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Minireview

Molecular biology of surface colonization by *Listeria monocytogenes*: an additional facet of an opportunistic Gram-positive foodborne pathogen

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Summary

The opportunistic and facultative intracellular pathogenic bacterium *Listeria monocytogenes* causes a rare but severe foodborne disease called listeriosis, the outcome of which can be fatal. The infection cycle and key virulence factors are now well characterized in this species. Nonetheless, this knowledge has not prevented the re-emergence of listeriosis, as recently reported in several European countries. *Listeria monocytogenes* is particularly problematic in the food industry since it can survive and multiply under conditions frequently used for food preservation. Moreover, this foodborne pathogen also forms biofilms, which increase its persistence and resistance in industrial production lines, leading to contamination of food products. Significant differences have been reported regarding the ability of different isolates to form biofilms, but no clear correlation can be established with serovars or lineages. The architecture of listerial biofilms varies greatly from one strain to another as it ranges from bacterial monolayers to the most recently described network of knitted chains. While the role of polysaccharides as part of the extracellular matrix contributing to listerial biofilm formation remains elusive, the importance of eDNA has been demonstrated. The involvement of flagella in biofilm formation has also been pointed out, but their exact role in the process remains to be clarified because of conflicting results. Two cell–cell communication systems LuxS and Agr have been shown to take part in the regulation of biofilm formation. Several additional molecular determinants have been identified by functional genetic analyses, such as the (p)ppGpp synthetase RelA and more recently BapL. Future directions and questions about the molecular mechanisms of biofilm formation in *L. monocytogenes* are further discussed, such as correlation between clonal complexes as revealed by MLST and biofilm formation, the swarming over swimming regulation hypothesis regarding the role of the flagella, and the involvement of microbial surface components recognizing adhesive matrix molecules in the colonization of abiotic and biotic surfaces.

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Introduction

Listeria monocytogenes is a Gram-positive pathogen involved in numerous foodborne disease outbreaks. Although its involvement in food poisoning lags behind that of *Salmonella* and *Campylobacter*, which remain the prominent threats to food safety worldwide, *L. monocytogenes* is singular because of its high mortality rate, which ranges from 20% to 35% (Fratamico *et al.*, 2005; Riemann and Cliver, 2006). Without any obvious single reason, but rather a combination of several factors, cases of listeriosis have increased in several European countries in recent years (Allerberger and Wagner, 2010). Listeriosis mainly affects high-risk groups, including immunocompromised patients, pregnant women, newborns and the elderly (Farber and Peterkin, 1991). *Listeria monocytogenes* infection can manifest as (i) a mild non-invasive gastrointestinal illness which can be misdiagnosed in healthy adults, or (ii) an invasive disease which manifests as septicemia or neuropathic disease (Vazquez-Boland *et al.*, 2001). In France, the recent re-emergence of listeriosis is mainly attributable to bacteremia in people over 60 years of age, particularly those with a predisposition, i.e. co-morbidities (ANSES, 2009). The infectious cycle, key virulence factors and pathogenesis mechanisms of *L. monocytogenes* have been extensively investigated and are now clearly defined (Cossart,

2002; Dussurget *et al*., 2004; Hamon *et al*., 2006). Briefly, the major steps of intracellular parasitism involve the cell wall proteins InlA (internalin A) and InlB for adhesion to the surface of the eukaryote cell and entry into the host cell via phagocytosis. Released listeriolysin O (LLO) and phospholipases C (PlcA and PlcB) then enable bacteria to escape from the phagocytic vacuole, whereas membrane-anchored ActA (actin assembly) is responsible for actin-based motility allowing for cell-to-cell spread.

Although at first glance *L. monocytogenes* is generally regarded as a pathogen, it should primarily be considered as a saprophytic bacterium well adapted for survival in the environment (Fenlon, 1999). It is able to contaminate and thrive in the food-processing environment thanks to its ability to grow in a wide range of temperatures (–0.4 to 45°C), pH (4.3 to 9.6) and salt concentrations (up to 10% NaCl) as well as at low water activity (*Aw* down to 0.90) (Seeliger and Jones, 1986; ICMSF, 1996). Moreover, *L. monocytogenes* adheres to food contact surfaces, such as glassware, metal (stainless steel), rubber and plasticware (polystyrene) where it can further establish a biofilm (Frank and Koffi, 1990; Blackman and Frank, 1996; Norwood and Gilmour, 1999; Beresford *et al*., 2001; Chae *et al*., 2006; Di Bonaventura *et al*., 2008). Biofilm-coated surfaces are particularly difficult to decontaminate, since biofilms protect the embedded bacteria from desiccation, antimicrobials and sanitizing agents (Folsom and Frank, 2006; Tessema *et al*., 2009). This ability potentially allows the persistence of *L. monocytogenes* for long periods of time in the processing environment and is therefore a food safety hazard since biofilms are an important source of contamination when food products come into contact with them (Moretro and Langsrud, 2004; Gounadaki *et al*., 2008; Poimenidou *et al*., 2009; Koutsoumanis *et al*., 2010). *Listeria monocytogenes* has been isolated from an extensive range of food products, including vegetables, milk, soft and farmhouse cheeses, fish and meat products as well as various ready-to-eat products (Ells and Truelstrup Hansen, 2006; 2010; Mataragas *et al*., 2008; Panagou and Nychas, 2008; Cordano and Jacquet, 2009; Kushwaha and Muriana, 2009; O'Brien *et al*., 2009; Pintado *et al*., 2009; Takahashi *et al*., 2009; Little *et al*., 2010). The elimination of *L. monocytogenes* biofilms in processing plants appears critical for improving food safety. This review summarizes current knowledge on the molecular mechanisms responsible for the process whereby *L. monocytogenes* colonizes food products and food-processing environments. A better understanding of the molecular determinants responsible for its ability to colonize biotic (i.e. of biological origin) and abiotic supports is a prerequisite for the development of new strategies that could limit and even prevent contamination as well as subsequent food infections.

