Identification of Sphingomonads on the Basis of Polymerase Chain Reaction Amplified 16S rRNA Gene

Sima Tokajian,* Maya Farah, and Fuad Hashwa

Department of Biology, Lebanese American University, Byblos, Lebanon

Sphingomonas is a genus that is basically of environmental origin but can also be associated with health hazards, especially in the hospital environment where there is a great need to properly monitor water sources. The abundance and frequent isolation of derivatives of yellow pigmented colonies from drinking water samples in Lebanon—where an intermittent mode of supply is employed, and which induces frequent biofilm sloughing—necessitated the establishment of a rapid and feasible assay to screen specifically for sphingomonads. In this study, 50 isolates recovered from drinking water with yellow- to orange-pigmented colonies were used to establish a polymerase chain reaction-based (PCR-based) screening assay. The use of sphingomonad specific modified primers gave one common band with a size of 320 bp in all presumptive and sequence confirmed sphingomonads. However, no amplification was observed with Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa. Applying the PCR-based assay described in this paper increased both the efficiency and the reliability of screening for sphingomonads in water samples, thereby minimizing related risk factors.

Key words: drinking water, sphingomonads, PCR, yellow colonies, biofilm, intermittent supply

Introduction

Sphingomonads are Gram-negative, chemoheterotrophic, nonsporeforming, straight rods, strictly aerobic, and characterized by an outer membrane that contains glycosphingolipids as cell envelope components, but lacks lipopolysaccharide (Yabuuchi et al. 1990; White et al. 1996). Colonies are yellow-pigmented or whitish brown (Takeuchi et al. 1993). Sphingomonas is widespread in water, soil, sediments, and in association with plants (White et al. 1996; Balkwill et al. 1997). Large numbers of phenotypically and phylogenetically similar strains of Sphingomonas have been isolated from various environments and described as novel species. The nomenclature of the genus Sphingomonas was revised and the genus was divided into four genera: Sphingomonas, Sphingobium, Novosphingobium, and Sphingopyxis (Takeuchi et al. 2001).

Organisms isolated from a drinking water distribution network and water storage tanks in Lebanon were previously defined to be mainly Gram-negative, pigmented, and as belonging to the a-Proteobacteria within the confines of Sphingomonadaceae (Tokajian and Hashwa 2004a, 2004b, 2004c; Tokajian et al. 2005). The high incidence of sphingomonads was consistent with their diverse metabolic capabilities, enabling them to inhabit a wide range of environments (Pollock and Armentrout 1999). The isolation of Sphingomonas from drinking water is not desirable. Members of this group are known to be pathogens or opportunistic pathogens, produce extracellular polymers, enhance biofilm formation and resistance towards disinfection, and some are known to induce corrosion in copper pipes (Arens et al. 1995; White et al. 1996; Busse et al. 1999). Most sphingomonads are environmental microorganisms, but 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 (ultraviolet light irradiated water used in surgery and dental unit water lines) (Yabuuchi et al. 1990; Barbeau et al. 1996; Lemaitre 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). Sphingomonas paucimobilis associated with septicaemias in a haematological unit of a university hospital was directly linked to bacterial colonization of the hospital water system (Perola et al. 2002). Furthermore, Hsueh et al. (1998) revealed that S. paucimobilis was widely distributed in hospital environments and could be involved in causing recurrent infections, in hospitalized patients, such as intravascular catheter-related bacteremia, bactereemic biliary tract infection, urinary tract infection, ventilator-associated pneumonia, and wound infection.

The identification of heterotrophic bacteria indigenous to the environment, including sphingomonads, has been based on morphological and physiological characterization of each organism after isolation (Amy et al. 1992). Commercial kits with different diagnostic substrates have also been employed either as an alternative or as a complementary approach to the conventional identification methods (Braun-Howland et al. 1993). The use of these commercial kits for the identification of environmental isolates is not very useful because of their limited databases (Amy et al. 1992; Tokajian and

* Corresponding author: stokjian@lau.edu.lb
Hashwa 2003; Tokajian et al. 2005). On the other hand, identification of bacteria based on rRNA gene sequences has in recent years emerged as a more reliable alternative to traditional identification (Böttger 1989; Boye et al. 1999).

