The attachment efficiency of cell-walled and L-forms of *Listeria monocytogenes* to stainless steel

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Abstract

There is limited knowledge regarding the attachment of cell wall-deficient forms of *Listeria monocytogenes* to abiotic and food contact surfaces. A typical L-form phenotype of *Listeria monocytogenes* was induced, propagated and recovered in both broth and plate culture by exposure to sub-lethal concentrations of ampicillin. Attachment of both cell-walled and L-forms to stainless steel were compared using viable counts. L-forms possessed no cell wall material and were present as spherical cells or small rods. L-form cells had the capacity to attach sufficiently well to stainless steel (0.3% of cells attached) with a higher efficiency than parental cells (0.002%) after 8 h exposure to culture. After longer exposure times of 18 h, the parental cells attached with slightly higher efficiency (0.8%) than L-forms cells (0.625%). The ability of *Listeria monocytogenes* L-forms to attach to stainless steel may suggest that a classical rigid cell wall structure is not a prerequisite for cell adhesion *in vitro*.

Key words: Surface-attached bacteria, *Listeria monocytogenes* L-form, ampicillin.

Introduction

*Listeria monocytogenes* has long been known as a cause of meningitis and other invasive infections in immunocompromised hosts. The natural habitat of these bacteria is thought to be decomposing plant matter, in which they live as saprophytes. In general, the bacterium is associated with foodborne outbreaks and has been isolated from a substantial portion of raw and ready-to-eat meats, poultry, seafood, dairy products, vegetables and also from sewage sludge and river water and has been found to survive even in fermented foods; for example cheese, yoghurt and sausage. The attachment of *L. monocytogenes* and other microorganisms to food contact surfaces is a concern in the food industry. It was found that vegetables/fruits and abiotic (stainless steel, glass, polypropylene and rubber) surfaces display many possible sites for attachment of *L. monocytogenes* at 4 to 20°C after a contact time as short as 20 minutes to 1 h, with extracellular material being observed on all surfaces. Under suitable conditions attached organisms may form biofilms, and it is these biofilms that are of concern because they are more resistant to sanitizers than are planktonic cells. The attachment of *L. monocytogenes* to stainless steel at various temperatures and pH values and biofilm formation by *L. monocytogenes* on stainless steel and buna-n rubber has been demonstrated. Furthermore, it was found that *L. monocytogenes* utilizing *Pseudomonas fragi* as a primary colonizing organism can form a biofilm on glass cover slips. Studies on attachment and biofilm formation by cell wall-deficient, the so-called “L-form”, organisms have been limited. In this study, we describe the attachment efficiency of *Listeria monocytogenes* L-form cells to stainless steel and compare it to the parental cell types.

Materials and Methods

**Bacterial strain and growth conditions:** *Listeria monocytogenes* ATCC 23074 serotype 4b was maintained on tryptic soy agar (TSA, Difco, Detroit, MI) and grown in tryptic soy broth (TSB, Difco, Detroit, MI) for 18 h at 30°C when needed. L-form cultures were maintained on TSA supplemented with 0.5 µg/ml ampicillin. Lambda buffer (MgSO4-7H2O, 2.5 g, gelatin, 0.05 g, Tris 1 M, pH 7.2, 6 ml per L) was used for all rinsing. L-forms were induced by treating 100 µl of classical culture (10⁷ CFU) in 10 ml of TSB containing 50 µg/ml ampicillin for 18 h at 30°C. The culture (10⁷ CFU/ml) was then plated onto TSA (0.5 µg/ml ampicillin) and incubated at 30°C for up to 3 weeks. Viable count measurements of both culture types were performed using the method of Miles et al. Colonial morphologies were compared to those shown by Brem and Eveland. *Transmission electron microscopy:* Classical and L-form culture were examined by transmission electron microscopy (TEM). Appropriate cultures were grown overnight. Bacteria were pelleted and washed 2 times with Hepes buffer, pH 6.8 (Research Organics Inc., Cleveland, OH), at a concentration of 0.5 M for classical culture and 1.5 M for L-forms. They were fixed in 2% glutaraldehyde for 2 h at 4°C and then washed 3 times with the Hepes buffer, pH 6.8 (0.05 M for classical culture and 0.15 M for L-forms). This was followed by fixing and staining with 2% OsO₄ for 2 h at 4°C. Samples were then washed 3 times with Hepes buffer and enrobbed in 2% Nobel agar (Difco, Detroit, MI). The samples were again fixed and stained with 2% uranyl acetate and this was followed by washing 3 times with Hepes buffer. Samples were dehydrated through an ethanol series 25, 50, 75, 90, and 2 times 100% for 15 minutes at each concentration. They were then placed in 50/50 absolute EtOH/LR White resin (London Resin Co., Berkshire, U.K.)
on a rotator at 4°C followed by 100% LR White resin on a rotator for 8 h at 4°C. After embedding in a gelatin capsule in 100% LR White, samples were polymerized at 60°C for 1 h. Samples were then sectioned and post-stained with 1% uranyl acetate (5 minutes) and Reynolds Lead Citrate (1 minute) and viewed on a Philips EM 300 (Philips Electronics, Holland).

