Sodium chloride affects Listeria monocytogenes adhesion to polystyrene and stainless steel by regulating flagella expression.

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ORIGINAL ARTICLE

Sodium chloride affects *Listeria monocytogenes* adhesion to polystyrene and stainless steel by regulating flagella expression

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Abstract

Aim: To study the adhesion capability of seven strains of *Listeria monocytogenes* to polystyrene and stainless steel surfaces after cultivation at various NaCl concentrations.

Methods and Results: Determination of growth limits indicated that all seven strains were able to grow in up to 11% NaCl in rain heart infusion and 3 g l^{-1} yeast extract–glucose at 20°C, but no growth was detected at 15% NaCl. Adhesion of *L. monocytogenes* was estimated after 4-h incubation at 20°C in 96-well microtitre plates. Statistical results revealed no significant difference between adhesion to polystyrene and stainless steel although surface properties were different. Adhesion between 0% and 6% NaCl was not different, whereas adhesion at 11% NaCl was significantly lower. This discrepancy in adhesion was correlated with the down-regulation of flagella at 11% NaCl.

Conclusions: Only high salinity levels, close to nongrowth conditions, repressed the expression of flagella, and consequently, decreased the adhesion capability of *L. monocytogenes*.

Significance and Impact of the Study: Adhesion of *L. monocytogenes* to inert surfaces depends on environmental conditions that affect flagellum expression. High salinity concentrations would delay biofilm formation.

Introduction

Listeria monocytogenes is a ubiquitous, Gram-positive, motile rod that causes listeriosis, a severe foodborne infection. The virulence of *L. monocytogenes* in humans is characterized by meningitis, gastroenteritis, miscarriage and septicemia with an overall mortality rate of 20–30% (Rocourt *et al.* 2000). *Listeria monocytogenes* is able to defy food protection conditions such as refrigeration, acidic pH and high salinity by maintaining its multiplication ability in these conditions (Farber and Peterkin 1991). Despite considerable focus on preventing the contamination of food products, *L. monocytogenes* still remains a threat to consumer health. In 2006, listeriosis was reported in 23 European Union Member States and was the fifth most common zoonotic infection in Europe (Anon 2007). Furthermore, in European countries, the incidence rate of listeriosis has recently increased (Anon 2007). This pathogen is most often reported above the legal safety limit in ready-to-eat (RTE) fishery products, followed by cheeses and other RTE products. *Listeria monocytogenes* can not only be found in food products but also be attached to food-processing facilities and equipment such as floors, walls, salt hoppers, brine containers, drain grids, store boxes, gaskets, conveyor belts, slicing, dicing and packaging machines (Holah and Gibson 2000; Tresse *et al.* 2007). This is explained by its ability to adhere to inert surfaces and to form biofilms that become less susceptible to cleaning procedures (Stewart *et al.* 2000; Stopforth *et al.* 2002). The biofilm eventually constitutes a reservoir of dissemination and cross-contamination in foods. The pre-requisite step for its formation is the initial adhesion of cells to inert surfaces (Bryers 2000). Biofilm development depends on

bacterial history and environmental conditions. To determine the adhesion ability of *L. monocytogenes*, the effects of various environmental conditions on adhesion and biofilm formation have been studied (Moreto and Langsrud 2004 and references therein). The presence of other bacteria, nutrients and surface conditioning also affect the adhesion ability of *L. monocytogenes*. However, the influence of salt on cell adhesion and biofilm formation has been poorly investigated. Although the effect of NaCl during biofilm formation has been explored (Cataldo *et al.* 2007; Jensen *et al.* 2007), the effect of salt prior to initial adhesion has not been evaluated.

The biological molecular mechanisms behind the adhesion of *L. monocytogenes* have not yet been elucidated. The best evidence is the contribution of flagella during the initial step of biofilm formation (Vatanyopaisarn *et al.* 2000; Tresse *et al.* 2006; Lemon *et al.* 2007). *Listeria monocytogenes* is a remarkable bacterium in the sense that its flagellum expression can be regulated by temperature and pH (Peel *et al.* 1988; Tresse *et al.* 2006). In this study, we have investigated the adhesion capacity of seven *L. monocytogenes* strains on two inert surfaces and examined their flagellation ability after cultivation at different NaCl concentrations.

