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Expression of cellular antigens of *Listeria monocytogenes* that react with monoclonal antibodies C11E9 and EM-7G1 under acid-, salt- or temperature-induced stress environments

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ABSTRACT

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Aims: To study the expression of cellular antigens of *Listeria monocytogenes* that react with monoclonal antibodies (MAbs) C11E9 and EM-7G1 under acid-, salt- or temperature-induced stress environments.

Methods and Results: The reaction patterns of antibodies to *L. monocytogenes* held in stressful environments for a short duration (3 h) or grown for extended periods (16–72 h) were investigated. During both short or prolonged exposure to stress environments of high temperature (45°C) and NaCl (>1·5%, w/v), reactions of whole cells of *L. monocytogenes* to antibodies were severely affected as determined by ELISA and by the reduced expression of the antibody-reactive 66 kDa antigen in the Western blot assay. Conversely, cold (4–15°C) or acid (pH 2–3) stress environments had very little effect on antigen expression or antibody reaction. Additionally, heat-killed cells showed reduced reactions to these antibodies when compared with unheated cells. Artificially created stress environments in hotdog slurry also affected the antigen expression in *L. monocytogenes*. Immunoelectron microscopy revealed that the antibody-reactive antigens were uniformly present on the surface of the cells. Morphological characteristics following growth in stressed environments revealed that heat stress at 45°C caused *L. monocytogenes* cells to be elongated and to form clumps; whereas, osmotic stress (5·5% NaCl, w/v) caused filamentous appearance with multiple septa along the length of the cell.

Conclusions: These results indicated that MAb C11E9 or EM-7G1 could detect *L. monocytogenes* from cold or acid-stress environments; however, they may show weaker reactions with heat or osmotically stressed cells or cells grown at 4°C.

Significance and Impact of the Study: Bacteria in food are routinely subjected to various stresses, induced by cold, heat, salt or acid during processing and storage. Whether stresses would modify the expression of cellular antigens of *L. monocytogenes* is of a great concern for immunodetections in food products.

Keywords: antibody reaction, antigen expression, *Listeria monocytogenes*, salt, stress, temperature.

INTRODUCTION

Listeria monocytogenes is a Gram-positive, rod-shaped food-borne pathogen with significance for human infections. It is of great concern to the food industry because of its ability to grow in extreme environments. It is often present in food processing environments and is responsible for postprocessing contamination of ready-to-eat (RTE) foods. In general, *L. monocytogenes* will grow in the pH range of 4·4–9·2 (George *et al.* 1988), in temperature ranges of 3–45°C (Walker *et al.* 1990) and at water activity values as low as 0·92 (Petran and Zottola 1989). *Listeria monocytogenes*

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could grow at NaCl concentrations as high as 10% (McClure *et al.* 1989) and survive in 22% salt brine (Ryser and Marth 1989). It is important to identify analytical tools that are capable of direct detection of *L. monocytogenes* originating from harsh growth environments.

Environmental temperatures and salt concentrations are significant factors that influence the expression of virulence genes in many bacterial species (Mekalanos 1992). It has been well-documented that the expression of virulence factors for *L. monocytogenes*, such as transcriptional activator protein (PrfA), internalin, listeriolysin, phospholipases, metalloprotease and actin polymerization proteins, are influenced by salt concentrations and temperatures (Dramsi *et al.* 1993; Sokolovic *et al.* 1993; Buncic and Avery 1996). Thus, virulence proteins may not be suitable candidates for antigenic detection.

Specificity and avidity of antibody binding to target antigen is critical for the success of antibody-based pathogen detection methods (Bhunia 1997). Likewise, it is critical that the antibody is able to react with bacterial pathogens originating from healthy or stressful growth environments of food. Monoclonal antibodies (MAbs), C11E9 (Bhunia *et al.* 1991) and EM-7G1 (Bhunia and Johnson 1992), are potential for immunocapture (Naschansky 2001) and various immunosensor- or biosensor-based applications for detection of *L. monocytogenes* in food (Bhunia *et al.* 2001; Gomez *et al.* 2001; Huang *et al.* 2003). The MAb C11E9 (IgG_{2b}) reacts with *L. monocytogenes* and *L. innocua* without any cross-reactions to other *Listeria* spp. (Bhunia *et al.* 1991). The epitopes for this MAb are located in several protein bands of 76, 66, 56 and 52 kDa with the 66 kDa being the predominant protein that shows the strongest reaction (Bhunia *et al.* 1991). The MAb EM-7G1 also reacts with the 66 kDa proteins, indicating that the epitope for EM-7G1 is unique and is located in the 66 kDa antigen (Bhunia and Johnson 1992). The gene for 66 kDa protein has been sequenced and identified as an aminopeptidase C (*pepC*) (Winters *et al.* 2000).

It is important to understand the capacity of *L. monocytogenes* to survive and multiply under conditions associated with food processing, preservation and distribution. Bacteria in food are routinely subjected to various stresses, induced by cold, heat, salt or acid during processing and storage (Petran and Zottola 1989; Vasseur *et al.* 1999). These stresses could occur over a brief period or could accompany the lifetime growth of bacteria (Becker *et al.* 2000). It is essential to monitor the expression of antigenic surface proteins that are essential for specific antibody-based detection as the physiological or metabolic activities in the cells are often altered in response to a stress. Therefore, the focus of this study is to evaluate reaction patterns of MAb C11E9 or EM-7G1 to *L. monocytogenes* cells exposed to various physical or chemical stresses. This information will help in the design of suitable assay procedures so that optimum signal is achieved during immunosensor or immunoassay-based detection.

