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Effect of osmotic stress and culture density on invasiveness of *Listeria monocytogenes* strains

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ABSTRACT

The effect of osmotic stress on its capacity to invade the human enterocytic cell line HT-29 was studied in the early log through the stationary phase in 10 *L. monocytogenes* strains representing three genetically independent lineages. The results demonstrate that the transition of the bacteria from the log to the stationary phase results in a stepwise reduction of invasiveness. This effect was heterogeneous in the studied *L. monocytogenes* population, as the range of invasiveness reduction between the log and stationary phases varied from 10- to 380-fold depending on the strain. Ten-minute exposure to 0.3 M NaCl was sufficient to generate invasiveness alteration. No significant change in invasiveness induction caused by osmotic stress was found between the different points of the log phase (OD_{600} 0.4–1.2), being significantly different in the early log phase (OD_{600} 0.2–0.3) and in the stationary phase after 18 h of culture. The level of internalins and *opuCA* transcripts in response to osmotic stress did not correlate with invasiveness alteration in most *L. monocytogenes* strains.

Prolongation of stress exposure to 1 h and an increase in NaCl concentration from 0.3 to 1.8 M had no significant effect on a further increase in invasiveness. Short exposure times and low NaCl concentrations were sufficient for the generation of maximal invasiveness response of *L. monocytogenes*. It appears that although stationary-phase bacteria exhibit lower invasiveness than log-phase bacteria, they have a greater capacity to enhance their pathogenicity in response to stress.

1. Introduction

Listeria monocytogenes is an intracellular pathogen responsible for about 28% of all deaths from food-borne diseases in the USA (Mead et al., 1999). About 99% of human listeriosis is food-borne and the main foods involved in *L. monocytogenes* transmission are soft cheeses, dairy products, seafood, salads, smoked fish, sausages, and ready-to-eat (RTE) food (Vázquez-Boland et al., 2001). Increased RTE food consumption makes *L. monocytogenes* a serious threat since the pathogen is able to survive a wide range of environmental stress conditions, such as low temperature, acidic pH, and high osmolarity (Sue et al., 2004). To survive these adverse conditions, the bacteria must sense the stress and change their metabolism during food processing or gastrointestinal passage (Boor, 2006). It was shown that bacteria which modify their metabolism in response to stress alter their virulence. Thus it could be hypothesized that strains efficiently responding to environmental changes should also be more virulent (Roche et al., 2005). Stress-induced activation of virulence genes was shown to be mediated by the alternative σ^B factor. σ^B partially controls the expression of a range of virulence genes, including *inlA* and *inlB* encoding internalin A and B, and virulence-associated genes e.g. *opuCA* encoding carnitine transporter (Kazmierczak et al., 2006). Internalins are key virulence factors of *L. monocytogenes* as they promote entry into non-phagocytic mammalian cells (Bierne et al., 2007). *opuCA* is involved in transport of carnitine to the cells in response to osmotic stress (Sue et al., 2004). It has so far been shown that salt stress increases the capability of *L. monocytogenes* to invade a human colon epithelial cell line (Garner et al., 2006; Olesen et al., 2009). Adaptation to organic acid has also been shown to alter its invasion efficiency of human epithelia (Conte et al., 2000; Werbrouck et al., 2009; Olesen et al., 2009). So far the effect of stress on *L. monocytogenes* invasiveness was investigated in few strains and little is known about the factors influencing its invasiveness in response to stress.

Our aim was to investigate the effect of growth phase, NaCl concentration, and exposure time to NaCl on the invasiveness of *L. monocytogenes* strains representing three genetically independent lineages.

2. Materials and methods

2.1. L. monocytogenes strains

Of the 10 strains tested in this study, 3 were of human clinical origin and 7 were isolated from food (Table 1). All the strains were

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Table 1
L. monocytogenes strains used in the study.

Strain number	Source	Lineage	Serotype group
L28	Human	I	4b (d,e)
L84	Human	I	4b (d,e)
L41	Human	I	1/2b (d,e)
L45	Food	I	1/2b (d,e)
L56	Food	II	1/2a (3a)
L71	Food	II	1/2a (3a)
L83	Food	II	1/2a (3a)
L1057	Food	II	1/2c (3c)
L4	Food	III	4c (a)
L2061	Food	III	4c (a)

classified into five serotype groups, i.e. 1/2a (3a), 1/2b (3b), 1/2c (3c), 4b (d,e), and 4c (a), using PCR (Doumith et al., 2004). *actA* gene sequence analysis and lineage assignment was performed according to Zhou et al. (2005).

2.2. Growth of L. monocytogenes

Single colonies of *L. monocytogenes* were seeded in 5 mL of BHI and grown at 37 °C with shaking at 180 rpm for 8 h. Then 10-μL aliquots were transferred into 50 mL of BHI and grown for 12 h. Fifty-μL aliquots from these cultures were used to inoculate 5 mL of fresh BHI and the bacteria were grown at 37 °C with shaking at 220 rpm for up to 18 h. Bacteria at different stages of growth were used to study the effect of osmotic stress on *L. monocytogenes* invasiveness. For this, 1 ml of bacterial culture was inoculated into an equal volume of BHI containing different concentration of NaCl to give final concentrations of 0.3, 0.8, and 1.8 M NaCl and incubated at 37 °C for 10 min or 1 h together with non-treated bacteria. Then 2 and 4 log CFU of treated and non-treated bacteria (control) were used for infection of the HT-29 human cell line and plated in duplicate onto BHI agar.