This paper describes the use of a 16S rRNA gene-based polymerase chain reaction (PCR) assay for the identification of yellow pigmented colonies, which allows for the accurate detection of all known sphingomonads isolated on R2A from drinking water. We also compared for the accurate detection of all known sphingomonads identification of yellow pigmented colonies.

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Materials and Methods

Water Samples

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 2004b).

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 seven days. Pure colonies were kept at -20ºC on glycerol. A total of 50 derivatives of yellow pigmented colonies, out of around 200, representing all the different morphological entities within this population were chosen and used for biotyping and 16S rRNA gene-based studies.

Biotyping

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) based on the metabolic profiles obtained for each of the tested isolates.

DNA Extraction

Chosen colonies were designated as ST-1 through ST-50. DNA extractions were made by suspending several colonies from R2A plates that had grown for a minimum of 5 days at 28°C in 1 ml of sterile water. DNA was then released by boiling for 15 min.

16S rRNA Gene Amplification

16S rDNA gene was amplified using the gene sequence specific universal forward 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primers 1492R (5'-GTT TAC CTT GTT ACG ACT T-3') (TIBMolBiol, Germany) (Lane et al. 1985). 16S rRNA gene amplification with a product of 1,500 bp was used as a positive PCR control ensuring the integrity of all DNA samples used for the identification of yellow pigmented colonies.

The amplification was performed on a 1.5 µl-DNA extract in 20 µl using 1U of AmpliTag Gold polymerase (Applied Biosystems, U.S.A.), 2.5 mM MgCl2, 1x PCR buffer, 0.4 mM of each deoxynucleoside triphosphate (dNTP), and 0.25 µM of the forward and reverse primers. DNA amplifications were performed on a Perkin Elmer GeneAmp 9700 thermal cycler. The cycles used were as follows: 1 cycle at 95°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s and elongation step at 72°C for 2 min. PCR amplicons were visualized by ethidium bromide staining on a 1% agarose gel.

Sphingomonas-Specific 16S rRNA-Based PCR Assay

The Sphingomonas-specific primer set consisted of the modified forward primer Sphingo 108f (5'-GGCGT AAGCCGCTAAGGCCCTTC-3’) and the reverse primer Sphingo 420r (5'-TACACATCTAAGCCCTTC-3’) (Leys et al. 2004). The PCR mixture contained 2 µl of DNA, 1 U of AmpliTag Gold polymerase (Applied Biosystems, U.S.A.), 20 pmol of the forward and reverse primers, 10 nmol of each deoxynucleoside triphosphate (dNTP), 2.5 mM MgCl2, and 1X PCR buffer in a final volume of 50 µl. All PCR assay runs incorporated a reagent control (without template DNA) and a negative PCR control using DNA extracted from Escherichia coli and Staphylococcus aureus. PCR amplification was comprised of the following three steps: heating at 95°C for 5 min; 50 cycles of denaturation at 95°C for 5 s, annealing at 62°C for 10 s, and extension at 74°C for 30 s; 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.

16S rDNA Sequencing

The 16S rDNA was amplified in a total volume of 20 µl using the primers SSU-bact-27f (5'-AGA GTT TGA TCC TGG CTC AG -3’) and SSU-bact-519r (5’- GWA TTA CCG CGG CKG CTG -3’) (Lane 1991). The reaction contained 2 µl of 50 ng DNA, 200 µM dNTPs, 0.4 pmol of each primer, 1x PCR Buffer II (Applied Biosystems), 2.5 mM MgCl2, and 0.1 U of AmpliTag Gold DNA polymerase (Applied Biosystems). The thermal cycling reaction consisted of an initial denaturation for 12 min

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at 95°C followed by 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 60°C), and extension (1 min at 72°C), with a single final extension for 10 min at 72°C.

