Teichoic acid is the major polysaccharide present in the Listeria monocytogenes biofilm matrix

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One sentence summary: This original article described for the first time the nature of the major carbohydrate present in the extracellular matrix of Listeria monocytogenes biofilm.

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ABSTRACT

The aim of this study was to characterize the Listeria monocytogenes biofilm and particularly the nature of the carbohydrates in the biofilm extracellular matrix and culture supernatant versus to cell wall carbohydrates. Listeria monocytogenes serotype 1/2a and 4b strains were able to form complex biofilms embedded in an extracellular matrix. The soluble carbohydrates from biofilm extracellular matrix and culture supernatant were identified as teichoic acids, structurally identical to cell wall teichoic acids. In addition, the DSS 1130 BFA2 strain had a serotype 1/2a teichoic acid lacking N-acetyl glucosamine glycosylation due to a mutation in the lmo2550 gene. Consequently, we hypothesized that the extracellular teichoic acids in L. monocytogenes biofilms have the same origin as cell wall teichoic acid.

Keywords: Listeria; biofilm; carbohydrate structure; teichoic acid; glycosyltransferase; sequencing

INTRODUCTION

Listeria monocytogenes is a Gram-positive ubiquitous bacterium and foodborne pathogen. This bacteria causes listeriosis, which ranges from febrile gastroenteritis to more severe life-threatening invasive diseases, in particular among at-risk population such as immunocompromised individuals, elderly people and pregnant women. Listeria monocytogenes strains differ in their epidemic potential and in their ability to cause disease. For example, among the 13 serotypes, serotype 4b strains caused the majority of human listeriosis outbreaks worldwide while most of L. monocytogenes isolates recovered from foods belong to the serotypes 1/2a and 1/2b. The food contamination may result from cross-contamination with surfaces contaminated with L. monocytogenes biofilms. Theses biofilms are communities of microorganisms adhering to a surface and generally surrounded by an extracellular (EC) matrix capable of functioning as both structural scaffold and protective barrier (Sutherland 2001; Häussler and Parsek 2010). The biofilm EC matrix consists of a hydrated gel with a complex mixture of polysaccharides, proteins, DNA and other polymeric substances. Polysaccharides are a crucial component of the EC matrix of numerous bacterial biofilms. They carry out a range of functions including promoting attachment to surfaces and other cells, building and maintaining biofilm structure, as well as protecting the cells from antimicrobials (Stewart and Costerton 2001).
More than 30 different polysaccharidic components of biofilms (Gram positive and Gram negative) have been characterized (Flemming and Wingender 2010). Well-known examples include poly-N-acetylglucosamine (PNAG) produced by Staphylococcus epidermidis, S. aureus and Escherichia coli (Mack et al. 1996; Wang, Preston and Romeo 2004; O’Gara 2007). Furthermore, many species secrete several exopolysaccharides: Pseudomonas aeruginosa, for example, was reported to produce at least three EC polysaccharides that can be important in biofilm development (Ryder, Byrd and Wozniak 2007). For L. monocytogenes, no studies have characterized the composition or the structure of the exopolysaccharides from biofilm EC matrix. Several earlier studies using mainly various colorimetric techniques demonstrated the presence of EC carbohydrates (Borucki et al. 2003; Zameer et al. 2010). For Deng et al. (2010), none of the genes known to be involved in the biosynthesis and transport of exopolysaccharides specific for the biofilm matrix were detected in the sequenced genomes of 26 L. monocytogenes strains representing the three lineages. This wasn’t in agreement with the recent findings of Chen et al. (2014) and Koseoglu et al. (2015) which described the c-di-GMP induced exopolysaccharide that was synthesized by the pssA-E operon in L. monocytogenes. To date, there is no data on chemical identity of EC polysaccharides of L. monocytogenes wild-type strains, and in particular of biofilm-associated polysaccharides. In order to address this question, carbohydrates from the 48 h-biofilm EC matrix and culture supernatant, as well as cell wall carbohydrates from strains of L. monocytogenes, were isolated, purified and identified by GC-MS and 13C and 1H nuclear magnetic resonance (NMR) spectroscopy. For that, we selected six strains previously shown to produce relatively high level of 48 h biofilms (Combrouse et al. 2013); the presence of EC matrix in 48 h biofilms on polysyntyne surfaces was confirmed by scanning electron microscopy (SEM).