Listerial phylogeny and biofilm formation

Listeria monocytogenes exhibits a high level of heterogeneity from one strain to another, and several techniques have been developed over the years to discriminate isolates (Chen and Knabel, 2008). Among phenotypic methods, serotyping has been the most widely used and can differentiate four serogroups and 13 distinct serovars, named 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7. This classification is based on serological reactions with variations of the 15 somatic (O-factor subtyped from I to XV) and 5 flagellar (H-factor subtyped from A to E) antigens with specific antisera (Seeliger and Hohne, 1979; Seeliger and Jones, 1986). While *L. monocytogenes* is generally regarded as a pathogen, the level of virulence is highly variable from one *L. monocytogenes* strain to another, as a significant proportion of isolates is hypovirulent and even apathogenic (Roche *et al*., 2001; 2003). The degree of virulence of *L. monocytogenes* strains could correlate with some serovars, as the majority of human listeriosis cases are caused by three serovars, i.e. 1/2a, 1/2b and 4b (Schuchat *et al*., 1991). A genoserotyping technique based on multiplex PCR has been developed to facilitate and speed up discrimination of the isolates (Doumith *et al*., 2004). Five genoserotypes (or PCR groups) are distinguished: genoserotype IIa (serovars 1/2a and 3a), genoserotype IIb (serovars 1/2b, 3b and 7), genoserotype IIc (serovars 1/2c and 3c), genoserotype IVb (serovars 4b, 4d and 4e) and genoserotype L (containing other serovars).

With the development of more reliable methods based on molecular tools, this bacterial species was separated into three major divisions, i.e. Genomic Division I (also designated Lineage II), Genomic Division II (also designated Lineage I) and Genomic Division III (or Lineage III) (Cheng *et al*., 2008). Based on the screening of *L. monocytogenes* strain libraries, the different serovars appeared to be associated with specific lineages: (i) Lineage I contains serovars 1/2b, 3b, 4b, 4d and 4e; (ii) Lineage II contains serovars 1/2a, 1/2c, 3a and 3c; and finally, (iii) Lineage III contains serovars 4a and 4c. The distribution between serovars and lineages (and probably genoserotypes) should not be considered as strict and absolute as, for example, some serovars were under-represented or not represented at all (e.g. 4ab or 7) or were distributed between different lineages, e.g. 4b found in both lineages I and III (Liu *et al*., 2006). More recently, an additional step in genotypic methods was reached using MLST (multilocus sequence typing) where the *L. monocytogenes* species emerged as distributed into 23 clonal complexes and 22 singletons (Ragon *et al*., 2008).

Several studies have noted that *L. monocytogenes* strains show significant differences in their ability to

adhere to food-processing surfaces (Norwood and Gilmour, 1999; Lunden *et al*., 2000). The hypothesis that the ability to adhere and then to form biofilms might be conserved within phylogenetic lineages was therefore formulated. The study of a large number of isolates suggested that serovars presented significant differences in their ability to adhere to stainless steel; strains within serovar 1/2c especially showed the highest levels of attachment (Norwood and Gilmour, 1999; Lunden *et al*., 2000). Moreover, this high capacity for adherence was associated with strain persistence. Subsequent investigations found a correlation between phylogeny and the ability to form biofilms, but their conclusions differed (Djordjevic *et al*., 2002; Borucki *et al*., 2003). Djordjevic and colleagues (2002) observed that strains associated with Lineage I (serovars 1/2b and 4b) produced more biofilm than did strains from Lineage II (serovars 1/2a and 1/2c), whereas Borucki and colleagues (2003) reported exactly the opposite. These investigations also disagree regarding the relationships between persistence and the ability to form biofilms. However, neither of them observed a direct correlation between biofilm formation and serovars. Conflicting conclusions might result from differences in sample sizes and strains used in the analyses, but not in methodology; eight common strains were tested in biofilm formation following their respective protocols and the results of the two methods were not statistically different (Borucki *et al*., 2003). In a recent report, however, a correlation between serovars and the ability to form biofilms was noted, but involved only 1/2a and 4b *L. monocytogenes* strains (Pan *et al*., 2009).

In view of these results, no conclusion about the putative correlation between the ability to form biofilms and lineage can be established. While a high capacity for adhesion seems to be correlated with persistence, the same cannot be said for the capacity to form biofilms. This is not that surprising considering *L. monocytogenes* most likely exists as a member of a complex bacterial community in the natural environment or in food-processing plants. *Listeria monocytogenes* could take advantage of other bacterial biofilms, which could explain the persistence of low biofilm producers when co-cultured with *Pseudomonas fragi*, for example (Sasahara and Zottola, 1993). It has also been shown that low biofilm producers, i.e. *L. monocytogenes* 4b strains, can form higher-density biofilm in the presence of a high biofilm producer, e.g. *L. monocytogenes* SK1387 1/2a strain, in mixed-culture biofilm. It has been suggested that the extracellular matrix produced by *L. monocytogenes* SK1387 strain sticks to the surface and improves sessile growth of serovar 4b *L. monocytogenes* strains, conferring greater protection against environmental stresses (Pan *et al*., 2009). These data highlight the need to take into account the interaction of *L. monocytogenes* with other bacterial species in biofilm communities in order to determine how they influence each other. For example, in co-culture with *Staphylococcus aureus*, the sessile population of *L. monocytogenes* increased significantly (Rieu *et al*., 2008a); conversely, some isolates of *Enterococcus durans*, *Lactobacillus plantarum* and *Lactococcus lactis* ssp. *lactis* were found to greatly inhibit *L. monocytogenes* biofilm formation (Zhao *et al*., 2004).

Biofilm architecture

A biofilm can be broadly defined as a community of microorganisms adhering to a surface (Costerton *et al*., 1995). Bacterial cells in a biofilm are generally surrounded by a self-produced extracellular matrix. Biofilm formation can be divided into several key steps in which the adhesion of planktonic bacteria is followed by their subsequent proliferation to form microcolonies (Fig. 1). The next step is the maturation of the biofilm in a three-dimensional structure, and finally some bacteria are released from the biofilm and dispersed, enabling cells to colonize other surfaces (O'Toole *et al*., 2000; Hall-Stoodley and Stoodley, 2002).