Previously, Nannapaneni *et al.* (1998a,b) indicated that *L. monocytogenes* surface epitopes for EM-7G1 or EM-6E11 (Bhunia and Johnson 1992) could be affected when bacteria were cultured in stressful environments of enrichment media containing various selective antimicrobial agents. In this project, we investigated the expression of MAb C11E9 and EM-7G1-reactive antigens in *L. monocytogenes* in response to different concentrations of NaCl, a broad range of temperatures and acidic environments that *L. monocytogenes* may encounter during growth and survival in the food processing environments or in the RTE foods.

MATERIALS AND METHODS

Cultures and media

L. monocytogenes V7 (serotype $\frac{1}{2}$a) strain was maintained on brain–heart infusion (BHI) agar (1·5%) slants (Difco Laboratories) at 25°C. The fresh cultures for all experiments were obtained by inoculating BHI broth and incubating them at 37°C for 16–18 h.

Preparation of monoclonal antibodies

Frozen-stored hybridoma cell line C11E9 (Bhunia *et al.* 1991) and EM-7G1 (Bhunia and Johnson 1992) were grown in Dulbecco's modified Eagles medium (DMEM) (Sigma Chemical Co., St Louis, MO, USA) with 10% foetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA) in a cultivation chamber of CELLine 1000 (Integra Biosciences, East Dundee, IL, USA) at 37°C in a humidified incubator with 7% CO_2. The medium was aspirated at 3-day intervals, centrifuged ($300 \times g$ for 20 min) and partially purified by ammonium sulphate precipitation (Harlow and Lane 1988). A Protein G column (ActaPrime, Pharmacia-Amersham, Uppsala, Sweden) was used for affinity purification of antibodies and the final concentration was adjusted to 1·2 mg ml^{-1}.

Indirect ELISA

L. monocytogenes cells obtained from various stress treatments were harvested by centrifugation ($6000 \times g$, 4°C, 10 min), washed, and adjusted to $A_{595} = 0{\cdot}37$ to obtain uniform cell concentrations. Cells were resuspended in 0·05 mol l^{-1} carbonate coating buffer, pH 9·6, and used to coat 96-well flat-bottomed microtitre plates (Dynatech Lab, Chantilly, VA, USA) at 4°C for 12–14 h. The plates were washed four times with 0·02 mol l^{-1} phosphate-buffered saline (PBS), pH 7·0 and 0·5% Tween 20 (PBS-T) then

0·1 ml, of diluted MAb C11E9 (1 : 200) or EM-7G1 (1 : 100) was added to each well and incubated at 37°C for 1 h in a shaker incubator. The plates were washed, reacted with a goat anti-mouse IgG horseradish peroxidase conjugate (Sigma), developed with a substrate solution containing *o*-phenyldiamine and H_2O_2, and the colour reaction was measured in a plate reader (Bio-Rad, Hercules, CA, USA) at 490 nm (Bhunia *et al.* 1991).

Western blotting and quantification of proteins

L. monocytogenes cells obtained from different growth conditions were harvested by centrifugation (6000 × *g*, 10 min) and washed three times with PBS and cell concentration was adjusted as mentioned above. The washed cell pellets were resuspended with sodium dodecyl sulphate buffer (5% SDS, 0·5% β-mercaptoethanol, PBS, pH 7·0) and incubated at 37°C for 60 min (Bhunia *et al.* 1991). After centrifugation (10 000 × *g*, 10 min), the cellular protein extracts were analysed in SDS-PAGE (7·5% polyacrylamide), transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) and immunoprobed with MAbs C11E9 (1 : 200) or EM-7G1 (1 : 100). Membranes were developed with horseradish-peroxidase conjugated goat anti-mouse antibody (1 : 2000) utilizing diamino benzidine tetrahydrochloride (DAB-4HCl) (Sigma) containing H_2O_2 as a substrate (Bhunia and Johnson 1992). The reaction intensity (sum intensity) of MAbs C11E9 or EM-7G1 with the 66 kDa bands was analysed by a Kodak 1D Image Analysis System (Eastman Kodak Co., Rochester, NY, USA).

Reaction of antibodies to heat-inactivated *Listeria monocytogenes* cells

Cultures (2 ml) from stationary phase growth at 37°C in BHI broth (4×10^9 CFU ml^{-1}) were heated at 80°C for 20 min, centrifuged (6000 × *g*, 10 min), and cell pellets were resuspended in carbonate buffer for ELISA analyses.

Reaction of antibodies to *Listeria* exposed to stressful environments of heat, cold, saline and acid for a short duration (3 h)

L. monocytogenes cultures from late log phase (18 h) of growth (37°C) were centrifuged (6000 × *g*, 10 min), washed in PBS, resuspended in BHI broth, and held at 45°C for 3 h. PBS washed cultures were also resuspended in BHI broth containing 5·5% NaCl and held at 37°C for 3 h. To analyse the effect of acid-induced stress, cultures from BHI broth (final pH 5·5) were harvested by centrifugation as described above and resuspended in acidified tryptic soy broth (TSB; Difco) to pH 5·5 or 3 or 2 and held at 37°C for 3 h (Gahan *et al.* 1996; Barker and Park 2001). The pH of the TSB were adjusted with concentrated HCl. All cultures were held in a shaker incubator (New Brunswick Scientific Co., Inc., Edison, NJ, USA) with constant agitation (~120 rpm) during this experiment.

From each treatment, 2 ml,of cultures were removed at 0, 0·5, 1, 2 and 3 h, centrifuged (6000 × *g*, 10 min), and resuspended in carbonate coating buffer to sensitize microtitre plates. ELISA was performed with MAb C11E9 and EM-7G1 as mentioned above.

