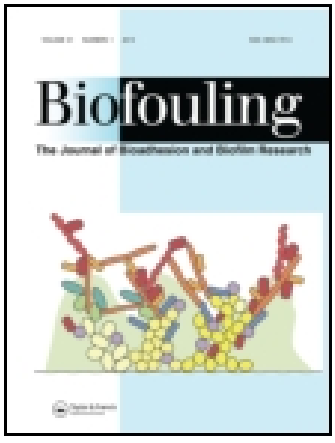

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Effect of culture conditions on the resistance of *Pseudomonas aeruginosa* biofilms to disinfecting agents

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The relationship between the environmental conditions of biofilm formation and resistance to disinfectants was studied. Anti-biofilm assays were performed against biofilms grown at 20, 30 and 37°C on stainless steel and polycarbonate, over 24 and 48 h. A rise in growth temperature increased the resistance of 24 h biofilms to disinfectants containing didecyldimethylammonium chloride and decreased it to a disinfectant containing alkyldimethylbenzylammonium chloride. The increase in growth temperature coupled with an incubation time of 24 h promoted increases in both matrix production and the membrane rigidity of sessile cells. An increase in incubation time also increased both matrix production and the membrane rigidity of sessile cells. Such phenomena resulted in an increased resistance to disinfectants of biofilms grown at 20 and 30°C. The resistance of 48 h biofilms to disinfectants decreased with an increase in growth temperature despite the increase in matrix production and the membrane rigidity of sessile cells.

Keywords: *Pseudomonas aeruginosa*; abiotic surfaces; environmental conditions; biofilm resistance; disinfectants

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium, commonly isolated from a range of environments including water, soil, animals and plants (Anaissie et al. 2002; Folkesson et al. 2012). This opportunistic human pathogen is prevalent in hospital environments and continues to be among the leading causes of nosocomial infections despite the advances made in the medical sectors (Mesaros et al. 2007; Tao et al. 2011). Therefore, this bacterium has attracted special attention due to its involvement in a broad spectrum of infections and its capacity to form biofilms (Donlan & Costerton 2002; Martínez-Solano et al. 2008). Moreover, there is a regular occurrence of infections which are often linked to medical devices colonized by *P. aeruginosa* biofilms (Donlan 2001).

A biofilm is defined as a structured community of microbial cells, attached to an inert or living surface, and embedded in a self-produced extracellular polymeric matrix which provides a protective environment against antimicrobial attacks (Donlan & Costerton 2002). Bacterial cells growing in biofilms can be up to 1,000 times more resistant to biocides than their planktonic counterparts (Bridier et al. 2011a). Hence, much attention has been paid to understanding biofilm formation on abiotic surfaces. Many studies have reported that environmental factors commonly encountered in hospital environments, such as temperature, pH, nutrient availability, oxygen, surface type and strain variability, may influence biofilm formation (Oh et al. 2009; da Silva Meira et al. 2012; Abdallah et al. 2014c). Such environmental factors also influence biofilm resistance to disinfectant agents (Nguyen & Yuk 2013; Abdallah et al. 2014c). Thus, a study investigating the effect of environmental conditions on the mechanisms that may control biofilm resistance to disinfectants can be helpful in understanding this resistance and in reducing the microbiological risk in affected sectors.

In order to prevent biofilms forming, various disinfecting agents are used in medical settings. However, the efficacy of such products is often based on planktonic cell assays and does not take into account the high resistance of sessile cells to biocides. This underestimation of biofilm resistance to biocides increases the probability of further disinfection failure which leads to development and spread of bacterial infections. Several mechanisms have been proposed for biofilm resistance to biocides. However, the precise mechanism of such resistance remains unclear to date (Bridier et al. 2011a). Biofilm resistance is thought to be related to the restricted penetration of antimicrobials through the biofilm matrix. Furthermore, it has been reported that the physiological state of sessile cells, and the presence of persister cells, are potentially important causes of biofilm resistance to biocides (Campanac et al. 2002; Stewart 2002; Lewis 2007; Abdallah et al. 2014a). However, it is

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now established that bacteria in natural and man-made ecosystems are constantly exposed to changes in their physical and chemical environments. In addition, bacteria can sense changes in the environment and respond through changes in cell physiology, to adapt to new environmental conditions. An important adaptive response of bacterial cells to non-optimal growth conditions is through modifications of membrane lipids (Chihib et al. 2005). The bacterial membrane, composed primarily of phospholipids and proteins, constitutes the first line of bacterial defense against antimicrobial attack. Moreover, the phospholipid fatty acyl chains, which are also influenced by environmental conditions, determine the fluidity of bacterial membranes and may hinder the efficacy of antimicrobials (Álvarez-Ordóñez et al. 2008; Dubois-Brissonnet et al. 2011). Thus, studies investigating the adaptation of bacteria to environmental conditions, and to the biofilm lifestyle, are crucial in order to understand the sessile phenotype and to reduce the microbiological risk related to biofilms.

The purpose of this work is to study the effect of environmental conditions, such as growth temperature (20, 30 and 37°C) and incubation time (24 and 48 h), on the formation of *Pseudomonas aeruginosa* biofilms on stainless steel and polycarbonate. These conditions aimed to mimic biofilm formation, under static conditions, on equipment used in operating rooms and on devices connected to human bodies (eg in 'dead-leg' areas). This study also aimed to evaluate the efficacy of disinfectant products against these biofilms. The characterization of biofilm matrices and the membrane fluidity of sessile *P. aeruginosa* were also studied, in order to understand the mechanisms of biofilm resistance to disinfectants. These results will contribute to an understanding of the relationship between the environmental conditions and the mechanisms of biofilm resistance to disinfectants.

Material and methods

Bacterial strain and culture conditions

The bacterial strain used for this study was *Pseudomonas aeruginosa* CIP 103467. The strain was stored at –80°C in tryptic soy broth (TSB; Biokar Diagnostics, Pantin, France) containing 40% (v/v) glycerol. Pre-cultures were prepared by inoculating 100 µl from frozen stock culture tubes into 5 ml of TSB and incubating at 20, 30 or 37°C. The pre-culture at 20°C was incubated for 48 h, while those at 30 and 37°C were incubated for 24 h. The cultures used in each experiment were then prepared by inoculating 5×10^4 CFU ml^{-1} from the pre-culture broths into 50 ml of TSB in sterile 500 ml flasks. Cultures were incubated under shaking (160 rpm) at 20, 30 or 37°C and stopped at the late exponential phase.

