Exploring the diversity of *Listeria monocytogenes* biofilm architecture by high-throughput confocal laser scanning microscopy: predominance of honey-comb-like morphotype

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Running title: *Listeria monocytogenes* biofilm architecture
ABSTRACT

Listeria monocytogenes is involved in foodborne illness with a high mortality rate. Persistence of this pathogen along the food chain can be associated with its ability to form biofilms on inert surfaces. While most of the phenotypes associated with biofilms are related to their spatial organization, most published data comparing biofilm formation of L. monocytogenes isolates are based on the quantitative crystal violet assay that does not give access to structural information. Using a high-throughput confocal imaging approach, the aim of this work was to decipher the structural diversity of biofilms formed by 96 L. monocytogenes strains isolated from various environments. Prior large-scale analysis, an experimental design was performed to improve L. monocytogenes biofilm formation in microscopic grade microplates, with special emphasis on the growth media composition. Microscopic analysis of biofilms formed in the selected condition by the 96 isolates revealed only weak correlation between the genetic lineages of the isolates and the structural properties of the biofilms. However, a gradient in their geometric descriptors (biovolume, mean thickness, roughness), ranging from flat multilayer to complex honey-comb-like structures was shown. The dominant honey-comb-like morphotype was characterized by hollow voids hosting free-swimming cells and localized pockets containing mixtures of dead cells and eDNA.
INTRODUCTION

Listeria monocytogenes still represents an important risk for public health; 1740 listeriosis cases were reported in the EU in 2011 with a mortality rate of 12.7% (1). Listeriosis is particularly dangerous for pregnant women, elderly or immunocompromised people. Persistence of L. monocytogenes strains on food plant surfaces can occur due to maladapted design of equipment and biofilm formation (2, 3). L. monocytogenes is able to attach and colonize various surfaces such as stainless steel, glass, polystyrene, and to contaminate food products during the processing (4-6). Biofilms of L. monocytogenes were associated with important ecological advantages such as a protection against biocide action (7). Several molecular determinants such as flagella, biofilm-associated proteins (Bap), SecA2 or cell-cell communication systems have been shown involved in biofilm construction within this species (8, 9). While no exopolysaccharidic components have been evidenced in L. monocytogenes biofilm matrix (8), extracellular DNA (eDNA) has been shown to participate to cellular initial adhesion and biofilm organization under specific growth conditions (10). Biofilm formation of this species is highly dependent on environmental conditions such as variations of temperature, pH and nutrients (11, 12). L. monocytogenes is structured into four major phylogenetic lineages, each of which is genetically heterogeneous and sub-structured into highly recognizable clonal complexes as defined by MLST (13, 14). Attempts to relate biofilm formation to strain origin, lineage or persistence status led to contradictory results. Currently, the association of biotype structure with lineages or clonal complexes of L. monocytogenes is unknown.

Limited data is available on the intra-specific diversity of the architecture of L. monocytogenes biofilms. Indeed, most published reports focusing on the biofilm formation of several strains are based on global quantitative measurements (15-19).
The only few studies focusing on the structure of *L. monocytogenes* biofilm showed a variety of architectures including monolayer of adherent cells, flat unstructured multilayers, knitted-chains network depending on the strains and experimental setup used (5, 9, 19-22). Early characterization by scanning electron microscopy (SEM) evidenced multilayers and honeycomb-like organizational structures of *L. monocytogenes* biofilms (21). However, this ultra-structural technique is time consuming and involves drastic artefactual preparation steps like chemical fixation and dehydration that can alter native spatial organization. So far, reports on the investigation of the three-dimensional structures of *L. monocytogenes* biofilms by confocal laser scanning microscopy (CLSM) are scarce. The coupling of CLSM with flow-cell devices has highlighted the formation of a complex structure by the EGD-e strain composed of ball-shaped microcolonies surrounded by a network of knitted chains (22).

Recently, a high throughput method based on CLSM combined with the use of 96-wells microtiter plates was successfully applied in our lab to explore the biofilm architecture of 60 pathogens (23). In this contribution, we selected culture conditions adapted to the growth of static *L. monocytogenes* biofilms and we deciphered the diversity of the architecture of the biofilms formed by a selection of 96 *L. monocytogenes* strains collected from diverse origins (food, animal, human and soil).
MATERIALS AND METHODS

Bacterial strains

The 96 L. monocytogenes isolates used in this study were selected according to their diverse origins and are listed in Table S1. The collection named ListRA (Listeria monocytogenes Reference collection A) is constituted of 37 human isolates (13 from healthy human carriage and 24 from patients), 8 strains isolated from animal, 40 from the food industry and 11 from soil samples. L. monocytogenes 10403S WT and its isogenic ΔflaA (HEL-304) mutant (24) were used to evaluate the role of flagella in biofilm architecture. For real time confocal observation, autofluorescent variants (25) harboring the pNF8 plasmid encoding GFPmut1 (26) or pJEBAN6 encoding DsRedExpress (27) were used. All strains were stored at -80°C in TSB (Tryptone Soya Broth, OXOID, France) containing glycerol 20% (v/v).