Reaction of antibodies to *Listeria monocytogenes* grown under stressful environments for extended period of time (>16 h)

Temperatures. Reaction characteristics of each antibody were analysed with *L. monocytogenes* cells harvested from log-phase cultures grown at 45, 37, 25, 15 or 4°C with constant agitation. Prior to this experiment, log-phase of each culture was determined from the growth curves, which were generated for each temperature. BHI broths (100 ml each) were preheated or precooled to 45, 37, 25, 15 or 4°C and inoculated (1%) with *L. monocytogenes* and allowed to grow until the end of the log-phase, which were about 36, 16, 24, 48 or 72 h, respectively. Cell concentrations were adjusted and analysed either by ELISA or by Western blotting.

Osmotic stress. Brain heart infusion broths (100 ml) were added with NaCl (Sigma) to obtain final salt concentrations of 0·7, 1·5, 3·5, 5·5 or 10·5%, and inoculated (2%) with *L. monocytogenes* and incubated at 37°C with constant shaking. Cultures grown in the BHI broth containing 0·5% NaCl as part of its formulation were used as controls. Bacterial cells were harvested from the log phase of growth, which was 16, 16, 18, 24 or 36 h of incubations at salt concentrations of 0·7, 1·5, 3·5, 5·5 or 10·5%, respectively. The cell concentrations were adjusted before ELISA and Western blotting analyses.

Reaction of antibodies to *Listeria monocytogenes* from spiked hotdog slurry subjected to different stresses

Packages of hotdogs were purchased from local grocery stores and sterilized by boiling in PBS for 10 min. Each hotdog (~46 g) was placed in 100 ml of BHI broth, which was previously adjusted to pH 5·5 with 1 M HCl or added with NaCl to give a final salt concentration of 3·5% (w/v) or without any treatment. Hotdogs were homogenized using a stomacher for 1 min in stomacher-bags with built-in filter lining (Seward, Cincinnati, OH, USA) and *L. monocytogenes* cells were added to each bag to obtain approximate cell concentration of 10^4 cells per millilitre. Meat suspensions were incubated with constant shaking in a shaker incubator

either at 37°C or at 45°C to induce thermal stress. Sample without any added stress (control) or with osmotic stress, acid or thermal stress were incubated for 16, 18, 22 or 36 h, respectively. Samples were collected from the bag, and bacterial cells were harvested by passing through Whatman filter paper 1 and centrifugation ($3900 \times g$, 10 min). Cell concentrations in each filtrate was adjusted ($A_{595} = 0{\cdot}37$) and analysed by ELISA.

Light and transmission electron microscopy

The morphological features of *L. monocytogenes* cells that manifested reduced expression of antibody-reactive antigens were examined microscopically. *Listeria monocytogenes* grown in BHI broth at 37, 45°C or broth containing 5·5% NaCl at 37°C for 16–18 h were viewed under a phase-contrast light microscope (Leica DMLB, Wetzlar, Germany) or prepared using the procedure of Van Tuinen and Riezman (1987) for imaging with a Philips EM-400 Transmission Electron Microscope (FEI Company, Inc., Hillsboro, OR, USA).

Immunoelectron microscopy was performed to determine the surface localization of antibody-reactive antigens (Beesley *et al.* 1984). The bacterial cultures were washed and resuspended in PBS and a drop of cell suspension was absorbed onto formavar plus carbon coated nickel grids. The grids were incubated in PBS containing 1% bovine serum albumin for 10 min, then immunoprobed with 1 mg ml^{-1} MAb C11E9 or EM-7G1 overnight at 4°C. The grids were then washed with PBS and incubated with anti-mouse IgG conjugated to 12 nm gold particles (Jackson Immuno Research, West Grove, PA, USA). The preparations were then stained with 1% uranyl acetate and viewed with a transmission electron microscope (Philips EM-400).

Statistical analysis

In all ELISA experiments, data were presented as mean ± S.D. Statistical differences for antibody reactions to heat-inactivated or viable cells were analysed by using a two-independent-sample *t*-test. Differences in antigen expression during various temperatures and salt concentrations were calculated by least significant difference (LSD) test for comparison of multiple samples. Significance was accepted at the level of $P < 0{\cdot}05$.

RESULTS

Surface localization of epitopes

It is essential that the antibody-reactive antigens are present on the surface of the cells for antibody-mediated direct detection or capture of viable bacterial cells from a sample. Immunoelectron microscopic analysis revealed uniform distribution of C11E9 and EM-7G1-reactive antigens on the surface of the viable *L. monocytogenes* cells (Fig. 1a,b); however, the average amount of C11E9-reactive antigens (192 gold particles per cell) in two cells were about four times higher than the EM-7G1-reactive antigens (47 gold particles per cell) from the same number of cells. Bacterial cells reacted with gold-conjugated secondary antibody without the primary antibody did not show any background gold particles on the cell (data not shown). Level of expression of antibody-specific antigens on surface of *L. monocytogenes* cells as indicated by gold particles is in conformity with antigen expression in other bacteria such as *Vibrio parahemolyticus* (Nagayama *et al.* 1995) and *Escherichia coli* (Hiemstra *et al.* 1986). Immunoelectron microscopy data also corroborated with ELISA data (Fig. 2) that showed C11E9 reaction to viable cells to be two to three times higher than the EM-7G1. The discrepancy in reactivity for these two antibodies could be attributed to the number of epitopes recognized by both antibodies. The epitopes recognized by C11E9 are located primarily on a 66 kDa antigen, and also present in 76, 56 and 52 kDa antigens (Bhunia *et al.* 1991). The epitope for MAb EM-7G1 is located only in 66 kDa antigen and appears numerically to be much lower than the C11E9-reactive epitopes. These antibodies would therefore

