FULL PAPER *Public Health*

Survival of *Salmonella* Strains Differing in Their Biofilm-Formation Capability upon Exposure to Hydrochloric and Acetic Acid and to High Salt

Akio HASEGAWA[1)], Yukiko HARA-KUDO[2,3)*] and Susumu KUMAGAI[2)]

[1)]*Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657,*
[2)]*Research Center for Food Safety, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657 and* [3)]*Division of Microbiology, National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan*

(Received 24 February 2011/Accepted 2 May 2011/Published online in J-STAGE 16 May 2011)

ABSTRACT. Acidic and osmotic treatments are part of hurdle systems to control pathogens such as *Salmonella* in food. In the current study, *Salmonella enterica* isolates previously shown to differ in their ability to form biofilms were grown in diluted tryptic soy broth (TSB) (1:5 dilution in distilled water) and subsequently exposed to phosphate-buffered saline (PBS) adjusted to pH 3.0 with HCl, PBS adjusted to pH 3.9 with acetic acid or rice vinegar diluted 1:15 with distilled water (pH 3.9). Cells grown in diluted TSB were also exposed to distilled water, pH 7.6, containing 5 M NaCl. No differences in survival upon exposure to PBS adjusted to pH 3.0 with HCl or distilled water containing high salt were observed between the isolates; however, exposure to acetic acid and rice vinegar resulted in lower survival levels of isolates previously shown to be poor biofilm formers. The numbers ($\log_{10}$ cfu/m*l*) of surviving cells after exposure for 36 hr to acetic acid and rice vinegar were 4.43 ± 0.24 vs. 2.27 ± 0.87 (P<0.05) and 5.19 ± 0.12 vs. 2.33 ± 0.93 (P<0.05) for isolates with a high vs. low biofilm-forming ability. The survival data could be fitted with the Weibull model. The data suggest that the ability of *Salmonella* strains to survive in the presence of acetic acid and rice vinegar parallels their ability to form biofilms. Thus, *Salmonella* with a high biofilm-formation capability might be more difficult to kill with acetic acid found in foods or cleaning solutions.
KEY WORDS: acid, biofilm, high osmolality, *Salmonella*, tolerance.

J. Vet. Med. Sci. 73(9): 1163–1168, 2011

Bacterial survival under various stresses is a great concern to food manufacturing and processing. Frequently, acidic and/or osmotic conditions are used for food preservation and to control hazardous bacteria such as *Salmonella.* Acids are also commonly applied in washes for foods and food contact surfaces.

Many bacteria are able to grow as biofilms when in contact with surfaces or at the air/medium interface [23, 36]. The biofilms are characterized by the presence of polymeric matrices produced by the bacteria, and these matrices together with physiological changes in the biofilm bacteria are known to alter the susceptibility of these bacteria to exogenous stresses and agents [18]. For example, it is known that *Escherichia coli, Streptococcus mutans* and *Vibrio cholerae* cells embedded in biofilms differ in their acid and osmotic tolerance from free-living cells [22, 25, 27, 38, 43] and that *Salmonella* cells embedded in biofilms show reduced susceptibility to trisodium phosphate [23], desiccation [21] and chlorination [36].

It is known that isolates of *Salmonella* differ in their ability to form biofilms [21, 39]. The connection between biofilm-forming ability and risk of foodborne outbreaks has been suggested in *Salmonella* and *Listeria* [10, 28, 42]. *Salmonella* strains with high biofilm-forming abilities were also more frequently observed in fish meal and feed factories than in wild life [42]. To determine if strains differing in their ability to from biofilms also differ in their ability to cope with acidic and osmotic stress, cells were exposed to phosphate buffered saline adjusted to pH 3.0 with HCl and to pH 3.9 with acetic acid, to diluted rice vinegar and to distilled water containing 5 M NaCl, and their survival was determined.

* CORRESPONDENCE TO: HARA-KUDO, Y., Division of Microbiology, National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan.
e-mail: ykudo@nihs.go.jp

MATERIALS AND METHODS

Bacterial strains: Seven *Salmonella* strains were used (Table 1). These strains were previously classified as strains having high (OD_{595}>1.0) or low (OD_{595}<0.03) biofilm-formation capability based on data from a microtiter plate assay [21].

