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ORIGINAL PAPER

Culturable bacterial diversity from a feed water of a reverse osmosis system, evaluation of biofilm formation and biocontrol using phages

D. R. B. Belgini · R. S. Dias · V. M. Siqueira · L. A. B. Valadares · J. M. Albanese · R. S. Souza · A. P. R. Torres · M. P. Sousa · C. C. Silva · S. O. De Paula · V. M. Oliveira

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Abstract Biofilm formation on reverse osmosis (RO) systems represents a drawback in the application of this technology by different industries, including oil refineries. In RO systems the feed water maybe a source of microbial contamination and thus contributes for the formation of biofilm and consequent biofouling. In this study the planktonic culturable bacterial community was characterized from a feed water of a RO system and their capacities were evaluated to form biofilm in vitro. Bacterial motility and biofilm control were also analysed using phages. As results, diverse Protobacteria, Actinobacteria and Bacteroidetes were identified. Alphaproteobacteria was the predominant group and *Brevundimonas*, *Pseudomonas* and *Mycobacterium* the most abundant genera. Among the 30 isolates, 11 showed at least one type of motility and 11 were classified as good biofilm formers. Additionally, the influence of non-specific bacteriophage in the bacterial biofilms formed in vitro was investigated by action of phages enzymes or phage infection. The vB_AspP-UFV1 (*Podoviridae*) interfered in biofilm formation of most tested bacteria and may represent a good alternative in biofilm control. These findings provide important information about the bacterial community from the feed water of a RO system that may be used for the development of strategies for biofilm prevention and control in such systems.

Keywords Bacterial diversity · Biofilms · Motility · Bacteriophages · RO systems

D. R. B. Belgini · V. M. Siqueira (✉) · V. M. Oliveira
Microbial Resources Division, Research Center for Chemistry, Biology and Agriculture (CPQBA), Campinas University - UNICAMP, CP 6171, Campinas, SP CEP 13081-970, Brazil
e-mail: virginiamedeiros@hotmail.com

R. S. Dias · L. A. B. Valadares · J. M. Albanese · C. C. Silva · S. O. De Paula
General Biology Department, Federal University of Viçosa – UFV, Viçosa, MG CP 36570-000, Brazil

R. S. Souza · A. P. R. Torres · M. P. Sousa
PETROBRAS Research and Development Center (CENPES), Biotechnology Management, Av. Horácio Macedo, 950, Expansão, Ala C, Ilha do Fundão, Rio de Janeiro, RJ 21941-915, Brazil

Introduction

The current trend in wastewater management by industries focuses on pollution prevention, either by the reduction of the use of natural resources or the application of clean technologies with low environmental impacts (Stepnowski et al. 2002). Although recycling of the total water is not applicable, and may not be required in all cases, this is an alternative for industries with high water consumption. In this sense, the technology of reverse osmosis (RO) membrane has been widely used by various industries, such as petroleum refineries (Salahi et al. 2010). The RO membrane technology offers several advantages such as durability, low power consumption, high productivity and efficiency in removing a variety of contaminants. Nevertheless, the adhesion and growth of microorganisms, and subsequent biofouling, represent a drawback associated with the use of this technology (Melo and Flemming 1993).

Biofilms are microbial communities that develop adhered to a surface and surrounded by extracellular polysaccharides substances (EPS) (Hughes et al. 1998a; Stoodley et al. 2002). As microbial communities, biofilms are assemblages of diverse species occupying the same functional discrete environment. Biofilms have a complex

level of organization with a distinctive and specialized structure and particular activities, which depend on the relationships between their constituents (Wimpenny 2000). Many factors are associated to biofilm formation on RO membrane surface, including properties of the feed water, e.g. its microbial planktonic community composition (Ridgway and Safarik 1991).

The kinetics of biofilm formation is often described by the initial adhesion of planktonic bacteria on the surface, and subsequent multiplication of microorganisms, with the formation of microcolonies and EPS production (Allison 2003; Stoodley et al. 2002). Then, other bacteria can be incorporated into the pre-formed biofilm, in which the water supply can be considered a source of microorganisms participating in both initial formation and maturation of the biofilm (Momba et al. 2000). Additionally, bacterial motility mediated by flagellum and *pili* is reported as an important feature linked to early biofilm formation. These motility structures are responsible for the transportation and fixation of microorganisms on the surface (Houry et al. 2010; Lemon et al. 2007; O'Toole and Kolter 1998; O'Toole et al. 2000).

Since biofilms are very difficult to eradicate, the ability of bacteria to form biofilms poses a major problem in various industrial settings, being a persistent source of (re)contamination. The impenetrable character of the biofilm, the slow growth rate of the constituent organisms and the induction of resistance are examples of mechanisms proposed to explain the observed increased endurance of biofilms to antimicrobial and disinfectant agents (Abee et al. 2011). On the other hand, initial steps of biofilm formation and subsequent dispersal of bacteria from the established biofilm are recently starting to be unraveled and may help to formulate strategies to prevent and control biofilm development.

