Online Journal of Health and Allied Sciences

Published Quarterly
Mangalore, South India
ISSN 0972-5997
Volume 6, Issue 2; Apr -Jun 2007

Original Article

Survivability of *Salmonella typhimurium L1388* and *Salmonella enteritidis L1225* under stressful growth conditions

Authors
Ngwai YB,
Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, PMB 071, Yenagoa, Bayelsa State, Nigeria
Wambebe C,
International Biomedical Research in Africa (IBRIA), P.M.B. Garki, Abuja, Nigeria
Adachi Y,
Animal Health Laboratory, School of Agriculture, Ibaraki University, 3-21-1 Ami, Ami-machi, Ibaraki, 300-0393, Japan

Address For Correspondence
Dr. Yakubu B. Ngwai,
Department of Pharmaceutical Microbiology and Biotechnology,
Faculty of Pharmacy,
Niger Delta University, Wilberforce Island,
P.M.B. 071 Yenagoa,
Bayelsa State, Nigeria
E-mail: ybngwai@yahoo.com

Citation
Ngwai YB, Wambebe C, Adachi Y. Survivability of *Salmonella typhimurium L1388* and *Salmonella enteritidis L1225* under stressful growth conditions *Online J Health Allied Scs.* 2007;2:4

URL
http://www.ojhas.org/issue22/2007-2-4.htm

Open Access Archives
http://cogprints.ecs.soton.ac.uk/view/subjects/OJHAS.html
http://openmed.nic.in

Submitted Feb 24, 2007; Suggested revision: Jun 17, 2007; Resubmitted: Jul 18, 2007; Accepted: Nov 7, 2007; Published: Nov 10, 2007

Abstract:

In an earlier study with *Salmonella typhimurium* L1388 (ST) and *Salmonella enteritidis* L1225 (SE) isolated from diseased chickens, we found that SE formed more biofilm than ST on abiotic surfaces in a time-dependent manner. Since the ability of salmonellae to survive extreme environment is related to their virulence, the present study examined the survival of *Salmonella typhimurium* L1388 and *Salmonella nteritidis* L1225 under the usual stresses that salmonellae encounter during their life cycle. This is with a view to understanding the strains' stress tolerance that could be used to explain their virulence. Incubation at 37°C for various time periods was done for: i) stationary phase (SP) cells at pH 2.6; ii) log-phase (LP) cells at pH 4.0; log-phase or stationary phase cells in broth containing iii) hydrogen peroxide, iv) sodium chloride and v) ethanol; vi) stationary phase cells in Hank's balanced salt solution (single strength) containing 10% human serum; and vii) prolong stationary phase cells. Stationary phase cells were also incubated at 52°C for 15 min. Surviving cells at the various incubation times were counted on trypticase soy agar (TSA) after appropriate dilution in saline and overnight incubation at 37°C. Growth iron-poor medium was determined by growing a single bacterial colony in Medium A with shaking at 37°C or 40°C for 24 h. Statistics was done by one-way analysis-of-variance (ANOVA) at P = 0.05. Differences in the survival of ST and SE were insignificant ($p>0.05$) in acid pH at both pH 4.0 (p = 0.3783) and pH 2.6 (p = 0.4711); at high salinity for log-phase (p = 0.1416) and stationary phase (p = 0.1816) cells; in ethanol (p = 0.5984), human serum (p = 0.8139), prolonged stationary phase (p = 0.3506); and under heat (p = 0.5766). SE was significantly ($p<0.05$; p = 0.0031) more tolerant to oxidative-killing by hydrogen peroxide. Culturable growth of the ST and SE in an iron-poor medium A revealed insignificant differences at 37°C (p = 0.8381) but marginally significant at 40°C (p = 0.0508). Thus, with the exception of survival in hydrogen peroxide, SE had similar response pattern with ST to the usual stresses that salmonellae encounter during their life cycle, despite the former's preferential ability to form biofilm on abiotic surfaces. The relationship between the observed enhanced ability of SE to survive in hydrogen peroxide and virulence need to be investigated in subsequent study.

