International Journal of Food Microbiology 214 (2015) 38–47

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Impact of growth temperature and surface type on the resistance of *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms to disinfectants

Marwan Abdallah [a,b], Oussama Khelissa [a,d], Ali Ibrahim [c], Corinne Benoliel [b], Laurent Heliot [c], Pascal Dhulster [a], Nour-Eddine Chihib [a,d,*]

[a] Laboratoire Régional de Recherche en Agroalimentaire et Biotechnologies: Institut Charles Viollette, Bâtiment Polytech'Lille, Université Lille 1, Avenue Paul Langevin, Cité Scientifique, 59655 Villeneuve d'Ascq Cedex, France
[b] Laboratoire SCIENTIS, Parc Biocitech — 102, Avenue Gaston Roussel, 93230 Romainville, France
[c] IRI — Institut de Recherche Interdisciplinaire, Parc de la Haute Borne — 50 av de Halley, BP70478-59658 Villeneuve d'Ascq Cedex, France
[d] INRA-UMR UMET 8207 — Equipe PIHM, CNRS-INRA, Université de Lille, 369 rue jules Guesde, BP20039, 59651 Villeneuve d'Ascq Cedex, France

ARTICLE INFO

Article history:
Received 29 October 2014
Received in revised form 12 July 2015
Accepted 18 July 2015
Available online 19 July 2015

Keywords:
Biofilm resistance
Food environment
Pseudomonas aeruginosa
Staphylococcus aureus
Disinfectant

ABSTRACT

Biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus* on food-contact-surfaces represents a significant risk for the public health. In this context, the present study investigates the relationship between the environmental conditions of biofilm formation and the resistance to disinfectants. Therefore, a static biofilm reactor, called NEC-Biofilm System, was established in order to study the effect of growth temperature (20, 30 and 37 °C), and of the surface type (stainless steel and polycarbonate), on biofilm resistance to disinfectants. These conditions were selected to mimic the biofilm formation on abiotic surfaces of food processing industries. The antibiofilm assays were performed on biofilms grown during 24 h. The results showed that the growth temperature influenced significantly the biofilm resistance to disinfectants. These data also revealed that the growth temperature has a significant effect on the biofilm structure of both bacteria. Furthermore, the increase of the biofilm growth temperature increased significantly the *algD* transcript level in sessile *P. aeruginosa* cells, whereas the *icaA* one was not affected in *S. aureus* cells. Overall, our findings show that the biofilm structure and matrix cannot fully explain the biofilm resistance to disinfectant agents. Nevertheless, it underlines the intimate link between environmental conditions, commonly met in food sectors, and the biofilm resistance to disinfectants.

1. Introduction

Pseudomonas aeruginosa and *Staphylococcus aureus* are important opportunistic human pathogens, causing major problems in food sectors. In fact, *S. aureus* is among the common known cause of foodborne infections worldwide and the involvement of *P. aeruginosa* in such infections and food spoilage is also reported (Kim and Wei, 2007; Newell et al., 2010). Furthermore, most bacteria, in their natural and man-made ecosystems, are attached to surfaces and form a complex three-dimensional structure, called biofilm (Donlan and Costerton, 2002). The biofilm formation on food-contact-surfaces, in turn, leads to contamination of food products, which reduces their shelf-life or results in human foodborne diseases, and causes significant economic losses (Sharma and Anand, 2002; von Holy, 2006). Moreover, the environmental conditions encountered in food sectors have also been found to promote the biofilm formation and influence the biofilm resistance to disinfecting agents (Abdallah et al., 2014b, 2014c; Belessi et al., 2011; da Silva Meira et al., 2012; Nguyen and Yuk, 2013).

