Effects of Short-Term Alkaline Adaptation on Surface Properties of *Listeria monocytogenes* 10403S

Efstathios S. Giotis*, Ian S. Blair and David A. McDowell

Food Microbiology Research Group, University of Ulster, Northern Ireland, UK

Abstract: The changes in cell surface properties associated with alkali stress can significantly disrupt cell metabolism and structures, preventing effective interactions between bacterial cells and their environment. *Listeria monocytogenes* is known to display an adaptive response to alkali stress that enhances its capacity to more effectively survive subsequent severe alkali challenge. In this study, we examined the effects of adaptation to alkali conditions (pH 9.5/ 1h) in reducing detrimental effects in hydrophobicity and cell morphology in *Listeria monocytogenes* 10403S during severe (subsequent) short-term alkali challenge (pH 12.0/ 1h). Severe alkali challenge induced larger reductions in hydrophobicity (i.e., lower MATH values) in non adapted control cells than in mild alkali adapted cells. SEM revealed greater morphological diversity in suspensions of non alkali adapted cells than in suspensions of mild alkali adapted cells, i.e., a larger proportion of alkali adapted cells retained the normal (bacillary) morphology. Non adapted cells displayed considerable morphological heterogeneity including elongation, rupture and cellular deformation. Short-term alkaline adaptation in *L. monocytogenes* involves direct changes in the cell surface properties and organisation which facilitate the well recognised phenotypic abilities of this pathogen to persist and/or grow in alkaline conditions. Alkali adaptation may be significant in the persistence of this pathogen in the presence of alkali detergents in food processing environments and alkali natural habitats, and has direct clinical implications in relation to virulence and response to mammalian defence mechanisms.

Keywords: *Listeria,* alkali, stress, hydrophobicity, surface, morphology.

Listeria monocytogenes is an important food borne pathogen due to its ability to resist environmental stresses and initiate high mortality rate human infections [1]. In particular, its considerable capacity to resist alkali stress may explain its persistence in food processing environments where decontamination procedures are significantly dependent on the use of alkali detergents [2-5]. Similarly, alkali resistance is suggested as important in enabling this pathogen to survive and establish infection in humans where it is challenged by pancreatic secretions [6], and the alkaline phase of phagocytosis [7].

L. monocytogenes is known to display an adaptive response in which exposure to non lethal environmental stress, enables it to more effectively survive subsequent [more severe] challenges by the same or different environmental stress(es) [8-12]. Such adaptation has been noted in response to alkali stress [13-17], and has also been reported to confer cross protection against subsequent thermal stress, ethanol and alcohol stress [4, 11, 18].

Alkali conditions can induce the solubilisation of bacterial surface proteins [19, 20], resulting in exposure of hydrophobic sites of adjacent lipids to the extracellular environment [21]. Alkali may also directly attack the structure of the cell membrane by saponification of membrane lipids or alteration of the membrane fatty acids ratio [22, 23]. The changes in cell surface properties and structures associated with alkali and/or other environmental induced damage can significantly disrupt cell metabolism and structure, preventing effective interactions between bacterial cells and their environment [24].

Considerable information is available about the general structure and biochemistry of the surface of *L. monocytogenes*, although less is known about the direct physical effects of environmental stresses and in particular alkali stress, on these structures and processes. To our knowledge this is the first study to investigate the relationship between alkaline adaptation and the associated changes in cell size and surface characteristics in order to determine possible mechanisms of resistance to short term alkali stress. A previous study [5] dealt with the long term alkali- induced change in bacterial size and morphology while this follow-up study reports the short term (i.e. 1 h) alkali- induced bacterial size changes as a resistance mechanism in combination with surface properties (CSH) alterations.

Listeria monocytogenes 10403S cells (BHI, pH 7.2, 30°C, $OD_{600}\approx0.4$), were pelleted by centrifugation and resuspended into alkali adjusted BHI (pH 9.5 with 2M NaOH, 30°C) or into (30°C) BHI (pH 7.2). After incubation (30°C/60 min), cells were recovered and placed in BHI (30°C) adjusted to pH 12.0 (2M NaOH) for 60 mins. Control cultures were adapted to pH 7.2, recovered by centrifugation, and resuspended in fresh BHI. Specimens were examined/photographed with a Hitachi S3200N scanning electron as previously described [5]. Cell dimensions (length, width, equatorial radius of spheroid caps) were recorded, and used to calculate cell volumes in the equation V (μm^3) = $\pi/4$ $w^2L + \pi/3$ W^2R, which assumes cells to be cylindrical with an hemisphere at each end [25]. W represented cell width of the