Survival experiments under acid solutions: The strains were grown in tryptic soy broth (pH 7.6) (TSB; Difco, Becton, Dickinson and Company, Sparks, MD, U.S.A.) at 37°C for 24 hr and a loop (10 μ*l*; Sarstedt Group, Germany) of the culture was transferred into TSB (pH 7.6) diluted 1:5 with

Table 1. *Salmonella* strains used in this study

Strain	Serovar	Origin
High capability of biofilm formation		
SEC 54	Enteritidis	Patient
SEC 55	Enteritidis	Patient
SEC 105	Enteritidis	Chicken eggs
SEC 153	Mbandaka	Chicken eggs
Low capability of biofilm formation		
SEC 280	Enteritidis	Chicken eggs
SEC 282	Cerro	Chicken feed
SEC 284	Cerro	Chicken feed

distilled water (1/5 TSB) and incubated at 25°C for 24 hr in a water bath. When grown in 1/5 TSB at 25°C, *Salmonella* can efficiently form biofilm on plastic wells [21]. Under this condition, *Salmonella* cells were confirmed to reach the stationary phase and attain 10^8 cfu/m*l*. After incubation, 1 m*l* of culture was combined with 9 m*l* of phosphate buffered saline (pH 7.6) (Dulbecco's PBS(–), Nissui Pharmaceutical Co., Ltd., Japan) adjusted to pH 3.0 with 1.0 M hydrochloric acid (HCl; Wako Pure Chemical Industries, Ltd, Tokyo, Japan) or pH 3.9 with acetic acid (Wako Pure Chemical Industries, Ltd, Tokyo, Japan). Also, 1 m*l* of incubated culture was mixed with 9 m*l* of commercial rice vinegar (Japanese Consumer Co-operative Union, Tokyo, Japan) diluted 1:15 with distilled water (adjusted to pH 3.9). This rice vinegar, which contained about 0.8 M acetic acid, was purchased at a local supermarket in Tokyo. After mixing the solutions, the pH of the mixed solutions was measured and found not to be changed (HCl, pH 3.0, acetic acid, pH 3.9, 1:15 rice vinegar, pH 3.9). The samples were kept at 25°C in a water bath, and the cell numbers were counted every 6 hr. To count the cell numbers, each sample was diluted serially by 10-fold using PBS (pH 7.6). Fifty or 200 μl of the diluted samples were spread on tryptic soy agar (TSA; Difco, Becton, Dickinson and Company) using a spiral plater (EDDY JET, IUL Instruments, Barcelona, Spain). The plates were incubated at 37°C for 24 hr, and then colonies were counted. The pH value of the solution was measured after exposure to 48 hr, and it was found that pH did not change during the experiments. The experiments were performed in triplicate.

Survival experiments under high osmolar solutions: One m*l* of culture grown in 1/5 TSB culture for 24 hr at 25°C was combined with 9 m*l* of saturated saline (distilled pure water with 5 M NaCl; Wako Pure Chemical Industries, Ltd, Tokyo, Japan) (pH 7.6). The samples were kept at 25°C in a water bath, and the cell numbers were counted every 1 to 3 days by serial dilution and plating on tryptic soy agar. Colonies were counted after 24 hr of incubation at 37°C. The experiments were done in triplicate.

Survival modeling: Due to the increasing use of risk analyses aiming to control foodborne diseases, seeking the best survival model is important. To identify an appropriate survival model for the survival data, the GInaFit software was used [19]. This software is mainly used for fitting the model to the bacteria inactivation.

The Weibull model is widely used as a statistical model of the distribution of inactivation times. In this model, lethal events are considered as probabilities, and survival curves are considered a cumulative form of the distribution of lethal events [33, 41]. This model is defined as follows [11, 26].

$$\log_{10} N = \log_{10} N_0 - \left(\frac{t}{\delta}\right)^p$$

In this formula, N_0, δ and p are the initial microbial cell density (cfu/m*l*), the exposure time (hours) for the first log reduction and the shape parameter, respectively.

Albert *et al.* developed the Weibull with the tail model as follows [1].

$$\log_{10} N = \log_{10}\left[(N_0 - N_{\text{res}}) \cdot 10^{\left(-\left(\frac{t}{\delta}\right)^p\right)} + N_{\text{res}}\right]$$

N_{res}, δ and p are the residual population density (cfu/m*l*), the exposure time (hours) for the first log reduction and the shape parameter, respectively.

