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Influence of food processing environments on structure initiation of static biofilm of *Listeria monocytogenes*

Tereza Pilchová [a,b,c], Mathieu Hernould [b,c], Hervé Prévost [b], Kateřina Demnerová [a], Jarmila Pazlarová [a], Odile Tresse [b,c,*]

[a] Department of Biochemistry and Microbiology, Faculty of Food and Biochemical Technology, Institute of Chemical Technology in Prague, Prague 6, 166 28, Czech Republic
[b] LUNAM Université, Oniris, UMR-1014 SECALIM, Nantes F-44307, France
[c] INRA, Nantes F-44307, France

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ABSTRACT

Deciphering the initial steps and the initial structure of biofilm formation is important to understand their effects on the subsequent formation of mature listerial biofilms on food-processing equipment as well as on remedial options to control their proliferation. In this research the achievement of an initial 2-D biofilm structure, observed using fluorescence microscopy, could be divided into four steps: (i) cell cluster formation with attached cells side-by-side; (ii) branch elongation by cell division; (iii) open geometric forms connected through branches and (iv) honeycomb-like structure. Among the four test strains able to reach a honeycomb-like structure, there was one (EGD-e) which could not enter to the second step and subsequently could not develop complex biofilms. Only time-shift differences according to growth medium and surface were observed except for EGD-e when incubated on plastic surface. Interestingly, the first step of the primary structure of *Listeria monocytogenes* was impaired for all strains in acidic conditions. The biofilm primary structure is a critical stage for the development of listerial biofilms with elaborated structure. Some environmental conditions such as acidic pH could impair the structure of the biofilm and prevent complex biofilm formation.

1. Introduction

Listeria monocytogenes, the microorganism responsible for listeriosis, is considered as one of the major foodborne pathogens with a fatality rate in humans as high as 30% (Rocourt, Jacquet, & Reilly, 2000). Listeriosis is also associated with late-onset complications such as meningitis, encephalitis, abortion in pregnant women and infection of central nervous system in neonates and immunocompromised patients (Farber & Peterkin, 1991; McLauchlin, 1997; Rocourt, 1996; Swaminathan & Gerner-Smidt, 2007). *L. monocytogenes* is frequently isolated from wastewater, soil and food plant material (Freitag, Port, & Miner, 2009). This ubiquitous pathogen is well adapted to the environment. Its ability to grow at refrigeration temperatures, high-salt concentration and low pH range constitutes a real problem for the food-processing industry (Farber & Peterkin, 1991; Gandhi & Chikindas, 2007; Herald & Zottola, 1988; Liu, 2006).

In response to adverse conditions, *L. monocytogenes* could develop biofilms either on abiotic or biotic surfaces resulting in persistence on food-processing surfaces and subsequent cross- or re-contamination of food products (Beresford, Andrew, & Shama, 2001; Renier, Hébraud, & Desvaux, 2011; Tresse et al., 2007). Biofilms are described as surface adhering bacterial cells organized in clusters and enclosed in self-produced extracellular polymeric substances (EPS), mainly composed of polysaccharides and proteins (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995; Costerton et al., 1994; Djordjevic, Wiedmann, & McLandsborough, 2002; Watnick & Kolter, 2000). In general, antimicrobial treatments using detergents and disinfectants are less efficient on these organized structures than on planktonic counterparts (Hood & Zottola, 1997). Consequently, control of biofilms of *L. monocytogenes* remains difficult all the more since the cleaning is not performed adequately (Pan, Breidt Jr., & Kathariou, 2006; Rajkovic et al., 2009).

Formation of biofilm results from a continuous process from attachment to a surface to the development of a bacterial community structure and detachment. The initial step for biofilm

* Corresponding author. UMR-INRA 1014 SECALIM, Oniris, Route de Gachet BP 40706, F-44307 Nantes Cedex 3, France. Tel.: +33 2 40 68 78 11.
E-mail address: odile.tresse@oniris-nantes.fr (O. Tresse).

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formation is the bacterial adhesion to the surface which could occur within only 3–5 s for some *L. monocytogenes* strains (Takhistov & George, 2004). This reversible adhesion becomes irreversible through stronger bonds leading to the initiation of biofilm formation (Bryers, 2000; van Loosdrecht, Lyklema, Norde, & Zehnder, 1989; Marshall, 1992). The cell attachment to the surface is influenced by physico-chemical properties of the material including charge, liquid velocity (static/dynamic flow biofilms), rugosity, hydrophobicity (nonpolar surface such as teflon and other plastics) and hydrophilicity (glass or metals) (Donlan, 2002; Kumar & Anand, 1998). Then, the biofilm growth is mainly characterized by aggregate formation and EPS secretion. Biofilm maturation is reached when these three dimensional structures (3-D structures) are crossed by water channels or pores which ensure nutrients exchange and removal of bacterial waste products (Bryers, 2000; van Loosdrecht et al., 1989; Sauer, Rickard, & Davies, 2007). The bacteria involved in the biofilm formation undergo transitions from planktonic and sessile forms and the net accumulation of biomass in a mature biofilm depends on cell growth and cell detachment. Mushroom-shaped, honeycomb-like and ball-shaped structures are the main architectures described for listerial biofilms (Hall-Stoodley & Stoodley, 2002; Rieu et al., 2008).