Materials and methods

Bacterial strains, media and culture conditions

Seven strains from diverse origins were used in this study. Three clinical strains were isolated from patients with listeriosis (Lm ScottA, serotype 4b, Lm EGD-e, serotype 1/2c and Lm 00/10/17-12, serotype 4b), one strain was isolated from seafood (Lm CL297, serotype 1/2a or 3a) by AFSSA (Boulogne-sur-mer, France), two strains were isolated from dairy plants (Lm X-Li-mo 500, serotype 1/2a and Lm X-Li-mo 506, serotype 4e) by the Pasteur Institute (Lille, France), and one strain was isolated from pork (Lm 111, serotype 1/2a or 3a) by ADRIANOR (Arras, France). Each strain was subcultured by adding 400 μl of the stock bacterial culture stored in cryotubes at −80°C to 2 ml of brain heart infusion, 3 g l^{-1} yeast extract–glucose (BHIYE; Biokar Diagnostics, Beauvais, France) and 2 g l^{-1} glucose and incubated at 20°C for 8 h. Half of the 8-h subculture was transferred to 10 ml of BHIYE-glucose and incubated at 20°C overnight. Cultures were obtained by inoculating 2 ml of the second subculture in 100 ml BHIYE-glucose supplemented with 0%, 6%, 11% or 15% NaCl (w/v), corresponding to 0, 1·0, 1·9 and 2·6 mol l^{-1} NaCl and incubated at 20°C in a rotary shaker at 150 rev min^{-1}. Growth parameters of each culture were obtained by following the growth at 20°C by optical density (OD) at 550 nm using a spectrophotometer.

Bacterial adhesion to polystyrene and stainless steel

Cells were harvested in the stationary phase at the point corresponding to the time needed to reach the stationary phase + 25% of this time. Each bacterial culture was centrifuged at 3000 ***g*** for 20 min at 20°C. The cell pellet was washed with 30 ml physiological medium (TS) [8·5 g tryptone and 0·85% (w/v) NaCl], centrifuged at 3000 ***g*** for 20 min at 20°C and resuspended in 10 ml of TS supplemented with chloramphenicol at a sub-bactericidal concentration (100 μg ml^{-1}) to prevent further cell growth, multiplication and adaptation during the assay, as previously described by Tresse *et al.* (2006). The bacterial adhesion was performed in 96-well sterile polystyrene microtitre plates (Greiner Bio-One, Courtaboeuf, France) and in 96-well stainless steel 316L microtitre plates manufactured by MECA-CN (Widehem, France). Assays were conducted according to the method as previously described by Tresse *et al.* (2007). Briefly, adhesion assays were carried out during 4 h at 20°C in eight replicates (eight wells) repeated three times from independent cultures for each condition. The initial bacterial population (X) was calculated with the mean of colonies enumerated on both oxford and trypticase soy agar and 6 g l^{-1} of yeast extract. After being stained with 1% (w/v) crystal violet, the resulting coloured solution, reflecting attached cells, was transferred to a 96-well sterile polystyrene microtitre plate and assessed using a Multiskan Spectrum microtitre plate reader (Thermo Life Science, Cergy-Pontoise, France) at OD 595 nm. Then, the number of attached cells after 4 h (Y) was determined from a calibration curve that correlates the number of cells to the crystal violet intensity determined at $OD_{595\ nm}$. The percentage of attached cells was calculated using the following equation:

$$\text{Attached cells}\,(\%) = \frac{Y}{X} \times 100$$

Flagella detection

Flagella of *L. monocytogenes* were detected using the Leifson staining method (Leifson 1951). A quantity of 1 ml of culture, before each adhesion assay, was centrifuged at 3000 ***g*** for 20 min at 20°C. The pellet was suspended and diluted 100 times in the physiological medium adjusted to the same pH of the cell culture. Ten microlitres of the suspension was loaded in triplicate in 4-mm diameter circles on a glass slide manufactured for immunofluorescence assays. After air-drying, the cells were stained according to the Leifson protocol for a contact time of 8 min. Cells and flagella were then observed at 1250× using a BH-2 microscope (Olympus, Rungis, France) and photographed with a Camedia digital camera (Olympus).

Statistical analyses

Results were analysed with Statgraphics Plus 5.1 software (StatPoint Inc., Herndon, VA, USA) using the general linear models designed to obtain the analysis of variance (ANOVA). The significance level was chosen at 99%; consequently an effect was considered significant if its *P*-value was <0·01. To determine the individual effect of each variable and potential interacting effects between strains, the surface of the substrata, and NaCl concentrations, main effects and all combinations at the second order were calculated based on the values obtained from the three independent trials. The combination NaCl × Surfaces was excluded from the model as NaCl concentration varied in cultures but not during adhesion assays. The statistical data were completed by a Scheffé multiple comparison with a significance level at 95%.