Performance of these models was evaluated using a prediction zone (APZ) method [31], which simultaneously assesses prediction bias and accuracy and predictive model performance. The performance factor of this method is the percentage of residuals (observed minus predicted values) that fall within an APZ from -1 (fail-safe) to 0.5 (fail-dangerous) $\log_{10}$ units. When ≥70% of the residuals are in the APZ, the model is considered to provide acceptable predictions of the test data.

Statistic analysis: The bacterial cell number observed at each time point was expressed as the mean log cfu of bacteria ± standard error of the mean (SEM) using Microsoft Office Excel. Significant differences were determined with an unpaired Student's *t*-test using the Systat 11.0 software (Cranes Software International Limited). The detection limit was set to 50 cfu/m*l*, and if the detection was under the limit, the cell number was assumed to be 25 cfu/m*l* and used in the statistic analysis.

RESULTS

Figure 1 shows survival curves in HCl solutions (pH 3.0) of strains with a high and low ability to produce biofilm. The initial average cell number ($\log_{10}$ cfu/m*l*) of the 2 groups was approximately the same (7.47 ± 0.04 (SEM) vs. 7.50 ± 0.08) at 0 hr after exposure to acid. The cell number decreased to 4.27 ± 0.30 vs. 2.90 ± 1.09 ($\log_{10}$ cfu/m*l*, P=0.22) at 48 hr of exposure for high vs. low biofilm-forming ability. At 48 hr 1 strain with low biofilm-formation capability (SEC 282) was under the detection limit. When fitted to the Weibull model, the δ values were 9.21 ± 1.45 vs. 6.39 ± 1.41 for high vs. low biofilm-forming ability, but no significant difference in the survival numbers was found during the exposure period.

Figure 2 shows survival curves of the *Salmonella* strains in acetic acid solutions (pH 3.9). Like under the HCl solutions, the initial average cell numbers of the 2 groups having high and low biofilm-forming ability were approximately the same, but significant differences were found at 24 hr (5.47 ± 0.25 vs. 2.88 ± 1.01, P<0.05) and 36 hr (4.43 ± 0.24 vs. 2.27 ± 0.87, P<0.05) for high vs. low biofilm-forming ability. At 48 hr, one strain with high capability (SEC 55) and 2 strains with low capability (SEC 280 and 282) were under the detection limit. Overall, obvious statistic differences for high vs. low biofilm-forming capability were noted from 24 hr of exposure onward. The Weibull model fitting showed this difference more clearly; the δ values were 11.42 ± 1.72 vs. 4.75 ± 3.86 for high vs. low biofilm-forming ability.

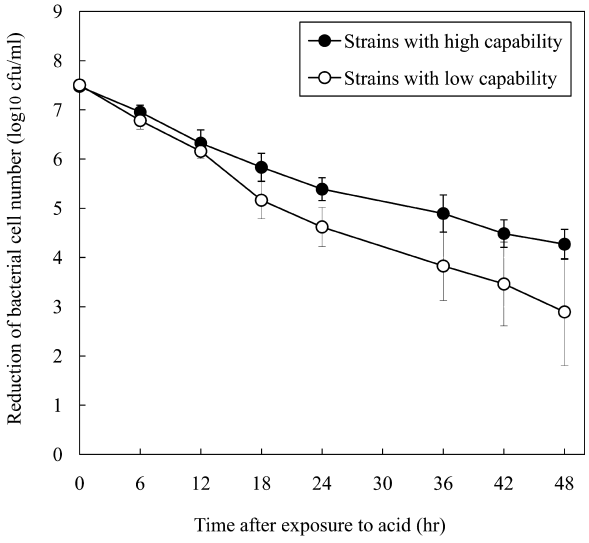

Fig. 1. Survival curves of *Salmonella* in PBS adjusted to pH 3.0 with HCl. Strains with high and low biofilm-formation capability were used. Each point on vertical bars indicates the mean±SEM (n=4 for strains with high capability, and n=3 for strains with low capability), which was calculated assuming the number below the detection limit (50 cfu/m*l*) to be 25 cfu/m*l*.