The amplicons were sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing products were purified using the Centri-Sep Columns (Princeton Separations, Adelphia, NJ) and analyzed using the ABI 3130xl Genetic Analyzer (Applied Biosystems) according to the manufacturer’s instructions.

Results

Isolation and Purification of Yellow Pigmented Colonies

Figure 1 shows different forms and derivatives of yellow pigmented colonies representing some of the morphological entities recovered from drinking water samples collected from water storage tanks and the water distribution network over a period of two years.

Biotyping

The metabolic profiles obtained, based on the utilization of 95 carbon sources, from the Biolog microplates were compared with ATCC *Sphingomonas* species as well as some other sphingomonads that were previously isolated and identified through 16S rDNA sequencing (Tokajian and Hashwa 2003; Tokajian and Hashwa 2004b). Results obtained revealed that there was considerable variation among the isolates. Biotype 1 (7 isolates: ST-3, 4, 5, 7, 8, 9, and 10) was found to be most similar to the ATCC strains *Sphingomonas macrogoiabida* and *Sphingobium yanokiyae* (Fig. 2). Biotype 2 (2 isolates: ST-12 and 33) was closely associated with *Sphingomonas* sp. strain SRS7. Biotype 3 (4 isolates: ST-21, 39, 41, and 49) was clustered with *Sphingomonas adhaesiva* and *Sphingomonas* sp. strain JSS-7. Biotype 4 (10 isolates: ST-11, 18, 19, 23, 24, 25, 26, 27, 47, and 50) was found to be closely related to *Sphingomonas chungbukensis*. Biotype 5 (9 isolates: ST-34, 35, 36, 37, 38, 40, 42, 43, and 48) and Biotype 6 (3 isolates: ST-15, 20, and 31) did not cluster with any of the used reference ATCC strains. Biotype 7 (3 isolates: ST-28, 32, and 46) was found to be closely related to *Sphingomonas* sp. strain BN6. Biotype 8 (ST-30) and Biotype 9 (ST-22) were closely related to *Sphingobium* sp. strain S10 and *Sphingomonas natatoria*, respectively. Finally, the remaining isolates ST-17 (*Sphingomonas* sp. strain IFO 11), ST-14, and ST-2 each represented a separate branch, and the overall similarity index was very low (Fig. 2).

16S rDNA Amplification

The quality of the extracted DNA from all sphingomonads was evaluated by the amplification of the 16S rDNA (Fig. 3). All samples except for the reagent control showed a clear band at 1500 bp.

16S rDNA Sequence Analysis

ST-39 and ST-41 consensus sequences were analyzed against the nucleotide collection database using the NCBI Blast. ST-39 was found to be most similar to *Mycobacterium* sp. and a few soil and Actinobactria sp., while ST-41 was similar to *Bacillus* sp. and a few marine sediment bacteria, with maximum identities ranging between 96 and 100%.

Discussion

The autochthonous microbial population of drinking water resources, representing an oligotrophic nutrient-deprived habitat, remains largely uncharacterized (Kalmbach et al. 1999). Only a small percentage of
Sphingomonas macrogoltabida
Sphingobium yanoikuyae
ST-13 (Sphingomonas sp. strain SRS7)
ST-1 (Sphingomonas adhaesiva)
ST-21
ST-41
ST-39
ST-16 (Sphingomonas sp. strain JSS-7)
ST-49
ST-24
ST-26
ST-25
ST-27
ST-23
ST-18
ST-19
ST-47
ST-11
ST-44 (Sphingomonas chungbukensis)
ST-50
ST-43
ST-34
ST-40
ST-42
ST-38
ST-48
ST-37
ST-35
ST-36
ST-20
ST-31
ST-15
ST-32
ST-46 (Sphingomonas sp. strain BN6)
ST-28
ST-29 (Sphingobium sp. strain S10)
ST-30
ST-17 (Sphingomonas sp. strain IFO 15917)
ST-14
ST-2
ST-22
ST-6 (Sphingomonas natatoria)