*Address correspondence to this author at the Centre for Endemic, Emerging and Exotic Diseases (CEEED), Royal Veterinary College, London, Hawkshead Lane, AL9 7TA, UK; Tel: ++44(0)1707667038, 028 90 366697; Mobile: ++44(0)7896498097; Fax: +44(0)1707667051, 028 90 368811; E-mail: egiotis@rvc.ac.uk

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central part of the cylindrical cell, *L* represents cell length of the central part of the cell, and *R* represents equatorial radius of spheroid caps [26]. Surface hydrophobicity was measured by a modification of the microbial adhesion to hydrocarbons (MATH) assay [27, 28]. Alkali challenged cells recovered from BHI (pH 12.0), and control cells recovered from BHI (pH 7.2) were resuspended in 1.2 ml of PBS (pH 7.4) to absorbance values between 0.9-1.0 [λ=400 nm], and vortex mixed with 100 µl *p*-xylene (Sigma) for 120 secs. The absorbance at 400 nm was estimated using a UV/Vis spectrophotometer (Shimatzu UV-1201), and the percentage adherence to *p*-xylene was calculated as $(abs_1\text{-}abs_2)/ abs_1 \times 100$, where abs_1 is the absorbance of the initial bacterial suspension and abs_2 is the absorbance of the aqueous phase. Experiments were done in triplicate.

EFFECT OF PH ON CELL MORPHOLOGY

Control (pH 7.2) cultures displayed little or no morphological heterogeneity after the growth, centrifugal recovery, and PBS washing processes. However, alkali challenged (pH 12.0) (adapted and non adapted) cultures exhibited considerable morphological heterogeneity, including ruptured, deformed, wrinkled and/or elongated cells in comparisons with control cultures (Fig. **1**). There was a significant difference (P=0.005) between the mean cell volume of alkali challenged (pH 12.0) non adapted cultures, and the mean cell volumes of control (pH 7.2) populations. There was no significant difference between the mean cell volume of alkali challenged (pH 12.0) adapted cultures, and control (pH 7.2) populations (Table **1**).

The results obtained in this study confirmed that pH 12.0 presented a very hostile environment to *L. monocytogenes*. SEM analysis revealed that alkali stress induced significant diversification of cell morphologies including cell deformation and elongation. This is in agreement with a previous SEM study of *Listeria monocytogenes* subjected to extreme (pH 12.0) alkali conditions [5]. Another study also reported distinct clear and dark zones in *Listeria* cells and a bulging cytoplasmic membrane against the cell wall [22]. The extent of the disruption observed in these studies provide some indication of the extent of physical disruption which underlies previous reports of severe and abrupt cessation and/or dysfunctions of cell division under high pH conditions, as previously discussed in the proposed bacterial suicidal response hypothesis [29]. The elongated forms of *Listeria* cells observed in this study have also been previously reported [5].

The scale of the above changes occurred to different extents in adapted and nonadapted populations of *L. monocytogenes.* Alkaline adapted cells did not significantly change their mean cell volumes during exposure to pH 12.0 for 60 mins, but under the same conditions non adapted cells showed a significant reduction in mean cell volume. Similarly, adapted populations displayed less diversity of cell shape during severe short-term alkaline challenge.

Alkali conditions similar to the conditions examined in this study are commonly found in food industry environments and in the human gastrointestinal system. Thus, further work should be undertaken to estimate the extent of morphological changes in the global stress modification of *L. monocytogenes.*

EFFECT OF PH ON CELL SURFACE HYDROPHOBICITY

Cell surface hydrophobicity (CSH) values for cultures adapted/or not adapted at pH 9.5 are presented in Table **1**. Cells from alkaline challenged (pH 12.0) cultures (adapted and not adapted) had significantly (P<0.05) lower MATH scores than cells from control (pH 7.0) cultures. The average MATH scores of cells from adapted cultures were significantly higher than the MATH scores of cells from non adapted cultures, suggesting less disruption of normal hydrophobicity status in adapted cells.

