View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Universidade do Minho: RepositoriUM

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 2007, p. 6192–6200 Vol. 73, No. 19
0099-2240/07/$08.00+0 doi:10.1128/AEM.00837-07
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

Biofilm Interactions between Distinct Bacterial Genera Isolated from Drinking Water[▽]

Lúcia Chaves Simões, Manuel Simões, and Maria João Vieira*

IBB—Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Received 13 April 2007/Accepted 28 July 2007

In the environment, multiple microorganisms coexist as communities, competing for resources and often associated as biofilms. In this study, single- and dual-species biofilm formation by, and specific activities of, six heterotrophic intergeneric bacteria were determined using 96-well polystyrene plates over a 72-h period. These bacteria were isolated from drinking water and identified by partial 16S rRNA gene sequencing. A series of planktonic studies was also performed, assessing the bacterial growth rate, motility, and production of quorum-sensing inhibitors (QSI). This constituted an attempt to identify key attributes allowing bacteria to effectively interact and coexist in a drinking-water environment. We observed that in both pure and dual cultures, all of the isolates formed stable biofilms within 72 h, with specific metabolic activity decreasing, in most cases, with an increase in biofilm mass. The largest single- and dual-biofilm amounts were found for *Methylobacterium* sp. and the combination of *Methylobacterium* sp. and *Mycobacterium mucogenicum*, respectively. Evidences of microbial interactions in dual-biofilm formation, associated with appreciable biomass variation in comparison with single biofilms, were found for the following cases: synergy/cooperation between *Sphingomonas capsulata* and *Burkholderia cepacia*, *S. capsulata* and *Staphylococcus* sp., and *B. cepacia* and *Acinetobacter calcoaceticus* and antagonism between *S. capsulata* and *M. mucogenicum*, *S. capsulata* and *A. calcoaceticus*, and *M. mucogenicum* and *Staphylococcus* sp. A neutral interaction was found for *Methylobacterium* sp.-*M. mucogenicum*, *S. capsulata*-*Staphylococcus* sp., *M. mucogenicum*-*A. calcoaceticus*, and *Methylobacterium* sp.-*A. calcoaceticus* biofilms, since the resultant dual biofilms had a mass and specific metabolic activity similar to the average for each single biofilm. *B. cepacia* had the highest growth rate and motility and produced QSI. Other bacteria producing QSI were *Methylobacterium* sp., *S. capsulata*, and *Staphylococcus* sp. However, only for *S. capsulata*-*M. mucogenicum*, *S. capsulata*-*A. calcoaceticus*, and *M. mucogenicum*-*Staphylococcus* sp., dual-biofilm formation seems to be regulated by the QSI produced by *S. capsulata* and *Staphylococcus* sp. and by the increased growth rate of *S. capsulata*. The parameters assessed by planktonic studies did not allow prediction and generalization of the exact mechanism regulating dual-species biofilm formation between the drinking-water bacteria.

Drinking-water systems are known to harbor biofilms, even though these environments are oligotrophic and often contain a disinfectant. Control of these biofilms is important for aesthetic and regulatory reasons (34, 51, 52). The interaction of pathogens with existing biofilms has predominantly been a concern with man-made water systems, particularly drinking-water distribution systems (5, 34, 49). Microorganisms are generally less of a problem in planktonic phase due to the increased susceptibility to disinfection (42, 43). The examination of a drinking-water distribution system reveals the complexity of such a technical system. There are not only many different materials used for the transportation and regulation of the water flow but also dramatic variations in the flow conditions between different locations (3, 41). Obviously microorganisms face a diversity of habitats with distinct physicochemical and nutritional conditions during treatment, storage, and distribution of drinking water (41, 49). Bacteria are affected not only by the environment they live in but also by the variety of other species present. By performing studies on the interactions present in multispecies biofilms, basic knowledge on several aspects of sociomicrobiology can be gained (7, 31). A range of interactions has been observed among microorganisms in biofilms, including antagonistic, mutualistic, competitive, and commensal relationships (6, 7, 9, 11, 29, 36, 50). For instance, competition among microorganisms for space and nutrients is a powerful selective force which has led to the evolution of a variety of effective strategies for colonizing and growing on surfaces (6, 49). The mechanisms that control microbial interactions in multispecies biofilms are yet not fully understood (22, 49). The ecology of a biofilm is a complex equation of physicochemical and biological parameters. As with all levels of evolution, a complex web of interactions is central to the structure, composition, and function of these or any communities (17). Optimizing the management of drinking water distribution systems and controlling microbial growth are difficult due to the complexity of these systems. The study of bacterial ecology and ethology might help to improve our understanding of the persistence of biofilms and associated pathogens in drinking-water distribution systems. There is evidence that biofilm community diversity can affect disinfection efficacy and allow pathogens to survive within biofilms (7, 15). The assessment of microbial mechanisms regulating multispecies biofilm

* Corresponding author. Mailing address: Centro de Engenharia Biológica, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal. Phone: 351 253604404. Fax: 351 253678986. E-mail: mjv@deb.uminho.pt.

▽ Published ahead of print on 3 August 2007.

formation becomes a very important tool for the determination of the composition of drinking-water bacteria because they likely lead to the predominance of the best-adapted species for that set of conditions. The knowledge of biofilm biodiversities and its species physiology may facilitate the development of drinking-water disinfection and biofilm control processes.

The aim of this study was to assess the role of interspecies interactions in dual-species biofilm formation and characteristics. Furthermore, we sought to assess possible key factors (growth rate, motility, and production of quorum-sensing antagonists) regulating microbial interactions between intergeneric drinking-water bacteria.

MATERIALS AND METHODS

Bacterial isolation and identification. The microorganisms used throughout this work were isolated from a model laboratory drinking-water distribution system, as described by Simões et al. (40). Briefly, two consecutive granular activated carbon filter columns were directly plugged into the normal tap water from the Braga (Portugal) water distribution network. The first granular activated carbon filter eliminated the free chlorine contained in the tap water, while the second was a biological activated filter furnishing a continuous bacterial inoculum to a Perspex chemostat (volume, 1.6 liter; diameter, 16.8 cm). The system was sterile until filled with potable water and operated so as to prevent immigration of microorganisms other than via the tap water feeding. The flow rate of tap water gave a dilution rate of 3.125 h^{-1}. Microorganisms were isolated by collecting 100 μl of the chemostat water and plating on both trypticase soy agar (Merck, VWR) and R2A (Oxoid) aerobically at room temperature (23 ± 2°C) for 15 days. These two media were already tested successfully in the recovery of heterotrophic bacteria from drinking water (37, 40).

Preliminary, presumptive bacteria identification was done using selective media Chromocult TBX agar (Merck), *Pseudomonas* isolation agar (Difco), and methanol minimum medium according to the method of Kim et al. (21), Gram staining, and biochemical methods (API 20 NE and API ID32 GN systems; Biomerieux) according to the manufacturer's instructions. Further identification tests for determination of the 16S rRNA gene sequence were performed for putative bacteria according to the procedure described by Simões et al. (41).

