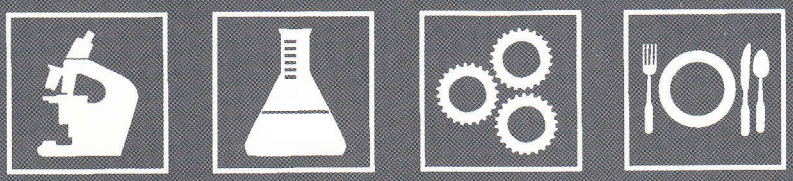
November, 2006
Volume 69, No. 11
Pages 2565–2820
CODEN: JFPRDR 69(11)2565–2820(2006)
ISSN:0362-028X

Journal of Food Protection®



"The mission of the International Association for Food Protection is to provide food safety professionals worldwide with a forum to exchange information on protecting the food supply."

JFP Online
foodprotection.org

Published Monthly by
International Association for Food Protection®
6200 Aurora Avenue, Suite 200W
Des Moines, Iowa 50322-2864 USA
www.foodprotection.org

Journal of Food Protection®

ISSN: 0362-028X
Official Publication

International Association for Food Protection®

Reg. U.S. Pat. Off.

Vol. 69 November 2006 No. 11

* Asterisk indicates author for correspondence.

Research Notes

Reviews

Journal of Food Protection (ISSN-0362-028X) is published monthly by the International Association for Food Protection, 6200 Aurora Avenue, Suite 200W, Des Moines, IA 50322-2864, USA. Each volume consists of 12 issues. Periodical postage paid at Des Moines, Iowa 50318, and additional entry offices. Claims for missing issues must be submitted to the Association within 30 days (US, Canada, and Mexico). International claims must be submitted within 60 days.

Postmaster: Send address changes to *Journal of Food Protection*, International Association for Food Protection, 6200 Aurora Avenue, Suite 200W, Des Moines, IA 50322-2864, USA.

Scope of the Journal: The *Journal of Food Protection* is intended for publication of research and review articles on all aspects of food protection and safety. Major emphases of *JFP* are placed on studies dealing with (i) causes (microorganisms, chemicals, natural toxicants) and control of all forms of foodborne illness; (ii) contamination (microorganisms, chemicals, insects, rodents) and its control in raw food and in foods during processing, distribution, preparation, and service to consumers; (iii) causes of food spoilage and its control through processing (low or high temperatures, preservatives, drying, fermentation, irradiation, pressure, and other innovative technologies); (iv) food quality and microbiological, chemical, and physical methods to assay food quality; and (v) wastes from the food industry and means to use or treat the wastes.

Submission of Manuscripts. All manuscripts must be submitted at http://foodprotection.allentrack.net. Letters to the Editor must be submitted to Didi Loynachan, Administrative Assistant, International Association for Food Protection, 6200 Aurora Avenue, Suite 200W, Des Moines, IA 50322-2864, USA. Instructions for Authors are available at www.foodprotection.org or from the *Journal of Food Protection* Editorial office.

Journal of Food Protection is available by institutional subscription for $345 US, $365 Canada/Mexico, and $395 International. *JFP* Online subscription rate is $600 per volume year. Call the Association for individual membership information. Single copies are available for $39 US and $48 other countries. All rates include shipping and handling. No cancellations accepted. Members of the International Association for Food Protection have the option of receiving *JFP* and *JFP* Online at a substantial discount. Membership information can be obtained from our Web site at www.foodprotection.org.

Request single reprints of articles published in the Journal from the corresponding author at the address listed in the footnote of each article. Electronic reprints are available at www.ingentaselect.com. Microfilm of *Journal of Food Protection* is available from Bell and Howell, 300 N. Zebb Road, Ann Arbor, MI 48106-1346, USA. All rights reserved.

Journal of Food Protection, Vol. 69, No. 11, 2006, Pages 2681–2686

Survival, Elongation, and Elevated Tolerance of *Salmonella enterica* Serovar Enteritidis at Reduced Water Activity

JASPER KIEBOOM,† HARSHI D. KUSUMANINGRUM,‡ MARCEL H. TEMPELAARS, WILMA C. HAZELEGER, TJAKKO ABEE, AND RIJKELT R. BEUMER*

Laboratory of Food Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

MS 05-578: Received 15 November 2005/Accepted 27 May 2006

ABSTRACT

Growing microorganisms on dry surfaces, which results in exposure to low water activity (a_w), may change their normal morphology and physiological activity. In this study, the morphological changes and cell viability of *Salmonella enterica* serovar Enteritidis challenged to low a_w were analyzed. The results indicated that exposure to reduced a_w induced filamentation of the cells. The amount of filamentous cells at a_w 0.94 was up to 90% of the total number of cells. Surviving filamentous cells maintained their membrane integrity after exposure to low a_w for 21 days. Furthermore, cells prechallenged to low a_w, obtained with an ionic humectant, demonstrated higher resistance to sodium hypochlorite than control cells. These resistant cells are able to survive disinfection more efficiently and can therefore cause contamination of foods coming in contact with surfaces. This points to the need for increased attention to cleaning of surfaces in household environments and disinfection procedures in processing plants.

Salmonella enterica serovar Enteritidis is the most important cause of *Salmonella* infections associated with the consumption of shelled eggs and poultry in Europe *(25)* and the United States *(21)*. Cross-contamination directly from raw poultry to ready-to-eat products or indirectly through contaminated surfaces or niches in the household kitchen is the predominant mode of infection *(9)*. In general, microorganisms often experience environmental stresses, such as nutrient starvation, osmotic shock, or temperature variation during transmission. The risks associated with cross-contamination of *Salmonella* Enteritidis from surfaces have been recognized because this microorganism has the ability to survive on stainless steel surfaces for hours or days, depending on the initial counts and the presence of food residues *(11, 13)*. On surfaces, the cells are exposed directly to the air that may lead to water removal from the cells and adjustment of cytoplasmic solvent composition. Osmotic stress is one consequence of the initial stage of the air-drying of cells on surfaces *(4, 20, 22)*. Little is known about the response of *Salmonella* Enteritidis to dry or drying surfaces or surfaces with low water activity (a_w) (e.g., $a_w < 0.96$) and the consequences of cellular adaptation on these surfaces to subsequent stress exposure, such as disinfection with sodium hypochlorite. Hypochlorite is generally used as chemical sanitizer because of its efficient action against a wide variety of microorganisms.