The architecture of *L. monocytogenes* biofilm has been investigated by several techniques as scanning electron microscopy (Kalmokoff *et al*., 2001; Chavant *et al*., 2002; Borucki *et al*., 2003), epifluorescence microscopy (Lunden *et al*., 2000; Kalmokoff *et al*., 2001; Carpentier and Chassaing, 2004; Monk *et al*., 2004; Pan *et al*., 2006) and laser-scanning confocal microscopy (LSCM) (Chae and Schraft, 2000; Rieu *et al*., 2008b). Several attempts have also been made to model *L. monocytogenes* biofilm formation (Kreft and Wimpenny, 2001; Zameer *et al*., 2010). Pioneering work on the structure of *L. monocytogenes* biofilm using scanning electron microscopy and epifluorescence microscopy revealed the ability of this bacterial species to colonize hydrophilic (stainless steel) and hydrophobic (polytetrafluoroethylene [PTFE]) surfaces (Chavant *et al*., 2002). In static culture conditions, some strains of *L. monocytogenes* produced three-dimensional mushroom-shaped biofilms with well-distributed channels and pores, whereas other ones produced only sparse aggregates of cells or a bacterial monolayer (Chae and Schraft, 2000; Kalmokoff *et al*., 2001; Chavant *et al*., 2002; Borucki *et al*., 2003). In similar conditions using either stainless steel or plasticware surfaces, a honeycomb-like biofilm structure has also been reported for some other strains (Marsh *et al*., 2003). Using LSCM, a novel three-dimensional structure has been characterized in *L. monocytogenes* EGD-e under dynamic conditions (flow-cell), where ball-shaped microcolonies are surrounded by a network of knitted chains (Rieu *et al*., 2008b). Following prolonged sessile growth

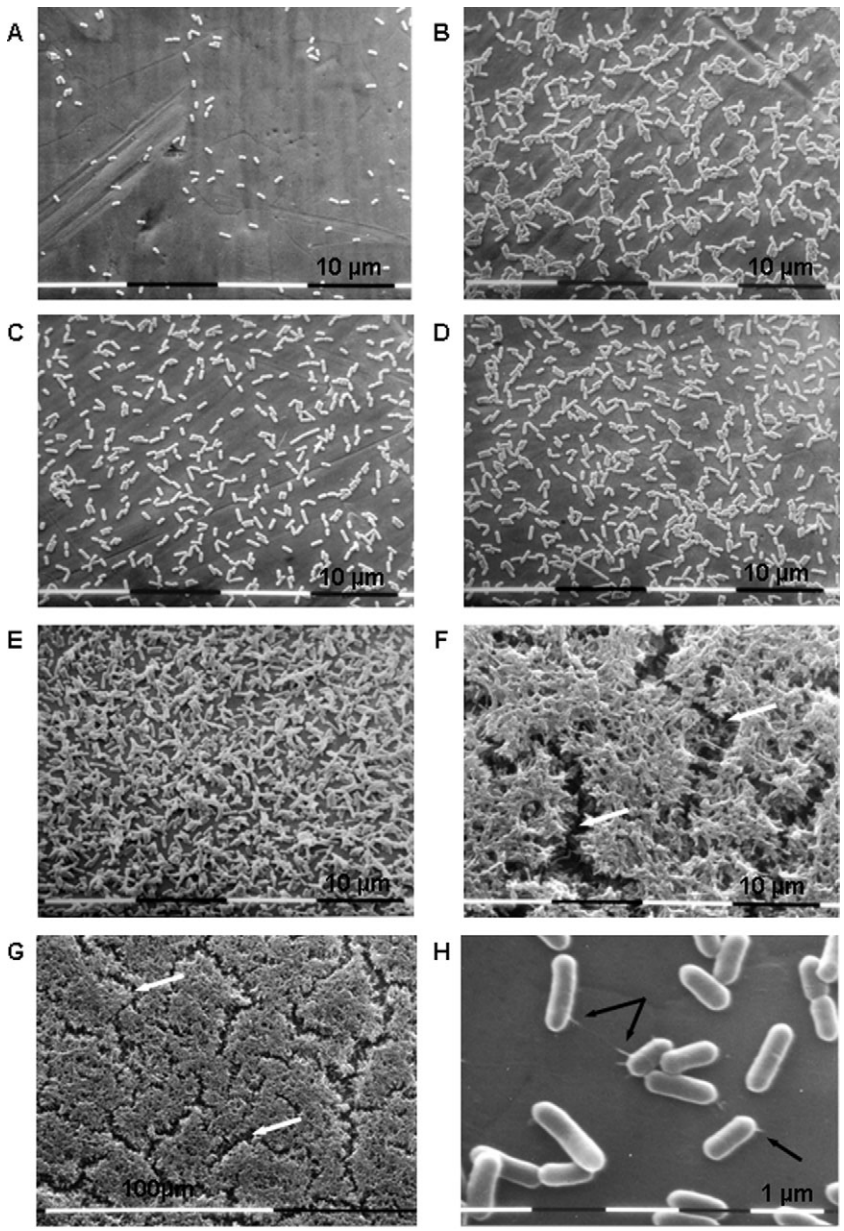

Fig. 1. Scanning electron microscopy observations of *L. monocytogenes* biofilm formation. *Listeria monocytogenes* was grown at 20°C in BHI on stainless steel chips after 10 s (A), 5 h (B), 8 h (C), 24 h (D), 5 days (E) and 7 days (F and G) of adhesion. White arrows indicate the putative water channel (F and G). Detail of 30-minute sessile cells with fimbriae-like structures as indicated by black arrows (H).

on stainless steel coupons in a bioreactor, morphotypic conversion from the common smooth colony morphology to a succession of rough colony variants has been reported to occur in the course of *L. monocytogenes* biofilm formation (Monk *et al*., 2004). Bacterial cell variants exhibiting rough colony morphology appeared spontaneously in the biofilm and formed chain cell structures allowing increased surface colonization (Monk *et al*., 2004). In the end, it appears that the biofilm structure of *L. monocytogenes* depends on a multitude of parameters, namely the strain, the type of surface as well as other environmental conditions, such as pH, medium and temperature.