Coating a surface by *L. monocytogenes* depends also on environmental conditions including medium, temperature, pH, hydrodynamic conditions and strains (Borucki, Peppin, White, Loge, & Call, 2003; Di Bonaventura et al., 2008; Djordjevic et al., 2002; Kumar et al., 2009; Tresse et al., 2007). For instance, the presence or the motility of flagella play a predominant role, at least in the early stages of biofilm formation. In *L. monocytogenes*, the temperature regulates the flagellation of the cells (van Houdt & Michiels, 2010; Lemon, Higgins, & Kolter, 2007; Todhanakasem & Young, 2008; Vatanyoopaisarn, Nazli, Dodd, Rees, & Waites, 2000). Consequently, temperature effect on biofilm formation of *L. monocytogenes* has been widely investigated (Moens & Vanderleyden, 1996; Peel, Donachie, & Shaw, 1988; Vatanyoopaisarn et al., 2000). Tresse et al. (2007) have also reported a pH dependence for *L. monocytogenes* flagellation and consequences on cell attachment. Most of the knowledge on monospecies biofilm formation of *L. monocytogenes* has been gained by studying either the bacterial adhesion to surfaces or biofilm architecture (Borucki et al., 2003; Chae & Schraft, 2000; Djordjevic et al., 2002; Kalmokoff et al., 2001; Lunden, Miettinen, Autio, & Korkeala, 2000; Norwood & Gilmour, 1999). Between single cell adhesion to surface and the development of a 3-D structure, the biofilm initiation goes through a two dimensional initial structure (2-D structure) which has not been clearly described for *L. monocytogenes*. In addition, there is no evidence for a correlation between *L. monocytogenes* strains, environmental conditions and this initial 2-D structure of biofilms.

The objective of this study was to decipher the initial 2-D structure of *L. monocytogenes* biofilm to assess the influence of pH 5 and 7, growth conditions (MWB and TSBYE) and surface (glass and plastic) on this structure from cells grown in culture chambers and detected using fluorescence microscopy. Temperature was fixed at 20 °C to imitate the environmental temperature in a food-processing plant.

2. Materials and methods

2.1. Bacterial strains and growth experiments

Four strains of *L. monocytogenes* were used in this study. *L. monocytogenes* Scott A (serotype 4b) and *L. monocytogenes* EGD-e (serotype 1/2c) were isolated from listeriosis outbreaks, *L. monocytogenes* X-Limo 500 (serotype 1/2a) was isolated from a dairy plant from brine room floor by Pasteur Institute (Lille, France) and *L. monocytogenes* 111 (serotype 1/2a) was isolated from a pork meat by ADRIANOR (Arras, France). All strains were maintained in fresh sterile trypticase soy broth supplemented with 6 g l^{-1} of yeast extract (TSBYE, Biokar Diagnostic, Beauvais, France) with 20% glycerol at −80 °C. Each strain, subcultured twice for 24 h on two consecutive days at 20 °C in TSBYE, was cultured at 20 °C until reaching the stationary phase plus 25% of this time as previously described by Tresse, Lebret, Benezech, and Faille (2006). Constant pH of 5 (acidic pH) and 7 (neutral pH) were obtained by adding lactic acid (Sigma–Aldrich, Saint-Quentin-Fallavier, France) and 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (Sigma–Aldrich) to the culture medium as previously described by Tresse et al. (2006).

2.2. Biofilm assay using 96-well polystyrene microtiter plates

Biofilm assay were performed according the protocol developed by Djordjevic et al. (2002) to assess biofilm formation in MWB (modified Welshimer's broth) according to Premaratne, Lin, and Johnson (1991) and TSBYE (trypticase soy broth supplemented with yeast extract) media at pH 5 and 7 obtained as previously described by Tresse et al. (2006). Briefly, stationary phase grown cells were harvested by centrifugation at 3000 *g* for 20 min at 20 °C, washed and re-suspended in MWB or TSBYE depending on the experiments. A 300 µl of suspended cells diluted to an OD_{550} of 0.5 were loaded to each well of a sterile polystyrene-microtitre plate (Greiner bio-one, Courtaboeuf, France). After incubating for 24 h and 72 h at 20 °C in static conditions for pH 7 and pH 5, respectively, the suspended cells were removed and the attached cells were stained with crystal violet (Sigma–Aldrich). The crystal violet intensity was measured at a wavelength of 595 nm using the Multiskan Spectrum microtitre plate reader (Thermo Life Science, Cergy-Pontoise, France). The experiment was done in triplicate for three independent cultures. Differences using two-sided Student's *t*-test comparisons with a $P < 0.05$ were considered statistically significant.

2.3. Biofilm assay using culture chambers

The cell adhesion assays were performed in 8-well culture chamber Nunc Lab-TEK (Thermo Fisher Scientific, Brebières, France). Two different culture chambers were chosen for this study to test surfaces with different properties. Soda lime glass was chosen as a hydrophilic surface model and plastic (Permanox®) surface was selected as a hydrophobic surface. After cultivation, 300 µl of suspended cells diluted to an OD_{550} of 0.5 (approx. 6.45×10^8 CFU/ml) were transferred into 8 chamber slide wells per strain and incubated at 20 °C up to 6 days in static conditions. The medium was replaced each day by fresh sterile medium. Experiments were made in duplicate for each condition. A chamber with a fresh medium was used as a control. In this study, MWB and TSBYE were tested with two different pH (neutral pH and acidic pH 5) and two contact surfaces (glass and plastic). After incubation, culture media and unattached cells were removed followed by washing of the remaining attached cells five times with sterile milliQ water. Samples from the incubating chambers and unattached cells and culture medium were removed and attached cells were washed five times using sterile milliQ water.

2.4. Attached cells staining

After leaving the chambers under the fume hood for 45 min, each well was stained with 150 µl of 0.01% acridine orange (Sigma–Aldrich) for 15 min at room temperature under foil and kept in