L. monocytogenes biofilm formation in microscopic grade microplates

Different factors including medium dilution, glucose supplementation and buffer solution addition were analysed to select growth conditions allowing L. monocytogenes static biofilm formation in microscopic grade microplates. As nutrient concentration is a critical parameter for L. monocytogenes biofilm formation (28), nutrient-rich or nutrient-poor media were tested using respectively TSB and ten time diluted TSB. Glucose supplementation was also tested as it has been shown previously to increase biofilm biomass (29). In order to avoid acidification of the medium, buffering with MOPS (3-(N-morpholino)propanesulfonic acid pH 7.4) was also tested. For all conditions, frozen stocks of the EGDe strain were subcultured twice in TSB at 25°C under vigorous orbital shaking (180 rpm). Subcultures were diluted to approximately 5×10⁶ CFU/mL in the medium used for the growth analysis prepared by combinations of three factors with two levels: dilution of TSB (1 or 1:10), addition of a buffered solution of MOPS (0 or 0.1M) and glucose supplementation 0 or 1% (wt/vol). 250 μL of these suspensions were used to inoculate the wells of a polystyrene 96-well microtiter plate with a μclear® base (Greiner Bio-one, France). After 1h of adhesion at 25°C,
supernatants containing non-adherent cells were removed and wells were refilled with 250 μL of the medium. Biofilms were analysed after 48h of static incubation at 25°C. A complete factorial design $2^4$ was constructed and analysed with the dedicated module of Statgraphics to identify the combination of factors enabling the best biofilm formation within the tested conditions.

Confocal Laser Scanning Microscopy (CLSM)
The biofilm fluorescent labelling was performed at 25°C for 20 min with a combination of two dyes: SYTO 9 (3 μM), a green cell permeant nucleic acid marker, and propidium iodide (20μM), a red impermeant nucleic acid marker (live/dead viability kit from Molecular probe). After biofilm staining, image acquisition was performed using a Leica SP2 AOBS Confocal Laser Scanning Microscope (LEICA, LEICA-Microsystems, France) at the MIMA2 microscopy platform (www6.jouy.inra.fr/mima2). All biofilms were scanned at 800 Hz using a ×63 oil immersion objective lens with a 488 nm argon laser set at 25% intensity. Emitted fluorescence was recorded within the range 500-600 nm to collect SYTO 9 emission fluorescence and 610-710 nm to collect propidium iodide emitted fluorescence. Two stacks of horizontal plane images with a z-step of 1 μm were acquired per well. Assays were all repeated on a different day from independent cultures (4 image series for each strain). Three-dimensional projections of the biofilms were constructed from the CLSM acquisitions using the easy 3D function of the IMARIS 7.1 software (Bitplane, Switzerland). Trajectories of motile bacteria from time-series acquisition were analysed with the IMARIS tracking function. Quantitative structural parameters (biovolume, thickness, roughness) were extracted from confocal image series with PHLIP (30), a freely available Matlab-based image analysis toolbox (http://sourceforge.net/projects/phlip/).

Scanning electron microscopy (SEM)
L. monocytogenes biofilms were prepared for scanning electron microscopy by immersing glass coupons in the wells of a 24-well polystyrene plate with $10^7$ CFU. After a 1h-adhesion, supernatant containing non-adherent cells was removed and wells were refilled with 1 mL of TSB supplemented with 1% (wt/vol) glucose and MOPS 0.1M pH 7.4. After 48h of static incubation at 25°C, biofilms were fixed 24h at 4°C in a solution containing 2.5% glutaraldehyde, 0.1 M sodium cacodylate (pH 7.4). Then, coupons were positioned into a new plate and washed three times for 10 min with a solution containing 0.1M sodium cacodylate. After a transfer into 50% ethanol, samples were progressively dehydrated by passage through a graded series of ethanol solutions from 50% to 100%. The samples were then critical-point dehydrated (Emitech K850, UK) using carbon dioxide as the transition fluid and finally coated with gold-palladium in an automatic sputter coater (Polaron SC7640, UK). The samples were observed with scanning electron microscope FE-SEM Hitachi S4500 (Hitachi, Tokyo, Japan).