Effect of environmental factors on biofilm formation

The switch from the planktonic to sessile state requires profound physiological changes, which occur after the regulation of gene expression in response to different environmental signals. The importance of environmental conditions, such as the nature of the surfaces, the growth temperature and medium, on biofilm formation of various *L. monocytogenes* strains has been highlighted (Moltz and Martin, 2005). The most recent investigation of the structural dynamics of *L. monocytogenes* biofilm formation using LSCM revealed different biofilm architectures when grown in static versus dynamic conditions (Rieu

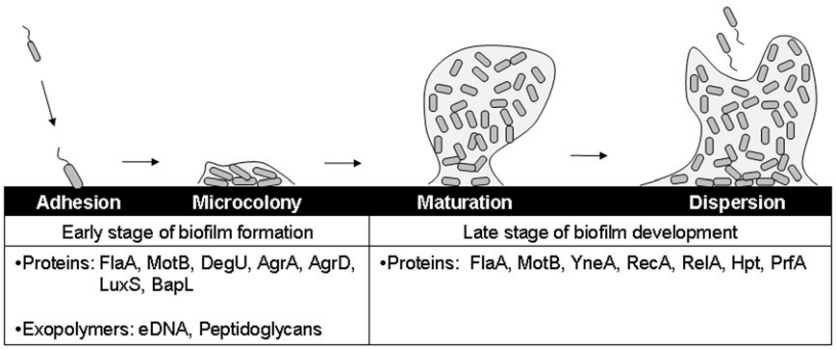

Fig. 2. Schematic representation of molecular determinants as yet uncovered and involved in early and late stages of biofilm formation in *L. monocytogenes*. Detailed descriptions of the roles of the different molecular determinants are provided in the text.

et al., 2008b). This investigation pointed out the importance of culture conditions in biofilm formation and the need for complementary approaches.

In food-processing environments, biotic factors also play a role in biofilm development (Zottola and Sasahara, 1994). Resident biofilm could have negative or positive effects on biofilm formation by *L. monocytogenes* (Carpentier and Chassaing, 2004). The inhibition of *L. monocytogenes* development could occur by competition for nutrients (Gnanou Besse *et al*., 2008; Guillier *et al*., 2008) or by the secretion of antimicrobial agents like bacteriocins produced by *Lactococcus lactis*, for example (Leriche *et al*., 1999; Liu *et al*., 2008). In contrast, the presence of microorganisms like *Staphylococcus capitis* or *S. aureus* could improve *L. monocytogenes* biofilm formation (Carpentier and Chassaing, 2004; Rieu *et al*., 2008a). Addition of cell-free supernatant from either *S. capitis* or *S. aureus* biofilm was sufficient to stimulate biofilm development of *L. monocytogenes*. Interestingly, this positive effect was abolished when supernatant was treated with proteinase K, but not after ultrafiltration (3 kDa cut-off), suggesting that peptide molecules within the supernatant of *S. aureus* could be involved in the improvement of biofilm formation (Rieu *et al*., 2008a). *Pseudomonas fragi* has also been shown to be necessary for the establishment of *L. monocytogenes* biofilm (Sasahara and Zottola, 1993). In order to determine the biofilm properties that influence the initial fixation of *L. monocytogenes*, a set of *L. lactis* model resident biofilms with different architectures, porosities, types of matrices and individual cell surface properties has been created (Habimana *et al*., 2009). This study suggests that the porous structure of resident biofilms improves the adhesion of *L. monocytogenes*, whereas exopolysaccharides produced by resident biofilms prevent its adhesion.

During infection of the gastrointestinal tract, *L. monocytogenes* is in a particular environment with suboptimal conditions, including exposure to bile. Nevertheless, *L. monocytogenes* is able to survive, colonize and enter epithelial cells. Bile has recently been shown to improve the initial attachment to plastic surfaces and biofilm formation of *L. monocytogenes* cells. So, during infection, the exposure to bile may enhance biofilm formation of *L. monocytogenes*, and consequently may contribute to its survival and facilitate colonization of the gastrointestinal tract (Begley *et al*., 2009).

Molecular determinants of biofilm formation

Common molecular determinants are seen in biofilm formation by Gram-positive bacteria, namely exopolysaccharides, Bap (biofilm-associated protein) and bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) (Götz, 2002; Lasa, 2006). However, some of these determinants clearly differ or have not yet been considered for biofilm development in *L. monocytogenes*. Molecular determinants characterized to date at the different stages of the biofilm formation process in *L. monocytogenes* are shown in Fig. 2. They include essentially motility factors, cell transduction signal systems, exopolymers, BapL as well as some other molecular determinants, such as second messengers.

Flagella

Flagella are very important for biofilm formation in several bacterial species (O'Toole *et al*., 2000). In *L. monocytogenes*, the flagellum is composed of thousands of flagellin monomers encoded by the *flaA* gene. *Listeria monocytogenes* has four to six peritrichous flagella per cell, the biosynthesis of which is regulated by temperature (Peel *et al*., 1988). The transcription of *flaA* is stopped at temperatures above 30°C, where *L. monocytogenes* strains are not motile. Control of flagella biosynthesis is rather complex as it involves at least five regulators, namely FlaR (flagellin regulator) (Sanchez-Campillo *et al*., 1995), PrfA (positive regulatory factor A) (Michel *et al*., 1998), DegU (degradation enzymes regulator) (Knudsen *et al*., 2004), MogR (motility gene repressor) (Gründling *et al*., 2004) and GmaR (glycosyltransferase and motility antirepressor) (Shen *et al*., 2006). Like PrfA (Scortti *et al*., 2007), most of these regulators also control expression of

virulence factors required for full virulence of *L. monocytogenes*. Interestingly, GmaR is bifunctional as it also glycosylates FlaA with β-O-linked N-acetylglucosamine (Schirm *et al*., 2004). So far, FlaA is the first and only surface protein reported to be glycosylated in *L. monocytogenes*.