Over the last two decades various studies have been focused on the development of methods for membrane biofouling control, including the use of biocides, enzymes and UV irradiation (Simões et al. 2010). The application of bacteriophages is nowadays seen as a good alternative to prevent and control biofilms in wastewater treatment plants and RO systems (Goldman et al. 2009). Phages are viruses that infect bacteria and, by their nature, are good candidates for biofilm control because of their high specificity, affecting only the target bacteria, their non-toxicity to animals and plants, and their simple, rapid and relatively inexpensive production (Clark and March 2006; Cornelissen et al. 2011; Azeredo and Sutherland 2008). Most phages for biofilm-forming bacteria yield polysaccharide depolymerases; either phage or enzyme, both could have a potential use in determining the role of single bacterial species and of their exopolysaccharides in mixed biofilm (Hughes et al. 1998a). Phages and enzymes have been the subject of extensive research on the control of bacterial biofilms formed in various environments such as hospital, food processing and industries (Ahiwale et al. 2011; Siringan et al. 2011; Soni and Nannapanen 2010) and your action can be affected by subtle changes in the EPS composition what may prevent them from degrading. It is unlikely to find phage in high concentrations. Nevertheless it is believed that it is possible that a single phage can infect a host, multiply and cause the collapse of the biofilm (Hughes et al. 1998b).

Thus, for a better understanding of the whole process of biofilm formation it is crucial to know the planktonic bacterial community, as well as the characteristics inherent to their microbial constituents, e.g. motility, which may be directly related to the development of these communities in such systems. In this context, this work aims to investigate the cultivated fraction of the bacterial diversity from feed water of a RO membrane system from a petroleum refinery, and evaluate their motility, capability to form biofilm and the use of phages in biofilm control.

Materials and methods

Bacterial isolation and identification

Bacteria were isolated from feed water sampled from a RO system at Gabriel Passos Refinery (REGAP) of Petrobras, located in the city of Betim, Minas Gerais State, Brazil. Samples were kept under refrigeration during transportation to the laboratory and stored at 4 °C prior to isolation. Briefly, 100 μL of the sample were directly inoculated onto tryptone soya agar (TSA; pancreatic digest of casein 15 g/L, enzymatic digest of soya bean 5 g/L, NaCl 5 g/L, agar 15 g/L), nutrient agar (NA; peptone 5 g/L, yeast extract 3 g/L, NaCl 5 g/L, agar 15 g/L) and yeast malt extract agar (ISP2; yeast extract 4.0 g/L, malt extract 10 g/L, glucose 4 g/L, agar 15 g/L) culture media surface in triplicate, and incubated at 30 °C up to 5 days. After this period, bacteria were selected based on macro and micromorphology for further purification and identification.

The bacterial genomic DNA was extracted according to the protocol described by Pitcher et al. (1989). DNA integrity and concentration were estimated through electrophoresis in 0.8 % agarose gel stained with SYBR Safe 10.000x in DMSO (Invitrogen) using the intact phage lambda DNA as standard. The DNA obtained was used in polymerase chain reaction (PCR) reactions for amplification of 16S rRNA using the primers 10f (5′-GAG TTT GAT TCA GGC CCT G-3′) and 1100r (5′-GTT GTG AGG GTT GGG G-3′), which are homologous to conserved regions of the 16S rRNA for the Domain Bacteria (Weisburg et al. 1991). The PCR amplification program

consisted of one cycle at 95 °C for 2 min, 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min, and 1 cycle of final extension at 72 °C for 3 min. Amplification was performed in 50 µL-reactions containing 2.0 U of *Taq* DNA polymerase (Invitrogen), 1× Taq cap (Invitrogen), 1.5 mM magnesium chloride, 0.2 mM dNTP mixture, 0.4 µM each primer and 50–100 ng genomic DNA. The results of PCR amplification were confirmed using 1 % agarose gel, stained with SYBR Safe 10.000x in DMSO (Invitrogen). PCR products were subsequently purified using mini-columns (GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) and subjected to sequencing in an automated sequencer (ABI 3500XL) with primers 10f and 1100r.

Partial 16S rRNA sequences obtained with each primer were assembled in a contig (unique sequence obtained by combining the different fragments) using the program phredPhrap (Ewing et al. 1998; Gordon et al. 1998). Identification was achieved by comparing the contiguous 16S rRNA sequences obtained with 16S rRNA sequence data from reference type strains available in the public databases GenBank (http://www.ncbi.nlm.nih.gov) and RDP (Ribosomal Database Project, Wisconsin, USA http://www.cme.msu.edu/RDP/html/index.html) using the BLASTn and Classifier routines, respectively. The sequences were align using the CLUSTAL X program (Thompson et al. 1997) and analyzed with MEGA software v.4 (Tamura et al. 2007). The evolutionary distances were derived from the sequence-pair dissimilarities, calculated as implemented in MEGA using the DNA substitution model reported by Kimura (1980) and the phylogenetic reconstruction was done using the neighbor joining (NJ) algorithm (Saitou and Nei 1987), with bootstrap values calculated from 1,000 replicate runs.

Motility assay

The protocol described by Déziel et al. (2001) was followed for the motility evaluation. Bacteria were rinsed from an overnight culture, suspended in distilled and sterilized water and inoculated on King B (peptone 20 g/L, $MgSO_47H_2O$ 1.5 g/L, K_2HPO_4 1.5 g/L, agar 15 g/L) culture medium containing 1.5, 0.5 and 0.3 % of agar for twitching, swarming and swimming tests, respectively, and incubated at 35 °C for 48 h. Three plates were used to evaluate each of the bacterial motility character. For twitching and swarming tests, bacteria were point-inoculated with a sterile toothpick on the agar surface. For swimming, bacteria were inoculated with a sterile toothpick through the culture medium. Motility was then assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation.

