LISTERIA MONOCYTOGENES FROM MEAT AND MEAT-PROCESSING ENVIRONMENTS: BIOFILM FORMATION, MOTILITY AND HYDROPHOBICITY

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Introduction

When bacteria attach to surfaces and colonise them, their phenotypes change. They show, for instance, a decreased susceptibility to antimicrobials, increased exopolysaccharides production, and the community they form is called a biofilm (Costerton *et al.,* 1987). Biofilms create major problems in the food industry, since they, represent an important source of contamination of materials or foodstuffs coming into contact with them (Mattila-Sandholm and Wirtanen, 1992). *Listeria monocytogenes* is part of the microflora most commonly found in the meat industry (Roberts and Wiedmann, 2003). It has been shown that *L. monocytogenes* strains that persist in food industry environments form thicker biofilms than isolates found only sporadically (Lunden *et al.,* 2000), indicating that biofilm formation is important for the survival of *L. monocytogenes*. The quantity of microrganisms transferred to an inhert surface or food depends on the physicochemical properties of both surfaces and microrganisms and factors such as temperature (Fletcher, 1977), and hydrophobicity (Hood and Zottola, 1995). Further, it has been shown that flagella can positively modulate adhesion to both living and non-living surfaces (Vatanyoopaisarn *et al.,* 2000). The aim of this study was to evaluate the ability of 23 strains of *L. monocytogenes*, from meat and meat industry-environments, to form biofilms on three different surfaces commonly used in food-processing plants (polystyrene, glass, and stainless steel) with regard to different temperatures (4, 12, 22, 37°C), hydrophobicity and motility.

Materials and Methods

Bacterial strains. Twenty-three *L. monocytogenes* strains were tested: 19 from meat, and 4 from meat-processing plants by swabbing food and other contact surfaces and equipment. Biofilm production assay. Overnight cultures of *L. monocytogenes* were prepared, from o/night Trypticase Soy Agar-growth, in Trypticase Soy Broth (TSB) by incubating at selected temperature (4, 12, 22, or 37°C) under dynamic conditions. Cultures were then washed twice with phosphate-buffered saline (PBS; pH 7.3) and diluted with fresh TSB to reach a concentration of about 1 x 10^8 CFU/ml. Food-processing surfaces (glass, polystyrene, and stainless steel) were exposed to a standardized inoculum in 35-mm-diameter polystyrene tissue culture plates, and incubated at selected temperatures. After 24h-incubation nonadherent cells were removed by three washings in sterile PBS. *L. monocytogenes* biofilm biomass was determined spectrophotometrically (OD_{492}) by the crystal violet-based colorimetric method of Christensen *et al.* (1985). Considering the different growth surfaces of test materials, results were normalized by calculating the "biofilm production index" (BPI) = [$OD_{meaubiofilm}$/surface (mm^2)] x 1.000. Cell surface hydrophobicity assay. *L. monocytogenes* hydrophobicity was evaluated by Microbial Adherence to n-Hexadecane (MATH) test. Briefly, 4 ml of OD_{550} = 0.8 standardized inoculum in PBS were overlayed with 0.4 ml of n-hexadecane. After agitation for 1 min by vortexing, the phases were allowed to separate for 15 min at room temperature. The results were expressed as the proportion of the cells which were excluded from the aqueous phase, determined by the equation: $[(A_0 - A)/A_0] \times 100$, where A_0 and A are the initial and final optical densities of the aqueous phase, respectively. Strains were classified as: highly hydrophobic, for values >50%; moderately hydrophobic, for values ranging from 20 and 50%; and hydrophilic, for values <20%. Cell motility. Swimming, swarming, and twitching assays were performed at selected temperatures by inoculating a single colony from an agar growth. i) Swimming. Media used for assay was tryptone broth (10g/liter tryptone, 5 g/liter NaCl) that contained 0.3% agar. After incubation for 24 h, results were expressed as diameter (mm) of growth zone. ii) Swarming. Media used for assay consisted of 0.5% agar with 8 g/liter nutrient broth and 5 g/liter glucose. Swarm plates were typically allowed to dry at room temperature o/night before being used. Results were expressed as diameter (mm) of growth zone. iii) Twitching. Assay was performed with LB broth (10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl) solidified with 1% agar. Twitch plates were briefly dried and strains were inoculated on the bottom of the Petri dish plate. After incubation for 24 h, the zone of motility at the agar/Petri dish interface was measured by staining with crystal violet. Statistical analysis. Assays were carried out in triplicate and repeated on two different occasions. Data were shown as mean ± standard deviation (SD). The significance of differences in biofilm formation was assessed by ANOVA, followed by Newman-Keuls Multiple Comparison Test. Relationship between biofilm formation and MATH% or motility was evaluated by calculating correlation Pearson r coefficient (r_P). Statistical significance was evaluated at a P value of < 0.05.

Results and Discussion

Biofilm, expressed as OD_{492}, was produced at significantly ($P<0.05$) higher levels on glass at 4°C (0.210±0.119), 12°C (0.265±0.124), and 22°C (0.392±0.167). At 37°C biofilm levels were comparable, regardless of substrate (0.571±0.262, 0.491±0.226, and 0.454±0.279 on glass, stainless steel, and plastic, respectively). According to previous studies (Blackman and Frank, 1996; Lunden *et al.*, 2000), our results showed that *L. monocytogenes* is able to adhere and form biofilm on commonly used surfaces in food-industry, regardless of the temperature tested. However, the ability to form biofilms was extremely variable in the population studied, making it difficult to select strong biofilm-producers strains with increased probability to persist in a food-processing environment. Bacterial adhesion to substrate is a consequence of complex physicochemical interactions, such as cell surface hydrophobicity (Hood and Zottola, 1995). Cell surface hydrophobicity level increased with temperature, although hydrophobicity at 37°C (mean MATH%, 32.3±10.1) was significantly ($P<0.001$) higher than those produced at 22°C (16.6±17.6), 12°C (12.4±6.0), and 4°C (8.7±10.6). It is plausible to hypothesize that temperature influences cell surface proteins and, consequently, the resulting hydrophobicity level (Chavant *et al.*, 2002). At 4°C, hydrophobicity levels negatively correlated with biofilm formation on both plastic (Pearson $r=-0.491$; $P<0.05$) and glass (Pearson $r=-0.499$; $P<0.05$). No strains were mobile by swarming or twitching, regardless of temperature. Conversely, 12 (52.2%) out of 23 strains tested exhibited flagellar motility (swimming) at 22°C. Motility by flagella, overcoming electrostatic repulsive forces, could facilitate the interaction between microrganism and substrate and so improve biofilm formation (Vatanyoopaisarn *et al.*, 2000). However, our results showed that swimming motility never correlated with biofilm produced, regardless of substrate considered.

Conclusions

The present work suggests that *L. monocytogenes* is able to organise itself as biofilm on surfaces commonly used in the food industry, regardless of temperature considered. This ability was exhibited also at 4°C, so raising a relevant hygienic/sanitary issue. Polystyrene is in use as a packaging material but is not much used in equipment in the food industry (Moretro *et al.*, 2003). However, our results suggest the use of polystyrene in food-processing plants since it might minimize biofilm formation, regardless of temperature considered.

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