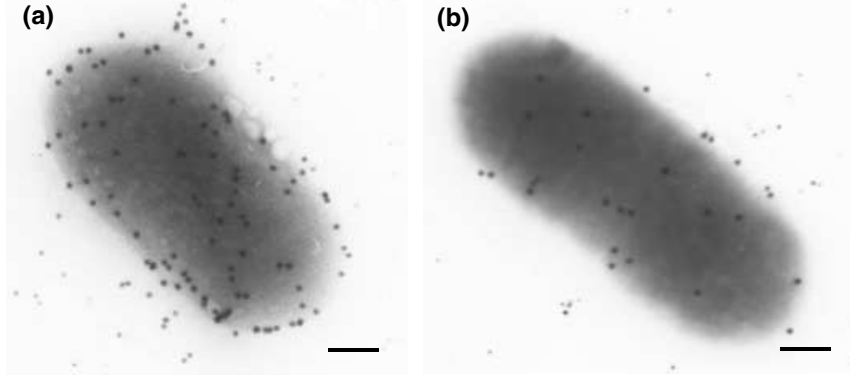

Fig. 1 Transmission electron microscopic analysis of surface localization and distribution patterns of antigens for (a) MAb C11E9 and (b) EM-7G1 on the surface of viable *Listeria monocytogenes* cells. A 12-nm gold-conjugated goat anti-mouse antibody was used as a secondary antibody. C11E9-reactive antigens were at least 3·5 times more than the EM-7G1 reactive antigens on the surface. Control cells treated only with gold-conjugated secondary antibody did not give any background binding of gold particles. Magnifications, 27 000×, Bar = 0·5 μm

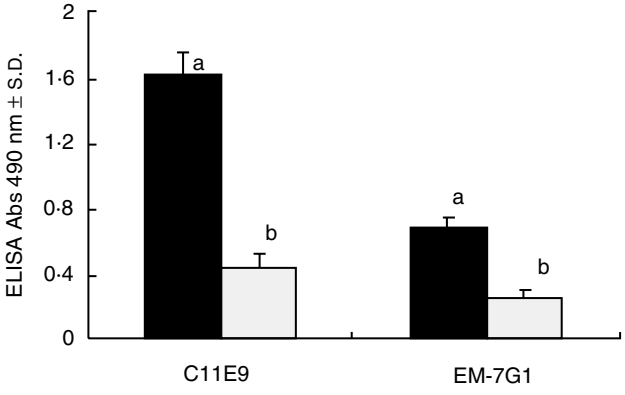

Fig. 2 ELISA reactions of MAbs C11E9 or EM-7G1 to viable (■) or heat killed (80°C for 20 min) (□) *Listeria monocytogenes* V7 cells. Bars with different letters (a and b) are significantly ($P < 0{\cdot}05$) different for a given antibody. Each datum point represents the average of three independent tests

be suitable for direct detection of intact viable *L. monocytogenes* cells. In fact, some of our recent studies demonstrated that these antibodies could capture *L. monocytogenes* cells from selective enrichment broth by an immunobead separation method (Naschansky 2001) or could detect viable cells on the surface of the C_{18}-coated silicon dioxide biochip (Huang *et al.* 2003).

Reaction patterns of antibodies to heat-killed *Listeria monocytogenes* cells

ELISA analyses of the reaction of MAbs C11E9 and EM-7G1 to heat-killed (80°C for 20 min) *L. monocytogenes* cells showed that C11E9 reaction was about four fold lower than the untreated control while EM-7G1 reaction was three fold lower than the control (Fig. 2). Previously Nannapaneni *et al.* (1998b) also reported similar reduced effect of heat treatment on EM-7G1 reaction. Others observed similar effects when *Listeria* cells were heated (Gavalchin *et al.* 1991; Torensma *et al.* 1993; Nannapaneni *et al.* 1998a). These reduced reactions could be due to temperature-induced denaturation of antibody-reactive epitopes on the surface of *L. monocytogenes* cells. Reduced reaction to heat-killed cells is a desirable property as these cells are not a concern in a food product and the assay could detect the viable cells.

Reaction patterns of antibodies to *Listeria monocytogenes* cells exposed to temperature-, salt- or acid-induced stresses for 3 h

The antibody reaction remained unchanged for *L. monocytogenes* cells held at 45°C until 60 min of exposure. The antibody reaction, however, progressively decreased thereafter until the end of the experiment (3 h) (Fig. 3a,d). The antibody reaction remained unchanged when cells were held at 37°C (control). These results indicate that temperature-induced stress possibly interfered with antibodies binding with the epitopes possibly because of conformational changes in the reactive proteins (epitopes).

The reaction of C11E9 decreased eight fold within 30 min of exposures (Fig. 3b) when *L. monocytogenes* was osmotically stressed by exposure to 5·5% of NaCl for 3 h. Similarly, reaction of EM-7G1 decreased more than three fold in 30 min and maintained similar reaction patterns until the end of the 3 h experiment (Fig. 3e). These data indicate that NaCl adversely affected the epitopes, thereby, interfered with antibody binding. It is known that NaCl removes water from proteins rendering them to be hydrophobic, and this may have caused conformational changes in the epitopes.

The reaction of C11E9 and EM-7G1 to acid-adapted (pH 5·5) *L. monocytogenes* cells or acid-adapted cells exposed to acid stress environment of pH 2 or 3 showed identical results without any adverse effects on the antibody reaction (Fig. 3c,f). This ELISA data indicated that the acid-stress environments had no detectable effect on the cellular antigens over the 3 h period. In addition, when Western blotting was performed with surface protein extracts of cells held at pH of 5·5 or acid-adapted cells in pH 2 or 3, C11E9 and EM-7G1 showed the same reaction patterns to 66 kDa antigens without any visible reduction in reactions (data not shown). These data further suggest that the expression of the 66 kDa antigen is not affected by acid-induced stress.

Reaction of antibodies to cellular antigens of *Listeria monocytogenes* grown at different stressful growth environments

Temperatures. ELISA analysis of cultures grown at 4, 15, 25, 37 or 45°C showed that both C11E9 and EM-7G1 had highest reactions with cultures grown at 37°C (control) followed by 25, 15, 4 and 45°C (Fig. 4a). Cultures grown at 45°C had the least reaction with the antibodies. This is significantly ($P < 0{\cdot}05$) lower than the other temperatures, suggesting that the expression of C11E9 and EM-7G1 reactive antigens could be affected by temperature-induced stress.