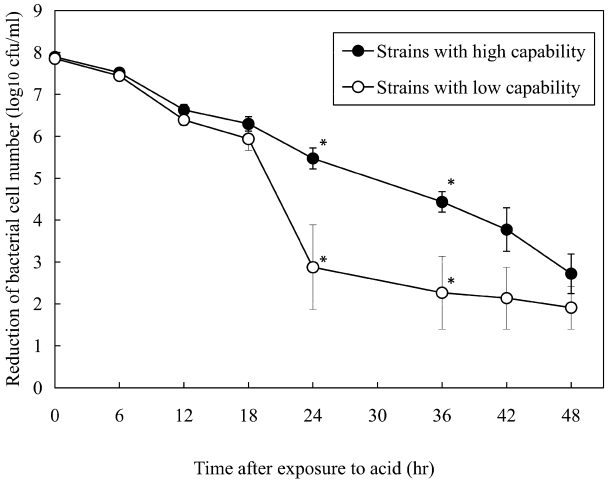

Fig. 2. Survival curves of *Salmonella* in PBS adjusted to pH 3.9 with acetic acid. Strains with high and low biofilm-formation capability were used. Each point on vertical bars indicates the mean±SEM (n=4 for strains with high capability, and n=3 for strains with low capability), which was calculated assuming the number below the detection limit (50 cfu/m*l*) to be 25 cfu/m*l*. * A significant difference was observed between the 2 groups of strains having different abilities to form biofilm at the same exposure time (P<0.05).

Figure 3 shows survival curves for 1:15 diluted rice vinegar (pH 3.9). The initial average cell numbers of the 2 groups having high and low biofilm-forming ability were approximately the same. Significant differences were found at 24 hr (6.03 ± 0.05 vs. 4.75 ± 0.51, P<0.05), 36 hr (5.19 ± 0.12 vs. 2.33 ± 0.93, P<0.05), 42 hr (4.92 ± 0.15 vs. 2.26 ± 0.86, P<0.05) and 48 hr (4.27 ± 0.22 vs. 2.03 ± 0.63, P<0.05) for high vs. low biofilm-forming ability. At 48 hr, 2 strains with low capability (SEC 280 and 282) were under the detection limit. The δ values were 13.19 ± 1.57 vs. 8.83 ± 3.82 for high vs. low biofilm-forming ability, but significant statistical differences in the survival numbers were not found at 42 hr and 48 hr of exposure. The reason for this seems to be that three strains (SEC 55, 280 and 282) were under the detection limit at 42 hr.

Survival curves under high hyperosmotic solutions: Figure 4 shows survival curves in saturated saline (pH 7.6) of strains with high and low biofilm-formation capability. The initial average cell numbers ($\log_{10}$ cfu/m*l*) of the 2 groups having high and low biofilm-forming ability were approximately the same (8.09 ± 0.06 vs. 8.13 ± 0.07) at 0 d. The cell numbers decreased to 1.95 ± 0.38 vs. 1.51 ± 0.11 ($\log_{10}$ cfu/m*l*, P=0.39) at 20 d of exposure for high vs. low biofilm-forming ability. At 20 d, 2 strains with high capability (SEC 54 and 55) and 2 strains with low capability (SEC 280 and 282) were under the detection limit. The average number for strains with high capability tended to be larger than for strains with low capability, but the δ values were not different (0.43 ± 0.12 vs. 0.12 ± 0.08 for high vs. low biofilm-forming ability), and no significant difference in survival numbers was observed.

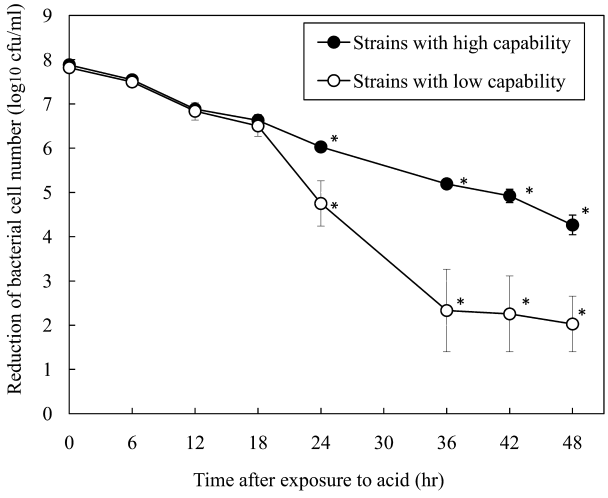

Fig. 3. Survival curves of *Salmonella* in 1:15 rice vinegar solution (pH 3.9). Strains with high and low biofilm-formation capability were used. Each point on vertical bars indicates the mean±SEM (n=4 for strains with high capability, and n=3 for strains with low capability), which was calculated assuming the number below the detection limit (50 cfu/m*l*) to be 25 cfu/m*l*. * A significant difference was observed between the two groups of strains having different abilities to form biofilm at the same exposure time (P<0.05).