Key Words: *Salmonella typhimurium*; *Salmonella enteritidis*; Survival; Stress

Introduction:

Members of the genus *Salmonella* (Family: Enterobacteriaceae) have capacity for survival in diverse environments as well as the capability of infecting a wide range of vertebrate hosts.(1) During their life cycles, salmonellae encounter diverse environmental stresses, such as nutrient deprivation, pH extremes, oxidative stress, osmotic shock, DNA damage and heat shock, (2) which may significantly influence their survival and virulence.(3) These stresses, depending on severity and duration of exposure, usually inhibit either the growth or survival, or cause a loss of viability of the cells.(4) Consequently, the ability of salmonellae to mount up an effective stress response is also critical to their virulence.

Over 2,400 serotypes of the genus *Salmonella* are believed to exist; and of this number, the serotypes Typhimurium and Enteritidis are more frequently isolated in human and animal salmonellosis.(5,6) The consumption of infected poultry meat and eggs is a major source of human cases of infections caused by Typhimurium and Enteritidis.(7) Therefore, these serotypes remain a Public health problem worldwide,

ojhas Online Journal of Health and Allied Sciences

particularly with respect to food safety.(8)

Salmonella typhimurium L1388 and *Salmonella enteritidis* L1225 strains were isolated from diseased chicken at the Japanese National Institute of Infectious Diseases. We have found that SE produces more biofilm on abiotic surfaces than ST in vitro.(9) In this study, we examined the survival of *Salmonella typhimurium* L1388 and *Salmonella enteritidis* L1225 under the usual stresses that salmonellae encounter during their life cycle. This is with a view to understanding the strains' stress tolerance. Studies of this nature have application in explaining virulence of bacteria.

Materials and Methods:

Bacterial isolates and culture media

Salmonella typhimurium L1388 and *Salmonella enteritidis* L1225, both of which were isolated from chicken in Japan, were used in this study. Both strains were propagated in either trypticase soy broth (TSB; BBL, U.S.A.) or Luria-Bertani broth (LB; Daigo, Inc., Japan) as indicated in the text. Solid media culture was grown on trypticase soy agar (TSA; BBL, U.S.A.) or LB agar (Daigo, Inc., Japan). Unless otherwise indicated, all chemicals used were from Wako Chemical Company, Japan.

Survival in acidic medium

Survival of log-phase (LP: 4-h LB culture) and stationary phase (SP: overnight LB culture) cells in pH-adjusted LB broth was assayed based on Fang et al.(10) with modifications. 2 ml of LB (pH 4.0 or 2.6; adjusted with 2 N HCl) was inoculated with 100 µl of overnight LB broth culture of bacteria and incubated with shaking at 37°C. Samples were drawn at times T = 0 h (before incubation) and T = 2 h of incubation (pH 4.0) or T = 5 min (pH 2.6) of incubation, diluted in normal saline and viable cells were counted after 18-h incubation on TSA at 37°C. Survival (%) was calculated from ([Bacterial CFU per ml at T = 2 h or 5 min / Bacterial CFU per ml at T = 0 h] x 100). Results are means of six independent experiments.

Survival in hydrogen peroxide

The ability of isolates to tolerate reactive oxygen intermediates produced by hydrogen peroxide (H_2O_2) was evaluated by their survival in LB broth containing 15 mM H_2O_2. Briefly, 2 ml of LB-15 mM H_2O_2 broth was inoculated with 100 µl of overnight LB broth culture of bacteria and incubated with shaking at 37°C. Samples were drawn at times T = 0 h (before incubation) and T = 1 h of incubation, diluted in normal saline and viable cells were counted after overnight incubation on TSA at 37°C. Survival (%) was calculated from ([Bacterial CFU per ml at T = 1 h / Bacterial CFU per ml at T = 0 h] x 100). Results are means of six independent experiments.

Survival at high salinity

Survival of log-phase (LP: 4-h TSB culture) and stationary phase (SP: overnight TSB culture) cells in NaCl-adjusted TSB broth as described previously, (11) with modifications. Briefly, 2 ml of TSB supplemented with NaCl to a final concentration of 10% (equivalent to water activity [a_w] of <0.94) (12) was inoculated with overnight broth culture of bacteria, and incubated at 37°C with shaking for 2 h. Samples were taken at times T = 0 h (before incubation) and T = 2 h of incubation, and viable cells determined as before. Survival (%) of bacteria was calculated by comparing the bacterial CFU at T = 2 h and T = 0 h as in other survival assays described earlier. Results are means of three experiments.