Western blot analysis with C11E9 indicated that reaction intensities of the antibody-reactive 66 kDa protein bands were variable with the weakest reaction observed with protein preparations from 45°C-grown cultures (Fig. 4b). These data were comparable with the ELISA data (Fig. 4a). Expression of the 66-kDa protein was used to determine the effect of various stresses as it is the predominant reactive protein for C11E9. MAb C11E9 also reacted with other proteins (76 and 52 kDa) from *L. monocytogenes*; however, we did not observe much variation in reaction to those proteins (data not shown). This experiment again suggests

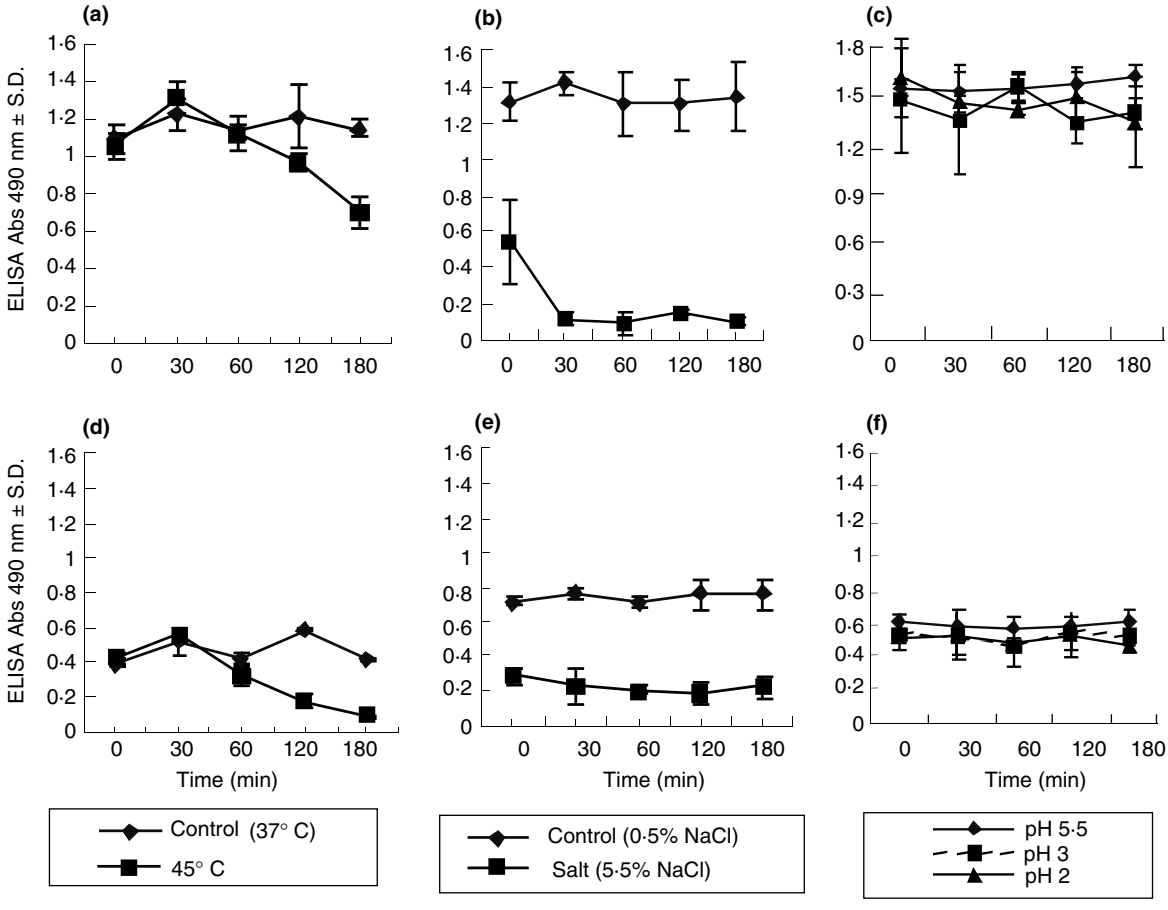

Fig. 3 ELISA reaction of monoclonal antibodies C11E9 (a, b and c) or EM-7G1 (d, e and f) to *Listeria monocytogenes* V7 cells held in brain heart infusion broth at 45°C (a, d) or broth containing 5·5% NaCl at 37°C (b, e) or in acidic environment (pH 5·5, 3 or 2) (c, f) for 3 h. Cultures were collected every 30 min and analysed by indirect ELISA. Each datum point represents the average of three independent tests

that expression of 66 kDa protein could be severely affected by temperature induced stress conditions of 45°C. As expected, Western blot reaction profiles of EM-7G1 to 66 kDa proteins (Fig. 4c) from cells from different growth temperatures were also very similar to those from C11E9 (Fig. 4b).

Osmotic stress (NaCl). In ELISA, expression of C11E9 and EM-7G1-reactive antigens was adversely affected when grown in BHI broth containing NaCl at concentrations of 1·5–10·5%. There were no significant differences ($P < 0·05$) at NaCl concentrations of 3·5–10·5% for C11E9 and 1·5–3·5% and 5·5–10·5% NaCl for EM-7G1 (Fig. 5a). Protein expressions in cells grown in BHI broth containing standard 0·5% (control) or 0·7% NaCl were not affected. The Western blot analyses with C11E9 and EM-7G1 showed a similar pattern of expression of the 66 kDa proteins (Fig. 5b,c). This study indicated that growth in the saline environment could severely affect the expression of the antigens in a concentration dependent manner.