2.3. Cell line and culture conditions

The human adenocarcinoma cell line HT-29 (Institute of Immunology and Experimental Therapy, Wrocław, Poland) was cultured in DMEM (Dulbecco's modified Eagle's medium; Sigma-Aldrich, Poznań, Poland) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Warsaw, Poland), 2 mM glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich) at 37 °C in 5% CO_2.

2.4. Plaque-Forming Assay (PFA)

HT-29 cells were seeded onto 6-well plates and grown to obtain almost confluent monolayers. Twenty-four hours before infection, the standard culture medium was replaced by DMEM without antibiotics. HT-29 cells were infected with 2 to 4 log CFU of treated and non-treated bacteria for 2 h, then the medium was replaced by DMEM containing 100 μg/mL gentamicin (Sigma-Aldrich) and incubated for 1.5 h. Each well was then overlaid with DMEM containing 10 μg/mL gentamicin and 1.0% low-melting-point agarose (Prona, Gdańsk, Poland). The plaque number was determined after 2–3 days of culture. Each assay was performed in duplicate and repeated three times. The results were expressed as the number of plaques per 4 log CFU deposited per well. The effect of NaCl on *L. monocytogenes* invasiveness was calculated as the quotient of plaque number in treated and non-treated bacteria expressed as a percentage (induction value).

2.5. RNA isolation

Bacterial pellets from 2-mililiter cultures were suspended in 100 μL of 0.1 M Tris–HCl pH 7.4 containing 2 μg/mL of lyzosyme, and incubated for 15 min at 37 °C. One mL of TRI-reagent (Sigma-Aldrich) was added, and RNA was extracted according to the producer's protocol. RNA was treated with RNAse free DNAse I (Sigma-Aldrich) for 30 min at room temperature to remove the DNA.

2.6. RT-PCR

cDNA synthesis was performed on 1 μg of RNA using the Superscript III First Strand Synthesis System (Invitrogen) with random hexamer primers according to the manufacturer's instructions. No-RT controls, used thereafter to check for DNA contamination, were prepared from 1 μg of RNA with the Superscript III First Strand Synthesis System in which no reverse transcriptase was added. A relative amount of *inlA*, *inlB* and *opuCA* transcripts were measured using the iQ5 Optical System (BioRad, Warsaw, Poland). Primers for *inlA* and *inlB* were from Werbrouck et al. (2006, 2009), and for *opuCA* from Sue et al. (2004). Primers for the housekeeping gene *gap* used for normalization of cDNA amount i.e. gap-for: TGGTGTTGTTGAAGGTCTAATG and gap-rev: GCAGCTCCGTCTAATTTACC were designed based on known *gap* gene sequences using Molecular Beacon software (BioRad). Real time PCR was performed from 3 independent RNA preparations and PCRs were run in duplicate. PCRs were run in a total volume of 20 μL containing 1 μL of appropriate cDNA, 450 nM primers for *gap* or 900 nM primers for *inlA*, *inlB* and *opuCA*, and 18 μL of iQ SYBR Green Supermix (BioRad) using the protocol: initial denaturation at 95 °C for 3 min., followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. Specificity of PCR was evaluated by melt curve analysis in a temperature range from 90 to 50 °C performed for each reaction. Residual DNA contamination was checked in each RNA sample by running no-RT controls. PCR efficiencies for each primer pair were determined on genomic *L. monocytogenes* DNA by running serial 5-fold dilutions of the template. Determined efficiencies were taken into account when calculating relative transcript levels according to Pfaffl (2001).

2.7. Statistical analysis

Each experiment was repeated at least three times. Two-way analysis of variance (ANOVA) was performed and the Fisher test was used with Statistica software (StatSoft, Poland) to determine whether statistical differences existed between different experimental groups. Significance was set at p level of <0.05.

3. Results

3.1. Growth of L. monocytogenes strains

Ten *L. monocytogenes* strains, including four strains from lineage I, four from lineage II, and two from lineage III, were inoculated into BHI and grown to determine the time-dependence of OD_{600}. The OD_{600} ranges 0.2–0.4 and 0.4–0.6, correspond to early stages of the log phase and OD_{600} 1.0–1.2 corresponds to the mid-log phase. At OD_{600} 2.8–3.5 the bacteria enter the stationary phase. After 18 h of growth, representing a later stage of the stationary phase, the OD values remained unchanged (Fig. 1).

3.2. Effect of growth phase on stress response of the L. monocytogenes strains

The ten *L. monocytogenes* strains from overnight culture were inoculated into BHI and grown for up to 18 h. The bacteria at OD_{600} ranges of 0.2–0.3, 0.4–0.6, and 1.0–1.2 and the bacteria after 18 h of culture (OD_{600} 2.8–3.5) were treated with 0.3 M NaCl for 10 min and then tested by the plaque-forming assay together with non-treated controls. It was shown that the osmotic stress influences the plaque number in a growth phase-dependent manner (Fig. 2). Decreased invasiveness in response to NaCl was observed in 6 strains (strains