Survival in ethanol

Ethanol is an acceptable food additive. Hence, the need to investigate the ability of ethanol to kill ST and SE. Survival in ethanol was investigated in accordance with St. John et al. (13)

ojhas Online Journal of Health and Allied Sciences

with minor modifications. 2 ml of LB broth containing 10 % ethanol (LB_{EtOH}) was inoculated with 0.1 ml of overnight broth culture of bacteria, and incubated with shaking at 37°C for 3 h. Samples were taken at times, T = 0 h (before incubation) and T = 3 h of incubation, and viable cells determined as described above. Results are means of three experiments.

Survival at elevated temperature

The method described by Jørgensen et al. (14) was used. Briefly, 2 ml of TSB was inoculated with 0.1 ml of overnight broth culture of bacteria, and incubated at 52°C with shaking. Samples were taken at times T = 0 min (before incubation) and T = 15 min, diluted in saline, and the number of viable cells were counted after overnight incubation on TSA at 37°C. Survival (%) was determined from ([Bacterial CFU per ml at T = 15 min / Bacterial CFU per ml at T = 0 h] x 100); Results are averages of three separate determinations.

Survival in prolonged stationary phase

Survival in prolonged stationary phase was examined as previously described, (10) with slight modifications. Briefly, one colony of a strain was grown in 2 ml of LB broth for 24 h at 37°C; 100 µl samples were taken, diluted appropriately in normal saline, spread on TSA, and viable cells counted after overnight incubation at 37°C. The 24-h broth cultures were incubated further for another 144 h (i.e. a total 168 h), and the number of viable cells was determined after appropriate dilution and overnight incubation on TSA as before. Survival (%) after 168 h of incubation was calculated from ([Bacterial CFU per ml after 168 h / bacteria CFU per ml after 24 h incubation] x 100). Experiments were done twice using duplicate samples (i.e., n = 4), and error bars are standard deviations of individual results from their respective means.

Survival in human serum

Bacterial survival in human serum was assayed as previously described. (15) Counts were determined at times T = 0 h and T = 2 h following addition of the inoculum to 10% normal human serum in single strength Hank's balanced salt solution (HBSS: 137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 5.6 mM D-glucose, 0.02% phenol red, distilled water to 1000 ml; membrane filter-sterilized (pore size: 0.45 µm). Survival, expressed as Serum Resistance Factor, was determined from the relation: CFU at T = 2 h / CFU at T = 0 h. Results are means of three independent determinations.

Growth in iron-poor Medium A

Growth of bacteria under iron-limited condition was monitored in an iron-poor medium. (16) Single colony of bacteria reactivated from a -80°C stock culture was inoculated into 2 ml of a membrane filter-sterilized (pore size: 0.4 µm) iron-poor medium (Medium A: 0.7 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $(NH_4)_2SO_4$, 0.02 g $MgSO_4.7H_2O$, 0.5% D-glucose, Distilled water to 100 ml; pH 6.95). The inoculated medium was then incubated (with shaking) at 37°C for 24 h; and number of colony forming units was counted on TSA, after appropriate dilution with normal saline and overnight incubation at 37°C. Growth was expressed as CFU of bacteria per milliliter of culture. Results are means of four independent experiments.

Statistical analysis

Data were analyzed by the one-way analysis of variance (ANOVA) using Smith Statistical Package, version 2.5 and significance of results determined at the 5 % probability level (that is, at *P = 0.05*).

Results

Survival in acidic medium

For both isolates, survival at pH 2.6 was less than at pH 4.0 (Fig. 1). However, differences in the survival of ST and SE were insignificant (*p>0.05*) at both pH 4.0 (p = 0.3783) and pH 2.6 (p = 0.4711).