MATERIALS AND METHODS

Bacterial strains and culture conditions

Six strains of L. monocytogenes were studied: Scott A and LmA (serotype 4b) (Carpentier and Chassaing 2004; Tresse et al. 2007) and DPF 234 HG2, CL 297 AS1, DSS 1130 BFA2 and EGD-e (serotype 1/2a) (Glaser et al. 2001; Midelet-Bourdin, Leleu and Malle 2007). Cultures were prepared into 5 mL of Tryptone Soya Broth Yeast extract medium (TSBYe, Oxoid, Dardilly, France) at 37 °C for 24 h then were transferred into 35 mL of TSBYe at 37 °C for 24 h.

Biofilm analysis

The polystyrene Petri dish (Grosseron, Saint Herblain, France) was filled with 10 mL of inoculum at 10⁶ CFU mL⁻¹ in the MCDB 202 medium (CryoBioSystem, L’Aigle, France) (Hébraud and Guzzo 2000) and incubated statically at 37 °C for 48 h. The growth medium was then removed and the contaminated surfaces were rinsed three times with 5 mL of sterile distilled water. The biofilms on the entire Petri dish surface were detached by thoroughly swabbing of the surface with a moistened swab, and was re-suspended in 1 mL of sterile distilled water by vigorous vortexing for 20 s. The number of detached CFUs was counted on Tryptone Soya Yeast agar (TSAYe), incubated at 37 °C during 24 h. The 48 h-biofilm architecture was observed by epifluorescence microscopy (Imager.Z1, Zeiss, Marly-le-Roi, France) connected to a CCD camera (Axiocam – MRm, Zeiss) after staining by a 5 μg mL⁻¹ solution of 4’, 6 diamidino-2-phenyl-indole (DAPI, Sigma-Aldrich, Saint-Quentin Fallavier, France). Measurements of biofilm coverage were carried out using ImageJ 1.22d. The biofilm structures were observed by SEM (Joel, JSR-35 CF, Japan) after fixing during 20 min in glutaraldehyde 3% (v/v) 0.02 M cacodylate buffer, dehydrating in a series of ethanol bath of 70% (v/v), 80% (v/v), 90% (v/v) and 100% (v/v) for 10 min each, and coating with gold-palladium for 1.5 min.

Preparation of cell wall, EM matrix and culture supernatant soluble carbohydrate from 48 h biofilm

Culture supernatant of 48 h biofilm was collected in a sterilized vial. The biofilm was washed three times with 5 mL of sterilized distilled water, and the water from the washing was added to the vial. Biofilms on the entire Petri dish were scraping by the finger with a sterile glove and harvested with 4 mL of sterilized distilled water in a Falcon tube (Combrouse et al. 2013). The biofilm EM matrix was detached from the bacterial cells by sonication on ice (IKA Labotecniksonicator, Staufen, Germany) for 3 × 30 s, 50% cycle, at a 0.5 intensity as previously described by Sadovskaya et al. (2005). Cells were removed by centrifugation at room temperature (10 000 g, 10 min) and kept for extraction of cell wall carbohydrates. The biofilm EC matrix was deproteinated by addition of trichloroacetic acid (TCA) at a final concentration of 5%, dialyzed (Visking dialysis tubing; 12–14 kDa cutoff, Dutscher, Brumath, France) and lyophilized. The residue was taken in 2 mL of water and additionally deproteinated by extraction with phenol (0.7 mL) and chloroform (0.5 mL), and then fractionated on a Sephadex G-50 column (1 × 40 cm), eluted with acetic acid (0.1%) (Sadovskaya et al. 2004). Fractions were screened for the presence of neutral sugars (Dubos et al. 1956). High molecular weight carbohydrate fractions (-10 kDa, void volume) were pooled and lyophilized to give EM matrix carbohydrates. Culture supernatant was clarified by centrifugation (6000 g, 10 min), and the cell pellet was kept for extraction of cell wall carbohydrate. The supernatant was deproteinated, dialyzed, lyophilized, treated with phenol and chloroform as described above and fractionated on a Sephadex G-50 column. Cell pellets were resuspended in 5% TCA and stirred for 48 h at 4 °C. Cell debris was removed by centrifugation (10 000 g, 10 min), the supernatant was dialyzed and lyophilized. Cell wall carbohydrates were further purified on Sephadex G-50 column.