The first studies of the involvement of flagella as a putative adhesive structure in biofilm formation showed that the absence of flagella affected the initial stage of adhesion, but did not influence final levels of attachment achieved over longer periods of time (Vatanyoopaisarn *et al*., 2000). Following the generation of unflagellated as well as nonmotile *L. monocytogenes* mutant strains, it was demonstrated that flagellated but non-motile bacterial cells do not adhere to or invade human epithelial cells more efficiently than nonflagellated listerial cells (O'Neil and Marquis, 2006). Rather than acting as adhesins, the flagella as mediators of motility enhance the adhesion of *L. monocytogenes* to targeted host cells. In the colonization of the intestines, motile bacteria appear to outcompete nonmotile bacteria, suggesting that flagellum-mediated motility enhances infectivity soon after bacterial ingestion (O'Neil and Marquis, 2006). However, it should be stressed that in this study the rapid transfer of *L. monocytogenes* from a temperature enabling flagella expression to 37°C for *in vitro* invasion assay would allow the presence of motile flagella in a significant proportion of the bacterial population. Moreover, the *L. monocytogenes* 10403S strain used in this study is a peculiar model as it exhibits deregulation of *flaA* expression at 37°C (Gründling *et al*., 2004). Altogether, results and conclusions drawn from this investigation might not be generalized to other *L. monocytogenes* strains. Using the same *L. monocytogenes* strain, though, further investigations using abiotic surfaces confirmed the involvement of flagella in the early stages of attachment, but not as surface adhesins (Lemon *et al*., 2007). A motile mutant of *L. monocytogenes* with an altered flagellar surface formed biofilm as well as the wild-type. In addition, comparison of adhesion between flagellum-minus cells and paralysed-flagellum cells after centrifugation revealed a significant difference, suggesting that paralysed-flagella interfere slightly with contact between putative surface adhesin(s) and abiotic surfaces. However, comparison of biofilm formation in wild-type bacteria and both non-motile mutants showed a biofilm-defective phenotype, suggesting that flagellum-mediated motility plays a predominant role in biofilm formation in *L. monocytogenes* (Lemon *et al*., 2007). Swarming ability, which is a specialized form of movement that enables flagellated bacteria to coordinately move atop solid surfaces, is distinct from the simple swimming ability conferred by flagella, which is an individual and non-cooperative movement (Henrichsen, 1972). Swarming over swimming regulation might further explain the importance of flagella as motile determinants rather than adhesins in biofilm formation (Desvaux and Hébraud, 2009).

Many later studies have confirmed the importance of motility in the first stages of biofilm formation (Tresse *et al*., 2006; 2009; Gueriri *et al*., 2008), except for one which demonstrated that defects in flagella or motility have a more complex effect on biofilm development. Using flow cells, mutant strains lacking flagella (*ΔflaA* and *ΔflgL*) or affected for motility (*ΔmotB*) were impaired in initial bacterial attachment but subsequently displayed a hyper-biofilm phenotype (HB) (Todhanakasem and Young, 2008). This HB phenotype was not previously found under static conditions using microtitre plate assays, probably because of differences due to the numerous changes that occur when bacteria reach a high density under static conditions, as changes in pH and/or nutrient availability. So, although flagellum-mediated motility improves initial attachment under both static and dynamic conditions, it is not necessary for attachment and biofilm formation and interferes with biofilm development under dynamic conditions.

Cell–cell communication

During biofilm development and maturation, complex cellular mechanisms are involved requiring coordinated regulation of gene expression by cell–cell communication (Dunny and Leonard, 1997; Hardman *et al*., 1998; Waters and Bassler, 2005; von Bodman *et al*., 2008). Among cell–cell signalling systems, quorum sensing (QS) has been the most investigated and specifically refers to cell density-linked coordinated gene expression in populations that experience threshold signal concentrations to induce a synchronized population response. In other terms, QS is just an example of multicellular behaviour in prokaryotes leading to regulation of diverse physiological processes, which is only induced when bacteria are at high cell population densities. This mechanism is based on the production and release of signal molecules named auto-inducers. The subsequent detection of auto-inducers from a certain threshold concentration leads to bacterial responses. There are two archetypal QS systems in *L. monocytogenes*: the auto-inducer 2 (AI-2) LuxS system found in both Gram-negative and Gram-positive bacteria, and the peptide-mediated QS signalling system Agr characteristic of Gram-positive bacteria (Dunny and Leonard, 1997; Miller and Bassler, 2001; Waters and Bassler, 2005).

Listeria monocytogenes is able to produce AI-2-like molecules via the LuxS orthologue (Challan Belval *et al*., 2006). Depending on the bacterium, the *luxS* mutation affects biofilm formation differently (Blehert *et al*., 2003; Cole *et al*., 2004; Wen and Burne, 2004). In *L. monocy-*

togenes, the *luxS* mutant strain forms denser biofilm than the parental strain (Challan Belval *et al*., 2006; Sela *et al*., 2006). However, the addition of synthetic AI-2 to the *luxS* mutant supernatant does not restore the phenotype of the parental strain. S-ribosyl homocysteine (SRH), the precursor of AI-2, may explain the denser biofilm phenotype, and was able to increase the number of attached cells. Furthermore, SRH was found in larger quantities in *luxS* mutant supernatant than in parental strain supernatant (Challan Belval *et al*., 2006). These results suggest that the *luxS* gene participates in repression of biofilm formation, probably by converting SRH to AI-2. It would be interesting to elucidate the mechanisms by which SRH allows an increase of attached cells and the precise role of *luxS* in attachment and biofilm formation in *L. monocytogenes*, by comparing genetic expression between sessile and planktonic cells, for example. Besides SRH, a toxic precursor, S-adenosyl homocysteine (SAH), also accumulated in culture supernatant of *luxS* mutant (Challan Belval *et al*., 2006). As AI-2 though, addition of SAH could not increase the number of attached cells. From this point of view, the physiological role of AI-2 may be limited to the detoxification of SAH in *L. monocytogenes*.

The Agr system is a peptide-mediated QS system initially described in *S. aureus* and present in *L. monocytogenes*. The *agr* locus is composed of four genes (*agrB*, *agrD*, *agrC* and *agrA*) organized as an operon (Autret *et al*., 2003). These genes encode the histidine kinase AgrC and the response regulator AgrA of a two-component system, as well as a precursor peptide AgrD, which is matured into an autoinducing peptide by AgrB. Whereas the *agr* system was known to be involved in the production of virulence factors, it has recently been shown to play an important role in biofilm formation in *L. monocytogenes* (Rieu *et al*., 2007; Riedel *et al*., 2009). It has been shown that the adhesion and the first stage of biofilm formation are affected in *agrA* and *agrD* mutant strains, within the first 24 h of incubation, but not afterwards (Rieu *et al*., 2007). Interestingly, *agr* gene expression increased progressively over the incubation period in flowing conditions and its activity was maximal in cells outside ball-shaped microcolonies (Rieu *et al*., 2008b). Using another culture medium (10-fold diluted BHI versus TSB), biofilm formation of a mutant Δ*agrD* was affected more than originally shown (Riedel *et al*., 2009). The wild-type biofilm phenotype could be restored by adding supernatant of *L. monocytogenes* EGD-e grown in full-strength BHI to the Δ*agrD* strain or by mixing Δ*agrD* cells with wild cells in a cell ratio of 90:10 respectively (Riedel *et al*., 2009). Considering that the *agr* system probably controls the expression of genes involved in different physiological processes, it will be interesting to identify *agr*-dependent mechanisms allowing biofilm formation. An interesting consequence of *agrD* deletion is the decrease of internalin A (InlA) in the cell wall compared with both wild-type and the complement strain (Riedel *et al*., 2009), indicating that the expression of virulence genes is also regulated by the *agr* system. *Listeria monocytogenes* strains expressing truncated InlA exhibited significantly enhanced biofilm-forming ability compared with those expressing full-length InlA (Franciosa *et al*., 2009). As observed in other systems (Davies *et al*., 1998; Hall-Stoodley and Stoodley, 2002; Sauer *et al*., 2002), developmental regulations involved during biofilm formation appear complex and probably transitory. As a general trend, these signalling systems are most certainly involved in physiological regulation beyond biofilm formation itself. Moreover, there is no demonstration that the *agr* system is a mechanism to assess cell density in order to coordinate behaviour of the whole population in *L. monocytogenes* (Garmyn *et al*., 2009); in other words, evidence of a QS system is still awaited in this species.