Survival model performance: The Weibull model provided acceptable predictions (≥70% of residuals) for all data. However, the APZ value of the strains with low biofilm-forming capability in acetic acid and rice vinegar was

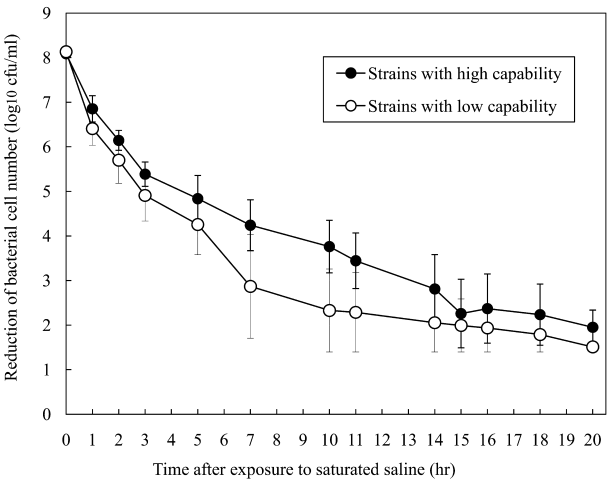

Fig. 4. Survival curves of *Salmonella* in saturated saline (pH 7.6). Strains with high and low biofilm-formation capability were used. Each point on vertical bars indicates the mean±SEM (n=4 for strains with high capability, and n=3 for strains with low capability), which was calculated assuming the number below the detection limit (50 cfu/m*l*) to be 25 cfu/m*l*.

smaller (APZ=71.4). When the Weibull with the tail model was used, the APZ values of these strains raised to 100.0, which showed more adequate fitting performance.

DISCUSSION

The ability of bacteria to form a biofilm is one of the important factors that must be considered to avoid food-borne diseases [10]. The advantage of a biofilm for bacteria is that it protects them against severe stresses such as poor nutrition and low temperature [12]. Acid and osmotic conditions are also involved in such stresses [22, 38, 43]. In this study, we used 2 groups of *Salmonella* strains, one showing a high ability to form biofilm and another showing a low ability. Previously, we have reported that biofilm formation by these strains can be facilitated on plastic wells by growing the bacteria in 1/5 TSB at 25°C for 24 hr [21]. Therefore, this growth condition was also used in this study. If the bacterial ability to form biofilm plays a key role in bacterial protection against acidic or hyperosmotic stress, such strains having high ability to form biofilm would be more tolerable to low pH and hyperosmotic conditions than strains showing a low ability.

The survival of *Salmonella* strains in HCl solutions, the pH of which was adjusted to pH 3.0 to mimic gastric acid [3], shows no significant difference in the survival curves between the 2 groups of strains having different abilities to form biofilm. However, the survival of *Salmonella* strains in acetic acid shows that the cell numbers of strains with low biofilm-formation capability decreased faster than those of strains with high capability. Acetic acid has been used as a cleanser detergent and a main material for a variety of vinegars and sources. Also, commercial rice vinegar has long been used for seasoning in Japanese and Chinese dishes. The pH value was adjusted to 3.9 for both acetic acid and rice vinegar solutions. Many studies have shown significant killing by acetic acid within minutes [9]. However, in this study, reductions of about 2 logs were observed 12 hr after acid treatment. Such a relatively slow reduction may be caused by using relatively mild acid, such as one with a pH of 3.9, and *Salmonella* cells at the stationary phase. Judging from the surviving cell numbers, acetic acid had a more severe effect on *Salmonella* survival than rice vinegar (the surviving number of cells with high capability at 36 hr was 4.43 ± 0.24 vs. 5.19 ± 0.12). Under pH 3.0 acid conditions, lactic acid has a more severe effect on cells than acetic acid and HCl [8], but Bjornsdottir, *et al.* reported that exposure to 1–20 mM of lactic acid aided the survival of *Escherichia coli* in severe acidic environments with a pH of 3.2 [7]. Since lactic acid produced by lactic acid bacteria exists at a concentration of 0.73–3.42 mM in commercial rice vinegar [17]; such low concentrations of lactic acid existing in rice vinegar solutions may also in some way have aided the survival of *Salmonella* strains in the acidified solutions.