Slide preparation

The stainless steel (SS) and polycarbonate (PC) slides, (2.5 × 5 × 0.1 cm) were cleaned by soaking in 95% ethanol (Fluka, Sigma-Aldrich, Saint-Quentin-Fallavier, France) overnight to remove grease. The SS and PC have surface energy values of 47.1 and 43.4 mJ m^{-2}, and root mean square (RMS) values of 20 and 2.2 nm, respectively (Abdallah et al. 2014b). Thereafter, slides were rinsed in water, then soaked in 500 ml of TDF4 detergent (5%) (Franklab SA, Billancourt, France) for 20 min at 50°C under agitation. The slides were thoroughly rinsed five times for 1 min with agitation in 500 ml of distilled water at room temperature to eliminate detergent, followed by three washes with ultrapure water (Milli-Q® Academic, Millipore, Guyancourt, France). The clean SS slides were air-dried and sterilized by autoclaving at 121°C for 15 min. PC slides were sterilized for 10 min with ethanol (100%) (Fluka, Sigma-Aldrich).

Cell suspension preparation and biofilm formation assays

Cells from 20, 30 and 37°C cultures were harvested by centrifugation for 10 min at 3,500 × *g* (20°C) and washed twice with potassium phosphate buffer (PB; 100 mM, pH 7) (Figure 1). Thereafter, cells were resuspended in 20 ml of PB in 50 ml conical tubes and dispersed by sonication at 37 kHz for 5 min at 25°C (Elmasonic S60H, Elma, Singen, Germany). Subsequently, bacteria were diluted in the PB to a cell concentration of 1.10^8 CFU ml^{-1} by adjusting the $OD_{620\ nm}$ to 0.110 ± 0.005 using a UV/visible light spectrophotometer (Ultrospec 1100 pro, GE Healthcare, Little Chalfont, UK). Standardized cell suspensions were diluted 10-fold for the bacterial adhesion experiments (10^7 CFU ml^{-1}). Sterile slides were placed in the horizontal position in Petri dishes. The upper face was covered with 3 ml of cell suspensions (10^7 CFU ml^{-1}), and incubated at 20°C for 60 min. Slides were removed using sterile forceps and gently rinsed by dipping into 30 ml of PB to remove excess liquid droplets and loosely attached cells. Thereafter, the upper face was covered with 2 ml of TSB and the slides were incubated under static conditions, at the same temperature as the cultures (ie 20, 30 or 37°C), for incubation times of 24 or 48 h (Figure 1). For the biofilm grown for 48 h, the culture medium was changed after 24 h. After 24 and 48 h, slides were withdrawn and rinsed twice by gently dipping into 30 ml of PB to remove loosely attached cells. These slides were used for the quantification of bacterial cells, for the anti-biofilm assays and for the quantification of proteins and carbohydrates. For the quantification of bacterial biofilm biomass, cells were detached in 30 ml of PB in 50 ml conical tubes by vortexing for 30 s followed by a

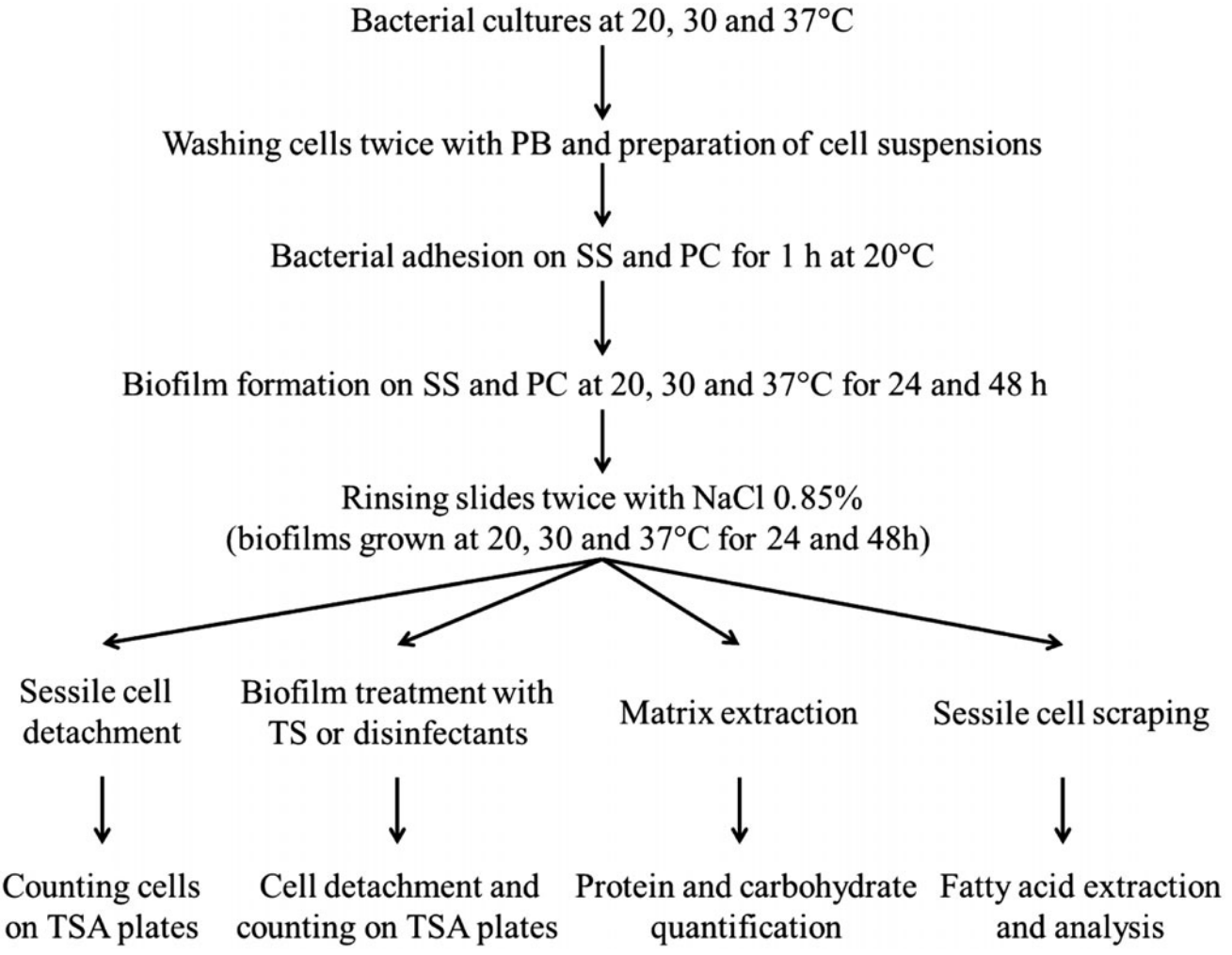

Figure 1. A flow chart illustrating the experimental steps in the study. PC: polycarbonate, PB: phosphate buffer, SS: stainless steel and TS: tryptone salt broth.

sonication for 5 min. Tubes were vortexed again for 30 s and serial dilutions were made in tryptone salt broth (TS) (Biokar Diagnostics, Pantin, France). Samples of 100 µl were spread onto tryptic soy agar (TSA) plates (Biokar Diagnostics) and incubated at 37°C for 24 h. After incubation for 24 or 48 h, the number of viable and culturable cells was counted on the plates and the results are expressed in log CFU cm^{-2}. The results represent the means of three independent experiments and in each experiment two slides were used.