Structural elucidation of carbohydrates

Monosaccharides were detected as reduced and acetylated derivatives (alditol acetates) by GC-MS performed on a Trace GC ULTRA system (Thermo Scientific, Villebon sur Yvette, France) equipped with a capillary column NMR-5MS (30 m × 0.25 mm) using a temperature gradient of 170 °C (3 min) → 250 °C at 5 °C min⁻¹ and with a DSQ II MS detector. NMR experiments were carried out on Bruker AVANCE® III 900 MHz spectrometer equipped with a 3 mm gradient probe (Centre Commun de Mesures RMN de l’Université Lille 1, Lille, France). All experiments were recorded for 2–5 mg of polysaccharides in 0.5 mL of D2O at 25 °C with acetone internal reference (2.225 ppm for ²H) and 31.45 ppm for ¹³C) using standard pulse sequences COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 400 ms) and ¹³C HSQC (¹H) was taken at 142.8 Hz). AQ time was kept at 0.8–1 s for H–H correlations and 0.13 s for HSQC, 256 increments were acquired for t1. Assignment of spectra was performed using Topspin 2 (Bruker Biospin®) program for spectra visualization and overlap.
**Sequencing lmo2549 and lmo2550 genes of serotype 1/2a L. monocytogenes strains**

We studied two genes, lmo2549 and lmo2550, involved in GlcNAc ramification of teichoic acid from L. monocytogenes serotype 1/2a (Eugster et al. 2011). Strains were grown on TSAYe during 24 h at 37°C. Colonies were removed with an inoculating loop and placed in the PCR mix. The PCR reactions were performed in a final volume of 40 μL. Each reaction mixture contained 1 μL of 10 mM DNTP (Sigma-Aldrich), 0.3 μL of Taq DNA Polymerase (Taq DNA Pol 5 U μL⁻¹, MP Biomedical), Santa Ana, California, USA), 5 μL of 10 x PCR buffer (10X Incubation T:pol Mix with MgCl₂, MP Biomedical), 32.7 μL of sterilized distilled water, 0.5 μL of 500 nM primer F and 0.5 μL of 500 nM primer R. A pair of primers for lmo2549 gene was used: lmo2549F (5′-GGG AAT GGA GTC ATT TGG TT-3′) and lmo2549R (5′-TGC CGT CAT CTT CCC ATT TA-3′). Four primers for the lmo2550 gene were used: lmo2550F (5′-CTT AAT TTT ATG TAC TAC AAG AGG A-3′), lmo2550R (5′-GTA TAC CAC GGA ATC TTG TC-3′), lmo2550F2 (5′-CTG GTT AAA GAA AGC AAC TTC A-3′) and lmo2550R2 (5′-TCT ACG CAT CTT CTA TCGA G-3′). The lmo2550F and lmo2550R primers were used in PCR and were performed in an iCycler thermocycler (Biorad, Marnes La Coquette, France) under the following conditions: preincubation at 94°C for 3 min, 35 cycles of repetition with a phase of DNA denaturation at 93°C for 45 s, a primer annealing phase at 54°C for 45 s and an elongation step at 72°C for 1 min, and finally a final elongation at 72°C for 10 min. PCR products were sequenced by the Genoscreen private company (Lille, France) using the amplification primers for lmo2449 and the amplification primers added of lmo2550F2 and lmo2550R2 for the lmo2550 gene. Alignments of nucleic acid and amine acid sequences were performed by using Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

**Statistical analysis**

All the experiments on biofilm were replicated three times. A variance analysis was used to determine the significant difference between strain populations. All calculations were performed with Statgraphics centurion XVI (Sigma plus, Paris, France). Statistical significance was evaluated at P < 0.05.