The observed relationship between acid tolerance and biofilm-formation capability also indicates the possibility that the acid tolerance mechanism might be related to biofilm-forming mechanism. The survival curves under acidic conditions showed that the death rate decreased with incubation time in both strains, especially in strains with high biofilm-forming ability. Exposure of bacteria to low pH stress is known to induce acid shock proteins, which are involved in acid tolerance response [2, 5, 16, 29, 40]. Among these proteins, RpoS, the functional stress-responsive sigma factor, is known to be involved in the resistance of stationary phase cells to various stress-including acids [13, 24] and in the formation of biofilm by regulating CsgD expression [34, 35]. A higher level of RpoS causes a higher level of stress resistance, but the expression of this protein is widely different depending on the stress [6]. In *Listeria monocytogenes,* loss of functional stress-responsive alternative sigma factor *sigB* reduced the survival of the cells at pH 2.5 to a greater extent in the presence of organic acid (acetic acid) than in the presence of inorganic acid alone (HCl), suggesting that the protection against organic and inorganic acid may be mediated through different mechanisms [15]. Thus, *Salmonella* cells with high biofilm-formation capability might be under the high influence of acetic acid tolerance mechanisms during exposure to acetic acid, resulting in an increase in the tolerance level with incubation time. Other organic acids might also be linked to the tolerance mechanism, so further study using various organic acids will be necessary.

The results for tolerance against osmotic stress indicate that tolerance against hyperosmotic stress is independent of the biofilm-formation capability of *Salmonella*. For *Pseudomonas aeruginosa* and *Vibrio cholerae*, biofilm development was observed to be under the influence of osmotic conditions [4, 37]. Further studies using various strains and approaches will be needed to elucidate the link-

age of biofilm formation and osmotic tolerance of *Salmonella*.

In order to estimate the D-value accurately, it is necessary to seek the best survival model in the food industry. Using the Weibull model showed adequate fitting performance in each experiment. A good fit of the inactivation curve is important to obtain more valuable estimates for the controlling the survival of bacteria. The Weibull model has been shown to be a very useful model for simulating the bacteria inactivation model [20, 32]. Also, in this experiment using *Salmonella,* this model was found to be very adequate for simulation of survival under acid and high osmolar stress. The strains with low biofilm-forming capability in acetic acid, and rice vinegar showed a characteristic decrease from 24 hr of exposure onward. By using the Weibull with the tail model, these types of survival curves were found to simulate clearly.

In conclusion, the data obtained in this experiment suggest that the ability of *Salmonella* strains to survive in the presence of acetic acid and rice vinegar parallels their ability to form biofilms. The relation between biofilm-forming ability and risk of foodborne outbreaks has previously been suggested [28, 42]. Therefore, it is important to consider strain-dependent variations in biofilm-formation capability when developing strategies for prevention of *Salmonella* contamination of foods. In particular, considering that various foods with low pH have caused outbreaks of *Salmonella* infection [14, 30], this notion should be emphasized.

ACKNOWLEDGMENT. This study was supported by a research grant of the Ministry of Health, Labour and Welfare.