Anti-biofilm assays

The disinfectant effect was evaluated on 24 and 48 h biofilms (Figure 1). To remove loosely attached cells, slides were rinsed as explained earlier and then placed vertically in 30 ml of the disinfectant solution in 50 ml conical tubes at 20°C for 5 or 15 min as recommended by the manufacturer (SCIENTIS, Romainville, France). The composition and characteristics of the disinfectants are shown in Table 1. Afterwards, the slides were withdrawn from the disinfectant solution and immersed in a neutralizing solution to stop the antibacterial action. The neutralizer contains a combination of Tween® 80 (30 g l^{-1}), saponin (30 g l^{-1}), lecithin (30 g l^{-1}), sodium thiosulfate (5 g l^{-1}), L-histidine (1 g l^{-1}) and TS broth (9.5 g l^{-1}). The viable and culturable cells were detached and counted as described above. For the control assays, the disinfectant solutions were replaced by TS broth solution. The results represent the average of three independent experiments and in each experiment two slides were used. For each experiment, the antimicrobial reduction was calculated from the difference between the biofilm biomass on untreated slides (control) and the treated slides. The antimicrobial reduction is presented as the average of these differences.

Table 1. Composition and characteristics of the disinfectant products.[a]

Disinfectant	Antimicrobial	Final concentration (μg g^{-1} and %)	Action time (min)
P1	DMPAP	315	15
	PHMB	100	
P2	DDAC	137.5	15
P3	DDAC	125	5
	ADBAC	475	
P4	ADBAC	5,000	5
P5	DDAC	490	15
	BDA	180	
P6	Ethanol	62%	5

[a]Products preformulated by the manufacturer (SCIENTIS).
ADBAC: alkyldimethylbenzylammonium chloride
BDA: bis (3-aminopropyl) dodecylamine
DDAC: didecyldimethylammonium chloride
DMPAP: N-didecyl-N-methyl-poly(oxyethyl) ammonium propionate
PHMB: polyhexamethylene biguanide

Quantification of carbohydrate and protein in the biofilm matrix

The protein and carbohydrate concentrations in the matrix of *P. aeruginosa* biofilms grown on SS and PC were quantified after incubation times of 24 and 48 h (Figure 1). The protein concentrations were quantified using the Bradford method with bovine serum albumin as the standard (Bradford 1976). The total carbohydrate content was measured using the phenol-sulfuric acid method with glucose as the standard (DuBois et al. 1956). After rinsing with physiological saline solution (0.85% NaCl), biofilms were recovered by scraping the surface, aspirating and expelling at least 10 times with 6 ml of ultrapure water. The suspensions were homogenized for 30 s, followed by sonication (5 min, 37 kHz). The cells were removed by centrifugation at 5,000 × *g* for 15 min and the supernatants were filtered through 0.2 μm Millipore filters and used for biochemical assays. The results were presented in μg cm^{-2} as the means of three independent experiments and in each experiment two slides were used.

Cellular fatty acid extraction and analysis

Sessile cells were collected after scraping cells from the washed biofilms and resuspended in 10 ml of PB (Figure 1). The suspensions were vortexed for 30 s. Subsequently, suspensions were sonicated for 5 min at 37 kHz and cells were dispersed by vortexing for 30 s. The cells were harvested by centrifugation (10,000 × *g*, 10 min at 4°C). The pellets, containing about 10^9 CFU, were washed twice with distilled water. One ml of the saponification solution (45 g sodium hydroxide, 150 ml methanol, and 150 ml distilled water) was used to resuspend the washed pellet and transferred to the extraction tube. Subsequently, cells were submitted to saponification and methylation. Fatty acid methyl ester extraction was realized as described previously (Chihib et al. 2003). Methyl ester analysis was performed on a GC-2014 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a Zebron ZB-FFAP (30 m × 0.25 mm) capillary column (Phenomenex, Pennant Hills, Australia), and connected to Thermo-Finnigan Trace dual-stage-quadrupole (DSQ) mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were injected in split mode. Chromatographic separation was carried out by a temperature gradient program beginning with a 5-min isothermal step at 70°C followed by an increase to 250°C at 4°C min^{-1}. Helium was used as the carrier gas. The injector and mass spectrometry interface were maintained at 260 and 280°C, respectively. Electron impact mass spectra were recorded at 70 eV. Masses were acquired in total ion current (TIC) between m/z 50 at 600 when the acceleration voltage was turned on after a solvent delay of three min. All data were processed by Xcalibur software (Thermo Fisher Scientific). All compounds were identified by comparing both the MS spectra and retention index with those available in the National Institute of Standards and Technology library. The results represent the average of three independent experiments and each experiment was done in duplicate.

Data analysis

The results are presented as the mean value ± the standard error of the mean (SEM). Data analysis was performed using Sigma Plot 11.0 (Systat Software, San Jose, CA, USA), using one-way ANOVA (Tukey's method) to determine the significance of the differences. Results were considered significant at a p-value of < 0.05.

Results

Effect of growth temperature and surface and incubation time on biofilm formation by P. aeruginosa *under static conditions*

The bacterial density of biofilms grown on SS and PC at 20, 30 and 37°C under static conditions after rinsing twice with saline solution is shown in Table 2. The results show that the final biomass of biofilms after incubation for 24 and 48 h were similar whatever the growth temperature and the surface type used (Table 2). The average biofilm biomass was of ~ 8 log CFU cm^{-2} after an incubation time of 24 or 48 h.

Effect of growth temperature and surface type on biofilm resistance to disinfectant agents

Treatment of biofilms grown under static conditions with TS broth (ie the negative control) slightly reduced the initial population whatever the growth temperature and the surface type. Indeed, the log reduction in viable cells within biofilms after the TS treatments did not exceed 0.9 log CFU cm^{-2} whatever the conditions studied (Table 3). The increase in growth temperature from 20 to 37°C, with an incubation time of 24 h, decreased the biofilm sensitivity to both P1 and P2 products (Table 3). The treatment of biofilms grown on SS with the P1 product for 15 min resulted in a 4.7, 1.7 and 2.4 log reduction in viable cells in biofilms grown at 20, 30 and 37°C, respectively (Table 3). When the biofilms were grown at 20, 30 and 37°C, the treatment with the P2 product for 15 min promoted, respectively, a 4.6, 3.5 and 2.3 log reduction in viable cells within SS biofilms, and a 4.3, 2.4 and 1.7 log reduction in viable cells within PC biofilms (Table 3). The treatment of the 20°C biofilms with P3 and P4 products for 5 min reduced the viable count of the initial population by, respectively, 1.3 and 2.1 log CFU cm^{-2} on SS and 2.8 and 3.1 log CFU cm^{-2} on PC (Table 3). Furthermore, the P3 and P4 products reduced the viable count of the 37°C biofilms by 3.1 log CFU cm^{-2} on SS and by 2.3 log CFU cm^{-2} on PC (Table 3). Both the P5 and P6 products reduced the viable and culturable counts below the detection limit whatever the growth temperature and the surface type (Table 3).