Results

Growth occurring in BHIYE-glucose at 20°C at different concentrations of NaCl indicated that all strains were able to multiply in up to 11% NaCl (Table 1). No growth was detected at 15% NaCl after 60 days of incubation at 20°C in BHIYE-glucose for all strains (data not shown). The lag phase was similar for all strains at 0% NaCl (0–1 h), 6% NaCl (3 h) and 11% NaCl (25–27 h) except for EGD-e (2, 9 and 60 h, respectively), Lm 00/10/17-12 (85 h) and Lm X-LiMo 500 (42 h) at 11% NaCl. For all conditions, the stationary phase was achieved within a minimum of 14 h at 0% NaCl and a maximum of 227 h at 11% NaCl. As expected, the growth rate decreased with the increase in NaCl concentration (Table 1).

For adhesion assays, cells were harvested in the stationary phase at the same growth evolution state (stationary phase + 25%) for all NaCl concentrations. Figure 1 presents the adhesion results obtained on polystyrene and stainless steel, and Table 2 indicates the statistical analysis results. The ANOVA analysis showed that the main effects on adhesion were mainly because of NaCl concentration and strains ($P < 0{\cdot}01$) but not because of surfaces ($P = 0{\cdot}0483$). Independent factor interactions at the second order showed a significant effect ($P < 0{\cdot}01$) for NaCl × Strains but not for Strains × Surfaces ($P = 0{\cdot}1378$).

Table 1 Growth parameters of seven strains of *Listeria monocytogenes* in brain heart infusion, 3 g l^{-1} yeast extract–glucose at 20°C in the presence of 0%, 6% and 11% NaCl

	μmax (h^{-1})			Lag phase (h)			Beginning of stationary phase (h)		
NaCl (%)	0	6	11	0	6	11	0	6	11
Lm CL297	0·61	0·29	0·08	0	3	25	14	25	124
Lm 00/10/17-12	0·55	0·19	0·05	1	3	85	14	27	227
Lm X-Li-Mo 506	0·58	0·27	0·08	0	3	25	14	25	124
Lm X-Li-Mo 500	0·58	0·29	0·06	0	3	42	14	25	130
Lm ScottA	0·60	0·34	0·07	0	3	25	15	25	124
Lm EGD-e	0·56	0·11	0·08	2	9	60	20	31	177
Lm 111	0·55	0·29	0·06	0	3	27	14	25	130

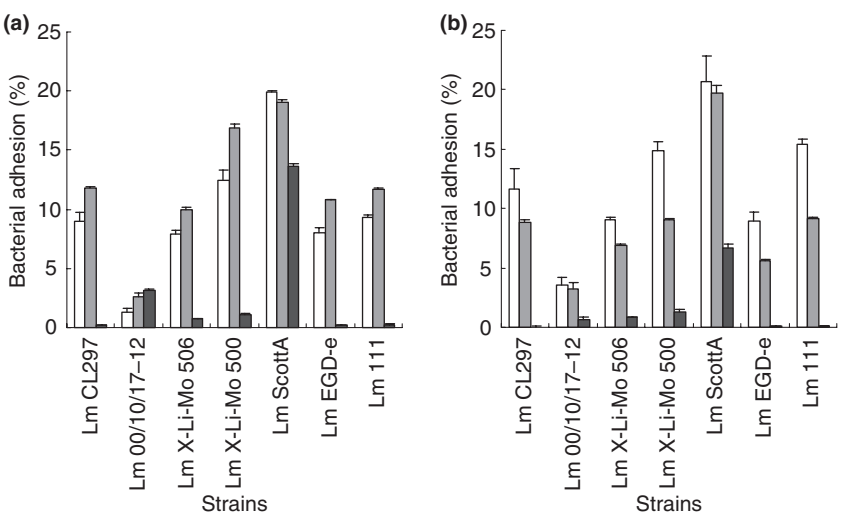

Figure 1 Adhesion of *Listeria monocytogenes* to polystyrene (a) and stainless steel (b) after cultivation at 20°C in brain heart infusion, 3 g l^{-1} yeast extract–glucose at 0% (white bars), 6% (grey bars) and 11% (black bars) NaCl. Error bars represent standard deviation ($n = 3$).