**Genotyping of the ListRA isolates**

In order to detect potential correlation between genotype and biofilm architecture, genotyping was achieved for the 96 isolates by sequencing two housekeeping genes, *cat* and *dapE*. (13). These two genes were selected because their allele variation is strongly associated with major lineages and clonal complexes (14). The combination of *cat* and *dapE* alleles was used to deduce the clonal complex of the isolates, based on knowledge of allelic variation of these two genes (www.pasteur.fr/mlst).

**Statistical analysis**

All statistical analysis (experimental design, discriminant analysis and ANOVA variance analyses) were performed using Statgraphics v16.1 software (Manugistics, Rockville, USA).
RESULTS

All 96 *L. monocytogenes* strains from the ListRA collection were characterised by sequencing of MLST genes *cat* and *dapE* (Table S1). 36 isolates were grouped in lineage I, 59 isolates in lineage II and one strain in lineage III. 18 different clusters were distinguished, and their correspondence with previously described clonal complexes (14) was established (Table S1).

Using TSB as a basis of growth medium, a complete 2\(^4\) factorial design was applied to determine the influence of three parameters on the EGD-e strain biofilms formation as evaluated by the biofilm biovolume extracted from CLSM images, namely (i) medium dilution, (ii) glucose supplementation and (iii) buffer concentration. Maximum biofilm biovolumes were obtained when glucose and MOPS were simultaneously added to undiluted TSB (Fig. 1).

**Structural diversity of the *L. monocytogenes* biofilms**

The 96 *L. monocytogenes* isolates were investigated for static biofilm formation with the selected growth protocol (TSB supplemented with glucose 1% and MOPS 0.1 M.). CLSM image acquisition showed that all strains were able to form three-dimensional structures after 48h of incubation (Fig. 2). Biofilm architecture ranged from flat homogeneous layer of cells to honey-comb-like structure. Within the full data set, diversity was particularly evident when considering biofilm roughness and biovolume (Fig. 3). Statistical analysis showed a positive correlation between biofilm thickness and their biovolumes (*P*<0.05), that are both anti-correlated with biofilm roughness (*P*<0.05). Of note, two of the collection strains (CIP 103574 and CIP 104794) exhibited the highest biofilm thickness and biovolume, whereas the two non-motile strains (CIP82110 and H6) formed flat multilayers structures. A discriminant analysis was used to classify strains in the MLST clonal complexes using the biofilm structural parameters (thickness, roughness and biovolume) (Fig. S1). The developed discriminant function significantly improved correct classification of the strains in the right
clonal complex from 5.6% (at random) to 25.0% ($P<0.05$). However no discriminant function helped predict origin of the strains using the biofilm structural parameters ($P>0.05$).

**Flat multilayer biofilms**

Within the collection, only the two non-motile strains (CIP82110 and H6) formed flat biofilms characterized by low roughness and relatively high biovolume. The iso-surface representation and the section view of a representative strain for this type of architecture (CIP 82110) showed dense and homogeneous biofilm with scattered damaged or dead cells as stained by propidium iodide (Fig. 4A and 4B). In accordance with the non-motile phenotype determined using soft agar assay (Fig. 4C), no visible flagella were observed with SEM on the bacteria forming these biofilms (Fig. 4D).