Effect of growth temperature, surface type and incubation time on biofilm resistance to disinfectant agents

The effect of incubation time on biofilm resistance was investigated only with the P2, P5 and P6 disinfectants, three products containing different active agents. The results shown in Table 4 indicate that treatment of the 48 h biofilms grown under static conditions with TS slightly reduced the initial population. The log reduction average of viable and culturable cells within biofilms after treatment with TS for 15 min was ~ 0.8 log CFU cm^{-2} on both surfaces (Table 4). The increase in the incubation time from 24 to 48 h at 20°C resulted in a significant decrease in biofilm sensitivity to the P2 and the P5 products on both surfaces ($p < 0.05$) (Table 4). When biofilms were grown at 20°C for 48 h (Table 4), the treatment with P2 and P5 products for 15 min reduced the viable and culturable count by 0.7 and 1.7 log CFU cm^{-2}, respectively, on SS and by 2.9 and 2.6 log CFU cm^{-2}, respectively, on PC. The increase in incubation time from 24 to 48 h at 30 and 37°C had no significant effect on the efficacy of the P2 product, while it decreased the sensitivity of these biofilms to the P5 product. The results also show that the rise in growth temperature with an incubation time of 48 h increased the sensitivity of the biofilm to both P2 and P5 products. However, the P6 product reduced the initial viable biomass of 48 h biofilms to below the detection limit on both surfaces whatever the temperature studied (Table 4).

Effect of growth temperature, surface type and incubation time on the major components of the biofilm matrix

The effect of growth temperature, incubation time and surface type were studied simultaneously on the major components of biofilm matrix (Table 5). The increase in the growth temperature from 20 to 37°C, with an incubation time of 24 h, led to a twofold increase in the protein concentration of the biofilm matrix on both surfaces ($p < 0.05$) (Table 5). When biofilms were grown at 20 and 30°C, the rise in incubation time from 24 to 48 h increased ~ 1.5 times the protein concentration of the biofilm matrix on both surfaces. However, the protein

Table 2. Bacterial densities of *P. aeruginosa* biofilms grown at 20, 30 and 37°C.

	Log CFU cm^{-2}					
	20°C		30°C		37°C	
	SS	PC	SS	PC	SS	PC
24 h	8.1 ± 0.2	8.2 ± 0.2	7.7 ± 0.1	7.9 ± 0.3	7.9 ± 0.1	8.1 ± 0.2
48 h	8.2 ± 0.1	8.5 ± 0.1	7.9 ± 0.1	8.4 ± 0.1	8.2 ± 0.2	8.3 ± 0.1

Biofilms were grown for 24 and 48 h on stainless steel (SS) and polycarbonate (PC). The bacterial density is presented as log CFU cm^{-2} ± SEM.

Table 3. Effect of disinfectants on *P. aeruginosa* biofilms grown at 20, 30 and 37°C after 24 h.

	Reduction in *P. aeruginosa* (log CFU cm^{-2})					
	20°C		30°C		37°C	
	SS	PC	SS	PC	SS	PC
TS	0.9 ± 0.3^{x}	0.9 ± 0.4^{x}	0.3 ± 0.2^{ax}	0.1 ± 0.5^{ax}	0.3 ± 0.1^{ax}	0.2 ± 0.5^{ax}
P1	4.7 ± 0.1	*	1.7 ± 0.2^{a}	*	2.4 ± 0.7^{a}	*
P2	4.6 ± 0.2^{ax}	4.3 ± 0.1^{x}	3.5 ± 0.3^{a}	2.4 ± 0.2	2.3 ± 0.1	1.7 ± 0.2
P3	1.3 ± 0.1	2.8 ± 0.2	1.7 ± 0.8^{x}	1.5 ± 0.7^{x}	3.1 ± 0.3	2.4 ± 0.2
P4	2.1 ± 0.1^{a}	3.1 ± 0.3	1.6 ± 0.2^{a}	4.0 ± 0.2	3.1 ± 0.2	2.3 ± 0.1
P5	ND	ND	ND	ND	ND	ND
P6	ND	ND	ND	ND	ND	ND

The biofilms were grown for 24 h on SS and PC. The results are presented as log reductions (LR), log (CFU cm^{-2}) ± SEM, relative to untreated biofilms. Between growth temperatures (a, b), attachment surfaces (x) under the same condition, the mean values with the same letters are not significantly different ($p > 0.05$). ND = viable cells not detected (ie below the detection limit).
*Product not recommended by the manufacturer for the polycarbonate surface.

Table 4. Effect of disinfectants on *P. aeruginosa* biofilms grown at 20, 30 and 37°C for 48 h.

	Reduction in *P. aeruginosa* (log CFU cm^{-2})					
	20°C		30°C		37°C	
	SS	PC	SS	PC	SS	PC
TS	0.6 ± 0.5^{ax}	1.1 ± 0.2^{ax}	0.6 ± 0.3^{ax}	0.9 ± 0.4^{ax}	0.9 ± 0.2^{a}	0.7 ± 0.3^{ax}
P2	0.7 ± 0.2^{x}	2.9 ± 0.1^{x}	3.2 ± 0.5^{z}	2.5 ± 0.8^{z}	3.0 ± 0.3^{z}	3.4 ± 0.3^{x}
P5	1.7 ± 0.1^{x}	2.6 ± 0.1^{x}	3.3 ± 0.3^{x}	4.2 ± 0.4^{z}	8.0 ± 0.1^{z}	5.4 ± 0.3^{x}
P6	ND	ND	ND	ND	ND	ND

Biofilms were grown for 48 h on SS and PC. The results are presented as log reductions (LR), log (CFU cm^{-2}) ± SEM, relative to untreated biofilms. Between growth temperatures (a, b), attachment surfaces (x) under the same condition, the mean values with the same letters are not significantly different ($p > 0.05$). Mean values with the letter (z) are not significantly different ($p > 0.05$) from the results of 24 h biofilms grown under the same conditions. ND = viable cells not detected (ie below the detection limit).