**RESULTS**

All strains formed heterogeneous biofilms

The ability to form biofilm of the six strains tested was first confirmed, while significant differences (P < 0.05) in the contamination level were observed between strains (Fig. 1). In the experimental conditions used in this study, the number of CFUs in the biofilm ranged from 5.07 (CL297 AS1 strain) to 7.45 log CFU cm⁻² (Scott A strain). Significant differences were also observed between strains from the same serotype, between 6.09 and 7.45 log CFU cm⁻² for the serotype 4b and between 5.07 and 6.67 log CFU cm⁻² for the serotype 1/2a. Conversely, with the difference in the CFUs numbers, all strains exhibited similar biofilm architectures (Figs 2 and S1, Supporting Information). The bacteria were not distributed homogenously on the surface. Some areas were devoid of bacteria, while others were covered with a more or less dense bacterial lawn composed of isolated bacteria. Cell aggregates with a three-dimensional structure were also observed on the different views and the only significant difference observed between strains concerned their density and width, as observed by epifluorescence microscopy. As indicated by the white arrow on the SEM views (Fig. 2), an EC matrix was also observed as diffuse material heterogeneous distributed on the surface, and the amount of this matrix clearly differed between strains. The highest production was observed with Scott A strain while the lowest production was observed with the DPF 234 HG2 strain.

Identification of teichoic acid in the biofilm EC matrix and the biofilm culture supernatant similar to cell wall teichoic acid

The GC-MS analysis showed that the three preparations (cell wall, biofilm EC matrix and biofilm culture supernatant) of L. monocytogenes ScottA strain (serotype 4b) contained glucose (Glc), galactose (Gal), N-acetyl glucosamine (GlcNAc) and ribitol in approximately equimolar ratios (data not shown). The ¹H-NMR spectra of the three preparations (Fig. 3) were very similar, with only minor differences in the contaminating signals (e.g. alkyls groups in high field zone, between 1 and 2 ppm). These three preparations were further studied by 1- and 2D NMR experiments, and their ¹H and ¹³C NMR spectra were completely assigned (Fig. 3, Table 1; Fig. S2, Supporting Information). NMR analysis revealed that these polymers were composed of repeating units containing a ribitol residue, glycosylated in the position 2 with the β-GlcNAc, which was in turn substituted at C3 by β-Glc and at C6 with α-Gal residues. The repeating units were linked via a phosphodiester linkage between C5 of ribitol and C4 of the β-GlcNAc. The structure was consistent with the one of L. monocytogenes serotype 4b cell wall teichoic acid (Uchikawa, Sekikawa and Azuma 1986) (Fig. 3). Similar results were obtained with LmA, the other strain belonging to the serotype 4b (data not shown).

The same analyses were performed with the four L. monocytogenes strains belonging to the serotype 1/2a: EGD-e, DPF 234 HG2, DSS1130 BFA2 and CL297 AS1. ¹H-NMR spectra of carbohydrate polymers from biofilm matrix, biofilm culture supernatant and cell wall were identical for each strain (data not shown). ¹H-NMR profiles and composition analyses of biofilm matrix carbohydrates of serotype 1/2a strains indicated the presence of ribitol, GlcNAc and rhamnose (Rha) for the three strains: EGD-e, DPF 234 HG2 and CL297 AS1 (Fig. 4). The complete chemical structure of carbohydrate polymers from EC matrix of EGD-e biofilm was established by 2D ¹H- and ¹³C-NMR techniques (Fig. 4, Table 1; Fig. S3, Supporting Information). It was composed of repeating units containing a ribitol-1-phosphate,
Figure 2. SEM observations of EC matrix from biofilms. Biofilms were cultivated for 48 h at 37°C on polystyrene in MCDB 202 poor medium. White arrows indicate the EC matrix.

Figure 3. Comparison of 1H-NMR spectra of carbohydrate from biofilm culture supernatant (A), EC matrix (B) and cell wall (C) of L. monocytogenes Scott A strain. Roman numbers correspond to unit.