Biofilm formation assay and biomass quantification

Surface-adhered biofilm formation was assayed using bacterial cells from an overnight culture grown in Nutrient Broth (NB; peptone 5 g/L, yeast extract 3 g/L, NaCl 5 g/L) medium at 37 °C and 150 rpm. Bacterial cell suspensions of an optical density of approximately 0.1 at 600 nm were inoculated into wells of a polystyrene flat-bottomed microtiter-plate and incubated at 37 °C for 24 h.

The biomass quantification was performed using the staining method previously described (Extremina et al. 2011; O'Toole 2011). Briefly, after 24 h the culture medium was removed from each well and the adherent cells were washed three times with PBS buffer (pH 7.2). These were dried for 1 h and 200 µL of 0.1 % (w/v) crystal violet (CV) solution were added. After 30 min, the excess stain was removed. The biofilms were distained by adding 250 µL of ethanol/acetone solution (80:20; v/v) to each well. The ethanol/acetone solution was gently pipetted to completely solubilize the CV, transferred into a clean 96-well microtiter plate and the OD_{600} was read using a microtiter plate reader (VersaMax, Molecular Devices). The OD_{600} values are proportional to the quantity of biofilm biomass, which comprises cells and extracellular polymeric material (the greater the quantity of biological material, the higher the level of staining and absorbance). All the experiment was done in triplicate and result values were averaged.

Phages isolation

Bacteria were isolated from activated sludge collected at REGAP (Betim, MG, Brazil), using the culture media: saline nutrient broth (Mod) modified (17.8 g NaCl, 0.1 g $MgS0_4{\cdot}7H_20$, 0.036 g $CaCl{\cdot}2H_20$, 0.2 g of KCl, 0.006 g NaHCO, 0.023 g NaBr, trace of $FeCl{\cdot}6H_20$, 0.5 g proteose-peptone, 1.0 g yeast extract, 0.1 g glucose, pH adjusted to 7.2) (Rohban et al. 2009), and R2A (0.5 g yeast extract, 0.5 g proteose-peptone, 0.5 g casamino acids, 0.5 g glucose, 0.5 g soluble starch, 0.3 g K_2HPO_4, 0.05 g $MgSO_4{\cdot}7H_2O$, 0.3 g sodium pyruvate, and 15 g agar per liter, pH adjusted to 7.2) (Reasoner and Geldreich 1985), both specific for halophilic bacteria. These culture media were chosen because bacteria forming biofilm on RO membranes are present mostly in contact with the saline concentrate produced during the process of water treatment. These bacteria were then used to isolate lytic phages employed in this work. Four morphologically different bacterial colonies were selected from each culture medium and were identify as *Arthrobacter soli* and *A. nicotianae*.

Phages were isolated from activated sludge of wastewater treatment system, step just prior to reverse osmosis. The phage isolation was done following the protocol described

by Tanji et al. (2008), with some modifications. Briefly, a solution of concentrate activated sludge (10 %; v/v) in water was supplemented with the same volume of nutrient broth and maintained at 37 °C for 24 h for the enrichment of the phages by a number of cycles of infection, replication, lysis and reinfection. After the incubation period, chloroform 10 % (v/v) was added to cause bacterial lysis and release of the virions, and NaCl up to a concentration of 1 M was also added to the suspension in order to release the bacterial cells that adsorbed the virus. The total volume was incubated for 1 h at 4 °C under agitation, and centrifuged at 4,000*g* for 20 min in order to precipitate larger particles, cells and cellular debris. The aqueous phase was removed and added of polyethylene glycol 8000 (PEG-8000) 10 % (w/v). The solution was incubated at 4 °C for 24 h and centrifuged at 11,000*g* for 20 min. The supernatant was discharged and the precipitate was suspended in SM buffer (5.8 g NaCl; 2 g $MgSO_2{\cdot}7H_2O$; 50 mL Tris–HCl pH 7.5 1 M; 5 mL gelatin 2 %; and H_2O to 1,000 mL). PEG was removed using equal volume of chloroform. The aqueous phase was separated after centrifugation at 4,000*g* for 20 min and used for phage isolation.

Screening and titration of lytic phage were perform by the double-layer agar method (Sambrook and Russell 2001) using the four bacteria isolated from activated sludge in Mod (Rohban et al. 2009) and R2A (Reasoner and Geldreich 1985) media. One hundred microliters of the previous viral suspension were added to equal volume of bacterial suspension at high concentration. For this, bacteria were grown to an OD600 of 0.7, the suspensions were centrifuged at 4,000×*g* for 20 min, supernatant was discarded and the pellet suspended in $MgSO_4$ 10 mM, and adjusted to an OD_{600} of 2. This final suspension was mixed with 3 mL LB-agar at 0.7 % and plated on 1.4 % LB-agar layer. The plates were incubated at 30 °C and lytic plaques formed on the upper layer were isolated by sequential plating, as described by Dias et al. (2013) and further propagated according to the methodology described by Eller et al. (2012).

Random amplified polymorphic DNA—RAPD

RAPD, a technique that consists on the amplification of random segments of genomic DNA by PCR using short primers of arbitrary sequence (Willians et al. 1990), was performed aiming at the differentiation of identical isolates at the infra-specific level.

Bacterial isolates

The molecular technique RAPD was employed in order to genetically differentiate the bacterial isolates belonging to the same species.

