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SPHINGOMONAS PAUCIMOBILIS AS A BIOFILM PRODUCER

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Abstract - The aim of this study was, for the first time in our country, to identify the capability of isolates of *Sphingomonas paucimobilis* to form a biofilm. In the 3-month period from January 1st to March 31st 2010, a total of 2630 samples of drinking water were microbiologically examined in the Institute of Public Health of Vojvodina, Serbia. From all examined samples of drinking water, non–fermentative Gram-negative oxidase positive bacilli were identified in 113 samples (4.30%). The bacteria isolates were identified as *Sphingomonas paucimobilis* (4 isolates), based on analysis by the automated VITEK 2 Compact system; biofilm formation was examined according to the modified method of Stepanović et al. (2000). All 4 *Sphingomonas paucimobilis* strains tested showed a strong biofilm-producing ability. Considering the potential pathogenic features of *Sphingomonas paucimobilis*, the presence of these strains in drinking water distribution systems is not desirable. Therefore, adequate biofilm degradation and management of drinking-water distribution networks that will guarantee microbiologically safe drinking water is recommended.

Key words: *Sphingomonas paucimobilis,* biofilm, drinking water

INTRODUCTION

Water intended for drinking and household purpose should be free of microbes posing a potential health hazard. It is well known that the quality and safety of drinking water continues to be an important public health issue, because its contamination has often been the way of transmission of serious infectious diseases associated mortality worldwide. Pathogens, coliforms and other bacteria can survive and grow within biofilms on the inner surfaces of distribution pipes, causing the deterioration of the microbiological quality of the water. Other hygienic aspects of biofilms in water systems are the deterioration of aesthetic water quality through discoloration, turbidity and malodour (Vilchez et al., 2007).

Sphingomonas paucimobilis is widely distributed in nature and has been isolated from drinking water and drinking water distribution systems. As it is ubiquitous in the environment, it can be frequently found in surface biofilms. Some *Sphingomonas* strains are well known for metabolizing complex organic pollutants, but some are opportunistic human pathogens (Gusman et al., 2010)

In recent years, it has become evident that biofilms in drinking water distribution networks and other man-made water systems can become transient or long-term habitats for hygienically relevant microorganisms. *Sphingomonas paucimobilis* can produce biofilms or attach to preexisting biofilms, where they become integrated and survive for days, weeks or even longer, depending on the biology and ecology of the organism and the environmental conditions (Koskinen et al., 2000).

The aim of this study was to identify for the first time in Serbia, the capability of the isolates of *Sphin-*

gomonas paucimobilis from drinking water to form a biofilm.

MATERIALS AND METHODS

In the 3-month period from January 1st to March 31st 2010, a total of 2630 samples of drinking water were microbiologically examined in the Department of Sanitary Bacteriology, Center for Microbiology, Institute of Public Health of Vojvodina in Novi Sad, Serbia.

After the sample water was inoculated into tubes containing double strength of lactose broth, they were incubated at 35°C-37°C for 48 h. If the liquid medium became turbid and/or changed color, subcultures were made by transferring the lactose broth on blood agar by a sterile wire loop; after incubation the biochemical activities of typical colonies was examined.

From all examined samples of drinking water, non-fermentative Gram-negative oxidase positive bacilli were identified in 113 samples (4.30%).

Subcultures that yielded a pure growth of yellow colonies of non-fermentative non-spore-forming, Gram-negative, rod-shaped bacteria were identified by the automated VITEK 2 Compact system (BioMerieux, France) as *Sphingomonas paucimobilis* (4 isolates). Bacterial strains were stored as stock cultures.

For further examination of biofilm production, prior to inoculation all strains were transferred from the stock cultures to blood agar and incubated aerobically at 37°C for 24 h. Then all the strains were subcultured once more under the same conditions. Thus, refreshed strains were transferred to tryptic soy agar, incubated aerobically at 37°C for 24 h and then resuspended in the volume of tryptic soy broth necessary to achieve cellular density of $1x10^8$ cells/ml.

Biofilm production was detected according to the modified microtiter plate test proposed by Stepanović et al. (2000). For each bacterial strain, at least 16 wells of a sterile 96-well flat tissue polystyrene culture plate were filled under aseptic conditions with 200 µl of bacterial suspension. The content of each well was removed and washed three times with 250 µl of sterile physiological saline to remove nonadherent and weakly adherent bacteria. The plates were air dried for 30 min, with the remaining attached bacteria being analyzed in terms of biomass adhering to the inner walls of the wells. Negative controls were obtained by incubating the wells only with broth without adding any bacterial cells. All experiments were performed in triplicate.