Extracellular matrix

The extracellular matrix, which encompasses communities of cells in biofilm, is a complex mixture of exopolysaccharides, DNA, proteins, and other extracellular polymeric substances (polyglutamate, teichoic acids, etc.) that plays a structure-stabilizing and protective role in biofilm (Sutherland, 2001). The presence, respective proportions and contribution of these different compounds are highly variable from one bacterial species to another and can even be strain-dependent. While exopolysaccharides have been described as the main component of the extracellular matrix of numerous bacterial biofilms (Whitfield, 1988; Guedon *et al*., 2000; Desvaux and Petitdemange, 2001; Desvaux *et al*., 2001a,b; Desvaux, 2005; 2006; Vu *et al*., 2009; Flemming and Wingender, 2010), their systematic presence and contribution in *L. monocytogenes* biofilm remain somewhat contentious. It is true that a thick and gummy extracellular polymeric substance as observed in some model biofilm-forming bacteria, e.g. *Staphylococcus epidermidis* (Götz, 2002), is not present in *L. monocytogenes*. The presence of fibres binding individual *L. monocytogenes* cells to another and to the surface has been reported on the basis of electron microscopy observations (Borucki *et al*., 2003; Marsh *et al*., 2003; Hefford *et al*., 2005; Zameer *et al*., 2010). It has been speculated though that these structures result from the complete dehydration used in processing samples for electron microscopy, leading to massive shrinkage of the polymeric matrix, leaving only thin fibrillar structures. In parallel, ruthenium red staining has revealed the presence of extracellular carbohydrate in the close surroundings of the *L. monocytogenes* cells, which is consistent with the presence of exopolysaccharides

(Borucki *et al*., 2003; Zameer *et al*., 2010). Nevertheless, because microscopically observed loose meshes of fibrils have not been directly identified as exopolysaccharides and considering that ruthenium red may also bind carbohydrates on the bacterial cell surface that are unrelated to exopolysaccharides, e.g. glycosylated compounds such as peptidoglycan, teichoic acids or proteins, these data are not conclusive. So far, genomic analyses in *L. monocytogenes* have not revealed the presence of biosynthetic pathways for exopolysaccharides like alginate in *Pseudomonas* or poly-*N*-acetylglucosamine in *Staphylococcus* (Harmsen *et al*., 2010). An alternative interpretation of these fibrils, which requires further investigation, resides in the report of putative fimbriae-like structures on the *L. monocytogenes* cell surface (Folio, 2003) (Fig. 1).

As for several bacteria species, including *Staphylococcus* (Qin *et al*., 2007; Rice *et al*., 2007; Izano *et al*., 2008), *Pseudomonas* (Whitchurch *et al*., 2002; Allesen-Holm *et al*., 2006) and *Bacillus* (Vilain *et al*., 2009), it has been recently shown that biofilm matrix of *L. monocytogenes* contains extracellular DNA (eDNA) that plays an important role in initial adhesion and the early stage of biofilm formation (Harmsen *et al*., 2010). The addition of DNaseI significantly reduces cellular attachment resulting in reduced biofilm formation. In contrast, enzymatic removal of both RNA and proteins does not alter the adhesion capacity. While the presence of exogenous DNA alone has no effect, cell attachment can be restored by the additional supply of culture supernatant enzymatically treated with DNaseI and proteinase K. When combined with eDNA, components other than proteins would then permit bacterial adhesion (Harmsen *et al*., 2010). The identification of the peptidoglycan, and more specifically *N*-acetyl glucosamine (NAG), as the causative agent also raises contradictions. It has been shown that the addition of peptidoglycan alone to DNaseI-treated bacteria appears sufficient to restore initial attachment, but eDNA has been described as having a major role in initial attachment. However, the growth medium used was different from one set of experiments to another, i.e. the complex medium BHI and the chemically defined medium HTM respectively. Nonetheless, the length of eDNA is quite important for attachment of *L. monocytogenes* cells since cell adhesion is abolished when low-molecular-weight (LMW) DNA is added to the peptidoglycan. Thus, LMW DNA may act as an inhibitor of components involved in adhesion. Further investigations are required to define at a molecular level the interactions of these components (eDNA, LMW DNA, peptidoglycan, NAG) and their roles in adhesion and biofilm formation in *L. monocytogenes*.

While the importance of eDNA in the early stages of biofilm formation has been demonstrated in *L. monocytogenes*, other studies have shown that surface/extracellular proteins play an essential role at least in the initial attachment to a surface (Smoot and Pierson, 1998; Longhi *et al*., 2008). It has been shown that the adhesion of *L. monocytogenes* to stainless steel and synthetic rubber is reduced by 99% when trypsin is added to the medium (Smoot and Pierson, 1998). Moreover, *L. monocytogenes* fails to produce biofilm following treatment with serratiopeptidase, an extracellular metalloprotease produced by *Serratia marcescens* (Longhi *et al*., 2008), while *L. monocytogenes* biofilm detachment occurs following treatment with endopeptidase K (Franciosa *et al*., 2009).