Planktonic bacterial growth. Assays were performed with six representative drinking-water bacteria, *Acinetobacter calcoaceticus* (031), *Burkholderia cepacia* (010), *Methylobacterium* sp. (029), *Mycobacterium mucogenicum* (017), *Sphingomonas capsulata* (003), and *Staphylococcus* sp. (052). The bacterial genera used in this study represented more than 80% of the total genera isolated and identified (results not shown).

Bacterial cells were grown overnight in batch culture using 100 ml of R2A broth at room temperature under agitation (150 rpm). Cells were harvested by centrifugation (20 min at 13,000 × *g*), washed three times in 0.1 M of saline phosphate buffer (KH_2PO_4, Na_2HPO_4, NaCl), and resuspended in a certain volume of R2A broth necessary to achieve a cellular density of 1×10^8 cells/ml.

Biofilm formation in microtiter plates. Biofilms were developed according to the modified microtiter plate test proposed by Stepanović et al. (47). Briefly, for each bacterium at least 16 wells of a sterile 96-well flat tissue culture plate (polystyrene; Orange Scientific) were filled under aseptic conditions with 200 μl of cell suspension (1×10^8 cells/ml in R2A broth). To promote biofilm formation, the plates were incubated aerobically on a shaker at 150 rpm at room temperature for 24, 48, or 72 h. Every 24 h, the growth medium was carefully discarded and replaced by a fresh one. At each sampling time, the content of each well was removed and washed three times with 250 μl of sterile distilled water 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 adhered on the inner walls of the wells and in terms of their respiratory activity. Negative controls were obtained by incubating the wells only with R2A broth without adding any bacterial cells. All experiments were performed in triplicate with three repeats.

Mass quantification by CV. The bacterial biofilms in the 96-well plates were fixed with 250 μl/well of 98% methanol (Vaz Pereira) for 15 min. Afterwards, the plates were emptied and left to dry. Then, the fixed bacteria were stained for 5 min with 200 μl/well of crystal violet (CV) (Gram color-staining set for microscopy; Merck). Excess stain was rinsed out by placing the plate under low-running tap water (47). After the plates were air dried, the dye bound to the adherent cells was resolubilized by 200 μl/well of 33% (vol/vol) glacial acetic acid (Merck). The optical density (OD) of the obtained solution was measured at 570 nm (OD_{570}) using a microtiter plate reader (Model Synergy HT; BIO-TEK), and biofilm mass was presented as OD_{570}.

Bacteria were classified using the scheme of Stepanović et al. (47) as follows: nonbiofilm producer (0), OD less than or equal to OD_c; weak biofilm producer (+), OD greater than OD_c and less than or equal to $2 \times OD_c$; moderate biofilm producer (++), OD greater than $2 \times OD_c$ and less than or equal to $4 \times OD_c$; strong biofilm producer (+++), OD greater than $4 \times OD_c$. This classification was based upon the cutoff OD (OD_c) value, defined as three standard deviation values above the mean OD_{570} of the negative control.

Activity assessment by XTT staining. The sodium 3,3′-[1[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) colorimetric method was applied to determine the bacterial activity of the biofilms as described previously by Stevens and Olsen (48) with some modifications. Briefly, 200 μl of a combined solution of XTT (Sigma) and phenazine methosulfate (Sigma) was added to each well in order to obtain a final concentration of 50 μg/ml of XTT and 10 μg/ml phenazine methosulfate. Then, the microtiter plates were incubated for 3 h and 150 rpm at room temperature in the dark. The OD of the formazan supernatant of each well was measured at 490 nm using a microtiter plate reader (Model Synergy HT; BIO-TEK). The biofilm-specific respiratory activity was expressed as OD_{490}/OD_{570} (biofilm respiratory activity/biofilm mass).

Bacterial screening for QSI and acyl-homoserine lactone (AHL) production. Test bacteria were streaked on the centers of R2A agar plates and grown overnight at room temperature. Indicator microorganisms were grown overnight in LB broth (*Chromobacterium violaceum* O26 and *C. violaceum* 12472) or LB plus 50 μg/ml spectinomycin (Sigma) and 4.5 μg/ml tetracycline (*Agrobacterium tumefaciens* A136). Following overnight growth, the test bacteria were overlaid with 5 ml LB soft agar (full-strength LB broth containing 0.5% [wt/vol] agar) cooled to 45°C, containing 10^6 CFU/ml of the indicator microorganism *C. violaceum* ATCC 12472. *Pseudomonas aeruginosa* PAO-1 was used as a positive control for quorum-sensing inhibition (QSI), since its two signal molecules, 3-oxo-dodecanoyl homoserine lactone and *N*-butanoyl homoserine lactone (C_4-HSL), competitively bind and inhibit the receptor for the cognate signal *N*-hexanoyl homoserine lactone (C_6-HSL) in both indicator microorganisms. *C. violaceum* ATCC 12472 was used as a negative control, since it produces the cognate C_6-HSL and would therefore not inhibit its own quorum-sensing signal. A positive QSI result was indicated by a lack of pigmentation of the indicator microorganism in the vicinity of the test microorganism.

A bioassay for AHL production was performed in order to detect the type of molecule responsible for QSI (24). Two biosensor microorganisms, *A. tumefaciens* A136 and *C. violaceum* CVO26, that directly respond to AHLs were used. The *A. tumefaciens* biosensor is highly sensitive to a variety of AHL chains, ranging from C_6 to C_{14}, while *C. violaceum* is unable to synthesize its endogenous C_6-HSL inducer but retains the ability to respond to C_4-HSL and C_6-HSL. For the bioassay, test bacteria were grown on R2A as described above. Following overnight growth, the bacteria were overlaid with LB soft agar containing 5 μl of overnight cultures of *C. violaceum* CVO26 or the *A. tumefaciens* A136 biosensor and incubated overnight at 30°C. Following incubation, 50 μl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma) solution (20 mg/ml in dimethylformamide) was added to the *A. tumefaciens* A136 assay plates and color development due to X-Gal hydrolysis allowed to proceed for 15 min at room temperature. *A. tumefaciens* KYC6, a 3-oxo-C_8-HSL overproducer, was used as a positive control for the *A. tumefaciens* biosensor. *C. violaceum* 31532 was the positive control for the *C. violaceum* CVO26 assay. The biosensor strains themselves were used as negative controls, since both strains lack AHL synthase genes. A positive test for AHLs was indicated by a blue coloration from X-Gal hydrolysis in the *A. tumefaciens* biosensor or by a purple CVO26 pigmentation. Negative tests for AHLs were indicated by a lack of coloration (24).