Reaction with *Listeria monocytogenes* from spiked food sample

Reaction of antibodies to *L. monocytogenes* was also affected when bacteria were grown in the presence of selective stress environment in homogenized meat slurry (Fig. 6). For MAb C11E9, thermal stress (45°C) affected the most followed by the osmotic stress (3·5% salt) and acid stress (pH 5·5). Whereas all three stress conditions uniformly affected the expression of EM-7G1-reactive antigens. These results clearly suggest that stress in food milieu can affect the antigen expression in *L. monocytogenes* cells.

Morphological analyses

Light microscopic examination showed that *L. monocytogenes* cells, heat stressed at 45°C, appeared elongated and had a tendency to form clump (Fig. 7b) compared with the cells grown at 37°C (Fig. 7a). Transmission electron microscopic images revealed that these elongated cells were often doublets

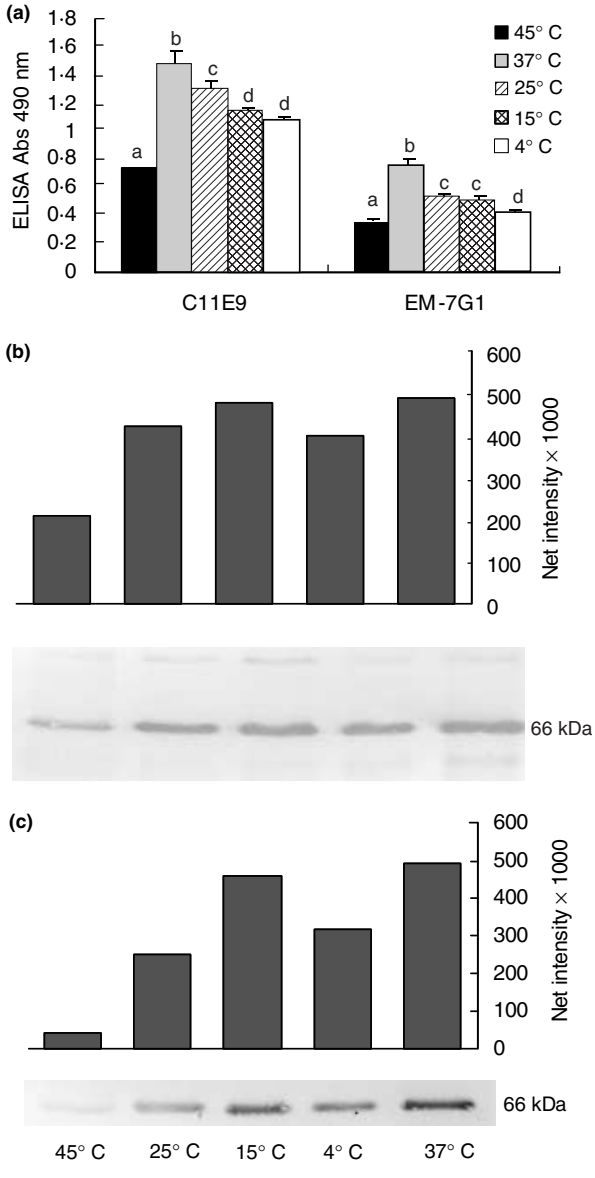

Fig. 4 Reaction analyses of monoclonal antibodies C11E9 or EM-7G1 to *L. monocytogenes* V7 obtained from different growth temperatures by (a) ELISA, (b) Western blotting for C11E9 and (c) EM-7G1. ELISA was performed with cells from log-phase grown cultures of *Listeria monocytogenes* V7 following growth at 4, 15, 25, 37 and 45°C for 72, 44, 24, 16 and 48 h, respectively, while Western blotting was carried out with protein extracts from similar cell preparations. Cell concentrations of each culture were adjusted prior to ELISA and Western blotting (see text). The ELISA data are the average of three independent tests and bars marked with different letters (a, b, c, d) are significantly ($P < 0{\cdot}05$) different for a given antibody. In the Western blot analysis, antibody reaction intensities of only 66 kDa bands were measured as this protein is the predominant band that reacts with both antibodies. The reaction intensities were measured by the Kodak 1D Image Analysis Program to compare the effects of various temperatures