The bacterial genomic DNA was extracted according to the protocol described by Pitcher et al. (Pitcher et al. 1989). The PCR amplification program consisted of one cycle at 95 °C for 2 min, 30 cycles at 94 °C for 30 s, 36 °C for 30 s and 72 °C for 90 s, and 1 cycle of final extension at 72 °C for 3 min. Amplification was performed in 25 µL-reactions containing 2.0 U of *Taq* DNA polymerase (Invitrogen), 1× *Taq* cap (Invitrogen), 1.5 mM magnesium chloride, 0.2 mM dNTP mixture, 0.4 µM primer and 5–10 ng genomic DNA. The results of PCR amplification were confirmed using 1.5 % agarose gel, stained with SYBR Safe 10.000x in DMSO (Invitrogen). The DNA was amplified by RAPD with the oligonucleotide primers UCB#12 (5′-CCTGGG TCCA-3′), UCB#13 (5′-CCTGGGTGGA-3′, UCB#25 (5′-A CAGGGCTCA-3′ and UCB#31 (5′-CCGGCCTTCC-3′) (Set 100/1; University of British Columbia, Vancouver, Canada), in independent reactions.

Phages

RAPD was applied for assessing genetic diversity and polymorphism of phages. The phage genomic DNA was extracted by the Proteinase K method, according to the protocol described by Sambrook and Russell (2001). The PCR amplification program consisted of four cycles at 94 °C for 45 s, 30 °C for 120 s and 72 °C for 60 s; 26 cycles at 94 °C for 5 s, 36 °C for 30 s and 72 °C for 30 s (the extension step was increased by 1 s for every new cycle); and a final step of 10 min at 75 °C. Amplification was performed in 25 µL-reactions using 2X Taq Master Mix (Vivantis Technologies, Malaysia), 8 µM each primer and 10 ng genomic DNA. The results of PCR amplification were confirmed using 1.5 % agarose gel, stained with GelRed 10.000x (Biotium). The DNA was amplified by RAPD with the oligonucleotide primers: OPL5 (5′-ACGCAGGCAC-3′) and P2 (5′-AACGGGCAGA-3′), as described by Gutierrez et al. (2011).

Biocontrol assay

Bacteria isolated from feed water sampled from a RO system that showed greater ability to form biofilm, i.e. higher biomass, were select to be used in the biocontrol assay employing phages against biofilm formation, as described by Kelly et al. (2012), with some modifications. A phage suspension of a multiplicity of infection (MOI) of 0.01 was added into 96-well microtiter plate containing bacterial suspension, and biofilm formation was measured using biomass quantification with CV staining as already described. The ratios evaluated ranged from 0.00001, 0.0001, 0.001, 0.01, 0.1 to 1 PFU/CFU, as described by Sun et al. (2012). The lowest ratio capable to inflict

reduction in bacterial biofilm was use, what proves susceptible to bacteriophages action.

Electron microscopy

Transmission electron microscopy (TEM) was used to characterize the morphology of isolated phages. For this, ten microliters of a viral suspension were added to a 200-mesh grid, previously covered with FormVar® for 5 min. The excess was removed with filter paper, and then the grid was covered with 10 μL of 2 % uranyl acetate for 15 s. A transmission electron microscopy Zeiss EM 109 TEM operating at 80 kV was used in the analyses, at the nucleus of microscopy and microanalyses (NMM) at UFV. Dilutions were performed for better quality images, when necessary. In order to classify the phages, morphological analyses were made, such as the presence of tail, tail length and diameter of the viral particle.

Results

Bacterial diversity

Based on macroscopic characteristics (e.g. colony size, shape, margin, elevation, surface, chromogenesis, etc.), a total of 30 bacterial colonies were selected and purified. Among them, 26 isolates were classified as Gram negative and 4 as Gram positive (Table 1).

Sequencing and phylogenetic analysis of their partial 16S rRNA gene revealed the presence of five taxonomic main groups: Actinobacteria, Bacteroidetes, Alpha, Beta and Gammaproteobacteria (Fig. 1). The most abundant group was Alphaproteobacteria (13), followed by Gammaproteobacteria (7), Actinobacteria (5), Betaprotebacteria (3) and Bacteroidetes (2). In total, 18 bacterial genera were identified among all isolates, in which *Brevundimonas* (4) was the most frequent, followed by *Pseudomonas* (3), *Mycobacterium* (3), *Sphingomonas* (2), *Azospirillum* (2), *Bosea* (2), *Flavobacterium* (2), *Lysobacter* (2), *Pseudoxanthomonas* (1), *Dokdonella* (1), *Burkholderia* (1), *Diaphorobacter* (1), *Acidovorax* (1), *Novosphingobium* (1), *Sphingopyxis* (1), *Agrobacterium* (1), *Nocardioides* (1) and *Gordonia* (1).

Phylogenetic analysis permitted the identification of many bacterial isolates at the species level (Fig. 1). The Alphaproteobacteria species were the most representative group with 5 isolates identified as *Sphingomonas xenophaga*, *Novosphingobium nitrogenifigens, Sphingopyxis soli* and *Bosea eneae*. Two isolates were recovered in a tight cluster (71 % bootstrap value) with the Gammaproteobacteria species *Lysobacter brunescens* and another one could be clearly identified as *Pseudoxanthomonas mexicana*. One isolate was recovered together with the type strain of the Betaproteobacteria species *Diaphorobacter nitroreducens*, presenting 100 % sequence similarity. Another Betaproteobacteria isolate could be clearly identified as *Acidovorax soli*, with 100 % sequence similarity. The Actinobacteria isolates could not be identified at the species level, maybe due to the extremely conserved nature of the 16S rRNA gene for this taxonomic group. Nonetheless, 5 isolates could be affiliated to the genera *Nocardioides* (1), *Gordonia* (1) and *Mycobacterium* (3).