Motility assays. R2B overnight cultures were used to assay motility in plates containing 1% tryptone, 0.25% NaCl, and 0.3% agar. The motility halos were measured at 8, 16, 24, and 48 h (45). Three plates were used to evaluate each bacterium motility; experiments were conducted with two independent cultures.

Statistical analysis. The data were analyzed using the statistical program SPSS, version 14.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. Because low sample numbers contributed to uneven variation, the nonparametric Wilcoxon test was used to compare biofilm characteristics. Statistical calculations were based on a confidence level equal or higher than 95% (a P value of <0.05 was considered statistically significant).

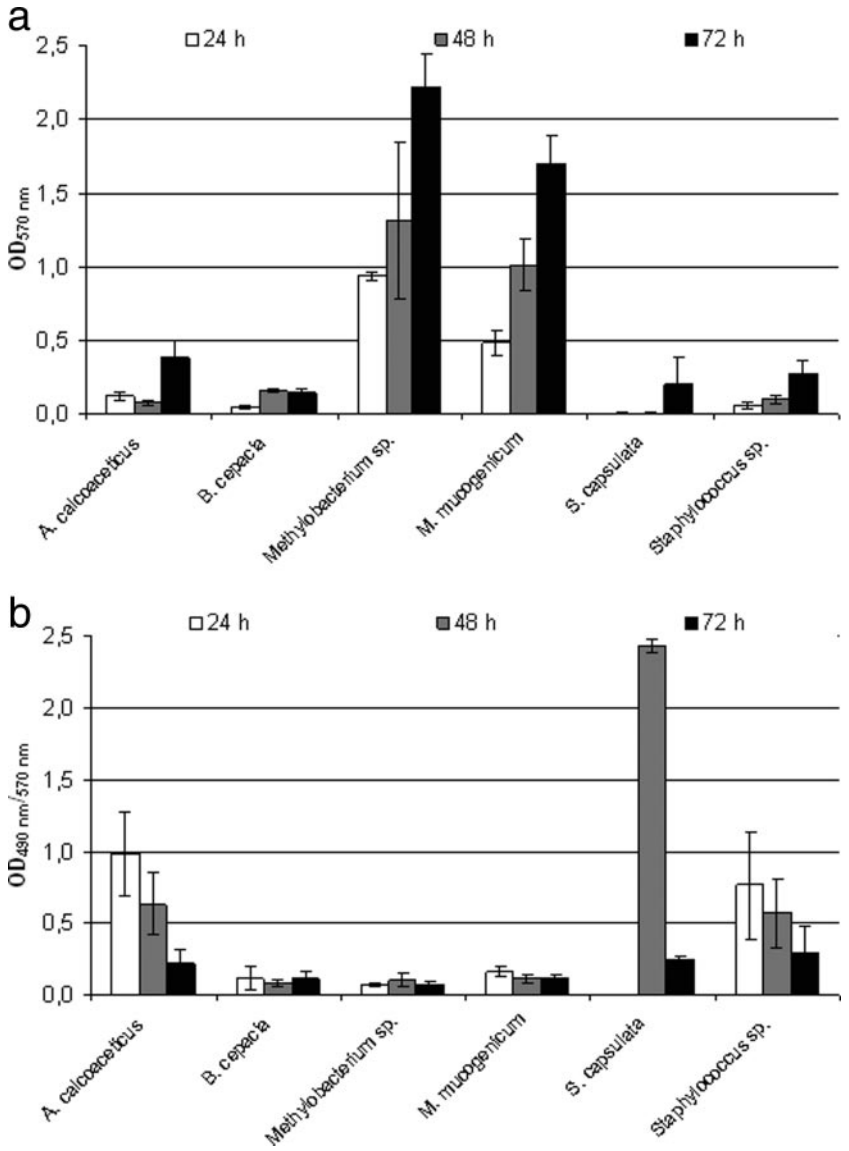

FIG. 1. OD_{570} and OD_{490}/OD_{570} values as a measure of single-species biofilm mass (a) or specific respiratory activity (b) for drinking-water bacteria. The means ± standard deviations for at least three replicates are illustrated.

RESULTS

Single-species biofilm formation and specific respiratory activity. In order to assess the biofilm formation abilities and specific respiratory activities of the several bacteria isolated from drinking water, the standard 96-well microtiter plates with CV and XTT staining were used to characterize biofilms (Fig. 1). Figure 1a shows that the tested bacteria formed biofilms, with *Methylobacterium* sp. producing the largest biomass amount for all sampling times. *M. mucogenicum* was the second-strongest biofilm producer. A directly proportional time-biomass formation relationship was found for the various bacteria ($P < 0.05$) except for *B. cepacia* ($P > 0.05$). *S. capsulata* showed biofilm formation ability only for sampling times greater than 48 h. The biofilm amount was statistically similar only between *A. calcoaceticus* and *Staphylococcus* sp. when results for the various sampling times were compared ($P > 0.1$).

Figure 1b shows that *B. cepacia*, *Methylobacterium* sp., and *M. mucogenicum* biofilms maintained their specific respiratory activities with similar values for the three bacteria ($P > 0.1$). *A. calcoaceticus* and *Staphylococcus* sp. formed biofilms with similar specific respiratory activities ($P > 0.05$), but their values decreased along time ($P < 0.05$). *S. capsulata* formed small biomass amounts (Fig. 1a), with an OD_{570} that was always smaller than 0.5. However, 48-h-aged biofilms had the highest specific respiratory activities (OD_{490}/OD_{570} higher than 2.3) compared with those of the other bacteria. *S. capsulata* specific respiratory activity from the 48-h biofilms sharply decreased, 1 day after, for OD_{490}/OD_{570} values smaller than 0.5 (Fig. 1b).

A ranking of biofilm formation was produced according to

TABLE 1. Biofilm-forming abilities of drinking-water-isolated bacteria[a]

Bacterium	Biofilm formation at sampling time (h)		
	24	48	72
A. calcoaceticus (031)	+	0	+++
B. cepacia (010)	0	+	+
Methylobacterium sp. (029)	+++	+++	+++
M. mucogenicum (017)	+++	+++	+++
Sph. capsulata (003)	0	0	++
Staphylococcus sp. (052)	0	0	++

[a] According to classification proposed by Stepanović et al. (47): 0, non-biofilm producer; +, weak biofilm producer; ++, moderate biofilm producer; +++, strong biofilm producer.

the method of Stepanović et al. (47), classifying test bacteria as non-biofilm producer, weak biofilm producer, moderate biofilm producer, or strong biofilm producer (Table 1). *Methylobacterium* sp. and *M. mucogenicum* showed a strong biofilm-producing ability for the various sampling times. *S. capsulata* and *Staphylococcus* sp. presented biofilm formation ability (moderate) only for the 72-h sampling time. *B. cepacia* formed weak biofilms after 48 h, while *A. calcoaceticus* showed a variability in biofilm formation ability by forming weak biofilms at 24 h, being classified as a non-biofilm producer at 48 h and as a strong biofilm producer at the 72-h sampling time.