Mattick et al. *(18)* have shown that filamentous cells were formed by three wild-type strains of *Salmonella* Enteritidis in low a_w broth. Lowering of a_w values to 0.95 was achieved by addition of sucrose, glycerol, and sodium chloride (NaCl). With the latter compound in the medium, the elongated cells appeared to be longer and more numerous *(18)*. Furthermore, in another study, it has been shown that air-dried *Salmonella* cells become more tolerant to heat *(12)*.

In this study, as a model for dry or drying surfaces, we investigated the morphological changes and cell viability of eight wild-type strains of *Salmonella* Enteritidis of different phage types after exposure to reduced a_w at different temperatures. Agar surfaces with reduced a_w, obtained with NaCl, were used as a model to study the response of bacteria to low a_w. The cross-protection against hypochlorite solutions as a result of prechallenge to reduced a_w was studied in suspension tests.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Six human isolates of *Salmonella* Enteritidis (1438 [phage type, PT, 13], 1439 [PT 4], 1444 [PT 25], 1391 [PT 21], 1389 [PT 1] and 1514 [PT 28]), a chicken isolate (1138, PT 28), and a chicken meat isolate (1448, PT 4) were obtained from the National Institute of Public Health and the Environment, The Netherlands. The stock cultures were maintained at −80°C in cryovials (Greiner Bio-One GmbH, Frickenhausen, Germany) containing a stationary-phase culture in brain heart infusion (BHI; Difco, Becton Dickinson, Sparks, Md.)

* Author for correspondence. Present address: Laboratory of Food Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands. Tel: +31 (317) 48 32 13; Fax: + 31 (317) 48 49 78; E-mail: rijkelt.beumer@wur.nl.

† Present address: TNO Defence, Security and Safety, Business Unit Biological and Chemical Protection, P.O. Box 45, 2280 AA Rijswijk, The Netherlands.

‡ Present address: Laboratory of Food Microbiology, Department of Food Technology and Human Nutrition, Faculty of Agricultural Engineering and Technology, Bogor Agricultural University, P.O. Box 220, Bogor 16002, Indonesia.

broth with 25% (vol/vol) glycerol (Fluka-Chemica, Buchs, Switzerland) and glass beads (diameter, 2 mm; Emergo, Landsmeer, The Netherlands). Strains were precultured by transferring one glass bead to 10 ml of BHI broth, followed by overnight incubation (20 to 22 h) at 37°C.

Survival in environments with reduced a_w. Survival at low a_w was studied on tryptone soy agar (TSA; Oxoid, Basingstoke, UK) containing 4, 6, and 8% NaCl (Merck, Darmstadt, Germany) in petri dishes, resulting in a_w of 0.97, 0.95, and 0.94, respectively. The a_w was measured with a water activity meter (Novasina, Zurich, Switzerland) that was based on the relative vapor pressure. A total of 50 μl of the appropriate dilutions of the preculture in peptone saline solution (PSS; NaCl 8.5 g/liter and neutralized bacteriological peptone [Oxoid] 1 g/liter) was applied on agar surfaces with a spiral inoculation apparatus (Eddy Jet; IUC, Barcelona, Spain), and the plates were sealed with parafilm to avoid evaporation of water during incubation at 25 and 37°C. The colony counts were determined when the visible colonies were observed: after 2, 4, or 6 days, depending on the a_w of the media and the incubation temperature. The recovery percentages were calculated as quotients of the colony counts at reduced a_w and those on TSA without additional humectants.

On sterilized glass surfaces (2 by 7 cm^2), 0.2 ml of overnight culture of *Salmonella* Enteritidis diluted in fresh BHI broth to a concentration of approximately 10^5 CFU/ml was applied and spread with a sterile loop, then incubated at 25 and 37°C in petri dishes. Microscopic examination of the glass surfaces with a Zeiss standard 20 light microscope was performed 24, 48, and 72 h after incubation.

Morphology changes. The morphology changes of the cells (i.e., cell elongation) were observed by viewing the cells from agar plates prepared on a microscopic slide with a ×100 phase contrast objective of a Zeiss standard 20 light microscope. Images recorded by a Sony Hyper HAD, CCD-Iris/RGB color video camera. A Protocol computer system (Synoptics Ltd., Cambridge, UK) was used to generate digital photomicrographs. The percentages of elongated cells, calculated from the total cells, were determined by the direct microscopic count procedure with a Bürker-Türk counting chamber (Schreck, Hofheim, Germany). Results are displayed as averages from two experiments with five observations for each experiment. Because under optimal growth condition *Salmonella* Enteritidis cells were found to be between 2 to 3 μm in length, cells longer than two times the maximal length (i.e., 6 μm) were considered to be elongated cells.

Cell viability. The cells grown at a_w 0.95 and a_w 0.94 and incubated for 6 days at 25°C were transferred with a sterile loop into an Eppendorf tube (Greiner Bio-One) containing 1 ml of PSS. The cell suspensions were centrifuged (BHG-Hermle, Gosheim, Germany) at 4,000 × *g* at 4°C for 5 min, and the pellets were resuspended in PSS to a concentration ranging from 1.0 to 1.5 × 10^8 CFU/ml. The culturability of these cells was determined on TSA and mannitol lysine crystal violet brilliant green agar (MLCB; Oxoid), a selective medium for isolation of *Salmonella*.