Source	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value
Model	4572·14	27	169·34	52·30	<0·001
Main effects					
NaCl	2054·96	2	1027·48	317·35	<0·001
Strains	2121·23	6	353·54	109·19	<0·001
Surfaces	12·95	1	12·95	4·00	0·0483
Interactions					
NaCl × Strains	350·66	12	29·22	9·03	<0·001
Strains × Surfaces	32·34	6	5·39	1·66	0·1378
Residual	317·30	98	3·27		
Total (corrected)	4889·44	125			

R^2 (adjusted for d.f.) = 91·72%.

Table 2 Significance of linear model analysed by ANOVA; the factors and their second-order interactions for adhesion (%)

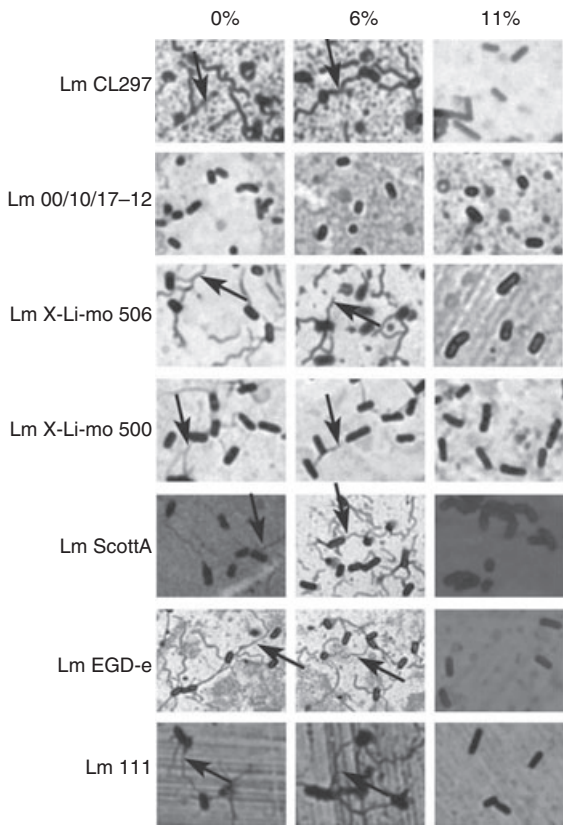

Figure 2 Microscopic images (1250X) of Leifson-stained *Listeria monocytogenes* after cultivation at 20°C at 0%, 6% and 11% NaCl. Black arrows indicate flagella.

The Scheffé grouping analysis ($P < 0·05$) indicated that Lm 00/10/17-12 was significantly less adherent than the other strains, and Lm ScottA was readily more adherent. Concerning the NaCl effect, growth in 0% and 6% NaCl did not affect adhesion, while growth in 11% NaCl significantly affected it ($P < 0·05$).

Microscopic observations of flagella on strains cultivated at different NaCl concentrations are shown in Fig. 2. Flagella were present on all strains, except Lm 00/10/17-12, at 0% and 6% NaCl. In the presence of 11% NaCl, no strain displayed any detectable flagellum.

Discussion

Growth limits in the presence of NaCl were determined for seven strains of *L. monocytogenes*. All strains could multiply in up to 11% NaCl in rich medium at 20°C with a stationary phase reached at a maximum of 227 h. However, no growth was detected at 15% NaCl after 1440 h (60 days) in the same conditions. This is consistent with the reported growth of *L. monocytogenes* in rich media (Conner *et al.* 1986; Cole *et al.* 1990; Painter and Slutsker 2007). Recently, growth limits have been evaluated in 127 (Shabala *et al.* 2008) and 138 strains (van der Veen *et al.* 2008) of *L. monocytogenes* in BHI at 25 and 30°C, respectively. These surveys showed that the majority of strains were able to grow in the presence of 11·3–11·6% NaCl, a few strains grew in 12·2% or 13% NaCl, and only 4·7% were able to grow in 13·9% NaCl. Therefore, the seven strains selected in our study were found to have growth limits in NaCl similar to those described in general for *L. monocytogenes*.

The influence of NaCl on the adhesion capacity of these seven strains of *L. monocytogenes* was assessed on 96-well microtitre plates of polystyrene and stainless steel. Previous determinations of physico-chemical properties of these surfaces indicated that polystyrene was uncharged and highly hydrophobic, while the stainless steel surface was slightly hydrophobic and negatively charged (Tresse *et al.* 2007). The absence of a contribution of the surface properties to adhesion after cultivation in the presence of salt is consistent with a previous study conducted on 101 strains of *L. monocytogenes* (Tresse *et al.* 2007). Beresford

et al. (2001) also pointed out the weak effect of the surface property on the adhesion capability of *L. monocytogenes* 10403S by comparing its adhesion to 17 different food-use surfaces. In addition, the present study showed that cells grown in the presence of NaCl did not favour adhesion to one type of surface. Statistical results also confirmed the strain variability in cell adhesion capability (Tresse *et al.* 2007). Overall, in the presence of NaCl, a significant decrease in adhesion was observed at 11% when compared to that observed at 0% and 6% NaCl.