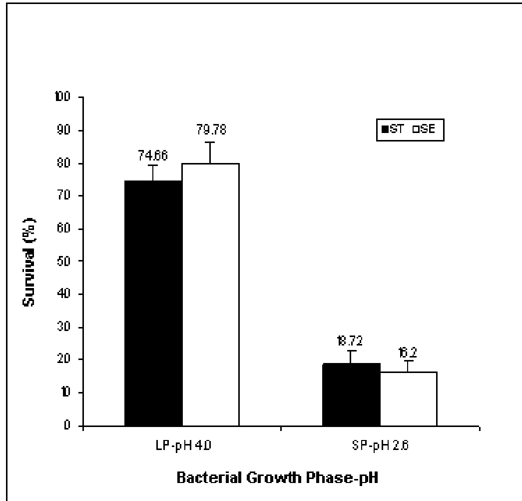

Figure 1: Survival of *Salmonella typhimurium* L1388 (ST) and *Salmonella enteritidis* L1225 (SE) in acid pH. Survival of bacteria was determined at 37°C for 5 min in LB broth (pH 2.6) or for 2 h in LB broth (pH 4.0). Viable cells were counted on TSA after dilution in saline and overnight incubation at 37°C; Survival (%) survival was determined from the relation: (Bacterial CFU ml-1 at stated incubation time / Bacterial CFU ml-1 at time, T = 0 h) x 100. Experiments were done three times, and error bars are standard deviations from mean values. LP-pH 4.0, log-phase cells exposed to pH 4.0 and SP-pH 2.6, stationary phase cells exposed to pH 2.6.

Survival in hydrogen peroxide

SE was significantly (*p<0.05*; p = 0.0031) more tolerant than ST to oxidative-killing by hydrogen peroxide (Fig. 2).

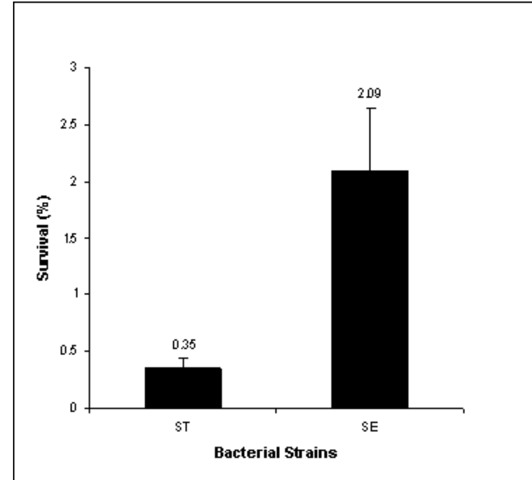

Figure 2: Survival of *Salmonella typhimurium* L1388 (ST) and *Salmonella enteritidis* L1225 (SE) in hydrogen peroxide. Bacterial survival was determined at 37°C in LB-15 mM H_2O_2 for 2 h, and viable cells were counted on TSA after appropriate dilution in saline and overnight incubation at 37°C. Survival (%) was calculated from the relation: (Bacterial CFU ml-1 T = 2 h / Bacterial CFU ml-1 at time, T = 0 h) x 100. Experiments were done three times, and error bars are standard deviations of individual results from their respective means.

Survival at high salinity

Survival at high salinity is as shown in Figure 3. The results indicate that differences in the tolerance of ST and SE to the osmotic up-shift generated by high salinity were insignificant (*p>0.05*) for log-phase (p = 0.1416) and stationary phase (p = 0.1816) cells.

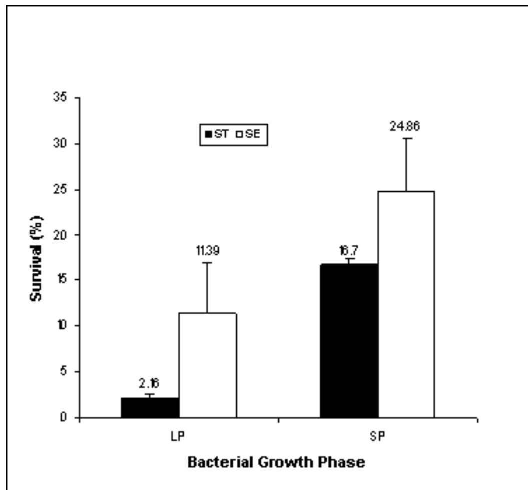

Figure 3: Survival of *Salmonella typhimurium* L1388 (ST) and *Salmonella enteritidis* L1225 (SE) in high salinity. Bacterial survival was determined at 37ºC in TSB-10% NaCl for 2 h, and viable cells were counted on TSA after appropriate dilution in saline and overnight incubation at 37ºC. Survival (%) was calculated from the relation: (Bacterial CFU ml-1 T = 2 h / Bacterial CFU ml-1 at time, T = 0 h) x 100. Experiments were done three times, and error bars are standard deviations of individual results from their respective means. LP, log-phase cells; SP, stationary phase cells.