The membrane integrity of the cells grown at reduced a_w was determined with LIVE/DEAD BacLight Bacterial Viability Kits (Molecular Probes, Inc., Eugene, Oreg.) according to the protocols provided with the kit. This kit uses mixtures of 3.34 mM SYTO 9 green fluorescent nucleic acid stain and 20 mM of the red fluorescent nucleic acid stain propidium iodide (PI). With a 1:1 mixture of the SYTO 9 and PI stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The cell suspensions were microscopically analyzed with an Axioskop epifluorescence microscope equipped with a 50 W mercury arc lamp, a fluorescein isothiocyanate filter set (excitation wavelength, 450 to 490 nm; emission wavelength, >515 nm), a ×100 1.3-numerical aperture Plan-Neofluar objective lens, and a camera (Carl Zeiss, Oberkochen, Germany). Photomicrographs were made with simultaneous light and epifluorescence microscope, a low light intensity, a magnification of ×1,000, and an exposure time of 15 to 45 s on Kodak 400 ASA color film. In these photomicrographs, both the SYTO 9 and PI-labeled cells could be counted. Depending on the number of cells, 10 to 20 microscopic fields were counted.

Flow cytometry. The cells grown at reduced a_w, labeled with BacLight bacterial viability kits, were analyzed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, Calif.) equipped with a 15 mW blue light at 488 nm, air-cooled argon ion laser. The side scatter signal was used as a trigger signal. The green fluorescence from SYTO 9–stained cells was detected through a 515- to 545-nm band-pass filter (FL1 channel), and the red fluorescence of the PI signal was collected in the FL3 channel (>670 nm long-pass filter). FACSFlow solution (Becton Dickinson) was used as the sheath fluid. The cells were measured at a low flow rate corresponding to 150 to 500 cells per second, and 10,000 events were collected for further analysis. A combination of forward scatter (FSC) and side scatter (SSC) signals was used to discriminate bacteria from the background and to characterize the morphology of the cells. All signals were collected by logarithmic amplifications. Data from the flow cytometer were analyzed by WinMDI (Joseph Totter, Salk Institute for Biological Studies, La Jolla, Calif.; available at http://facs.scripps.edu/software.html).

Cross-protection to sodium hypochlorite challenges. The cell suspensions were prepared from the population on TSA with reduced a_w and incubated at 25°C for 6 days to a concentration of 1.0 to 1.5 × 10^8 CFU/ml, as described for examination of the cell culturability. Sodium hypochlorite (Acros Organics, Morris Plains, N.J.) solutions were prepared at chlorine concentrations of 4.2 mM (300 ppm) and 5.6 mM (400 ppm). The available chlorine concentrations were confirmed by titration *(1)*. The preparation of the solutions and the suspension tests was carried out following European Norm 1276 *(4)*. Bovine serum albumin (3 g/liter from Sigma-Aldrich, Steinheim, Germany) was used as an interfering substance to simulate dirty conditions. The reductions of the log numbers were determined at 10, 30, and 60 min of exposure to hypochlorite.

Statistical analyses. Each experiment was carried out at least twice on different days, and no fewer than two replications were performed for each experiment. Except flow cytometry data, data analyses were performed on the statistical software package SPSS for Windows 95/98/NT/2000, release 10.1 (SPSS Inc., Chicago, Ill.). A P value of <0.05 was considered to be statistically significant.

RESULTS

Survival in reduced a_w environments. In BHI broth (a_w 0.999), all strains formed visible colonies 2 days after incubation at 37°C at a_w 0.97 and 0.95, whereas at 25°C, the colonies appeared after 4 and 6 days, respectively. Strikingly, at a_w 0.94 no separate colonies were formed, but a thin layer of bacterial growth appeared. The recovery percentages, calculated as quotients of the colonies at reduced a_w and those on TSA without additional humectants, were

broth with 25% (vol/vol) glycerol (Fluka-Chemica, Buchs, Switzerland) and glass beads (diameter, 2 mm; Emergo, Landsmeer, The Netherlands). Strains were precultured by transferring one glass bead to 10 ml of BHI broth, followed by overnight incubation (20 to 22 h) at 37°C.

Survival in environments with reduced a_w. Survival at low a_w was studied on tryptone soy agar (TSA; Oxoid, Basingstoke, UK) containing 4, 6, and 8% NaCl (Merck, Darmstadt, Germany) in petri dishes, resulting in a_w of 0.97, 0.95, and 0.94, respectively. The a_w was measured with a water activity meter (Novasina, Zurich, Switzerland) that was based on the relative vapor pressure. A total of 50 μl of the appropriate dilutions of the preculture in peptone saline solution (PSS; NaCl 8.5 g/liter and neutralized bacteriological peptone [Oxoid] 1 g/liter) was applied on agar surfaces with a spiral inoculation apparatus (Eddy Jet; IUC, Barcelona, Spain), and the plates were sealed with parafilm to avoid evaporation of water during incubation at 25 and 37°C. The colony counts were determined when the visible colonies were observed: after 2, 4, or 6 days, depending on the a_w of the media and the incubation temperature. The recovery percentages were calculated as quotients of the colony counts at reduced a_w and those on TSA without additional humectants.

On sterilized glass surfaces (2 by 7 cm^2), 0.2 ml of overnight culture of *Salmonella* Enteritidis diluted in fresh BHI broth to a concentration of approximately 10^5 CFU/ml was applied and spread with a sterile loop, then incubated at 25 and 37°C in petri dishes. Microscopic examination of the glass surfaces with a Zeiss standard 20 light microscope was performed 24, 48, and 72 h after incubation.

