphology (Biotype) clustered together indicting that those were closely related. However, some of the biotypes did not cluster with any reference strain and many reference strains did not cluster with any biotype. Thus, the biotyping approach used in this study, when combined with the enrichment of the Biolog database to include those environmental isolates, could definitely enhance identification using the Biolog system. Finally, some of the samples that were identified as sphingomonads using Biolog system did not cluster with their corresponding reference strains. This should be further investigated to identify the source of error.

The size and sequence of the ITS can be extremely variable between species, strains, and even among different operons within the same cell (intercisronic) and has been used to resolve phylogenetic relationships among closely related microorganisms (Garcia-Martinez *et al.*, 1999). In this study, the level of ITS polymorphism

and thus its ability to discriminate between the different sphingomonads isolated from drinking water in Lebanon has been investigated in two steps. The first step was PCR amplification of the ITS, followed by RFLP (restriction fragment length polymorphism) of the amplified ITS using restriction endonucleases. A highly variable PCR product of a size ranging between 400 and 1100 bp was obtained which was consistent with the expected length of the 16S-23S intergenic spacer region of members of the α-*Proteobacteria* (130- 1529 bp) which includes all sphingomonads (Garcia-Martinez *et al.*, 1999). Most of the reference strains and 50% of the isolates gave an intense band at position 900 bp. Based on the primary amplification products alone, it was not possible to differentiate among the different sphingomonads at the species level. However, some of the strains gave additional 2-3 bands, which appeared weaker and more variable. When these bands were included in the identification scheme, we were able to differentiate between some of the ATCC strains having similar primary product size. These secondary products could be due to non-specific amplification, with similar results being reported by Jensen *et al.* (1993) when the ITS was amplified from various species. The number of secondary bands could be reduced by decreasing the number of cycles and annealing time. In this study, this was not possible since reducing the number of cycles led to faint primary bands. On the other hand, the source of these bands might be the presence of more than one rRNA operon in these strains with the larger bands containing several tRNAs and the smaller bands containing one or less tRNA sequences (Condon *et al.*, 1995; Kabadjova *et al.*, 2002). Similarly, PCR amplification from all *Carnobacterium* strains analyzed by Kabadjova *et al.* (2002) yielded more than one band. The source of

these bands was investigated by sequencing. Two tRNA coding genes were invariably found in the larger bands, one was found in medium sized bands, and none in the smaller ones. On the other hand, the number of rRNA operons in sphingomonads was only investigated in *Sphingomonas* sp. strain RB2256. It was found that RB2256 contains a single copy of the rRNA operon, but this does not necessarily apply for all sphingomonads, since the copy number depends on the growth rate and is not necessarily conserved among closely related species (Fegatella *et al.*, 1998). Moreover, ITS sequences for all sphingomonad species are not currently available, thus it is not possible to know the number and type of tRNAs within the spacer, although the order of the ribosomal genes in *Sphingopyxis alaskensis* and *Novosphingobium aromaticivorans* was found to be conserved. The rRNA transcription units in both organisms consisted of 16S rRNA, $tRNA^{Ile}$, $tRNA^{Ala}$, 23S rRNA, 5S rRNA, and the $tRNA^{fMet}$ gene. *Sphingopyxis alaskensis* contained one copy of the rRNA operon, while *Novosphingobium aromaticivorans* contained three. The 16S-23S ITS in all three operons had the same size and sequence (Kwon and Kim, 2007). It might be possible that all sphingomonads contain the same rRNA organization and thus two tRNA coding sequences within the 16S-23S ITS since functional units within the ITS are conserved among closely related organisms. However, the number of rRNA operons in each species can only be investigated by whole genome sequencing. Thus, unless sequenced, the primary utility of these secondary bands should be to confirm identification based on the primary amplification products.

According to the results of this study, the ITS size was not sufficient to subtype sphingomonad species, and consequently the amplified ITS was subjected to restriction digestion for the purpose of possible