Table 1 Bacterial motility assay

Isolate number	Bacteria	Motility assay		
		Tw	Swa	Swi
AO1-01	*Mycobacterium* sp.	−	−	−
AO1-02	*Acidovorax soli*	+	−	−
AO1-04	*Mycobacterium* sp.	−	−	−
AO1-05	*Gordonia* sp.	−	−	−
AO1-06	*Flavobacteria* sp.	−	−	−
AO1-07	*Pseudomonas* sp.	+	−	+
AO1-08	*Diaphorobacter nitroreducens*	+	−	+
AO1-10	*Sphingomonas xenofaga*	−	−	−
AO1-12	*Nocardioides* sp.	−	−	−
AO1-13	*Lysobacter brunescens*	−	−	−
AO1-14	*Sphingopyxis soli*	−	−	−
AO1-15	*Bosea eneae*	−	−	−
AO1-16	*Azospirillum irakense*	−	−	−
AO1-17	*Pseudoxanthomonas mexicana*	−	−	+
AO1-18	*Flavobacterium* sp.	−	−	−
AO1-19	*Doknodella* sp.	−	−	−
AO1-20	*Azospirillum* sp.	−	−	+
AO1-21	*Brevundimonas* sp.	−	−	+
AO1-22	*Lysobacter brunescens*	−	−	−
AO1-23	*Mycobacterium* sp.	−	+	−
AO1-25	*Pseudomonas* sp.	+	−	+
AO1-26	*Brevundimonas* sp.	−	−	−
AO1-27	*Pseudomonas* sp.	+	−	+
AO1-28	*Agrobacterium tumefaciens*	−	+	+
AO1-29	*Bosea eneae*	−	−	−
AO1-30	*Brevundimonas* sp.	−	−	−
AO1-31	*Burkholderia* sp.	+	+	+
AO1-32	*Novosphingobium nitrogenifigens*	−	−	−
AO1-33	*Brevundimonas* sp.	−	−	−
AO1-34	*Sphingomonas xenophaga*	−	−	−

Bacterial motility

After motility tests, 11 out of 30 isolates showed at least one type of motility (Table 1). Six isolates were positive

Fig. 1 Neighbor-joining matrix tree based on partial 16S rRNA sequences gene of planktonic bacteria isolated from feed water of a RO system and related species. Evolutionary distances were based on the Kimura 2p model (Kimura 1980). Bootstrap values (1,000 replicate runs, shown as %) >70 % are listed. GenBank accession numbers are listed after species names. *Haloarcula* sp. was used as outgroup

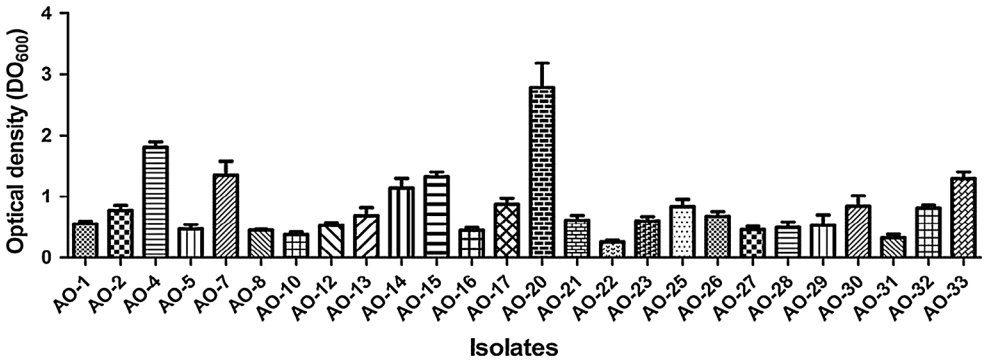

Fig. 2 Values of OD_{600} as a measure of biomass of biofilm producing bacteria. The means ± standard deviations for triplicates are illustrated

for twitching, three for swarming and nine for swimming. Four isolates were positive for both twitching and swimming motilities while only one isolate showed swarming and swimming motilities. *Burkholderia* sp. (AO1-31) was positive for the three types of motilities tested.

Bacterial biofilm formation

Because some isolates did not grow after being consecutively plated, among 30 bacteria isolated, 26 were test regarding their capability to form biofilm. Among the 26 isolates tested, 11 (42 %) were classified as good biofilm formers, though in different levels (Fig. 2).

Azospirillum sp. (AO1-20) produced the highest biomass amount, followed by *Mycobacterium* sp. (AO1-04), *Pseudomonas* sp. (AO1-07), *Sphingopyxis soli* (AO1-14), *Bosea eneae* (AO1-15), *Brevundimonas* sp. (AO1-26; AO1-30; AO1-33), *Acidovorax soli* (AO1-02) and *Lysobacter brunescens* (AO1-13) (Fig. 2).

Phage isolation

Four bacteriophages were isolated from activated sludge. Each phage was named according to the characteristics of the bacterium and the culture medium in which it was isolated: UFVhalophage R2, UFVhalophage R3, UFVhalophage M3 and UFVhalophage M4.

All isolated phages showed the same morphological characteristics (Fig. 3), with an average size of 30 nm, a 5 nm-short tail, and an isometric head approximately 25 nm in diameter. These small dimensions may be the result of the reduced phage genome, with 21.4 kb, approximately, composed by deoxyribonucleic acid (DNA) (Fig. 4). All these characteristics and mainly the presence of a small tail classify these phages as a member of the C1 morphotype, family *Podoviridae*, order *Caudovirales*.