Jensen *et al.* (2007) reported that biofilm production in plastic microtitre plates was equally low at 35°C for LM2 *L. monocytogenes* at NaCl concentrations ranging from 0% to 10%, and Cataldo *et al.* (2007) found that biofilm production increased at 37°C with NaCl concentrations up to 5% because of cell aggregation. However, cell aggregation was not observed at temperatures used in food-processing conditions. In these studies, the effect of salt on cell physiology could not be distinguished from the effect of surface conditioning by salt. Surface conditioning by salts of divalent cations and NaCl was shown to enhance the attachment of bacterial cells (Barnes *et al.* 1999). This was explained by the increase in the overall ionic strength of the adhesion medium (Briandet *et al.* 1999). In the present study, only the first step of biofilm formation (adhesion) was evaluated. This enabled changes in ionic strength to be avoided as no salt was added during adhesion. Thus, only salt effects on cell physiology prior to cell adhesion were measured. The unchanged adhesion capability observed after cell cultivation at 0% and 6% NaCl indicated that salt had no effect on cell adhesion at these concentrations. At 11% NaCl, the decrease in adhesion was correlated with the down-regulation of flagella for six strains. The last strain (Lm 00/10/17-12) was found to be aflagellated in all conditions. The adhesion capability was equally affected at very high salt concentrations in strains from diverse origins (clinical, food or food-processing) and different serotypes (1/2a or 3a, 1/2c, 4b, 4e) indicating that variation in adhesion was not linked to either processing history or different flagellar antigens expressed at the cell surface.

The role of flagella in the biofilm development of *L. monocytogenes* is extremely puzzling. By analysing differences in protein patterns between biofilm and planktonic cells, Tremoulet *et al.* (2002) observed that flagellin, the major protein of the flagellum appendage, was down-regulated during the biofilm growth of *L. monocytogenes*. Using mutants defective for flagellum formation or for motility and chemically paralysed strains, Lemon *et al.* (2007) found that motility was critical for the late steps of biofilm formation, while Todhanakasem and Young (2008) recently observed that the loss of flagellum-based motility of *L. monocytogenes* 10403S resulted in the formation of hyperbiofilms in dynamic conditions (flow cells). However, the contribution of flagella to the biofilm initiation of *L. monocytogenes* is more consensual (Vatanyopaisarn *et al.* 2000; Lemon *et al.* 2007; Todhanakasem and Young 2008). Flagella were also described as contributing to the adhesion to inert surfaces of other microorganisms such as *Campylobacter jejuni* (Joshua *et al.* 2006), *Achromobacter piechaudii* (Nejidat *et al.* 2008) and *Aeromonas caviae* (Kirov *et al.* 2004). Generally, it is assumed that flagellum expression in *L. monocytogenes* is repressed at temperatures above 25°C (Peel *et al.* 1988). It was also demonstrated that flagella of *L. monocytogenes* were repressed at pH below 6 and that repression was responsible for less adhesion of *L. monocytogenes* to inert surfaces (Tresse *et al.* 2006). This indicates that diverse environmental conditions could influence the regulation of flagella of *L. monocytogenes*. Responding to osmotic or pH stresses requires metabolic energy to restrict water loss by accumulating osmoprotectants (Shabala *et al.* 2006) or to maintain intracellular homoeostasis (O'Byrne and Booth 2002). Although the cell benefit of flagella down-regulation by temperature is not explained, the down-regulation of flagella at pH and NaCl concentrations close to nongrowth conditions could be an energy-saving mechanism to resist acidic pH and hyperosmotic environments.

In conclusion, very high salt concentrations repressed the expression of flagella thus decreasing the adhesion capability of *L. monocytogenes* to both polystyrene and stainless steel. In the food industry, especially in the fish and cheese sectors, high salinity levels are used for food processing or food storage. However, only salinity concentrations close to nongrowth conditions will prevent adhesion and probably delay the formation of a biofilm of *L. monocytogenes* on inert surfaces.

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