**Honey-comb-like biofilms**

The vast majority of the tested strains formed complex honey-comb-like architecture decorated with hollow voids. Structures with the largest hollow voids generally presented the highest roughness and the smallest bacterial biovolume. *L. monocytogenes* H25 is a telling example of strains forming a honey-comb-like biofilm (Fig. 5 and supplementary movies S1 and S2). Large holes or channels were scattered in the biofilm as seen in the 3D reconstruction (Fig. 5A) and the section view (Fig. 5B). To investigate whether those “hollow voids” were filled with unseen materials, 2 μm latex green fluorescent beads were deposited at the top of the biofilms stained in red with SYTO 61 and their sedimentation was followed in time. After less than 10 minutes, beads were observed at the bottom of the voids showing the absence of a compact matrix in the voids (Fig. 5C). Staining the biofilm with propidium iodide showed the presence of red pockets of materials likely formed by a mixture of dead cells and eDNA (Fig 5A and movie S1). Direct time-series observations showed the presence of swimming bacteria in the hollow voids, with an average speed of 2.3 μm/s (Movie S2).
Trajectories of swimming bacteria (Fig. 5D) showed that motile cells were almost exclusively located in those holes. A low-agar swimming test confirmed the high motility of this strain (Fig. 5E). SEM observations of this honey-comb-like biofilm showed many filamentous materials between the cells (Fig. 4F). Those filaments were presumed to be flagella as they were also observed in the biofilms formed by the strain 10403S WT, but not in the biofilms formed by its isogenic flagella deficient mutant 10403S flaA (Fig 6). Biofilms formed by the non-motile mutant exhibited a flat unstructured architecture compared to the honey-comb-like biofilms formed by the motile WT strain (Fig. 6), suggesting a role of flagella in the honey-comb-like architecture. Taking advantage of a *L. monocytogenes* 10403S mutant expressing GFP, dynamic of honey-comb-like formation was continuously visualized for 48h by real-time CLSM (Movie S3). Over-time, surface-associated bacteria expanded as clusters, while the number of planktonic motile cells decreased until 48h, when motile cells became undetectable in the bulk.
DISCUSSION

The objective of this study was to evaluate the biofilm structural diversity within the \textit{L. monocytogenes} species. To this aim, a medium for static biofilm growth in microscopic grade microplates was selected from a $2^4$ experimental design. Using this medium (TSB supplemented with glucose and MOPS), the biofilm architecture of the 96 isolates of the ListRA collection was analyzed by CLSM.

Various 3D structures of static \textit{L. monocytogenes} biofilm have been reported: unorganized architectures with multicellular layers or aggregates (17, 22), clustered biofilms (31) and 3D network of cells (19, 21). However, differences in the protocols and data analyses used render comparisons between reports impossible. Here, with the same protocol, all strains were able to form 3D structures. Architectures formed by the 96 isolates were quite diverse as highlighted with the quantitative biofilm roughness and biovolume parameters. This intra-species diversity in the ability to form biofilms is in accordance with previous works where quantitative methods were used to evaluate biofilm formation (19, 32, 33). The 3D reconstruction of CLSM images showed that the majority of strains formed honey-comb-like structures consisting in layers of cohesive cells decorated with hollow voids with diameters ranging from 5 to 50 $\mu$m. This type of spatial organization was described only once for biofilms of \textit{L. monocytogenes} (21). Honey-comb-like biofilm architectures were previously described for other species including \textit{Staphylococcus epidermidis} (34) and \textit{Staphylococcus aureus} (23). Surprisingly, hollow voids were maintained up to 72h and were not colonized by sessile bacteria. Time-course observations of 48h-honey-comb-like biofilms showed progressive invasion of the surface by the bacteria resulting from cell multiplication. These hollow voids likely originate both from privileged colonization locations and localized cell death as previously described for \textit{Pseudomonas aeruginosa} (35). Bacteria in movements were visible in the hollow voids. The high average speed of those motile cells (2.3 $\mu$m/s) excluded
Brownian motion as the driving force for this subpopulation. Coexistence of sessile and motile cells on the surface has been described previously in different species and was often associated with active biofilm dispersal phenomena (36-38).

The observation of the presence of eDNA in the matrix is in accordance with previously published reports about the key role of DNA for *L. monocytogenes* cell adhesion and biofilm structuration (10, 33). The origin of the eDNA composing *L. monocytogenes* matrix remains unclear but could involve cellular lysis or the release of small vesicles as for other species (39). DNA pockets observed in the case of honey-comb-like structures are similar to previously described localized cell death resulting from quorum sensing driven processes (40). This phenomenon can provide nutrients for the starved surviving subpopulation. The release of eDNA can also influence the spatial organization of the biofilm and contributes to the stability of its structure. The observation of bacteriophages associated to sessile cells using SEM, and in biofilm supernatants using Transmission Electron Microscopy (TEM) (Fig. S2) suggests that localized cell death could result from prophages activity as already observed with some strains of *P. aeruginosa* (41).