Fig. 2. Clustering of drinking water sphingomonads on the basis of the utilization and hydrolysis of carbon sources (UPGMA algorithm). The positions of relevant reference strains are also shown.
Fig. 3. PCR-mediated amplification of the 1500-bp segment of 16S rRNA gene. DNA samples were analyzed on a 1% agarose gel. Well 1: O’RangeRuler 500 bp DNA ladder (Fermentas); Well 2: negative reagent control; Well 3: *Staphylococcus aureus* positive control; Well 4: *Escherichia coli* positive control; Lanes 1 through 50 presumptive ST-1 through ST-50 sphingomonads.

Fig. 4. PCR-mediated amplification of 320-bp *Sphingomonas*-specific 16S rRNA gene. DNA samples were analyzed on a 2.5% agarose gel. Well 1: Molecular weight marker VIII (Roche); Well 2: negative reagent control; Well 3: *Staphylococcus aureus* Gram-positive organisms used as a negative PCR control; Well 4: *Escherichia coli* Gram-negative organisms used as a negative PCR control; Lanes 1 through 50 presumptive ST-1 through ST-50 sphingomonads.
these bacteria encountered in water distribution systems are identifiable, as many fail to grow on conventional media used for biochemical characterization (Spinno 1985). Yellow pigmented colonies are common in water samples collected both from water storage tanks and distribution networks in Lebanon. The identification of HPC (heterotrophic plate count) isolated from drinking water samples, based on the sequencing of the 16S rRNA gene, revealed that some members of the family Sphingomonadaceae (Tokajian and Hashwa 2004a; Tokajian et al. 2005).

Sørensen et al. (2001) used the Biolog system for the identification of Sphingomonas sp. (SRS2), and Yang et al. (2006) tested the ability S. chlorophenolica to utilize (oxidize) various carbon sources, while Pollock (1994) identified isolates secreting gellan-related polysaccharides as Sphingomonas. The API 20NE and Biotype 100 were also used by Yabuuchi et al. (2002) for biochemical characterization of different sphingomonads. All biochemical identification schemes including the Biolog (Biolog, Inc., Hayward, California) and the API (bioMerieux, Marcy-L’Etoile, France) may yield ambiguous and misleading results (Tokajian et al. 2005). The tests used in those schemes may not lead to reproducible results and the phenotype of a species is not an absolute property but may exhibit remarkable variability. The database of phenotypic characteristics is only limited to common species (Springer et al. 1996; Tang et al. 1998), not necessarily suitable for environmental isolates. However, we tried to base phenotyping on individual Biolog test results to overcome database related constraints, but the low similarity indices (Fig. 2) obtained upon clustering those isolates again confirmed the general irreproducibility associated with biochemical identification schemes. Consequently, and since the Biolog system was not useful even to identify those isolates at the genus level, we have established a more applicable 16S rRNA gene-based PCR assay that can be easily employed for the screening of sphingomonads amongst colonies growing on R2A agar. The modified primer set used can be specifically employed to differentiate and confirm the number of colonies belonging to this genus in samples collected from drinking water networks as well as storage tanks. Isolates designated as ST-1, 4, 6, 9, 10, 13, 16, 17, 25, 29, 44, and 45 were previously identified based on 16S rDNA sequencing. ST-4, 6, 9, 10, and 25 were identified as Sphingomonas natatoria, ST-13 and ST-45 as Novosphingobium subarcticum, ST-16 as Sphingomonas aquatilis, ST-17 as Sphingomonas sp. strain IFO 15917, ST-18 as Sphingomonas adhaesiva, ST-29 as Sphingomonas sp. strain MBIC 3990, and ST-44 as Sphingomonas chungbukensis (Tokajian and Hashwa 2004a; Tokajian et al. 2005).