Dual-species biofilm formation and specific respiratory activity. Dual-species biofilms studied showed time-dependent biofilm formation ability (Fig. 2a) with a statistical level of significance ($P < 0.05$) for the following biofilms: *S. capsulata* (003)-*B. cepacia* (010), *S. capsulata* (003)-*Methylobacterium* sp. (029), *B. cepacia* (010)-*M. mucogenicum* (017), *B. cepacia* (010)-*Methylobacterium* sp. (029), *B. cepacia* (010)-*A. calcoaceticus* (031), *B. cepacia* (010)-*Staphylococcus* sp. (052), *M. mucogenicum* (017)-*Methylobacterium* sp. (029), and *M. mucogenicum* (017)-*A. calcoaceticus* (031). Dual *S. capsulata* (003)-*A.*

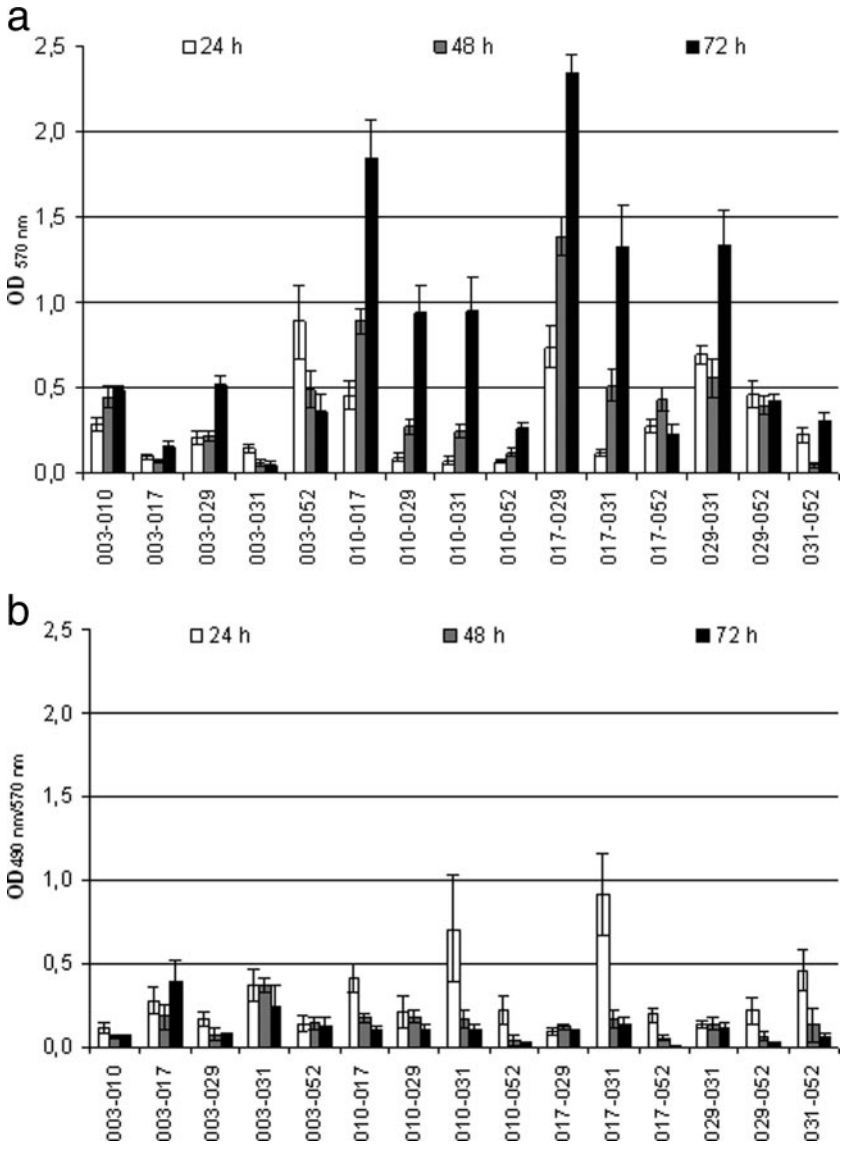

FIG. 2. OD_{570} and OD_{490}/OD_{570} values as a measure of dual-species biofilm mass (a) or specific respiratory activity (b) for drinking-water bacteria. The means ± standard deviations for at least three replicates are illustrated.

TABLE 2. Biofilm formation ability of dual species of bacteria[a]

Interaction[b]	Biofilm formation at sampling time (h)		
	24	48	72
003-010	++	++	++
003-017	0	0	+
003-029	+	+	+++
003-031	+	0	0
003-052	+++	++	++
010-017	+++	+++	+++
010-029	0	++	+++
010-031	0	++	+++
010-052	0	0	++
017-029	+++	+++	+++
017-031	+	+++	+++
017-052	++	++	+
029-031	+++	+++	+++
029-052	++	++	++
031-052	+	0	++

[a] According to classification proposed by Stepanović et al. (47): 0, non-biofilm producer; +, weak biofilm producer; ++, moderate biofilm producer; +++, strong biofilm producer.

[b] Bacterial species are as follows: 003, *S. capsulata*; 010, *B. cepacia*; 017, *M. mucogenicum*; 029, *Methylobacterium* sp.; 031, *A. calcoaceticus*; 052, *Staphylococcus* sp.

calcoaceticus (031) and *S. capsulata* (003)-*Staphylococcus* sp. (052) biofilms decreased in mass over time ($P < 0.05$). The remaining biofilms maintained a stable biomass over time, since biofilm mass differences for the different sampling times did not reach a level of statistical significance ($P > 0.05$).

XTT biofilm reaction for specific biofilm respiratory activity assessment showed that for 11 of the 15 dual biofilms studied (Fig. 2b), a statistically significant ($P < 0.05$) decrease of the OD_{490}/OD_{570} value occurred over time. This was found for the following dual biofilms: *S. capsulata* (003)-*B. cepacia* (010), *S. capsulata* (003)-*Methylobacterium* sp. (029), *S. capsulata* (003)-*A. calcoaceticus* (031), *B. cepacia* (010)-*M. mucogenicum* (017), *B. cepacia* (010)-*Methylobacterium* sp. (029), *B. cepacia* (010)-*A. calcoaceticus* (031), *B. cepacia* (010)-*Staphylococcus* sp. (052), *M. mucogenicum* (017)-*A. calcoaceticus* (031), *M. mucogenicum* (017)-*Staphylococcus* sp. (052), *Methylobacterium* sp. (029)-*Staphylococcus* sp. (052), and *A. calcoaceticus* (031)-*Staphylococcus* sp. (052). The other biofilms maintained statistically similar specific respiratory activity values for the various sampling times ($P > 0.05$).