Biofilm-associated protein (Bap)

Bap (biofilm-associated protein) belongs to a family of surface proteins involved in biofilm formation (Lasa and Penadés, 2006). Bap was first identified in a *S. aureus* mastitis isolate and then found in diverse bacterial species like *Enterococcus faecalis*, but also Gram-negative bacteria, e.g. *Pseudomonas fluorescens* and *Salmonella enterica* sv. *typhimurium* (Cucarella *et al*., 2001; Toledo-Arana *et al*., 2001; Hinsa *et al*., 2003; Latasa *et al*., 2005). All Bap-related proteins have common structural features. They are surface proteins of high molecular weight that contain a core domain of tandem repeats and confer upon bacteria the capacity to form a biofilm. They play a relevant role in bacteria infectious processes and can occasionally be contained in mobile elements (Lasa and Penadés, 2006).

Recently, an *in silico* analysis of the genome of *L. monocytogenes* revealed an open reading frame (Lmo0435) for a protein similar to Bap (Jordan *et al*., 2008). This protein was designated BapL because it presents Bap-like structural features and is required for cell attachment to abiotic surfaces. The *lmo0435* mutant of *L. monocytogenes* 10 403 s shows a significant reduction in attachment level compared with its isogenic parent. In contrast with other Bap-related proteins, BapL is not required for virulence (Jordan *et al*., 2008). Although BapL seems to play an important role in adhesion, only four out 17 *L. monocytogenes* isolates tested possessed the gene encoding this protein. Furthermore, some *bapL*-negative strains adhered significantly better than *bapL*-positive strains such as *L. monocytogenes* 10 403 s and EGD-e, while other strains were strongly impaired in their attachment ability. BapL can contribute to the attachment of some *L. monocytogenes* strains, but its role in biofilm development has not been clearly established. A reduced level of attachment does not prevent the *bapL* mutant from forming a biofilm. This is in marked contrast to all other bacterial species encoding Bap, where deletion of the *bap*-related gene systematically led to defective biofilms (Lasa and Penadés, 2006). Consequently, the capacity of the *lmo0435* mutant to form biofilm should be tested in different conditions to confirm whether or not that BapL is

really functional and actually belongs to the Bap family. Moreover, other proteins with putative Bap structural features should be sought throughout the *L. monocytogenes* genome available to date.

Other molecular determinants

By screening a transposon bank of *L. monocytogenes*, two mutants disrupted in the *relA* and *hpt* genes show impaired biofilm formation (Taylor *et al*., 2002). The *relA* gene encodes a guanosine pentaphosphate synthetase catalysing the formation of the alarmone (p)ppGpp, whereas the *hpt* gene encodes a 6-oxopurine phosphoribosyltransferase that converts the purine base (guanine) into the corresponding nucleotide, i.e. guanosine monophosphate (GMP). Both mutants have been shown to adhere to microtitre plates to a degree comparable to that of the wild-type during the first hour of incubation. However, subsequently the attached bacteria were apparently unable to grow. This suggests that both genes are essential for *L. monocytogenes* growth after attachment to an inert surface. Interestingly, *relA* and *hpt* mutants were unable to synthesize (p)ppGpp in response to nutritional starvation (Taylor *et al*., 2002). Moreover, the level of transcription of the gene *relA* increased after initial attachment, proving that a stringent response is established after attachment. These results strongly suggest that the ability to establish a stringent response and undergo physiological adaptations to nutrient deprivation is essential for the subsequent growth of the adhered bacteria. It would be interesting to determine the role of (p)ppGpp during biofilm formation and understand its regulation in *L. monocytogenes*.

The cyclic diguanylate (c-di-GMP) is another guanine derivative, which plays a crucial role as second messenger, especially for transition between motile planktonic and sedentary biofilm-associated modes of growth in a wide variety of bacteria (Lasa, 2006; Hengge, 2009). This signal transduction system involves multiple diguanylate cyclase and phosphodiesterase enzymes, i.e. proteins exhibiting GGDEF and EAL/HD-GYP motifs respectively. According to the Pfam database, the genome of *L. monocytogenes* EGD-e encodes four proteins exhibiting a GGDEF domain (PF00990: Lmo1912, Lmo2174, Lmo0531 and Lmo1911) and three proteins with an EAL domain (PF00563: Lmo1914, Lmo0111 and Lmo0131). In *Listeria*, though, the c-di-GMP signalling network in biofilm formation has not been deciphered nor has its role even been questioned. An additional secondary signal molecule, i.e. cyclic diadenosine monophosphate (c-di-AMP), has recently been shown in *L. monocytogenes* to trigger a cytosolic pathway of innate immunity in the course of infection (Woodward *et al*., 2010). c-di-AMP has scarcely been investigated in bacteria, but it is thought to regulate bacterial sporulation (Bejerano-Sagie *et al*., 2006). While sporulation does not occur in *L. monocytogenes* (Seeliger and Jones, 1986; Payot *et al*., 1999; Desvaux and Petitdemange, 2002), the possible role of c-di-AMP in listerial physiology, including biofilm formation, merits further investigation.

Under continuous flow conditions, *L. monocytogenes* forms biofilm composed of a network of knitted chains (Rieu *et al*., 2008b). And yet the SOS response factor YneA is specifically activated during continuous flow biofilm formation, with RecA required for its activation (van der Veen and Abee, 2010). The deletion of the *yneA* and *recA* genes leads to a significantly reduced biofilm under flowing conditions, whereas no significant differences were observed under static biofilm conditions. Compared with the wild-type strain which formed a biofilm composed of a complex structure of elongated cells forming a network of knitted chains, *yneA* and *recA* mutants presented some patches of adhered cells after 24 h, which developed to very small microcolonies after 48 h. Elongated cells were not observed for these mutants. The SOS response factors YneA and RecA were not required for initial attachment but were essential for this type of biofilm development.

Recently, the virulence regulator PrfA has been shown to be involved in the regulation of biofilm development (Lemon *et al*., 2010). The *prfA* mutant presents a defective biofilm compared with the wild-type. However, the mutant and wild-type *L. monocytogenes* showed similar adhesion to glass at 20°C, suggesting that biofilm defects occurred after initial surface adhesion. Considering regulation by PrfA is temperature-dependent, adhesion tests were unfortunately not performed at higher temperatures, especially 30°C and 37°C. As PrfA positively regulates both virulence genes and genes involved in biofilm formation, it may play a global role in modulating *L. monocytogenes* lifestyle (Lemon *et al*., 2010).