The bacterial biofilms in the 96-well plates were fixed with 250 µl/well of 99% methanol for 15 min. After this, the plates were emptied and left to dry. The fixed bacteria were stained for 5 min with 200 µl/well of crystal violet (Gram color-staining set for microscopy, Merck). Excess stain was rinsed out by placing the plate under low-running tap water. After the plates were air dried, the dye bound to the adherent cells was resolubilized by 200 µl-well of 33% glacial acetic acid. The optical density of the obtained solution was measured at 570nm (OD_{570}) using a microtiter plate reader and the biofilm mass was presented as $OD_{570.}$

Bacteria were classified using the scheme of Stepanović et al. (2000) as follows: non-biofilm producer, weak, moderate and strong biofilm producer.

RESULTS AND DISCUSSION

From all examined samples of drinking water, non-fermentative Gram-negative oxidase positive bacilli were identified in 113 samples (4.30%), and among them, 4 isolates of *Sphingomonas paucimobilis* were found.

Based on intensity of color, ie OD measured for each well at 620nm using ELISA reader, and compared with the OD of the negative control, all isolates could be referred to as non-adherent, weakly, moderately and strongly adherent. All 4 *Sphingomonas paucimobilis* isolates tested showed

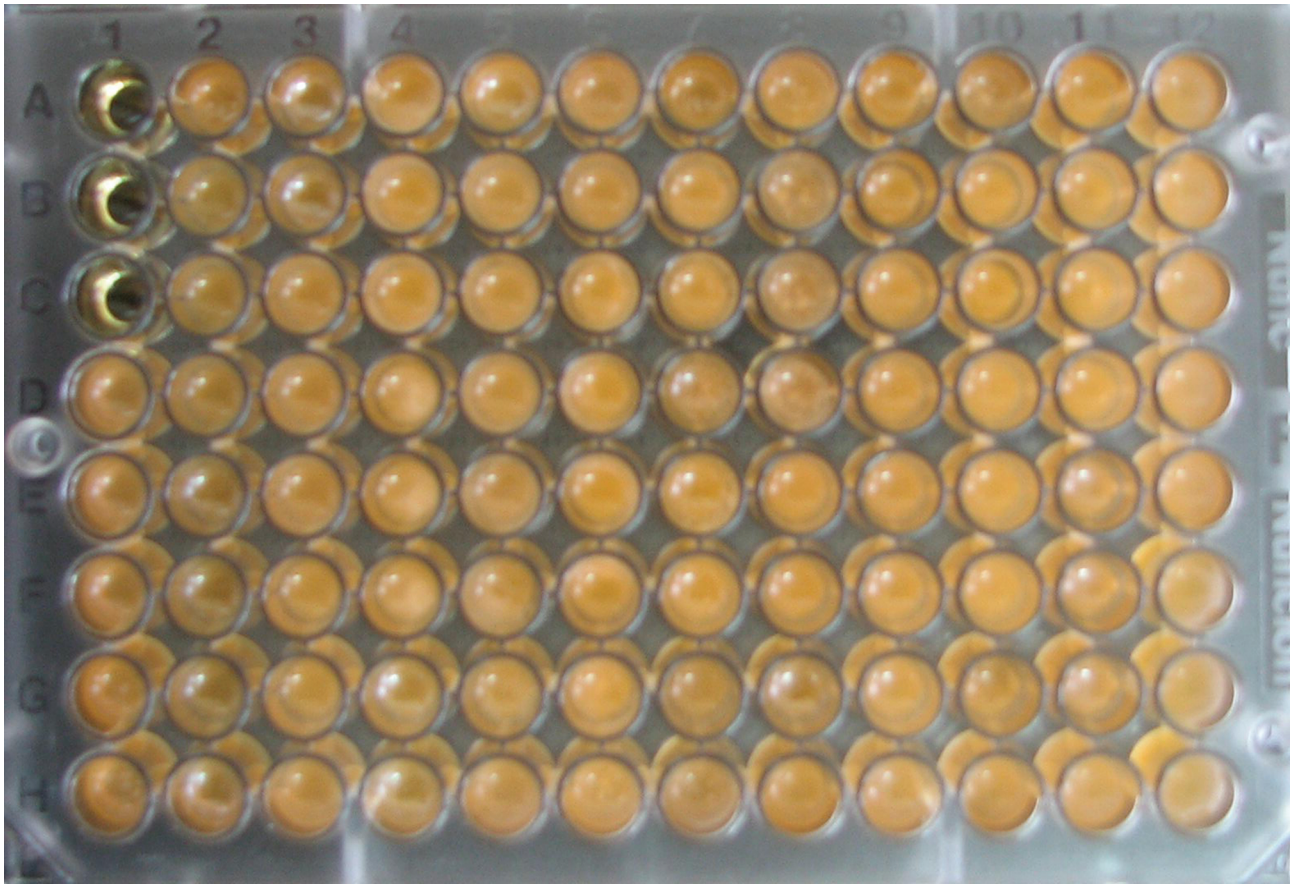

Fig 1. Biofilm production in isolates of *Sphingomonas paucimobilis* isolated from drinking water after 24 h incubation.

a strong biofilm-producing ability and the control ATCC 31461 strain was non-adherent. Strongly adherent isolates had 4 times higher OD values than the control.