Survival in ethanol

At 10% ethanol that is a common level in many beverages such as wine, survival of both ST and SE was dramatically reduced within 3 h (Figure 4). However, differences in the survival of the two isolates were insignificant ($p > 0.05$; p = 0.5984).

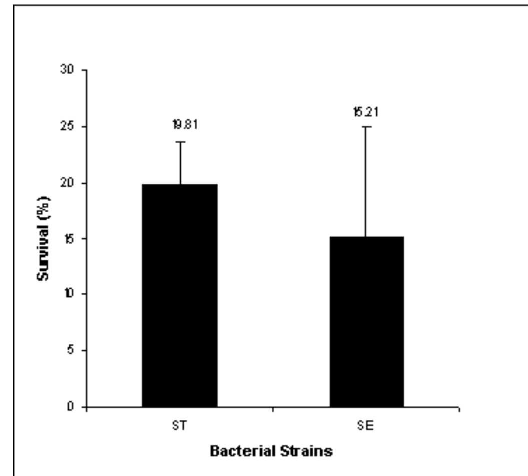

Figure 4: Survival of *Salmonella typhimurium* L1388 (ST) and *Salmonella enteritidis* L1225 (SE) in ethanol. Bacterial survival was determined at 37ºC in LB-10% Ethanol for 3 h, and viable cells were counted on TSA after appropriate dilution in saline and overnight incubation at 37ºC. Survival (%) was calculated from the relation: (Bacterial CFU ml-1 T = 3 h / Bacterial CFU ml-1 at time, T = 0 h) x 100. Experiments were done three times, and error bars are standard deviations of individual results from their respective means.

Survival at elevated temperature

Survival of both ST and SE were significantly reduced on exposure to 52ºC (Figure 5). However, differences in survival between the isolates were insignificant ($p>0.05$; p = 0.5766).

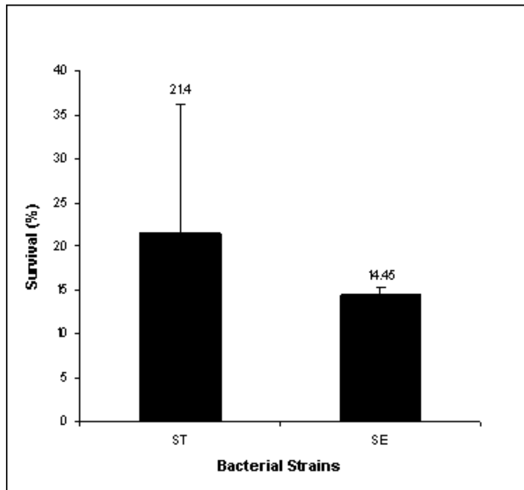

Figure 5: Survival of *Salmonella typhimurium* L1388 (ST) and *Salmonella enteritidis* L1225 (SE) at elevated temperature. Bacterial cells were incubated (with shaking) for 15 min at 52ºC in TSB, and viable cells were counted on TSA after appropriate dilution in saline and overnight incubation at 37ºC. Survival (%) was calculated from the relation: (Bacterial CFU ml-1 T = 15 min / Bacterial CFU ml-1 at time, T = 0 h) x 100. Experiments were done three times, and error bars are standard deviations of individual results from their respective means.

Survival in prolonged stationary phase

Our findings on prolonged stationary phase survival (shown in Figure 6) indicated that differences in the survival of ST and SE were insignificant ($p>0.05$; p = 0.3506).