The biofilm populations have several advantages over their free-living counterparts, including the resistance to antimicrobial agents (Donlan and Costerton, 2002). The biofilm resistance is often linked to the biofilm matrix. In fact, the biofilm resistance mechanism involves antibacterial sequestration by matrix and prevents its penetration in biofilm deeper layers (Davison et al., 2010; Jang et al., 2006). The biofilm matrix is mainly composed of exopolysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender, 2010). Different studies have shown that *P. aeruginosa* cells produce at least three exopolysaccharides: Alginate, Psl and Pel. The Alginate is a linear polyanionic exopolysaccharide composed of uronic acids and involves at least 24 genes for its biosynthesis (*algA*, *B*, *D*, *8*, *44*…) (Rehm, 2009). The Psl polysaccharide, which is synthesized by the polysaccharide synthesis locus (*psl*), consists of a repeating pentasaccharide, containing D-mannose, D-glucose and L-rhamnose (Byrd et al., 2009). Eleven

* Corresponding author at: Laboratoire Régional de Recherche en Agroalimentaire et Biotechnologies: Institut Charles Viollette, Bâtiment Polytech'Lille, Université Lille 1, Avenue Paul Langevin, Cité Scientifique, 59655 Villeneuve d'Ascq Cedex, France.
E-mail address: nour-eddine.chihib@univ-lille1.fr (N.-E. Chihib).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.07.022
0168-1605/

genes, *pslACDEFGHIJKL*, are believed to be required for the Psl synthesis. The Pel polysaccharide, a glucose-rich polysaccharide is synthesized by the *pel* locus containing seven genes (*pelA* to *pelG*) (Ghafoor et al., 2011). Many *S. aureus* strains produce a poly-N-acetylglucosamine (PNAG) as a main exopolysaccharide and involve the intercellular adhesion (*icaADBC*) locus for the PNAG production (Arciola et al., 2012). Furthermore, different studies underlined that *P. aeruginosa* and *S. aureus* exopolysaccharides provide the structural scaffold of the biofilm and increase the resistance to antimicrobials such as antibiotics and disinfectants (Arciola et al., 2012; Yang et al., 2011). Subsequent studies showed that the biofilm resistance is related to the physiological state of sessile cells (Abdallah et al., 2014a, 2015; Campanac et al., 2002; Simoes et al., 2011). Therefore, a more thorough understanding of biofilm resistance mechanisms, as a function of environmental conditions, is necessary in order to improve the antibiofilm treatments in food processing industries.

In this regard, a static biofilm reactor was developed in order to study the biofilm resistance of *P. aeruginosa* and *S. aureus* to disinfecting agents This investigation is also intended to elucidate the effect of growth temperature (20, 30 and 37 °C), and surface type (stainless steel and polycarbonate), on the biofilm resistance to disinfectants formulated by Scientis laboratory (Scientis, France). The selected experimental conditions aimed to mimic the biofilm formation, under static conditions, on food processing equipment. The effect of these growth conditions was studied on the biofilm three-dimensional structure, using the confocal laser scanning microscopy. The expression of genes involved in the biosynthesis of exopolysaccharides was also quantified using the real time PCR in sessile cells grown under the different growth conditions. The present work thus endeavors to understand the relationship between the environmental conditions of biofilm formation and the resistance to disinfectant products in order to reduce the issues associated with the biofilm persistence.

2. Material and methods

2.1. Bacterial strain and culture conditions

The bacterial strains used for this study were *P. aeruginosa* CIP 103467 and *S. aureus* CIP 4.83. The strains were stored at −80 °C in Tryptic Soy broth containing 40% (v/v) of glycerol (TSB; Biokar Diagnostics, France). To prepare precultures, 100 mL from frozen stock cultures was inoculated into 5 mL of TSB and then incubated at the culture temperature (i.e., 20, 30 or 37 °C). The 20 °C pre-culture was incubated for 48 h, whereas those at 30 and 37 °C were incubated for 24 h. 1 mL of these preculture, containing 5×10^4 CFU, was used to inoculate 50 mL of TSB medium in 500 mL sterile flasks for bacterial cultures. Cultures were then incubated at 20, 30 and 37 °C, under shaking conditions at 160 rpm, and bacterial cells were harvested in the late exponential phase.