Glycosylated at positions 2 and 4 with α-Rha (II) and α-GlcNAc (I). The glycosylated ribitol residues were linked to each other via 1,5-phospho-diester bonds. This structure corresponded to the one of the cell wall teichoic acid of L. monocytogenes serotype 1/2 a, described earlier by Uchikawa, Sekikawa and Azuma (1986). Taken together, these data have demonstrated the major carbohydrate components, extracted from biofilm EC matrix and the biofilm culture supernatants of two L. monocytogenes serotype 4b and three L. monocytogenes serotype 1/2 a strains were identical to the corresponding cell wall teichoic acids.

Similarly to other strains, the carbohydrate extracted from the biofilm EC matrix of DSS 1130 BFA2 strain (serotype 1/2a) was identical to the cell wall teichoic acid of this strain. However, 1H-NMR spectra showed that this teichoic acid lacked one anomeric signal of GlcNAc I at δ 5.08 ppm, the signals corresponding to the N-acetyl group (δ 2.1 ppm) and the pseudo-triplet signals H4 of this residue (Table 1). Further analyses (data not shown) established that the teichoic acids extracted from cell wall, biofilm medium supernatant and biofilm EC matrix of DSS 1130 BFA2 serotype 1/2a strain contain a ribitol residue substituted with a single α-Rha residue.

The DSS 1130 BFA2 strain present a nonsense mutation in the lmo2550 gene

Two genes, lmo2549 and lmo2550, were involved in the decoration of cell wall serotype 1/2a teichoic acid with GlcNAc (Fig. 5A). These two genes in DSS 1130 BFA2 strain were sequenced and compared to those of the three other serotype 1/2a strains of L. monocytogenes used in this study (DPF 234 HG2, EGD-e and CL 297 AS1) (Fig. 5B and C). All the sequences of lmo2549 gene appeared identical (data not shown). For the DSS 1130 BFA2 strain producing a major teichoic acid without GlcNAc, a substitution of an adenine by a thymine in position 757 creating a stop codon after the 253th amino acid was shown resulting in a C-terminally modified protein lacking the last 62 amino acids and explaining the absence of GlcNAc residue.
DISCUSSION

Under the experimental conditions used in this study, the six strains of L. monocytogenes were able to form biofilms on polystyrene at 37 °C during 48 h in MCDB 202 poor medium. The number of cultivable cells within biofilms was strain dependent but the differences did not seem to be related to the strain serotype or origin, in accordance with previously reported works (Borucki et al. 2003; Combrouse et al. 2013). In our study, epifluorescence microscopy observations and SEM analysis showed that the six strains formed heterogeneous biofilms with isolated cells as well as multilayered aggregates of bacteria. Similar observations have been already reported (Percival et al. 2011; Combrouse et al. 2013; Guilbaud et al. 2015). The density and the structure of L. monocytogenes biofilms were also found to be affected by the substratum and the environmental conditions (Borucki et al. 2003). Production of EC matrix was suggested as being one of the parameters involved in biofilm formation (Flemming and Wingender 2010). In this study, some EC material heterogeneously distributed on the biofilm surface was observed for all strains, the quantity of this matrix depending on the strain as already shown (Combrouse et al. 2013). Several earlier studies using mainly various colorimetric techniques demonstrated aspecific presence of an EC carbohydrates, for example, when staining of biofilms with ruthenium red (Borucki et al. 2003; Zameer et al. 2010) and Congo red stains (Tienku et al. 2013; Chen et al. 2014), fluorescein-conjugated lectin binding (Helford et al. 2005), fluorescent dye-conjugated antibody binding (Cywes-Bentley et al. 2013) or phenol-sulfuric acid assay in crude EC matrix extracts (Combrouse et al. 2013). In this study, we analyzed the biofilm EC matrix and culture supernatant of the six strains for their content in soluble carbohydrates after deproteinization and fractionation on a Sephadex G-50 column. To ensure minimal cell lysis, cell viability was checked in preliminary experiments by counting viable cells before and after sonication at different intensities (data not shown). For each strain, the results showed that the carbohydrate extracted from the EC matrix and culture supernatants were identical to their corresponding cell wall teichoic acids described by Uchikawa, Sekikawa and Azuma (1986) and Eguster et al. (2011).