identification to the genus, species, and subspecies level. Previously, ITS PCR-RFLP was used by Matar *et al.*, (1993) to subtype the *Rochalimaea* species when the ITS size was not variable enough for species differentiation. Our results revealed that the four main genera (*Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis*) had a unique and reproducible restriction patterns. The restriction patterns were variable among species of the same genus (except for *N. stygium* and *N. subterraneum* which had the same pattern), and strains of the same species (since the two strains of *S. chlorophenolicum*: ATCC 33790 and 53874 had two different restriction patterns). Moreover, isolates belonging to the same biotype generated the same restriction pattern, while isolates belonging to different biotypes generated different restriction patterns. Thus, confirming the close association between isolates having similar colony morphology. In addition, several isolates had restriction patterns that were similar to those generated by the reference strains, which indicated that those were closely related to the corresponding ATCC strains. Thus, although ITS size appeared to be similar among most ATCC strains, restriction sites appeared to be extremely variable. Consequently, ITS PCR-RFLP is a rapid, reproducible, and feasible two-steps technique that can be used to generate molecular fingerprints of sphingomonads for the purposes of subtyping and identification. In addition, since 16S rDNA sequencing is expensive and cannot be used for routine identification, the ITS PCR-RFLP technique is equally efficient yet simpler and less expensive. The only limitation to the use of this technique in subtyping sphingomonads is the absence of data related to rRNA copy number, and the lack of information on the ITS sequence and size in databases. The presence of such databases can provide information as to the number of rRNA operons present in each species and the number of tRNA sequences

per ITS (Fegatella *et al.*, 1998; Garcia-Martinez *et al.*, 1999). Thus we recommend sequencing the ITS in sphingomonads as part to increase the feasibility of using it as a means for identification.

The use of biochemical and phenotypic approaches for sphingomonads identification is necessary to characterize sphingomonad species but has many limitations when used alone. However, the use of sphingomonas-specific 16S rRNA based PCR and 16S-23S ITS PCR-RFLP assay, is rapid, feasible and reproducible for identification of closely related sphingomonad species. The polyphasic approach employed in this study proved to be efficient in overcoming problems usually faced when attempting to identify organisms especially those of environmental origin. To the extent of our knowledge, this study is the first comprehensive record of the different 16S-23S ITS sizes that can be found in the four major sphingomonad genera (*Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis*). Moreover, this is the first study that describes the use of 16S-23S ITS PCR-RFLP for subtyping the different sphingomonad species. We recommend, however, sequencing the ITS region in the future to create a comprehensive database for the size, and number of tRNAs per spacer prevalent in sphingomonads.

Chapter 6

CONCLUSION

- Sphingomonas-specific 16S rRNA based PCR assay is a rapid and reproducible assay to screen for the presence of sphingomonds in drinking water. All reference strains and around 88% of yellow-orange pigmented colonies gave the expected band size.

- The most predominant biotype was dark yellow colonies (26%), while the least was light translucent yellow colonies (2%).

- Seventeen out of 113 sphingomonas-specific 16S rRNA PCR positive isolates were identified as sphingomonads using the Biolog system.

- The phenogram generated using metabolic profiles obtained by the Biolog system of reference strains and drinking water isolates helped in determining the association between all tested isolates.

- The sizes of ITS, obtained from 16S-23S ITS PCR amplification, was extremely variable and ranged between 400 and 1100 bp. The majority of sphingomonas-specific 16S rRNA PCR positive isolates (50%) gave a primary product of 900 bp.

- Based on the primary PCR products, most of the isolates belonging to the same biotype gave the same size and most biotypes yielded different ITS sizes.

- The reference strains didn't show much size variability to allow differentiation among the four main genera: *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis*.

- Taking into consideration the secondary ITS PCR products was helpful to differentiate some of the strains with similar primary product size.

- Electrophoresis and fragment size analysis of restriction products revealed extensive variability in the number and size of bands obtained. Sixteen distinct banding patterns among the reference strains and twelve among isolates were observed.

- Each of the four genera (*Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis*) had a unique and reproducible restriction pattern. The restriction patterns were variable among species of the same genus (except for *N. stygium* and *N. subterraneum* which had the same pattern), and strains of the same species.

- Isolates belonging to the same biotype generated the same restriction patterns, while those belonging to different biotypes generated different restriction patterns.

- Several isolates had restriction patterns that were similar to those generated by the reference strains revealing their close association.

- Although ITS size appeared to be similar among most sphingomonads, restriction sites appeared to be extremely variable. Consequently, ITS PCR-RFLP is a rapid, reproducible and feasible two-steps technique that can be used to generate molecular fingerprints of sphingomonads for the purposes of subtyping and identification to the strain level.

- A polyphasic approach, which combines molecular, biochemical, and phenotypic assays, proved to be efficient to identify and characterize not only sphingomonads but also other organisms especially those of environmental origin.