No polysaccharides could be detected in the honey-comb-like biofilms when tested with two different fluorescent lectins (WGA and Concanavalin A). SEM observations showed the presence of extracellular fibrils in the honey-comb-like structures. Those filaments are likely flagella as they were not observed in a biofilm formed by the non-motile CIP82110 strain, neither with the 10403S mutant defective in flagella production. Previous studies described that *L. monocytogenes* strains affected for motility displayed a reduced capacity to form static biofilms (42, 43). Under our specific experimental conditions, lack of flagella did not impede biofilm formation, but resulted in a flat unstructured architecture. These results suggested that flagella play a structural role in the complex architecture of *L. monocytogenes* honey-comb-like biofilms.
Genotyping of the 96 isolates from the ListRA collection revealed that most isolates were grouped into lineage I and II (only 1 strain from lineage III). Indeed, lineage III strains are scarce and poorly represented in collections (19, 28, 44). Previous work showed variable capacity of the strains to form biofilms depending on their lineage (28). Some works showed that lineage I strains produced more biofilms compared to lineage II strains (15, 16) and others reached the opposite conclusion (17, 19). In our study, a weak correlation was detected between the strain lineage and the structure of the biofilms. In accordance with a recent work (33), we observed no correlation between strain origin and the structure of the biofilms formed (Table S1).

All together, we have shown in this report that most of the *L. monocytogenes* isolates form spatially structured honey-comb-like biofilm morphotype in static condition. This morphotype involves both eDNA and flagella as structural components. The link between this biofilm architecture and the persistence of *L. monocytogenes* on surfaces remains to be elucidated.

**ACKNOWLEDGMENTS**

We thank Thierry Meylheuc and Christine Longin for electron microscopy observations (INRA MIMA2 imaging center). The authors are grateful to Jens Bo Andersen and Tine Rask Licht (Technical University of Denmark, Soeborg) for kindly providing multiple fluorescence labelling system in *L. monocytogenes* as well as Hélène Marquis (Cornell University, USA) for the nonflagellated mutant strain and Abdelkader Boubetra (Institut Scientifique d’Hygiène et d’Analyse) for *Listeria monocytogenes* food strains. The “Essonne department” contributed to the acquisition of the confocal microscope. This work was supported by Institut National de la Recherche Agronomique funding.
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FIG 1: Biovolumes of *L. monocytogenes* EGD-e biofilms depending on their growth conditions. Biofilms were grown for 48h at 25°C. Media used are described on x axis (TSB10: ten times dilution of TSB; glu 1%: addition of 10 g/L of glucose; MOPS: addition of 3-(N-morpholino)propanesulfonic acid 0.1 M, pH 7.4). The error bars indicate the standard error from four experiments. * Optimal combination as determined by the experimental design.

FIG 2: IMARIS easy 3D projections from CLSM images of the biofilms form by the 96 isolates of the ListRA collection showing the predominance of the honey-comb-like morphotype. Biofilms were labelled in green with SYTO 9 and in red with propidium iodide. All biofilms were grown at 25°C using the selected media: TSB supplemented with glucose 1% and MOPS 0.1M.

FIG 3: Correlation between the biofilms structural parameters of the ListRA collection. Distribution of the mean thickness (A) and roughness (B) as a function of the biovolume and distribution of roughness as a function of the mean thickness (C) for the 96 isolates. All values were extracted from CLSM images with the PHLIP Matlab routine and were averaged from 4 values for each case from 2 independent sets of experiments.

FIG 4: CIP82110 strain as a representative of non-motile strains forming flat biofilms. Imaris iso-surface representation (A) and section view (B) of the CLSM images of the biofilms stained with SYTO 9 and propidium iodide. White scale bars represent 20 μm. SEM observation of the biofilm formed with a $2 \times 10^4$ magnification (C). 24h swimming plate (TSB+0.25% agar) of the non-motile strain CIP82110 compared to the swimming phenotype of the H25 strain represented by the white dashed line circle (D).
FIG 5: H25 strain as a representative of motile strains forming biofilms with honey-combs morphotype. Imaris iso-surface representation (A) and section view (B) of CLSM Images from biofilms forming honey-comb-like structure stained in green with SYTO 9 and in red with propidium iodide. Imaris 3D projection of the same biofilm stained with the red SYTO 61 in presence of green fluorescent 2 μm fluorescent latex microbeads (C). Tracking of motile bacteria in the hollow voids, white arrows focus the cell trajectories (D). 24h swimming plate (TSB+0.25% agar) of the strain H25 (E). SEM image with a $2 \times 10^4$ magnification (F). White scale bars represent 30μm.

FIG 6: Microscopic observations of the biofilms formed by the motile L. monocytogenes 10403S WT strain and its isogenic non-motile 10403S flaA mutant. (A) Iso-surface representation obtained from the confocal image series using the IMARIS software (green SYTO 9 staining). (B) SEM image with a $1.5 \times 10^4$ magnification. White scale bars correspond to 30 μm and yellow bars to 2 μm.