An understanding of the microbial ecology of water distribution systems is necessary to design innovative and effective control strategies that will ensure safe and high-quality drinking water. Recent investigations of the microbial ecology of drinking water distribution systems have found that pathogen resistance is affected by the community biodiversity and interspecies relationships.

Sphingomonas paucimobilis possess unique abilities in degrading refractory organic pollutants and simple aromatic and polyaromatic hydrocarbons. Because of their metabolic diversity, they are potential microbial agents for bioremediation. Despite its potential as a bioremediation agent, it is an opportunistic pathogen causing nosocomial infections such as bacteremia, catheter-related sepsis, meningitis, peritonitis, diarrheal diseases and tracheal infections. Because of its ability to survive in low nutrient conditions, oligotrophic niches such as hospital tap water supply systems, reverse osmosis systems, dialisate and ventilators have been implicated as sources of infection. Furthermore, *Sphingomonas* strains have been identified in the flight potable water supply and humidity condensate samples on board of the international Space Station (Castro, 2004).

Another undesirable property of *Sphingomonas paucimobilis* is that the bacteria is capable of forming a biofilm that can cause metal corrosion of plumbing systems and biofouling of drinking water and industrial water distribution systems. The biofilm-forming property of *Sphingomonas paucimobilis* may explain its ability to survive in chlorinated municipal and industrial water distribution systems. As a result, both municipal and industrial water systems can be vulnerable to contamination by this type of bacteria.

Some authors have investigated the effects of pipe materials (steel, copper, stainless steel, polyvinyl chloride, polystyrene) on biofilm accumulation and water quality, and have concluded that regardless of the pipe materials, *Sphingomonas* was the predominant species in all biofilms (Jang et al., 2011, Simoes et al., 2010).

In this study, *Sphingomonas paucimobilis* isolates are recognized as biofilm producing and problematic opportunistic bacteria. The selected bacterial species were also detected in drinking-water biofilms. In fact, biofilms on surfaces exposed to drinking water in distribution systems may be the main source of planktonic bacteria, since up to 1000 sessile microorganisms can be present for each planktonic cell detected. Microbial growth control is a key issue in fulfilling drinking water quality standards.

Similar findings have been reported by other authors in different countries, including Canada, where Kurissery has found that *Sphingomonas* were the most common bacterial species in biofilm that showed the highest production of extracellular polymeric substances and very good adhesion properties (Kurissery et al., 2010). In Finland, Rasimus has detected that 75% of the bacterial isolates from analyzed biofilms were identified as members of the genus *Sphingomonas* (Rasimus et al., 2011). In the Hungarian study by Bohus, analyzing bacterial communities in ultrapure water, *Sphingomonas* dominated in outlet water samples (Bohus et al., 2011).

As under natural conditions, true monospecies biofilms are rare, occurring mostly as complex communities, the physiology and metabolism of multispecies biofilm communities are immensely complex. Diversity in microbial communities leads to a variety of complex relationships involving inter- and intraspecies interactions; the specific mechanisms for multispecies biofilm formation and organization still remain unclear, and most studies were carried out under the experimental conditions used for single biofilms (Venungopalan et al., 2005). Even though in this study we have examined the capability of *Sphingomonas* to produce monospecies biofilm, Min and Rickard have pointed that coaggregation promotes biofilm integration by facilitating attachment to partner species and likely contributes to the expansion of coaggregating Sphingomonas populations in dual-species biofilms through competitive interactions (Min and Rickard, 2009), while Simoes, Kim and Han, in separate studies, have demonstrated that Sphingomonas has a high potential for inhibiting the growth of counterpart biofilm (Simoes et al., 2011, Kim and Han, 2011).

From an ecological point of view, both competition and cooperation can exist in drinking water distribution systems. Beneficial interactions in biofilms can include coaggregation and plasmid conjugation, contributing to the protection of one or several layers of microorganisms from eradication even when the biofilm is exposed to external stress factors.

This is the first study in our country revealing *Sphingomonas paucimobilis* as a potential biofilm producer in a drinking water distribution system.

CONCLUSION

The presented results show that *Sphingomonas paucimobilis* isolates are capable of forming biofilm. Considering the potential pathogenic features of *Sphingomonas paucimobilis*, the presence of these strains in drinking water distribution systems is not desirable. Adequate biofilm degradation and management of drinking-water distribution networks that will guarantee microbiologically safe and thereby high-quality drinking water, are therefore recommended. Further studies are being carried out to find key fac-

tors regulating biofilm occurrence and community composition in drinking water distribution systems and to improve disinfection strategies against biofilm formation.

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