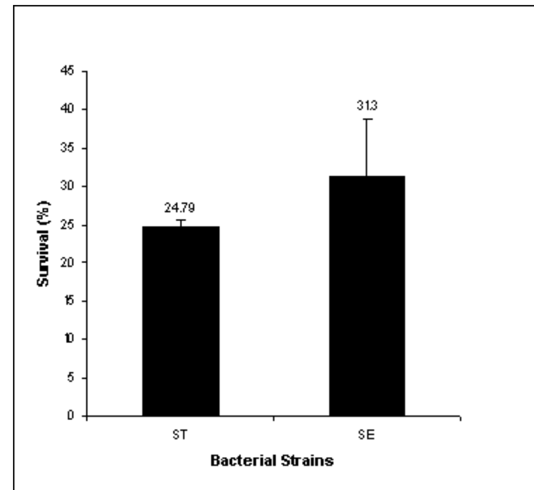

Figure 6: Survival of *Salmonella typhimurium* L1388 (ST) and *Salmonella enteritidis* L1225 (SE) in prolonged (7 days) stationary phase. Bacteria were grown in TSB at 37ºC for seven days. Viable cells were counted (both on Day 1 and Day 7) on TSA after appropriate dilution in saline and overnight incubation at 37ºC. Survival (%) was determined from the relation: (Bacterial CFU ml^{-1} on Day 7 / Bacterial CFU ml^{-1} on Day 1) x 100. Experiments were done twice using duplicate samples (i.e., n = 4), and error bars are standard deviations of individual results from their respective means.

Survival in human serum

The resistances of ST and SE to the complement-killing action of human serum were generally similar (Fig. 7) as the relative differences observed were insignificant ($p>0.05$; p = 0.8139).

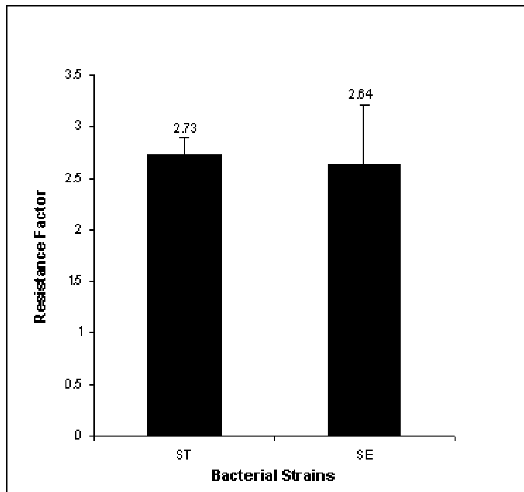

Figure 7: Serum resistance of *Salmonella typhimurium* L1388 (ST) and *Salmonella enteritidis* L1225 (SE). Counts were determined at times T = 0 h and T = 2 h following addition of the inoculum to 10% normal human serum in single strength Hank's Balanced Salt Solution (HBSS) at 37°C. Resistance factor was calculated from the relation: CFU at T = 2 h / CFU at T = 0 h. Error bars are standard deviations from means of three independent experiments.

Growth in iron-poor Medium A

Culturable growth of the ST and SE in an iron-poor medium A is as shown in Figure 8. Although for both isolates, growth was uninhibited at 37°C and 40°C, growth at 37°C was siginificantly ($p<0.05$) more than at 40°C. The differences between the isolates in growth were insignificant at 37°C (p = 0.8381) and marginally significant at 40°C (p = 0.0508).

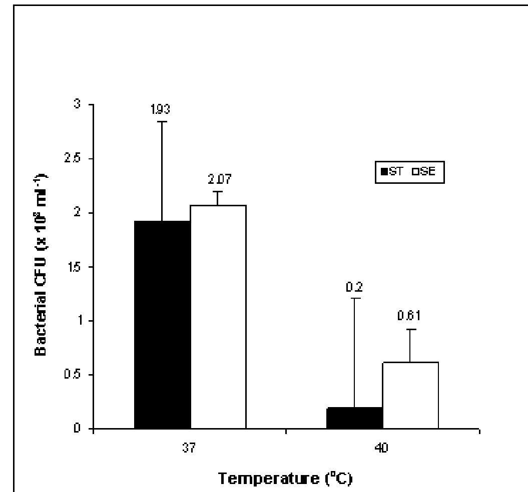

Figure 8: Growth of *Salmonella typhimurium* L1388 (ST) and *Salmonella enteritidis* L1225 (SE) in iron-poor medium. Bacteria were grown in low-iron Medium A (Pollack et al., 1970), then incubated (with shaking) at 37°C or 40°C for 24 h; and the number of colony forming units was counted on TSA, after appropriate dilution with normal saline and overnight incubation at 37°C. Growth was expressed as CFU of bacteria per milliliter of culture. Results are means of four independent experiments.