Table 5. Total proteins and carbohydrates in the matrix of *P. aeruginosa* biofilms grown at 20, 30 and 37°C.[a]

	Protein and carbohydrate content (μg cm^{-2})					
Growth temperature	20°C		30°C		37°C	
Incubation time (h)	24	48	24	48	24	48
Proteins (SS)	13.6 ± 2.2	20.7 ± 2.5	16.7 ± 1.5	24.3 ± 0.4	27.5 ± 1.4	24.8 ± 1.8
Proteins (PC)	12.9 ± 2.5	18.9 ± 2.3	17.3 ± 1.2	20.4 ± 2.1	28.6 ± 3.1	29.2 ± 2.2
Carbohydrates (SS)	5.9 ± 1.0	10.0 ± 1.0	13.6 ± 1.5	18.6 ± 1.5	11.0 ± 1.0	17.3 ± 1.4
Carbohydrates (PC)	7.3 ± 1.1	7.7 ± 1.3	13.2 ± 0.9	20.5 ± 1.6	13.1 ± 2.0	20.7 ± 1.2

[a]The biofilms were grown for 24 and 48 h on SS and PC. The concentrations of proteins and carbohydrates are presented in μg cm^{-2} ± SEM.

concentration of the biofilm matrix at 37°C was not significantly influenced by an increased incubation time ($p > 0.05$). The protein concentration in the biofilm matrix was about 24.8 and 29.2 μg cm^{-2} on SS and PC, respectively, after 48 h at 37°C (Table 5).

After 24 h, the concentration of carbohydrates in the matrix of the 20, 30 and 37°C biofilms was, respectively, ~ 5.9, 13.6 and 11 μg cm^{-2} on SS and, respectively, ~ 7.3, 13.2 and 13.1 μg cm^{-2} on PC. In addition, the rise in the incubation time from 24 to 48 h resulted in a 1.6-fold increase in the carbohydrate concentration of the biofilms matrix, except for the biofilms grown on PC at 20°C. Under this condition, the carbohydrate concentrations of the 48 h biofilm matrices were similar to those of the 24 h-grown biofilm (Table 5).

The increase in growth temperature from 20 to 37°C for the 24 h biofilm resulted in a twofold increase in matrix production on both surfaces ($p < 0.05$) (Figure 2A, B). When the growth temperature was increased from 20 to 37°C with an incubation time of 48 h, matrix production increased 1.4-fold and 1.9-fold, respectively, on SS and PC ($p < 0.05$) (Figure 2A, B). The increase in biofilm age

also resulted in a significant increase in matrix production except for the biofilm grown on PC at 20°C ($p > 0.05$) and for the biofilms grown on both surfaces at 37°C ($p > 0.05$) (Figure 2A, B).

Effect of growth temperature on the membrane fatty acid profile of sessile cells

The increase in the growth temperature from 20 to 37°C significantly increased the saturated fatty acids (SFA) from 25% to 37% for the 24 h biofilms, whatever the surface used ($p < 0.05$) (Figure 3A, B). Under the same conditions, the unsaturated fatty acids (UFA) decreased from 62% to 46.9% ($p < 0.05$) on SS (Figure 3A) and from 63.5% to 41.6% ($p < 0.05$) on PC (Figure 3B). The results also showed a significant increase in the amounts of cyclic fatty acids (CFA) from 0.6 to 4.8% ($p < 0.05$) with the increase in growth temperature from 20 to 37°C (Figure 3A, B). The relative amount of total hydroxy fatty acids (HFA) increased from 11.6 to 15.8% ($p < 0.05$) when the growth temperature was increased from 20 to 30°C on both surfaces (Figure 3A, B). At 37°C, the HFA amounts were ~ 10.1% and 16.6% on SS and PC, respectively (Figure 3A, B).

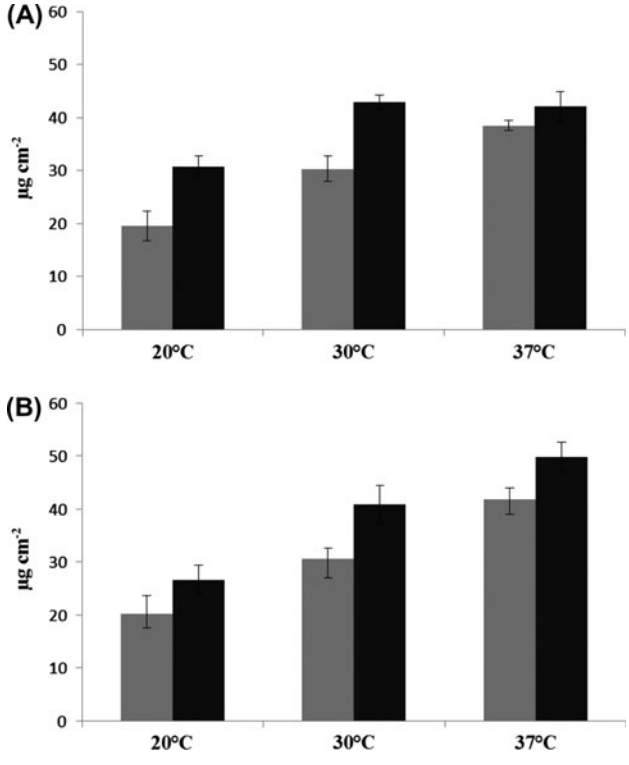

Figure 2. Matrix production in biofilms grown at 20, 30 and 37°C for 24 h (dark gray) and 48 h (black) on SS (A) and PC (B). Data represent the sum of the protein and carbohydrate concentrations ± SEM.

When the incubation time was increased from 24 to 48 h, no significant effect was observed on the SFA amount in cells grown at 20, 30 and 37°C, except for those grown on SS at 37°C (Figure 3A, C). Under this condition, the rise in growth temperature from 20 to 37°C decreased the SFA amount by 5% ($p < 0.05$). When biofilms were grown at 20 and 37°C, the increase in the incubation time from 24 to 48 h decreased the UFA amount in the sessile *P. aeruginosa* cells. The UFA amount in cells grown at 20°C decreased from 62.1% to 57.4% ($p < 0.05$) on SS (Figure 3A, C) and from 63.5% to 50.7% ($p < 0.05$) on PC (Figure 3B, D). However, the UFA amount was similar for both 24 and 48 h sessile cells when the biofilms were grown at 30°C (Figure 3). For the 48 h biofilms, the rise in the growth temperature from 20 to 37°C resulted in a decrease in the SFA/UFA ratio from 2.3 to 1.2 whatever the surface used (Figure 3C, D). The 48 h biofilm cells also showed an increase in the CFA amount from 2% to 4.4% on SS and from 1.8% to 5.7% on PC when the growth temperature was increased from 20 to 37°C (Figure 3C, D). In addition, the 48 h biofilm cells showed significant increases in the amounts of HFA ($p < 0.05$) (Figure 3C, D).