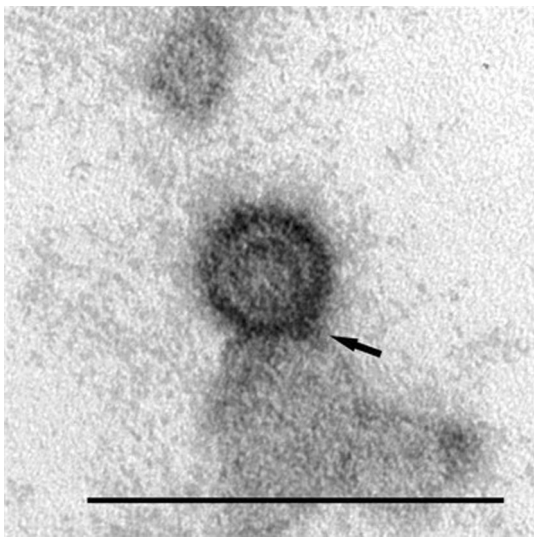

Fig. 3 Transmission electron microscopy of isolated phage vB_AspP-UFV1. *Arrow* indicates the small tail. *Bar* 100 nm

Random amplified polymorphic DNA—RAPD

Bacterial isolates

The bacterial isolates submitted to RAPD typing were AO1-22/AO1-13, AO1-25/AO1-27, AO1-16/AO1-20, AO1-34/AO1-10, AO1-29/AO1-15, AO1-21/AO1-26/AO1-30/AO1-33 and AO1-06/AO1-18.

RAPD fingerprints allowed us to successfully discriminate the genetically distinct isolates (Table 2). Except for *Lysobacter brunescens* (AO1-22/AO1-13), *Azospirillum* sp. (AO1-16/AO1-20), and *Flavobacterium* sp. (AO1-06/AO1-18), all the taxa surveyed presented more than one RAPD profile, revealing a great genetic diversity among the isolates recovered from the feed water sampled from the RO system.

Table 2 Number of RAPD profiles using four primers

Bacteria	Number of isolates	Number of RAPD profiles
Lysobacter brunescens	02 (AO1-22/AO1-13)	01
Pseudomonas sp.	02 (AO1-25/AO1-27)	02
Azospirillum sp.	02 (AO1-16/AO1-20)	01
Sphingomonas xenophaga	02 (AO1-34/AO1-10)	02
Bosea eneae	02 (AO1-29/AO1-15)	02
Brevundimonas intermedia	04 (AO1-21/AO1-26/ AO1-30/AO1-33)	02
Flavobacterium sp.	02 (AO1-06/AO1-18)	01

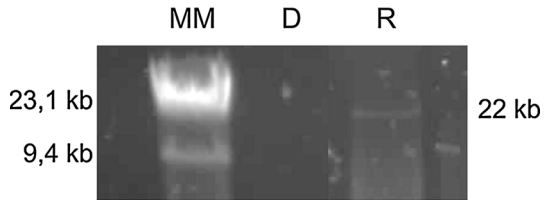

Fig. 4 Phage genome composed by deoxyribonucleic acid (DNA) with c.a. 21.4 kb

Phages

RAPD fingerprints revealed 4 phages that the profile of the fragments obtained was in the range from 500 to 5 kb with 14 polymorphic bands for P2 primer and 12 bands for OLP5 primer (Fig. 5). The four isolates showed to be identical in polymorphism, which, along with morphological analysis led us to believe that they are of the same phage.

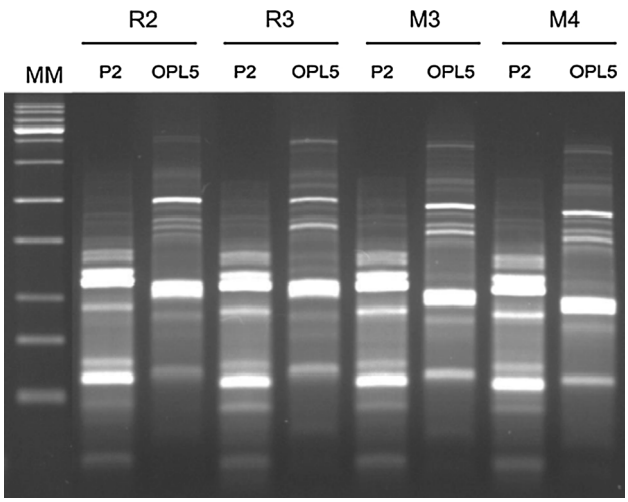

Fig. 5 Band profile of the four isolated phages based on DAPD analysis. *P2* and *OPL5*: RAPD primers. *MM*—1 kb Ladder

Biocontrol assay

Based on the morphology and RAPD results, the four phage isolates were grouped and renamed as vB_AspP-UFV1 (UFV1). This phage was used for biocontrol analyses. After the addition of phage, statistically significant reduction in the biofilm biomass was observed for some of the strains tested, e.g. *A. soli* (AO1-02), *Pseudomonas* sp. (AO2-07) and *Brevundimonas* sp. (AO1-30 and AO1-33). The phage did not affect biofilm formation of *Azospirillum* sp. (AO-20) (Fig. 6).