Bacterial adhesion: One hundred µl of classical or L-form overnight culture were inoculated into 10 ml fresh TSB (containing 0.5 µg/ml ampicillin for L-form). Clean sterile stainless steel squares (No 304, 1/2” x 1/2” x 1/8”) were employed as the surface for attachment. Triplicate squares were immersed in 10 ml of appropriate culture in sections of sterile divided Petri plates containing 1 piece of stainless steel per section. Viable counts were performed for each initial culture. After incubation for 8 h and 18 h at 30°C and 25 rpm, the treatments were terminated by removing unattached bacteria and washing the stainless steel three times in a reproducible manner with copious quantities of phosphate-buffered saline (PBS). Rinsed stainless steel squares were each placed in 50 ml centrifuge tubes. Attachment was assessed for each square by viable count of bacteria dislodged from surface by 15 minutes contact with 1 ml of PBS supplemented with 2% Tween 80 followed by scraping and vigorous vortex mixing for 30 second to remove adhered bacteria 27. Viable count for the final 18 h broth culture was also performed.

Results

Confirmation of the L-form: Typical L-form phenotypes were observed by plating cultures onto TSA supplemented with 0.5 µg/ml ampicillin. L-colonies were rough, diffuse, and granular (Fig.1) and required at least 72 h to be visible. Classical and L-form cells were compared by TEM. Classical cells displayed characteristic intact cell walls (Fig. 2a) and L-form cells were short and oval or spherical (0.5 µm in diameter) with no cell walls (Fig.2b).

Adhesion of classical and L-forms to stainless steel: Classical and L-form cultures were compared for their ability to adhere to and produce biofilms on stainless steel squares. Both culture types formed biofilms on the squares after 8 and 18 h incubation (Table 1). In spite of the fact that the initial culture levels used to form biofilms for the classical form was 1 log cycle higher than the L-form culture, L-form attachment to stainless steel squares was consistently 1 log cycle higher than that for parent cells exposed to culture for 8 h and with a greater efficiency (0.3% of cells attached) than for the cell walled cells (0.002%). After 18 h exposure, the classical cells appeared to adhere more readily to stainless steel than the L-forms (Table 1) and, during this treatment, both cell types attached with almost similar efficiency (0.8% for parental strains and 0.625% for L-forms cells).

Discussion

Ampicillin present in liquid or solid culture generates cell wall-deficient forms of L. monocytogenes14. They are pleomorphic, of variable size and often contain vacuoles and granules 14, 28.
Attachment of L. monocytogenes L-form cells was greater than the parental cell types to stainless steel during the first 8 h exposure to culture. Jass et al. 23 suggest that L-forms may be as successful as the parent at attaching to surfaces within the body, such as on catheters or other implants. The classical cells appeared to require a longer period, up to 18 h, to attach with almost equivalent efficiency to L-forms on stainless steel surfaces. Poor biofilm-forming capabilities of the L. monocytogenes “cell-walled” forms were reported 22,29,30 and 9-10 days were required to achieve significant attachment on stainless steel 22,30.

The role of L-forms in disease, for some organisms, is well understood 11. Since virulence of pathogenic organisms, including L. monocytogenes, may be linked to their surface-attachment ability 32,33. With this in mind, and as our results show a greater capability of L. monocytogenes L-form to attach to stainless steel in vitro, we hypothesize that the L-form phenotype of Listeria monocytogenes may emerge as stealth pathogens.

Conclusions
Overall, this study showed that L-forms of Listeria monocytogenes attached more readily to stainless steel with significantly greater attachment capability to that of cell-walled parent strain, which may suggest that a rigid cell wall of Listeria monocytogenes is not a prerequisite for cell attachment in vitro. This is a crucial aspect of biofilm investigation, as the phenotypic behavior of L-form cells may be quite distinct from classical cells in terms of their extracellular microbial structure that assists in initial adhesion, and maintenance of biofilm structure. Further studies should attempt to identify molecular factors involved in the attachment of and development of biofilms by L. monocytogenes cell wall-deficient forms. The importance of L. monocytogenes L-forms as environmental contaminants in food processing plants and their clinical implications remains to be determined, but this study may indicate that L-forms have the potential to colonize food contact surfaces.

References