As for the single biofilms (Table 1), an equivalent ranking was attributed for dual biofilms (Table 2). This ranking showed that *B. cepacia-M. mucogenicum*, *M. mucogenicum-Methylobacterium* sp., and *Methylobacterium* sp.-*A. calcoaceticus* interactions produced strong biofilms for the three sampling times. Strong biofilms were also found at 24 h for *S. capsulata-Staphylococcus* sp. interactions, at 48 h for *M. mucogenicum-A. calcoaceticus*, and at 72 h for *S. capsulata-Methylobacterium* sp., *B. cepacia-Methylobacterium* sp., *B. cepacia-A. calcoaceticus*, and *M. mucogenicum-A. calcoaceticus*. *S. capsulata-B. cepacia* and *Methylobacterium* sp.-*Staphylococcus* sp. formed moderate biofilms at the various sampling times. Other bacterial interactions producing moderate biofilms were found for *S. capsulata-Staphylococcus* sp. at 48 and 72 h, *B. cepacia-Methylobacterium* sp. at 48 h, *B. cepacia-A. calcoaceticus* at 48 h, *B. cepacia-Staphylococcus* sp. at 72 h, *M. mucogenicum-Staphylococcus* sp. at 24 and 48 h, and *A. calcoaceticus-Staphylococcus* sp. at the 72-h sampling time. Weak biofilm production was found for *S. capsulata-M. mucogenicum* interactions at 72 h, *S. capsulata-Methylobacterium* sp. at 24 and 48 h, *S. capsulata-A. calcoaceticus* at 24 h, *M. mucogenicum-A. calcoaceticus* at 24 h, *M. mucogenicum-Staphylococcus* sp. at 72 h, and *A. calcoaceticus-Staphylococcus* sp. at 24 h. The remaining microbial interactions/sampling times drew the non-biofilm producer classification.

TABLE 3. Bacterial planktonic growth rates

Bacterium	Growth rate (h^{-1})
A. calcoaceticus	0.0313 ± 0.008
B. cepacia	0.174 ± 0.005
Methylobacterium sp.	0.112 ± 0.004
M. mucogenicum	0.0757 ± 0.015
S. capsulata	0.119 ± 0.003
Staphylococcus sp.	0.0893 ± 0.014

The comparisons between single and dual biofilms showed the existence of interspecies microbial interactions. Biofilm interspecies relationships were based on the comparison between dual-species biofilm characteristics (ranking) and those from each single biofilm. The existence of synergistic or antagonistic interactions in dual biofilm formation was considered whether the biofilm formation category of each single bacterium (Table 1) was lesser or greater, respectively, than that found for dual biofilms (Table 2). Accordingly, evident antagonistic interactions were found for 72-h-aged biofilms of *S. capsulata-M. mucogenicum*, *S. capsulata-A. calcoaceticus*, and *M. mucogenicum-Staphylococcus* sp. Cooperation in biofilm formation, increasing biomass, was found for *S. capsulata-B. cepacia* and *S. capsulata-Staphylococcus* sp., both 24- and 48-h-aged biofilms, and for *B. cepacia-A. calcoaceticus* (48 h). Neutral interaction was found for *Methylobacterium* sp.-*M. mucogenicum* for all sampling times and for 72-h-aged biofilms of *S. capsulata-Staphylococcus* sp., *M. mucogenicum-A. calcoaceticus*, and *Methylobacterium* sp.-*A. calcoaceticus*, since the resultant biofilms had biomasses and specific metabolic activities similar to the averages for each ancestral biofilm.

Bacterial growth rate, motility, and production of QSI and AHL. To determine which factors influenced the interaction of the several bacteria in a dual-species biofilm, a series of planktonic experiments was performed. Planktonic studies were performed with the test bacteria to assess the bacterial growth rate in R2B (Table 3), motility (Table 4), and production of QSI and AHLs (Table 5) according to the methodology described by McLean et al. (24). The referred parameters were evaluated

TABLE 4. Assessment of bacterial motility

Bacterium	Motility (cm) at sampling time (h)			
	8	16	24	48
A. calcoaceticus	0.5	1.0	1.0	1.5
B. cepacia	1.5	7.5	9.5	48.5
Methylobacterium sp.	1.0	1.0	1.0	1.5
M. mucogenicum	0.5	1.0	1.0	1.0
S. capsulata	0.5	0.5	1.0	1.5
Staphylococcus sp.	1.0	1.0	1.0	1.0

TABLE 5. Screening for QSI and AHL molecules[a]

Bacterium	QSI	Presence of AHLs	
		C_4-HSL and C_6-HSL	C_6-HSL to C_{14}-HSL
A. calcoaceticus	−	−	−
B. cepacia	+	+	+
Methylobacterium sp.	+	+	+
M. mucogenicum	−	−	−
S. capsulata	+	−	+
Staphylococcus sp.	+	−	−

[a] −, not detected; +, detected.

in order to assess their role in dual-species biofilm formation and activity. According to results presented in Table 3, *B. cepacia* showed the highest growth rate and *A. calcoaceticus* the lowest. *Methylobacterium* sp. and *S. capsulata* had similar growth rates ($P > 0.05$), which were higher than those of *M. mucogenicum* and *Staphylococcus* sp. ($P < 0.05$).

Table 4 shows motility results of the test bacteria. All presented an increase in motility over time, from 8 to 48 h, except *Staphylococcus* sp. This bacterium had an invariable motility for the various times. A significantly time-increased motility was verified for *B. cepacia*, the bacterium with the highest motility values for all of the sampling times. The remaining species had very close motility values.

Studies of QSI screening (Table 5) showed that *B. cepacia*, *Methylobacterium* sp., *Staphylococcus* sp., and *S. capsulata* produced QSI molecules. Only for *Staphylococcus* sp. were QSI not related to AHLs. This was expected due to the peptide-like molecules involved in gram-positive-bacterium quorum-sensing (QS) events not detected by the methodology used.

DISCUSSION

An understanding of the microbial ecology of distribution systems is necessary to design innovative and effective control strategies that will ensure safe and high-quality drinking water. Recent investigations into the microbial ecology of drinking water distribution systems have found that pathogen resistance to chlorination is affected by the community biodiversity and interspecies relationships (3). In this study, some of the bacterial isolates tested (*B. cepacia*, *M. mucogenicum*, and *Staphylococcus* sp.) are recognized as problematic opportunistic bacteria (10, 39, 46, 54). The selected bacterial species were also detected in drinking-water biofilms (results not shown). In fact, biofilms on surfaces exposed to drinking water in distribution systems may well be the main source of planktonic bacteria, since up to 1,000 sessile microorganisms can be present for each planktonic cell detected (27). Microbial growth control is a key issue in fulfilling drinking water quality standards. All of the isolated bacteria, belonging to distinct genera, had the ability to form biofilms (Fig. 1a) during the 72-h study. In some particular cases (*A. calcoaceticus* and *Staphylococcus* sp.), the specific metabolic activity (Fig. 1b) was determined to be inversely related to the biofilm mass increase. In fact, following microbial attachment, the formation of a complex extracellular polymeric matrix increased the nonmetabolically active biofilm mass (42, 43, 44), consequently decreasing the specific respiratory activity ($OD_{490}/OD_{570\ nm}$). *Methylobacterium* sp. and *M. mucogenicum* showed the greatest biofilm formation ability, while *A. calcoaceticus*, *B. cepacia*, *S. capsulata*, and *Staphylococcus* sp. produced small biofilm mass amounts, with an OD_{570} of <0.5 (Fig. 1a). This fact is also sustained by the biofilm formation ability score shown in Table 1. Contradicting these single-species biofilm results, in a previous study (41) assessing bacterial adherence to selected polymeric surfaces, it was found that only *A. calcoaceticus* was strongly adherent to drinking-water-related materials while only *Staphylococcus* sp. was moderately adherent. This allows the speculation that adhesion ability results do not necessarily predict the best biofilm-forming bacteria and final biofilm characteristics, a fact also observed by other authors (19, 20).