Discussion

The first major stress that *Salmonella* encounters after an oral infection is exposure to acidic gastric contents; but the most clinically relevant acid exposure occurs after invasion of the intestinal mucosa, within the phagolysosome, where the internal pH is 3 or 4.(17) Therefore, the similar acid tolerance of ST and SE is probable indication that they may survive within the phagolysosome.

The greater tolerance of SE to hydrogen peroxide than ST suggests possible differences in the response of ST and SE to the lethal effect of reactive oxygen species formed as by products of respiratory burst that occurs in the phagolysosome after invasion of the intestinal mucosa.(2) It has been reported that resistance to killing by reactive oxygen (RO) or nitrogen (RN) in-

termediates is associated with increased virulence of *Salmonella* Typhimurium.(18)

Salmonella grow optimally at a water activity (a_w) value of 0.99.(19) An osmotic up-shift usually lowers a_w, and consequently impairs growth. The insignificant differences in the tolerance of ST and SE to the osmotic up-shift generated by high salinity indicates a similar capacity for persistence in low a_w environments.

The insignificance of the differences observed in the survival of the two isolates in ethanol is relevant in the food industry, which strongly relies on the acid or alcoholic conditions to inactivate pathogens.

High-temperature heating is known to induce protein denaturation through vibration of water molecules to break disulfide and hydrogen bonds of intracellular proteins.(20) The similarity in heat tolerance of ST and SE suggests possible similarity in their protein composition.

The similarity in serum sensitivity could be accounted for by the smooth lipopolysaccharide (LPS) structure of the isolates (Data not shown). The ability of *Salmonella* to withstood host defense mechanisms is determined by certain structural and physiological attributes that act together or independently to promote the survival and growth of *Salmonella* in host cells. One of such attributes is serum resistance,(7) which is complement-mediated, LPS structure-dependent and plasmid-encoded. (21) Human serum was used for the test because it is known to function more efficiently in bacterial systems than either rabbit or guinea-pig serum.(22)

Since both strains survived to a similar extent under conditions of nutrient limitation, such as that observed in prolonged stationary phase, they may have similar persistence within the phagosome.(23) It is known that nutrient limitation is a key regulatory signal of some virulence genes in salmonellae.(24)

When deprived of iron, *Salmonella typhimurium* synthesizes the high-affinity iron chelator enterochelin (enterobactin), as well as produce a specific transport system for this siderophore.(25) Like in many Gram-negative bacteria, *Salmonella typhimurium* do not grow well at elevated temperatures unless the growth medium is supplemented with iron.(26) In *S. typhimurium*, the requirement for additional iron is due, at least in part, to decreased biosynthesis of the phenolate siderophore enterochelin at the elevated temperature.(26)

In conclusion, but for the significant variation in response to hydrogen peroxide, little insignificant variations were observed in the stress tolerance response of ST and SE. These observations will need to be correlated with virulence of the strains investigated.

Acknowledgements

We are grateful to Rikkyo University, Japan for The Bishop William's Fellowship support to Yakubu B. Ngwai.

References

1. Taylor J, McCoy JH. *Salmonella* and Arizona infections and intoxications. In Food-borne Infections and Intoxications. New York: Academic Press; 1969. p. 3-71.
2. Foster JW, Spector MP. How *Salmonella* survive against the odds. *Annual Review of Microbiology* 1995; 49: 145-174.
3. Archer DL. Preservation microbiology and safety: evidence that stress enhances virulence, and triggers adaptive mutations. *Trends in Food Science and Technology* 1996; 7: 91-95.
4. Kwon YM, Ricke SC. Induction of Acid Resistance of *Salmonella typhimurium* by Exposure to Short-Chain Fatty Acids. *Applied and Environmental Microbiology* 1998; 64: 3458-3463.