Morphology changes. The morphology changes of the cells (i.e., cell elongation) were observed by viewing the cells from agar plates prepared on a microscopic slide with a ×100 phase contrast objective of a Zeiss standard 20 light microscope. Images recorded by a Sony Hyper HAD, CCD-Iris/RGB color video camera. A Protocol computer system (Synoptics Ltd., Cambridge, UK) was used to generate digital photomicrographs. The percentages of elongated cells, calculated from the total cells, were determined by the direct microscopic count procedure with a Bürker-Türk counting chamber (Schreck, Hofheim, Germany). Results are displayed as averages from two experiments with five observations for each experiment. Because under optimal growth condition *Salmonella* Enteritidis cells were found to be between 2 to 3 μm in length, cells longer than two times the maximal length (i.e., 6 μm) were considered to be elongated cells.

Cell viability. The cells grown at a_w 0.95 and a_w 0.94 and incubated for 6 days at 25°C were transferred with a sterile loop into an Eppendorf tube (Greiner Bio-One) containing 1 ml of PSS. The cell suspensions were centrifuged (BHG-Hermle, Gosheim, Germany) at 4,000 × *g* at 4°C for 5 min, and the pellets were resuspended in PSS to a concentration ranging from 1.0 to 1.5 × 10^8 CFU/ml. The culturability of these cells was determined on TSA and mannitol lysine crystal violet brilliant green agar (MLCB; Oxoid), a selective medium for isolation of *Salmonella*.

The membrane integrity of the cells grown at reduced a_w was determined with LIVE/DEAD BacLight Bacterial Viability Kits (Molecular Probes, Inc., Eugene, Oreg.) according to the protocols provided with the kit. This kit uses mixtures of 3.34 mM SYTO 9 green fluorescent nucleic acid stain and 20 mM of the red fluorescent nucleic acid stain propidium iodide (PI). With a 1:1 mixture of the SYTO 9 and PI stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The cell suspensions were microscopically analyzed with an Axioskop epifluorescence microscope equipped with a 50 W mercury arc lamp, a fluorescein isothiocyanate filter set (excitation wavelength, 450 to 490 nm; emission wavelength, >515 nm), a ×100 1.3-numerical aperture Plan-Neofluar objective lens, and a camera (Carl Zeiss, Oberkochen, Germany). Photomicrographs were made with simultaneous light and epifluorescence microscope, a low light intensity, a magnification of ×1,000, and an exposure time of 15 to 45 s on Kodak 400 ASA color film. In these photomicrographs, both the SYTO 9 and PI-labeled cells could be counted. Depending on the number of cells, 10 to 20 microscopic fields were counted.

Flow cytometry. The cells grown at reduced a_w, labeled with BacLight bacterial viability kits, were analyzed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, Calif.) equipped with a 15 mW blue light at 488 nm, air-cooled argon ion laser. The side scatter signal was used as a trigger signal. The green fluorescence from SYTO 9–stained cells was detected through a 515- to 545-nm band-pass filter (FL1 channel), and the red fluorescence of the PI signal was collected in the FL3 channel (>670 nm long-pass filter). FACSFlow solution (Becton Dickinson) was used as the sheath fluid. The cells were measured at a low flow rate corresponding to 150 to 500 cells per second, and 10,000 events were collected for further analysis. A combination of forward scatter (FSC) and side scatter (SSC) signals was used to discriminate bacteria from the background and to characterize the morphology of the cells. All signals were collected by logarithmic amplifications. Data from the flow cytometer were analyzed by WinMDI (Joseph Totter, Salk Institute for Biological Studies, La Jolla, Calif.; available at http://facs.scripps.edu/software.html).

Cross-protection to sodium hypochlorite challenges. The cell suspensions were prepared from the population on TSA with reduced a_w and incubated at 25°C for 6 days to a concentration of 1.0 to 1.5 × 10^8 CFU/ml, as described for examination of the cell culturability. Sodium hypochlorite (Acros Organics, Morris Plains, N.J.) solutions were prepared at chlorine concentrations of 4.2 mM (300 ppm) and 5.6 mM (400 ppm). The available chlorine concentrations were confirmed by titration *(1)*. The preparation of the solutions and the suspension tests was carried out following European Norm 1276 *(4)*. Bovine serum albumin (3 g/liter from Sigma-Aldrich, Steinheim, Germany) was used as an interfering substance to simulate dirty conditions. The reductions of the log numbers were determined at 10, 30, and 60 min of exposure to hypochlorite.

Statistical analyses. Each experiment was carried out at least twice on different days, and no fewer than two replications were performed for each experiment. Except flow cytometry data, data analyses were performed on the statistical software package SPSS for Windows 95/98/NT/2000, release 10.1 (SPSS Inc., Chicago, Ill.). A P value of <0.05 was considered to be statistically significant.

RESULTS

Survival in reduced a_w environments. In BHI broth (a_w 0.999), all strains formed visible colonies 2 days after incubation at 37°C at a_w 0.97 and 0.95, whereas at 25°C, the colonies appeared after 4 and 6 days, respectively. Strikingly, at a_w 0.94 no separate colonies were formed, but a thin layer of bacterial growth appeared. The recovery percentages, calculated as quotients of the colonies at reduced a_w and those on TSA without additional humectants, were

TABLE 1. *Percentages of elongated cells (>6 μm) at reduced a_w after 6 days at 25°C, determined by direct microscopic counting*[a]

Strain	Percentage of elongated cells at:	
	a_w 0.95	a_w 0.94
1138	29 ± 6	88 ± 4
1389	24 ± 5	94 ± 2
1391	40 ± 5	90 ± 3
1444	38 ± 6	94 ± 2
1448	24 ± 5	93 ± 2
1438	34 ± 4	82 ± 6
1439	29 ± 4	87 ± 3
1514	35 ± 3	90 ± 6

[a] Data are expressed as average ± SD percentages.

in a range of 70 to 95% at a_w 0.97, and 30 to 70% at a_w 0.95. The overall recovery at 25 or 37°C was not dependent on the temperature ($P = 0.45$) but was only affected by the a_w ($P = 0.01$).