The classification of L. monocytogenes into distinct serotype is based on serological reactions of group-specific somatic (O) and flagella (H) antigens (Garmyn et al. 2009). Cell wall teichoic acids represent the major somatic antigens (Dubail et al. 2006). These cell wall carbohydrate polymers are also important for bacteriophage attachment (Wendlinger, Loessner and Scherer 1996) and recognition and binding of bacteriophage endolysins (Eguster et al. 2011). Our results are reminiscent of several works that reported the presence of teichoic acid-like components in the biofilms EC matrix of S. epidermidis and S. aureus (Hussain, Hastings and White 1992; Sadovskaya et al. 2005; Vinogradov et al. 2005; Jabouiri and Sadovskaya 2010). This is in agreement with the fact that a large fraction of the cell wall teichoic acid is located in a ‘fluffy’ layer region beyond the cell wall (Neuhaus and Baddiley 1996). It also indicates that the molecules of staphylococcal teichoic acid could be released from the cell surface and become a component of the EC ‘slime’, or a biofilm, when the cell aggregate is attached to a solid surface (Kogan et al. 2006).

The EC teichoic acids were the only carbohydrate polymers which could be identified in L. monocytogenes biofilm matrix of all strains studies. We did not find the insoluble cell-bound poly-β-(1,4)-N-acetylmannosamine decorated with terminal α-1,6-linked galactose, recently identified in a constructed
aggregate-forming mutant strain of EGD-e, ΔpdeB/C/D (Koseoglu et al. 2015). One explanation would be that this polysaccharide could be identified only in this aggregate-forming mutant, where intracellular c-di-GMP levels were elevated compared with the wild type. Neither wild-type EGD-e strain nor the ΔpdeB/C/D ΔpsSC mutant, used by the authors as a negative control, was found to produce this insoluble polysaccharide.

Structural studies of EC carbohydrates and cell wall teichoic acid of the serotype 1/2a DSS 1130 BFA2 strain indicated that this strain expressed a serotype 1/2a teichoic acid lacking GlcNAc substitution. We identified two genes described in the study of Eugster et al. (2011) that are implicated in the decoration of cell wall teichoic acid with GlcNAc. The first gene was the lmo2549 gene identified in L. monocytogenes serotype 1/2a EGD-e strain and coded a glycosyltransferase. The lmo2549 gene was flanked by the lmo2550 gene that coded a protein of unknown function. In the DSS 1130 BFA2 strain, we identified a nonsense mutation of lmo2550 gene explaining the absence of GlcNAc residue. The results obtained with the DSS 1130 strain and the other strains studied showed that EC teichoic acids present in the culture supernatant and in the biofilms matrix have the structure identical to that of the cell wall teichoic acids. As for many components of the matrix, it is difficult to establish the origin of these EC teichoic acids. It can be suggested that EC teichoic acid in large part comes from autolysis and peeling of bacteria (dead and/or viable but not cultivable and/or viable cultivable). It would be interesting to search for a potential active secretion pathway of these teichoic acids. To date, this has never been demonstrated for any bacterial genus.

In conclusion, we showed that the strains of L. monocytogenes were able to form complex biofilms made of cells sometimes embedded in a polymeric matrix. Using a protocol similar to one described by Sadovskaya et al. (2005), which identified the PNAG and teichoic acid in S. epidermidis and S. aureus biofilms, we described the presence of a major soluble carbohydrate, the teichoic acid, in L. monocytogenes biofilms. We showed that this biofilm major soluble carbohydrate was identical to this present in the cell wall. Interestingly, the DSS 1130 BFA2 strain had a serotype 1/2a teichoic acid lacking GlcNAc glycosylation. Since the teichoic acids have been characterized as cell wall binding domains of bacteriophage endolysins (Eugster et al. 2011; Biellmann et al. 2015), their presence in the biofilm ECM matrix could possibly have a protective effect of L. monocytogenes cells by blocking the phage receptors.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSLE online.

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**Conflict of interest** None declared.

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