RESPONSE OF *LISTERIA MONOCYTOGENES* BIOFILMS TO DESICCATION STRESS

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Abstract - *Listeria monocytogenes* is an important foodborne human pathogen associated with high mortality rates. Even if good manufacturing processes are followed, contamination can occur after processing making *Listeria* a major concern for food industry. As part of the EcoSec ANR program whose overall purpose is to reduce the environmental impact of hygiene operations in workshops producing chilled food, this work is more specifically focused on the adaptive response of *L. monocytogenes* to a desiccation stress. For that, first aim of this study was to propose a standard protocol optimized for the growth of cells in biofilm and the application of a desiccation stress that could be compared with the conditions encountered in the industry. Viabilty of bacteria measured with classical plate count method and confocal microscopy revealed clear viability differences between desiccated and control biofilm. Identification of intracellular and surface proteins involved in bacterial desiccation tolerance was investigated using different proteomic approaches.

Foodborne pathogen, sessile cells, viability, relative humidity, subproteome.

I. INTRODUCTION

Listeria monocytogenes is ubiquitous in the environment and of major concern for the food industry, since it is the causal agent of the serious foodborne illness *listeriosis*. Its entrance and persistence in food-processing premises is unavoidable even if cleaning and disinfection (C&D) are periodically applied [1]. Procedures of C&D require large amount of water, cleaning agents and biocides that are not eco-friendly. Reducing the available water by air drying after C&D to dry surfaces and control bacterial growth is already realized in several food industries. Persistent strains adhere to numerous materials commonly used in the food industry surfaces and form biofilms that are known to enhanced stress resistance [2]. *L. monocytogenes* is also able to survive desiccation for extended periods of time (up to 91 days) under conditions resembling the food processing environment [3]. Although the desiccation stress is omnipresent in the environment, less is known on the impact of anhydrobiosis on bacterial death, resistance or adaptation. This process of adaptation is mainly mediated by a striking combination of transcriptional regulatory networks, which allow bacteria to sense and convert extracellular stimuli into a specific cellular response, resulting in altered gene expression and enzyme activities. Considering intracellular proteins is necessary to understand mechanisms involved. In addition to cytoplasmic proteins, surface proteins are also known to play an important role. These proteins are surface-associated and constitute the so-called surfaceome of a bacterium [4]. This surfaceome represents a group of molecules involved in diverse important processes and are part of the interface between the bacterium and its environment. Important roles for surface proteins including bacterial growth, sensing of and resistance from environmental stresses, signaling and biofilm formation are documented. This study propose to develop a protocol of biofilm growth reproducing at best the conditions encountered in food industry and to apply a stress of desiccation subsequent to pre-adaptation of cells to low temperature used in food plants. Taking into account (i) the ability of *L. monocytogenes* to persist during desiccated conditions and (ii) the significant role of proteins in stress resistance, this prompts us to investigate the importance of intracellular and surface proteins in biofilm resistance during desiccation. To explore cells viability, characterize biofilm structure and monitoring evolution throughout the process, confocal laser scanning microscopy techniques have been implemented.

II. MATERIALS AND METHODS

Bacterial strain. The *L. monocytogenes* EGD-e strain, serogroup 1/2a was routinely pre-

culturing and culturing in Tryptic Soy Broth (TSB, Difco, Fisher Scientific) at 25°C and 150 rpm.

Biofilm formation. Precultured cells in stationary phase were used to inoculate cultures in order to obtain a final OD_{600} of 0.01 and strain was grown during 6 h. Cells were harvested by centrifugation (7500 × *g*, 15 min) and resuspended in TSB diluted 1:5 such that the final cell concentration was approximatively 8-9 log CFU/ml. Seven milliliters of the bacterial suspension was poured into a stainless steel (SS) disc (38.5 cm^2) and incubated at 25°C. The medium was replaced after 3 h and then every 24 h. After 24 h at 25°C, biofilms were pre-adapted to 10°C during 24 h.

Biofilm desiccation. Stainless steel supporting biofilms were placed for 3 and 24 h in a ventilated desiccation chamber where the RH (Relative Humidity) was stabilized with saturated NaCl solution to obtain an RH of 75%. During the desiccation experiments the RH and temperature were recorded using data logger (EL-USB-2-LCD, Lascar electronics, UK). Control and desiccation-stressed biofilms were detached in 10 ml of TS by scraping the SS discs with a sterile spoonbill. At each step of the experiment, cell enumeration was evaluated by plated serial dilutions on Tryptic Soy Agar (TSA, Difco, Fisher Scientific).