Morphology changes. Microscopy revealed that all tested strains of *Salmonella* Enteritidis formed elongated cells (longer than 6 μm) at a_w 0.95 and a_w 0.94. At a_w 0.97, no elongated cells were found. Direct microscopic counting indicated that at a_w 0.95, the percentage of the elongated cells was between 24 and 40%, and at a_w 0.94 between 82 and 90% of the total cell numbers (Table 1). Particularly at 25°C and a_w 0.94, elongated cells with a size of 50 μm or more were found (Fig. 1). Microscopical analysis of cells in liquid low a_w medium, obtained with NaCl and glucose (data not shown), both showed the formation of elongated cells. This suggested that reduced a_w resulted in filamentation and that this was not due to surface effects or the presence of high NaCl concentrations.

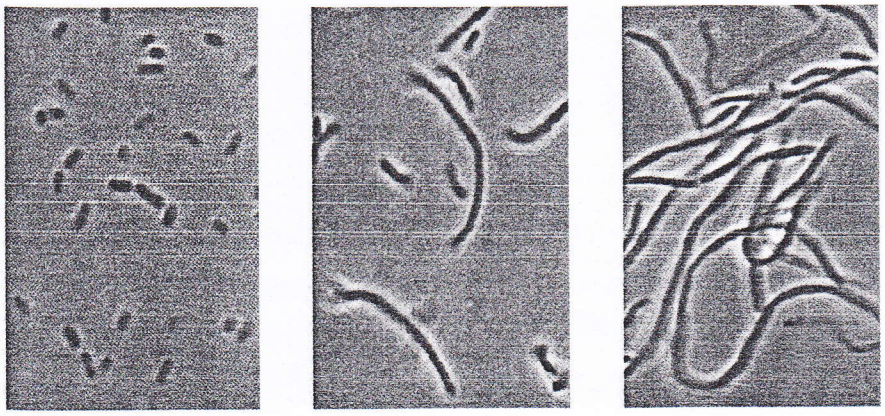
Our study also indicated that when an aliquot of bacterial suspension in fresh BHI broth was applied on glass surfaces, elongated cells were microscopically observed after slow air-drying for 24 h at 37°C and for 48 h at 25°C. The elongated cells were found on these glass surfaces in low percentages (approximately 3% of the cell population), indicating that filamentation of the cells may occur on *Salmonella*-contaminated wet surfaces after a slow-drying process.

Analysis of data obtained by flow cytometry indicated that cells challenged to a_w 0.95 showed slightly higher signals on FSC and SSC (Fig. 2B) compared with the control cells grown on TSA (Fig. 2A). A noticeable increase of FSC and SSC signals was observed in cell populations grown at a_w 0.94, which resulted in a complete shift of a population on both detector signals (Fig. 2C). These findings indicated that particularly at a_w 0.94 cells with large dimensions were observed, which was confirmed by the microscopy analysis.

Cell viability. The culturability on TSA and MLCB of cells prechallenged at a_w 0.95 and 0.94 at 25°C for 6 days are shown in Figure 3. In a control experiment, we demonstrated equal colony counts on MLCB and TSA, indicating that the viability of cells was not affected by the selective agents present in MLCB.

Epifluorescence microscopy of the stressed cells revealed the existence of four subpopulations composed of viable short and elongated cells as well as nonviable short and elongated cells (Fig. 4). The viability was based on assessment of intact or damaged membrane of individual cells. Dead cells with damaged membrane accumulated PI and were stained fluorescent red. At a_w 0.95 at 25°C, about 80 and 70% of the total number of the cells were still viable after 6 and 21 days, respectively. Among the elongated cells, the percentage of the viable cells was approximately 75% after 6 days and decreased to 50% after 21 days of exposure (Fig. 4). At a_w 0.94, about 50% of the cell population lost their viability after 6 days. Exposure at a_w 0.94 resulted in more rapid loss of viability than at higher a_w. Furthermore, when the elongated cells were recovered in BHI broth and incubated at 37°C, the majority of the filaments split up, and the separation was complete within approximately 3 h, as was observed under the microscope (data not shown).

Figure 2 shows dot plots of events collected by flow cytometry of the control cells (D) and cells challenged at a_w 0.94 (E), subsequently stained with the BacLight kit. FL1 is a measure for green fluorescence of SYTO 9–stained cells. The control cells demonstrated low signals on SSC



A B C

FIGURE 1. *Impact of reduced water activities on cell morphology of* Salmonella *Enteritidis strain 1448. (A) Control cells, (B) cells at a_w 0.95, and (C) cells at a_w 0.94, after 6 days at 25°C.*