2.2. Slide preparation

The circular slides of stainless steel (304 L, Equinox, France), and polycarbonate (Plexilux, France), 41 mm in diameter and 1 mm thick, were soaked overnight in ethanol 95% (Fluka, Sigma-Aldrich, France) and then rinsed with distilled water. Rinsed slides were then soaked in 500 mL of 5% TDF4 detergent (Franklab SA, France), for 20 min at 50 °C under agitation conditions. The slides were then thoroughly rinsed 5 times, for 1 min under agitation in 500 mL of distilled water at 20 °C to eliminate detergent residues, followed by three washes with ultrapure water (Milli-Q® Academic, Millipore, France). Stainless steel slides were air-dried and sterilized by autoclaving at 121 °C for 15 min. The polycarbonate slides were sterilized in 95% ethanol for 10 min.

2.3. Description of NEC Biofilm System

The system consists of several assembled pieces of stainless steel and a rubber O-ring (Fig. 1A). The lower part (1) is made of stainless steel and constitutes the circular basis of the system. On the upper flat face, the O-ring (2) can be used to fit perfectly one circular test slide (3). Then a stainless steel cylinder (4) can be placed in order to form the well of the biofilm formation. This cylinder has two orifices on its lateral wall in order to ensure oxygen supply for the bacterial growth. A collar clamp (5) was used to provide tightness and a metal cover (6) was used to ensure the sterility of the closed system (7). This system is called NEC-Biofilm System (Fig. 1B). All system parts are autoclavable at 121 °C for 20 min.

2.4. Cell suspension preparation

Cells of 20, 30 and 37 °C cultures were harvested by centrifugation for 10 min at 3500 *g* (20 °C). Bacteria were washed twice with 20 mL of potassium phosphate buffer (PB; 100 mM, pH 7) and finally resuspended in 20 mL of PB. To disperse cells, a sonication at 37 kHz was carried out for 5 min at 25 °C (Elmasonic S60H, Elma, Germany). Subsequently, bacteria were resuspended in the PB to a cell concentration of 1×10^8 CFU/mL by adjusting the optical density to $OD_{620\ nm} = 0.110 \pm 0.005$ (Ultrospec 1100 pro, GE Healthcare, formerly Amersham Biosciences, United Kingdom). Standardized cell suspensions were diluted 10 fold in order to make a cell concentration of 10^7 CFU/mL for bacterial adhesion experiments.

2.5. Biofilm formation assay

The biofilm formation was initiated by the deposition of 3 mL of bacterial suspension (10^7 CFU/mL) in the sterile well of each reactor and then incubated at 20 °C for 60 min. After the bacterial adhesion, the 3 mL were removed and the slides were gently washed twice using 5 mL of PB in order to remove loosely adherent cells. Then 5 mL of TSB

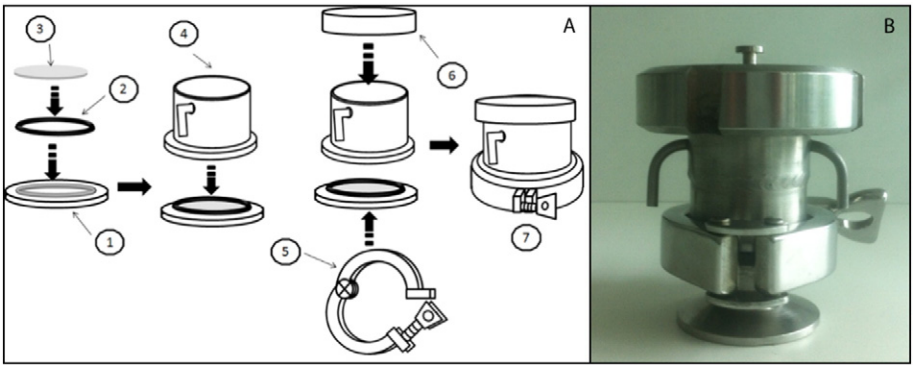

Fig. 1. Description of the static biofilm system. A presents the different pieces of the assembled system. B presents NEC Biofilm System.