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ANNEX I

Isolate Number	Colony Type	Biolog	ITS size
1	Brown	*Burkholderia cocovenens*	900
2	Yellowish orange	*Burkholderia cocovenens*	900
3	Dark Yellow	*Pseudomonas boreopolis*	900
4	Yellowish orange	*Vibrio Tubiashii*	900
5	Brown	*Breundimonas vesicularis*	900
6	Dark Yellow	*Acintobacter calcoaceticus*	1000
7	Dark Yellow	*Burkholderia cocovenens*	1000
8	Brown	*Aeromonas veronii*	1000
9	Orange (fuzzy)	*Pseudomonas boreopolis*	400
10	Yellowish orange	*Aquaspirillum peregrinum*	1000
11	Dark Yellow	*Xanthomonas campesteris*	1000
12	Dark Yellow	*Capnocytophaga gingivalis*	900
13	Orange (fuzzy)	*S. paucimobilis B*	400
14	Orange (fuzzy)	*Acintobacter calcoaceticus*	400
15	Whitish yellow	*Xanthomonas campesteris*	800
16	Dark Yellow	*S. sanguis*	1100
17	Dark Yellow	*S. macrogoltabidus*	900
18	Orange (Translucent)	*Xanthomonas campesteris*	400
19	Large Yellow Mucoid	*Xanthomonas campesteris*	600, 800, 900
20	Dark Yellow	*Burkholderia cepacia*	800, 900
21	Whitish yellow	*Acintobacter calcoaceticus*	800
22	Small Yellow Mucoid	*Breundimonas vesicularis*	900
23	Yellowish orange	*Acintobacter calcoaceticus*	1000
24	Yellowish orange	*Pseudomonas boreopolis*	1000
25	Dark Yellow	*Burkholderia cocovenens*	900
26	Dark Yellow	*Acintobacter calcoaceticus*	900
27	Dark Yellow	*Acintobacter calcoaceticus*	900
28	Small Yellow Mucoid	*Pseudomonas boreopolis*	1000
30	Small Yellow Mucoid	*Acintobacter calcoaceticus*	1100
31	Dark Yellow	*Achromobacter cholinophagum*	1000
32	Dark Yellow	*Burkholderia vietnamensis*	900
33	Dark Yellow	*Acintobacter calcoaceticus*	1000

Isolate Number	Colony Type	Biolog	ITS size
34	Dark Yellow	*Acintobacter calcoaceticus*	800
35	Dark Yellow	*Acintobacter calcoaceticus*	900
36	Dark Yellow	*Pseudomonas boreopolis*	900
37	Dark Yellow	*Burkholderia cocovenens*	900
38	Dark Yellow	*Pseudomonas boreopolis*	1000
40	Yellowish orange	*Xanthomonas campesteris*	900
41	Orange (Fuzzy)	*Pseudomonas boreopolis*	400
43	Dark Yellow	*S. paucimobilis B*	900
44	Opaque light yellow	*S. yanoikuyae*	900
45	Dark Yellow	*Xanthomonas campesteris*	800, 900
46	Dark Yellow	*Acintobacter calcoaceticus*	1000
47	Dark Yellow	*Xanthomonas campesteris*	1000
48	Orange (Translucent)	*Acintobacter calcoaceticus*	900
49	Orange (Translucent)	*CDC group II-H*	900
50	Orange (Translucent)	*S. paucimobilis B*	800
51	Dark Yellow	*S. yanoikuyae*	900, 1100, 2500
52	Yellowish orange	*Pseudomonas boreopolis*	500, 700, 900
53	Dark Yellow	*Burkholderia vietnamiensis*	500, 700, 900
54	Yellowish orange	*Burkholderia cocovenens*	400, 700, 900
55	Yellowish orange	*Bordetella pertussis*	900
56	Yellowish orange	*Xanthomonas campesteris*	900, 1100, 2500
57	Yellowish orange	*Xanthomonas campesteris*	900
58	Brown	*Pseudomonas boreopolis*	900, 1100, 2500
59	Whitish yellow	*Stenotrophomonas malophilia*	400, 700, 900
60	Whitish yellow	*Stenotrophomonas malophilia*	400, 700, 900
62	Whitish yellow	*S. paucimobilis B*	900
63	Orange (Translucent)	*Brevudimonas vesicularis*	900, 1100, 2500