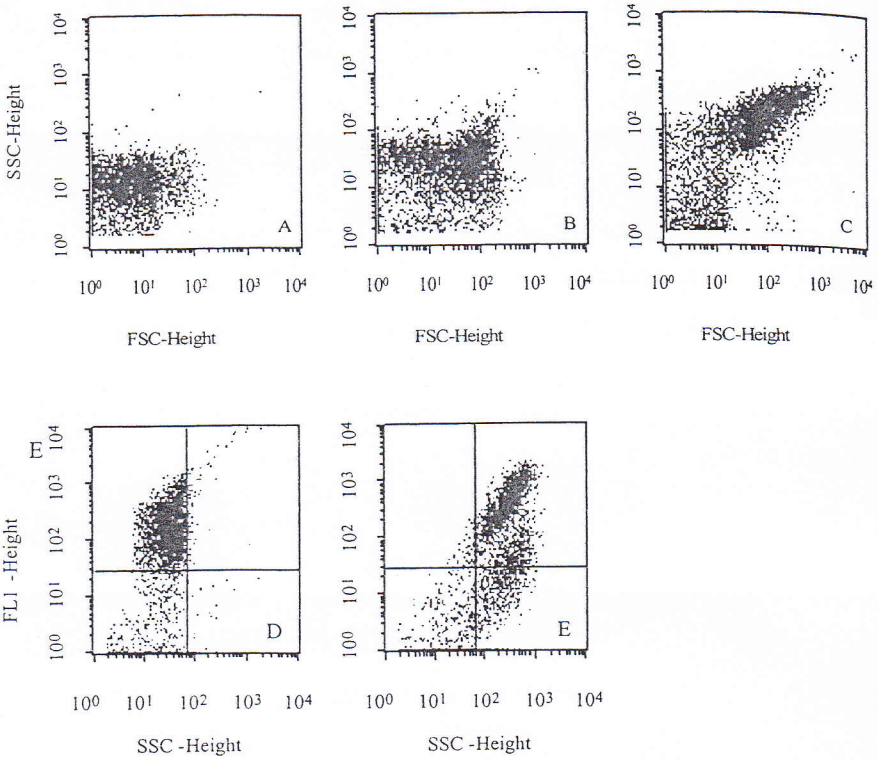


FIGURE 2. *Flow cytometry analysis of low a_w stressed* Salmonella *Enteritidis strain 1448 after 6 days at 25°C. Forward side scatter (FSC) versus side scatter signals (SSC) of (A) cells grown on TSA, (B) cells challenged at a_w 0.95 and (C) at a_w 0.94, and green fluorescence (FL1) versus side scatter signals (SSC) of (D) cells grown on TSA, and (E) cells challenged at a_w 0.94.*

with high signals on FL1, indicating that almost all cells were viable. Cells challenged at a_w 0.94 revealed a shift in population with higher SSC signals, indicating formation of elongated cells as also shown before in Figure 2C, and this consisted of two populations (approximately 50% of the total counts for each population) either with high and low signals on FL1 (Fig. 2E). Moreover, the population with the low FL1 signals also displayed high FL3 signals (data not shown), indicating that these PI-stained cells are dead. These results confirm the findings obtained by the fluorescence microscopy analysis, which indicated that after exposure at 0.94 for 6 days at 25°C, the majority of the cells were elongated, and approximately 50% of the cells were viable.

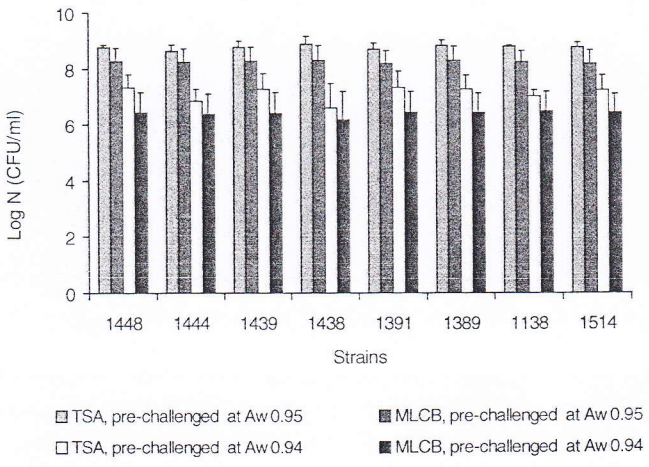


FIGURE 3. *Culturability on TSA and MLCB agar of* Salmonella *Enteritidis strains prechallenged for 6 days at 25°C at a_w 0.95 and a_w 0.94* (n = 2).

Cross-protection to sodium hypochlorite. The tolerance of cells grown on TSA (control) and prechallenged at reduced a_w, obtained with NaCl, to hypochlorite solutions at 25°C is shown in Figure 5. Experiments showed that the NaCl downshock by serial dilutions in PSS did not influence the results (data not shown). At a chlorine concentration of 300 ppm (4.2 mM), a 3-log reduction was observed for control cells after 60 min of exposure. Cells that were

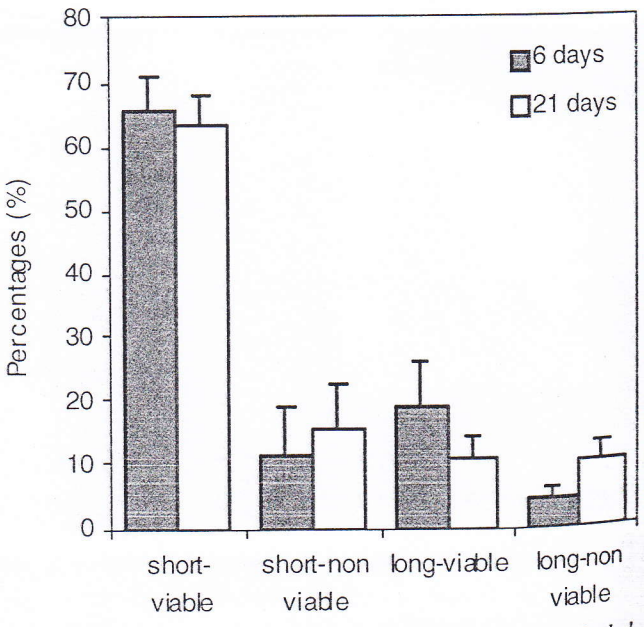


FIGURE 4. *Viability of* Salmonella *Enteritidis strain 1448 challenged at a_w 0.95 at 25°C, determined by direct microscopy counting after staining with Live/Dead BacLight viability kit* (n = 2). *Cells longer than 6 μm were considered as elongated cells.*