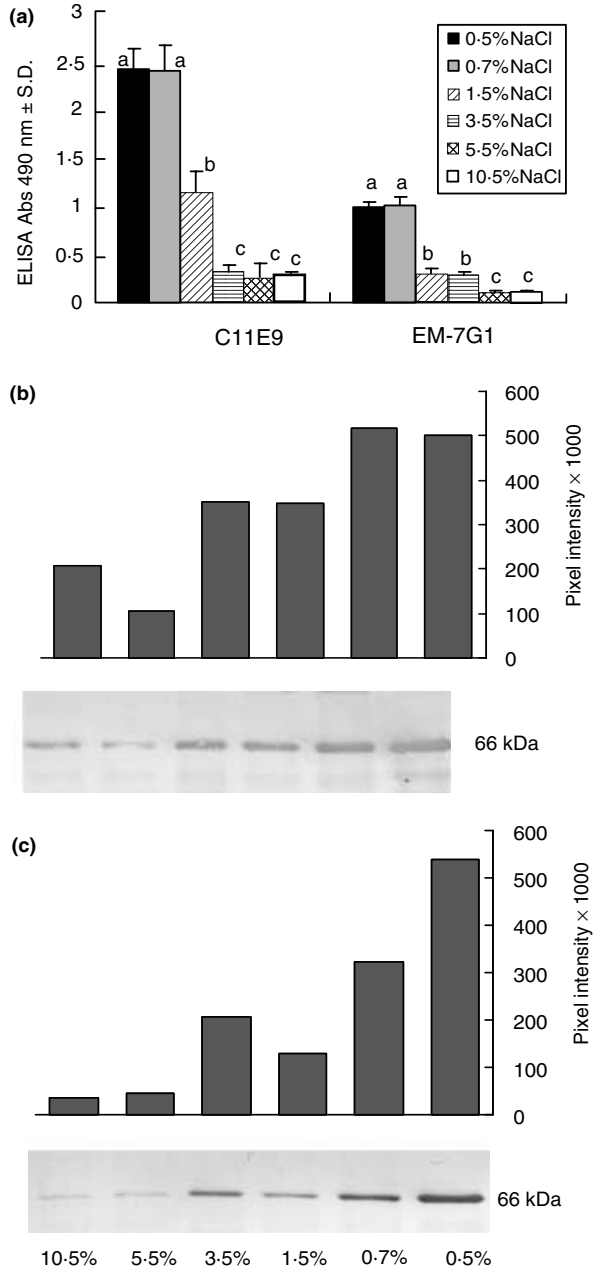

Fig. 5 Reaction analyses of monoclonal antibodies C11E9 or EM-7G1 to *Listeria monocytogenes* V7 cultured at 37°C in the presence of various concentrations of NaCl by (a) ELISA, (b) Western blotting for C11E9 and (c) EM-7G1. NaCl concentrations in brain heart infusion broth were adjusted to be 0·5% (control), 0·7, 1·5, 3·5, 5·5 and 10·5%. Cell concentrations from each treatment were adjusted ($A_{595} = 0{\cdot}37$) to uniform values before analyses by ELISA and Western blotting. The ELISA data are average of three independent tests and bars marked with different letters (a, b, c) are significantly ($P < 0{\cdot}05$) different for a given antibody. In the Western blot, expression of only 66 kDa was analysed (Kodak 1D Image Analysis Program) as this protein is the most predominant band that reacts with both C11E9 and EM-7G1

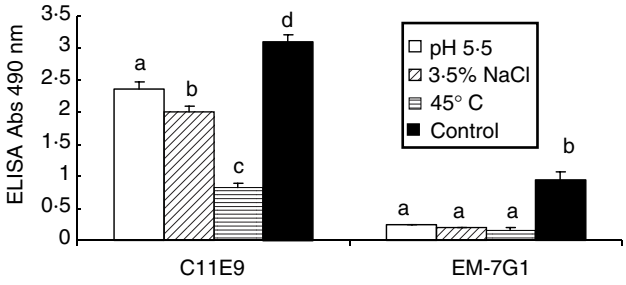

Fig. 6 ELISA analysis of reaction of monoclonal antibodies C11E9 or EM-7G1 to *Listeria monocytogenes* V7 cells grown in homogenized hotdog slurry prepared in brain heart infusion broth, which was adjusted to pH 5·5 or added with 3·5% NaCl (w/v) or incubated at 45°C. Cultures with acid-, osmotic- or thermal-stress were incubated for 22, 18 or 36 h, respectively, while control samples without any stressor were incubated at 37°C for 16 h. Cell concentrations were adjusted to uniform value (A_{595} = 0·37) before ELISA. The data are average of three independent tests and bars marked with different letters (a, b, c, d) are significantly ($P < 0·05$) different for a given antibody

and containing one septum (Fig. 7b). In contrast, cells grown in the presence of 5·5% NaCl appeared extremely filamentous (Fig. 7c) and contained numerous septa along the length of the cells (Fig. 7c). This suggests that NaCl interfered with the formation of the more conventional single cell appearance.

DISCUSSIONS

During food processing or storage, bacteria in foods are routinely exposed to various stress stimuli, such as salinity, acids or high or low temperatures for several hours to several days. Reports suggest that *L. monocytogenes* employs various mechanisms to overcome stress induced by salts (Rouquette *et al.* 1996; Sleator *et al.* 2001), acids (Wiedmann *et al.* 1998; Phan-Thanh *et al.* 2000) or temperatures (Bayles *et al.* 1996; Rouquette *et al.* 1996; Becker *et al.* 2000). Also during exposure to stress, physiological activities of the bacterial cells could be altered affecting expression of surface antigens that, in turn, may affect antibody-specific reaction. Antibodies are central to the success of many immunoassays or