Isolate Number	Colony Type	Biolog	ITS size
64	Yellowish orange	*Ochrobacterium anthropi*	800, 900
65	Dark Yellow	*Pantoea dispersa*	900
66	Translucent light yellow	*S. paucimobilis B*	500, 700, 900
67	Orange (Fuzzy)	*Acintobacter calcoaceticus*	900
68	Yellowish orange	*Bordetella pertussis*	500, 700, 800, 900
69	Whitish yellow	*S. sanguinis*	900
70	Small Yellow Mucoid	*Vibrio metschnikovii*	900
71	Brown	*Burkholderia cocovenens*	800
72	Whitish yellow	*Pseudomonas boreopolis*	900
73	Orange (translucent)	*S. paucimobilis B*	900
74	Dark Yellow	*Neisseria subflava*	800
75	Dark Yellow	*Agrobacterium tumefaciens*	900
76	Small Yellow Mucoid	*Burkholderia cocovenens*	500, 700, 1100
77	Dark Yellow	*Xanthomonas campesteris*	800
78	Yellowish orange	No ID	900
79	Yellowish orange	*Stenotrophomonas maltophilia*	900
80	Yellowish orange	*S. paucimobilis B*	900
90	Orange (Fuzzy)	No ID	400
91	Orange (Fuzzy)	No ID	400, 900
92	Orange (Fuzzy)	No ID	400
93	Orange (Fuzzy)	No ID	400
94	Orange (Fuzzy)	No ID	400
95	Orange (Fuzzy)	No ID	400
96	Orange (Fuzzy)	No ID	400, 700, 600
97	Small Yellow Mucoid	*Comamonas terrigena*	400
98	Orange (Translucent)	*Yersinia aldovae*	700, 1100
99	Orange (Fuzzy)	*Vibrio Tubiashii*	400, 700, 600
100	Orange (Fuzzy)	*Vibrio Tubiashii*	400
101	Orange (Fuzzy)	No ID	400

Isolate Number	Colony Type	Biolog	ITS size
102	Orange (Fuzzy)	No ID	400
103	Whitish yellow	*Pseudomonas fluorescens*	400
104	Opaque light yellow	*S. adhaesiva*	500, 900
105	Orange (Translucent)	*S. paucimobilis B*	900
107	Opaque light yellow	No ID	900, 1000
108	Yellowish orange	*Burkholderia cocovenens*	1000
109	Brown	No ID	900
110	Brown	*Xanthomonas campesteris*	900
111	Yellowish orange	No ID	900
112	Small Yellow Mucoid	No ID	1000
113	Brown	*Burkholderia cocovenens*	900
114	Brown	*Burkholderia cocovenens*	900
115	Yellowish orange	No ID	900
116	Dark Yellow	*S. yanoikuyae*	1100
118	Brown	No ID	900
119	Large Yellow Mucoid	*Vibrio metschnikovii*	400, 600
120	Yellowish orange	No ID	900
121	Large Yellow Mucoid	*Burkholderia cocovenens*	400, 600
122	Opaque light yellow	*Acintobacter calcoaceticus*	800
124	Dark Yellow	*Pseudomonas boreopolis*	500, 700, 900
125	Yellowish orange	*CDC group II-E*	1000
126	Yellowish orange	*Ochrobacterium anthropi*	1000
127	Yellowish orange	*Brevundimonas vesicularis*	900
128	Translucent light yellow	*S. sanguinis*	500, 700, 900,
129	Yellowish orange	*S. macrogoltabidus*	900

Reference strain	Colony Type	Biolog	ITS size
S. paucimobilis	Large yellow Mucoid	*S. Paucimobilis A*	350, 500
S. macrogoltabida	Translucent light yellow	*S. macrogoltabida*	500, 900
S. sanguinis	Dark Yellow	*S. sanguis*	1000
S. yanoikuyae	Whitish yellow	*S. yanoikuyae*	400, 500, 600
N. subterraneum	Translucent light yellow	*Xanthomas campestris*	500, 700, 900
N. stygium	Opaque light yellow	*S. sanguinis*	500, 700, 900,
S. pruni	Translucent light yellow	No ID	900
S. parapaucimobilis	Opaque light yellow	*S. parapaucimobilis*	900
S. mali	Translucent light yellow	*S. sanguinis*	900
S. chloromphenicum 1	Translucent light yellow	*S. yanoikuyae*	500, 700, 900
S. chloromphenicum 2	Bright Yellow	No ID	900
S. assacharolytica	Translucent light yellow	*Photobacterium logei*	900
S. adhaesiva	Dark Yellow	*Pseudomonas boreopolis*	400, 700
N. capsulatum	Large yellow Mucoid	*N. capsulatum*	500, 700, 900, 1000
S. terrae	Dark Yellow	*S. terrae*	900
S. rosa	Fuzzy Orange	*Agrobacterium rhizogenes*	400, 700
S. suberficience	Dark Yellow	*N. capsulatum*	1000
B. natatoria	Brown		900