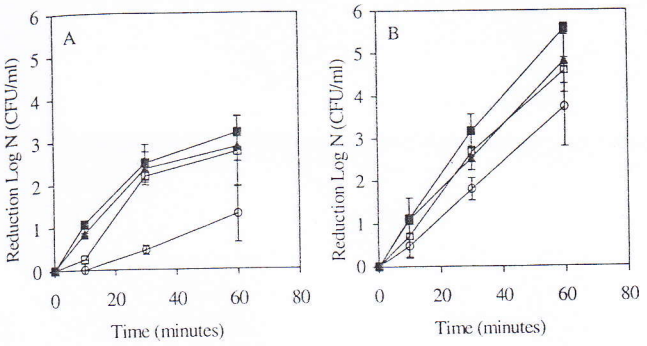


FIGURE 5. *Tolerance to sodium hypochlorite of* Salmonella *Enteritidis strain 1448 at (A) 300 ppm (4.2 mM) and (B) 400 ppm (5.6 mM)* (n = 3). ■, *Control cells;* ▲, *cells prechallenged at* a_w *0.97;* ○, *cells prechallenged at* a_w *0.95;* □ *cells prechallenged at* a_w *0.94.*

prechallenged to a_w 0.95 were the most tolerant to the treatment, followed by the cells challenged to a_w 0.94 and 0.97.

At 400 ppm (5.6 mM), a higher killing efficiency was found with the same trend. After 60 min of exposure, more than a 5-log reduction was observed for the control cells, whereas cells prechallenged to a_w 0.95 were reduced by approximately 3 log units. The cells that were prechallenged to a_w 0.97 and 0.94 decreased by approximately 4.5 log units. Overall, the cells challenged to reduced a_w demonstrated better tolerance to hypochlorite than the control cells.

DISCUSSION

In this study, the responses of eight wild-type strains of *Salmonella* Enteritidis to reduced a_w environments were analyzed. Challenge at a_w 0.95 and 0.94 resulted in cell elongation of all tested *Salmonella* Enteritidis strains.

Mattick et al. *(19)* demonstrated that filamentous salmonellas contained regularly spaced nucleoids, which indicated that cells were probably blocked in septation. It is conceivable that the cells elongation resulted from inactivation or inhibition of cell division proteins, which in turn blocks the septation during the cell division *(16, 23)*. It has been reported that FtsZ is by far the best-conserved cell division protein; it is also present in most species of bacteria *(16, 23)*. Next to FtsZ, when any one of the cell division proteins in *Escherichia coli,* including FtsA, FtsI, FtsK, FtsL, FtsN, FtsQ, FtsW, and ZipA, is nonfunctional or absent, cells grow without dividing, which leads to the formation of filaments *(3, 6)*.

Because not all cells were elongated, as was particularly observed at a_w 0.95, the response of *Salmonella* Enteritidis strains to a_w reduction occurs at the level of the individual cell. Booth *(2)* suggested that response to stress largely takes place at the single cell level and can lead to heterogeneity in a bacterial population. This heterogeneity is a recognized property of bacterial populations and allows adaptation to a diversity of niches. Any protein that is required for survival is capable of contributing to the heterogeneity *(2)*.

Cellular parameters essential to survival under stress conditions are the integrity of the cell membrane, maintenance of the folding of proteins, and the integrity of the DNA *(2, 5)*. Discrimination between intact and permeable cells by fluorescent stains has been used in many studies on viability of bacteria *(6)*. Examining the cell viability by means of fluorescent techniques highlighted the heterogeneity of *Salmonella* Enteritidis populations in response to challenge to low a_w because both viable and nonviable short and elongated cells were observed. The filamentation of the cells resulted in higher signals on FSC and SSC by flow cytometry. FSC light is laser light diffracted around the cells and is related to cell surface area. SSC light is reflected and refracted laser light; is related to the internal complexity or granularity of a cell *(6)*. A large population of cells (i.e., 10,000 events) was measured by flow cytometry, which offered substantial information on the morphological heterogeneity of this particular bacterial population, allowing sorting and subsequent characterization of filamentous cells in future experiments.

Studies have demonstrated the fact that *Salmonella* cells adapted to certain stress conditions show cross-protection against other stresses *(17, 24)*. In this study, we investigated the effect of sodium hypochlorite on cells prechallenged to low a_w obtained with an ionic humectant. When mixed with water, sodium hypochlorite dissociates and forms hypochlorous acid (HOCl), an active form of chlorine. HOCl is an effective disinfectant partly because most microorganisms do not posses specific enzymes for detoxification of HOCl, like they do for other oxidants such as reactive oxygen species *(14)*. This study indicated that the cells prechallenged to low a_w show better tolerance against sodium hypochlorite than the control cells with ionic humectant. Cross-protection to hypochlorite may be conferred by the expression of the stress sigma factor *rpoS* and the subsequent synthesis of stress-related proteins in the cells exposed to low a_w *(8)*. Moreover, the addition of the ionic humectant NaCl may induce the accumulation of compatible solutes such as betaine that may confer protection against the detrimental effects of sodium hypochlorite by maintaining cellular protein conformation and enzyme activities and supporting cell membrane integrity *(7, 10, 15)*.

The survival of *Salmonella* Enteritidis at reduced a_w—as low as 0.94—increases the risk of cross-contamination because these tolerant cells can come into contact with foodstuffs placed on these surfaces. In this study, we observed that the filamentation of the cells resulted in an increase of the optical density in broth without apparent increase in colony-forming units (data not shown), indicating that filamentous cells form single colonies on plates. However, when these elongated cells were recovered under favorable conditions, the filaments could split up and form numerous single cells. The possible presence of elongated cells on surfaces should be considered a potential infection risk because these filaments are viable for several days and can rapidly split up under favorable conditions in foodstuffs, resulting in a large number of viable cells. Furthermore, the existence of a population tolerant to hypochlorite after challenge to low a_w poses an important risk for public health. These cells can survive disinfection in processing

plants or on household surfaces more efficiently. Therefore, increased attention should be paid to the cleaning and disinfection procedures used on surfaces in these environments.