REFERENCES

1. Albert, I. and Mafart, P. 2005. A modified Weibull model for bacterial inactivation. *In. J. Food Microbiol.* **100**: 197–211.
2. Arnold, C. N., McElhanon, J., Lee A., Leonhart, R. and Siegele, D. A. 2001. Global analysis of *Escherichia coli* gene expression during the acetate-induced acid tolerance response. *J. Bacteriol.* **183**: 2178–2186.
3. Arthur, C., Guyton, M. D. and John, E. H. 2006. p. 797. Textbook of Medical Physiology (11 ed.). Elsevier Saunders, Philadelphia.
4. Bazire, A., Diab, F., Jebbar, M. and Haras, D. 2007. Influence of high salinity on biofilm formation and benzoate assimilation by *Pseudomonas aeruginosa*. *J. Ind. Microbiol. Biotechnol.* **34**: 5–8.
5. Bearson, B. L., Wilson, L. and Foster, J. W. 1998. A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. *J. Bacteriol.* **180**: 2409–2417.
6. Becker, G., Klauck, E. and Hengge-Aronis, R. 1999. Regulation of RpoS proteolysis in *Escherichia coli*: the response regulator RssB is a recognition factor that interacts with the turnover element in RpoS. *Proc. Natl. Acad. Sci.* **96**: 6439–6444.
7. Bjornsdottir, K., Breidt, F. Jr. and McFeeters, R. F. 2006. Protective effects of organic acids on survival of *Escherichia coli* O157:H7 in acidic environments. *Appl. Environ. Microbiol.* **72**: 660–664.
8. Buchanan, R. L. and Edelson, S. G. 1999. pH-dependent stationary-phase acid resistance response of enterohemorrhagic *Escherichia coli* in the presence of various acidulants. *J. Food Prot.* **62**: 211–218.
9. Chang, J. M. and Fang, T. J. 2007. Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovars Typhimurium in iceberg lettuce and the antimicrobial effect of rice vinegar against *E. coli* O157:H7. *Food Microbiol.* **24**: 745–751
10. Clouser, C. S., Knabel, S. J., Mast, M. G. and Doores, S. 1995. Effect of type of defeathering system on *Salmonella* cross-contamination during commercial processing. *Poult. Sci.* **74**: 732–741.
11. Coroller, L., Leguerinel, I., Mettler, E., Savy, N. and Mafart, P. 2006. General model, based on two mixed Weibull distributions of bacterial resistance, for describing various shapes of inactivation curves. *Appl. Environ. Microbiol.* **72**: 6493–6502.
12. Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. and Lappin-Scott, H. M. 1995. Microbial biofilms. *Annu. Rev. Microbiol.* **49**: 711–745.
13. Dodd, C. E. and Aldsworth, T. G. 2002. The importance of Rpos in the survival of bacteria through food processing. *Int. J. Food Microbiol.* **74**: 189–194.
14. Evance, M. R., Salmon, R. L., Nehaul, L., Mably, S., Wafford, L., Nolan-Farrell, M. Z., Gardner, D. and Ribeiro, C. D. 1999. An outbreak of *Salmonella typhimurium* DT170 .associated with kebab meat and yoghurt relish. *Epidemiol. Infect.* **122**: 377–383.
15. Ferreira, A., Sue, D., O'Byrne, C. P. and Boor, K. J. 2003. Role of *Listeria monocytogenes* sigma(B) in survival of lethal acidic conditions and in the acquired acid tolerance response. *Appl. Environ. Microbiol.* **69**: 2692–2698.
16. Foster, J. W. 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. *J. Bacteriol.* **175**: 1981–1987.
17. Furukawa, S. and Ueda, R. 1963. Studies on Non-volatile Organic Acids in Vinegar (I) Contents of Non-volatile Organic Acids in Commercial Vinegars. *J. Biosci. Bioeng.* **41**: 14–19.
18. Fux, C. A., Costerton, J. W., Stewart, P. S. and Stoodley, P. 2005. Survival strategies of infectious biofilms. *Trends Microbiol.* **13**: 34–40.
19. Geeraerd, A. H., Valdramidis, V. P. and Van Impe, J. F. 2005. GInaFiT, a freeware tool to assess non–log-linear microbial survivor curves. *In. J. Food Microbiol.* **102**: 95–105.
20. González, M., Skandamis, P. N. and Hänninen, M. L. 2009. A modified Weibull model for describing the survival of *Campylobacter jejuni* in minced chicken meat. *Int. J. Food. Microbiol.* **136**: 52–58.
21. Iibuchi, R., Hara-Kudo, Y., Hasegawa, A. and Kumagai, S. 2010. Survival of *Salmonella* on a polypropylene surface under dry conditions in relation to biofilm-formation capability. *J. Food Prot.* **73**: 1506–1510.
22. Kapfhammer, D., Karatan, E., Pflughoeft, K. J. and Watnick, P. I. 2005. Role for glycine betaine transport in *Vibrio cholerae* osmoadaptation and biofilm formation within microbial communities. *Appl. Environ. Microbiol.* **71**: 3840–3847.
23. Korber, D. R., Choi, A., Wolfaardt, G. M., Ingham, S. C. and Caldwell, D. E. 1997. Substratum topography influences susceptibility of *Salmonella enteritidis* biofilm to trisodium phosphate. *Appl. Environ. Microbiol.* **63**: 3352–3358.
24. Lee, I. S., Lin, J., Hall, H. K., Bearson, B. and Foster, J. W. 1995. The stationary-phase sigma factors S (RpoS) is required