Under natural conditions, true monospecies biofilms are rare, occurring mostly as complex communities. Because multispecies interactions prevail in the environment, dual-biofilm studies were carried out but with the experimental conditions used for single biofilms. The dual-biofilm approach was an attempt to get closer to the reality of naturally occurring biofilms. Although still being a great simplification of true biofilms, not fully replicating reality, it is still a more exact approach. The physiology and metabolism of multispecies biofilm communities are immensely complex (38). Diversity in microbial communities leads to a variety of complex relationships involving inter- and intraspecies interactions (3). The specific mechanisms for multispecies biofilm formation and organization still remain unclear. Nevertheless, a more complete picture of microbial community diversity and interspecies relationships should facilitate and contribute to the understanding of the biofilm formation process and persistence in drinking-water systems and other systems. From an ecological point of view, both competition and cooperation can exist in drinking water distribution systems (49). In a recent study (17) concerning biofilm interspecies interactions, it was found that a derived dual-species (*Acinetobacter* sp. and *Pseudomonas putida*) community was more stable and productive than the ancestral community. Previously Møller et al. (26) revealed a metabolic synergy between *P. putida* and *Acinetobacter* sp. community members for the biodegradation of toluene and related aromatic compounds. Cooperative interactions between bacteria have been demonstrated mainly for degradation processes (11, 26, 49). According to Burmølle et al. (7), beneficial interactions in multispecies biofilms can include coaggregation and plasmid conjugation, contributing to the protection of one or several species from eradication even when the biofilm is exposed to external stress factors. In low-nutrient environments, such as drinking water, it may be assumed that oligocarbophilic-obligate bacteria are the first colonizers of the system and that copiotrophs are the second colonizers, using remnants and excretions of the original colonizers (49).

Most research into interspecies interactions within biofilms has focused on the beneficial aspects of these relationships. However, not all interactions will be advantageous for the several interacting microorganisms. Antagonistic interactions may play an important role in the development and structure of microbial communities. Competition for substrates is considered to be one of the major evolutionary driving forces in the bacterial world, and numerous experimental data obtained in the laboratory under well-controlled conditions show how different microorganisms may effectively outcompete others as

a result of a better utilization of a given energy source (9, 22, 36). The production of antagonistic compounds also seems to be a common phenomenon for some bacteria (4, 36, 50). The present results show that the association of bacteria forming larger single-biofilm mass amounts also formed dual biofilms (*M. mucogenicum-Methylobacterium* sp.) with the highest OD_{570} for the differently aged biofilms (Fig. 2a). Other bacterial interactions/associations producing considerable biofilm amounts over time were found for *B. cepacia-M. mucogenicum* and *Methylobacterium* sp.-*A. calcoaceticus*. Cooperation in biofilm mass development does not necessarily lead to the highest specific respiratory activity. For the most significant situations, specific metabolic activity was inversely proportional to biofilm mass formation, a fact even more evident than for single-species biofilms (Fig. 2b). Stabilization of the interaction between the various dual-species biofilms is likely to have implications not only for the community composition, structure, and activity but also for the interspecies relationships and function of the community. Evidence of intraspecies relationships was assessed by the analysis of the differential biomass formation of single (Table 1) and dual (Table 2) biofilms. By this approach, mutualism/synergy in biofilm formation was found with the association of *S. capsulata-B. cepacia*, *S. capsulata-Staphylococcus* sp., and *B. cepacia-A. calcoaceticus*, a fact probably related to the establishment of intergeneric metabolic cooperation. Competition/antagonistic interactions allowing the formation of smaller biofilm amounts were found for the following associations: *M. mucogenicum-Staphylococcus* sp., *S. capsulata-M. mucogenicum*, and *S. capsulata-A. calcoaceticus* (Table 2). Neutral interaction was apparently existent for *Methylobacterium* sp.-*M. mucogenicum*, *S. capsulata-Staphylococcus* sp., *M. mucogenicum-A. calcoaceticus*, and *Methylobacterium* sp.-*A. calcoaceticus* biofilms, since the resultant coculture had phenotypic characteristics similar to the average of each single biofilm (Fig. 1 and 2).

The mechanisms that control biofilm microbial interactions in the environment are not fully understood (22, 38). The existence of multiple interactions or even the simple production of a metabolite can interfere with the development of what seem to be structurally organized communities existing within a biofilm. To determine which factors may influence the interaction of the studied dual-species biofilms, a series of batch experiments was performed, allowing the assessment of the bacterial growth rate, motility, QSI, and AHL production. These features are recognized as important factors regulating biofilm formation and interspecies interactions (12, 18, 22, 24, 28, 35). According to existing descriptions of multispecies biofilm population dynamics (1, 53), the faster-growing bacteria should outcompete those that grow more slowly. QS, a cell density-related communication mode between one or more species, is a significant factor adding complexity to the interactions between biofilm bacteria. It is also known to influence bacterial community development in aquatic biofilms (25). QS plays a role in cell attachment and detachment from biofilms (12, 13, 14). It involves an environmental sensing system that allows bacteria to monitor and respond to their own population densities. The bacteria produce a diffusible organic signal, generally called an autoinducer molecule, differing from gram-negative to gram-positive bacteria. It accumulates in the bacterial surrounding environment during growth (16). QS systems are known to be involved in a range of important microbial activities. These include extracellular enzyme biosynthesis, biofilm development, antibiotic biosynthesis, biosurfactant production, extracellular polymeric substance synthesis, and extracellular virulence factors in gram-negative bacteria (2, 8, 12, 13, 32, 33). In a dual biofilm, interference with this system through QSI of one bacterial species to another will arguably influence the proportion of each species in the biofilm. Motility is known to be essential for biofilm formation, overcoming the electrostatic repulsion of cells and surfaces (18, 35). High bacterial motility constitutes a surface colonization advantage (18). Predictive biofilm bacterium dominance results, assessed by planktonic tests (Tables 3 to 5), show that *B. cepacia* had the highest growth rate, motility, and produced QSI (AHL-related molecules). It was apparently the bacteria with the best competitive advantage in cocultured biofilms. *Methylobacterium* sp. and *S. capsulata* also presented considerably high growth rates and motilities compared with the remaining bacteria, being also QSI producers. However, none of the bacteria showing a competitive advantage in terms of an increased growth rate (*B. cepacia*, *Methylobacterium* sp., and *S. capsulata*), motility (*B. cepacia*), or QSI production (*B. cepacia*, *Methylobacterium* sp., *S. capsulata*, and *Staphylococcus* sp.) conferred their specific characteristics to dual biofilms. In fact, antagonistic interactions in biofilm formation were found only for the *S. capsulata-M. mucogenicum*, *S. capsulata-A. calcoaceticus*, and *M. mucogenicum-Staphylococcus* sp. dual biofilms. For these situations, biofilm formation seems to be regulated by the QSI produced by *S. capsulata* and *Staphylococcus* sp. and by the increased growth rate of *S. capsulata*. Moreover, in these same cases, planktonic tests demonstrate the inefficiency of the tested parameters in predicting microbial interactions in mixed biofilms, since dual biofilms formed by bacteria with a competitive advantage, i.e., *S. capsulata-B. cepacia* and *S. capsulata-Staphylococcus* sp., produced greater biomass than each single biofilm, showing the existence of intergeneric synergistic interactions in biofilm formation. This result demonstrates that the parameters assessed by planktonic experiments did not allow prediction and generalization of the exact mechanism regulating mixed biofilm formation. Other cell-cell events, such as intergeneric coaggregation, may play a significant role in the formation and interspecies interactions in drinking-water biofilms.