It is worth mentioning that ST-4, 9, 10 (Sphingomonas natatoria), and isolate ST-18 (Sphingomonas adhaesiva) showed at least two additional bands with a size of around 500 bp upon amplification using the sphingomonad-specific primer set. This characteristic pattern distinguished those sequenced isolates from all other sequenced sphingomonads, with the same pattern being also consistent with isolates ST-1, 3, 8, 23, 24, 27, 35, and 36. All the isolates that showed additional bands formed dark yellow to orange colonies. At this stage it is not clear whether this additional band is due to nonspecific amplification or due to the presence of more than one rRNA operon (Condon et al. 1995; Kabadjova et al. 2002). Finally, it is noteworthy that two of the presumptive sphingomonads tested in this study having yellow pigmented colonies (ST-39 and ST-41) did not show any amplification products using the Sphingomonas-specific PCR assay. 16S rDNA sequencing revealed that ST-39 and ST-41 were closely related to Mycobacterium sp. and Bacillus sp., respectively. This indicates that it is very much likely to have other than sphingomonads appearing as yellow pigmented colonies on R2A agar, which additionally supports the use of a PCR-based assay to screen for sphingomonads from drinking water recovered on R2A agar.

Growing sphingomonads requires 4 to 5 days on R2A agar, and employing the PCR-based assay will not reduce the time needed but will definitely increase the testing reliability, since all tested isolates (presumptive sphingomonads, previously sequenced isolates and ATCC strains) gave one common amplification product (320 bp). This is especially true when taking into consideration that biotyping (which currently is the only available method for identifying isolates of environmental origin including sphingomonads) through the use of commercial kits such as the Biolog, and even when based on metabolic profiles to overcome database limitations, resulted in very low similarity indexes.

It is well established now that the presence of sphingomonads in drinking water distribution systems is not desirable, with some strains being considered as potential pathogens. Waterborne bacteremia among neutropenic patients in a hospital in Finland was caused by Sphingomonas paucimobilis (Perola et al. 2002). We have previously reported, in agreement with Koskinen et al. (2000), that the presence of sphingomonads in drinking water may be much more common than has been reported (Tokajian and Hashwa 2004a; Tokajian et al. 2005). The intermittent mode of supply, which is the strategy employed in all countries suffering from water shortage in the Middle East, is characterized by water stagnation and flow interruption, that on start up is associated with biofilm detachment (Tokajian et al. 2005). The intermittent mode of supply with high and fluctuating pressure causes the frequent detachment of bacterial clumps into the water column, including Gram-negative biofilm–forming bacteria such as Stenotrophomonas, Sphingomonas spp., Acidovorax sp., and Pseudomonas (Tokajian and Hashwa 2004a; Tokajian et al. 2005). Establishing a rapid and feasible molecular assay for the identification of yellow pigmented colonies isolated from drinking water samples in Lebanon is important to minimize health-related risk factors, taking into
consideration that phenotypic methods (Biolog system) usually fail to generate reproducible phenograms.

**Conclusion**

The abundance and frequent isolation of derivatives of yellow pigmented colonies from drinking water samples in Lebanon raised the need to establish a feasible assay to identify sphingomonads. Amplification of 16S rRNA gene in sphingomonads, which are known to be likely drinking water contaminants originating from distribution network biofilms, provides an accurate and sensitive assay for their screening and accurate identification. The method also overcomes the problems associated with identifying organisms of environmental origin based on their metabolic profiles using commercially available kits such as the Biolog. Finally, we recommend testing the feasibility of this assay in identifying sphingomonads directly from water samples without the need for prior cultivation to improve practicability of this assay.

**References**


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