This study noted that short term severe alkaline stress led to significant reductions in cell surface hydrophobicity in *L. monocytogenes*. This study is the first to report the semi protective effects of short alkali adaptation in limiting the impact of severe alkali challenge on cell hydrophobicity. This suggests that the process of adaptation to mild alkali stress involves direct physical and/or chemical changes which limit

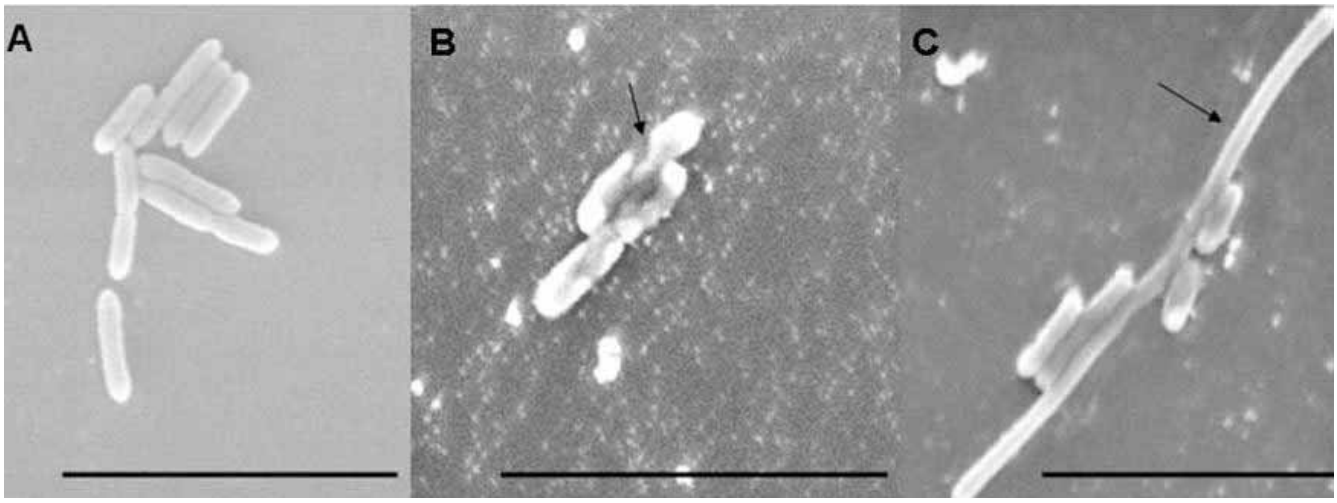

Fig. (1). Electron micrographs of non alkali adapted (pH 7.2) and alkali adapted (pH 9.5) *L. monocytogenes* after alkali challenge (pH 12.0/1 hr) (Bar =5 µm). **A**: Control cells (pH 7.2/ pH 7.2), **B**: Non alkali adapted cells (pH 7.2/ pH 12.0) (Arrow indicates cell rupture), **C**: Alkali adapted cells (pH 9.5/ pH 12.0) (Arrow indicates elongated cell).

Table 1. Mean Cell Sizes and Surface Hydrophobicity Values of *L. monocytogenes* Populations After Alkali Challenge (pH 12.0/1 h)

Population (Treatments)	Length	Width	Spherical Cap Radius	Volume	% Adherence to *p*-xylene
Control (pH 7.2/ pH 7.2)	1.65 ± 0.03	0.42 ± 0.05	0.19 ± 0.04	0.26 ± 0.07	23.08 ± 2.25
Non Adapted (pH 7.2/ pH 12.0)	1.49 ± 0.05	0.38 ± 0.07	0.18 ± 0.07	0.19 ± 0.02	5.29 ± 0.97
Adapted (pH 9.5/ pH 12.0)	1.62 ± 0.06	0.40 ± 0.04	0.19 ± 0.06	0.24 ± 0.05	10.17 ± 1.47

*Values represent the means ± S.D. of three separate experiments.

the impact of subsequent severe alkali challenge on those components of the cell surface which contribute to cell surface hydrophobicity.

Higher CSH values have been correlated with enhanced adherence to host epithelial cells [30] and effective formation of biofilms [31]. Therefore, the impact of adaptation in limiting the extent by which alkali challenge may reduce CSH values may indicate a broader protective effect of adaptation in limiting cell surface disruption and/or maintaining a wider range of such cell surface activities. However, it is important to note that the significant surface components which contribute to CSH in *Listeria* are different from the types of surface of components which dictate CSH values in Gram-negative bacteria CSH values that are principally dictated by polysaccharides or (glyco-) proteinaceous material [32]. In *Listeria*, such materials are very limited or completely absent, and stress related changes in CSH in this organism involve changes in teichoic acids and/or peptidoglycans [33]. Interestingly, alterations in teichoic acids have also been reported to contribute to the alkaline tolerance of species of *Bacillus* [34].

Further work is needed to characterise the changes that take place on the cell surface of *Listeria* under alkali stress in order to better understand the impact of alkali stress, and the adaptive response of this pathogen to such stress, in attachment, persistence, and virulence.

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