In conclusion, biofilms are recognized as focal points where bacteria and other microorganisms interact (3, 20, 23, 27). This study provides evidences of some mechanisms by which different species interact in biofilms and should therefore help in developing strategies for their elimination from the specific problem source. The development of multispecies biofilms may help to determine optimal operational parameters and lead to knowledgeable decisions regarding the management of drinking-water distribution networks that will guarantee microbiologically safe and thus high-quality drinking water (3, 23, 30). The identification of the main bacteria forming more complex biofilms (*B. cepacia-M. mucogenicum*, *M. mucogenicum-Methylobacterium* sp., and *Methylobacterium* sp.-*A. calcoaceticus*) may provide new information necessary for improv-

ing water quality for the consumer. Further studies are being carried out to find other key factors regulating biofilm occurrence and community composition in drinking-water distribution systems, such as intergeneric coaggregation studies, and to improve disinfection strategies against multispecies biofilms.

ACKNOWLEDGMENTS

We acknowledge the financial support provided by the Portuguese Foundation for Science and Technology (SFRH/BD/31661/2006 to Lúcia C. Simões and SFRH/BPD/20582/2004 to Manuel Simões).

We gratefully acknowledge Robert J. C. McLean (Department of Biology, Texas State University) for providing the microorganisms for screening of QSI and AHL production.