Discussion

Cells in biofilms are more resistant to antimicrobial agents than those grown under planktonic conditions. In addition, it has been reported that the healthcare sector constitutes a suitable environment for biofilm formation. Several studies have shown the persistence of biofilms on medical devices despite the use of disinfection procedures. This study aimed to investigate the effect of growth temperature (20, 30 and 37°C) and incubation time (24 and 48 h) on biofilm formation under static conditions by *P. aeruginosa* on SS and PC, two surfaces commonly encountered in medical devices. These culture conditions have been used to mimic some ecosystems encountered in the medical field. The temperatures 20°C and 37°C represent the temperatures in operating rooms and the human body. The efficiency of disinfectant products against biofilms grown at these temperatures was studied. The biofilm matrix and the membrane fluidity of sessile cells were characterized to study the effect of the environmental conditions on the mechanisms of biofilm resistance.

Growth temperature and surface type had no clear effect on the biomass of the *P. aeruginosa* biofilms. These results are in agreement with previous reports on the effect of growth temperature and surface type on biofilm formation by *Staphylococcus aureus* under static conditions (Abdallah et al. 2014c) and dynamic flow conditions (Waines et al. 2011). However, these results disagree with previous findings (Cappello & Guglielmino 2006; Oh et al. 2009; Choi et al. 2013), which indicated

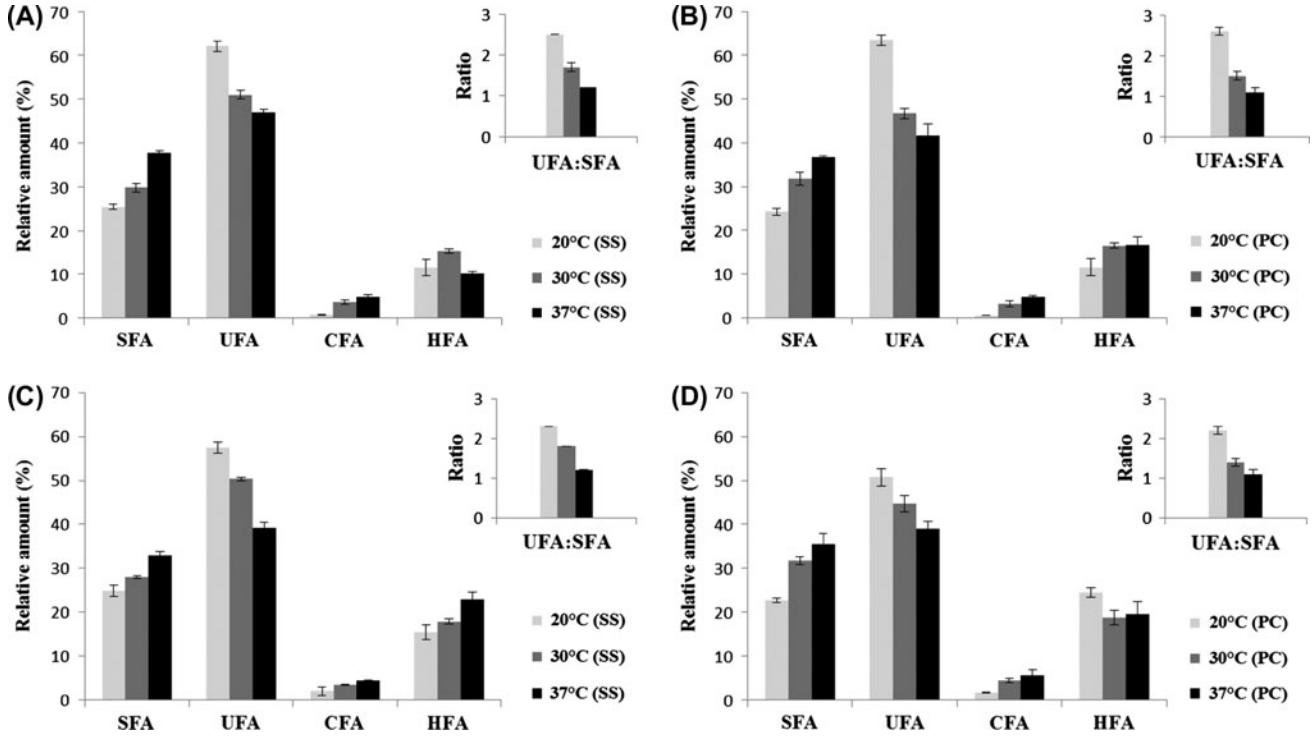

Figure 3. Membrane fatty acid profile of sessile *P. aeruginosa* grown at 20, 30 and 37° for (A) 24 h on SS; (B) 24 h on PC; (C) 48 h on SS; and (D) 48 h on PC. Values present the relative amount ± SEM. UFA: unsaturated fatty acids; SFA: saturated fatty acids; CFA: cyclic fatty acids; HFA: hydroxy fatty acids.

that the growth temperature and the substratum may influence biofilm formation of *P. aeruginosa*, under static conditions. It should, however, be pointed out that the differences in experimental procedures used to examine biofilm formation, including the culture medium, substrata and strains, may also account for the discrepancies observed between the different studies. In addition, Buckingham-Meyer et al. (2007) concluded that biofilm growth conditions significantly influence biofilm formation.

The results of the anti-biofilm assays showed that the growth temperature influenced the resistance of *P. aeruginosa* biofilms to disinfectants. These results are in agreement with previous studies (Nguyen & Yuk 2013; Abdallah et al. 2014c). In addition, the results showed that the effect of growth temperature on biofilm resistance was dependent on the active agents in each disinfectant product. The increase in the growth temperature from 20 to 37°C, with an incubation time of 24 h, increased biofilm resistance to disinfectants containing didecyldimethylammonium chloride (DDAC) and polyhexamethylene biguanides (PHMB) as the main active agents. However, the increase in the growth temperature resulted in an increase in biofilm sensitivity to both P3 and P4 products, containing alkyldimethylbenzylammonium chloride (ADBAC) as the main active agent. Ioannou et al. (2007) reported that DDAC presented a high-affinity binding to the membrane cells, while ADBAC exhibited a moderate affinity-binding. These authors also showed that the ADBAC molecules formed a single monolayer at the end of primary uptake, while the DDAC formed a double monolayer. Thus, the discrepancies found in the effect of growth temperature could be also related to the structure and the mode of action of each active agent.