Discussion

In this study, the diverse planktonic bacteria isolated from feed water of a RO membrane system were identified and characterized regarding their ability to form biofilm. With some minor differences, the results presented are in agreement with previous reports that identified most of bacteria isolated from RO systems as Proteobacteria (Bereschenko et al. 2008; Huang et al. 2008; Ivnitsky et al. 2007; Wagner and Loy 2002). Among all isolates, representatives of the classes Alpha-, Beta- and Gammaproteobacteria, as well as of the phyla Actinobacteria and Bacteroidetes, were identified. These findings show that feed water of the RO system contains a broad diversity of typical freshwater phylotypes (Zwart et al. 2002). Within Proteobacteria, Alphaproteobacteria was the most abundant class found in this study. These bacteria are known to be highly adapted to survive in RO membrane systems due to their tendency to proliferate in oligotrophic ecosystems (Chen et al. 2004; Zhang et al. 2011).

Despite the predominance of Proteobacteria in most related studies, some variation at the species level has been observed among them. The differences in process configuration and operating conditions (e.g. hydrodynamics and previous feed water treatment), as well as general environmental features such as water flow, osmolality, pH, and level of disinfectant may interfere in the bacterial community composition. In this study, *Sphingomonas*, *Brevundimonas*, *Mycobacterium*, *Lysobacter*, *Pseudomonas* and *Burkholderia* were the genera mostly found, and represent the core group of bacteria isolated. These same genera were also detected in RO system by other researchers (Bereschenko et al. 2007; Huang et al. 2008; Schafer et al. 2005). These genera, particularly *Sphingomonas*, are known as metabolically versatile organisms with high-affinity uptake systems under low nutrient condition (Eguchi et al. 2001). The presence of *Sphingomonas* living in its planktonic phase and forming biofilms on RO membranes has been reported (Bereschenko et al. 2010; Chen et al. 2004; Ivnitsky et al. 2007). Bereschenko et al.

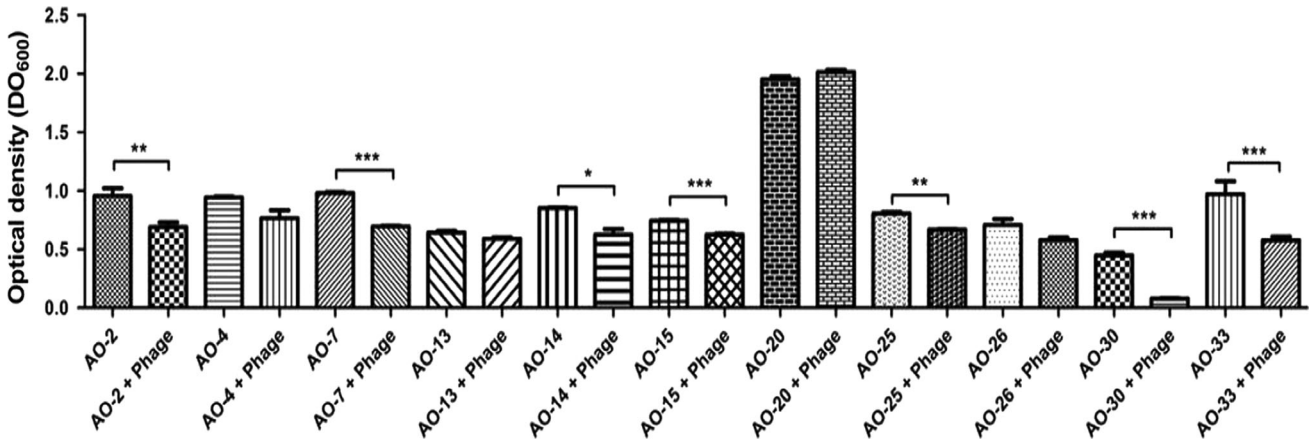

Fig. 6 Effect of the bacteriophage vB_AspP-UFV1 on the biofilms at MOI 0.01. The means ± standard deviations for triplicates are illustrated (*$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$)

(2010) suggested an important role of *Sphingomonas* in the biological fouling of spiral-wound membrane elements applied in the RO systems: when *Sphingomonas* finds a suitable microenvironment, it irreversibly attaches by producing exopolysaccharides around their cells spreading of over the surface, and effectively colonizing the membrane area. *Sphingomonas* was also detected in the present study, although it was not classified as a good biofilm former under the conditions applied in this work.

Since the feed water can be a source of contaminating microorganisms in RO systems, its microbial community may directly influence biofilm formation on membrane surface and further appearance of biofouling. Although not all feed water bacteria are capable of active colonization on membrane surfaces, some of them have been correlated to the early phases of biofilm formation, mainly due to their motility, capability to produce different kinds of EPS and to survive and proliferate in oligotrophic conditions (Bereschenko et al. 2010; Pollock and Armentrout 1999).

In several bacterial species, flagellum and *pili* were shown to play an important role for transportation, adhesion and fixation of bacteria on solid surfaces (Kirov et al. 2004; Vatanyoopaisarn et al. 2000). In biofilm formation these appendages serve to assist in initial attachment of cells to surfaces and in additional stages of biofilm development (Shrout et al. 2011). In this study, three types of bacterial motility (i.e. swarming, swimming and twitching) were investigated. Among the Gram-negative bacteria isolated, *Burkholderia* sp. and *Pseudomonas* sp. showed at least two types of motility. The genus *Burkholderia* comprises aerobic rod-shaped bacteria that are best-known as pathogenic motile species such as *B. cepacia*, *B. mallei* and non-motile species such as *B. pseudomallei*. Although originally identified as a plant pathogen, *Burkholderia* is an ubiquitous genus found in soil, fresh water, distillated water and oligotrophic environments (Vongphayloth et al. 2012). The *Burkholderia* strain (AO1-31) isolated in this study was the only bacterium that showed the three types of motility. *B. cepacia* is also reported forming biofilms in water systems (Torbeck et al. 2011) and on membranes of RO systems (Bereschenko et al. 2008).