Proteins extraction. Sessile cells were harvested by centrifugation (7500 g, 45 min, 4°C) and washed twice with 1 ml Tris-EDTA (TE, pH7). The protein lysate of control and desiccated cells were obtained by mechanical disruption. Cells debris were removed by centrifugation (4500 g, 10 min, 4°C) and the supernatants were ultracentrifugated (200 000g, 30 min, 4°C) to separate intracellular proteins. The concentration of proteins was measured using the Bio-Rad Bradford protein assay. Intracellular proteins were analyzed by two-dimensional gel electrophoresis. Preparation of proteins surface was made using three protein extraction methods: trypsin-enzymatic shaving, powerful fractionation and biotin labeling of exposed proteins on the surface of intact cells. Peptides separation and identification was then performed by liquid chromatography tandem mass spectroscopy (LC-MS/MS).

Confocal Laser Scanning Microscopy (CLSM). The biofilm fluorescent labeling was performed at 25°C for 15 min with a combination of two dyes: SYTO 9 (3 µM) a green cell permeant nucleic acid marker and Propidium Iodide (20 µM) a red impermeant nucleic acid marker (live/dead viability kit, invitrogen). After biofilm staining, image acquisition was performed using a Leica TCS SP5 Confocal Laser Scanning Microscope (Leica-microsystem, France) at the ICCF microscopy platform (www.gred-clermont.fr).

III. RESULTS AND DISCUSSION

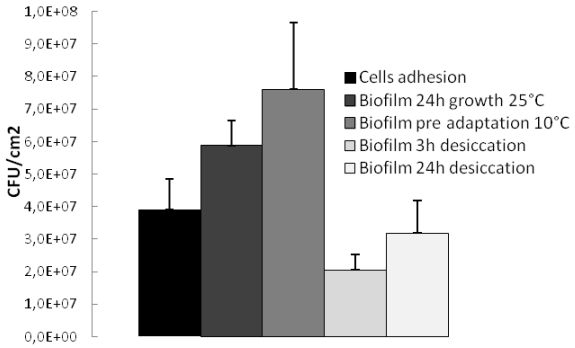
A large set of parameters have been tested and led to define a methodology for the study of the desiccation stress on *L. monocytogenes* biofilms. Among the experimental conditions tested, different media for pre-culture, culture, cells adhesion and biofilm formation have been experimented. The optimal final protocol to obtain desiccated and control biofilms is detailed in Table 1.

Table 1. Protocol of biofilm desiccation.

Pre-culture	Culture	Cells adhesion	Biofilm growth	Biofilm pre-adaptation	Stress desiccation
TSB 25°C		TSB 1/5 25°C		TSB 1/5 10°C	75% HR 10°C
14H	6H	3H	24H	24H	3H 24H

Results showed that the most favorable adhering and growing conditions for the biofilms on SS discs (data not shown) were obtained when planktonic cells were diluted in 1:5 TSB medium and allow to adhere during three hours at 25°C. Non-adherent cells were removed before adding new 1:5 TSB medium. Enumerations showed that 8 to 15% of the initial inoculum adheres on SS discs, corresponding to 7.4 log CFU/cm^2 (Figure 1).

Figure 1: Growth kinetics of *L. monocytogenes* biofilm on stainless steel and survival after 3 h and 24 h stress desiccation at 75% RH.



After 24 h of incubation at 25°C and 24 h of pre-adaptation at 10°C, the biofilms formed on the SS discs reached 7.9 log CFU/cm^2. Two exposure times to desiccation (3 and 24 h) were selected to observe respectively fast and slow changes taking place in the bacterial cells. Bacterial enumeration (Figure 1) showed that following desiccation to 75% RH for 3 h, survivors decreased by 70%. After 24 h exposition to 75% RH, bacterial population increases by a factor of 1.5 compared to a 3 h exposition. This would indicate that surviving bacteria are able to adapt to stress and multiply.
CLSM image acquisition (Figure 2) showed that cells were able to form three-dimensional structures after 24 h incubation to 25°C followed by 24 h incubation to 10°C (Control).

Figure 2: CLSM images of non-stressed (control) and desiccated-biofilms during 3 h and 24 h.