The results presented in this work demonstrate that the effect of biofilm age on resistance to disinfectants depends on the growth temperature and surface type. These results are in agreement with a previous report by Nguyen and Yuk (2013). Overall, the rise in incubation time resulted in an increase in biofilm resistance to the P5 product containing DDAC as a main active agent, independently of the growth temperature. However, the increase in biofilm age from 24 to 48 h, with a growth temperature of 30 and 37°C, had no significant effect on biofilm resistance to the P2 product. The results showed that the surface type also influenced biofilm resistance and this effect was dependent on the growth temperature of biofilm development. This effect could be due to the influence of surface properties on the biofilm shield and architecture (Singh et al. 2011), or to its influence on the effectiveness of cleaning and sanitizing

(Chaturongkasumrit et al. 2011). Furthermore, the concentration of ADBAC in the P4 product was 10 times higher than its concentration in the P3 product. However, the efficiency of both products in killing biofilm cells was broadly similar. In accordance with previous studies, the efficiency of disinfectant products seems to depend on the time of action rather than the concentration of antibacterials in the products (Bridier et al. 2011b).

The effect of environmental conditions such as growth temperature and surface type on the resistance of *P. aeruginosa* biofilms to disinfectants is insufficiently studied. Nevertheless, the findings presented in this study highlight the significant effects of environmental conditions and bacterial background on the capacity of biofilms to resist disinfectant products. In order to study the mechanisms of biofilm resistance to disinfectants, matrix production was evaluated as a function of growth temperature, surface type and incubation time of biofilm formation. The *P. aeruginosa* biofilm matrix has been reported to play an important role in resistance to antimicrobial attacks (Colvin et al. 2012). The data indicated that matrix production increased when the biofilm growth temperature increased from 20 to 37°C with an incubation time of either 24 or 48 h. These results conflict with those of Kiliç and Dönmez (2008), which showed a decrease in matrix production with an increase in growth temperature from 20 to 30°C. On the other hand, the present results indicate that the surface type had no significant effect on matrix production after 24 h. The increase in the incubation time resulted in a significant increase in matrix production despite the steady state of the biofilm biomass. These results are in agreement with those of Myszka and Czaczyk (2009), who showed an increase in matrix production with an increase in incubation time. These results suggest that at maturity there is increased matrix production rather than increased bacterial biomass. This rise in matrix production may increase electrostatic and hydrophobic interactions between exopolymeric substances and disinfectant molecules. These interactions may hinder the penetration of antimicrobials into the biofilm and increase the resistance of the biofilm to antimicrobials (Bridier et al. 2011b). Thus, the increase in biofilm resistance with a rise in growth temperature and biofilm age is probably the result of the changes observed in matrix production. However, the decrease in biofilm resistance under certain conditions with an increase in growth temperature highlights the involvement of another mechanism of biofilm resistance to disinfectant agents.

The cell membrane is a potential target of antimicrobial agents used in disinfectant products. Bacterial membranes act as barriers which prevent antimicrobials penetrating the lipid bilayers. The cationic antimicrobials used for this study use the cell membranes as a common target but with different modes of action (Gilbert & Moore 2005). The hydrophilic moiety of quaternary ammonium compounds, such as ADBAC and DDAC, has been reported to interact with the negatively charged groups of membrane cells. The hydrophobic tails strongly interact with membrane fatty acids, which induce disruption of lipid bilayers. PHMB interacts superficially with negatively charged acidic phospholipids, inducing their aggregation and therefore the disruption of the lipid bilayers (Gilbert & Moore 2005). Thus, the membrane fatty acid composition, controlling membrane fluidity, could be involved in the resistance to membrane active agents. A rise in growth temperature for biofilms grown over 24 h resulted in a decrease in the fluidity of the cell membranes through an increase in SFA and CFA, and a decrease in the amount of UFA. The straight-chain saturated fatty acids are linear and known to pack together to produce a bilayer with a high phase transition (Zhang & Rock 2008). The decrease in fatty-acid unsaturation with the increase in biofilm growth temperature probably decreased the membrane fluidity of sessile cells. In addition, the decrease in the UFA was accompanied by an increase in the CFA, which was likely derived from the corresponding monounsaturated fatty acid by addition of a methyl group from *S*-adenosyl-l-methionine across the double bond (Grogan & Cronan 1997). The significant increase in the amounts of CFA measured with the increase in growth temperature may increase the stability of the structural and the dynamic properties of biological membranes (Brown et al. 1997). In accordance with previous studies, the present results showed that an increase in growth temperature reduced the membrane fluidity of sessile cells (Kim et al. 2005). Such rigidification may explain the increased resistance of the biofilm to P1 and P2 disinfectants when the growth temperature was increased for the 24 h biofilms. However, the decrease in biofilm sensitivity to P3 and P4 products when the growth temperature was increased from 20 to 37°C for 24 h suggests that the involvement of membrane fluidity in the resistance depended on the structure of the disinfectant molecule.

The increase in incubation time for biofilms did not significantly affect the ratio of UFA:SFA whatever the surface and the temperature. However, the rise in growth temperature with an incubation time of 48 h decreased membrane fluidity on both surfaces (ie decreased the UFA:SFA ratio). Again, neither the increase in matrix production nor the decrease in membrane fluidity can explain the decrease in the resistance of 48 h biofilms to disinfectant with the increase in the growth temperature. It should be noted that the rise in incubation time resulted in a significant increase in HFA for sessile cells on both surfaces. The HFA, in Gram-negative bacteria, are structural components in lipid-A of the lipopolysaccharides, which anchors the polysaccharide to the exterior of the outer cellular membrane (Parsons & Rock

2013). Such changes in the amount of HFA seem to be a way by which sessile *P. aeruginosa* cells adapt to increases in biofilm age. However, the involvement of the HFA in the resistance of sessile *P. aeruginosa* cells to disinfectants remains unclear.

In conclusion, the results showed that the resistance of biofilms to disinfectants depended on several environmental conditions commonly found in the healthcare sectors. It is therefore important to be conscious of the environmental conditions of biofilm formation, such as growth temperature, surface type and biofilm age, when testing the anti-biofilm efficacy of disinfectant products. In addition, this study showed that the biofilm matrix and the membrane fluidity of biofilm cells could not fully explain the resistance of biofilms grown under different environmental conditions.

Conflict of interest disclosure statement

No potential conflict of interest was reported by the author(s).

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References

Abdallah M, Benoliel C, Drider D, Dhulster P, Chihib NE. 2014a. Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. Arch Microbiol. 196:453–472.

Abdallah M, Benoliel C, Jama C, Drider D, Dhulster P, Chihib NE. 2014b. Thermodynamic prediction of growth temperature dependence in the adhesion of *Pseudomonas aeruginosa* and *Staphylococcus aureus* to stainless steel and polycarbonate. J Food Prot. 77:1116–1126.

Abdallah M, Chataigne G, Ferreira-Theret P, Benoliel C, Drider D, Dhulster P, Chihib NE. 2014c. Effect of growth temperature, surface type and incubation time on the resistance of *Staphylococcus aureus* biofilms to disinfectants. Appl Microbiol Biotechnol. 98:2597–2607.