In biofilms of RO systems, Bereschenko et al. (2008) detected *Burkholderia* spp. as secondary colonizers (i.e. bacteria mainly present in the feed water as free-living cells), growing on microbial or decay products from primary colonizers. Thus, this bacterial genus may play an important role in biofilm formation on membranes of such systems. In this study, *Burkholderia* sp. strain AO1-31 was not one of the isolates that showed higher biomass values after quantification by CV staining. These results corroborate with the fact that *Bulkholderia* spp. have been detected as secondary colonizers and may depend on a preformed biofilm to attach and remain adhered on a surface (Bereschenko et al. 2008).

Pseudomonas is a genus composed of ubiquitous metabolically versatile Gram-negative bacteria, capable to grow in a variety of environmental conditions. Swarming, twitching and swimming motilities have been previously observed for *P. aeruginosa* (Murray and Kazmierczak 2008). The three *Pseudomonas* strains isolated in this study presented both twitching and swimming motilities, but were negative for swarming motility. In the last decade, various studies that correlate motility and biofilm formation by *Pseudomonas* have been published (Boles et al. 2005; Caiazza et al. 2007; Shrout et al. 2006; Toutain et al. 2007; Verstraeten et al. 2008). *Pseudomonas* sp. strain (AO1-07) isolated in this work was classified as a good biofilm former. Therefore, twitching and swimming motilities may influence positively in biofilm formation.

Species of *Azospirillum* are known as nitrogen-fixing bacteria that produce plant beneficial effects through interaction with plant surface, forming biofilms (Burdman

et al. 2000). *Azospirillum* sp. strain AO1-20 was the isolated that showed the best biofilm formation, indicating its intrinsic characteristic to adhere to surfaces and form biofilm. Interestingly, *Azospirillum* sp. strain A01-20 was the only isolate classified as a good biofilm former for which the use of phage did not interfere in the development of its biofilm. Besides the high ability of *Azospirillum* to form biofilm, and consequently its alleged role in biofouling of RO systems, these results emphasizes the importance to apply specific phage to control biofilm formation.

Diverse strategies that aim to prevent and control biofilm formation in RO systems have been studied in the last two decades, e,g. the use of biocides, UV radiation and nutrient limitation (Nguyen et al. 2012). More recently, the use of phages has been seen as a promising and "green" alternative (Azeredo and Sutherland 2008). The infection of biofilm cells by phages is extremely conditioned by diverse factors, including biofilm chemical composition, environmental conditions (pH, temperature, nutrients), and phage features such as concentration and specificity (Sillankorva et al. 2004).

The ability of the phage vB_AspP-UFV1 to reduce biofilm formation in most bacteria tested reveals a broad action spectrum, what makes it a viable alternative to control biofilm. For better results, it may also be used with specific lytic phage mixed in cocktails preparation (Hughes et al. 1998a, b; Fu et al. 2010). Xiong and Liu (2010) highlighted that specific parasitic characteristics of phages would eventually pose a challenge to their application in large-scale wastewater treatment, including RO systems. However, Goldman et al. (2009) observed a reduced microbial attachment to ultra-filtration membrane surface after the addition of specific bacteriophages. Beyond their specific lytic action, phages can control biofilm by the production of enzymes that degrade EPS causing biofilm slough off (Cornelissen et al. 2011). Thus, the reduction in biofilms of some bacterial isolates observed in this work is apparently related to the exopolysaccharide degradation by enzymes produced by the phage. Although phages have not been effective against the isolated AO-20 (great biofilm formation capacity), in complex communities, the destabilization of the biofilm by the action of the enzyme on certain exopolysaccharides, can take to the complete collapse (Hughes et al. 1998b). UFV1 was not specific to the bacterial isolates tested, causing no decrease in bacterial growth. Thus, its interference in biofilm formation may be due to the action of depolymerase or infection of the cell without necessarily causing cell lysis. Bacteriophages can interfere with host gene expression by up- or down-regulating some genes, reaching fitness changes of 20–30 % per generation (Chen et al. 2005). These fitness changes may be result of the presence of phage sigma factors (Schuch and Fischetti 2009) or by the action of proteins expressed by lysogenic phages, which function is not established yet (Ainsworth et al. 2013).

Although there is an evident efficiency of phages in biofilm control, in addition to the advantage as a clean technology, few studies have applied phages in RO membrane systems against microbial biofouling and further research is needed to successfully develop this biofilm control strategy.

The results gathered in this work showed that diverse free-living bacteria inhabit the feed water of a RO system, and that among them some bacteria are motile and thus may be more adapted to form biofilm on membranes of the RO system. The presence of such diverse bacteria highlights the importance of an effective pretreatment of the feed water of RO systems. In addition, a direct correlation between motility and ability of biofilm formation was observed, although motility is reported as a no-determinant factor to biofilm formation. Further research is clearly needed in order to elucidate which planktonic bacteria living in the feed water are also composing biofilms on membrane osmosis surface, as well as which bacteria are primary or secondary biofilm formers. For this purpose, our research group is currently carrying out the in situ characterization of microbial composition of biofilms on membranes of a RO system, using specific probes for fluorescent in situ hybridization (FISH). The advances in our understanding of the different mechanisms involved in biofilm formation will certainly provide information and support the search for compounds to prevent and control biofouling in industrial settings.

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