Biofilm covered entirely and heterogeneously the surface with an average thickness of 8 µm (data not shown) and biofilm architecture appeared not disturbed by desiccation under our experimental conditions. Within non-stressed biofilm, no damaged or dead cells were detected as stained by SYTO 9. On the contrary, after 3 h and 24 h exposition to 75% HR, a large proportion of damaged or dead cells were visible as stained in red. These observations were in accordance to cells enumeration even if the higher amount of non-viable cells enumerated after 24 h, by comparison with 3 h post-stress, was not obvious on CLSM images.
Proteomic analysis were performed by 2-DE with two pH gradients (IPG strips 3-10 and pH 4-7) in the first dimension. Comparison of intracellular proteins of *L. monocytogenes* control and desiccated-biofilm revealed that 46 protein spots displayed significant different protein spots level (data not shown). These 46 protein spots corresponded to 28 distinct proteins. Among these spots, 26 were down-expressed and 20 were appeared over-expressed. Interestingly, a protein similar to adhesion binding protein, named LpeA, was over-expressed after 3 h of desiccation while a protein similar to transcription elongation factor (GreA) was over-expressed after 24 h desiccation. The expression of GroEL molecular chaperone protein increased to both time of exposure. Table 2 summerized the differentially expressed proteins identified by mass spectrometry. The analysis of the surfaceome subproteome is underway by using a «label-free» LC-MS/MS approach.

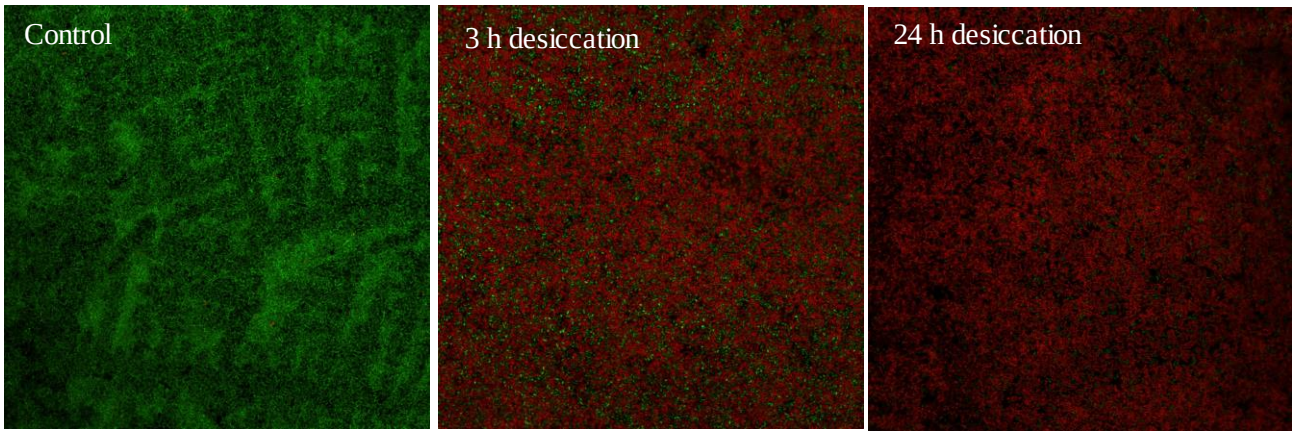


Table 2: Some of the 28 intracellular proteins differentially expressed during a stress of desiccation on *L. monocytogenes* biofilm.

Fonction of identified protein	**Protein**
Metabolism	Pyruvate dehydrogenase, phosphohoglycerate mutase, dihydrodipicolinate reductase, adenylate kinases, fructose-1,6-bisphosphate aldolase, similar to phosphotransferase system (PTS) beta-glucoside-specific enzyme IIB component, similar to lipases,
Detoxification	Superoxide dismutase
Chaperone/Protease/Stress proteins	ATP-dependent Clp protease proteolytic subunit, GroEL, ferritin
Transcription	Transcription elongation factor
Traduction	Ribosomal protein L10

IV. CONCLUSION

This work allowed to standardize a protocol to form biofilms on SS surfaces and applied desiccation stresses in conditions mimicking the food plants environments. This protocol was implemented to assess the viability of cells in stresses conditions and to explore the molecular mechanisms that allow adaptation of sessile cells to desiccation. In our experimental conditions, the viability of 70% of the cells was affected, which means that a significant part of the biofilm population is able to resist and to adapt to 75% RH. The proteomic approach revealed variation in expression for 28 intracellular proteins. The analysis of the surfaceome is underway to evaluate the effect of desiccation on the cell surface protein content. This work will lead to a better understanding of *L. monocytogenes* biofilms cellular and molecular responses to hydric stress. This focus on stress response mechanisms is important to help food industry to fight against *L. monocytogenes*.

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