Álvarez-Ordóñez A, Fernández A, López M, Arenas R, Bernardo A. 2008. Modifications in membrane fatty acid composition of *Salmonella* typhimurium in response to growth conditions and their effect on heat resistance. Int J Food Microbiol. 123:212–219.

Anaissie EJ, Penzak SR, Dignani MC. 2002. The hospital water supply as a source of nosocomial infections: a plea for action. Arch Intern Med. 162:1483–1492.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248–254.

Bridier A, Briandet R, Thomas V, Dubois-Brissonnet F. 2011a. Resistance of bacterial biofilms to disinfectants: a review. Biofouling. 27:1017–1032.

Bridier A, Dubois-Brissonnet F, Greub G, Thomas V, Briandet R. 2011b. Dynamics of the action of biocides in *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemother. 55:2648–2654.

Brown JL, Ross T, McMeekin TA, Nichols PD. 1997. Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. Int J Food Microbiol. 37:163–173.

Buckingham-Meyer K, Goeres DM, Hamilton MA. 2007. Comparative evaluation of biofilm disinfectant efficacy tests. J Microbiol Methods. 70:236–244.

Campanac C, Pineau L, Payard A, Baziard-Mouysset G, Roques C. 2002. Interactions between biocide cationic agents and bacterial biofilms. Antimicrob Agents Chemother. 46:1469–1474.

Cappello S, Guglielmino SPP. 2006. Effects of growth temperature on polystyrene adhesion of *Pseudomonas aeruginosa* ATCC 27853. Braz J Microbiol. 37:205–207.

Chaturongkasumrit Y, Takahashi H, Keeratipibul S, Kuda T, Kimura B. 2011. The effect of polyesterurethane belt surface roughness on *Listeria monocytogenes* biofilm formation and its cleaning efficiency. Food Control. 22:1893–1899.

Chihib NE, Ribeiro da Silva M, Delattre G, Laroche M, Federighi M. 2003. Different cellular fatty acid pattern behaviours of two strains of *Listeria monocytogenes* Scott A and CNL 895807 under different temperature and salinity conditions. FEMS Microbiol Lett. 218:155–160.

Chihib NE, Tierny Y, Mary P, Hornez JP. 2005. Adaptational changes in cellular fatty acid branching and unsaturation of *Aeromonas* species as a response to growth temperature and salinity. Int J Food Microbiol. 102:113–119.

Choi NY, Kim BR, Bae YM, Lee SY. 2013. Biofilm formation, attachment, and cell hydrophobicity of foodborne pathogens under varied environmental conditions. J Korean Soc Appl Biol Chem. 56:207–220.

Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC, Ryder C, Howell PL, Wozniak DJ, Parsek MR. 2012. The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. Environ Microbiol. 14:1913–1928.

Da Silva Meira QG, de Medeiros Barbosa I, Alves Aguiar Athayde AJ, de Siqueira-Júnior JP, de Souza EL. 2012. Influence of temperature and surface kind on biofilm formation by *Staphylococcus aureus* from food-contact surfaces and sensitivity to sanitizers. Food Control. 25:469–475.

Donlan RM. 2001. Biofilms and device-associated infections. Emerging Infect Dis. 7:277–281.

Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 15:167–193.

DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem. 28:350–356.

Dubois-Brissonnet F, Naïtali M, Mafu AA, Briandet R. 2011. Induction of fatty acid composition modifications and tolerance to biocides in *Salmonella enterica* serovar typhimurium by plant-derived terpenes. Appl Environ Microbiol. 77:906–910.

Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Hoiby N, Molin S. 2012. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat Rev Microbiol. 10:841–851.

Gilbert P, Moore LE. 2005. Cationic antiseptics: diversity of action under a common epithet. J Appl Microbiol. 99:703–715.

Grogan DW, Cronan JE Jr. 1997. Cyclopropane ring formation in membrane lipids of bacteria. Microbiol Mol Biol Rev. 61:429–441.

Ioannou CJ, Hanlon GW, Denyer SP. 2007. Action of disinfectant quaternary ammonium compounds against *Staphylococcus aureus*. Antimicrob Agents Chemother. 51:296–306.

Kiliç NK, Dönmez G. 2008. Environmental conditions affecting exopolysaccharide production by *Pseudomonas aeruginosa*, *Micrococcus* sp. and *Ochrobactrum* sp. J Hazard Mater. 154:1019–1024.

Kim BH, Kim S, Kim HG, Lee J, Lee IS, Park YK. 2005. The formation of cyclopropane fatty acids in *Salmonella enterica* serovar Typhimurium. Microbiology. 151:209–218.

Lewis K. 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol. 5:48–56.

Martínez-Solano L, Macia MD, Fajardo A, Oliver A, Martinez JL. 2008. Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pulmonary disease. Clin Infect Dis. 47:1526–1533.

Mesaros N, Nordmann P, Plésiat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, Van Laethem Y, Jacobs F, Lebecque P, Malfroot A, et al. 2007. *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. Clin Microbiol Infect. 13:560–578.

Myszka K, Czaczyk K. 2009. Characterization of adhesive exopolysaccharide (EPS) produced by *Pseudomonas aeruginosa* under starvation conditions. Curr Microbiol. 58:541–546.

Nguyen HDN, Yuk HG. 2013. Changes in resistance of *Salmonella* Typhimurium biofilms formed under various conditions to industrial sanitizers. Food Control. 29:236–240.

Oh YJ, Lee NR, Jo W, Jung WK, Lim JS. 2009. Effects of substrates on biofilm formation observed by atomic force microscopy. Ultramicroscopy. 109:874–880.

Parsons JB, Rock CO. 2013. Bacterial lipids: metabolism and membrane homeostasis. Prog Lipid Res. 52:249–276.

Singh AV, Vyas V, Patil R, Sharma V, Scopelliti PE, Bongiorno G, Podesta A, Lenardi C, Gade WN, Milani P. 2011. Quantitative characterization of the influence of the nanoscale morphology of nanostructured surfaces on bacterial adhesion and biofilm formation. PLoS One. 6:26.

Stewart PS. 2002. Mechanisms of antibiotic resistance in bacterial biofilms. Int J Med Microbiol. 292:107–113.

Tao L, Hu B, Rosenthal VD, Gao X, He L. 2011. Device-associated infection rates in 398 intensive care units in Shanghai, China: International Nosocomial Infection Control Consortium (INICC) findings. Int J Infect Dis. 15:e774–e780.

Waines PL, Moate R, Moody AJ, Allen M, Bradley G. 2011. The effect of material choice on biofilm formation in a model warm water distribution system. Biofouling. 27:1161–1174.

Zhang YM, Rock CO. 2008. Membrane lipid homeostasis in bacteria. Nat Rev Microbiol. 6:222–233.