Conclusion and perspectives

Further investigations are clearly necessary to decipher the molecular mechanisms specific to biofilm formation in *L. monocytogenes* (Table 1). Thanks to recent advances in phylogenetic clustering of *L. monocytogenes* isolates, especially MLST, correlations with biofilm-forming capacity as well as biofilm architecture can be formulated, and require further investigation. Future studies to establish more precisely the role of flagella in biofilm formation should test the swarming over swimming regulation hypothesis. As recently demonstrated in *Caulobacter crescentus* where eDNA masks the adhesive properties of newly synthesized holdfast to enable the escape of motile flagellated cells from the biofilm (Berne *et al*., 2010; Kirkpatrick and Viollier, 2010), differential release of DNA

Table 1. Key questions for future research on molecular mechanisms of biofilm formation in *L. monocytogenes*.

Question	Rationale
Is there a relationship between clonal complexes and the ability to form biofilm?	At the moment, MLST analysis of *L. monocytogenes* is the most representative of the biodiversity of the species. Investigating the correlations between these clonal complexes and biofilm formation is more pertinent than considering the serovars or lineages.
What are the different natures and contributions of the potential exopolysaccharides and other exopolymers?	While the presence of exopolysaccharides is suggested, their secretion must be ascertained following biochemical identification and deciphering of the biosynthetic pathway(s). The contribution of exopolysaccharides to biofilm formation with respect to other alternative exopolymers such as eDNA should also be investigated.
What are the different roles for the flagella in biofilm formation?	Contradictory results emerge from different investigations on the role of the flagella in biofilm formation. While environmental conditions such as growth media or temperatures may account for the contradictory results, the swarming over swimming regulation hypothesis should be tested, as should the interaction with eDNA and other putative exopolymers.
What is the contribution of the secretome to biofilm development?	The secretion systems and secreted proteins, which can remain anchored to the cell envelope or are released into the extracellular milieu, could be involved at different stages of biofilm formation. This involves the study of MSCRAMM, which mediate colonization of both abiotic and biotic surfaces, including food products.
How is the signalling and regulation network engaged in the course of sessile growth?	Signal transduction seems to occur at least via the LuxS and Agr systems. Together with the involvement of second messengers (p)ppGpp, c-di-GMP and possibly c-di-AMP, much remains to be learned about the regulation network associated with biofilm development.

depending on culture conditions might explain the contradictory results regarding the role of the flagella in biofilm development in *L. monocytogenes*. Besides exopolymers produced by some bacteria exhibiting anti-adhesion and biofilm inhibition properties (Valle *et al*., 2006), the use of synthetic or natural compounds that could outcompete QS and then prevent biofilm formation (Rice *et al*., 1999; 2005; de Nys *et al*., 2006; Chorianopoulos *et al*., 2010) has not yet been investigated in *L. monocytogenes*.

As functional determinants interfacing the cell with its surroundings (Desvaux *et al*., 2003; 2005a,b; 2006a; 2009a,b; Henderson *et al*., 2004), secreted proteins certainly deserve more careful attention, especially MSCRAMM (microbial surface components recognizing adhesive matrix molecules), which can both take part in colonization of abiotic and biotic surface and in bacterial virulence (Desvaux and Hébraud, 2006; 2008; Desvaux *et al*., 2006b). Protein secretion and its consequences are under active investigation in our laboratory. While the infection cycle of *L. monocytogenes* does not leave room for surface colonization (Tilney and Portnoy, 1989; Sleator *et al*., 2009), the presence of several genes coding for MSCRAMM suggests that interactions might occur at some stage in the lifetime of this bacterial species. In other words, interactions with biotic surfaces could occur not only in the course of infection but also in the environment. When considering a foodborne pathogen, its involvement in the colonization of food products and food-processing environments is a legitimate and exciting hypothesis, which has so far been overlooked, but is currently being tested by our group. Several MSCRAMM proteins putatively involved in adhesion to fibronectin, mucin and collagen have been reported in *L. monocytogenes* (Bierne and Cossart, 2007). Few have been functionally and experimentally characterized, but internalins InlB, InlC and InlJ have been shown to bind human mucin MUC2 (Lindén *et al*., 2008). FpbA (fibronectin binding protein A) is a surface protein associated with the membrane by an undetermined mechanism (Dramsi *et al*., 2004). Nonetheless, FpbA has been confirmed to bind human fibronectin, contributing to eukaryotic cell adhesion and participating in bacterial virulence. The example of other investigated fibronectin-binding proteins suggests that MSCRAMM proteins may also be involved in adhesion and colonization of abiotic surfaces such as polystyrene (Shimoji *et al*., 2003; O'Neill *et al*., 2008).

Apart from proteins directly involved in bacterial adhesion to and colonization of both biotic and abiotic surfaces, characterization of gene determinants responsible for the regulation of biofilm formation remains a key challenge in *L. monocytogenes*. Considering the multifactorial nature of biofilm formation, obtaining a clear and unequivocal altered phenotype is often hampered when mutating a single gene encoding a structural protein. However, such a phenotype could most certainly be observed by mutating genes encoding signal transducers or transcriptional regulators controlling the expression of several genes encoding proteins physically engaged in cell attachment or the colonization process. In the context of microbial food safety, the influence of environmental conditions on biofilm formation and the regulation network engaged in the course of sessile growth are of crucial

importance for the development of practices and policies to limit and even prevent the contamination of food-processing plants and ultimately of food products. Multidisciplinary strategies, such as genomics, transcriptomics, proteomics, functional genetics and state-of-the-art microscopic techniques, are undoubtedly required to identify, target and characterize these gene products (Tremoulet *et al*., 2002; Planchon *et al*., 2007; 2009; Dumas *et al*., 2008; 2009a,b; Stewart and Franklin, 2008; Wood, 2009; Desvaux *et al*., 2010). The implementation of these complementary and readily available approaches promises major findings ahead in the field of molecular biology of surface colonization by *L. monocytogenes*. In the fight against listeriosis and following the adage 'mieux vaut prévenir que guérir' or 'an ounce of prevention is worth a pound of cure', research with the long-term aim of prevention complements investigations of listerial virulence whose shorter-term purpose is curative with the development of new treatments for infected people.

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