REFERENCES

1. **Banks, M. K., and J. D. Bryers.** 1991. Bacterial species dominance within a binary culture biofilm. Appl. Environ. Microbiol. **57:**1974–1979.
2. **Beck von Bodman, S., and S. K. Farrand.** 1995. Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. J. Bacteriol. **177:**5000–5008.
3. **Berry, D., C. Xi, and L. Raskin.** 2006. Microbiol ecology of drinking water distribution systems. Curr. Opin. Microbiol. **17:**297–302.
4. **Bhattarai, H. D., Y. K. Lee, K. H. Cho, H. K. Lee, and H. W. Shin.** 2006. The study of antagonistic interactions among pelagic bacteria: a promising way to coin environmentally friendly antifouling compounds. Hydrobiologia **568:** 417–423.
5. **Block, J. C.** 1992. Biofilms in drinking water distribution systems, p. 469–485. *In* T. R. Bott, L. Melo, M. Fletcher, and B. Capedeville (ed.), Biofilms—science and technology. Kluwer, Dordrecht, The Netherlands.
6. **Burgess, J. G., E. M. Jordan, M. Bregu, A. Mearns-Spragg, and K. G. Boyd.** 1999. Microbial antagonism: a neglected avenue of natural products research. J. Biotechnol. **70:**27–32.
7. **Burmølle, M., J. S. Webb, D. Rao, L. H. Hansen, S. J. Sørensen, and S. Kjelleberg.** 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Appl. Environ. Microbiol. **72:**3916–3923.
8. **Chatterjee, A., Y. Cui, Y. Lui, C. K. Dumenyo, and A. K. Chaterjee.** 1995. Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulases, and proteases in *Erwinia carotovora* subsp. c*arotovora* in the absence of the starvation/cell density-sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. Appl. Environ. Microbiol. **61:**1959–1967.
9. **Christensen, B. B., J. A. J. Haagensen, A. Heydorn, and S. Molin.** 2002. Metabolic commensalism and competition in a two-species microbial consortium. Appl. Environ. Microbiol. **68:**2495–2502.
10. **Conway, B.-A. D., V. Venu, and D. P. Speert.** 2002. Biofilm formation and acyl homoserine lactone production in the *Burkolderia cepacia* complex. J. Bacteriol. **184:**5678–5685.
11. **Cowan, S. E., E. Gilbert, D. Liepmann, and J. D. Keasling.** 2000. Commensal interactions in a dual-species biofilm exposed to mixed organic compounds. Appl. Environ. Microbiol. **66:**4481–4485.
12. **Daniels, R., J. Vanderleyden, and J. Michiels.** 2004. Quorum sensing and swarming migration in bacteria. FEMS Microbiol. Rev. **28:**261–289.
13. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295–298.
14. **Donlan, R. M.** 2002. Biofilms: microbial life on surfaces. Emerg. Infect. Dis. **8:**881–890.
15. **Elvers, K. T., K. Leeming, and H. M. Lappin-Scott.** 2002. Binary and mixed population biofilms: time-lapse image analysis and disinfection with biocides. J. Ind. Microbiol. Biotechnol. **29:**331–338.
16. **Fuqua, C., and E. P. Greenberg.** 2002. Listening in on bacteria acyl-homoserine lactone signalling. Nat. Rev. Mol. Cell Biol. **3:**685–695.
17. **Hansen, S. K., P. B. Rainey, J. A. J. Haagensen, and S. Molin.** 2007. Evolution of species interactions in a biofilm community. Nature **445:**533–536.
18. **Harshey, R. M.** 2003. Bacterial motility on a surface: many ways to a common goal. Annu. Rev. Microbiol. **57:**249–273.
19. **Heilmann, C., O. Schweitzer, C. Gerke, N. Vanittanakon, D. Mack, and F. Gotz.** 1996. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. Mol. Microbiol. **20:**1083–1091.
20. **Heilmann, C., M. Hussain, G. Peters, and F. Gotz.** 1997. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. Mol. Microbiol. **24:**1013–1024.
21. **Kim, S. W., P. Kim, and J. H. Kim.** 1999. Production of poly(3-hidroxybutyrate-co-3-hydroxyvalerate) from *Methylobacterium organophilum* by potassium-limited fed-batch culture. Enzyme Microb. Technol. **24:**555–560.
22. **Komlos, J., A. B. Cunningham, A. K. Camper, and R. R. Sharp.** 2005. Interaction of *Klebsiella oxytoca* and *Burkholderia cepacia* in dual-species batch cultures and biofilms as a function of growth rate and substrate concentration. Microb. Ecol. **49:**114–125.
23. **LeChevallier, M. W., N. J. Welch, and D. B. Smith.** 1996. Full-scale studies of factors related to coliform regrowth in drinking water. Appl. Environ. Microbiol. **62:**2201–2211.
24. **McLean, R. J. C., L. S. Pierson III, and C. Fuqua.** 2004. A simple screening protocol for the identification of quorum signal antagonists. J. Microbiol. Methods **58:**351–360.
25. **McLean, R. J. C., M. B. Barnes, M. K. Windham, M. Merchant, M. R. J. Forstner, and C. Fuqua.** 2005. Cell-cell influences on bacterial community development in aquatic biofilms. Appl. Environ. Microbiol. **71:**8987–8990.
26. **Møller, S., C. Steinberg, J. B. Andersen, B. B. Christensen, J. L. Ramos, M. Givskov, and S. Molin.** 1998. *In situ* gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. Appl. Environ. Microbiol. **64:**721–732.
27. **Momba, M. N. B., R. Kfir, S. N. Venter, and T. E. Cloete.** 2000. An overview of biofilm formation in distribution systems and its impact on the deterioration of water quality. Water SA **26:**59–66.
28. **Moons, P., R. Van Houdt, A. Aertsen, K. Vanoirbeek, Y. Engelborghs, and C. W. Michiels.** 2006. Role of quorum sensing and antimicrobial component production by *Serratia plymuthica* in formation of biofilms including mixed biofilms with *Escherichia coli*. Appl. Environ. Microbiol. **72:**7294–7300.
29. **Nielsen, A. T., T. Tolker-Nielsen, K. B. Barken, and S. Molin.** 2000. Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. Environ. Microbiol. **2:**59–68.
30. **Norton, C. D., and M. W. LeChevallier.** 2000. A pilot study of bacteriological population changes through potable water treatment and distribution. Appl. Environ. Microbiol. **66:**268–276.
31. **Parsek, M. R., and E. P. Greenberg.** 2005. Sociomicrobiology: the connections between quorum sensing and biofilms. Trends Microbiol. **13:**27–33.
32. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260:**1127–1130.
33. **Pearson, J. P., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1995. A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:**1490–1494.
34. **Percival, S. L., and J. T. Walker.** 1999. Potable water and biofilms: a review of the public health implications. Biofouling **42:**99–115.
35. **Pratt, L. A., and R. Kolter.** 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. **30:**285–293.
36. **Rao, D., J. S. Webb, and S. Kjelleberg.** 2005. Competitive interactions in mixed-species biofilms containing the marine bacterium *Pseudoalteromonas tunicata*. Appl. Environ. Microbiol. **71:**1729–1736.
37. **Reasoner, D. J., and E. E. Geldrich.** 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. **49:**1–7.
38. **Rickard, A. H., P. Gilbert, N. J. High, P. E. Kolenbrander, and P. S. Handley.** 2003. Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol. **11:**94–100.
39. **Rickard, A. H., A. J. McBain, A. T. Stead, and P. Gilbert.** 2004. Shear rate moderates community diversity in freshwater biofilms. Appl. Environ. Microbiol. **70:**7426–7435.
40. **Simões, L. C., N. Azevedo, A. Pacheco, C. W. Keevil, and M. J. Vieira.** 2006. Drinking water biofilm assessment of total and culturable bacteria under different operating conditions. Biofouling **22:**91–99.
41. **Simões, L. C., M. Simões, R. Oliveira, and M. J. Vieira.** 2007. Potential of the adhesion of bacteria isolated from drinking water to materials. J. Basic Microbiol. **47:**174–183.
42. **Simões, M., H. Carvalho, M. O. Pereira, and M. J. Vieira.** 2003. Studies on the behaviour of *Pseudomonas fluorescens* biofilms after ortho-phthalaldehyde treatment. Biofouling **19:**151–157.
43. **Simões, M., M. O. Pereira, and M. J. Vieira.** 2005. Action of a cationic surfactant on the activity and removal of bacterial biofilms formed under different flow regimes. Water Res. **39:**478–486.
44. **Simões, M., M. O. Pereira, S. Sillankorva, J. Azeredo, and M. J. Vieira.** 2007. The effect of hydrodynamic conditions on the phenotype of *Pseudomonas fluorescens* biofilms. Biofouling **23:**249–258.
45. **Sperandio, V., A. G. Torres, and J. B. Kaper.** 2002. Quorum sensing Escherichia coli regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. Mol. Microbiol. **43:**809–821.
46. **Stelma, G. N., D. J. Lye, B. G. Smith, J. W. Messer, and P. Payment.** 2004. Rare occurrence of heterotrophic bacteria with pathogenic potential in potable water. Int. J. Food Microbiol. **92:**249–254.
47. **Stepanović, S., D. Vuković, I. Dević, B. Savić, and M. Švabić-Vlahović.** 2000.

A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Methods **40:**175–179.
48. **Stevens, M. G., and S. C. Olsen.** 1993. Comparative analysis of using MTT and XTT in colorimetric assays for quantitating bovine neutrophil bactericidal activity. J. Immunol. Methods **157:**225–231.
49. **Szewzyk, U., R. Szewzyk, W. Manz, and K. H. Schleifer.** 2000. Microbiological safety of drinking water. Annu. Rev. Microbiol. **54:**81–127.
50. **Tait, K., and I. W. Sutherland.** 2002. Antagonistic interactions amongst bacteriocin-producing enteric bacteria in dual species biofilms. J. Appl. Microbiol. **93:**345–352.
51. **Tsai, Y.-P.** 2005. Impact of flow velocity on the dynamic behaviour of biofilm bacteria. Biofouling **21:**267–277.
52. **Volk, C. J., and M. W. LeChevallier.** 1999. Impacts of the reduction of nutrient levels on bacterial water quality in distribution systems. Appl. Environ. Microbiol. **65:**4957–4966.
53. **Wanner, O., and W. Gujer.** 1986. A multispecies biofilm model. Biotechnol. Bioeng. **28:**314–328.
54. **Zanetti, F., G. De Luca, and S. Stampi.** 2000. Recovery of *Burkolderia pseudomallei* and *B. cepacia* from drinking water. Int. J. Food Microbiol. **59:**67–72.