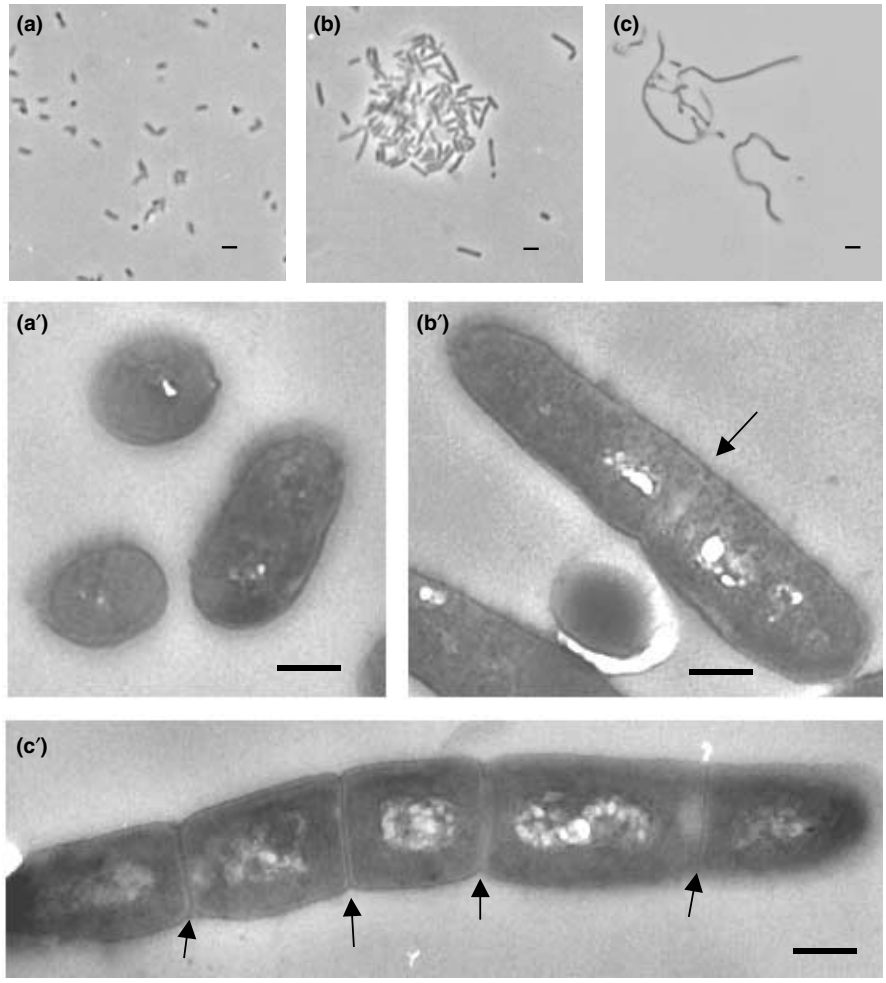

Fig. 7 Light and transmission electron microscopic analyses of morphological characteristics of *Listeria monocytogenes* cells grown in brain heart infusion broth at 37°C for 18 h (a and a′), 45°C for 16 h (b and b′) and broth containing 5·5% NaCl at 37°C (c and c′). The arrows show the septa along the length of the cell. a, b, c: 1000×, Bar = 5 μm. a′, b′, c′: 18 000×, Bar = 0·5 μm

biosensor-based tools that have the potential to be used for detection of bacteria from food (Bhunia 1997; Vo-Dinh and Cullum 2000; Bhunia *et al.* 2001; Gomez *et al.* 2001). Reliable performance of antibodies and their reactions to viable *L. monocytogenes* cells from various stressful environments are essential to obtain unambiguous rapid results. Therefore, the reactions of C11E9 and EM-7G1 were evaluated with *L. monocytogenes* cells that were subjected to several common physical and chemical stresses. Stress conditions such as osmotic stress or high temperature severely affected the antigen expression and, subsequently, affected the antibody performance, whereas low temperatures (4–15°C) or acidic environments (pH 2 or 3) did not affect the antibody reaction. Thermal or osmotic stress factors possibly down-regulated the expression of the antigen during a brief stress exposure and/or caused denaturation of the surface antigens. This could result in conformational changes in the epitopes, thus showing progressively reduced reaction with antibodies (Fig. 3). When the bacteria were allowed to grow in the stressed environments of meat slurry or the BHI broth also resulted in weaker reaction. This could be due to the reduced production of C11E9 or EM-7G1 reactive 66-kDa antigens resulting from down-regulation of the *pepC* gene that encodes for the 66-kDa-antigen (aminopeptidase) (Winters *et al.* 2000).

In a previous study, Sokolovic and Goebel (1989) reported that expression of listeriolysin O was increased several fold when subjected to heat-shock temperature of 48°C for 1 h. However, under the same condition, the expression of p60 was decreased. This suggests differential expression of *L. monocytogenes* proteins under heat-shock conditions.

Reduced reaction of antibodies to stressed bacteria was also reported for *Salmonella* (Duffy *et al.* 2000). In that study, reaction was severely affected when bacteria were subjected to heat (56·9°C for 4 min), high concentration of NaCl (4%) or low pH (pH 3·5). While low concentration of NaCl (2%), pH (5·0) or freezing treatment (−20°C) did not affect antibody-binding. In case of *Listeria* spp., some MAbs were reported to react with heat-killed cells (Ziegler and Orlin 1984; Butman *et al.* 1988; Siragusa and Johnson 1990), while some to viable cells (Torensma *et al.* 1993; Kathariou *et al.* 1994; Sølve *et al.* 2000). Antibody reaction to *Listeria* cells subjected to nonthermal stress conditions of H_2SO_4 (pH 1–4) or sanitizers (quarternary ammonium compound, chlorhexidin and iodophor) also severely affected the reaction while treatment with NaOH (pH 12) or H_2O_2 (3%) did not (Sølve *et al.* 2000).

Stress conditions, particularly treatment with 45°C or NaCl (5·5%), also affected the morphology of cells resulting in filamentous appearance with long chain. In a previous study, Jorgensen *et al.* (1995) reported that growth of *L. monocytogenes* in NaCl concentrations of 8·7% resulted in filamentous cells. When NaCl concentration was reduced to 0·5%, the culture regained its normal single cell appearance. It has been reported before that the rough variant of *L. monocytogenes* with defective *iap* (invasion associated protein, p60), a housekeeping gene with murein hydrolase activity, spontaneously form long chains under normal growth conditions (Wuenscher *et al.* 1993; Rowan *et al.* 2000). It is possible that thermal or osmotic stress down-regulate the expression of such genes, thus rendering cells to become filamentous (Rowan *et al.* 2000).

To negate the effect of stress on morphology or antigen expression, bacterial cells could be held at a growth environment for a period sufficient to regenerate surface antigens fully. This would result in increased efficiency of antibody detection of target cells. Among the stress treatments, cold (4–15°C) or acidic environments (pH 2 or 3), did not show any detrimental effects on the expression of antibody-specific cellular antigens, suggesting that bacteria obtained from these environmental conditions may not need to be resuscitated before testing. The failure or success of any immunoassays to detect target bacterial cells may partly depend on the expression of antigens in cells subjected to different stress producing environments. Therefore, surface-protein expression patterns of target pathogens subject to stress producing environments need to be thoroughly examined before antibody-based methods could be proposed for reliable detection of food-borne pathogens.

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