5. Popoff MY, Le Minor L. Antigenic formulae of the *Salmonella* serovars. WHO Collaborating Center for Reference and Research, Institut Pasteur, Paris, France; 1997.
6. Schwartz KJ. Salmonellosis. In Straw DE, D`Allaire S, Mengeling WL, Taylor DJ, editors. Diseases of Swine, 8[Th] Ed. Ames: Iowa State University Press; 1999. p. 535-551.
7. D`Aoust JY. *Salmonella* and the international food trade. *International Journal of Food Microbiology* 1994; 24: 11-31.
8. Fierer J, Swancutt M. Non-typhoid *Salmonella*: a review. *Current Clinical Topics in Infectious Diseases* 2000; 20: 134-157.
9. Ngwai YB, Onaolapo JA, Ehinmidu JO, Adachi Y, Ogawa Y. Interaction of *Salmonella typhimurium* and *Salmonella enteritidis* with polystyrene does not correlate with virulence in young chickens. *African Journal of Biotechnology* 2006; 5: 1122-1130.
10. Fang FC, Libby SJ, Buchmeier NA, Loewen PC, Switala J, Harwood J, Guiney DG. The alternative sigma factor KatF (RpoS) regulates *Salmonella* virulence. *Proceedings of The National Academy of Sciences USA* 1992; 89: 11978-11982.
11. McCann MP, Kidwell JP, Matin A. The Putative Sigma Factor KatF Has a Central Role in Development of Starvation Mediated General Resistance in *Escherichia coli*. *Journal of Bacteriology* 1991; 173: 4188-4194.
12. Jørgensen F, Stephens PJ, Knochel S. The effect of osmotic shock and subsequent adaptation on the thermotolerance and cell morphology of *Listeria monocytogenes*. *Journal of Applied Bacteriology* 1995; 79: 274-281.
13. St. John G, Brot N, Ruan J, Erdjument-Bromage H, Tempst P, Weissbach H, Nathan C. Peptide methionine sulfoxide reductase from *Escherichia coli* and *Mycobacterium tuberculosis* protects bacteria against oxidative damage from reactive nitrogen intermediates. *Proceedings of The National Academy of Sciences USA* 2001; 98: 9901-9906.
14. Jørgensen F, Leach S, Wilde SJ, Davies A, Stewart GSAB, Humphrey T. Invasiveness in Chickens, stress resistance and RpoS status of wild-type *Salmonella enterica* subsp. enterica serovar Typhimurium definitive type 104 and serovar Enteritidis phage type 4 strains. *Microbiology* 2000; 146: 3227-3235.
15. Pai CH, DeStephano L. Serum resistance associated with virulence in *Yersinia enterocolitica*. *Infection and Immunity* 1982; 35: 605-611.
16. Pollack JR, Ames BN, Neilands JB. Iron transport in *Salmonella typhimurium*: mutants blocked in the biosynthesis of enterobactin. *Journal of Bacteriology* 1970; 104: 635-639.
17. Foster JW, Hall HK. Adaptive Acidification Tolerance Response of *Salmonella typhimurium*. *Journal of Bacteriology* 1990; 172: 771-778.
18. De Groote MA, Testerman T, Xu Y, Stauffer G, Fang FC. Homocysteine antagonism of nitric oxide-related cytostasis in *Salmonella typhimurium*. *Science* 1996; 272: 414-417.
19. Tamminga SK, Beumer KK, Kampelmacher EH. Survival of *Salmonella eastborne* and *Salmonella typhimurium* in chocolate. *Journal of Hygiene* 1976; 76: 41-47.
20. Earnshaw RG, Appleyard J, Hurst RM. Understanding physical inactivation processes: combined preservation opportunities using heat, ultrasound and pressure. *International Journal of Food Microbiology* 1995; 28: 197-219.
21. Guiney DG, Fang FC, Krause M, Libby S. Plasmid-mediated virulence genes in non-typhoid *Salmonella* serovars. *FEMS Microbiology Letters* 1994; 124: 1-9.
22. Ogata RT, Levine RP. Characterization of complement resistance in *Escherichia coli* conferred by the antibiotic resistance plasmid R100. *Journal of Immunity* 1980; 125: 1494-1498.
23. Rathman M, Sjaastad MD, Falkow S. Acidification of Phagosomes Containing *Salmonella typhimurium* in Murine Macrophages. *Infection and Immunity* 1996; 64: 2765-2773.
24. Gentry DR, Hernandez VJ, Nguyen LH, Jensen DB, Cashel M. Synthesis of stationary phase sigma factor is positively regulated by ppGpp. *Journal of Bacteriology* 1993; 175: 7982-7989.
25. Neilands JB. Microbial envelope proteins related to iron. *Annual Review of Microbiology* 1982; 36: 285-309.
26. Garibaldi JA. Influence of temperature on the biosynthesis of iron transport compounds by *Salmonella typhimurium*. *Journal of Bacteriology* 1972; 110: 262-265.