for a sustained acid tolerance response in virulent *Salmonella* typhimurium. *Mol. Microbiol.* **17**: 155–167.
25. Li, Y. H., Hanna, M. N., Svensäter, G., Ellen, R. P. and Cvitkovitch, D. G. 2001. Cell density modulates acid adaptation in *Streptococcus mutans*: implications for survival in biofilms. *J. Bacteriol.* **183**: 6875–6884.
26. Mafart, P., Couvert, O., Gaillard, S. and Leguerinel, I. 2002. On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. *In. J. Food Microbiol.* **72**: 107–113.
27. McNeill, K. and Hamilton, I. R. 2004. Effect of acid stress on the physiology of biofilm cells of *Streptococcus mutans*. *Microbiology* **150**: 735–742.
28. Møretrø, T. and Langsrud, S. 2004. *Listeria monocytogenes*: biofilm formation and persistence in food-processing environments. *Biofilms* **1**: 107–121.
29. Nakayama, S., Kushiro, A., Asahara, T., Tanaka, R., Hu, L., Kopecko, D. J. and Watanabe, H. 2003. Activation of *hilA* expression at low pH requires the signal sensor CpxA, but not the cognate response regulator CpxR, in *Salmonella enterica* serovar Typhimurium. *Microbiology* **149**: 2809–2817.
30. Ortega-Benito, J. M. and Langridge, P. 1992. Outbreaks of food poisoning due to *Salmonella typhimurium* DT4 in mayonnaise. *Public Health* **106**: 203–208.
31. Oscar, T. P. 2006. Validation of a tertiary model for predicting variation of *Salmonella* Typhimurium DT104 (ATCC 700408) growth from a low initial density on ground chicken breast meat with a competitive microflora. *J. Food Prot.* **69**: 2048–2057.
32. Oyarzabal, O. A., Oscar, T. P., Speegle, L. and Nyati, H. 2010. Survival of *Campylobacter jejuni* and *Campylobacter coli* on retail broiler meat stored at -20, 4, or 12 degrees C and development of Weibull models for survival. *J. Food Prot.* **73**: 1438–1446.
33. Peleg, M. and Cole, M. B. 1998. Reinterpretation of microbial survival curves. *Crit. Rev. Food Sci.* **38**: 353–380.
34. Romling, U., Rohde, M., Olsen, A., Normark, S. and Reinkoster, J. 2000. AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella* typhimurium regulates at least two independent pathways. *Mol. Microbiol.* **36**: 10–23.
35. Romling, U., Bokranz, W., Rabsch, X., Zogaj, M., Nimtz, M. and Tschape, H. 2003. Occurrence and regulation of the multicellular morphotype in *Salmonella* serovars important in human disease. *Int. J. Med. Microbiol.* **293**: 273–285.
36. Scher, K., Romling, U. and Yaron, S. 2005. Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. *Appl. Environ. Microbiol.* **71**: 1163–1168.
37. Shikuma, N. J. and Yildiz, F. H. 2009. Identification and characterization of OscR, a transcriptional regulator involved in osmolarity adaptation in *Vibrio cholerae*. *J. Bacteriol.* **191**: 4082–4096.
38. Skandamis, P. N., Stopforth, J. D., Ashton, L. V., Georgnaras, I., Kendall, P. A. and Sofos, J. N. 2009. *Escherichia coli* O157:H7 survival, biofilm formation and acid tolerance under simulated slaughter plant moist and dry conditions. *Food Microbiol.* **26**: 112–119.
39. Solomon E. B., Niemira B. A., Sapers G. M. and Annous, B. A. 2005. Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. *J. Food Prot.* **68**: 906–912.
40. Testerman, T. L., Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Libby, S. J. and Fang, F. C. 2002. The alternative sigma factor sigma E controls antioxidant defences for *Salmonalla* virulence and stationary phase survival. *Mol. Microbiol.* **43**: 771–782.
41. Van Boekel, M. A. 2002. On the use of Weibull model to describe thermal inactivation of microbial vegetative cells. *In. J. Food Microbiol.* **74**: 139–159.
42. Vestby, L. K., Møretrø, T., Langsrud, S., Heir, E. and Nesse, L. L. 2009. Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal- and feed factories. *BMC Vet. Re.* **27**: 5–20.
43. Welin-Neilands, J. and Svensater, G. 2007. Acid tolerance of biofilm cells of *Streptococcus mutans*. *Appl. Environ. Microbiol.* **73**: 5633–5636.