ACKNOWLEDGMENTS

We thank Kaouther Ben-Amor for her valuable comments on flow cytometry assessments. This project was partly funded by the Health, Safety and Sustainability program of The Netherlands Organization for Health Research and Development.

REFERENCES

1. Bender, D. F., J. P. Gould, J. D. Johnson, and A. T. Palin. 1985. Determination of inorganic nonmetallic constituents: chlorine (residual). *In* A. E. Greenberg, R. R. Trussell, and L. S. Clesceri (ed.), Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
2. Booth, I. R. 2002. Stress and the single cell: intrapopulation diversity is a mechanism to ensure survival upon exposure to stress. *Int. J. Food Microbiol.* 78:19–30.
3. Chen, J. C., and J. Beckwith. 2001. FtsQ, FtsL and FtsI require FtsK, but not FtsN, for co-localization with FtsZ during *Escherichia coli* cell division. *Mol. Microbiol.* 42:395–413.
4. Comité Européen de Normalisation, European Committee for Standardization (CEN). 1997. European Norm 1276. Chemical disinfectants and antiseptics—quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas—test method and requirements (phase 2, step 1). British Standard Institute, London.
5. Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* 53:121–147.
6. Davey, H. M., and D. B. Kell. 1996. Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiol. Rev.* 60:641–649.
7. Diamant, S., D. Rosenthal, A. Azem, N. Eliahu, A. P. Ben-Zvi, and P. Goloubinoff. 2003. Dicarboxylic amino acids and glycine-betaine regulate chaperone-mediated protein-disaggregation under stress. *Mol. Microbiol.* 49:401–410.
8. Dodd, C. E., and T. G. Aldsworth. 2002. The importance of RpoS in the survival of bacteria through food processing. *Int. J. Food Microbiol.* 74:189–194.
9. Dufrenne, J., W. Ritmeester, E. Delfgou-van Asch, F. van Leusden, and R. de Jonge. 2001. Quantification of the contamination of chicken and chicken products in The Netherlands with *Salmonella* and *Campylobacter*. *J. Food Prot.* 64:538–541.
10. Gutierrez, C., T. Abee, and I. R. Booth. 1995. Physiology of the osmotic stress response in microorganisms. *Int. J. Food Microbiol.* 28:233–244.
11. Humphrey, T. J., E. Slater, K. McAlpine, R. J. Rowbury, and R. J. Gilbert. 1995. *Salmonella*-Enteritidis phage type-4 isolates more tolerant of heat, acid, or hydrogen-peroxide also survive longer on surfaces. *Appl. Environ. Microbiol.* 61:3161–3164.
12. Kirby, R. M., and R. Davies. 1990. Survival of dehydrated cells of *Salmonella* Typhimurium LT2 at high-temperatures. *J. Appl. Bacteriol.* 68:241–246.
13. Kusumaningrum, H. D., G. Riboldi, W. C. Hazeleger, and R. R. Beumer. 2003. Survival of foodborne pathogens on stainless surfaces and cross-contamination to foods. *Int. J. Food Microbiol.* 25:227–236.
14. Leyer, G. J., and E. A. Johnson. 1997. Acid adaptation sensitizes *Salmonella typhimurium* to hypochlorous acid. *Appl. Environ. Microbiol.* 63:461–467.
15. Lloyd, A. W., J. A. Baker, G. Smith, C. J. Olliff, and K. J. Rutt. 1992. A comparison of glycine, sarcosine, N,N-dimethylglycine, glycinebetaine and N-modified betaines as liposome cryoprotectants. *J. Pharm. Pharmacol.* 44:507–511.
16. Margolin, W. 2000. Themes and variations in prokaryotic cell division. *FEMS Microbiol. Rev.* 24:531–548.
17. Mattick, K. L., F. Jorgensen, J. D. Legan, M. B. Cole, J. Porter, H. M. Lappin-Scott, and T. J. Humphrey. 2000. Survival and filamentation of *Salmonella* Enteritidis PT4 and *Salmonella enterica* serovar Typhimurium DT104 at low water activity. *Appl. Environ. Microbiol.* 66:1274–1279.
18. Mattick, K. L., F. Jorgensen, J. D. Legan, H. M. Lappin-Scott, and T. J. Humphrey. 2000. Habituation of *Salmonella* spp. at reduced water activity and its effect on heat tolerance. *Appl. Environ. Microbiol.* 66:4921–4925.
19. Mattick, K. L., L. E. Phillips, F. Jorgensen, H. M. Lappin-Scott, and T. J. Humphrey. 2003. Filament formation by *Salmonella* spp. inoculated into liquid food matrices at refrigeration temperatures, and growth patterns when warmed. *J. Food Prot.* 66:215–219.
20. O'Byrne, C. P., and I. R. Booth. 2002. Osmoregulation and its importance to food-borne microorganisms. *Int. J. Food Microbiol.* 74:203–216.
21. Olsen, S. J., L. C. MacKinon, J. S. Goulding, N. H. Bean, and L. Slutsker. 2002. Surveillance for foodborne disease outbreaks—United States, 1993–1997. MMWR surveillance summaries 49 (SS01). Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/ss4901a1.htm. Accessed 25 July 2006.
22. Potts, M. 1994. Desiccation tolerance of prokaryotes. *Microbiol. Rev.* 58:755–805.
23. Rothfield, L., S. Justice, and J. Garcia-Lara. 1999. Bacterial cell division. *Annu. Rev. Genet.* 33:423–448.
24. Saby, S., P. Leroy, and J. C. Block. 1999. *Escherichia coli* resistance to chlorine and glutathione synthesis in response to oxygenation and starvation. *Appl. Environ. Microbiol.* 65:5600–5603.
25. Schmidt, K., and C. Tirado (ed.). 2001. WHO surveillance programme for control of foodborne infections and intoxications in Europe, 7th report, 1993–1998. Federal Institute for Health Protection of Consumers and Veterinary Medicine, Berlin, Germany.